

FATZ, a Filamin-, Actinin-, and Telethonin-binding Protein of the Z-disc of Skeletal Muscle*

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We report the identification and characterization of a novel 32-kDa protein expressed in skeletal muscle and located in the Z-disc of the sarcomere. We found that this protein binds to three other Z-disc proteins; therefore, we have named it FATZ, γ -filamin/ABP-L, α -actinin and telethonin binding protein of the Z-disc. From yeast two-hybrid experiments we are able to show that the SR3-SR4 domains of α -actinin 2 are required to bind the COOH-terminal region of the FATZ as does γ -filamin/ABP-L. Furthermore, by using a glutathione S-transferase overlay assay we find that FATZ also binds telethonin. The level of FATZ protein in muscle cells increases during differentiation, being clearly detectable before the onset of myosin. Although FATZ has no known interaction domains, it would appear to be involved in a complex network of interactions with other Z-band components. On the basis of the information known about its binding partners, we could envisage a central role for FATZ in the myofibrillogenesis. After screening our muscle expressed sequence tag data base and the public expressed sequence tag data bases, we were able to assemble two other muscle transcripts that show a high level of identity with FATZ in two different domains. Therefore, FATZ may be the first member of a small family of novel muscle proteins.

The Z-disc of vertebrate striated muscle is a region where the antiparallel actin filaments spanning the sarcomere are cross-linked. This supramolecular structure plays an important role in the regulation of contraction both in skeletal and cardiac muscle. Variation of the Z-disc structure is observed during development and differentiation of muscle cells and can be correlated with specific pathological or degenerative conditions associated with muscle injuries or atrophies.

The number of different protein components of the Z-disc is

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The nucleotide sequences reported in this paper have been submitted to the GenBank/EMBL/DDJB data bases with accession numbers AJ278124 and AJ005620.

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far from being complete, and many of the newly discovered muscle proteins appear to be localized in this region, and very generally they can be divided into two groups based on their location. In the first group are proteins that are only partially in the Z-disc while extending into other portions of the sarcomere or the sarcolemma. An example of the first group is titin that acts as a ruler for the ordered distribution of sarcomeric proteins and is particularly important in Z-disc assembly. The NH₂-terminal portion of titin extends into the Z-disc, where two different sub-domains have been shown to interact specifically with α -actinin (1–3) and also with the muscle-specific protein telethonin (4). Although telethonin binds to the NH₂-terminal domain of titin, it also acts as one of the substrates of the titin serine kinase domain that is located outside of the Z-disc (5).

The second group is composed of proteins, many of which have been recently discovered and characterized, that appear to be entirely located in the Z-disc (e.g. α -actinin, Nspl1, telethonin, ZASP1, and CapZ). Among these proteins, α -actinin plays a central role by directly cross-linking the actin molecules. There are four α -actinin genes, two non-skeletal muscle isoforms, actinin 1 and 4 (6, 7), and two skeletal muscle isoforms, actinin 2 and 3 (8). The general structure of the skeletal muscle-specific α -actinin 2 can be divided in three functionally distinct domains as follows: the NH₂-terminal region of 252 amino acids that mediates the interaction with the actin rod, a central region that is composed of four spectrin-like motifs, and finally, the COOH terminus that contains a calmodulin-like domain (9, 10). In muscle the sarcomeric actinin isoforms are capable of forming both homodimers and heterodimers (11). Structural studies have demonstrated that the dimer is organized with antiparallel orientation (12), held together by the internal spectrin-like repeats, whereas the NH₂-terminal domains of the dimer cross-link two opposite actin molecules.

In this paper we present the identification and functional characterization of a novel structural component of the Z-disc that is expressed at a very high level in adult skeletal muscle and to a lesser extent in heart, testis, and prostate. In muscle, this novel protein appears to be involved in a complex network of interactions with other Z-disc components despite the absence of known interaction domains. By using a variety of methodologies, we demonstrate the specific interaction of this novel protein with α -actinin 2, telethonin, and γ -filamin/ABP-L. Therefore, we have named this novel γ -filamin/ABP-L, α -actinin-2 and telethonin binding protein of the Z-disc of skeletal muscle, FATZ. On the basis of the information known about its binding partners, we could envisage a central role for FATZ in the myofibrillogenesis.

EXPERIMENTAL PROCEDURES

Culture of Primary Myoblasts

Primary human myoblasts (CHQ5B) were obtained from Dr. Vincent Mouly (URA, Paris, France). The cells were isolated and grown as described previously (13). Differentiation medium was Dulbecco's modified Eagle's medium supplemented with 0.4% Ultrosor G (Life Technologies, Inc.). The mouse myoblast cell line C2C12 (14) was obtained from Dr. S. Soddu (CRS, Rome, Italy).

