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The Centrosome: Identification of New Components, a Study of Behaviour, and its Role in Human Autoimmune Disease.

by

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ABSTRACT

During cell division chromosome segregation relies on the coordinated action of several components of the mitotic apparatus, including the chromosome (kinetochore), the spindle microtubules, and the centrosome. In this dissertation the centrosome was studied (1) in human autoimmune disease, (2) during the post-mitotic period, and (3) as a focus for a number of unique proteins.

In the first phase of this work I identified a group of human autoimmune sera reactive with the centrosome and characterized their autoantibody specificity to centrosomal proteins. Patient sera reactive with the centrosome were found to have autoantibodies directed against multiple centrosomal components. These components include the known centrosomal proteins pericentrin and PCM-1, and proteins previously identified in mouse but unknown as centrosomal antigens in human autoimmune disease: Cep76, and ninein, and two novel centrosomal components Cep250 and Cep110. No correlation between having autoantibodies to the centrosome and a specific rheumatic diseases was found.

In the second phase, autoantibodies to the centrosome were used to characterize the movement of the centrosome following karyokinesis but preceding cytokinesis. During this time the centrosome was found to move from its polar location in each daughter cell to a region adjacent to the intercellular bridge. This movement was shown to require both intact microtubules and a microfilament network. The latter network was shown to specify the direction of movement.

Finally, I cloned and characterized the two novel centrosome proteins Cep250 and Cep110. Both of these proteins have extensive regions of coiled-coil structure and numerous leucine zipper motifs. Furthermore, both of these proteins were found to have unique cell cycle centrosome associations. Cep250 is associated with the centrosome during interphase, but disassociates at the beginning of mitosis. Cep110 is only detected at one centriole pair at interphase, suggesting that there is a compositional difference between the centrioles. My findings implicate these proteins in microtubule nucleation and/or centrosome maturation.

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DEDICATION

To my mom, dad, brother and sister

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bp	base pair
cDNA	complementary DNA
CENP	centromere protein
Cep	centrosome protein
Da	dalton
DAPI	4',6-diamindino-2-phenyl-indole
DPBS	Dulbecco's phosphate buffered saline
°C	degrees celsius
GFP	green fluorescent protein
h	hour
kDa	kilodalton
kb	kilobase
mM	millimolar
min	minute
MSA	mitotic spindle antigen
NGS	normal goat serum
NuMA	nuclear protein of the mitotic apparatus
PAGE	polyacrylamide gel electrophoresis
PCM	pericentriolar material
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate

SECTION I

INTRODUCTION

AUTOIMMUNITY

The human immune system is a complex and tightly controlled network of organs, cells, proteins, and signalling molecules that defends the body from infection. One of the cardinal properties unique to the immune system is its ability to discriminate between self and non-self antigens (Abbas, A.K. et al. 1994). The necessity of maintaining this unresponsiveness to self-proteins, called self tolerance, is underappreciated. The loss of self tolerance can often lead to immune reactions targeted against self proteins, resulting in debilitating and sometimes fatal autoimmune diseases.

A salient feature of autoimmune disease is the productions of autoantibodies. These autoantibodies have high specificity and are usually directed against conserved epitopes, often representing the functional or active sites of proteins (Tan, E.M. 1989). Furthermore, the autoantibody response has been shown to be polyclonal, with antibodies directed against more than one epitope on a single protein, and is often disease restricted, with the clustering of certain antibodies to specific autoimmune diseases (Fritzler, M.J. 1997, Tan, E.M. 1989). These and other properties of autoantibodies have led to the suggestion that they are the products of an antigen driven process (Fritzler, M.J. 1997, Tan, E.M. 1989).

Models of autoimmunity

With an ever increasing number of autoantibodies being identified, the elucidation of the biological mechanisms underlying autoimmunity will continue to pose a challenge to investigators (Tan, E.M. 1989). Any models or hypothesis of autoimmunity must be able to explain the diverse properties of the autoantibodies and their unique disease restrictions.

One possible theory of how an autoimmune response might be generated involves programmed cell death or apoptosis. Apoptosis, first described by (Kerr, J.F.R. et al. 1972), is an energy driven process of cellular suicide that is characterized by cell shrinkage, chromatin condensation, nuclear fragmentation, cytoplasmic blebbing and cellular fragmentation into small apoptotic bodies (Kerr, J.F.R. et al. 1972, Tan, E.M. 1994, Casciola-Rosen, L.A. et al. 1994). One hallmark of the apoptotic process is the degradation of chromosomal DNA to nucleosomal fragments. Apoptosis is known to occur in a variety of cell types and may occur in response to many different stimuli such as DNA damage, growth factor deficiency, and bacterial or viral infection (Casciola-Rosen, L.A. et al. 1994).

That apoptosis may have a role in autoimmunity is supported by the report that mice defective for the cell surface receptor Fas (*lpr/lpr*) develop multiple autoantibodies and a disease analogous to human lupus (Tan, E.M. 1994, Nagata, S. et al. 1995). The Fas receptor and ligand (FasL) are well known mediators of apoptosis (Hartman, J.J. et al. 1998, Nagata, S. et al. 1995). The deficiency of cell death in these mice results in the persistence of autoreactive T lymphocytes, which in turn stimulate autoreactive B cells to produce autoantibodies (Singer, G.G. et al. 1994). Mutations in the Fas gene have been found in patients with autoimmune lymphoproliferative syndromes (Rieux-Laucat, F. et al. 1995, Fisher, G.H. et al. 1995, Drappa, J. et al. 1996, Hetts, S.W. 1998). It is likely that some form of genetic predisposition, such as defects in apoptosis or mutations in the MHC class II locus (Vyse, T.J. et al. 1996) are involved in the process of autoimmunity.

An alternative role for apoptosis in the generation of autoimmunity is in the unique

way that self proteins may be clustered together and presented to the immune system during cell death. Casciola-Rosen, L.A. et al. 1994 have shown that two distinct populations of surface blebs on apoptotic cells contain clusters of autoantigens (DNA, fragments of ER, ribosomes, and the nucleoprotein antigens Ro, La and snRNPs). Autoantibodies to these antigens are frequently found in patients with the multi-system autoimmune disease known as systemic lupus erythematosus (SLE) (Tan, E.M. 1994, Casciola-Rosen, L.A. et al. 1994). The clustering and modification of these antigens could potentially be involved in challenging self tolerance. Further work has also shown the clustering of viral and self antigens in apoptotic blebs (Rosen, A. et al. 1995). These blebs of foreign and self protein may create unique environment that could challenge self tolerance. The fate of centrosomal antigens during the apoptotic process has yet to be investigated. Although apoptosis could provide an efficient method of clustering, modifying and presenting self proteins to the immune system, very little is known about its overall contribution to autoimmunity. Most researchers studying apoptotsis will be quick to point out that a cell undergoing apoptosis is rapidly and efficiently removed from the cell population by phagocytosis before lysis, and the release of potentially toxic or immunogenic cellular components can occur (Fadok, V.A. et al. 1998).

A second theory developed to address the generation of autoimmunity is based on the immune system being exposed to a bacterial superantigen. Superantigens are capable of activating large numbers of T and B cells irrespective of their antigen specificity (Schiffenbaurer, J. et al. 1998). In this case the superantigen could cross-link the MHC class II molecules on the B cell with a T cell receptor. The T cell will then release

cytokines in the vicinity of the autoreactive B cell, and initiate the production of autoantibodies (Schiffenbaurer, J. et al. 1998). Superantigens could also activate autoreactive T cells and antigen presenting cells that mediate destruction with the release of cytokines and other mediators of inflamation (Schiffenbaurer, J. et al. 1998).

A "two hit" hypothesis has been put forward to explain how superantigens may be involved in the development of autoimmune disease (Schiffenbaurer, J. et al. 1998). In this model, exposure to an infectious or toxic agent may activate autoreactive T cells via molecular mimicry (first hit). This is where a foreign agent mimics host cell proteins by having an identical or similar epitope. These activated autoreactive T cells are then amplified by the superantigen (second hit) resulting in an autoimmune response.

It is likely that no one model of autoimmunity is capable of explaining the myriad of features and consequences of this disease, and it is possible that several different mechanisms contribute to its genesis.

The clinical and biological significance of autoantibodies

Using autoantibodies as diagnostic markers for diseases such as SLE, scleroderma, Sjögrens syndrome, mixed connective tissue disease and drug induced lupus has been well established (Fritzler, M.J. 1997, Tan, E.M. 1989). Many autoantibodies, such as anti-topoisomerase I or anti-centromere appear months or years before any clinical manifestations of the disease, suggesting that they are not epiphenomena that follow an inflammatory event (Fritzler, M.J. 1997). Furthermore, sera titers of these autoantibodies have been correlated with disease activity, rising during flare-ups and falling during extended remission (Fritzler, M.J. 1997). Not only are autoantibodies useful in the

clinical setting, but they have also proven invaluable for basic cell biology. Further, many protein components of structures as diverse as the kinetochore (Earnshaw, W.C. et al. 1987, Saitoh, N. et al. 1992), centrosome (Balczon, R. et al. 1994, Doxsey, S.J. et al. 1994, Mack, G.J. et al. 1998) and Golgi apparatus (Fritzler, M.J. et al. 1995) have been identified and characterized with the aid of autoantibodies. Many proteins that are present in low amounts or are found only during specific stages of the cell cycle have been identified with human autoantibodies (Rattner, J.B. et al. 1993). The high specificity and conserved epitope binding properties of autoantibodies make them ideal probes for cell biology, and their use has resulted in the identification and characterization of many components of the mitotic apparatus.

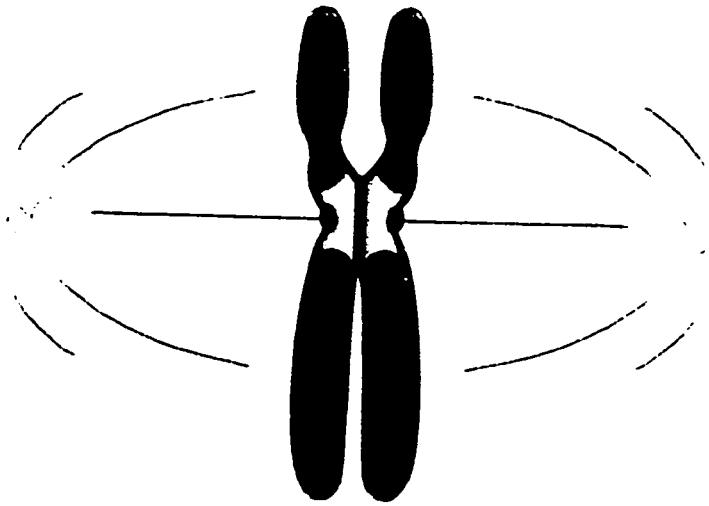
THE MITOTIC APPARATUS

The segregation of the genome (karyokinesis) and the division of the cytoplasm (cytokinesis) are the result of the coordinate interactions between components of the mitotic apparatus. The mitotic apparatus consists of three main components, the chromosomes, the spindle microtubules, and the centrosome (Rattner, J.B. et al. 1998, Fig. 1.0). A brief introduction to the components of the mitotic apparatus is presented.

The chromosome

During cell division the genomic material must be conveniently packaged so that it is easily transmissible to daughter cells and yet dynamic enough to quickly convert to a functional state after division. The chromosome is well suited for this purpose, and although models of its organization are still controversial, several lines of evidence indicate

Figure 1.0. The mitotic apparatus. Diagram of the mitotic apparatus illustrating the centrosomes and PeriCentriolar Material (PCM, yellow) with centrioles (white) at the spindle poles, spindle microtubules and the metaphase chromosome. Within the chromosome specific domain are highlighted: light purple-telomeres, black-surface domain, blue-nucleolus organizer, red-the body of the chromosome, yellow-centromere, and purple-kinetochore. Taken from Rattner, et al. 1998.



that a chromosome is formed from a series of packing and folding hierarchies (Rattner, J.B. 1992d). The basic unit of the chromosome consists of DNA and histone proteins assembled into a 10 nm chromatin fiber. The compaction of the chromosome results in the formation of at least five distinct domains (Fig.1.0): the centromere or primary constriction, the Nucleolar Organizer Region (NOR) or secondary constriction (present on a subset of chromosomes), the telomeres at each chromosome end, the body of the chromosome including the chromosomal arms, and the surface domain or chromosome periphery (Rattner, J.B. et al. 1998). The surface domain can be further subdivided into two functional domains: the paring domain where sister chromatids physically contact each other, and the kinetochore domain which is responsible for chromosome segregation. Interestingly, all of these domains with the exception of the telomeres have components that have been reported as autoantigens (Rattner, J.B. et al. 1998). The NOR has a series of autoantigens associated with it including RNA polymerase I (Reimer, G. et al. 1987), NOR-90 (Rodriguez-Sanchez, J.L. et al. 1987), NO 55 (Ochs, R.L. et al. 1996), and ASE-1 (Whitehead, C.M. et al. 1997). The central domain of the chromosome is principally composed of DNA, histones, and topoisomerase II, all of which are autoantigens (Tan, E.M. 1989, Hoffman, A. et al. 1989). The surface domain is frequently defined by autoantibodies to fibrillarin (Ochs, R.L. et al. 1985), Ki 67 (Bloch, D.B. et al. 1995), and GU/RH-II (Valdez, B.C. et al. 1996). The pairing domain can be defined by autoantibodies to the Chromatid Linking Proteins (CliPs) (Rattner, J.B. et al. 1988). Finally, the primary constriction or centromere houses numerous autoantigens, some of which are listed in Table 1.0 As the centromere is a specialized domain within the chromosome and is

intimately associated with the kinetochore, it is considered in more detail below.

The centromere

The constricted appearance of the centromere in relation to the remainder of the chromosome is believed to result from the absence of at least one step of chromatin condensation and coiling which occurs in the arms of the chromosome (Rattner, J.B. et al. 1987, Busch, S.J. et al. 1990, Wong, A.K.C. et al. 1992). The DNA within the centromere generally consists of repetitive sequences. In humans this DNA consists largely of alphoid DNA (Willard, H.F. et al. 1989). Although the reason that the structure of the centromere is distinct from the rest of the chromosome is unclear, it has been proposed that it may facilitate the interactions between sister chromosomes at the surface domain, and be required for the proper configuration of the kinetochore (Rattner, J.B. 1991b).

The kinetochore

Accurate chromosome segregation requires the proper attachment, positioning, and movement of chromosomes by microtubules of the mitotic and meiotic spindle. The structure responsible for mediating the link between microtubules of the spindle and the chromosomes is the kinetochore. Each kinetochore is a macromolecular structure that is located on the surface of the centromere. Ultrastructurally, it appears as a tri-laminar disk with inner, middle, outer plates that are approximately 30 nm thick (Wong, A.K.C. et al. 1992). A fourth kinetochore layer, the fibrous corona is found immediately outside the outer plate, and is believed to mediate the initial contact of the kinetochore with microtubules (Rieder, C.L. 1982a), Figure 2.0.

Figure 2.0. Diagram of the mammalian kinetochore. Diagram illustrating the centromere and kinetochore region of a metaphase chromosome. The kinetochore consists of three layers the inner plate, middle plate (space), and outer plate. The fibrous corona sits on the outside of the outer kinetochore plate. Taken from Wong et al. 1992.

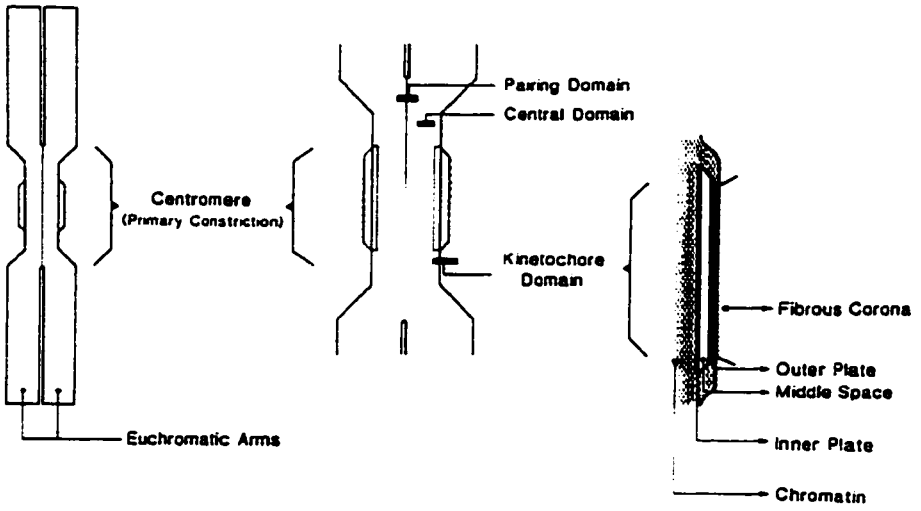

Pairing Domain
Central Domain
Centromere
(Primary Constriction)
Kinetochore
Domain
Fibrous Corona
Outer Plate
Middle Space
Inner Plate
Euchromatic Arms
Chromatin

Table 1.0. Human centromere/kinetochore associated proteins. A list of the major proteins, invariant and transient, found at the mammalian centromere/kinetochore.

Invariant proteins	Location	Reference
Cenp A	inner kinetochore plate, centromere	Vafa, O. et al. 1997
Cenp B	centromere	Cooke, C.A. et al. 1990a
Cenp C	inner kinetochore plate	Saitoh, N. et al. 1992
Cenp D	kinetochore	Kingwell, B. et al. 1987
Cenp E	outer kinetochore plate	Cooke, C.A. et al. 1997
Cenp F	outer kinetochore plate	Rattner, J.B. et al. 1993
Cenp G	inner kinetochore plate	He, D. et al. 1998
HP1HSα	centromere	Saunders, W.S. et al. 1993
p26	centromere	Nicol, L. et al. 1994
Transient proteins	**Location**	**Reference**
p55cdc	kinetochore	Kallio, M. et al. 1998
Mad2	kinetochore	Water, J.C. et al. 1998
hzw10	kinetochore	Williams, B.C. et al. 1996
Bub1	kinetochore	Taylor, S.S. et al. 1997
Bub 3	kinetochore	Taylor, S.S. et al. 1998
3F3/2 epitope	kinetochore	Gorbsky, G.J. et al. 1993
Dynactin	kinetochore	Echeverri, C.J. et al. 1996
Dynein	kinetochore	Steuer, E.R. et al. 1990, Pfarr, C.M. et al. 1990
CLIP-170	kinetochore	Dujardin, D. et al. 1998
p34cdc2	kinetochore	Rattner, J.B. et al. 1990
MCAK	centromere	Wordeman, L. et al. 1995
MSA36	centromere	Rattner, J.B. et al. 1992a

How the kinetochore is assembled on the centromere is still largely unknown, most likely either specific DNA sequence or DNA conformation is needed to initiate its formation. Using electron spectroscopic imaging (ESI) DNA has been found within the inner kinetochore plate, but not within the outer plate or corona (Cooke, C.A. et al. 1993). Furthermore, analysis of the complete DNA sequence of a *Drosophila* centromere has failed to identify any unique "kinetochore formation" sequence (Sun, X. et al. 1997). This has led the authors to speculate that the proper placement of the kinetochore could be determined by an epigenetic three dimensional higher order structure of heterochromatic DNA within the centromere (Sun, X. et al. 1997), and this is the favoured view at the present time.

The complex ultrastructure of the centromere/kinetochore is reflected in the number of proteins found to reside within this structure. These proteins are categorized by their spatial and temporal distribution patterns (Pluta, A.F. et al. 1995) (Table 1.0). Proteins that are a part of the centromere/kinetochore throughout the cell cycle were some of the first to be identified using human autoimmune sera (Moroi, Y. et al. 1980). The centromere protein CENP-A is a histone H3 variant (Sullivan et al. 1994) that has been found within a subset of nucleosomes associated with the centromere (Plamer, D.K. et al. 1987) and inner kinetochore plate (Vafa, O. et al. 1997). CENP-B is a helix-loop-helix DNA binding protein that recognizes a 17 base pair DNA sequence (CENP-B box) that is found within a subset of alphoid DNA at the centromere (Muro, Y. et al. 1992). Immunoelectron microscopy has shown CENP-B to be localized to the centromeric heterochromatin (Cooke, C.A. et al. 1990). The role of CENP-B at the

centromere remains unresolved, although a role in the folding of DNA repeats into a heterochromatin structure has been suggested (Sugimoto, K. et al. 1994a). Recently, mice null for CENP-B were shown to be mitotically and meiotically normal but have a decrease in body and testis weight (Hudson, D.F. et al. 1998). CENP-C has been shown to be a component of the inner kinetochore plate (Saitoh, N. et al. 1992) and may bind DNA (Sugimoto, K. et al. 1994b). CENP-D was identified with a human autoimmune serum (Kingwell, B. et al. 1987), and has been suggested to be the protein RCC1, a protein initially characterized as a regulator of chromatin condensation (Bischoff, F.R. et al. 1990). CENP-G, the most recently identified centromere protein is localized to the inner kinetochore plate, but its DNA sequence has not been determined (He, D. et al. 1998).

A second group of proteins is found to associate with the centromere/kinetochore in a cell cycle dependent fashion (Table 1.0) Many of these proteins are believed to regulate the assembly and function of the kinetochore through mitosis. Of these transient proteins, CENP-E has been the most thoroughly studied. CENP-E can be found on the kinetochore from prometaphase through metaphase, and at early anaphase, a sub-population of CENP-E is found to redistribute from the kinetochore to overlapping microtubules at the spindle midzone (Yen, T.J. et al. 1991). Molecular cloning of CENP-E revealed it to be a 312 kDa protein with a predicted kinesin-like motor at its NH_2-terminus (Yen, T.J. et al. 1992). CENP-E has been shown to co-purify with a minus-end directed microtubule motor activity (Thrower, D.A. et al. 1995), although experiments with a bacterially expressed fragment of CENP-E show it

to have a plus end directed motor activity (Wood, K.W. et al. 1997). Microinjection of CENP-E antibody into cells prevents proper chromosome alignment on the mitotic spindle, suggesting that it functions in the congression of chromosomes to the metaphase plate (Schaar, B.T. et al. 1997, Grancell, A. et al. 1998). Recently, the kinetochore binding domain of CENP-E has been located near its COOH terminus (Chan, G.K.T. et al. 1998). A two hybrid screen with this domain has shown that CENP-E specifically interacts with CENP-F and the human homolog of yeast spindle checkpoint kinase (hBUBR1) (Chan, G.K.T. et al. 1998). The nuclear matrix protein CENP-F is found to associate with the kinetochore during late G2, before CENP-E (Rattner, J.B. et al. 1993), and may be a key protein in the maturation of the kinetochore structure. CENP-E may participate with hBUBR1 in monitoring the spindle checkpoint pathway, and its association with CENP-F is the first clear evidence of the step-wise assembly of the kinetochore from spatially and temporally regulated proteins. Interestingly, autoantibodies to CENP-E and F do not appear together, and each is correlated with a distinctly different type of disease.

Three general functions of the kinetochore during cell division have been established. First, it mediates the attachment of replicated chromosomes to opposing poles of the mitotic spindle. Second, it is responsible for positioning the chromosome within the spindle, and third, it monitors the chromosomes to ensure that they are properly attached and positioned within the spindle (Grancell, A. et al. 1998). A single unattached chromosome or kinetochore will delay mitosis until it is attached to the spindle (Rieder, C.L. et al. 1998, Grancell, A. et al. 1998). Molecular details of the first

two functions of the kinetochore are still lacking, but it has been established that the microtubule motor proteins CENP-E, MCAK, and dynein are necessary for capturing and positioning chromosomes within the spindle (Grancell, A. et al. 1998, Rieder, C.L. et al. 1998). The kinetochore's role in the spindle checkpoint pathway has been extensively investigated in the last few years. Specific proteins at the kinetochore appear to be phosphorylated depending whether or not the kinetochore is attached to the spindle and the structure is placed under tension (Gorbsky, G.J. et al. 1993, Li, X. et al. 1997). For example, the kinetochores of chromosomes that have achieved a bipolar spindle attachment fail to stain with an antibody to a phosphoepitope, whereas unattached kinetochores, stain brightly (Gorbsky, G.J. et al. 1993). Furthermore, applying tension to an unattached kinetochore with a micromanipulation needle reduces this antibody staining and allows mitosis to proceed (Li, X. et al. 1997). Thus, distinct biochemical differences exist between attached and unattached kinetochores. How this difference between kinetochores arises appears to involve a complex of proteins which are homologous to yeast proteins that are genetically established as spindle checkpoint components (Straight, A.F. 1997, Glotzer, M. 1996, Gorbsky, G.J. et al. 1998, Taylor, S.S. et al. 1997, Taylor, S.S. et al. 1998, Water, J.C. et al. 1998, Li, Y. et al. 1996). How this complex of regulatory proteins and kinases actually prevents mitosis from occurring is still under debate. However, it is likely that the signal produced by an unattached kinetochore inhibits the ubiquitin-mediated proteolysis pathway (Rieder, C.L. et al. 1998). A large multiprotein assemblage known as the anaphase promoting complex (APC) is at the heart of this pathway, and is responsible for degrading

anaphase inhibitors and mitotic cyclins (King, R.W. et al. 1996). Recently the protein p55CDC has been shown to mediate the association of the spindle checkpoint complex on the kinetochore with the APC (Kallio, M. et al. 1998).

In light of the recent work on the identification of the regulatory proteins that transiently associate with the kinetochore, it is interesting to note that no structural proteins of the kinetochore plates has yet been identified with certainty. Clearly, much more work is needed in order to completely understand the formation and function of the kinetochore. Many of the centromere/kinetochore proteins have been identified with the use of human autoimmune sera. The protein CENP-G was recently identified with serum from a patient with an uncommon autoimmune disease (He, D. et al. 1998). Thus, it is possible that more centromere/kinetochore proteins could be identified with this approach, and should focus on sera from patients with autoimmune disease not commonly associated with producing antibodies to the centromere.

The spindle

Chromosome segregation during mitosis and meiosis is mediated by a complex microtubule based structure called the spindle (Mcintosh, J.R. et al. 1989, Galgio, T. et al. 1997). The dynamic properties of microtubules directly contribute to the formation of the spindle in a spatially and temporally regulated fashion. Each microtubule is composed of 13 protofilaments of alternating α and β tubulin subunits. Furthermore, the microtubule has a defined polarity, with a slow growing minus end at the centrosome and a fast growing plus end in the cytoplasm. During interphase long stable microtubules are nucleated by the centrosome. However, during late G2-prophase this

microtubule network is disassembled and replaced with shorter more dynamic microtubules from the duplicated centrosomes. Centrosome separation at prophase is believed to result from the force of microtubule motors pushing against microtubules between the centrosomes (Merdes, A. et al. 1997, Walczak, C.E. et al. 1996b). Microtubules from opposing centrosomes will then search the cytoplasm until the kinetochores of each chromosome have been captured, and a bipolar spindle established.

Mitotic microtubules can be divided into five classes. The first class are astral microtubules which extend away from the spindle and contact the cell membrane, where they play a role in properly orienting the spindle. The second class, pole-to-pole microtubules, are nucleated by opposing centrosomes and overlap each other in the middle of the spindle. These microtubules participate in the separation of the duplicated centrosomes, and in the elongation of the spindle during anaphase B, and will become sequestered into the intercellular bridge during telophase. The third class of microtubules interact with motor proteins (chromokinesins) on the surface of the chromosomes, and are a responsible for pushing the chromosomes into the spindle and away from the poles. The fourth class of microtubules interact with the kinetochore on each chromosome and are responsible for directing its movement to the poles during anaphase. Finally, the fifth class of microtubules are responsible for moving the centrosomes from their polar location at the end of mitosis to a region adjacent to the intercellular bridge (Mack, G. et al. 1993).

The role of centrosomes in nucleating microtubules for the spindle has been

well established (Waters, J.C. et al. 1997, Merdes, A. et al. 1997, Walczak, C.E. et al. 1996b). However, several examples of meiotic spindles or early embryonic mitotic spindles in animals have been found without centrosomes (Merdes, A. et al. 1997). Furthermore, all plant cells have also been shown to lack recognizable centrosomes (Smirnova, E.A. et al. 1992). Recent work on the assembly of spindles *in vitro* has shown that both centrosomal and acentrosomal cells organize a spindle through a common mechanism that requires both non-centrosomal structural proteins and microtubule motors (Compton, D.A. 1998, Rodionov, V.I. et al. 1993), (Table 2.0). Cells without preexisting centrosomes organize a spindle by first coalescing microtubules around chromosomes, and then reorganizing these microtubules so their minus ends point away from the chromosomes (Merdes, A. et al. 1997, Waters, J.C. et al. 1997). This process involves the action of plus end directed chromatin bound kinesin-like proteins such as chromokinesin (Wang, S.Z. et al. 1995), nod (Afshar, K. et al. 1995), and kid (Tokai, N. et al. 1996). Recently, spindle assembly in the absence of centrosomes was carried out *in vitro* using *Xenopus* egg extracts and DNA coated beads (Heald, R. et al. 1996). These results suggested that neither centrosomes or specific centromere DNA sequences are necessary for the formation of a bipolar spindle (Merdes, A. et al. 1997). Cells containing centrosomes are not faced with the challenge of reorganizing microtubules since they are nucleated from the centrosome with the correct polarity. After the nucleation and reorganization of microtubules, the formation of a bipolar spindle appears to proceed through a similar mechanism in both centriolar and acentriolar systems, and involves the convergence of the microtubules

Table 2.0. Major motor proteins involved in spindle assembly. A list of the major motor proteins known to be involved in the focussing and assembly of the spindle. Also listed is the direction of movement on microtubules.

Protein	Direction of movement	Reference
Eg5	plus	Sawin, K.E. et al. 1992
Xklp1	plus	Vernos, I. Et al. 1995
Xklp2	plus	Boleti, H. et al. 1996
XCTK2	minus	Walczak, C.E. et al. 1997
XKCM1	n/a	Walczak, C.E. et al. 1996a
ncd	minus	Endow, S.A. et al. 1994
nod	plus	Afshar, k. et al. 1995
kid	plus	Tokai, N. et al. 1996
CHO1	plus	Nislow, C. et al. 1992
CHO2	minus	Kuriyama, R. et al. 1995
dynein	minus	Steuer, E.R. et al. 1990, Pfarr, C.M. et al. 1990

into a spindle pole. The minus end directed microtubule motor protein dynein appears to be necessary for this process, as antibodies to dynein can block the focussing of microtubule arrays on DNA coated beads in frog extracts (Heald, R. et al. 1996). Similarly, if a complex of dynein, its regulatory complex dynactin, and NuMA, is immunodepleted from *Xenopus* extracts, the assembled spindle has a splayed out appearance instead of focussed spindle pole (Merdes, A. et al. 1996). Cytoplasmic dynein is proposed to dimerize and tether the microtubules together as it motors toward the minus end of each microtubule (Merdes, A. et al. 1997). In addition to this dynein complex, several kinesin-like proteins such as ncd (Endow, S.A. et al. 1994) and Eg5 (Sawin, K.E. et al. 1992) are necessary for proper spindle formation. Depletion of Eg5 from mammalian spindle assembly extracts results in spindle poles that remain unfocussed (Gaglio, T. et al. 1996), and has led to the suggestion that it is required to cross link microtubules at the spindle pole (Compton, D.A. 1998). Furthermore, microinjection of antibodies to Eg5 in animal cells also results in the failure of the centrosomes to separate (Blangy, A. et al. 1995). Recently, microinjection of HsEg5 antibody into HeLa cells has been shown to prevent the formation of microtubule arrays associated with pre and post-mitotic centrosome movement, and to disrupt the distribution the spindle protein NuMA (Whitehead, C.M. et al. 1998). The role of non-motor structural proteins such as NuMA in the formation of the spindle is largely unknown, however it has been proposed that these proteins may form an insoluble matrix that could anchor microtubule minus ends at the spindle poles (Compton, D.A. 1998).

The assembly of a functional bipolar spindle in cells is a complex process that requires the cooperative action of both plus and minus-end directed motor proteins, as well as non motor structural proteins. Furthermore, spindle assembly appears to occur in a similar manner in cells that are either centriolar or acentriolar. Although many protein components of the spindle have been identified, only two (NuMA and HsEG5) have been shown to act as antigens in autoimmune disease (Andrade, L.E.C. et al. 1996, Whitehead, C.M. et al. 1996a).

The centrosome

Distinct structures at the poles of the mitotic spindle were discovered over 100 years ago by Van Beneden and Boveri (Fulton, C. 1971). These structures were first termed polar corpuscles, central bodies, and then centrosomes. Theodor Boveri (Fulton, C. 1971, Stearns, T. et al. 1997, Davis, T.N. 1997) defined the centrosome as the "dynamic center of the cell", or "an independent permanent cell-organ, which, exactly like the chromic elements, is transmitted by division to the daughter cells." Although more modern definitions of the centrosome would also include its function in the regulation and organization of microtubules, not much else has been universally agreed upon. The difficulty in defining a centrosome could in part be due to the fact that it is more of a cellular focus than an organelle (Rattner.J.B 1992c), and has thus been referred to as the central enigma of cell biology (Wheatley, D.N. 1982). Even though large volumes of material have been published on the ultrastructural morphology of the centrosome, information regarding its biochemical composition and function lags far behind.

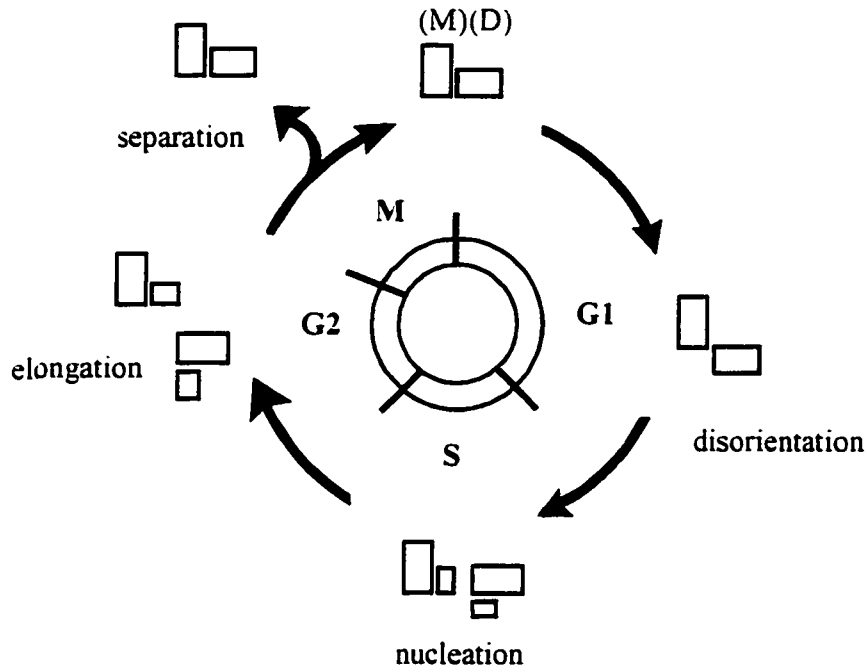
The centrosome generally consists of one or two centrioles and their surrounding pericentriolar material (PCM). Each centriole is ~ 0.2μm in diameter and ~ 0.5μm long, and is composed of nine triplet microtubule blades that are tilted with respect to each other around a central circle (Fulton, C. 1971, Vorobjev, I.A. et al. 1987). Each triplet is assembled from one complete microtubule and two incomplete tubules that share their walls with the preceding tubule (Fulton, C. 1971). Triplets are linked together throughout the centriole and give it a pinwheel-like appearance. Furthermore, the centriole is a polar structure, with a amorphous hub-like structure within the centriole lumen at its distal end, and a cartwheel-like structure connecting the nine tubules at the proximal end (Fulton, C. 1971). Immediately surrounding all of the triplet microtubules in a centriole is a large amount of electron dense material (Rattner.J.B 1992c, Vorobjev, I.A. et al. 1987). Centriolar structure is know to vary between different species (Vorobjev, I.A. et al. 1987, Fulton, C. 1971), and even within different cell types of a single species (Gonzalez, C. et al. 1998).

Generally the two centrioles of a centrosome (diplisome) will be oriented at right angles (Fig.3.0) to each other throughout mitosis until mid telophase (Fulton, C. 1971). The older (mother, parent, or mature) centriole can be defined using the right-angle rule (Rieder, C.L. et al. 1982b). In this relationship the axis of the parent centriole does not intersect the younger (daughter or immature) centriole, but the axis of the daughter centriole will intersect the parent. This rule applies to the centriole because of the nature of its duplication. During mitosis the single centrosome of the cell must duplicate such that each daughter cell will receive one. During G1-S phase of

the cell cycle the two centrioles of the centrosome will lose their orthogonal relationship and separate from each other by a small distance, a process called disorientation (Koschanski, R.S. et al. 1990, Rattner, J.B. et al. 1973). Then synchronously each centriole will begin to nucleate a second small centriole called a procentriole at a right angle from it (Fulton, C. 1971, Vorobjev, I.A. et al. 1987, Rattner, J.B. et al. 1973, Alvey, P.L. 1985). The procentriole forms toward the proximal end of the parent but is separated from it by 50-100μm (Fulton, C. 1971). The procentriole appears to condense from the cytoplasm in a stepwise fashion (Fulton, C. 1971), and appears to require protein (Phillips, S.G. et al. 1976) but not DNA (Rattner, J.B. et al. 1973) synthesis. Each procentriole will elongate during the G2 and M phase of the cell cycle until it reaches the same size as the parent, usually by mid to late mitosis (Vorobjev, I.A. et al. 1987, Rattner, J.B. et al. 1973). The complete maturation of a centriole/centrosome into a microtubule organizing center takes one and a half cell cycles (Vorobjev, I.A. et al. 1982, Rieder, C.L. et al. 1982b, Vorobjev, I.A. et al. 1987). Figure 3 illustrates centriole duplication and separation.

The other major element of a typical centrosome is the ill-defined dense staining PCM. The PCM begins just outside of the electron dense material of the centrioles and extends as a halo or cloud around it (Rattner.J.B 1992c). The PCM is directly responsible for microtubule nucleation (Gould, R.R. et al. 1977), and can function independently of centrioles (Gould, R.R. et al. 1977, Calcarco-Gillam, P.D. et al. 1983).

Figure 3.0. The centriole cycle. A diagram illustrating the centriole cycle in mammalian cells. In G1 phase of the cell cycle two centrioles are in a right angle orientation. One of these centrioles, the mother (M) is the older of the centrioles and is a microtubule organizing center (MTOC). The daughter centriole (D) was formed one cell cycle ago and is not an MTOC. During late G1 early S phase, mother and daughter centrioles lose their right angle orientation (disorientation) and each centriole nucleates a procentriole its proximal end. The procentriole will elongate through S and G2, and will reach the same size as the parent centriole by metaphase, at which time it is considered mature. In the following cell cycle each procentriole will have matured to a mother centriole and a functional MTOC.


(M)(D)
separation
M
G2
G1
elongation
disorientation
S
nucleation

In fact, irradiation of centrioles with a laser microbeam has no obvious effect on the centrosomes' function as a microtubule nucleating center (Berns, M.W. et al. 1977). During interphase the mature centriole will be surrounded by more PCM and pericentriolar structures (satellites and appendages) than the immature centriole, and thus has the sole capacity to act as a microtubule organizing center (MTOC) (Rieder, C.L. et al. 1982b, Vorobjev, I.A. et al. 1987). During mitosis the PCM becomes more extensive around each parent centriole and is correlated with an increase in the microtubule nucleating capacity of the centrosome (Gould, R.R. et al. 1977, Rieder, C.L. et al. 1982b). The nature of the PCM has remained obscure for over 100 years and only recently has its architecture been more throughly investigated. Using high resolution immunofluorescence imaging, two protein components of the PCM, pericentrin and γ-tubulin, were shown to exist in an organized reticular network around the centrioles (Dictenberg, J.B. et 1998). This lattice-like structure may be responsible for the arrangement of microtubule nucleating sites at the centrosome, and has been shown to undergo a dramatic rearrangement in size and complexity from interphase to mitosis (Dictenberg, J.B. et al. 1998).

From its discovery, the centrosome has been known as the cells primary site for the organization of microtubules (Brinkley, B.R. 1985). The microtubule cytoskeleton is essential for maintaining cell shape and the distribution of cellular organelles such as the ER and Golgi apparatus. In addition, microtubules are required for accurate cell division and for development and maintenance of polarized cell structures (Joshi, H.C. 1993).

Original models for microtubule nucleation have suggested that the microtubule minus end is anchored in the PCM preventing its disassembly, while the plus end undergoes continuous polymerization and depolymerization events. However, it has been observed that microtubules can undergo a poleward flux, where polymerization and growth occur at its plus end and disassembly occurs at the centrosome attached minus end (Mitchison, T. 1989). Refinement of the model for centrosome nucleation must now account for this, and the observation that microtubule detachment occurs from the centrosome *in vivo* (Yu, W. et al. 1993, Keating, T.J. et al. 1997) and *in vitro* (Belmont, L. et al. 1990).

Recently, McNally, F.J. et al. (1993) identified a heterodimeric ATP dependent microtubule severing protein called katanin from sea urchin eggs. Katanin is localized at the centrosome throughout the cell cycle in a region surrounding the γ-tubulin containing PCM (McNally, F.J. et al. 1996). The 60 kDa subunit of katanin has recently been shown to be a member of the AAA family of ATPases and contains the microtubule severing activity (Hartman, J.J. et al. 1998). An 80 kDa subunit appears to direct katanin to the centrosome through a WD40 protein-protein interaction domain (Hartman, J.J. et al. 1998). Katanin activity at the centrosome may explain the observations of poleward microtubule flux and the distribution of microtubule minus ends throughout the centrosome. It has been proposed that after nucleation from the PCM, a microtubule is severed by katanin to expose its minus end. The remaining microtubule is held within the PCM by the pulling force of a kinesin-like protein, while polymerization continues at its plus end resulting in the poleward flux phenomenon

(McNally, F.J. et al. 1996). Why microtubule severing is needed at the centrosome is not fully known. However, it has been suggested to provide an efficient and rapid means of reorganizing the cytoskeleton in response to internal or external cues (Vale, R.D. 1991)

An additional function of the centrosome is in dictating the number of spindle poles during mitosis (Compton, D.A. 1998). Little is known about the mechanisms used by animal cells to regulate centrosome replication at G1/S. However, for cell division to occur properly, the centrosome must duplicate only once during each cell cycle. Abnormal centrosome duplication could result in the formation of monopolar or multipolar spindles. Recently, Balczon, R. et al. (1995) have shown that if CHO cells are arrested at the G1/S boundary of the cell cycle with hydroxyurea, multiple rounds of centrosome duplication occurred in the absence of DNA synthesis and cell division. It has been suggested that centrosome duplication is possible in these blocked cells only at G1/S because centrosome subunits are maximally and constantly expressed. The authors go on to speculate that centrosome duplication is controlled by the activation and inactivation of the centrosome replication machinery at the appropriate times during the cell cycle. Alternatively, Sluder, G. et al. (1996) have suggested that strict control over centrosome duplication during S phase is maintained because the cell only provides the daughter centrioles with components or conditions necessary for the construction of replication templates only after G1/S when other centrosome subunits are in low abundance.

Finally, a third function for centrosomes has recently been suggested from

experiments designed to assemble spindles *in vitro* around chromatin containing beads (Heald, R. et al. 1997). As explained in the previous section, both plus and minus end directed microtubule motor proteins are required for the formation of a bipolar spindle, whereas the centrosome is not required. If, however, centrosomes are included within the extract, spindle assembly is more efficient (Heald, R. et al. 1997). Therefore, centrosomes could provide a means to concentrate microtubule nucleation material, thereby suppressing microtubule self-assembly and direct the site of efficient spindle formation (Heald, R. et al. 1997).

As mentioned above, the centrosome is a component of the mitotic apparatus. Alteration or mutation in centrosomal components could have a profound effect on the organization and regulation of cell division. Defects in cell division frequently result in cancer, which is often characterized as a state of genomic instability. This instability can be in the form of gene deletions, amplifications, and translocations. Additional genetic damage, such as aneuploidy and tetraploidy are also observed in cancerous cells, and are likely the result of mitotic spindle defects and or cell cycle checkpoint errors (Winey, M. 1996).

Since the function of the centrosome is critical to cell division, its contribution to the genesis of cancer is now coming under investigation. For example, the tumor suppressor protein p53 is known to be involved in the maintaining cell cycle checkpoints, promoting apoptosis (Encoch, N.T.C. 1995), and is frequently mutated in human and rodent cancers (Ziegler, A. et al. 1994, Hollstein, M. et al. 1991). The p53 protein has been shown by immunofluorescence to be associated with the centrosome

during interphase but not during mitosis (Blair Zajdel, M.E. et al. 1988). A role for p53 in cancer was demonstrated using p53 null mutations in mouse cells. If normal mouse embryonic fibroblasts are treated with the microtubule disrupting drug nocodazole they will arrest the cell cycle immediately after S-phase, the result of the triggering a spindle checkpoint. However, p53 deficient mouse cells can override this checkpoint and inappropriately reinitiate DNA synthesis, suggesting that p53 is an essential component of the spindle checkpoint machinery (Cross, S.M. et al. 1995). Furthermore, an additional study has also shown that p53 deficient mouse cells have defects in centrosome duplication (Fukasawa, K. et al. 1996). More than 30% of interphase cells from p53 null mice have more than two centrosomes, and more that half of the mitotic cells have spindles organized by multiple centrosomes. This increase in the number of centrosomes was shown to result from premature duplication during G1, and multiple duplication during each cell cycle (Fukasawa, K. et al. 1996). Together, this work demonstrates that cells that are deficient in p53 are more likely to have defects in centrosome number and proceed to form defective spindles which in turn results in chromosome mis-segregation because of a defective spindle checkpoint (Winey, M. 1996).

Another tumor suppressor gene that appears to have a role in mitotic abnormalities is TSG101. Inactivation of this gene in mouse cells leads to cell transformation and the ability to form metastatic tumors (Li, L. et al. 1996). TSG101 has been localized to the nucleus and Golgi complex during G1, and the centrosomes, spindle and midbody during mitosis. Cells in which TSG101 has been functionally

inactivated display multiple spindles and centrosomes as well as an abnormal configuration of metaphase chromatin. Extensive aneuploidy was also observed in some of these cells. If TSG101 function is restored to these cells, the mitotic abnormalities appear to be reversed, although in some cells the cancerous transformation is irreversible. This has led to the suggestion that TSG101 may have a "caretaker" role in the cell, and that alteration of this role may lead to other genetic damage promoting cancer progression (Xie, W. et al. 1998).

Recently, the centrosome-associated kinase STK15/BTAK has been found to be amplified and overexpressed in multiple human tumor cell types (Zhou, H. et al. 1998). Ectopic expression of this kinase in mouse cells has been shown to induce centrosome duplication errors that often lead to aneuploidy and transformation (Zhou, H. et al. 1998), suggesting that centrosomal kinases are key regulatory factors that ensure proper chromosome segregation.

Finally, by examining 35 human breast carcinomas, Lingle, W.L. et al. 1998 has shown that these tumor cells have increased centrosome numbers, an accumulation of excess of PCM, supernumerary centrioles, and inappropriate phosphorylation levels of the centrosomal protein, centrin. Furthermore, these abnormal centrosomes can nucleate unusually large and numerous microtubule arrays. As a result of these changes, cells will loose their organization and polarity and often have an increase in multipolar mitoses, leading to further genomic instability (Lingle, W.L. et al. 1998). Thus, the proper regulation of centrosome function is essential for proper cell division.

An understanding of the function of the centrosome also requires an

understanding of its protein composition. Several centrosome proteins have been identified by various means, and can be grouped into two classes, those that are principally found at the centrosome, and those that are found at the centrosome but also in other areas of the cell. Table 3.0 lists some of the known proteins that are residents of the centrosome throughout the cell cycle and may be responsible for its organization and function.

One of these centrosomal proteins, γ-tubulin, is essential for microtubule nucleation and has been found in virtually all eukaryotes (Oakley, C.E. et al. 1989, Raff, J.W. 1996, MacDonald, D. et al. 1995). Gamma tubulin can be found in a high molecular weight complex in the cytoplasm of *Xenopus* and mammalian cells, but appears to be inactive (Hartman, J.J. et al. 1998, Bao, L. et al. 1998, Stearns, T. et al. 1994). This complex has a ring-like appearance in electron micrographs (Zheng, Y. et al. 1995), and consists of at least six other polypeptides (Zheng, Y. et al. 1995). A ring-like structure of γ-tubulin has also been observed at the centrosome (Mortitz, M. et al. 1995). A model has been proposed for microtubule nucleation where the γ-tubulin ring complex provides a template for the formation of a microtubule with the correct polarity (Zheng, Y. et al. 1995). Recently, two other protein components of the *S. cerevisiae* ring complex, Spc97 and Spc98, have been shown to dock with the spindle pole body, through an interaction with a coiled-coil protein Spc110 (Knop, M. et al. 1997). Mammalian homologs of Spc97 and Spc98 have recently been identified through immunoprecipitation of the γ-tubulin ring complex (Murphy, S.M. et al. 1998), and a putative homolog of Spc110 has been detected immunologically at the

Table 3.0. Proteins found only at the centrosome. List of some of the proteins known to reside only at the centrosome.

Protein/Antigen	Location	Reference
Cep250 (C-Nap1)	Centrioles	This dissertation Fry, A.M. et al. 1998a
Cep110	Centrioles	This dissertation
Centrin	Centrioles	Errabolu, R. et al. 1994
Cenexin	Centrioles	Lange, B.M.H. et al. 1995
Nek-2	Centrioles	Fry, A.M. et al. 1998b
PCM-1	PCM, centrioles ?	Balczon, R. et al. 1994
CCD41	Centrioles ?	Rothbarth , K. et al. 1993
Pericentrin	PCM	Doxsey, S.J. et al. 1994
Ninein	PCM	Bouckson-Castaing, V. et al. 1996
1133/5p (Cep76)	PCM	Angiolillo, A. et al. 1996
Centrosomin A	PCM	Joswig, G. et al. 1991
Katanin	PCM	Hartman, J.J. et al. 1998
Centrin-like	PCM	Baron, A.T. et al. 1988
hGCP2	PCM	Murphy, S.M. et al. 1998
hGCP3	PCM	Murphy, S.M. et al. 1998
hsSpc98	PCM	Tassin, A.M. et al. 1998
γ-tubulin	PCM	Zheng, Y. et al. 1991
HSP70-like	PCM	Perret, E. et al. 1995
CDC16Hs	PCM	Tugendreich, S. et al. 1995
CDC27Hs	PCM	King, R.W. et al. 1995
Centractin	PCM	Clark, S.W. et al. 1992

centrosome (Tassin, A.M. et al. 1997). The resident centrosomal protein pericentrin is also essential for microtubule nucleation (Doxsey, S.J. et al. 1994) and has recently been found to be a part of the *Xenopus* γ-tubulin complex (Dictenberg, J.B. et al. 1998). Pericentrin may act as the backbone for tethering the cytoplasmic γ-tubulin complex to the centrosome (Dictenberg, J.B. et al. 1998).

The second group of centrosomal proteins show a complex distribution throughout the cell, and often associate with the centrosome in a cell cycle dependent fashion (Table 4.0). Many of these proteins are known regulatory factors that may be required for specific cell cycle functions of the centrosome.

One regulatory protein that can be found at the centrosome (PCM and centrioles) is the cyclin dependent kinase cdc2 (Bailly, E. et al. 1989, Rattner, J.B. et al. 1990, Pockwinse, S.M. et al. 1997). Immunologically cdc2 has been observed throughout the cytoplasm and nucleus (Bailly, E. et al. 1989), and at the kinetochore, intercellular bridge, and kinetochore to pole microtubules (Rattner, J.B. et al. 1990). When complexed with cyclin B, cdc2 is an essential kinase that is required for regulating the entry into mitosis in mammalian cells (Morgan, D.O. 1995). Furthermore, cdc2/cyclinB has been implicated in controlling microtubule nucleation and dynamics during mitosis (Verde, F. et al. 1990, Verde, F. et al. 1992, Ohta, K. et al. 1993). During most of interphase, the cdc2 /cyclin B kinase is maintained in an inactive form by inhibitory phosphorylation (Norbury, C. et al. 1991). At the G2-M transition, the cdc25B phosphatase removes two inhibitory phosphates resulting in an active protein kinase (Dunphy, W.G. et al. 1991), which can then modify the

Table 4.0. Proteins found at the centrosome and elsewhere in the cell. List of the protein that associate with the centrosome in addition to other areas within the cell.

Protein/Antigen	Cellular distribution	Reference
Protein 4.1	Centrosome, nucleus, spindle	Krauss, S.W. et al. 1997a, b
PPX phosphatase	Centrosome, nucleus, cytoplasm	Brewis, N.D. et al. 1993
Enolase determinant	Centrosome, cytoplasm	Rattner, J.B. et al. 1991c
P21/23 (turkey)	Centrosome, nuclear membrane	Woods, C.M. et al. 1995
Mitotin	Centrosomes, nucleus, midbody	Todorov, I.T. et al. 1992
p34cdc2	Centrosomes, cytoplasm, kinetochores, intercellular bridge, nucleus	Rattner, J.B. et al. 1990, Bailly, E. et al. 1989
FKBP59-HBI	Centrosomes, nucleus, spindle	Perot-Applanat, M. et al. 1995
Cytocentrin	Centrosomes, cytoplasm, midbody	Paul, E.C.A. et al. 1993
CHO2	Centrosome, spindle	Kuriyama, R. et al. 1995
Protein phosphatase 2A	Centrosome, microtubules, spindle	Sontag, E. et al. 1995
Protein phosphatase 1α	Centrosomes, nuclear matrix,	Andreassen, P.R. et al. 1998
Protein phosphatase γ1	Spindle poles, nucleoli	Andreassen, P.R. et al. 1998
HsEg5	Centrosomes, spindle, intercellular bridge	Blangy, A. et al. 1995
Casein kinase I/II	Centrosome, cytoplasm, spindle	Brockman, J.L. et al. 1992, Krek, W. et al. 1992
Calmodulin dependent kinase II	Centrosome, nucleus, cytoplasm, midbody	Ohta, Y. et al. 1990
Polo-like kinase	Centrosome, intercellular bridge	Golsteyn, R.M. et al. 1995
Aurora-related kinase (IAK1)	Centrosome, midbody	Gopalan, G. et al. 1997
p53	Centrosome, nucleus	Blair Zajdel, M.E. et al. 1988
TSG101	Spindle poles, nucleus, Golgi	Xie, W. et al. 1998
NuMA	Nucleus, centrosomes	Andrade, L.E.C. et al. 1996
hsp70	Cytoplasm, centrosome	Rattner, J.B. 1991a

microtubule nucleating capacity of the centrosome through phosphorylation (Keryer, G. et al. 1995). Recently, Gabrielli, B.G. et al. 1996 have shown that overexpression of a catalytically inactive cdc25B results in a delay of G2-M transition due to a lack of prophase microtubule nucleation, and that overexpression of wildtype cdc25B leads to the formation of mini-spindles without chromosome condensation or nuclear envelope breakdown. Thus, it has become clear that control over the number, spatial distribution and length of microtubules resides within the centrosome and surrounding cytoplasm (Brinkley, B.R. et al. 1981), and this likely requires the activity of many of these centrosomal associated regulatory proteins.

The complex organization and function of the centrioles and PCM suggests that it is assembled from an extensive collection of protein components. It has been estimated that approximately 150-200 proteins are necessary for a functional mammalian centrosome and spindle pole (Salisbury, J.L. 1995). By comparison, the spindle pole body of the yeast *S. cerevisiae*, which has a similar microtubule nucleation function as that of the centrosome, is composed of at least 27 proteins (Wigge, P.A. et al. 1998). Thus, it is likely that we have identified only a small fraction of the protein components of the centrosome. This may be due to several factors, one of which is that the centrosome represents only 0.01% of the average cell volume (Vorobjev, I.A. et al. 1987), making its isolation and purification impractical. To circumvent this problem other methods of protein identification such as the use of human autoimmune sera, have been used successfully to identify some centrosome proteins. However, it is apparent that this approach has not been used to its fullest extent. This may in part be due to the

difficulty in recognizing centrosome reactivity, or the misinterpretation of its reactivity when characterizing serum samples in clinical labs.

Many questions about centrosome, organization, duplication, regulation, and function still remain unanswered. Clearly, further investigation of the centrosome is required in order to answer these and other questions. The goal of this dissertation was to obtain more information on the clinical relevance and cell biology of the centrosome. To do this, I investigated the centrosome from three different perspectives, clinical, biological and biochemical.

Clinical: I have characterized a group of human sera with autoantibody specificity to centrosomal proteins. This work directly led to the identification of two novel centrosomal proteins, Cep250 and Cep110, and the finding that two previously known centrosomal proteins, ninein and Cep76, are human autoantigens. I found that autoimmune sera that react with the centrosome have autoantibodies to multiple centrosome proteins. This observation is consistent with the suggestion that a centrosomal complex acts as an antigenic target in autoimmune disease. This work has allowed us to determine if there are any specific rheumatic diseases associated with having autoantibodies to the centrosome.

