Abstract

Deborah L. Ambert. THE EXPRESSION OF A PUTATIVE CYTOSKELETAL PROTEIN IN HYPERTROPHYING SKELETAL MUSCLE. (Under the co-direction of Dr. George J. Kasperek and Dr. Gerhard W. Kalmus). Department of Biology, July 2002.

The purpose of this study was to determine if the expression of the putative cytoskeletal protein temporarily named pseudopaxillin (pPax) is regulated by skeletal muscle growth or skeletal muscle development. Pseudopaxillin was found with a monoclonal antibody to paxillin during skeletal muscle exercise studies. Paxillin is a cytoskeletal focal-adhesion docking protein with which pPax has some homology. They share an epitope and possibly other common amino acid sequence. Previous studies in the laboratory have indicated that pPax is only expressed in skeletal muscle and that its protein levels are highest in young animals.

The hypothesis for this research is that the pPax protein levels are developmentally regulated. This would indicate that pPax could be an important component of muscle restructuring that occurs during development.

To test this hypothesis hypertrophy studies were done with adult male Sprague Dawley rats to determine if the increased pPax protein levels were due to muscle development or muscle growth.

Hypertrophy is defined as an increase in muscle growth. To induce hypertrophy, the right gastrocnemius and plantaris muscles were removed by surgical ablation. This allowed the soleus muscle to increase in size by 136%

over the left non-operative control leg after two weeks and 147% after four weeks of induced hypertrophy. Pseudopaxillin (pPax) protein levels in these hypertrophied soleus muscles did not increase indicating that pPax expression is not associated with growth and thus is consistent with the hypothesis that pPax plays a developmental role in skeletal muscle.

The cytoskeletal proteins α-actinin and talin showed a significant decrease with hypertrophy at four weeks. These results follow the same trend as pPax indicating that pPax could be a developmentally regulated skeletal-muscle cytoskeletal protein.

THE EXPRESSION OF A PUTATIVE CYTOSKELETAL PROTEIN IN HYPERTROPHYING SKELETAL MUSCLE

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In Partial Fulfillment

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DEDICATION

I would like to dedicate this thesis to my husband Gary for his love and support, and to Dr. Gerhard Kalmus who gave me the opportunity that allowed me to receive this Master's degree.

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LIST OF ABBREVIATIONS

AUP	Animal Use Protocol
BCA	Bicinchoninic Acid
CTL	Control
ECL	Enhanced Chemiluminescence
ECM	Extracellular Matrix
FAC	Focal Adhesion Complex
FAK	Focal Adhesion Kinase
FGF	Fibroblast Growth Factor
HRP	Horse Radish Peroxidase
HYP	Hypertrophy
IGF	Insulin-Like Growth Factor
kDa	Kilo Dalton
pPAX	Pseudopaxillin
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

INTRODUCTION

Pseudopaxillin (pPax) Background

Pseudopaxillin (pPax) is a 136kDa skeletal muscle protein, originally detected with the monoclonal antibody to paxillin, a known 68kDa-cytoskeletal focal-adhesion-docking protein during, skeletal-muscle exercise experiments. Paxillin and pPax share an epitope and possibly other common amino acid sequences indicating that they could possibly share common functions. Previous studies conducted with other tissues indicate that pPax is only expressed in skeletal muscle and that its protein levels are elevated in young animals. Experiments on older animals showed a marked decrease in pPax protein levels.

The Hypothesis

The purpose of this research project was to determine whether pPax protein levels were increasing during skeletal muscle development or during skeletal muscle growth in young animals. Since development and muscle growth occur at the same time in young animals, hypertrophy studies were done with adult male Sprague Dawley rats whose muscles were no longer developing. Based on data from whole skeletal muscle and tissue culture studies of differentiating myoblasts and myotubes, the hypothesis of this thesis is that the putative cytoskeletal protein pPax is developmentally regulated.

Muscle: A General Overview

Muscle is the body's largest tissue. It is a contractile tissue and makes up 35 percent of the body weight in women and 45 percent of the body weight in

men. To do even the simplest of tasks requires from 10-200 muscles. Muscle is also extremely efficient. With the help of the nervous system's electrical input, muscle can convert chemical energy into mechanical work. In the process it gives off heat and allows movement, using both fats and carbohydrates as fuel (Siegel, 2000).

Skeletal Muscle Development/Differentiation and the Extracellular Matrix

In vertebrates, muscle precursor cells called myoblasts arise from somites, which are groups of tissue masses segmentally arranged on both sides of the body (Bray, 2001). The myoblasts migrate from the somite and take up positions somewhere in the body such as a limb region. At this point the myoblasts are mixed with connective tissues where they reside until they begin to differentiate. After differentiation, the myoblasts begin to make muscle-specific proteins such as actin and myosin, as well as the acetylcholine receptor. As the myoblasts become long and spindle-shaped, fusing with each other to form myotubes, rearrangement of their cytoskeleton occurs.

This process of myoblast fusion into myotubes requires specific communication between myoblasts, since myoblasts do not fuse with any other cells. Also important for the functioning of differentiating muscle cells is the integration of the myofibrils with each other, the sarcolemma, and the extracellular matrix (ECM) (Lee, et al., 1999). Growth factors such as fibroblast growth factor (FGF) and insulin-like growth factor (IGF) help control these developmental events either negatively or positively. The ECM also helps

regulate muscle differentiation by regulating the migration, morphogenesis, gene expression and organization of the contractile apparatus (Lee, et al., 1999). The next step in muscle development is the accumulation of the thick (myosin) and thin (actin) filaments in the myotube cytoplasm where they align into myofibrils and become fully differentiated skeletal muscle fibers. (Bray, 2001).

Once the muscle is fully differentiated it will not divide again and will survive for the life of the animal. If, however, it is damaged or destroyed, new muscle tissue can take its place through the process of regeneration, which also involves the ECM. New muscle fibers are able to assemble via a set of undifferentiated myoblast-precursor cells called satellite cells that are already present in the mature muscle (Lee, et al., 1999). Satellite cells are small mononucleated skeletal-muscle stem cells that are located between the basal lamina of the muscle and the sarcolemma of myofibers. These cells are mobilized in response to increases in loading (hypertrophy) of the muscle or after injury of the muscle cells. The mechanism of recruitment for these cells after hypertrophy or muscle injury is not known, but it is assumed that satellite cells provide new myonuclei by entering the cell cycle and differentiating (Adams, et al., 1999).

Muscle formation is under the control of the MyoD family of transcription regulating genes which are only expressed in myoblasts and mature muscle fibers (Bray, 2001). The expression of myogenin, a MyoD family member, may function as a muscle-specific marker of differentiation.

Skeletal Muscle Structure

Skeletal muscle cells, called myofibers, are very long cylindrical unbranched single cells formed during development by the fusion of many separate cells. These cells have many nuclei lying just beneath the plasma membrane (sarcolemma). The bulk of the cytoplasm (sarcoplasm) is made up of myofibrils which lie along the length of each myofiber. All myofibers and bundles of myofibers are held together by connective tissue. There are three types of connective tissue associated with muscle fibers: the endomysium, perimysium and epimysium. The endomysium is a layer of reticular fibers surrounding each individual muscle fiber. Small capillaries and nerve branches are found in the endomysium. The perimysium is a thicker connective tissue layer that encompasses groups of fibers to form a bundle (fascicle). Large blood vessels and nerves run through the perimysium. The last and thickest connective tissue layer is the epimysium, which surrounds a collection of bundles (fascicles) made up of muscle fibers. The major nerve and blood supply to the muscle runs through the epimysium (Ross, et al., 1995). The endomysium, perimysium and the epimysium are components of the ECM. The ECM, myofibrils and the sarcolemma must be integrated with each other for muscle formation to occur (Vellerman, 2000).

Myofibrils are the structural and functional units of skeletal muscle. They contain the contractile component of skeletal muscle called the sarcomere, which is the segment of the myofibril between the two Z-disks. Cross striations can be

seen when the muscles are examined under a light microscope. These striations are seen as alternating light and dark bands, which are named the A band (dark bands), the I band (light bands), and the Z line, which is a dense zone that bisects the I band. There is also an H zone which bisects the A band and an M line that is seen in the middle of the H band (Ross, et al., 1995). The arrangement and density differences of the thick (myosin) and thin (actin) filaments cause these striations. Both cardiac and skeletal muscles contain these striations of light and dark bands, and are considered to be striated muscles.

The thin filaments are made up of actin, and they are associated with α-actinin at the Z-disk. Each actin molecule contains a myosin-binding site. Actin (the thin filament) also contains two major proteins, tropomyosin and troponin, which cover the myosin binding sites on actin in muscle fibers that are relaxed. The thick filaments are composed of myosin and are held in register in the middle of the H zone by myomesin filaments, which makeup the M line (Ross, et al. 1995).

There are other essential proteins necessary for regulating attachment and alignment of the myofilaments. Among these are titin, which connects the thick filaments to the Z disk; nebulin, which runs parallel to the actin myofilaments and is attached to the Z disk; and α-actinin, a protein that bundles actin filaments and helps to anchor them at the Z disk (Ross, et al. 1995).

