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The Association of Nuclear Pore Complex Proteins with Chromatin-binding Membrane Vesicles

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "The Association of Nuclear Pore Complex Proteins with Chromatin-binding Membrane Vesicles" submitted by Dee Ann Warren in partial fulfilment of the requirements for the degree of Master of Science.

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ABSTRACT

Cell-free extracts of *Xenopus* eggs can assemble nuclear envelopes around demembranated sperm chromatin. Nuclear envelope assembly requires soluble cytosolic proteins and two particulate fractions, one with vesicles that bind chromatin and the other with vesicles that fuse to the chromatin-bound vesicles. Several nucleoporins, the peripheral proteins of the nuclear pore complex, were found to be tightly-associated surface components of the chromatin-binding vesicles. The nucleoporins were also found in a soluble form in the cytosolic fraction. During an early stage of nuclear envelope assembly, only the nucleoporins in association with the chromatin-binding vesicles became bound to sperm chromatin. When vesicles are prevented from binding by metaphase cytosol or okadaic acid, nucleoporins do not associate with the chromatin. Thus, I propose that there are two pools of nucleoporins that play different roles during the formation of the nuclear pore complex when nuclear envelopes are assembled.

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For John

and

my parents

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LIST OF ABBREVIATIONS

apL10	affinity purified antiserum from rabbit L10 (anti-p62)
ATP	adenosine-5'-triphosphate
BSA	bovine serum albumin
DAPI	4',6-diamidine-2-phenylindole dihydrochloride
$DiOC_6$	3,3'-dihexyloxacarbocyanine iodide
DTT	dithiothreitol
EM	electron microscopy
FITC	fluorescein isothiocyanate
g	gravitational force (where g=9.81 meters/second^2)
GTP	guanosine-5'-triphosphate
GTPγS	guanosine-5'-O-(3-thiotriphosphate)
kDa	kilodalton
L10	rabbit antiserum against nuclear pore complex protein p62
LII	lamin II
LIII	lamin III
LAP	lamina-associated polypeptide
LBR	lamin-B receptor
mAb414	monoclonal antibody 414
MDa	megadalton
MPF	maturation-promoting factor
NEM	*N*-ethylmaleimide
NEP-A	nuclear envelope precursor-A
NEP-B	nuclear envelope precursor-B
NLS	nuclear localization sequence
NPC	nuclear pore complex
PBS	phosphate-buffered saline
PMSF	phenylmethyl-sulfonylfluoride
POM	pore membrane protein
PVDF	polyvinylidene difluoride
S_{200}	supernatant generated by centrifugation at 200,000 *g* for 75 min
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
WGA	wheat germ agglutinin

CHAPTER I: INTRODUCTION

I-A. Background

The nuclear envelope is a dynamic structure found only in eukaryotic cells that marks the outer boundary enclosing a cell's genetic material and nuclear contents. The nuclear envelope is comprised of three distinct but interacting components; the nuclear lamina, nuclear membrane and nuclear pores. The nuclear membrane is itself composed of three regions: 1) the inner membrane, 2) outer membrane and 3) the pore membrane domain, a membrane region that is fused where nuclear pores are located. During cell division the nuclear envelope undergoes a dramatic rearrangement in cells with an open mitosis as each of these constituent parts are disassembled and dispersed throughout the cytoplasm. A new nuclear envelope is assembled from the components of the disassembled nuclear envelope without a need for new protein or lipid synthesis. An understanding of the structural, functional and biochemical makeup of the nuclear envelope is well under way, but the dynamics of the nuclear envelope during cell division remains largely unknown. This introduction will serve to review the progress that has been made in nuclear envelope research as well as to provide background information on the nuclear pore complex proteins and nuclear membranes that were the focus of my research.

The approaches that have been used to study the nuclear envelope are based on three predominant systems. One approach is to study the nuclear envelope in mammalian cells. Although it is difficult to study nuclear envelope assembly in mammalian cells, this system is very useful for subcellular fractionations and protein purification. In addition, many monoclonal antibodies have been prepared from the nuclear envelope material in mammalian cells. Mammalian cell lines are also useful for the study of protein localization by immunofluorescence.

The second system that is commonly used in nuclear envelope research takes advantage of yeast cell genetics. Although yeast nuclei undergo a closed mitosis and are therefore not useful for the study of assembly/disassembly, in this system specific nuclear envelope proteins can be genetically added or deleted. This has already provided a wealth of information about nuclear pore complex proteins (see section I-D.1.2).

The third system that is used for nuclear envelope research is a cell-free system that can be prepared from *Xenopus* eggs (Lohka and Masui, 1983). Unfertilized *Xenopus* eggs are arrested at the second meiotic metaphase. At this stage, they contain numerous precursors of the nuclear envelope that are largely derived from the disassembly of the germinal vesicle, a specialized nucleus in amphibian oocytes. The nuclear envelope precursors that are stored in the egg will become incorporated into the nuclei that are assembled in the rapid cell division events that follow fertilization (Laskey et al., 1979). Thus, the unfertilized *Xenopus* egg is a stockpile of nuclear envelope material that makes this organism an excellent system for the study of nuclear envelope assembly. The main disadvantage in using *Xenopus* to study the nuclear envelope is that this organism cannot be genetically manipulated.

Although each of the three systems have their own advantages and disadvantages, together they have contributed essentially everything that we understand about the nuclear envelope today.

I-A. The Nuclear Lamina

I-A.1. Interphase structure and function. The nuclear lamina is a proteinaceous layer that lies to the nuclear side of the inner nuclear membrane. The lamina is essential to the cell nucleus for post-mitotic growth, maintenance of nuclear envelope structure, and organization of the genome by direct contact with the chromatin

during interphase (Newport et al., 1990; Meier et al., 1991; Luderus et al., 1992; Glass et al., 1993). It is composed of a family of proteins, termed lamins, which share regions of extensive homology with intermediate filament proteins (Aebi et al., 1986; Fisher et al., 1986; McKeon et al., 1986). Like intermediate filament proteins, lamins contain an amino-terminal head and a carboxyl-terminal tail region separated by an internal α-helical rod domain. Molecules with this type of protein sequence are known to dimerize and form strong and structurally supportive assemblies.

Lamin proteins differ in their distribution during metaphase; A-type lamins become soluble and disperse throughout the cytoplasm while the B-type lamins associate with the disassembled nuclear membranes. In mammalian cells, the B-type lamin is lamin B. Lamins A and C are mammalian A-type lamins, as is lamin LIII in *Xenopus* oocytes and early-stage eggs. Until recently it was thought that LIII was the only lamin present in early-stage *Xenopus* cells. However, lamin II was recently identified and a small percentage of both LII and LIII were found on disassembled nuclear membranes (Lourim and Krohne, 1993). Both A- and B-type lamin proteins undergo extensive post-translational modifications, including isoprenylation, carboxyl methylation, and proteolytic cleavage of their carboxyl-terminal end (McKeon, 1986; Georgatos et al., 1994). The isoprenyl residues on the lamins help to direct them to the inner nuclear membrane after they have entered the nucleus. After entering the nucleus for the first time, the lamins are proteolytically cleaved. The lamins are not cleaved, however, when they re-enter the nucleus, such as during nuclear envelope assembly.

I-B.2. Nuclear lamina assembly/disassembly. The nuclear lamina is disassembled along with the nuclear membranes and pore complexes as cells enter into mitosis. Both A- and B-type lamins are hyperphosphorylated by $p34^{cdc2}$ when the lamina is disassembled (Ottaviano and Gerace, 1985; Peter et al., 1990). The

hyperphosphorylation of the lamins directs their disassembly into the A-type soluble lamins and the B-type membrane bound lamins (Gerace and Blobel, 1980). It is unclear how the lamins are reassembled into the nascent nuclear envelopes, although in *in vitro* assays, the soluble mammalian lamins A and C are able to associate with metaphase chromosomes (Glass and Gerace, 1990). Since lamin B is vesicle-bound, a model for the initiation of nuclear envelope assembly was proposed whereby lamins A/C at the chromosome surface recruit the lamin B, which then brings the membrane vesicles to the surface of the chromatin. Since other proteins have been identified that could potentially mediate the vesicle-chromatin interaction (section I-C.2.2), the above model for the initiation of nuclear envelope assembly by the lamins is speculative.

I-C. The Nuclear Membrane

I-C.1. Interphase structure and function. The nuclear membrane is composed of an inner membrane that is in contact with the nuclear lamina, and an outer membrane that is continuous with the endoplasmic reticulum. The inner and outer membrane are fused at regions called the pore membrane domain. The pore membrane domain anchors the NPC to the nuclear envelope and has its own distinct set of proteins associated with it (see section I-D.1.2). The outer nuclear membrane is marked with numerous ribosomes, but no nuclear envelope-relevant proteins have been identified on the outer membrane. In contrast, the inner nuclear membrane contains several known integral membrane proteins. A group of proteins called lamina-associated polypeptides (LAPs), have been recently identified (Foisner and Gerace, 1990; Newport and Spann, 1987). Among these are the three LAP1-type proteins, LAP1A, LAP1B, and LAP1C, that interact with both A- and B-type lamins. The other LAP protein, LAP2, binds both lamin B and chromatin. Another integral membrane protein of the inner nuclear membrane, lamin-B receptor

(LBR), also selectively binds to lamin B (Worman et al., 1988; Bailer et al., 1991). Whether the LAPs and LBR function in attaching the nuclear lamina to the nuclear membrane is unclear. Another integral inner membrane protein, otefin, has been identified, although it is not known what nuclear envelope proteins are associated with otefin (Padan et al., 1990).

I-C.2. Nuclear membrane assembly/disassembly. When the nuclear envelope is disassembled during prophase, the nuclear membrane becomes dispersed into the cytoplasm in the form of membrane vesicles (Zeligs and Wollman, 1979). Since *Xenopus* egg extracts are very suitable for studying nuclear envelope assembly, the majority of research on assembly and disassembly has been done using this system. The extracts that are prepared from *Xenopus* eggs can be made in either an interphase or a mitotic state (Lohka and Masui, 1983, 1984; Lohka and Maller, 1985; Newport, 1987; Newport and Spann, 1987), which has allowed the study of both nuclear envelope assembly and disassembly, respectively, around a DNA template. Nuclei that are assembled *in vitro* are similar to endogenous cell nuclei in that they can replicate their DNA (Blow and Laskey, 1986; Blow and Watson, 1987), import karyophilic proteins into the nucleus (Newmeyer et al., 1986a, 1986b; Newmeyer and Forbes, 1988), and respond to the mitotic regulator, maturation-promoting factor (MPF; Lohka and Maller, 1985; Miake-Lye and Kirschner, 1985; Newport and Spann, 1987). When examined ultrastructurally, these nuclei are also morphologically indistinguishable from a typical eukaryotic cell nucleus (Forbes et al., 1983; Lohka and Masui, 1983, 1984).

Although the process of nuclear membrane assembly and disassembly is not completely understood, much progress has been made in this area. It is generally agreed upon that there are three main events during nuclear membrane assembly. They include:

1) decondensation of the metaphase chromosomes, 2) binding of the membrane vesicles to the chromatin, and 3) fusion of the vesicles into an intact double membrane structure.

I-C.2.1. Decondensation of metaphase chromosomes. To date, an intact nuclear envelope has not been found to assemble around any material other than DNA. Demembranated sperm nuclei were used as the template in the first experiments where nuclear envelopes were assembled *in vitro* (Lohka and Masui, 1983). The assembly of a nucleus around the sperm chromatin mimics the process of pronuclear formation that occurs shortly after egg fertilization. This prototype experiment was followed by the injection of bacteriophage λ DNA into unfertilized *Xenopus* eggs (Forbes et al., 1983). The λ DNA supported assembly of a pseudo-nucleus that could also be disassembled when initiators of mitosis were added. However, these nuclei assemble at a slower rate than when demembranated sperm nuclei are used as the template (Newport, 1987). This slower rate is thought to be due to a delay in assembly while the protein-free λ DNA is remodeled into a nucleosomal structure. These experiments could be repeated using both linear bacteriophage T4 DNA and circular plasmid pBR322 DNA, which established that nuclear envelope assembly and disassembly occur independently of specific DNA sequences. When the carbohydrate, glycogen, was depleted from these extracts, the nuclei that formed around the λ DNA were functionally inactive for DNA replication and protein import (Hartl et al., 1994). However, if the glycogen was depleted at a stage after the assembly of the protein-free DNA into a chromatin-like structure, the nuclei were completely functional. Although glycogen was found to be necessary for the formation of nuclei when using protein-free DNA as the template, it was not necessary for nuclear envelope assembly around sperm chromatin. For this reason, the importance of the glycogen cannot be extended to nuclear envelope assembly except for those cases where nuclear envelopes were assembled *in vitro* around the protein-free DNA.

Before nuclear envelope assembly can begin, the chromatin must be decondensed to an extent that the membrane vesicles can become bound. This early event in nuclear envelope assembly requires the presence of cytosol. Nucleoplasmin, a soluble protein in the *Xenopus* egg extracts, is necessary and sufficient to decondense sperm chromatin, and also contributes to nucleosome assembly (Laskey et al., 1978; Philpott et al., 1991). The nucleoplasmin protein contains large tracts of polyglutamic acid (Dingwall et al, 1987). Since polyglutamic acid alone is sufficient for the decondensation of sperm chromatin, this is the region on the nucleoplasmin that is likely to be responsible for chromatin decondensation (Pfaller et al., 1991). The decondensation of sperm chromatin with polyglutamic acid provides a way to study the membrane vesicle binding in the absence of cytosolic proteins. Without a proper decondensation of the chromatin by nucleoplasmin or polyglutamic acid, vesicles will not bind and nuclear envelope assembly cannot be initiated.

I-C.2.2. Vesicle-chromatin interaction. After the decondensation of chromatin, membrane vesicle binding is the next identified event during nuclear envelope assembly. Two groups have determined that this event can be abolished when the vesicles have been treated with trypsin, indicating that a proteinaceous component on the vesicles mediates their interaction with the chromatin (Wilson and Newport, 1988; Vigers and Lohka, 1991). However, when these vesicles were subjected to treatments that disrupt protein-protein interactions, such as high salt (0.9 M KCl), membrane binding was prevented in some cases (Vigers and Lohka, 1991), while not in others (Wilson and Newport, 1988). These contradictory results make it difficult to predict whether the protein(s) that mediates the binding interaction on the vesicles is an integral or peripheral membrane protein.

Several candidate proteins on the vesicles that mediate the vesicle-chromatin interaction have been identified, including both peripheral and integral membrane

proteins. One of these candidates is the previously mentioned B-type lamin, that is thought to interact with the A and C lamins at the chromosome surface (Gerace and Blobel, 1980). In support of this, when either lamins A/C or lamin B was depleted from an *in vitro* mammalian nuclear envelope assembly system derived from HeLa cells, nuclear envelopes failed to form around the endogenous chromosomes (Burke and Gerace, 1986). However, nuclear envelope assembly reactions using a *Xenopus* extract that was depleted of lamins were competent for assembly, although the lamins were essential for nuclear envelope growth and DNA replication (Newport et al., 1990; Dabauvalle et al., 1991; Hartl et al., 1994). Thus, the mammalian system for nuclear envelope assembly predicts that lamins are essential, and the amphibian system predicts that assembly is independent of the lamin proteins. However, the lamin independent model will need to be re-evaluated since at the time of the experiment, the only lamin to be identified in *Xenopus* was lamin LIII. Since this time, LII has been identified in early-stage *Xenopus* cells and was probably not depleted during the earlier experiments (Lourim and Krohne, 1993).

Another candidate protein is the integral membrane protein, LAP2, which becomes concentrated at the chromosome surface early in nuclear envelope assembly when vesicles become bound to the chromatin (Foisner and Gerace, 1993; Newport and Spann, 1987). Recent sequencing of LAP2 reveals potential $p34^{cdc2}$ phosphorylation sites, and when LAP2 is phosphorylated by $p34^{cdc2}$ *in vitro*, it no longer binds either lamin B or chromatin. Through its ability to bind a vesicle-associated protein (lamin B) and chromatin, LAP2 may play a key role in initial nuclear envelope membrane assembly. However, LAP2 may simply serve to attach the interphase chromatin to the nuclear lamina through its ability to bind both chromatin and lamin B.

Another potential mediator of the vesicle-chromatin interaction during early nuclear envelope assembly is the integral membrane protein, LBR (Worman et al. 1988;

Bailer et al., 1991). Like LAP2, LBR binds to both lamin B and DNA and when phosphorylated, binding to both is abolished (Worman et al., 1988; Applebaum et al., 1990; Courvalin et al., 1992; Smith and Blobel, 1994; Ye and Worman, 1994). Also like LAP2, LBR is associated with a population of membrane vesicles that associate with the chromatin at an early stage of nuclear envelope assembly, before the association of an integral membrane protein of the pore membrane domain, gp210 (Chaudhary and Courvalin, 1993). In addition to the putative importance of LBR in vesicle targeting, vesicles that have been stripped of lamin B are unable to bind to chromatin (Maison et al., 1995). Whether it is the lamin B itself or the loss of the ability to associate with LBR that abolishes the vesicle-chromatin interaction remains to be determined.

