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Characterization of Epitopic Structure Similarities Found Between the Immunoglobulin-type Domains of Titin and the Actin-binding Site of Caldesmon

by

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ABSTRACT

Characterization of anti-Ti II monoclonal antibodies (mAbs) has shown a cross-reactivity to caldesmon. This finding suggested that caldesmon and titin share epitope structural similarity. To further investigate the structural similarity, Western blotting was carried out on titin and caldesmon or their fragments using anti-Ti II mAb Ti104 and anti-caldesmon mAb C21. Characterization of Ti104 and C21 on titin and caldesmon from various species showed similar reaction pattern of the mAbs to both proteins. Western blotting on Ti II chymotryptic fragments and caldesmon CNBr fragments demonstrated the epitopes recognized by Ti104 and C21 are localized to a 3 kDa Ti II fragment and a 10 kDa caldesmon fragment. Amino acid sequence comparison between the 3kDa Ti II fragment and the actin-binding site in the 10 kDa caldesmon fragments showed a lack of significant primary structure similarity. This suggests that the similarity between the two protein fragments is found in their three dimensional structures. Several bacterially-expressed deletion mutants and Trp/Ala mutants of the CaD39 COOH-terminal human fibroblast caldesmon showed the epitope recognized by Ti104 and C21 to be localized to the COOH-terminal actin-binding site of caldesmon. Furthermore, labelling of titin by Ti104 on actin-extracted sarcomeres showed localization of the epitope recognized by Ti104 in the actin-binding sites of titin. The experimental results demonstrate that an epitope structural similarity was found between the actin-binding site of caldesmon and the actin-binding site of titin. The convergent structure found in caldesmon and titin suggests an analogous function between the two proteins.

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Dedicated to my husband and my son

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LIST OF ABBREVIATIONS

ABTS	2,2'-azino-di(3-ethylbenzothiazoline sulfonate)
AP	Alkaline phosphatase
BCIP	5-bromo-4-chloro-3 indolyl phosphate
BSA	Bovine serum albumin
CaD	Caldesmon
CaD39	COOH-terminal fragment of l-caldesmon
CNBr	Cyanogen bromide
ddH_2O	Double distilled H_2O
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbant assay
FCS	Fetal calf serum
GAM-HRP	Goat anti mouse-horse radish peroxidase
HAT	Hypoxanthine aminopterin thymidine
HMM	Heavy meromyosin
HPRT	Hypoxanthine guanine phosphoribosyl transferase
HT	Hypoxanthine thymidine
IPTG	Isopropyl-1-thio-β-D-galactopyranoside
LB	Luria bertani
LMM	Light meromyosin
NBT	Nitro blue tetrazolium
PBS	Phosphate-buffered saline
PEG	Polyethylene glycol
PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinylidene difluoride
SD	Sprague-dawley

SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TBS	Tris-buffered saline
Ti I	Titin class I motif
Ti II	Titin class II motif

CHAPTER ONE:

GENERAL INTRODUCTION

Muscle Structure and Contraction

Muscles found in vertebrates and many invertebrates are classified into striated muscle and smooth muscle. Striated muscle includes skeletal muscle and cardiac muscle. Skeletal muscle consists of fused long fibers cells. It is involved in complex coordinated activities such as walking and positioning, under voluntary control (Darnell *et al.*, 1990). Cardiac muscle consists of unfused single myocites that are connected by the intercalated discs. The cardiac muscles perform involuntary contractions to circulate the blood. Smooth muscles form the contractile portion of internal organs such as the stomach, intestines, arterial walls and the uterus. Smooth muscle is under involuntary control of the central nervous system to maintain slow and sustainable contraction (Darnell *et al.*, 1990; Alberts *et al.*, 1994).

A. Striated Muscles

A.1. Contractile Filaments in the Sarcomere

In the striated muscles, the organization of the major contractile elements has been extensively studied. The contractile elements, actin and myosin, are present in a highly ordered arrangement. Each muscle cell or myofiber is multinucleated and contains filament bundles called myofibrils, each of which is comprised of repeating arrays of sarcomeres. Sarcomeres, the contractile unit of striated muscle, contain thin filaments and thick filaments. Thin filaments are made up of actin, tropomyosin, and troponin complex, consisting of

troponin-C (TnC, Ca^{2+}-binding subunit), troponin-I (TnI, inhibits actomyosin ATPase activity) and troponin-T (TnT, tropomyosin-binding subunit). Thick filaments are made up of myosin (Darnell *et al.*, 1990) and its associated-proteins such as C-protein and X-protein (Morimoto and Harrington, 1973; Etlinger *et al.*, 1976; Starr and Offer, 1983). When seen under the electron microscope, the region of the sarcomere that covers the entire length of myosin shows anisotropic formation, which is called the A band (Fig.1.1) (Darnell *et al.*, 1990). The centre of this region is designated as the M-line. The region that only contains the actin filament is isotropic and called the I-band, which is bisected by the Z-line (Darnell *et al.*, 1990), which is mainly composed of α-actinin (Small *et al.*, 1992). The region that contains both actin and myosin is called the A-I junction. By light microscopy, the thin filaments appear as light bands and the thick filaments appear as dark bands. The array of light and dark bands that lie side by side form the striation of the muscle (Darnell *et al.*, 1990).

Actin filaments (F-actin) are polymers of globular actin (G-actin), a single polypeptide chain with a molecular weight of about 45 kDa (Darnell *et al.*, 1990; Alberts *et al.*, 1994). Polymerization of globular actin monomers into filaments is induced by Mg^{2+}, and K^+ or Na^+. Actin polymerization consists of nucleation and elongation stages. In the nucleation stage, several actin molecules form a small stable aggregate, whereas in the elongation stage, actin monomers are added to both ends of actin filaments (Berhadsky and Valisilev, 1988). The monomers interact with each other to form a helical actin filament, in which each monomer is oriented nearly perpendicular to the helix axis (Darnell *et al.*, 1990).

Myosin that is present in the muscle cells is type II myosin, which has the ability to hydrolyze ATP into ADP + Pi when stimulated by binding to actin. It consists of two

polypeptide chains called heavy chains, with molecular weights of about 200 kDa, and two pairs of light chains with molecular weights of 20 kDa and 17 kDa (Berhadsky and Valisilev, 1988; Darnell *et al.*, 1990). The heavy chain consists of a globular head at its NH_2-terminus and a rod-like α–helical tail at its COOH-terminus. The rods of the heavy chains coil around each other to form a rigid coiled-coil tail. This part has two domains called hinges, where the myosin molecule is flexible (Darnell *et al.*, 1990). Two major fragments produced by trypsin or chymotrypsin digestion are named heavy meromyosin (HMM) with a molecular weight of about 350 kDa and light meromyosin (LMM) with a molecular weight of about 150 kDa (Szent-Györgyi, 1953; Mueller and Perry, 1962). HMM possesses actin-binding and ATPase activity. Further digestion with papain degrades HMM into subfragment-1 (S-1), which retains the actin-binding and ATPase activity, and subfragment-2 (S-2) which is the fibrous part (Berhadsky and Valisilev, 1988; Darnell *et al.*, 1990). The fibrous tails of 300-400 myosin dimers pack together laterally to form the thick filament, a specific bipolar aggregate (Berhadsky and Valisilev, 1988; Darnell *et al.*, 1990). The central zone of the thick filament is composed merely of antiparallel overlapping arrays of the tails, whereas the terminal region contains globular heads protruding from the surface to form a helical array at intervals of 14 nm (Darnell *et al.*, 1990).

During muscle contraction, actin and myosin slide past each other (Huxley and Hanson, 1954). The protruding heads of myosin that retain actin-stimulated ATPase activity (S-1 myosin) bind to and move along the actin filament (Darnell *et al.*, 1990). The contraction is driven by ATP hydrolysis. Myosin by itself can hydrolyse ATP at a low rate, but when bound to actin, the rate of ATP hydrolysis increases rapidly and facilitates movement

(Chalovich, 1992). The phosphate release is a very important step, since the muscle is in a force producing state following that step (Chalovich, 1992). Striated muscle contraction is stimulated by a rise in cytoplasmic Ca^{2+}, released from the sarcoplasmic reticulum, a network of smooth membrane that surrounds each myofibril (Darnell *et al.*, 1990).

An actin-linked regulatory system, the troponin-tropomyosin system, is required in the activation of contraction. Tropomyosin is a coiled-coil of two parallel α-helical polypeptides with molecular weight ~35 kDa (Darnell *et al.*, 1990). It binds to F-actin with a stoichiometry of one tropomyosin dimer to seven actin monomers (Spudich and Watt, 1971; Bremel *et al.*, 1972). Tropomyosin molecules polymerize head-to-tail to form filaments that lie along the groove of the actin helix (O'Brien *et al.*, 1971; Darnell *et al.*, 1990). The primary structure of tropomyosin shows 14 groups of clustered acidic amino acid residues that are thought to be involved in actin binding (Parry, 1975; McLachlan and Stewart, 1976). The acidic residues can be grouped into α and β sites (McLachlan and Stewart, 1976). The troponin complex consists of TnI, TnT and TnC. TnI binds to TnT, TnC and actin, and inhibits ATP hydrolysis by actomyosin (Darnell *et al.*, 1990). The inhibition of ATP hydrolysis is enhanced by the presence of tropomyosin, and the stoichiometry of binding required for the inhibition is 1 troponin : 7 actin monomers, similar to that of tropomyosin to actin (Chalovich, 1992). TnT is an elongated molecule that binds to tropomyosin. Its NH_2-terminus binds to 1/3 the length of the COOH-terminal end of tropomyosin, extends beyond it and interacts with the adjacent tropomyosin (White *et al.*, 1987). TnT also binds to TnI but not to actin. TnC is the Ca^{2+}-binding subunit; it has four Ca^{2+}-binding sites (Ikemoto *et al.*, 1974; Potter and Gergely, 1975). It binds to both TnI and TnT. The TnC-Ca^{2+} complex reverses the actomyosin ATPase

activity inhibition of TnI (Chalovich, 1992). In regulating contraction, the binding of Ca^{2+} to TnC causes conformational changes in TnC that make the troponin subunits bind more tightly with each other and less tightly with actin and tropomyosin. These changes cause an alteration in the binding of tropomyosin to actin, and tropomyosin moves slightly towards the centre of the actin filament, exposing the myosin-binding site of actin, and enabling myosin to bind strongly to actin (Darnell *et al.*, 1990; Chalovich, 1992). This strong binding of myosin to actin promotes cross-bridge formation and cycling (Chalovich, 1992).

A.2. The Third and Fourth Filaments as the Sarcomere Cytoskeleton

In addition to the thick and thin filaments, a possible existence of filamentous constituents within the sarcomere has been recognized since the 1960's (Wang, 1984). Pringle (1978) demonstrated a continuity between the ends of thick filaments and adjacent Z-lines in insect flight muscle. Locker and Daines (1980) demonstrated gap filaments in highly stretched beef muscle. In 1976, Locker and Leet proposed a model in which a gap filament serves as the core of two thick filaments in adjacent sarcomeres, linking them through the Z-line and passing between the thin filaments. The presence of structural continuity within the muscle cell was also suggested by some aspects of mechanical and structural behaviour of resting muscle (Wang, 1984). The two-filament model shows that the structural and mechanical continuity of the contractile elements is brought about only by the dynamic and transient interactions of two sets of discontinuous filaments in active muscle (Wang, 1984). This model encounters a difficulty in explaining the contractile elements-related resistance to passive tension in relaxed muscle, where the interaction of thick and thin filaments is expected to be

absent. It also encounters a difficulty in explaining the A-band position that remains in the centre of regularly spaced Z-lines when resting muscle is stretched even to a length where thick and thin filaments no longer overlap (Wang, 1984). In 1977, Maruyama *et al.* obtained a crudely extracted muscle residue that was considered to represent an elastic protein, named connectin. Connectin was associated with the gap filaments, and was later also named titin. Wang *et al.* (1979), characterized three major protein bands above the myosin heavy chain in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of muscle extracts. The top doublet was collectively designated as titin (from titan) because of their gigantic size. These two bands (T1 and T2) were proposed to be immunologically and possibly, chemically related. The third band was designated as nebulin because it is associated with the N_2-line, a nebulous striation within the I-band (Wang and Williamson, 1980; Wang and Ramirez-Mitchell, 1981). The sizes of those proteins were approximately $1x10^6$ Da for titin and $5x10^5$ Da for nebulin as determined under the dissociative conditions of high porosity SDS gels (Wang and Ramirez-Mitchell, 1981). It was proposed that titin forms the third filament in the sarcomere (Wang, 1984) and nebulin forms the fourth filament, and that both proteins function as cytoskeletal elements to stabilize the contractile apparatus and maintain sarcomere integrity (Small *et al.*, 1992; Fürst and Gautel, 1995). Nebulin has only been found in skeletal muscle, but a smaller immunologically and structurally related protein, nebulette, has been identified in the cardiac muscle (Moncman and Wang, 1995). Nebulin has been proposed as a set of inextensive longitudinal filaments attached to the Z-line and coextensive with the actin filament (Wang and Wright, 1988). It has been demonstrated that nebulin binds to F-actin and may function as an actin-binding template (Jin and Wang, 1991). Several

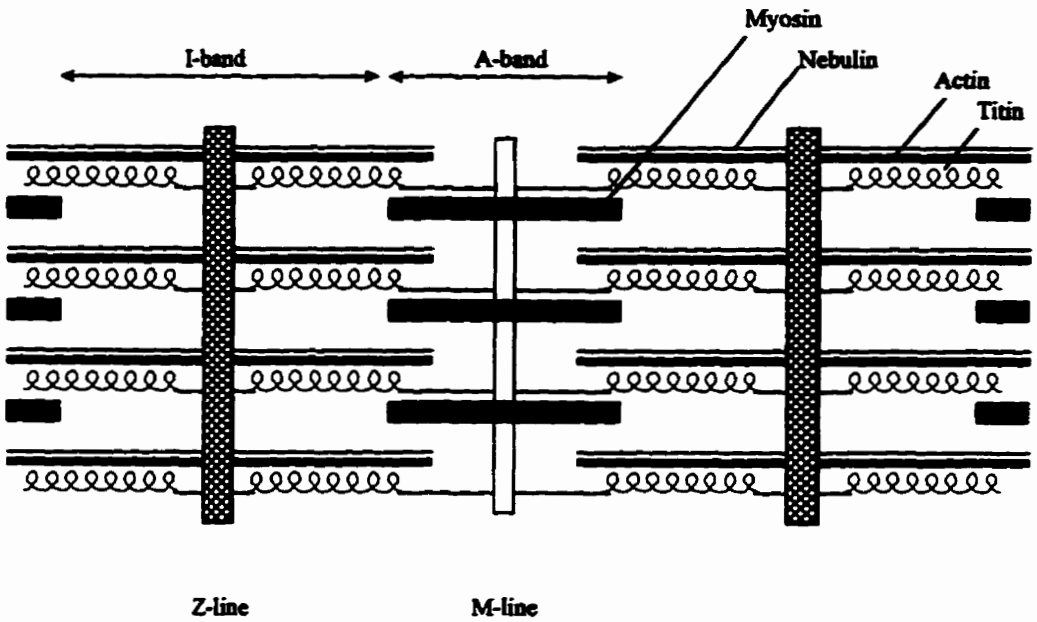


Figure 1.1. Schematic representation of the sarcomere cytoskeleton and contractile filaments in skeletal muscle.

Actin, tropomyosin and the troponin complexes form the thin filament, whereas myosin forms the thick filament in the sarcomere. Titin and nebulin form the third and fourth filaments, and function as the sarcomere cytoskeleton. Titin serves as an elastic connector spanning from M-line to Z-line. Its function is to keep the myosin filament centered in the sarcomere during muscle contraction and when the muscle is stretched. Nebulin, anchored at the Z-line, forms an inextensible filament that may act as a protein ruler and actin-binding template. (Based on Darnell *et al.*, 1990. Molecular Cell Biology 2nd ed. pp 859-902. Scientific American Books Inc, New York).

studies have shown that single titin molecules extend from the M-line to the Z-line in each half of the sarcomere (Fürst *et al.*, 1988; Itoh *et al.*, 1988). It also has been demonstrated that titin keeps myosin filaments centered in the sarcomere during muscle contraction (Wang, 1985; Horowits and Podolsky, 1987).

B. Smooth Muscles

In smooth muscles, the organization of the contractile elements is not clearly understood. The smooth muscle has spindle-shaped cells, each with a single nucleus. By electron microscopy, smooth muscle cells demonstrate three kinds of filaments, actin, myosin and desmin (or vimentin) (Small, 1995). Immunocytochemical analysis has revealed that smooth muscle cells contain multiple sets of two types of domains; contractile and cytoskeletal domains (Small, 1995). Different isoforms of actin have been found as components of both contractile and cytoskeletal domains. It was shown that β-cytoplasmic actin, a major non-muscle isoform of avian gizzard, codistributes with the intermediate filaments in the cytoskeletal domain, whereas γ-smooth muscle actin was found as a part of the contractile domain (North *et al.*, 1994).

B.1. Contractile Filaments in Smooth Muscle Cells

Actin and myosin contractile filaments occupy the majority of the cytoplasm of the cells. They slide relative to each other during muscle contraction in a manner similar to that of striated muscle (Small, 1995). They are distributed throughout the cells without the strictly ordered arrangement found in striated muscles (Alberts *et al.*, 1994; Small, 1995). Unlike

striated muscle, smooth muscle does not contain a developed sarcoplasmic reticulum membrane, and changes in the cytosolic Ca^{2+} level are much slower than that in striated muscle, allowing a slow, steady response in contractile tension (Darnell *et al.*, 1990). Smooth muscle contains tropomyosin but not troponin, and the Ca^{2+} regulation of contraction mainly involves a myosin light chain phosphorylation-dephosphorylation system. In the presence of Ca^{2+}, calmodulin-dependent myosin light chain kinase (MLCK) is activated, which in turn catalyses myosin phosphorylation, triggering cross-bridge cycling and the development of force or contraction of the smooth muscle (Walsh, 1994).

In 1981, Sobue *et al.* isolated a smooth muscle protein from chicken gizzard. This protein, with a molecular weight of 150 kDa as revealed by SDS-PAGE, bound to calmodulin and was named caldesmon. Caldesmon also bound to F-actin. The interaction of caldesmon with calmodulin was Ca^{2+} dependent, whereas the interaction of caldesmon with actin was not. The Ca^{2+} acts as a flip-flop switch between the formation of the two complexes (Sobue, 1981). Several studies have revealed that caldesmon binds to tropomyosin and myosin (Sobue *et al.*, 1982; Smith *et al.*, 1987; Ikebe and Reardon, 1988). Furthermore, in *in vivo* studies (Marston and Lehman, 1985; Fürst *et al.*, 1986), caldesmon is characterized as an integral component of the thin filament of the contractile apparatus of smooth muscle.

B.2. The Cytoskeleton of Smooth Muscle Cells

Desmin or intermediate filament forms the major constituent of the cytoskeleton. Intermediate filaments are grouped into bundles that run longitudinally along the cell axis and form lateral connections to the cell membrane (Small *et al.*, 1992; Small, 1995). Structural

studies suggest that a linkage between the cytoskeleton and the contractile apparatus occurs at the cytoplasmic dense bodies (Small *et al.*, 1992). Dense bodies are ovoid-shaped, electron-dense structures that are distributed relatively uniformly throughout the cell. They contain the actin cross-linking protein α-actinin that is also found in the Z-lines of the striated muscle. Dense bodies serve as filament anchorage sites that allow the contractile apparatus to exert force at the cell surface and produce contraction (Small *et al.*, 1992; Small, 1995).

Titin Structure and Function

Titin is a giant protein found as the third filament in addition to the thick and thin filaments in striated muscle. This 3 megadalton protein extends from the M-line into the Z-line in the cardiac sarcomeres. The cDNA sequence from human heart shows that 90% of titin contains 244 copies of 100 amino acid residues, encoding 132 fibronectin-like domains (class I) and 112 immunoglobulin C2-like domains (class II) (Labeit and Kolmerer, 1995). It was demonstrated that each domain possesses seven anti parallel β-sheet conformations (Erickson, 1994; Politou *et al.*, 1994). The M-line region of titin consists of a 200 kDa portion of the COOH-terminal region. This portion of the molecule is composed of a complex array of class II motifs, interspersed with unique titin sequence insertions (Small, 1995). About 200 kDa of the NH_2-terminal of titin enters the Z-line as shown by T12 anti-titin antibody epitope mapping (Labeit and Kolmerer, 1995).

In the A-band region, the two classes of titin domains form 11 super repeat domains, I-I-I-II-I-I-I-II-I-I-II, with a periodicity resembling the 43 nm cross bridge repeat of the thick filament (Trinick, 1991; Labeit *et al.*, 1992). The presence of 43 nm super repeat domains

suggests that the A-band region of titin binds to the thick filament. The binding to myosin predominantly involves the light meromyosin part of the molecule that forms the backbone of the thick filament. The A-band region of titin also binds other thick filament proteins, such as C-protein and X-protein, that are also composed of class I and class II motifs (Labeit *et al.*, 1992; Soteriou *et al.*, 1993; Trinick, 1994). It has been suggested that the A-band region of titin is likely to be an integral part of the thick filament (Whiting *et al.*, 1989). This A-band region is not stretchable within the physiological sarcomere length range (Itoh *et al.*, 1988; Fürst *et al.*, 1989; Whiting *et al.*, 1989). The function of titin in this region is possibly as a protein ruler to regulate myosin assembly (Whiting *et al.*, 1989).

The I-band region is composed mainly of tandem class II and non-modular regions rich in proline, hydrophobic and charged residues, termed the PEVK region (Fig.1.2) (Labeit and Kolmerer, 1995). Titin stretches without changing the thick filament structure when the sarcomere length is increased. This was shown by Funatsu *et al.* (1990), using electron microscopy of gelsolin-treated myofibrils. Trinick (1991) demonstrated that the titin epitopes in the I-band moved away from the Z-line and M-line as the sarcomere length was increased. It was then postulated that this I-band of titin forms an elastic connector between the thick filament and the Z-line (Fürst *et al.*, 1988; Itoh *et al.*, 1988). This elastic filament may function to keep the thick filament centered in the sarcomere. In addition, it may provide the passive tension to restore force when the muscle is stretched (Horowits and Podolsky, 1987; Wang, 1985). The latter is of particular importance in the myocardium. For example, this function determines to what extent the myocardium wall can be stretched during diastole thus enabling the myocardium to function more effectively (Trombitas *et al.*, 1995). It is thought

that the elasticity of the I-band titin is contributed by class II motifs and the PEVK region (Labeit and Kolmerer, 1995). Politou *et al.* (1996), using circular dichroism, fluorescence spectroscopy and nuclear magnetic resonance, demonstrated that immunoglobulin-like modules and module pairs which form the I-band were independently folded in solution and shared the same type of fold. It was demonstrated that the module-module interactions are very weak, but they behave as an autonomous cooperative unit upon unfolding (Politou *et al.*, 1996). Erickson (1994) showed that the mechanism of stretch involves the unravelling of the β-strands of the immunoglobulin-like domain initiated by peeling off of the weakest one. The A-I junction contains a complex domain composed of class I and class II motifs.

Using solid-phase binding assays, Soteriou *et al.* (1993) reported weak binding of titin to nitrocellulose-bound actin at an ionic strength of ~ 225 mM. It was also demonstrated by previous researchers that the T2 titin fragment (β-connectin) interacted with actin and myosin under physiological conditions (Kimura and Maruyama, 1983; Kimura *et al.*, 1984). Jin (1995), using an enzyme-linked immunosorbant (ELISA)-mediated solid phase binding assay, has demonstrated interactions between single class I, class II motifs and joined class I-II motifs of titin to F-actin. The concentration of the titin motifs required for 50% maximal binding to F-actin was 2.5×10^{-7} M for Ti I and 1.9×10^{-6} M for Ti II and 3×10^{-8} M for Ti I-II. The binding affinity of Ti I-II to F-actin was ~ 10 fold higher than Ti I. Co-sedimentation assays also showed stronger F-actin binding to Ti I-II than to Ti I in solution. The stoichiometry was 1 Ti I-II (at a concentration of 10 μM) to 2.6 F-actin and 1 Ti I (at a concentration of 40 μM) to 3.9 F-actin. No significant interaction between Ti II and F-actin was observed under these experimental conditions and the binding stoichiometry was 1 Ti II

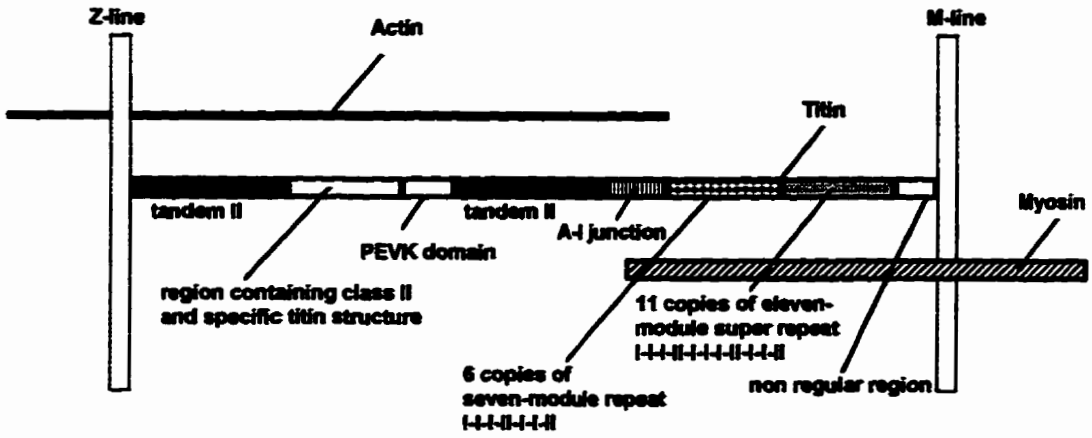


Figure 1.2. The domain structure of titin in the I-band, A-band and A-I junction. The I-band region of titin contains tandem immunoglobulin-type domains (class II motif) and a PEVK region that contribute to its elasticity. The A-band contains 6 copies of 7-module super repeats and 11 copies of 11-module super repeats containing class I and class II motifs that bind the thick filament and a non-regular region from the A-band entering the M-line. In the A-I junction, a complex domain containing class I and class II motifs of titin was demonstrated. (Based on Labeit and Kolmerer, 1995. Science 270, 293-296).

