G3.5 ANTIGEN APPEARS TO BE A FORM OF α-ACTININ WHICH CO-ISOLATES AND CO-LOCALIZES WITH TYPE III INTERMEDIATE FILAMENTS

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ABSTRACT

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The G3.5 monoclonal antibody (mAB) was raised against multiple sclerotic plaque material from human brain and spinal cord. The antigen of this antibody has been previously designated an α-actinin-like protein with the unusual features of localizing separately from skeletal muscle α-actinin, localizing with desmin in muscle and with GFAP in astrocytes, and simultaneously binding actin and desmin *in vitro*. The physical properties of the antigen are essentially identical to the published properties of α-actinin, and initial amino acid sequencing showed a high degree of identity to isoform 2 of the α-actinin family except for three deviant amino acids. Attempts to use amino acid sequence data to isolate a unique gene sequence for the G3.5 antigen have failed.

Recent studies have demonstrated that α-actinin has a more complex role in cellular structure and development than previously thought. α-Actinin has been shown to be subject to tyrosine kinase phosphorylation and associate with many proteins in addition to actin, including intermediate filaments. Because of this new information, we reisolated the G3.5 antigen from rat skeletal muscle as before, using a modified desmin isolation protocol. The G3.5 antigen was then released and separated from the insoluble desmin fraction by addition of a reducing agent and centrifugation. Limited proteolysis and sequencing of the G3.5 antigen has provided 7 fragments totaling 72 residues, which share a high degree of identity in primary sequence to regions found in rat α-actinins. Immunohistochemical analysis of the G3.5 antigen was also performed, producing results that suggest some co-localization of the G3.5 mAB and anti-α-actinin antibodies while areas of separate localization are also present. The preponderance of evidence therefore suggests that mAB G3.5 reccognizes α-actinin, and ascribes to α-actinin the novel function of associating with type III intermediate filaments as well as actin. The source of a deviant sequence motif remains unresolved.

CHAPTER I

INTRODUCTION

Cells of eukaryotic organisms contain a skeleton of filamentous proteins that form a three dimensional network. This sub-cellular skeleton allows eukaryotic cells to have complex and diverse structural and physiological properties. These proteins, collectively called the cytoskeleton, provide eukaryotic cells with structural and mechanical support as well as the means for locomotion and intracellular transport. The proteins that make up the filaments and tubules of the cytoskeleton require the presence of accessory proteins in order to form complex structures and bundles and attach to cellular components. The purpose of this study was to further characterize the antigen of monoclonal antibody (mAB) G3.5, which has been reported to recognize one of these accessory proteins.

The G3.5 mAB was raised in Balb-C female mice by injection of plaques from human brain and spinal cord obtained at autopsy from patients who died as a result of multiple sclerosis (Malhotra, 1984). The antigen recognized by G3.5 mAB has been found in a variety of mammalian tissues, including brain, liver, and skeletal and cardiac muscle. This antibody has also been shown to recognize structures in neural retina and RPE in bluegill fish (Bolanos, 1998; Jeffcoat, 1995; Malhotra, 1984; Price, 1993). Several previous studies have suggested the antigen of the G3.5 mAB is an intermediate filament associated protein (IFAP) of type III intermediate filaments (IF)

(Bolanos, 1998; Jeffcoat, 1995; Malhotra, 1984; Price, 1993). In brain and spinal cord tissue the G3.5 mAB labels structures in astrocytes that are also labeled by antibodies to glial fibrillary acidic protein (GFAP) (Bolanos, 1998; Herrera, 1995; Malhotra, 1984). Immunofluorescence microscopy using the G3.5 mAB in longitudinal sections of rat skeletal and cardiac muscle suggested the localization of this antibody in a pattern consistent with the parallel Z-bands of striated muscle. However, immunofluorescence microscopy of transverse sections of striated muscle as well as electron microscopy using immunogold techniques revealed that the G3.5 mAB did not label structures within the Z-discs (Price, 1993). It was shown that this antibody localized in the exosarcomeric space next to the Z-discs, along the desmin filament network connecting the Z-discs of adjacent myofibrils, and on filaments associating with the mitochondria, nuclear envelope, and sarcolemma (Price, 1993). These results led Price to adapt a desmin purification protocol for the purpose of isolating the G3.5 antigen (Lazarides, 1980; Price, 1993). The antigen of the G3.5 mAB was found to co-isolate with the insoluble IF fraction of skeletal muscle and could then be released into solution by the addition of reducing agents. The isolated G3.5 antigen appears as a single band of M_r 100 by SDS-PAGE, and initial primary sequence analysis has revealed a 19mer, a 12mer and an 11mer with 80%, 100%, and 91% sequence identity, respectively, to α-actinin isoform 2 (Bolanos, 1998; Jeffcoat, 1995; Price, 1993). Visualization of the G3.5 antigen by electron microscopy revealed a dimer with globular ends and a rod-like central region with approximate length of 30-40 nm and width of 2-3 nm, similar to the physical characteristics of α-actinin visualized using the same methods (Jeffcoat, 1995). However, double-labeling immunofluorescence studies using the G3.5 mAB and different anti-α-actinin antibodies showed limited co-

localization between α-actinins and the G3.5 antigen (Bolanos, 1998; Jeffcoat, 1995). Based on sequence identity and physical characteristics, it has been inferred that the G3.5 antigen is an isoform of the actin cross-linking protein α-actinin (Bolanos, 1998; Jeffcoat, 1995; Price, 1993) having the ability to cross-link IFs and microfilaments.

α-Actinin belongs to the spectrin superfamily of actin cross-linking and bundling proteins which also includes spectrin, dystrophin and utrophin (Davison, 1989; Djinovic-Carugo, 1999; Viel, 1999; Wasenius, 1987). Each monomer subunit of α-actinin comprises an highly conserved actin-binding region at the N-terminus, a central region consisting of four spectrin-like repeats, and a C-terminal region containing two EF-hand Ca^{++} binding sites (Baron, 1987). The spectrin-like repeats in the central region of the α-actinin monomers are considered to form the basis of the interactions which bring about the dimerization of α-actinin (Djinovic-Carugo, 1999; Viel, 1999). In addition to cross-linking actin filaments, α-actinin also functions in the association of the cortical cytoskeleton with membrane-associated structures in non-muscle cells. α-Actinin has been recently shown to have many more binding partners than actin. These new interactions include regulatory proteins and kinases as well as receptors components, stress fiber dense regions and cell adhesion sites (Bang, 2001; Chi, 2005; Hance, 1999; Izaguirre, 1999; Izaguirre, 2001; Jo, 2001; Mologni, 2005; Nave, 1990; Ohtsuka, 1997; Otey, 2004). Intermediate filament (IF) proteins have also been suggested to be binding partners of α-actinin (Bellin, 1999; Bolanos, 1998; Kjorell, 1982).

Four known isoforms of α-actinin encoded by separate genes have been found. These isoforms include ACTN1 and ACTN4, which are found only in non-sarcomeric cytoplasm, and isoforms ACTN2 and ACTN3, which are present in the sarcomeres of

myocytes (Beggs, 1992; Dixson, 2003; Honda, 1998; Landon, 1985). The functionality of the EF-hand Ca^{++} binding sites and the consequent effects on the binding of filamentous actin by α-actinin is a major functional difference between α-actinins found in the sarcomeres of striated myocytes and those found in non-sarcomeric cytoplasm (Baron, 1987; Noegel, 1987). The binding of Ca^{++} to the functional EF-hand domains of the non-sarcomeric isoforms of α-actinin causes conformational changes in the α-actinin dimer, leading to the release of actin from its binding sites in the N-terminal calponin homology regions (Noegel, 1987).

The different isoforms of α-actinin contain isoform-specific peptide sequences as well as common antigenic determinants (Baron, 1987). Although the four known isoforms of α-actinin form homodimers, heterodimers have also been shown to form from the products of different α-actinin genes. Monomers of α-actinin isolated from human blood platelets form heterodimers when combined *in vitro* (Chan, 1998; Gache, 1984). Sarcomeric α-actinin isoforms 2 and 3 from skeletal myocytes also form heterodimers when expressed simultaneously *in situ* and when combined *in vitro*. Tissue-specific alternative splicing of the mRNA from α-actinin genes can also lead to the production of functionally different isoforms of α-actinin and to tissue specific expression of certain α-actinin isoforms (Kremerskothen, 2002; Mills, 2001; Suzuki, 2002; Waites, 1992). The difference in calcium sensitivity of some α-actinin isoforms has been attributed to the alternative splicing of mutually exclusive exons encoding regions of the EF-hand calcium binding domains (Waites, 1992). The rat α-actinin 1 transcript contains mutually exclusive smooth muscle and non-muscle exons, which are subject to tissue-specific alternative splicing, leading to the formation of two different

isoforms of α-actinin 1. The combination of these exons in brain tissue results in the production of yet another tissue-specific isoform of α-actinin 1 (Kremerskothen, 2002; Suzuki, 2002).

Based on the results of previous attempts to characterize the G3.5 mAB, I hypothesized that the G3.5 antigen is a previously undescribed isoform of α-actinin that may cross-link IFs and microfilaments. To test my hypothesis, amino acid sequence analysis was performed to more clearly identify the G3.5 antigen. Immunofluorescence microscopy and immunogold electron microscopy techniques were also used to further characterize the *in situ* localization of the G3.5 antigen and α-actinins. I found that fragments of the G3.5 antigen isolated from rat skeletal muscle share a high degree of identity with rat α-actinins. The G3.5 mAB and anti-α-actinin pAB label some distinct structures but also co-localize on certain structures within brain and liver tissue as recorded by immunofluorescence and immunogold electron microscopy. In cardiac muscle the G3.5 mAB and anti-α-actinin antibodies do not co-localize but appear to label the same striated structures in the sub-sarcolemmal space of cardiac myocytes.