Biological: the understanding of centrosome behaviour and function during the cell cycle has concentrated mainly on the events leading to mitosis. However, the centrosome continues to function after mitosis, and these event have received little attention. Therefore, I undertook an investigation to characterize centrosome behaviour immediately following cell division. This work led to the description of the post-mitotic

movement of the centrosome to a defined location within the cell. This movement occurs in a spatially and temporally regulated manner, and is dependent on both microtubule and the microfilament networks. Thus, the centrosome's role during the cell cycle is far more complex than previously described.

Biochemical: since the centrosome is a complex structure with a dynamic role during the cell cycle, determining its protein composition is essential for understanding its organization and function. Therefore, using human autoimmune sera I identified and characterized two previously unknown centrosomal proteins, Cep250 and Cep110. These proteins provided us with a more complete picture of the dynamics of the centrosome during the cell cycle, and give us additional tools to investigate centrosome structure and function.

SECTION II

GENERAL METHODS AND MATERIALS

Indirect immunofluorescence (IIF)

Monolayer cultures of HeLa, chinese hamster ovary (CHO), or mouse L929 cells (American Type Tissue Collection, Bethesda, MD.) were grown in Joklik's MEM supplemented with 10% fetal calf serum. Cells were seeded onto coverslips 24 - 48 h prior to use. To prepare cells for IIF, cells were fixed in either 100% methanol at -20°C for 10 min and air dried, or 3% paraformaldehyde in Dulbecco's phosphate buffered saline (DPBS) for 5 min followed by permeablization in 0.5% Triton X-100 in DPBS for 5 min. Methanol fixed cells were rehydrated in DPBS prior to incubation with antibody. In some instances cells were extracted by incubation in the extraction buffer (60 mm PIPES, 25 mM HEPES, 10 mM EGTA, 2mM $MgCl_2$ (pH 6.9), 0.5 % Triton-X 100) for 1-2 min prior to fixation. Cells were then blocked with a 1:200 dilution of normal goat serum in DPBS (NGS-DPBS) for 20 min at room temperature. Each coverslip was incubated in the appropriate dilution of antibody in NGS for 30 min to 1 h in a humid chamber at 37°C. After 2-3 washes in D-PBS the coverslips were incubated for 30 min in a 1:400 dilution of the appropriate secondary antibody in NGS-DPBS: either fluorescein conjugated goat anti-human IgG (Dimension labs, Mississauga, Ont.), Cy3 conjugated goat anti-human (Dimension labs), fluorescein conjugated goat anti-rabbit (Jackson Labs, Oak Grove PA), Cy3 conjugated goat anti-rabbit (Dimension labs), Cy3 conjugated goat anti-mouse (Jackson Labs), fluorescein conjugated goat anti-mouse (SIGMA, Oakville, Ont.), or a rhodamine conjugated goat anti-mouse (Dimension labs). After incubation the coverslips were washed in DPBS and counterstained with DAPI (4',6'-diamidino-2-phenylindole) in DPBS, mounted in 90% glycerol containing paraphenylenediamine as an anti-fade agent.

Cells were observed using a Nikon Optophot fluorescence microscope or a Leica DMRB microscope using a 100X objective . Images were recorded on Ilford HP-5 film or a RTE/CCD detector (Princeton Instruments, Trenton, NJ.) using IPLab Spectrum software (Signal Analytics Co. Vienna, VA).

SDS-PAGE/Western blotting

Protein samples were resuspended in SDS sample buffer, boiled for 5 min, and resolved on either 4, 6, or 10% polyacrylamide gels. High molecular weight protein markers were purchased from Bio Rad Labs (Hercules, CA.) or Amersham (Arlington Hts, IL.). Staining of protein gels was carried out in 0.25% coumassie blue in 10% methanol/acetic acid for1-2 min followed by a 30 min destain in 10% methanol/acetic acid.

Protein gels prepared for Western blotting were electroblotted to nitrocellulose membrane (Bio Rad Labs, Richmond, CA.) according to the procedure of Towbin, H.S. et al. (1987). The nitrocellulose membranes were then blocked in 3% nonfat milk in Tris Buffered Saline and 0.1% Tween-20 (TBST) for 30-60 min. Membranes were then incubated for 1 h at room temperature in the appropriately diluted antibody in TBST. Unbound antibody was removed by three 10 min washes in TBST. Subsequently, the membranes were incubated in a 1:2000 dilution of horse radish peroxidase conjugated goat anti-rabbit IgG secondary antibody (Zymed Labs, South San Francisco, CA.) for 1 h at room temperature. Membranes were then washed three times 10 min each in TBST and immunoreactive band were detected on Kodak X-OMAT MR film using enhanced chemiluminescence (ECL) (Amersham) following the procedure recommended by the manufacturer.

Antibody library screening

A single bacterial colony of Y1090 was grown overnight in 10 ml of LB or NZY media supplemented with 0.2% maltose and 10 mM $MgSO_4$ at 37°C. Bacteriophage λgt11 from either a human breast carcinoma cDNA library or a HeLa cDNA library (Clontech, Palo Alto, CA.) were diluted to 10^3 pfu/μl in lambda dilution buffer (LDB, 100 mM NaCl, 10 mM $MgSO_4 \cdot 7H_2O$, 35 mM Tris-HCl pH 7.5). Two to three μl of the diluted phage (final titer of 50000 pfu/150 mm plate) were then mixed with 100μl of LDB and 600μl of y1090 bacterial culture at 37°C for 15-20 min. During this incubation, 0.7% LB or NZY top agarose was melted on a heat bock and cooled to ~ 40°C. Seven to eight ml of top agarose was quickly added to each tube of diluted phage and bacteria and poured evenly on individual 150mm LB or NZY bacterial plates. After 10 plates had been poured they were inverted and incubated at 42°C for 3 h or just until pin prick plaque lysis could be observed. During this incubation, circular nitrocellulose filters (Schleicher & Schuell, Keene, NH.) were incubated in 10mM IPTG (isoproplythio-β-galactoside, GIBCO BRL, Burlington Ont.) for 10 min in the dark and air dried. When plaques were observed, one nitrocellulose circle was laid evenly on the surface of each plate and further incubated for 4 h at 37°C right side up. Following this incubation, the circles were marked asymmetrically with needle holes and lifted carefully from the surface of the lysed bacterial plate. The filters were washed for 5-10 min in TBST and then blocked for 1 h in 3% milk-TBST at room temperature. The filters were then briefly rinsed in TBST and incubated in a sealed plastic bag with a 1:1000 dilution of the appropriate human autoimmune serum in TBST-0.5% sodium azide for 1 h while gently rocking. The filters were then washed three times

for 10 min each in TBST, and then further incubated in a sealed bag with a 1:2000 dilution of horseradish peroxidise conjugated goat anti-human secondary antibody (Zymed Labs) for 1 h at room temperature. Unbound antibody was removed with a final wash three times for 10 min each in TBST.

Reactive plaques were detected by incubating the filters two at a time for 1 min in ECL solution in a 150 mm Petrie dish in the dark. When all the filters had been incubated a 2-5 min exposure to Kodax X-OMAT film was carried out. Alignment of the filter holes on film with the holes in the bacterial plate allowed for the identification and removal of reactive plaques into 1 ml of LDB containing two drops of chloroform.

Secondary and tertiary screens were carried out as above except that the dilution of the phage was empirically determined and 200µl of Y1090 cells were used for each 82 mm bacterial plate. The resulting pure phage clones were removed into 500µl LDB containing no chloroform. Each library screen with a human serum was carried out on a minimum of 1 million bacteriophage.

Polymerase Chain Reaction (PCR)

All PCR amplifications were carried out in a standard 20 µl reaction mix consisting of the following components: PCR buffer (20 mM Tris-HCl (pH 8.4), 50 mM KCl), 2 mM $MgCl_2$, 0.2 mM each dGTP, dATP, dTTP, dCTP, 0.5 µM each primer, 1µl of a pure λ phage clone solution or 1-10 ng of DNA template, 2.5 units Taq DNA Polymerase (GIBCO BRL or LABQUIP LTD, Woodbridge, Ont.). Cycling was carried out in a Perkin Elmer 2400 Geneamp or Ericomp power block I/II system. A typical reaction consisted of 30 cycles with the following parameters: 94°C for 20 sec, 65 °C for 30 sec, 72 °C for 2 min.

This followed by a final 7 min extension at 72°C. High fidelity PCR using cloned PFU DNA polymerase (Stratagene, La Jolla CA.) was carried out according to the manufacturers recommendations in a total volume of 20 µl per reaction tube. Long PCR products were amplified using the expand long template PCR system from Boehringer Mannheim (Laval, Que) according to the manufacturers suggestions. All annealing temperatures and extension times were dependent on the primer Tm and template length respectively.

PCR amplification of the complete open reading frame of desired cDNA was carried out using human placenta and HeLa Marathon-Ready cDNA (Clontech) according to the manufacturers recommendations.

Agarose gel electrophoresis

DNA fragments were resolved on 0.5-1% agarose gels in TAE (40 mM Tris-Acetate, 2mM EDTA pH 8.5) with 10ng/ml ethidium bromide. DNA ladders (1 kb and 100bp) were purchased from GIBCO BRL and used according to the manufacturers instructions.

Restriction enzyme digestion

All restriction enzymes were purchased from the following companies: Boehringer-Mannheim, GIBCO BRL, Pharmacia and Promega. DNA digestion was carried out according to the manufacturers instructions.

DNA fragment isolation and purification

DNA fragments were isolated on agarose gels using one of the following procedures: DNA fragments of interest were electrophoresed on to DEAE paper (Schleicher & Schuell, Keene, NH.) and recovered according to the manufacturers

recommendations. Alternatively, DNA fragments were excised from the agarose gel and purified using the QIAquick gel extraction kit (Qiagen, Mississauga Ont.). All DNA solution were quantitated in GeneQuant DNA/RNA calculator (Pharmacia, Que.).

Preparation of competent bacterial cells

E. coli strain JM109 was streaked on a Luria-Bertani medium (LB) plate and incubated at 37°C overnight. One colony was used to inoculate 5 ml of LB media shaking overnight at 37°C. This culture was then used to innoculate 100 ml of LB and shaken at 37°C until an OD_{600} of 0.4-0.6 was reached. The cells were then chilled for 10 min on ice, and then dispensed into two 50 ml tubes and pelleted for 5 min at 4000 x g at 4°C. The pellet was resuspended in 50 ml of 0.1M $MgCl_2$ and incubated for 20 min on ice. Cells were pelleted as before, resuspended in 5 ml 0.1M $CaCl_2$ and incubated at 4°C for 1 h on ice. Sterile 80% glycerol was added to the cells to a final concentration of 15% and 100 µl aliquots were frozen in liquid nitrogen and then stored at -70°C.

DNA ligation

1. Cohesive ligation. 10ng of the appropriate linearized vector DNA was mixed with a three fold molar excess of insert DNA, 2 µl of 5X DNA ligase reaction buffer (250 mM Tis-HCl (pH 7.6), 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, and 25% (w/v polyethylene glycol-8000), 1 U of T4 DNA ligase (GIBCO BRL), and ddH_2O to 10 µl. The reaction was incubated at 14°C overnight.

2. Blunt ligation. 50-100ng of the appropriate linearized vector DNA was mixed with a 3-10 fold molar excess of insert DNA, 1ul of 5X DNA ligase reaction buffer minus ATP, 0.5 mM ATP, 1U T4 DNA ligase (GIBCO BRL), and ddH_2O to 5 µl. The reaction

was incubated at room temperature overnight. The reaction was then supplemented with 1 µl of 5X ligase reaction buffer minus ATP, 0.5 µl 10 mM ATP, 1 µl T4 DNA ligase and 2.5 µl ddH_2O, and further incubated at room temperature for 2-3 h. Alternatively, the rapid DNA ligation kit (Boehringer Mannheim) was used for blunt end ligations according to the manufacturers recommendations, except that all reaction volumes were 10 µl.

3. **PCR product cloning.** Ten micrograms of the vector pGEMT (Promega, Madison WI.) was mixed with a 3 fold molar ratio of the purified PCR product and ligated at 16°C according to the manufacturers recomodations.

Transformation

The 10 µl ligation reaction was mixed with 100 µl of competent bacteria and incubated on ice for 20 min. The cells were then heat shocked at 42°C for 90 sec, chilled on ice, and 1 ml of LB was immediately added. The cells were then rotated at 37°C for 1 h, followed by centrifugation at 3000 x g in a table top centrifuge to pellet the cells. The supernatant was decanted, and the cells resuspended in 200 µl of LB. The resuspended bacteria were then spread on the appropriate antibiotic supplemented LB plate and incubated at 37°C overnight.

Identification of recombinant clones

Bacterial colonies containing the recombinant vector were determined by using PCR and either gene specific or vector primers. Briefly, colonies were picked off the plate with a pipette tip and spotted on a master plate. The tip was then washed of bacteria in 10 µl of ddH_2O. Each 10 µl sample was then boiled for 10 min, chilled on ice and then centrifuged at 6000 x g for 1 min. 2 µl of the liquid was then use for PCR. Alternatively,

each bacterial colony was picked off the plate with a tip and resuspended in 10 µl of ddH_2O. 2 µl of this solution was then used directly for PCR. Positive samples were then used to innoculate 5-10 ml LB antibiotic cultures.

Plasmid DNA preparations

One to five ml of a overnight culture of the appropriate bacterial culture was harvested of plasmid using the alkali lysis procedure described in (Sambrook, J. et al. 1989). Alternatively, plasmid DNA was prepared using the QIAprep spin miniprep kit (Qiagen, Mississauga Ont.) according to the manufacturers recommendations.

DNA sequencing

PCR fragments and plasmid DNA were sequenced using the dsDNA cycle sequencing system from GIBCO BRL. All reactions were carried out according to the manufacturer except that the radioisotope γ ^{33}P-ATP (Dupont) was substitute in place of γ ^{32}P-ATP. Five microliters of each sequencing reaction (G, A, T, C) was loaded into a separate well and resolved on a 6 % polyacrylamide sequencing gel. The DNA sequence was manually read from an overnight Kodak X-OMAT film exposure of the gel.

Additional DNA sequence was determined using the dye terminator cycle sequencing ready reaction kit (Applied Biosystems) according to the manufacturers recommendations, except that 10 µl reactions were preformed. Reactions were analysed by the University of Calgary DNA sequencing facility.

Recombinant protein production

Small scale protein production. DNA fragments to be expressed were cloned into the appropriate pGEX expression vector (Pharmacia) in the correct reading frame, and

transformed into the *E. coli* strain JM109. A single colony of transformed bacteria was used to innoculate 2 ml of 2XYT media plus 100μg/ml ampicillin overnight at 37°C. Forty microliters of this culture was used to innoculate 2 ml of 2XYT media plus ampicillin and was shaken in a bacterial culture shaker at 37°C for 3-4 h. GST (glutathione S transferase) fusion protein was induced by adding IPTG to final concentration of 0.1 mM with continued shaking at 37°C for 2 h. One ml of this culture was centrifuged at 6000 x g to pellet the bacterial cells, and the supernatant discarded. The bacterial pellet was resuspended in one hundred microliters of SDS sample buffer, boiled for 5 min, and 5 μl was resolved on a 10 % SDS PAGE gel.

Large scale protein production. Ten ml of an overnight culture of the appropriate bacteria was used to innoculate 500 ml of sterile 2XYT plus 100μg/ml ampicillin in a 1000 ml fluted flask shaking vigorously at 37°C. The culture was grown to an OD_{600} of 0.6-0.7 and IPTG was added to a final concentration of 0.1 mM, followed by continues shaking at 37°C for an additional 2 h. The GST fusion protein was harvested from the bacteria according to the batch isolation procedure (Pharacia) recommended by the manufacturer.

Fusion protein amounts were determined using the Bio Rad colormetric protein assay reagent (Bio Rad Labs) according to the manufacturer.

Antibody production

Antibodies to GST fusion proteins were produced in rabbits using one of two methods: First, for insoluble fusion proteins, total bacterial protein was separated out on 10% SDS PAGE and stained for 10 min in 0.05 % coumassie blue in water, followed by

destaining in water. The fusion protein was located and excised from the gel with a scalpel. The gel was frozen in liquid nitrogen and ground into powder with a mortar and pestle. The powder was resuspended in DPBS and approximately 100 µg of the fusion protein was mixed with an equal volume of complete Freund's adjuvant (DIFCO, Detroit, MI.) and injected subcutaneously into a New Zealand white rabbit. Three weeks later the rabbit was test bled and boosted with 100µgs of fusion protein in incomplete Freund's adjuvant (DIFCO, Detroit, MI.), followed by subsequent boosts/bleeds every three weeks until the appropriate titer of antibody was achieved. Second, soluble fusion proteins were quantitated and 100-500µgs was mixed with SDS to a final concentration of 0.5 % and boiled for 5 min to denature the protein. The protein was then mixed with Freund's complete adjuvant (DIFCO) and injected as previously described. All animal bleeds and injections were carried out by the University of Calgary vivarium staff or the Southern Alberta Cancer Research Hybridoma facility staff.

Antibody affinity purification

For insoluble fusion proteins, a 500 ml induced bacterial culture was centrifuged at 6000x g for 10 min at 4°C. The bacterial pellet was resuspended in 4.5 ml DPBS and 0.5 ml 10mg/ml lysozyme (SIGMA) in 25 mM Tris-HCl (pH 8.0), after an 5-10 min incubation the bacteria were frozen in dry ice and thawed at 55°C. The volume was brought to 20 ml with DPBS and $NaCl_2$ and DTT was added to final concentration of 0.5 M and 5 mM respectively. The cells were lysed by sonication with a large probe four times for 30 sec each. Insoluble material was collected by centrifugation at 12000 x g for 20 min and resuspended in 1 ml 2 X SDS sample buffer minus β-mercaptoethanol and bromophenol

blue, followed by the addition of 4 ml 1 x SDS sample buffer minus β-mercaptoethanol and bromophenol blue. The insoluble protein was dissolved by boiling for 30 min with vigorous vortexing.

For soluble proteins, the bacterial pellet from a 500 ml culture was resuspended in 25 ml DPBS and sonicated to lyse the cells. Insoluble material was removed by centrifugation at 12000x g for 10 min. The soluble fraction was then collected and the fusion protein was purified with glutathione sepharose 4b beads (Pharmacia) according to the manufacturer.

The protein sample, (insoluble or soluble) was then dialysed against 100 mM MOPS (3-[N-Morpholino] propanesulfonic acid) (pH 7.0) overnight with two buffer changes at room temperature (insoluble proteins) or 4°C (soluble proteins).

The dialysed sample was then coupled to 1 ml of Affigel 10 beads (Bio Rad Labs) according to the manufacturer in a total volume of 5 ml overnight at room temperature. Protein coupling efficiency was determined by SDS PAGE.

Four ml of rabbit serum plus 0.5 % sodium azide was preincubated with control bacterial GST protein coupled beads for 2-3 h to remove antibacterial protein and GST antibodies. This precleared serum was then incubated with the fusion protein coupled beads in a 10 ml econo column (Bio Rad Labs) for 2-4 h at room temperature. The beads were then washed with 25 bed volumes of TBS. Antibody was eluted by passing 15 ml of elution buffer (5 % acetic acid. 0.15M $NaCl_2$) over the column and collecting 1 ml fractions into 150 µl of 1 M Tris pH 9.0. The absorbency of each fraction was then read at OD_{280}, and all protein containing fractions were pooled and dialysed against DPBS

overnight.

The dialysed antibody was then concentrated in centricon 30 (AMICON, Oakville, Ont.) at 5000 x g for 30 min until a final volume of 100-200 µl was achieved.

DNA hybridization screening

Lawns of the bacterial strain y1090 were infected with λ phage from an appropriate cDNA library to a density of 50,000 pfu per plate. After a 3-4 h incubation at 37°C a single positively charged nylon membrane (Hybond-N$^+$, Amersham) was placed on the surface of each plate for one minute, asymmetrically marked, and then denatured/renatured according to the manufacturer. DNA was fixed on the membrane by oven baking at 80°C for 2 h. Probe hybridization and washing was carried out exactly as described in the Hybond-N$^+$ instruction manual. Alternatively, Northern blots were prehybridized and hybridized in Rapid-Hyb buffer (Amersham) according to the manufacturer.

The membranes were exposed to Kodak X-OMAT MS film at -70°C for 1-24 h. Positive plaques were removed and reinfected until pure plaques were obtained. A total of 1 million plaques were screened for each probe.

Exonuclease III deletions for DNA sequencing

Exonuclease III digestion was carried out using a protocol modified from that reported in Ausubel, F.M. et al. (1994). Briefly, DNA fragments to be sequenced were cloned into pBluescript (Stratagene) in both orientations and linearized with the appropriate restriction enzymes that leave a 3' overhang adjacent to vector DNA (sequencing primer site), and a 5' overhang adjacent to the insert. DNA (2.5 µg) was diluted into 1 X exonuclease III buffer (50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$) to give a

final volume of 25 µl. This DNA was incubated at 30°C for 1 min, followed by the addition of 150 U of exonuclease III (GIBCO BRL) per pmol susceptible ends. Three microliters was removed at 1 min intervals into 14.75 µl ddH_2O, 2 µl 10 X mung bean buffer (100 mM sodium acetate (pH 5.0), 1 mM zinc acetate, 500 mM NaCl, 10 mM L-cysteine, 50 % glycerol), and immediately frozen on dry ice. When all time points had been taken, the tubes were incubated at 70°C for 10 min. The samples were then chilled on ice, centrifuged, and 9.5 U (0.25 µl) mung bean nuclease (GIBCO BRL) added to each tube. This was followed by a 30 min incubation at 30°C. Five microliters was removed and run an a 1% agarose gel to resolve the deletions

The deletions were then precipitated with 1/10 volume Sodium acetate (pH 5.0) and 2.5 volumes ice cold 95% ethanol at -70°C for 2-3 h. Pelleted DNA was then resuspended in water, ligated and transformed.

Preparation of ^{32}P DNA probes for plaque hybridizations

DNA fragments to be used as probes were labelled with ^{32}P-dCTP (10 mCi/ml, ICN, Costa Mesa CA.) using the random primer labelling kit (Stratagene) according to the manufacturer. The probe was purified from free nucleotides using microspin G-50 columns (Pharmacia).

Preparation of ^{32}P DNA probes for Northern blot hybridizations

High specific activity probes for Northern blot hybridizations were produced by PCR. The following reagents were mixed on ice: 1-5 ng template DNA, 100 ng 5' primer, 100 ng 3' primer, 1 X Taq buffer, 25 µM d(AGT)TPs, 50µCi α ^{32}P-dCTP, and 1 U Taq Polymerase to a total volume of 10 µl and PCR carried out for 30 cycles. Alternatively,

radioactively labelled probes were produced using the random primer labelling kit (GIBCO BRL) as described by the manufacturer. Each probe was purified from free nucleotides using microspin G-50 columns (Pharmacia). Following this procedure, each probe was further purified using standard DNA precipitation techniques (Sambrook, J. et al. 1989).

Northern blot hybridization

Northern hybridizations were performed on a human multiple tissue blot (Clontech) using Rapid-hyb buffer (Amersham) according to manufacturers instructions. A rotating hybridization oven (Micro- 4, Labnet) was used to incubate the membranes at 65 °C for the desired time. Following hybridization the membranes were washed as described in the Hybond-N$^+$ instruction manual. The membrane was wrapped in saran wrap or sealed in a plastic bag and exposed to a phosphoimager screen (Molecular Dynamics, Sunnyvale CA.). for 1h to several days at room temperature. Images were collected using a STORM 860 phosphoimager and ImageQuant software (Molecular Dynamics, CA.).

Transfections

HeLa cell seed coverslips (20-50% confluent) were transfected with 1-5μgs of control or recombinant pEGFP plasmid (Clontech) and 5-10 μl of lipofectin (GIBCO BRL) for 5 h in serum free Opti-MEM (GIBCO BRL) at 37°C according to the manufacturer. Cells expressing GFP for 24-72 h were then processed for immunofluorescence as described above.

Immunoprecipitation

HeLa cells were grown to 50-70% confluency in T75 tissue culture flasks. For mitotic cell collection, colcemid (GIBCO BRL) was added to 10 ml of media to a final

concentration of 0.1µg/ml, and incubated overnight. Mitotic cells were collected by shake off and interphase cells were collected by trypsin detachment. The cells were pelleted and washed once in ice cold DPBS. The pelleted cells were then resuspended in 1ml NP-40 lysis buffer (1% NP-40, 100 mM NaCl, 50 mM Tris (pH 8.0)), with 10µg/ml leupeptin, antipain, chymostatin, pepstain and 10 µl of a mammalian protease inhibitor cocktail (SIGMA), for 10 min on ice. The suspension was then centrifuged at 14000 x g for 10 in at 4°C. Five micrograms of antibody was added to the supernatant and it was rotated at 4°C overnight. Protein A agarose (Calbiochem, San Diego CA.) was added, and further rotated for 3 h. The agarose was pelleted at 500 x g and washed three times in NP-40 lysis buffer. SDS sample buffer was added, and boiled for 5 min.

SECTION III

Characterization of Human Autoimmune Sera that Recognize the Centrosome

Portions of this section have been published in Arthr. Rheum. 41:551-558. 1998

INTRODUCTION

Human autoimmune sera reactive with the centrosome have provided the only consistent means to investigate centrosome composition in higher eukaryotes. Known centrosomal autoantigens include the proteins: pericentriolar material 1 (PCM-1), pericentrin, γγ-enolase and NuMA. Of these autoantigens only PCM-1 and pericentrin are exclusively found at the centrosome. Additional proteins that are involved in the organization and function of the centrosome remain to be identified. Their characterization is necessary for a complete understanding of the centrosome and its role during the cell cycle.

Studies of human sera suggest that anti-centrosome antibodies are rare, and have been found to occur in ~ 0.1% of female Red Cross blood donor sera and in less than 0.5 % of sera from scleroderma patients (Fritzler, M.J. et al. 1985, Rattner, J.B. et al. 1996a). Information regarding antibody specificity or clinical correlations of centrosome autoantibodies are fragmentary. Therefore, I have undertaken a study of 21 human sera that recognize the centrosome by indirect immunofluorescence, in order to more completely address the nature of the autoimmune response to the centrosome. I found that most patients produce autoantibodies to multiple centrosome proteins, and autoantibodies to pericentrin, ninein and Cep250 occur more frequently than autoantibodies to PCM-1. Furthermore, this work has also led to the identification of two novel centrosome proteins, and the finding that the human homologs of the mouse proteins ninein and Cep76 are human centrosome autoantigens. Retrospective chart review revealed no correlation

between having autoantibodies to the centrosome and specific rheumatic diseases.

METHODS AND MATERIALS

Human sera and clinical information

Twenty one centrosome reactive sera were identified from a bank of 25,000 autoimmune sera collected for testing at the Advanced Diagnostic Laboratory at the University of Calgary. Clinical information was obtained by retrospective chart review.

Recombinant protein production

A 1.4 kb partial cDNA clone for the human centrosomal protein ninein was cloned into the expression vector pGEX-5X1. This encoded protein has significant identity with amino acids 32-506 of the mouse ninein protein (Mack, G.J. et al. 1998). A 1.7 kb partial cDNA clone for human pericentrin representing amino acids 142-592 of mouse pericentrin was cloned into pGEX-5X1. A 1.6 kb partial cDNA clone (17A1) encoding the COOH terminus of PCM-1 was provided to us in the expression vector pMAL-c2 by Dr. R. Balczon (Dept. of Structural and Cellular Biology, Mobile, AL.). Two partial cDNA clones for Cep250 (SM 8 and SM1) spanning amino acids 1065-1841 were cloned into pGEX-5X1.

The expression and purification of recombinant protein was carried out according to the manufacturers recommendations.

Western blot analysis

Purified fusion proteins for ninein, pericentrin, PCM-1 and Cep250 were separated on 10% SDS-PAGE and transferred to nitrocellulose as described in the GENERAL METHODS AND MATERIALS section of this dissertation. Each blot was cut into strips

and incubated with a 1:1000 dilution of the appropriate human serum. Immunoreactive bands were detected using the ECL system.

RESULTS

Expression library screening

A prototype centrosome reactive serum (patient 11, Table 5.0) was chosen to screen a HeLa cDNA expression library because of high titer reactivity to the centrosome throughout the cell cycle (Fig. 3.1). From this screen 18 reactive clones were identified. DNA sequence analysis of these partial clones allowed them to be grouped into four categories (Table 6.0). The first category consisted of seven clones that encode the human homolog of the mouse centrosomal protein pericentrin. The second category consisted of a single clone representing the human homolog of the mouse centrosomal protein ninein. The third category of four clones (SM 1, 8, 12, 23) were found to encode for a protein with no homology in the GenBank database. Subsequent work has shown these clones encode a centrosomal protein designated Cep250 (Centrosome protein 250, see SECTION V A). Finally, the fourth category consisted of six known clones that were determined not to be significant in this study, as they have been shown not to localize to the centrosome.

Centrosomal reactive autoimmune sera have autoantibodies to multiple centrosome proteins

The finding that our prototype anti-centrosome sera has antibodies to multiple centrosome proteins prompted us to investigate whether other centrosome reactive autoimmune sera also recognize multiple centrosomal proteins. Western blot analysis against partial fusion proteins of the centrosomal antigens ninein, pericentrin, PCM-1 and Cep250 was performed with 21 human anti-centrosome reactive autoimmune sera.

Table 5.0. Clinical features of patients whose sera recognize centrosomal autoantigens. Twenty one patients with autoantibodies to the centrosome, were scored for reactivity against four centrosomal antigens. No correlation between autoantibodies to the centrosome and a specific clinical feature were detected. SS = Sjögrens's syndrome; OA = osteoarthritis; MS = multiple sclerosis; RP = Raynaud's phenomenon; TB = tuberculosis; SLE = systemic lupus erythematosus; RA = rheumatoid arthritis.

Patient/age/sex	Diagnosis	Ninein	Pericentrin	PCM-1	Cep250
1/67/F	rash, SS, OA	+	+	-	+
2/48/F	transverse myletis, MS	+	+-	+	+
3/37/F	RP, pituitary tumor, livedo reticularis, photosensitivity	+	+	+	+
4/72/M	inactive TB, viral infection	-	+	-	+
5/28/F	Lofgren's syndrome, SS	+	+	-	-
6/38/M	myositis	-	+	-	-
7/46/F	RP, fibromyalgia, malar rash	++	-	-	+
8/41/F	alopecia, SS	+	-	++	+
9/52/F	RP, fibromyalgia, family history SLE, RA, and MS	+	+	-	-
10/23/F	arthritis	-	+	-	+
11/37/F	arthritis, RP	+++	+++	+	+++
12/38/F	arthritis	+	+	+	+
13/40/F	arthritis	+	+	-	+
14/51/F	arthritis	+	+	-	+
15/52/M	arthritis, rash, weakness	+-	+	-	+
16/67/F	arthritis, rash	+	+	-	+
17/78/F	arthritis	+	+	+-	+
18/1/M	postinfectious ataxia	+	+	+	-
19/6/M	postinfectious ataxia	+	+	-	+
20/7/M	postinfectious ataxia	++	+	+	+
21/15/M	postinfectious ataxia	-	+	-	+

Figure 3.1. Immunofluorescent staining of cells using human autoimmune serum SM 2555. HeLa cells were doubled stained with anti-β-tubulin (B, E, H, K) and serum SM 2555 (C, F, I, L), then counter stained for DNA with DAPI (A, D, G, J). An interphase cell (A) shows two dots representing the duplicated centrosomes. Prophase cell (F) shows bright centrosome staining at each centrosome while aster microtubules form (E). A cell in metaphase with a bipolar spindle (H), and intense spindle pole staining (I), note the high cytoplasmic staining with SM 2555. Telophase cell (K) showing microtubules of the intercellular bridge. Single centrosome staining is observed within each daughter cell (L). Bar = 10μm.

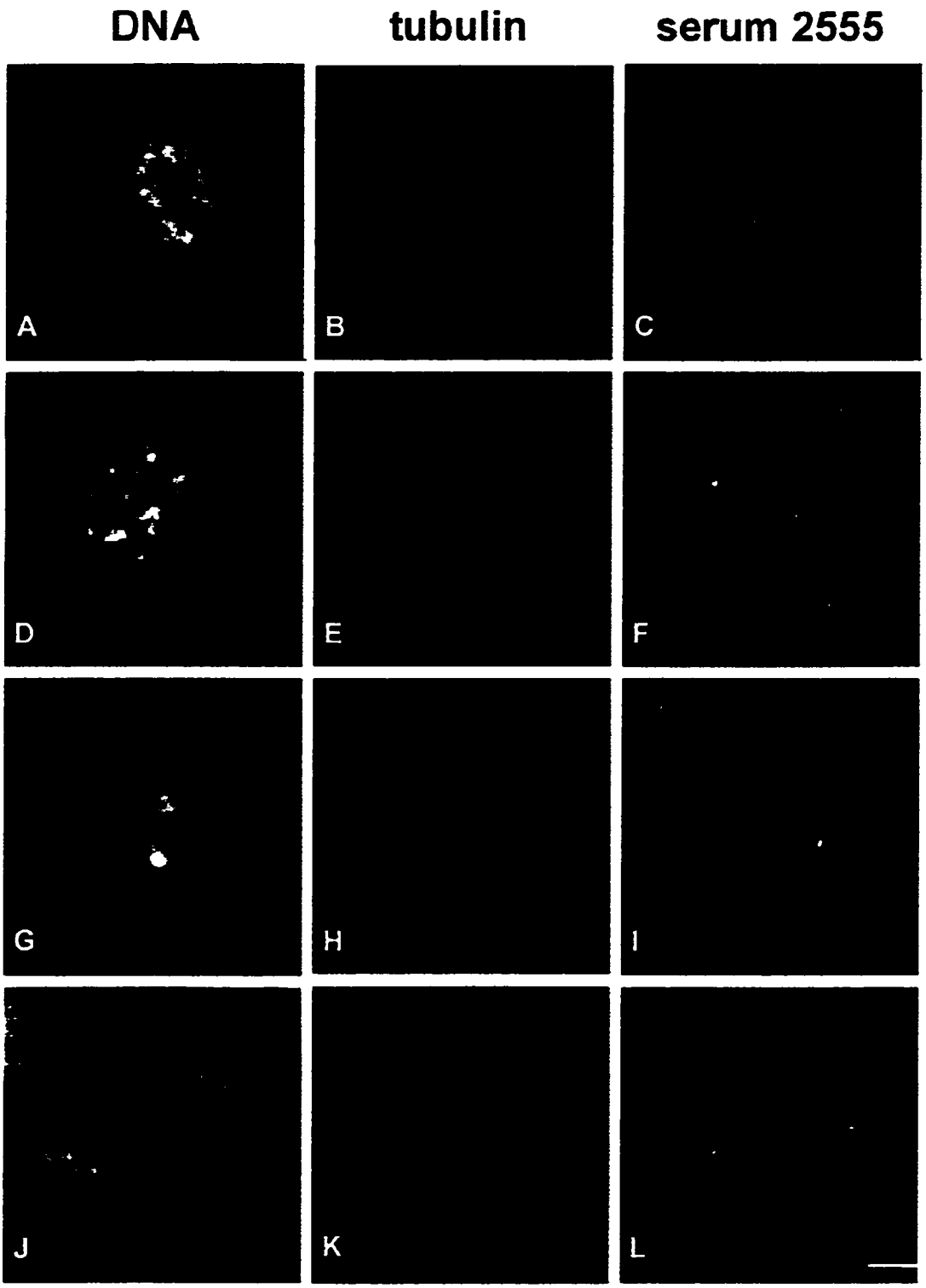

DNA
tubulin
serum 2555
A
B
C
D
E
F
G
H
I
J
K
L

Table 6.0. cDNA clones identified with a prototype anti-centrosome serum. Four categories of cDNA clones that were identified from a HeLa cDNA expression library immunoscreened with a human serum (SM 2555, patient 11) that recognizes the centrosome.

Category	Number of cDNA clones identified	Characterization
1	7	Homolog of the mouse centrosomal protein pericentrin
2	1	Homolog of the mouse centrosomal protein ninein
3	4	Novel human centrosomal protein Cep250
4	6	Not significant to this study

Western blot results obtained for a representative sample of these sera is shown in Figure 3.2. Autoantibody reactivity to pericentin, ninein, Cep250 and PCM-1 was not determined at different dilutions. Therfore, no information is known about a correlation between centrosome antigen reactivity and serum titer.

When reactivity to the different centrosomal fusion proteins is considered, (Table 7.0), 19 of 21 (90%) reacted with pericentrin, 17 of 21 (81%) reacted with ninein and Cep250, and 8 of 21 (38%) reacted with PCM-1. When these results are scored for reactivity to multiple antigens, 13 of 21 (62%) were found to react with at least three of these antigens, whereas 6 of 21 (29%) reacted with all four of the antigens. These values may be underestimated since only partial regions of each protein antigen were used to test for autoantibody reactivity.

To determine if the complexity of the autoimmune response seen in anti-centrosome sera was established early or developed over time, sera from our prototype patient collected over a ten year period was tested against the four centrosomal antigens. Western blot analysis showed that each of the serum samples reacted with all four of the fusion proteins (data not shown). This suggests that in some individuals, the complexity of the autoantibody response to the centrosome was established early and persists throughout the clinical course.

Clinical data on anti-centrosome reactive patients

Retrospective chart review of all 21 patients with centrosome reactive sera (Table 5.0) revealed no correlation between serum autoantibody reactivity and the clinical diagnosis. The most common diagnosis was symmetric polyarthritis (8 of 21 [38%]). Four

Figure 3.2. Representative Western blot of centrosomal autoantigens. Recombinant protein to PCM-1 (lanes 1-6), Cep250 (lanes 7-12), pericentrin (lanes 13-18), and ninein (lanes 19-24). Patient serum 11 (lanes 1, 7, 13, and 19), a normal healthy control (lanes 2, 8, 14, and 20), patient 6 (lanes 3, 9, 15, and 21), patient 10 (lanes 4, 10, 16, and 22), patient 17 (lanes 5, 11, 17, and 23), and patient 18 (lanes 6, 12, 18, 24). Multiple protein bands in the pericentrin and ninein blots are breakdown products of the fusion proteins. All fusion proteins were separated on 10% SDS-PAGE, and each serum sample was diluted 1:1000.

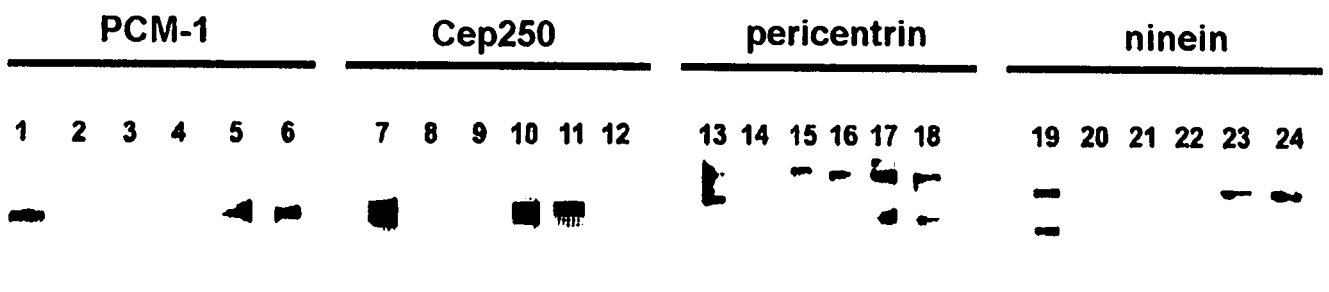

PCM-1
Cep250
pericentrin
ninein
1 2 3 4 5 6
7 8 9 10 11 12
13 14 15 16 17 18
19 20 21 22 23 24

Table 7.0. Autoimmune sera reactivity with centrosomal antigens. The frequency of autoantibody reactivity to partial fusion proteins of four known centrosomal autoantigens, Ninein, Pericentrin, PCM-1 and Cep250.

Reactivity of autoimmune sera with centrosomal antigens			
Ninein	Pericentrin	PCM -1	Cep250
17/21	19/21	8/21	17/21
81%	90%	38%	81%

patients had Raynaud's phenomenon or skin rash, three patients had xerosomia and xerophthalmia and a provisional diagnosis of Sjögren's syndrome. Four children were found to have post infectious (*Mycoplasma pneumoniae)* ataxia. In this study, 14 patients were female and seven male, with an average age of 40 years (range 1-78).

DISCUSSION

Characterization of a prototype anti-centrosome autoimmune serum

Using a high titer autoimmune serum reactive with the centrosome to screen a cDNA expression library, we have identified a family of centrosomal proteins that act as autoantigens, two of these proteins, ninein and Cep250, were not previously know as human autoantigens.

The majority of the centrosomal clones (7/12) identified with this prototype serum encode for portions of the human centrosomal protein pericentrin. This protein was identified from a mouse B cell cDNA library using an autoimmune sera from a scleroderma patient (Doxsey, S.J. et al. 1994). Pericentrin is a large 220 kDa protein with a long central coiled-coil segment flanked by non-coiled domains (Doxsey, S.J. et al. 1994). Autoepitope mapping of pericentrin has not been investigated, however, it is interesting to note that the single cDNA clone identified from the mouse library encodes a portion of the central coiled-coil region. The seven human pericentrin cDNA clones identified from this study all encode portions of the NH_2-terminal region with non-coiled and coiled-coil domains. The difference in the location of these two library fusion proteins suggests that pericentrin may have multiple autoepitopes. That the majority of the cDNA clones identified in this study are human pericentrin is unusual. Previous work on pericentrin has indicted that its mRNA transcript is rare in mouse culture cells and tissue (Doxsey, S.J. et al. 1994). This is further reflected by the fact that only one cDNA clone

encoding a portion of pericentrin was identified from a mouse library (Doxsey, S.J. et al. 1994). It is possible that our cDNA library (HeLa) was more efficiently constructed than the mouse B cell library, resulting in more in-frame pericentrin inserts. Alternatively, pericentrin message may be more abundantly expressed in human cancer cells. Since this library was made from a human cancer cell line, it could possibly have an increased representation of pericentrin cDNA.

This work is the first to report the human homolog of the mouse centrosomal protein ninein, and that this protein is an antigen in human autoimmune disease. Ninein is an alternatively spliced protein of 245 and 249 kDa that contains a central coiled-coil segment flanked by non-coiled ends (Bouckson-Castaing, V. et al. 1996). One cDNA clone encoding human ninein was identified in this study, and it encodes a portion of the NH_2-terminal section of ninein containing non-coiled and coiled-coil domains as well as a predicted calcium binding region and four GTP-binding domains. As only one clone of ninein was identified, no further information about the presence of other autoepitopes in ninein could be determined. Northern blot data indicates that ninein is encoded by two transcripts and is relatively abundant as compared to actin levels in mouse tissue (Bouckson-Castaing, V. et al. 1996). Since only one cDNA clone for ninein was identified in this work, it is possible that the ninein message is expressed at low levels in HeLa cells, resulting in an under representation of these clones in the library. Alternatively, the method used for immunoscreening was not sensitive or complete enough to identify additional ninein clones. Recombinant antibody raised against this human ninein fusion protein reacts with the centrosome throughout the cell cycle, in agreement with what is

reported for mouse ninein (Bouckson-Castaing, V. et al. 1996).

Of the centrosomal cDNA clones identified in this study, clones encoding portions of the centrosomal protein Cep250 are the second most abundant (4/12). The clones, SM 12 and 23 are identical and encode for amino acids 976-1706 of Cep250. Clone SM 8 encodes for amino acids 1066-1623, and clone SM1 encodes for amino acids 1443-1840 of Cep250 (see SECTION V A). Interestingly, two additional clones (283 and 39) were identified with a second autoimmune serum, and encode for amino acids 560-1433 of Cep250 (see SECTION V B). Although precise autoepitope mapping for Cep250 has not been investigated, it is likely that two or more autoepitopes exist between amino acids 560 and 1840. The majority of this region of Cep250 is predicted to adopt a coiled-coil conformation (see SECTION V A). Northern blot analysis of Cep250 from HeLa cells has been unsuccessful, and is likely due to the rarity of this mRNA transcript in tissue culture cells (data not shown). The fact that several clones for Cep250 were identified in this study might reflect the presence of multiple autoepitopes throughout the protein, increasing the chances that Cep250 inserts will be identified. The presence of autoepitopes at the extreme NH_2 or COOH terminus of Cep250 has not been investigated.

Finally, six of the 18 reactive clones identified with the prototype sera were shown by DNA sequencing to encode proteins that are not localized to the centrosome. These include the proteins alkaline phosphatase and glycoprotein 3A. It is possible that this autoimmune serum contains specific autoantibodies to these proteins. Alternatively, these proteins may contain epitopes that are similar to centrosomal antigens. Precedence for this suggestion have been found in one cDNA clone identified while immunoscreening for the

fibrillarin autoantigen. This clones was not fibrillarin, yet it encoded a short polypeptide with fibrillarin autoepitopes (Dr. M. Pollard, Scripps Research Institute, personal communication).

Centrosome reactive autoimmune sera have autoantibodies to multiple centosome antigens.

Since our prototype anticentrosome sera reacts with multiple centrosome proteins, we reasoned that this may be a common feature of all centrosome reactive autoimmune sera. To investigate this, we tested 21 human autoimmune sera reactive with the centrosome against four centrosomal antigens, pericentrin, ninein, Cep250 and PCM-1 using immunoblot analysis. The protein PCM-1 was included in this study as it also represents an additional centrosomal antigen (Balczon, R. et al. 1994). PCM-1 is a 228 kDa protein that was identified from a human fetal liver library using an anticentrosome sera from a patient with systemic sclerosis and Raynaud's phenomenon (Balczon, R. et al. 1991, Balczon, R. et al. 1994).

When reactivity against these four proteins is analysed, 80-90% of the patients are seen to have autoantibodies directed against pericentrin, ninein, and Cep250. Only 38% of the patients have autoantibodies to PCM-1. It must be pointed out that these values are likely to be underestimated as only partial fusion protein for each antigen was used to test for autoantibody reactivity. Additional autoepitopes may be found in regions of these proteins not tested in this study.

The high frequency with which anticentrosome sera have autoantibodies to pericentrin, ninein and Cep250 over PCM-1 is striking. It is possible that pericentrin,

ninein and Cep250 are equally antigenic but more so than PCM-1. Pericentrin, ninein, and Cep250 are very similar in structure, and all contain large regions of coiled-coil structure. This motif is a preferential target for the immune system (Rattner, J.B. et al. 1996b, Rattner, J.B. et al. 1993, Fritzler, M.J. et al. 1995, Whitehead, C.M. et al. 1996a, Mack, G.J. et al. 1998), and the fusion proteins for pericentrin, ninein, and Cep250 used in this study all contain regions of coiled-coil structure. Thus, a common autoepitope within the coiled-coil region of these fusion proteins could explain the similar frequency of autoantibody reactivity between them. The lack of extended coiled-coil structure in PCM-1 (G. J. Mack, personal observation using the COILs program) could result in it being targeted by the immune system to a lesser extent than that of highly coiled-coil proteins. PCM-1 is the only centrosomal protein in which a detailed autoepitope analysis has been preformed (Bao, L. et al. 1995, Bao, L. et al. 1998). These studies have shown that there are three individual autoepitopes in PCM-1, amino acids 506-545, amino acids 1434-1465, and amino acids 1661-1686 (Bao, L. et al. 1998). Only the autoepitope between amino acids 506-545 lies with a region of PCM-1 predicted to adopt a coiled-coil conformation (G. J. Mack, unpublished observation using the COILs program). Further work has shown that the tripeptide KDC in the third autoepitope is all that is required for autoantibody recognition (Bao, L. et al. 1998). No information is known about which autoepitope if any, is dominant. The partial fusion protein to PCM-1 used in our study encompasses the second and third autoepitope of PCM-1, but does not contain the autoepitope found in the coiled-coil region. Hence, the low frequency of autoantibody reactivity detected against this PCM-1 fusion protein may be a reflection of the fact that most autoantibodies to the

centrosome are directed against coiled-coil epitopes. Alternatively, the abundance and cell cycle distribution of these centrosomal proteins may reflect their potential to be targeted by the immune system for an autoantibody response. It has been suggested that the release of autoantigens, such as in macromolecular complexes in cells undergoing programmed cell death, may be the source of autoantigens that drive the autoantibody response (Tan, E.M. 1994, Casciola-Rosen, L.A. et al. 1994). Both ninein and pericentrin are found at the centrosome throughout the cell cycle (Bouckson-Castaing, V. et al. 1996, Doxsey, S.J. et al. 1994), with pericentrin becoming more abundant as the cell progress further toward mitosis. Cep250 is also found at the centrosome throughout the cell cycle, although the majority of it appears to dissociate into the cytoplasm during prophase to anaphase (see SECTION V A). PCM-1 shows a very similar cell cycle distribution as Cep250, being tightly associated with the centrosome from G1 through G2, and then dissociating into the cytoplasm during mitosis, only to reappear at the centrosome during the following G1 phase (Balczon, R. et al. 1994). PCM-1 is only weakly if at all observed at the mitotic spindle poles (Balczon, R. et al. 1994). It is possible that an interphase centrosome protein complex could contain pericentrin, ninein, Cep250 and PCM-1, whereas a mitotic centrosome complex would contain pericentrin and ninein, but lower amounts of Cep250 and PCM-1. The complexity of the autoantibody response would then depend on the type of centrosome complex the immune system targets. Our results would seem it suggest that a mitotic centrosome complex is targeted in patients with anti-centrosome antibodies, as very few autoantibodies to PCM-1 are detected. However, in this scenario, autoantibodies to Cep250 would also have been expected to be found at lower frequencies than that

observed.

Autoantibodies to multiple centrosome proteins may arise as a result of epitope spreading. Since pericentrin, ninein , Cep250, and PCM-1 all localize within the centrosome, and may even interact, it is possible that an immune reaction against one of these proteins could broaden to include all of them. For example, rabbits immunized against specific epitopes of the splicesome Sm B/B' polypeptide have been shown to produce autoantibodies to these epitopes and to other proteins of the splicesome complex through a phenomenon of epitope spreading (James, J.A. et al. 1995).

Clincial features of anti-centrosome reactive patients

Review of the clinical features of the patients in this study group did not disclose any unifying clinical diagnosis or feature. However, four patients with autoantibodies to pericentrin were found to have post infectious ataxia. A similar study has also shown centrosomal antibodies in children with viral or mycoplasmal infection (Cimolai, N. et al. 1993). Other studies noting centrosome autoantibodies in patients with autoimmune disease have been reported. Most of these patients have Raynaud's phenomenon and features of systemic sclerosis (Osborn, T.G. et al. 1986, Rattner, J.B. et al. 1996a, Balczon, R. et al. 1994). That autoantibodies to the centrosome have no disease correlation is not unprecedented, many autoantibodies to cellular proteins, such as the heat shock proteins, have no specific disease association (Rowley, M.J. et al.1996). Furthermore, the lack of clinical association of anticentrosome antibodies should be viewed with caution as so few patients have been reported (Rattner, J.B. et al. 1996a, Mack, G.J. et al. 1998). It is possible that the low frequency of patients reported to have anti-centrosome autoantibodies

is due to the difficulty in detecting the small size of centrosome reactivity, or in the misinterpretation of its reactivity. Furthermore, centrosome reactivity can vary depending on the cell cycle, and on the cell substrate. More sensitive diagnostic techniques such as Western blot analysis or micro bead flow cytometry may be required to identify additional patients with anti-centrosome antibodies. It is possible that a larger patient population with autoantibodies to the centrosome is required before any clinical correlation can be discovered.

Of further interest, our prototype anti-centrosome serum was also found to have autoantibodies to the centrosomal antigen γγ-enolase (Rattner, J.B. et al. 1991c). The presence of autoantibodies to enolase in our patient group was not investigated due to the lack of purified enolase protein. Autoantibodies to enolase have also been reported in a group of children which mycoplasma infection (Cimolai, N. et al. 1993). Furthermore, one of the human sera characterized in this study was used in the identification of two additional centrosomal proteins, Cep110 and Cep76. The protein Cep76 is a known mouse centrosomal protein, and its identification in this library screen indicates that it is also a human autoantigens. Cep110 is a novel centrosome protein and will be discussed in detail in SECTION V C. Neither Cep110 or Cep76 were included in this study as they were characterized as centrosomal autoantigens after its publication.

In summary, we have characterized 21 human autoimmune sera that react with the centrosome. One of these sera, our prototype anti-centrosome serum was used to screen a cDNA expression library. From this screen several known centrosome associated proteins were identified, including pericentrin and ninein. The protein pericentrin is known as a

centrosome autoantigen (Doxsey, S.J. et al. 1994), and this is the first report that the protein ninein can also act as a autoantigen. A previously unknown centrosomal antigen designated Cep250 was also identified in this study. Characterization of the autoantibody specificity in these 21 sera showed that 80-90% have autoantibodies to pericentrin, ninein, and Cep250. Only half this amount, 38%, have autoantibodies to the centrosome antigen PCM-1. Furthermore, the majority of these sera have autoantibodies directed against multiple centrosome proteins. In this respect, patients with anti-centrosome antibodies are similar to patients with autoantibodies to the centromere/kinetochore, and may prove useful in the identification of a multi-protein centrosome complex that is targeted for an autoimmune response. Retrospective chart review of these patients did not reveal an unifying clinical diagnosis or feature associated with having autoantibodies to the centrosome.

SECTION IV

Post-Mitotic Centrosome Behaviour

Portions of this section have been published in Cell Molility Cytoskeleton, 26:239-247.

INTRODUCTION

The centrosome is the major site of assembly and organization of microtubules, and its position within the cell is correlated with both the cell cycle and the state of the cell (Rieder, C.L. 1990, Wong, M.K. et al. 1988). Both microtubules and microfilaments have been implicated in centrosome positioning and separation (Gotlieb, A.I. et al. 1993, Euteneuer, U. et al. 1985). For example, centrosome re-orientation during wound healing in endothelial cells is perturbed by microtubule and microfilament inhibitors (Gotlieb, A.I. et al. 1993).

In rapidly cycling cells, one of the most dynamic periods of centrosome movement precedes cell division when replicated centrosomes separate to establish the poles of the mitotic spindle. Centrosome separation is accompanied by the appearance of an astral array of microtubules that concentrates within the region between the separating centrosomes (Rattner, J.B. et al. 1976, Aubin, J.E. et al. 1980). While the mechanism of separation remains undefined, this movement does appear to depend on microtubules since microtubule inhibitors disrupt this process (Brinkley, B.R. et al. 1970, Rieder, C.L. 1982b). Furthermore, several microtubule motor proteins such as dynein, Xklp2, and HsEg5 have been shown to be required for centrosome separation, (Vaisberg, E.A. et al. 1993, Boleti, H. et al. 1996, Blangy, A. et al. 1995)

While the centrosome retains its polar location until the completion of anaphase A and B, there are fragmentary reports of centrosomes found at the opposite side of the cell in the region adjacent to the intercellular bridge prior to the completion of cytokinesis

(Rattner, J.B. et al. 1976). However, to date there has been no systematic study of the behaviour of the centrosome following cell division. Since the centrosome is the focal point for the organization of the interphase cytoskeleton and its position determines cell polarity, we undertook a study to document the behaviour of the post-mitotic centrosome in tissue culture cells using anti-centrosome antibodies and indirect immunofluorescence. We find that following karyokinesis, but preceding cytokinesis, sister centrosomes routinely undergo an orderly repositioning to the region immediately adjacent to the intercellular bridge. Studies using colcemid and cytochalasin D suggest that centrosome repositioning is dependent on microtubules while the correct positioning of the centrosome-microtubule complex requires an intact actin filament network.

MATERIALS AND METHODS

Tissue culture

Forty-eight hours prior to use, CHO cells were seeded onto coverslips as described in the GENERAL METHODS AND MATERIALS section. For some experiments colcemid (0.1-0.4 μg/ml) or cytochalasin D (Sigma: 0.3-0.9 μg/ml) were added as described in the Results section. In some experiments a skin fibroblast line of the Indian muntjac (a gift from C.C. Lin, The University of Alberta), HeLa cells, or a mouse L929 cell line were used.