(at a concentration of 13 μM) to ~30 F-actin (Jin, 1995). Jin's experiment also demonstrated Ti I-II binding to myosin with a ~10 fold greater affinity than that of Ti I. The single class Ti II did not bind to myosin (Jin, 1995). Considering the tandem class II motif in the I-band, it is possible that the weak interaction between the titin class II motif and F-actin may be combined and may produce significant actin binding within this region (Jin, 1995). Using *in vitro* motility assay, Li *et al.* (1995) demonstrated that Ti I-II inhibits actin filament sliding on heavy meromyosin. The binding of Ti I-II to actin probably blocks the myosin-binding site of actin, decreasing the number of myosin heads that can bind to actin. This mechanism is similar to the effect of caldesmon binding to actin in the smooth muscle cell, blocking myosin binding to actin (Haeberle *et al.*, 1992). The inhibition of actin filament sliding by Ti I-II also suggests potential regulation of striated muscle contraction by titin, similar to the possible role of caldesmon on smooth muscle contraction. This hypothesis has yet to be investigated.

To investigate the function of the class II titin motif, a cloned fragment of rat cardiac titin class II motif (Ti II) has been constructed and monoclonal antibodies (mAbs) against Ti II have been raised (named Ti102 and Ti104 , Jin, 1995). Immunofluorescence microscopy, using Ti102 and Ti104 and using rhodamine-conjugated anti mouse IgG as a secondary antibody to stain cardiac myofibrils, showed Ti102 labelled the I-band whereas Ti104 did not label the cardiac myofibril. These results suggest that epitopes recognized by Ti102 and Ti104 are located in different sites of Ti II. The epitope recognized by Ti102 is located in the accessible site of Ti II, whereas the epitope recognized by Ti104 was probably covered by other protein that came in contact with Ti II in the intact myofibril. Considering the finding that the class II motif is prominent in the I-band, a region which consists mainly of actin

filaments, and that Ti II bound to F-actin but not to myosin, it has been proposed that the epitope recognized by Ti104 may be located in the actin-binding site of Ti II.

In preliminary studies, it has been demonstrated that Ti104 interacts with C-protein, a myosin-binding protein composed of class I and class II motifs similar to that of titin (Labeit *et al.*, 1992; Soteriou *et al.*, 1993; Trinick, 1994). Interestingly, Ti104 was also found to interact with caldesmon. The cross-reactivity of Ti104 to caldesmon suggests a structural similarity shared between titin and caldesmon. The interaction of Ti104 with caldesmon is a basic link to the hypothesis that, in striated muscle and smooth muscle/non-muscle cells, titin and caldesmon are analogous proteins.

Caldesmon Structure and Function

Caldesmon is a calmodulin-binding protein that was first isolated from chicken gizzard (Sobue *et al.*, 1981), and which is stable to heat and acid denaturation (Bretscher, 1984). The tissue content of caldesmon is variable. Tonic vascular smooth muscles have the lowest caldesmon content, whereas phasic smooth muscles have the highest (Haeberle *et al.*, 1992). In addition, caldesmon was also found in non-muscle cells (Bretscher and Lynch, 1985). Two isoforms of caldesmon have been found based on their mobilities on SDS-PAGE. h-Caldesmon has a molecular mass of 120-150 kDa and l-caldesmon has a molecular mass of 70-80 kDa (Sobue *et al.*, 1981; Bretscher, 1984; Owada *et al.*, 1984; Bretscher and Lynch, 1985; Sobue *et al.*, 1988). The molecular masses calculated from the deduced amino acid sequence of the full length cDNA from chicken smooth muscle/non-muscle tissues are in the range of 87-89 kDa and 59-60 kDa for h- and l-caldesmon respectively (Bryan *et al.*, 1989;

Hayashi *et al.*, 1989a; Bryan and Lee, 1991; Hayashi *et al.*, 1991). h-Caldesmon has been found in smooth muscle tissues such as chicken gizzard (Sobue *et al.*, 1981; Bretscher, 1984), rabbit uterus (Marston, 1989), stomach and trachea (Marston and Lehman, 1985); whereas l-caldesmon has been found in non-muscle tissues such as platelets (Kakiuchi *et al.*, 1983; Dingus *et al.*,1986), fibroblasts (Yamashiro *et al.*, 1991), erythrocytes (der Terrossian *et al.*, 1989) and many cultured cell lines (Owada *et al.*, 1984; Lin *et al.*, 1988). The two caldesmon isoforms have identical sequences in their NH_2- and COOH-terminal domains. They differ only in the central repeating domain that is absent in l-caldesmon (Fig. 1.3) (Ball and Kovala, 1988; Hayashi *et al.*,1991). Payne *et al.* (1995) reported that the two isoforms are generated from a single gene by alternative mRNA splicing. In the h-caldesmon transcript they found the presence of an exon, 3b, that is not present in l-caldesmon (Hayashi *et al.*,1992; Payne *et al.*, 1995).

Both h- and l-caldesmons have the same functional domains in their amino and carboxyl termini. The 35 kDa COOH-terminal chymotryptic fragment of caldesmon binds to calmodulin, tropomyosin and actin, and inhibits the actomyosin ATPase (Szpacenko and Dabrowska, 1986; Hayashi *et al.*, 1989b). A further study using cyanogen bromide (CNBr) cleavage revealed that a 10 kDa fragment from the COOH-terminal of caldesmon binds to actin and calmodulin (Bartegi *et al.*, 1990). Mezgueldi *et al.* (1994), using reverse-phase high performance liquid chromatography on chymotryptic caldesmon fragments, demonstrated that the essential determinants for both F-actin binding and inhibition of the actomyosin ATPase are located in the COOH-terminus between Leu693 and Trp722 (corresponding to the region including Leu495 to Trp524 in l-caldesmon). The function of the central repeating domain

of caldesmon is not known. This domain may serve as a spacer between the actin- and myosin-binding sites (Matsumura and Yamashiro, 1993). In non-muscle cells, actin and myosin serve both structural and contractile functions (Darnell *et al.*, 1990). It was suggested that caldesmon cross-links actin and myosin contractile filaments. It is possible that the binding of actin to myosin in the non-muscle cells is in a ratio of 1:1. Therefore, a shorter caldesmon molecule, without the central repeating domain, is sufficient to cross-link the actin to myosin. Whereas in smooth muscle cells, actin binds to several myosin molecules that form the thick filament. It has been suggested that a longer caldesmon molecule containing the central repeating domain is required to cross-link actin to all myosin molecules. CaD39 is a COOH-terminal human fibroblast caldesmon fragment (residues 244-538). This fragment was used in the experimental work in this study. CaD40 is a NH_2-terminal human fibroblast caldesmon fragment (residues 1-243). CaD40 was used to generate anti-caldesmon mAb, C57 (Warren *et al.*, 1994).

In the smooth muscle cells, it has been shown that caldesmon resides along with actin in the contractile domain (Fürst *et al.*, 1986), whereas in non-muscle cells, caldesmon is associated with actin and tropomyosin in stress fibres and membrane ruffles (Bretscher and Lynch, 1985; Dingus *et al.*, 1986; Lin *et al.*, 1988). Anti-caldesmon antibody has been used to study the structure of the thin filaments in the smooth muscle tissue. It was shown that native thin filament is made up of actin, tropomyosin and caldesmon at a ratio of 1 caldesmon molecule to 17 actin molecules (Lehman *et al.*, 1989). In non-muscle cells, microfilaments were extracted that were also composed of actin, tropomyosin and caldesmon (Ngai and Walsh, 1987; Yamashiro-Matsumura and Matsumura, 1988). It has been reported that in non-

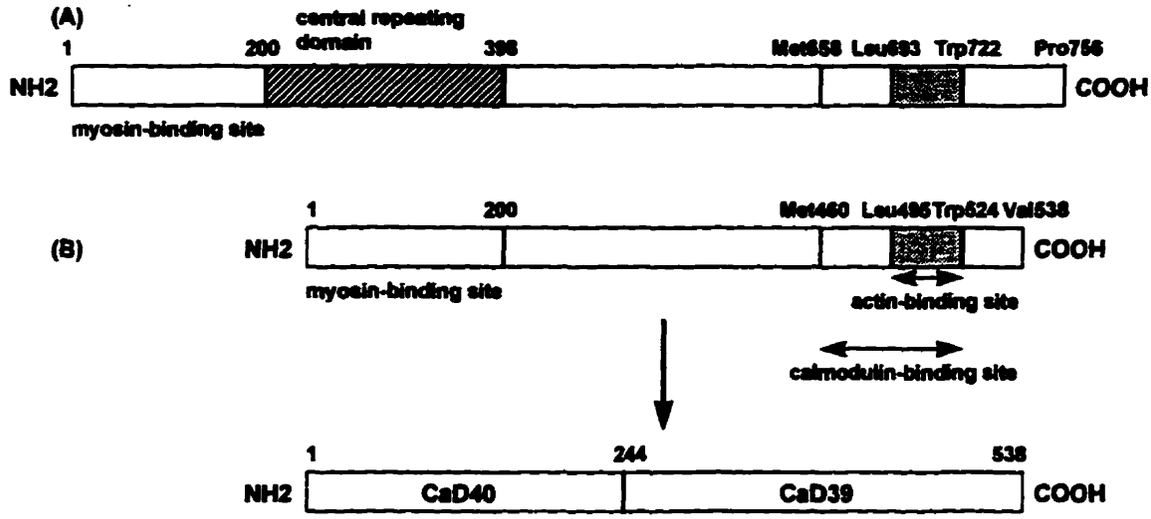


Figure 1.3. The domain structure of h- and l-caldesmon.

The cDNA sequence of chicken gizzard (h-caldesmon, A) (Bryan *et al.*, 1989) and human fibroblast caldesmon (l-caldesmon, B) (Novy *et al.*, 1991) showed identical sequence at their NH_2- and COOH-terminal domains. The l-caldesmon lacks the central repeating domain. The myosin-binding site is located in the 27 kDa NH_2-terminal domain whereas calmodulin- and actin-binding sites are located in the 10 kDa COOH-terminal domain (residues Met658-Trp722 in h-caldesmon corresponds to Met460-Trp524 in l-caldesmon). CaD39 is a COOH-terminal fragment of l-caldesmon (residues 244-538) and CaD40 is a NH_2-terminal fragment of l-caldesmon (residues 1-243). (Based on Sobue and Sellers, 1991. J. Biol. Chem. 266, 12115-12118; Warren *et al*, 1994. J. Cell Biol. 125, 359-368).

muscle, caldesmon binds to actin with a stoichiometry of 1 caldesmon to 9 actins (Yamashiro-Matsumura and Matsumura, 1988). Electron microscopy combined with antibodies were used to show the possible configuration of caldesmon in the thin filament. A polyclonal antibody against caldesmon was found to label the entire surface of the filaments (Marston *et al.*, 1988; Lehman *et al.*, 1989). It was then suggested that caldesmon binds to the thin filaments in an extended configuration (Marston and Redwood, 1991). Moody *et al.*(1990) using high resolution electron microscopy of native thin filament demonstrated elongated molecules following the long-pitch helices with no evidence of lateral projections. This suggested that caldesmon and tropomyosin lie side by side in the thin filament. Caldesmon has no significant sequence similarities to any currently known actin-binding protein (Marston and Redwood, 1991). Nonetheless, it binds to the same region near the NH_2-terminal of actin as do many other actin-binding proteins (Marston and Redwood, 1991).

It has been shown that interaction of calmodulin with the COOH-terminal of h-caldesmon occurs at three sites, A (residues 658-666), B (residues 687-695) and B' (residues 717-725), each of which contains a Trp residue (Marston *et al.*, 1994; Mezgueldi *et al.*, 1994). Mutation of each Trp residue to Ala has been carried out and the effect on calmodulin-binding has been studied by fluorescence measurements and using immobilized calmodulin. The results show that Trp659 and Trp692 play a much greater role in binding to calmodulin than Trp722 does (Graether *et al.*, 1997). The binding of caldesmon to actin and calmodulin is termed as a "flip-flop" mechanism depending upon the free Ca^{2+} concentration. Caldesmon interacts with calmodulin in a Ca^{2+}-dependent manner and with actin, tropomyosin and myosin in Ca^{2+}-independent manner (Sobue *et al.*, 1981; Bretscher, 1984; Sobue *et al.*, 1985; Fürst

et al., 1986).

A 27 kDa NH_2-terminal fragment of caldesmon binds to myosin. The affinity of this interaction is weakened in the presence of Ca^{2+}/calmodulin (Katayama *et al.*, 1989; Sutherland and Walsh, 1989; Hemric, 1990; Velaz *et al.*, 1990; Marston and Redwood, 1991). This binding occurs predominantly through the S-2 region of myosin (Ikebe and Reardon, 1988; Hemric and Chalovich, 1990). Like the interaction of caldesmon with actin, caldesmon-myosin interactions are reversed by the binding of caldesmon to calmodulin (Hemric *et al.*, 1993). According to Marston and Redwood (1991), the ability of caldesmon to cross-link actin and myosin may be of physiological significance in maintaining the correct orientation and spatial relationship between actin and myosin and to enable the contractile filaments to perform efficient contraction upon stimulation. Caldesmon's ability to cross-link actin and myosin suggests it has a cytoskeletal role in organizing smooth muscle and non-muscle contractile elements.

A potential role of caldesmon in the regulation of smooth muscle contraction has been proposed. *In vitro* studies of caldesmon have revealed that caldesmon inhibits the actin-activated Mg^{2+} ATPase activity of myosin without affecting myosin phosphorylation (Ngai and Walsh, 1984; Marston and Lehman, 1985; Sobue *et al.*, 1985). This property of caldesmon is central to the Ca^{2+}-dependent control of thin filament activity (Marston *et al.*, 1988) and the effect of caldesmon on contractility (Taggart and Marston, 1988). The presence of tropomyosin increases the potency of inhibition (Dabrowska *et al.*, 1985; Moody *et al.*, 1985; Smith *et al.*, 1987). This inhibitory mechanism involves a competition between caldesmon and myosin for binding to actin (Hemric and Chalovich, 1988; Velaz *et al.*, 1990),

and is reversed by caldesmon binding to calmodulin in the presence of Ca^{2+}.

Several studies showed that phosphorylation of caldesmon also inhibits the interaction of caldesmon with actin (Ngai and Walsh, 1984; Yamashiro *et al.*, 1991) and with myosin (Sutherland and Walsh, 1989; Hemric *et al.*, 1991). Caldesmon has been shown to be a substrate of several protein serine/threonine kinases in *in vitro* experiments. The phosphorylation sites are clustered within the myosin-binding and actin-binding domains of caldesmon (Walsh, 1994). Phosphorylation at sites within the COOH-terminal domain by calmodulin kinase II reduces the ability of caldesmon to inhibit actomyosin ATPase (Walsh, 1994).

Two mAbs, C21 and C23, raised against chicken gizzard caldesmon were produced. C21 has been demonstrated to cross-react to non-muscle caldesmons from a variety of species (Lin *et al.*, 1988). Epitope mapping of C21 indicates that it recognizes a 10 kDa fragment of the COOH-terminal domain of caldesmon. Solid-phase actin-binding assays demonstrated that C21 competitively inhibits the binding of caldesmon to F-actin or F-actin-tropomyosin. These findings demonstrate that the epitopes recognized by C21 are within the F-actin binding domain of caldesmon. Epitope mapping of C23 showed the epitope it recognizes is localized to the region between residues 230 to 446 of gizzard caldesmon (Lin *et al.*, 1991). Monoclonal antibodies against human fibroblast caldesmons have been produced, named C94 and C98. The epitope mapping and characterization of these mAbs have not been yet determined.

A monoclonal antibody is a homogenous antibody that responds specifically to a single epitope (Goding, 1995). Monoclonal antibodies are useful to identify and characterize

conformational changes of the antigen (Goldberg, 1991). The production of mAbs was carried out using hybridoma cell culture following fusion of the spleen cells of a mouse injected with a certain antigen and mouse myelome cells. The latter contribute to long term survival of the hybridoma cells in the culture medium (Goding, 1995). Single clones were obtained by diluting the hybridoma cells in the culture medium.

Hypothesis

In reviewing titin and caldesmon structure and function, some important features are highlighted:

1). Titin and caldesmon have been shown to interact with actin and myosin contractile filaments. Titin binds to myosin in its A-band region. A single titin class II motif fragment was shown to weakly interact with F-actin. However, the tandem repeat of titin class II motifs that is present in the I-band region of titin suggests that a combined interaction may produce a higher avidity of actin binding in that region. Caldesmon has been shown to interact with actin at its COOH-terminal domain and with myosin at its NH_2-terminal domain.

2). Titin's mechanical function is well established. Titin has been shown to serve as an elastic connector between the thick filament and the Z-line; and to keep the thick filament centered in the sarcomere. It is also responsible for the passive tension of the muscle and restores force when muscle is stretched.

3). The regulation of caldesmon-actin interactions has been extensively studied. Several protein kinases have been shown to phosphorylate the actin-binding site of caldesmon.

The features found in titin and caldesmon raise the following questions:

1). Are titin and caldesmon analogous proteins?

2). Is the titin-actin interaction regulated in the same way as the caldesmon-actin interaction?

3). Does caldesmon play a role in organizing smooth muscle and non-muscle cell contractile elements as titin does in striated muscle?

Cross-reactivity of the anti-titin mAb Ti104 to caldesmon indicates that titin and caldesmon have a convergent structure. It suggests that titin and caldesmon may have analogous function.

Objectives

The objectives of this study are to investigate the possibility that titin and caldesmon are analogous proteins in striated muscle and smooth muscle/non-muscle cells, based on the fact that titin and caldesmon share a similar epitope recognized by Ti104.

CHAPTER TWO:

CHARACTERIZATION OF ANTI-TITIN AND ANTI-CALDESMON MONOCLONAL ANTIBODIES

INTRODUCTION

Anti-titin and anti-caldesmon mAbs were used in all experiments in this research project. Before using the mAbs in each experiment, characterization of each mAb needed to be carried out. Using Western blotting, anti-titin mAbs, Ti102, Ti104, Ti106 and Ti108 were characterized and their specificities to titin or titin fragments were investigated. Anti-caldesmon mAbs, C21, C23 and C57, were also characterized and their specificities to caldesmon were investigated.

In our laboratory, several anti-titin mAbs have been raised against cloned rat cardiac titin class II motifs (Ti II) using hybridoma technology. Four different hybridoma cell lines were obtained which secreted mAbs specific for the antigen, named Ti102, Ti104 (Jin, 1995), Ti106 and Ti108. Ti102, Ti104 and Ti106 are of the IgG1 subclass. The subclass of Ti108 was not conclusively determined. Those mAbs were used in Western blotting to probe several titin motif fragments containing Ti I, Ti II or joined combinations of both motifs.

Anti-caldesmon mAbs, C21 and C23, were raised against chicken gizzard caldesmon (Lin *et al.*, 1988). The epitope mapping of C21 showed the epitope localized to the actin-binding site of caldesmon in the 10 kDa COOH-terminal domain, whereas epitope mapping of C23 indicated a localization to the central repeating domain of h-caldesmon (Lin *et al.*, 1991). C57 was raised against CaD40, a NH_2-terminal human fibroblast caldesmon fragment

(Warren *et al.*, 1994). The isotyping of the mAbs are IgG1 for C21 and C57, and IgM for C23 (Warren *et al.*, 1994).

The characterization of anti-titin and anti-caldesmon mAbs using Western blotting showed anti-Ti II mAbs Ti102, Ti104,Ti106 and Ti108 were specific for fragments containing the titin class II motif, and anti-caldesmon mAbs C21,C23 and C57 were specific for h-caldesmon in various smooth muscle tissues.

MATERIALS AND METHODS

A. Protein Samples of Titin Fragments and Caldesmon

The samples of titin protein fragments which were used in these experiments were purified from bacterially-expressed rat cardiac titin class I or class II cDNA or from cDNA's coding for a combination of these motifs. The construction of single class Ti I, Ti II cDNA's, and cDNA's in which these motifs are combined, was described by Jin (1995). Samples of titin protein fragments containing a single class Ti I motif or a combination of Ti I and Ti II motifs were available in the laboratory. The samples of single class Ti II motif protein fragments were obtained by expressing cloned rat cardiac Ti II in *E. coli* and the purification of the expressed protein from *E. coli* was carried out as described in Jin (1995).

Protein extracts from samples of smooth muscle from fish vein, fish stomach, frog stomach, turtle blood vessel, turtle stomach, chicken aorta, chicken gizzard, rabbit aorta, bovine stomach, rat aorta, rat stomach and mouse stomach were used to document the caldesmon expression pattern. One hundred miligram of fresh frozen tissue from each source was homogenized in 1 mL of 3x sample buffer containing 150 mM Tris-HCl pH 6.8, 6% w/v

SDS, 0.3% w/v bromophenol blue, 30% v/v glycerol. β-mercaptoethanol was added to 3% v/v prior to use. The samples were heated to 80°C for 10 min, and centrifuged at 14,000 rpm in a table-top microcentrifuge for 5 min at 4°C. Supernatants containing crude proteins were used for SDS-PAGE. Samples were stored at -70°C when not in use.

B. Expression and Purification of a Cloned Rat Cardiac Titin Class II Motif

The calculated pI of the Ti II motif from its amino acid sequence was 9.35 with a predicted molecular weight of 10.900 kDa. Protein expression and purification was carried out as follows. The Ti II cDNA was cloned into a pET vector plasmid and was transformed into the *E. coli* strain BL21(DE3)pLysS and plated on Luria-Bertani (LB) agar pH 7.3 (10 g tryptone, 5 g yeast extract, and 5 g NaCl in 1 L medium), containing 100 μg/mL ampicillin and 25 μg/mL chloramphenicol. Cells were incubated overnight at 37°C. A single colony was taken and suspended in 500 μL LB medium and vortexed to obtain a homogenous suspension. One L of 2x TY medium pH 7.3 (16 g tryptone, 10 g yeast extract, 5 g NaCl, and 2.5 g disodium phosphate in 1 L), containing 100 μg/mL ampicillin and 25 μg/mL chloramphenicol was inoculated with 125 μL of the suspension. A total of 4 L of 2x TY medium was inoculated with the bacteria. The culture was incubated at 37°C with vigorous shaking (250 rpm) for 10 hours until $OD_{600\ nm}$ = 0.832. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to 0.4 mM and the culture was incubated for another 3 hours. The bacterial cells were collected by centrifugation at 3,500 rpm at 4°C for 15 min in a table-top clinical centrifuge and resuspended in 35 mL of 50 mM Tris-HCl pH 8 and 2.5 mM EDTA. Lysate from the cells was obtained following three passages through a French

cell press at 500-1,000 p.s.i. The lysate was diluted to 200 mL with the same buffer. Ammonium sulfate fractionation was carried out at 50% saturation for 30 min at 4°C, and the lysates were centrifuged in a JA14 rotor at 10,000 rpm (4°C) for 10 min. The supernatant was then subjected to 80% ammonium sulfate precipitation for 30 min at 4°C. The precipitate from this fractionation was dissolved in 10 mL of 0.1 mM EDTA and dialysed against two changes of 4 L of 0.1 mM EDTA pH 8. After dialysis, the volume was adjusted to 100 mL with the addition of double-distilled water (ddH_2O), and the pH was adjusted to 4.3 using 1 M sodium acetate (pH 4.3). The solution was centrifuged at 10,000 rpm, 4°C in a JA14 rotor for 15 min. The supernatant was loaded on a 49 mL CM-52 cation-exchange column (2.5 x 10 cm) equilibrated in 10 mM sodium acetate (pH 4.3) containing 0.1 mM EDTA. After washing with 100 mL of equilibration buffer, the column was eluted at 1 mL/min with a linear gradient of 0-300 mM KCl in the same buffer for a total volume of 300 mL. Elution of proteins from the column was determined by measurement of absorbance at 280 nm. Fractions containing protein peaks were resolved by 15% w/v SDS-PAGE with an acrylamide:bisacrylamide ratio of 29:1 and with a 4% w/v stacking gel (Laemmli, 1970). Fractions containing Ti II (assay described below) were collected and lyophilized. To remove contaminating high molecular weight proteins, the lyophilized protein was dissolved in 3-5 mL of a buffer containing 6 M urea, 10 mM imidazole pH 7, 0.1 mM EGTA and 6 mM β–mercaptoethanol. The solution was centrifuged at 10,000 rpm, 4°C for 10 min in a JA20 rotor. The supernatant was loaded onto a 589 mL G-75 column (2.5 x 120 cm) equilibrated in the same buffer. The column was eluted with the equilibration buffer at 0.5 mL/min and fractions containing the $A_{280\ nm}$ peaks were analyzed by 15% w/v SDS-PAGE. Fractions

containing pure Ti II protein were collected and dialysed against 3 changes of 2 L of 0.5% v/v formic acid. The purified protein was lyophilized and stored at -20°C.

C. Characterization of the mAbs on Titin Motif Fragments and Caldesmon Samples

Samples of Ti I, Ti I-I, Ti I-II, Ti I-II-I, Ti II, Ti II-I and Ti II-II protein fragments were resolved by small pore 4-16.7% w/v gradient SDS-PAGE with an acrylamide:bisacrylamide ratio of 19:1 (Schaegger and von Jagow, 1987). Crude protein extracts of smooth muscle samples were resolved by 10% w/v SDS-PAGE with an acrylamide:bisacrylamide ratio of 29:1 (0.75 mm thick). A representative SDS-PAGE gel containing titin samples and one containing caldesmon samples were stained in 0.25% w/v Coomassie Brilliant Blue R-250 (ICN) for 1 hour and destained in 7% v/v methanol/7% v/v acetic acid for 2 hours to reveal the protein profiles. Identical copies of these SDS-PAGE gels were processed for Western blotting.