The Cytoskeleton and Myogenesis

The main focus of this thesis is to determine if the putative cytoskeletal protein temporarily called pseudopaxillin (pPax) is regulated by skeletal muscle growth or by the development of skeletal muscle. A secondary aim is to determine how the regulation of pPax expression may relate to a known cytoskeletal cell-adhesion-docking protein called paxillin, as well as to other actin cytoskeletal proteins α-actinin, talin, and vinculin.

Myogenesis involves the precise regulation of many developmental events, which include cell adhesion and cell-cell recognition (Miller, 1992 and Buckingham, 1994). These processes are critical for myoblast alignment and fusion into multinucleated myotubes. The formation of these myotubes is a critical event in skeletal muscle development (Vellerman, 2000).

The main function of the ECM is to communicate information back to the cell that could affect cellular gene expression, cell shape, cell migration, and cell proliferation and differentiation (Vellerman, 2000). The communication from the ECM to the cell is carried out through heterodimeric transmembrane receptors called integrins. The integrins function to link the ECM and the cell's cytoskeleton, because the cell needs to integrate signals coming from its surrounding environment (Carson and Wei, 2000). Studies have indicated that it is the cytoplasmic domain of the β1 integrin subunit, not the α subunit, which is required for interaction with the cytoskeleton. A muscle-specific splice variant called β1D is expressed in both skeletal and cardiac muscle. This integrin

isoform shift is necessary for myoblast differentiation during muscle development (Carson and Wei, 2000).

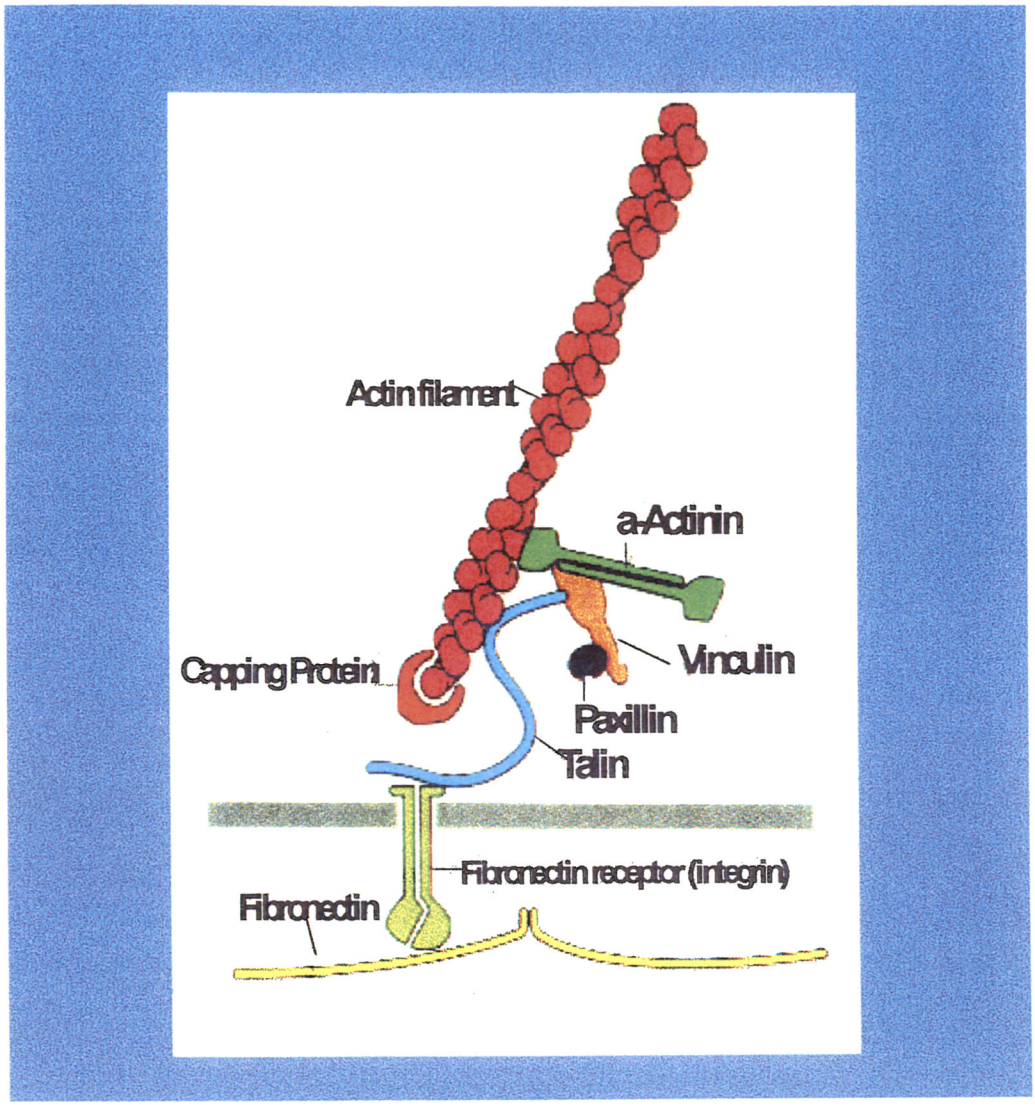
Induction of Hypertrophy and its Affects on the Formation of the Focal Adhesion Complex

It has been shown that loading of skeletal muscle results in an adaptive increase in the expression of focal-adhesion complex proteins. These proteins include focal adhesion kinase (FAK) and paxillin (Fluck, et al., 1999). During muscle contraction, skeletal muscle fibers transmit load from their cytoskeleton to the ECM by using focal adhesions (Fluck, et al., 1999). The function of focal adhesion complexes is to form molecular bridges between actin filaments, integrin receptors, and the ECM. This occurs through stabilization of cell adhesions that provide a pathway for signals to be transferred across the cell membrane. Increased tyrosine phosphorylation of FAK promotes cytoskeletal reorganization. Paxillin (a target of phosphorylated FAK) is sent to focal adhesion complexes when integrins cluster due to occupancy of their receptors. Occupancy of integrin receptors by ECM proteins causes the accumulation of vinculin, talin and α-actinin, which help attach the integrins to the focal adhesion complexes allowing the phosphorylation of FAK (Fluck, et al., 1999) (See Figure 1).

Focal Adhesion Protein Levels During Hypertrophy of the Soleus Muscle

It was hypothesized that loading of skeletal muscles (in this case the soleus muscle) using the surgical ablation model to induce hypertrophy results in

Figure 1 A model of focal adhesion complex formation with the intracellular attachment proteins that mediate linkage between an integrin and an actin filament (Alberts, et al. 1994).


Actin filament
a-Actinin
Capping Protein
Vinculin
Paxillin
Talin
Fibronectin receptor (integrin)
Fibronectin

an increase in the expression of two focal-adhesion complex proteins FAK and paxillin (Fluck, et al., 1999).

The results showed that paxillin levels per total protein were significantly increased by 87 percent within one day of surgically induced overload. They remained elevated (431 percent) after eight days of overload. Focal adhesion kinase protein content per total protein was significantly increased after one day of overload and at eight days of overload the levels were increased by 611 percent (Fluck, et al., 1999).

Skeletal Muscle Hypertrophy Model

Skeletal muscle hypertrophy is defined as an increase in the diameters of muscle fibers (Banks, et al., 1990). This increase in muscle mass results in increased amino acid transport, increased satellite cell proliferation, increased protein synthesis, decreased protein degradation and fiber type switching (Adams, et al., 1999 and Carson, et al., 2002). In this thesis, the model used to induce hypertrophy was surgical ablation, which induces hypertrophy by overloading the soleus muscle after removal of its synergists (the plantaris and gastrocnemius). Two different time courses of hypertrophy, one for two weeks and one for four weeks were used. The goal was to induce as much hypertrophy as possible in older rats to measure pPax levels due to growth, not development. These time courses were based on the literature results for actual induction of significant amounts of hypertrophy (Mozdziak, et al., 1998). The two-week experiment was chosen because it was near the middle of the time courses used

in reported hypertrophy studies. The four-week experiment was chosen because it was near the end of the reported studies and has been shown to produce more hypertrophy. Both time courses should allow for induction of hypertrophy without causing hyperplasia.

In order to test the hypothesis, the expression of pPax protein levels in hypertrophied skeletal muscle will be measured. The model used will cause growth of the soleus muscle. If pPax levels are increased in these muscles then the increases seen in young animals are due to growth. If the levels of pPax do not increase in the hypertrophied soleus muscles the increases in pPax levels could be due to development. Other cytoskeletal proteins will be measured to determine if they have similar increases and decreases in hypertrophied skeletal muscle. This will help to determine if pPax is a putative cytoskeletal protein.

MATERIALS AND METHODS

All chemicals, reagents and solutions were of analytical or electrophoresis grade and were purchased from several different companies as noted in parenthesis.

Model

Adult outbred 250-275 gram male Sprague Dawley rats purchased from Harlan (Indianapolis, IN) were used for all experiments. They were housed in standard plastic rat cages with wire tops and fed standard rat chow in the Department of Comparative Medicine, Brody School of Medicine at East Carolina University before, during and after surgical treatment. Hypertrophy of the soleus muscle was induced by surgical ablation of its synergists, the gastrocnemius and the plantaris. These experiments were conducted for periods of two weeks and four weeks, respectively.

Surgical Procedures

The rats were acclimated for five days prior to surgery. Food and water were removed the night before surgery and the day of surgery each rat was anesthetized with Ketamine 90mg/ml and Xylazine 10mg/ml (concentration =0.1ml/gm of body weight) given IP.