Indirect immunofluorescence

To prepare cells for indirect immunofluorescence (IIF), coverslips were washed in DPBS and fixed in methanol as described in the GENERAL METHODS AND MATERIALS section. The coverslips were then incubated with primary antibody, which included: a human autoimmune serum (SM 2555) reactive with the centrosome (see previous section), a monoclonal antibody to tubulin (Boehringer Mannheim), a human autoimmune serum (MSA-35) reactive with the kinesin-like protein HsEg5 (Rattner, J.B. et al. 1992b), and a monoclonal antibody to the microtubule associated proteins (MAP) TAU (Sigma). In some experiments cells were reacted with FITC conjugated phalloidin (Sigma) at a dilution of 1:100 in DPBS. After three washes in DPBS, the coverslips were incubated with the appropriate secondary antibody at a dilution of 1:20 . After 1 h at 37°C, the coverslips were washed three times in DPBS, counterstained for DNA with DAPI, mounted and observed using a Nikon Optophot fluorescent microscope. Images were recorded on

Ilford HP-5 film.

RESULTS

Centrosome repositioning following cell division

In our initial studies we investigated the behaviour of the centrosome in a CHO cell line. At the completion of anaphase B, the centrosome of each daughter cell was positioned at the spindle poles (Fig. 4.1, A-C). Concurrent with the initial formation of the intercellular bridge and the reformation of the nucleus, microtubules in association with each centrosome depolymerize so that only short microtubules extend from the centrosomal region. A survey of cells at a slightly later stage, based on the morphology of both the nucleus and the intercellular bridge, revealed that in some cells a bundle of microtubules can be seen along one margin of the centrosome (Fig. 4.1, D-F). The shape of this microtubule bundle is generally either cylindrical or triangular (compare Fig. 4.1, F and I) and extends from the centrosome to the plasma membrane (Fig. 4.1, D-I). An increase in the length of the microtubule bundle accompanies the repositioning of the centrosome towards the intercellular bridge side of the cell (Fig. 4.1, D-I). The path of the microtubule bundle varies so that the centrosome-microtubule complex may be positioned across the nucleus as seen in the lower daughter cell shown in (Fig. 4.1, I) or around the nucleus as seen in the upper daughter cell in (Fig. 4.1, I). In many cells with a well formed intercellular bridge and nucleus, sister centrosomes are found in a region adjacent to the intercellular bridge (Fig. 4.1, J-L). In these cells the microtubule bundle, associated with the centrosome, is no longer detected.

The position of the centrosome was scored in 100 cells containing an intercellular

bridge found in logarithmically growing cultures. Of these cells, 30% displayed centrosome repositioning with the centrosome placed at different regions within each daughter cell (Fig. 4.1, D-F), while 23% contained centrosomes displaying a similar position in both daughter cells (Fig. 4.1, G-H). Thus, there was not a strict correlation between the time or path of centrosome repositioning between daughter cells. Twenty-six percent of telophase cells had both centrosomes positioned adjacent to the intercellular bridge (Fig. 4.1, J-L) and 20% showed centrosomes that were still at the spindle pole position (Fig. 4.1, A-C).

To determine if centrosome repositioning observed in our CHO line was unique to this cell line or a reflection of a more universal phenomena, we carried out IIF studies using three other cells lines. In cells of the Indian muntjac we observed a pattern similar to that observed in the CHO line. Figure 4.2, A-B illustrates one telophase cell from an Indian muntjac culture in which both daughter cells display prominent post-mitotic centrosome associated microtubule bundles. In the lower cell the centrosome still has a polar position while in the upper cell the centrosome has relocated to a region adjacent to the intercellular bridge. Similarly, Fig. 4.2, C-D illustrates sister centrosomes displaced to the intercellular bridge region in a post-mitotic cell obtained from an HeLa cell culture. In contrast to both the Indian muntjac and CHO cells, the centrosome-associated microtubule bundle found in HeLa cells more often appeared as a triangular rather than a linear array of microtubules (Fig. 4.2, C). Indirect immunofluorescent studies of mouse L929 cells also confirmed the repositioning of the centrosome to the intercellular bridge region in this cell line (Fig. 4.2, E-F). However, in this cell line we found it difficult to detect a prominent

Figure. 4.1. Indirect immunofluorescence of CHO cells at various stages after karyokinesis. Cells stained with DAPI (A, D, G, J), and antibody to the centrosomes (B, E, H, K), or an anti-tubulin antibody (C, F, I, L). Small arrows denote position of the centrosome. Panels A-C illustrate cells prior to centrosome repositioning. In the cells shown in panel D-F, centrosome repositioning has begun in one daughter cell (upper cell, double arrows). Both daughter cells display centrosome repositioning in the cells displayed in panels G-H (double arrows). Panels J-L illustrate a pair of daughter cells in which centrosome repositioning has been completed and the centrosomes has come to reside adjacent to the intercellular bridge. Bar = 10μm.

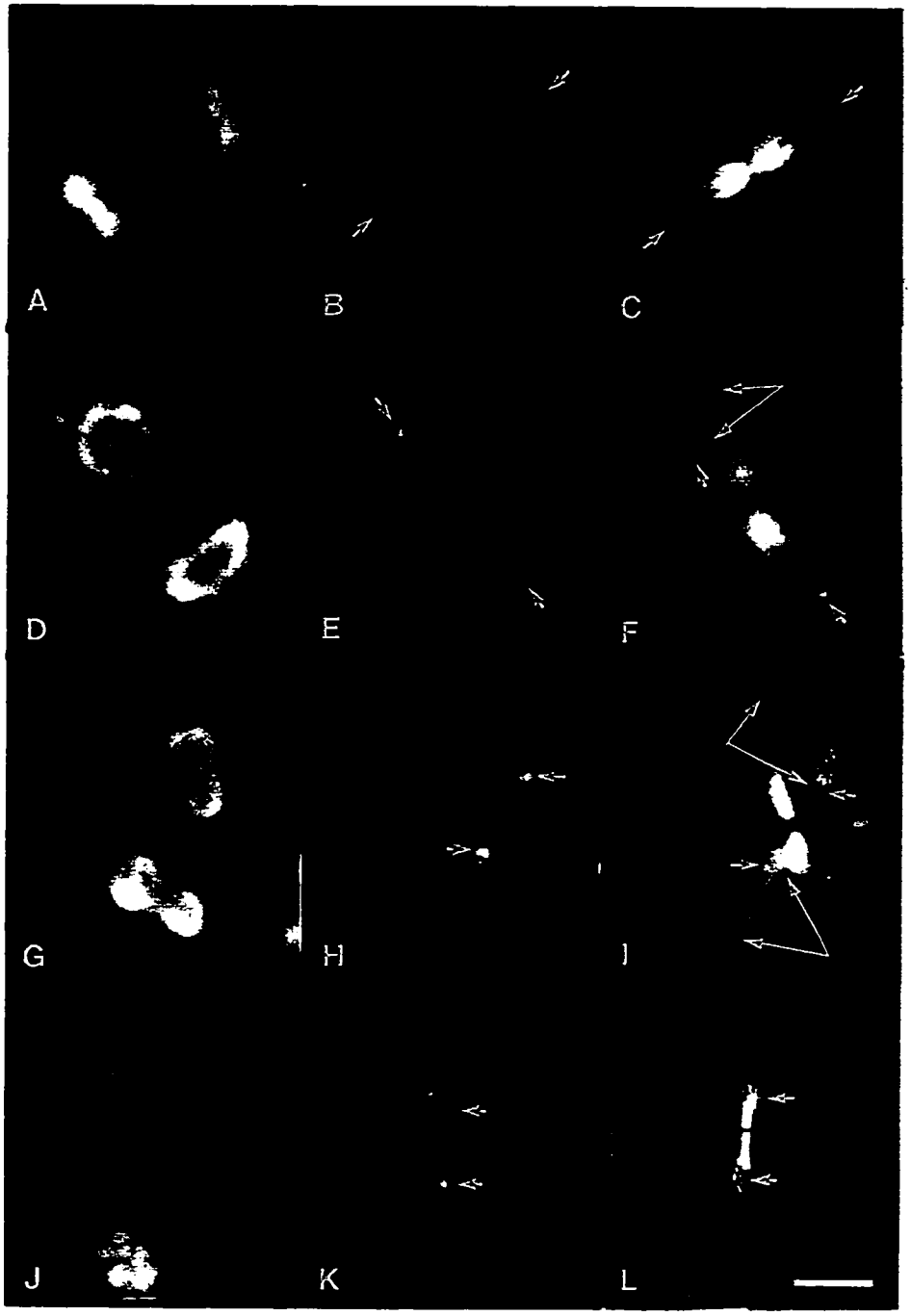
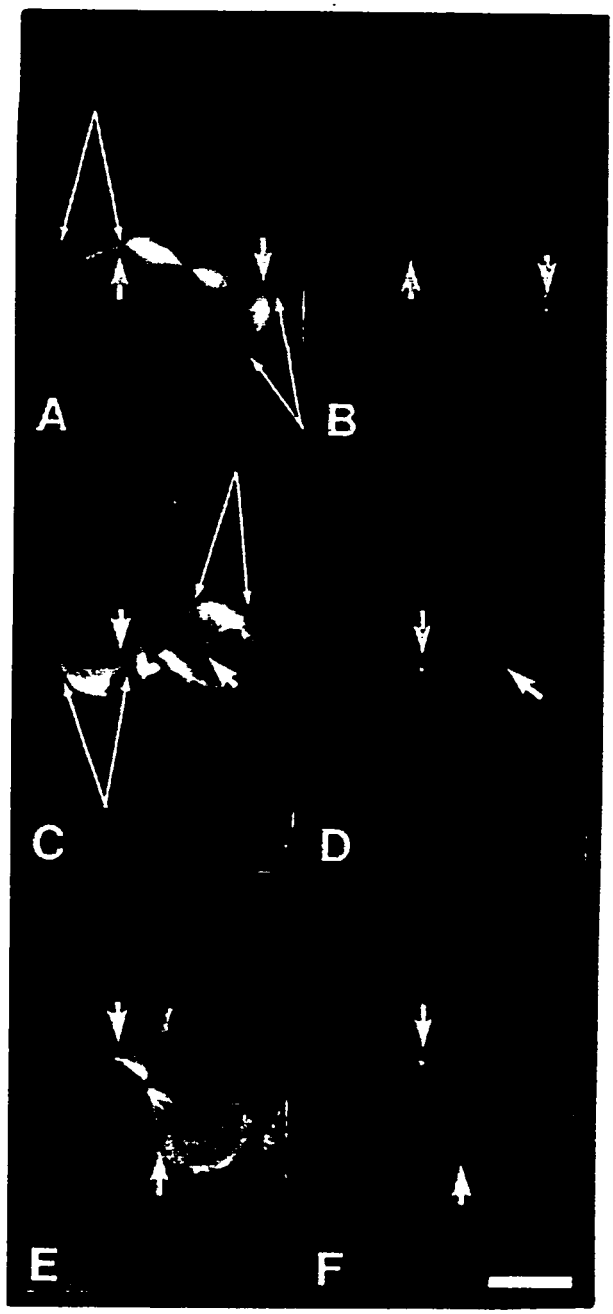

A
B
C
D
E
F
G
H
I
J
K
L

Figure. 4.2. Centrosome repositioning in various cell lines. Indian muntjac (A-B), human: HeLa (C-D), and mouse: L929 (E-F). Cells were stained with anti-tubulin (A, C, E) and anti-centrosome (B, D, F) antibodies. Small arrows denote the position of the centrosome and double arrows denote the extent of the centrosome associated microtubule array. Bar = 10μm.


A
B
C
D
E
F

microtubule array associated with centrosomes showing intermediate positions between the poleward and intercellular bridge regions of the cell (see lower cell Fig. 4.2, E-F). Further, we observed that the centrosome at the intercellular bridge side had a more juxtanuclear position than that seen in the other cell lines. Thus, although the repositioning of the centrosome was a common feature in all of the cell lines we investigated, we noted subtle variations in the morphology and characteristics of the microtubule-centrosome complex between these cell lines.

Affects of cytochalasin D on centrosome repositioning

To further characterize the organization of the cytoskeleton in cells immediately following karyokinesis, CHO cells were stained with fluorescein conjugated phalloidin. Figure 4.3, A-B illustrate that in cells containing a well formed intercellular bridge, prominent actin bundles can be detected traversing the cell and several bundles appear aligned along the axis of the intercellular bridge (Fig. 4.3, A, arrows). Similarly, filaments were detected oriented parallel to the longitudinal axis of the bridge in thin sections prepared for electron microscopy (data not shown). These filaments were often found adjacent to the microtubules bundle extending from the bridge as well as within the microtubule array.

The correlation between the orientation of the actin network and the path of centrosome repositioning led us to investigate whether an intact actin filament network was essential for centrosome repositioning. Logarithmical growing cultures were treated with cytochalasin D for periods of 15, 30, 45 or 60 minutes. Cells treated in this manner were then processed for IIF and reacted with both anti-centrosome and anti-tubulin antibodies.

Figure 4.3, C-F shows that cells treated with cytochalasin D continue to display centrosome-microtubule complexes in various stages of formation, even in the absence of furrowing (Fig. 4.3, C). However, following 30 minutes of exposure to the drug, telophase cells displaying centrosome repositioning had an aberrant morphology with centrosomes and their associated microtubule bundle displaying a random orientation with respect to the intercellular bridge (Fig. 4.3, C-F). In most cells the bundles and their associated centrosome extended away from the cell body. This morphology was commonly found in both daughter cells. These images suggest that intact actin filaments are required for maintaining the proper path of centrosome repositioning, but not for centrosome movement from the poles.

Measurements of the distance from the pole region to the intercellular bridge-cell interface in 25 late telophase CHO cells was an average of 10.8 μm. An average length of 12.5 μm was measured in 25 tubulin stained images for microtubule bundles associated with centrosomes that had moved to a location adjacent to the intercellular bridge. Larger measurements were obtained from cells in which the centrosome-microtubule path was around rather than across the nucleus. When the length of the microtubule bundle associated with centrosomes in 25 cytochalasin D treated cells were measured in IIF photographs after tubulin staining, the bundles were found to have an average length of 11.6 μm. Bundle length did not increase with increased exposure times to cytochalasin. Thus, the length of the microtubule bundle associated with repositioning centrosomes was roughly equivalent in treated and untreated cells, suggesting that there is a finite length that can be achieved by these microtubule bundles. A similar observation was made by

Brinkley, B.R. et al. 1981 using purified brain tubulin in the nucleation of microtubules from the centrosomes of lysed cells.

Affects of colcemid on centrosome repositioning

To investigate the role of microtubules in post-mitotic centrosome repositioning, logarithmical growing cultures were treated with the microtubule disrupting drug colcemid for periods of 5, 10, 15, 30, 45 or 60 minutes over a series of concentration ranges (see Materials and Methods). In late telophase cells treated for period of 10-60 minutes, microtubule or microtubule bundles were no longer detected in association with the centrosome. Initially the microtubules associated with the intercellular bridge remained intact at the concentrations used. However, during longer exposures the microtubules associate with the intercellular bridge shortened but staining in the region adjacent to the midbody persisted (Fig. 4.3, G-H). Following exposure to colcemid for 45 minutes, cells fixed and reacted with anti-centrosome antibodies were scored for the position of the centrosome within the cells. In 100 cells scored, 24% showed centrosomes at a polar location, 76% showed centrosomes within the cell body and none showed centrosomes adjacent to the intercellular bridge (compared to 26% in control cultures). No instances of centrosomes occupying equivalent positions in daughter cells were observed. Comparison of these centrosome patterns to those observed in untreated cells (see above), suggests that the movement of centrosomes to the region adjacent to the intercellular bridge and the maintenance of this location within the cell is directly correlated with the presence of microtubules.

Proteins associated with the microtubule bundle

The finding that the centrosome is repositioned both preceding and following mitosis raises questions as to the similarity in the mechanisms underlying these two events. To approach this question we compared the protein composition of the post-mitotic complex with that of the pre-mitotic centrosome microtubule complex using a series of antibody probes. Perhaps not surprisingly, monoclonal antibodies to the Microtubule Associated Protein (MAP) TAU commonly found in association with most known microtubule arrays were found to react along the length of the post-mitotic microtubule bundle (Fig. 4.4, A-B). However, autoantibodies to several proteins found in association with centrosomes and/or microtubules from the onset of centrosome separation, including calmodulin, NuMA and MSA-36 (Dedman, J.R. et al. 1980, Lydersen, B.K. et al. 1980, Rattner, J.B. et al. 1992a), failed to react with the post-mitotic centrosome and its associated microtubules (data not shown). However, one spindle associated protein, MSA-35 (Rattner, J.B. et al. 1992b), did show reactivity. Subsequent cloning and characterization of the MSA-35 antigen has shown it to be the kinesin-like protein HsEg5 (Whitehead, C.M. et al. 1996a).

HsEg5 is first detected in association with microtubules and centrosomes in cells prior to the onset of cell division (Rattner, J.B. et al. 1992b, Blangy, A. et al. 1995, Whitehead, C.M. et al. 1996a). This protein is also found in conjunction with kinetochore microtubules throughout their appearance. HsEg5 is transiently associates with pole-to-pole microtubules following anaphase and displays a complex temporal distribution within the intercellular bridge (Rattner, J.B. et al. 1992b, Whitehead, C.M. et

al. 1996a). As shown in Fig. 4.4, C-D, anti-HsEg5 antibodies recognize the microtubule bundle associated with post-mitotic centrosome repositioning. This reactivity is not distributed throughout the bundle but is confined to the region adjacent to the centrosome. The detection of HsEg5 in the post-mitotic centrosome-microtubule complex indicates that at least one component of the mitotic spindle other than tubulin and TAU is incorporated into this array. HsEg5 is not detected in association with the interphase cytoskeleton.

Studies using antibodies to the centromere and spindle proteins CENP- E and F (Yen, T.J. et al. 1992, Rattner, J.B. et al. 1993) failed to show reactivity with the microtubule bundle (data not shown). Further, the centrosomal and mitotic spindle component $p34^{cdc2}$ (Rattner, J.B. et al. 1990) also failed to show reactivity with the relocating centrosome (data not shown). Taken together, there appears to be limited homology in terms of protein composition between the post-mitotic centrosome-microtubule complex and that complex found at the onset of cell division. This preliminary information may suggest that different mechanisms are utilized to reposition the centrosome before and after mitosis.

Figure. 4.3. Cytochalasin D and colcemid treated cells. Late telophase (A-B) CHO cell reacted with FITC conjugated phalloidin (A) and DAPI (B). Prominent actin bundles are observed extending along the axis of the intercellular bridge (arrows). (C-F) CHO cells treated with cytochalasin D for 45 minutes and reacted with tubulin (C, E) and centrosome (D, F) antibodies. Two types of telophase cells are seen, one containing a well formed intercellular bridge (upper right panel C and panel E) and one in which furrowing has been disrupted by drug treatment (panel C centre cell). Both cells display prominent projections containing microtubules (double arrows) and a distally placed centrosome (small arrows). (G-H) CHO telophase cell from a culture treated with colcemid for 45 minutes. Short microtubules are still detected in association with the midbody although microtubules are absent from the cell body. A randomly placed centrosome is present within the cell body of each daughter cell (panel H). Bar = 10 μm.

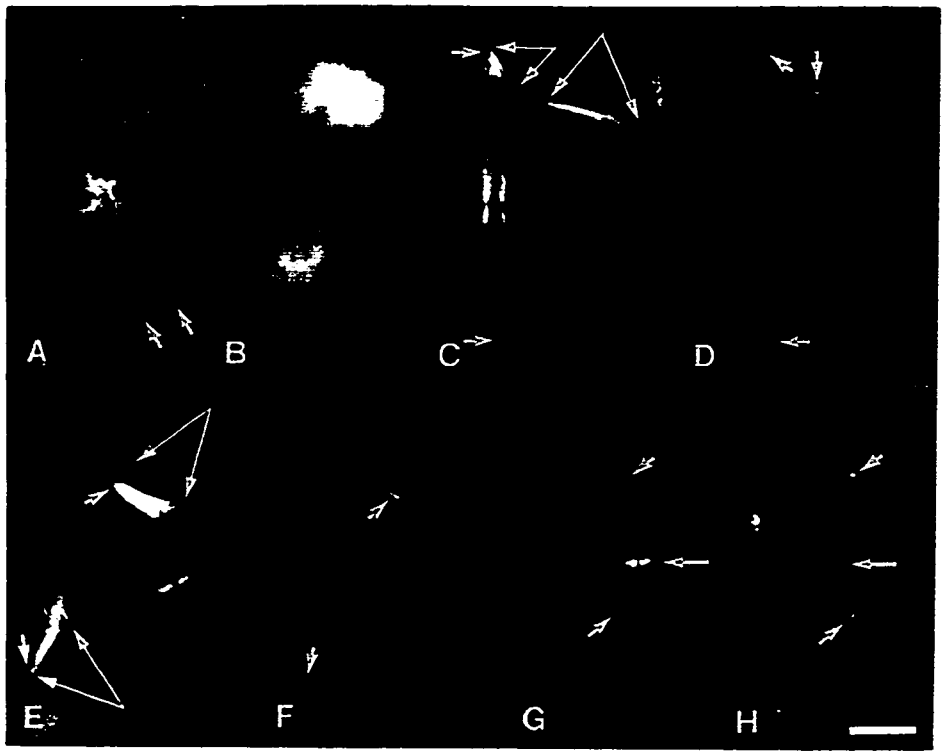
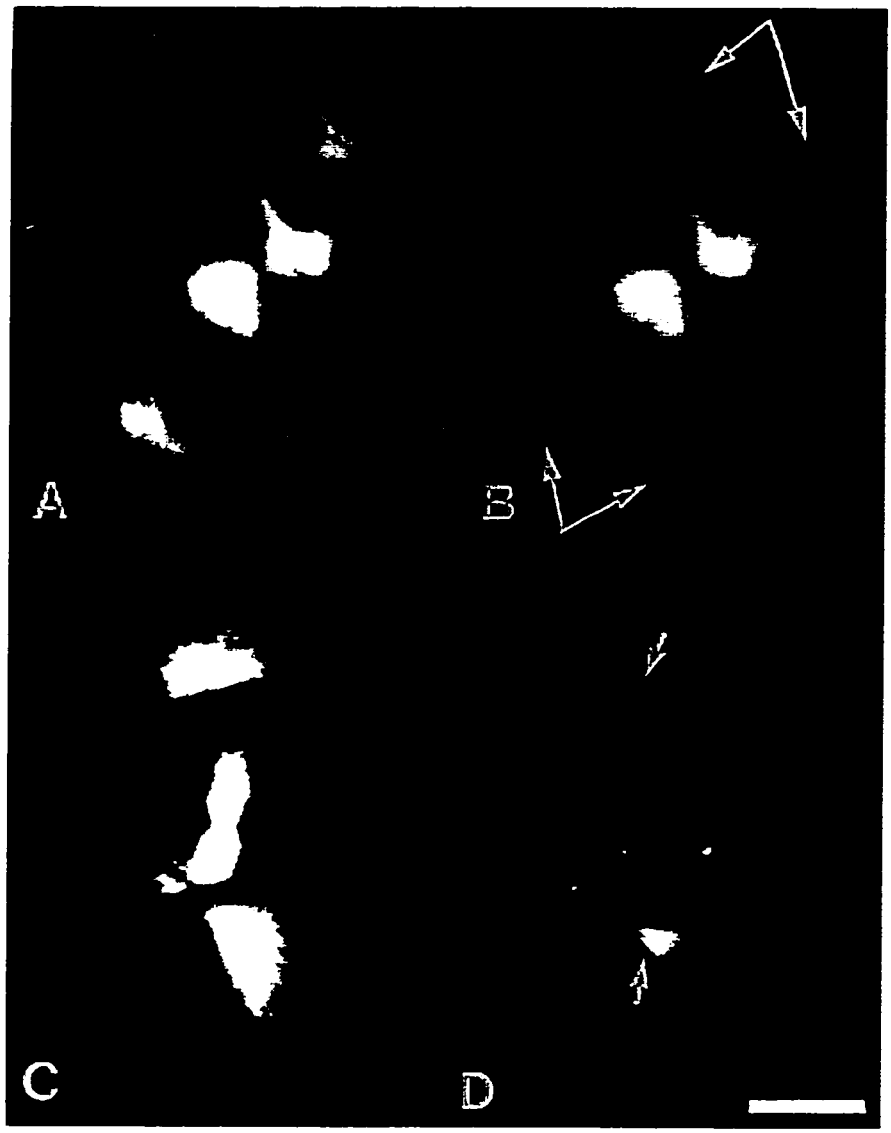

A
B
C
D
E
F
G
H

Figure. 4.4. Proteins at the moving centrosome. CHO cells stained with anti-β-tubulin (A, C) and an antibody to TAU (B) or MSA-35 (D). TAU reacts with the entire bundle of microtubules associated with the centrosome (panel B double arrows) while MSA-35 reacts only with the region of the bundle adjacent to the centrosome (arrow). The antigen MSA-35 has recently been shown to be the kinesin-like protein HsEg5 (Whitehead, C. M. et al. 1996a). Bar = 10 μm.


A
B
C
D

DISCUSSION

The investigation of centrosome movement and function during the cell cycle has concentrated on the events leading to mitosis. Many of the proteins believed to be responsible for centrosome separation during prophase have been characterized as microtubule motors. Two general models have been proposed for centrosome separation. In the first, separation results from the pushing apart of anti-parallel microtubules between duplicated centrosomes. The plus end directed microtubule motor proteins HsEg5 and Xklp2 have been proposed to provide the force necessary for this movement (Blangy, A. et al. 1995, Boleti, H. et al. 1996). The second model proposes that pulling forces from the cell cortex act on astral microtubules of duplicated centrosomes resulting in their separation. The minus end-directed microtubule motor protein dynein may supply the force necessary for this movement (Ault, J.G. et al. 1994). Very little has been reported about the role of the centrosome following chromosome segregation. Thus, the purpose of this study was to describe the behaviour and function of the centrosome following karyokinesis but prior to cytokinesis.

Repositioning of the centrosome.

We observed that following mitosis in tissue culture cells the centrosome undergoes an orderly and directed repositioning to a region adjacent to the intercellular bridge. The highly conserved nature of this event between cell lines of different species suggests that the relocation of the centrosome is an integral part of the re-establishment of the interphase cytoskeleton following cell division. It is unclear why it is necessary to relocate the

centrosome following mitosis. A variety of studies have lent support to the idea that the centrosome functions as a determinate of cell polarity (Albrecht-Buehler, G. et al. 1979, Malech, H.L. et al. 1977, Koonce, M.P. et al. 1984). The repositioning of the centrosome following karyokinesis relative to a fixed marker, the intercellular bridge, may function to ensure that daughter cells develop equal and opposite polarity. In addition, it is also possible that this movement ensures the termination of all spindle pole functions. The positioning of the centrosome adjacent to the intercellular bridge may also act as a signal for the initiation of the final phase of cytokinesis and may reflect an interrelationship between cytokinesis and cytoskeletal organization. Furthermore, the movement of the centrosome may be required for the polarization of cellular organelles.

The period during which the centrosome is positioned adjacent to the intercellular bridge may be relatively brief. We observed many instances when cells in the terminal phases of cytokinesis, as judged by the morphology of the intercellular bridge, had centrosomes in a juxtanuclear position. These observations suggest that, following relocation to the region adjacent to the bridge, the centrosome moves to a more central position within the cell. These observations are compatible to the movement documented in L929 cells by electron microscopy (Rattner, J.B. et al. 1973).

Centrosome repositioning is dependent on microfilaments and microtubules

Experiments using colcemid and cytochalasin D indicate that centrosome repositioning is dependent on microtubules. Further, the correct positioning of the centrosome-microtubular complex appears to require an intact actin filament network. The actin network may act as a track to guide centrosome relocation. Alternatively, it is

possible that the cell cortex plays a role in constraining the direction of movement. The loss of the mechanical integrity of the cell cortex as a result of cytochalasin treatment may affect this ability. Nevertheless, these results are consistent with the finding that there is a dynamic relationship between actin networks and microtubules during positioning and motility of centrosomes in cultured human polymorphonuclear leukocytes exposed to the tumour promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) (Euteneuer, U. et al. 1985). Furthermore, Whitehead, C.M. et al. (1996b) have shown that proper mitotic centrosome movement also requires an intact microfilament network. Prophase cells treated with cytochalasin D also show uncoordinated centrosome movement, and were often seen to extrude from the cell by deforming the plasma membrane. Interestingly, prometaphase centrosome movement was found to be unaffected by cytochalasin D, and may be due to the overriding mechanisms of centrosome separation associated with formation of the spindle (Whitehead, C.M. et al. 1996b). The detection of actin bundles parallel to the axis of the intercellular bridge suggests that the intercellular bridge may play a role in the organization and/or determination of the path of these bundles. The orientation of these bundles towards the intercellular bridge may in turn serve to insure the repositioning of the centrosome toward the intercellular bridge. Recently an actin homolog, centractin, has been localized to the centrosome (Clark, S.W. et al. 1992). The discovery of this homolog has raised the possibility that it may function in centrosome movement, perhaps by linking the centrosome to actin. This may suggest that centractin or similar proteins may play a role in integrating the centrosome-microtubular complex with the actin tracks oriented towards the intercellular bridge.

Proteins associated with the moving centrosome.

While these IIF studies represent a limited attempt to characterize the protein components associated with the post-mitotic centrosome-microtubule complex, they do point out some interesting features of this complex. For example, detection of at least one spindle associated protein within the complex suggests that some spindle proteins are recycled to this specific post-mitotic microtubule array. The spindle reactive human autoimmune serum used in this study was originally proposed to react with the protein MSA-35 (Rattner, J.B. et al. 1992b). Additional characterization of this serum demonstrated that the spindle reactivity was due to antibodies to the kinesin-like protein HsEg5 (Whitehead, C.M. et al. 1996b). This protein has been suggested to participate in centrosome separation by crosslinking antiparallel microtubules, and microinjection of HsEg5 antibody into animal cells can inhibit centrosome separation (Blangy, A. et al. 1995). In this study, HsEg5 was found within a small region of the microtubule bundle adjacent to the centrosome. This bundle has no association with antiparallel microtubules. Thus, it is possible that HsEg5 may not strictly require antiparallel microtubules to function. Recently, Whitehead et al. 1998, have shown that microinjection of antibodies to HsEg5 into HeLa cells prevents the post-mitotic movement of the centrosome by inhibiting the formation of the microtubule bundle required for this movement. Furthermore, by inhibiting the post-mitotic movement of the centrosome, the Golgi apparatus which normally associates with this microtubule bundle, fails to coalesce into a single focus. Thus, HsEg5 may be necessary for the stabilization of both mitotic and post-mitotic microtubule bundles. Further, the formation and function of the post-mitotic bundle

directly influences the reorganization of the Golgi apparatus and establishes the polarity of the daughter cells.

Lastly, the absence of several proteins from the centrosome microtubule bundle which are found in association with separating prophase centrosomes (Calmodulin, NuMA, MSA-36, CENP-E and -F, and $p34^{cdc2}$) suggests that some protein interactions and mechanisms that occur during centrosome repositioning following cell division may differ significantly from that found at the onset of spindle formation.

In summary, this is the first detailed documentation that following karyokinesis but preceding the completion of cytokinesis there is a series of orderly changes in the position of the centrosome resulting in its repositioning to the region adjacent to the intercellular bridge. This repositioning is dependent on the presence of an unique microtubule array and the integrity of the actin filament network.

SECTION V

NOVEL PROTEINS OF THE CENTROSOME

PART A: Identification and Characterization of Cep250

INTRODUCTION

Critical to the understanding of the centrosome is the identification and characterization of its molecular components. Biochemical purification of eukaryotic centrosomes is possible (Mitchison, T. et al. 1984, Moudjou, M. et al. 1994), but the low abundance of centrosomal proteins and the difficulty in obtaining pure fractions has made this approach impractical. Although, recent purification of the spindle pole body from the yeast *S. cerevisiae* has led to the identification of eleven new proteins not previously known to localize to the spindle pole (Wigge, P.A. et al. 1998). Antibodies against enriched centrosome fractions, or human autoimmune sera reactive with the centrosome are two methods commonly used to identify eukaryotic centrosome components (Kalt, A. et al. 1993). A human autoimmune serum was responsible for the identification of the centrosome protein pericentrin (Doxsey, S.J. et al. 1994), which is conserved from *Xenopus* to humans, and has been shown to be essential for microtubule nucleation (Doxsey, S.J. et al. 1994, Dictenberg, J.B. et al. 1998). The cell cycle dependent centrosome protein PCM-1 is another antigen identified with a human serum (Balczon, R. et al. 1994). The function of PCM-1 remains unknown, however, it has been suggested to have a role in the regulation of microtubule nucleation (Balczon, R. et al. 1994). An additional autoantigen that is found at the centrosome after nuclear envelope breakdown is NuMA (Nuclear protein associated with the Mitotic Apparatus) (Lydersen, B.K. et al. 1980). NuMA is believed to have roles in the interphase nucleus (Merdes, A. et al. 1998), and within the mitotic spindle (Galgio, T. et al. 1997).

The characterization of a group of human sera reactive with the centrosome in SECTION III of this dissertation directly led to the identification of several novel overlapping cDNA clones, in addition to the known centrosomal proteins pericentrin and ninein. Since autoimmune sera react with multiple centrosome proteins, it seemed possible that these novel clones encode for a unknown centrosome antigen. In this SECTION of my dissertation I describe the characterization of these clones, and show they encode a centrosome protein that I have given the designation Cep250. Cep250 is a high molecular weight coiled-coil protein with five leucine zipper motifs. Antibodies to Cep250 react with the centrosome throughout the cell cycle, and show that a population of Cep250 dissociates from the centrosome during mitosis. Furthermore, green fluorescent protein (GFP) tagging of various domains of Cep250 have shown that the COOH terminal region of the protein is required for centrosome targeting.

METHODS AND MATERIALS

Identification of cDNA clones

A human autoimmune serum (SM 2555) reactive with the centrosome throughout the cell cycle was used to immunoscreen a HeLa cDNA expression library at a dilution of 1:1000, as described in the general METHODS AND MATERIALS section of this dissertation. Eighteen reactive clones were identified (Table 6.0), and four overlapping clones (SM 1, 8, 12, 23) were chosen for further analysis. Two additional cDNA clones (283 and 39) that overlap and extend these four clones (Fig. 5.1) were found during a subsequent library screen with a second autoimmune serum and were included in this study (see SECTION V C).

Additional cDNA clones that overlap and extended these clones were obtained by rescreening the HeLa cDNA library by DNA hybridization as described in the general METHODS AND MATERIALS section of this dissertation. Positive clones identified from these library screens were further characterized through PCR to determine which clones contained inserts with the largest amount of additional DNA sequence information. A 200 bp probe for the 5' end of clone 283 was obtained through PCR using primer 302 (TGGAGAAGCTGCTGGTTCAG) and the λ forward primer (GGTGGCGACGACTCCTGGAGCCCG). From this library screen, three clones were identified and one clone, 638, was chosen for further investigation. A second DNA hybridization screen using a 100 bp Bcl I fragment of 638 was used as a probe. From this screen, eleven clones were identified, and a single clone designated, B, was further

analysed. A 500 bp probe for the 3' end of clone SM 1 was generated by digesting the SM 1 insert with Xho I and EcoR I. From this library screen, seventeen positives were identified and a single clone, 43, was chosen for further investigation.

The complete DNA sequence of these clones was determined by a combination of restriction enzyme digestion cloning, exonuclease III deletion, manual and dye termination sequencing methods as described in the general METHODS AND MATERIALS section of this dissertation. All nucleic acid and protein sequence searches and analysis were carried out on the local network server using the BLAST search program (Altschul, S.F. et al. 1990).

Recombinant protein production and antibody generation

The 2.0 kb cDNA insert of clone SM 23 was digested out of the λ vector with EcoR I and cloned into the EcoR I site of the expression vector pGEX 5X-2 (Pharmacia). Cells expressing this construct produce a 100 kDa insoluble GST-fusion protein. Large scale protein inductions were carried out, and the protein was resolved on SDS-PAGE as described in the GENERAL METHODS AND MATERIALS section. SM 23 fusion protein was excised from the gel, pulverized and injected into one rabbit by the SACRC hybridoma facility to generate a polyclonal antibody.

A 525 bp EcoR I/Rsa I digestion fragment of clone 283 was cloned into the EcoR I and Sma I sites of the expression vector pGEX 5X-2 (Pharmacia). Cells expressing this construct produced a 46 kDa soluble GST-fusion protein. Large scale protein inductions were carried out, and the fusion protein was purified according to the manufacturer. Fusion protein 283 was injected into two mice by the SACRC hybridoma facility to

generate a polyclonal antibody.

A 1340 bp Ecl136 II/Rsa I digestion fragment of clone B was cloned into the Sma I site of the expression vector pGEX 5X-2 (Pharmacia). Cells expressing this construct produce a 76 kDa soluble GST-fusion protein. Large scale protein inductions were carried out, and the fusion protein was purified according to the manufacturer. Fusion protein B was injected into two rabbits by the University of Calgary vivarium staff to produce a polyclonal antibody.

Clone 43 was PCR amplified with T3 and T7 primers and PFU polymerase (Stratagene) to generate bunt ends. The 2.5 kb product was digested with Ecl136 II and a 950 bp fragment was cloned into the Sma I site of the expression vector pGEX 5X-2. Cells expressing this construct produce a 52 kDa soluble GST-fusion protein. Large scale protein inductions were carried out, and the fusion protein was purified according to the manufacturer. Fusion protein 43 was then injected into two rabbits by the University of Calgary vivarium staff to produce a polyclonal antibody.

Northern blot hybridization

A 525 bp EcoR I/Rsa I fragment of clone 283 was PCR amplified from the GST expression vector construct described above using the primers pGEXF and pGEXR. The DNA fragment was purified and quantitated. A ^{32}P labelled probe was produced from this template using the random primer procedure described in the GENERAL METHODS AND MATERIALS section of this dissertation.

A human multiple tissue Northern blot (Clontech) was prehybridized in 10 ml Rapid-hyb (Amersham) for 2 h at 65°C. Radioactively labelled probe was then added to

the membrane at 1 x 10^6 cpm/ml and rotated in a hybridization oven for 4 h at 65 °C. The hybridization solution was removed and the membrane washed as described by the manufacturer. The membrane was then sealed in a plastic bag and exposed to a phosphoimager screen (Molecular Dynamics) for three days.

Indirect immunofluorescence

HeLa cell seeded coverslips were fixed in methanol, and blocked with a 1:200 dilution of NGS-DPBS at room temperature for 30 min. After rinsing, the coverslips were incubated with affinity purified anti-Cep250 (anti-m23, B, and 43) at a dilution of 1:50 in NGS-DPBS for 30 min at 37°C. Following a wash in DPBS, the coverslips were incubated with either 1:14,000 anti-pericentrin in NGS-DPBS or 1:1000 anti-β-tubulin (SIGMA) for 30 min at 37°C . The coverslips were washed for 2 min, and incubated with a mixture of Cy3-conjugated anti-mouse (1:400) and FITC-conjugated anti-rabbit (1:400) secondary antibody in NGS-DPBS for 30 min at 30°C. The coverslips were washed well, counterstained with DAPI, and mounted in glycerol. In some instances commercially prepared monkey tissue sections (MeDiCa, Carlsbad, CA.) were used in immunofluorescence studies. Human sperm collected from a healthy male (G.J.M) was diluted in DPBS and centrifuged onto a glass coverslip on a paraffin mounted chamber. The coverslips were then fixed in methanol for 10 min and processed for immunofluorescence as described above.

Western blot and immunoprecipitation

HeLa mitotic protein was prepared by selective detachment of mitotic cells from monolayer cultures treated with or without colcemid (10μg/ml) followed by resuspension

in SDS sample buffer. HeLa interphase protein was prepared by trypsin detachment of adherent cells after mitotic shake off into SDS sample buffer. Alternatively, interphase protein was prepared by trypsin detachment of adherent cells treated with thymidine (2 mM) for 12-16 h to block the cells in S phase, followed by resuspension in SDS-sample buffer. Total HeLa protein was prepared by scraping as logarithmically growing culture into 200µl of SDS sample buffer using a rubber policeman.

Insoluble and soluble protein fractions from interphase and mitotic cells were prepared by lysing the cells in 1% NP-40, 150 mM NaCl, 50 mM Tris (pH 8.0), for either 1 or 10 min at 4°C. Protein samples were centrifuged at 12000 x g at 4°C for 10 min. Soluble and insoluble fractions were separated and mixed with and equal volume of 2x SDS sample buffer. The relative amount of each protein fraction was determined by comparision on stained polyacrylamide gels.

Either mitotic, interphase or total protein was separated on 4 or 6% SDS-PAGE and transblotted to nitrocellulose as described in the GENERAL METHODS AND MATERIALS section. Membranes were then incubated with either anti-M23 (1:500), anti-B (1:500), or anti-43 (1:500), as described in the GENERAL METHODS AND MATERIALS section.

Cep250 protein was immunoprecipitated with 5µg of affinity purified antibody (anti-B) from mitotic cells as described in the GENERAL METHODS AND MATERIALS section.

HeLa cells grown in a 15 cm petrie dish were transfected with the appropriated GFP construct and incubated overnight. Cells were washed once in DPBS and scraped into 100

µl of SDS sample buffer using a rubber policeman.

Green Fluorescent Protein (GFP) fusion and transfection

Various fragments of protein encoding cDNA of Cep250 were cloned in-frame into pEGFP-N1 (Clontech) a red-shifted GFP eukaryotic expression vector (Fig. 5.18). The full length coding region of Cep250 (7322 bp) was amplified using PFU PCR and the primers CEPF (ATGGAGACAAGAAGCCCTGGG) and CEPR (GGAGGCGGCTTGGGTAGTGC) from HeLA cDNA (Clontech) as described in the general METHODS AND MATERIALS section of this dissertation. This fragment was gel isolated and the 5' and 3' ends were phosphorylated using T4 polynucleotide kinase (GIBCO BRL) for 30 min at 37°C according to the manufacturer. The fragment was then gel isolated and ligated into the Ecl136 II site of pEGFP-N1 creating the construct Cep250 GFP. A 1440 bp Bgl II digestion fragment of clone B was ligated into the Bgl II site of pEGFP-N1, creating the construct Del A GFP. A 1341 bp fragment of clone 283 was generated by PFU PCR amplification using the primers 283FL (AAGCTAGTGCCTTAAATG) and λ reverse. Following EcoR I digestion, the purified fragment was ligated into the Ecl136 II and EcoR I sites of pEGFP-N1, creating the construct Del B GFP. A 4580 bp fragment was generated using PFU polymerase and the primers CEP39F (GCCCTATGCCAGATGCAG) and CEPR. This fragment was isolated and cloned into the Ecl 136 II site of pEGFP-N1 to create the construct Del C GFP. A 4022 bp fragment spanning the COOH terminus of Cep250 was generated using PFU PCR and the primers MIDCEP (GCTCAGATGGAATTACTAAGGC) and CEPR. This fragment was isolated and cloned into the Ecl136 II site of pEGFP-N1 producing the construct Del D GFP. A 837 bp Bgl

II/Sac I digestion fragment of clone SM 1 was ligated into the Bgl II and Sac I sites of pEGFP-N1, creating the construct Del E GFP. A 2058 bp fragment of clone 43 was generated by PFU PCR using the primers M1REV (GGAGCAGCAGCTCCAGGGCC) and CEPR. This fragment was purified and phosphorylated using T4 polynucleotide kinase (GIBCO BRL) for 30 min at 37°C according to the manufacturer. The phosphorylated fragment was then gel isolated and ligated into the Eco 47 III and Ecl136 II sites of pEGFP-N1, creating the construct Del F GFP. A 553 bp fragment of clone 43 was generated using PFU PCR and the primer CEPLEU1 (CCTAGTCCTGATGGAATGG) and CEPR. This fragment was purified and cloned into the Ecl136 II site of pEGFP-N1, creating the construct Del G GFP. Finally a 112 bp fragment of the COOH terminus was generated by digesting the PFU polymerase PCR product of the primers CEPLEU and CEPR with Rsa I. This fragment was cloned into the Ecl 136 II site of pEGFP-N1 to create the construct Del H GFP.

The various DNA constructs were transformed into competent cells, recombinant clones were identified and miniprep DNA was made using the QIAprep spin columns (Qiagen) as described in the GENERAL METHODS AND MATERIALS section of this dissertation. HeLa coverslips were transfected with 1-2 μg of plasmid DNA, and allowed to express for 24 h. Coverslips were fixed in 4% paraformaldehyde (SIGMA) in DPBS and mounted, or further processed for additional IIF using a mouse anti-pericentrin antibody (1:10,000) as described in the GENERAL METHODS AND MATERIALS section. Figure 5.18 illustrates the location of each Cep250 GFP construct.

RESULTS

Identification of cDNA clones encoding a novel human autoantigen

In an effort to further characterize human autoimmune sera reactive with the centrosome, a prototype serum (SM 2555) that reacts with the centrosome throughout the cell cycle (Fig. 3.1) was used to immunoscreen a HeLa cDNA expression library. Eighteen reactive clones were identified. From these clones, 8 were found to encode portions of the known centrosomal proteins, ninein and pericentrin (see SECTION III). Six clones were not significant to this study and were not further investigated. Four overlapping clones designated SM 1, 8, 12, and 23 (Fig. 5.1) were shown by DNA sequence analysis not to have significant homology to known sequences in the GenBank database as of June 1998. Two additional cDNA clones, designated 283 and 39, were isolated using another human autoimmune serum reactive with the centrosome (see SECTION V C), and were found to overlap clones SM 8, 12, and 23 (Fig. 5.1). Sequence analysis also showed that 283 and 39 had no significant homologues in the GenBank database as of June 1998. Together these 6 cDNA clones span 3845 bp, and encode a long open reading frame (ORF) with no translation initiation or termination codons. Since human autoimmune sera have autoantibodies to multiple centrosome proteins (SECTION III) I reasoned that these cDNA clones could encode for an unknown centrosomal antigen.

As no translation initiation codon was present in these clones, the isolation of additional upstream DNA sequence was necessary. To identify clones extending further 5', a probe to clone 283 was made and used in a DNA hybridization screen of the HeLa cDNA

library. One clone, 638, was identified and found to extend the sequence of clone 283 by approximately 600 bp. This clone had an ORF continuous with that of clone 283, but contained no translation initiation codon. An additional DNA hybridization screen was carried out using a probe specific for the 5' end of clone 638. One clone, B, was found to extend the sequence of clone 638 by approximately 1400 bp. An ORF initiating at nucleotide position 222 was identified in this clone, and it is preceeded by three in-frame stop codons (Fig. 5.1).

Since no translation termination codon was present in the ORF of any of these clones, it was necessary to find additional downstream DNA sequences. To isolate clones that would extend clone SM 1 further 3', an SM 1 specific probe was generated and used in a DNA hybridization screen of the HeLa cDNA library. One clone, designated 43, was found to extend SM 1 by approximately 2000 bp and contained a continuous ORF continuous terminating at an amber stop codon (TAG) at position 7548 (Fig. 5.1).

All together these cDNA clones encompass 7814 bp, and encode for a protein that I have designated as Cep250 (Centrosome protein 250). The complete nucleotide and predicted amino acid sequence of Cep250 is shown in Appendix I, and is available from GenBank under accession number AF022655. A cDNA identical to Cep250 has recently been reported by Fry, A. M. et al. (1998a).

Northern blot analysis of Cep250

Multiple cDNA clones were used in assembling the full length Cep250 DNA sequence. To confirm that the final 7814 bp cloned cDNA is correct, Northern blot analysis was performed on HeLa RNA. Two to four μg of poly(A)$^+$ RNA from

logarithmically growing HeLa cells was transferred to Nylon membrane and probed for Cep250. No hybridization signal was detected after exposure to film for several weeks (data not shown). Hybridization with an actin control probe confirmed the integrity as well as the quantity of the mRNA. The inability to detect a hybridizing signal suggests that Cep250 has a low abundance mRNA in HeLa cells. As a result, this prompted us to probe other tissues/cells which may express more Cep250 mRNA. For this purpose, Northern blot hybridization was repeated with a human multiple tissue Northern blot (Clontech). Poly(A)$^+$ RNA from heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas is represented on this membrane. When probed for Cep250, a single weak signal was detected at ~ 8.0 kb at equal levels in all tissue samples (Fig. 5.2). Hybridization with an actin probe revealed that all lanes contained approximately equal amounts of RNA (Fig. 5.2). Thus, Cep250 is expressed more abundantly in human tissues, and the ~ 8 kb signal is in good agreement with the assembled Cep250 cDNA clones.

Features of the deduced amino acid sequence of Cep250

Analysis of the cloned DNA sequence identified an open reading frame (ORF) of 7326 nucleotides encoding 2442 amino acids. The initiator codon, corresponding to nucleotides 222-224 is in good agreement with the Kozak consensus sequence (CCA/GXXATGG) (Kozac, M. 1984). This initiation codon is preceded by a short untranslated region containing multiple inframe stop codons. A termination codon is found at nucleotide position 7548-7550, but no poly(A)$^+$ initiation signal or poly(A)$^+$ tail is found. From the deduced amino acid sequence, the molecular weight of Cep250 was calculated to be 281,002 Da. GenBank database searches with the amino acid sequence revealed some

Figure 5.1 Schematic diagram depicting the cDNAs identified in the complete cloning of Cep250. Clones designated by SM (red) were identified with the human autoimmune serum SM 2555. The cDNA clones 283 and 39 (green) were identified with the autoimmune serum 2688. Clones B, 638 and 43 (black) were identified through DNA hybridization screening of a HeLa cDNA library. The complete Cep250 clone is shown at the top (from 1 to 7814 bp) with the blue box representing the predicted open reading frame (from base 222 to 7548).

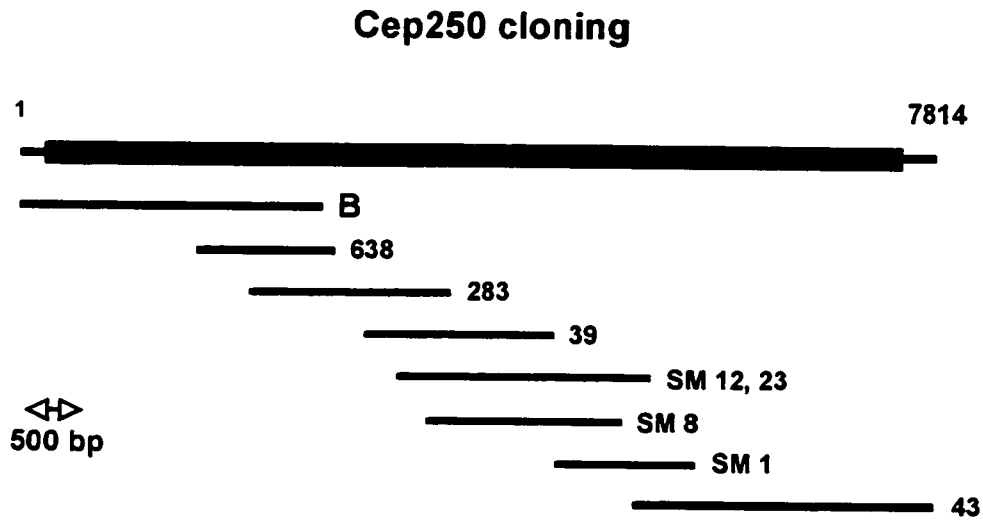
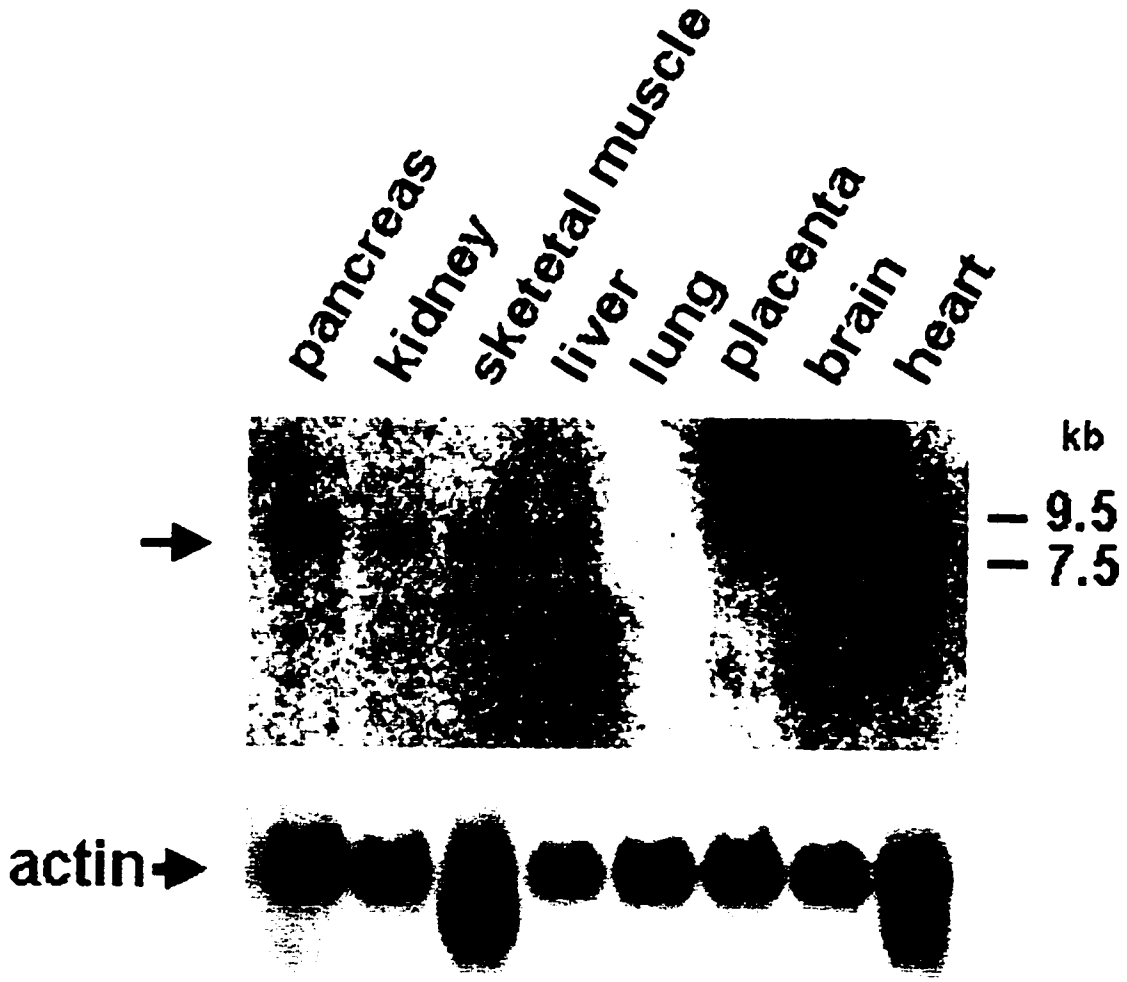

Cep250 cloning
1
7814
B
638
283
39
SM 12, 23
SM 8
SM 1
43
500 bp

Figure 5.2. Northern blot analysis of Cep250. A human multiple tissue Northern blot was probed for Cep250 and actin. A single Cep250 transcript at ~ 8.0 kb is weakly detected in all human tissues tested (arrow). A control hybridization of actin reveals that all tissues have approximately equal amount of RNA. Molecular size markers are 9.5 and 7.5 kb.


pancreas
kidney
sketetal muscle
liver
lung
placenta
brain
heart
kb
9.5
7.5
actin

weak homology to a mouse intranuclear matrix protein INMP (Menz, K. et al. 1996). Furthermore, small regions of similarity can be observed with various coiled-coil cytoskeletal proteins such as myosin and trichohyalin. Analysing the amino acid sequence with the program COILS (Lupas, A. et al. 1991) shows that Cep250 is predicted to have stretches of coiled-coil structure throughout much of its sequence, except for the NH_2-terminal 250 amino acids, between residues 1168-1248, and the COOH terminal 50 amino acids (Fig. 5.3). Breaks in the coiled-coil structure of Cep250 are a result of proline residues, which usually disrupt α helices (Doxsey, S.J. et al. 1994).

A protein MOTIF search reveals one amidation site, 12 N-myristoylation sites, and four N-glycosylation sites. Furthermore, there are two potential cAMP-phosphorylation sites, 49 casein kinase II phosphorylation sites, 34 protein kinase C phosphorylation sites, one tyrosine kinase phosphorylation site, and five potential cyclin dependent kinase (cdk) sites. In addition, there are five predicted leucine zippers at amino acid positions 309-330, 433-496, 580-622, 994-1015, and 2275-2296. Each of the first, fourth and fifth leucine zipper motifs consist of four leucine residues separated by six amino acids, whereas the second leucine zipper motif contains 11 leucine residues, and the third leucine zipper motif has seven leucine residues. The location of each potential leucine zipper lies within a regions predicted to adopt a coiled-coil structure (Fig. 5.4). Each predicted leucine zipper region is underlined, and the five potential cdk phosphorylation sites of Cep250 are boxed in Appendix I. There are no consensus sequences in Cep250 for microtubule or nucleotide binding. Overall Cep250 is an acidic protein with a predicted pI of 5.00. However, the COOH terminal 250 amino acid are more basic with a predicted pI of 8.09.