CHAPTER II

MATERIALS AND METHODS

Chemical and Reagents

All chemical and reagents were supplied by Sigma Laboratories (St. Louis, MO) unless otherwise specified. G3.5 hybridoma cells were obtained from the American Type Culture Collection (ATCC, #CRL-2252, Manassas, VA).

I. Purification of G3.5 Antigen

Use of animals in this study was approved by the Texas State IACUC (#04535C6F03). Skeletal muscle was harvested from laboratory rats purchased from Charles River Laboratories (Houston, TX). The tissue was trimmed into small pieces and placed in 10 volumes of cold homogenization buffer consisting of 1.5 mM EDTA, 100 μg/ml phenylmethylsulfanylfluoride (PMSF), and 0.5 M NaCl in 50 mM Na_2HPO_4 (pH 7.4). The tissue was held on wet ice, then minced using a blender with 5 pulses of 15 seconds with cooling time allowed in between. The suspension was filtered through 4 layers of cheesecloth to remove adipose and connective tissue. Triton X-100 was added to the suspension to a final concentration of 1% and stirred at 4°C for 2 hours. The homogenate was centrifuged at 5000 x *g* for 60 minutes using a Sorvall RC 28S centrifuge (Ashville, NC). A small sample of the supernatant was collected and the remainder discarded. The pellet was resuspended in 5 volumes of homogenization buffer with 1% Triton X-100 using a ULTRA-TURRAX T8 tissue homogenizer from Fisher Scientific (Pittsburg, PA)

and centrifuged at 4000 x *g* for 30 minutes. This step was repeated once and a sample of the supernatant was collected. From here on all suspensions of the insoluble pellet were performed using the aforementioned tissue homogenizer with 3 pulses at 10 seconds each.

The pellet was resuspended in 5 volumes of a high salt extraction buffer containing 0.6 M KI, 1 mM EGTA, and 10 mM $Na_2S_2O_3$ in 10 mM Na_2HPO_4 (pH 8.0) and centrifuged at 10,000 x *g* for 10 minutes. This step was performed a total of four times for the extraction of desmin intermediate filaments as described by Lazarides and Granger (Lazarides, 1980). Small samples of the supernatants were collected and the remainders were discarded.

The final pellet was washed twice by resuspension in 5 volumes of phosphate buffered saline (PBS) containing 1 mM EDTA and centrifuged at 10,000 x *g* for 10 minutes. The PBS contained 11.0 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl at pH 7.4. The G3.5 antigen was released from the insoluble desmin fraction by resuspension of the pellet from the second wash in 5 volumes of an uncoupling buffer containing 5% β-mercaptoethanol, and 1 mM EDTA in PBS and centrifugation at 10,000 x *g* for 20 minutes. This step was repeated 2 times and the supernatants from all three centrifugations were pooled. The combined supernatant was clarified by centrifugation at 100,000 x *g* for 30 minutes. The final pellet was discarded, and the supernatant was concentrated to 25% of the original volume by dialysis against sucrose or using Millipore Centriplus centrifugal filter devices from Fisher Scientific.

To confirm the identity of the G3.5 antigen, samples of the final concentrated isolate as well as samples of the supernatants of some earlier steps were subjected to

SDS-PAGE and western blot analysis using the G3.5 mAB as described by Bolanos *et al.* (1997). Positive controls were performed using chicken gizzard α-actinin and an anti-cytoplasmic-α-actinin antibody.

II. Limited Proteolysis and Amino Acid Sequencing

Samples of the G3.5 isolate were diluted 1:5 with electrophoresis sample buffer containing 60 mM Tris-HCl, 25% glycerol, 2% SDS, 14.4 mM β-mercaptoethanol, and 0.1% bromophenol blue. The samples were then boiled for 10 minutes and loaded onto SDS polyacrylamide (10% acrylamide, Tris-HCl) pre-made gels from Bio-Rad (Hercules, CA). The samples were subjected to 200V for approximately 45 minutes using a Bio-Rad Mini Protean 3 cell. The protein bands were stained in Coomassie gel stain (1.2 mM Coomassie Blue R-250, 45% methanol, 10% acetic acid) and destained in Coomassie gel destain (10% methanol, 10% acetic acid). The bands corresponding to the G3.5 antigen were excised, packaged cold, and shipped to University of Texas Medical Branch Protein Chemistry Laboratory (Galveston, Texas) for limited tryptic digestion and primary sequence analysis.

III. Amino Acid Sequence Analysis

All G3.5 primary sequence data from the current and previous studies were analyzed against the recently published *Rattus norvegicus* genome using the Ensembl genomic search tool (http://www.ensembl.org/Rattus_norvegicus/blastview). Primary sequence data was also analyzed against UNIPROT protein database using the FASTS3 protein database search tool (//ww://www.ebi.ac.uk/fasta33). Protein alignments were performed using the T-COFFEE multiple protein alignment tool (http://www.ch.embnet.org/software/TCoffee.html).

IV. Immunocytochemistry

A. Immunofluorescence Microscopy

Ventricular cardiac muscle, liver, and brain tissue were harvested from laboratory rats purchased from Charles River Laboratories and cut into 0.5 cm^3 cubes in 4% paraformaldehyde in PBS (PFA). The tissue was then transferred to fresh PFA and incubated overnight at room temperature. The fixed tissue was then washed three times in PBS for a total period of 90 minutes. Following the wash, the tissue was incubated in 30% sucrose in PBS for approximately 24 hours at room temperature for cryoprotection. The tissue was then removed from this solution and mounted in Tissue-Tek OCT compound at -20°C for cryotomy. Tissue sections were cut at 20°C with thickness of 14 μm using a cryotome and mounted on 0.5% gelatin coated coverslips. The sections were allowed to dry overnight at 4°C.

Once dry, the sections on coverslips were washed with PBS containing 0.1% Tween-20 (PBST) three times for 5 minutes each. The sections were then blocked to prevent non-specific antibody binding by incubation in PBST containing 20% non-fat dry milk (NFDM) for 60 minutes at room temperature. The blocking solution was removed by dipping the coverslips in PBST, and the sections were incubated in G3.5 mAB hybridoma culture supernatant overnight at 4°C. Negative controls were incubated in mouse serum diluted 1:100 with 0.5% bovine serum albumin (BSA). Tissue sections were washed three times for 5 minutes each with PBST and incubated in the appropriate secondary antibody conjugated to AlexaFluor 568 fluorescent dye from Invitrogen (Carlsbad, CA) for 2 hours at room temperature. The sections were again washed three times at 5 minutes each with PBST and blocked against non-specific antibody binding as

stated previously. Following the removal of blocking solution the sections were incubated in a second primary antibody specific for α-actinins at 4°C overnight. Negative controls were again incubated in mouse serum diluted 1:100 with 0.5% BSA. Sections were washed three times for 5 minutes each with PBST and incubated in the appropriate secondary antibody conjugated to AlexaFluor 488 fluorescent dye for 2 hours at room temperature. The secondary antibody solution was removed and the sections were washed once for 10 minutes with PBST and incubated in a 1:1000 dilution of TO-PRO-3 Nucleic Acid Binding Probe stain in PBST for 30 minutes at room temperature. Following this incubation the sections were washed three times for 5 minutes each with PBS and mounted in 90% glycerol in PBS containing 1% ρ-phenylenediamine. The immunolabeled tissue was viewed and images acquired using an Olympus IX-70 microscope (Melville, NY) fitted with a Bio-Rad MRC 1024 confocal scan-head. The images were processed for presentation using NIH Image-J (National Institute of Health, Bethesda, MD) and Adobe Photoshop software (San Jose, CA).

B. Immunogold Electron Microscopy

Brain tissue grey matter was harvested from mixed breed rats and trimmed appropriately for TEM processing in 4% PFA in 0.05 M sodium cacodylate buffer at pH 7.5. The tissue was transferred to fresh fixative and incubated at room temperature overnight. The tissue was washed three times for 15 minutes each in cacodylate buffer, post-fixed in 1% OsO_4 in cacodylate buffer for 2 hours at room temperature, and washed three times for 15 minutes each in the same buffer. The tissue was then dehydrated by three 15 minute incubations in 60% ethanol, followed by three 15 minute incubations in 80% ethanol. The final volume of 80% ethanol was removed and the tissue was

incubated in LR White embedding resin at room temperature overnight. The tissue was incubated in fresh LR White from Electron Microscopy Sciences (Hatfield, PA) for 30 minutes at room temperature. This step was repeated one time. For embedding, the tissue in LR White was placed in gelatin capsules, closed to avoid exposure to oxygen, and the LR White was polymerized for 24 hours at 55°C.

The embedded tissue was sectioned at 70 nm thickness for electron microscopy and collected on 400 mesh nickel grids. The grids were washed with PBS three times for 5 minutes. The tissue sections were blocked using 5% NFDM in PBS for 1 hour at room temperature and washed three times for 5 minutes with the same buffer. The grids were incubated in G3.5 mAB hybridoma culture supernatant overnight at 4°C. Negative controls were incubated in mouse serum diluted 1:100 with 0.5% bovine serum albumin (BSA). The grids were washed three times for 5 minutes each with PBS and incubated in the appropriate secondary antibody with 6 nm colloidal gold conjugate from Electron Microscopy Sciences for 2 hours at room temperature. The tissue sections were washed three times for 5 minutes with PBS and again blocked as previously described. The grids were washed three times for 5 minutes with PBS and incubated in a solution of an anti-α-actinin antibody overnight at 4°C. The grids were washed three times for 5 minutes each with PBS and incubated in the appropriate secondary antibody with 10 nm colloidal gold conjugate for 2 hours at room temperature. The grids were washed three times for 5 minutes with PBS and allowed to air dry. The immunogold labeled sections were viewed and images were acquired using a JEOL 1200 EX II transmission electron microscope (Peabody, MA). Photomicrograph negatives were scanned at 2400 dpi using an Epson high resolution scanner (Long Beach, CA) connected to an Apple Macintosh G5

computer (Cupertino, CA). The scanned images were processed for presentation using Adobe Photoshop software.