All surgery was performed in a sterile environment with the surgical leg being shaved and swabbed with Betadine (ECU Medical Storeroom, Greenville, N.C.).

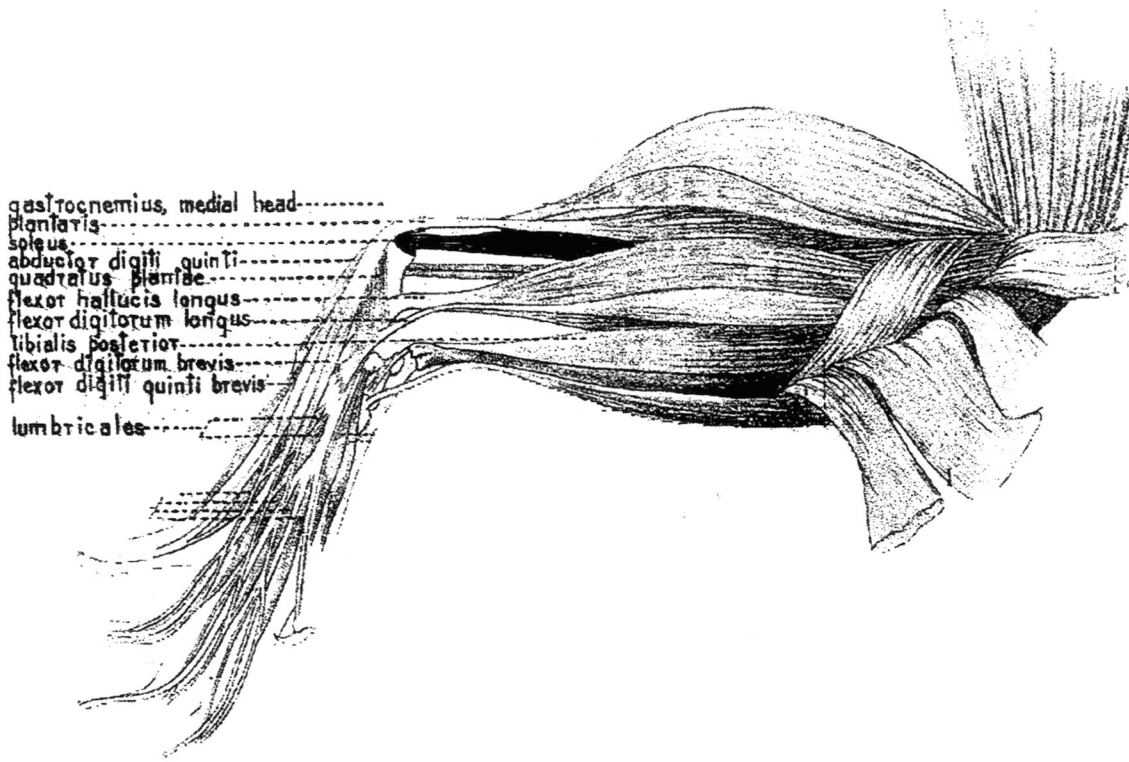
Ablation

Hypertrophy of the soleus muscle was induced by surgical ablation of its synergists. The surgical incision was made longitudinally through the skin and fascia along the lateral aspect of the tibia where the distal two-thirds of the right plantaris and gastrocnemius muscles were removed (see Figure 2). The left leg was used as a non-surgical control. Care was taken to insure that both the blood and nerve supplies remained intact during the surgical procedure (Baldwin, et al., 1982).

Postoperative care and monitoring include administration of two doses of Ampicillin 50mg IM and two doses of Toridol for analgesia (0.1ml/gm body weight) one immediately after surgery and one four hours later. The rats were continuously monitored until they were fully awake. All procedures were performed in accordance with the guidelines set forth by the animal care and use committee and an AUP #C055. The Ketamine/Xylazine used for surgical anesthesia/euthanasia and the Ampicillin and Toridol used to treat infection and for analgesia, respectively, were purchased from the Department of Comparative Medicine, Brody School of Medicine at East Carolina University, Greenville, NC.

At two weeks and four weeks post-surgery the animals were given a lethal dose of Ketamine /Xylazine 90/10mg/kg (0.3ml/100gm of body weight) and the soleus muscle was surgically removed from both legs and weighed. The muscles were then quick-frozen in liquid nitrogen (ECU Medical Storeroom, Greenville,

Figure 2 Muscles of the medial surface of the lower leg and the flexor surface of the rat foot. The soleus muscle (black) is hypertrophied by surgical ablation of its synergists (Greene, E.C., 1968).


gastrocnemius, medial head
plantaris
soleus
abductor digiti quinti
quadratus plantae
flexor hallucis longus
flexor digitorum longus
tibialis posterior
flexor digitorum brevis
flexor digiti quinti brevis
lumbricales

NC) and stored in a -70°C Cryo-Fridge (Baxter Scientific Products, Charlotte, NC) for further analysis with Western blotting (Laemmli, 1970).

Tenotomy

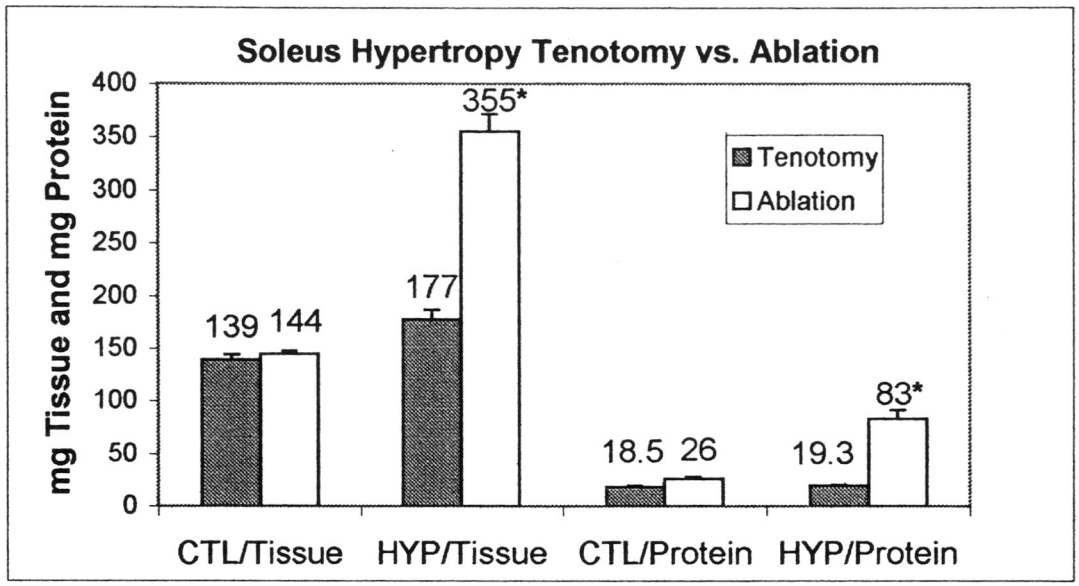
An initial four-week set of hypertrophy experiments was carried out using the technique of surgical tenotomy (Horne and Hesketh, 1990). This experiment was performed exactly the same way as the ablation experiments except that the tendons of the gastrocnemius and the plantaris muscles were severed, leaving the muscles intact. The four week tenotomy procedure produced only a 27 percent increase in muscle wet weight, while the four week surgical ablation technique produced a 147 percent increase in muscle weight (see Figure 3). Based on the data in Figure 3 the surgical ablation model was used in the studies reported in this thesis.

Both the tenotomy animals and the ablation animals were allowed to walk freely on the floor each day to check their range of motion and use of the surgical leg. They were also carefully monitored for infection.

Protein Concentration Determination

The Pierce Bicinchoninic Acid (BCA) protein assay (Pierce, Rockford, IL) was used to determine the total protein concentrations of the samples in preparation for western blotting. A standard curve was run in triplicate using 2 μg/μl Bovine Serum Albumin (Pierce, Rockford, IL) at concentrations of 0, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 μg/μl. Supernatants (100μl) from the muscle preparation were removed and added to 900μl of 0.5M NaCl to be used for this

Figure 3 A comparison of soleus muscle hypertrophy due to surgical tenotomy and surgical ablation at four weeks post surgery based on wet weight and total protein in milligrams. The * indicates $p<0.001$.


Soleus Hypertropy Tenotomy vs. Ablation
mg Tissue and mg Protein
400
350
300
250
200
150
100
50
0
139
144
177
355*
18.5
26
19.3
83*
Tenotomy
Ablation
CTL/Tissue
HYP/Tissue
CTL/Protein
HYP/Protein

assay. The 1:10 dilution of the sample in 0.5M NaCl removed the possibility for interference that would occur with this assay due to the high amounts of SDS and EDTA that are present in the sample homogenization buffer. All samples and standards were run in triplicate using 10μl of sample or standard/well in a 96 well Costar plate (Corning, Corning, NY). After the samples/standards were added to the wells the BCA reagents A and B are mixed together (50:1) and 200μl was added to each sample/standard well. The plates were then incubated at 37°C for 30 minutes or at room temperature (23° C) for one hour. The absorbance was then read at 562nM in a Thermo-max microplate reader (Molecular Devices, Sunnyvale, CA).