The proteins that mediate reassembly of the vesicles with the chromatin are likely targets for phosphorylation by protein kinases during nuclear envelope disassembly. The regulation of these proteins by phosphorylation and dephosphorylation provides a way for the cell to regulate when nuclear envelope assembly should be initiated and prevents the premature initiation of assembly. Although $p34^{cdc2}$ has many potential protein targets in the nuclear envelope, when added in a purified and active state to vesicles that are bound to chromatin, the vesicles are not released (Pfaller et al., 1991). Thus, it does not appear to be solely responsible for directing disassembly. In addition, when vesicles are incubated in the presence of phosphatase inhibitors, the binding of the vesicles to chromatin is prevented. This indicates that to begin the process of reassembly protein phosphatases must act to dephosphorylate those proteins that were phosphorylated during disassembly. Thus, antagonistic events regulate nuclear envelope dynamics; protein kinases initiate disassembly while protein phosphatases regulate reassembly. Recently both the protein kinase and protein phosphatase have been partially purified from *Xenopus* egg extracts (Pfaller and Newport, 1995). The protein kinase that regulates disassembly is distinct from $p34^{cdc2}$, yet the presence of $p34^{cdc2}$ and the as yet

unidentified protein kinase together enhance the speed at which disassembly occurs, and are therefore thought to work in concert. Unexpectedly, this unidentified protein kinase is present in the soluble cytosolic fraction and on the membrane vesicles. Although this work reveals a new potential protein kinase and protein phosphatase, further studies are necessary to determine the targets of these regulatory proteins.

I-C.2.3. Membrane vesicle fusion. The next event during nuclear envelope assembly is membrane vesicle fusion, that results in the formation of an inner and outer double membrane. There is an absolute requirement for the presence of cytosol during the fusion event, since vesicles cannot fuse in its absence (Vigers and Lohka, 1991; Bowman et al., 1992; Newport and Dunphy, 1992). A distinct membrane population has been found that when added to vesicles that are pre-bound to the chromatin, allows fusion to proceed (Vigers and Lohka, 1991, 1992). Without the addition of this membrane fraction, the pre-bound vesicles are unable to fuse. Other research groups have found that the fusion between membrane vesicles can be prevented by the addition of a nonhydrolyzable analog of GTP, such as GTPγS (Bowman et al., 1992; Newport and Dunphy, 1992). At present, the biochemical events during nuclear membrane fusion remain largely unknown.

I-D. The Nuclear Pore Complex

I-D.1. Interphase structure and function. The nuclear pore complex (NPC) is a massive assembly of proteins located at each nuclear pore that act as a gated channel between the cytoplasm and the nucleoplasm. The pores are located at regions of the nuclear membrane called the pore membrane domain. At the pore membrane domain, the inner and outer membranes are fused so that the pore complex proteins fill the space

between the fused membrane regions. These complexes allow the passive diffusion of proteins and other molecules that are less than 9nm in size or have an approximate molecular mass of 40-60 kDa (Feldherr et al., 1984). Any molecule that is bigger than this must contain a proper signal for its export from or import into the nuclear interior. Without such a signal, a molecule cannot be transported across the nuclear envelope. Thus, the large proteinaceous complexes that are located at the nuclear pores play a regulatory role by maintaining the proper compartmentalization of cellular molecules, such that they are always located on the proper side of the nuclear envelope. With such an important role to play in cell maintenance, the pore complex is understandably a huge structure of properly positioned proteins. This section will review the 1) structure, 2) function and 3) biochemical make-up of the NPCs.

I-D.1.1. NPC structure. When viewed by electron microscopy (EM), the NPC is viewed as a tripartite assembly consisting of the cytoplasmic ring, nucleoplasmic ring, and a central ring containing thick spokes (Unwin and Milligan, 1982). Each of these three rings exhibit eight-fold rotational symmetry when viewed at a plane that is perpendicular to the nuclear membranes. The spokes that make up the central ring of the NPC were found to be composed of many individual subunits when the samples were prepared using negative staining or cryofixation techniques, which are thought to provide a more preserved NPC structure (Hinshaw et al., 1992; Akey and Radermacher, 1993). Hinshaw et al. (1992) determined that each of the eight spokes per NPC is itself composed of annular, columnar, ring, and lumenal subunits present in two copies per spoke. There are two copies of each subunit per spoke, due to the two-fold mirror symmetry of each NPC relative to a plane that traverses the center of each complex and the lumen of the nuclear membrane, making a total of at least 64 subunits. They also determined that between the eight spokes, there are eight peripheral channels that may be

the sites of passive diffusion for molecules smaller than 9 nm. Alternatively, Akey and Radermacher (1993) have proposed that the channels for passive diffusion are located internal to the eight spokes. The NPC model proposed by Akey and Radermacher (1993) also differs from the model proposed by Hinshaw et al. (1992) in that a central barrel-like transporter was observed in the central channel between the eight spokes. This NPC transporter or central plug has been a subject of great controversy, as it is believed that the plug could be material in transit or even the distal ring of the nuclear basket (see below; Jarnik and Aebi, 1991). All of the components of the NPC described up to this point make up the central core of the NPC.

While transmission EM was useful to determine the symmetry of the NPC, several groups have used high resolution scanning EM to look at other aspects of the NPC structure (Jarnik and Aebi, 1991; Ris, 1991; Goldberg and Allen, 1992). They have found that unlike the symmetry attributed to the central core of the NPC, there are structures that project into the nucleus and the cytoplasm from the nucleoplasmic and cytoplasmic ring, respectively. There are eight filamentous structures that project into the cytoplasm that are thought to originate from each one of the eight spokes. Eight filament-like structures also project into the nucleoplasm, but are joined at their ends by a distal ring. These filaments plus the distal ring have been termed the nuclear baskets or fishtraps. The cytoplasmic filaments and nuclear baskets are both thought to participate in the transport of molecules through the pores.

Quantitative scanning EM has been used to calculate the mass of the NPC. The total mass was calculated at 124 megadaltons (MDa; Reichelt et al., 1990). The mass of the central core, cytoplasmic ring and filaments, and the nucleoplasmic ring and nuclear baskets were 52, 21, and 32 MDa, respectively. The fact that these three numbers do not collectively equal 124 MDa indicates that the mass estimates may not be completely accurate or that additional unidentified components of the NPC may exist.

The use of such powerful electron microscopy techniques has not only elucidated the NPC structure but has provided additional clues to its relationship with other components of the nuclear envelope. For example, the lumenal subunit of the spoke complex may extend into the pore membrane domain (Akey and Radermacher, 1993). This subunit may then be the means through which the NPC attaches itself to the pore membrane domain. Another possible attachment site for the NPC to the nuclear envelope is through the nuclear envelope lattice, a structure seen only in *Triturus cristatus* oocytes using high resolution scanning EM (Goldberg and Allen, 1992). The nuclear envelope lattice is located just to the nucleoplasmic side of the nuclear lamina and seems to be attached to the distal ring of the nuclear baskets. This structure between the NPC and the nuclear lamina may help to stabilize the protruding nuclear baskets. These two sites of attachment are only the beginning to our understanding of the way in which the NPC structure ties in to the overall structure of the nuclear envelope.

I-D.1.2. Proteins of the NPC. The apparent eightfold rotational symmetry and a twofold mirror symmetry of the NPC suggest that many proteins are present in multiple copies. With a molecular mass of 124 MDa, it is has been estimated that there are approximately 100 different proteins in each NPC. Since it has proven difficult to isolate intact NPCs from the rest of the nuclear envelope (the nuclear lamina in particular), a large percentage of these proteins may still be unaccounted for. However, intact NPCs have been isolated from the yeast, *Saccharomyces cerevisiae*, where at least 80 uncharacterized proteins have been found (Rout and Blobel, 1993). This is a significant achievement in the search for NPC proteins, although it will remain some time before these proteins will be identified and compared to homologous proteins in other species. The NPC proteins that have been characterized to date fall into four groups; integral membrane proteins, nucleoporins with XFXFG-type repeats, nucleoporins with GLFG-

type repeats, and nucleoporins without repeat motifs. Nucleoporins refer to peripheral membrane proteins of the NPC.

The integral membrane proteins have been collectively termed POMs, for pore membrane proteins. The three proteins that so far fall into this class all contain hydrophobic regions that localize them to the membranes. The first of these to be identified, gp210, was also the first NPC protein to be identified (Gerace et al., 1982). gp210 contains a single transmembrane domain that is sufficient for its targeting to the pore membrane domain (Wozniak and Blobel, 1992). The other mammalian POM to be identified is POM 121, which also falls into the group of pore proteins with XFXFG repeats (Hallberg et al., 1993). POM 152 is a POM protein identified in *Saccharomyces cerevisiae*, that when expressed in mammalian cells also targets to the pore membrane domain, suggesting that a metazoan homolog may exist or that signals that target POMs to the NPC are conserved (Wozniak et al., 1994). All three proteins are thought to anchor the pore complex to the pore membrane domain.

All NPC proteins, except POM121, that contain XFXFG or GLFG repeats are peripheral membrane proteins. A group of eight major and many minor nucleoporins were first identified by a set of monoclonal antibodies that were raised against a mammalian nuclear lamina/NPC fraction (Davis and Blobel, 1986; Park et al., 1987; Snow et al., 1987). This group of nucleoporins, as well as all other nucleoporins, are modified by the addition of single O-linked *N*-acetylglucosamine residues to serine or threonine groups (Davis and Blobel, 1987; Hanover et al., 1987; Holt et al., 1987). Although it is not known how these sugar residues contribute to the function of the nucleoporins, the addition of terminal galactosamine residues to the *N*-acetylglucosamine does not affect either NPC formation or nuclear protein import (Miller and Hanover, 1995). The lectin wheat germ agglutinin (WGA) can bind to the *N*-acetylglucosamine residues on the nucleoporins (Finlay et al., 1987).

Nucleoporins with the degenerate XFXFG pentapeptide repeat have been found in yeast and metazoan species. Among the yeast proteins are NUP1p, NUP2p and NSP1p (Hurt, 1988; Davis and Fink, 1990; Loeb et al., 1993). Only NSP1p has an identified metazoan homolog, p62 (Carmo-Fonseca et al., 1991). NSP1p and p62 share regions of homology in their carboxyl-terminal region, which consists of heptad repeats in the form of alpha helical coiled-coils. This carboxyl-terminal structure indicates that these proteins may form complexes with themselves or other proteins. The only region of homology in their amino-terminal domain is the 15-25 XFXFG repeats. Otherwise, p62 is thought to form an overall β-sheet conformation at the amino-terminal end (Starr et al., 1990; Carmo-Fonseca et al., 1991; Cordes et al., 1991). p62 takes on the conformation of a rod shaped dimer when expressed *in vitro* and, in addition to the amino-terminal β-sheet and carboxy-terminal coiled-coil, contains a serine/threonine rich linker region in the middle of its sequence which probably is the site of *N*-acetylglucosamine addition (D'Onofrio et al., 1988; Cordes and Krohne, 1993; Buss et al., 1994).

Other XFXFG nucleoporins include the zinc finger protein, nup153, and a protein that shares homology to the oncoprotein CAN, nup214/nup250 (Sukegawa and Blobel, 1993; Kraemer et al., 1994; Pante et al., 1994). Immunogold EM revealed that nup153 is located at the distal ring of the nuclear baskets (Pante et al., 1994) or on the filamentous structures that lead to the distal ring (Sukewaga and Blobel, 1993; Wente and Blobel, 1994). Nup214/p250 has been named by two independent groups and probably represents the same protein. It is located on the cytoplasmic filaments that protrude into the cytoplasm from the NPC (Kraemer et al., 1994; Pante et al., 1994). Many of the XFXFG nucleoporins have been found in complexes with other XFXFG nucleoporins. For example, p62 is found in a 230 kDa complex with the nucleoporins p54 and p58 (Finlay et al., 1991; Kita et al., 1993; Pante et al., 1994; Buss and Stewart, 1995; Guan et al., 1995). nup250 was found in a complex with a protein that has a molecular mass of

75 kDa (Pante et al., 1994). The fact that some XFXFG proteins form a complex with other XFXFG proteins may indicate that the XFXFG repeats are involved in protein-protein interactions. Yeast genetic systems have attempted to address this issue by creating deletion mutants of NSP1p and NUP1p/NUP2p that lack the XFXFG repeats (Karchenko and Nalivaeva, 1980; Loeb et al., 1993). The yeast cells grew regardless of whether or not the repeats were present, indicating that the repeats were not required for cell viability. However, of the strains that were mutated, one had only an XFXFG-deleted NSP1p (Nehrbass et al., 1990) while the other strain had only XFXFG-deleted NUP1p and NUP2p (Loeb et al., 1993). The viability of both strains could reflect a functional redundancy between these XFXFG proteins. To thoroughly test their effect on cell structure or function, all proteins should lack the XFXFG repeat motif.

Most of the GLFG type nucleoporins were identified in yeast cells, although the antibodies that were used to recognize some of the yeast GLFG proteins were originally made against mammalian NPC proteins (Wente et al., 1992). The first group of GLFG nucleoporins to be identified were NUP49p, NUP100p and NUP116p (Wente et al., 1992). At the same time another group identified NUP49p and NUP116p and called them NSP49 and NSP116 (Wimmer et al., 1992). Of these, only NUP49p is essential for cell viability (Wente et al., 1992). NUP49p binds to the XFXFG nucleoporin NSP1p and together they are associated in a complex with NUP57p (which also contains GLFG repeat motifs) and NIC96 (Grandi et al., 1993; Grandi et al., 1995). Another GLFG-type nucleoporin, NUP145p, as well as NUP100p and NUP116p, bind RNA (Fabre et al., 1994). All three perform a redundant RNA-binding function, and so it is thought that they share a common role in the nucleocytoplasmic transport of RNA (Fabre et al., 1994). A newly identified *Xenopus* protein may potentially be a homolog of NUP116p (Powers et al., 1995).

Like the XFXFG repeat motif, it is not known what function the GLFG motif plays when present on a protein. When NUP116p or NUP145p were mutated or deleted from a yeast cell line, the nuclei underwent severe structural abnormalities, suggesting a structural role for these two GLFG nucleoporins (Wente and Blobel, 1993, 1994). A deletion in the GLFG repeats on NUP145p did not affect function or structure in the yeast cells (Fabre et al., 1994). However, when vertebrate p97 was depleted from nuclear reconstitution assays, the nuclei were defective for nuclear growth and replication (Powers et al., 1995). The interactions between NPC proteins and the redundancy in function makes the analysis of the function of any one protein difficult.

Peripheral membrane proteins that do not contain repeat motifs are members of the fourth type of NPC proteins. This group includes a number of diverse NPC proteins. Among these are the RNA binding protein, NUP133p (Grandi et al., 1993), the leucine zipper protein, NUP107p (Radu et al., 1994), a protein that localizes to the cytoplasmic fibrils, p180 (Wilken et al., 1993), and a protein that localizes to both sides of the NPC, NUP155p (Radu et al., 1993). Two proteins that have already been found in protein complexes with other XFXFG or GLFG nucleoporins, NIC 96 and p75, also lack repeat motifs (Grandi et al., 1993; Pante et al., 1994).

I-D.1.3. Nuclear protein import. Transport of proteins and RNAs into and out of the nucleus occurs in cells exclusively through the NPCs. Unless a protein is small enough to freely diffuse through the eight channels for passive diffusion, they require a signal that will direct their transport. This signal has been called a nuclear localization signal (NLS). The protein sequence of an NLS may take the form of a small segment of basic amino acids or a longer bipartite sequence that is disrupted by a stretch of about ten amino acids (reviewed in Dingwall and Laskey, 1991; Garcia-Bustos et al., 1991).

Protein import into the nucleus was found to involve two distinct steps; docking to the cytoplasmic side of the NPC and transport through the complex (Newmeyer and Forbes, 1988; Richardson et al., 1988; Akey and Goldfarb, 1989). The docking step can take place at 0° C and does not depend on the presence of ATP. However, translocation of the bound protein through the NPC is inhibited at 0° C and is energy dependent. When WGA was added to a transport assay, even though NLS containing proteins could bind to the NPC, the transport of proteins was blocked at the translocation step (Finlay et al., 1987; Newmeyer and Forbes, 1988). Also, when WGA-binding proteins were depleted from a nuclear assembly extract, nuclear envelopes were assembled, but the newly assembled nuclei were defective for transport (Finlay and Forbes, 1990). In fact, when three of the WGA binding proteins, p62/p58/p54, were depleted from a nuclear assembly extract, or when an antibody against p62 was incubated with nuclei, import was also prevented (Dabauvalle et al., 1988; Finlay et al., 1991). Thus, some of the WGA binding proteins must play a role in protein import, likely at a step after the NLS containing protein docks to the exterior of the pore complex.

Moore and Blobel (1992) fractionated *Xenopus* extracts and found that separate cytosolic fractions were needed for the docking and translocation steps. Fraction A was required for docking while fraction B could could support the translocation of a protein that had already docked. In independent searches, a 54/56 kDa protein from bovine erythrocyte cytosol and a protein termed NIF1 from *Xenopus* extracts were able to support the docking step (Adam et al., 1989; Newmeyer and Forbes, 1990; Adam and Gerace, 1991). Surprisingly, these proteins were located in the cytosol and provided evidence that the NLS receptor is actually a cytosolic protein, and binds to the NLS on a protein before docking at the NPC. Like the proteins in fraction A, both the 54/56 kDa protein and NIF1 are sensitive to NEM treatment, and are likely to be the important proteins in fraction A. Another cytosolic protein, with a molecular mass of 97 kDa, was

found to enhance binding of the 54/56 kDa NLS receptor-protein complex to the NPC, although its exact role is not known (Adam and Adam, 1994; Chi et al., 1995).