Figure 5.3. Coils output for the predicted amino acid sequence of Cep250. The probability of forming a coiled coil structure is plotted for the complete amino acid sequence of Cep250. The majority of Cep250 is predicted to form a coiled coil structure except for regions at the NH_2 terminus (amino acids 1-250), COOH terminus (amino acids 7498-7548), and between residues1168-1248. The calculated window size is 28 residues using the program COILS (Lupus, A. et al. 1991).

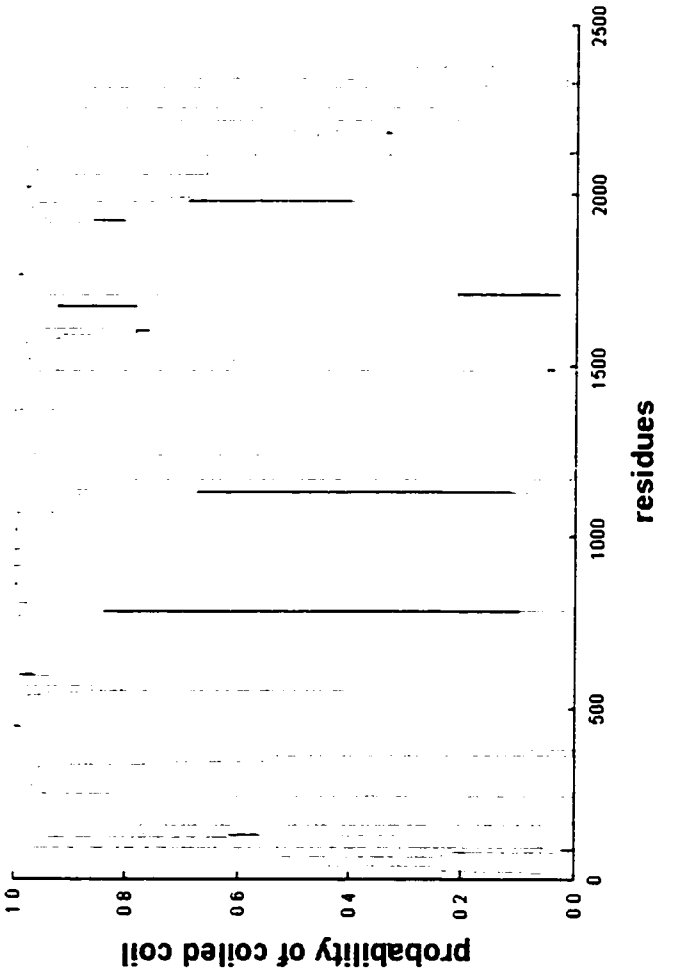
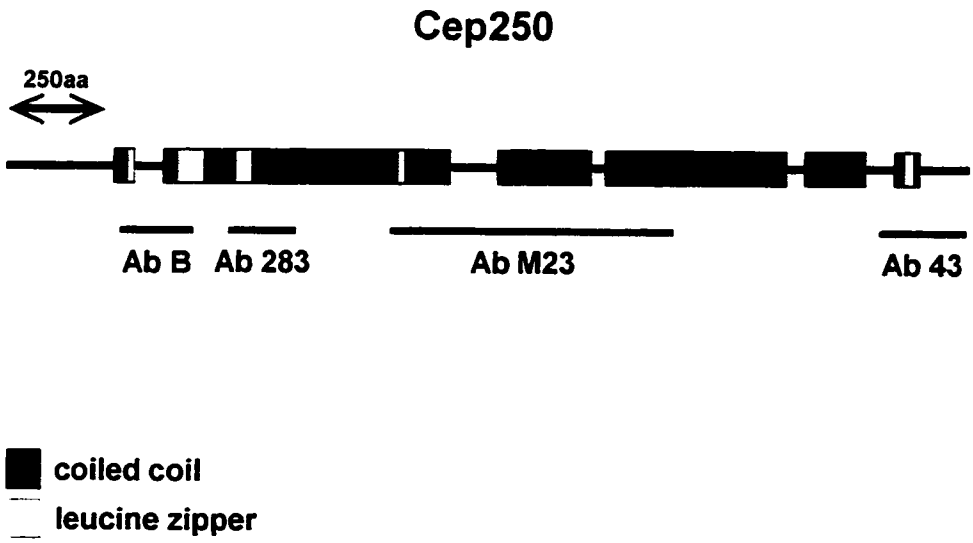

probability of coiled coil
0.0
0.2
0.4
0.6
0.8
1.0
residues
0
500
1000
1500
2000
2500

Figure 5.4. Characterization of the Cep250 protein. A stylized diagram of the Cep250 protein is shown at the top. Blue boxes are regions of coiled coil structure, and yellow boxes are predicted leucine zipper motifs. Shown underneath are regions of Cep250 that were used to generate polyclonal antibodies.


Cep250
250aa
Ab B
Ab 283
Ab M23
Ab 43
coiled coil
leucine zipper

Immunolocalization of Cep250 during the cell cycle

Even though the human serum used for immunoscreening in this study reacted with the centrosome, it was possible that the isolated clones may encode an antigen that was reacting with additional noncentrosome-specific autoantibodies. Thus, to determine if Cep250 is a centrosomal protein, antibodies were produced in rabbits against different domains of the protein (Fig. 5.4), and used for indirect immunofluorescent studies.

Immunostaining of HeLa cells was carried out using affinity purified rabbit antibody against Cep250 (anti-m23) and a mouse antibody raised against the human centrosomal protein pericentrin. Preimmune rabbit sera did not react with the centrosome at the dilutions used in this study (data not shown).

As shown in Figure 5.5, in early interphase cells Cep250 was observed as two closely opposed compact foci adjacent to the nuclear envelope and colocalized with pericentrin reactivity (Fig. 5.5, A-C). Cep250 reactivity at the centrosome is observed to be smaller than that of pericentrin. Figure 5.6 shows overlapping images of the Cep250 signal (green) and the pericentrin (red) or γ-tubulin (red) signal. Cep250 is often found on the periphery of pericentrin or γ-tubulin reactivity and is significantly smaller in size. In late interphase, Cep250 is observed as a single discrete foci tightly associated with each duplicated and separated centrosome (Fig. 5.5, D-F). During early prophase, Cep250 was no longer observed as a compact foci at the centrosome, rather it appeared loosely organized and diffuse around each centrosome (Fig. 5.7, A-C). During a later stage of prophase Cep250 was barely detectable at the centrosomes, however, weak reactivity can be seen throughout the entire cytoplasm of the cell (Fig. 5.7, D-F). During metaphase

Cep250 remains barely detectable at the spindle pole, and cytoplasmic reactivity is more prominent especially in the area of the mitotic spindle (Fig. 5.8, A-C). In early to late anaphase cells a prominent diffuse dot of Cep250 was observed at the centrosome (Fig. 5.8-5.9, C), and cytoplasmic reactivity often appeared more intense beneath the cleavage furrow (Fig. 5.9, C). Finally, in late telophase early G1 cells, Cep250 is once again observed as a compact foci at each centrosome, and midbody reactivity is seen within the intercellular bridge (Fig. 5.9, D-F). In some late telophase cells two compact Cep250 foci are observed at the centrosome (Fig. 5.9, F left hand cell) whereas in others a single foci is observed (Fig. 5.9, F right hand cell).

As mentioned above, Cep250 was observed to be tightly associated with the centrosome during interphase. However, in early prophase, as judged by nuclear morphology, Cep250 reactivity changes, such that it appears loosely organized around the centrosome. In order to further define the cell cycle distribution of Cep250 during interphase, HeLa cells were double stained for Cep250 and CENP-F. CENP-F is a kinetochore associated protein that has a unique cell cycle distribution. During G1 phase of the cell cycle, CENP-F is not detectable in the cell. At S phase, CENP-F shows weak nuclear staining, and by G2, nuclear staining is very prominent (Liao, H. et al. 1995, Rattner, J.B. et al. 1993). In Figure 5.10, three cells are shown to be in G1, S, and G2 of the cell cycle by CENP-F nuclear reactivity (Fig. 5.10, A), in each of these cells Cep250 is observed as a discrete foci at the centrosome (Fig. 5.10, B). The cell in G2 (Fig. 5.10, B far right) has centrosomes that have duplicated and separated to near opposite sides of the nucleus and Cep250 remains tightly associated each centrosome. Thus, Cep250 remains as

a tightly associated centrosome protein throughout the duration of interphase.

The weak reactivity of Cep250 during prophase and at the mitotic spindle poles might possibly be due to epitope inaccessibility of this antibody (anti-m23). To address this concern, two additional antibodies directed against the NH_2-terminus (anti-B) and COOH terminus (anti-43) of Cep250 were used to stain HeLa cells at metaphase. Using both antibodies only weak spindle pole reactivity was observed (Fig. 5.11). In addition, both antibodies also reveal prominent Cep250 reactivity throughout the entire cytoplasm (Fig. 5.11, B and D). During interphase these antibodies show specific centrosome staining and no cytoplasmic reactivity is observed (data not shown). Thus, multiple antibodies to Cep250 reveal that it has a cell cycle dependent centrosome association and that it is not due to antibody inaccessibility. Furthermore, the loss of staining at the centrosome observed during mitosis is concurrent with the release of Cep250 into the cytoplasm.

In summary, Cep250 is a novel centrosome protein identified with a human autoimmune serum reactive with the centrosome. Cep250 is tightly associated with the centrosome during interphase. As its reactivity does not completely colocalize with pericentrin or γ-tubulin, components of the PCM, Cep250 may associate with the centrioles. During mitosis, most of Cep250 dissociate from the centrosome into the cytoplasm and concentrates under the cleavage furrow. By telophase Cep250 is observed as a discrete foci at the centrosome and within the midbody in the intercellular bridge.

Immunolocalization of Cep250 in differentiated tissue and centrioles

Northern blot analysis has indicated that Cep250 mRNA is more abundantly expressed in differentiated human tissue as compared to HeLa cells. To determine if

Figure 5.5. Immunolocalization of Cep250 in interphase. HeLa cells were double stained for pericentrin (B, E), and Cep250 (C, F), and then counterstained for DNA with DAPI (A, D). In early interphase Cep250 is observed as two small focussed dots at the centrosome (C). Pericentrin reactivity at the centrosome is significantly larger than that of Cep250 (compare B and C). In a later interphase cell, the centrosomes have duplicated and are separating (E). Cep250 reactivity is seen at both centrosome pairs (F). Bar = 10μm.

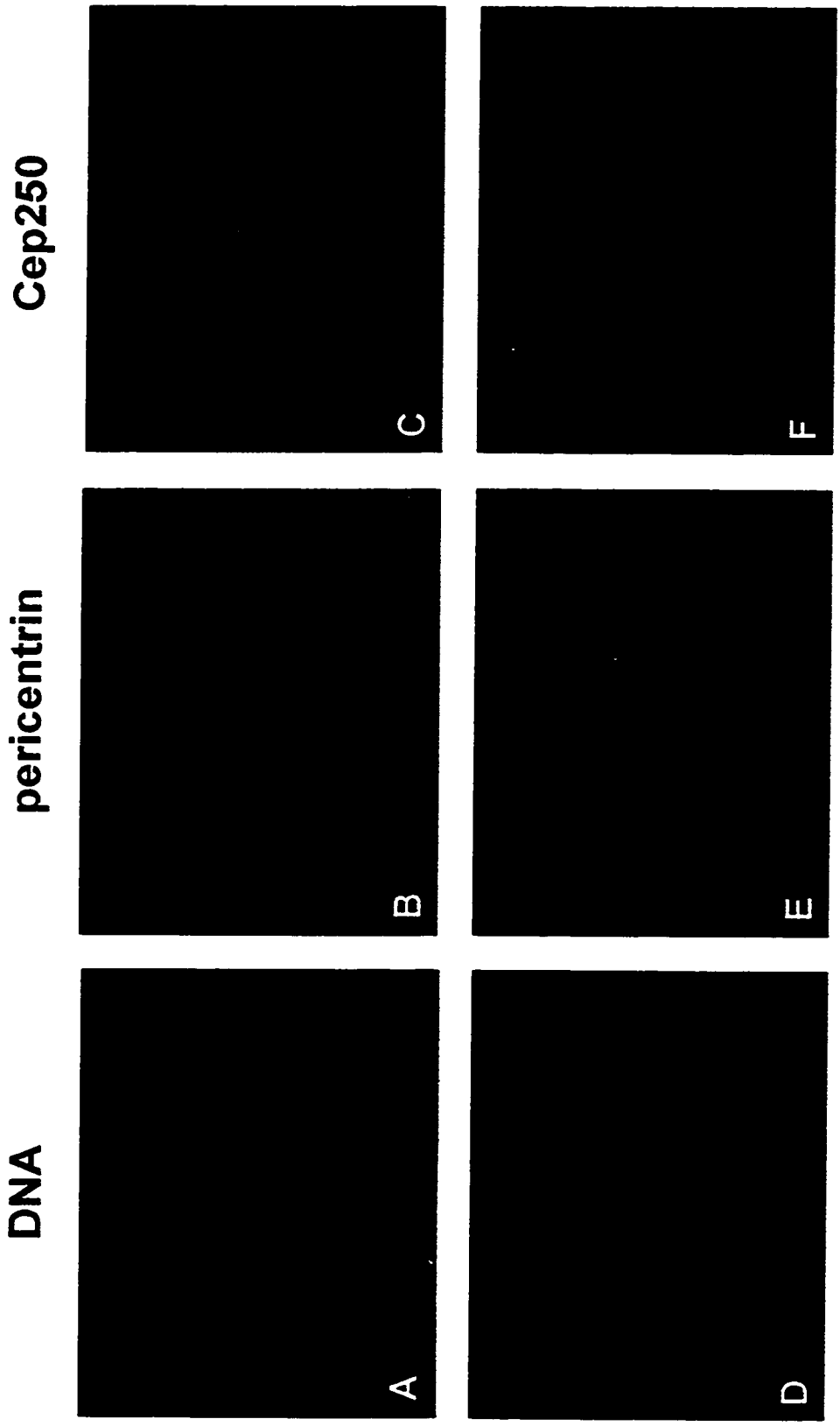

DNA
pericentrin
Cep250
A
B
C
D
E
F

Figure 5.6. A composite image of Cep250 and pericentrin or γ- tubulin. HeLa cells were double stained for Cep250 (green) and pericentrin (red) or Cep250 and γ-tubulin (red). Images were collected separately and merged together using Photoshop 4.0. Cep250 reactivity is seen adjacent to pericentrin reactivity in (A), and at the periphery of γ-tubulin in (B). Colocalization of Cep250 and γ-tubulin is seen as yellow in (B). Cep250 signal is always observed to be much smaller than that of pericentrin or γ-tubulin, and likely reflects its association with the centrioles.

pericentrin - Cep250

gamma tubulin - Cep250

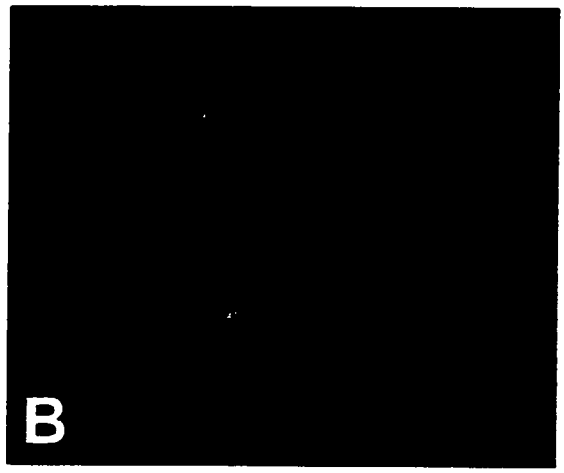


Figure 5.7. Immunolocalization of Cep250 during prophase. HeLa cells were double stained for pericentrin (B, E) and Cep250 (C, F), and then counterstained for DNA with DAPI (A, D). During early prophase pericentrin reactivity at the centrosome increases as a reflection of increased microtubule nucleation (B). At the same time Cep250 reactivity is observed to expand and become more diffuse around the centrosome (C). In late prophase pericentrin reactivity at the centrosome remains prominent, but Cep250 reactivity is diminished (F). Concurrent with the lose of Cep250 staining at the centrosome is an increase in cytoplasmic Cep250 reactivity (F). Bar = 10μm

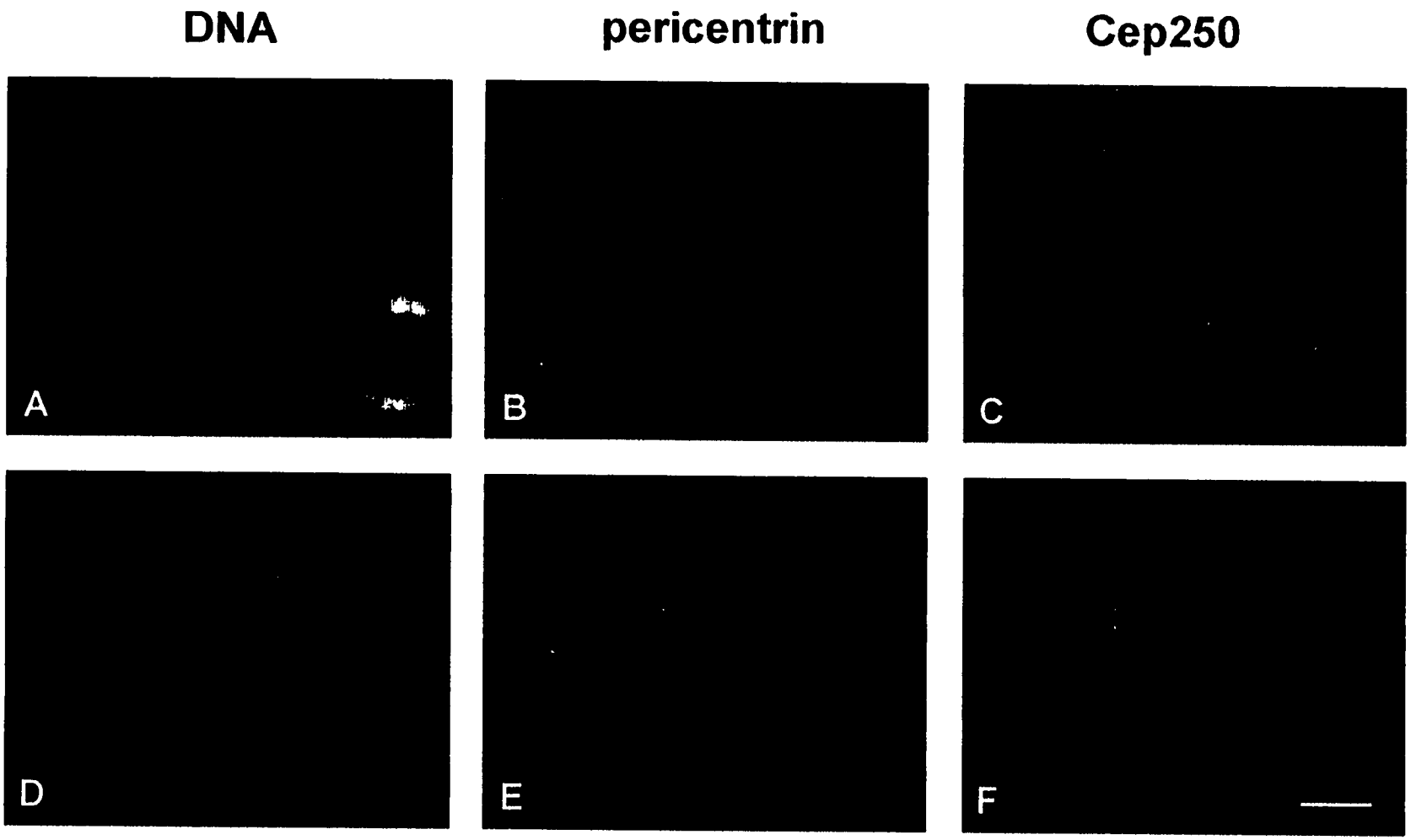

DNA
pericentrin
Cep250
A
B
C
D
E
F

Figure 5.8. Immunolocalization of Cep250 during metaphase and anaphase. HeLa cells were double stained for pericentrin (B, E), and Cep250 (C, F), and then counterstained for DNA with DAPI (A, D). During metaphase pericentrin is prominently detected at the spindle poles (B) whereas Cep250 is only weakly observed at the poles (C). Significant cytoplasmic Cep250 reactivity is observed throughout the cell and in the region of the mitotic spindle (C). In early anaphase Cep250 is barely detectable at the spindle poles and cytoplasmic reactivity is still prominent (F). Bar = 10μm

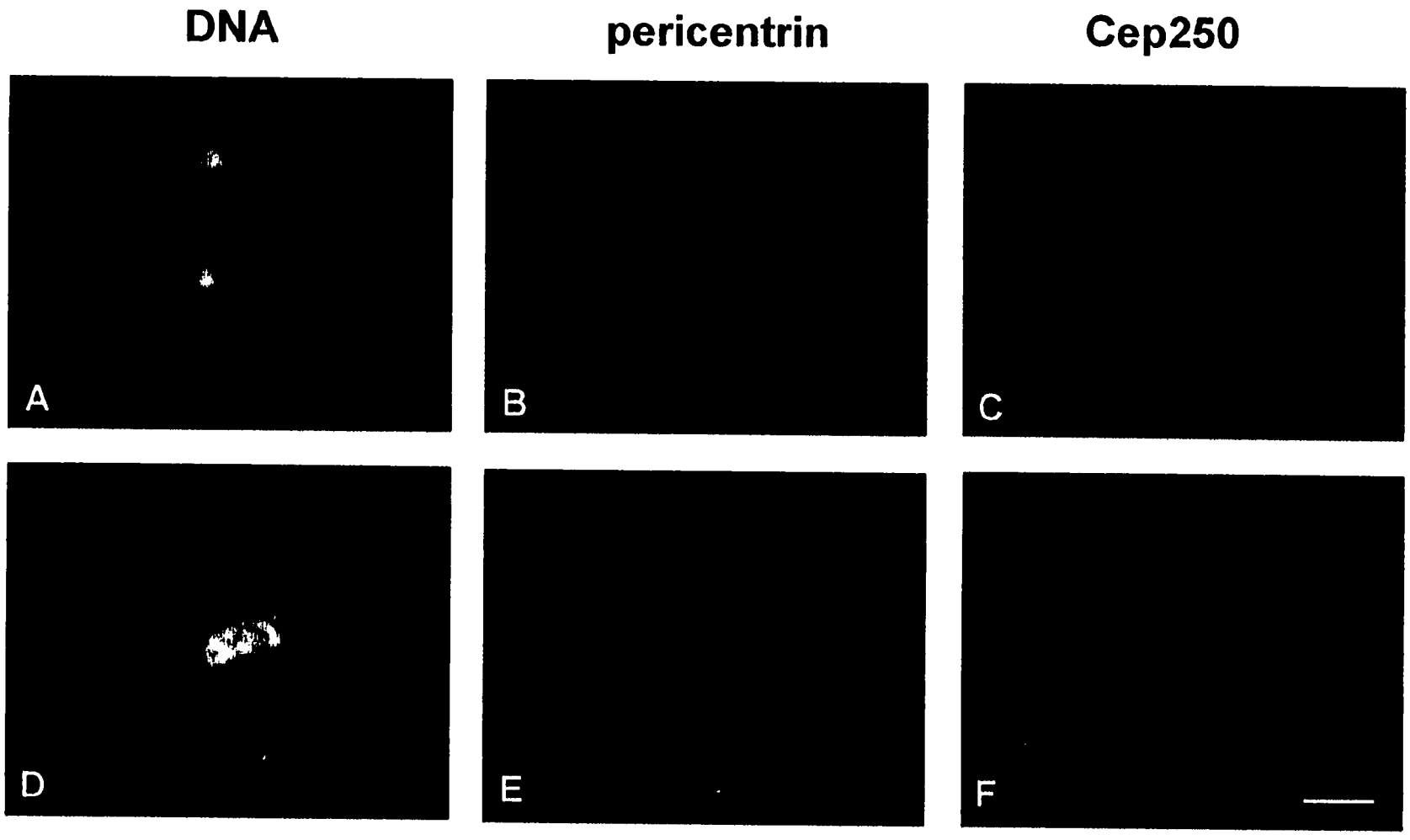

DNA
pericentrin
Cep250
A
B
C
D
E
F

Figure 5.9. Immunolocalization of Cep250 during late anaphase and telophase. HeLa cells were double stained for pericentrin (B, E) and Cep250 (C, F), and then counter stained for DNA with DAPI (A, D). During late anaphase Cep250 reactivity is more prominent at each centrosome as a diffuse dot, and cytoplasmic reactivity is strongly observed under the cleavage furrow (C). In late telophase early G1 cells Cep250 is now observed as a tightly associated foci at each centrosome (F). Two dots of reactivity are observed in one daughter cell, and likely reflect the centrioles of duplicated centrosomes. Furthermore, Cep250 reactivity is observed within the intercellular bridge at the midbody. (F). Bar = 10μm

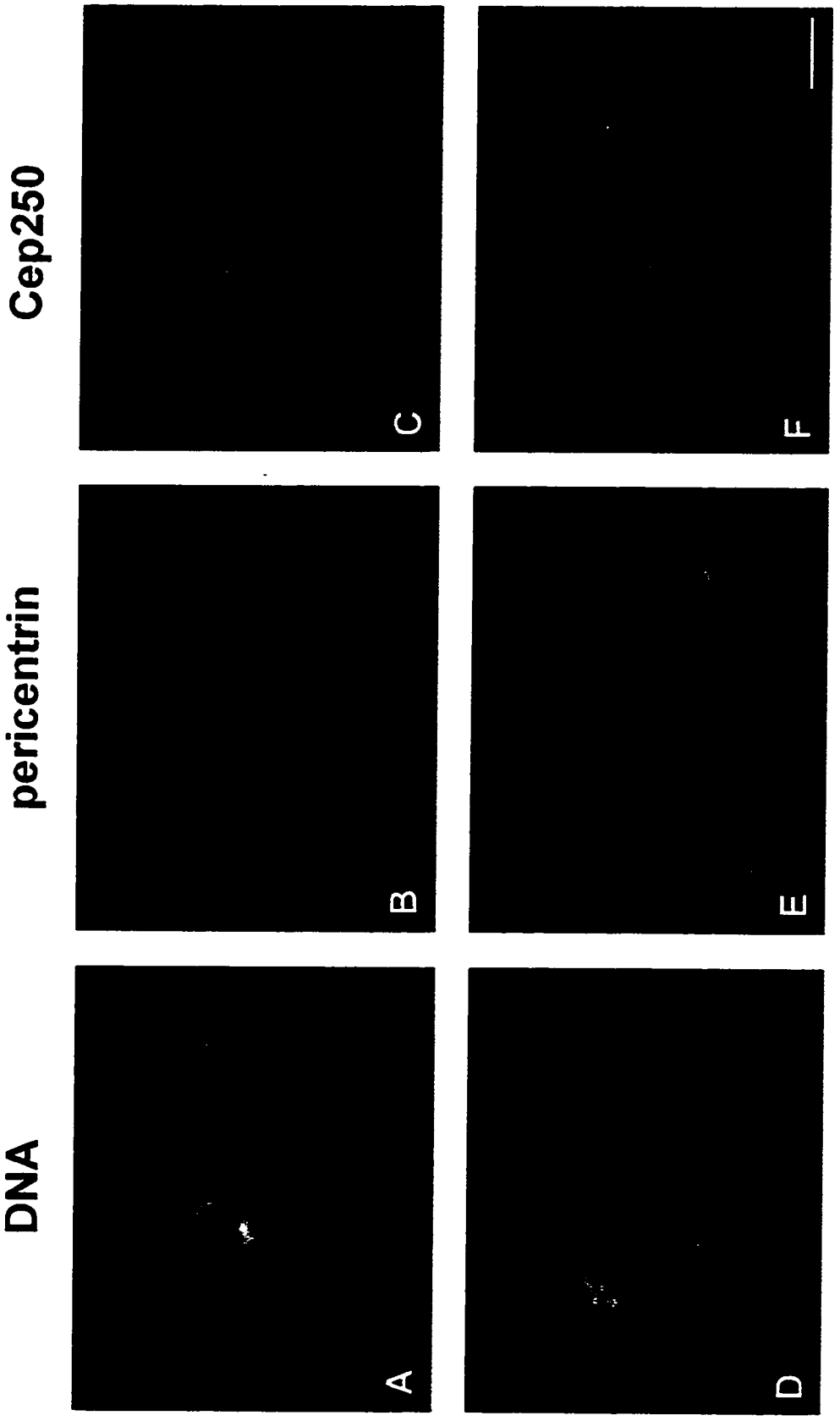

DNA
pericentrin
Cep250
A
B
C
D
E
F

Figure 5.10. Cep250 is a tightly associated centrosome protein during interphase. HeLa cells were double stained for CENP-F (A) and Cep250 (B). CENP-F nuclear reactivity is cell cycle regulated, showing no reactivity in G1 cells, weak reactivity in S cells and prominent nuclear staining at G2. Three cells in G1, S, and G2 show an increase in CENP-F nuclear reactivity (A). The same cells stained for Cep250 show prominent focussed centrosome reactivity (B). In the lower right cell (B) centrosomes have duplicated and separated to near opposite sides of the nucleus. Bar = 10μm

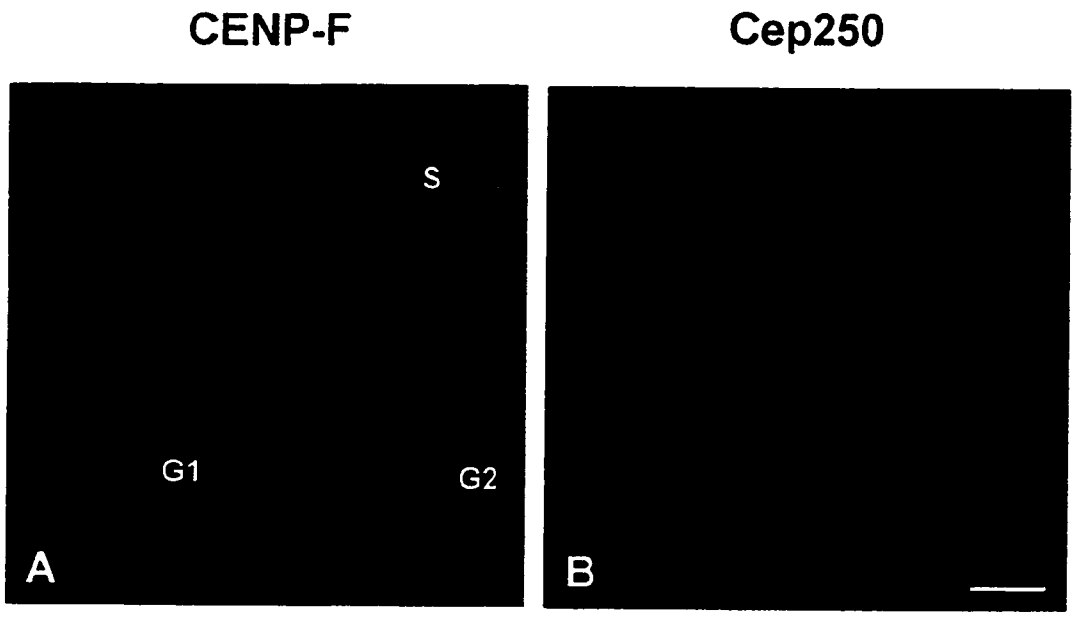
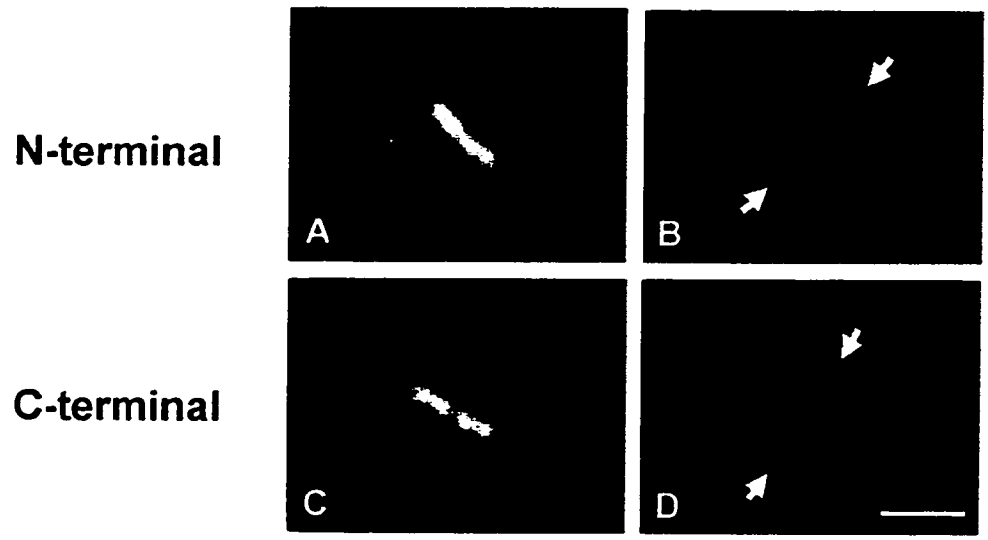

CENP-F
Cep250
S
G1
G2
A
B

Figure 5.11. The loss of Cep250 staining at the spindle poles is not due to epitope masking. HeLa cells were stained with antibody to the NH_2 terminus (anti-B) (B) or the COOH terminus (anti-43) (D) of Cep250, and counterstained for DNA with DAPI (A, C). Both antibodies show weak spindle pole staining (arrows), and prominent cytoplasmic reactivity (B, D). The pattern of reactivity observed with these antibodies is consistent with antibodies raised to other regions of Cep250. Bar = 10μm.


N-terminal
C-terminal
A
B
C
D

Cep250 protein was also abundant in differentiated tissue, indirect immunofluorescence was preformed on commercially prepared monkey cerebellum and testis tissue sections.

In cerebellum sections, Cep250 was observed in many cells as either one or two closely opposed foci adjacent to the nucleus (Fig. 5.12, B and B'). The size of the area stained by the Cep250 antibody in these cells is considerably larger that seen at the centrosome in G1 HeLa cells (compare Fig. 5.12, B or B' with Fig. 5.5, C). Furthermore, in monkey testis sections, Cep250 reactivity was also observed at the centrosome in specific cells. Smooth muscle cells of the seminiferous tubule and pachytene prophase cells all showed Cep250 reactivity as one or two foci (data not shown). Each of these foci also appeared larger that the centrosome reactivity seen in G1 HeLa cells. Primary spermatocytes, as judged by their nuclear morphology and location within the seminiferous tubule showed Cep250 reactivity as a large dot or "V "shaped structure adjacent to the nucleus (data not shown). Thus, immunostaining reveals that Cep250 retains its centrosome association in differentiated tissues and appears to be more abundant at the centrosome in comparison to HeLa cells.

Finally, in indirect immunofluorescence of HeLa cells, Cep250 reactivity is often observed to be much smaller than that of pericentrin or γ-tubulin. One possible explanation is that Cep250 is associated with the centriole rather than the PCM. In order to determine if Cep250 is present on centrioles, human spermatozoa were isolated and immunostained for Cep250. In sperm cells the proximal centriole is oriented transversely beneath the nucleus, and the remnant of the distal centriole is oriented in the axis of the sperm flagellum (Bloom, W. et al. 1986). In isolated sperm cells no detectable Cep250

reactivity was observed at either centriole (Fig. 5.13, F). Anti-pericentrin antibody also failed to stain the sperm centrioles (data not shown). In contrast, sperm cells stained with anti-β tubulin showed both proximal centriole and distal-centriole flagella staining (Fig. 5.13, C). Thus, Cep250 does not appear to associate with the centrioles found in sperm cells. However, as these are specialized cells, we can not rule out the possibility that Cep250 is discarded from the centrioles, or is present below the limits of detection by immunofluorescence.

Characterization of the Cep250 protein

DNA sequence analysis of the Cep250 cDNA clone predicts that it encodes a large protein of approximately 281 kDa. The large molecular size of Cep250 is not unexpected as other centrosomal antigens including pericentrin, PCM-1, and NuMA are all large proteins (Doxsey, S.J. et al. 1994, Balczon, R. et al. 1994, Lydersen, B.K. et al. 1980). Thus, to verify the complete cloning of Cep250, and confirm its predicted ORF and molecular weight, immunoblot analysis was preformed on HeLa protein using affinity purified antibody against the NH_2-terminus (anti-B), the COOH terminus (anti-43) and middle region (anti-m23) of Cep250. As shown in Figure 5.14 (lanes i), each antibody recognized a prominent protein band at approximately 280 kDa. Preimmune sera for each of these antibodies showed no reactivity with this band (Fig. 5.14, lanes p). Given that the DNA sequence upstream of the putative transcription initiation codon cannot extend the ORF due to the presence of multiple stop codons, and the estimated size of Cep250 of 281 kDa is in good agreement with its observed molecular weight of 280 kDa, I conclude that the complete open reading frame of Cep250 has been cloned.

Figure 5.12. Cep250 staining of monkey brain centrosomes. Monkey cerebellum sections were immunostained for Cep250 using anti-m23 (B, B'), and counterstained for DNA with DAPI (A, A'). Cep250 reactivity was seen as two closely opposed foci (centrioles) that are adjacent to the nuclear material (B, B'). Intact nuclei or cells were not observed in the tissue section.

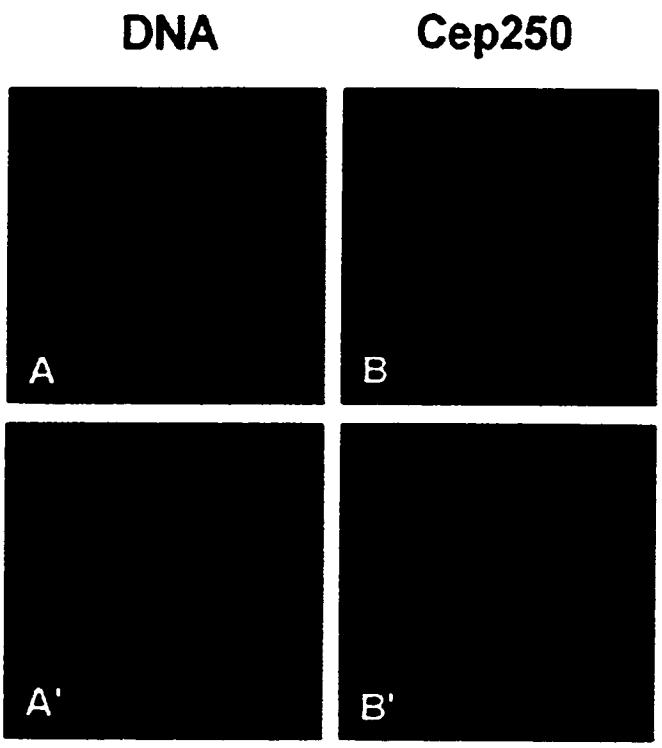
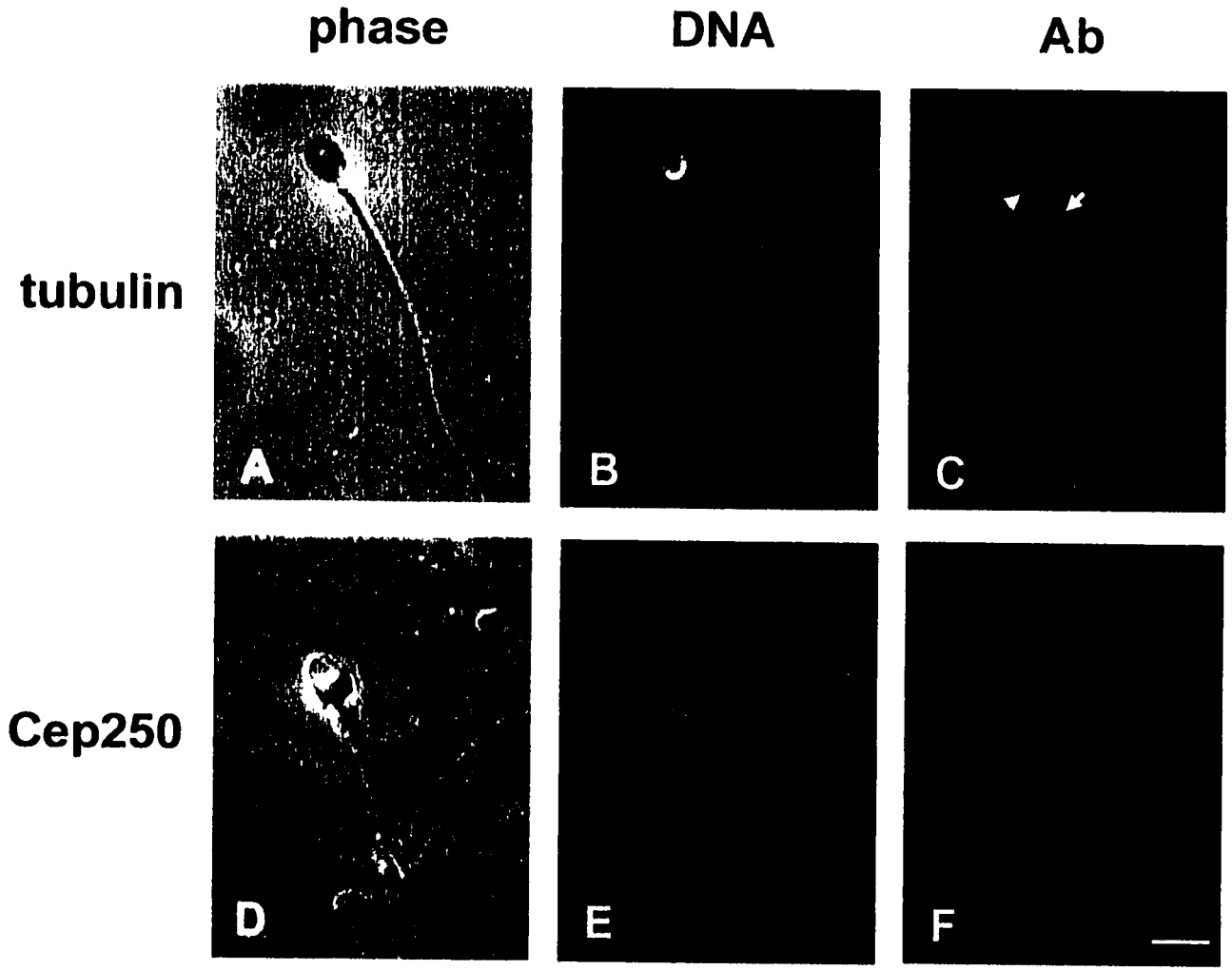

DNA
Cep250
A
B
A'
B'

Figure 5.13. Cep250 is not detected at the centrioles of human sperm. Isolated human sperm were immunostained for Cep250 (F) and β-tubulin (C), and counterstained for DNA with DAPI (B, E). Individual sperm cells were identified under phase contrast (A, D). Immunostaining of sperm cells with anti-β-tubulin identified the proximal (arrowhead) and distal centriole and flagella tail (arrow, C). No staining of either the proximal or distal centriole is observed when sperm cells are reacted with antibody to Cep250 (F). Bar = 4 μm.


phase
DNA
Ab
tubulin
Cep250
A
B
C
D
E
F

Using indirect immunofluorescence, Cep250 was prominently observed at the interphase centrosome, but was only barely detectable at the mitotic spindle poles. It is possible that the inability to detect Cep250 at mitosis may actually represent a change in the amount of the protein during the cell cycle. Therefore, to test the abundance of Cep250 during the cell cycle, immunoblot analysis was performed total interphase and mitotic protein. As seen in Figure 5.15, the relative levels of Cep250 between interphase and mitotic cells remain unchanged. Thus, even though Cep250 changes its cellular distribution from the centrosome to the cytoplasm during mitosis, its protein levels remain constant.

The loss of Cep250 staining at the mitotic spindle poles is also accompanied by an increase in total cytoplasmic reactivity. To investigate this further, soluble protein fractions of interphase and mitotic cells were collected after gentle permeablization in lysis buffer. The amount of Cep250 in each fraction was then determined by immunoblot analysis. As shown in Figure 5.16, Cep250 was detected in the soluble protein fractions of both interphase and mitotic cells. However, more Cep250 was found within the mitotic soluble protein fraction as compared to an equivalent amount of interphase soluble protein. This observation confirms the immunostaining results which show that most of Cep250 is released into the cytoplasm during mitosis.

The release of Cep250 from the centrosome into the cytoplasm prompted us to investigate whether this could be due to post-translational modification of the protein during mitosis. Often modification of a proteins results in an increase in its molecular weight. Soluble protein fractions of interphase and mitotic cells were separated by 6 %

Figure 5.14. Multiple antibodies to Cep250 recognize the same protein. Total HeLa protein was separated by 4 % SDS-PAGE and transblotted. Western blot analysis was preformed with antibody raised against the NH_2 terminus (anti-B), the COOH terminus (anti-43) or middle region (anti-m23) of Cep250. Each antibody (i) recognizes a high molecular weight protein at ~ 280 kDa (arrow). Preimmune sera (p) show no reactivity with this protein. Molecular weight markers are 220 and 97.4 kDa.

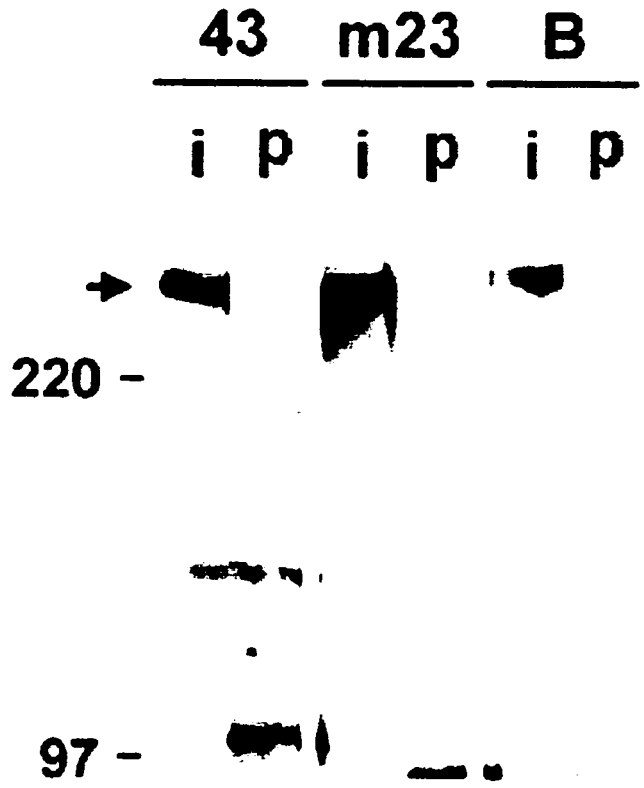

43
m23
B
i p i p i p
220 -
97 -

Figure 5.15. Cep250 protein levels are constant throughout the cell cycle. Equal amounts of total HeLa protein from interphase and mitotic cells was separated by 4 % SDS-PAGE and immunoblotted. Western blot analysis was carried out with a 1:500 dilution of antibody to Cep250 (anti-m23). A single band at ~ 280 kDa is detected in equivalent amounts in both the interphase and mitotic protein fractions. Molecular weight marker is 200 kDa.

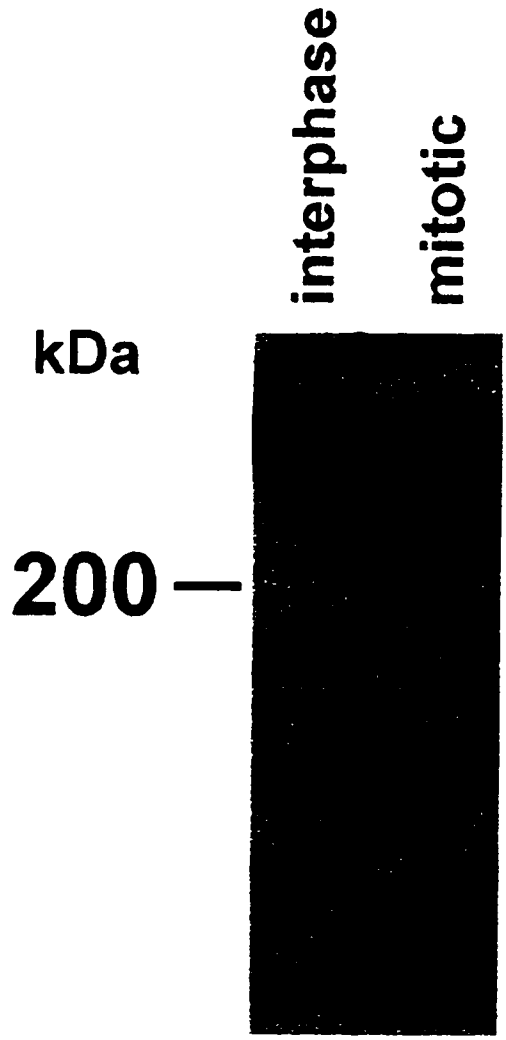
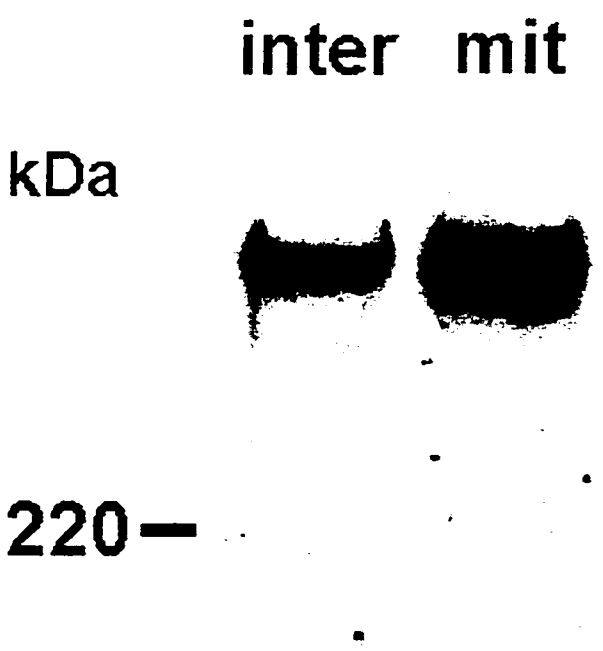

interphase
mitotic
kDa
200

Figure 5.16. Cep250 is abundant in the mitotic cytoplasm. Cep250 protein levels were determined in soluble fractions of HeLa interphase and mitotic cells after treatment for 1.0 min in lysis buffer (see methods and materials). More Cep250 protein is detected in the mitotic soluble fraction as compared to an equal amount of interphase soluble protein. Proteins were resolved on a 4% polyacrylamide gel and detected with anti-m23 antibody. Molecular weight marker is 220 kDa.


inter mit
kDa
220

SDS-PAGE, transferred to nitrocellulose, and immunoblotted for Cep250. Comparison of the Cep250 protein in mitotic cells with that found in interphase cells revealed that mitotic Cep250 had a slight decrease in electrophoretic mobility, indicating that its molecular size had increased (Fig. 5.17, mit s). A similar decrease in mobility is also seen when Cep250 is immunoprecipitated from mitotic soluble protein fractions (Fig. 5.17, ip). In addition, a single Cep250 protein band at 280 kDa was detected in the insoluble fractions of interphase and mitotic cells, whereas additional band(s) slightly above 220 kDa were detected in the soluble protein fractions. These additional bands may represent breakdown products of Cep250 or proteins that cross react with Cep250 antibody. Interestingly, these proteins also show a similar decrease in mobility in the mitotic soluble fraction (compare inter, s with mit, s, Fig. 5.17). Thus, the observed change in mobility of soluble Cep250 during mitosis could reflect a modification of the protein associated with its release into the cytoplasm.

Fusion of Cep250 to green fluorescent protein (GFP)

Sequence analysis of Cep250 revealed two major protein motifs, coiled-coil structure, and leucine zippers. Both types of motifs have been shown to mediate protein-protein interactions. For example, a coiled-coil region of CENP-E is required for its interaction with CENP-F and HBUBR1 at the kinetochore (Chan, G.K.T. et al. 1998), and leucine zippers are necessary for the dimerization of several transcription factors (Busch, S.J. et al. 1990). Thus, to determine which region(s) of Cep250 are required for its interaction with the centrosome, various domains of Cep250 were tagged with green fluorescent protein (GFP) and transfected into HeLa cells. After 24 hours, the transfected

cells were fixed and immunostained with antibody to pericentrin to serve as a centrosome marker. The colocalization of GFP signal and pericentrin (Cy3) on extracted and unextracted cells was scored for each transfection. Figure 5.18 illustrates the location of each Cep250 GFP construct and its ability to localize to the centrosome.

HeLa cells transfected with vector alone showed only nuclear and cytoplasmic GFP reactivity, no localization of GFP was observed at the centrosome (Fig. 5.19, A-C). However, in cells transfected with the full length Cep250 GFP construct, GFP reactivity was routinely observed as one or two dots at the centrosome (Fig. 5.19, F). In certain cells, Cep250 GFP was overexpressed and accumulated as large masses around the centrosome and in other areas of the cytoplasm (data not shown). Thus, GFP tagging confirms the immunostaining results, that indicate that Cep250 is a centrosome associated protein. To ensure that Cep250-GFP is properly expressed in these transfected cells, Western blot analysis was carried out on transfected cell protein. Anti-GFP antibody detected a single fusion protein of approximately 290 kDa in the transfected cell sample (Fig. 5.20 Cep250-GFP), whereas no reactivity was observed in protein from cells transfected with vector alone (Fig. 5.20, GFP). The 290 kDa GFP fusion protein is in good agreement with its expected size of 308 kDa (281 plus 27 kDa).

To define the region of Cep250 required to target it to the centrosome, various NH_2-terminal regions of Cep250 were tagged with GFP and transfected into HeLa cells. Cells expressing the constructs Del A-B GFP (Fig. 5.18) showed no GFP localization to the centrosome, rather GFP reactivity was observed throughout the cell cytoplasm (data not shown). Thus, the NH_2-terminal 1000 amino acids including the first four leucine zippers

are not required for Cep250 localization to the centrosome. However, cells expressing the constructs Del C-D GFP and Del F-G GFP did show GFP reactivity at the centrosome colocalizing with pericentrin reactivity (data not shown). All of these constructs contain a 178 amino acid stretch of the COOH terminus of Cep250 (amino acids 2263-2441). This region is predicted to adopt a coiled-coil structure, and contains the fifth leucine zipper of Cep250 between amino acids 2275 and 2296 (Fig. 5.18). To more precisely identify the amino acid sequence required to target these GFP fusion proteins to the centrosome, cells were transfected with the Del H GFP construct, consisting of 35 amino acids of Cep250 including the fifth leucine zipper fused to GFP (Fig. 5.18). Cells expressing this construct show GFP localization to the centrosome (Fig. 5.19, I). Weak cytoplasmic GFP reactivity is also observed in unextracted cells transfected with Del H GFP, and GFP reactivity can be seen at the midbody in telophase cells (data not shown). Therefore, Cep250s association with the centrosome appears to mediated by the fifth leucine zipper motif at its COOH terminus. Furthermore, this motif is also required for Cep250s localization to the midbody in the intercellular bridge.

Figure 5.17. Cep250 may be post-translationally modified during mitosis. Soluble (s) and insoluble (p) protein fractions from interphase and mitotic HeLa cells were collected after treatment for 10 min in lysis buffer (see methods and materials) and immunoblotted. Cep250 was detected in each fraction with antibody directed against the NH_2 terminus (anti-B). Insoluble protein show a single Cep250 band at ~ 280 kDa, whereas additional proteins bands (possible breakdown products) are seen in the soluble fractions. Cep250 from the mitotic soluble fraction appears slightly larger than Cep250 from the soluble interphase protein, and may be suggestive of a post-translationally modified protein. A similar shift in size is also observed for the ~ 220 kDa breakdown product, and Cep250 immunoprecipitated (ip) from mitotic HeLa protein (arrow). All proteins were resolved on a 6 % polyacrylamide gel. Molecular weight markers are 220 and 97.4 kDa.