The titin protein fragments which had been resolved by SDS-PAGE were transferred onto 0.2 μm nitrocellulose membranes (Schleicher & Schuell) using a current of 5 mA/cm^2 gel for 30 min in a Transblot semi dry transfer cell (Bio-Rad). The small pore nitrocellulose was used to allow efficient retention of small protein fragments. The protein extracts of smooth muscle samples were transferred onto 0.45 μm nitrocellulose (Trans-blot, Bio-Rad). To assess the efficiency of the transfer, a representative nitrocellulose membrane was stained in 0.2% w/v amido black for 2 min and destained in 50% v/v methanol/5% v/v acetic acid for 5 min. Other membranes were soaked in 1% w/v bovine serum albumin in tris-buffered saline blocking solution (BSA/1x TBS pH 7.4, 80 gram NaCl, 2 gram KCl, and 30 gram Tris-base

in 1 L of 10x) for 1 hour at room temperature.

The mAbs used in Western blotting were obtained from supernatants of the hybridoma cells or from the ascites fluids of BALB/c mice injected with hybridoma cells. Immunoblotting using the anti-Ti II mAbs on the titin motif fragments was carried out using a 1/50 dilution of the Ti102 hybridoma supernatant, a 1/2,000 dilution of Ti104, a 1/50 dilution of Ti106 hybridoma supernatant or a 10^{-5} dilution of Ti108. The nitrocellulose membranes were incubated with the primary antibodies overnight at 4°C on a horizontal platform shaker. Incubation with the secondary antibody, an alkaline phosphatase (AP)-labelled goat anti mouse immunoglobulins (mixed IgG and IgM, Sigma), was carried out for 1 hour at room temperature using a 1/4,000 dilution. Blots were washed with 1x TBS for 10 min, 3 changes of TBS + 0.5% v/v Triton-X 100 + 0.05% w/v SDS for 10 minutes each and two changes of 1x TBS for 5 min each. To develop the blot, the membranes were incubated with 165 µL of 5% w/v nitro blue tetrazolium (NBT) and 82.5 µL of 5% w/v 5-bromo-4-chloro-3 indolyl phosphate (BCIP) mixed in 25 mL substrate buffer 10 mM NaCl, 5 mM $MgCl_2$ and 100 mM Tris, pH 9.5, for 20 min.

Immunoblotting of protein extracts of smooth muscle tissues was carried out using a 1/500 dilution of C21, a 10^{-4} dilution of C23 or a 10^{-3} dilution of C57 with overnight incubation on horizontal platform shaker at 4°C. The secondary antibody was 0.5 µCi/mL ^{125}I-labelled sheep anti mouse IgG (total of 5 µCi) which was incubated with the blot for 1 hour at room temperature. Unbound antibodies were washed as described above. Nitrocellulose membranes were exposed to X-ray film with intensifying screen for 2 days at -70°C.

RESULTS

A. Expression and Purification of Ti II, a Cloned Rat Cardiac Titin Class II Motif

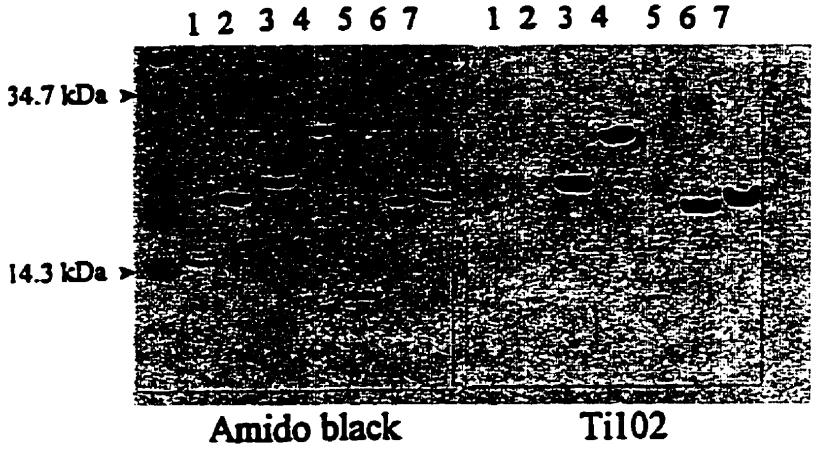
Samples from every step in the purification method were analyzed by 15% w/v SDS-PAGE. All samples from the steps before the CM-52 column contained contaminating bacterial proteins. The fractions eluted from the CM-52 column gave a single absorbance peak at $A_{280\,nm}$. Analysis of the fractions around the peak showed some high molecular weight contaminating proteins (fractions 47-61). Those samples were collected and further purified by G-75 gel filtration. After G-75 gel filtration, the fractions around the $A_{280\,nm}$ peak (fractions 14-30) showed pure Ti II protein in fractions 21-30, and Ti II with a few contaminating of higher molecular weight proteins in fractions 14-20. The molecular weight of the expressed protein was consistent with that calculated from the cDNA sequence. The pure Ti II was collected separately from the impure Ti II. As the final result, purification of 4 L of culture of cloned rat cardiac Ti II using cation-exchange and gel filtration chromatography yielded 74.2 mg of pure protein and 27.5 mg of impure protein. The pure Ti II was used in the following experiment. Although some contaminating proteins were present in some fractions after G-75 gel filtration, the purification method applied for obtaining Ti II was effective in yielding a large amount of pure protein. The important step in this method was to prepare a large volume of culture (4 L) to obtain a large amount of expressed protein.

B. Characterization of the mAbs on Titin Motif Fragments and h-Caldesmon Samples

The immunoblotting using anti-Ti II mAbs, Ti102, Ti104, Ti106 or Ti108 on different titin fragments showed all of the mAbs interacted with Ti II. The anti-Ti II mAbs did not

Figure 2.1. Reactivities of anti-Ti II mAbs to Ti I, Ti II, or combination of Ti I and Ti II fragments.

The titin fragment samples were loaded as follows: 1, Ti I; 2, Ti I-I; 3, Ti I-II; 4, Ti I-II-I; 5, Ti II; 6, Ti II-I; 7, Ti II-II. The primary antibodies were Ti102, Ti104, Ti106 and Ti108. The secondary antibody was AP-labelled goat anti mouse immunoglobulins (IgG and IgM, Sigma). The blot was developed using NBT and BCIP substrate. Anti-Ti II mAbs, Ti102, Ti104, Ti106 and Ti108 reacted against fragments containing the Ti II motif (lanes 3-7). Fragments containing only Ti I (lanes 1 and 2) were not recognized by any of the mAbs. This result shows the specificity of Ti102, Ti104, Ti106 and Ti108 for the titin class II motif which is the immunoglobulin-type domain of titin.


1 2 3 4 5 6 7 1 2 3 4 5 6 7
34.7 kDa
14.3 kDa
Amido black
Ti102

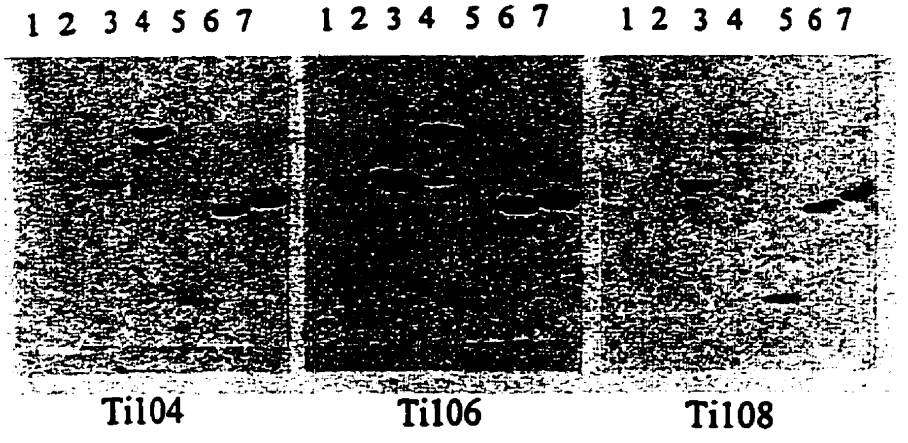
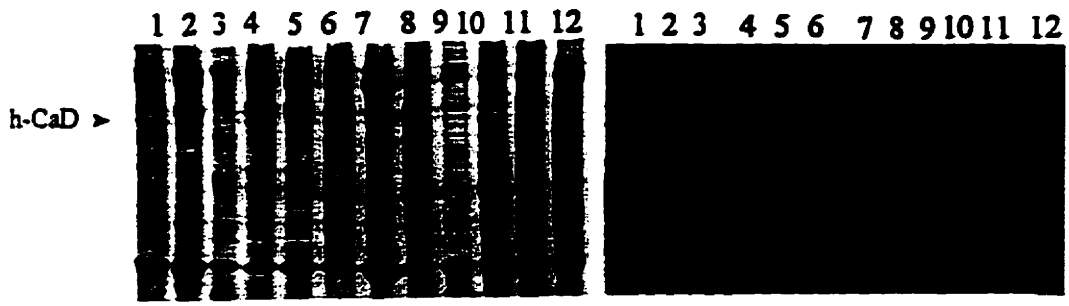

1 2 3 4 5 6 7 1 2 3 4 5 6 7 1 2 3 4 5 6 7
Ti104
Ti106
Ti108

Figure 2.2. Reactivities of anti-caldesmon mAbs to caldesmon in the protein extracts of smooth muscle samples.

The loading pattern of the samples is: 1, fish vein; 2, fish stomach; 3, frog stomach; 4, turtle vessel; 5, turtle stomach; 6, chicken aorta; 7, chicken gizzard; 8, rabbit aorta; 9, bovine stomach; 10, rat aorta; 11, rat stomach; 12, mouse stomach. The primary antibodies were C21, C23 and C57. The secondary antibody was ^{125}I-labelled sheep anti mouse IgG. Autoradiography was performed using an intensifying screen for 2 days at -70°C. Anti-caldesmon mAbs, C21, C23 and C57 showed reactivities to caldesmon in chicken gizzard (7), bovine stomach (9), rat stomach (11) and mouse stomach (12). C57 showed very weak interaction with both rat stomach and mouse stomach caldesmons. In addition, C23 interacted with frog stomach caldesmon (3) very strongly, and C57 interacted with turtle stomach caldesmon (5).



SDS-PAGE C21

C23 C57

show any reactivity to fragments containing only the Ti I. The mAbs showed strong reactivities to fragments containing Ti II, a joined combination of Ti I and Ti II or Ti II and Ti II, and weaker reactivities to the single Ti II fragment (Fig. 2.1).

Immunoblotting of C21 against smooth muscle protein extracts which had been separated by SDS-PAGE showed its reacted against chicken gizzard, bovine stomach, rat stomach and mouse stomach caldesmons. C23 reacted against frog stomach and chicken gizzard caldesmons very strongly and against bovine stomach, rat stomach and mouse stomach caldesmons. C57 reacted against frog stomach, chicken gizzard, bovine stomach, rat stomach and mouse stomach caldesmons (Fig. 2.2).

DISCUSSION

Several monoclonal antibodies against titin and caldesmon have been characterized and their specificities have been investigated. The result in Fig. 2.1 showed anti-Ti II mAbs Ti102, Ti104, Ti106 and Ti108 were specific for the titin class II motif. However, it could not be discounted that the antibodies might recognize different surface sites on the Ti II protein, as those mAbs were produced by different hybridoma cell lines. It is therefore very possible that each mAb interacts with a different specific surface site on the antigen although there is also a possibility that some mAbs interact with the same surface site, and thus, recognize the same epitope. Epitope mapping of Ti102, Ti104, Ti106 and Ti108 could be done by cleaving the Ti II into smaller protein fragments using other proteolytic or chemical methods, characterizing the fragments by Western blotting. The mAbs interact weakly with single Ti II fragments in comparison with their reactivities to the multimerized Ti II fragments. This

result indicates that Ti II, when combined with either a Ti I or another Ti II, forms more defined epitopes than a single Ti II, in that they are recognized better by the mAbs.

Anti-caldesmon mAbs C21 and C23 were shown to be specific for chicken gizzard caldesmon. However, their epitope localization on h-caldesmon is different. Lin *et al.*(1991) have demonstrated that the epitope which C21 reacts against is localized to the actin-binding site, in a 10 kDa COOH-terminal caldesmon CNBr fragment. Epitope mapping of C23 showed it reacted to an epitope which was localized within the central domain of caldesmon. C21 also recognizes caldesmon from bovine stomach, rat stomach and mouse stomach. This result is consistent with the result of Lin *et al.* (1991) that demonstrated that C21 reacted broadly against caldesmon from different tissues from various species. C23 and C57 also showed broad reactivities against caldesmon from different sources. The reactivities of C21, C23 and C57 to caldesmon in chicken gizzard, bovine stomach, rat stomach and mouse stomach suggest possible structural similarities which could be shared among h-caldesmon in these tissues.

In conclusion, anti-Ti II mAbs, Ti102, Ti104, Ti106 and Ti108 have been shown to interact specifically with Ti II, a titin class II motif. Anti-caldesmon mAbs, C21, C23, and C57 have been demonstrated to interact with h-caldesmon in various smooth muscle tissues.

CHAPTER THREE:

SPECIFIC CROSS-REACTIVITY AND EPITOPE MAPPING OF ANTI-TITIN AND ANTI-CALDESMON MONOCLONAL ANTIBODIES

INTRODUCTION

The purpose of the experiments described in this chapter was to further investigate the structural similarity found between titin and caldesmon by analyzing the cross-reactivity of anti-Ti II mAbs to caldesmon, and cross-reactivity of anti-caldesmon mAbs to titin or to titin motif fragments using Western blotting. The level of structural similarity between titin and caldesmon was investigated by comparing the amino acid sequence of the smallest chymotryptic fragment of Ti II to the CNBr fragment of caldesmon that was still recognized by both anti-Ti II and anti-caldesmon mAbs. The possible location of the similar structure was also investigated.

In preliminary studies, an anti-Ti II mAb, Ti104, was demonstrated to cross-react to C-protein. C-protein is a myosin-binding protein found in striated muscle (Moos *et al.*, 1975; Starr and Offer, 1978). Its position along the A-band has been characterized (Craig and Offer, 1976; Dennis *et al.*, 1984; Bennett *et al.*, 1986). Like titin, C-protein belongs to a superfamily of protein which shares sequence similarity to fibronectin type III and immunoglobulin C2 domains (Einheber and Fischman, 1990). This protein exists as multiple isoforms in striated muscles of various fiber types (Callaway and Bechtel, 1981; Reinach *et al.*, 1982). Fürst *et al.*, (1992) proposed that C-protein could connect the missing link of the titin strings at multiple sites of the A-band.

The cross-reactivity of anti-Ti II mAb to C-protein was studied. To confirm the positive binding of anti-Ti II mAb to C-protein, anti C-protein mAbs, MF1 and ALD66, were used as controls. Cross-reactivities of anti-Ti II mAb to h-caldesmon and anti-caldesmon mAbs to titin or to titin motif fragments were also investigated. Epitope mapping of anti-Ti II mAbs, Ti102, Ti104, Ti106 and Ti108 was carried out on Ti II chymotryptic fragments. Epitope mapping of anti-caldesmon C94 was carried out on caldesmon CNBr fragments.

The experimental results indicate a structural similarity between the immunoglobulin-type domain of titin and the actin-binding site of caldesmon. This structural similarity was found to be in their three dimensional structures. The result suggests an analogous function of titin and caldesmon. Epitope mapping of Ti102, Ti104, Ti106 and Ti108 on the Ti II chymotryptic fragments did not specify their epitope localizations. Epitope mapping of C94 showed the epitope localized to the actin-binding site of caldesmon.

MATERIALS AND METHODS

A. Protein Samples of Titin, Titin Motif Fragments and Caldesmon

Titin samples were obtained from crude protein extracts of various skeletal and cardiac muscle tissues. Caldesmon samples were obtained from crude protein extracts of various smooth muscle tissues. Other caldesmon sample was purified from chicken gizzard. Titin motif fragments, a single Ti I motif or coupled Ti I motifs and combinations of Ti I and Ti II motifs were available in the laboratory. Large quantities of Ti II motif fragments were obtained by purification of bacterially-expressed protein from cloned rat cardiac titin class II motif cDNA as described in the Materials and Methods B section of Chapter 2. Small

fragments of the Ti II motif and caldesmon were obtained by digestion of the intact Ti II fragment using α–chymotrypsin and of caldesmon using CNBr. The COOH-terminal caldesmon synthetic peptide was a gift from Dr. A.S. Mak, Queen's University. The molecular weight of the synthetic peptide is ~ 6.4 kDa and its amino acid sequence is N S M W E K G N V F S S P G G T G T P N K E T A G L K V G U S S R I N E W L T K T P E G N K S P A P K S P D L C, similar to that of l-caldesmon residues 456-512.

B. Monoclonal Antibodies

The cross-reactivity of anti-Ti II mAb Ti104 to C-protein was investigated. To confirm the positive binding of anti-Ti II mAb to C-protein, anti C-protein mAbs, MF1 and ALD66, were used as controls. MF1 was raised using a crude myosin preparation from chicken pectoralis major as the immunogen. ALD66 was raised using a crude myosin preparation from chicken anterior latissimus dorsi as the immunogen (Reinach *et al.*, 1982). Both mAbs, MF1 and ALD66, were observed to interact with C-protein of chicken posterior latissimus dorsi muscle (Reinach *et al.*, 1982). The immunoglobulin subclass of these mAbs was not described. MF1 and ALD66 used in this experiment were hybridoma supernatant, gifts from Dr. D.A. Fischman, Cornell University Medical College, New York, USA.

Cross-reactivity of Ti104 to h-caldesmon of various smooth muscle samples was studied. Anti-caldesmon mAbs, C21, C23 (Lin *et al.*, 1988) and C57 (Warren *et al.*, 1994) were used to confirm the positive binding of Ti104 to h-caldesmon. Cross-reactivity of anti-caldesmon mAbs, C21, C94 and C98 to titin motif fragments was also studied. The epitope localization of C94 and C98 are not yet determined. C94 and C98 were gifts from Dr. J.J.-C.

Lin, University of Iowa, Iowa City, USA.

To investigate the cross-reactivity of the anti-caldesmon mAb to titin, C21 was used to probe titin from various skeletal and cardiac muscle samples, and Ti104 was used to confirm the positive binding of C21 to titin.

C. Obtaining Titin from Protein Extracts of Skeletal and Cardiac Muscles, and Caldesmon from Protein Extracts of Smooth Muscle

Cardiac and skeletal muscle tissues were taken from frog, turtle, chicken, bovine, rat and mouse. Smooth muscle samples were taken from fish vein, fish stomach, frog stomach, turtle blood vessel, turtle stomach, chicken aorta, chicken gizzard, rabbit aorta, bovine stomach, rat aorta, rat stomach and mouse stomach as also described in the Material and Methods A section of Chapter 2. The protein extracts of muscle tissues were obtained by homogenizing 0.1 gram fresh frozen tissues in 1 mL of 3x sample buffer. After the samples were heated to 80°C for 10 min, they were centrifuged at 4°C for 5 min at 14,000 rpm in a micro centrifuge. The supernatant was used as gel samples and stored at -70°C to prevent protein degradation.

D. Purification of Caldesmon from Chicken Gizzard

Chicken gizzard (100 g) was cut into small pieces and boiled in 50 mM imidazole pH 7.0, 0.3 M KCl and 1 mM EGTA. After cooling on ice for 30 min, PMSF (0.5 mM), DTT (1 mM), leupeptin (10 μg/mL) and 1 mM sodium tetrathionate were added. The heat-treated chicken gizzard tissue was homogenized using a high-speed blender for 1 min, then

centrifuged in a JA14 rotor at 14,000 rpm, 4°C for 20 min. Into the supernatant, ammonium sulfate was added to 30% saturation, the reaction was stirred on ice for 30 min and spun in a JA14 at 14,000 rpm, 4°C for 25 min. The second step precipitation was carried out by adding ammonium sulfate to the supernatant to 50% saturation. After stirring on ice for 30 min, the solution was centrifuged in a JA14 rotor at 14,000 rpm, 4°C for 25 min. The precipitate was then dissolved in 20 mM sodium acetate pH 5.6, 0.1 mM EGTA and 6 mM β-mercaptoethanol, and dialysed against 2 changes of 2 L of the same buffer. After dialysis, urea (6 M) and β-mercaptoethanol (6 mM) were added and the solution was centrifuged in a JA17 rotor at 15,000 rpm, 4°C for 25 min. The supernatant was loaded on a 147 mL CM-52 cation-exchange column (2.5 x 30 cm) in a buffer containing 6 M urea, 20 mM sodium acetate pH 5.6, 0.1 mM EGTA and 6 mM β-mercaptoethanol, and eluted by a linear gradient of 0-300 mM KCl. The fractions revealed in the $A_{280\,nm}$ peak were analyzed by 12% w/v SDS-PAGE. The fractions containing caldesmon were collected, dialysed against 2 changes of 4 L ddH_2O and lyophilized. After dissolving the caldesmon powder in a buffer containing 6 M urea, 10 mM imidazole pH 7.0, 0.1 mM EGTA and 6 mM β-mercaptoethanol, the protein was subjected to a 589 mL G-75 gel filtration in a column (2.5 x 120 cm) equilibrated in the above buffer. The column was eluted with the equilibration buffer at 0.5 mL/min and fractions showing peak absorbance at $A_{280\,nm}$ were analyzed by 12% w/v SDS-PAGE. The purified fractions were collected and dialysed against 3 changes of 2 L of 0.5% v/v formic acid. The protein was lyophilized and stored at -20°C.

E. Preparation of Chymotryptic Fragments of Ti II and CNBr Fragments of Caldesmon

Ti II was digested using α–chymotrypsin. One mg of Ti II dissolved in ddH_2O was incubated with 5 μg of α–chymotrypsin in a 500 μL reaction for 2 hours at room temperature. The pellet of protein was obtained by centrifuging the solution in a SpeedVac sample drier. The pellet was washed with 2 changes of 500 μL ddH_2O. The pellet was dissolved in 50-100 μL of 3x sample buffer containing 3% v/v β-mercaptoethanol and stored at -20°C. The smallest fragment of Ti II resolved by small pore 4-16.7% w/v gradient SDS-PAGE was transferred onto a polyvinylidene difluoride (PVDF) membrane, stained with 0.2% w/v amido black and was sent to the Protein Sequencing Facility, University of Calgary, for amino acid sequence analysis.

Digestion of caldesmon was carried out using cyanogen bromide (CNBr). Five nanomoles of caldesmon (~ 450 μg) in 70% formic acid was incubated with 2 mg of CNBr overnight at room temperature (as described by Lin *et al.*, 1991). The solution was pelleted as above. The protein pellet was dissolved in 50-100 μL of 3x sample buffer and stored at -20°C.

F. Gel Electrophoresis of Titin, Caldesmon, Titin Motif Fragments and COOH-terminal Caldesmon Synthetic Peptide

SDS-PAGE (as described by Jin, 1995) was performed using a Bio-Rad minigel apparatus. Electrophoresis was carried out using a current of 20 mA/gel in 1x running buffer (30.2 g Tris-base, 188 g glycine and 1% w/v SDS in 1 L of 10x buffer) for 0.75 mm Laemmli gels, and in 1x FBX buffer (20 mM EDTA, 0.4 M Tris, 0.2 M sodium acetate and 1% w/v

SDS, pH 7.5, in 10x buffer) for 0.75 mm large pore gradient gel. Electrophoresis for the small-pore gradient gel (0.75 mm) was carried out using a current of 40 mA/gel in 1x top buffer (1 M Tris, 1 M Tricine and 1% w/v SDS in 10x buffer) and 1x bottom buffer (2 M Tris pH 8.9 in 10x buffer).

Laemmli gels. Smooth muscle tissue caldesmons were resolved by 10% w/v SDS-PAGE. The protein purification of caldesmon was examined on 12% w/v SDS-PAGE. The stacking gel was 4% w/v.

Small-pore gradient gels. For the purpose of analyzing Ti II and caldesmon digestion fragments as well as the COOH-terminal caldesmon synthetic peptide, small-pore 4-16.7% w/v gradient polyacrylamide gel was used, omitting SDS in the gel. The gels were prepared in a multigel caster with a linear gradient maker. The ratio of acrylamide:bisacrylamide was 19:1 and Tris-Tricine buffer containing 0.1% w/v SDS was used in the electrophoresis.

Large-pore gradient gels. To resolve the very high molecular weight intact titin in the striated muscle tissues, large pore low cross-linker 2-12% w/v gradient gels containing 0.3% w/v low melting agarose and 7% v/v glycerol in the Fairbanks' continuous buffer system (Jin, 1995) were prepared in a multigel caster with a linear gradient maker. The ratio of acrylamide:bisacrylamide was 50:1. Gels were stained in Coomassie Brilliant Blue R-250 for 1 hour and destained in 7% v/v methanol/7% v/v acetic acid for 2 hours.

G. Western Blotting of Titin, Titin Motif Fragments and Caldesmon

Proteins resolved by SDS-PAGE were transferred onto a nitrocellulose membrane using a Transblot semi-dry transfer cell (Bio-Rad) with a current of 5-6 mA/cm^2 gel. Proteins

with molecular weight greater than 15 kDa were transferred onto 0.45 μm nitrocellulose (Trans-blot, Bio-Rad), whereas proteins with molecular weight less than 15 kDa were transferred onto a 0.20 μm nitrocellulose membrane (Schleicher & Schuell). Titin was transferred for 90 min using a three buffer system. The first buffer, 1x transfer buffer (20.2 g Tris and 188 g glycine, pH 8.3, in 1 L of 10x buffer) containing 20% v/v methanol, was used to soak the bottom layer (+ polarity) of filter paper and the nitrocellulose membrane. The second buffer (25 mM Tris pH 10.8, 10% v/v methanol and 0.05% w/v SDS) was used to soak the SDS gel and the upper layer of filter paper. The most upper layer (- polarity) of filter paper was soaked in the third buffer (300 mM Tris pH 10.8, 10% v/v methanol and 0.05% w/v SDS). β-mercaptoethanol (1/1,400) was added to all transfer buffers. Other proteins were transferred for 30 min using 1x transfer buffer containing 20% v/v methanol. After the proteins were transferred, the nitrocellulose membranes were soaked in a blocking solution containing 1% BSA/TBS for 1 hour at room temperature or overnight at 4°C. To assess the efficiency of the transfer replica, a membrane was stained in 0.2% w/v amido black followed by destaining in 50% v/v methanol/5% v/v acetic acid.