Sample Preparation

The muscles were weighed and homogenized at a concentration of 25mg muscle/ml of Homogenization buffer (10mM Tris pH 8.5, 1mM EDTA, 3.3% SDS, 10% glycerol and protease inhibitors added just before use) using a Polytron (Brinkman Instruments, Westbury, NY). A sample of whole homogenate (1.5ml) was then placed in a 2ml microcentrifuge tube and centrifuged at 10,000 X g for 20 minutes at room temperature (23°C). After the centrifugation was completed the muscle supernatants were analyzed by the BCA protein assay as reported above, and placed in 2X SDS-PAGE sample buffer (156mM Tris-HCL, pH 6.8, 1.25% glycerol, 5% SDS, 1.25-% β-Mercaptoethanol and 3mg of Bromophenol.

Blue). The muscle supernatants were boiled for five minutes cooled and were used for analysis by Western blotting

Western Blotting

Using an Hoefer SE600 vertical gel apparatus (Amersham Biosciences, Piscataway, NJ) 30μg of each muscle sample was loaded onto a 6% Sodium Dodecyl Sulfate-Polyacrylamide Gel (SDS-PAGE) and run in Laemmli Electrode Buffer (25mM Tris, 19.2mM Glycine and 2% SDS) overnight for 17 hours at 20mA (Laemmli, 1970). Following SDS-PAGE the gels were transferred for 2 hours at 24 volts in Towbin's blotting buffer (25mMTris, 192mM glycine, 20% methanol, and 0.02% SDS, pH 8.3, (Towbin, et al., 1979) using a semi-dry blotter from (IDEA Scientific Company, Minneapolis, MN).

The blotted PVDF-Immobilon-P membranes (Millipore Corporation, Bedford, MA) containing the muscle proteins were blocked in 5% non-fat dry milk in 1X Basic Buffer (10mM Tris, 154mM NaCl and.01% Tween-20). The proteins were then analyzed for the actin cytoskeletal proteins paxillin, pPax, vinculin, talin, and α-actinin by Western blotting (Towbin et al., 1979). All antibodies used were monoclonal. The paxillin antibody (BD Biosciences-Pharmingen, San Diego, CA) was diluted 1:5000, Vinculin (Chemicon International, Temecula, CA), 1: 2000, Talin 1: 5000, and α-actinin (Sigma Chemical Company, St. Louis, MO) 1:500 in 5% non-fat dry milk /1X basic Buffer. After incubation for at least one hour at room temperature (23°C) or four hours at 4°C the blots were washed for 15 minutes (5 minutes X 3 washes) in 1X Basic Buffer. They were then probed

with a secondary antibody (Anti-Mouse Ig Horse Radish Peroxidase (HRP)-linked whole antibody from sheep) (Amersham Pharmacia Biotech UK Limited) which was diluted 1:5000 in 5% milk in 1X Basic Buffer for one hour at room temperature (23°C). The secondary antibody was removed and the blots were washed for twenty five minutes (5 minutes X 5 washes) in 1X Basic Buffer.

After washing the blots were prepared for Enhanced Chemiluminescense (ECL) by placing them into ECL buffer (100mM Tris, pH 8.5) for at least 5 minutes before entering the dark room. The blots were developed using ECL Luminol Detection Reagent (100mM Tris, pH 8.5, 0.68mM p-Coumeric acid, 30% hydrogen peroxide and 100mg/ml Luminol with a total volume of 88.24ml). This method of ECL detection on immunoblots was adapted from the Amersham Biosciences ECL kit (Amersham Biosciences, Piscataway, NJ). The blots are placed in the ECL reagent for one minute and wrapped in plastic wrap (Saran, Dow Chemical, Midland, MI). They were then exposed for 1-5 minutes to Fuji Medical x-ray film 100 NIF 13 X 18 cm (Fisher Scientific Company, Pittsburgh, PA). The films were developed in a Konica SRX-101A Medical Film Processor (Fisher Scientific, Pittsburgh, PA), scanned using Deskscan 2 software (Hewlett Packard Co., Boise Idaho), and quantified using ImageQuant 3.3 software (Molecular Dynamics, Sunnyvale, CA). Results were expressed as relative units per milligram protein and per milligram tissue. Variance between blots was corrected for by running four identical samples on each blot. The ECL reagent is reusable and is made up fresh in the laboratory on a monthly basis.

Statistical Methods

Group means, standard deviations, and standard errors, were calculated using Excel and Quattro Pro statistical programs. Testing of the significant differences between means of control and treatment groups were calculated using the program T-Test (Surgery Research Laboratory Statistics Program, Harvard Medical School, D.R. Araneda, Version 2). A $p<0.05$ was accepted as statistically significant.

RESULTS

Verification of Soleus Muscle Hypertrophy at Two Weeks and Four Weeks after Surgical Ablation of its Synergists

Hypertrophy of the soleus muscle was induced by the surgical removal of the gastrocnemius and plantaris muscles. At two weeks (N=8 rats) and at four weeks (N=10 rats) post surgery, the muscles were removed and weighed (wet weight). The muscles were then homogenized and a protein assay was run to determine the amount of total protein in both the control and hypertrophied muscles.

Both the two week and the four week hypertrophy experiments showed significant differences ($p<0.001$) in wet weights (measured in milligrams of protein) and total protein per muscle (measured in milligrams of muscle). Figure 4 shows an actual picture comparing a hypertrophied muscle to a control muscle. The percentage differences between the hypertrophied muscles and the control muscles in wet weight are 135 percent (two weeks) and 146 percent (four weeks). In total protein per muscle the percentage differences between hypertrophied and control muscles are 126 percent and 219 percent at two and four weeks, respectively (Figures 5 and 6).

The Effect of Two and Four Weeks of Hypertrophy on Protein Levels of Pseudopaxillin (pPax) and Four Other Actin Cytoskeletal Proteins

To determine the levels of pseudopaxillin (pPax) in both the control and hypertrophied soleus muscles the two and four-week muscles were run on 6%

Figure 4 Surgical ablation results of an actual two-week soleus control and hypertrophied Muscle.

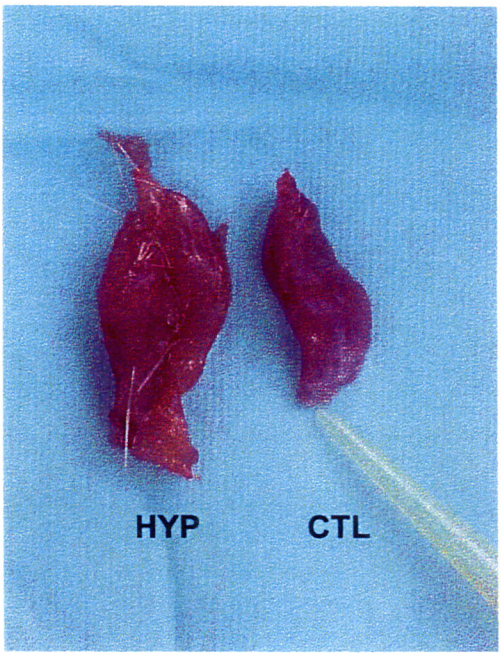
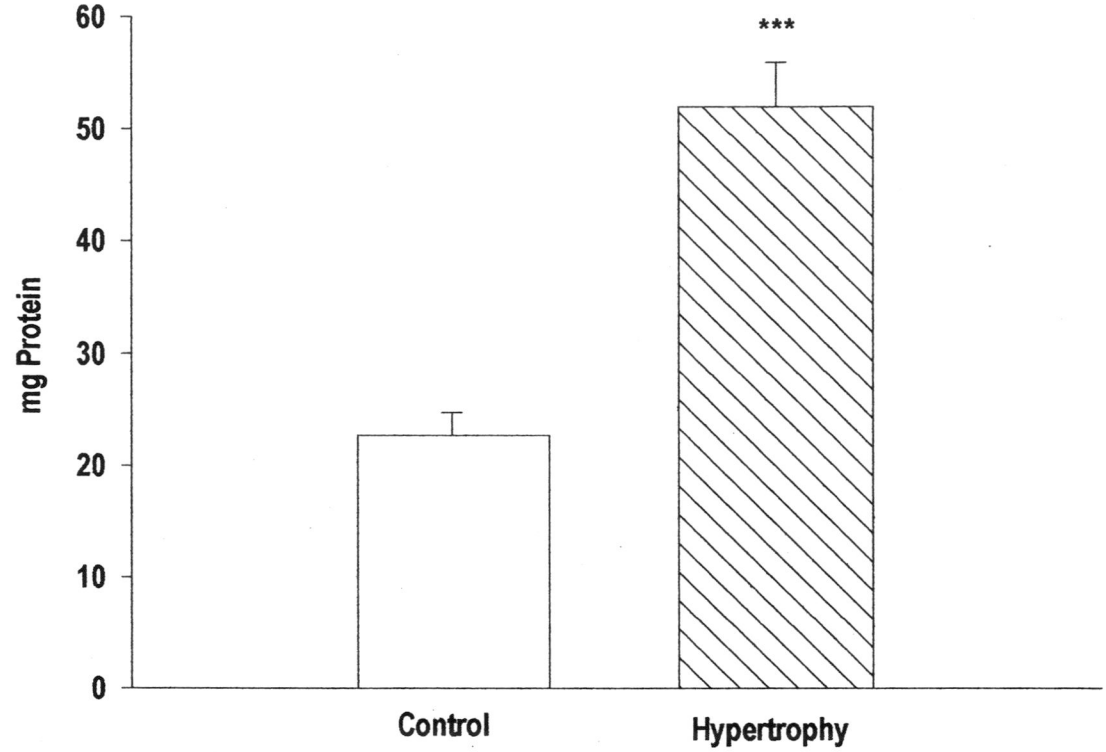

HYP
CTL

Figure 5 Two-week hypertrophy induced by surgical ablation. Graphs depict total protein per muscle in milligrams and soleus wet weight in milligrams immediately after surgical removal from the animal. Each bar represents an overall average and standard error of control and hypertrophied muscles. The *** indicates $p<0.001$.