Table 1. Antibodies used

Antibody	Conjugate	Dilution	Source
G3.5 mAB (mouse IgM)	None	None	ATCC Hybridoma
mouse anti-sarcomeric-α-actinin (IgG)	None	1:500	Sigma Laboratories
mouse anti-cytoplasmic-α-actinin (IgM)	None	1:200	Sigma Laboratories
rabbit anti-α-actinin (IgG)	None	1:500	Sigma Laboratories
goat anti-mouse IgM	AlexaFluor 568	1:200	Molecular Probes Inc.
goat anti-rabbit IgG	AlexaFluor 488	1:200	Molecular Probes Inc.
goat anti-mouse polyvalent Ig	alkaline phosphatase	1:3000	Sigma Laboratories
goat anti-mouse IgM	6 nm colloidal gold	1:20	Electron Microscopy Sciences
goat anti-mouse IgM	10 nm colloidal gold	1:20	Electron Microscopy Sciences
goat anti-mouse IgG	10 nm colloidal gold	1:20	Electron Microscopy Sciences
goat anti-rabbit IgG	10 nm colloidal gold	1:20	Electron Microscopy Sciences

CHAPTER III

RESULTS

I. Purification of G3.5 antigen

In order to determine the identity of the isolated protein, SDS-PAGE and western blot analysis were performed. The protein isolate obtained from rat muscle using the procedures described previously contained primarily the G3.5 antigen as well some amount of other proteins of mostly lower molecular weight as revealed by SDS-PAGE (Fig. 1 lane 3). Immunoblotting confirmed the identity of the G3.5 antigen as single band with M_r of approximately 100 (Fig. 1 lane 8). The G3.5 mAB recognized chicken gizzard α-actinin and the G3.5 antigen was recognized by the anti-cytoplasmic-α-actinin antibody (Fig. 1 lane 7, Fig. 2 lane 8). None of the protein bands present in the supernatant of the second homogenization step other than the 100 kD band were labeled by the G3.5 mAB (Fig. 1 lane 9).

II. Limited Proteolysis and Amino Acid Sequencing

The limited tryptic digest of the G3.5 antigen produced several small fragments. Of these, 7 fragments consisting of a hexamer, four decamers, one dodecamer, and one 14-mer were sequenced (Table 2).

III. Amino Acid Sequence Analysis

Fragments of the G3.5 antigen sequenced in the current study shared a high degree of primary sequence identity with rat α-actinins (Table 3). A series of BLAST

searches performed on all sequenced G3.5 fragments using Ensembl genomic search tool revealed that the primary sequence of the fragments in question matched most closely to members of the α-actinin family within the rat genome. Fragments 1 and 7 (1: DLLLDPAWEK, 7: GISQEQ) are 100% identical with regions found in all rat α-actinins (Table 3). Fragment 2 (YLDIPKMLDAEDIV) is 100% identical to regions found in rat α-actinins 1, 3, 4 and is 100% similar to a region in rat α-actinin 2 (Table 3, Fig. 3). Fragment 3 (LASELLEWIR) is 100% identical to rat α-actinin 2 and 3 regions and 100% similar to regions in rat α-actinins 1 and 4 (Table 3). Fragment 4 (GYEEWLLXEI) is 100% identical to regions of

rat α-actinins 1, 2, and 4 and 100% similar to a region of α-actinin 3 among its identified residues (Table 2, Table 3). G3.5 fragment 5 is 100% identical to a region of rat α-actinin 1, and 100% similar to a region of α-actinin 2. This fragment is 90% and 80% identical to regions of rat α-actinins 3 and 4, respectively (Table 3). The final G3.5 fragment produced in the current study (TINEVENQVFTR) shares 83%, 75%, 91%, and 83% identity with regions of rat α-actinins 1-4 respectively (Table 3). This fragment contains one residue not present in any of the four rat α-actinin proteins (Fig. 3). The predicted rat α-actinin isoform 2 is the highest match for all sequenced G3.5 fragments from previous studies (Table 3, Fig. 3).

IV. Immunocytochemistry

A. Immunofluorescence Microscopy

Double-labeling immunofluorescence microscopy was used to further characterize the *in situ* localization patterns of the G3.5 antigen and α-actinin. In PFA-fixed cryosections of rat cardiac muscle the G3.5 mAB localized in a pattern of parallel

stripes consistent with the Z-bands of parallel myofibrils (Fig. 4). However, the G3.5 mAB did not localize within the Z-discs themselves but rather in the sub-sarcolemmal space as seen in previous studies (Bolanos, 1998; Price, 1993). A pattern of lighter bands was visible between the more heavily labeled lines, suggesting the localization of the G3.5 mAB in a pattern consistent with the M-bands in addition to the Z-bands (Fig. 4 C, D). The Sigma anti-α-actinin pAB, which was raised against smooth muscle α-actinin from chicken gizzard, also localized in a striped pattern consistent with the Z-bands of striated muscle. This anti-α-actinin pAB localized beneath the sarcolemma of cardiac myocytes as well (Fig. 4). Although co-localization between the two antibodies is not clearly evident, it does appear that both the G3.5 mAB and the anti-α-actinin pAB are localized along the same structures in the sub-sarcolemmal region of cardiac myocytes (Fig. 4).

In cryosections of PFA-fixed liver tissue the G3.5 mAB appears to localize diffusely throughout the cytoplasm of hepatocytes and strongly in the sub-membrane space outlining the hepatocytes (Fig. 5 A, D, G). The anti-α-actinin pAB also appears to localize diffusely within the cytoplasm of hepatocytes and strongly in the sub-membrane space of the hepatocytes (Fig. 5 B, E, H). Although both antibodies label distinct structures not recognized by the other, areas of co-localization do exist. Co-localization of the G3.5 mAB and anti-α-actinin pAB appears to be limited to the sub-membrane space and cell junctions of hepatocytes (Fig. 5 C, F, I).

In cryosections of rat brain tissue fixed with PFA, the G3.5 mAB appeared to label the processes of astrocytes (Fig. 6 A, D, G) as expected by the findings of previous studies (Malhotra, 1984). The anti-α-actinin pAB localized diffusely throughout the

brain tissue sections and more strongly in structures that also appear to be some of the processes of the same astrocytes labeled by the G3.5 mAB (Fig. 6 B, E, H). As in liver tissue, areas of co-localization of the G3.5 mAB and anti-α-actinin pAB are evident although both antibodies also bind distinct structures not labeled by the other. The areas where these antibodies co-localize are limited to the processes of what appear to be astrocytes (Fig. 6 C, F, I).

Tissue sections incubated in normal serum exhibited no specific binding of either secondary antibody in any of the tissues tested (Fig. 7).

B. Immunogold Electron Microscopy

Double-labeling immunogold electron microscopy was used to confirm the results of immunofluorescence experiments. Sections of LR White embedded PFA fixed rat brain tissue appeared to show specific labeling of the G3.5 mAB and anti-ctyoplasmic-α-actinin mAB in areas that correspond to the plasma membranes of what are assumed to be astrocytic processes (Fig. 8). The G3.5 mAB and anti-cytoplasmic-α-actinin mAB were seen to label in close packed bundles in areas near plasma membranes (Fig. 8 B, D, F, G).

Figure 1. SDS-PAGE and Immunoblot Probed with G3.5 mAB

SDS-PAGE and immunoblot analysis of muscle cell lysate, G3.5 isolate, and chicken gizzard α-actinin. MW standards (1,6); α-actinin (2); G3.5 isolate (3); supernatant of second homogenization step (4); immunoblot of α-actinin with G3.5 mAB (7); immunoblot of G3.5 isolate with G3.5 mAB (8); immunoblot of proteins present in the supernatant of second homogenization step with G3.5 mAB (9). Lane 5 served as a spacer and was loaded only with sample buffer.