The monoclonal antibodies used for immunoblotting were in the form of hybridoma ascites fluids from BALB/c mice, or the supernatant of the hybridoma cell cultures. Immunoblotting of titin motif fragments using anti-caldesmon mAbs was carried out with a 1/500 dilution of C21, a 1/2,000 dilution of C94 or a 1/500 dilution of C98 overnight incubation at 4°C. AP-labelled goat anti mouse Igs (Sigma) was used as secondary antibody with dilution of 1/4,000 for blot which had been probed with primary antibody C21, 1/2,000 for blot which had been probed with primary antibody C94 and 1/1,000 for blot which had

been probed with primary antibody C98 for 1 hour incubation at room temperature. Blots were washed with 1x TBS for 10 min, 1x TBS + 0.1% v/v NP40 for three changes at 10 min each and 1x TBS for two changes at 5 min each. NBT and BCIP substrate were used to reveal the positive antibody binding, as described earlier.

Immunoblotting of protein extracts of cardiac and skeletal muscle tissues was carried out using anti-Ti II mAb, Ti104, anti C-protein mAbs, MF1 and ALD66, and anti-caldesmon mAb, C21. Nitrocellulose membranes were incubated with the primary antibodies overnight at 4°C on a horizontal platform shaker. Dilution of the primary antibodies was 1/50 for Ti104, 1/2 for the hybridoma supernatants of MF1 and ALD66, and 1/50 for C21. Secondary antibody was a 1/4,000 dilution of AP-labelled goat anti mouse Igs for blots which had been probed with primary antibodies MF1 and ALD66, and ^{125}I-labelled sheep anti mouse IgG (1 μCi/mL for a total of 10 μCi) for blots which had been probed with primary antibodies Ti104 and C21 with a 1 hour incubation at room temperature. The washing conditions were 1x TBS for 10 min, 1x TBS + 0.5% v/v Triton-X 100 + 0.05% w/v SDS for three changes for 10 min each and 1x TBS for two changes for 5 min each. As an exception, the washing of the blots after probed with primary antibodies Ti104 and C21, was 1x TBS for 10 min, 1x TBS + 0.5% v/v Triton-X 100 + 0.05% w/v SDS for 10 min and 1x TBS for two changes for 5 min each. Positive antibody binding was detected using NBT and BCIP substrate for MF1 and ALD66. Nitrocellulose blots probed with Ti104 were exposed to X-ray film for 2 days and the blot probed with C21 was exposed for 9 days with an intensifying screen at -70°C.

Immunoblotting of protein extracts of smooth muscle tissues was carried out using dilutions of 10^{-3} for Ti104, 1/500 for C21, 10^{-4} for C23 and 10^{-3} for C57 with overnight

incubation on a horizontal platform shaker at 4°C. The secondary antibody was ^{125}I-labelled sheep anti mouse IgG (0.5 μCi/mL for a total of 5 μCi) with a 1 hour incubation at room temperature. Unbound antibodies were removed by washing with 1x TBS for 10 min, 1x TBS + 0.5% v/v Triton-X 100 + 0.05% w/v SDS for three changes for 10 min each and 1x TBS for two changes for 5 min each. Nitrocellulose blots were exposed to X-ray film with an intensifying screen for 3 days (for blots which had been probed with Ti104 as primary antibody) and 2 days for blots which had been probed with primary antibodies C21, C23 and C57 at -70°C.

For Ti II and caldesmon peptide fragments, immunoblotting was carried out using dilutions of 1/100 for C21, 1/50 for Ti104 and using undiluted hybridoma supernatant containing Ti106 in overnight incubations at 4°C. The AP-labelled goat anti mouse Igs secondary antibody was used at a 1/2,000 dilution for blots which had been probed with primary antibodies Ti106 and C21, and a 1/1,000 dilution for blot which had been probed with primary antibody Ti104. Washing conditions were 1x TBS for 10 min, 1x TBS + 0.1% v/v NP40 for 10 min and 1x TBS twice for 10 min each for blot probed with Ti104. Nitrocellulose blots probed with Ti106 and Ti108 were washed with 1x TBS for 10 min, 1x TBS + 0.5% v/v Triton-X 100 + 0.05% w/v SDS for 10 min and 1x TBS for two changes at 5 min each. NBT and BCIP substrate was used to detect the positive antibody binding.

H. Identification of Structural Similarity Between Titin Motif II and Actin-binding Site of Caldesmon or COOH-terminal Caldesmon Synthetic Peptide

To investigate the level of structural similarity between caldesmon and Ti II, amino

acid sequence alignment was carried out on the 3 kDa chymotryptic fragment of Ti II (smallest fragment that was recognized by both Ti104 and C21), the actin-binding domain of caldesmon (Leu495-Trp523 in l-caldesmon) and the 6.4 kDa COOH-terminal domain caldesmon synthetic peptide.

RESULTS

A. Cross-reactivity of Anti-Ti II mAb to C-protein and Anti-caldesmon mAbs to Titin and Titin Motif Fragments

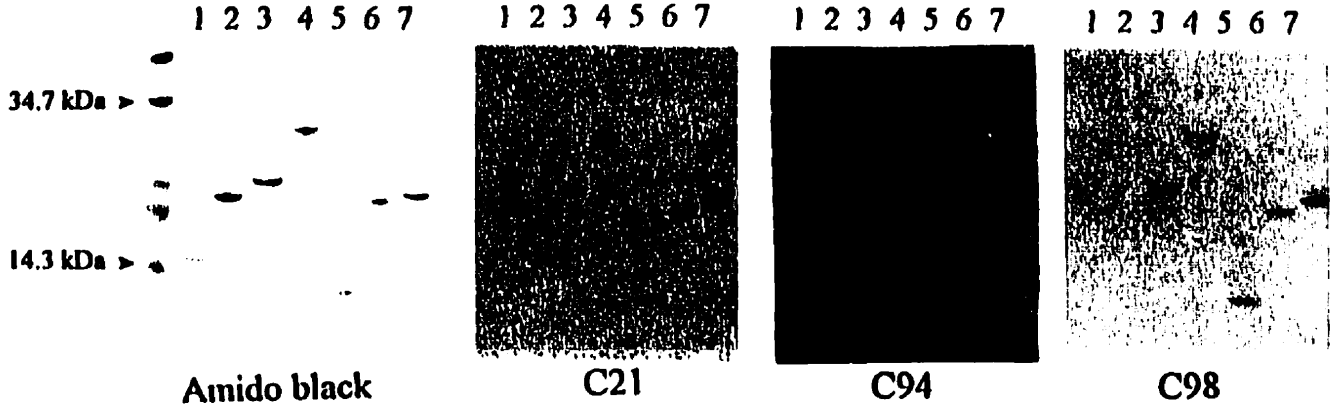
Immunoblotting using C21, C94 and C98 on titin motif fragments showed reactivities of all anti-caldesmon mAbs to fragments containing titin motif II (Ti II) only, similar to the reaction seen with anti-Ti II mAbs (Fig. 2.1 in Chapter 2). Cross-reactivity of C98 to the fragments containing Ti II was weak. All fragments containing titin motif I (Ti I), either single or multimerized, were not recognized by anti-caldesmon mAbs. The epitope recognized by C21 was known to be localized to the actin-binding site of caldesmon. The epitope recognized by C94 and C98 had not been identified before. This result suggests a similar epitopic structure is shared between caldesmon and Ti II but not Ti I (Fig. 3.1.A).

Western blotting of crude protein extracts of cardiac and skeletal muscles using Ti104 showed reactivities to titin and cross-reactivities to C-protein as confirmed by anti C-protein mAbs, MF1 and ALD66 (Fig. 3.1.B). Ti104 recognized titin in all tissue samples, very strongly in rat cardiac, rat skeletal muscle and bovine cardiac muscle. In addition, Ti104 interacted strongly with C-protein of frog skeletal muscle, turtle cardiac and turtle skeletal muscle; moderately with C-protein of chicken cardiac, chicken skeletal, bovine cardiac,

Figure 3.1. Cross-reactivity of anti-Ti II mAb to C-protein and anti-caldesmon mAbs to titin, titin motif fragments and C-protein.

(A). Titin motif fragments, Ti I, Ti II, or combined Ti I and Ti II motif fragments were resolved by 4-16.7% small-pore gradient polyacrylamide gels with the following loading pattern: 1, Ti I; 2, Ti I-I; 3, Ti I-II; 4, Ti I-II-I; 5, Ti II; 6, Ti II-I; 7, Ti II-II and transferred onto 0.2 μm nitrocellulose membranes. The primary mAbs were C21, C94 and C98; secondary Ab was AP-labelled goat anti mouse Igs. Positive antibody binding was revealed by NBT and BCIP substrate. All anti-caldesmon mAbs showed cross-reactivities to fragments containing Ti II motifs (lanes 3-7) but not to Ti I (lanes 1 and 2). (B). Protein extracts of various cardiac and skeletal muscle tissues were resolved by large-pore 2-12% gradient SDS-PAGE and transferred onto nitrocellulose membranes. The samples were loaded as follows: 1, frog heart; 2, frog skeletal muscle; 3, turtle heart; 4, turtle skeletal muscle; 5, chicken heart; 6, chicken skeletal muscle; 7, bovine heart; 8, bovine skeletal muscle; 9, rat heart; 10, rat skeletal muscle; 11, mouse heart; 12, mouse skeletal muscle. Primary antibodies were Ti104, C21, MF1 and ALD66. Secondary antibodies were ^{125}I-labelled sheep anti mouse IgG (for blots probed with Ti104 and C21) and AP-labelled goat anti mouse Igs (for blots probed with MF1 and ALD66). Autoradiography was carried out at -70°C using intensifying screen for 2 days (Ti104) and 9 days (C21). Positive binding of MF1 and ALD66 was revealed by NBT and BCIP substrate. Ti104 showed specific reactivities to titin and cross-reactivities to C-protein, as confirmed by anti C-protein mAbs, MF1 (interacted with C-protein of turtle and chicken skeletal muscle) and ALD66 (interacted with C-protein of chicken skeletal muscle). C21 showed cross-reactivities to titin and C-protein from various samples, similar to Ti104.

A


1 2 3 4 5 6 7
34.7 kDa
14.3 kDa
Amido black
1 2 3 4 5 6 7
C21
1 2 3 4 5 6 7
C94
1 2 3 4 5 6 7
C98

B

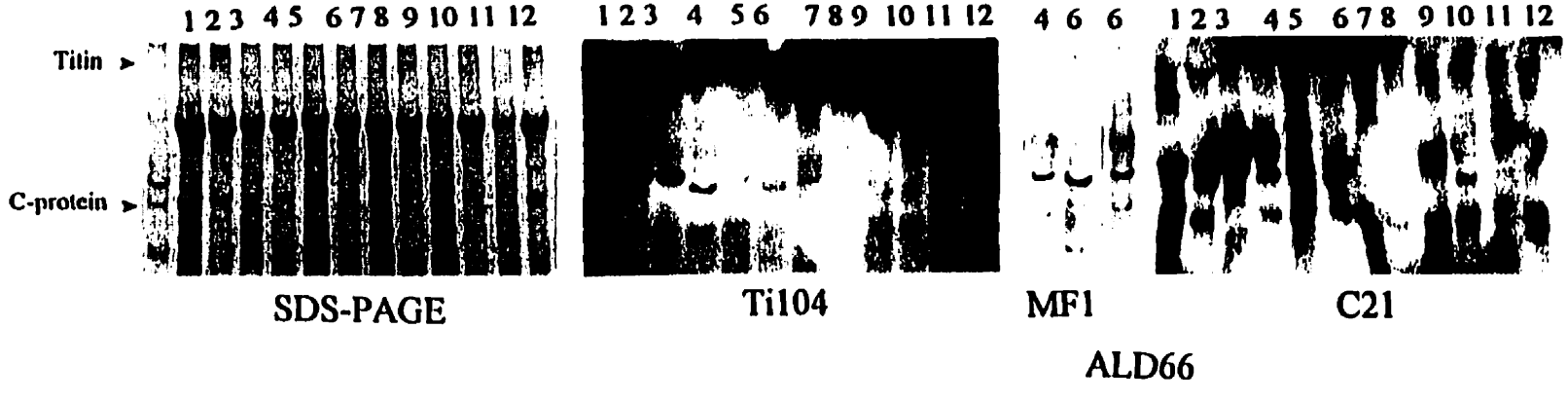
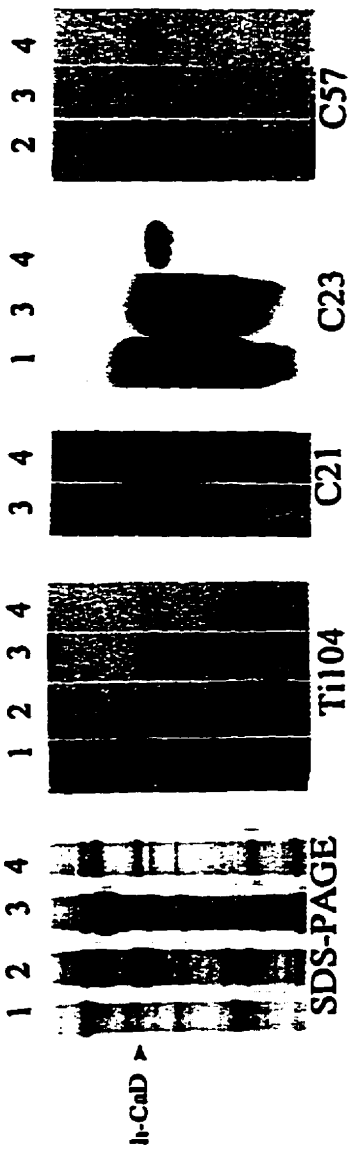

1 2 3 4 5 6 7 8 9 10 11 12
Titin
C-protein
SDS-PAGE
1 2 3 4 5 6 7 8 9 10 11 12
Ti104
4 6 6
MF1
1 2 3 4 5 6 7 8 9 10 11 12
C21
ALD66

Figure 3.2. Cross-reactivity of anti-Ti II mAb to h-caldesmon.

Protein extracts of various smooth muscle tissues were resolved by 12% w/v SDS-PAGE and transferred onto nitrocellulose membranes with the following loading pattern: 1, frog stomach; 2, turtle stomach; 3, chicken gizzard; 4, bovine stomach. Primary antibodies were Ti104, C21, C23 and C57. The secondary antibody used was ^{125}I-labelled sheep anti mouse IgG. Autoradiography was performed at -70°C using an intensifying screen for 3 days (Ti104) and 2 days (C21, C23 and C57). Ti104 showed cross-reactivities to h-caldesmon from different samples. The positive binding of Ti104 to h-caldesmon was confirmed by reactivities of anti-caldesmon mAbs C21 (interacted with chicken gizzard and bovine stomach caldesmons), C23 (interacted with frog stomach, chicken gizzard and bovine caldesmons) and C57 (interacted with turtle stomach, chicken gizzard and bovine stomach caldesmons) to h-caldesmon.


1 2 3 4
SDS-PAGE
1 2 3 4
Ti104
3 4
C21
1 3 4
C23
2 3 4
C57
h-CaD

bovine skeletal, rat cardiac, rat skeletal and mouse skeletal muscle; and weakly with frog cardiac and mouse cardiac muscles. The binding of Ti104 to C-proteins was confirmed by MF1 and ALD66 which showed their reactivities to C-proteins of turtle and chicken skeletal muscles. The cross-reactivity of Ti104 to C-protein can be explained because titin and C-protein consist of the same types of structural fibronectin III-like and immunoglobulin C2-like domains.

Immunoblotting using C21 mAb indicated moderate cross-reactivities to titin of all samples and also to C-protein of the samples (Fig. 3.1.B). This result is consistent with the result in Fig. 3.1.A that shows cross-reactivity of C21 to Ti II, an immunoglobulin-type motif of titin. C-protein also consists of immunoglobulin-type domains and is also recognized by C21.

B. Cross-reactivity of Anti-Ti II mAb to Caldesmon in Smooth Muscle (h-Caldesmon)

Western blotting of smooth muscle proteins using Ti104 showed it cross-reacted to caldesmon of frog stomach, turtle stomach, chicken gizzard and bovine stomach. The reactivity of Ti104 to h-caldesmon was confirmed by immunoblotting using anti-caldesmon mAbs, C21, C23 and C57. C21 confirmed the Ti104 cross-reactivities to chicken gizzard and bovine stomach caldesmons. C23 confirmed Ti104 cross-reactivities to frog stomach caldesmon, also to chicken gizzard and bovine stomach caldesmons; whereas C57 confirmed Ti104 cross-reactivities to turtle stomach, chicken gizzard and bovine stomach caldesmons (Fig. 3.2). The cross-reactivities of Ti104 to some h-caldesmons that interacted with different anti-caldesmon mAbs indicated that titin and caldesmon share similar epitopic structure.

C. Purification of Caldesmon from Chicken Gizzard

The purification method used in this experiment produced pure caldesmon in large quantities. Samples taken from all steps before the CM-52 column showed a lot of contaminating proteins in the samples. When the samples were loaded onto a CM-52 column, 2 peaks were revealed by $A_{280\ nm}$. The first peak consisted of fractions 1-12. Second peak consisted of fractions 50-62. Analysis of the samples from both peaks by 12% w/v SDS-PAGE showed that fractions in the first peak contained contaminating proteins of lower molecular weight such as actin, similar to the samples taken from the steps before CM-52 column. The fractions from the second peak contained less contaminating proteins. To remove the contaminating proteins, these fractions (50-62) were further purified by G-75 gel filtration column. In the G-75 column, only 1 peak was revealed at $A_{280\ nm}$. Analysis of fractions around that peak (6-20) by 12% w/v SDS-PAGE showed pure caldesmon in all fractions. From 100 g chicken gizzard, 71.1 mg pure caldesmon was obtained by this purification method. This 150 kDa caldesmon was used in the following experiment. The pure caldesmon obtained by this method indicates that the purification method applied in this experiment is reliable in providing a high yield of pure caldesmon.

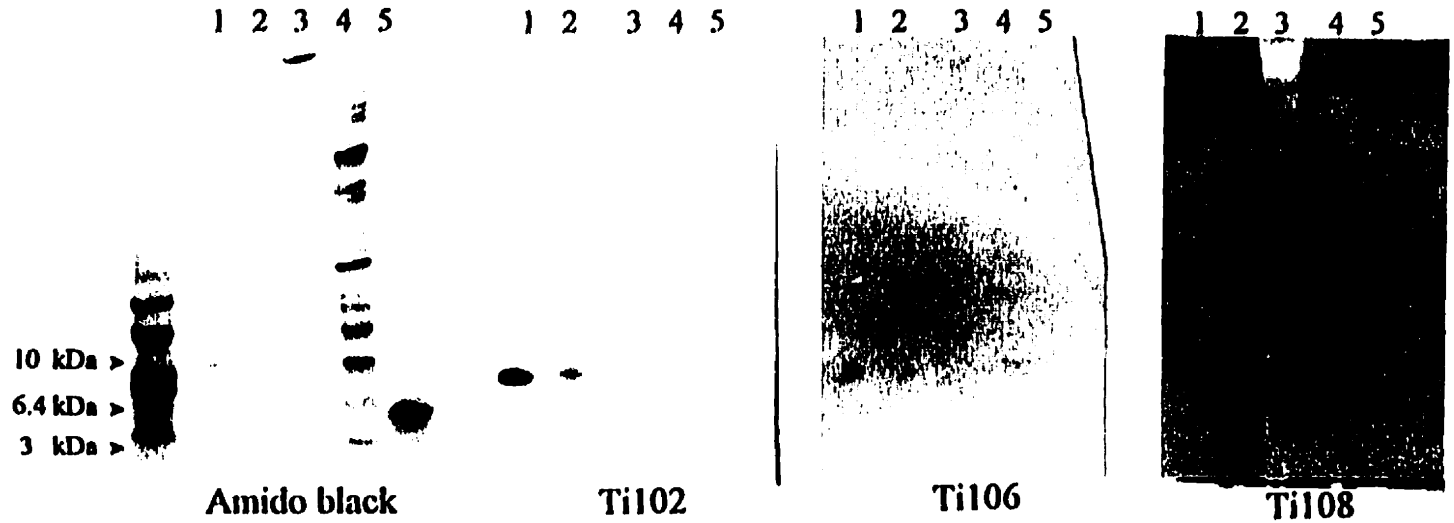
D. mAbs Epitope Mapping on Ti II Chymotryptic Fragments, Caldesmon CNBr Fragments and the COOH-terminal Caldesmon Synthetic Peptide

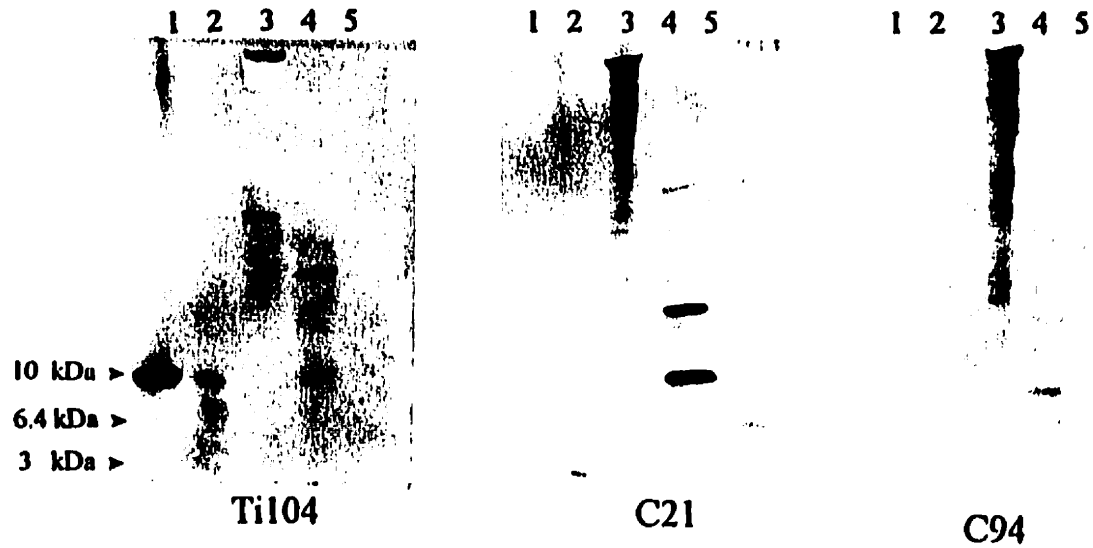
Immunoblotting of intact Ti II, Ti II chymotryptic fragments, intact caldesmon, caldesmon CNBr fragments and COOH-terminal caldesmon synthetic peptide using Ti104 and C21 showed similar reactivities to intact Ti II, the 3 kDa Ti II chymotryptic fragment, intact

caldesmon and the 10 kDa caldesmon CNBr fragment (Fig. 3.3). These cross-reactivities strongly suggest that both mAbs recognize a similar epitope in the Ti II 3 kDa fragment and the caldesmon 10 kDa fragment. According to Lin *et al* (1991), this 10 kDa CNBr fragment of caldesmon which is recognized by C21 is the actin-binding domain of the COOH-terminal domain of caldesmon. Mezgueldi *et al.* (1994) have demonstrated the precise location of this actin-binding site between residues Leu495 to Trp524 in l-caldesmon. Amino acid sequence alignment of those fragments could be used to determine the level of the primary structural similarity. The result also showed that C21 interacted with the 6.4 kDa caldesmon synthetic peptide whereas Ti104 did not interact with that peptide. This result suggests that C21 is still able to recognize the actin-binding domain retained in the synthetic peptide, but since the folding arrangement of the synthetic peptide may not be exactly the same as the one in native protein, Ti104 failed to recognize that epitope. This indicates that the similar epitope recognized by both Ti104 and C21 is the one retained in the native Ti II and caldesmon. Ti102 and Ti106 showed reactivities to intact Ti II but not its 3 kDa chymotryptic fragment. This indicates that the epitopes recognized by Ti102 and Ti106 are different from that of Ti104. Both mAbs interact weakly with intact caldesmon and Ti106 also shows weak reactivity to the 10 kDa caldesmon fragment. Ti108 shows strong reactivity to Ti II and its chymotryptic fragments but not to intact caldesmon, and reacts weakly to the 10 kDa caldesmon CNBr fragment. Immunoblotting using C94 showed it cross-reacted to intact Ti II, it reacted to the 10 kDa caldesmon CNBr fragment and it interacted weakly with the 6.4 kDa caldesmon synthetic peptide.

Figure 3.3. Reactivities of anti -Ti II and anti-caldesmon mAbs to Ti II chymotryptic fragments, caldesmon CNBr fragments, and the COOH-terminal caldesmon synthetic peptide.

Ti II, Ti II chymotryptic fragments, caldesmon, caldesmon CNBr fragments and the COOH-terminal caldesmon synthetic peptide were resolved by small pore 4-16.7% w/v gradient polyacrylamide gel and transferred onto 0.2 μm nitrocellulose membrane. The sample loading pattern was: 1, intact Ti II; 2, Ti II chymotryptic fragments; 3, intact caldesmon; 4, caldesmon CNBr fragments; 5, COOH-terminal caldesmon synthetic peptide. For immunoblotting, the primary antibodies used were Ti102, Ti104, Ti106, Ti108, C21 and C94. The secondary antibody used was AP-labelled goat anti mouse Igs and positive binding was revealed by NBT and BCIP substrate. Anti-caldesmon mAb, C21, and anti-Ti II mAb, Ti104, showed similar reactivities to intact Ti II, the 3 kDa Ti II chymotryptic fragment, caldesmon, and the 10 kDa caldesmon CNBr fragment (lanes 1-4). C21 also interacted with the 6.4 kDa COOH-terminal caldesmon synthetic peptide (5). Ti106 and Ti108 interacted weakly with the 10 kDa caldesmon CNBr fragment (4). Ti102 did not interact with the 10 kDa caldesmon CNBr fragment, whereas C94 showed its reactivity to the 10 kDa caldesmon CNBr fragment and to the synthetic peptide (4 and 5).