After the NLS receptor-protein complex binds to the NPC, the addition of fraction B allowed protein translocation (Moore and Blobel, 1992). The two proteins that mediate this process were later isolated; one of them is the GTPase, Ran/TC4, and the other is a protein called B-2 (Melchior et al., 1993; Moore and Blobel, 1993, 1994). Recently, a protein that binds Ran/TC4, RanBP2, was identified that is located at the NPC and contains XFXFG-type repeats (Yokoyama et al., 1995). It is possible that this protein is present in the central annulus of the NPC and assists Ran/TC4 in the translocation of proteins through the NPC. Since Ran/TC4 is a known GTPase, this raises the possibility that GTP hydrolysis may play a role in releasing the imported protein from its NLS receptor after translocation.

A 60 kDa protein called importin has been cloned, and found to share homology with both the yeast protein SRP1 and the mammalian 54/56 kDa NLS receptor (Gorlich et al., 1994; Powers and Forbes, 1994). Interestingly, SRP1 interacts with the yeast pore proteins, NUP1p and NUP2p (Belanger et al., 1994). When assayed by immunofluorescence microscopy, SRP1 was found at the NPC (Cordes et al., 1993). Thus, these results have provided the first link between the NLS receptor and interaction with proteins of the NPC. The potential now lies ahead to understand the interactions at the pore complex that govern protein import, and to assign a function to specific nuclear pore proteins.

I-D.2. NPC assembly/disassembly. An understanding of the composition and function of the NPC has greatly advanced in the past decade. However, significantly less is known about what happens to the NPC when it is disassembled during cell division. Likewise, it is not clear how NPC assembly is accomplished during the assembly of the

nuclear envelope following cell division. Another type of NPC assembly, that occurs during interphase, will not be discussed until secion IV-C. In this thesis, all discussion of NPC assembly will refer to the assembly that takes place after an open mitosis.

The only major attempts to understand NPC assembly have addressed whether or not glycoproteins were necessary for NPC assembly. In one study, when p62 was depleted from a *Xenopus* nuclear assembly extract, nuclear envelopes were assembled without NPCs (Dabauvalle et al., 1990). The same held true if all WGA binding proteins were depleted from the assembly extract. These results indicate that NPC assembly occurs independently of nuclear membrane assembly. In another study where WGA-binding proteins were depleted from a *Xenopus* extract, nuclear envelopes and NPCs were assembled, but this time the NPCs were incapable of import (Finlay and Forbes, 1990). The discrepancy between these two results could have been due to the effectiveness of WGA depletion, or perhaps to the fact that in one case λ DNA was used as the template, whereas in the other, demembranated sperm chromatin was the template. Thus, the roles that these WGA proteins may play during nuclear envelope/NPC assembly is still unresolved.

Since NPCs are disassembled when the nuclear envelope breaks down, the NPC proteins must undergo reversible modifications that regulate their state of assembly. This has been found to be the case with a few of the NPC proteins. Three NPC proteins were found in separate complexes when mitotic cytosol that contained disassembled NPC proteins was separated by column chromatography. The NPC proteins p200, p97 and p60 (a newly named version of p62), were found in 1000, 450, and 600 kDa complexes, respectively (Macaulay et al., 1995). The p200 and p97 proteins were also found to be phosphorylated by $p34^{cdc2}$, suggesting that phosphorylation may play an important role in mediating protein-protein interactions of the NPC during mitosis. Likely, many other

NPC proteins are disassembled into distinct complexes through reversible phosphorylation.

The above studies have either found that NPC and nuclear membrane assembly are independent events during nuclear envelope assembly or they have not addressed this issue altogether. The experiments that were conducted in this thesis will help to determine whether these two topics, nuclear membrane and NPC assembly, are independent or coincident during nuclear envelope assembly. Specifically, the question of how NPC proteins are targeted to the chromatin surface during nuclear envelope assembly is addressed. For these experiments, the *Xenopus* egg fractionation protocol developed by Vigers and Lohka (1991, 1992) was used. Briefly, *Xenopus* eggs were collected and a low speed extract was prepared that, when subjected to differential high speed centrifugation, generated three fractions that were necessary for nuclear envelope formation around demembranated sperm chromatin (Vigers and Lohka, 1991, 1992). Two of these nuclear envelope precursor fractions, NEP-A and NEP-B (for nuclear envelope precursor A and B), are particulate, and the other is a soluble cytosolic fraction. Both the NEP-A and NEP-B fractions contain membrane vesicles. However, only the NEP-B fraction contains membrane vesicles that bind to chromatin, but upon binding do not fuse into an intact nuclear envelope. Like all nuclear envelope assembly reactions, the vesicles in NEP-B cannot bind in the absense of the cytosol, which is necessary for chromatin decondensation. Accordingly, the same result is obtained if S_{200}, which consists of the NEP-B vesicles and cytosol, is incubated with demembranated sperm chromatin. That is, vesicles in NEP-B bind to the chromatin but do not fuse into an intact nuclear envelope. When the NEP-A fraction is added to either NEP-B and cytosol or S_{200} in the presence of sperm chromatin, an intact nuclear envelope is formed. Thus, NEP-A appears to contribute a component(s) that allows membrane fusion to proceed.

As the ratio of NEP-B to NEP-A is decreased, the number of NPCs in the nuclear envelopes that form is decreased (Vigers and Lohka, 1991). In this regard, the NEP-B fraction appears to play a role in NPC assembly, as well as providing a membrane vesicle population that is targeted to the chromatin surface. For this reason, the NEP-B fraction was examined for the presence of nuclear pore complex proteins using mAb414, a well characterized monoclonal antibody that recognizes many XFXFG-type nucleoporins. Three mAb414-reactive nucleoporins have been found in the NEP-B fraction: p62, a 180 and a 210 kDa protein that correspond to NUP153 and NUP214 (Vigers and Lohka, 1992). The experiments in this thesis address the role that the NEP-B fraction plays in NPC assembly by testing the hypothesis that the nucleoporin, p62 and other mAb414-reactive nucleoporins, are components of the NEP-B vesicles. The approaches that were used to test this hypothesis were: 1) to examine the distribution of p62 in nuclear envelope precursor fractions, 2) to determine whether the presence of p62 in the NEP-B fraction was due to its association with the vesicles in that fraction, 3) to follow the association of p62 with sperm chromatin when NEP-B vesicles bind, and 4) to determine whether the NEP-B vesicles could be captured by beads coated with anti-nucleoporin antibodies.

CHAPTER II: MATERIALS AND METHODS

II-A. Preparation of nuclear envelope precursor fractions from *Xenopus* eggs.

II-A.1. Low-speed extract. Low speed extracts of *Xenopus* eggs were prepared, with minor modifications, as described by Lohka and Maller (1985) and Vigers and Lohka (1991,1992). Female *Xenopus laevis* frogs were injected in the dorsal lymph sac with 100 IU pregnant mares' serum gonadotropin 48-72 hr before use. The frogs were then injected with 915 IU human chorionic gonadotropin 13 hr before ovulation. Eggs were collected, dejellied in 2% cysteine (pH 7.9) and washed in gluconate buffer (10 mM hemi-magnesium gluconate, 25 mM potassium gluconate, 20 mM HEPES, pH 7.5) containing 1 mM dithiothreitol (DTT), 15 μg/ml leupeptin and 300 μM phenylmethyl-sulfonylfluoride (PMSF; Vigers and Lohka, 1992). The eggs were transferred to 3 ml centrifuge tubes and lysed by centrifugation at 10,000 *g* for 10 min in an HB-4 swinging bucket rotor (Sorvall Instruments, Dupont Co., Wilmington, DE). After removal of the lipid cap, the low-speed supernatant was collected, cytochalasin B was added to a concentration of 50 μg/ml and the supernatant was again centrifuged at 10,000 *g* for 10 min. The supernatant obtained after the second centrifugation was fractionated as described in section II-A.2. In some experiments, extracts were prepared in extraction buffer A consisting of 100 mM KCl, 5 mM $MgCl_2$, 20 mM HEPES (pH 7.5), 1 mM DTT, 15 μg/ml leupeptin, and 300 μM PMSF (Vigers and Lohka, 1991). There were no detectable differences in the results obtained with the two buffers.

II-A.2. S_{200} and NEP-A fractions. The low-speed extracts were fractionated by differential centrifugation as described by Vigers and Lohka (1991, 1992). The low-speed extracts were centrifuged at 200,000 *g* for 75 min in a TLS-55 rotor (Beckman

Instruments, Inc., Palo Alto, CA) to generate a crude NEP-A fraction in the pellet and a high-speed supernatant (S_{200}). A lipid cap on the surface of the S_{200} was removed and the NEP-A fraction was collected by side puncture of the centrifuge tube with a syringe and an 18G needle. The NEP-A was frozen in 30% sucrose (wt/vol in gluconate buffer) and stored at -80° C until further use. The S_{200} fraction was collected with a pasteur pipette, divided into 100 µl aliquots, frozen in liquid nitrogen and stored at -80° C until further use.

The crude NEP-A pellet could be further fractionated by centrifugation through a discontinuous sucrose gradient. One ml of the NEP-A pellet that had been frozen in 30% sucrose was thawed and layered over a sucrose density step gradient that consisted of 2 ml 35% sucrose and 2 ml 40% sucrose (wt/vol in gluconate buffer). The gradient was centrifuged at 300,000 *g* for 4 hr in an SW55 rotor (Beckman Instruments Inc., Palo Alto, CA) at 4° C. Material at the 35-40% interface was collected with a syringe and 18G needle, diluted up to 1.5 ml with gluconate buffer and centrifuged at 10,000 *g* for 10 min to pellet the purified NEP-A. The pellet was resuspended in sample buffer (Laemmli, 1970) for immunoblot assays.

II-A.3. Cytosol and NEP-B fractions. The cytosol fraction was obtained by centrifugation of 2 ml S_{200} at 200,000 *g* for 4 hr in a TLS-55 rotor. After centrifugation, a lipid cap was removed and the cytosol was collected with a pasteur pipette and stored at -80° C. For some experiments, the cytosol was centrifuged a second time using the same centrifugation conditions.

The NEP-B fraction was isolated from the S_{200} fraction in one of two ways, both of which produced pellets containing vesicles that bind to sperm chromatin and can complement cytosol and NEP-A in nuclear envelope assembly reactions around sperm chromatin (Vigers and Lohka, 1991, 1992). Therefore, a functional NEP-B fraction

could be obtained by either procedure. In one protocol (method A), 2 ml of the S_{200} fraction was transferred to an ultracentrifuge tube treated with Sigma-cote (Sigma Chemical Co., St. Louis, MO) and centrifuged for 4 hr at 200,000 *g* in a TLS-55 rotor. The NEP-B fraction, the fluffy, membrane layer above the gelatinous, golden pellet, was collected by side puncture of the tube. This fraction was either mixed with sample buffer or used for floatation through sucrose gradients (see section II-A.4).

In the second protocol (method B), 100 µl samples of S_{200} were diluted to 1 ml with gluconate buffer containing DTT, leupeptin and PMSF, and centrifuged at 10,000 *g* for 10 min in an Eppendorf microcentrifuge (Brinkmann Instruments, Inc., Westbury, NY) to sediment residual NEP-A. The supernatant was transferred to an ultracentrifuge tube treated with Sigma-cote, diluted up to 2 ml with the same buffer, and centrifuged in a TLS-55 rotor at 25,000 *g* for 1 hr. The supernatant was removed and the NEP-B fraction in the pellet was solubilized in sample buffer for immunoblot assays or processed for electron microscopy (see section II-H).

For some experiments, a portion of the supernatant was also solubilized in sample buffer for immunoblot assays. In other experiments, the supernatant from the 25,000 *g* centrifugation was recentrifuged for 4 hr at 200,000 *g* in a TLS-55 rotor. The pellet was either solubilized in sample buffer for immunoblot assays or processed for electron microscopy. A sample of the second supernatant was also taken for immunoblot assays. In other experiments, the S_{200} fraction was mixed with Triton X-100 to a final concentration of 2%, and incubated at 4° C for 30 min. The detergent-treated S_{200} was then diluted with gluconate buffer and centrifuged as described above.

II-A.4. Sucrose density gradient centrifugation of NEP-B vesicles. 250 µl of a NEP-B fraction prepared by method A was mixed with 250 µl of 80% sucrose in extraction buffer A, and transferred to a 5 ml ultracentrifuge tube. The sample was

overlaid with 2 ml of 30% sucrose, 2 ml of 20% sucrose and 0.5 ml of 10% sucrose (all in extraction buffer A). The tubes were centrifuged in an SW55 rotor at 180,000 *g* for 16 hr as described by Chaudhary and Courvalin (1993). Material at each of the interfaces and in the pellet was collected separately by side puncture of the tube with an 18 gauge needle. Each sample was diluted to 2 ml, for a final sucrose concentration of 10% (extraction buffer A), and centrifuged for 4 hr at 200,000 *g* in a TLS-55 rotor. The pellets from each interface were either mixed with Laemmli sample buffer for immunoblotting, or processed for electron microscopy (section II-H).

In some experiments, the pellets from the 20-30% interfaces were divided into four separate tubes, and treated with 1.0 M NaCl, 4.0 M urea or 0.1 M NaOH (final concentrations in extraction buffer A) at 4° C for 45 min. The samples were centrifuged at 200,000 *g* in a TLS-55 rotor for 1 hr, and the pellets were resuspended in sample buffer for immunoblot assays or SDS-PAGE stained with silver.

II-B. Antibodies.

Mouse monoclonal antibody 414 (mAb414) hybridoma supernatant and ascites were gifts from J. Aris and G. Blobel (Rockefeller University), and L. Davis (Duke University), respectively. The proteins recognized by mAb414 have been described previously (Davis and Blobel, 1986, 1987; Sukegawa and Blobel, 1993; Kraemer et al., 1994). A rabbit antiserum against full-length, bacterially-expressed, rat p62 (anti-p62) was a gift of Dr. F. Buss and Dr. M. Stewart (Cambridge, U. K.). The anti-p62 antiserum has been described previously (Buss and Stewart, 1995).

An anti-peptide antiserum against the last 20 amino acids of the carboxyl-terminal end of *Xenopus* p62 (Cordes et al., 1991) was raised in rabbits. This antiserum is referred to as L10. The L10 antiserum was affinity purified using CNBr-Sepharose (Pharmacia LKB Biotechnology) to which the peptide had been coupled. One ml of the

L10 was passed through the column, washed with PBS (137 mM NaCl, 10 mM Na_2HPO_4, 2.5 mM KCl, 1.8 mM KH_2PO_4, pH 7.2), eluted with 0.1 M glycine, pH 2.5, and the eluate was neutralized with 1.0 M Tris methylamine, to a final pH of 7.2. The affinity purified serum is referred to as apL10.

II-C. Sperm chromatin binding reactions.

The binding of NEP-B vesicles to chromatin was assayed in a reaction that consisted of 100 µl S_{200} and lysolecithin-treated *Xenopus* sperm (Lohka and Masui, 1983) incubated at 18° C for 30 min. In control experiments, 100 µl of cytosol was incubated with the lysolecithin-treated *Xenopus* sperm under the same incubation conditions. The concentration of sperm used for all of the binding reactions was 2.5 x 10^5 sperm/reaction for fluorescence microscopy and 8 x 10^5 sperm/reaction for immunoblot analysis. DNA was visualized with 1 µg/ml 4',6-diamidine-2-phenylindole dihydrochloride (DAPI; Boehringer Mannheim) and membrane vesicles were detected using the lipophilic fluorochrome 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$; Eastman Kodak Company) at a concentration of 5 µg/ml. The association of p62 and other mAb414-reactive nucleoporins with sperm chromatin was assayed by immunoblotting or indirect immunofluorescence as described in sections II-D and II-E.

Metaphase cytosol or okadaic acid added to a sperm chromatin binding reaction were assayed by both indirect immunofluorescence and immunoblotting for their effect on the association of vesicles and p62 with chromatin. The effect of metaphase cytosol was assayed by incubating 100 µl of the S_{200} fraction for 1 hr at 18° C with 25 µl of a 0-33% ammonium sulfate fraction from metaphase cytosol (Lohka et al., 1988), in the presence of 1 mM ATP and an ATP regeneration system, consisting of 6 mM phosphocreatine and 150 µg/ml creatine kinase (Schlossman et al., 1984). Metaphase cytosol that had been heat-treated at 65° C for 15 min was added to control reactions.

Permeabilized sperm was added and incubated for 30 min at 18° C. The effects of okadaic acid treatment were examined by incubating 100 µl S_{200} with 1 µM okadaic acid (Pfaller et al., 1991) for 1 hr, followed by the addition of sperm and a 30 min incubation. In both cases, control incubations were carried out in parallel without the metaphase cytosol or without okadaic acid.

II-D. Immunoblotting.