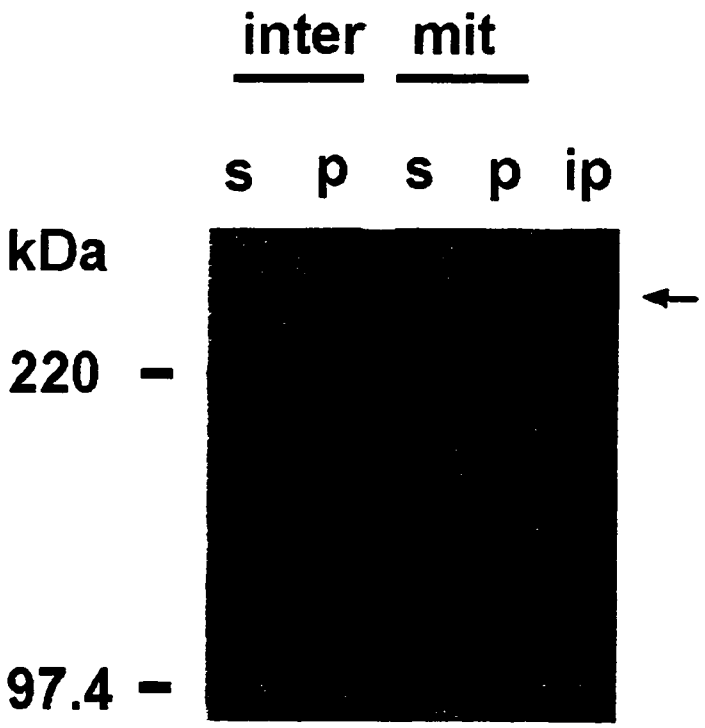

inter
mit
s
p
s
p
ip
kDa
220
97.4

Figure 5.18. Schematic diagram of Green Fluorescent Protein (GFP) tagged Cep250 constructs. Various region of Cep250 were tagged with GFP at the COOH terminus, transfected and scored for their ability to target the centrosome. Each deletion (DEL) construct was tested for centrosome targeting in two separate experiments on extracted and unextracted cells. Colocalization of GFP with the centrosome protein pericentrin is scored as positive (+), whereas no colocalization is scored as negative (-). Amino acid positions are shown above each construct.

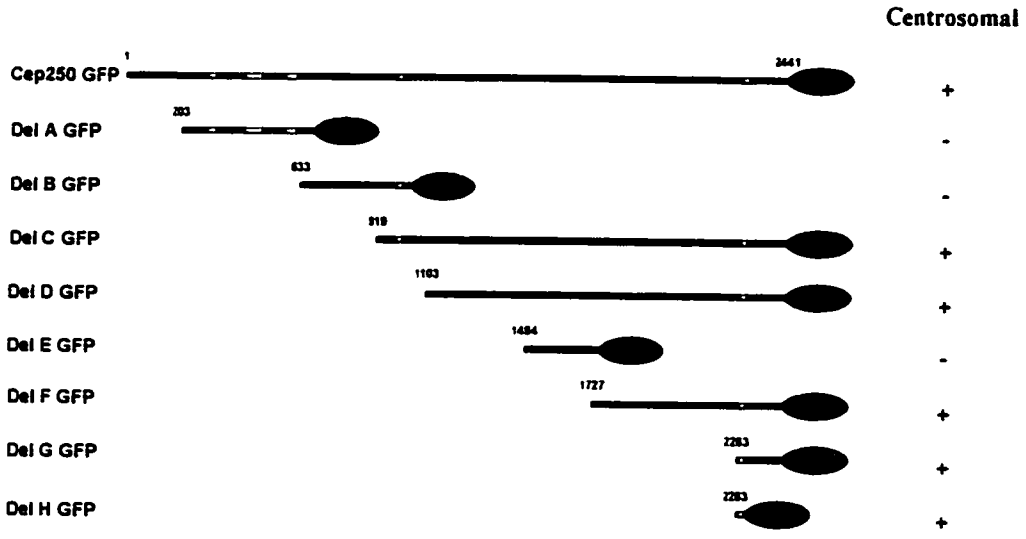

Centrosomal
Cep250 GFP
1
3441
+
Del A GFP
283
-
Del B GFP
533
-
Del C GFP
919
+
Del D GFP
1163
+
Del E GFP
1494
-
Del F GFP
1727
+
Del G GFP
2283
+
Del H GFP
2283
+

Figure 5.19. Targeting of GFP tagged Cep250 to the centrosome. HeLa cells were transfected with the vector pEGFP-N1 alone, or the constructs Cep250-GFP or Del H-GFP. Cells expressing the GFP constructs (C, F, I) were fixed and double stained for the centrosomal protein pericentrin (B, E, H,), and counterstained for DNA with DAPI (A, D, G). Cells expressing the vector alone do not show centrosome targeting of GFP (C). Cells expressing the GFP tagged Cep250 construct show GFP centrosome staining (F) that colocalized with pericentrin reactivity (E). Centrosome targeting of GFP is observed for Del H-GFP (I) where 35 amino acids of the COOH terminus of Cep250 including the fifth leucine zipper are fused to GFP. Bar = 10μm.

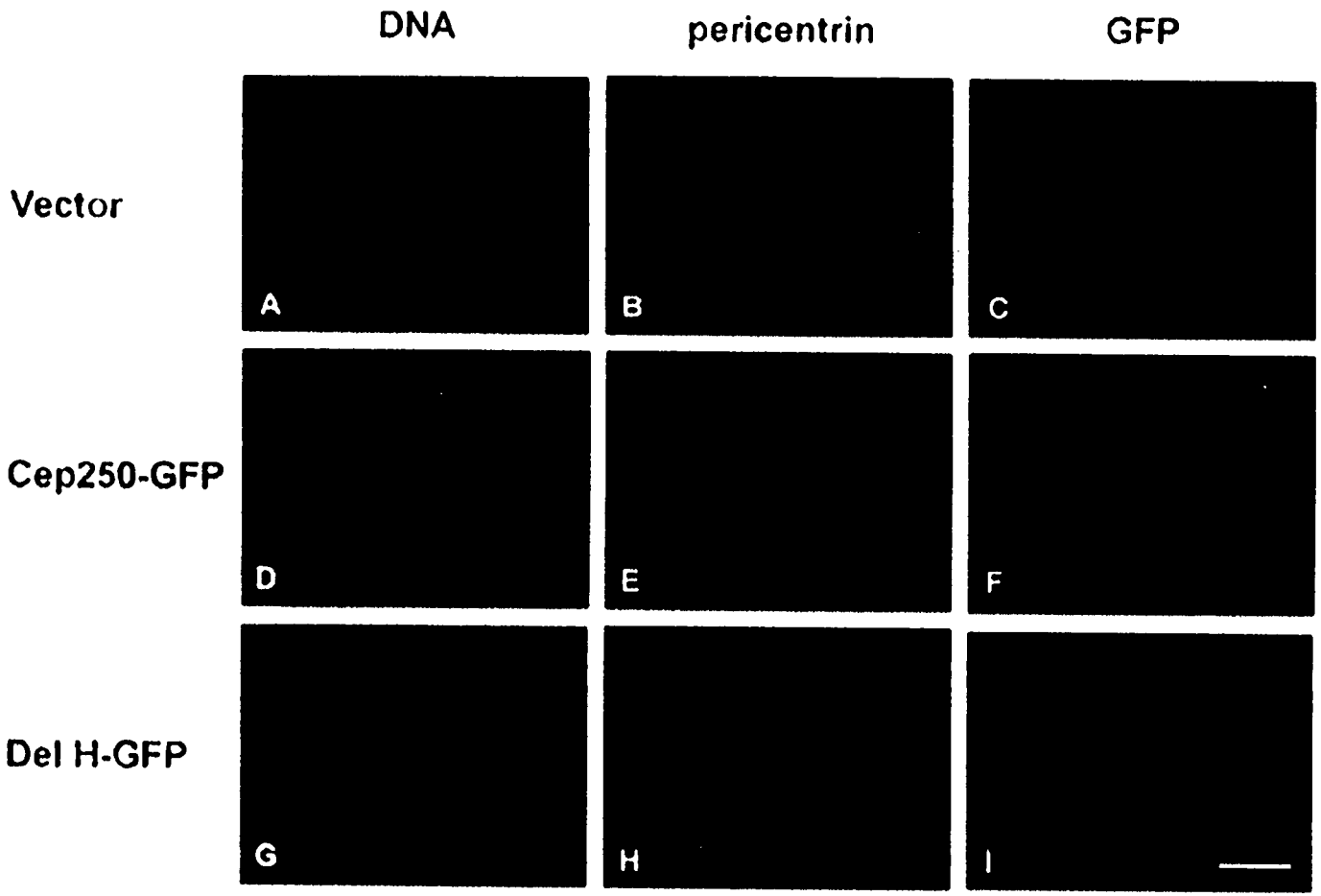
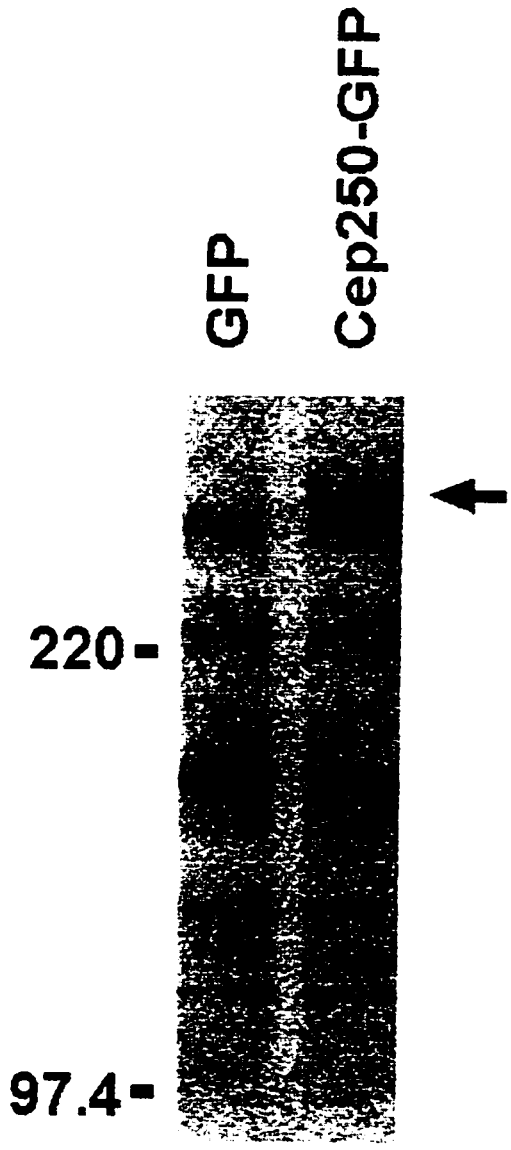

DNA
pericentrin
GFP
Vector
Cep250-GFP
Del H-GFP
A
B
C
D
E
F
G
H
I

Figure 5.20. Western blot analysis of GFP tagged Cep250. HeLa cells transfected with the construct Cep250-GFP or vector alone were allowed to express for 24 h before total protein was collected and separated by 6 % SDS-PAGE. Western blot analysis with an anti-GFP antibody (Clontech) detected a single strong band at ~ 290 kDa in Cep250-GFP transfected cell protein. An equivalent band was not detected in protein from cells transfected with vector alone. Molecular weight marker is 220 kDa.


GFP
Cep250-GFP
220
97.4

DISCUSSION

Identification of a novel centrosome protein

A human autoimmune serum reactive with the centrosome throughout the cell cycle was used to identify a new centrosomal protein, Cep250. Human autoimmune sera have been successfully used to identify other centrosomal proteins such as PCM-1 (Balczon, R. et al. 1994), pericentrin (Doxsey, S.J. et al. 1994), and Cep110 (see SECTION V C). The complete cDNA encoding Cep250 has been cloned and analysed. Cep250 is a 281 kDa protein that is predicted to have extensive regions of coiled-coil structure. The large size and coiled-coil nature of Cep250 is not uncommon for centrosomal proteins. Ninein, PCM-1, and pericentrin are all examples of other large centrosomal proteins, and both ninein and pericentrin are predicted to adopt a coiled-coil structure (Bouckson-Castaing, V. et al. 1996, Doxsey, S.J. et al. 1994). Proteins sharing these similar characteristics have also been identified in the yeast spindle pole body (Marschall, L.G. et al. 1997, Lane, H.A. et al. 12-1996), and in the mammalian kinetochore (Liao, H. et al. 1995, Yen, T.J. et al. 1992), both of which are highly ordered multi-protein complexes like the centrosome. Interestingly, of the 27 known spindle pole body proteins identified in the yeast *S. cerevisiae*, 19 have coiled-coil structure (Wigge, P.A. et al. 1998). Thus, this motif appears to have an essential function in the formation of microtubule organizing centers.

Cep250 is also found to have five leucine zipper domains, three at the NH_2-terminus, one in the middle region, and one at the COOH terminus. Leucine zippers have been well established as motifs involved in protein-protein binding (Busch, S.J. et al.

1990). Two other mammalian centrosome proteins, ninein and Cep110, have also been shown to have leucine zippers (Bouckson-Castaing, V. et al. 1996 , and this dissertation). At this point it is not known if ninein or Cep110 can interact with Cep250 through the leucine zipper motif, but it is conceivable that this motif plays an important role in establishing protein interactions within the centrosome (see below).

DNA sequence analysis with the COOH terminus of Cep250 revealed some homology to a proposed mouse intranuclear matrix protein INMP (Menz, K. et al. 1996). This protein was identified with an antibody raised to nuclear matrix preparations. Antibody to INMP reacts with the nucleus during interphase and the cytoplasm during mitosis using indirect immunofluorescence and identifies a 68 kDa protein in a Western blot (Menz, K. et al. 1996). In analysing the INMP DNA sequence, it became apparent that the predicted ORF for the protein incorrectly terminated, and in fact was a portion of mouse Cep250 (G. J. Mack unpublished results). Reactivity within the nucleus has never been observed with antibodies raised against Cep250. Other than weak homology with other coiled-coil motifs, Cep250 is a novel protein.

The association of Cep250 with the centrosome is cell cycle regulated

As shown in Figures 5.5-5.9, Cep250 is a centrosome associated protein that exhibits a unusual cell cycle distribution in HeLa cells. Comparison of Cep250 reactivity with CENP-F nuclear staining has shown that Cep250 remains tightly associated with the centrosome throughout the duration of interphase. Once the cell has entered prophase, Cep250 undergoes a change in its organization and appears to dissociate from the centrosome into the cytoplasm. Near the completion of cell division, Cep250 is once again

observed as a discrete focus at the centrosome and is found within the midbody in the intercellular bridge. This cell cycle pattern of Cep250 was completely unexpected, as it differs significantly from the centrosome immunofluorescent pattern observed with the human autoimmune serum used in its identification. As shown in Figure 3.1, this autoimmune serum strongly reacts with the centrosome throughout the cell cycle. As described in SECTION III, this autoimmune serum contains autoantibodies to multiple centrosome proteins, some of which are known to be cell cycle invariant, such as pericentrin and ninein. Thus, autoantibodies to these centrosome proteins likely obscure the loss of Cep250 from the centrosome. However, prominent cytoplasmic reactivity during metaphase is observed with this autoimmune serum and is consistent with what is observed with in immunofluorescence with affinity purified Cep250 antibody. In addition this human autoimmune serum fails to react with the midbody during telophase, even though Cep250 can be found here using rabbit antibodies. This is not unexpected, as only one of the rabbit antibodies raised against Cep250 (anti-m23) reveals this distribution, suggesting that the other antibody epitopes on Cep250 are inaccessible in the midbody. What function Cep250 has in the intercellular bridge is unknown, however, its location to the midbody, where antiparallel microtubules of the spindle overlap, could suggest that it functions in holding or bundling these microtubules together. Alternatively, Cep250 may associate with the midbody as a method for its disposal at the completion of cell division. Cep250 and the kinesin-like protein CHO1 (Sellito, C. et al. 1988) are the only two centrosomal residents known to redistribute to the midbody during telophase.

Cep250 represents one of only a handful of centrosomal proteins known to undergo

a cell cycle change in distribution. The protein PCM-1 shows a similar cell cycle distribution as Cep250. It is found at the centrosome through most of interphase and then dissociates into small foci throughout the cytoplasm during mid to late G2. Very little spindle pole staining is observed, and centrosome reactivity only returns during G1 (Balczon, R. et al. 1994). The kinesin-like protein CHO1 is also observed to associate with interphase but not mitotic centrosomes (Sellito, C. et al. 1988). Cell cycle changes in centrosome morphology have also been documented. For example, dense granules have been observed to associated with the centrosome during interphase, but are absent from mitotic centrosomes (Rattner, J.B. 1992c). Furthermore, the PCM is known to expand and increase in complexity as the cells enters mitosis. This change in the PCM is correlated with the increase in microtubule nucleation that occurs during mitosis (Rieder, C.L. et al. 1982b, Gould, R.R. et al. 1977). The PCM proteins pericentrin and γ-tubulin are involved in microtubule nucleation and show an increase in reactivity at mitotic centrosomes (Doxsey, S.J. et al. 1994, Joshi, H.C. 1993). Thus, the cell cycle change in Cep250 reactivity at the centrosome further reflects the dynamic nature of the centrosome during the cell cycle.

Concurrent with the decrease in Cep250 staining at the centrosome at mitosis is an increase in reactivity in the cell cytoplasm. This cytoplasmic staining is likely a result of the release of Cep250 from the centrosome. Antibodies directed against different epitopes on Cep250 show an identical immunofluorescence pattern with respect to centrosome and cytoplasmic staining during the cell cycle. Thus, it is unlikely that the unusual cell cycle distribution of Cep250 is a result of the masking of antibody epitopes.

Direct comparison of pericentrin and γ-tubulin reactivity at the centrosome with that of Cep250 indicates that Cep250 occupies a small part of the centrosome and is probably a component of the centriole(s). Cep250 reactivity is often found at the periphery of pericentrin or γ-tubulin reactivity may simply reflect the three-dimensional organization of the centrosome, where two right angled centrioles can be oriented in any direction within the PCM.

The reduction of Cep250 at the centrosome during mitosis suggests that it is most likely not required for a general centriole/centrosome function, rather it may have a specific cell cycle dependent function. Further analysis and discussion of Cep250 function will be presented in SECTION V B.

Expression of Cep250 is differentiated tissues

Northern blot analysis of Cep250 from differentiated human tissues shows that it is ubiquitously expressed, and at comparable levels. Confirmation of these results by RNase protection have been done on mouse tissue for a protein identical to Cep250 (Fry, A.M. et al. 1998a). The single ~ 8 kb signal that is observed on the human tissue blot is also in excellent agreement with the 7814 bp cDNA cloning results. Furthermore, since only one hybridizing signal is detected, it is likely that Cep250 is not alternatively spliced, although difficulty in detecting these forms can not be ruled out at this time.

Cep250 has also been immunolocalized in brain and testis tissue sections of monkey. In both cases specific centrosomal staining was observed. The finding of Cep250 in brain cells, which are characterized as being in G0 stage of the cell cycle, and in early meiotic cells, suggests that Cep250 may have a centrosomal function that is common to

both quiescent and dividing cells. The larger area of centrosome reactivity seen with Cep250 antibody in brain and testis cells reflects the more abundant mRNA levels that are detected by Northern blot in brain and other differentiated tissues. In addition, this may also indicate an alteration in centrosome morphology that accompanies the passage of a cell into G0.

Cep250 reactivity was not observed at either the proximal or distal centrioles in human sperm cells. It is possible that during the maturation of this cell, Cep250 is discarded. Immunofluorescence of testis sections support this idea, as centrosome staining was not observed in cells that had progressed beyond the prophase stage of pachytene. Thus, Cep250 appears to have a similar cell cycle regulated centrosome association during meiosis. Upon fertilization, Cep250 may be restored to the forming centrosome by the oocyte, although its presence in the oocyte cytoplasm has not been investigated. Alternatively, Cep250 may be present at the sperm centrioles, but below the limits of detection by immunofluorescence.

Characterization of the endogenous Cep250 protein

Multiple antibodies raised against different regions of recombinant Cep250 fusion protein have been used in immunoblot analysis of HeLa protein. Antibodies directed against either the NH_2, middle, or COOH region epitopes all recognize the same ~ 280 kDa protein. This indicates that the predicted ORF from the assembled cDNA clones does encode a high molecular weight protein, and the translation initiation and termination sites have been properly identified. The high molecular weight of Cep250 makes it the largest centrosomal protein yet identified. Immunoblot analysis has also shown Cep250 to be

present in equivalent amounts in total interphase and mitotic protein fractions and suggests that Cep250 protein levels are constant throughout the cell cycle, even though it undergoes a change in cellular distribution. The cell cycle dependent centrosomal protein PCM-1 also shows a constant level of protein throughout the cell cycle (Balczon, R. et al. 1994). Western blot analysis has also indicated that Cep250 is more abundant in the cytoplasm of mitotic cells as compared to interphase cells. This is consistent with the observation that Cep250 cytoplasmic staining increases at the beginning of prophase and remains prominent until late anaphase-telophase. Because both Cep250 and PCM-1 are released from the centrosome into the cytoplasm during mitosis, but are not degraded, it is possible that they have, (1), an alternative function in the cytoplasm apart from their role at the centrosome, or (2), they are required by the centrosome for a specific function during late telophase-early interphase, before new proteins can be re-synthesized. Consistent with this idea is the absence of PEST (Proline, Glutamic acid, Serine, Threonine) sequences in Cep250 or PCM-1, which usually indicate proteins that are rapidly degraded in stage dependent fashion (Rodger, S. et al. 1986). An explanation for Cep250 release from the centrosome during mitosis is currently unknown and will be further discussed in SECTION V B.

The release of Cep250 from the centrosome only during mitosis prompted an investigation of the nature of mitotic Cep250 protein. Comparison of Cep250 from soluble interphase and mitotic protein preparations revealed Cep250 from mitotic cells to have a slightly larger molecular weight. One possible explanation is that Cep250 is post-translationally modified during mitosis, and this modification may be responsible for its release from the centrosome. Both protein glycosylation and phosphorylation are

modification processes that may effect the mobility of proteins. Protein sequence analysis indicates that Cep250 has four potential glycosylation sites, and circumstantial evidence for protein glycosylation has been reported for the CCD41 centrosomal protein (Rothbarth , K. et al. 1993). Protein phosphorylation is also a well known method of modifying proteins, and specific mitotic phosphorylation has been described for a number of cytoskeletal protein such as NuMA (Compton, D.A. et al. 1995), HsEg5 (Blangy, A. et al. 1997) and the centrosome protein centrin (Lingle, W.L. et al. 1998). Cep250 contains several sites for potential phosphorylation by cA/GMP dependent protein kinase, casein kinase II, protein kinase C, tyrosine kinase and cyclin dependent kinase. Many protein kinase such as cA/GMP dependent kinase, Ca^{2+}/calmodulin-dependent kinase, casein kinase I/II, Ipl1/aurora related kinase, polo-like kinase, Nek2 kinase and p34cdc2 have also been shown to localize to the centrosome (Browne, C.L. et al. 1980, Ohta, Y. et al. 1990, Brockman, J.L. et al. 1992, Krek, W. et al. 1992, Gopalan, G. et al. 1997, Golsteyn, R.M. et al. 1995, Fry, A.M. et al. 1998b, Bailly, E. et al. 1989, Rattner, J.B. et al. 1990). Furthermore, direct evidence for the phosphorylation of centrosomal proteins is also found in the report that the monoclonal antibody, MPM-2, against a phosphorylated epitope (Davies, F.M. et al. 1983), reacts with phosphoproteins at the centrosome (Vandre, D.D. et al. 1984). Intense centrosomal MPM-2 staining is observed at the prophase-prometaphase transition, and persisted until the following G1 phase (Vandre, D.D. et al. 1984). Finally, both cAMP dependent kinase and p34cdc2 have been shown to phosphorylate proteins in enriched centrosome protein fractions (Keryer, G. et al. 1995). Although the role that phosphorylation plays in the function of any specific centrosomal protein has not yet been

characterized, phosphorylation of centrosomal components is known to promote microtubule nucleation at mitosis (Centonze, V.E. et al. 1990). Recently (Fry, A.M. et al. 1998a) has identified a protein identical to Cep250 using a two hybrid screen with the Nek 2 kinase. Subsequently, they have shown that this protein is phosphorylated at its COOH terminus *in vitro* by the Nek 2 kinase. Further discussion of the results of Fry, A. M. et al. (1998a) will be presented in SECTION V B.

In soluble protein fractions from interphase and mitotic cells, Cep250 antibody also recognizes a protein doublet at ~220 kDa. This protein doublet may be breakdown products of Cep250, alternatively spliced forms, or cross reactive proteins. An additional ~ 190 kDa protein band has been observed on Western blots of human T-lymphoblast protein with antibodies raised against the COOH terminus of a protein identical to Cep250 (Fry, A.M. et al. 1998a).

Although Figure 5.17 shows Cep250 immunoprecipitated from soluble mitotic protein, any attempt to repeat this experiment or to immunoprecipitate Cep250 from interphase cells has been unsuccessful. At this time it is not known if this is a reflection of the low abundance of Cep250 in cells or the inadequacy of recombinant antibodies to recognize nondenatured protein.

Analysis of the Cep250 protein has revealed it to consist essentially of non-coiled-coil ends separated by a long central coiled-coil domain. In addition, five leucine zippers are found within its protein sequence. Thus, it was not obvious which domain(s) were required for its interaction with the centrosome. In order to locate the centrosome targeting domain of Cep250, various regions of the protein were tagged to GFP. By fusing full

length Cep250 to GFP, we confirmed that Cep250 is a centrosome associated protein. Furthermore, molecular dissection and GFP tagging demonstrated that a 35 amino acid sequence, encompassing the COOH terminal leucine zipper, is all that is required for the centrosomal targeting of Cep250. In addition, this sequence is responsible for localization to the midbody during telophase. It is possible that this leucine zipper may interact with two proteins, one at the centrosome and another at the midbody.

Alternatively, this leucine zipper motif may interact with one protein throughout cell division, and it is this protein that initiates a cell cycle redistribution from the centrosome to the cytoplasm and midbody. The protein binding domains for other mammalian centrosome proteins have not been reported, and the small size of Cep250s targeting domain was unexpected. However, equally small targeting domains have been characterized in other cytoskeletal proteins such as TAU (Butner, K.A. et al. 1991), MAP2 (Cleveland, D.W. 1990), and MAP4 (Olson, K.R. et al. 1995). Furthermore, only 42 amino acids at the NH_2-terminus of the inner centromere proteins (INCENPs) has been shown to localize these proteins to spindle midzone and midbody during anaphase (Mackay, A.M. et al. 1993). The four remaining leucine zippers that are not required for the centrosome association of Cep250 may function in homo or hetero dimerization of the protein at either the centrosome, cytoplasm or midbody.

The Cep250-GFP fusion protein will likely be an important tool for the continued investigation of this protein and of the centrosome in general. Cell cycle analysis of Cep250-GFP has not been rigorously investigated as optimal conditions for transfected cell viability and cycling have not yet been established.

In conclusion, this work has led to the identification of a novel centrosome protein that I have designated Cep250. This protein is 281 kDa in size and is highly coiled-coil in structure. Antibodies raised to Cep250 reveal that it has a unique cell cycle distribution, being strongly centrosome associated during interphase, and only weakly detectable at the centrosome during mitosis. Concurrent with the loss of Cep250 staining at the mitotic spindle poles is the increase in Cep250 reactivity in the cytoplasm. In addition, Cep250 has been observed at the midbody within the intercellular bridge. Characterization of Cep250 protein reveals that it contains five leucine zipper motifs, of which only the COOH terminal leucine zipper is required for centrosome targeting. Furthermore, Cep250 has many potential phosphorylation sites, and may be post-translationally phosphorylated or glycosylated during mitosis. Northern blot experiments have demonstrated that Cep250 is ubiquitously expressed in most human tissues, and Cep250 protein has been detected at the centrosome in monkey brain and testis.

SECTION V

PART B : Function of Cep250 at the Centrosome

INTRODUCTION

The centrosome is responsible for organizing and regulating the microtubule cytoskeleton throughout the cell cycle. A number of changes are observed in the microtubule cytoskeleton as the cell progresses through division. During interphase relatively long and stable microtubules are nucleated from the centrosome. These microtubules are essential for various intercellular transport processes and for maintaining cell shape. During mitosis these microtubules are disassembled and replaced with short dynamic microtubules that are necessary for centrosome separation, the attachment of the chromosome at the kinetochore, and the formation of the bipolar spindle. At the completion of mitosis, the interphase microtubule array is once again reestablished after the centrosomes have migrated back to the intercellular bridge.

The change in microtubule organization and dynamics during the cell cycle is also reflected within the centrosome. The PCM directly nucleates microtubules (Gould, R.R. et al. 1977, Rieder, C.L. et al. 1982b), and has been shown to be a dynamic substance with a composition that fluctuates during the cell cycle (Gould, R.R. et al. 1977, Rieder, C.L. et al. 1982b, Rattner.J.B 1992c). During interphase the PCM around the centrioles is sparse, and dramatically increases in size and complexity during mitosis. How the centrosome regulates microtubule dynamics is still a matter of debate. However, it has been shown that the increase in microtubule nucleation that accompanies the entry into mitosis is a result of PCM protein phosphorylation (Centonze, V.E. et al. 1990).

As shown in SECTION V A, Cep250 associates with the centrosome in a cell cycle

dependent manner. During interphase Cep250 remains tightly associated with the centrosome, however, during mitosis Cep250 is released from the centrosome into the cytoplasm. In this part of the dissertation, I describe the association of Cep250 with microtubules and microtubule nucleation. Cep250 is found to associate with the centrosome independent of microtubules and its immunolocalization during the cell cycle is suggestive of a role in the nucleation of mitotic microtubules, and/or in the maturation of the centrioles during the cell cycle. Furthermore, an identical protein to Cep250 has been identified by Fry, A. M. et al. (1998a). Their proposed function of this protein, is discussed in relation to the data obtained for Cep250.

METHODS AND MATERIALS

Drug treatment and indirect immunofluorescence

HeLa cells were seeded onto coverslips 24- 48 h prior to use. In some instances HeLa cell coverslips were treated with nocodazole (SIGMA, 6μg/ml) for 4 hr at 37°C. Coverslips were fixed in methanol at -20°C, and blocked with1:200 NGS-DPBS at room temperature for 30 min. After rinsing, the coverslips were incubated with antibody to Cep250 (anti-B) at a dilution of 1:50 in NGS-DPBS for 30 min at 37°C. Following a wash in DPBS, the coverslips were incubated with 1:1000 anti-β-tubulin (SIGMA) or 1:10000 anti-pericentrin for 30 min at 37°C . The coverslips were washed for 2 min, and incubated with a mixture of FITC-conjugated anti-mouse (1:400) and Cy3-conjugated anti-rabbit (1:400) secondary antibody in NGS-DPBS for 30 min at 37°C. The coverslips were washed well, counterstained with DAPI, and mounted in glycerol.

RESULTS

Cep250 is a core component of the centrosome during interphase

Cep250 is tightly associated with the centrosome during interphase. To determine if this association is dependent upon microtubules, HeLa cells were grown in the microtubule depolymerizing drug nocodazole for 4 h, and immunostained for β-tubulin and Cep250. In control cells grown without nocodazole Cep250 is observed as a discrete foci at the center of a complex microtubule network (Fig. 5.21, A-C). When HeLa cells are treated with nocodazole, no organized microtubule cytoskeleton is observed, and tubulin reactivity is seen to redistribute throughout the entire cytoplasm (Fig. 5.21, D-F). When these cells are double stained for Cep250, reactivity is always observed as a focussed dot similar to that seen in untreated cells (Fig. 5.21, D-F). Thus, Cep250 remains associated with the centrosome independently of microtubules, and by this criterion is considered as a core component of the centrosome (Oegema, K. et al. 1995).

Relationship of Cep250 to the microtubule cytoskeleton

The redistribution of Cep250, from the interphase centrosome to the cytoplasm during mitosis (SECTION V A), appears to coincide with the reorganization of the microtubules during the G2-prophase transition. To further explore the relationship of Cep250 and microtubule dynamics, HeLa cells were double stained with antibody to Cep250 and β-tubulin.

During interphase, a complex network of microtubules are found surrounding the nucleus and extending radially into the cytoplasm (Fig. 5.22, C). The focal point for these

microtubules is the centrosome. In these cells, Cep250 is observed as a compact foci tightly associated with the duplicated centrosomes (Fig. 5.22, B). During the G2-prophase transition, the interphase microtubule array is disassembled, and in early prophase each duplicated centrosome nucleates an extensive array of microtubules (Fig. 5.22, F). During this same period Cep250 reactivity decreases at the centrosome as it leaves the centrosome core (Fig. 5.22, E). The loss of Cep250 at the centrosome during prophase does not appear to be a direct result of the microtubule nucleation, as prophase cells treated with nocodazole do not show a reassociation of Cep250 on to the centrosomes (data not shown). During metaphase extensive microtubule nucleation from the centrosome or spindle pole has been completed and a bipolar spindle is established (Fig. 5.22, I). Cep250 is barely detectable at the spindle poles, but its cytoplasmic reactivity has substantially increased (Fig. 5.22, H). During anaphase both pole to pole and kinetochore microtubules are easily distinguished (Fig , L), and prominent Cep250 reactivity is observed at each centrosome (Fig. 5.22, K). During early telophase, kinetochore microtubules breakdown, and the remnants of the pole to pole microtubules become sequestered within the intercellular bridge. Furthermore, each centrosome migrates from its polar location to a region adjacent to the intercellular bridge (Mack, G. et al. 1993). During this repositioning the centrosome nucleates a long microtubule array that extends to the cell membrane (Fig. 5.22, O and SECTION IV). In these post-mitotic cells, Cep250 reactivity is observed at the centrosome at the leading edge of this microtubule array (Fig. 5.22, N).

Thus, during the cell cycle three different microtubule nucleation events take place from the centrosome. The first arises in interphase and is responsible for the long stable

microtubules of the interphase cytoskeleton. The second nucleation event occurs during prophase and is responsible for the separation of the centrosomes and formation of the bipolar mitotic spindle. The final nucleation event occurs during telophase, and as I have shown in SECTION IV, is necessary for the migration of the centrosome to a region adjacent to the intercellular bridge. Comparison of these microtubule nucleation events with Cep250 staining have revealed that Cep250 remains associated with the centrosome only during the interphase and telophase nucleation events.

Figure 5.21. Cep250 associates with the centrosomes independent of microtubules. HeLa cells were incubated in either the presence (D, E, F) or absence (A, B, C) of nocodazole for 4 h. Cell were then fixed and double stained for β-tubulin (B, E) and Cep250 (C, F), and counterstained for DNA with DAPI (A, D). Cells incubated without nocodazole show an intact microtubule cytoskeleton (B), and a focussed dot of Cep250 reactivity at the centrosome (C). Cells treated with nocodazole do not have an intact microtubule cytoskeleton (E), but Cep250 staining at the centrosome remains as a focussed dot of reactivity (F). Bar = 10μm.

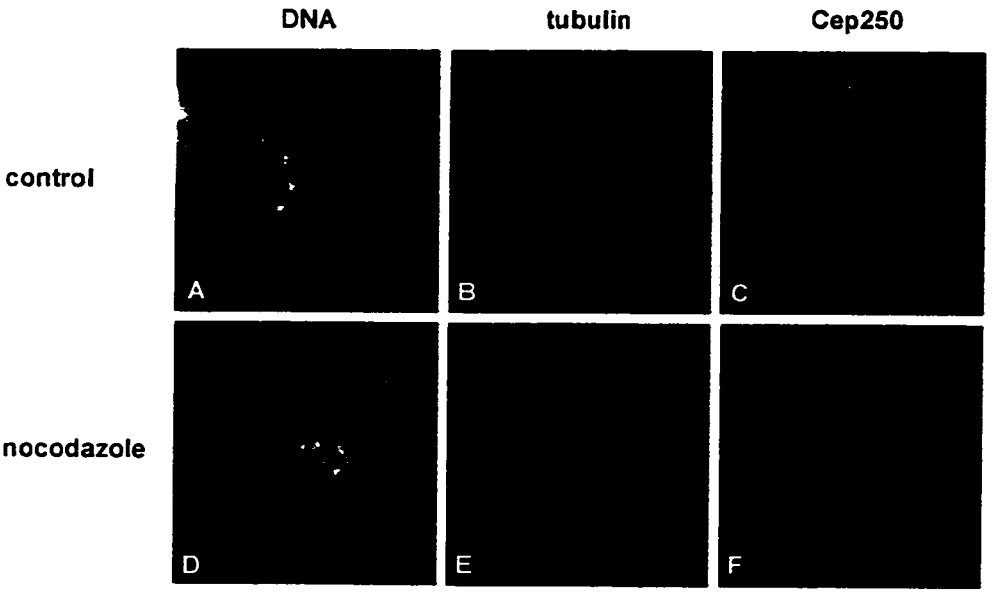
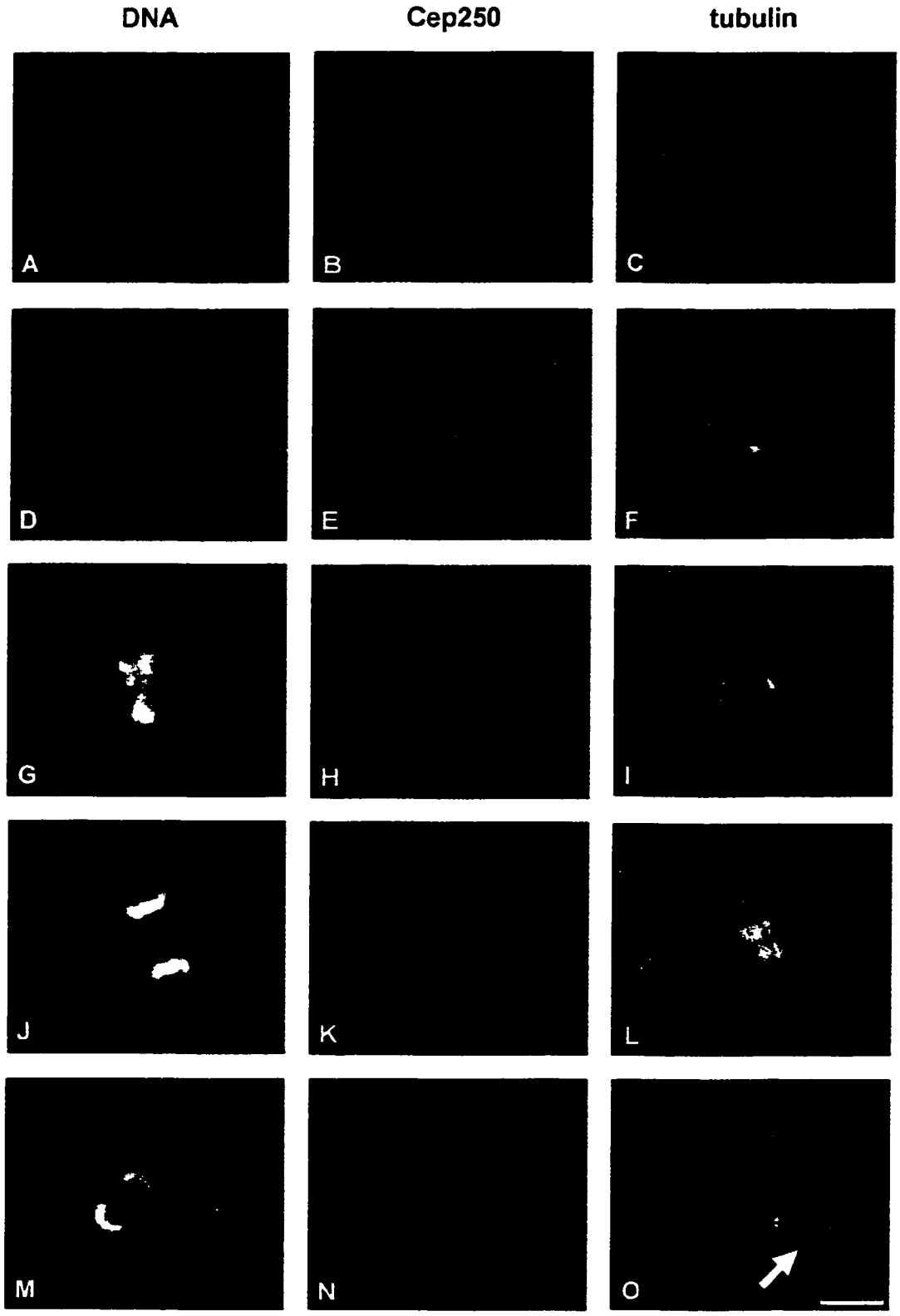

DNA
tubulin
Cep250
control
nocodazole
A
B
C
D
E
F

Figure 5.22. The relationship of Cep250 to microtubule nucleation. HeLa cells were doubled stained with Cep250 (B, E, H, K, N) and β-tubulin (C, F, I, L, O), and then counterstained for DNA with DAPI (A, D, G, J, M). In interphase a complex microtubule cytoskeleton is observed around the nucleus and throughout the cytoplasm (C). Cep250 is observed as a focussed dot of reactivity at the centrosome in these cells (B). During prophase the microtubule profile changes and extensive amounts of short dynamic microtubules are nucleated by duplicated centrosomes (F). Cep250 reactivity at the centrosomes in these cells is decreased (E). In metaphase a bipolar spindle is established (I), and Cep250 reactivity is barely detectable at the spindle poles (H), but prominent Cep250 reactivity is observed in the cytoplasm. In anaphase, microtubules that associate with the kinetochore and with the spindle midzone are observed (L). Cep250 reactivity becomes more prominent on the centrosomes in these cells (K). During telophase microtubules are sequestered in the intercellular bridge and a "V" shaped microtubule bundle forms behind the moving centrosomes (arrow, O). Cep250 is observed as a compact foci at the centrosome as it moves to the intercellular bridge (N). Bar = 10μm.


DNA
Cep250
tubulin
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O

DISCUSSION

Cep250 and microtubule nucleation

Centrosome proteins can be divided into two groups: those proteins that require microtubules to associate with the centrosome, and those that can associate with the centrosome independent of microtubules. Members of the first group of proteins include the kinesin-like protein ncd (Endow, S.A. et al. 1994), and the nuclear mitotic apparatus protein, NuMA (Price, C.M. et al. 1986). The latter group of proteins are exemplified by γ-tubulin (Zheng, Y. et al. 1991) and pericentrin (Doxsey, S.J. et al. 1994). When HeLa cells are treated with the microtubule depolymerizing drug nocodazole, Cep250 staining remains as a focussed dot. This indicates that like γ-tubulin and pericentrin, Cep250 is a core component of the centrosome, and does not require microtubules to maintain its localization at the centrosome. This is further supported by the finding of a distinct leucine zipper domain in Cep250 that is required for centrosome association and the absence microtubule binding motifs (see SECTION V A).

Although Cep250 does not require microtubules for its centrosomal targeting, its unusual cell cycle redistribution appears to coincide with specific microtubule nucleation events. During the establishment and maintenance of the interphase microtubule network, Cep250 remains as a tightly associated centrosome protein. However, from prophase to metaphase, when the centrosome is nucleating an extensive and dynamic microtubule array, the majority of Cep250 is released from the centrosome into the cytoplasm. When this mitotic nucleation ceases at anaphase, Cep250 is once again detected at the

centrosome. Furthermore, during telophase when the centrosome nucleates a final microtubule array, Cep250 is found as a tightly associated protein (see SECTION V A). Each of the three microtubule nucleation events is likely to be regulated by different proteins or protein modifications. In light of this, Cep250 could possibly act as a specific inhibitor of mitotic microtubule nucleation at the centrosome. In this role, Cep250 would prevent extensive microtubule nucleation until it is released from the centrosome during prophase. Phosphorylation of Cep250 may be responsible for its release from the centrosome, and several phosphorylation sites for $p34^{cdc2}$ are found surrounding the leucine zipper responsible for its centrosome targeting. Furthermore, evidence for the modification of Cep250 during mitosis has been shown, and is consistent with the phosphorylation of Cep250. Thus, during prophase when the cdc2/cyclin B kinase is activated, Cep250 may be phosphorylated and released from the centrosome. This in turn removes the inhibition of mitotic microtubule nucleation. During late metaphase early anaphase Cep250 could be dephosphorylated and reassociate with the centrosome to terminate mitotic microtubule nucleation.

Alternatively, because Cep250 is not detected within the PCM, but may in fact be associated with the centrioles (further evidence for this is discussed below), it is possible that Cep250 may act as a protein tether between the centriole and the PCM. The long rod-like coiled-coil region of Cep250 flanked by non-coiled ends maybe predictive of this function. During interphase Cep250 could bind the centriole through its COOH terminus and interact with components of the PCM through its NH_2-terminal domain. During prophase, the phosphorylation of Cep250 could result in its release from the centriole in

order for the PCM to nucleate the mitotic microtubule array. At the completion of mitosis, Cep250 could be dephosphorylated and return to the centriole in order to re-establish the interphase PCM. In this model, Cep250 acts as a structural protein at the centrosome during interphase and late mitosis. Furthermore, since Cep250 is not degraded during mitosis, it is possible that it is an essential protein required for the formation of the interphase centrosome before a functional nucleus is established.

Finally, Cep250 may not have a role in either microtubule nucleation or centrosome organization, but may be involved in other centrosomal events, such as centriole maturation. The centrosome of a G1 cell consists of two orthogonally arranged and equal sized centrioles (mother and daughter). The mother centriole is primarily associated with the PCM and appendages, and is considered as the MTOC. During S phase, the centrioles will separate from each other by a short distance and begin nucleating a procentriole at their proximal ends. Throughout S and G2 the procentriole will continue to elongate as each centriole duplex moves to opposite sides of the nucleus. By prophase the centriole duplex consisting of the daughter centriole and procentriole acquires the ability to become a MTOC. Thus, there are now two independent MTOCs that will form the spindle poles. By late mitosis each procentriole elongates to the same length as its parent, and is considered a mature centriole. Figure 3.0 illustrates centriole duplication and maturation. It is conceivable that Cep250 functions in this maturation process. During G1, Cep250 is often observed as a two compact foci at the centrosome (Fig. 5.5, C). Although indirect immunofluorescence does not provide us with enough resolution, Cep250 could be associated with both centrioles. During S and G2 phases of the cell cycle, Cep250 remains

associated with each centriole as it nucleates the procentrioles. Thus Cep250 reactivity should appear as a single foci at each centrosome. This is consistent with what is observed at S-G2 (Fig. 5.5, F). During prophase however, a portion of Cep250 could be released from the parent centriole into the cytoplasm. At the end of mitosis, Cep250 reassociates with both centrioles at the spindle poles, and indicates that each centriole is mature. Thus, by telophase two Cep250 foci should be expected at the centrosome. As shown in Figure 5.9, F (left hand cell) at late telophase, a single centrosome is observed with pericentrin antibody, but two compact foci are detected with Cep250 antibody. Thus, Cep250 could be a structural protein that is required for the complete growth and maturation of the procentrioles. The model for Cep250 reactivity at the centrosome is shown in Figure 5.23.

Is Cep250 a "glue" protein holding centrioles together?

Following the publication of our results on the characterization of centrosome reactive autoimmune sera in which we also describe the cloning of Cep250, we were contacted by Dr. A. M. Fry and colleagues at the University of Geneva. They indicated they had identified a centrosomal protein identical to Cep250. This protein, which they gave the designation C-Nap1(Centrosome Nek2 associated protein), was identified in a two hybrid screen using the mammalian kinase Nek2 as bait (Fry, A.M. et al. 1998a). Nek2 is the closest known relative to the mitotic regulator kinase NIMA of *Aspergillus nidulans* (Osmani, A.H. et al. 1991) and has been shown to be localized to the centrosome throughout the cell cycle (Fry, A.M. et al. 1998b). Overexpression of Nek2 appears to induce centrosome splitting, leading to the suggestion that Nek2 may be necessary for centrosome separation at G2 (Fry, A.M. et al. 1998b). Interestingly, cells overexpressing

Nek2, have no focussed MTOC, yet they appear to progress through mitosis normally (Fry, A.M. et al. 1998b). C-Nap1 is identical to Cep250 in nucleotide and amino acid sequence. Antibodies raised to the COOH terminus of C-Nap1 were shown to react with the centrosome during interphase, but not during mitosis. Furthermore, no midbody reactivity is observed with antibodies to C-Nap1. Antibody to C-Nap1 identify a ~ 250 kDa protein and a second protein at ~ 190 kDa in KE37 cells. Furthermore, Fry A. M. et al (1998a) show that the COOH terminal 464 amino acids target C-Nap1 to the centrosome and is phosphorylated by Nek2 *in vitro*. Lastly, immunoelectron microscopy with antibodies to C-Nap1 and Nek2 show that they are both concentrated at the proximal ends of mother and daughter centrioles (Fry, A.M. et al. 1998a). This has led the authors to propose a model for C-Nap1 and for the centrosome splitting phenomena associated with Nek2 overexpression. In this model, C-Nap1 is a component of the "molecular glue" bridging together duplicated centrioles at their proximal ends (see Fig. 5.24). When C-Nap1 is phosphorylated by Nek2, it is either depolymerized or degraded leading to the weakening of centriole-centriole adhesion, thus freeing the centrosomes for separation (Fry, A.M. et al. 1998a).

Several of our findings with Cep250 argue against this model of Fry, A.M. et al. (1998a). First, immunolocalization of Cep250 through the cell cycle has shown that it remains tightly associated with the centrosome/centriole even after it has duplicated and separated. In some instances separated centrosomes have been observed near opposite sides of the nucleus, and Cep250 staining remains as a discrete foci (Fig. 5.10, B). As a component of "centriole glue", the Nigg model would have predicted that Cep250 should

have been disassociated from the centriole before separation was clearly visible. Furthermore, Cep250 protein is not degraded through the cell cycle, and reassociates with the centrosome/centriole after mitosis, suggesting that Cep250 is needed for an immediate function after mitosis but before protein synthesis can be reinitiated. If Cep250 were a glue protein it is likely that it would not be required on the centriole until its duplication during S phase. In addition, Cep250 is found at the centrosome in G0 brain and muscle cells. These cells have one centrosome and will not undergo cell division. It appears unlikely that a protein required to hold two centriole pairs together would be found in cells with only one pair of centrioles. Lastly, Fry, A.M. et al. (1998a) do not show an immunoelectron micrograph of two attached centriole pairs with Cep250 staining at the proximal ends of each parent (as in Fig. 5.24), yet this is a central part of their model for C-Nap1-Nek2 function. Thus, several lines of evidence are inconsistent with the suggestion that C-Nap1/Cep250 holds together duplicated centrioles.

In conclusion, Cep250 is centrosome protein that does not require microtubules for its localization. However, comparison of the cell cycle distribution of Cep250 with that of microtubule nucleation has reveals some similarities. First, Cep250 remains tightly associated with the centrosome while the interphase microtubule network is intact. Second, Cep250 dissociates from the centrosome only during the prophase microtubule nucleation event. Third, Cep250 reassociates with the centrosome immediately after mitotic microtubule nucleation, and remains associated during a third microtubule nucleation event that results in the post-mitotic movement of the centrosome.

While the precise function of Cep250 remains to be addressed, evidence collected

Figure 5.23. Model of Cep250s function in centriole maturation. In this model Cep250 (red) is associated with both mother and daughter centrioles in G1 centrosome. During duplication, Cep250 remains associated with the each parent centriole as it nucleates a procentriole (white). During G2-prophase, most of Cep250 is released from the parent centrioles into the cytoplasm (red circles). Red shading of the parent centrioles indicates that some Cep250 remains associated with the mature centrioles during mitosis. At late metaphase early anaphase cytoplasmic Cep250 returns on both mother and daughter centrioles, and indicates that the daughter centriole is mature.

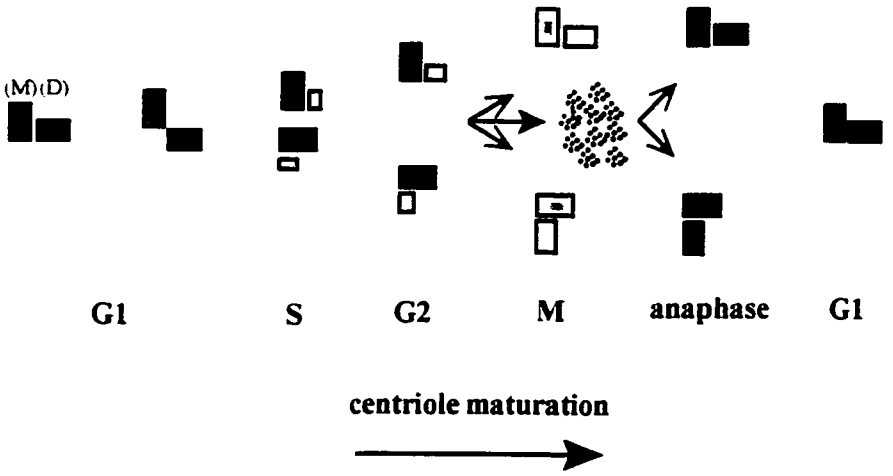
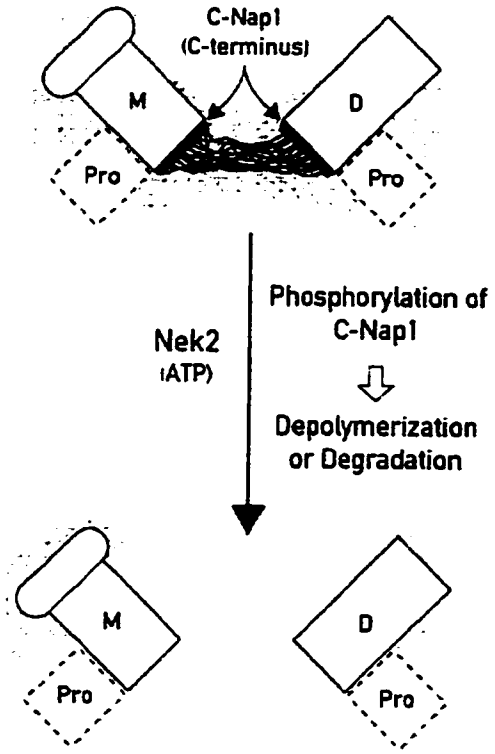

(M)(D)
G1
S
G2
M
anaphase
G1
centriole maturation

Figure 5.24. Alternative model of Cep250/C-Nap 1 function. A model depicting the function of Cep250 as proposed by Fry, A. M. et al. (1998a). Cep250 is proposed to be a component of the molecular glue that holds duplicated centrioles together at their proximal ends. Upon phosphorylation by Nek2 kinase, Cep250 is depolymerized or degraded and centriole pairs are free to separate from each other. The mother centriole (M) is shown with a distal appendage, adjacent to the daughter (D) centriole. Procentrioles are depicted by dashed boxes (Pro), and pericentriolar material is shaded around each centriole pair.


C-Nap1
(C-terminus)
M
D
Pro
Pro
Nek2
(ATP)
Phosphorylation of
C-Nap1
Depolymerization
or Degradation
M
D
Pro
Pro

thus far supports a role for Cep250 as a linker protein between the centriole and PCM. Cep250s protein structure is well suited for this role, having non-coiled-coil ends flanking a long coiled-coil central region. Cep250 could act as bridge between two protein complexes. Furthermore, a break in coiled-coil structure of Cep250s central region (see SECTION V A) may allow it to act as a hinge and bringing these two protein complexes together during the cell cycle. The cell cycle distribution of Cep250 parallels changes observed in the PCM with respect to microtubule nucleation and organization. When the PCM is sparce at interphase and telophase, Cep250 is observed as a discrete focus at the centrosome, when the PCM expands in mitosis, Cep250 disassociates from the centrosome into the cytoplasm. Furthermore, this release of Cep250 could be due to mitotic phosphorylation. Alternatively, Cep250 could have a role as an inhibitor of mitotic microtubule nucleation, or as protein factor required for the timely growth and maturation of the centrioles. Further investigation of Cep250 will be required to distinguish between these possibilities.

SECTION V

PART C: Identification of a Novel Centrosome Associated Protein, Cep110

INTRODUCTION

During each cell cycle, the centrosome duplicates so that each daughter cell will receive one functional centrosome. Duplication begins at S phase when each centriole nucleates a small procentriole at its proximal end (see Fig. 3.0). Following this duplication, the centriole duplexes separate from each other as the procentrioles elongate and mature. One cell cycle later, each procentriole will have become the parent centriole and become a MTOC. Although centriole mature has been well characterized ultrastructurally, the molecular events of this process remain unknown.

Using a human autoimmune serum, characterized for its reactivity to the centrosome in SECTION III of this dissertation, I have identified three centrosomal proteins. These proteins include the previously unknown centrosome antigen Cep250, the human homolog of a centrosomal protein not previously known as an antigen in autoimmune disease, Cep76, and a novel centrosome antigen Cep110. Characterization of Cep110 reveal it to be a highly coiled-coil proteins with numerous leucine zipper motifs. Using indirect immunofluorescence, Cep110 is found as a single foci at the centrosome at early interphase. During centrosome duplication, Cep110 is only detected at one centriole pair until late interphase, when the second centriole pair acquires Cep110 reactivity. Furthermore GFP fusion of various domain of Cep110 have demonstrated that the COOH terminal half of the protein is required to target it to the centrosome. The Cep110 gene locus has recently been shown to be a site for a translocation event involving the fibroblast growth factor receptor. Fusion of Cep110 to the tyrosine kinase domain of this receptor is

suspected to be involved in the genesis of certain cancers.