Full-length cDNA Cloning and Sequencing of Human and Mouse FATZ

Two full-length skeletal muscle cDNA libraries were screened to complete the human FATZ transcript. The first was prepared from human adult pectoral muscle mRNA and cloned into pCDNAII plasmid vector (Invitrogen), and the second, from a commercial source (CLONTECH), was cloned into the pGAD10 yeast expression vector. Both these libraries were arrayed in 96-well plates containing 50 recombinant clones per well. Two rounds of PCR¹ screening were performed on bacterial lysates from the plate wells to isolate the longest FATZ cDNA clones (13). Six full-length cDNA clones were isolated and sequenced from the two libraries.

A similar approach was used to isolate the mouse FATZ transcript. Five independent full-length clones were isolated from a mouse diaphragm muscle cDNA library cloned in the Uni-Zap XR λ vector (Stratagene).

The sequences were produced from full-length cDNA clones using a primer-walking strategy and analyzed on a PE Applied Biosystems 377 automated DNA sequencer with fluorescent BigDye[®] terminators. Sequences were assembled using the SeqMan II computer program (DNASar, Madison WI).

GST Overlay and Pulldown Experiments

GST Overlay—Proteins from lysates (60 μ g) of muscle cells and tissues were separated by SDS-PAGE and blotted onto Immobilon P membrane (Millipore). The membrane was blocked with milk buffer (PBS, 10% milk powder, 0.05% Tween 20) for 1 h, washed, and incubated for 2 h at room temperature with either the GST protein (6 μ g) alone or with the GST-FATZ protein (6 μ g) in milk buffer. Then the membrane was washed, incubated with goat anti-GST antibody (1/1000) for 2 h at room temperature, washed again, and incubated with rabbit anti-goat conjugated with alkaline phosphatase (AP).

Cell Lysate Overlay—Purified recombinant proteins (1 μ g) were separated by SDS-PAGE and blotted onto Immobilon P membrane that was incubated for 2 h at room temperature with cell lysates (60 μ g per blot). The lysate was prepared by sonication of the cells in PBS containing 1% Triton X-100, 0.2% SDS, 0.5% Nonidet P-40, and 0.1% Tween 20 and then diluted 200-fold in 5% milk buffer plus 0.05% Tween 20. The membrane was washed and incubated for 2 h at room temperature with either preimmune sera or monoclonal anti-sarcomeric α -actinin antibody (Sigma A7811) at 1/500 dilution. Goat anti-mouse conjugated with AP (1/20,000) was used as the second antibody.

GST Pulldown Assay—Differentiated C2C12 cells were starved in Dulbecco's modified Eagle's medium minus methionine for 3 h and then labeled with [³⁵S]methionine overnight. The cells were harvested, resuspended in buffer (50 mM Hepes, pH 8.0, 250 mM NaCl, 0.1% Nonidet P-40) plus protease inhibitor mixture (Roche Molecular Biochemicals), and sonicated briefly on ice. Equal amounts (2 μ g) of the GST protein alone or the GST-FATZ protein bound to glutathione-Sepharose 4B were mixed with [³⁵S]methionine-labeled cell lysate (150 μ g) and incubated for 1 h at 4 °C. The samples were washed and run on 15% SDS-acrylamide gels. The dried gels were exposed to SR Packard phosphor screens, and the analysis was done on a Packard Cyclone Phosphor Imager (Packard Instrument Co.). Both Bio-Rad pre-stained precision (Bio-Rad) and rainbow ¹⁴C-methylated protein (Amersham Pharmacia Biotech) molecular weight markers were used in these experiments.

Immunoelectron Microscopy

Sections of heart and skeletal muscle fibers were prepared using an ultramicrotome (Reichert Ultratuc S, Leica). The samples were fixed in

paraformaldehyde (4%), dehydrated, embedded in Lowicryl resin K4M (Sigma), and then ultrathin sections (0.1 μ m) cut. The muscle sections were blocked in 1% bovine serum albumin plus 0.05% Tween 20 and incubated with mouse polyclonal antibody to the recombinant FATZ protein used at a 1/25 dilution for heart and a 1/50 dilution for skeletal muscle samples. Anti-mouse IgG whole molecule conjugated with 5-nm gold particles (Sigma G-7527) was used as the secondary antibody, at a 1/20 dilution. After counter-staining the sections were visualized using a transmission EM (Zeiss 255/230, Carl Zeiss, Germany).