At the end of each incubation, all binding reactions were centrifuged through a 1.0 M sucrose cushion (in gluconate buffer) in an HB-4 rotor at 1,000 *g* for 10 min to separate the sperm chromatin and bound vesicles from the unbound vesicles, which do not sediment through the sucrose. Six binding reactions of 100 µl each were pooled for the immunoblots, diluted to 1 ml with gluconate buffer and layered over 3 ml of the 1 M sucrose solution in a 4 ml centrifuge tube. After centrifugation, the gluconate buffer and sucrose layers were carefully removed, the walls of the tubes were cleaned with a cotton swab and the pellets were resuspended in sample buffer. Samples were electrophoresed on 10% SDS-PAGE and transferred onto nitrocellulose. For the immunoblots shown in Figures 8 and 10, samples were separated by 7.5% SDS-PAGE and transferred to PVDF membrane. The membranes were blocked in 10% powdered milk in TBS (137 mM NaCl, 25 mM Tris, 2.5 mM KCl, pH 7.4) at room temperature for 1 hr, and incubated overnight at 4° C with a 1:100 dilution of mAb414 hybridoma supernatant or a 1:1000 dilution of mAb414 ascites in 5% powdered milk in TBS. Anti-p62 and L10 antisera were used at a dilution of 1:1000. Affinity-purified L10 was used at a dilution of 1:100. The blots were washed 3 times for 15 min with 5% powdered milk and incubated for 2 hr at room temperature with alkaline phosphatase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA), depending upon the primary antibody or antisera used, at a dilution of 1:1000 in 5%

powdered milk. The blots were washed three times, and developed for alkaline phosphatase activity in 100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris (pH 9.5) containing 0.1 mg/ml ρ-nitro tetrazolium chloride and 0.05 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim).

II-E. Indirect immunofluorescence.

For indirect immunofluorescence assays, a binding reaction was diluted to 1 ml with gluconate buffer and layered over 5 ml of a 1 M sucrose solution in an inverted 15 ml centrifuge tube (Corning Inc., Corning, NY), that had been modified so that a 12 mm circular coverslip could be supported in the cap of the tube. The binding reaction was centrifuged at 1000 *g* in an HB-4 rotor onto poly-L-lysine (1 mg/ml) coated coverslips. After the gluconate buffer and sucrose layers were removed, as described above, coverslips were fixed for 20 min in 2% paraformaldehyde in gluconate buffer (pH 7.0) at room temperature. Coverslips were washed 3 times in gluconate buffer for 1 min, blocked in 1% ovalbumin and 1% BSA in PBS for 1 hr, and incubated in undiluted mAb414 hybridoma supernatant or 1:250 ascites overnight at 4° C. Anti-p62 antiserum or pre-immune serum (Buss and Stewart, 1995) was used at a dilution of 1:250. Coverslips were again washed 3 times in PBS for 10 min each, reblocked for 30 min, and incubated in the dark for 1 hr with a Texas Red-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Jackson ImmunoResearch Inc.) at a dilution of 1:1000 in PBS. Specimens were washed in PBS, stained with DAPI, with or without $DiOC_6$, and mounted in 70% glycerol containing 1 mg/ml phenylene diamine and 100 mM $NaHCO_3$. Samples were examined with a Leitz Aristoplan microscope equipped with a 100W mercury bulb for immunofluorescence. Images were photographed on Ektachrome 400 film and black and white prints were prepared by Communications Media, The University of Calgary.

II-F. Confocal microscopy and image analysis.

Optical sections through specimens that had been labeled with both mAb414/Texas Red-conjugated goat anti-mouse IgG and $DiOC_6$ were scanned simultaneously in the rhodamine and FITC channels with a Leica laser scanning confocal microscope (Department of Anatomy and Cell Biology, University of Alberta). Images from each channel were captured separately for subsequent analysis. Images were digitally subtracted from each other by Dr. D. Bazett-Jones (Department of Medical Biochemistry and Anatomy, University of Calgary), as described in the text. Images representing the distribution of p62 and membranes were prepared by Parsec Productions Ltd., Calgary, AB.

II-G. Immunomagnetic depletion of vesicles from S_{200}.

For experiments in which NEP-B vesicles were depleted from S_{200} fractions, mAb414 was first attached to sheep anti-mouse IgG that was covalently linked to magnetic beads (Dynabeads M-280, Dynal Inc., Great Neck, NY). The beads were washed in PBS to remove the manufacturer's buffer before use and were collected to the side of a Sigma-coated microcentrifuge tube using the Dynal Magnetic Particle Concentrator. After the removal of PBS, the beads were resuspended in mAb414 ascites that had been diluted 1:40 in PBS. This mixture was incubated with gentle rotation overnight at 4° C. The beads were washed 3 times in PBS and added immediately to the S_{200} fraction. Control beads were prepared in the same way using control mouse IgG antibodies (Jackson ImmunoResearch Laboratories, Inc.). 6-7 x 10^7 control or mAb414 beads were incubated with 100 µl S_{200} for 3 hr at 4° C and the immunomagnetic beads were separated from the S_{200} fraction using the Dynal Magnetic Particle Concentrator. Sperm nuclei were added to the depleted S_{200} fraction to a concentration of 2.5 x 10^5 sperm/reaction and incubated at 18° C for 30 min. The sperm chromatin was stained

with DAPI and $DiOC_6$ to observe DNA and chromatin-bound vesicles, respectively, by fluorescence microscopy.

II-H. Electron microscopy.

Immunomagnetic beads were prepared as described above with the following exceptions: after removal of the S_{200}, beads were washed 3 times in gluconate buffer to remove loosely associated material, concentrated with the magnet and mixed with 10 µl of 2% low melting agarose made up in gluconate buffer. Samples were cooled on ice, diced into small pieces, and fixed *en bloc* with 2% glutaraldehyde in 0.1M cacodylate buffer, pH 7.2, overnight at 4° C. Specimens were post-fixed in 1% OsO_4 in 0.1M cacodylate buffer, pH 7.2, and stained in the dark with 2% uranyl acetate in H_20. The samples were then dehydrated in a graded series of ethanol, propylene oxide and embedded in JEMBED 812 (J.B. EM Services Inc., Dorval, Quebec) for sectioning. Sections were prepared by M. Herfort (Department of Anatomy, University of Calgary). Sections were post-stained in 2% uranyl acetate and 0.35% lead citrate, and viewed on a Hitachi H-7000 transmission electron microscope using an accelerating voltage of 75 kV (Faculty of Medicine, University of Calgary). The images were recorded onto Kodak 4489 film, objective aperature set at 80 µm. The film was developed in Kodak D-19 that had been diluted 1:2 for 4 min.

When fractions of S_{200} were examined by electron microscopy (see sections II-A.3 and II-A.4), the samples were resuspended in 2% low melting agarose in gluconate buffer, fixed and processed as described above.

II-I. Image Acquisition and Printing.

Black and white photographs of indirect immunofluorescence and EM images, and the original immunoblots were scanned at 300 dpi using a Hewlett Packard ScanJet

IIc, and saved as PICT files. PICT files were opened in Adobe Photoshop, where they were adjusted to the appropriated size, brightness, or contrast. The files were transferred to MacDraw Pro, labeled, and printed by a Radius Proof Positive dye sublimation printer.

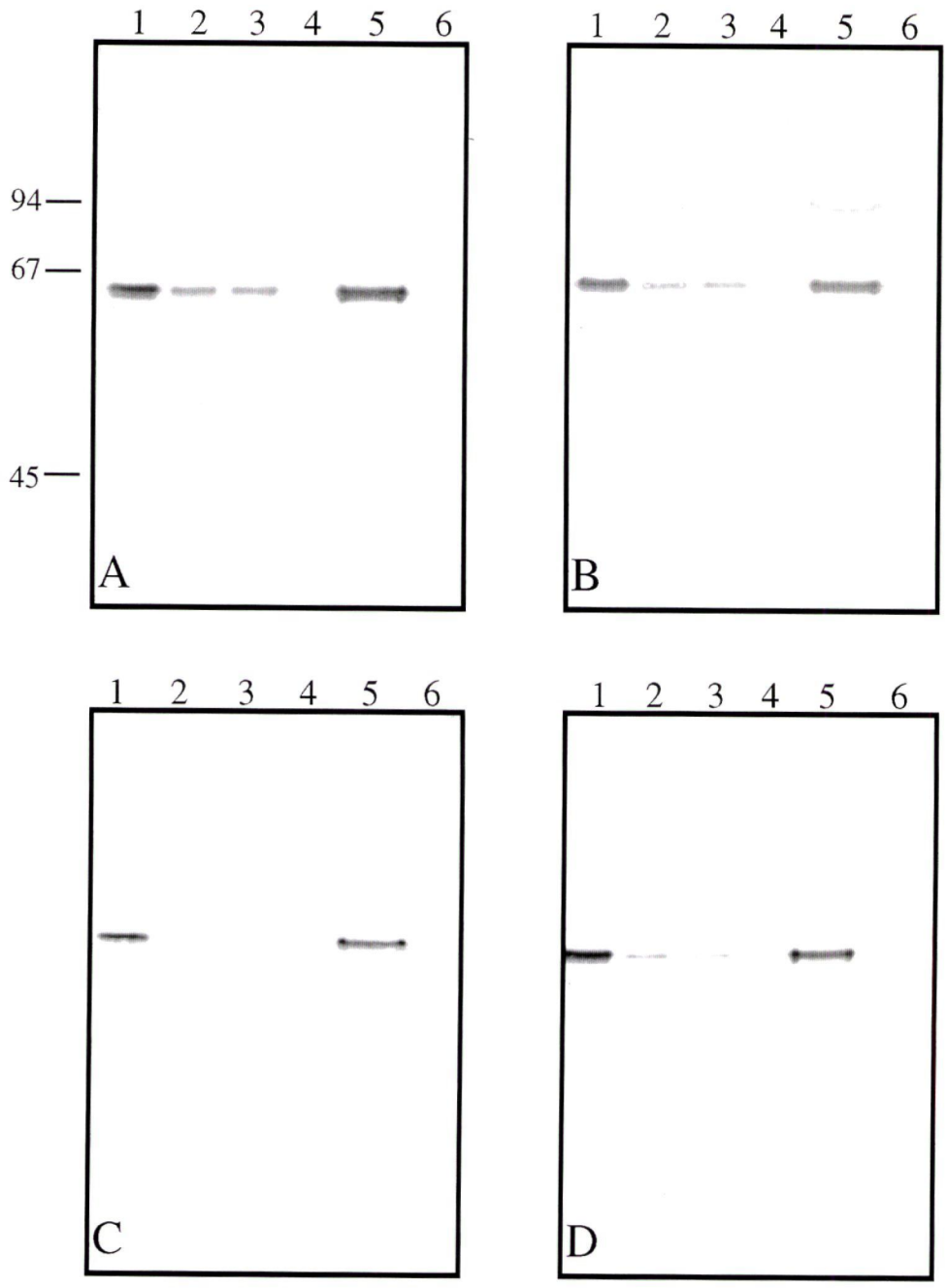
CHAPTER III: RESULTS

III-A. The distribution of p62 in nuclear envelope precursor fractions.

III-A.1. p62 is present in S_{200}, NEP-B, and cytosol. The presence of p62 in nuclear envelope precursor fractions was previously assayed by immunoblotting with mAb414 (Vigers and Lohka, 1992), which recognizes several XFXFG-type nucleoporins. Vigers and Lohka (1992) found that p62 and two other nucleoporins, nup153 and nup214, were present in the S_{200} and NEP-B fractions, but not in NEP-A or cytosol. Although they interpreted their results to indicate that the p62 and other nucleoporins are enriched in the NEP-B fraction, this conclusion is compromised due to unequal loading of the different fractions; the S_{200} and cytosol lanes were each loaded with 3 egg equivalents, whereas, NEP-A and NEP-B were loaded with 30 and 60 egg equivalents, respectively. One egg equivalent refers to the amount of material that is present in a single egg. Due to the uncertainty of these results, their experiments were repeated using equivalent amounts of material from each fraction and with more than one antibody against p62. In addition to mAb414, rabbit antisera against the whole p62 protein (Buss and Stewart, 1995) or against the carboxyl-terminal end (L10) were used.

mAb414 and the two polyclonal antisera recognized a 62 kDa protein in 5 egg equivalents of the S_{200} fraction and of the NEP-B fraction (figure 1A-D, lanes 1 and 3, respectively). Since three independent antibodies raised against p62 recognize the same protein in egg extracts, this protein is referred to as the nucleoporin, p62. In contrast to previous results, p62 was also detected when 5 egg equivalents of the cytosolic fraction was used (figure 1A-D, lane 2). The intensity of p62 staining in the NEP-B fraction was lower than that seen in the S_{200} fraction. Only when 20 egg equivalents of the NEP-B fraction was loaded (figure 1A-D, lane 5), was the signal intensity similar to that seen in

Figure 1. Immunoblot analysis of the distribution of p62 in nuclear envelope precursor fractions from *Xenopus* egg extracts. p62 was detected in the S_{200} fraction (lane 1), the NEP-B fraction (lanes 3 and 5), and the cytosol fraction (lane 2), but not in the NEP-A fraction (lanes 4 and 6). Lanes 1 through 4 contain 5 egg equivalents of sample, and lanes 5 and 6 contain 20 egg equivalents. Panel A, mAb414. Panel B, L10 antiserum. Panel C, affinity purified L10 antiserum. Panel D, anti-p62.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5 egg equivalents of S_{200}. Although under the conditions used, immunoblots are not an accurate measure of protein quantity, the intensity of p62 in the NEP-B and cytosol lanes together is approximately equal to that of the S_{200} lane. Thus, p62 distributes into both the cytosol and the NEP-B fraction when S_{200} is centrifuged. p62 and other nucleoporins were not detected in the NEP-A fraction, even when 20 egg equivalents of material was loaded (figure 1A-D, lane 6).

p62 was the most prominent band to be detected by mAb414, although as seen by Vigers and Lohka (1992), two higher molecular weight bands, corresponding to NUP153 and NUP214 were also present (Sukegawa and Blobel, 1993; Kraemer et al., 1994; Pante et al., 1994). The L10 antiserum detected p62 but not the other nucleoporins that are recognized by mAb414, since the antiserum was prepared against a region of p62 that is not shared between nucleoporins. However, another band at approximately 95 kDa was recognized by L10 (figure 1B). When L10 was affinity purified using the 20 amino acid peptide to which the antibody was raised, only p62 was detected on immunoblots, indicating that the 95 kDa band was nonspecific (figure 1C). Anti-p62 recognized p62 in the same nuclear envelope precursor fractions as mAb414 and L10 (figure 1D). Since all three antibodies detected p62 in the same nuclear envelope precursor fractions, including the cytosol fraction, the inability to detect p62 in the cytosol in the previous report (Vigers and Lohka, 1992) was likely due to the small amount of material that was loaded.

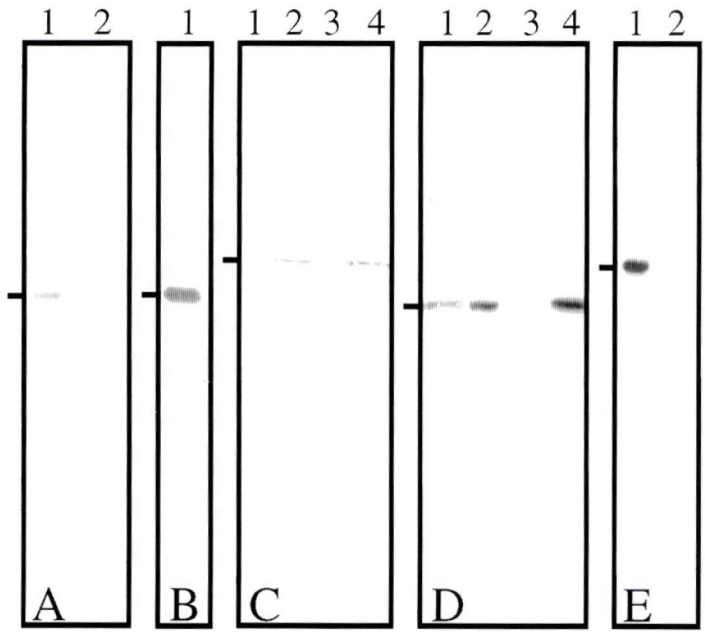
III-A.2. S_{200} contains both soluble and particulate p62. The p62 in the cytosol is in a soluble form since membrane vesicles are not found in this fraction (Vigers and Lohka, 1991; and data not shown). However, it is possible that the p62 in the NEP-B fraction is associated with membrane vesicles or with high molecular weight complexes that sedimented out of the cytosol during the preparation of the NEP-B fraction. p62 has been found in soluble high molecular weight complexes by others (Dabauvalle et al.,

1990; Finlay et al., 1991; Kita et al., 1993; Pante et al., 1994; Buss and Stewart, 1995). In support of this idea, when cytosol was subjected to a second centrifugation, nearly all of the p62 was pelleted, and most of the p62 was cleared from the supernatant (figure 2A, lanes 1 and 2, respectively). Since the same centrifugation conditions were used for the first and second centrifugations, it is possible that a portion of the p62 could have also been pelleted during the first centrifugation, and contributed to the observed signal detected in the NEP-B fraction. To examine which form p62 is found in each of these fractions, a series of centrifugation experiments was performed.

According to the original definition of the NEP-B fraction by Vigers and Lohka (1991), NEP-B could be prepared by two procedures (diagrammed in figure 3). In both cases, the NEP-B that is prepared contains membrane vesicles that bind to sperm chromatin in the presence of cytosol, and will assemble into nuclear envelopes in the presence of NEP-A and cytosol. By the first method (method A), undiluted S_{200} is centrifuged at high speeds for four hours, which generates cytosol and the pelleted NEP-B fraction. In the second method (method B), a small amount of S_{200} can be diluted and then centrifuged at a low speed for one hour, resulting in a NEP-B pellet and a supernatant that is discarded. The advantages of method B over method A are that much less starting material (S_{200}) is required and the preparation time is much shorter.