METHODS AND MATERIALS

Identification of cDNA clones

A human autoimmune serum (2688) that reacts with the centrosome throughout the cell cycle and with the mitotic spindle (Fig. 5.25) was used at a dilution of 1:1000 to immunoscreen a HeLa 5' stretch cDNA expression library as described in the GENERAL METHODS AND MATERIALS section. From this screen four reactive clones were identified and designated 21, 282, 283, and 39. The cDNA clone 21 was chosen for further investigation.

Additional cDNA clones that overlap and extended the 21 clone were obtained by rescreening the HeLa cDNA library by DNA hybridization as described in the GENERAL METHODS AND MATERIALS section. Positive clones identified from these library screens were further characterized through PCR to determine which clones contained inserts with the largest amount of additional DNA sequence information. A 500 bp probe for the 5' end of 21 was obtained from a Pst I/EcoR I digestion of clone 21. From this library screen a total of seven positive clones were identified. Two clones designated 6 (800 bp) and 2 (2 kb) were chosen for further analysis. A 300 bp probe for the 3' end of clone 21 was obtained from a Sac I/Sma I digestion of clone 21. From this library screen a total of eleven positive clones were identified. Two clones designated 72 (1.6 kb) and 5 (1.0 kb) were chosen for further analysis

All the cDNA clones were cloned into the vector pBS SK$^-$(Stratagene), and the DNA sequence determined using exonuclease deletion, and dye termination sequencing as

described in the GENERAL METHODS AND MATERIALS section of this dissertation. All nucleic acid and protein sequence searches and analysis were carried out on the local network server using the BLAST search program (Altschul, S.F. et al. 1990).

PCR amplification from cDNA

In order to confirm the complete cloning of this cDNA, PCR amplification was performed on HeLa and human placenta Marathon RACE ready cDNA (Clontech). For PCR on human placenta cDNA the primers GM1 (TTCGTTCTTCAAAACCCAGGC) and GM4 (CCACATGTTGGTTTAAATTCCC) were used with 2µl of cDNA, following standard protocol number three for the expand long template PCR system (Boehringer Mannheim). For PCR using HeLa cDNA the primers SPINF (AAGCGGGAAGAAAGGTGG) and SPINR (GGCTGAGGCATTCTTTTCC) were used with 2µl of cDNA, following the protocol described in the GENERAL METHODS AND MATERIALS section.

Recombinant protein production and antibody generation.

The 1.4 kb cDNA insert of clone 21 was digested out of the λ vector with EcoR I and cloned into the EcoR I site of the expression vector pGEX 5X-2 (Pharmacia). Cells expressing this construct produce a 78 kDa insoluble GST-fusion protein. Large scale protein inductions were carried out, and the protein was separated on SDS-PAGE. The fusion protein band was excised from the gel, pulverized and injected into one rabbit by the SACRC hybridoma facility to generate a polyclonal antibody.

A 510 bp Ecl136 II/Pvu II digestion fragment of clone 72 was clone into the Sma I site of the expression vector pGEX 3X (Pharmacia). Cells expressing this construct

produce a 46 kDa soluble GST-fusion protein. Large scale protein inductions were carried out, and the fusion protein was purified according to the manufacturer. Fusion protein was injected into two rabbits for the production of polyclonal antibody.

A 300 bp Dra I/Ecl136 II digestion fragment of clone 2 was cloned into the Sma I site of the expression vector pGEX-5X-2 (Pharmacia). Cells expressing this construct produce a 37 kDa soluble GST-fusion protein. Large scale protein inductions were carried out, and the fusion protein was purified according to the manufacturer. Fusion protein was injected into two rabbits and two mice for the production of polyclonal antibody.

Northern blot hybridization

The vector pGEX-5X-2 containing the 300 bp Dra I/Ecl 136 II fragment of clone 2 was used as a template to generate a PCR probe for hybridization as described in the GENERAL METHODS AND MATERIALS section. A ^{32}P labelled actin probe was also generated by random primer labelling as described in the GENERAL METHODS AND MATERIALS section. A human multiple tissue Northern blot (Clontech) was prehybridized in 10 ml of Rapid-hyb (Amersham) for 4 h at 65°C. The radioactively labelled probes were added to the membrane at 1 x 10^6 cpm/ml, and rotated in a hybridization oven for 4 h at 65°C. The hybridization solution was removed and the membrane was washed as described by the manufacturer. The membrane was then sealed in a plastic bag and exposed to a phosphoimager screen (Molecular Dynamics). for three days.

Western blot analysis

Insoluble HeLa interphase protein was separated by 6 % SDS-PAGE and

electroblotted to nitrocellulose as described in the GENERAL METHODS AND MATERIALS section. Western blotting was preformed as described in the GENERAL METHODS AND MATERIALS section using a 1:100 dilution of affinity purified Cep110 (anti-72) antibody.

Indirect immunofluorescence

Hela cells grown on coverslips were extracted in 0.5% Trition X-100 for 2 min as described in the GENERAL METHODS AND MATERIALS section, fixed in methanol, and blocked with1:200 NGS-DPBS at room temperature for 30 min. The cells were then incubated with a 1:50 dilution of affinity purified anti-Cep110 antibody (anti-72) in NGS-DPBS for 30 min at 37°C. After washing, the coverslips were incubated with a 1:10000 dilution of anti-pericentrin in NGS-DPBS and further incubated for 30 min at 37°C. The coverslips were then washed and incubated with a mixture of Cy3-conjugated anti-rabbit (1:400) and FITC-conjugated anti-mouse (1:400) secondary antibody in NGS-DPBS for 30 min at 37°C. The coverslips were then throughly washed, counterstained with DAPI, and mounted.

Green Fluorescent Protein (GFP) fusion and transfection

Various portions of the protein encoding cDNA of Cep110 were cloned inframe into pEGFP-N1 (Clontech) a red-shifted GFP eukaryotic expression vector (Fig. 5.39). The full length coding region of Cep110 (2957 bp) was amplified using PFU PCR and the primers SPINF and SPINR. This fragment was gel isolated and cloned into the Ecl136 II site of pEGFP-N1 to create the construct Cep110-GFP. A 2339 bp fragment amplified by PFU PCR using the primers SPINF and 21REV3 (CCGCTCATGGTTGTCCTG) was gel

isolated and cloned into the Ecl 136 II site of pEGFP-N1 to create the construct Del A GFP. A 1703 bp fragment generated by PFU PCR using the primers SPINF and 21R4 (CTTCTGGAGTCGGGTCTGC) was gel isolated and cloned into the Ecl 136 II site of pEGFP-N1 to create the construct Del B GFP. A 1232 bp NH_2-terminal fragment of clone 2 was produced by Dra I/EcoR V digestion, and cloned into the Eco 47 III site of pEGFP-N1 to create the construct Del C GFP. A 1734 bp COOH terminal fragment was generated by digesting the 2957 bp SPINF/SPINR PFU PCR product with EcoR V. The fragment was gel isolated and cloned into the Eco 47 III site of pEGFP-N1 to create the construct Del D GFP. A 1167 bp COOH terminal fragment of clone 21 was produced by digesting clone 21 with EcoR V and Ecl 136 II. This fragment was gel isolated and cloned into the Eco 47 III site of pEGFP-N1 to create the construct Del E GFP. A 1257 bp COOH terminal fragment was amplified by PFU PCR using the primers 21F4 (GAAGGACATCAGTGAATGGG) and SPINR. This fragment was gel isolated and cloned into the Ecl 136 II site of pEGFP-N1 to create the construct Del F GFP. Finally, the construct Del G GFP was created by cloning a 342 bp Ecl 136 II/Hae III digestion fragment of clone 72 into the Ecl 136 II site of pEGFP-N1.

The various DNA constructs were transformed into competent cells, recombinant clones were identified and miniprep DNA was made using the QIAprep spin columns (Qiagen) as described in the GENERAL METHODS AND MATERIALS section. HeLa coverslips were transfected with 1-2 μg of plasmid DNA, and allowed to express for 24 h. Coverslips were then fixed in 4% paraformaldehyde (SIGMA) in DPBS and mounted, or further processed for additional IIF using a mouse anti-pericentrin antibody (1:10,000) as

described in the GENERAL METHODS AND MATERIALS section. Figure 5.39 illustrates the location of each Cep110 GFP construct.

RESULTS

Identification of cDNA clones encoding a novel centrosome protein

Our previous work with human autoimmune sera reactive with the centrosome has proven it to be a useful tool in the identification of novel centrosome proteins (see SECTION V A). Furthermore, I have shown that the majority of human sera reactive with the centrosome have autoantibodies to multiple centrosome proteins. Therefore, to identify additional centrosome proteins I began the characterization a human sera reactive with the centrosome throughout the cell cycle and mitotic spindle (Fig. 5.25).

Immunoscreening a HeLa cDNA expression library with this human autoimmune serum resulted in the identification of four reactive clones. DNA sequencing revealed that two of these clones (283 and 39) overlapped each other and were part of a larger cDNA clone encoding Cep250 (see SECTION V A, Fig. 5.1). A cDNA clone, 282, was found to have significant homology to the mouse centrosomal protein 1133/5p (Angiolillo, A. et al. 1996). This cDNA clone has been completely sequenced by Dr. Yong Ou, and I have designated it Cep76 (Centrosome protein 76). The final cDNA clone, 21, showed no significant homology in the GenBank database as of January 1998. No translation initiation or termination codons were found within this clone indicating that it is not a complete cDNA clone.

Since this human serum resulted in the identification of several centrosome proteins, it is possible that clone 21 may encode for a novel centrosome antigen. To investigate this possibility, I identified the full length cDNA encoding this antigen. As no

translation initiation codon was found in clone 21, identification of additional upstream DNA sequence was required. To identify clones extending further 5', a probe to clone 21 was used in a DNA hybridization screen of the HeLa cDNA library. Two clones designated 6 and 2 were chosen for further analysis (Fig. 5.26). DNA sequencing showed that clone 6 extended the DNA sequence of clone 21 by approximately 600 bp. This clone contained an ORF continuous with that in clone 21, but did not contain a potential translation initiation codon. Clone 2 extended the sequence of clone 6 by approximately 975 bp, an contained an ORF initiating at nucleotide position 473 and is preceeded by two in-frame stop codons. Furthermore, the ORF in clone 2 is continuous with that of clone 6 (Fig. 5.26).

Since the ORF in clone 21 contained no translation stop codons, additional downstream DNA sequence information was required to complete the ORF. Therefore, a probe to the 3' end of clone 21 was generated and used in a DNA hybridization screen of the HeLa cDNA library. A single clone, 72, extended the sequence of clone 21 by approximately 981 bp. DNA sequencing revealed an ORF continuous with that of clone 21 and terminating at a ochre codon (TGA) at nucleotide position 3455. An additional clone, 5, was also characterized and found to be identical to that of 72, except that it contained a 141 bp in-frame deletion between nucleotide positions 2755-2896 (Fig. 5.26), thus there appears to be a long form and a short form of this antigen.

All together these cDNA clones span 3893 bp for the long form and 3752 bp for the short form, and encode for proteins that I have designated Cep110 (Centrosome protein 110). The complete nucleotide and predicted amino acid sequence of Cep110 is shown in Appendix II, and is available in GenBank accession number AF083322.

Figure 5.25. Immunofluorescent staining of cells with human autoimmune serum 2688. HeLa cells were double stained with antibody to β-tubulin (B, E, H, K) and autoimmune sera 2688 (C, F, I, L), and counterstained for DNA with DAPI (A, D, G, J). Specific centrosome staining is observed with the human serum throughout the cell cycle. Weak mitotic spindle and cytoplasmic staining is observed during metaphase (I). At telophase weak intercellular bridge reactivity is seen adjacent to the midbody (L). Bar = 10μm.

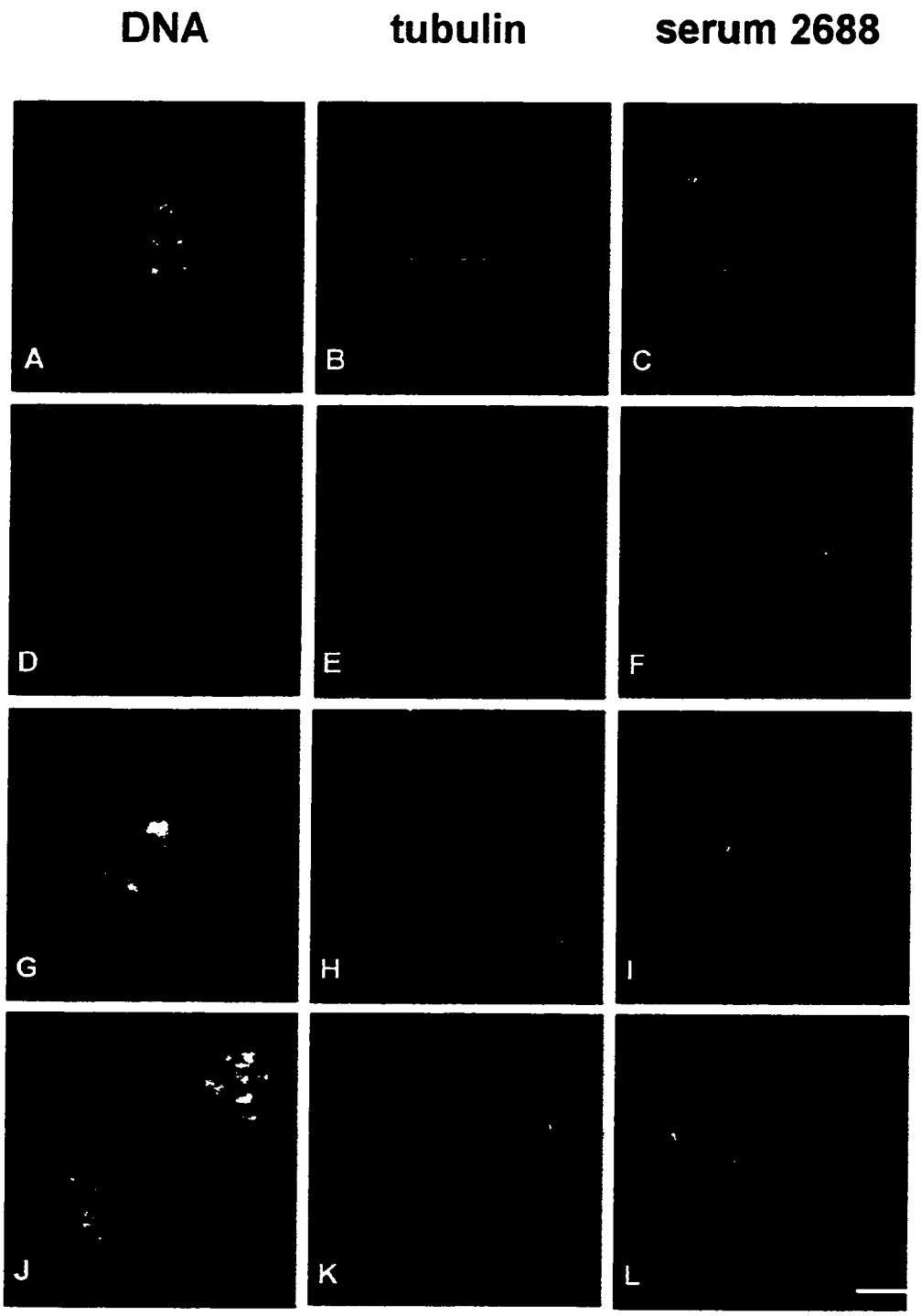
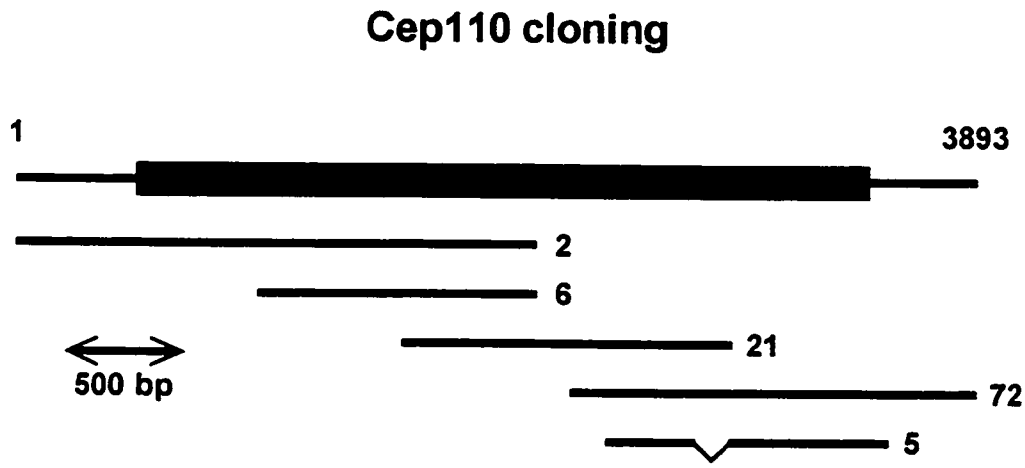

DNA
tubulin
serum 2688
A
B
C
D
E
F
G
H
I
J
K
L

Figure 5.26. Schematic drawing of the cloning of Cep110. The complete Cep110 cDNA clone (long form) is shown on top from base pair 1 to 3893, with the blue box representing the predicted open reading frame. Clone 21 was identified with the human autoimmune serum whereas all other clones were identified using DNA hybridization screening techniques (see methods and materials). Clone 5 was determined to have a 141 bp in frame deletion with respect to clone 72.


Cep110 cloning
1
3893
2
6
21
72
5
500 bp

Features of the deduced amino acid sequence of Cep110

Analysis of the cloned sequences identified an open reading frame (ORF) of 2982 nucleotides encoding 994 amino acids in the long form of Cep110, and an ORF of 2841 nucleotides encoding 947 amino acids in the short form. The likely initiator codon for both forms is found at nucleotides 473-475, and is preceded by a untranslated region containing multiple inframe stop codons. A termination codon is found at nucleotide position 3455-3457 in the long form of Cep110, and at position 3514-3516 in the short form of Cep110. Two potential poly(A)$^+$ initiation signals (AATAAA) are found at position 3866-3871 and 3887-3892 in the long form of Cep110, but no poly(A)$^+$ tail was found. From the deduced amino acid sequence, the molecular weight of the long form of Cep110 was calculated to be 116,813 Da, and the short form of Cep110 to be 111,105 Da. All subsequent database searches and analysis were done with the long form of Cep110. A GenBank database search (October 1998) with the amino acid sequence revealed no significant homology with known proteins, however regions of weak similarity can be observed with the coiled-coil proteins CENP-E and myosin heavy chain. Analysing the amino acid sequence with the program COILS (Lupas, A. et al. 1991) shows that Cep110 is predicted to have extensive stretches of coiled-coil structure throughout much of its sequence, except between amino acids 65-80, 295-320, and last 114 amino acids of the COOH terminus (Fig. 5.27).

A protein MOTIF search reveals the possibility of one amidation site, two N-myristoylation sites, and one N-glycosylation site. Furthermore, there are two potential cAMP-phosphorylation sites, 19 casein kinase II phosphorylation sites, and 20 protein

kinase C phosphorylation sites. In addition, there are four predicted leucine zippers at amino acid positions 28-49, 97-118, 496-517, and 689-710. Each leucine zipper region has four leucine residues separated by six amino acids. The location of each potential leucine zipper lies within a region predicted to adopt a coiled-coil structure (Fig. 5.28). Each potential leucine zipper is underlined in the amino acid sequence of Cep110 in Appendix II. There are no consensus sequences for microtubule or nucleotide binding. Overall Cep110 is an acidic protein with a predicted pI of 5.43.

Multiple forms of Cep110

As described above, during the cloning of Cep110 a single cDNA clone was isolated that contained a 141 bp deletion in the COOH terminus. This deletion was inframe such that 47 amino acids were removed from the long form of Cep110. To confirm that this clone was not an artifact, and that Cep110 may be an alternatively spliced protein, PCR amplification was preformed on HeLa and human placenta cDNA.

Using two primers that lie within the predicted 5' UTR (GM1) and 3' UTR (GM4) of Cep110, two major cDNA products, 5.0 kb and 4.0 kb, were amplified from placenta cDNA (Fig. 5.29, A). Cloning and DNA sequencing revealed the 5 kb product to be the long form of Cep110 with a ~ 1.0 kb intron between nucleotides 3227-3228. This intron has no ORF and contains sequences identical to the consensus 5' and 3' splice sites. Cloning of the ~ 4.0 kb product revealed that it consisted of both long form and short forms of Cep110 (data not shown). Furthermore, PCR using primers at the extreme NH_2 (SPINF) and COOH (SPINR) terminal ends of the Cep110 ORF, resulted in the amplification of two products slightly below 4.0 kb (Fig. 5.29, B). Both products have been cloned and

Figure 5.27. Coils output of the amino acid sequence of Cep110. The probability of forming a coiled coil is plotted for the complete amino acid sequence of Cep110. Coiled coil structure is seen throughout most of Cep110 except between residues 65-80, 295-320, and the last 114 amino acids of the COOH terminus. The calculated window size is 28 residues using the COILS program of Lupus, A. et al. (1991).

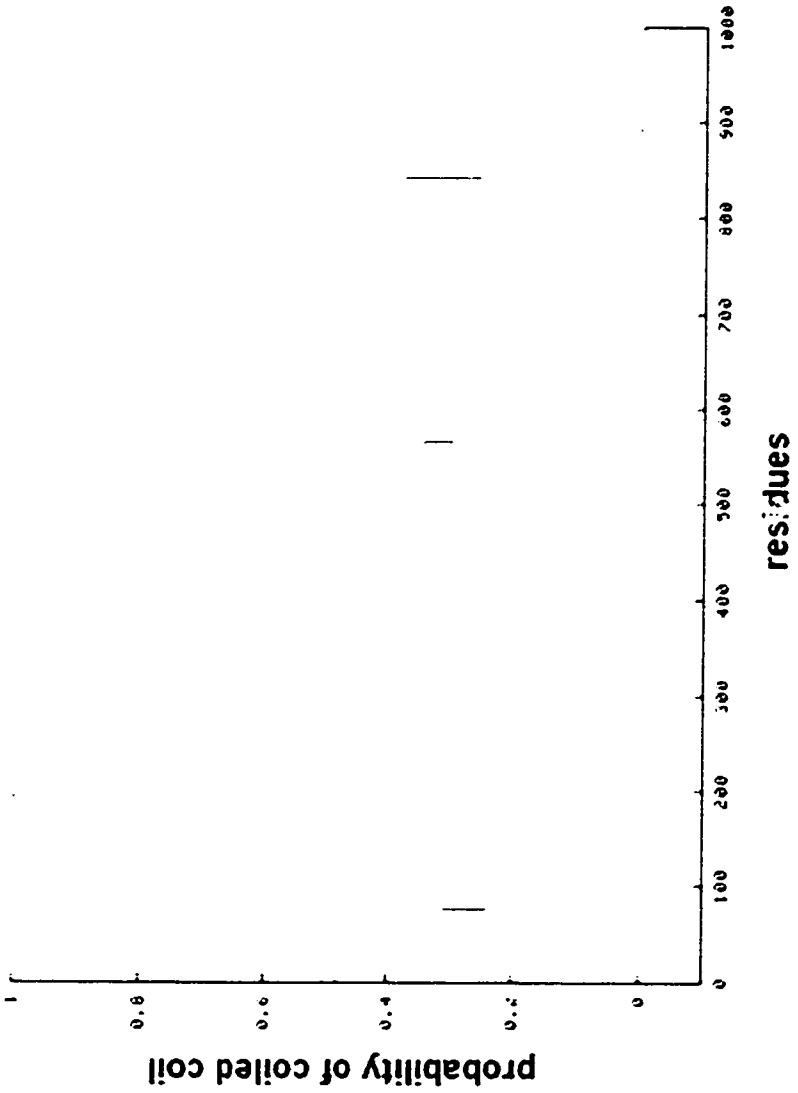

probability of coiled coil
residues

Figure 5.28. Stylized drawing of the Cep110 protein. The complete Cep110 protein is shown on top, with blue boxes representing regions of coiled coil structure, yellow boxes are predicted leucine zipper motifs, and the red region represents the 47 amino acids of Cep110 that are removed in the short form of the protein. Three different regions of Cep110 that were used to generate polyclonal antibodies are shown underneath. Anti-72 is the only antibody that has been affinity purified and used in this study.

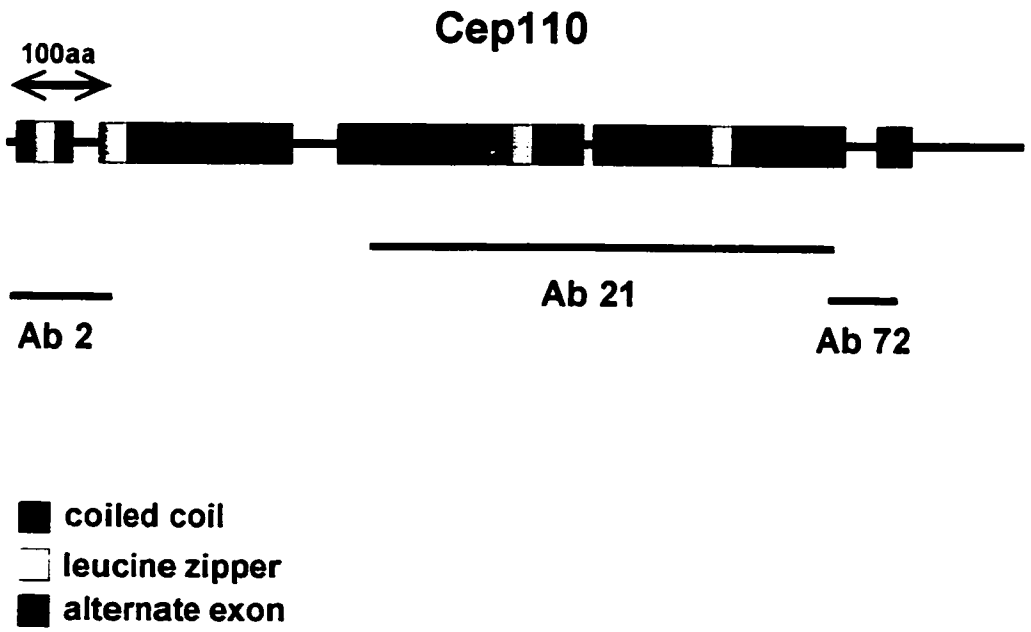
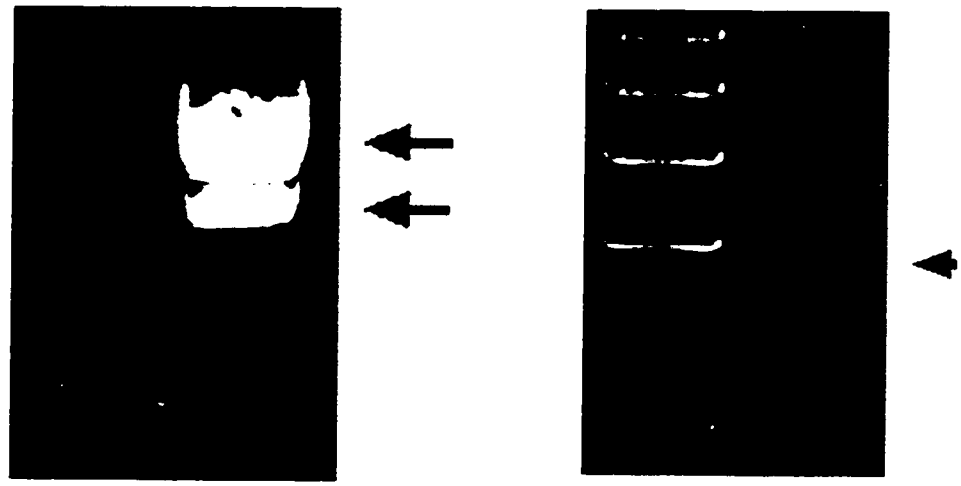

Cep110
100aa
Ab 21
Ab 2
Ab 72
coiled coil
leucine zipper
alternate exon

Figure 5.29. Multiple forms of Cep110. The short and long forms of Cep110 were PCR amplified from placenta (A) or HeLa (B) cDNA. PCR using primers within the 5' and 3' untranslated region of Cep110 amplified two products of ~ 4 and 5 kb (arrows). The ~ 4 kb product consists of both short and long forms of Cep110, and the ~5 kb product has a 1.0 kb intron within the long form of Cep110. PCR using primers within the coding region of Cep110 amplified both short and long forms of Cep110 in HeLa cDNA (B, arrow head). DNA ladder from the bottom is 1636, 2036, 3054, 4072, 5090, 6108, 7126, and 8144 bp.



A B

sequenced, and shown to differ by 141 bp. These results indicate that the short form of Cep110 is not a cloning artifact, but an alternatively spliced form of the Cep110 transcript in both placenta and Hela cDNA sources.

Northern blot analysis of Cep110

Both cDNA cloning and PCR amplification indicate that Cep110 has a least two forms that differ by 47 amino acids at their COOH terminus. It is possible that Cep110 may have other forms that were not identified during the cloning or PCR procedures. Therefore, to investigate this possibility, and to confirm that the complete cDNA obtained for Cep110 is the correct size, Northern blot hybridization was performed.

A human multiple tissue Northern blot (Clontech) was probed with a fragment of the 5' end of Cep110. As shown in Figure 5.30, multiple weak transcripts were detected in most tissues. In heart, a single transcript of 3715 kb was detected. In placenta two transcripts of 3715 and 4400 kb were observed. In lung, two transcripts of 3090 and 3715 kb were detected. Skeletal muscle showed three transcripts of 3805, 5011, and 7413 kb. In both kidney and pancreas a single transcript of 3801 kb was detected. No signal was observed for Cep110 in either brain or liver. Hybridization with an actin probe revealed that all tissue lanes contained approximately equal amounts of RNA (Fig. 5.30). Thus, Cep110 appears to have a complex arrangement of mRNA transcripts in several tissues, and its weak hybridization signal suggests that its mRNA is in low abundance. Cep110 transcripts at approximately 3.7-4.0 kb were identified in each tissue where a signal was detected, and is in good agreement with the size of the full length Cep110 cDNA clones (3.9 kp for the long form of Cep110, and 3.7 kp for the short form of Cep110).

Characterization of the Cep110 protein

DNA sequence analysis of the Cep110 cDNA clones predicts that a 116 and 111 kDa protein should be encoded by the long and short forms of Cep110 respectively. To confirm the complete cloning of the Cep110 ORFs, Western blot analysis was carried out on HeLa protein extracts. Using an affinity purified Cep110 antibody (anti-72) that was generated against an epitope common to both forms of Cep110, a single protein at approximately 120 kDa was detected in insoluble HeLa interphase protein (Fig. 5.31, I). Preimmune sera showed no reactivity with this protein (Fig. 5.31, p). The ~ 120 kDa protein identified in this Western blot is in good agreement with the estimated size of the long form of Cep110 of 116 kDa, and confirms that the complete ORF of Cep110 has been cloned. It is possible that the short form of Cep110 was not detected on this immunoblot due to its low abundance.

Immunolocalization of Cep110 during the cell cycle

The human autoimmune serum used to identify Cep110 reacts with the centrosome and mitotic spindle using indirect immunofluorescence. It is possible that the Cep110 may encode for an antigen of either the centrosome, spindle, or both. Alternatively, Cep110 may be an antigen that is not localized to either of these structures. Thus, to determine the localization of Cep110 in the cell, antibodies were produces in rabbits against different domains of the protein (Fig. 5.28). Affinity purified antibody (anti-72) to the COOH terminus of Cep110 was used in indirect immunofluorescence studies along with an antibody against the human centrosomal protein pericentrin. Preimmune rabbit sera showed no specific reactivity in these studies (data not shown).

Figure 5.30. Northern blot analysis of Cep110. A human multiple tissue Northern blot was probed for Cep110 and actin. Multiple Cep110 transcripts (*) were detected in placenta (3715 and 4400 kb), lung (3090 and 3715 kb), and skeletal muscle (3805, 5011,and 7413 kb). A single Cep110 transcript was observed in heart (3715 kb), kidney (3801 kb), and pancreas (3801 kb). No Cep110 transcripts were observed in either brain or liver. All Cep110 transcript were weak except for those observed in skeletal muscle. A control hybridization of actin (arrow) revealed all lane to have approximately equal amount of RNA. Molecular size markers are 9.5, 7.5, 4.4, 2.4, and 1.35 kb.

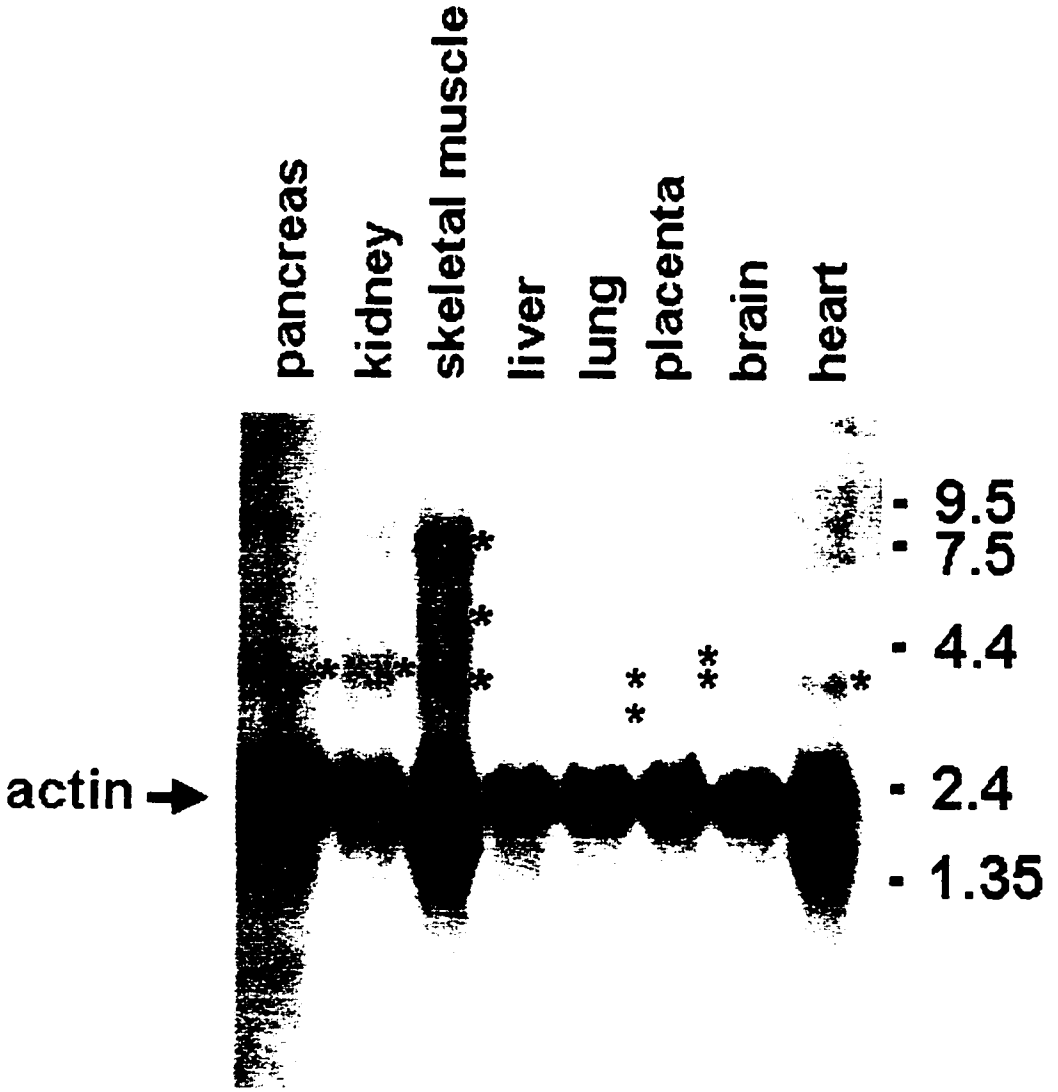
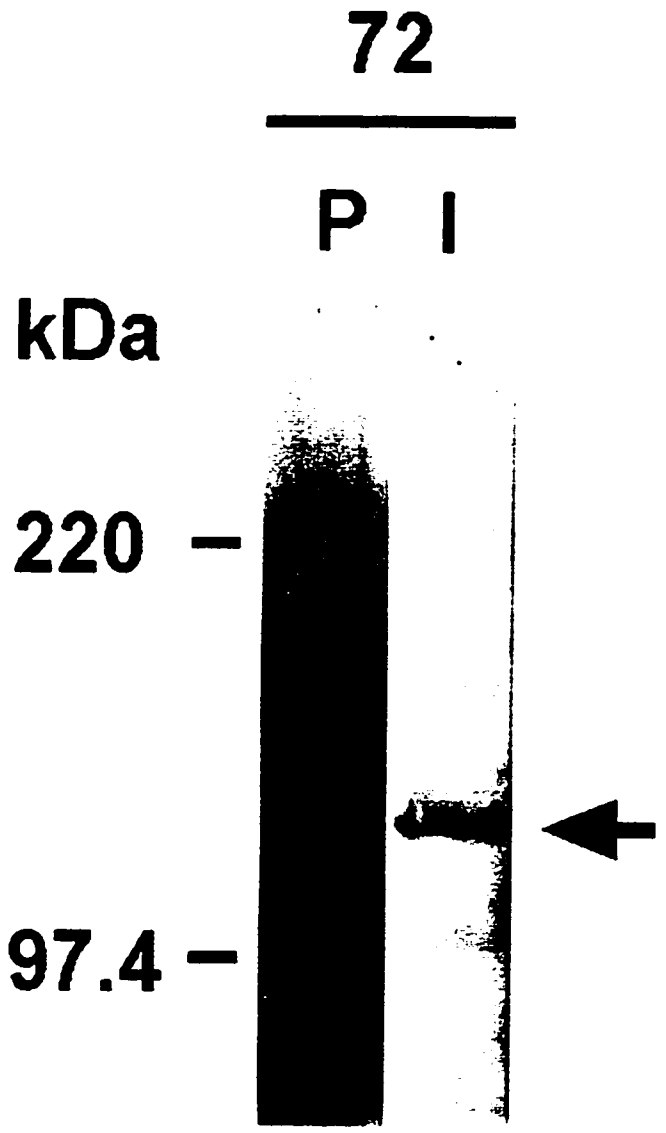

pancreas
kidney
skeletal muscle
liver
lung
placenta
brain
heart
9.5
7.5
4.4
2.4
1.35
actin

Figure 5.31. Western blot analysis of Cep110. Insoluble HeLa interphase protein was resolved by 6 % SDS-PAGE and immunoblotted using Cep110 antibody (anti-72). A single band at ~120 kDa is detected with the affinity purified immune sera (I, arrow), but not with the preimmune serum (1:500) (P). The molecular weight markers are 220 and 97.4 kDa.


72
P I
kDa
220
97.4

As shown in Figure 5.32, A-C, early interphase (G1) cells showed Cep110 staining as a single small foci at the centrosome, colocalizing with pericentrin reactivity. In all the cells Cep110 reactivity at the centrosome was significantly smaller than that seen with pericentrin (compare B and C), and may reflect its association with the centriole. A composite image of Cep110 and pericentrin reactivity is shown in Figure 5.33. Cep110 is often seen at the periphery or center of the pericentrin reactivity, and this may reflect the three dimensional orientation of the centriole cylinders in the PCM. During centrosome separation, Cep110 was observed in association with only one of the two centrosome duplexes (Fig. 5.32, D-F arrow and Fig. 5.33, B-C). However, before prophase, Cep110 reactivity could now be observed at both centrosome duplexes (Fig. 5.34, A-C). At early prophase as pericentrin reactivity at the centrosome increases, Cep110 reactivity at both centrosomes appeared to decrease, and was barely detectable in most cases (Fig. 5.34, C). From metaphase to anaphase, Cep110 was weakly observed at the spindle poles in most cells (Fig. 5.35, C and F). No increase in cytoplasmic Cep110 staining was observed. At the end of telophase and the beginning of G1, Cep110 was now easily observed as a small foci at each centrosome (Fig. 5.36, A-C). In some cases is was possible to observe recently duplicated centrosomes where Cep110 was found at only one centrosome (Fig. 5.36, C arrow). Cep110 reactivity was not observed at the intercellular bridge or midbody.

Thus, indirect immunofluorescence has shown that Cep110 is a centrosome associated protein. In summary, Cep110 is only detected at one of two centrosome duplexes in early interphase. In late interphase Cep110 can be found at both centrosomes, but during mitosis, it is barely detectable at the centrosome. Cep110s small area of

Figure 5.32. Immunolocalization of Cep110 during interphase. HeLa cells were doubled stained for pericentrin (B, E) and Cep110 (C, F), and then counterstained for DNA with DAPI (A, D). In early interphase Cep110 is observed as a small foci at the centrosome. In later interphase cells when duplicated centrosomes begin to separate (E), Cep110 reactivity is found associated with only one centrosome (F, arrow). Bar = 10μm.

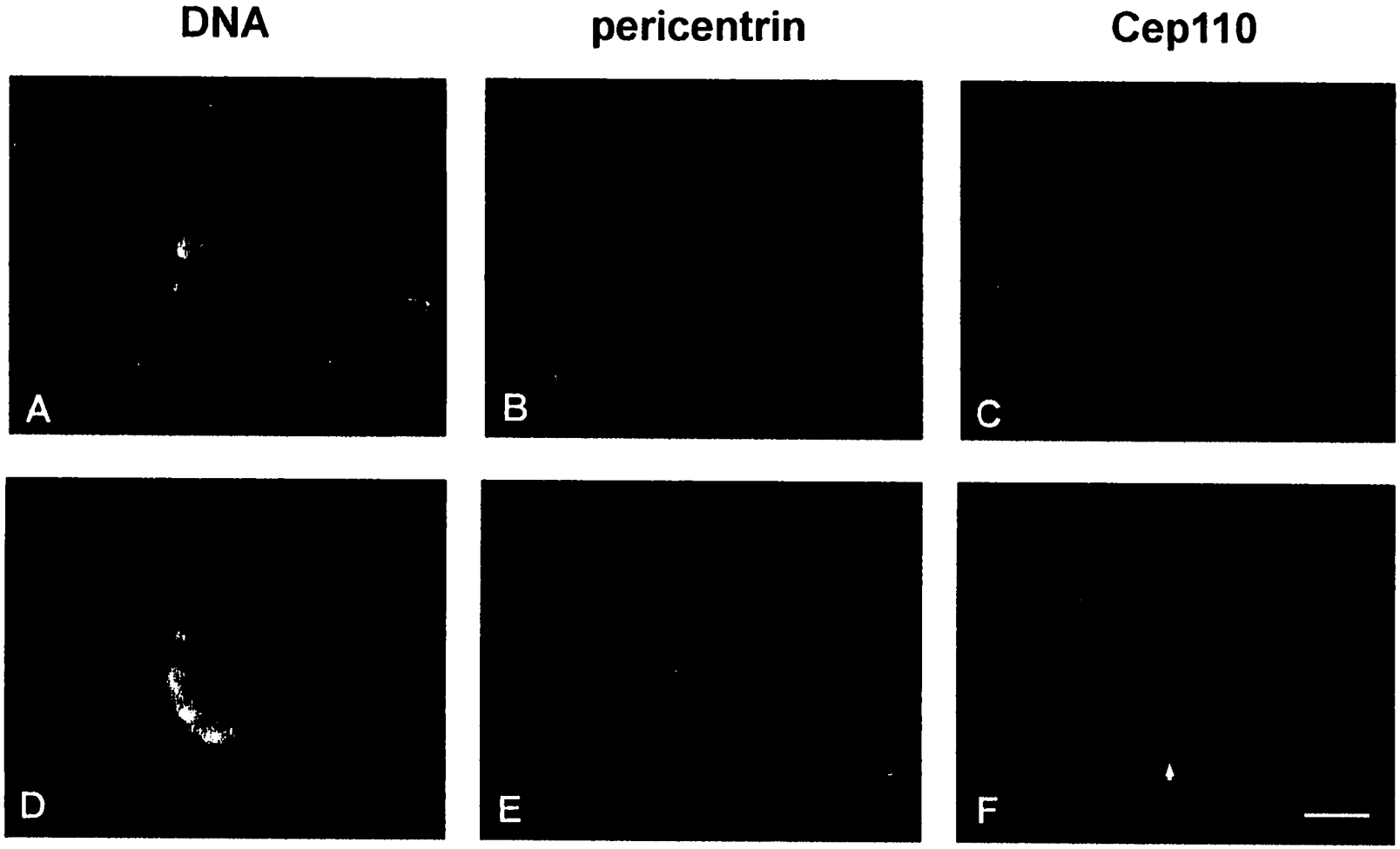

DNA
pericentrin
Cep110
A
B
C
D
E
F

Figure 5.33. Composite figure of Cep110 and pericentrin reactivity. HeLa cells were double stained for Cep110 (red) and pericentrin (green). Images were collected separately and merged together in Photoshop 4.0. Cep110 reactivity is always observed to be much smaller than pericentrin reactivity, and is often found at the periphery of pericentrin staining (A, B). In some cases Cep110 is observed to be associated with only one of two centrosomes, at either the periphery (B) or colocalizing with pericentrin (yellow in C).

pericentrin-Cep110

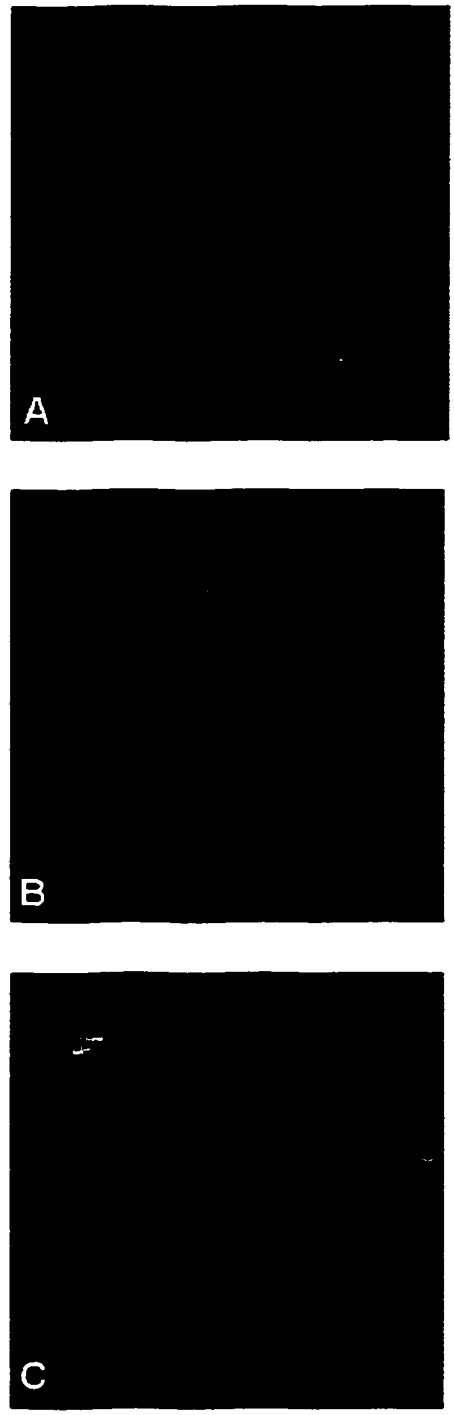


Figure 5.34. Immunolocalization of Cep110 during late interphase and prophase. HeLa cells were doubled stained for pericentrin (B, E) and Cep110 (C, F), and then counterstained for DNA with DAPI (A, D). In late interphase cells Cep110 reactivity can now be found on both duplicated and separated centrosomes. During prophase pericentrin reactivity increases at the centrosomes (E), but Cep110 reactivity is barely detectable (F). Bar = 10μm.

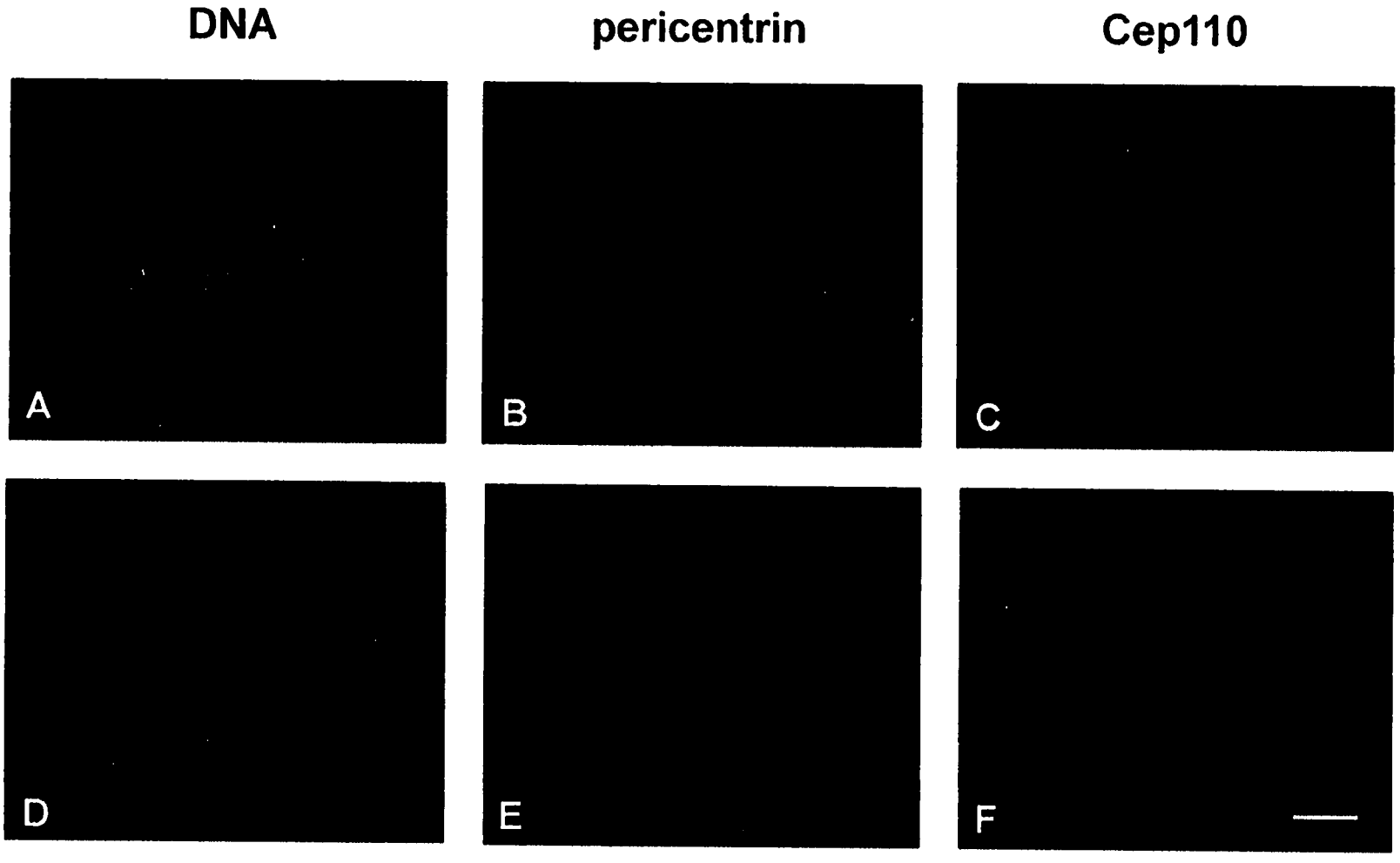

DNA
pericentrin
Cep110
A
B
C
D
E
F

Figure 5.35. Immunolocalization of Cep110 during metaphase and anaphase. HeLa cells were doubled stained for pericentrin (B, E) and Cep110 (C, F), and then counterstained for DNA with DAPI (A, D). Pericentrin reactivity is prominent at the spindle poles during metaphase and anaphase (B, E). Cep110 reactivity is barely detectable at the spindle poles in most cases (C, F), and no increase in cytoplasmic staining is observed. Bar = 10μm.

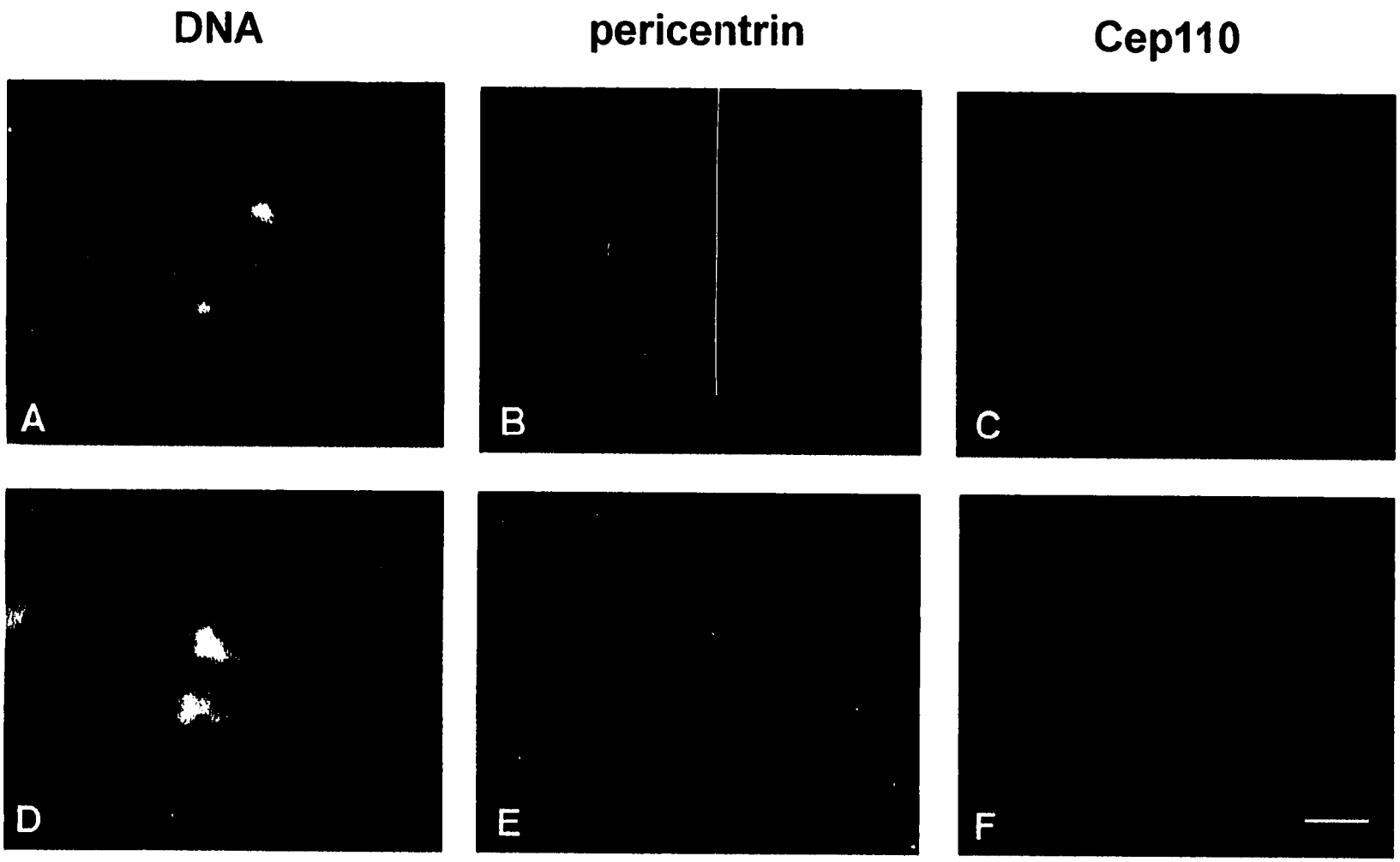
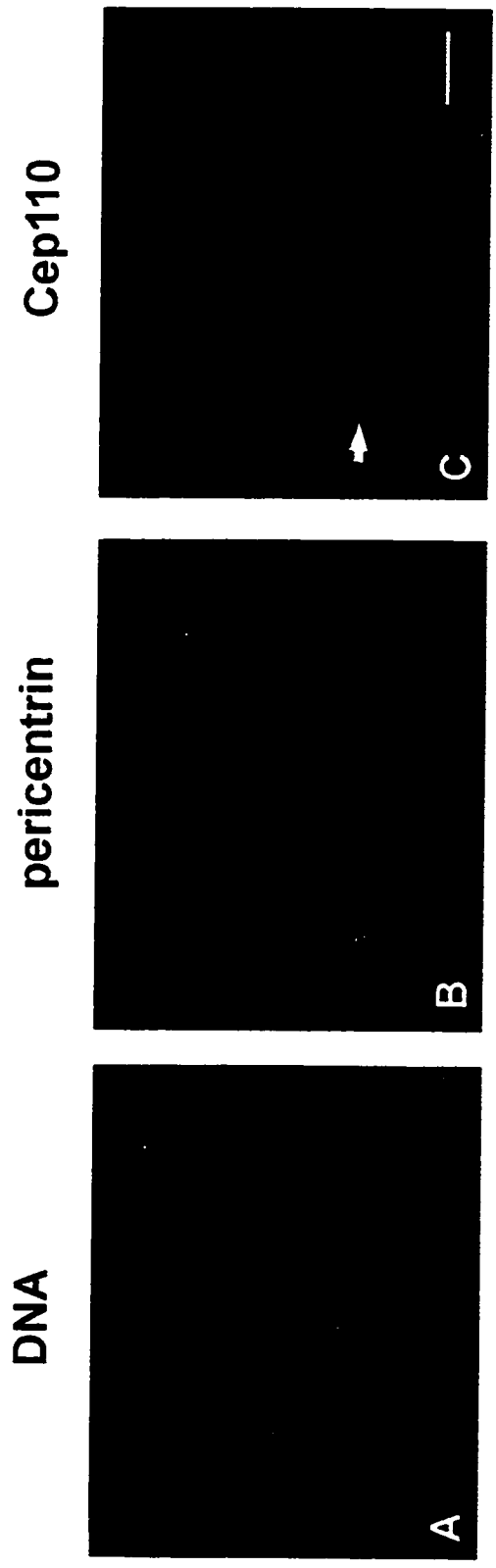

DNA
pericentrin
Cep110
A
B
C
D
E
F

Figure 5.36. Immunolocalization of Cep110 during telophase. HeLa cells were doubled stained for pericentrin (B) and Cep110 (C), and then counterstained for DNA with DAPI (A). In telophase Cep110 reactivity is strongly observed as a small foci at the centrosome (C). In the left most daughter cell two centrosomes are detected with pericentrin (B), and only one shows staining for Cep110 (C, arrow). Bar = 10μm.