Immunofluorescence Microscopy

Tissue sections as well as primary human and mouse muscle cells were fixed in paraformaldehyde (4%), permeabilized with Tween 20 (0.05%), and then blocked in PBS containing 1% bovine serum albumin and 0.05% Tween 20. The samples were incubated with mouse anti-FATZ or rabbit anti-actin (Sigma A2668) antibodies used at 1/30 and 1/50 dilutions, respectively. The secondary antibodies used for these experiments were fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Sigma F 4018) and Texas Red-conjugated goat anti-rabbit immunoglobulin (Calbiochem). An Axiovert 35 fluorescence microscope (Zeiss) at \times 40 magnification was used to view and photograph the slides.

Northern Blot Analysis and RT-PCR

Northern blot analysis was done using mRNA filters obtained from CLONTECH (Palo Alto, CA). Human and mouse tissue filters contained 1 μ g of mRNA per lane from different tissues as indicated in Fig. 2. Specific probes corresponding to the 3'-portion of the FATZ transcript were generated by PCR amplification of the cDNA clone with specific primers in the presence of 50 μ Ci of [³²P]dCTP (3000 Ci/mmol, PerkinElmer Life Sciences).

Reverse transcriptase PCR (RT-PCR) assay was performed on a panel of 16 different human tissue cDNAs (human MTC panels I and II, CLONTECH) using primers specific for the 3'-portion of the FATZ mRNA.

Recombinant Protein and Antibody Production

The cDNA for FATZ was cloned into both the pQE9 His tag (Qiagen) and pGEX 6P (Amersham Pharmacia Biotech) prokaryote expression vectors and then sequenced to confirm that there were no significant changes from the original transcript. FATZ was purified using nickel-nitrilotriacetic acid resin (Qiagen) for the His tag protein and glutathione-Sepharose 4B (Amersham Pharmacia Biotech) for the GST-tagged protein. The His-tagged FATZ protein was used to immunize rabbits and mice for the production of polyclonal antibodies.

Western Blotting and Quantitation

Mouse and human heart and skeletal muscle extracts were prepared as described (13). Human tissue extracts were obtained from CLONTECH (catalog number 7800-7808 and 7813), and all except skeletal muscle (10 μ g) were used at 60 μ g of total protein concentration. The extracts were run on 12 or 15% SDS-polyacrylamide gels (10–60 μ g of total protein per lane). Polyclonal antibody to the FATZ protein was used at a 1/200–1/400 dilution. The myosin monoclonal antibody MF 20 developed by Dr. D. A. Fischman was obtained from the Developmental Studies Hybridoma bank maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA. Goat anti-mouse immunoglobulin conjugated with alkaline phosphatase (Sigma A3562) was used as the second antibody.

As detailed previously (13, 15), the intensity of the signal obtained from Western blot analysis can be used to make an estimate of the relative amount of a specific protein in heart and skeletal muscle extracts. This procedure was used to estimate the amount of human α -actin (data not shown) and FATZ protein (Fig. 4) present in total skeletal muscle extract.

Yeast Two-hybrid Library Screening

Bait Construction—The FATZ coding sequence was PCR-amplified from human skeletal muscle cDNA with primers containing *Eco*RI and *Sal*I sites. The cDNA fragment was subcloned into pHybLex/Zeo (Invitrogen), and the resulting clone was sequenced to confirm it was in frame with LexA. Then the construct was transformed in the L40 yeast strain (16) using the lithium acetate protocol (17) and plated on YPAD plus zeocin (300 μ g/ml). The LexA fusion bait does not activate the His reporter when grown on selective medium without histidine. Western blotting using both the FATZ and LexA (Santa Cruz Biotechnology) antibodies confirmed that the expected fusion protein was translated.

¹ The abbreviations used are: PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; GST, glutathione S-transferase; AP, alkaline phosphatase; ACTN, actinin; EST, expressed sequence tag; RT, reverse transcriptase; ORF, open reading frame; AR LGMD, autosomal recessive limb-girdle muscular dystrophy; bp, base pair; kb, kilobase pairs.

Human Skeletal Muscle cDNA Fusion Library—Double-stranded cDNA was synthesized from human skeletal muscle mRNA (Invitrogen) using the Gubler and Hoffman procedure. After removing the molecules shorter than 400 bp by CLB4 spun column (Amersham Pharmacia Biotech), the cDNA was ligated to the B42 activation domain in pYES/Trp2 cut with *Bst*XI and *Not*I (Invitrogen). The library consists of 7.3×10^6 primary transformants. The plasmid DNA from 2.5×10^6 colonies was purified after amplification on solid medium and then used for the yeast two-hybrid screen.