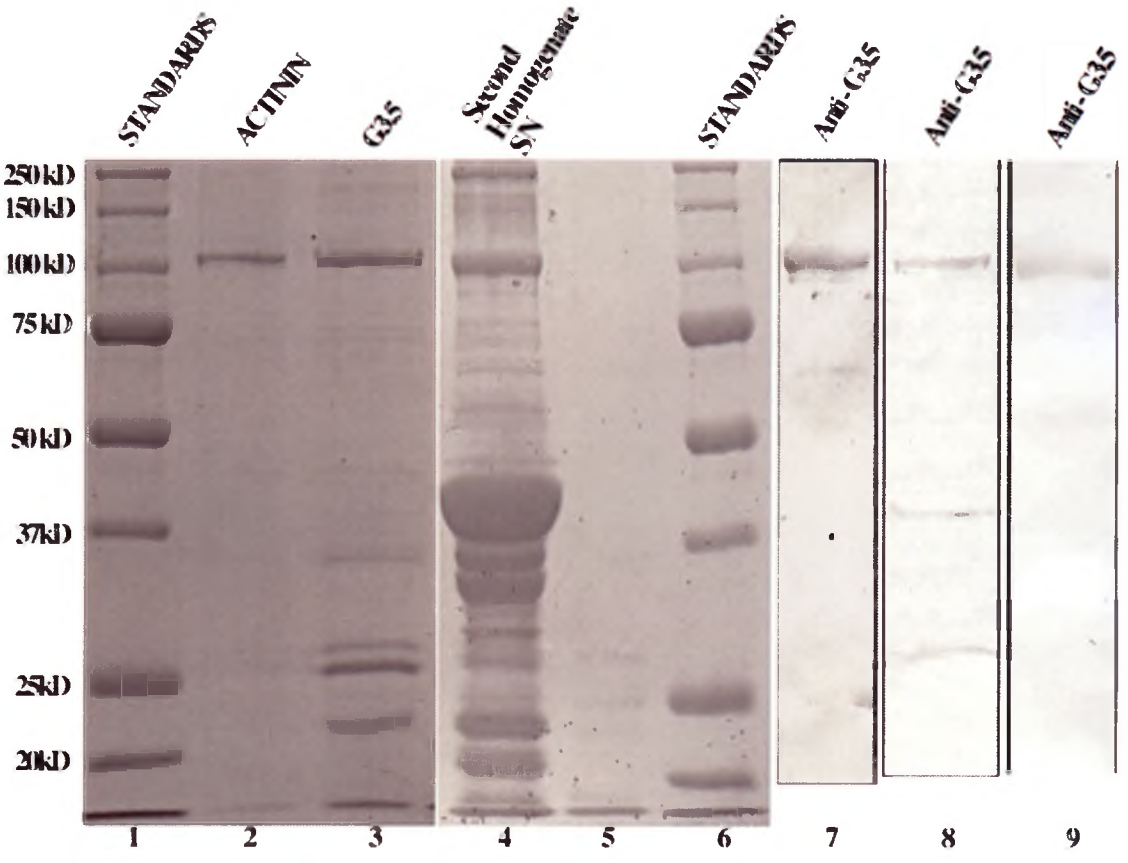

STANDARDS
ACTININ
G35
Second Homogenate SN
STANDARDS
Anti-G35
Anti-G35
Anti-G35
250kD
150kD
100kD
75kD
50kD
37kD
25kD
20kD
1
2
3
4
5
6
7
8
9

Figure 2. SDS-PAGE and Immunoblot Probed with Anti-α-Actinin pAB

SDS-PAGE and immunoblot analysis of muscle cell lysate, G3.5 isolate, and chicken gizzard α-actinin. MW standards (1,6); α-actinin (2); G3.5 isolate (3); Supernatant of second homogenization step (4); immunoblot of α-actinin with anti-cytoplasmic-α-actinin mAB (7); immunoblot of G3.5 isolate with anti-cytoplasmic-α-actinin mAB (8); immunoblot of proteins present in the supernatant of second homogenization step with anti-cytoplasmic-α-actinin mAB (9). Lane 5 served as a spacer and was loaded only with sample buffer.

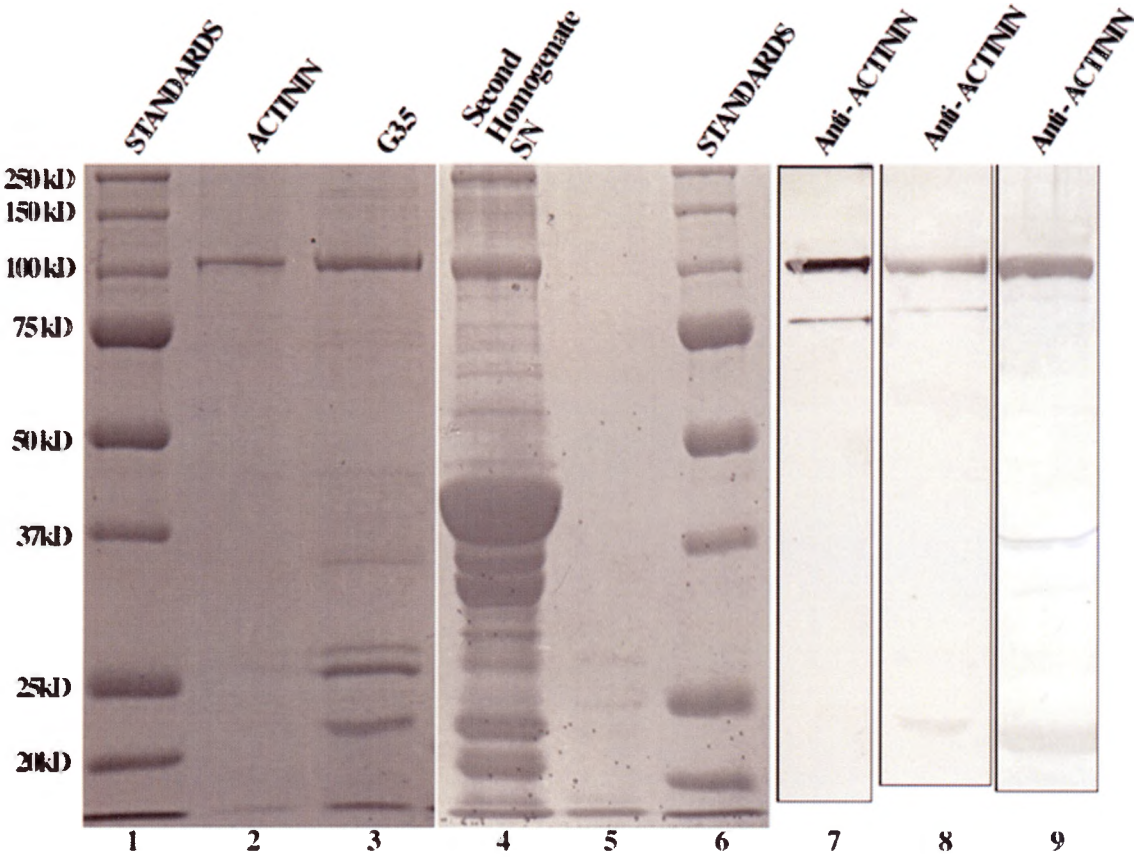

STANDARDS
ACTININ
G35
Second Homogenate SN
STANDARDS
Anti-ACTININ
Anti-ACTININ
Anti-ACTININ
250 kD
150 kD
100 kD
75 kD
50 kD
37kD
25kD
20kD
1
2
3
4
5
6
7
8
9

Table 2. G3.5 Protein Fragments

A listing of all sequenced G3.5 antigen fragments from the current study as well as previous studies.

G3.5 Fragment	Source
1) DLLLDPAWEK	Current Study
2) YLDIPKMLDAEDIV	Current Study
3) LASELLEWIR	Current Study
4) GYEEWLLXEI	Current Study
5) LLETIDQLYL	Current Study
6) TINEVENQVFTR	Current Study
7) GISQEQ	Current Study
8) KASTHETWAYGK	Bolanos et al. 1998
9) KQYXHNIINYK	Bolanos et al. 1998
10) DPIXNINLAMQIFVKHLDI	Price et al. 1993

Table 3. Analysis of G3.5 Protein Fragments and α-Actinins

The percentage of shared identity between sequenced G3.5 fragments and corresponding regions of known α-actinins as deduced using the ensembl.org genomic search tool against the published rat genome and the T-COFFEE protein alignment tool against the rat α-actinin sequences available at NCBI (http://www.ncbi.nlm.nih.gov/) (ACTN1: AF115386, ACTN2: XM_214499, ACTN3: NM_133424, ACTN4: Q90734). In cases of conservative amino acid substitutions, the overall percentage of similarity is provided in parentheses. The inclusion of an asterisk * signifies that the preceding percentage of identity was calculated among identified residues within a fragment.

G3.5 Fragment	ACTN1	ACTN2	ACTN3	ACTN4
1) DLLLDPAWEK	100%	100%	100%	100%
2) YLDIPKMLDAEDIV	100%	92.8% (100%)	100%	100%
3) LASELLEWIR	90% (100%)	100%	100%	90% (100%)
4) GYEEWLLXEI	100% *	100% *	80% (90%)	100% *
5) LLETIDQLYL	100%	90% (100%)	90%	80% (90%)
6) TINEVENQVFTR	83.3% (91.6%)	75% (83.3%)	91.6%	83.3% (91.6%)
7) GISQEQ	100%	100%	100%	100%
8) KASTHETWAYGK	66.6%	100%	66.6%	66.6%
9) KQYXHNIINYK	60% * (81.8%)	100% *	70% * (81.8%)	60% * (81.8%)
10) DPIXNINLAMQIFVKHLDI	42.1% (68.4%)	78.9% (84.2%)	52.6% (73.6%)	47.3%