1 2 3 4 5
10 kDa
6.4 kDa
3 kDa
Amido black
Ti102
Ti106
Ti108


1 2 3 4 5
10 kDa
6.4 kDa
3 kDa
Ti104
C21
C94

E. Comparison of Structural Similarities Between the 3 kDa Ti II Chymotryptic Fragment, and the Actin-binding Site of Caldesmon, or the 6.4 kDa COOH-terminal Caldesmon Synthetic Peptide

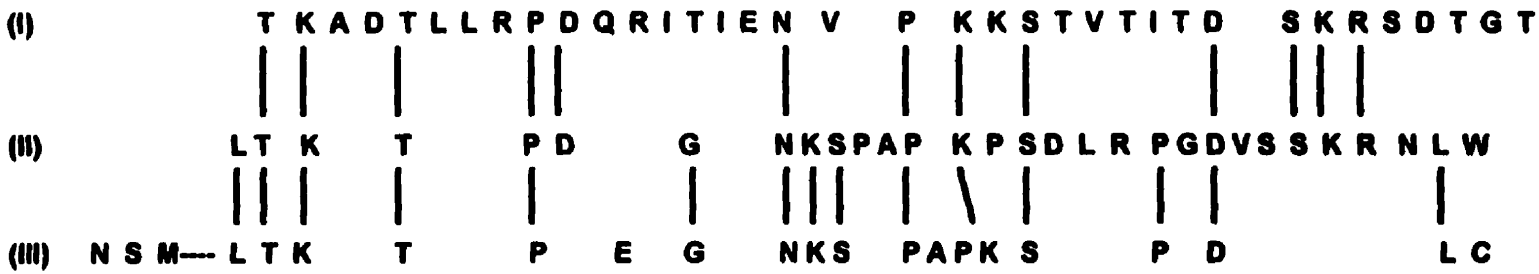
The amino acid sequence alignment carried out on the 3 kDa Ti II fragment (verified by microsequencing done at the Protein Analysis Facility, University of Calgary) and the actin-binding site of caldesmon and the 6.4 kDa caldesmon synthetic peptide showed there was no significant primary structural similarity. However, the 3 kDa Ti II chymotryptic fragment and the 10 kDa caldesmon CNBr fragment were recognized by the same two mAbs, C21 and Ti104. This result suggests that structural similarity occurs at the level of the tertiary structure of both the 3 kDa Ti II fragment and the actin-binding site of caldesmon. Protein data bank searching to match the 3 kDa Ti II fragment amino acid sequence with other proteins, including any actin-binding protein did not find any significant homologous sequence.

DISCUSSION

The structural similarity between titin and caldesmon was investigated. Several experiments show strong cross-reactivities of anti-titin mAbs to caldesmon and anti-caldesmon mAbs to titin. The immunoblotting of titin motif fragments (Fig. 3.1.A) showed that anti-caldesmon mAbs, C21, C94 and C98, cross-reacted to Ti II, the immunoglobulin-type of titin. This cross-reactivity indicates that the immunoglobulin-type domain of titin and caldesmon share similar epitopic structure. Considering that the epitope recognized by C21 has been localized to the actin-binding site of caldesmon (Lin *et al*, 1991), this result suggests

Figure 3.4. Amino acid sequence comparison between the 3 kDa Ti II chymotryptic fragment, the actin-binding site of caldesmon, and the 6.4 kDa COOH-terminal caldesmon synthetic peptide.

Amino acid sequence comparison showed a lack of significant primary structural similarity between the 3 kDa Ti II chymotryptic fragment, the actin-binding site of caldesmon and the 6.4 kDa COOH-terminal caldesmon synthetic peptide.



(I) : 3 kDa Ti II chymotryptic fragment

(II) : actin-binding site of 10 kDa CaD CNBr fragment

(III) : COOH-terminal CaD synthetic peptide

that the structural similarity lies in the actin-binding site of caldesmon. This result also suggests that the epitope recognized by C94 is possibly also located in the actin-binding site of caldesmon. The cross-reactivity of C98 to fragments containing Ti II is weak. This suggests the epitope recognized by C98 is possibly also located in the actin-binding site of caldesmon, or in the sites adjacent to the actin-binding site of caldesmon that still share similar structure with Ti II. The result in chapter 2, in which anti-Ti II mAbs, Ti102, Ti104, Ti106 and Ti108 specifically recognized the Ti II motif and that Ti104 is known to recognize caldesmon, suggests that C21 and Ti104 can be used to further investigate the structural similarity of titin and caldesmon.

The immunoblotting results on cardiac and skeletal muscle tissue samples showed reactivities of Ti104 to both titin and C-protein. This finding was confirmed by MF1 and ALD66 blotting. It has been demonstrated that titin and C-protein belong to a superfamily of proteins built from domains that share sequence similarity with immunoglobulin C2 and fibronectin type III motifs (Einheber and Fischman, 1990). The cross-reactivities of Ti104 to C-protein indicated the structural similarity in titin and C-protein (Fig.3.1.B).

Blotting results of proteins from striated muscle samples of various species using C21 showed reactivities similar to that of Ti104 (Fig 3.1.B). Consistent with the result in Fig. 3.1.A, this result indicates that the immunoglobulin-type motif present in Ti II and C-protein retains an epitopic structure similar to that of caldesmon's actin-binding site, which has an epitope recognized by C21.

The immunoblotting results of protein extracts of smooth muscle tissues showed cross-reactivities of Ti104 to caldesmon, that was confirmed by different anti-caldesmon

mAbs (Fig. 3.2). This result indicates that an epitope structure in caldesmon is recognized by an anti-Ti II mAb, and thus, this epitope structure is similar to a three dimensional structure on Ti II.

In Fig. 3.3, it is demonstrated that C21 and Ti104 recognized the same 3 kDa Ti II chymotryptic fragment and 10 kDa caldesmon CNBr fragment. The epitope recognized by C21 is located in the actin-binding domain of caldesmon in the 10 kDa COOH-terminal CNBr fragment (consistent with Lin *et al.*, 1991) suggesting that a structural similarity is shared between the 3 kDa Ti II chymotryptic fragment and the actin-binding site of caldesmon. Further, amino acid sequence analysis of the 3 kDa Ti II chymotryptic fragment and the actin-binding site of caldesmon showed a lack of significant primary structural similarity. Nevertheless, it has been demonstrated that both fragments are recognized by two different sources of mAbs. This result strongly indicates that these fragments possess epitopic similarities formed by their three dimensional structures. The existence of similar three dimensional structure suggests an analogous function performed by the actin-binding site of caldesmon and the immunoglobulin-type domain of titin. C94 showed reactivity to the 10 kDa caldesmon CNBr fragment and the 6.4 kDa synthetic peptide, suggesting the epitope it recognizes is also located in the 10 kDa COOH-terminal domain of caldesmon, possibly in the same location as the epitope recognized by C21. The synthetic peptide showed interaction with C21 but not with Ti104, suggesting that the actin-binding site retained in this peptide is recognizable by C21 but the folding arrangement is probably different from that formed by native caldesmon so that it can not interact with Ti104. The result in Fig. 3.3 also showed the epitopes recognized by anti-Ti II mAbs were different. Ti104 and Ti108 recognized the 3 kDa

chymotryptic fragment, suggesting the epitopes they recognize are located close to each other. Both Ti104 and Ti108 also interact with the 10 kDa caldesmon CNBr fragment, but Ti104 interacts with that fragment stronger than Ti108 does. The epitopes recognized by Ti102 and Ti106 were not found in the smaller fragments of Ti II. In general, to determine the epitope location recognized by each of anti-Ti II mAb, cleavage of Ti II by other proteolytic or chemical methods needs to be done and Western blotting on those fragments carried out using the different anti-Ti II mAbs.

In conclusion, cross-reactivities of anti-titin mAbs to caldesmon or caldesmon CNBr fragments, and cross-reactivities of anti-caldesmon mAbs to titin or Ti II chymotryptic fragments indicate a similar epitope structure found between the immunoglobulin-type domain of titin and the actin-binding site of caldesmon. The comparison of amino acid sequences of the 3 kDa Ti II chymotryptic fragment and the actin-binding site of caldesmon showed no significant primary structure similarity. These results suggest that the structural similarity between the immunoglobulin-type domain of titin and the actin-binding site of caldesmon, most likely, in their three dimensional structures, suggesting an analogous function between the two proteins. Epitope mapping of anti-Ti II mAbs, Ti102, Ti104, Ti106 and Ti108, on Ti II chymotryptic fragments does not specify the epitope location recognized by each of the mAb. Epitope mapping of anti-caldesmon mAb C94 on caldesmon CNBr fragments shows it recognizes an epitope which is located in the actin-binding site of caldesmon.

CHAPTER FOUR :

CHARACTERIZATION OF THE TI II/CALDESMON COOH-TERMINAL MONOCLONAL ANTIBODY EPITOPES USING CALDESMON COOH-TERMINAL DELETION MUTANTS

INTRODUCTION

Caldesmon 39 (CaD39) is a COOH-terminal domain fragment of human fibroblast caldesmon (Warren *et al.*, 1994). This domain is also conserved in smooth muscle cells with similar primary structure and function (Hayashi *et al*, 1991). The 10 kDa COOH-terminal of this domain retains actin- and calmodulin-binding sites (Bartegi *et al*, 1990).

Four deletion mutants of the 10 kDa COOH-terminal domain of caldesmon, deletions A (Met460-Ser468), B (Ser489-Lys497), C(combination of deletions A+B) and D (Leu495-Leu523) were constructed (Fig. 4.1). The amino acid removed in A and B have been proposed to be involved in the Ca^{2+}-calmodulin binding site, whereas the region deleted in D has been proposed to be involved in actin binding (Marston *et al.*, 1994; Mezgueldi *et al.*, 1994). The epitopes of these deletion mutants, as well as wild type CaD39, were characterized by anti-caldesmon mAbs, C21, C94, and C98. Cross-reactivities of the deletion mutants, as well as wild type CaD39, to anti-Ti II mAbs, Ti104 and Ti106, were also investigated.

To further characterize the 10 kDa COOH-terminal caldesmon fragment, a mutation of Trp residues to Ala was carried out in sites A (Trp461), B (Trp494), C (Trp461 and 494), and D (Trp524) (Graether *et al.*, 1997). The characteristic changes of these site mutations

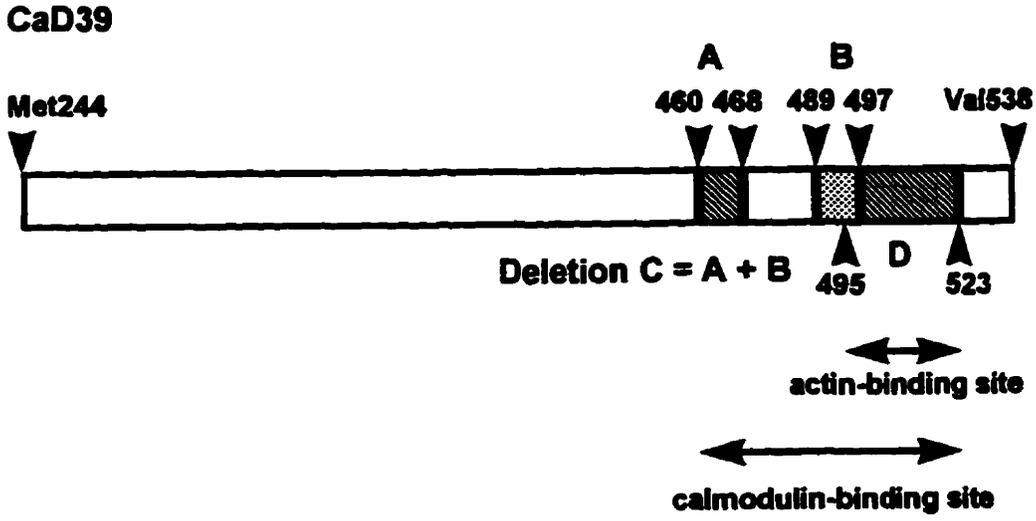


Figure 4.1. Deletion mutants designed in the CaD39 COOH-terminal domain fragment of caldesmon.

Four deletion mutants on the CaD39 COOH-terminal fragment of human fibroblast caldesmon. Deletion A (Met460-Ser468), B (Ser489-Lys497), C (del. A + del. B), and D (Leu495-Leu523). All deletion mutants are located within the 10 kDa COOH-terminal domain caldesmon.

were investigated using anti-caldesmon mAbs, C21, C94, and C98; and anti-Ti II mAb Ti106.

The results of the experiments on CaD39 deletion mutants and CaD39 Trp/Ala mutants demonstrate a similar epitopic structure found between the immunoglobulin-type domain of titin and caldesmon. The epitope in caldesmon has been shown to be in its actin-binding site. The epitope recognized by C94 is shown to be in the actin-binding site of caldesmon and the epitope recognized by C98 is located in the COOH-terminal domain of caldesmon, downstream and adjacent to the actin-binding site.

MATERIALS AND METHODS

A. Engineering of CaD39 Mutants

A.1. CaD39 Deletion Mutants

The CaD39 deletion mutants were constructed using 2 steps of recombinant PCR. Recombinant PCR was carried out using wild type CaD39 as a template according to the method described by Higuchi (1990). To obtain CaD39 deletion mutants, two vector primers, T7 and pETreverse, and three pairs of internal primers were used. The internal primers were:

Deletion A primers	:	A1	GTGGGGGAACTCTTGATGTTGCGTACA
		A2	TCAAGAGTTCCCCCACTGCAGCAG
Deletion B primers	:	B1	TCTGGGGTAGAAACCCCTACCTTCAAG
		B2	GGGTTTCTACCCCAGATGGAAACAAG
Deletion D primers	:	D1	CTTTTCCCACCATTCATTGATGCGGCT
		D2	AATGAATGGTGGGAAAAGCAATCTGTG

The complementary sequences between A1 and A2, B1 and B2, D1 and D2 are underlined.

In the first step of recombinant PCR, fragment A1 and A2 were constructed using primer pairs T7 and A1 to construct fragment A1; pETrev and A2 to construct fragment A2. Fragments B1, B2, D1 and D2 were constructed in the same way using their own specific internal primers. Each PCR reaction contained 100-200 ng DNA template, 10 μL of 10x PCR standard buffer (1 M $MgCl_2$, 3 M KCl, 0.5 M Tris-HCl pH 8.3, 0.2% w/v gelatin), 10 μL of 1 mM dNTPs, 0.5 U of Taq DNA polymerase, 10 pmoles of vector primer (either T7 or pETrev) and 10 pmoles of internal primer, with ddH_2O to make up volume to 100 μL. The PCR program used was 1 cycle at 94°C for 2 min, 55°C for 40 sec, 72°C for 1 min, followed by 24 cycles at 94°C for 1 min, 55°C for 40 sec, and 72°C for 1 min.

In the second step of recombinant PCR, fragments A1 and A2 were joined, as well as fragments B1 and B2, D1 and D2, to obtain the full length coding sequence with its specific deletion. Vector primers T7 and pETrev were used in this step. Each PCR reaction contained 100-200 ng of DNA template from the 1st PCR product, 10 μL of 10x PCR buffer, 12 μL of 1 mM dNTPs, 10 pmoles of each vector primer (added after first 5 cycles), 0.5 U Taq DNA polymerase, ddH_2O to make volume to 100 μL. The PCR program used was 5 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 3 min; followed by 20 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min. Minimal units of Taq DNA polymerase were used and a maximum of 25 PCR cycles was applied to avoid non-specific and undesired product containing mismatches or deletion errors.

Deletion C was constructed using deletion B as a template. Recombinant PCR was carried out using the above procedure with the internal primers A1 and A2, and vector primers T7 and pETrev.

The purification of the first PCR product was done by separating the DNA of interest on a 0.6% w/v agarose gel in 1x Tris acetate EDTA (TAE) buffer (242 g Tris base, 57.1 mL acetic acid, 10 mM EDTA pH 8.0 in 1 L of 50x buffer) followed by purification using the Prep-A-Gene kit from Bio-Rad, according to the protocol from the manufacturer. The second PCR product was purified by phenol-chloroform extraction and ethanol precipitation.

A.2. CaD39 Trp/Ala Mutants

The CaD39 Trp/Ala mutants cloned into pET3d were gifts from Dr. A.S. Mak, Queen's University. The site directed mutation of Trp to Ala was constructed according to the method described by Graether *et al.*, 1997. The mutants obtained were site A (Trp461Ala), site B (Trp494Ala), site C (Trp461Ala,Trp494Ala) and site D (Trp524Ala).

B. Construction of CaD39 Deletion Mutants

The CaD39 deletion mutants were cloned into the plasmid vector pET3d using the BamHI and XbaI sites. The final PCR products were digested by BamHI 10 U/μg of DNA in 1x buffer with an incubation time of 3 hours at 37°C. The second digestion was done using XbaI at 50 U/μg of DNA in 2x buffer with an incubation time of 3 hours at 37°C. The digested DNA was purified using the Prep-A-Gene kit after agarose gel electrophoresis, and ligated to the plasmid vector, which was similarly digested with BamHI and XbaI. The ligation reaction contained 1x ligation buffer, 1U of T4 DNA ligase, with an insert to vector ratio of 4:1 and ddH_2O to give a final volume of 20 μL. This reaction was incubated at 16°C overnight. Plasmids with deletion mutant inserts were transformed into *E. coli* strain JM109

and plated on LB agar containing 100 μg/mL ampicillin. To check the positive inserts, PCR was carried out on randomly chosen colonies using T7 and pETrev primers.

C. DNA Sequencing of CaD39 Deletion Mutants

For the purpose of DNA sequencing, the plasmids containing the deletion mutant inserts were recovered from JM109 using the Plasmid Mini Prep kit (Qiagen) and following the procedure supplied by the manufacturer. DNA sequencing was carried out by the dideoxy chain termination method (Sanger, 1977) using the T7 Sequencing kit from Pharmacia. Briefly, 3 μg DNA was denatured using 2 M NaOH. The denatured DNA was then neutralized by 3 M sodium acetate. Ethanol precipitation using 100% ethanol was carried out on the DNA and the mixture was centrifuged at 14,000 rpm in a microcentrifuge at 4°C for 15 min. The pellet was washed once using 70% ethanol and dried in a SpeedVac for 5 min. Prior to the annealing step, the DNA pellet was dissolved in ddH_2O, and 5 pmoles of primer was added and 2 μL annealing buffer from the kit were also added. Annealing was carried out after heating the DNA in 65°C water bath for 5 min, by then incubating the mixture at room temperature for an additional 5 min. Into the mixture, 3 μL of labelling mix A (supplied by the kit), 2 μL of diluted T7 DNA polymerase (supplied by the kit), and 5 μCi of ^{35}S dATP were added and the mixture was incubated at room temperature for 5 min. The mixture was divided and was added into 4 tubes, each already containing 2.5 μL of mixtures of dideoxy A, C, G and T nucleotides respectively, and incubated in a 37°C water bath for 5 min. Four μL of stop reaction (supplied by the kit) was added into each of the 4 tubes afterward. Before loading onto the polyacrylamide sequencing gel (7 M urea, 57 g acrylamide, 3 g bisacrylamide

and 0.5x TBE, in 1 L gel) , samples were heated at 80°C for 2 min to denature the extension products.

The sequencing mixture was loaded onto a polyacrylamide sequencing gel and electrophoresis was performed at 40 Watts for thin gels, and 60 Watts for wedge gels, in 0.5x Tris borate EDTA (TBE) buffer (108 g Tris base, 55 g boric acid, 10 mM EDTA in 1 L of 10x buffer). After the electrophoresis, the sequencing gel was fixed in 7% v/v methanol/7% v/v acetic acid for 1 hour and the gel was dried. The dried gel was exposed to X-ray film and autoradiography was performed at room temperature overnight. The sequencing data were read and matched to the complete sequence of human fibroblast caldesmon (Novy *et al.*, 1991).

D. Protein Expression and Western Blotting of CaD39 Deletion Mutants and CaD39 Trp/Ala Mutants

To express the deletion products, *E. coli* strain BL21(DE3)pLysS was used as the bacterial host (Studier *et al*, 1990). The vector plasmids containing the CaD39 deletion mutants and CaD39 Trp/Ala mutants were transformed into BL21(DE3)pLysS and plated on LB agar containing 100 μg/mL ampicillin and 25 μg/mL chloramphenicol and incubated overnight at 37°C.

To obtain the expressed protein, fresh transformed colonies from BL21(DE3)pLysS plates were used to inoculate 2 mL of 2x TY medium containing 100 μg/mL ampicillin and 25 μg/mL chloramphenicol. The culture was incubated at 37°C, until an $OD_{600\ nm}$ of 0.4 was reached. Into half of the culture, IPTG was added to 0.4 mM and the other half was used as

an uninduced control. Both uninduced and induced cultures were incubated for another 3 hours. The cells were then centrifuged and the supernatant was discarded. The pellets were dissolved in 100 μL of 3x sample buffer.

The proteins expressed from the CaD39 deletion mutants and CaD39 Trp/Ala mutants were resolved by 14% w/v SDS-PAGE with acrylamide:bisacrylamide ratio of 180:1 and 12% w/v SDS-PAGE with acrylamide:bisacrylamide ratio of 29:1. The gels were stained in Coomassie Brilliant Blue R250 for 1 hour and destained in 7% v/v methanol/7% v/v acetic acid for 2 hours. Proteins resolved on several copies of gels were transferred onto nitrocellulose membranes.

The transfer of protein onto 0.45 μm nitrocellulose membranes was carried out as described in Chapter 3. The nitrocellulose membranes were then blocked in 1% w/v BSA/TBS for 1 hour at room temperature. The efficiency of the transfer was assessed by staining the membrane in 0.2% w/v amido black for 2 minutes, followed by destaining in 50% v/v methanol/5% v/v acetic acid for 5 minutes.

The immunoblotting of CaD39 and its deletion mutants was carried out using anti-caldesmon mAbs, C21, C94 and C98, and anti-Ti II mAbs, Ti104 and Ti106. Nitrocellulose membranes were incubated with the primary antibodies overnight at 4°C for Ti104 and Ti106, and 3 hours at room temperature for C21, C94 and C98. The dilution of mAbs in 0.1% w/v BSA/TBS were as follows: for C21 10^{-3}, for C94 10^{-3}, for C98 10^{-3}, for Ti104 1/500 and Ti106 was used as undiluted hybridoma culture supernatant. Incubation with the secondary antibody, AP-labelled goat anti mouse 1/2,000, was carried out for 1 hour at room temperature. The washing conditions following primary and secondary antibody incubation

were 1x TBS for 10 min, 1x TBS + 0.1% v/v NP40 for 10 min, and 1x TBS for two changes for 5 min each. NBT and BCIP substrate was used to reveal the positive antibody binding.

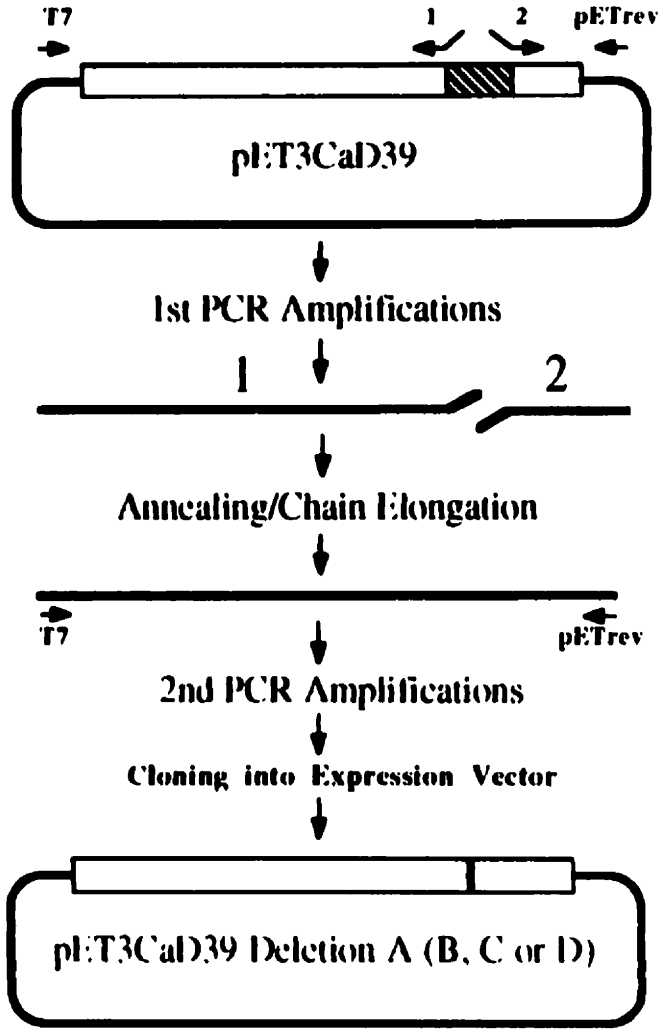
The immunoblotting of CaD39 and its Trp/Ala mutants was carried out using C21, C94, C98 and Ti106 mAbs. Incubation with the primary antibodies was done overnight at 4°C. The mAbs were diluted as follows: C21 1/2,000, C94 1/1,000, C98 1/2,000 and Ti106 was used as undiluted hybridoma culture supernatant. One hour incubation with the secondary antibody, AP-labelled goat anti mouse, was done with a dilution in 0.1% BSA/TBS of 1/4,000 for blots which had been probed with primary antibodies C21 and C98 and 1/2,000 for blots which had been probed with primary antibodies C94 and Ti106. Washing conditions were adjusted to obtain the best results. The nitrocellulose blots probed with C21 and C98 were washed with 1x TBS for 10 min, 1x TBS + 0.1% v/v NP40 for three changes for 10 min each, and 1x TBS for two changes for 5 min each. For the blot probed with C94, the washing conditions were 1x TBS for 10 min, 1x TBS + 0.5% v/v Triton-X 100 + 0.05% w/v SDS for 10 min, and 1x TBS for two changes for 5 min each. For the blot probed with Ti106 hybridoma supernatant, the washing was 1x TBS for 10 min, 1x TBS + 0.1% v/v NP40 for 10 min, and 1x TBS for two changes for 5 min. The positive binding was revealed by NBT and BCIP substrate.