60
50
40
30
20
10
0
mg Protein
***
Control
Hypertrophy

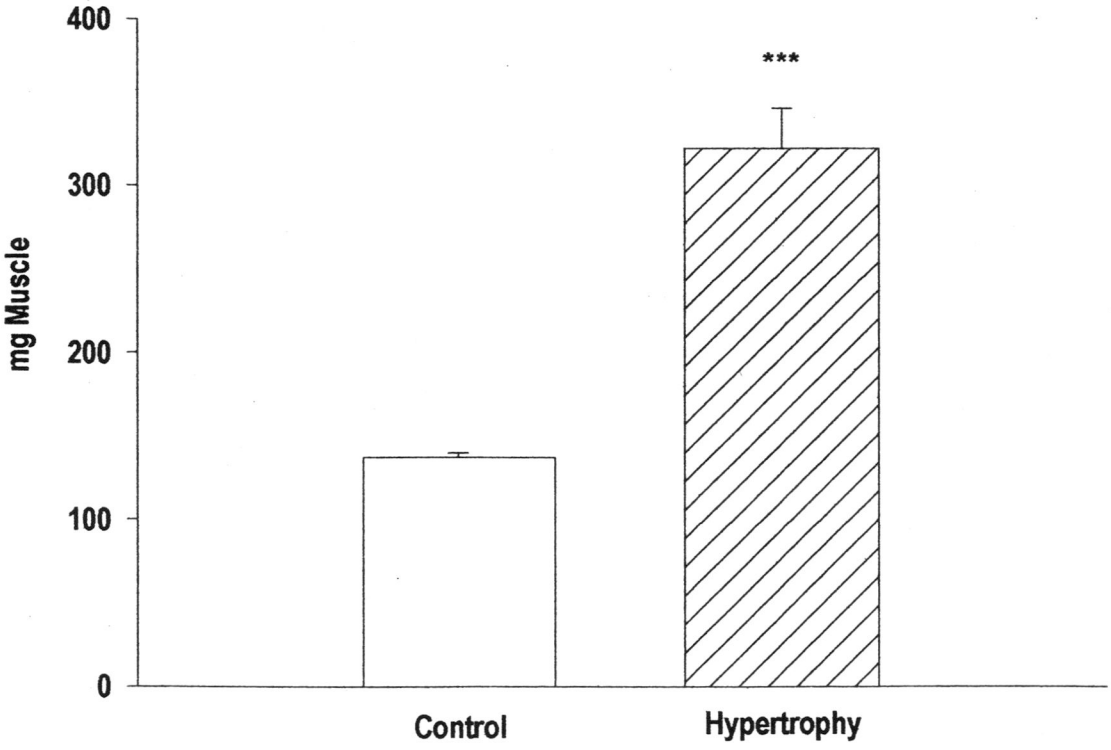
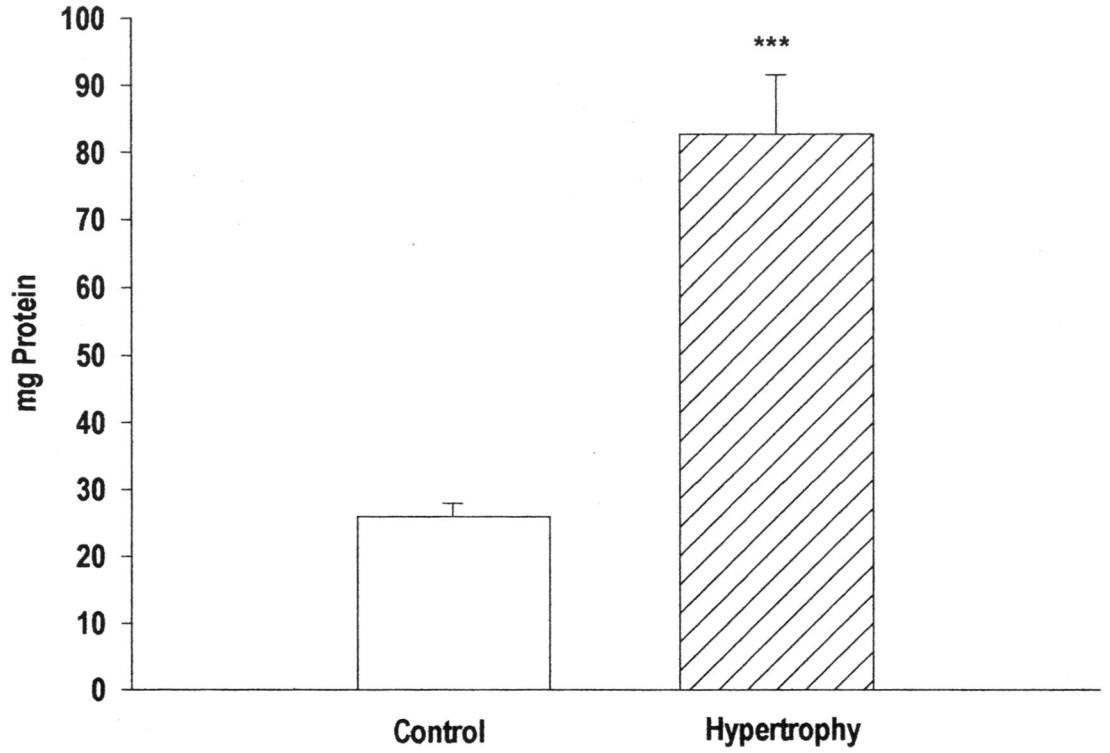
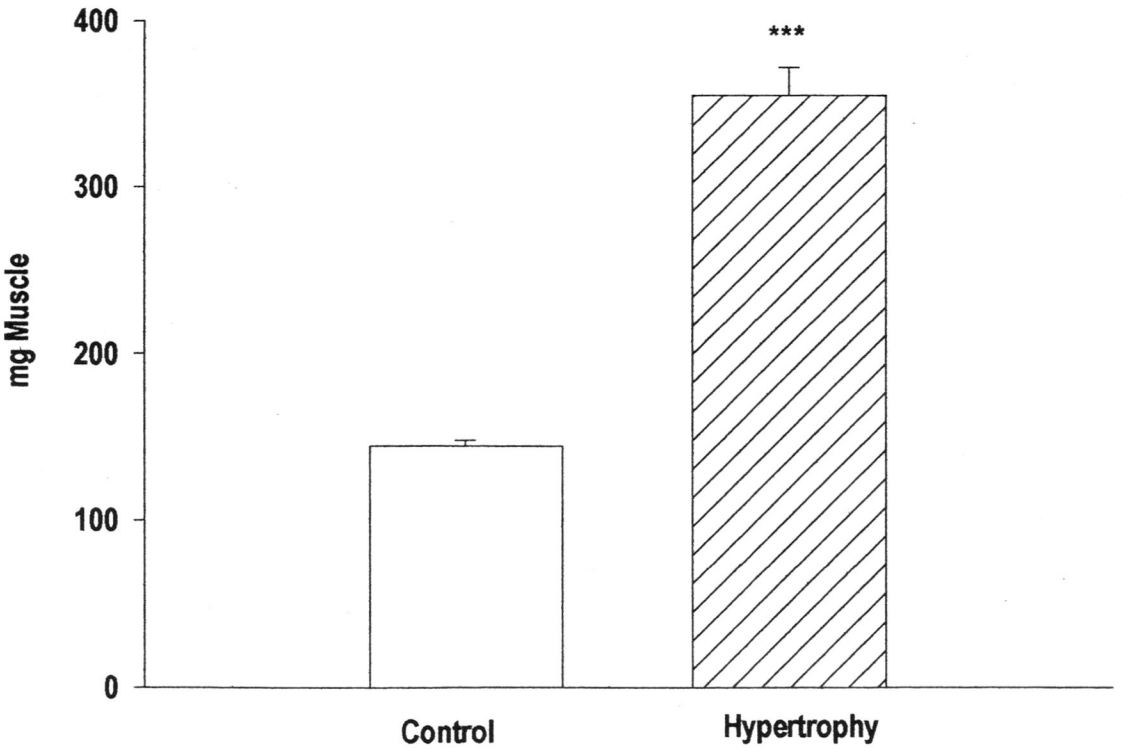

400
300
200
100
0
mg Muscle
***
Control
Hypertrophy

Figure 6 Four-week hypertrophy induced by surgical ablation. Graphs depict total protein per muscle in milligrams and soleus wet weight in milligrams immediately after surgical removal from the animal. Each bar represents an overall average and standard error of control and hypertrophied muscles. The *** indicates $p<0.001$.