Figure 3. Multiple Alignment of G3.5 Fragments with Rat α-Actinins 2 and 3

Multiple alignments of the primary sequence from the 7 fragments of the G3.5 antigen and rat α-actinin isoforms 2 (ACTN2) and 3 (ACTN3) obtained using T-COFFEE multiple alignment tool. The amino acid sequences of rat α-actinin 2 (XM_214499) and α-actinin 3 (NM_133424) were obtained from NCBI (http://www.ncbi.nlm.nih.gov/). Areas corresponding to the α-actinin N-terminal actin-binding domains are colored in red. Areas corresponding to the four, central spectrin-like repeats of α-actinin are colored in green. Areas corresponding to the α-actinin C-terminal EF-hand motifs are colored in blue. G3.5 antigen primary sequence obtained from the findings of previous studies is italicized while primary sequence obtained in the current study is underlined. The non-aligned N-terminal region (amino acids 1-300) of the predicted rat α-actinin 2 protein has been truncated.

```
ACTN2           HPSIHPSIHF SIRLSIPRAA LAPQSPQPPS AMNQIEPGVQ YNYVYDEDEY MIQEEEWDRD
G3.5            ---------- ---------- ---------- ---------- ---------- ---------D
ACTN3           ---------- ---------- -----MMMVM QPEGLGAGEG PFSGGGGGEY MEQEEDWDRD
                                                                       *

ACTN2           LLLDPAWEKQ QRKTFTAWCN SHLRKAGTQI ENIEEDFRNG LKLMLLLEVI SGERLPKPDR
G3.5            LLLDPAWEK- ---------- ---------- ---------- ---------- ----------
ACTN3           LLLDPAWEKQ QRKTFTAWCN SHLRKAGTQI ENIEEDFRNG LKLMLLLEVI SGERLPRPDK
                *********

ACTN2           GKMRFHKIAN VNKALDYIAS KGVKLVSIGA EEIVDGNVKM TLGMIWTIIL RFAIQDISVE
G3.5            ---------- ---------- ---------- ---------- ---------- ----------
ACTN3           GKMRFHKIAN VNKALDFIAS KGVKLVSIGA EEIVDGNLKM TLGMIWTIIL RFAIQDISVE

ACTN2           ETSAKEGLLL WCQRKTAPYR NVNIQNFHTS WKDGLGLCAL IHRHRPDLID YSKLNKDDPI
G3.5            ---------- ---------- ---------- ---------- ---------- -------DPI
ACTN3           ETSAKEGLLL WCQRKTAPYR NVNVQNFHTS WKDGLALCAL IHRHRPDLID YAKLRKDDPI
                                                                       ***

ACTN2           GNINLAMEIA EKHLDIPKML DAEDIVNTPK PDERAIMTYV SCFYHAFAGA EQAETAANRI
G3.5            XNINLAMQIF VKYLDIPKML DAEDIV---- ---------- ---------- ----------
ACTN3           GNLNTAFEVA EKYLDIPKML DAEDIVNTPK PDEKAIMTYV SCFYHAFAGA EQAETAANRI
                 *:* *:::   *:******* ******

ACTN2           CKVLAVNQEN ERLMEEYERL ASELLEWIRR TIPWLENRTP EKTMQAMQKK LEDFRDYRRK
G3.5            ---------- ---------L ASELLEWIR- ---------- ---------- ----------
ACTN3           CKVLAVNQEN EKLMEEYEKL ASELLEWIRR TVPWLENRVG EPSMSAMQRK LEDFRDYRRL
                          * *********

ACTN2           HKPPKVQEKC QLEINFNTLQ TKLRISNRPA FMPSEGKMVS DIAGAWQRLE QAEKGYEEWL
G3.5            ---------- ---------- ---------- ---------- ---------- ----GYEEWL
ACTN3           HKPPRVQEKC QLEINFNTLQ TKLRLSHRPA FMPSEGKLVS DIANAWRGLE QVEKGYEDWL
                                                                       ***:**

ACTN2           LNEIRRLERL EHLAEKFRQK ASTHETWAYG KEQILLQKDY ESASLTEVRA LLRKHEAFES
G3.5            LXEI------ ---------K ASTHETWAYG K--------- ---------- ----------
ACTN3           LSEIRRLQRL QHLAEKFQQK ASLHEAWTRG KEEMLNQHDY ESASLQEVRA LLRRHEAFES
                * **                * ** **:*: * *

ACTN2           DLAAHQDRVE QIAAIAQELN ELDYHDAVNV NDRCQKICDQ WDRLGTLTQK RREALERTEK
G3.5            ---------- ---------- ---------- ---------- ---------- ----------
ACTN3           DLAAHQDRVE HIAALAQELN ELDYHEAASV NSRCQAICDQ WDNLGTLTQK RRDALERMEK

ACTN2           LLETIDQLHL EFAKRAAPFN NWMEGAMEDL QDMFIVHSIE EIQSLITAHE QFKATLPEAD
G3.5            LLETIDQLYL ---------- ---------- ---------- ---------- ----------
ACTN3           LLETIDQLQL EFARRAAPFN NWLDGAIEDL QDVWLVHSVE ETQSLLTAHE QFKATLPEAD
                ******** *

ACTN2           GERQSILAIQ NEVEKVIQSY SIRISSSNPY STVTMDELRN KWDKVKQLVP VRDQSLQEEL
G3.5            ---------- ---------- ---------- ---------- ---------- ----------
ACTN3           RERGAILGIQ GEIQKICQTY GLRPKSGNPY ITLSSQDINN KWDTVRKLVP SRDQTLQEEL

ACTN2           ARQHANERLR RQFAAQANAI GPWIQNKMEE IARSSIQITG ALEDQMNQLK QYEHNIINYK
G3.5            ---------- ---------- ---------- ---------- ---------K QYXHNIINYK
ACTN3           ARQQVNERLR RQFAAQANAI GPWIQGKVEE VGRLAAGLAG SLEEQMAGLR QQEQNIINYK
                                                                  : *  :******

ACTN2           NNIDKLEGDH QLIQEALVFD NKHTNYTMEH IRVGWELLLT TIARTINEVE TQILTRDAKG
G3.5            ---------- ---------- ---------- ---------- ----TINEVE NQVFTR---G
ACTN3           SNIDRLEGDH QLLQEGLVFD NKHTVYSMEH IRVGWEQLLT SIARTINEVE NQVLTRDAKG
                                                             ****** .*::**   *

ACTN2           ISQEQMNEFR ASFNHFDRRK NGLMDHEDFR ACLISMGYDL GEAEFARIMT LVDPNGQGTV
G3.5            ISQEQ----- ---------- ---------- ---------- ---------- ----------
ACTN3           ISQEQLNEFR ASFNHFDRKR NGMMEPDDFR ACLISMGYDL GEVEFARIMT MVDPNAAGVV
                *****

ACTN2           TFQSFIDFMT RETADTDTAE QVIASFRILA SDKPYILAEE LRRELPPDQA QYCIKRMPPY
G3.5            ---------- ---------- ---------- ---------- ---------- ----------
ACTN3           TFQAFIDFMT RETAETDTAE QVVASFKILA GDKNYITPEE LRRELPAEQA EYCIRRMAPY

ACTN2           SGPGSVPGAL DYTAFSSALY GESDL
G3.5            ---------- ---------- -----
ACTN3           KGSGAPSGAL DYVAFSSALY GESDL
```

Figure 4. Confocal Images of Rat Cardiac Muscle Double Immunolabeled with G3.5 mAB and Anti-α-Actinin pAB

Confocal micrographs of rat cardiac muscle cryosections double immunolabeled with the G3.5 mAB (AlexaFluor 568 secondary antibody seen as red signal) and anti-α-actinin pAB (AlexaFluor 488 secondary antibody seen as green signal). Pannels B, D, F represent progressive 20° projections of areas selected from the stacks of images in panels A, C, E respectively. The G3.5 antigen is present near the Z-lines in the sub-sarcolemmal space. α-Actinin is present in the Z-discs and near the Z-discs in the sub-sarcolemmal space. The scale bars are expressed in micrometers.

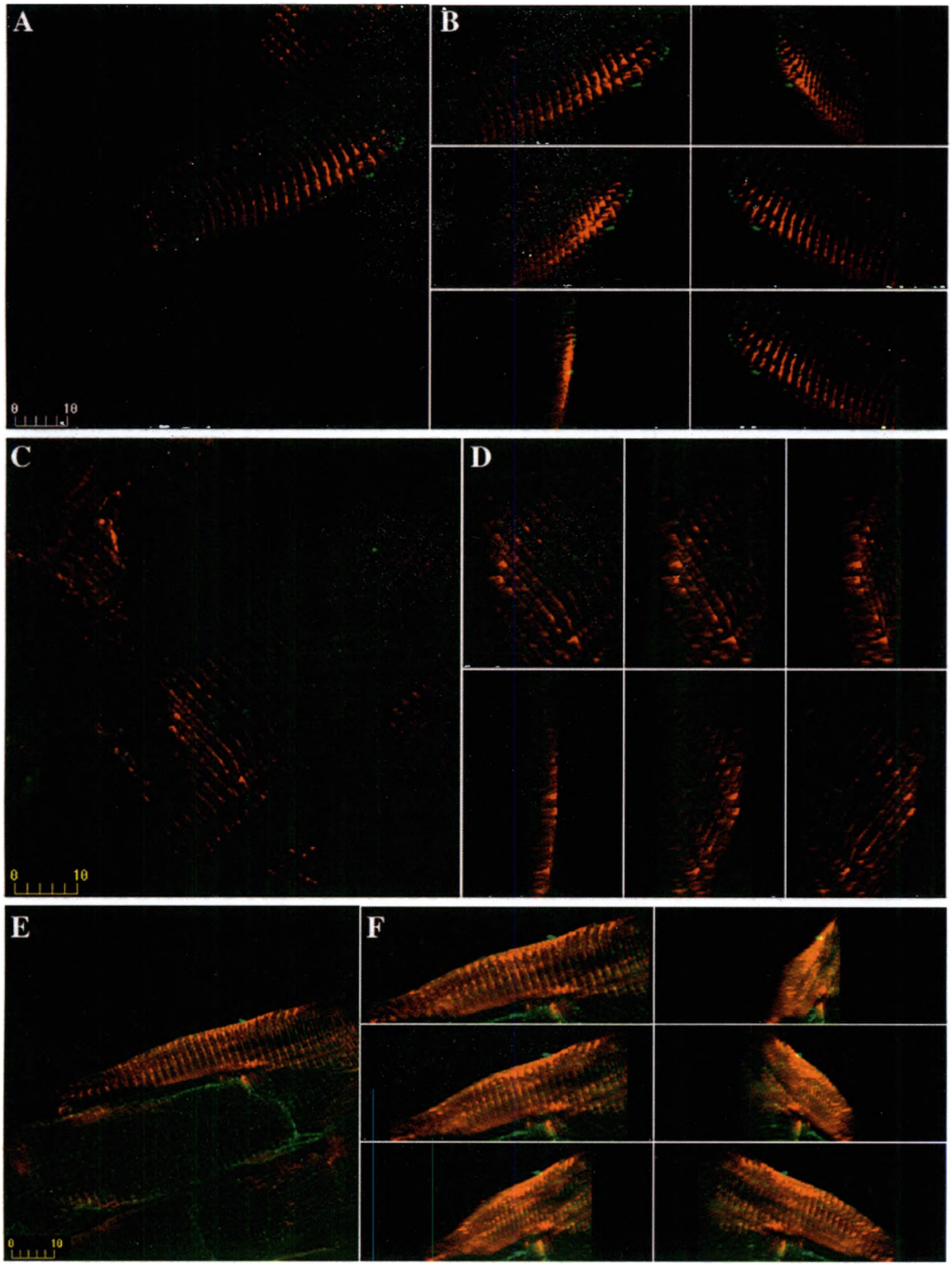

A
B
C
D
E
F

Figure 5. Confocal Images of Rat Liver Double Immunolabeled with mAB G3.5 and Anti-α-Actinin

Confocal micrographs of rat liver cryosections, double immunolabeled with the G3.5 mAB (AlexaFluor 568 secondary antibody seen as red signal) and anti-α-actinin pAB (AlexaFluor 488 secondary antibody seen as green signal). The G3.5 antigen is present diffusely in the cytoplasm of hepatocytes and more predominantly in the sub-membrane space and cell junctions as seen in the red channel (A, D, G). α-Actinin is also present diffusely in the cytoplasm and more predominantly in the sub-membrane space and cell junctions as seen in the green channel (B, E, H). Each antibody labeled some structures distinct from the other. Areas of co-localization are present in the sub-membrane spaces and cell junctions of hepatocytes as seen in the combined signals from the G3.5 antigen and α-actinin (C, F, I). The scale bars are expressed in micrometers.