Much less p62 was detected on immunoblots in the NEP-B fraction prepared by method B than was seen on immunoblots of the NEP-B fraction prepared by method A (figure 2B and 2D, lane 1). To determine why the amount of p62 in the NEP-B pellet prepared by method B was greatly reduced, the supernatant was examined for the presence of p62. If the p62 was not pelleted or proteolyzed during the centrifugation, then it should be found in the supernatant. p62 was in fact found in the supernatant after the S_{200} fraction was diluted and centrifuged for one hour (figure 2C and 2D, lane 2).

Figure 2. Immunoblot analysis of soluble and particulate p62. A: The distribution of p62 in the pellet (lane 1) and supernatant (lane 2) after recentrifugation of cytosol. B: p62 in the NEP-B fraction by method A, 16 egg equivalents. C and D: p62 in the NEP-B fraction by method B (lane 1) and the remaining supernatant (lane 2). The effect of Triton X-100 treatment on the distribution of p62 in the NEP-B fraction (lane 3) and the remaining supernatant (lane 4). C: lanes 1 and 3 contain 5 egg equivalents of sample, lanes 2 and 4 contain 2 egg equivalents of sample. D: lanes 1 and 3 contain 50 egg equivalents of sample, lanes 2 and 4 contain 2 egg equivalents of sample. E: Sedimentation of p62 from the supernatant prepared by method B during high speed centrifugation. Lane 1: pellet. Lane 2: supernatant. The position of p62 is indicated by the black bar for each panel. All immunoblots were treated with L10 and an alkaline phosphatase-conjugated secondary antibody.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1
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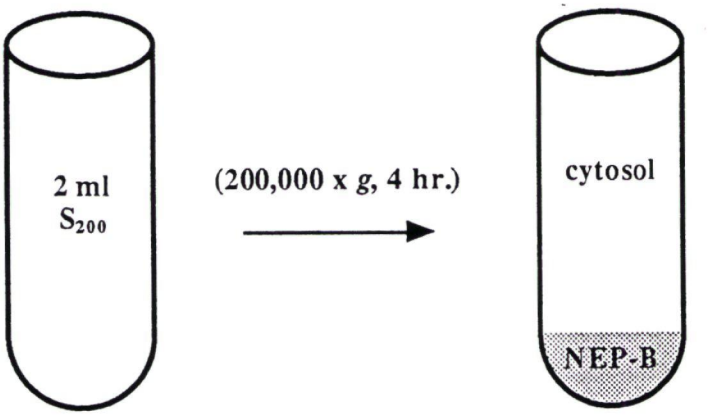
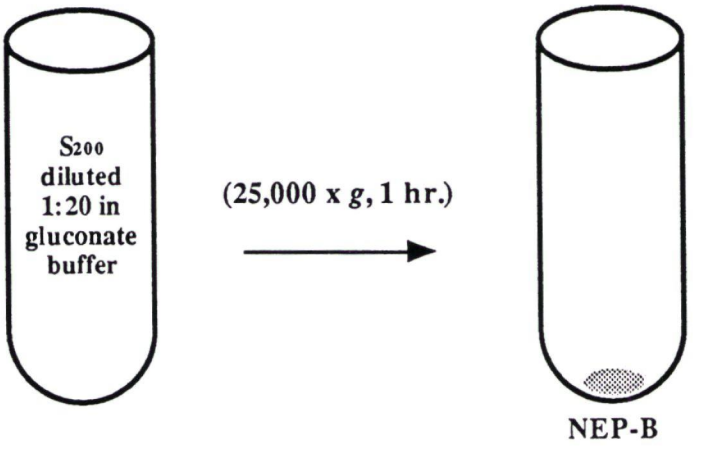
E

Figure 3. Two methods for the preparation of the NEP-B fraction from S_{200}. In method A, 2 ml of the S_{200} fraction is centrifuged at 200,000 *g* for 4 hr to generate cytosol and the NEP-B fraction. In method B, the S_{200} fraction is diluted and centrifuged at 25,000 *g* for 1 hr to generate the NEP-B fraction and a supernatant that is usually discarded. Both methods contain membrane vesicles in the NEP-B fraction that are functional for nuclear envelope assembly.

1. Method A



2. Method B

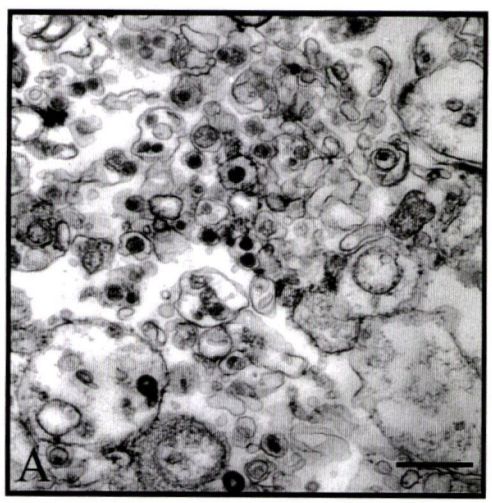


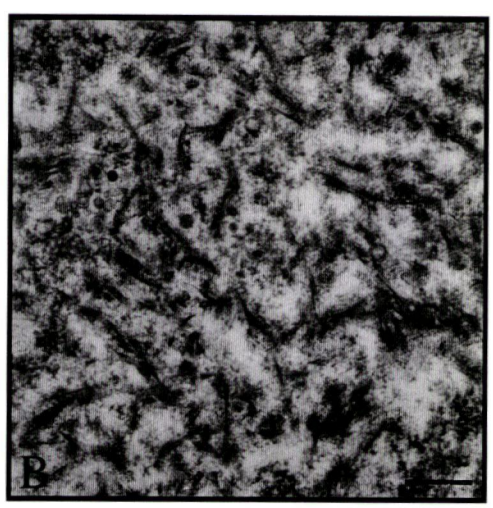
However, p62 could not be detected in the NEP-B pellet when two and one half times the amount of material was loaded, relative to the amount in the supernatant (figure 2C, lane 1). When twenty five times the amount, relative to the supernatant, was loaded onto the immunoblot, p62 was detected in the pellet (figure 2D, lane 1). The huge difference in the amount of material that could be detected between the supernatant and pellet indicates that a large amount of the p62 is not pelleted during the method B preparation of the NEP-B fraction from S_{200}. Nevertheless, a small amount of p62 was consistently found in the NEP-B fraction prepared in this manner, which has been shown to function in nuclear envelope assembly (Vigers and Lohka, 1991, 1992).

The supernatant from method B which was usually discarded, was instead subjected to a high speed centrifugation at 200,000 *g* for 4 hours to determine whether p62 could be pelleted. After the high speed centrifugation, p62 was not detected in the new supernatant (figure 2E, lane 2), but a strong signal could be found in the pellet (figure 2E, lane 1). The signal in the second NEP-B pellet was closer in intensity to that of the S_{200}. Thus, the NEP-B pellet after a low speed centrifugation did not contain much p62 because it was not completely centrifuged into the pellet.

The next step was to determine whether the p62 that did not pellet out of the supernatant was in a soluble or vesicle-associated form. To this end, the supernatant and pellet fraction derived from method B were examined by electron microscopy. As expected from previous results, numerous vesicles were present in the NEP-B pellet (figure 4A). To determine whether membrane vesicles remained in the supernatant prepared from S_{200} by method B, the supernatant was centrifuged a second time at high speeds and the pellet was examined by electron microscopy. In contrast to the first pellet, the second pellet was largely devoid of any membrane structures, but contained much amorphous material and rod-shaped structures of an unknown identity (figure 4B). Since electron microscopy revealed that very few vesicles were found in the second pellet, in

Figure 4. Electron micrographs of the NEP-B pellet and supernatant prepared by method B. A: the NEP-B fraction. B: material sedimented from the supernatant by centrifugation at 200,000 *g*. Numerous vesicles were present in the NEP-B fraction, whereas few were present in the supernatant. Scale bars represent 0.5 μm.


A


B

which p62 was abundant (figure 2E, lane1), most of the p62 in egg extracts appears not to be associated with membrane vesicles.

The possibility that the small amount of p62 present in the first pellet is associated with membrane vesicles is supported by observations that treatment of the S_{200} with Triton X-100 before centrifugation abolishes the presence of p62 in the NEP-B pellet (figure 2D, lane 3). Since high molecular weight complexes of p62 are resistant to detergent treatment (Finlay et al., 1991; Kita et al., 1993; Pante et al., 1994), the failure to detect p62 in the pellet suggests that some p62 may be associated with a detergent-sensitive structure, such as membrane vesicles. If so, there may be at least two pools of p62 in egg extracts; a large pool of soluble high molecular weight complexes and a smaller pool associated with membrane vesicles.

III-B. Nucleoporins are associated with the NEP-B population of membrane vesicles.

The experiments described above provided the first evidence that at least some of the p62 in the NEP-B fraction was membrane associated since it could be solubilized by detergent. However, it was possible that the p62 found in the NEP-B fraction was merely caught in the downward flow of vesicles during their centrifugation or may have been bound to these vesicles nonspecifically. Therefore, definitive proof was required that p62 was specifically associated with the NEP-B vesicles.

III-B.1. Sucrose floatation of NEP-B vesicles. Sucrose floatation is a method of centrifugation that is based upon layering a sucrose gradient over sample material that is suspended in a dense sucrose solution. If there is lipid in the sample at the bottom of the centrifuge tube, then during centrifugation it will "float" to a density in the sucrose gradient that is equal to its own. Since the density of proteins in the starting sample is

similar to that of the sucrose in the starting sample, proteins that are not associated with lipid will remain at the starting interface or they will be pelleted. This procedure was used to determine whether p62 is associated with vesicles in the NEP-B fraction (see diagram in figure 5). The starting material that was used for the floatation experiments was the NEP-B fraction that had been prepared by method A. This fraction was mixed with an equal volume of 80% sucrose, overlaid with 30, 20, and 10% sucrose solutions and then centrifuged. After centrifugation, material at each of the interfaces was collected, diluted to a 10% sucrose concentration and recentrifuged to pellet any material in the sample. Each sample was then examined by immunoblotting with the L10 antiserum or mAb414. Both L10 and mAb414 detected p62 at the 20-30% interface (figure 6A and 6B, lane 2). p62 was not found at the 10-20% interface (figure 6A and 6B, lane 1), indicating that p62 was associated with the vesicles that had floated to a density between the 20 and 30% sucrose solutions. On immunoblots where mAb414 was used, two major and several minor mAb414-reactive nucleoporins were also found at the 20-30% interface (figure 6A). The major nucleoporins have apparent molecular masses of 180 and 210 kDa, which correspond to NUP153 and NUP214, since these nucleoporins react with mAb414 (Davis and Blobel, 1986, 1987; Sukegawa and Blobel, 1993; Kraemer et al., 1994). In addition to p62, NUP153 and NUP214, proteins with molecular masses of 100, 135, 195, 205, 240 and 260 kDa were also detected by mAb414. Whether these represent other nucleoporins of the XFXFG family remains to be determined. None of the L10- or mAb414-reactive proteins were present at the 10-20% interface, but most could be found at the starting interface (30-40%; figure 6A and 6B, lane 3), and in the pellet (figure 6A and 6B, lane 4). The presence of p62 in the starting interface and pellet is consistent with the results described above that showed that much of the p62 is not associated with membrane vesicles.

Figure 5. Protocol for sucrose density centrifugation.

Sucrose Floatation of NEP-B prepared by method A

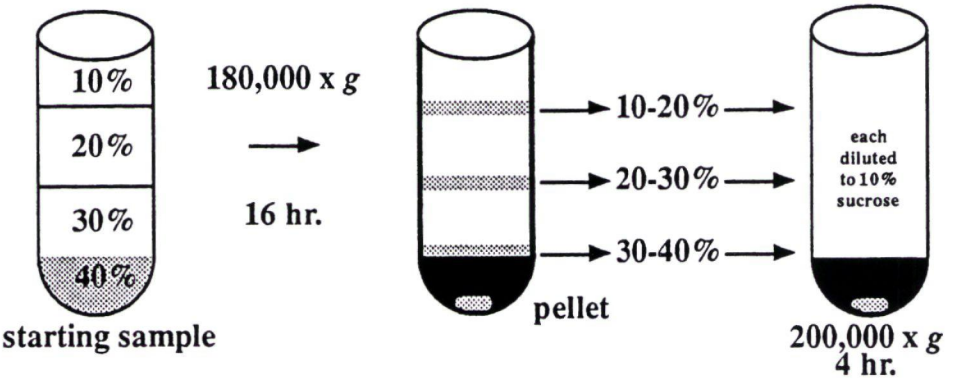
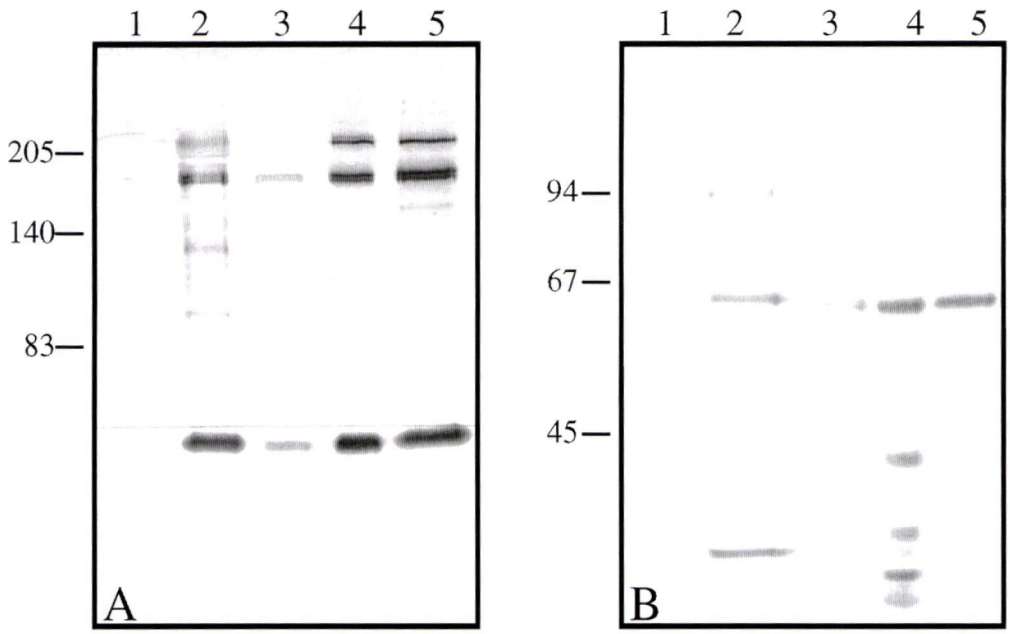


Figure 6. Immunoblot analysis of samples recovered following sucrose density centrifugation of the NEP-B fraction. The NEP-B fraction prepared by method A was mixed with sucrose to a final concentration of 40%, overlaid with a discontinuous sucrose gradient and centrifuged as described in Materials and Methods. Samples collected from the interfaces of the sucrose solutions and from the pellet were analysed by immunoblots with mAb414 (A) or the L10 antiserum (B). The membrane in panel A was cut in order for the region of the imunoblot that contains p62 to be developed independently from the region that contains higher molecular weight nucleoporins. The top region of the immunoblot was developed for approximately two times longer than the bottom region. Lane 1: 10-20% interface (8000 egg equivalents), lane 2: 20-30% interface (8000 egg equivalents), lane 3: 30-40% interface (1000 egg equivalents), lane 4: pellet (1000 egg equivalents), and lane 5: starting NEP-B fraction (35 egg equivalents). Egg equivalents were calculated assuming a 100% efficient recovery of material after centrifugation.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To confirm that vesicles were found only at the 20-30% interface, the pellets recovered from each interface were processed for electron microscopy. While vesicles were not found at either the 10-20% or 30-40% interfaces (figure 7A and B), numerous vesicles were found at the 20-30% interface (figure 7C). Thus, both mAb414-reactive nucleoporins and membrane vesicles were found at the 20-30% sucrose interface. Furthermore, the vesicles were morphologically similar to the NEP-B vesicles that were defined by Vigers and Lohka (1991).

III-B.2. Treatment of the NEP-B vesicles at the 20-30% interface. To show unequivocally that the mAb414-reactive nucleoporins are specifically associated with the NEP-B vesicles, the vesicles at the 20-30% interface were treated with high salt and chaotropic agents. Even when the vesicles were treated with 1.0 M NaCl and 4.0 M urea, the mAb414-reactive nucleoporins were still present (figure 8A, lanes 3 and 4, respectively). The fact that these nucleoporins were not dissociated from the vesicles by these treatments indicates that there is a tight association between the nucleoporins and the NEP-B vesicles. In contrast, all mAb414-reactive nucleoporins were removed from the vesicles by 0.1 M NaOH (figure 8A, lane 2), indicating that they are all peripheral membrane proteins. To confirm that proteins were present in all of the vesicle pellets that had been treated with the various reagents, a sample of each was examined by SDS-PAGE gels that had been stained with silver. In control vesicles, as well as those that had been treated with the NaCl and urea, numerous proteins were present (figure 8B, lanes 1, 3, and 4, respectively). The vesicle pellet that had been treated with NaOH showed a great reduction in the number of proteins present (figure 8B, lane 2). However, since a doublet of proteins is present at approximately 40 kDa, the vesicles were pelleted in a similar manner as the control vesicles. The overall results of the sucrose floatation experiments provide strong evidence that p62 and other mAb414-reactive nucleoporins

Figure 7. Electron micrographs of the samples recovered following sucrose density centrifugation of the NEP-B fraction. Fractions were prepared as described in figure 6. Panel A: material collected from the 10-20% interface; panel B: material collected from the 30-40% interface; panel C: material collected from the 20-30% interface. Scale bars represent 0.5 μm.