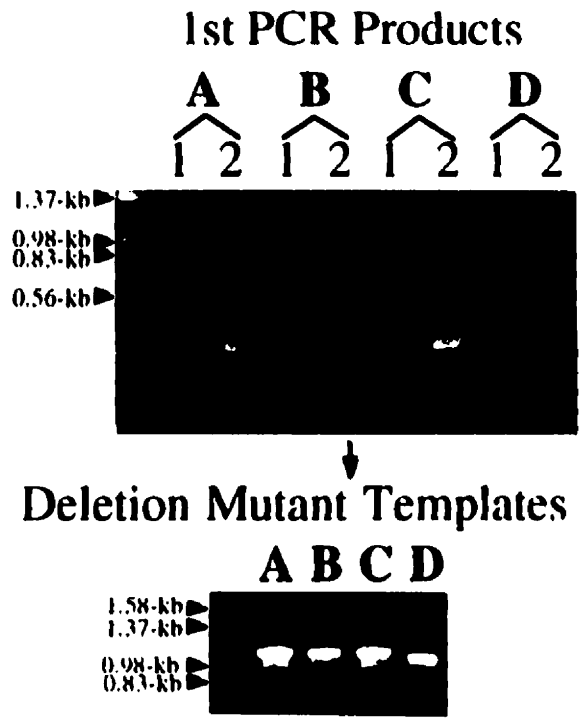
RESULTS

A. Engineering of the CaD39 Deletion Mutants

The first recombinant PCR produced the following DNA fragments: A1=728 bp, A2=320 bp, B1=815 bp, B2=233 bp, C1=728 bp, C2=293 bp, D1=834 bp, D2=155 bp. The

Figure 4.2. Construction of expression vectors encoding the deletion mutants of CaD39. CaD39 deletion mutants were constructed by recombinant PCR using the wild type CaD39 expression vector as template. In the first PCR step, T7 and internal primer 1 were used to obtain fragment 1; pETrev and internal primer 2 were used to obtain fragment 2. In the second PCR step, T7 and pETrev were used to obtain full length coding sequences with specific deletions. The first PCR yielded fragments with the following sizes: A1: 728 bp, A2: 320 bp, B1: 815 bp, B2: 233 bp, C1: 728 bp, C2: 293 bp, D1: 834 bp, D2: 155 bp. The second PCR step joined fragments 1 and 2, producing final result of A: 1048 bp, B: 1048 bp, C: 1021 bp and D: 989 bp.


T7
1
2
pETrev
pET3CalD39
1st PCR Amplifications
1
2
Annealing/Chain Elongation
T7
pETrev
2nd PCR Amplifications
Cloning into Expression Vector
pET3CalD39 Deletion A (B, C or D)


1st PCR Products
A B C D
1 2 1 2 1 2 1 2
1.37-kb
0.98-kb
0.83-kb
0.56-kb
Deletion Mutant Templates
A B C D
1.58-kb
1.37-kb
0.98-kb
0.83-kb

second PCR produced four coding sequences containing specific deletion mutants with the sizes of A=1048 bp, B=1048 bp, C=1021 bp and D=989 bp as determined by 0.6% w/v agarose gel electrophoresis. The recombinant PCR protocol and its products are shown in Fig. 4.2.

B. DNA Sequence Verification of the CaD39 Deletion Mutants

The DNA sequences of CaD39 deletion mutants were compared to the nucleotide sequence of human fibroblast caldesmon (Novy *et al.*, 1991). Several constructs using the recombinant PCR were found to contain either an unwanted mutation or an unwanted deletion. A single nucleotide deletion at nt 993 caused a frameshift mutation, resulting in a premature stop codon (TAA) at nt 1159. The premature stop codon caused truncated expression of the recombinant protein. One base substitution at nt 1674 (CGG to CGA) resulted in a silent mutation, as both triplet codons encode the same amino acid, arginine. Table 4.1 lists all the mutations and deletions yielded in this work. The locations of the unwanted mutation or deletion in the CaD39 are presented in Fig. 4.3. After several attempts, the intended deletion mutants were obtained. The nucleotide sequences outside the deletion area were confirmed. CaD39 del. A (A-6) has a 27 nucleotide deletion (nt 1489-1515) or a 9 amino acid deletion (Met460-Ser468); del. B (B-2) has a 27 nucleotide deletion (nt 1576-1602) or a 9 amino acid deletion (Ser489-Lys497); del. C (C-3) has a 54 nucleotide deletion (nt 1489-1515 and 1576-1602) or an 18 amino acid deletion (Met460-Ser468 and Ser489-Lys497) and del. D (D-4) has an 87 nucleotide deletion (nt 1594-1680) or a 29 amino acid deletion (Leu495-Leu523).

Table 4.1. Expression plasmids with unintended mutation or deletion in the CaD39 coding sequence.

Several plasmids containing unintended mutations were obtained using recombinant PCR in the attempts to construct the correct CaD39 deletion mutants. The most common error was a single base substitution which resulted in an incorrect amino acid. Deletion of a single nucleotide (nt 993) caused a premature stop codon at nucleotide 1159 (TAA) as shown in fragment B-3 and C-8, which used B-3 as a DNA template. The silent mutation is noted by an asterix. In the silent mutation, the triplet codon with the substituted base encodes the same amino acid. In fragment C-7, an ACT to ACC mutation codes for a threonine. In fragment C-3, a CGG to CGA mutation codes for an arginine.

DNA template	primer pairs in 1st step PCR	final clone sequenced	mutation or deletion	location of mutation/del.
CaD39 WT	T7/A1 and A2/pETrev	1. A-4 2. A-6	AGC→ GGC (-)	nt 898/aa 263
CaD39 WT	T7/B1 and B2/pETrev	1. B-3 2. B-10 3. B-2	GAA→ GA GCA→ ACA (-)	nt 993/aa 294 nt 1399/aa 430
CaD39 WT	T7/D1 and D2/pETrev	1. D-4	(-)	
CaD39 del. A	T7/B1 and B2/pETrev	1. C-3 2. C-7	CGA→ CTA ACT→ ACC*	nt 1124/aa 338 nt 1260/aa 383
CaD39 del. B	T7/A1 and A2/pETrev	1. C-8 2. C-10 3. C-6 4. C-2 5. C-3	GAA→ GA GTT→ GCT AGC→ GGC AGC→ GGC CGG→CGA*	nt 993/aa 294 nt 1724/aa 538 nt 988/aa 293 nt 1324/aa 405 nt 1674/aa 521

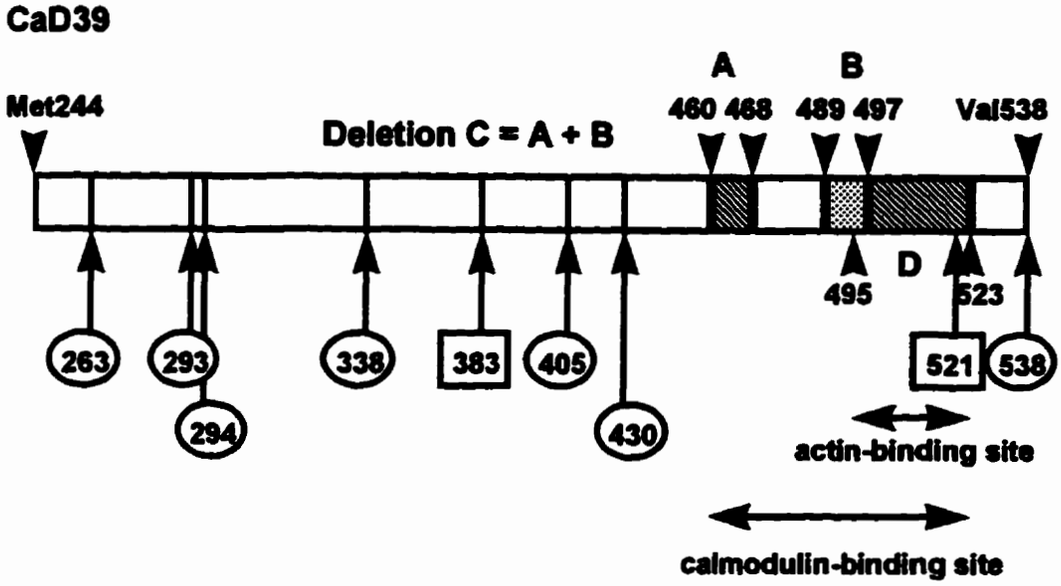


Figure 4.3. Locations of the unintended mutations or deletions in the CaD39.

Most of the unintended mutations or deletions occured outside the constructed CaD39 deletion mutants area. Numbers inside the circles indicate the amino acid position having a mutation or deletion that causes premature stop codon (aa 294) or incorrect amino acid. Numbers inside the rectangles indicate amino acid position having a silent mutation (aa 383 and aa 521).

C. Characterization of CaD39 Deletion Mutants and CaD39 Trp/Ala Mutants

C.1. CaD39 Deletion Mutants

The proteins expressed by the CaD39 deletion mutants were separated by 14% w/v SDS-PAGE using a ratio of acrylamide:bisacrylamide 180:1 produced bands with molecular weights which were consistent with those calculated from cDNA sequences.

Western blotting using C21 showed it interacted with proteins produced by wild type CaD39, del. A, del. B, del. C but not del. D (Fig. 4.4). This result confirms that the area within deletion D is the location of the actin-binding site of caldesmon. This result also allowed a more precise localization of the epitope recognized by C21. Anti-Ti II mAbs, Ti104 and Ti106 interacted with proteins of the wild type CaD39 and its deletion mutants A, B and C in a manner similar to C21, suggesting a similar epitopic structure is conserved between Ti II and the actin-binding site of caldesmon.

The anti-caldesmon mAb C94 reacted in a manner similar to C21, suggesting that the epitope recognized by C94 also lies on the actin-binding site of caldesmon. C98 interacted with the wild type CaD39 protein and all of its deletion mutants proteins. This suggests that epitope recognized by C98 is outside the area of the deletions. The result in Fig. 3.1 that shows the C98 mAb cross-reacts to the Ti II fragment suggesting the C98 epitope is possibly localized to the very end of the COOH-terminal domain of caldesmon which was not altered by the mutations which were constructed.

C.2. CaD39 Trp/Ala Mutants

The proteins expressed from the CaD39 WT and CaD39 Trp/Ala mutants were

resolved by 14% w/v SDS-PAGE using an acrylamide to bisacrylamide ratio of 180:1 and produced bands having molecular weights consistent with those calculated from the cDNA sequence.

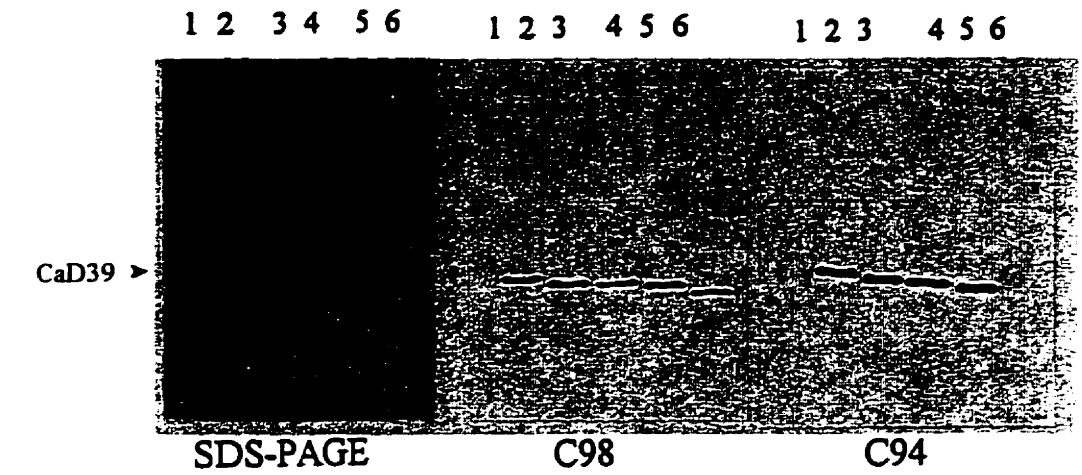
Western blotting using C21 showed it could react to the CaD39 WT protein, site A mutation protein, site B mutation protein, and site C mutation protein but not site D mutation protein and protein from CaD39 deletion D (Fig. 4.5). This result suggested that a mutation of one amino acid, tryptophan to alanine, in the actin-binding site of caldesmon changed the epitope being recognized by this mAb. C94 interacted with proteins from the CaD39 Trp/Ala mutants in a manner similar to C21, again showing that the epitope recognized by C94 is in the actin-binding site of caldesmon, similar to that of C21. The C98 mAb recognized an epitope which is located outside of the area which had been mutated. Ti106, an anti-Ti II mAb, also interacted with CaD39 WT protein, site A mutation product, site B mutation product, and site C mutation product in a manner similar to that of C21. This cross-reactivity confirms the previous result indicating that the actin-binding site of caldesmon and the immunoglobulin-type class II motif of titin share a similar epitopic structure.

DISCUSSION

In constructing the CaD39 deletion mutants, some spontaneous mutations or deletions were obtained as shown in Table 4.1. These resulted from the recombinant PCR methodology used where Taq DNA polymerase was utilized to amplify the DNA. Taq DNA polymerase is heat stable, making it a suitable enzyme for PCR. Nonetheless, Taq polymerase does not have proof reading activity and has been shown to incorporate 1 incorrect nucleotide for

Figure 4.4. Interaction of the CaD39 deletion mutants with anti-caldesmon and anti-Ti II mAbs.

Proteins expressed from the CaD39 construct and its deletion mutants were resolved by 14% w/v SDS-PAGE using a 180:1 ratio of acrylamide to bisacrylamide and transferred onto nitrocellulose membranes. For immunoblotting, the primary antibodies used were C21, C94, C98, Ti104 and Ti106. The secondary antibody used was AP-labelled goat anti mouse Igs. Antibody binding was revealed by NBT and BCIP substrate. The loading pattern of the samples was: 1, pET3d containing no recombinant protein; 2, CaD39 WT product; 3, CaD39 del. A product; 4, CaD39 del. B product; 5, CaD39 del. C product; 6, CaD39 del. D product. The C98 mAb interacted with wild type CaD39 protein and all the CaD39 deletion mutants proteins. The C94 and C21 mAbs interacted with CaD39 WT protein, del. A protein, del. B protein and del. C protein, but not del. D protein, which lacks the actin-binding site of caldesmon. The Ti104 and Ti106 mAbs interacted with the CaD39 WT product and the products from deletion mutants A, B and C only, similar to the C21 mAb.


1 2 3 4 5 6
1 2 3 4 5 6
1 2 3 4 5 6
CaD39
SDS-PAGE
C98
C94

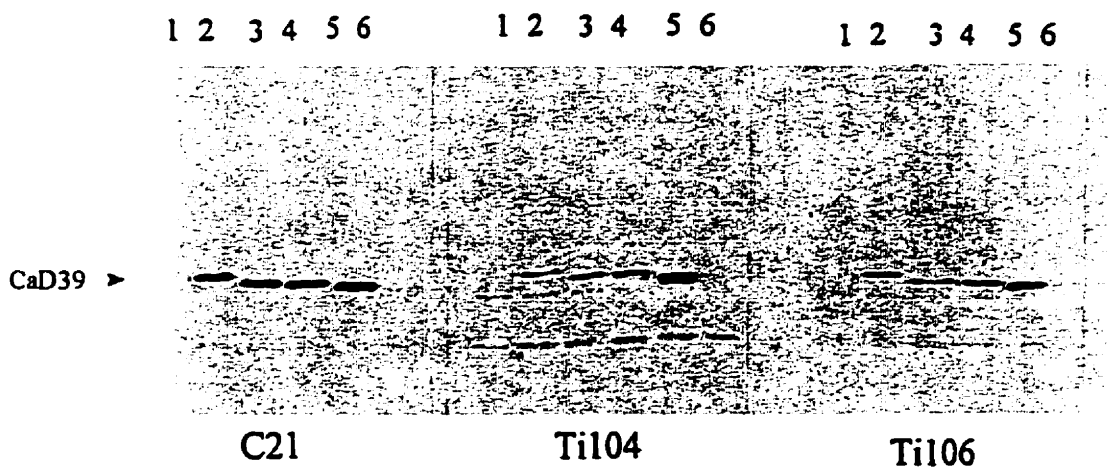
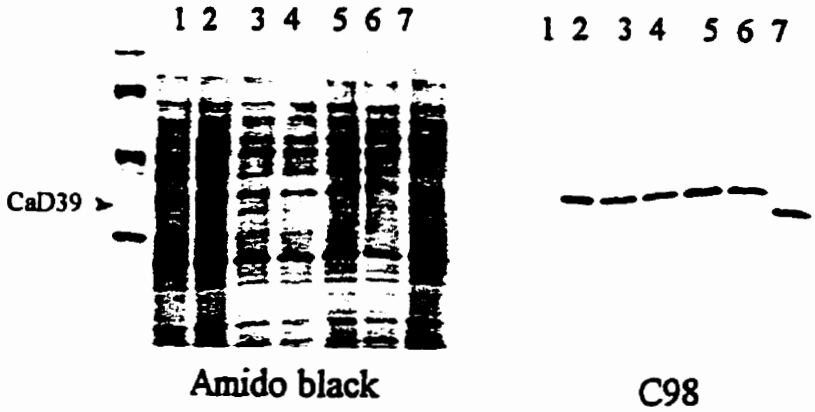
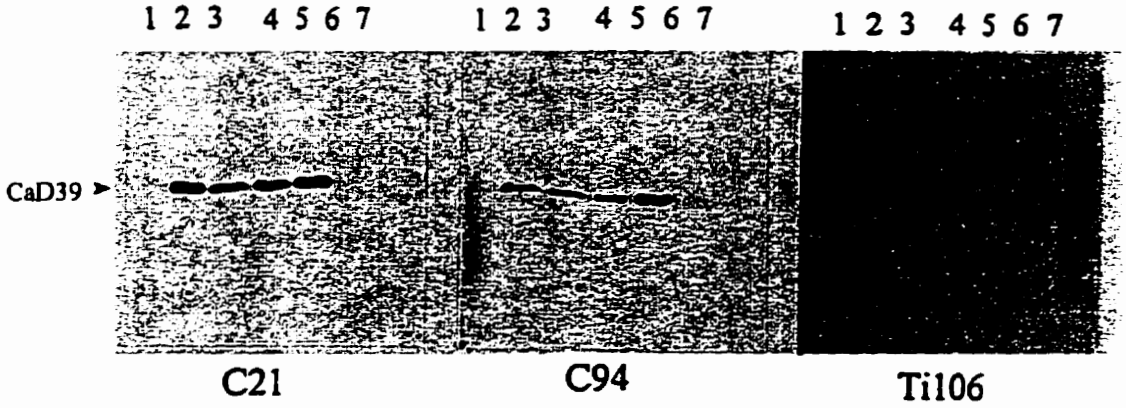

1 2 3 4 5 6
1 2 3 4 5 6
1 2 3 4 5 6
CaD39
C21
Ti104
Ti106

Figure 4.5. Interaction of the CaD39 Trp/Ala mutants with anti-caldesmon and anti-Ti II mAbs.

Proteins expressed by the CaD39 Trp/Ala mutants were resolved on 14% w/v SDS-PAGE as before together with the wild type CaD39 product and CaD39 del. D product as controls. A set of samples probed with the Ti106 mAb was resolved on 12% w/v SDS-PAGE using a 29:1 ratio of acrylamide to bisacrylamide. The expressed proteins were transferred onto nitrocellulose membranes and immunoblotting was performed using C21, C94, C98 and Ti106 mAbs. The secondary antibody was using AP-labelled goat anti mouse Igs. Antibody binding was revealed by NBT and BCIP substrate. The loading pattern was: 1, pET3d product; 2, CaD39 WT product; 3, Site A mutation product; 4, Site B mutation product; 5, Site C mutation product; 6, Site D mutation product; 7, CaD39 del D product. Immunoblotting using the C98 mAb as the primary antibody showed it interacted with CaD39 WT product, CaD39 Trp/Ala mutants products and CaD39 del D product. The C21 and Ti106 mAbs interacted similarly with site A, B and C mutation products but not site D mutation product. The C94 mAb interacted with the products from CaD39 Trp/Ala mutants in a similar way to that of the C21 mAb.


1 2 3 4 5 6 7
CaD39
1 2 3 4 5 6 7
Amido black
C98


1 2 3 4 5 6 7
1 2 3 4 5 6 7
1 2 3 4 5 6 7
CaD39
C21
C94
Ti106

every 2 x 10^4 nucleotides incorporated (Watson *et al.*, 1992). To overcome this problem, the number of PCR cycles used were reduced from 35 cycles to 25 cycles, and the units of Taq polymerase used were minimal.

In the CaD39 WT construct and deletion mutants, the results show that C21 recognizes CaD39 WT product, CaD39 del. A, B and C products, but not del. D product. In the CaD39 del. D product, the actin-binding site was deleted. The lack of recognition of C21 of CaD39 del. D product strongly supports the report (Lin *et al.*, 1991) which shows that the epitope recognized by C21 is in the actin-binding site of caldesmon. In the experiment described in this chapter, the epitope was found to be localized to a region within residues Leu495-Leu523 in l-caldesmon, as reported in Mezgueldi *et al.* (1994). The fact that there is cross-reactivity of Ti104 and Ti106 mAbs to the CaD39 WT protein and its deletion mutants proteins, and of C21 to the same products, confirms the previous finding that Ti104, Ti106 and C21 all bind to a similar epitope in caldesmon and titin. The result shown here indicates the precise position of that epitope to be in the actin-binding site of caldesmon. The precise location of the epitope (s) for the Ti104/Ti106 mAbs remains to be determined. The way in which C94 reacts to the CaD39 protein and its deletion mutants proteins, which is similar to that of C21, suggests that the epitope it recognizes is located in the actin-binding site of the COOH-terminal domain of caldesmon. The C98 mAb reacted to CaD39 WT product and all the products of its mutants, suggesting the epitope it recognizes is located in an area outside of the deletions. The result in Chapter 3 Fig. 3.1.A shows that C98 weakly recognizes the Ti II fragment. This experiment demonstrates that the Ti II motif fragment and the actin-binding site of caldesmon possess a similar epitopic structure. This suggests that the

epitope recognized by C98 must be located in the actin-binding site of caldesmon. Nevertheless, Fig. 4.1 shows that the epitope recognized by C98 is not localized to the actin-binding site of caldesmon but to a surface site of caldesmon that still has some structural similarity to the Ti II motif fragment. Also in Chapter 3, the results indicate that the anti-Ti II mAbs do not cross-react to the 6.4 kDa caldesmon synthetic peptide that contains the sequence of caldesmon upstream of the actin-binding site. This shows that the Ti II motif fragment does not have a structure similar to that site. Taken together, these results suggest the epitope recognized by C98 is likely to be located in the COOH-terminal of caldesmon (Glu525 to Val538 in l-caldesmon), downstream and adjacent to the actin-binding site.

Western blotting of the CaD39 Trp/Ala mutants proteins using C21 shows that substitution of a single amino acid at the very end of the actin-binding site of caldesmon changed the epitope recognized by C21, resulting in loss of recognition (Fig. 4.4). Immunoblotting using Ti106 shows consistently that this mAb recognizes an epitope that is similar to the structure recognized by C21. In other words, titin and caldesmon have been shown to share a similar structure as represented by the fact that they contain epitopes which are recognized by the Ti104/Ti106 and C21 mAbs. The ability of C94 and C98 to recognize CaD39 Trp/Ala mutants indicates that the epitope recognized by C94 is in the actin-binding site of caldesmon and the epitope recognized by C98 is in a region outside of the mutations. According to the results shown in Chapter 3 and in this chapter, the epitope recognized by C98 is located in the COOH-terminal of caldesmon, downstream and adjacent to the actin-binding site.

In conclusion, the experimental results described in this chapter indicate that the

immunoglobulin-type domains of titin and caldesmon share similar epitope structures which are located in the confirmed actin-binding site of caldesmon and in a putative actin-binding site of the Ti II motif fragment. The epitope recognized by C94 has been shown to be in the actin-binding site of caldesmon and the epitope recognized by C98 has been shown to be in the COOH-terminal of caldesmon, downstream and adjacent to the actin-binding site.

CHAPTER FIVE:

TI II TITIN EPITOPE LOCALIZATION IN THE SARCOMERE

INTRODUCTION

Several experiments described in the previous chapters have shown the cross-reactivities of anti-caldesmon mAbs to titin and anti-titin mAbs to caldesmon. Strong cross-reactivity is especially shown by C21 to titin or titin fragments, and Ti104 to caldesmon or caldesmon fragments. These results suggest a similar epitopic structure shared between the actin-binding site of caldesmon, recognized by C21, and the epitope recognized by Ti104 in the Ti II fragment.

The anti-Ti II mAbs Ti102, Ti104 and Ti106 were raised against titin class II motif which is present in the A-I junction. It has been reported that immunofluorescence staining of intact rat cardiac myofibrils using Ti102 and using rhodamine-conjugated anti mouse IgG as a secondary antibody showed wide I-band staining and A-I junction labelling in the intact cardiac sarcomere (Jin, 1995). Preliminary data from experiments in which intact myofibrils stained using Ti106 gave similar results to that using Ti102 staining. Immunofluorescence staining on intact myofibrils using Ti104, however, failed to show any staining within the sarcomere. This result suggests that Ti102, Ti104 and Ti106 each recognizes a different epitope within the Ti II fragment. It also suggests that the epitope within the Ti II fragment that is recognized by Ti104 is covered by other proteins within the sarcomere which come in contact with titin in the I-band.