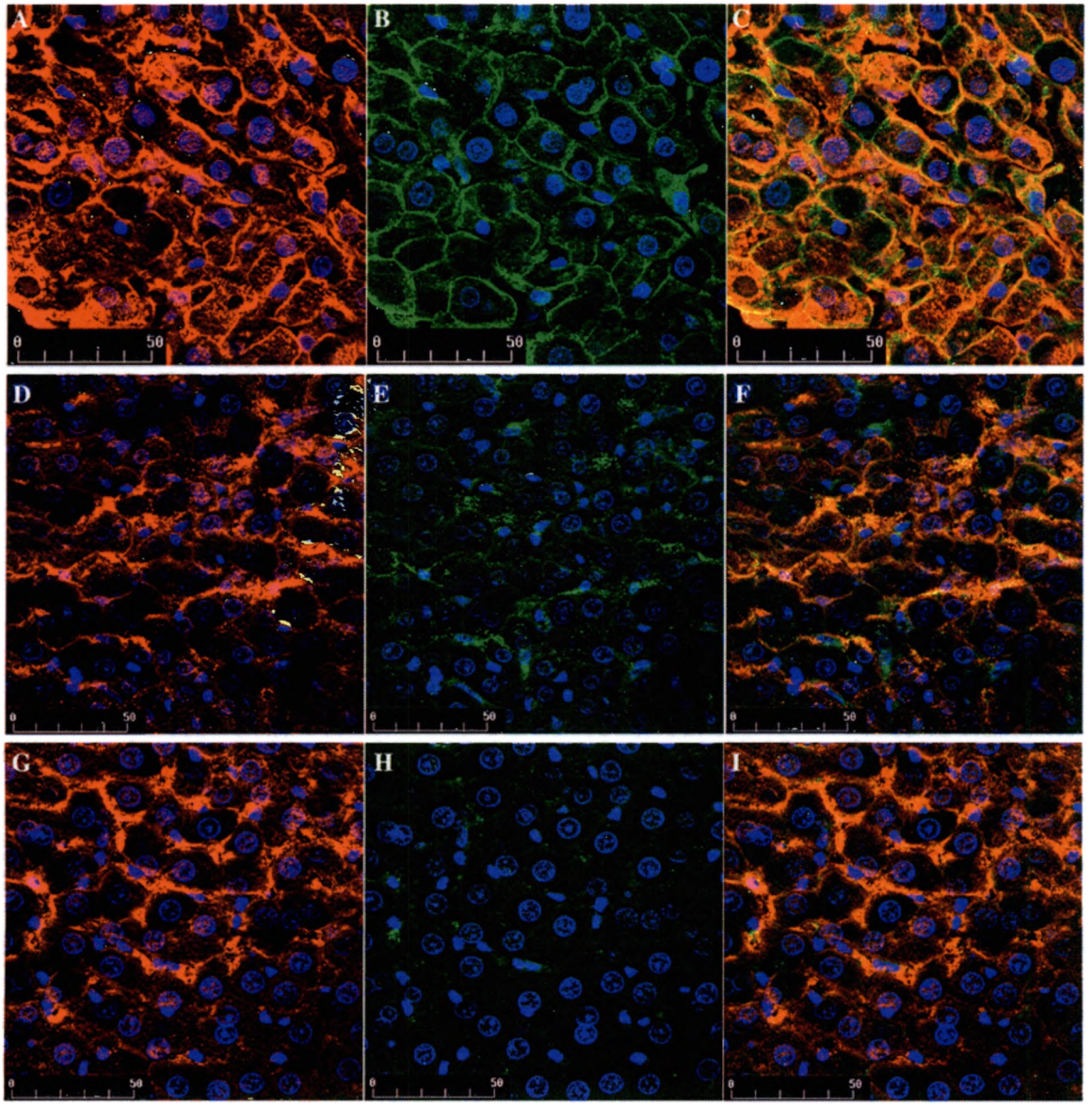

A
B
C
D
E
F
G
H
I
0
50

Figure 6. Confocal Images of Rat Brain Double Immunolabeled with mAB G3.5 and Anti-α-Actinin

Confocal micrographs of rat brain grey matter cryosections double immunolabeled with the G3.5 mAB (AlexaFluor 568 secondary antibody seen as red signal) and anti-α-actinin pAB (AlexaFluor 488 secondary antibody seen as green signal). The G3.5 antigen is present along the processes of astrocytes as seen in the red channel (A, D, G). α-Actinin is present diffusely throughout the tissue and more strongly in the processes of astrocytes as seen in the green channel (B, E, H). Both antibodies labeled some structures distinct from one another. Areas of co-localization are present in the processes of astrocytes as seen in the combined signals from the G3.5 antigen and α-actinin (C, F, I). The scale bars are expressed in micrometers.

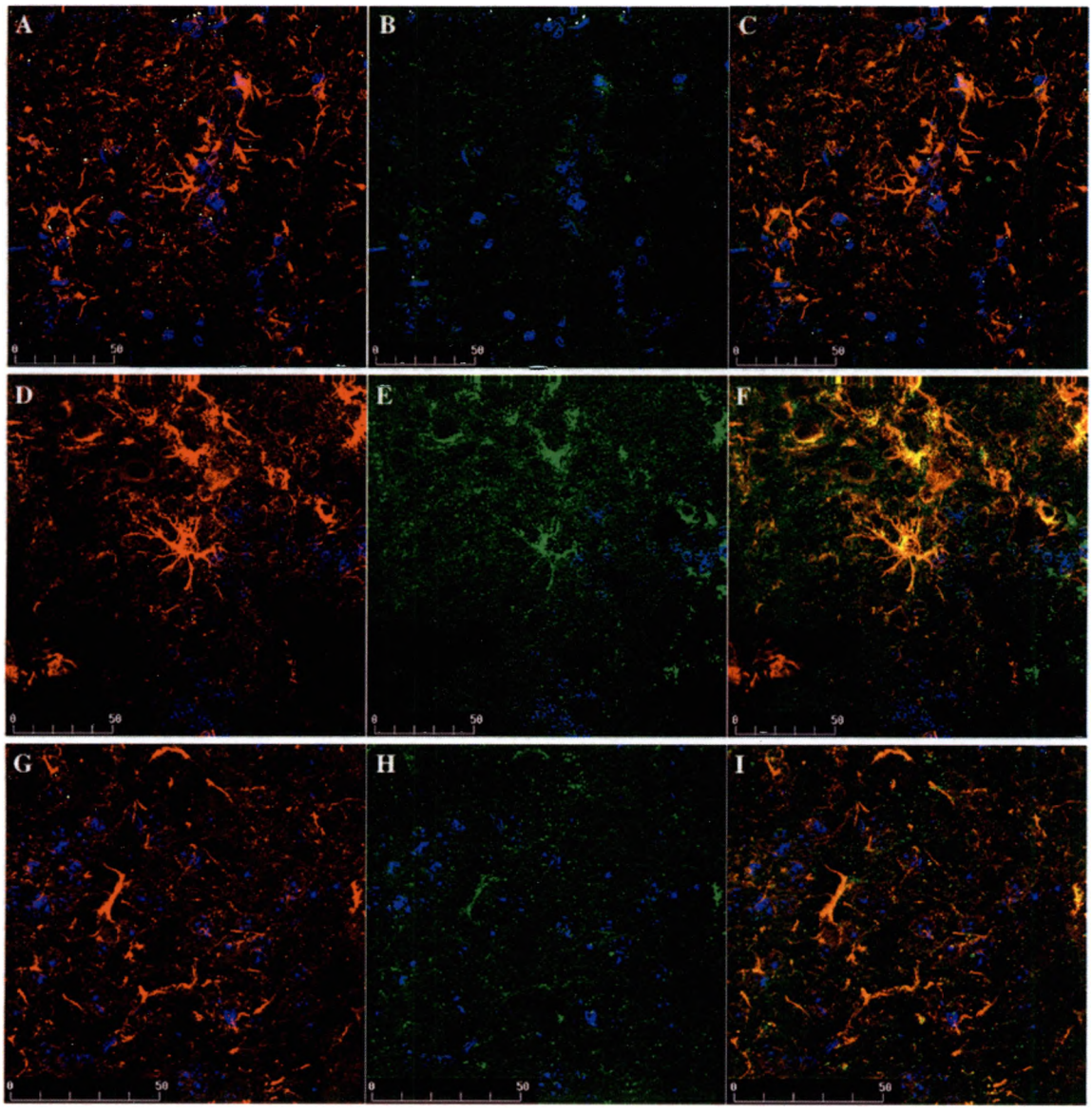

A
B
C
D
E
F
G
H
I

Figure 7. Immunofluorescence Negative Controls

Confocal micrographs of rat cardiac muscle (A, B), Liver (C, D), and brain (E, F) crysections. These samples are negative controls, which were treated using the same protocol as experimental samples, with the omission of both primary anti-bodies. None of the negative control samples exhibit specific binding of either Alexafluor conjugated secondary antibody. The scale bars are expressed in micrometers.