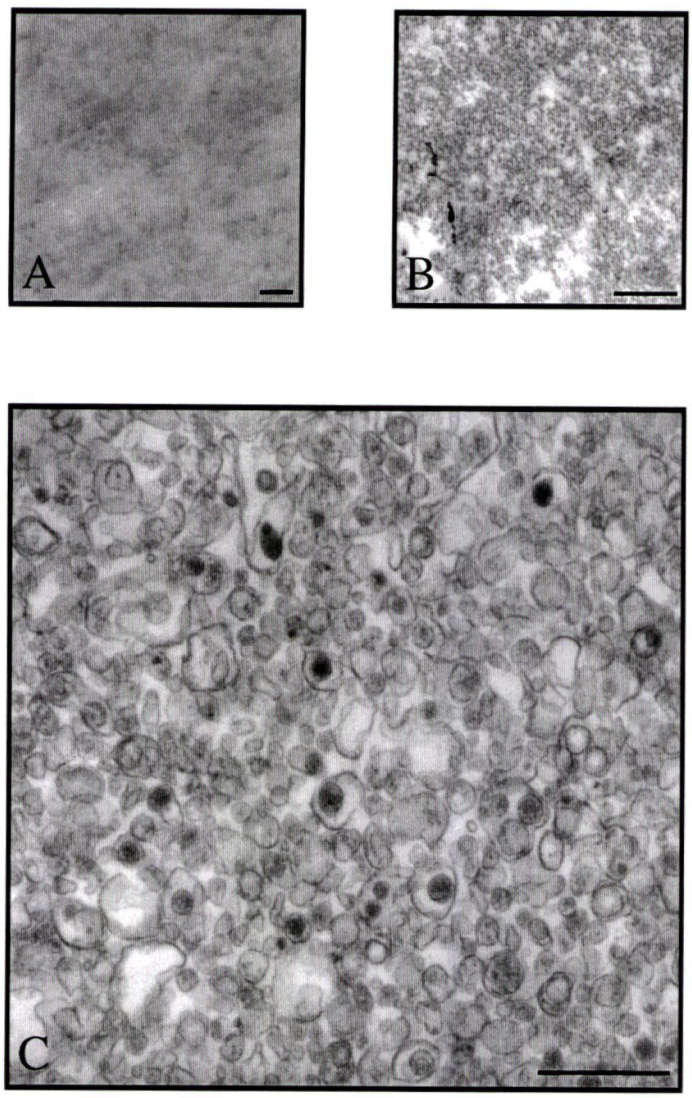
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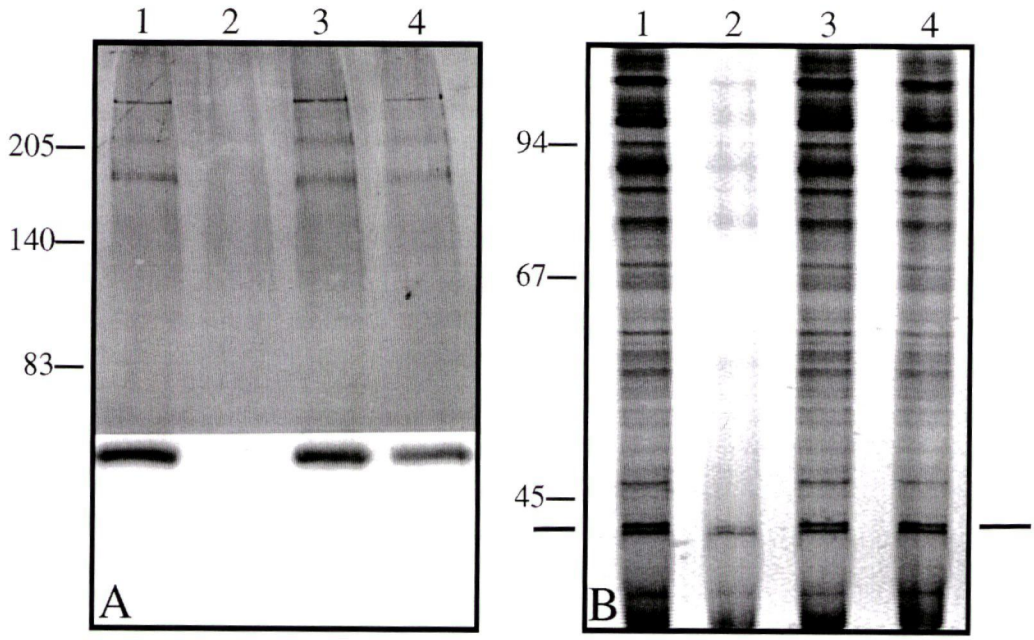
Figure 8. Treatment of the NEP-B vesicles with NaCl, NaOH and urea. Material that was collected from the 20-30% interface was treated with gluconate buffer (control in lane 1), 0.1M NaOH (lane 2), 1.0 M NaCl (lane 3), and 4.0 M urea (lane 4) and then centrifuged to pellet the treated sample. The protein content remaining on the vesicles from the 20-30% interface was analysed by immunoblotting with mAb414 (A) or by silver staining (B). Egg equivalents were calculated assuming a 100% efficient recovery of material after centrifugation: all lanes in panel A contain 3600 egg equivalents, all lanes in panel B contain 400 egg equivalents. The membrane in panel A was cut in order for the region of the immunoblot that contains p62 to be developed independently from the region that contains higher molecular weight nucleoporins. The top region of the immunoblot was developed for approximately two times longer than the bottom bottom region. Black bars in panel B point to a doublet of proteins at approximately 40 kDa that resist extraction by NaOH.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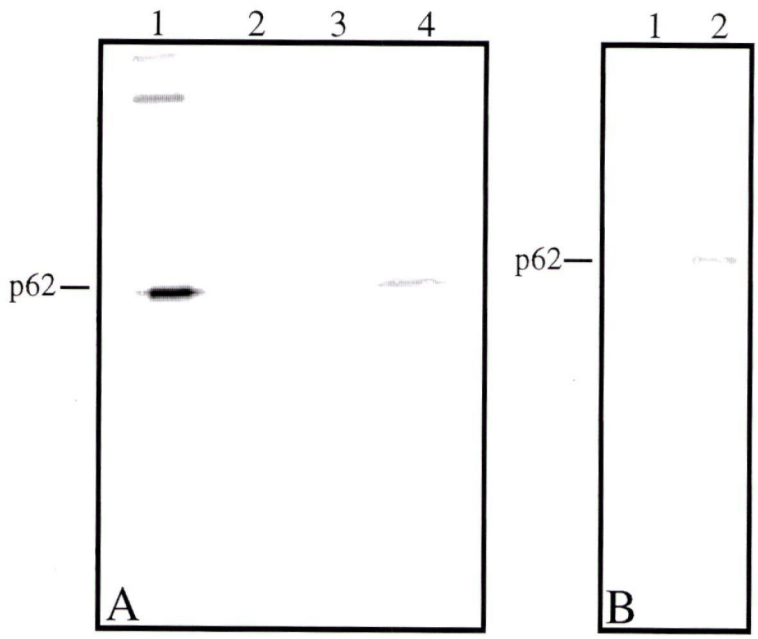
are specifically associated with the NEP-B vesicles.

III-C. p62 associates with sperm chromatin when NEP-B vesicles bind.

III-C.1. Sperm chromatin binding reactions. The results described above suggest that a pool of p62 and other nucleoporins are associated with NEP-B vesicles in the egg extracts. If this is so, one might predict that p62 would associate with sperm chromatin when the NEP-B vesicles bind. To test this hypothesis, an *in vitro* sperm chromatin binding reaction was developed that followed the mAb414-reactive nucleoporins by both immunoblotting and indirect immunofluorescence. For a typical binding reaction, S_{200} was incubated with demembranated sperm chromatin for 30 min. After incubation, the sperm chromatin was visualized with the DNA staining dye, DAPI, and the vesicles were visualized with the lipophilic dye, $DiOC_6$. When the sperm chromatin was examined following incubation with S_{200}, they were surrounded by many vesicles, even though at this early stage of nuclear envelope assembly the vesicles have not fused together to form the double membranes of the nuclear envelope. Since the vesicles were bound to the surface of the sperm chromatin, it was possible to separate them from the unbound material in the S_{200} fraction by centrifugation through a 1.0 M sucrose cushion. The unbound material remained at the surface of the sucrose cushion, while the sperm chromatin was pelleted into the bottom of the centrifuge tube where it could be processed for immunoblot analysis or for indirect immunofluorescence.

Sperm chromatin that had been centrifuged through the sucrose cushion without an incubation in S_{200} was not reactive with mAb414 on immunoblots, indicating that the nucleoporins were not associated with sperm chromtin at the begining of an incubation period (figure 9A, lane 2). However, when the sperm chromatin was incubated with

Figure 9. Immunoblot analysis of sperm chromatin binding reactions. mAb414 was used to examine the presence of p62 on sperm that had not been incubated in the S_{200} fraction, the S_{200} fraction without sperm, and sperm that had been incubated in the S_{200} fraction were centrifuged through 1M sucrose (panel A, lanes 2-4, respectively). In lane 1, proteins with molecular masses of approximately 180 and 210 kDa were recognized in addition to p62 in the starting S_{200} fraction. Permeabilized sperm were incubated in cytosol alone (panel B, lane 1) or in the S_{200} fraction (panel B, lane 2). p62 did not associate with sperm incubated in cytosol alone, despite the presence of soluble p62 in this fraction.


1
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p62
A
1
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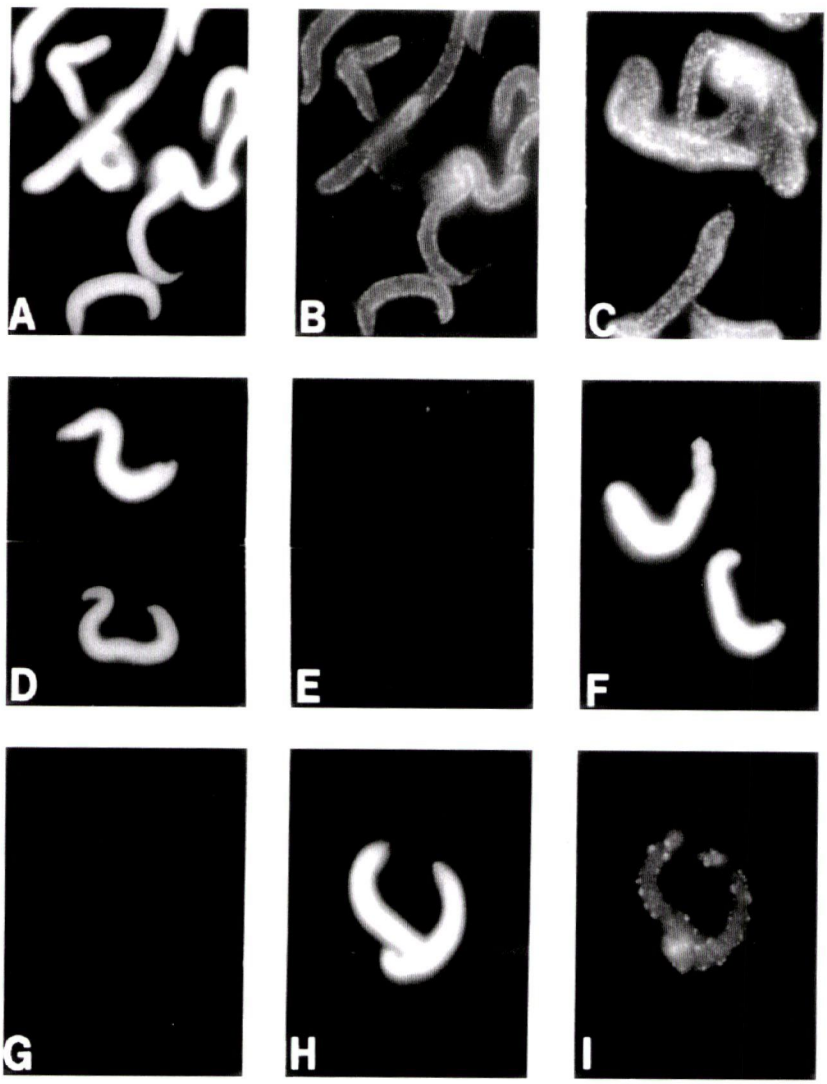
p62
B

S_{200}, the NEP-B vesicles were bound to the sperm chromatin (as observed with DAPI and DiOC6; see also figure 11), and p62 could be detected in association with the sperm (figure 9A, lane 4). The presence of p62 in the pellet was not due to any unbound material that centrifuged through the sucrose cushion since a signal could not be detected when S_{200} was loaded onto the sucrose cushion in the absence of sperm chromatin (figure 9A, lane 3).

Indirect immunofluorescence was also used to examine the association of p62 with the sperm chromatin. Sperm chromatin that had been incubated in the S_{200} fraction was brightly stained with mAb414 and a Texas Red conjugated secondary antibody (figure 10, panels A-C). The bright signal was not due to the secondary antibody alone since no staining was observed when the secondary antibody was used without mAb414 (data not shown). Since the results presented in section III-B.1 indicated that mAb414 reacts with several nucleoporins on the NEP-B vesicles recovered by density gradients, it is possible that the bright staining in panels B and C (figure 10) is due to one or all of these nucleoporins. To determine if p62 is present on the sperm chromatin, an antibody that recognizes only p62 (anti-p62) was used for indirect immunofluorescence (Buss and Stewart, 1995). The sperm chromatin binding reactions that were incubated with anti-p62 antiserum and a Texas Red-conjugated secondary antibody were also stained (figure 10, panels H and I), indicating that p62 bound to the surface of the sperm at this early stage of nuclear envelope assembly. This signal is specific for p62 since the pre-immune sera did not generate a signal on the sperm chromatin surface (figure 10, panels F and G). The staining of the sperm chromatin with mAb414 was much brighter than when anti-p62 was used, probably due to the recognition of other nucleoporins by mAb414. However, since we know that p62 is present on a sperm chromatin binding reaction by immunoblotting, it is likely that p62 is one of the nucleoporins that is recognized by mAb414 during indirect immunofluorescence assays.

Figure 10. Indirect immunofluorescence of sperm chromatin binding reactions. Sperm were stained with DAPI (A,D,F, and H), and with mAb414 and a Texas Red-conjugated goat anti-mouse secondary antibody (B,C, and E). In other experiments, sperm were stained with DAPI (F and H) and with either pre-immune rabbit serum (G) or with rabbit anti-p62 antiserum and a Texas Red-conjugated goat anti-rabbit secondary antibody (I). Sperm chromatin shown in A-C and F-I were incubated with S_{200}, and sperm chromatin shown in D-E were incubated in cytosol only.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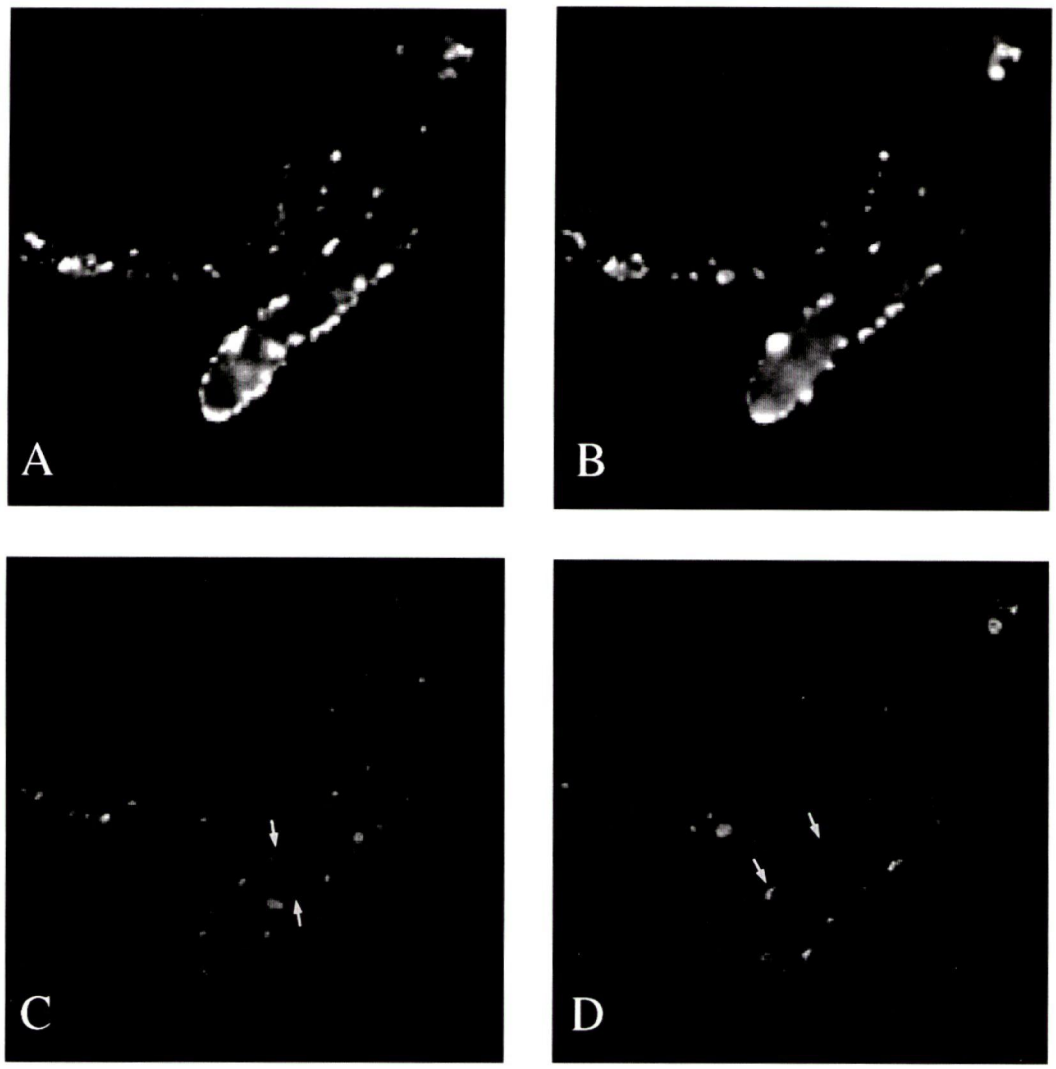
To determine if the soluble or the vesicle-associated p62 in the S_{200} fraction became associated with the sperm chromatin, sperm were incubated with the cytosol fraction, which contains only soluble p62, and centrifuged through the sucrose cushion. When the pellet was examined on an immunoblot, there were no mAb414-reactive proteins present (figure 9B, lane 1). In addition, when the pellet was examined by indirect immunofluorescence, no staining was observed on the sperm chromatin (figure 10, panels D and E). Since high molecular weight complexes that contain p62 are present in the cytosol, the immunoblot and indirect immunofluorescence results presented here suggest that it is unlikely that p62 can associate with the sperm chromatin in its soluble form. Therefore, the association of p62 with the sperm chromatin that was detected by immunoblotting and by indirect immunofluorescence was due to the presence of p62 on the NEP-B vesicles.