Based on the finding that titin class II motif is found in tandem repeats in the I-band

(Labeit and Kolmerer, 1995) and the finding that Ti II binds to F-actin but not to myosin in solid-phase binding assays and cosedimentation assays (Jin, 1995), it has been proposed that the epitope recognized by Ti104 is the actin-binding site of Ti II which in intact myofibrils is covered by the actin filament.

To reveal the epitope recognized by Ti104, the actin filament is removed and the extracted myofibrils are stained using Ti104 and rhodamine-conjugated anti mouse IgG as the secondary antibody. The fluorescence staining on those extracted myofibrils indicates that the epitope recognized by Ti104 is localized in the actin-binding site of Ti II.

To remove the actin filament from the myofibril, a 45 kDa NH_2-terminal gelsolin fragment (FX45) that contains amino acid residues 1-406 of intact gelsolin (Kwiatkowski *et al.*, 1986) was used. Gelsolin is an actin-modulating protein found in a number of organism from lower eukaryotes and to mammals, that severs the actin filament (Stossel *et al.*, 1985). Funatsu *et al.*, (1990) have introduced gelsolin for removing the actin filament from sarcomeres of skeletal muscles. However, the calcium that is required for optimal actin-severing activity of intact gelsolin would occasionally induce proteolysis and cause structural damage that complicates the interpretation (Granzier and Wang, 1993; Kruger *et al.*, 1991; Funatsu *et al.*, 1990). Yu *et al.* (1991), have demonstrated that substitution of intact gelsolin with FX45 effectively severs the actin filament in the absence of calcium. The small size of FX45 also facilitates accessibility to the actin filament (Granzier and Wang, 1993).

As a control for distinguishing intact myofibrils from the extracted myofibrils, an anti-TnI mAb was used to stain both the intact and extracted myofibrils. TnI is a thin filament protein that binds actin (Leavis and Gergely, 1984). The anti-TnI mAb was made by Fang-

Wei Yang in our laboratory, and has been shown to interact specifically with TnI from various striated muscle samples. In intact myofibrils, anti-TnI will stain the thin filament in the sarcomere. When the actin is extracted from the myofibril, TnI will also be removed. Thus, anti-TnI will not label these sarcomeres.

The results of the experiments show that the selective removal of actin filaments exposes the epitope recognized by Ti104. This demonstrates that the epitope recognized by Ti104 is localized in the actin-binding site of Ti II. Together with the findings in the previous chapters, this finding suggests that the structural similarity between titin and caldesmon is found to be in the actin-binding site of titin and the actin-binding site of caldesmon.

MATERIALS AND METHODS

A. Myofibril Preparation

Sprague-Dawley (SD) rat cardiac myofibrils were prepared at 4°C. Myofibrils were obtained from fresh SD rat cardiac muscle. Perfusion of the rat heart was carried out using a buffer containing 0.1 M KCl, 2 mM $MgCl_2$, 2 mM EGTA, 10 mM Tris-HCl pH 6.8, 2 mM $Na_4P_2O_7$, 0.5 mM DTT and 0.1 mM PMSF. The buffer was injected through the aortae and vena cava until the cardiac muscle was soft and pale. Rat cardiac muscle was then blended in 50 mL of the same relaxing buffer using a high-speed blender for three 15 second bursts with 1 min intervals in between. The blended cardiac was passed through 2 layers of cheese cloth and centrifuged in a JA14 rotor at 3,500 rpm, 4°C for 15 min. The precipitate was suspended in 50 mL relaxing buffer (0.1 M KCl, 2 mM $MgCl_2$, 2 mM EGTA, 10 mM Tris-HCl pH 6.8, 0.5 mM DTT and 0.1 mM PMSF) by gentle mixing and passed through 2 layers of cheese

cloth. The cardiac muscle suspension was centrifuged in a JA14 rotor at 3,500 rpm for 15 min at 4°C. This washing step was repeated 3 times. After the 3rd wash, the precipitate was resuspended in 50 mL of the same relaxing buffer containing 0.5% v/v Triton-X 100. To check if the myofibrils were well separated, a drop of myofibril was placed on a glass slide and examined under the light microscope. If the myofibrils were not well separated, the solution was blended and washed as before until the myofibrils were suitably separated. The last precipitate was resuspended in 20 mL of relaxing buffer containing 50% v/v glycerol and the myofibril solution was stored at -20°C.

B. Purification of Cloned FX45 Gelsolin Fragment

The cloned FX45 NH_2-terminal fragment of gelsolin (amino acid residues 1-406) was constructed in the pET3 expression vector (a gift from Dr. H.L. Yin, University of Texas Southwestern Medical Centre). The pI calculated from its sequence was 7.35 and the molecular weight was 45 kDa. The protein purification was as follows. Cloned FX45 in the pET3 vector was transformed into host *E. coli* BL21(DE3)pLysS and plated on the LB agar containing 100 μg/mL ampicillin and 25 μg/mL chloramphenicol for an overnight incubation at 37°C. Four fresh colonies were suspended by vortexing in 1 mL of 2x TY medium containing the ampicillin and chloramphenicol as before. The suspension (20 μL) was used to inoculate 1 L of 2x TY medium containing 100 μg/mL ampicillin and 25 μg/mL chloramphenicol. A total of 2 L of medium was inoculated with the bacteria. The culture was incubated in a shaker at 300 rpm, at 37°C for 9 hours until $OD_{600\ nm}$ = 0.796. IPTG was added to 0.4 mM and the culture was incubated for an additional 3 hours. It was followed by

centrifugation at 3,500 rpm, 4°C for 15 min in a clinical centrifuge, and the bacteria were resuspended in 40 mL of 50 mM Tris-HCl pH 8.0 and 2.5 mM EDTA. Three passages through a French press cell press at 500-1,000 p.s.i. was applied to the cell suspension. The lysate obtained was centrifuged at 3,000 rpm, 4°C for 15 min in a clinical centrifuge. The supernatant was again centrifuged in a JA20 rotor at 15,000 rpm, 4°C for 15 min to clear the lysate. The precipitate was dissolved in 100 mL of washing buffer (10 mM Tris pH 8, 0.1 mM EDTA and 0.05% v/v Triton-X 100) and centrifuged in a JA14 rotor at 12,000 rpm, 4°C for 10 min. This washing step was repeated once more. The precipitate from the second washing was dissolved in 20 mL ddH_2O by gentle mixing on ice. At this step the solution was milky and the pH measured was ~ 11. As soon as the precipitate was dissolved, Tris-HCl pH 9.0 was added to 100 mM until the solution was clear and the pH was 9.0. After centrifugation in a JA20 rotor at 12,000 rpm, 4°C for 10 min, the supernatant was loaded onto a 49 mL DE-52 anion-exchange column (10 x 2.5 cm) pH 9.0 equilibrated in 20 mM Tris-HCl pH 9.0 containing 0.1 mM EDTA. After washing with 100 mL of equilibration buffer, the column was eluted at 1 mL/min with a linear gradient of 0-300 mM KCl in the same buffer for a total of 300 mL. The fractions from the column identified by the $A_{280\ nm}$ absorbance peaks were resolved by 12% w/v SDS-PAGE. Fractions containing FX45 were collected according to its purity and divided into three different pools. All pools were dialysed against 3 changes of 4 L of 1 mM Tris-HCl pH 8.0 and 6 mM β-mercaptoethanol. The protein suspension was lyophilized and 50 mL of pool II was made as a working stock by dialysing in 50 mL of buffer containing 20 mM MOPS pH 7.0, 2 mM EGTA, 2 mM $MgCl_2$, 0.2 mM PMSF, 30 mM β-mercaptoethanol, 50 mM NaCl and 20% v/v glycerol. Glycerol was later added to 28.5% v/v.

C. Removal of the Actin Filament from Myofibrils Using FX45

To remove the actin filament from myofibrils, approximately 100 μg of FX45 was incubated with 250 μL of rat cardiac myofibrils that were adhered to a glass slide, in a humid petri dish at room temperature for 10 min. The myofibrils were then washed by three 5 min incubations in relaxing buffer (40 mM imidazole pH 7.0, 10 mM EGTA, 6.4 mM magnesium acetate, 5 mM sodium azide, 70 mM KOH, 70 mM propionic acid, 10 mM creatine phosphate, ATP to 5.9 mM and leupeptin to 20 μg/mL) followed by 30 min fixation in 3.7% v/v formaldehyde/relaxing buffer.

D. Immunofluorescence Microscopy

For the purpose of fluorescence staining, the myofibrils were washed once using the relaxing buffer. The procedure was done in a humid petri dish at room temperature. To attach the myofibrils onto the glass slide, 0.2% w/v gelatin was coated onto the glass slide for 15 min. Myofibrils were then sedimented on the glass slide for 20-30 min, and fixed with 3.7% v/v formaldehyde/relaxing buffer for 30 min. After washing twice for 2 min with the relaxing buffer, blocking solution containing 1% w/v BSA/relaxing buffer was applied for 30 min. Incubation with the primary antibodies was carried out for 1.5-2 hours using 1/30 dilution of Ti102 and Ti104, and 1/10 dilution of anti-TnI hybridoma culture supernatant as a control to test extractions. It was followed by three changes of 2 min washes with relaxing buffer and incubation of the slide in 1/30 dilution of rhodamine-conjugated anti mouse IgG as secondary antibody (Sigma) for 1 hour. After three final washes, the slide was mounted with a cover slip and viewed under a Nikon Optiphot X-2 phase contrast-epifluorescence microscope.

Photographs of the phase-contrast and fluorescence images were taken with CF phase Fluor DL 60x and 100x objective lenses with a Nikon Microflex UFX-DX automated photographic system.

The immunofluorescence staining of the extracted myofibrils was carried out by the same procedure as above. Actin filament removal was carried out before the fixation step, as described earlier.

RESULTS

A. Expression and Purification of FX45

Using the recombinant pETFX45 plasmid-transformed BL21(DE3)pLysS *E. coli* culture, FX45 was successfully expressed upon IPTG induction. In carrying out large scale culture, it was important to use freshly transformed bacterial colonies as well as a rich medium. Samples collected from the steps before DE-52 chromatography were resolved by 12% w/v SDS-PAGE. The samples showed a large amount of FX45 with contaminating bacterial proteins. Subsequent washes using Triton-X 100 removed many bacterial protein contaminants (Fig. 5.1 samples before column). After purification by DE-52 anion-exchange chromatography, samples from fractions around the $A_{280\ nm}$ peak were analyzed by SDS-PAGE and showed a large amount of pure FX45 protein, and some impure proteins. The purification method with a washing step using Triton-X 100 proved to be effective for proteins such as FX45 that are expressed in the bacterial inclusion bodies. After DE-52 chromatography, no further purification step was needed. The fractions containing pure FX45 were pooled separately from the impure FX45 and were used in the myofibril experiment.

B. The Conditions for Extraction of the Thin Filaments

Analysis of the conditions of extraction was carried out by examining the mAb staining on intact and extracted myofibrils. The analysis was done on several images, representing different fields, taken from two sets of experiments. All myofibrils present in the field of view were counted and the numbers of myofibrils stained by each of the mAbs were identified. As a control for the extraction conditions, the myofibrils stained by anti-TnI were initially analyzed. In intact myofibrils, anti-TnI was shown to stain all myofibrils, as indicated by fluorescence on all of the myofibrils (Table 5.1). This staining has been counted also as staining of actin, because TnI is a component of the thin filament that is mainly formed by the actin filament. Removal of actin by FX45 will also remove TnI from the myofibril. This is confirmed by the data in extracted myofibrils (Table 5.1), showing that the majority of myofibrils (87.5%) were not stained by anti-TnI mAb, as indicated by no fluorescence. A minority (12.5%) of myofibrils were not extracted, this is possibly because the amount of FX45 was not enough to sever all actin filaments. The result of the myofibril extraction by FX45 agrees the results of Granzier and Wang (1993) who used an actin antibody to show actin removal. These results suggest that the extraction procedure effectively removed actin and this approach has applied in subsequent experiments to investigate staining by Ti102 and Ti104.

Staining of intact and extracted myofibrils using Ti102 showed 98-100% of myofibrils in both conditions with fluorescence. This suggests that the epitope to which Ti102 reacts on the Ti II fragment is in an exposed area, not covered by the actin filament. Thus, the presence or absence of actin filaments does not influence Ti102 staining. In intact myofibrils stained

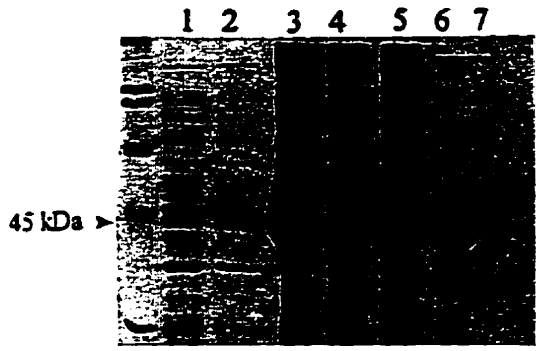
Figure 5.1. Purification of cloned FX45.

Cloned pETFX45 was transformed into host BL21(DE3)pLysS *E. coli* to obtain protein expression upon IPTG induction. The bacterial lysate was washed subsequently with Triton-X 100 and purified using DE-52 anion-exchange chromatography.

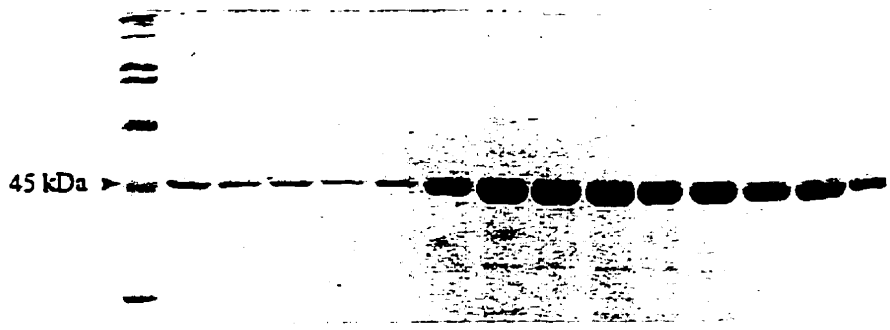
A. Protein expression in *E. coli* and samples from different steps of the purification were resolved by 12% w/v SDS-PAGE. Samples were loaded as follows: 1, protein expression in BL21(DE3)pLysS without IPTG; 2, protein expression in BL21(DE3)pLysS upon IPTG induction; 3, sample of French press lysate; 4, first spin precipitate after French press; 5, second spin precipitate after French press; 6, supernatant before DE-52 column; 7, precipitate before column. Lanes 3-5 showed a lot of contaminating bacterial proteins present in the samples which were reduced by subsequent washes with Triton-X 100 (lane 6). Lane 7 showed a small amount of FX45 in the precipitate.

B. The final result after DE-52 column chromatography from various fractions around the $A_{280\,nm}$ peak were resolved by 12% w/v SDS-PAGE, showing pure FX45 and impure FX45 containing a small amount of contaminant.

A



B



with Ti104, 90.5% of myofibrils were not stained by the antibody. This suggests that the epitope recognized by Ti104 in Ti II was not exposed. In analysing the extracted myofibrils stained by Ti104, it was found that only 38.2% of myofibrils showed fluorescence.

The analysis of extraction conditions assessed by no anti-TnI staining on the extracted myofibrils showed that 87.5% of myofibrils were extracted. The extracted myofibrils stained by Ti104 showed 38.2% fluorescence, suggesting that the Ti104 epitope was still covered by other protein (s) after actin removal.

C. Immunofluorescence Microscopic Localization of Anti-TnI, Ti102, and Ti104 on Intact and Extracted Myofibrils

The immunofluorescence staining on intact rat cardiac myofibril using anti-TnI showed the I-band labelled by this mAb (Fig. 5.2). This result indicates the presence of TnI, a thin filament protein that binds to actin, in the I-band of the intact sarcomere. Thus, it also indicates the presence of actin. Staining of FX45 extracted myofibrils using anti-TnI mAb showed no staining, indicating that TnI, as well as actin, were removed from the myofibrils. Ti102 labelled the I-band and A-I junction in intact sarcomeres. Ti102 was raised against Ti II which is present in the A-I junction of sarcomeres. This result indicates that the epitope recognized by Ti102 is localized in a wide area of the I-band and A-I junction, showing that titin class II motifs form a major portion of the I-band and are also present in the A-I junction as revealed by the sequence data (Maruyama, 1993; Labeit and Kolmerer, 1995).

Ti104 did not label the intact sarcomere. This suggests that the epitope recognized by Ti104 is localized in the actin-binding site in the intact sarcomere and was covered by the

mAb	conditi on of MF	# of exp.	# of images	total MF	(+) fluores cence	% of (+) fl.	(-) fluores cence	% of (-) fl.
α-TnI	intact	2	5	21	21	100%	0	0%
α-TnI	extract.	2	8	72	9	12.5 %	63	87.5%
Ti102	intact	2	5	59	58	98.3%	1	1.7%
Ti102	extract.	1	2	11	11	100%	0	0%
Ti104	intact	2	6	42	4	9.5%	38	90.5%
Ti104	extract.	2	6	34	13	38.2%	21	61.8%

Table 5.1. Analysis of mAbs staining on intact and extracted myofibrils.

Intact and extracted myofibrils were stained using anti (α)-TnI, Ti102 and Ti104 mAbs and rhodamine-conjugated anti mouse IgG as a secondary antibody. Anti-TnI mAb stained all intact myofibrils and 87.5% of extracted myofibrils, suggesting that the extraction conditions are correct. Ti102 mAb stained all intact and extracted myofibrils, suggesting the epitope it recognizes is localized in the exposed area, not covered by the actin filament. Ti104 mAb stained only 9.5% of intact myofibrils, suggesting that the epitope it recognizes was covered by other protein. After actin removal, only 38.2% of myofibrils were stained by Ti104. This indicates that beside actin, other proteins may cover the epitope of Ti104 on titin.

actin filament. Removal of the actin filament using FX45 allowed this epitope to be accessible to Ti104, as shown in Fig. 5.2. In the extracted myofibrils, Ti104 labelled an area in the I-band. By comparing the size of the thick and thin filaments in the phase-contrast and fluorescence picture, it is shown that the A-I junction is not clearly labelled by Ti104. This result indicates that the epitope to which the Ti104 mAb binds is in the actin-binding site of Ti II, and that is located in the I-band region. However, not all of the extracted myofibrils could be labelled by Ti104. As shown in Table 5.1, only 38.2% of the extracted myofibrils were labelled by Ti104, compared to 87.5% labelled by the anti-TnI mAb. This data suggests that removal of the actin filament did not completely reveal the epitope to which Ti104 binds in the sarcomere. It is possible that another protein may cover this epitope or more likely, the A-I junction structure is different from the I-band region.

DISCUSSION

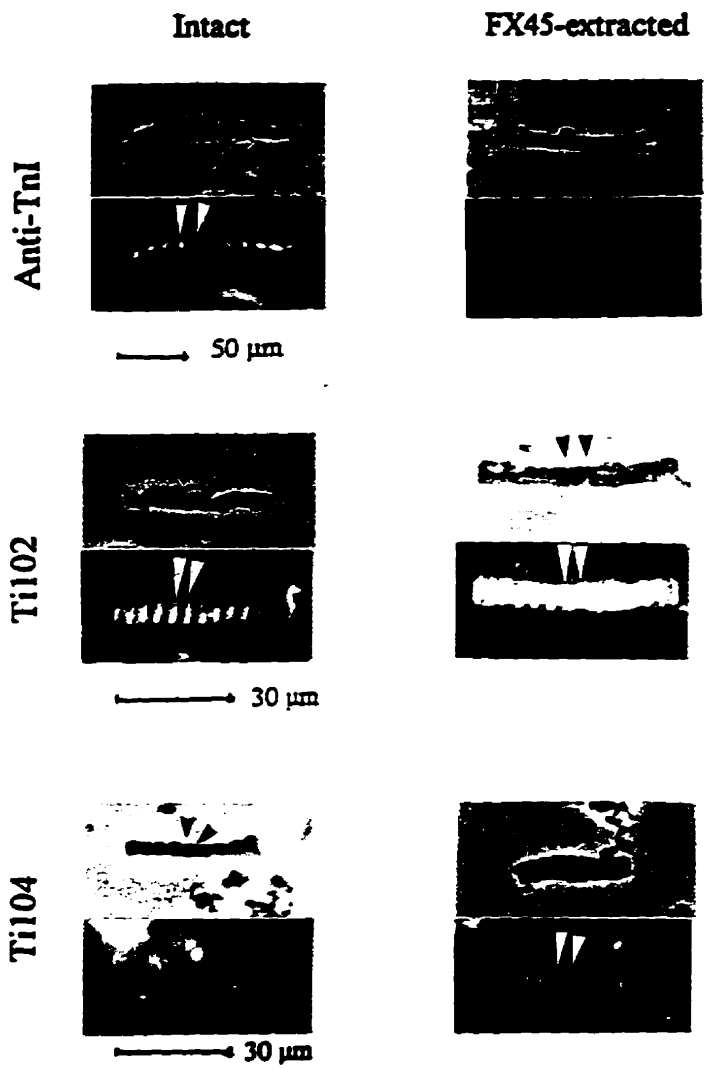
In this chapter, the epitope to which the anti-Ti II mAbs, Ti102 and Ti104 bind within the sarcomere was investigated. To reveal the epitope which Ti104 recognizes, hypothesized to be in the actin-binding site of Ti II, the actin filament was removed from the sarcomere using FX45.

As a 45 kDa NH_2-terminal gelsolin fragment, FX45 effectively severs actin filaments from myofibrils (Granzier and Wang, 1993b). The purification method applied for cloned FX45 yielded large quantities of highly purified FX45 fragment (Fig. 5.1).

The immunofluorescence staining on intact and extracted myofibrils using anti-TnI mAb with rhodamine-conjugated anti mouse IgG as secondary antibody showed the

Figure 5.2. Immunofluorescence microscopic localization of anti-TnI, Ti102 and Ti104 epitopes in the sarcomere.

Anti-TnI stained a wide region of the I-band, indicating the presence of TnI as a component of the thin filament along the entire I-band, thus also indicating the presence of the actin filament. In myofibrils extracted by FX45, actin was removed, as was TnI, resulting in no fluorescent staining on the I-band. Ti102 stained a region in the I-band, including the A-I junction in intact and extracted myofibrils, suggesting that the epitope it recognizes is localized in the exposed site of Ti II, not covered by other protein (s). Ti104 showed no staining within intact myofibrils, but fluorescence in the I-band in extracted myofibrils indicated that the epitope it binds to is localized in the actin-binding site of Ti II, and that in the intact myofibrils this site was covered by actin.


Intact
FX45-extracted
Anti-TnI
50 μm
Ti102
30 μm
Ti104
30 μm

effectiveness of thin filament removal from the myofibrils using FX45. Since TnI binds actin (Leavis and Gergely, 1984), it is considered that the lack of staining of anti-TnI mAb on extracted myofibrils indicates the absence of TnI and in turn the absence of actin in that myofibril. Therefore, these extraction conditions are suitable for use in experiments using Ti102 and Ti104 mAbs. However, the anti-TnI staining on the myofibrils can not be used to evaluate the actin removal in the A-I junction. This region contains thin and thick filaments and the TnI epitope is likely to be covered by the thick filaments. This makes anti-TnI mAb inaccessible to its epitope in this A-I junction region.

In comparing the result of Ti102 staining on intact and extracted myofibrils (Table 5.1 and Fig. 5.2), it can be seen that there was no difference in the results between the two conditions. The epitope to which Ti102 binds was in the exposed area of Ti II in the I-band and A-I junction of the sarcomeres. This result also suggests that Ti II forms a major part of I-band titin and is also present in the A-I junction region.

The Ti104 mAb did not stain the intact myofibrils, suggesting that its epitope was covered by other protein (s). It was proposed that the epitope to which Ti104 binds is localized in the actin-binding site of Ti II, which is covered by the actin filament in the intact myofibril. After actin removal, Ti104 labelled the I-band. This result strongly confirms the hypothesis that the epitope Ti104 recognizes is localized to and is indeed in the actin-binding site of Ti II. The result that only 38.2% of the extracted myofibrils were labelled by Ti104 whereas using the same conditions 87.5% of actin was extracted from the myofibril (as revealed by lack of staining of extracted myofibrils by anti-TnI mAb), indicates that there could be other protein (s) which need to be removed to completely reveal the epitope of

Ti104. This also indicates that the structure of the A-I junction is different from the I-band. To completely reveal the epitope to which Ti104 binds in the sarcomere, immunofluorescence microscopy could be done on fully stretched intact and FX45-extracted myofibrils. In fully stretched sarcomere, thin and thick filaments do not overlap each other and that makes Ti II epitopes in titin more accessible to Ti104. This finding invites further experimental studies to clarify the arrangements of the organizational proteins in the cardiac sarcomeres.

In conclusion, the experiment described in this chapter demonstrates that the similar structure found between the actin-binding site of the immunoglobulin-type domain of titin and the actin-binding site of caldesmon. This finding strongly supports the hypothesis that titin and caldesmon may perform analogous functions.

CHAPTER SIX:

SUMMARY

The experimental results described in this thesis could be summarized as follows:

1. Cross-reactivities of anti-Ti II mAbs to caldesmon and anti-caldesmon mAbs to titin demonstrate that titin and caldesmon have a convergent structure. It means that both proteins originate from different genes, but in the end they have a similar epitopic structure.

2. The convergent structure is found in the three dimensional structure. This is supported by the result that showed weak cross-reactivities between small fragments of Ti II (3 kDa chymotryptic fragment) to anti-caldesmon mAbs and small fragments of caldesmon (10 kDa CNBr fragment and synthetic peptide) to anti-Ti II mAbs. The amino acid sequence alignment also indicates no significant sequence homology between the small fragment of Ti II and the small fragment of caldesmon.

3. Using the CaD39 deletion mutants, it was shown that the convergent structure is found to be in the actin-binding site of caldesmon.

4. Immunofluorescence staining on FX45-extracted myofibrils showed that the convergent structure, as presented by epitope recognized by the Ti104 mAb, is located in the actin-binding site of Ti II.