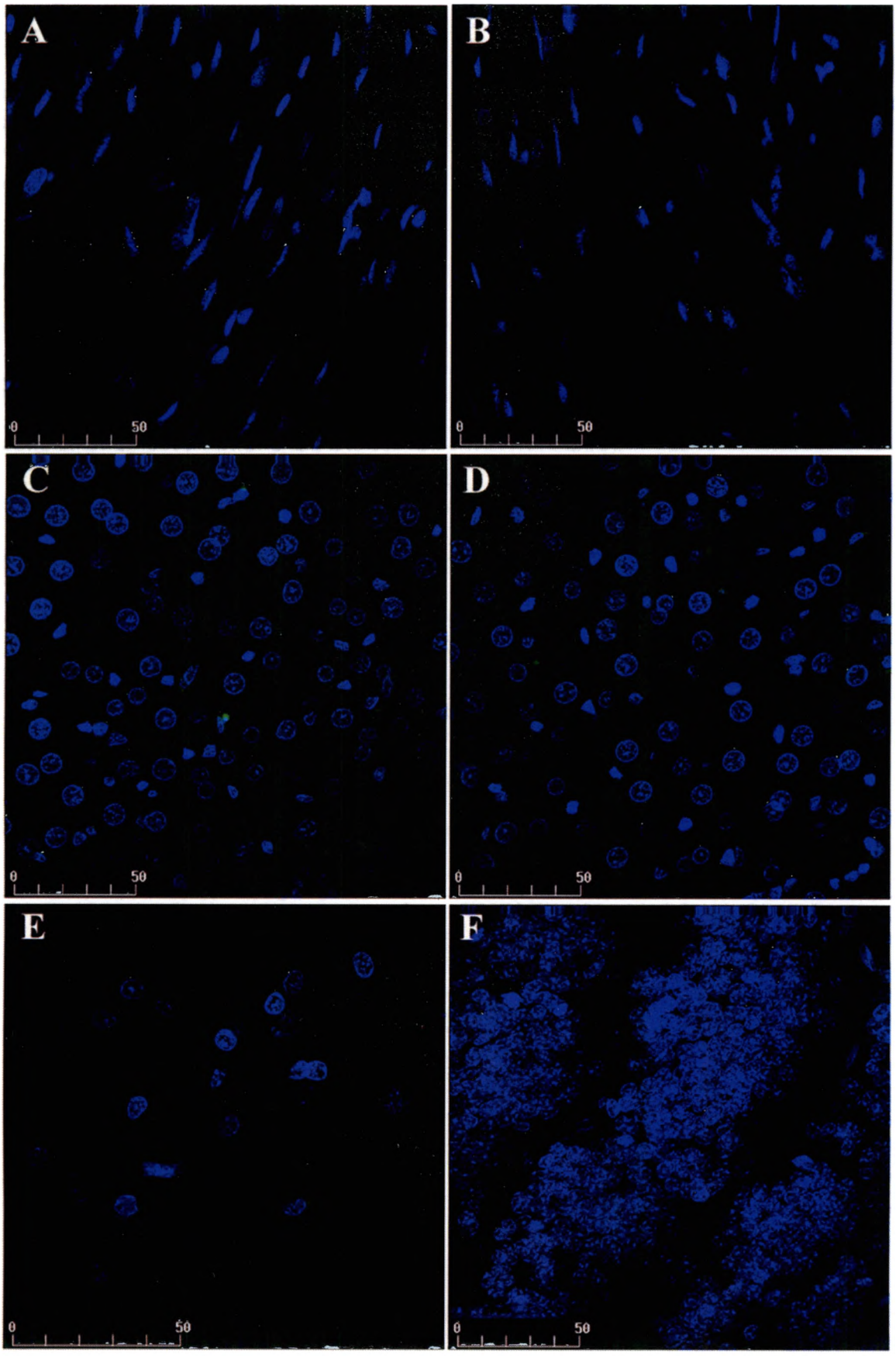
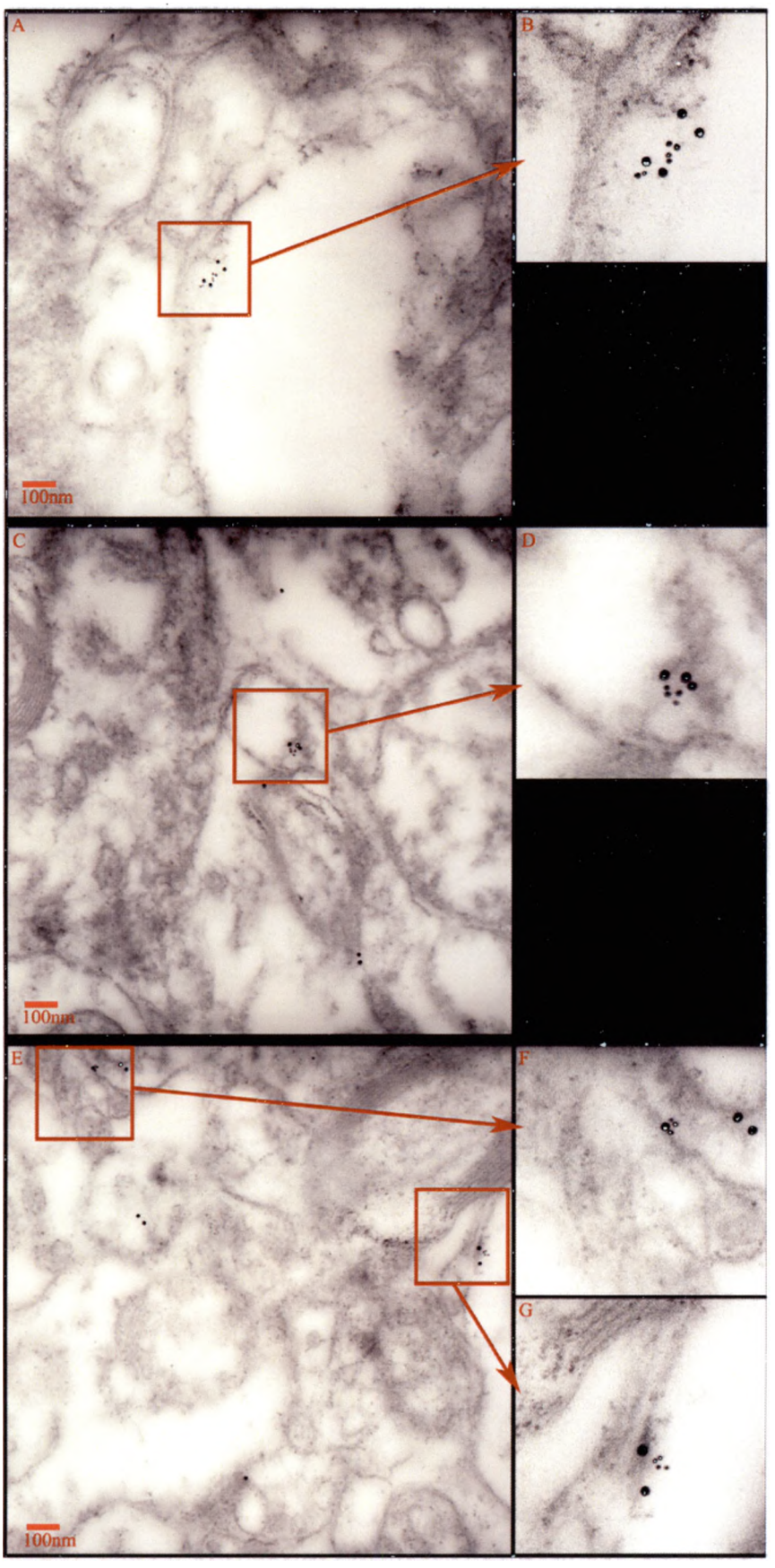

A
0
50
B
0
50
C
0
50
D
0
50
E
0
50
F
0
50

Figure 8. TEM Image of Rat Brain Double Immuolabeled with G3.5 mAB and Anti-α-Actinin

TEM micrograph of rat brain tissue double immunolabeled with the G3.5 mAB (6nm colloidal gold) and anti-cytoplasmic-α-actinin mAB (10nm colloidal gold) collected at 50,000X magnification (A, C, E). The anti-G3.5 and anti-α-actinin antibodies appear to co-localize near structures that are assumed to be the cell membranes of astrocytes (B, D, F, G). The scale bars are expressed in nanometers.


A
B
100nm
C
D
100nm
E
F
G
100nm

CHAPTER IV

DISCUSSION

The G3.5 mAB has been previously described to recognized an intermediate filament associated protein (IFAP) which has the additional characteristic of binding actin filaments *in situ* and *in vitro* as exhibited by immunofluorescence microscopy and overlay binding assays (Bolanos, 1998; Jeffcoat, 1995; Price, 1993). The antigen of the G3.5 mAB shares a high degree of primary sequence identity. Also, the G3.5 mAB exhibits cross-reactivity with anti-α-actinin antibodies when the antigens of these antibodies are subjected to SDS-PAGE and western blotting under denaturing conditions (Bolanos, 1998; Jeffcoat, 1995; Price, 1993). The G3.5 antigen and α-actinin also have a similar overall structure, as illustrated by electron microscopy, and produce the same pattern of fragments when treated with V8 protease (Bolanos, 1997; Jeffcoat, 1995). However, the presence of discrepant sequence (QIFV) in the 19-mer isolated by Price (1993) and the absence of co-localization between the G3.5 mAB and monoclonal antibodies raised against cytoplasmic and sarcomeric α-actinins in some tissues and under certain types of fixation in previous studies have prevented the recognition of the G3.5 antigen as a known isoform of α-actinin (Bolanos, 1998; Jeffcoat, 1995).