III-C.2. Nucleoporins co-localize with chromatin-bound vesicles. To further demonstrate that the mAb414-reactive nucleoporins associated with the sperm chromatin because of their presence on the NEP-B vesicles, the localization of mAb414-reactive nucleoporins and the membrane lipid of the chromatin-bound vesicles were compared. For this, sperm chromatin recovered from the binding reactions was examined by confocal microscopy to reduce background glare and to examine the lipid and nucleoporin staining in a single plane. The vesicle membranes that had been stained with $DiOC_6$ (figure 11, panel B) produced a staining pattern that was similar to that seen when sperm were stained with mAb414 and a Texas Red-conjugated secondary antibody (figure 11, panel A). To determine the degree of overlap between the bound vesicles and the nucleoporins, image analysis was used to subtract the separate images from each other. When the staining pattern of the vesicles was subtracted from the staining pattern of the nucleoporins, only regions where nucleoporins were found in the absence of membranes

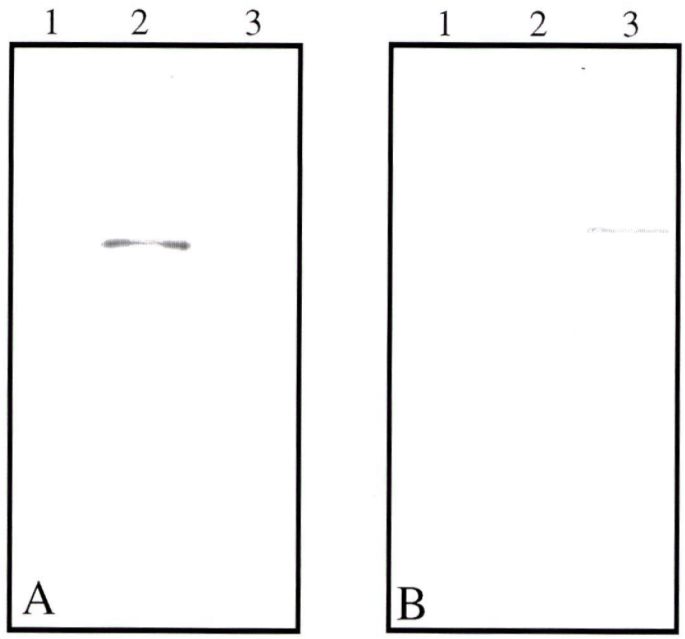
Figure 11. The co-localization of nucleoporins with membrane vesicles bound to sperm chromatin. Permeabilized sperm were incubated with the S_{200} fraction, stained with mAb414 and a Texas Red-conjugated secondary antibody (A), and with the lipophilic dye, $DiOC_6$ (B), and observed by laser scanning confocal microscopy. Using image analysis software, the staining pattern of vesicles was subtracted from that of p62 (C) and *vice versa* (D). The staining pattern that remains in C represents areas where p62 did not overlap with lipid. Arrows point to regions where p62 is in close proximity to the surface of the chromatin. The staining pattern that remains in D represents lipid staining that did not overlap with the staining of p62. Arrows point to regions where the remaining lipid staining is not adjacent to the chromatin, but is separated from the chromatin by an area where both lipid and p62 overlap.


A
B
C
D

remained (figure 11, panel C). Interestingly, the pattern of nucleoporins after vesicle subtraction, seems to be found directly at the chromatin surface in some cases (arrows in panel C). This may indicate that the nucleoporins that were initially associated with the periphery of the NEP-B vesicles accumulated close to the surface of the chromatin after vesicle binding. This idea holds true upon examination of the pattern of lipid staining after subtraction of the mAb414-reactive nucleoporins. When the staining pattern of nucleoporins was subtracted from the staining pattern of the vesicles, only regions containing lipid and no nucleoporins remained (figure 11, panel D). The lipid that remains appears to be located at a slight distance from the edge of the sperm chromatin, which indicates that the area of the heaviest nucleoporin concentration on the vesicles is at the periphery of the vesicles (arrows in panel D). Thus, the confocal images support the idea that the nucleoporins, when bound, are associated with the sperm chromatin through their interaction with the NEP-B vesicles.

III-C.3. Regulation of nucleoporin and NEP-B vesicle binding. It is thought that the regulation of nuclear envelope assembly and disassembly is dictated by states of protein phosphorylation. If an extract capable of nuclear envelope assembly is kept in a state where phosphatase activity is inhibited, nuclear envelope assembly cannot be initiated, and when proteins that regulate the vesicle-chromatin interactions are phosphorylated, the vesicles cannot bind to the sperm chromatin (Pfaller et al., 1991; Vigers and Lohka, 1992). To investigate whether the associations of the NEP-B vesicles and the nucleoporins with chromatin are regulated in a similar manner, sperm chromatin was incubated with the S_{200} fraction that had been pre-treated with okadaic acid. Okadaic acid is an inhibitor of phosphatases 1 and 2A. When the binding reactions were carried out in the presence of okadaic acid, p62 could not be detected in association with the sperm chromatin by either immunoblot analysis (figure 12A, lane 3), or by indirect

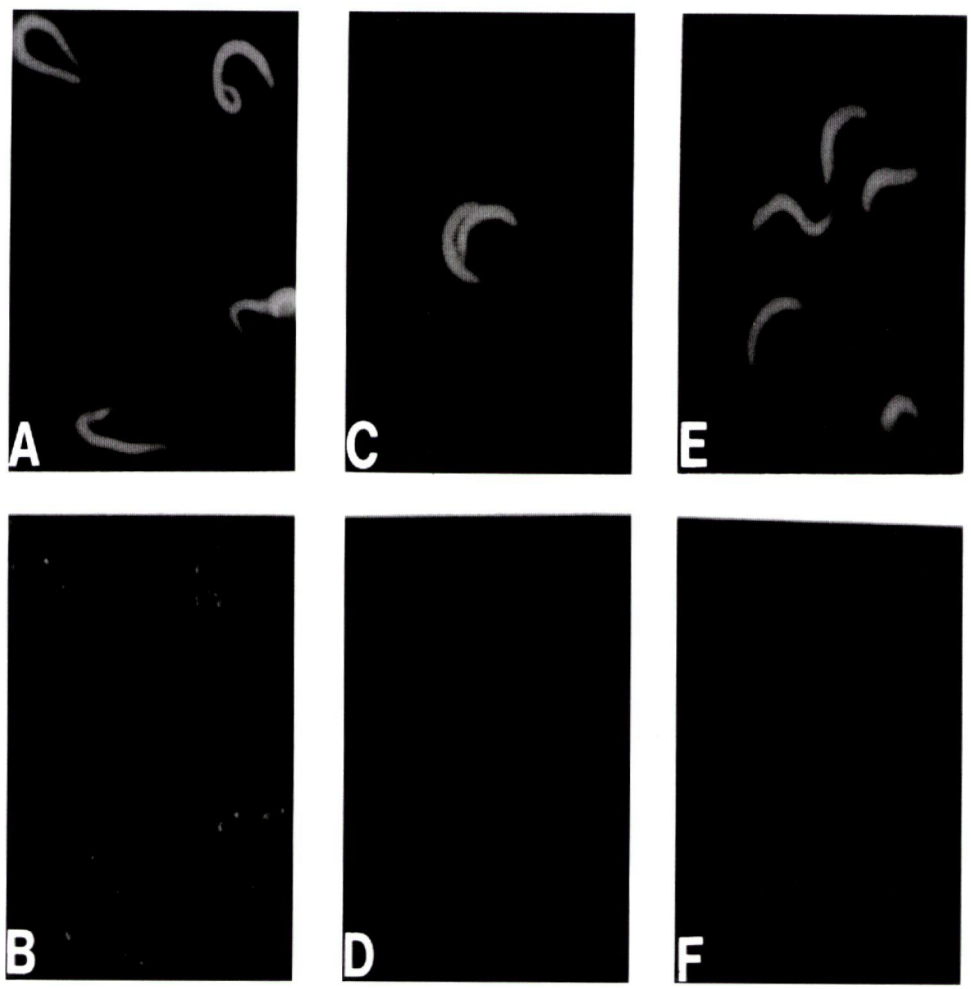
Figure 12. Immunoblot analysis of the effects of okadaic acid and metaphase cytosol on sperm chromatin binding reactions. A: The effect of okadaic acid. S_{200} fraction with 1 μM okadaic acid but no sperm (lane 1); sperm incubated with the S_{200} fraction but no okadaic acid (lane 2); sperm incubated with the S_{200} fraction and okadaic acid (lane 3). The amount of p62 that was detected in the pellet was greatly reduced by the presence of okadaic acid. B: The effect of metaphase cytosol. S_{200} and metaphase cytosol without sperm (lane 1); sperm incubated with S_{200} and an ammonium sulfate fraction of metaphase cytosol (lane 2); sperm incubated with S_{200} and a heat-inactivated ammonium sulfate fraction of metaphase cytosol (lane 3). The amount of p62 associated with sperm was reduced by an active ammonium sulfate fraction of metaphase cytosol. p62 was detected with the L10 antiserum and an alkaline phosphatase-conjugated secondary antibody.


1
2
3
A
1
2
3
B

immunofluorescence using mAb414 (figure 13, panels C and D). In control reactions that were run in parallel with the experimental reactions, p62 was detected on the sperm chromatin (figure 12A, lane 2 and figure 13, panels A and B). In other control experiments, the S_{200} fraction was first incubated with okadaic acid in the absence of sperm chromatin, and then the mixture was layered over the sucrose cushion and centrifuged. The NEP-B vesicles in the okadaic acid-treated S_{200} were not pelleted through the sucrose (figure 12A, lane 1).

Nuclear envelopes do not form and NEP-B vesicles fail to bind chromatin in extracts prepared from cells in metaphase. To examine the effects of metaphase extracts on the interactions between mAb414-reactive nucleoporins and chromatin, an ammonium sulfate fraction of metaphase cytosol was added to a S_{200} fraction before incubation with sperm chromatin. The addition of the metaphase cytosol pushes the S_{200} into a metaphase state. When sperm chromatin recovered from a binding reaction containing metaphase cytosol was assayed by immunoblotting (figure 12B, lane 2) or indirect immunofluorescence (figure 13, panels E and F), p62 was not found on the sperm chromatin. These results indicate that when NEP-B vesicles are prevented from binding by metaphase cytosol, mAb414-reactive nucleoporins fail to associate with the sperm chromatin. When the metaphase cytosol was heat inactivated, p62 associated with the sperm chromatin, indicating that the metaphase cytosol inhibited both the binding of NEP-B vesicles and the association of nucleoporins with the chromatin (figure 12B, lane 3). Thus, the regulation of nucleoporin association with sperm chromatin follows a similar cell cycle specific pattern as the regulation of the chromatin-binding vesicles with the sperm chromatin.

Figure 13. Indirect immunofluorescence of sperm chromatin binding reactions in the presence of okadaic acid or metaphase cytosol. Sperm chromatin was stained with DAPI (A, C, and E) or with mAb414 (B, D, and F). A-B: control incubation without added okadaic acid or metaphase cytosol. C-D: sperm chromatin binding reaction incubated in the presence of 1 μM okadaic acid. E-F: sperm chromatin binding reaction incubated in the presence of an ammonium sulfate fraction of metaphase cytosol.


A
C
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III-D. Nucleoporins are surface components of the NEP-B vesicles.

III-D.1. Depletion of NEP-B vesicles using immunomagnetic beads. To eliminate the possibility that the independent binding of nucleoporins and vesicles to the same sites on chromatin accounts for their co-localization, two sets of experiments were carried out. In the first experiment, immunomagnetic beads were coated with either mAb414 or a control mouse IgG. The beads were incubated with S_{200} for 2 hours and collected at the side of a microcentrifuge tube with a magnet. The S_{200} was recovered and mixed with sperm chromatin. When S_{200} was pre-incubated with the control IgG beads, many vesicles bound to the surface of the chromatin (figure 14, panels A and B). In contrast, when the sperm were incubated with S_{200} that had been pre-incubated with the mAb414 beads, the number of vesicles that bound to the chromatin was substantially reduced (figure 14, panels C-F). These results support the idea that the nucleoporins recognized by mAb414 are found on the surface of the NEP-B vesicles before the vesicles bind to the sperm chromatin.

III-D.2. mAb414-coated immunomagnetic beads bind NEP-B vesicles. In the second set of experiments, the S_{200} fraction was incubated with immunomagnetic beads that had been coated with mAb414 or with control mouse IgG. Instead of examining the treated S_{200} fraction, the immunomagnetic beads were recovered and processed for electron microscopy. In many cases only one or two vesicles were bound to the periphery of the mAb414-coated beads, but in other cases numerous membrane vesicles were observed on the bead surface (figure 15A). In contrast, vesicles were not found on the surface of beads that had been coated with control IgG (figure 15B). The ability of mAb414 to capture vesicles in the S_{200} fraction, together with the association of p62 and the other nucleoporins with vesicles after sucrose density gradient centrifugation, provides

Figure 14. Immunomagnetic depletion of NEP-B vesicles by beads coated with mAb414. Permeabilized sperm were incubated in S_{200} fractions that had been treated with immunomagnetic beads carrying sheep anti-mouse IgG antibodies and coated with either control mouse IgG (A-B) or mAb414 (C-F). Following incubation with the treated S_{200} fractions, unfixed sperm were stained with DAPI (A, C, and E) and $DiOC_6$ (B, D, and F). Treatment of the S_{200} fraction with mAb414-coated beads reduced the number of vesicles that bound to sperm chromatin.