100
90
80
70
60
50
40
30
20
10
0
mg Protein
***
Control
Hypertrophy


400
300
200
100
0
mg Muscle
***
Control
Hypertrophy

SDS-PAGE and western blotted (Figures 7 and 8) using the monoclonal antibody to paxillin. After two and four-weeks of induced hypertrophy calculations expressed in relative units per milligram tissue showed pPax levels were lower than the controls by 25 and 35 percent respectively ($p<0.001$) in the hypertrophied muscles (Figures 9 and 10). When expressed in relative units per milligram protein this same trend was seen but with less significance at two weeks of hypertrophy where the pPax levels were significantly lower than controls by 26 percent ($p<0.05$). The decrease in statistical significance at two weeks is likely due to variability between samples (Figure 11). After four weeks of hypertrophy, pPax levels were lower than the control muscles by 52 percent ($p<0.001$) (Figure12).

Since pPax is a putative cytoskeletal protein, four other actin cytoskeletal proteins (α-actinin, paxillin, vinculin, and talin) were measured at both two and four weeks to compare their protein levels with those of pPax after induction of hypertrophy by surgical ablation (Figures 9-12).

At two weeks post surgery, there were no significant differences between the four proteins when expressed per milligram tissue or per milligram protein. At four weeks post surgery there were no significant differences between these four proteins per milligram tissue but there were differences when expressed per milligram protein. Talin and α-actinin protein levels were decreased by 17.5 ($p<0.05$) and 28 percent ($p<0.01$), respectively. Thus, talin and α-actinin follow the same trend observed for pPax.

Figure 7 Western blot comparing the five different cytoskeletal proteins pseudopaxillin (pPax), paxillin, α-actinin, vinculin and talin that were measured after two weeks of surgical ablation. Note: C= control and H= hypertrophy.

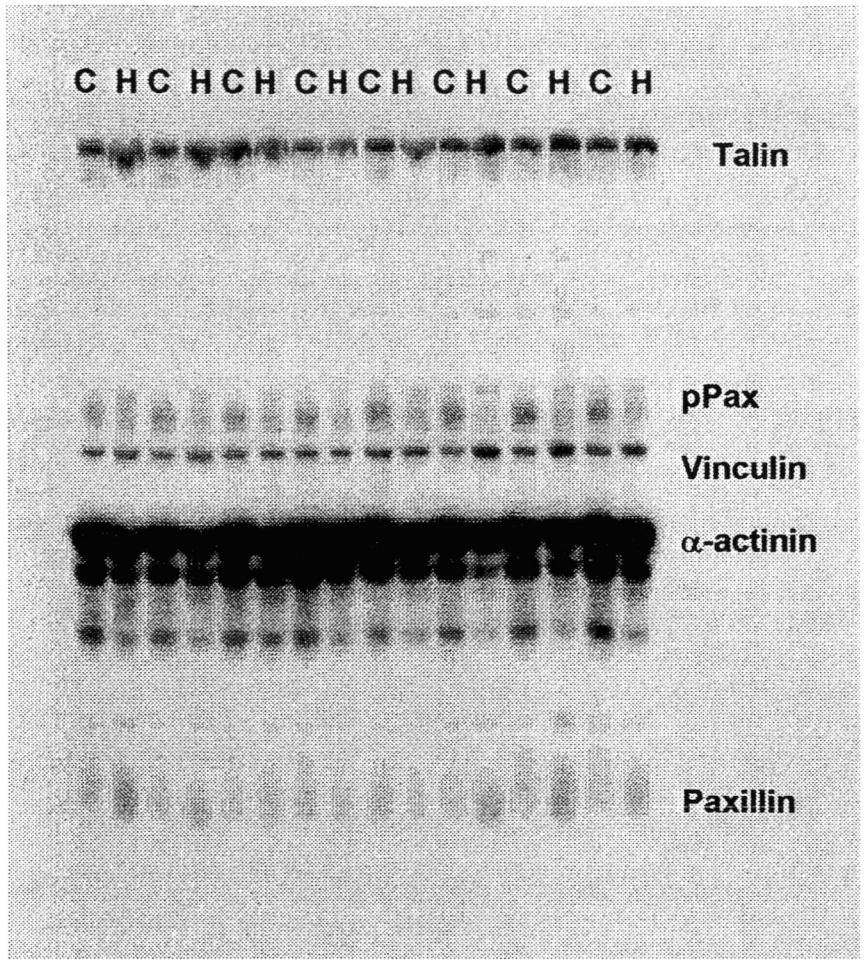

C H C H C H C H C H C H C H
Talin
pPax
Vinculin
α-actinin
Paxillin

Figure 8 Western Blot comparing the five different cytoskeletal proteins pseudopaxillin (pPax), paxillin, α-actinin, vinculin, and talin that were measured after four weeks of surgical ablation. Note: C= control and H= Hypertrophy.

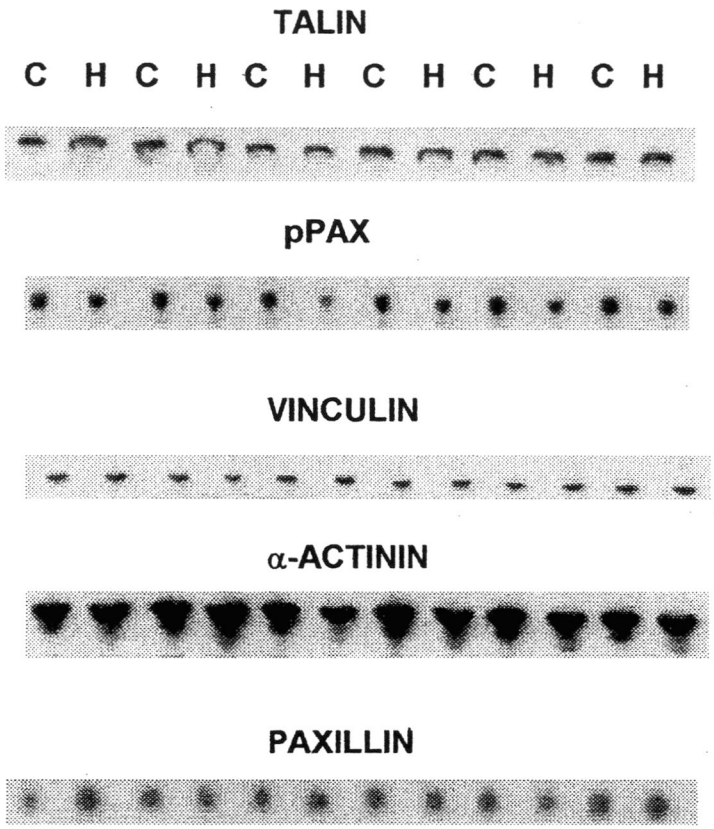

TALIN
C H C H C H C H C H C H
pPAX
VINCULIN
α-ACTININ
PAXILLIN

Figure 9 Two-week hypertrophy induced by surgical ablation. Graphs compare relative units per milligram tissue for five different actin cytoskeletal proteins, paxillin, pseudopaxillin (pPax), α-actinin, talin and vinculin. Each bar represents an overall mean and standard error of control and hypertrophied soleus muscles. *** Indicates $p<0.001$.

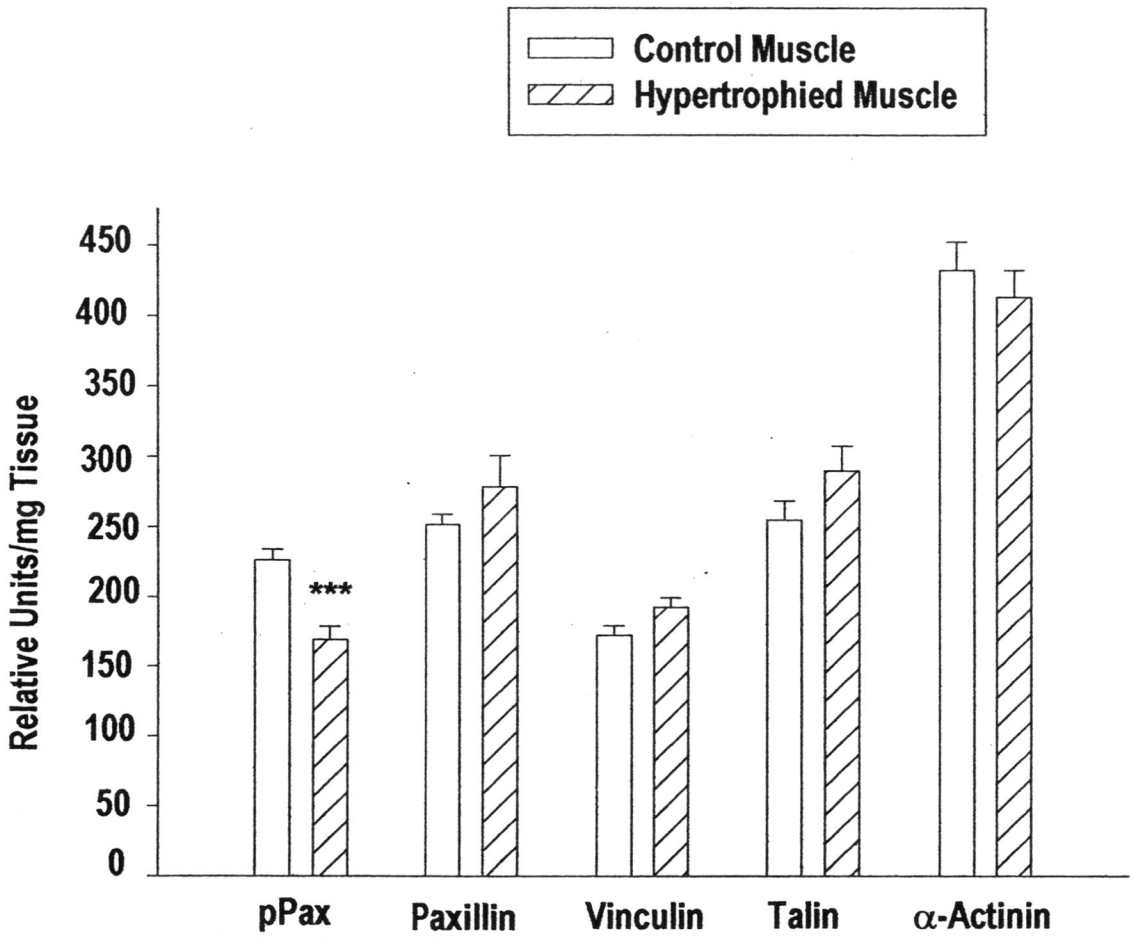

Control Muscle
Hypertrophied Muscle
Relative Units/mg Tissue
450
400
350
300
250
200
150
100
50
0
***
pPax
Paxillin
Vinculin
Talin
α-Actinin