To shed new light on this subject the G3.5 antigen was again isolated using an adapted desmin purification protocol (Bolanos, 1998; Lazarides, 1980; Price, 1993). Upon partial tryptic digestion, the G3.5 antigen yielded 7 fragments, which share a high degree of

primary sequence identity with rat α-actinins in line with the findings of the studies mentioned above (Table 2, Table 3). Analysis of newly obtained G3.5 amino acid sequence as well as previously published G3.5 sequence data using the automated genomic search tool provided by Ensembl.org confirmed the four known α-actinin genes as the primary matches within the rat genome for the G3.5 antigen fragments. All seven fragments correspond to regions that are highly conserved among the members of the α-actinin family (Table 3). Six of these fragments share 100% primary sequence identity with a corresponding sequence of at least one α-actinin isoform. None of the published rat α-actinins share 100% sequence identity with all 7 of the fragments produced in this study, suggesting the presence of multiple isoforms of α-actinin within the G3.5 isolate sample (Table 3). Fragment 6 (TINEVENQVFTR) is the only fragment from the current study with a residue not found in any of the published α-actinin primary sequences (Table 3). This fragment matches most closely with rat α-actinin isoform 3 with the exception of a phenylalanine residue, which sits against a leucine residue in the corresponding α-actinin 3 sequence (Fig. 3). This disparity may be due to an error during the sequencing process or represent a different allele of rat α-actinin 3 since a one nucleotide change in the third position of the leucine codon can account for a switch from leucine to phenylalanine during codon translation. α-Actinin isoform 2 appears to share the highest degree of primary sequence identity with the G3.5 fragments produced in previous studies (Table 3, Fig. 3) (Bolanos, 1998; Price, 1993). There are no significant matches for the newly and previously sequenced G3.5 protein fragments outside of the four known α-actinin genes. This finding rules out the possibility of the G3.5 antigen as a novel α-actinin isoform and suggests sequencing error as a possible source for the

presence of 3 amino acid residues in a previously sequenced G3.5 fragment which are not present in any of the known α-actinins (Table 2, Table 3) (Bolanos, 1998; Price, 1993).

The localization pattern of the G3.5 antigen and α-actinin was again analyzed using immunofluorescence light microscopy and immunogold electron microscopy double labeling techniques in attempts to better characterize the co-localization pattern of the G3.5 mAB and anti-α-actinin antibodies among members of the α-actinin family in different tissues. A polyclonal anti-α-actinin antibody was used to view the localization pattern of all α-actinins relative to the G3.5 mAB in rat cardiac muscle, liver, and brain tissue. The G3.5 mAB and anti-α-actinin pAB did not appear to co-localize in cryosections of rat cardiac muscle. The G3.5 mAB exhibited the expected labeling pattern along the Z-lines and M-band in the exosarcomeric space and beneath the sarcolemma (Fig. 4). The anti-α-actinin pAB also localized in a pattern overlying the Z-lines and beneath the sarcolemma as well as within the Z-discs. When rotated along the Y-axis and viewed at increments of 20°, the two antibodies appear to localize along the same structures in the sub-sarcolemmal space (Fig. 4 B, D, F). The occurrence of non-localized antibody labeling in striated muscle tissue may be explained by the presence of different isoforms of α-actinin within the sarcomere of certain myofiber subtypes (Mills, 2001). Both these isoforms, α-actinins 2 and 3, have been shown to be expressed in mammalian brain tissue (Mills, 2001). In cryosections of rat liver and brain tissue grey matter the G3.5 mAB and the anti-α-actinin pAB appeared to co-localize along some structures while each also labeled structures not recognized by the other antibody (Fig. 4, Fig. 5). The labeling pattern of the G3.5 mAB and an anti-cytoplasmic-α-actinin mAB in sections of LR White embedded PFA fixed rat brain also appear to suggest co-

localization when viewed by transmission electron microscopy (Fig. 8). Here the different sized colloidal gold particles corresponding to each antibody are seen together in bundles with diameters that fall within the known length of the α-actinin monomer (Fig. 8 B, D, F, G) (Tang, 2001). These areas of labeling are near plasma membranes. It is assumed these membranes belong to astrocytes as the G3.5 mAB has previously been shown to associate with astrocytes in the central nervous system (Malhotra, 1984). Also, the structures where labeling had occurred did not appear to be myelinated (Fig. 8).

The results obtained in this current study suggest that the G3.5 antigen is α-actinin. The sequenced fragments of the G3.5 antigen and corresponding regions of α-actinin not only share a high degree of primary sequence identity, but the G3.5 antigen and α-actinin also have the same molecular weight, dimeric protein structure and size, and produce similar cleavage patterns when subjected to V8 protease (Bolanos, 1997; Bolanos, 1998; Jeffcoat, 1995; Price, 1993; Tang, 2001). The G3.5 mAB appears to recognize several different isoforms of α-actinin. This antibody was raised against material from brain and spinal cord tissue (Malhotra, 1984). However, it recognizes α-actinins in brain and liver tissue *in situ* (Fig. 5, Fig. 6, Fig. 8) as well as isolated smooth and sarcomeric muscle α-actinins *in vitro* (Fig. 1, Fig. 2, Fig. 3) (Bolanos, 1998; Jeffcoat, 1995). These results suggest the epitope recognized by the G3.5 mAB is common to multiple isoforms of α-actinin. The G3.5 mAB and anti-cytoplasmic-α-actinin antibodies exhibit differential localization in bluegill sunfish RPE tissue sections when fixed by quick freezing. However under PFA fixation, colocalization does occur between these antibodies in this tissue (Bolanos, 1998). When applied to non-denaturing western blots the anti-cytoplasmic-α-actinin antibody does not recognize the G3.5 antigen (Bolanos,

1998). However, this antibody does label the isolated G3.5 antigen on western blots of denaturing polyacrylamide gels (Fig. 2 lane 7) (Bolanos, 1998). The anti-sarcomeric-α-actinin antibody, which was raised against sequences specific to α-actinin isoforms 2 and 3, also labels the G3.5 bands on blots of SDS-PAGE gels (Bolanos, 1998). However this antibody as well exhibits a lack of complete or significant co-localization with the G3.5 mAB in tissue sections of rat skeletal muscle (Bolanos, 1998). Although the G3.5 mAB and the anti-cytoplasmic and anti-sarcomeric-α-actinin antibodies do not co-localize under all conditions *in situ*, the cross-reactivity between these antibodies and their antigens under denaturing conditions suggests the epitopes recognized by the G3.5 mAB and anti-α-actinin antibodies are present in the same protein (Fig. 8) (Bolanos, 1998; Jeffcoat, 1995). The G3.5 epitope may therefore be hidden on certain isoforms of α-actinin while in their native dimeric configuration *in situ* and only becomes available for recognition upon denaturation. The appearance of normally masked epitopes upon denaturation of a protein is not an uncommon occurrence, having been observed in *B. thuringiensis* toxins, *E. coli* nonfimbrial adhesin CS31A, and human lipoprotein lipase. In all these cases, denaturation of the protein in question facilitates the unmasking of epitopes not presented when the protein is in its native configuration (Daniel, 2002; Mechin, 1996; Peterson, 1992). Heterodimerization of α-actinin monomers and tissue specific alternative splicing of α-actinin gene transcripts may also explain the presence of dissimilar antigenic patterns in the native structure of some α-actinin isoforms (Chan, 1998; Gache, 1984; Kremerskothen, 2002; Mills, 2001; Suzuki, 2002; Waites, 1992). Masked epitopes present on these α-actinin isoforms may become exposed following removal from the cellular environment and denaturation.

The co-purification of the G3.5 antigen with the insoluble desmin fraction of muscle cell lysates and subsequent release by the introduction of a reducing agent suggest that it associates with IFs. Although α-actinin is not considered an IFAP, interactions between this protein and the IF protein desmin have previously been reported. α-Actinin has been shown to cross-link filamentous actin and desmin *in vitro* (Bolanos, 1998; Kjorell, 1982). Desmin (skeletin), when isolated from bovine heart tissue, was also found to be present in a 150 kD complex with a sarcomere-associated isoform of α-actinin (Kjorell, 1982). Spectrin, which is another member of the spectrin superfamily of actin binding proteins and shares a great deal of homology with the repeating central rod domains of α-actinin, also binds desmin *in vitro* (Davison, 1989; Langley, 1986; Viel, 1999; Wasenius, 1987). In addition, the IF protein synemin has been shown to bind α-actinin when the two are mixed *in vitro* (Bellin, 1999; Bellin, 2001; Jing, 2005).

Future studies should attempt to identify the specific isoform(s) of the α-actinin, which present the G3.5 antigen using isoform-specific anti-α-actinin antibodies in double-labeling immunohistochemical experiments. This goal can also be achieved by isolation of the G3.5 antigen from different tissues such as skeletal muscle, brain and liver. Primary sequence analysis can be used to determine the identity of α-actinin isoforms obtained using the G3.5 isolation procedure in these tissues. A study of this nature may confirm the presence of the G3.5 epitope on multiple isoforms of α-actinin by producing several fragments that match the amino acid sequence of different α-actinin isoforms specifically.

Additional studies should focus on demonstrating a direct interaction of α-actinin with desmin *in situ.* Immunofluorescence light microscopy and immunogold electron

microscopy techniques using anti-α-actinin and anti-desmin antibodies should verify these interactions. The production of specific fragments of the various α-actinin proteins using bacteriophage vectors may allow for the characterization of the specific binding site for type III IFs on the α-actinin protein.

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