DNA
pericentrin
Cep110
A
B
C

reactivity as compared to pericentrin suggests that it is a component of the centriole.

Immunolocalization of Cep110 in differentiated tissue and centrioles

In Northern blot analysis, a Cep110 hybridizing signal was not detected in human brain or liver. We reasoned that since Cep110 transcripts were only weakly detected in other human tissues, Cep110 messages could be expressed in brain and liver at levels not detectable by Northern blot hybridization. To determine if Cep110 was expressed in the brain, monkey cerebellum tissue sections were immunostained for Cep110. Prominent Cep110 reactivity was detected as discrete foci adjacent to the nucleus in almost every cell (data not shown). Thus, since Cep110 protein is expressed in the brain our inability to detect Cep110 transcripts by Northern blot hybridization indicted that its mRNA is expressed at very low levels.

Using indirect immunofluorescence, the area of Cep110 reactivity is always observed to be much smaller than that of pericentrin in HeLa cells. One possible explanation is that Cep110 is associated with the centriole(s) rather than the PCM. In order to determine if Cep110 is a component of the centrioles, human spermatozoa were isolated and immunostained for Cep110. In the sperm cells, weak Cep110 reactivity in the form of a line or bar was observed beneath the sperm nucleus (Fig. 5.37, D). Double staining with anti-β-tubulin antibodies revealed that the proximal centriole of the sperm lies within this area (Fig. 5.37, C). An overlapping image of Cep110 and β-tubulin reactivity reveals that Cep110 lies on the periphery of the tubulin signal at the proximal centriole (Fig. 5.38, E). Cep110 staining was not observed in association with the distal centriole or flagella. Thus, Cep110 is observed in region around the proximal centriole of the sperm, but does not

completely colocalize with it.

Fusion of Cep110 to Green Fluorescent Protein (GFP)

The association of Cep110 with the centrosome/centriole is likely mediated through protein-protein interactions. However, several protein interaction motifs are found throughout Cep110, including four leucine zippers, and numerous coiled-coil regions. Thus, to define which region(s) of Cep110 are necessary for its interaction with the centrosome/centriole, various domains of Cep110 were fused with the green fluorescent protein (GFP) and transfected into HeLa cells. After 24 h, the transfected cells were fixed and immunostained with antibody to pericentrin to serve as a centrosome marker. The colocalization of GFP signal and pericentrin (Cy3) on extracted and unextracted cells was scored for each transfection. Figure 5.39 illustrates the location of each Cep110 GFP construct and its ability to localize to the centrosome.

HeLa cells transfected with the GFP vector alone showed only diffuse nuclear and cytoplasmic reactivity, no localization of GFP was observed at the centrosome (Fig. 5.40. A-C). However, in cells transfected with the full length Cep110 GFP construct, GFP reactivity was observed at the centrosome, colocalizing with pericentrin reactivity (Fig. 5.40, E-F). In certain cells, Cep110 GFP was overexpressed, and accumulated as large masses around the centrosome and in other areas of the cytoplasm (data not shown). Thus, GFP fusion of Cep110 confirms the immunostaining results, which indicate that Cep110 is a centrosome associated protein.

To ensure that Cep110-GFP is properly expressed in these cells, Western blot analysis was preformed on transfected cell protein. Anti-GFP antibody detected a single

fusion protein of approximately 140 kDa (Fig. 5.41, Cep110-GFP). No reactivity was detected in protein from cells transfected with vector alone (Fig. 5.41, GFP). The 140 kDa GFP fusion protein is in good agreement with the expected size of Cep110 GFP of 143 kDa (116 plus 27 kDa).

To identify the region(s) of Cep110 required for its centrosome association, various COOH terminal deletions of Cep110 were fused to GFP and transfected into HeLa cells (Fig. 5.39). Cells transfected with the construct Del A GFP showed specific GFP reactivity at the centrosome (data not shown). However, cells expressing the constructs Del B-C showed no reactivity at the centrosome, rather, GFP was observed throughout the cell cytoplasm (data not shown). The difference between the Del A GFP construct and the Del B construct is approximately 200 amino acids of the COOH terminus. Thus, I reasoned that the centrosome localization domain for Cep110 must lie within this stretch of 200 amino acids. To further define this region, various NH_2-terminal deletions of Cep110 (Fig. 5.39) were transfected into HeLa cells. Cells expressing the constructs Del D-F, showed GFP reactivity at the centrosome. Figure 5.40 illustrates the GFP reactivity at the centrosome for cells expressing the Del-F GFP construct. Cells expressing Del G GFP showed GFP reactivity only within the cytoplasm and nucleus, no centrosome localization of GFP was observed. DNA sequence comparison of the construct Del A GFP with that of Del F GFP, reveals that the centrosome targeting domain for Cep110 lies within a stretch of 170 amino acids between amino acids 617 and 787. This region is predicted to adopt a coiled-coil structure, and contains the fourth leucine zipper domain between amino acids 689-710 (Fig. 5.39). Thus, Cep110's association with the centrosome is mediated through

170 amino acids at its COOH terminus, and is likely due to protein interactions with the leucine zipper motif.

Figure 5.37. Immunostaining of Cep110 in human sperm cells. Isolated human sperm were double stained for β-tubulin (C) and Cep110 (D), and counterstained for DNA with DAPI (B). Isolated sperm cells were identified under phase contrast (A), and both proximal (arrow) and distal centrioles and flagella (arrowhead) was observed with anti-β-tubulin (B). The same cell show a small bar of Cep110 reactivity underneath the nucleus (D). Bar = 4 μm.

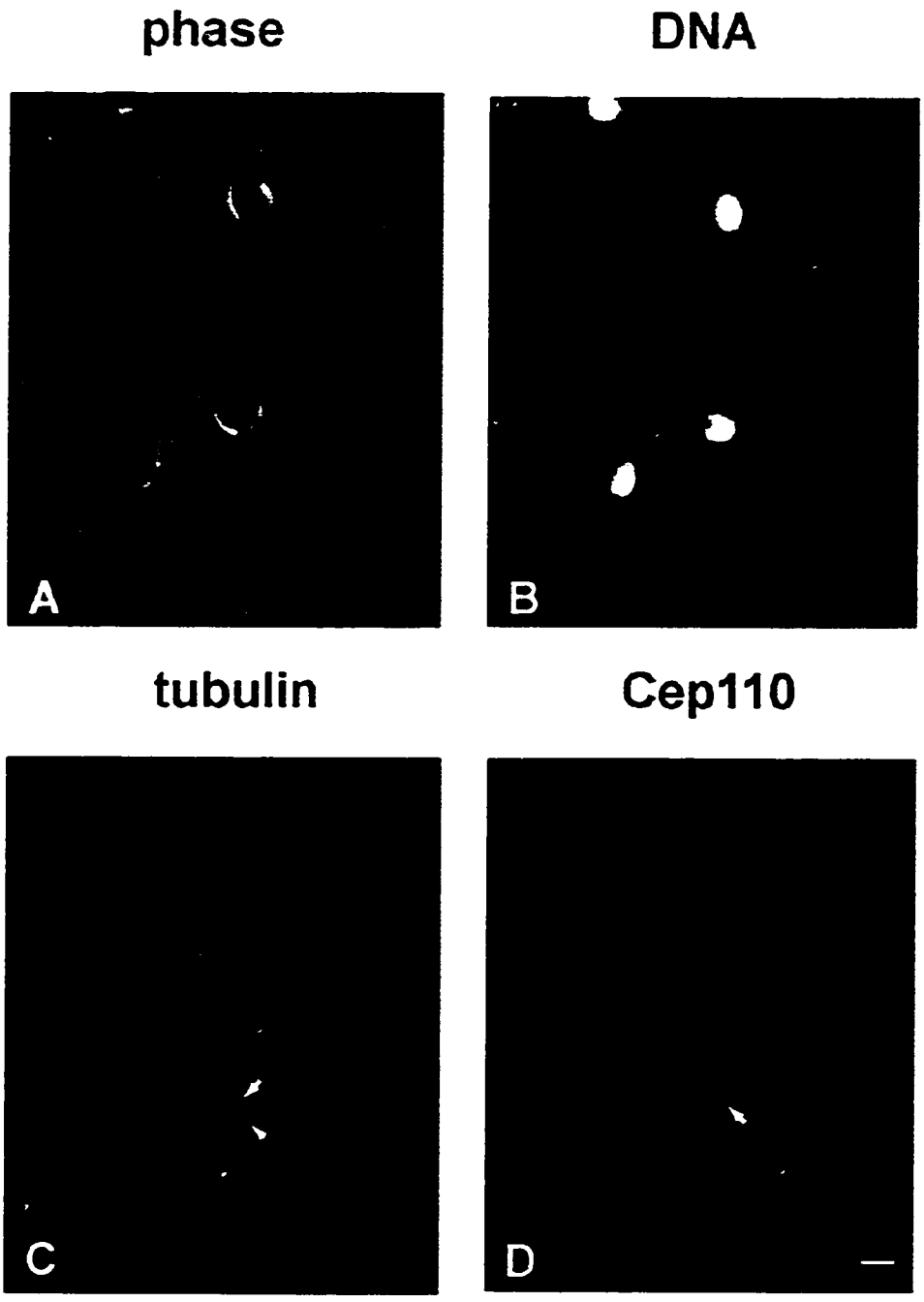

phase
DNA
A
B
tubulin
Cep110
C
D

Figure 5.38. Composite figure of tubulin and Cep110 reactivity in human sperm. A merge between the anti-β-tubulin (green) and anti-Cep110 (red) signals shown that Cep110 is found just above the proximal centriole (arrow, E). Sperm headpiece staining (line or dots across the nucleus) is an artifact of the Cep110 antibody. Bar = 4 μm.

merge

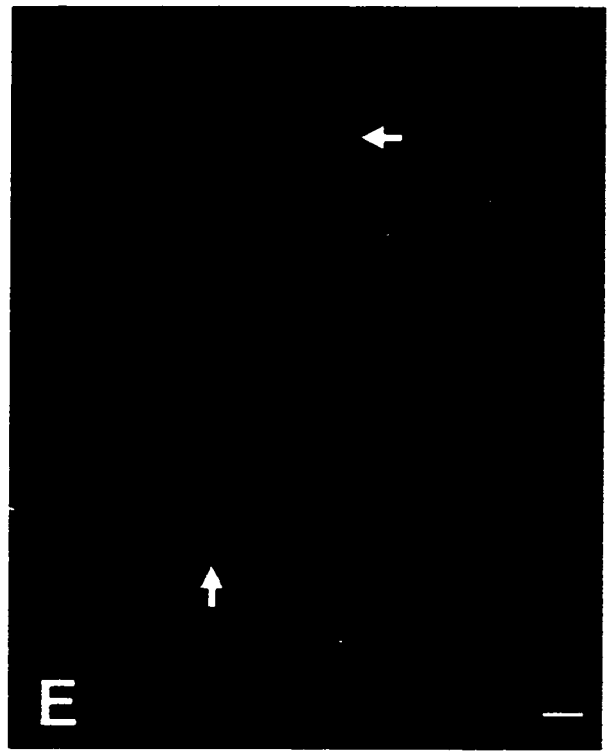


Figure 5.39. Schematic diagram of Green Fluorescent Protein (GFP) tagged Cep110 constructs. Various regions of Cep110 were tagged with GFP at the COOH terminus, transfected and scored for their ability to target the centrosome. Each deletion (DEL) construct was tested for centrosome targeting in two separate experiments on extracted and unextracted cells (see methods and materials). Localization of GFP to the centrosome was scored as positive (+), whereas no localization was scored as negative (-). Amino acid positions are shown above each construct, and yellow regions represent the predicted leucine zipper motifs.

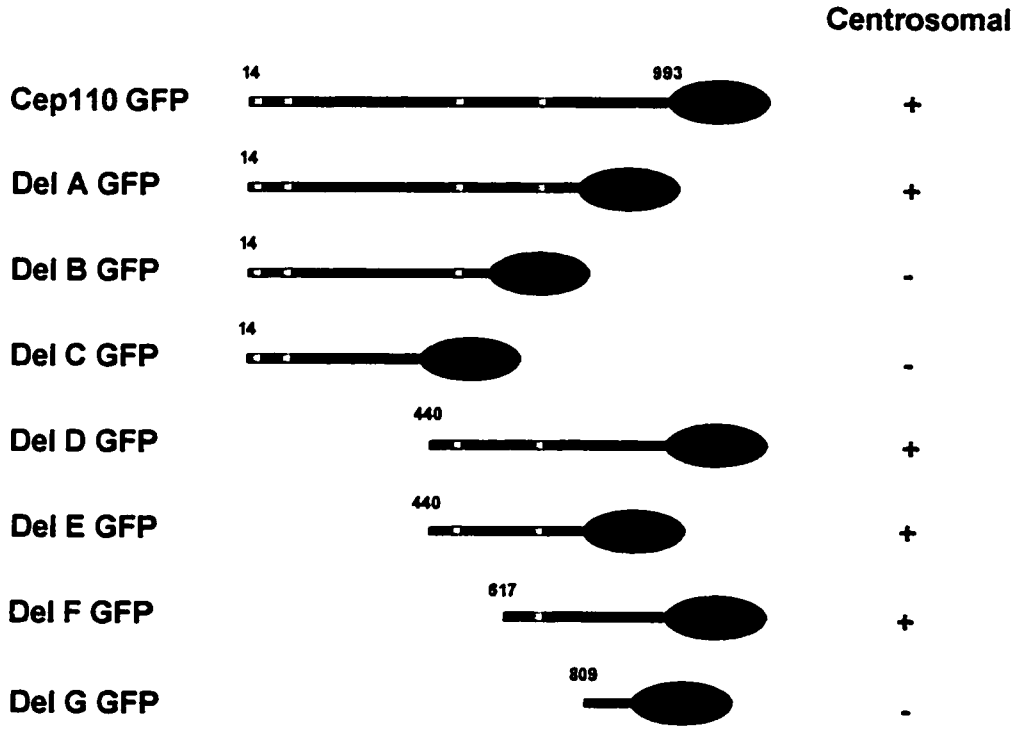

Centrosomal
Cep110 GFP
14
993
+
Del A GFP
14
+
Del B GFP
14
-
Del C GFP
14
-
Del D GFP
440
+
Del E GFP
440
+
Del F GFP
617
+
Del G GFP
809
-

Figure 5.40. Targeting of GFP tagged Cep110 to the centrosome. HeLa cells were transfected with the vector pEGFP-N1 alone, or the constructs Cep110-GFP or Del F-GFP. Cells expressing the GFP constructs (C, F, I) were fixed and double stained for the centrosomal protein pericentrin (B, E, H,), and counterstained for DNA with DAPI (A, D, G). Cells expressing the vector alone do not show centrosome targeting of GFP (C). Cells expressing the GFP tagged Cep110 construct show GFP centrosome staining (F) that colocalized with pericentrin reactivity (E). Centrosome targeting of GFP is observed for Del F-GFP (I) where 376 amino acids of the COOH terminus of Cep110 including the fourth leucine zipper are fused to GFP. Bar = 10μm.

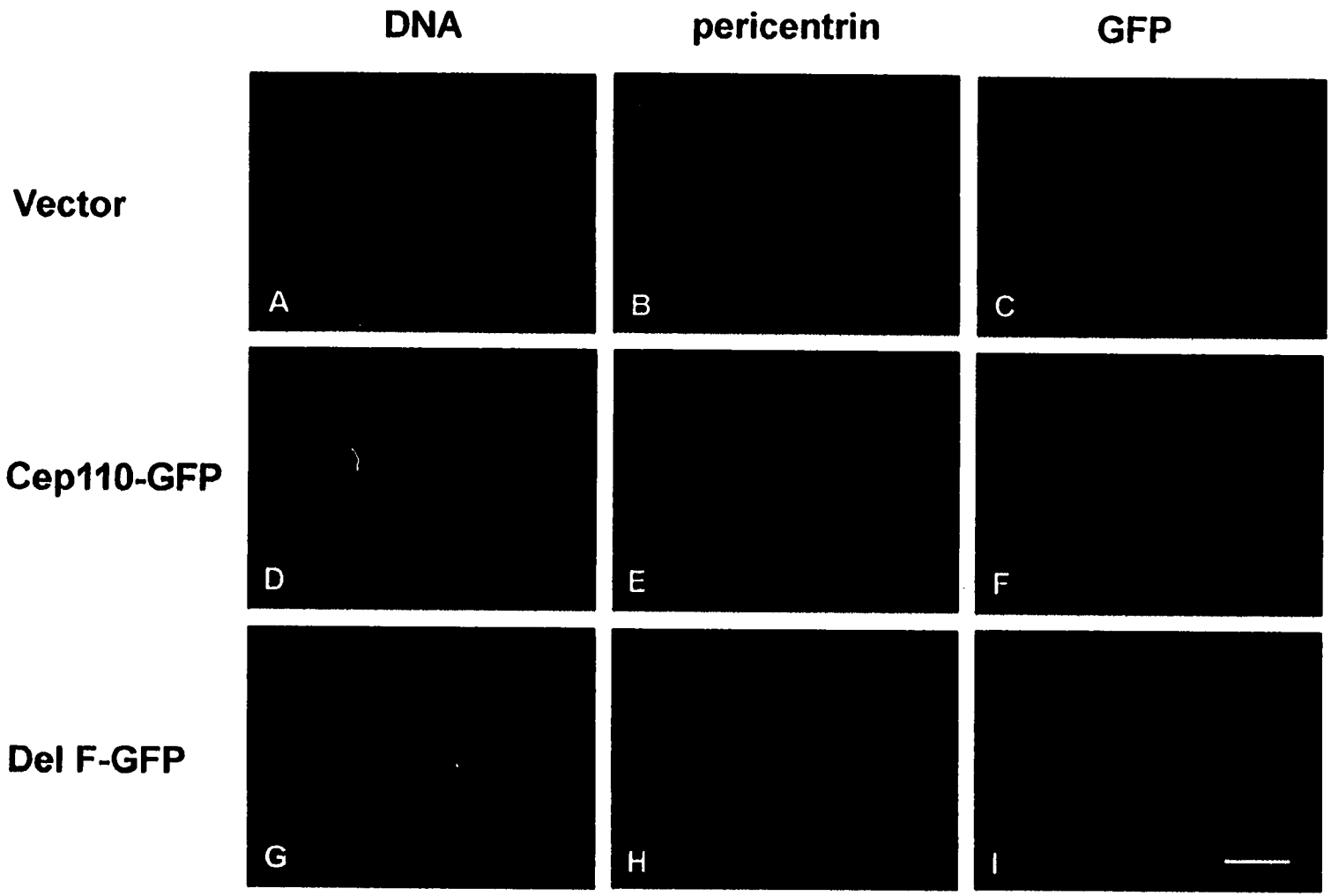
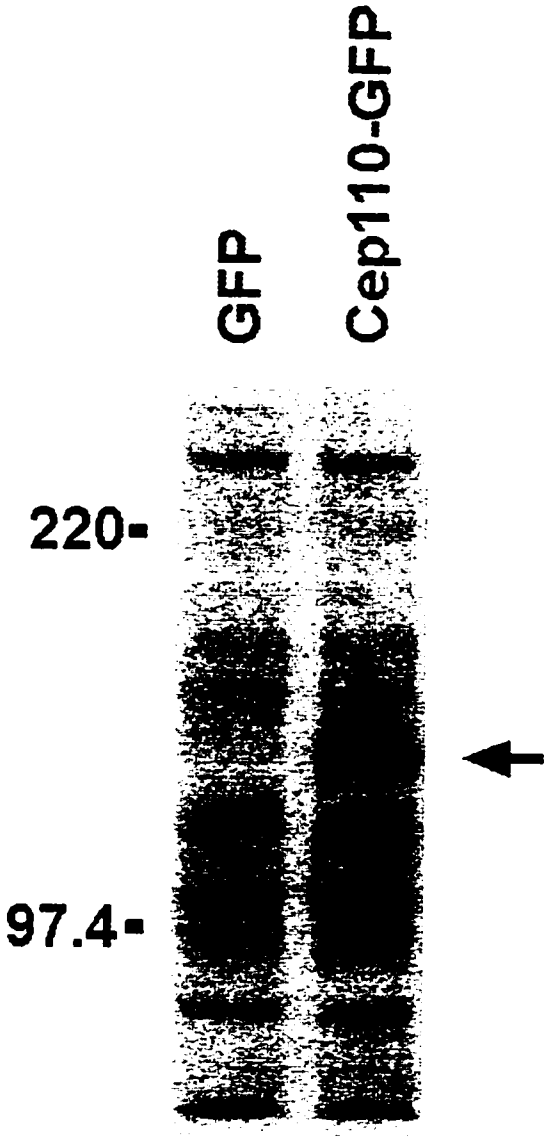

DNA
pericentrin
GFP
Vector
Cep110-GFP
Del F-GFP
A
B
C
D
E
F
G
H
I

Figure 5.41. Western blot analysis of GFP tagged Cep110. HeLa cells transfected with the construct Cep110-GFP or vector alone were allowed to express for 24 h before total protein was collected and resolved by 6 % SDS-PAGE. Western blot analysis with an anti-GFP antibody (Clontech) detected a single strong band at ~ 140 kDa in Cep110-GFP transfected cell protein. An equivalent band was not detected in protein from cells transfected with vector alone. Molecular weight markers are 220 and 97.4 kDa.


GFP
Cep110-GFP
220-
97.4-

DISCUSSION

Characterization of a human autoimmune sera reactive with the centrosome

In this study, a human autoimmune sera reactive with the centrosome throughout the cell cycle, and the mitotic spindle (Fig. 5.25) was used to screen a cDNA expression library. Four cDNA clones were identified, representing three different centrosome proteins. Two clones were found to encode for a portion of Cep250 between amino acids 559-1433 (see SECTION V A). A third complete cDNA clone encodes the human homolog of the 1133/5p centrosome protein of mouse. This is the first report that this protein is a human centrosome autoantigen. The 1133/5p protein was identified from a mouse expression library with a monoclonal antibody directed against antigens associated with extracted human sperm nuclei (Angiolillo, A. et al. 1996). Characterization of the 76 kDa 1133/5 protein revealed that it contains two NH_2-terminal domains potentially capable of forming short coiled-coils, and a COOH terminus with similarity to the Src homology region 3 (SH3). Antibody raised against the 1133/5p protein shows reactivity with the centrosome throughout the cell cycle (Angiolillo, A. et al. 1996). Our human cDNA clone of this protein has been completely sequenced, and I have designated the protein it encodes as Cep76 (Centrosome protein 76). The final cDNA clone identified from our library screen showed no significant homology in the GenBank database, and has subsequently been shown to encode a novel centrosomal antigen called Cep110.

Neither Cep76 or Cep110 were included in our investigation of centrosome autoantibody specificity in human autoimmune sera (SECTION III), as they have only

recently been characterized. A study on the frequency of autoantibodies to these antigens is now underway in our laboratory.

Characterization of a novel centrosomal antigen, Cep110

The complete cDNA of Cep110 has been cloned, and analysis of the deduced amino acid sequence reveal that Cep110 has extensive stretches of coiled-coil structure. In this respect Cep110 is similar to other centrosomal proteins such as pericentrin (Doxsey, S.J. et al. 1994), ninein (Bouckson-Castaing, V. et al. 1996) and Cep250 (this dissertation). It is interesting to note that all three centrosomal antigens identified with this autoimmune serum contain regions of coiled-coil structure, further illustrating the antigenicity of this protein motif. Further analysis also indicates that Cep110 has several potential sites for post-translational modification, including amidation, myristoylization, glycosylation, and phosphorylation. As yet no evidence for either of these modifications occurring on Cep110 has been obtained. Four leucine zipper motifs are also found in Cep110, two at the NH_2-terminus, one near the middle region, and one at the COOH terminus. The presence of leucine zipper motifs in centrosomal proteins is not unexpected, as they have also been found within the centrosomal proteins ninein (Bouckson-Castaing, V. et al. 1996) and Cep250 (this dissertation). The placement of leucine zippers in Cep110 is similar to that observed for Cep250, and maybe useful in grouping proteins with similar functions or distributions within the centrosome.

Affinity purified antibody to the COOH terminus of Cep110 detects a single protein at ~ 120 kDa in Western blot experiment, and is in good agreement with the estimated molecular weight of Cep110 (long form) of 116 kDa. Since this antibody was raised

against an epitope common to both the long and short form of Cep110, it was surprising that only a single protein was detected. It is possible that short form of Cep110 is rarely produced in HeLa cells, and may be more easily detected in preparations of enriched centrosomes. Western blot detection of pericentrin requires the use of enriched centrosome fractions (Doxsey, S.J. et al. 1994).

Characterization of Cep110 has shown that it contains four leucine zipper motifs and is highly coiled-coil. To determine if either of these motifs are responsible for targeting Cep110 to the centrosome, various domains of Cep110 were fused to GFP. When full length Cep110 is tagged with GFP, specific centrosome reactivity is observed confirming the immunolocalization results (see below). Furthermore, GFP tagged deletions of Cep110 have indicated that 170 amino acids near the COOH terminus is required for its centrosome association. This stretch of amino acids includes the fourth leucine zipper motif of Cep110. It is possible that this leucine zipper is all that is required for centrosome targeting. In this respect, Cep110 would appear to target to the centrosome in an identical fashion as the centrosome protein Cep250 (see SECTION V A). The remaining three leucine zipper motifs not required for the centrosome association of Cep110 may function in either homo or hetero dimerization of the protein at the centrosome. Evidence for the homodimerization of Cep110 has been obtained in collaboration with Dr. D. Birnbaum, and is discussed below. GFP reactivity was not observed within the intercellular bridge indicating that Cep110 does not undergo a redistribution during the cell cycle like Cep250. Cell cycle analysis of Cep250 GFP has not been rigorously investigated as optimal conditions for transfected cell viability and growth

have not yet been established.

Cep110 is a alternatively spliced protein

During the characterization of this protein it became apparent that two isoforms of Cep110 had been cloned, differing at their COOH terminus by 47 amino acids. The shorter isoform of Cep110 lacks a small region of coiled-coil which does not contain any significant protein motifs. Using specific primers within the non coding and coding regions of Cep110 we were able to confirm that both the long (3893 bp) and short (3752 bp) forms of Cep110 were present in cDNA derived from HeLa cells and human placenta, and are not cloning artifacts. The functional significance of this shorter isoform of Cep110 is not known.

The cloning of these two isoforms prompted us to determine if Cep110 was alternatively spliced in other tissues. Northern blot analysis indicated the presence of multiple Cep110 transcript sizes in most human tissues. The multiple forms of Cep110 may reflect alternative splicing within either the coding region or the 5' and 3' untranslated regions. Alternatively, the multiple Cep110 transcripts may result from heterogeneity within the poly $(A)^+$ tail. Evidence for alternative splicing within the coding region of Cep110 has already been discussed. In each tissue sample where a hybridization signal was detected a transcript between 3.7-3.8 kb was present, and likely represents either one or both of the cloned Cep110 isoforms. Surprisingly, skeletal muscle showed an abundant Cep110 transcript at ~7.4 kb whereas cardiac muscle did not. Centrosomes are not expected to have an important role in muscle cell biology. However, the association of the centrosome with actin filaments has been well established (Euteneuer, U. et al. 1985,

Mack, G. et al. 1993, Whitehead, C.M. et al. 1996b). The large size and abundance of Cep110 in skeletal muscle may be indicative of a specialized role for Cep110 at the centrosome in these cells. Other alternatively spliced centrosome proteins include ninein (Bouckson-Castaing, V. et al. 1996), centrosomin A (Petzelt, C. et al. 1997) and structural protein 4.1 (Krauss, S.W. et al 1997b). Protein 4.1 was first identified as a human red blood cell membrane protein, and is a member of a large family of proteins generated by extensive alternative RNA splicing (Krauss, S.W. et al. 1997a,). Immunoreactive proteins 4.1 species range in size from 30-210 kDa and are detected throughout the nucleus, mitotic spindle, midbody (Krauss, S.W. et al. 1997a), and centrosome at interphase (Krauss, S.W. et al. 1997b). The function of protein 4.1 appears very diverse, as it has been shown to bind to actin (Correas, I. Et al. 1986), myosin (Pasternack, G.R. et al. 1989) and tubulin (Correas, I. Et al. 1988). Cep110 transcripts were not detected in either brain or liver. It is possible that these tissues have Cep110 messages below the limit of detection by Northern blot hybridization, or they contain alternatively spliced forms that do not hybridize with the NH_2-terminal probe used in this study. However, immunostaining of monkey brain sections has revealed that Cep110 is present at the centrosome. Thus, it is possible that the brain has a low turnover of Cep110 protein, and therefore does not require Cep110 transcripts to be continually produced. As a result, Cep110 message is not easily detect in the brain. I have not been able to determine if Cep110 protein is expressed in liver cells, due to the unavailability of liver tissue sections.

Immunolocalization of Cep110

As shown in Figures 5.32-5.36, Cep110 is a protein that exhibits an unusual

centrosome association. In early interphase a single foci of Cep110 reactivity is detected at the centrosome. A composite image of Cep110 and pericentrin reactivity during interphase shows that Cep110 occupies a small part of the centrosome material, and most likely reflects its association with a centriole(s) (Fig. 5.33). Only one foci of reactivity is detected at the centrosome, suggesting that Cep110 is found at only one of the centriole pairs. However, we can not rule out the possibility that the second centriole is reacting with the antibody but is obscured due to the orientation of orthogonal centrioles. This is further supported by the finding of Cep110 reactivity at either the periphery or center of pericentrin reactivity in different samples (Fig. 5.33). When duplicated centrosomes begin their separation to opposite sides of the nucleus, Cep110 reactivity is only detected at one centrosome. After cells have progressed to late interphase-early prophase Cep110 reactivity is detected on both centrosomes. Thus, during a portion of interphase, duplicated centrosomes are not equal with respect to Cep110. As the cell proceeds through mitosis Cep110 reactivity at the centrosome decreases, and is barely detectable at each centrosome or spindle pole. Finally, during telophase Cep110 reactivity is once again prominently observed as a single compact foci at the centrosome. It is possible that the inability to detect Cep110 at one centrosome during interphase may be due to the absence of Cep110, or the inaccessibility of Cep110 epitopes on that centrosome (these possibilities will be discussed below).

Asymmetric centrosome reactivity has been noted for several other centrosome proteins such as centrin (Errabolu, R. et al. 1994), ninein (Bouckson-Castaing, V. et al. 1996), and the CCD41 antigen (Rothbarth , K. et al. 1993). However, for each of these

proteins, antibody reactivity appears stronger on one centrosome as compared to the other. Antibody to the centrosome protein cenexin reveal a similar cell cycle centrosome pattern as that of Cep110 (Lange, B.M.H. et al. 1995). Reactivity with the cenexin antibody is detected on only one centriole pair until early prophase, when it is detected on both centrosomes. From prophase through telophase cenexin reactivity at each centrosome or spindle pole remains prominent. Further investigation with the cenexin antibody has shown that it is only the mother centriole in an interphase centrosome that has cenexin reactivity. When this centrosome duplicates, cenexin reactivity follows the mother centriole. Only when the cell reaches the G2-prophase transition does the daughter centriole acquire cenexin reactivity (Lange, B.M.H. et al. 1995). The cenexin antigen has not been molecularly characterized and I believe it is unlikely that cenexin and Cep110 are the same protein. Monoclonal antibodies against cenexin identify a single 96 kDa protein in Western blots of isolated calf centrosomes, significantly smaller than the 120 kDa size observed for Cep110. However, cloning of the cenexin cDNA and comparison to Cep110 will be required to confirm the relationship between these two proteins. At this time we are not able to determine with confidence if Cep110 reactivity is confined to either the mother or daughter centriole during interphase. Colocalization of Cep110 with cenexin, or with a primary cilium nucleated from the mother centriole (Tucker, R.W. et al. 1979) will be necessary in order to distinguish these possibilities.

A composite image of Cep110 and β-tubulin antibody reactivity in human sperm cells shows Cep110 to be adjacent to, but not completely colocalizing with the proximal centriole. It is possible that this area of reactivity represents a population of Cep110 that

resides in the electron dense material just outside of the centriole proper. Alternatively, Cep110 reactivity throughout the proximal centriole may be prevented due to epitope inaccessibility. Antibodies raised to other Cep110 epitopes may be able to distinguish between these possibilities. It is the proximal centriole of the sperm that will be introduced into the oocyte during fertilization, and will be responsible for organizing the spindle poles for the subsequent mitotic divisions (Sathananthan, A.H. et al. 1996).

The unusual centrosome reactivity observed for Cep110 is not seen with the human autoimmune serum used to identify Cep110 (Fig. 5.25). It is likely that this is due to the presence of additional centrosome autoantibodies to the proteins Cep76 and Cep250 that obscure the Cep110 centrosome pattern. Interestingly, strong cytoplasmic staining is observed at metaphase with the human autoimmune serum, and is probably due to autoantibodies against Cep250 (see SECTION V A). As mentioned previously, this human serum also weakly reacts with the mitotic spindle. The antigen responsible for the reactivity has not been identified and indicates that only a portion of the autoantibody specificity within this serum has been identified. Alternatively, the autoantibodies against the centrosomal proteins Cep76, Cep110, or Cep250 may cross react with this mitotic spindle antigen.

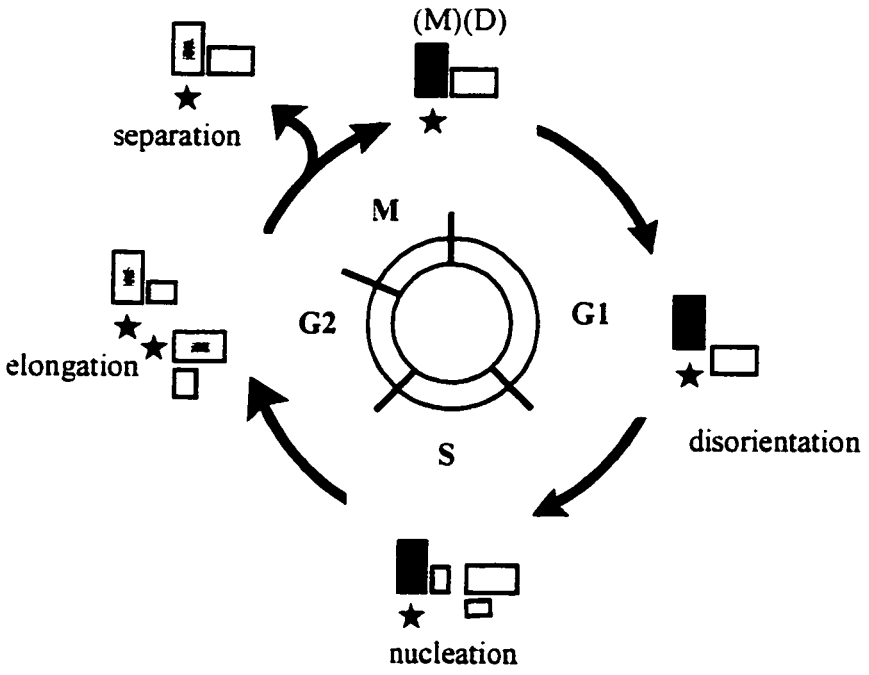
Models for Cep110 reactivity at the centrosome

Two models that could account for Cep110s unusual immunofluorescence pattern at the centrosome are present. In the first model, Cep110 is postulated to be a protein component of one centriole of a G1 centrosome, and is required for a centrosome to become a MTOC. Thus, for the purpose of this model Cep110 associates the mother

centriole which possess the capacity to act as a MTOC. During centrosome duplication and separation Cep110 remains associated with the mother centriole, and hence only one centrosome reacts with Cep110 antibody. The maturation of the daughter centriole into a functional MTOC occurs at the G2-prophase transition (Fulton, C. 1971, Rieder, C.L. et al. 1982b). During this maturation process Cep110 becomes associated with the daughter centriole and enables it to become an MTOC. Thus, at prophase both centrosomes reacts with Cep110 antibody, and are functional MTOCs (see Fig. 3.0). It is conceivable that Cep110 could be synthesized at the G2-M transition and then associates with the maturing daughter centriole. However, Cep110 reactivity has not been observed at any time with in the cytoplasm during the cell cycle. A diagram illustrating Cep110's association with the centrioles in this model is shown in Figure 5.42.

In an alternative model, Cep110 is a protein component of both centrioles (mother and daughter) of a G1 centrosome, but is altered in some fashion on the daughter centriole such that our Cep110 antibody will not detect it. During the G2-prophase transition molecular or regulatory processes occur at the daughter centriole associated with maturation in to a MTOC. As a result of these events previously hidden epitopes on Cep110 now become exposed. Therefore, antibody to Cep110 can detect both parent centrioles at duplicated centrosomes. The sudden finding of Cep110 reactivity on both centriole pairs in late interphase, and the gradual decrease in reactivity at the centrosome that is observed during mitosis, support this model in which Cep110 epitopes are modified during the cell cycle. It is possible that phosphorylation or dephosphorylation events may be responsible for these epitope changes, although protein interactions with Cep110 can

Figure 5.42. Model for Cep110 expression at the centriole. In this model, Cep110 (blue shading) is only observed at mother centriole (M) of a G1 centrosome. The mother is the older of the two centrioles and is a microtubule organizing center (MTOC, red star). As the centrioles duplicate and separate, Cep110 is only found on the mother centriole. At late G2-prophase, the daughter centriole (D) becomes an MTOC, and acquires anti-Cep110 reactivity. It is not known if this is a result of the synthesis and association of Cep110 at this phase of the cell cycle, or an unmasking of antibody epitopes. Cep110 is now found on both centrioles, but its staining intensity at each centriole decreases during mitosis (light blue shading). MTOC acitivity is indicated by a red star.


(M)(D)
separation
M
G2
G1
elongation
disorientation
S
nucleation

not be ruled out at this time. The antibody used in these studies is directed against the COOH terminus of Cep110, near the leucine zipper responsible for centrosome targeting (see Fig. 5.28). Thus, this region of Cep110 is in close contact with at least one other protein. An alteration in the interaction of Cep110 with this protein during the G2-prophase transition, could expose the epitopes recognized by this antibody.

Additional antibodies to other regions of Cep110 have been generated (Fig. 5.28) but have not yet been affinity purified. When completed, immunofluorescence studies with these antibodies should be able to distinguish between these models.

Cep110 and cancer

A mentioned previously, the centrosome is an important component of the mitotic apparatus, and is the location for a mitotic checkpoint that ensures the accurate segregation of the genome. Alterations in centrosome duplication, morphology and biochemistry can lead to genome instability, and are often characteristic of certain cancers (Lingle, W.L. et al. 1998). Cep110's role in cancer has only recently come to light with the finding that its gene locus is specifically involved in translocation events characteristic of stem cell myeloproliferative disorder (MPD). This disease results in the proliferation of lymphoblastic T or B cells, myeloid hyperplasia, eosinophilia, and often leads to myleoid leukemia (MacDonald, D. et al. 1995). The chromosome rearrangements characteristic of MPD always involve the fibroblast growth factor receptor 1 gene (FGFR1) on chromosome 8, and three different partners on chromosome 6, 9, and 13 (Popovici, C. et al. 1998, Chaffanet, M. et al. 1998). Characterization of the translocation event between chromosome 8 and 13 has revealed that the FGFR1 tyrosine kinase domain is fused in-

frame at its NH_2-terminus to a zinc finger containing protein called FIM (Popovici, C. et al. 1998). Cells with this translocation have a constitutively activated tyrosine kinase that is mediated by the dimerization of the NH_2-terminal portion of FIM. Activation of the kinase likely promotes hemopoetic stem cell proliferation and leukemogenesis (Popovici, C. et al. 1998).

In collaboration with Dr. D. Birnbaum (Institut de Cancerologie et d'Immunologie de Marseille) we have recently shown that the translocation of the FGFR1 gene into chromosome 9 occurs at nucleotide position 809 within the Cep110 sequence. As a result, the first 112 amino acids of Cep110 are fused to the tyrosine kinase domain of FGFR1. We believed that constitutive kinase activity results when this portion of Cep110 dimerizes. This NH_2-terminal region of Cep110 contains the first complete leucine zipper motif and truncates the second leucine zipper by six amino acids. These results suggest that either one or both of the leucine zipper motifs could be involved in Cep110 dimerization. It will be interesting to determine if cells expressing this Cep110-FGFR1 fusion protein have an altered centrosome morphology. Previous characterization of cancer cells has shown dramatic alterations in centrosome structure and microtubule nucleating capacity (Lingle, W.L. et al. 1998). Furthermore, in light of these recent findings, and the discovery that autoantibodies to cell cycle proteins are often found in patients with cancer (Casiano, C.A. et al. 1995, Rattner, J.B. et al. 1997), we are investigating whether Cep110 autoantibodies are more prevalent in patients with hemopoietic cancer.

In conclusion, immunolibrary screening with human autoimmune sera reactive with the centrosome has resulted in the identification of three centrosomal proteins, Cep250,

Cep76 and Cep110. This work is the first to report that Cep76 is a human autoantigen. Characterization of Cep110 has revealed that it is a ~ 120 kDa highly coiled-coil protein with four leucine zipper motifs. Centrosome targeting of Cep110 appears to reside within a region near the COOH terminus and likely requires the fourth leucine zipper motif. Northern blot analysis indicates that Cep110 is rare and has multiple forms in different tissues. The possibility exists that the two forms of Cep110 described in this dissertation are part of a larger protein family. Cep110 is likely a component of the centriole(s), and displays a unique immunofluorescence pattern that suggests it participates in centrosome maturation at the G2-M transition. Recent collaboration with Dr. D. Birnbaum has confirmed the correct cloning of Cep110, and has recently shown that the NH_2-terminal domain of Cep110 is fused in-frame to the kinase domain of the FGFR1. These translocation events are often associated with hemopoietic cancer and result from the dimerization of kinases leading to its constitutive activation.

SECTION VI

FINAL DISCUSSION

The focus of this dissertation was to investigate the centrosome from three different perspectives: clinical, biological and biochemical. By studying the centrosome in this manner a more complete picture of centrosome biology was obtained.

I. Human autoimmune sera reactive with the centrosome

In other studies of human sera reactive with the centrosome, autoantibody complexity and possible associations with disease were not fully addressed. Therefore, one of my goals was to determine which centrosome proteins function as human autoantigens, and determine if there exists a correlation between having autoantibodies to these antigens and having a specific type of rheumatic disease. By characterizing a group of 21 human sera reactive with the centrosome, I found that the majority have autoantibodies to multiple centrosomal proteins. Furthermore, most of these autoantibodies are directed against the centrosome antigens pericentrin, ninein and Cep250. Evidence that the autoimmune response to the centrosome is even more complex than this is shown by the identification and characterization of a human autoimmune sera with autoantibodies to two other centrosome antigens, Cep110 and Cep76, in addition to autoantibodies to Cep250. The complexity of the autoimmune response to the centrosome suggests that it is the centrosome or a centrosome protein complex(s) that is the target for the immune system. Interestingly, autoantibodies to PCM-1 appear at half the frequency of autoantibodies to pericentrin, ninein, or Cep250, and this may indicate that this centrosome antigen is a part different centrosome protein complex.

By reviewing the clinical features of patients in this study, we were not able to find a clear correlation between having autoantibodies to the centrosome and a specific clinical

diagnosis. However, it is possible that more patient sera with autoantibodies to the centrosome need to be investigated before any correlation with disease can be observed.

This work has not only contributed to the understanding of the autoimmune response to the centrosome, but will make the identification of additional sera reactive with the centrosome more straightforward. As a result, some of these sera could be used to identify additional centrosome proteins which will not only benefit cell biology, but also our understanding of how the immune system reacts against the centrosome.

II. Post-mitotic centrosome movement

Many studies looking into the function of proteins or structures involved in cell division have concentrated on the events leading to mitosis. While these events are important, very few studies have extended their investigations post-mitotically, when the cell returns to an interphase state. For example, centrosome movement in preparation for mitosis has been well describe both morphologically and functionally. Many microtubule motor proteins involved in this process have been identified and characterized, and microtubule arrays nucleated by the centrosome for separation have been mapped and modelled. However, almost nothing was known about centrosome function after mitosis. For example, at the completion of anaphase B centrosomes are in a polar location. However, there are fragmentary reports of centrosomes on the opposite side of the cell next to the intercellular bridge. How these centrosomes acquired this position was unknown. Therefore, the second goal of my work on the centrosome was to characterize the centrosome following mitosis.

By using human autoimmune sera that react with the centrosome, I was able to

follow the behaviour of the centrosome after mitosis. I discovered that shortly after telophase but before cytokinesis, the centrosome of each daughter cell moves from its polar location to a region adjacent to the intercellular bridge. Furthermore, a directionally nucleated microtubule bundle is required for this movement, and the centrosomes position next to the intercellular bridge is dependent on an intact microfilament network. How the centrosome is linked to the actin filament network still remains unknown. However, as illustrated in Figure 6.1, the centrosome may attach to an actin filament through the dynactin complex and protein 4.1. Where it was previously believed that the centrosome underwent one major movement during the cell cycle associated with mitosis, I have now shown that it undergoes a second major movement at the end of cell division. Furthermore, this post-mitotic centrosome movement is different than the pre-mitotic movement in that each centrosome moves independent of each other, and does require antiparallel microtubules. Because of these features, post-mitotic centrosome movement can be used as a means to investigate the function of a mitotic protein independent of its role in either the formation of the spindle or the in absence of antiparallel microtubules. Recently, Whitehead and Rattner. (1998) have used this approach to determine that the motor protein HsEg5 is required for post-mitotic centrosome movement, and that HsEg5 can functions in the absence of antiparallel microtubules. Furthermore, by inhibiting HsEg5 function, the microtubule array that is responsible for centrosome movement was also shown to be necessary for the correct repositioning of the Golgi apparatus after mitosis. Thus, by characterizing post-mitotic centrosome movement, we now understand more about the function of the centrosome in establishing (1) the polarity of cellular organelles, and (2) the

equal and opposite polarity of the interphase cytoskeleton in daughter cells. Furthermore, its discovery has given us a new appreciation for the function an essential motor protein.

III. Centrosome proteins, Cep250 and Cep110

The last goal for my investigation of the centrosome was to identify and characterize additional centrosome proteins. This has been the major focus of most of my graduate work as I feel it is essential to understanding of the centrosome. Any model used to describe the function or organization of the centrosome requires a complete knowledge of its protein components. Clearly then, models of centrosome function are incomplete as only a few centrosome proteins are known and well characterized.

Human autoimmune sera have been essential for this study, and have resulted in the identification of two novel centrosome proteins, Cep250 and Cep110. The characterization of these proteins has revealed several interesting possibilities. First, the centriole may be organized or integrated with the PCM by a family of structurally similar proteins. Both Cep250 and Cep110 could be members of the same family, as both have a similar structure, consisting of extensively coiled-coil regions and have numerous leucine zipper motifs. Furthermore, both Cep250 and Cep110 appear to be targeted to the centrosome with a single COOH terminal leucine zipper. Furthermore, these proteins may have a conserved function in MTOC, as similar large coiled-coil leucine zipper containing proteins are components of the yeast spindle pole body. Thus, even though the structure of the spindle pole body and the centrosome are very different, they may be organized with similar protein components.

My work also indicates that the structure of the centrosome is more dynamic than

previously believed. Much of what we know about the changes that do occur at the centrosome upon entry into mitosis has come from electron micrographs showing the static accumulation of PCM and the reorganization of microtubules. By following Cep250 through the cell cycle we can now observe that there is also a loss of protein components at the mitotic centrosome. The centrosome protein PCM-1 has an immunofluorescence pattern similar to Cep250, and may suggest that these two proteins interact or function in the same manner. Having the tools to identify both of these proteins, we can now address these possibilities.

Similarly, Cep110 undergoes a change in its reactivity at the centriole during G2-prophase, and further reflects the dynamics of the centrosome. Should Cep110 be involved in centriole maturation, it will be the first molecularly characterized protein that participates in this process. Cep110's immunofluorescence pattern at the centrosome also parallels that of cenexin, and may indicate that these proteins function together. Thus, with the identification of Cep250 and Cep110, other avenues of centrosome investigation have come to light. A diagrammatic representation of how Cep250 and Cep110 may function at the centrosome is shown in Figure 6.2. In this model Cep250 is shown to link the PCM to the centrioles during interphase, and is released from the centrioles at mitosis so that the PCM can expand and nucleate the dynamic microtubules of the spindle. Cep110 is strongly detected on the mature centrioles that are microtubule organizing centers, and its staining intensity at each centriole is cell cycle dependent.

Figure 6.1. Schematic representation of cytoplasmic dynein and the dynactin complex mediating the attachment of a post-mitotic moving centrosome to actin filaments. Cytoplasmic dynein (yellow circles), the intermediate chains (green ovals), and the light intermediate chains (pink circles) are attached to the dynactin complex: $p150^{Glued}$ (red ovals), dynamitin (dark purple squares), centractin and actin (blue circles), capping protein (orange triangle), and p62 (light purple square). After attachment to a microtubule, the dynein-dynactin complex is anchored to an actin filament through protein 4.1 (light pink squares). Also shown is the kinesin-like protein HsEg5 linking $p150^{Glued}$ and anti-parallel microtubules.

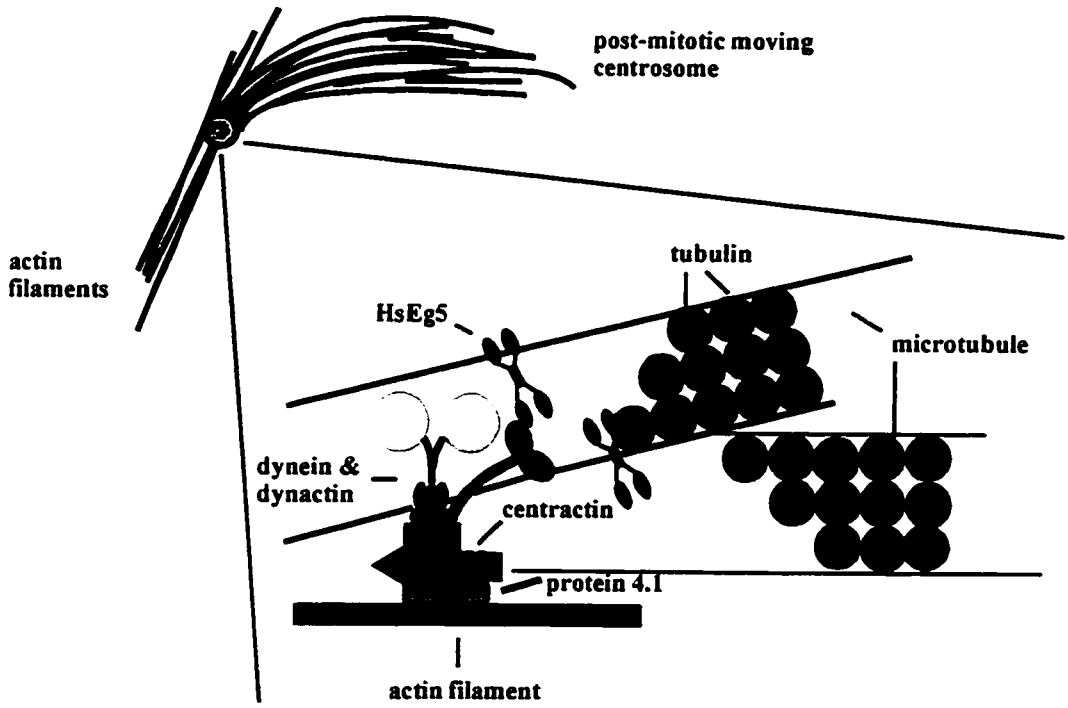
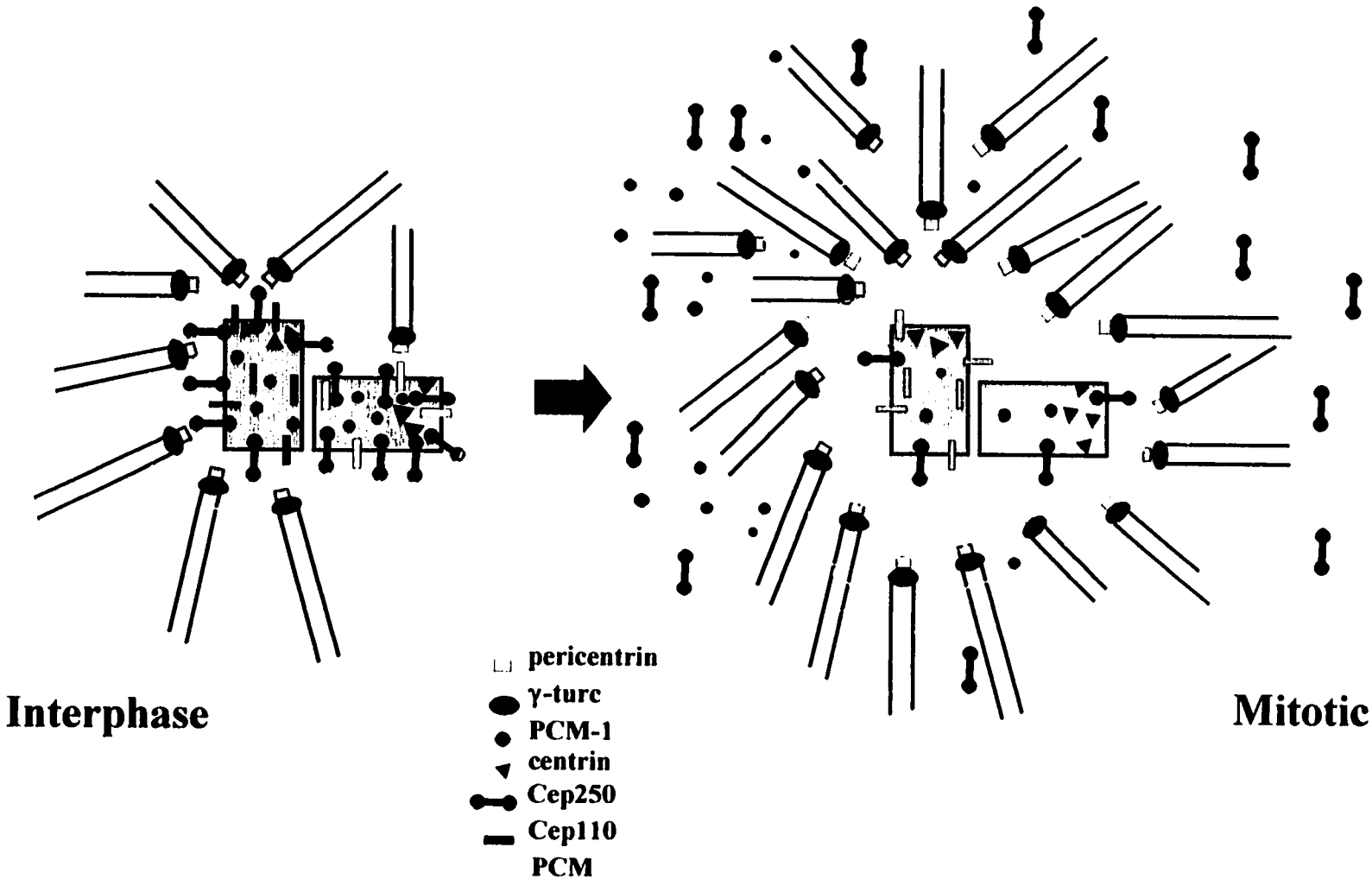

post-mitotic moving centrosome
actin filaments
tubulin
HsEg5
microtubule
dynein & dynactin
centractin
protein 4.1
actin filament

Figure 6.2. Schematic representation of the changes occurring at the centrosome during the transition from interphase to mitosis. Two right angled centrioles of an interphase centrosome have tightly associated Cep250 protein (red dumbbells) anchoring the pericentriolar material (PCM) close to the centrioles. The mother or mature centriole (light blue) has the majority of the PCM and nucleates microtubules with the γ-tubulin ring complex (γ-turc, blue ovals) and pericentrin (yellow boxes). Also shown is Cep110 reactivity (green bars) on the mother centriole indicating it is a microtubule organizing center (MTOC). Cep110 (white bars) may be found on the daughter centriole (peach), but anti-Cep110 antibody fail to recognize it. The protein centrin (black triangles) is asymmetrically distributed to the distal end of the mother and daughter centrioles. The protein PCM-1 (pink circles) may also be found on the centrioles. At mitosis, most of Cep250 is released from the centrioles into the cytoplasm possibly a result of mitotic phosphorylation. As a result, the PCM can expand to nucleate more microtubules for the forming spindle. The majority of PCM-1 is also released from the centrioles into the cytoplasm at mitosis. Cep110 remains attached to the mature mother centriole but its reactivity with anti-Cep110 antibodies is diminished (light green bars). The newly formed daughter centriole (light purple) has not acquired anti-Cep110 reactivity and is not a MTOC. At the end of mitosis, Cep250 may be dephosphorylated allowing it to interact with the centrioles again thereby modifying the PCM, and returning the centrosome to an interphase state.