Figure 10 Four-week hypertrophy induced by surgical ablation. Graphs compare relative units per milligram tissue for five different actin cytoskeletal proteins paxillin, pseudopaxillin (pPax), α-actinin, talin and vinculin. Each graph represents an overall mean and standard error of control and hypertrophied soleus muscles. *** Indicates $p<0.001$.

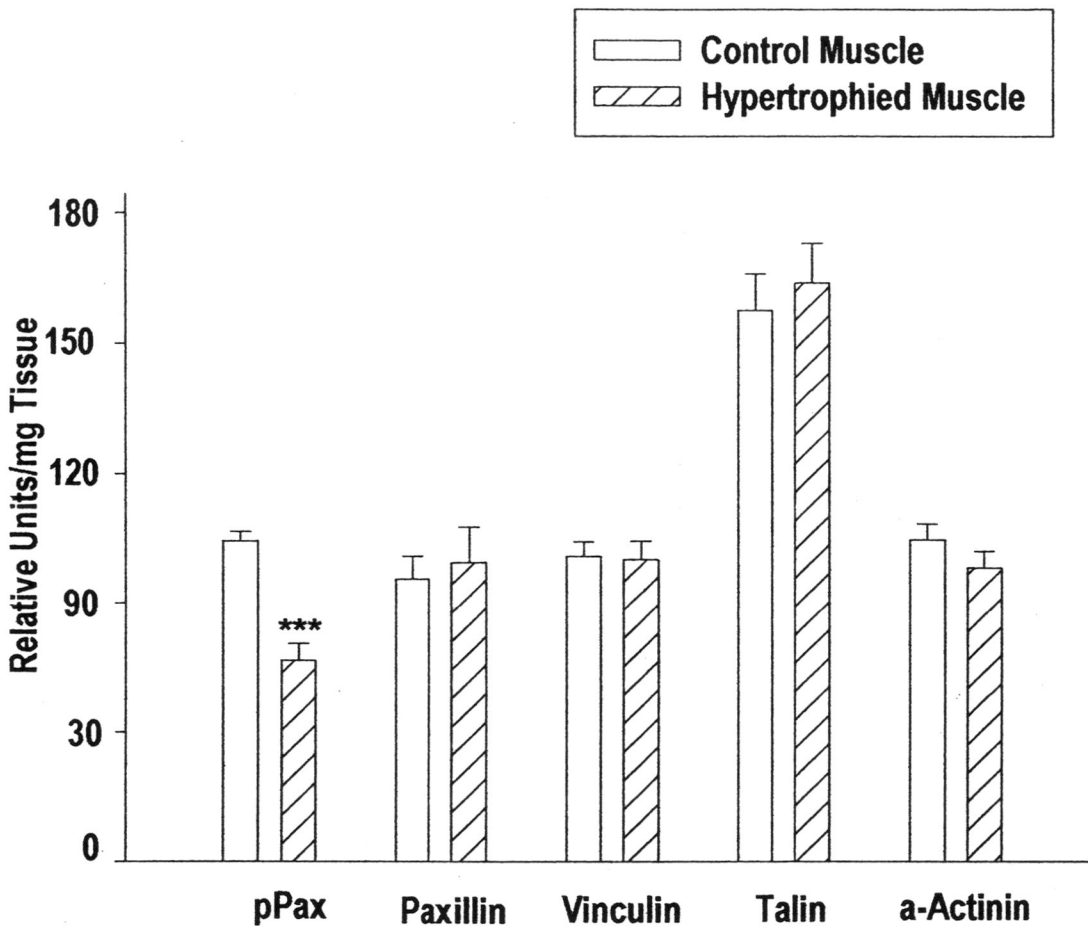

Control Muscle
Hypertrophied Muscle
Relative Units/mg Tissue
180
150
120
90
30
0
***
pPax
Paxillin
Vinculin
Talin
a-Actinin

Figure 11 Two-week hypertrophy induced by surgical ablation. Graphs compare relative units per milligram protein for five different actin cytoskeletal proteins, paxillin, pseudopaxillin (pPax), α-actinin, talin and vinculin. Each bar represents an overall mean and standard error of control and hypertrophied soleus muscles. *Indicates $p<0.05$.

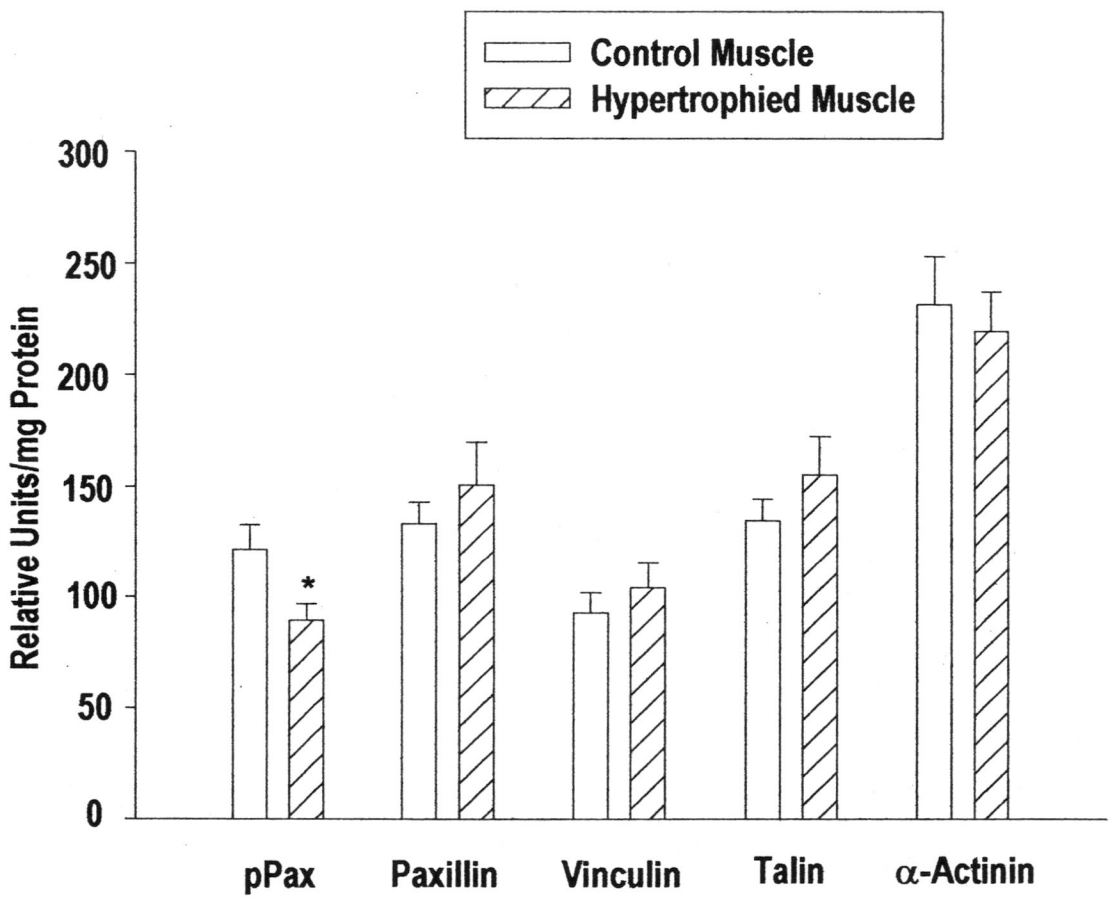
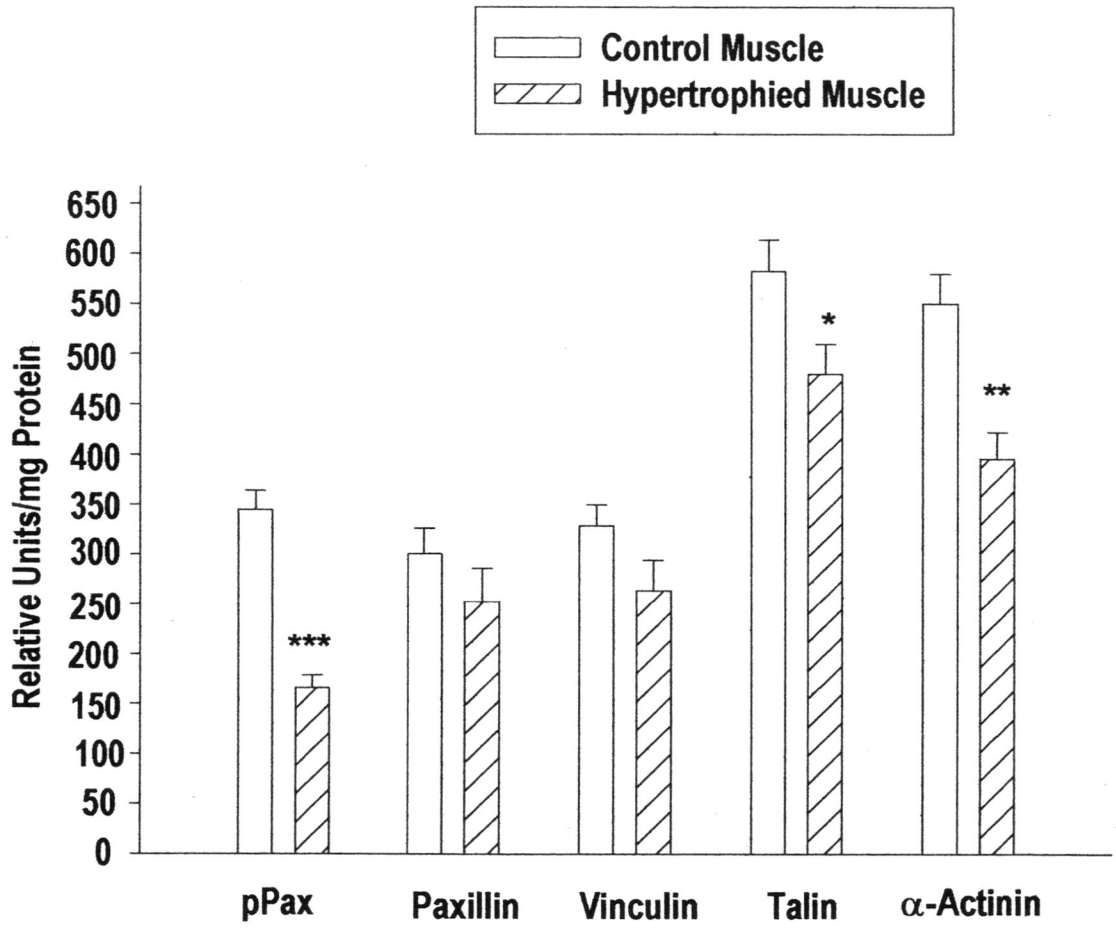