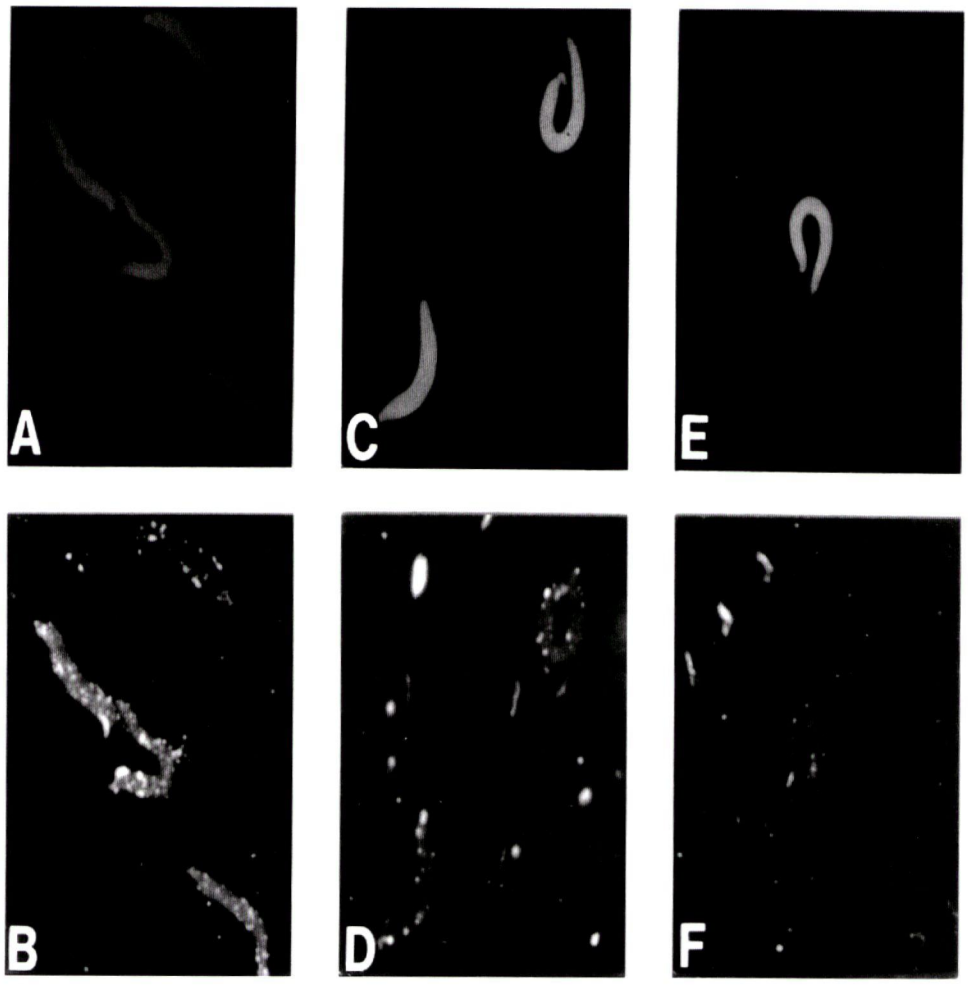
A
C
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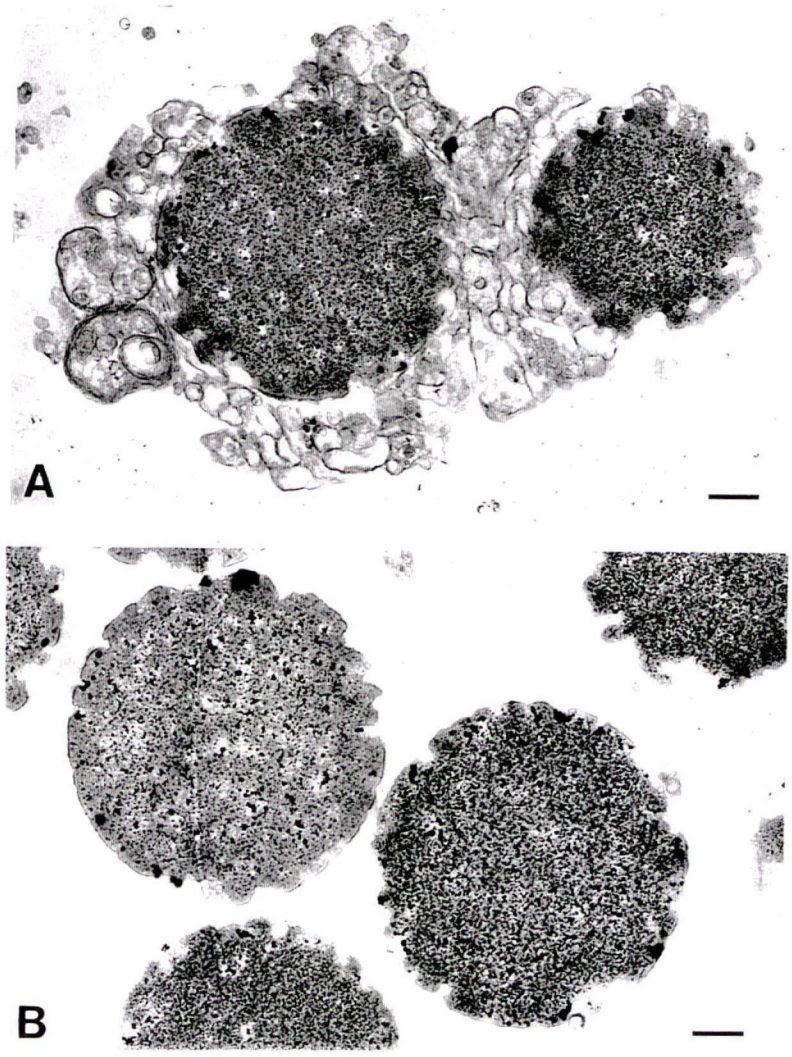
Figure 15. Electron micrographs of immunomagnetic beads that had been incubated in the S_{200} fraction. Immunomagnetic beads were coated with mAb414 (A) or control mouse IgG (B), incubated with the S_{200} fraction and processed for electron microscopy. Vesicles were associated with the mAb414-coated beads but not with the beads coated with control IgG. Scale bars represent 0.5 μm.


A
B

strong evidence that nucleoporins are directly associated with the surface of the NEP-B vesicles while they are still precursors of the nuclear envelope.

CHAPTER IV: DISCUSSION

In fractionated *Xenopus* egg extracts, the two membrane vesicle populations, NEP-A and NEP-B, together with the cytosolic fraction, can assemble nuclear envelopes around demembranated sperm chromatin (Vigers and Lohka, 1991, 1992). The membrane vesicle populations play functionally distinct roles during this process; the NEP-B vesicles are targeted to the surface of the sperm chromatin and the NEP-A vesicles mediate fusion into a double-membrane structure. The results presented in this thesis provide evidence that a portion of the total p62, and at least two other mAb414-reactive nucleoporins, are associated with the NEP-B population of membrane vesicles. Since the nucleoporins were not found with purified NEP-A vesicles, the two vesicle populations can now be regarded as being both biochemically and functionally distinct.

After initial observations indicated that p62 was present in the NEP-B fraction and that the p62 in this fraction was solubilized after detergent treatment, further evidence was necessary to determine whether the nucleoporins were associated with the NEP-B vesicles. This evidence was provided by the following observations: 1) the nucleoporins were detected in the vesicle-containing fraction that was recovered after floatation of the NEP-B fraction on a sucrose density gradient, 2) p62 and other mAb414-reactive nucleoporins were detected, by immunoblot analysis and by indirect immunofluorescence, on the surface of sperm chromatin when the NEP-B vesicles bound to chromatin, but not when binding was prevented by treatment with okadaic acid or an ammonium sulfate fraction of metaphase cytosol, 3) the immunofluorescence staining of chromatin-bound vesicles by mAb414 overlapped extensively with that of membrane vesicle lipids, 4) preincubation of extracts with immobilized mAb414 decreased markedly the number of membrane vesicles that bound to sperm chromatin during a subsequent incubation, and 5) vesicles were seen on the surface of

immunomagnetic beads that had been coated with mAb414 antibodies and incubated in fractions containing NEP-B vesicles. Thus, these results contribute to the field of nuclear envelope research by providing the first evidence that peripheral membrane proteins of the NPC become targeted to the chromatin surface through their association with chromatin-binding membrane vesicles.

IV-A. Soluble and membrane-associated nucleoporins.

The results in this thesis support previous studies where p62 was found in the S_{200} and NEP-B fractions, but not in the NEP-A fraction (Vigers and Lohka, 1992). However, they differ from those of Vigers and Lohka (1992), in that p62 was also found in the cytosol fraction. Perhaps this difference is due to the greater amount of material that was loaded on the immunoblots for the results presented here. The presence of p62 in the cytosol is not suprising, since to date, p62 has been said to be found only in the cytosol fraction from *Xenopus* eggs where it has been found in high molecular weight complexes with differing molecular masses. Complexes of 230-250 kDa (Dabauvalle et al., 1990; Kita et al., 1993), 500-600 kDa, (Finlay et al., 1991; Pante et al., 1994; Buss and Stewart, 1995; Macaulay et al., 1995), or 1000 kDa (Macaulay et al., 1995) have been found by different researchers. The various molecular masses of the complexes are not unexpected and could result from aggregation of the complexes, from the non-specific binding of other proteins to the complexes, or from differences in the chromatography conditions and material that was used. When the soluble pool of WGA-binding proteins from a *Xenopus* extract was examined by gel filtration chromatography, p62 eluted at molecular masses of 600 and 1000 kDa (Macaulay et al., 1995). Since the soluble fraction (cytosol) that is defined by Macaulay et al. (1995) was prepared in nearly the same way that I prepare S_{200} (which contains the NEP-B vesicles and cytosol), perhaps the p62 that elutes at 1000 kDa is membrane-associated and the p62 in the 600 kDa

complex is the soluble form of p62. The results of Macaulay et al. (1995) support the data presented here in that p62 is found in at least two different forms in a *Xenopus* egg extract. However, their results did not address whether or not the nucleoporins were associated with membrane vesicles.

The high speed centrifugations that were used to prepare the NEP-B fraction (method A) were likely to sediment both the vesicle-associated p62 and the p62 that is present in high molecular weight complexes. For this reason it was important to determine whether the p62 in the NEP-B fraction was present due to its sedimentation as a high molecular weight complex or in association with vesicles. The sucrose floatation experiments show that p62 is indeed found in the 20-30% interface which also contained the NEP-B vesicles. However, two types of experiments clearly demonstrated that some of the p62 that was not vesicle-associated could be sedimented by the high-speed centrifugation. 1) When the cytosol fraction was subjected to a second round of centrifugation, nearly all of the p62 in this fraction was sedimented. Since the same conditions were used for the preparation of cytosol in the first round of centrifugation, some of the soluble p62 could have been pelleted into the NEP-B fraction at this time. 2) Much of the p62 in the NEP-B fraction prepared by method A remained in the loading zone and the pellet after sucrose density centrifugation, even though the vesicles in the sample floated to a less dense position in the gradient. The p62 that remained in the 30-40% fraction was not associated with vesicles since they were not found when this interface was examined by electron microscopy. The p62 at the 30-40% interface and in the pellet confirmed the idea that some of the p62 in high molecular weight complexes was pelleting out of the cytosol when S_{200} was subjected to high speed centrifugations.

Even though much of the p62 in the NEP-B fraction is soluble, the soluble form of p62 and the other mAb414-reactive nucleoporins do not become bound to the sperm when the binding reactions were carried out with cytosol and sperm chromatin. Thus,

during the early stages of nuclear envelope assembly, only the p62 that is membrane-associated becomes bound to the sperm chromatin. What then are the roles of the membrane-associated and soluble p62 during nuclear envelope assembly? The p62 (and other nucleoporins) that is associated with the NEP-B vesicles bound to sperm chromatin may act as nucleation sites that attract soluble nucleoporin complexes to the chromatin surface during subsequent stages of NPC/nuclear envelope assembly. Thus, the targeting of the mAb414-reactive nucleoporins to the chromatin surface by the NEP-B vesicles may be the mechanism that brings other NPC proteins to the chromatin surface during nuclear envelope assembly. The α-helical coiled-coil rod domain at the carboxyl-terminal end of p62 enables p62 to dimerize and to interact with other NPC proteins (Buss and Stewart, 1995). This sequence on the p62 and others like it on the mAb414-reactive nucleoporins may be the way that the membrane-bound and soluble nucleoporins interact. Another possibility is that protein-protein interactions between the NPC proteins are mediated by the repeat motifs, XFXFG and GLFG. This possibility is supported by the finding that the yeast homolog of p62, NSP1, an XFXFG-type nucleoporin, can be found in a complex with two other GLFG-type nucleoporins, NUP49 and NUP57 (Grandi et al., 1993; Grandi et al., 1995).

IV-B. Differences in the nucleoporins that are complexed with p62 in its soluble and membrane-associated form.

Since p62 is found in both a soluble form and a membrane-associated form, it is conceivable that the proteins with which it associates are different for each case. The soluble high molecular weight complexes that have been reported by others are stable when treated with reagents that can disrupt protein-protein interactions; such as salts (Finlay et al., 1991; Kita et al., 1993), detergents (Finlay et al., 1991; Kita et al., 1993;

Pante et al., 1994), and urea (Finlay et al., 1991). This indicates that the soluble complexes are held together by strong protein-protein interactions.

The nucleoporins that are membrane-associated are also held onto the vesicles by strong protein-protein interactions since the p62 and the other mAb414-reactive nucleoporins remained on the vesicles when they were treated with 1 M NaCl and 4 M urea. Although the group of proteins that p62 associates with on the vesicles are likely to be different than those that it associates with when soluble, the complex on the vesicles is also highly resistant to disruption by salts and chaotropic agents.

One component that must be different between the soluble and vesicle-associated nucleoporins is the protein(s) that anchors these nucleoporins onto the vesicle membrane. Since the sequence of p62 does not reveal any regions that could serve as membrane spanning regions (D'Onofrio et al., 1988; Starr et al., 1990; Cordes et al., 1991), it is unlikely that p62 binds directly to the vesicle membrane. In addition, p62 and the other nucleoporins on the NEP-B vesicles can be removed by treatment with NaOH, suggesting that they are peripheral membrane proteins. NaOH is known to remove peripheral proteins but not integral proteins from the nuclear membrane (Senior and Gerace, 1988). The protein(s) that mediates the attachment of p62 to the vesicle membrane may either be an integral membrane protein, or a protein that is modified by the addition of lipid moieties, such as myristate, palmytilate, and isoprenoids. Whether p62, NUP153 and NUP214 are associated with the same membrane anchoring protein or with different proteins remains to be determined. A candidate for this anchoring protein is one or both of a pair of proteins of approximately 40 kDa that can be seen when NaOH treated vesicles are examined by SDS-PAGE and stained with silver. It will be interesting to determine whether these two proteins interact with the mAb414-reactive nucleoporins.

The sequence of p62 also suggests that it does not have a DNA-binding domain (D'Onofrio et al., 1988; Starr et al., 1990; Cordes et al., 1991). As the results described in this thesis have shown, the p62 in high molecular weight complexes is unable to directly associate with sperm chromatin. Therefore, the interaction between the vesicles and chromatin is not mediated by p62, but by an unidentified vesicle-associated protein. The interaction between the chromatin-binding vesicle protein and its chromatin target is likely to be regulated by states of protein phosphorylation since okadaic acid and a crude MPF-containing fraction of metaphase cytosol both prevent the association of vesicles with chromatin (Pfaller et al., 1991; Vigers and Lohka, 1992).

When the $DiOC_6$ staining pattern of the chromatin-bound vesicles is subtracted from that of the mAb414-reactive nucleoporins, the stained regions that remain (which represent regions of nucleoporins only), are often in close proximity to the surface of the chromatin. This localized concentration of mAb414-reactive nucleoporins at the chromatin surface suggests that the nucleoporins are concentrated near the proteins on the vesicles that bind to the chromatin, or the nucleoporins may have rearranged toward the surface of the sperm chromatin after the vesicles bind. This cluster of nucleoporins may have aggregated in preparation for NPC assembly. In other experiments where sperm chromatin with bound NEP-B vesicles was solubilized with Triton X-100, the vesicles were completely solubilized, as judged by the complete loss of $DiOC_6$ staining, but mAb414-reactive nucleoporins were still present on the sperm chromatin (Warren, unpublished data). The nucleoporins that remain after detergent treatment may be associated in a complex. This complex may contain the chromatin-binding protein and its target on the chromatin surface, the nucleoporin anchoring protein, and possibly other precursors of the NPC. The identification of the members of this complex will prove useful for further understanding the process of nuclear envelope assembly.

IV-C. NPC assembly vs. NPC biogenesis.

During the life cycle of a cell there are two times when NPCs are formed. The first is during nuclear envelope assembly around newly segregated metaphase chromosomes. The second is the *de novo* assembly of NPCs into the intact nuclear envelope. For the purposes of this discussion, this event will be called NPC biogenesis. NPC biogenesis has been found to take place before S-phase, since the number of NPCs in late G_1 doubles (Maul, 1977). NPC biogenesis is especially relevant to yeast cells which undergo a closed mitosis. Interestingly, the yeast homolog of p62, NSP1p, may play a role in the initiation of this event. When *NSP1* expression is repressed, a significant decrease in the formation and accumulation of NPCs occurs (Mutvei et al., 1992).

The results presented in this thesis also support the idea that p62 plays a significant role in the formation of NPCs during nuclear envelope assembly. The first event in nuclear envelope assembly that involves the vesicles is the targeting of the vesicles to the chromatin surface. In a *Xenopus* nuclear envelope assembly system, the NEP-B vesicles are targeted to the surface of the chromatin, but do not fuse into double membranes. The targeting of the NEP-B vesicles to the chromatin surface is important for two reasons: 1) the vesicles provide a source of lipid material that is used for the construction of the nuclear membrane, and 2) the NEP-B vesicles also target nucleoporins to the chromatin surface. The nucleoporins that are targeted to the chromatin surface can then act as nucleation sites for the recruitment of other soluble nucleoporins to the sites NPC assembly. In subsequent stages of nuclear envelope assembly the NEP-A vesicles are targeted to the chromatin around which a nuclear envelope is to be assembled, and contribute components that initiate membrane fusion. At some step during membrane fusion, the NPC is also assembled, since before fusion, intact NPCs are not seen on the NEP-B vesicles (Vigers and Lohka, 1991, 1992).

This mechanism may also be relevant to NPC biogenesis. One might imagine that when the proteins that anchor the nucleoporins to membranes are synthesized and inserted into the nuclear envelope, p62 and other nucleoporins become associated with it. These new complexes at the nuclear membrane could recruit other nucleoporins and serve as sites of NPC biogenesis when a sufficient amount of nucleoporins have accumulated. Thus, the formation of NPCs during nuclear envelope assembly and NPC biogenesis may be initiated by the same protein components.

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