Interphase
pericentrin
γ-turc
PCM-1
centrin
Cep250
Cep110
PCM
Mitotic

SECTION VII

FUTURE DIRECTIONS

I. Clinical

The study of human autoimmune sera reactive with the centrosome presented in this dissertation can be further expanded to a number of areas. For example, the frequency of autoantibody reactivity to the recently identified centrosome antigens Cep110 and Cep76 should be determined. This work will help not only in our understanding of the complexity of the autoimmune response to the centrosome, but also in how the immune system targets the centrosome. Furthermore, it is possible that the rarity of human autoimmune sera reactive with the centrosome is due to the difficulty in recognizing centrosome reactivity. Therefore, a better method of identifying centrosomal reactive sera is required. It is possible that centrosome antigen-coupled micro-beads will be a more sensitive method of detecting centrosome autoantibodies. By increasing the number of patients characterized with the centrosome antibody reactivity, specific clinical associations may become evident.

II. Cell Biology: microtubule nucleation

Post-mitotic centrosome movement provides us with a number of interesting questions to address. However, I feel that the focus of further investigation should concentrate on determining how the centrosome nucleate a spatially directed microtubule array. As microtubule nucleation *in vivo* is completely dependent on γ-tubulin, the investigation of post-mitotic centrosome microtubules should focus on this protein. It is possible that the microtubule bundle that arises behind the centrosome as it moves, is nucleated by an asymmetric distribution of γ-tubulin ring complexes. To investigate this, immuno electron microscopy (IEM) with γ-tubulin antibody could be carried out on post-

mitotically moving centrosomes. The concentration of γ-tubulin to one end of the centrosome would be evidence of this model. How γ-tubulin would achieve this distribution at the centrosome is the next question to consider. Since the γ-tubulin ring complex is known to exist in the cytoplasm, the specific recruitment of these complexes to one side of the centrosome at telophase is possible. The protein pericentrin has recently been suggested to tether the γ-tubulin complex to the centrosome. Thus, the specific placement of pericentrin in the PCM may mediate the binding of γ-tubulin. Pericentrin has been immunolocalized throughout the PCM, but this work has not concentrated on post-mitotic cells. Additional IEM with pericentrin antibody should be conducted.

Another possibility for the nucleation of a spatially directed microtubule array is the specific inhibition of nucleation sites on one side of the centrosome. If IEM with γ-tubulin antibody reveals that γ-tubulin is distributed throughout the centrosome during post-mitotic movement, then it possible that nucleation sites at the apex of the moving centrosome have been inhibited. This inhibition could be in the form of specific proteins or protein modification. Protein phosphorylation is known to be responsible for the microtubule nucleation at mitosis, and it is possible that only nucleation proteins on one side of the centrosome may be phosphorylated in telophase.

III. Function of Cep250 and Cep110

Because Cep250 and Cep110 are similar in many ways, the experiments designed for one protein can also be carried out on the other. In this manner direct comparison between the proteins is possible. The further investigation of these proteins should focus on addressing two main questions: what is their function at the centrosome, and with what

other centrosome proteins do they interact? These questions can be approached using microinjection and protein interaction strategies.

Antibody microinjection experiments: Cep250

Microinjection of antibody into cells may be the most straightforward way to directly determine function of Cep250. Interphase cells should be microinjected with affinity purified antibody against Cep250. After injection, cells should then be fixed and processed for indirect immunofluorescence using antibodies to PCM proteins such as pericentrin or γ-tubulin. Using this approach it should be possible to detect any significant alteration in centrosome morphology. Any abnormal centrosomes observed in injected cells might suggest that Cep250 is functional during interphase, possibly in either confining the PCM or in maintaining its integrity. Furthermore, it will be interesting to determine if centrosome separation occurs normally in injected cells, since this will allow a direct evaluation of the model proposed by Fry. A. M .et al. (1998) for Cep250/C-Nap1 function. If injected cells are not blocked during interphase, then observations of cells that have progressed into prophase will be possible. For these cells it will be interesting to see if Cep250 can dissociate from the centrosome and if prophase microtubule nucleation occurs normally. It is possible that antibody injection may prevent Cep250 from releasing from the centrosome, and this may in turn inhibit or reduce microtubule nucleation at prophase. Microinjection of Cep250 antibody into mitotic cells should prevent Cep250 from reassociating with the centrosome during late mitosis. This will allow the determination whether Cep250's absence at the centrosome affects its morphology at telophase or in the next G1 phase. If centrosome morphology is perturbed, it will be interesting to see if the

interphase microtubule network is normal.

Protein mutation and overexpression experiments

Although antibody microinjection is the most straightforward approach to determining the function of the proteins described here, it is dependent on the specificity of the antibody used, and its ability to inhibit the function of the protein. A second approach to assessing the role of Cep250 and Cep110 is to express various protein truncations and mutations to essentially "poison" the function of the endogenous protein. My work has already resulted in the construction of various GFP tagged deletions of Cep250 and Cep110. Although not the most ideal source of protein (possible deleterious effects of the GFP tag) it is a starting point to examine the effectiveness of this experimental plan.

The work reported in this dissertation has shown that COOH terminal deletions of the Cep proteins will not associate with the centrosome. However, these proteins likely interact other centrosome proteins at its NH_2-terminus. Thus, this mutant protein could out-compete the endogenous Cep proteins for these interactors. Alternatively, the mutant protein may interact and inhibit the function of the endogenous Cep protein through dimerization. Careful examination of these cells may reveal changes in centrosome structure and function. In addition, these cells may be blocked at a particular time in the cell cycle when these interactions are required by the endogenous Cep proteins. Overexpression of other domains and deletion products may results in different phenotypes and help in further defining the roles of specific regions of Cep250 or Cep110. The introduction of point mutations in phosphorylation sites could be useful in understanding how Cep250 and Cep110 are regulated during the cell cycle. Several potential

phosphorylation sites are clustered around the COOH terminal leucine zipper in Cep250. Their phosphorylation may disrupt the binding of Cep250 with the centriole resulting in its disassociation. Mutations in these sites could prevent Cep250 from being released, and may result in centrosome alterations, an inhibition of microtubule nucleation, or a block in the cell cycle.

Protein-protein interactions

Understanding the function of Cep250 and Cep110 is important, and deciphering how these proteins are organized within the centrosome will be essential for understanding the centrosome. One step toward this goal is to identify what other centrosome proteins Cep250 and Cep110 interact with. Characterization of these partners will likely shed additional light on the function of the Ceps, and provide us with other avenues in which to investigate the centrosome.

One method to identify interacting proteins is the yeast two-hybrid screen (Chien, C. et al. 1991). This approach has been successful in uncovering protein interactions in the yeast spindle pole body (Chen, X.P. et al. 1998) and the mammalian kinetochore (Chan, G.K.T. et al. 1998), among others. The most obvious regions of Cep250 and Cep110 to use as bait are the centrosome binding leucine zipper, and the NH_2-terminal leucine zipper clusters. The nucleotide sequence of any interactor is easily obtained using the two hybrid system, and confirmation of its centrosome location can be quickly determined using GFP tagging.

Because the two-hybrid system is notorious for identifying false positives, an complementary approach to identifying interactors is through co-immunoprecipitation.

Immunoprecipitation of Cep250 or Cep110 from interphase or mitotic protein extracts could result in the identification of other centrosome proteins. Furthermore, it will allow us to determine if there are different interactors during interphase and mitosis. Although the confirmation that any interactor is a genuine centrosome protein is time consuming, the approach has the benefit of being more direct. Overexpression of various tagged domains of Cep250 or Cep110 in cells followed by immunoprecipitation could be useful in identifying proteins that interact with specific regions of the Cep proteins. This approach has already been successful in identifying proteins and protein binding regions in the yeast spindle pole body (Knop, M. et al. 1997), and the mammalian centrosome (Murphy, S.M. et al. 1998).

In conclusion, the initial identification and characterization of Cep250 and Cep110 has been completed. Both proteins are genuine components of the centrosome, and are probably associated with the centrioles. Continued investigation of these proteins through the use of antibody microinjection and/or mutant protein expression will undoubtedly shed more light on the function of these proteins at the centrosome. These experiments and others designed to determine what other proteins Cep250 or Cep110 interact with, will also increase our understanding of how the centrosome is organized and functions through the cell cycle.

CONCLUDING REMARKS

The centrosome remains an enigma of modern cell biology. Understanding its organization and function during the cell cycle will keep scientists occupied for many more years. Human autoimmune sera has been extremely useful as an investigative tool for centrosome structure and function. Why the centrosome is a target in autoimmune disease still remains unknown. Key breakthroughs in the understanding of the centrosome will likely come from work focussed on identifying how the proteins of the centrosome interact. Furthermore, careful observation of the subtleties of centrosome behaviour during the cell cycle will probably open up additional areas of investigation that will tie the centrosome into previously unrelated cellular processes. Over 100 years after its discovery, the centrosome is only now beginning to reveal many of its mysteries.

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APPENDIX I

Nucleotide and amino acid sequence of Cep250.
The nucleotide sequence of Cep250 is shown, with the predicted amino acid sequence underneath. Leucine zipper motifs are underlined, and predicted cdk phosphorylation sites are boxed. These sequence data are available in GenBank under accession number AF022655.

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   1  GGAATTCCGGGAAATCCTGGGATAAGAGAATAGTTTCCTGGAAGATCTGTGCCTCCAACC
  61  AGCAGAGAGGGATTGAGCTTCATTGAACTCAACAGAGCCAACATTTCATAGCACCATGTT
 121  CAAGAGGAGGTTGAAGTGGCATGGCAATGGTTAGAGACCCTGCTGGGCGTGAACACCCTC
 181  TGGCTACCTAGGGACCTGTGGGCCTACCACCTGGTGCCCTCATGGAGACAAGAAGCCCTG
                                                  M  E  T  R  S  P  G

 241  GGTTGAACAACATGAAGCCCCAGTCACTGCAGCTGGTACTGGAAGAGCAGGTGCTGGCAC
        L  N  N  M  K  P  Q  S  L  Q  L  V  L  E  E  Q  V  L  A  L

 301  TACAGCAGCAGATGGCAGAGAATCAGGCAGCCTCCTGGCGGAAGCTGAAGAACTCCCAGG
        Q  Q  Q  M  A  E  N  Q  A  A  S  W  R  K  L  K  N  S  Q  E

 361  AGGCCCAGCAGAGACAAGCAACCCTTGTGAGGAAGCTGCAGGCCAAGGTGCTGCAGTACC
        A  Q  Q  R  Q  A  T  L  V  R  K  L  Q  A  K  V  L  Q  Y  R

 421  GAAGCTGGTGCCAAGAGCTGGAGAAGCGGCTAGAAGCCACTGGAGGACCAATCCCCCAGA
        S  W  C  Q  E  L  E  K  R  L  E  A  T  G  G  P  I  P  Q  R

 481  GGTGGGAAAATGTGGAGGAGCCAAACCTGGATGAGCTGCTGGTCCGATTGGAGGAGGAGC
        W  E  N  V  E  E  P  N  L  D  E  L  L  V  R  L  E  E  E  Q

 541  AACAGAGGTGTGAGAGTCTAGCAGAGGTGAACACCCAGATTCGACTGCACATGGAAAAAG
        Q  R  C  E  S  L  A  E  V  N  T  Q  I  R  L  H  M  E  K  A

 601  CTGACGTGGTGAATAAAGCCCTTAGGGCAGATGTGGAAAAACTGACAGTGGACTGGAGCC
        D  V  V  N  K  A  L  R  A  D  V  E  K  L  T  V  D  W  S  R

 661  GGGCCCGGGATGAGCTAATGAGGAAGGAGAGCCAGTGGCAGATGGAGCAGGAGTTCTTCA
        A  R  D  E  L  M  R  K  E  S  Q  W  Q  M  E  Q  E  F  F  K

 721  AGGGCTACCTGAAAGGGGAGCACGGTCGCCTTCTCAGTCTATGGCGGGAGGTTGTGACAT
        G  Y  L  K  G  E  H  G  R  L  L  S  L  W  R  E  V  V  T  F

 781  TCCGACGCCACTTCCTGGAAATGAAGTCAGCTACTGACAGAGATCTGATGGAGCTAAAAG
        R  R  H  F  L  E  M  K  S  A  T  D  R  D  L  M  E  L  K  A

 841  CTGAGCATGTGAGGCTTTCAGGGTCTCTGTTGACCTGTTGTCTGCGCTTGACTGTGGGAG
        E  H  V  R  L  S  G  S  L  L  T  C  C  L  R  L  T  V  G  A

 901  CACAGTCTCGGGAACCCAACGGATCTGGAAGAATGGATGGGCGGGAGCCGGCCCAGCTGC
        Q  S  R  E  P  N  G  S  G  R  M  D  G  R  E  P  A  Q  L  L

 961  TGCTGCTACTAGCCAAGACCCAGGAGCTGGAGAAGGAAGCCCATGAAAGGAGCCAGGAGT
        L  L  L  A  K  T  Q  E  L  E  K  E  A  H  E  R  S  Q  E  L

1021  TAATACAGCTGAAGAGTCAAGGGGATCTGGAGAAGGCTGAACTTCAGGACCGGGTGACCG
        I  Q  L  K  S  Q  G  D  L  E  K  A  E  L  Q  D  R  V  T  E

1081  AGCTCTCTGCTCTGTTGACCCAGTCTCAGAAGCAAAATGAAGATTATGAAAAGATGATAA
        L  S  A  L  L  T  Q  S  Q  K  Q  N  E  D  Y  E  K  M  I  K

1141  AGGCTCTGAGAGAGACAGTGGAGATCCTGGAGACAAATCACACAGAATTAATGGAACATG
        A  L  R  E  T  V  E  I  L  E  T  N  H  T  E  L  M  E  H  E

1201  AAGCATCTCTTAGTAGGAATGCGCAAGAGGAGAAGTTGTCTTTACAGCAGGTGATCAAGG
        A  S  L  S  R  N  A  Q  E  E  K  L  S  L  Q  Q  V  I  K  D
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1261  ATATAACCCAGGTCATGGTGGAAGAAGGGGACAATATAGCCCAAGGCTCTGGTCTTGAGA
        I  T  Q  V  M  V  E  E  G  D  N  I  A  Q  G  S  G  L  E  N

1321  ACTCTTTGGAATTGGAGTCTAGTATCTTCTCCCAGTTTGATTACCAAGATGCAGACAAGG
        S  L  E  L  E  S  S  I  F  S  Q  F  D  Y  Q  D  A  D  K  A

1381  CTCTTACTCTGGTGCGTTCAGTGCTGACTCGGAGACGCCAGGCTGTGCAGGACCTAAGGC
        L  T  L  V  R  S  V  L  T  R  R  R  Q  A  V  Q  D  L  R  Q

1441  AGCAGCTTGCAGGCTGTCAAGAGGCTGTGAACTTGTTGCAACAGCAGCATGATCAGTGGG
        Q  L  A  G  C  Q  E  A  V  N  L  L  Q  Q  Q  H  D  Q  W  E

1501  AGGAAGAGGGCAAAGCCTTGAGACAGCGGCTGCAGAAGCTCACTGGGGAGCGGGACACTC
        E  E  G  K  A  L  R  Q  R  L  Q  K  L  T  G  E  R  D  T  L

1561  TGGCAGGGCAGACTGTGGACCTCCAGGGAGAGGTGGACTCTCTCAGCAAGGAGCGAGAGC
        A  G  Q  T  V  D  L  Q  G  E  V  D  S  L  S  K  E  R  E  L

1621  TGCTGCAGAAGGCCAGGGAAGAGCTGCGGCAGCAGCTGGAGGTGCTAGAGCAGGAGGCAT
        L  Q  K  A  R  E  E  L  R  Q  Q  L  E  V  L  E  Q  E  A  W

1681  GGCGCCTGCGAAGGGTAAATGTGGAGCTTCAGCTGCAGGGGGACTCTGCCCAGGGCCAGA
        R  L  R  R  V  N  V  E  L  Q  L  Q  G  D  S  A  Q  G  Q  K

1741  AGGAGGAACAGCAGGAGGAGCTGCACCTGGCTGTCCGGGAGAGGGAGCGTCTTCAGGAGA
        E  E  Q  Q  E  E  L  H  L  A  V  R  E  R  E  R  L  Q  E  M

1801  TGCTGATGGGCCTGGAAGCCAAACAGTCAGAATCACTCAGTGAACTGATCACTCTTCGGG
        L  M  G  L  E  A  K  Q  S  E  S  L  S  E  L  I  T  L  R  E

1861  AAGCCCTGGAGTCAATTCACCTGGAAGGGGAGTTACTGAGGCAAGAGCAAACGGAAGTGA
        A  L  E  S  I  H  L  E  G  E  L  L  R  Q  E  Q  T  E  V  T

1921  CCGCAGCGCTGGCTAGGGCAGAGCAGTCAATTGCAGAGCTGTCGAGTTCTGAAAACACCC
        A  A  L  A  R  A  E  Q  S  I  A  E  L  S  S  S  E  N  T  L

1981  TGAAGACAGAAGTAGCTGATCTTCGGGCTGCAGCTGTCAAGCTCAGTGCCTTAAATGAGG
        K  T  E  V  A  D  L  R  A  A  A  V  K  L  S  A  L  N  E  A

2041  CTTTGGCGTTAGATAAAGTTGGGCTGAACCAGCAGCTTCTCCAGTTAGAGGAGGAGAACC
        L  A  L  D  K  V  G  L  N  Q  Q  L  L  Q  L  E  E  E  N  Q

2101  AGTCTGTGTGCAGCAGAATGGAGGCCGCAGAGCAGGCGAGAAATGCTTTGCAGGTCGACC
        S  V  C  S  R  M  E  A  A  E  Q  A  R  N  A  L  Q  V  D  L

2161  TGGCGGAGGCAGAGAAGAGGAGGGAAGCCCTGTGGGAAAAGAACACTCACCTGGAGGCTC
        A  E  A  E  K  R  R  E  A  L  W  E  K  N  T  H  L  E  A  Q

2221  AGCTGCAGAAAGCTGAGGAGGCTGGGGCTGAGCTGCAGGCAGATCTCAGGGACATCCAAG
        L  Q  K  A  E  E  A  G  A  E  L  Q  A  D  L  R  D  I  Q  E

2281  AAGAGAAGGAAGAAATTCAAAAGAAACTAAGTGAGTCACGTCACCAGCAGGAGGCAGCCA
        E  K  E  E  I  Q  K  K  L  S  E  S  R  H  Q  Q  E  A  A  T

2341  CGACTCAGCTGGAGCAGCTACATCAGGAGGCAAAGCGACAGGAAGAAGTGCTTGCCAGGG
        T  Q  L  E  Q  L  H  Q  E  A  K  R  Q  E  E  V  L  A  R  A
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2401  CAGTCCAGGAGAAGGAGGCCCTAGTACGAGAGAAAGCGGCTCTAGAGGTGCGGCTGCAGG
        V  Q  E  K  E  A  L  V  R  E  K  A  A  L  E  V  R  L  Q  A

2461  CCGTGGAGCGTGACCGGCAGGACCTCGCTGCACAACTACAGGGGCTCAGCTCAGCCAAGG
        V  E  R  D  R  Q  D  L  A  A  Q  L  Q  G  L  S  S  A  K  E

2521  AGCTACTGGAGAGCAGTCTGTTTGAAGCCCAACAACAAAATTCTGTGATAGACGAGCCGC
        L  L  E  S  S  L  F  E  A  Q  Q  Q  N  S  V  I  D  E  P  Q

2581  AGGGGCAGCTGGAGGTCCAGATTCAAACTGTCACTCAAGCCAAGGAAGTAATCCAAGGGG
        G  Q  L  E  V  Q  I  Q  T  V  T  Q  A  K  E  V  I  Q  G  E

2641  AAGTGAGGTGCCTGAAGCTGGAACTGGACACTGAACGGAGTCAGGCAGAGCAGGAGCGGG
        V  R  C  L  K  L  E  L  D  T  E  R  S  Q  A  E  Q  E  R  D

2701  ATGCTGCAGCCAGACAGCTGGCCCAGGCTGAGCAAGAAGGGAAGACTGCCTTGGAGCAGC
        A  A  A  R  Q  L  A  Q  A  E  Q  E  G  K  T  A  L  E  Q  Q

2761  AGAAGGCAGCCCATGAGAAAGAGGTGAACCAGCTCCGGGAGAAATGGGAGAAGGAGCGCT
        K  A  A  H  E  K  E  V  N  Q  L  R  E  K  W  E  K  E  R  S

2821  CCTGGCACCAGCAGGAGCTGGCAAAGGCTCTGGAGAGCTTAGAAAGGGAAAAAATGGAGC
        W  H  Q  Q  E  L  A  K  A  L  E  S  L  E  R  E  K  M  E  L

2881  TGGAAATGAGGCTAAAGGAGCAGCAGACAGAAATGGAGGCCATCCAGGCCCAGAGGGAAG
        E  M  R  L  K  E  Q  Q  T  E  M  E  A  I  Q  A  Q  R  E  E

2941  AAGAACGGACCCAGGCAGAGAGTGCCCTATGCCAGATGCAGCTGGAAACAGAGAAGGAGA
        E  R  T  Q  A  E  S  A  L  C  Q  M  Q  L  E  T  E  K  E  R

3001  GAGTATCCCTCCTGGAGACACTGCTGCAGACGCAGAAGGAGCTAGCAGATGCCAGCCAAC
        V  S  L  L  E  T  L  L  Q  T  Q  K  E  L  A  D  A  S  Q  Q

3061  AACTGGAACGACTGAGGCAGGACATGAAAGTCCAGAAATTAAAGGAGCAGGAGACCACTG
        L  E  R  L  R  Q  D  M  K  V  Q  K  L  K  E  Q  E  T  T  G

3121  GGATACTACAGACCCAGCTCCAGGAGGCTCAACGGGAGCTGAAGGAGGCAGCCCGGCAGC
        I  L  Q  T  Q  L  Q  E  A  Q  R  E  L  K  E  A  A  R  Q  H

3181  ACAGAGATGACCTTGCTGCCCTCCAAGAAGAGAGCAGCTCCCTGCTGCAGGATAAGATGG
        R  D  D  L  A  A  L  Q  E  E  S  S  S  L  L  Q  D  K  M  D

3241  ACCTGCAGAAGCAGGTGGAGGACTTGAAGTCTCAGCTGGTGGCCCAGGATGACTCCCAGA
        L  Q  K  Q  V  E  D  L  K  S  Q  L  V  A  Q  D  D  S  Q  R

3301  GGCTGGTGGAGCAGGAGGTTCAGGAGAAGCTGAGAGAGACCCAGGAGTATAACCGAATTC
        L  V  E  Q  E  V  Q  E  K  L  R  E  T  Q  E  Y  N  R  I  Q

3361  AGAAGGAGCTGGAGAGAGAGAAAGCCAGCCTGACTCTGTCACTGATGGAAAAGGAACAGA
        K  E  L  E  R  E  K  A  S  L  T  L  S  L  M  E  K  E  Q  R

3421  GACTCCTTGTTTTACAAGAAGCTGACTCTATTCGACAACAAGAGCTGAGTGCCCTGCGCC
        L  L  V  L  Q  E  A  D  S  I  R  Q  Q  E  L  S  A  L  R  Q

3481  AGGACATGCAGGAGGCCCAGGGAGAACAGAAAGAGCTCAGTGCTCAGATGGAATTACTAA
        D  M  Q  E  A  Q  G  E  Q  K  E  L  S  A  Q  M  E  L  L  R
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3541  GGCAAGAGGTGAAGGAAAAGGAGGCTGACTTTCTGGCCCAGGAAGCACAGCTGCTGGAGG
        Q  E  V  K  E  K  E  A  D  F  L  A  Q  E  A  Q  L  L  E  E

3601  AGCTGGAGGCGTCTCATATCACGGAGCAGCAGCTGCGAGCCTCCTTGTGGGCCCAGGAAG
        L  E  A  S  H  I  T  E  Q  Q  L  R  A  S  L  W  A  Q  E  A

3661  CCAAGGCAGCCCAACTACACCTGCGACTGCGCAGCACAGAGAGCCAGCTAGAAGCGCTGG
        K  A  A  Q  L  H  L  R  L  R  S  T  E  S  Q  L  E  A  L  A

3721  CCGCAGAGCAGCAGCCCGGGAACCAGGCCCAGGCCCAGGCCCAGCTGGCCAGCCTCTACT
        A  E  Q  Q  P  G  N  Q  A  Q  A  Q  A  Q  L  A  S  L  Y  S

3781  CTGCCCTGCAGCAGGCCCTGGGGTCTGTTTGTGAGAGCAGGCCTGAGCTGAGTGGTGGGG
        A  L  Q  Q  A  L  G  S  V  C  E  S  R  P  E  L  S  G  G  G

3841  GAGACTCTGCTCCTTCCGTCTGGGGCCTTGAGCCAGACCAGAATGGAGCTAGGAGCCTCT
        D  S  A  P  S  V  W  G  L  E  P  D  Q  N  G  A  R  S  L  F

3901  TTAAGAGAGGGCCCCTGCTGACTGCTCTCTCCGCTGAGGCAGTAGCATCTGCCCTCCTCA
        K  R  G  P  L  L  T  A  L  S  A  E  A  V  A  S  A  L  L  K

3961  AGCTTCATCAAGACCTGTGGAAGACTCAACAGACCCGGGATGTTCTGAGGGATCAGGTCC
        L  H  Q  D  L  W  K  T  Q  Q  T  R  D  V  L  R  D  Q  V  Q

4021  AGAAACTGGAAGAGCGTCTAACTGATACTGAGGCTGAGAAGAGCCAGGTCCACACAGAGT
        K  L  E  E  R  L  T  D  T  E  A  E  K  S  Q  V  H  T  E  L

4081  TGCAGGATCTGCAGAGACAGCTCTCCCAGAATCAGGAAGAGAAATCCAAGTGGGAAGGAA
        Q  D  L  Q  R  Q  L  S  Q  N  Q  E  E  K  S  K  W  E  G  K

4141  AGCAGAACTCCCTAGAATCTGAGCTGATGGAACTACATGAAACTATGGCATCCTTACAGA
        Q  N  S  L  E  S  E  L  M  E  L  H  E  T  M  A  S  L  Q  S

4201  GTCGCCTGCGGAGAGCAGAGCTACAGCGAATGGAAGCCCAGGGTGAGCGAGAGTTACTTC
        R  L  R  R  A  E  L  Q  R  M  E  A  Q  G  E  R  E  L  L  Q

4261  AGGCAGCCAAGGAGAACCTGACAGCCCAGGTGGAACACCTGCAAGCAGCTGTCGTAGAAG
        A  A  K  E  N  L  T  A  Q  V  E  H  L  Q  A  A  V  V  E  A

4321  CCAGGGCTCAGGCAAGTGCTGCTGGCATCCTGGAAGAAGACCTGAGAACGGCTCGCTCAG
        R  A  Q  A  S  A  A  G  I  L  E  E  D  L  R  T  A  R  S  A

4381  CACTGAAGCTGAAAAATGAGGAAGTAGAGAGTGAGCGTGAGAGAGCCCAGGCTCTGCAAG
        L  K  L  K  N  E  E  V  E  S  E  R  E  R  A  Q  A  L  Q  E

4441  AGCAGGGCGAACTGAAGGTGGCCCAAGGGAAGGCTCTGCAAGAGAATTTGGCCCTCCTGA
        Q  G  E  L  K  V  A  Q  G  K  A  L  Q  E  N  L  A  L  L  T

4501  CCCAGACCCTAGCTGAAAGAGAAGAGGAGGTGGAGACTCTGCGGGGACAAATCCAGGAAC
        Q  T  L  A  E  R  E  E  E  V  E  T  L  R  G  Q  I  Q  E  L

4561  TGGAGAAGCAACGGGAAATGCAGAAGGCTGCTTTGGAATTGCTGTCTCTGGACCTGAAGA
        E  K  Q  R  E  M  Q  K  A  A  L  E  L  L  S  L  D  L  K  K

4621  AGAGGAACCAAGAGGTAGATCTGCAGCAAGAACAGATTCAGGAGCTAGAGAAGTGTAGGT
        R  N  Q  E  V  D  L  Q  Q  E  Q  I  Q  E  L  E  K  C  R  S
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4681  CTGTTTTAGAGCATCTGCCCATGGCCGTCCAGGAGCGAGAGCAGAAGCTGACTGTGCAGA
        V  L  E  H  L  P  M  A  V  Q  E  R  E  Q  K  L  T  V  Q  R

4741  GGGAGCAGATCAGAGAGCCCGAGAAGGATCGGGAGACTCAGAGGAACGTCTTGGAGCATC
        E  Q  I  R  E  P  E  K  D  R  E  T  Q  R  N  V  L  E  H  Q

4801  AGCTTCTAGAACTTGAGAAGAAAAGACCAAATGATTGAGTCCCAGAGAGGACAGGTTCAGG
        L  L  E  L  E  K  K  D  Q  M  I  E  S  Q  R  G  Q  V  Q  D

4861  ACCTGAAAAAGCAGTTGGTTACTCTGGAATGCCTGGCCCTGGAACTGGAGGAAAACCATC
        L  K  K  Q  L  V  T  L  E  C  L  A  L  E  L  E  E  N  H  H

4921  ACAAGATGGAGTGCCAGCAAAAACTGATCAAGGAGCTGGAGGGCCAGAGGGAAACCCAGA
        K  M  E  C  Q  Q  K  L  I  K  E  L  E  G  Q  R  E  T  Q  R

4981  GAGTGGCTTTGACCCACCTTACGCTGGACCTAGAAGAAAGGAGCCAGGAGCTGCAGGCAC
        V  A  L  T  H  L  T  L  D  L  E  E  R  S  Q  E  L  Q  A  Q

5041  AAAGCAGCCAGATCCATGACCTGGAGAGCCACAGCACCGTTCTGGCAAGAGAGCTGCAGG
        S  S  Q  I  H  D  L  E  S  H  S  T  V  L  A  R  E  L  Q  E

5101  AGAGGGACCAGGAGGTGAAGTCTCAGCGAGAACAGATCGAGGAGCTGCAGAGGCAGAAAG
        R  D  Q  E  V  K  S  Q  R  E  Q  I  E  E  L  Q  R  Q  K  E

5161  AGCATCTGACTCAGGATCTCGAGAGGAGAGACCAGGAGCTGATGCTGCAGAAGGAGAGGA
        H  L  T  Q  D  L  E  R  R  D  Q  E  L  M  L  Q  K  E  R  I

5221  TTCAGGTTCTCGAGGATCAGAGGACCCGGCAGACCAAGATCCTGGAGGAGGACCTGGAAC
        Q  V  L  E  D  Q  R  T  R  Q  T  K  I  L  E  E  D  L  E  Q

5281  AGATCAAGCTGTCCTTGAGAGAGCGAGGCCGGGAGCTGACCACTCAGAGGCAGCTGATGC
        I  K  L  S  L  R  E  R  G  R  E  L  T  T  Q  R  Q  L  M  Q

5341  AGGAACGGGCAGAGGAAGGGAAGGGCCCAAGTAAAGCACAGCGCGGGAGCCTAGAGCACA
        E  R  A  E  E  G  K  G  P  S  K  A  Q  R  G  S  L  E  H  M

5401  TGAAGCTGATCCTGCGTGATAAGGAGAAGGAGGTGGAATGTCAGCAGGAGCATATCCATG
        K  L  I  L  R  D  K  E  K  E  V  E  C  Q  Q  E  H  I  H  E

5461  AACTCCAGGAGCTCAAAGACCAGCTGGAGCAGCAGCTCCAGGGCCTGCACAGGAAGGTAG
        L  Q  E  L  K  D  Q  L  E  Q  Q  L  Q  G  L  H  R  K  V  G

5521  GTGAGACCAGCCTCCTCCTGTCCCAGCGAGAGCAGGAAATAGTGGTCCTGCAGCAGCAAC
        E  T  S  L  L  L  S  Q  R  E  Q  E  I  V  V  L  Q  Q  Q  L

5581  TGCAGGAAGCCAGGGAACAAGGGGAGCTGAAGGAGCAGTCACTTCAGAGTCAACTGGATG
        Q  E  A  R  E  Q  G  E  L  K  E  Q  S  L  Q  S  Q  L  D  E

5641  AGGCCCAGAGAGCCCTAGCCCAGAGGGACCAGGAACTGGAGGCTCTGCAGCAAGAACAGC
        A  Q  R  A  L  A  Q  R  D  Q  E  L  E  A  L  Q  Q  E  Q  Q

5701  AGCAGGCCCAGGGACAGGAGGAGAGGGTGAAGGAAAAGGCAGACGCCCTCCAGGGAGCTC
        Q  A  Q  G  Q  E  E  R  V  K  E  K  A  D  A  L  Q  G  A  L

5761  TGGAGCAAGCCCATATGACACTGAAGGAGCGTCATGGAGAGCTTCAGGACCACAAGGAAC
        E  Q  A  H  M  T  L  K  E  R  H  G  E  L  Q  D  H  K  E  Q
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5821  AGGCACGAAGGCTGGAGGAAGAGCTGGCAGTGGAGGGACGGCGGGTCCAAGCCCTGGAGG
        A  R  R  L  E  E  E  L  A  V  E  G  R  R  V  Q  A  L  E  E

5881  AGGTGCTGGGAGACCTAAGGGCTGAGTCTCGGGAACAGGAGAAAGCTCTGTTGGCCCTCC
        V  L  G  D  L  R  A  E  S  R  E  Q  E  K  A  L  L  A  L  Q

5941  AGCAGCAGTGTGCTGAGCAGGCACAGGAGCATGAGGTGGAGACCAGGGCCCTGCAGGACA
        Q  Q  C  A  E  Q  A  Q  E  H  E  V  E  T  R  A  L  Q  D  S

6001  GCTGGCTGCAGGCCCAGGCAGTGCTCAAGGAACGGGACCAGGAGCTGGAAGCTCTGCGGG
        W  L  Q  A  Q  A  V  L  K  E  R  D  Q  E  L  E  A  L  R  A

6061  CAGAAAGTCAGTCCTCCCGGCATCAGGAGGAGGCTGCCCGGGCCCGGGCTGAGGCTCTGC
        E  S  Q  S  S  R  H  Q  E  E  A  A  R  A  R  A  E  A  L  Q

6121  AGGAGGCCCTTGGCAAGGCTCATGCTGCCCTGCAGGGGAAAGAGCAGCATCTCCTCGAGC
        E  A  L  G  K  A  H  A  A  L  Q  G  K  E  Q  H  L  L  E  Q

6181  AGGCAGAATTGAGCCGCAGTCTGGAGGCCAGCACTGCAACCCTGCAAGCCTCCCTGGATG
        A  E  L  S  R  S  L  E  A  S  T  A  T  L  Q  A  S  L  D  A

6241  CCTGCCAGGCACACAGTCGGCAGCTGGAGGAGGCTCTGAGGATACAAGAAGGTGAGATCC
        C  Q  A  H  S  R  Q  L  E  E  A  L  R  I  Q  E  G  E  I  Q

6301  AGGACCAGGATCTCCGATACCAGGAGGATGTGCAGCAGCTGCAGCAGGCACTTGCCCAGA
        D  Q  D  L  R  Y  Q  E  D  V  Q  Q  L  Q  Q  A  L  A  Q  R

6361  GGGATGAAGAGCTGAGACATCAGCAGGAACGGGAGCAGCTGCTGGAGAAGTCTCTGGCCC
        D  E  E  L  R  H  Q  Q  E  R  E  Q  L  L  E  K  S  L  A  Q

6421  AGAGGGTCCAAGAGAATATGATCCAAGAGAAGCAGAATCTGGGGCTAGAGAGAGAAGAGG
        R  V  Q  E  N  M  I  Q  E  K  Q  N  L  G  L  E  R  E  E  E

6481  AGGAGATAAGGGGCCTTCATCAGAGTGTAAGGGAGCTACAGCTGACTCTAGCCCAAAAGG
        E  I  R  G  L  H  Q  S  V  R  E  L  Q  L  T  L  A  Q  K  E

6541  AACAGGAGATTCTGGAGCTGAGGGAGACCCAGCAAAGGAACAACCTGGAAGCCTTACCCC
        Q  E  I  L  E  L  R  E  T  Q  Q  R  N  N  L  E  A  L  P  H

6601  ACAGCCACAAAACCTCCCCAATGGAGGAACAATCTCTAAAACTTGATTCTTTAGAGCCCA
        S  H  K  T  [S  P]  M  E  E  Q  S  L  K  L  D  S  L  E  P  R

6661  GGCTGCAGCGGGAGCTGGAGCGGCTACAGGCAGCCCTGAGACAGACAGAAGCCAGGGAGA
        L  Q  R  E  L  E  R  L  Q  A  A  L  R  Q  T  E  A  R  E  I

6721  TTGAGTGGAGGGAGAAGGCCCAGGACTTGGCACTCTCCCTAGCGCAGACCAAGGCCAGTG
        E  W  R  E  K  A  Q  D  L  A  L  S  L  A  Q  T  K  A  S  V

6781  TCAGCAGTCTGCAGGAGGTTGCCATGTTCCTACAAGCCTCTGTCCTGGAGCGGGACTCAG
        S  S  L  Q  E  V  A  M  F  L  Q  A  S  V  L  E  R  D  S  E

6841  AACAGCAAAGGCTGCAGGATGAACTGGAGCTCACCAGACGGGCTCTGGAGAAGGAGCGGC
        Q  Q  R  L  Q  D  E  L  E  L  T  R  R  A  L  E  K  E  R  L

6901  TACACAGCCCAGGTGCAACCAGCACAGCAGAACTGGGGTCCAGAGGGGAGCAGGGTGTGC
        H  [S  P]  G  A  T  S  T  A  E  L  G  S  R  G  E  Q  G  V  Q
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6961  AGCTGGGAGAGGTCTCAGGAGTGGAGGCTCAGCCTAGTCCTGATGGAATGGAGAAGCAGT
        L  G  E  V  S  G  V  E  A  E  P  S  P  D  G  M  E  K  Q  S

7021  CATGGAGACAAAGGCTTGAACACCTGCAGCAAGCAGTGGCCCGGCTGGAGATTGACAGGA
        W  R  Q  R  L  E  H  L  Q  Q  A  V  A  R  L  E  I  D  R  S

7081  GCAGGCTGCAGCGCCACAATGTCCAGCTGCGGAGTACCTTGGAGCAGGTGGAGCGAGAAC
        R  L  Q  R  H  N  V  Q  L  R  S  T  L  E  Q  V  E  R  E  R

7141  GGAGGAAGCTGAAGAGGGAGGCCATGCGTGCGGCCCAGGCAGGGTCCCTAGAGATCAGCA
        R  K  L  K  R  E  A  M  R  A  A  Q  A  G  S  L  E  I  S  K

7201  AGGCCACGGCTTCTTCACCCACACAGCAGGATGGGAGAGGACAGAAGAACTCAAATGCCA
        A  T  A  S  S  P  T  Q  Q  D  G  R  G  Q  K  N  S  N  A  K

7261  AGTGTGTGGCTGAACTGCAGAAAGAGGTGGTCCTGCTGCAAGCTCAGCTGACTTTGGAGC
        C  V  A  E  L  Q  K  E  V  V  L  L  Q  A  Q  L  T  L  E  R

7321  GGAAGCAGAAGCAGGACTACATCACCCGCTCAGCACAGACCAGCCGTGAGCTAGCAGGCC
        K  Q  K  Q  D  Y  I  T  R  S  A  Q  T  S  R  E  L  A  G  L

7381  TGCACCACAGCCTCTCACACTCACTTCTTGCCGTGGCCCAGGCCCCTGAGGCCACTGTCC
        H  H  S  L  S  H  S  L  L  A  V  A  Q  A  P  E  A  T  V  L

7441  TGGAGGCAGAGACCCGCAGGCTGGATGAGTCCCTGACTCAAAGTCTGACATCCCCAGGGC
        E  A  E  T  R  R  L  D  E  S  L  T  Q  S  L  T  S  P  G  P

7501  CAGTCCTGCTACACCCCAGCCCCAGCACTACCCAAGCCGCCTCCAGGTAGCAGCCACAGC
        V  L  L  H  P  S  P  S  T  T  Q  A  A  S  R

7561  CAGGAGCACACAGACAGAAGACTGTGTCATGGGTCATGGCCCCTCCGCACACCTACAGGT
7621  TTGCCAAAGGAAAAGCCTGGCTCTGTTAGGCACCCAGGAGCCCCAGGTCGGCGGGTGTTC
7681  CCAGGAAGAGGAAGTAAATCTGCAACCCTGGGGAGGACCCCAACTCACCTGGGAATGAGG
7741  CAAATTGCATTTGCTTGCTCCCTATGGAATCACCCAGAGGGGTGCCTTGCCCTGGCTGAG
7801  GGACCCGGAATTCC
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APPENDIX II

Nucleotide and amino acid sequence of Cep110.
The nucleotide sequence of Cep110 is shown, with the predicted amino acid sequence underneath. Leucine zipper motifs are underlined, and potential poly(A) signals are boxed. These sequence data are available in GenBank under accession number AF083322.

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   1  CACTTTTATACTTCCCTTCGTTTGCATGTGTTACTTCTTCAGCCTAGGTCTTTCCCCTTC
  61  CGTTCGTTCTTCAAAACCCAGGCAAAACACCTCTTCCTCTGAGCAGCCTTCTCAGAACTT
 121  TTCTCCCATGTGGCCAGAGGAAGGAGAGACTTCTGTCTATCTCTTCTGCTAGACCCCGAA
 181  CTCTTTGGAGGCAGAGGTTATCCACAAGAGGCTTGGCAAATGTTTCTCAGTCATAGTTGG
 241  ACTGTTTGAAAAATTAAAGGTTCTATTTGTCCGTCAGTTTCATCCGTGACATTTAACTTT
 301  TTATACCAAAGTCTTAGAATCTACCATCATTTTTTGGGCCATTGATACCACTTTAACCAC
 361  AGTTAATTTCAGCTTTTGGCAGGAAAGAGAGGAAACATCCTGTGATGTTTCAGCACTGCA
 421  TTTTTTTCCCCATCTCATTCAGGAGAATGAAGTTTCTAGATTAGAAGACATAATGCAGCA
                                                        M  Q  H

 481  TTTAAAATCAAAGAAGCGGGAAGAAAGGTGGATGAGAGCATCCAAGCGGCAGTCGGAGAA
        L  K  S  K  K  R  E  E  R  W  M  R  A  S  K  R  Q  S  E  K

 541  AGAAATGGAAGAACTGCATCATAATATTGATGATCTTTTGCAAGAGAAGAAAAGCTTAGA
        E  M  E  E  L  H  H  N  I  D  D  L  L  Q  E  K  K  S  L  E

 601  GTGTGAAGTAGAAGAATTACATAGAACTGTCCAGAAACGTCAACGGCAAAAGGACTTCAT
        C  E  V  E  E  L  H  R  T  V  Q  K  R  Q  R  Q  K  D  F  I

 661  TGATGGAAATGTAGAGAGTCTTATGACTGAACTAGAAATAGAAAAATCACTCAAACATCA
        D  G  N  V  E  S  L  M  T  E  L  E  I  E  K  S  L  K  H  H

 721  TGAAGATATTGTAGATGAAATTGAGTGCATTGAGAAGACTCTTCTGAAACGTCGCTCAGA
        E  D  I  V  D  E  I  E  C  I  E  K  T  L  L  K  R  R  S  E

 781  GCTCAGGGAAGCTGACCGACTCCTGGCAGAGGCTGAGAGTGAACTTTCATGCACTAAAGA
        L  R  E  A  D  R  L  L  A  E  A  E  S  E  L  S  C  T  K  E

 841  AAAGACAAAAAATGCTGTTGAAAAGTTCACTGATGCCAAGAGAAGTTTATTGCAAACTGA
        K  T  K  N  A  V  E  K  F  T  D  A  K  R  S  L  L  Q  T  E

 901  GTCAGATGCTGAGGAATTAGAAAGGAGAGCTCAGGAAACTGCTGTTAACCTCGTCAAAGC
        S  D  A  E  E  L  E  R  R  A  Q  E  T  A  V  N  L  V  K  A

 961  TGATCAGCAGCTAAGATCGCTCCAGGCTGATGCAAAGGATTTGGAGCAGCACAAAATCAA
        D  Q  Q  L  R  S  L  Q  A  D  A  K  D  L  E  Q  H  K  I  K

1021  GCAAGAAGAAATCTTGAAAGAAATAAACAAAATTGTAGCAGCAAAAGACTCAGACTTCCA
        Q  E  E  I  L  K  E  I  N  K  I  V  A  A  K  D  S  D  F  Q

1081  ATGTTTAAGCAAGAAGAAGGAAAAACTGACAGAAGAGCTTCAGAAACTACAGAAAGACAT
        C  L  S  K  K  K  E  K  L  T  E  E  L  Q  K  L  Q  K  D  I

1141  AGAGATGGCAGAACGCAATGAGGATCACCACCTGCAGGTCCTTAAAGAATCTGAGGTGCT
        E  M  A  E  R  N  E  D  H  H  L  Q  V  L  K  E  S  E  V  L

1201  TCTTCAGGCCAAAAGAGCCGAGCTGGAAAAGCTGAAAAGCCAGGTGACAAGTCAGCAGCA
        L  Q  A  K  R  A  E  L  E  K  L  K  S  Q  V  T  S  Q  Q  Q

1261  GGAGATGGCTGTCTTGGACAGGCAGTTAGGGCATAAAAAGGAGGAGCTGCATCTACTCCA
        E  M  A  V  L  D  R  Q  L  G  H  K  K  E  E  L  H  L  L  Q

1321  AGGAAGCATGGTCCAGGCAAAAGCTGACCTCCAGGAAGCTCTGAGACTGGGAGAGACTGA
        G  S  M  V  Q  A  K  A  D  L  Q  E  A  L  R  L  G  E  T  E

1381  AGTAACTGAGAAGTGCAATCACATTAGGGAAGTAAAATCTCTTCTGGAAGAACTGAGTTT
        V  T  E  K  C  N  H  I  R  E  V  K  S  L  L  E  E  L  S  F
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1441  TCAGAAAGGAGAACTAAATGTTCAGATTAGTGAAAGAAAAACTCAACTTACACTTATAAA
        Q  K  G  E  L  N  V  Q  I  S  E  R  K  T  Q  L  T  L  I  K

1501  GCAGGAAATTGAAAAAGAGGAAGAAAATCTTCAGGTTGTTTTAAGGCAGATGTCTAAACA
        Q  E  I  E  K  E  E  E  N  L  Q  V  V  L  R  Q  M  S  K  H

1561  TAAAACCGAACTACAGAATATTCTGGACATGTTGCAACTTGAAAACCATGAGCTACAAGG
        K  T  E  L  Q  N  I  L  D  M  L  Q  L  E  N  H  E  L  Q  G

1621  TTTGAAGCTACAACATGACCAAAGGGTATCTGAATTAGAGAAGACTCAGGTGGCAGTGCT
        L  K  L  Q  H  D  Q  R  V  S  E  L  E  K  T  Q  V  A  V  L

1681  AGAGGAGAAACTGGAGTTAGAGAATTTGCAGCAGATATCCCAGCAGCAGAAAGGGGAAAT
        E  E  K  L  E  L  E  N  L  Q  Q  I  S  Q  Q  Q  K  G  E  I

1741  AGAGTGGCAGAAGCAGCTCCTTGAGAGGGATAAACGAGAAATAGAACGAATGACTGCTGA
        E  W  Q  K  Q  L  L  E  R  D  K  R  E  I  E  R  M  T  A  E

1801  GTCCCGAGCTTTACAATCGTGTGTTGAGTGTTTGAGCAAAGAAAAGGAAGATCTCCAAGA
        S  R  A  L  Q  S  C  V  E  C  L  S  K  E  K  E  D  L  Q  E

1861  GAAATGTGACATTTGGGAAAAAAAGTTGGCACAAACCAAAAGGGTTTTAGCAGCAGCAGA
        K  C  D  I  W  E  K  K  L  A  Q  T  K  R  V  L  A  A  A  E

1921  AGAAAATAGCAAAATGGAGCAATCAAACTTAGAAAAGTTGGATTTGAATGTCAGAAAACT
        E  N  S  K  M  E  Q  S  N  L  E  K  L  D  L  N  V  R  K  L

1981  GCAGCAGGAACTAGACCAACTAAACAGAGACAAGTTGTCACTGCATAACGACATTTCAGC
        Q  Q  E  L  D  Q  L  N  R  D  K  L  S  L  H  N  D  I  S  A

2041  AATGCAACAGCAGCTCCAAGAAAAACGAGAAGCAGTAAACTCACTGCAGGAGGAACTAGC
        M  Q  Q  Q  L  Q  E  K  R  E  A  V  N  S  L  Q  E  E  L  A

2101  TAATGTCCAAGACCATTTGAACCTAGCAAAACAGGACCTGCTTCACACCACCAAGCATCA
        N  V  Q  D  H  L  N  L  A  K  Q  D  L  L  H  P  T  K  H  Q

2161  GGATGTGTTGCTCAGTGAGCAGACCCGACTCCAGAAGGACATCAGTGAATGGGCAAATAG
        D  V  L  L  S  E  Q  T  R  L  Q  K  D  I  S  E  W  A  N  R

2221  GTTTGAAGACTGTCAGAAAGAAGAGGAGACAAAACAACAACAACTTCAAGTGCTTCAGAA
        F  E  D  C  Q  K  E  E  E  T  K  Q  Q  Q  L  Q  V  L  Q  N

2281  TGAGATTGAAGAAAACAAGCTCAAACTAGTCCAACAAGAAATGATGTTTCAGAGACTCCA
        E  I  E  E  N  K  L  K  L  V  Q  Q  E  M  M  F  Q  R  L  Q

2341  GAAAGAGAGAGAAAGTGAAGAAAGCAAATTAGAAACCAGTAAAGTGACACTGAAGGAGCA
        K  E  R  E  S  E  E  S  K  L  E  T  S  K  V  T  L  K  E  Q

2401  ACAGCACCAGCTGGAAAAGGAATTAACAGACCAGAAAAGCAAACTGGACCAAGTGCTCTC
        Q  H  Q  L  E  K  E  L  T  D  Q  K  S  K  L  D  Q  V  L  S

2461  AAAGGTGCTGGCAGCTGAAGAGCGTGTTAGGACTCTGCAGGAAGAGGAGAGGTGGTGTGA
        K  V  L  A  A  E  E  R  V  R  T  L  Q  E  E  E  R  W  C  E

2521  GAGCCTGGAGAAGACACTCTCCCAAACTAAACGGCAGCTTTCAGAAAGGGAGCAGCAATT
        S  L  E  K  T  L  S  Q  T  K  R  Q  L  S  E  R  E  Q  Q  L
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2581  GGTGGAGAAATCAGGTGAGCTGTTGGCCCTCCAGAAAGAGGCAGATTCTATGAGGGCAGA
        V  E  K  S  G  E  L  L  A  L  Q  K  E  A  D  S  M  R  A  D

2641  CTTCAGCCTTCTGCGGAACCAGTTCTTGACAGAAAGAAAGAAAGCTGAGAAGCAGGTGGC
        F  S  L  L  R  N  Q  F  L  T  E  R  K  K  A  E  K  Q  V  A

2701  CAGCCTGAAGGAAGCACTTAAGATCCAGCGGAGCCAGCTGGAGAAAAACCTTCTTGAGCA
        S  L  K  E  A  L  K  I  Q  R  S  Q  L  E  K  N  L  L  E  Q

2761  AAAACAGGAGAACAGCTGCATACAAAAGGAAATGGCAACAATTGAACTGGTAGCCCAGGA
        K  Q  E  N  S  C  I  Q  K  E  M  A  T  I  E  L  V  A  Q  D

2821  CAACCATGAGCGGGCCAGGCGCCTGATGAAGGAGCTCAACCAGATGCAGTATGAGTACAC
        N  H  E  R  A  R  R  L  M  K  E  L  N  Q  M  Q  Y  E  Y  T

2881  GGAGCTCAAGAAACAGATGGCAAACCAAAAAGATTTGGAGAGAAGACAAATGGAAATCAG
        E  L  K  K  Q  M  A  N  Q  K  D  L  E  R  R  Q  M  E  I  S

2941  TGATGCAATGAGGACACTTAAATCTGAGGTGAAGGATGAAATCAGAACCAGCTTGAAGAA
        D  A  M  R  T  L  K  S  E  V  K  D  E  I  R  T  S  L  K  N

3001  TCTTAATCAGTTTCTTCCAGAACTACCAGCAGATCTAGAAGCTATTTTGGAAAGAAACGA
        L  N  Q  F  L  P  E  L  P  A  D  L  E  A  I  L  E  R  N  E

3061  AAACCTAGAAGGAGAATTGGAAAGCTTGAAAGAGAACCTTCCATTTACCATGAATGAGGG
        N  L  E  G  E  L  E  S  L  K  E  N  L  P  F  T  M  N  E  G

3121  ACCTTTTGAAGAAAAACTGAACTTTTCCCAAGTTCACATAATGGATGAACACTGGCGTGG
        P  F  E  E  K  L  N  F  S  Q  V  H  I  M  D  E  H  W  R  G

3181  AGAAGCACTCCGGGAGAAACTGCGTCACCGGGAAGACCGACTCAAGGCCCAACTCCGACA
        E  A  L  R  E  K  L  R  H  R  E  D  R  L  K  A  Q  L  R  H

3241  CTGTATGTCCAAGCAAGCAGAAGTATTAATTAAAGGAAAGCGGCAGACAGAGGGCACTTT
        C  M  S  K  Q  A  E  V  L  I  K  G  K  R  Q  T  E  G  T  L

3301  ACACAGTTTGAGGAGACAAGTAGATGCTTTAGGGGAATTGGTCACCAGCACCTCTGCAGA
        H  S  L  R  R  Q  V  D  A  L  G  E  L  V  T  S  T  S  A  D

3361  TTCAGCGTCATCACCCAGTCTGTCTCAGCTGGAGTCTTCCCTCACAGAGGACTCTCAACT
        S  A  S  S  P  S  L  S  Q  L  E  S  S  L  T  E  D  S  Q  L

3421  TGGACAAAATCAGGAAAAGAATGCCTCAGCCAGATGAGGAATACTGTCTTGTGTAAATAT
        G  Q  N  Q  E  K  N  A  S  A  R  *

3481  ATTCAAGGAAAACACCTCCACTACCTCACTGACTTCATAATTGGAATGTCACATGGTTTT
3541  TTTTTAATCAAGATGCAGTGAACTGAGATTCTGAAACTCCACTGTAGTTTACTTTGCCTG
3601  TACCATTAATGCCAATGTTTTTATAAAATCACTTGTACATAGTACATATGGGAATAGTTGC
3661  ATATGGGAATTTAAACCAACATGTGGCTGAGCCTTTTTTCTTTAATCTTCGTAACATGTT
3721  TAAAAAAAAAACAGTGATTTTAACTGCATATTTGAACCTACAAACTGGTAAATCTTATTA
3781  ACAAAAAGAATGTACGTAAGGCCCTCTTTCCTTATAGTGTCGAGTTATTTTTGAATTTTG
3841  CTTAAAATCTATTTTTTCATATGAAAATAAAAGATAACAATCAAAAAATAAAAC
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