Control Muscle
Hypertrophied Muscle
Relative Units/mg Protein
300
250
200
150
100
50
0
*
pPax
Paxillin
Vinculin
Talin
α-Actinin

Figure 12 Four-week hypertrophy induced by surgical ablation. Graphs compare relative units per milligram protein for five different actin cytoskeletal proteins, paxillin, pseudopaxillin (pPax), α-actinin, talin and vinculin. Each bar represents an overall mean and standard error of control and hypertrophied soleus muscles. * Indicates $p<0.05$, ** $p<0.01$ and *** $p<0.001$.


Control Muscle
Hypertrophied Muscle
Relative Units/mg Protein
650
600
550
500
450
400
350
300
250
200
150
100
50
0
***
*
**
pPax
Paxillin
Vinculin
Talin
α-Actinin

DISCUSSION

Pseudopaxillin (pPax) is a putative cytoskeletal protein with a molecular weight of approximately 136 kDa. It is found only in skeletal muscle and has increased protein levels in young animals and in differentiating myotubes. Pseudopaxillin shares some amino acid homology (an epitope) with the 68-kDa cytoskeletal focal adhesion docking protein paxillin (Fluck, et al., 1999), based on western blots of skeletal muscle, where both proteins are detected with the monoclonal antibody to paxillin.

To determine if pPax protein levels were increased due to muscle growth or to muscle development, hypertrophy (muscle growth) studies of the soleus muscle were undertaken in adult male Sprague Dawley rats. In an initial experiment, hypertrophy was induced by tenotomy, which involved severing the tendons of the gastrocnemius and plantaris muscles, but allowing the muscles to remain intact (Horne and Hesketh, 1990). However, the results of a four-week preliminary experiment on eight adult male Sprague Dawley rats produced only 27 percent hypertrophy. These results may be due to the reattachment of the gastrocnemius and plantaris tendons to areas other than their original insertions, allowing the gastrocnemius and plantaris muscles to function, which in turn decrease hypertrophy of the soleus muscle.

Since the tenotomy test experiment only produced moderate hypertrophy, the surgical ablation model was used to induce hypertrophy in the right soleus muscle (Baldwin, et al., 1982). This model required removal of the synergistic

muscles of the soleus, the gastrocnemius and plantaris muscles, causing the soleus muscle to work harder, resulting in greater hypertrophy. The left leg was used as a non-surgical control and hypertrophy studies were conducted for two and four week time periods. The two and four-week time periods were chosen because they produced significant amounts of hypertrophy (Kandarian, et al., 1992; Dunn, et al., 1999)

The goal for these studies was to determine what function or functions pPax has in the muscle cell. To do this, pPax protein levels in older rats were measured as they increased their muscle mass. These experiments were conducted to test the hypothesis that pPax protein levels are lower in older animals due to developmental regulation and not to regulation by skeletal muscle growth. This hypothesis was based on previously unpublished data that showed pPax protein levels were elevated in differentiating Rat Myoblast Omega (RMO) cells and then decreased in fully differentiated myotubes. The same increases were seen in young as compared to adult skeletal muscles of Sprague Dawley rats.

Hypertrophy of the right soleus muscle at two weeks post surgery showed a 135 percent increase in wet weight measured in milligrams over the non-surgical control and at four weeks the increase was 146 percent. The percentage differences in total protein per muscle between the hypertrophy and control muscles are 126 percent at two weeks post surgery and 219 percent after four weeks. These results indicate that the surgical ablation model was appropriate

for inducing hypertrophy of the soleus muscle in adult male Sprague Dawley rats.

After determining that significant amounts of hypertrophy (muscle growth) had occurred in the soleus muscle, the next step in elucidating the potential function of pPax in the muscle cell was to determine whether its protein levels had increased or decreased. Protein levels were also measured for the cytoskeletal proteins paxillin, vinculin, talin and α-actinin via western blots.

Pseudopaxillin protein levels were decreased by 25 percent after two weeks of induced hypertrophy and 35 percent after four weeks of induced hypertrophy when expressed in relative units per milligram tissue. When pPax levels were expressed in relative units per milligram protein, the same trend occurred but with a change of significance. Pseudopaxillin levels at two weeks were 26 percent lower than their control counterparts, but the significance levels decreased from $p<0.001$ when expressed per milligram tissue to $p<0.05$ when expressed per milligram protein. These differences are due to variability between the samples and possibly a lower number of rats (N=8) in the two-week group. At four weeks (N=10), pPax levels were 52 percent lower in the hypertrophied muscles than in the control muscles.

Since pPax protein levels were significantly decreased after two and four weeks of hypertrophy in the muscles of older rats, both in relative units per milligram tissue and in relative units per milligram protein, the increased levels of pPax that are seen in young animals could not be due to muscle growth.

Therefore, it is consistent with the hypothesis that the regulation of pPax is through a developmental mechanism.

Because pPax is a putative cytoskeletal protein, the protein levels of three actin cytoskeletal proteins, α-actinin, talin and vinculin as well as paxillin (a cytoskeletal-focal-adhesion docking protein) (Fluck, et al., 1999), were measured at two and four weeks post hypertrophy to compare their values with those of pPax. There were no differences in the levels of these four proteins at two weeks post hypertrophy, either in relative units per milligram tissue or in relative units per milligram protein. At four weeks post hypertrophy there were no significant differences between the four proteins when expressed per milligram tissue, but differences did occur when the data were expressed per milligram protein. The cytoskeletal proteins talin and α-actinin showed significant decreases in the hypertrophied muscles of 17.5 percent and 28 percent respectively following the same trend as pPax. Vinculin and paxillin levels were not significantly different in either the two-week or four-week groups.

These results show that paxillin expression is not significantly increased in hypertrophied muscles after two or four weeks of hypertrophy. Both (Fluck, et. al., 1999; and Gordon, et al., 2001) used hypertrophy time courses of one and eight days when measuring focal adhesion (FAK) and paxillin expression levels. Paxillin appears to show an acute response to muscle hypertrophy that lasts from one to twelve days. On day thirteen the levels of paxillin are markedly decreased in skeletal muscle (Fluck, et al., 1999). Thus the findings of the present study

show that during both the two and four-week hypertrophy experiments paxillin levels were not significantly increased was consistent with the data of Fluck and co-workers (1999).

The determination that pPax protein levels are not increased due to muscle growth supports the hypothesis that the observed increase in pPax levels seen in young rats is due to skeletal muscle development. At four weeks post hypertrophy, α-actinin and talin, two important cytoskeletal proteins, show the same protein expression patterns as pPax, indicating that pPax may in fact be a skeletal-muscle-cytoskeletal protein.

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