In conclusion, all the experiment results consistently showed that titin and caldesmon have a convergent structure. These results suggest that although titin and caldesmon originate from different genes, they may perform an analogous function. This finding leads to a new model in understanding the organization of the contractile filaments in smooth muscle/non-

muscle which could be similar to that of striated muscle. In other words, caldesmon may play a significant role in organizing smooth muscle/non-muscle contractile filaments as the role of titin in striated muscle. This finding also suggests a possibility that titin-actin interaction in striated muscle could be regulated in a manner similar to regulation of caldesmon-actin in smooth muscle.

For future studies, the analogous function of titin and caldesmon could be further investigated. For example, an indirect protein binding assay could be carried out to investigate the caldesmon-actin interaction. To investigate the regulation of titin-actin interaction, protein kinase assays could be performed.

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APPENDIX:

PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST BOVINE CALPONIN

INTRODUCTION

Monoclonal antibodies (mAbs) are widely used and are critical in this research. Therefore, it is advantageous to be familiar with the protocol. Since the mAbs used in this research were previously available in the laboratory, an alternative antigen, bovine calponin, was used to complete the mAb production/hybridoma protocol.

The purpose of producing mAbs is to obtain a homogenous antibody that responds specifically to a single epitope. In a typical immune response, most antigens that consist of a mixture of molecules will produce antibodies where the response is directed against many different regions or epitopes on each molecule (Goding, 1996). Monoclonal antibodies overcome this problem by producing an antibody specific to a single epitope on the antigen (Koehler and Milstein, 1975, 1976). In producing mAbs, the immune response is dissected into its component parts by culturing the single clones of antibody-forming cells (Goding, 1996).

The significant property of the immune system is its ability to distinguish self from non-self. An antigen that enters the body will be seen as non-self and the immune system will phagocytose it. Some phagocytic cells are specialized to present the antigen to the T and B lymphocytes. Within 2-3 days, the B cell response of specific IgM antibodies will be detectable. The avidity of IgM in binding to the antigen is very high and it is also efficient in

activating the complement system. Three or four days after the primary response, the immune response will switch to IgG with the help of T cells. Four subclasses (isotypes) of human IgG have been identified as IgG1, IgG2, IgG3 and IgG4 (Roitt *et al.*, 1993). The affinity of IgG will increase but if there is no additional input of the antigen, it will decrease gradually over a period of weeks (Nossal, 1992; Gray, 1993). However, the persistence of the small amounts of antigen that remain attached on the lymphoid follicles of spleen and lymph nodes will sustain the antigenic stimulation over long periods (Nossal, 1992; Gray, 1993; MacLennan, 1994).

The clonal selection theory that applies to B and T lymphocytes stated that the antigen selects for the specific clones of its own antigen-binding cells. Once the antigen binds to the few cells that can recognize it, it induces them to proliferate rapidly (Roitt *et al.*, 1993). The B lymphocytes that are the precursors of antibody-forming cells bear receptor forms of antibodies on their surfaces. The unique rearrangements of antibody genes in each cell ensures that the cell and its progeny are committed to that specificity during subsequent divisions (Goding, 1996). The T lymphocytes have antigen-specific membrane receptors that are similar to antibodies but are coded for by different genes. Each T cell also has a unique rearrangement of its antigen-receptor genes (Goding, 1996).

Most antigens are proteins. The binding site for an antibody or T cell receptor is known as the antigen determinant or epitope. The antigenicity is determined by the sequence difference between the protein and the self counterpart. The greater the difference, the more antigenic it likely is, the greater the number of epitopes available (Goding, 1996). Antigenicity may also be defined or determined by surface accessibility or flexibility. Laver *et al.*(1990) has

demonstrated that the great majority of antigenic determinants recognized by antibodies and B cells are conformational. The recognition of protein antigen by B cells depends on its conformation, because of the stereospecific complementarity of the antigen-antibody complex (Laver *et al.*, 1990). Benjamin *et al.* (1984) and Goodnow *et al.* (1989) have shown that activation and tolerance in B cells will be dependent on stereospecific recognition of the antigen. It has been observed that many antibodies to native proteins do not react with denatured forms of the same proteins, and *vice versa* (Arnon, 1973; Crumpton, 1974). T cell activation and tolerance, however, do not depend on the conformation of the protein antigen. It would be expected that T cell response to native and denatured proteins is equally good (Goding, 1996).

In order to maintain the level of antibody in the mouse during mAb production, the antigen is given in a depot form that is gradually released into the body of the mouse. For this purpose, Freund's adjuvant was used. There are 2 kinds of Freund's adjuvant; complete and incomplete. The complete adjuvant containing *Mycobacterium tuberculosis* which function is to enhance the immune response in a non-specific manner (Audibert and Lise, 1993; Gupta *et al.*, 1993), and this was used in the first immunization. For booster immunizations, incomplete adjuvant without bacteria was used to prevent possible hypersensitivity reactions to the bacteria (Goding, 1996).

To obtain single clones of antibody-forming cells, spleen cells from a mouse immunized with a certain antigen were grown in a culture medium and the clones were diluted. However, spleen cells can only survive in the culture for a few days. In order to maintain the spleen cells in culture, they were fused with myeloma cells that grow well in the

continuous culture. The fusion of the spleen cells and the myeloma cell Sp2/0 is mediated by polyethylene glycol (PEG), and fused cells are grown in a culture medium containing hypoxanthine, aminopterin and thymidine (HAT). The fusion of the membrane of spleen cells and myeloma cells is facilitated by PEG, yielding multinucleate cells called heterokaryons (Ringertz and Savage, 1976; Abbott and Povey, 1995). At the next cell division, the nuclei of the heterokaryons fuse and the daughter cells possess a more or less equal share of the genetic material (Goding,1996). The HAT medium permits only growth of the hybrid cells and unfused spleen cells or myeloma cells die. The selection procedure for the hybrids depends on the fact that when the main biosynthetic pathway for guanosine is blocked by the folic acid antagonist, aminopterin, the biosynthesis will use a salvage pathway in which the hypoxanthine or guanine are converted into guanosine monophosphate using the enzyme hypoxanthine guanine phosphoribosyl transferase (HPRT) (Goding, 1996). The unfused myeloma cells lacking HPRT will not survive in the HAT medium since the main biosynthetic pathway is blocked by aminopterin and the salvage pathway is not available without the HPRT enzyme. Although the spleen cells possess $HPRT^+$ they will not survive for a long time in the culture medium. Only the hybrid cells, possessing $HPRT^+$ from the spleen cells and long survival provided by the myeloma cells, will grow in the HAT medium.

In producing the mAbs described in this study, bovine calponin was used as the antigen. Calponin is a smooth muscle protein located on the thin filament (Walsh *et al.*, 1993) that binds actin (Takahashi *et al.*, 1986; Winder and Walsh, 1990), myosin (Lin *et al.*, 1993; Szymanski and Tao, 1993), tropomyosin (Takahashi *et al.*, 1988; Childs *et al.*, 1992) and calmodulin (Takahashi *et al.*, 1986; Takahashi *et al.*, 1988). Calponin binds to actin and

tropomyosin in Ca^{2+}-independent manner and to calmodulin in Ca^{2+}-dependent manner (Takahashi *et al.*, 1986; Takahashi *et al.*, 1988). It inhibits actin-activated myosin Mg^{2+}-ATPase activity which is alleviated by the phosphorylation of calponin (Winder and Walsh, 1990; Winder *et al.*, 1992). *In vitro* motility assays have demonstrated that calponin inhibits the relative movements of actin and myosin (Shirinsky, *et al.*, 1992; Haeberle, 1994). The association of calponin with actin and its regulation of actin-activated myosin Mg^{2+}-ATPase activity suggests a possible role as a thin filament-based regulatory system in smooth muscle (Lehman, 1991; Walsh, 1991) to modulate contractility. It would occur in addition to the myosin light chain phosphorylation mechanism (Hartshorne, 1987; Stull *et al.*, 1991; Somlyo and Somlyo, 1994). Several studies have reported cDNAs encoding *h1*- and *h2*-calponin (Takahashi *et al.*, 1991; Nishida *et al.*, 1993; Strasser *et al.*, 1993), and acidic calponin (Applegate *et al.*, 1994; Trabelski-Terzidis *et al.*, 1995), each of which is encoded by a different gene. *h1*-calponin has been found as the major calponin in smooth muscle and cDNAs encoding α- and β-isoforms of *h1*-calponin have been found as potential products of alternative mRNA splicing (Takahashi *et al.*, 1991). Gao *et al.* (1995) have isolated and sequenced the entire mouse *h1*-calponin revealing its structural organization and alternative splicing pattern.

MATERIALS AND METHODS

A. Immunization of the Antigen into the Mouse

The antigen bovine calponin was injected intra-peritoneally into a BALB/c mouse 3 times with 1 month intervals between each injection. Each injection contained 100 μg of

calponin in sterile phosphate-buffered saline (PBS, 50 g NaCl, 4.72 g KH_2PO_4, 11.12 g Na_2HPO_4 in 1 L of 10x buffer) to make the volume to 100 μL and mixed into an immersion with 100 μL Freund's adjuvant. The Freund's adjuvant used in the first injection was a complete adjuvant containing *Mycobacterium tuberculosis*. The other two booster immunizations were done with incomplete adjuvant.

B. Enzyme-linked Immunosorbant Assay

The immune response of the mouse was checked 2 weeks after the third injection of the antigen. Serum was taken from the mouse by bleeding the tail. The blood was stored at 4°C and the serum was obtained by centrifuging the blood at 2,500 rpm for 10 min.

Enzyme-linked immunosorbant assay (ELISA) was used to check the antibody titer and the hybridoma cell clones. Each well in the ELISA plate was coated with 100 μL of 10 μg/mL bovine calponin diluted in 1x ELISA coating buffer (0.05 M carbonate buffer pH 9.6). The plate was incubated overnight at 4°C. After incubation, the plate was washed with phosphate-buffered saline (PBS) containing 0.05% v/v Tween-20 three times. For checking the mouse serum, the primary antibody to coat each well of the plate was 100 μL of diluted mouse serum in 0.1% w/v BSA/PBS, with dilutions of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} . To test the hybridoma cell clones, the primary antibody was the hybridoma culture supernatant. The plate was then incubated for 3 hours at 37°C. After 3 times washing with 0.05% v/v Tween-20/PBS, each well of the plate was coated with 100 μL of goat anti mouse labelled with horseradish peroxidase (GAM-HRP) as secondary antibody at a 1/750 dilution in 0.1% w/v BSA/PBS, and incubated for 1 hour at 37°C. The plate was washed 3 times with 0.05% v/v

Tween-20/PBS and 100 μL of [2,2'-azino-di(3-ethylbenzothiazoline sulfonate)] (ABTS) substrate (0.4 g ABTS, 9.72 g sodium citrate, and 3.57 g citric acid in 1 L) containing 0.1% v/v H_2O_2 was added into the wells. The plate was incubated at room temperature for 20 min. The quantity of the titer was determined using an ELISA reader at 405 nm.

C. Cell Fusion Between Spleen Cells and Sp2/0 Cells

Two days before the cell fusion, the mouse was injected with 400 μg bovine calponin intra-muscularly. Cell fusions were performed between the mouse spleen cells and the Sp2/0 mouse myeloma cell line (Goding, 1996). The spleen was taken from a mouse that produced a high titer of antibody.

To prepare Sp2/0 cells, frozen cells were quick thawed in a 37°C water bath, put into a 15 mL tube and spun at 700 rpm for 5 min. The supernatant containing dimethylsulfoxamide (DMSO) was removed and the cells were mixed with 10 mL Dulbecco's modified Eagle's medium (DMEM) containing 1% v/v Penicillin/Streptomycin (PenStrep) and 15% v/v Fetal Calf Serum (FCS). The medium containing Sp2/0 was plated on a petri dish and incubated at 37°C. When the cells were 70% confluent, they were split into 3 petri dishes and incubated at 37°C for 1 day until 80% confluent (confluent cells in a 15 cm petri dish contain ~ 1×10^7 cells) and ready to be used for the cell fusion.

The spleen was taken from the mouse, cleaned of fat and rinsed in a washing medium (1% v/v PenStrep in DMEM medium). Blood was also taken from the mouse to obtain the polyclonal antiserum. The spleen was mashed on a cell strainer in a petri dish using a 1 mL syringe. The spleen cells were diluted in 20 mL washing media, and the total number of cells

was determined by counting the cells on a Neubauer improved field. The number of cells in 16 squares was multiplied by the total volume and the volume conversion factor of the field volume (0.1 μL) to mL.

The myeloma cells from 3 petri dishes were combined and spun at 700 rpm for 5 min. The supernatant was removed and the cells were diluted in 20 mL of washing media. The number of Sp2/0 cells were counted as above.

The spleen cells and Sp2/0 cells were combined in a ratio of 5 spleen cells to 1 Sp2/0 cells. The combined cells in the media were spun at 600 rpm for 10 min. The supernatant was removed and the bottom of the tube was immersed in 37°C water in a beaker. Prewarmed, 0.6 mL of PEG containing 8% v/v DMSO was added into the combined cells over a period of 1 min and the tube was shaken lightly. The cells were mixed with a pipette tip for 30 sec and the tube was further incubated in the warm water for 1 min. Washing media of 1 mL was added into the cells over a period of 1 min, while the tube was shaken. Another 1 mL of washing media was added in the same way. Finally, 8 mL of washing media was added for a period of 5 min. The tube was incubated at 37°C for 10 min. The solution was spun at 450 rpm for 10 min and the supernatant was removed. Cells were resuspended in 10 mL of HAT medium (1% v/v PenStrep, 20% v/v FCS and 1% v/v HAT in DMEM medium) with gentle mixing. This 10 mL of media containing the cells was added into 115 mL of the same media and mixed gently. The cells were spread into six 96-well culture plates. The culture was incubated at 37°C for 5 days.

D. Hybridoma Cell Cloning

Two days after the cell fusion, half of the medium in each well was replaced by the new HAT medium. The cells were further incubated. The medium was changed for the second times with HAT medium 6 days after the fusion, and by HT medium 10 days after fusion. The purpose of replacing the medium is to remove the waste products and replenish the nutrients. It will also dilute any antibody made by unfused spleen cells which may remain alive for a week. After several medium size colonies were seen, the supernatants of the hybridoma cells were tested by the ELISA method as above, using the mouse tail serum as a control. The cells with ELISA reading of greater than 0.050 were cloned. The cloning is important to reduce the risk of overgrowth by non-producer cells (myeloma cells) and to ensure that the antibodies are truly monoclonal (Goding, 1996).

To do the cell cloning, cells from a single well were taken from the cell culture and added into 1 mL of HT medium in 24 well culture plate. Three drops of cells were put back in the 96 wells plate used as back up. To obtain 3 cells/200 μL, the cells were diluted as follows. Cells were counted as explained previously. The average number of cells in a square of 16 squares field was identified. If the average was 1, a drop of cells was mixed with 7.5 drops HT medium (first dilution). A drop of cells from this mixture was then added into 3.5 mL HT medium and mixed. The diluted cells were spread into upper part of 1/3 of a 96 wells culture plate with 2 drops in each well. Another 1/2 drop of cells from the first dilution was added into the rest of the 3.5 mL HT medium, and were spread into the lower part of 1/3 of the culture plate as before. The plates were incubated at 37°C. Hybridoma cell cloning was carried out each time a single clone of medium size was obtained with high antibody titer as

tested by ELISA. Cloning was carried out until the fourth generation of each positive cell line was obtained with a high antibody titer. The cell line that survived entering the next generation was frozen in a freezing medium and stored in liquid nitrogen.

E. Preparation of Feeder Cells

The purpose of adding feeder cells into the hybridoma culture is to provide growth factors, that are important for the growing of the hybridoma cells. Feeder cells were prepared from the spleen of a 3-4 week old mouse. The mouse was sacrificed and disinfected in 70% ethanol. The spleen was taken and mashed on a cell strainer in a petri dish containing 10 mL of washing medium. The mixture was spun at 700 rpm for 5 min. The supernatant was removed and the spleen cells were suspended in 50 mL HT medium. The feeder cells were dropped into 1/3 of 96 wells culture plate, 3 drops in each well, and incubated at 37°C overnight before use.

F. Cell Freezing

The cells that survived cloning were frozen in liquid nitrogen to be used as a stock. The cells in the 24 well plate were blown out and put into a 15 mL tube. The cells were spun at 700 rpm for 5 min. The supernatant was taken and saved for Western blotting. The cells were dissolved in the freezing medium (1% v/v PenStrep, 30% v/v FCS and 10% v/v DMSO in DMEM medium). The cells were quickly stored at -70°C for 1 day and then transferred into liquid nitrogen for storage.

G. Injection of Hybridoma Cells into Mouse

The fourth generation of hybridoma cell lines were expanded into a petri dish with 15 mL of DMEM containing 1% v/v PenStrep and 20% v/v FCS, and incubated at 37°C until 80% confluent. All cells were blown from the petri dish, put into a tube and spun at 700 rpm for 5 min. The supernatant was taken and saved for Western blotting. The cells were resuspended in washing media and spun again for 5 min to remove all FCS from the cells. The cells were redissolved into 2.4 mL of washing medium, and 1/8 or 300 μL (containing ~ 1 x 10^6 hybridoma cells) was injected intra-peritoneally into a BALB/c mouse that had been injected with pristane one week before. Pristane is used to induce tumor development and ascites formation in the mouse. The rest of the cells were frozen into 5 cryo tubes according to the method described above.

Ascites was obtained 3-4 weeks after injection. The ascites was collected and stored at 4°C to separate it from the blood cells. The ascites was then spun at 2,500 rpm, 4°C for 15 min. The supernatant containing the mAb was stored at -20°C in 50% v/v glycerol as a working stock. Remaining aliquots were lyophilized and stored at -20°C.

H. Characterization of the mAbs

The specificity of the mAbs was tested using Western blotting as previously described. The supernatant of the fourth generation of each hybridoma cell line was used as the primary antibody. AP-labelled goat anti mouse Igs were used as the secondary antibody at a 1/4,000 dilution. A positive reaction was revealed by NBT and BCIP substrate. Western blotting was carried out on various samples resolved by 12% w/v SDS-PAGE: mouse *h1*- and *h2*-calponin,

and chicken gizzard, chicken aorta, bovine stomach, bovine aorta, mouse stomach, mouse aorta, SD rat stomach, SD rat aorta, Wistar rat stomach, and Wistar rat aorta total homogenate. Purified mouse *h1*- and *h2*-calponin were available in the laboratory. The rat samples were kindly provided by Dr. Rita Nigam in our laboratory. The immunoglobulin subclass was determined using an Isotyping kit (PharMingen) according to the protocol from the manufacturer.

RESULTS

A. Antibody Immunoresponse in the Immunized Mice

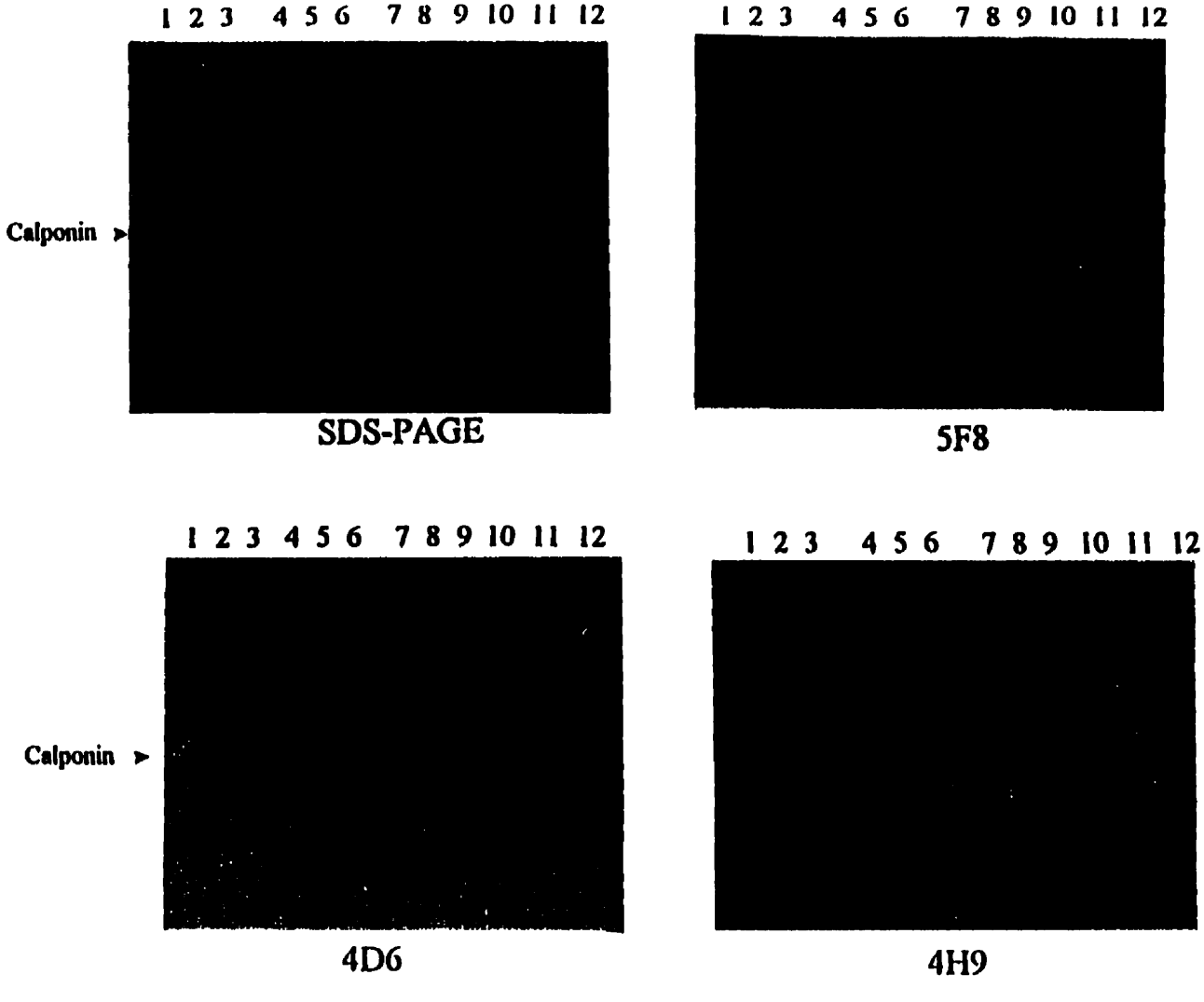
The mouse tail serum tested with ELISA produced a green color in all titers. The ELISA reading was 0.499, 0.337, 0.199 and 0.096 for the titer of 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} respectively.

B. Hybridoma Cell Culture and Ascites Production

The cell fusion successfully produced several hybridoma cell lines. In the first generation, 12 cell lines were obtained with ELISA results reading greater than 0.045 (control 10^{-3} of 0.156). In the second generation, only 3 cell lines, 4D6, 4H9 and 5F8 survived with an ELISA greater than 0.050 (control 10^{-3} of 0.386). These 3 cell lines also survived until the fourth generation with an ELISA reading of 0.345 for 4H9, 0.377 for 5F8 (control 10^{-3} of 0.525) and 0.129 for 4D6 (control 10^{-3} of 0.485). The three lines of the hybridoma cells were injected into separate BALB/c mice to obtain the ascites. Each mouse produced 5-10 mL of ascites which was separated from the blood cells and stored at -20°C in 50% v/v glycerol.

Figure A.1. Specificity of mAbs against bovine calponin.

Sample loading pattern was:1, mouse *h1*-calponin; 1, mouse *h2*-calponin; 3, chicken gizzard; 4, chicken aorta; 5, bovine stomach; 6, bovine aorta; 7, mouse stomach; 8, mouse aorta; 9, SD rat stomach; 10, SD rat aorta; 11, Wistar rat stomach; and 12, Wistar rat aorta homogenates. Primary antibodies were 1/10 dilution of hybridoma supernatant of 4D6, 4H9 and 5F8. The secondary antibody was AP-labelled goat anti mouse Igs. Positive antibody interaction was revealed by NBT and BCIP substrate. 5F8 showed very non-specific binding to many proteins in the crude protein extract. 4D6 showed interaction with bovine calponin and also *h2*-calponin, but also non-specific interaction with other proteins. 4H9 showed specificity to bovine calponin and reaction to *h1*- and *h2*-calponin.


1 2 3 4 5 6 7 8 9 10 11 12
Calponin
SDS-PAGE
1 2 3 4 5 6 7 8 9 10 11 12
5F8
1 2 3 4 5 6 7 8 9 10 11 12
Calponin
4D6
1 2 3 4 5 6 7 8 9 10 11 12
4H9

C. Immunological Characterization of the mAbs Against Bovine Calponin

Western blotting using 5F8 showed non-specific binding to many proteins in the extract samples. 4D6 also showed non-specific interaction with some proteins and specific interaction with calponin of different samples. 4H9 showed interaction with *h1*- and *h2*-calponin and also specific interaction with bovine calponin (Fig. A.1). These results show that from the three survived cell lines, only one shows specificity to bovine calponin. Immunoglobulin subclass determination shows 4H9 and 4D6 to be of IgM subclass. Due to its non-specificity, the immunoglobulin subclass of 5F8 was not tested.

DISCUSSION

Monoclonal antibody production using the method described above successfully produced a specific mAb against calponin. The hybridoma cell cloning result shows that not many cell lines can survive until the fourth generation. In this study, 10 cell lines did not produce enough titer of antibody in their second generation. The repeated cell cloning is to ensure that we obtain high titer of mAb. If the cell lines fail to survive until the next generation, the frozen stock can be recovered and used to produce antibody or to attempt to reclone. The critical step in this method is in the fusion part, which may not always produce hybrid clones. In some wells, only myeloma cells may grow. The cell feeding using fresh medium was also important to maintain the cell life. Another critical step is to establish clean working habits, so that contamination can be avoided. Care also should be taken not to cross-contaminate the cell lines and importantly not to contaminate the medium as it can spread contamination to all cells.