Yeast Two-hybrid Screen—L40 yeast strain containing the FATZ bait was transformed with library plasmid DNA (45 μ g), obtaining 3.75×10^6 yeast transformants. After 5 days, 160 of the largest growing colonies were pooled and the plasmids extracted and electroporated into the DH10B *Escherichia coli* strain. In order to screen for nonspecific interactions, these library plasmids were used to transform the L40 yeast strain containing the control vector pHybLex/Zeo-Laminin. Clones that did not activate the β -galactosidase reporter were pooled (575 of the 645 colonies tested) and, after a new DNA preparation, the library plasmids were finally retransformed into L40 yeast strain containing the FATZ bait. Double transformants (150) were tested for activation of the β -galactosidase reporter and then the cDNA inserts from 74 blue colonies were analyzed by sequencing.

Construction of Truncation Mutants—NH₂-terminal deletions of ACTN2 cloned in pGAD10 vector (CLONTECH) were recovered from a previous two-hybrid hunt (13). The COOH-terminal deletions were obtained by PCR amplification of human skeletal muscle cDNA using reverse primers designed to cover the functional domains of ACTN2 (ABD, SLR1, SLR2, SLR3, and SLR4). The forward and reverse primers were designed to contain *Eco*RI and *Xho*I sites for ligation into the pYESTrp2 vector. The structure of the truncation mutants obtained is described in Fig. 9.

Moreover, two deletion clones of FATZ were constructed, the NH₂-terminal deletion has residues 75–299 and the COOH-terminal preserves the region between residues 1 and 171. These regions were cloned using the *Eco*RI and *Sal*I restriction sites of pHybLex/Zeo.

RESULTS

Molecular Cloning and Characterization of FATZ mRNA—The FATZ transcript was discovered as part of a systematic sequencing project for human skeletal muscle ESTs at Centro di Ricerca Interdipartimentale per le Biotecnologie Innovative, Padova, Italy (18), as were telethonin (15) and ZASP (13) that are localized in the Z-band of skeletal muscle.

Transcript HSPD00355, hereafter named FATZ, was found at a frequency of 0.2% and appeared to be expressed only in heart and skeletal muscle based on the result of a preliminary RT-PCR experiment. Human and mouse transcripts of FATZ were obtained by screening full-length muscle cDNA libraries. The human and mouse FATZ nucleotide sequences are shown in Fig. 1 and correspond to 1533 and 1329 bp, respectively. The open reading frame (ORF) of human FATZ is 897 bases encoding a putative protein of 299 amino acids with a calculated molecular mass of 31,743 Da, whereas the ORF of mouse FATZ encodes a protein of 296 amino acids with a molecular mass of 31,456 Da.

The human and mouse coding sequences have a high degree of similarity; there are 9 bases less in the ORF of mouse FATZ and 85 base substitutions between mouse and human resulting in 27 changes in amino acid residues as can be seen in Fig. 1. The identity of human and mouse FATZ is 89.5% at the nucleotide level and 90% at the amino acid level.

An extensive search for known functional domains was done on the FATZ sequence using a variety of computer programs. No functional domains could be detected using SMART (19) and Pfam (20) programs. PredictProtein (21) and Psort program (22) did not detect any transmembrane or actinin-type actin-binding domain in the FATZ protein; however, a nuclear localization signal was detected, and the FATZ protein was predicted to have a 65% probability of being nuclear.

The genomic mapping was done using the radiation hybrid technique (23), placing the *FATZ* gene on human chromosome 10q22.1 at 8.56 cR distance from WI-2997.

FATZ Is Primarily Expressed in Skeletal Muscle—Northern blot analysis of different tissues using the 3'-untranslated region of FATZ as a probe revealed that skeletal muscle is the main site of expression of this gene. A major band was detected at 1.5 kb in human (Fig. 2, A and B) and 1.35 kb in mouse (Fig. 2C) skeletal muscle. However, on longer exposure, 24 h (*right panels*, Fig. 2, A and B) as opposed to 2 h (*left panels*, Fig. 2, A and B), a positive signal can be detected in heart, prostate, and although very weak, also in pancreas. Northern blot analysis of mouse tissues (Fig. 2C) confirms the muscle-restricted expression pattern of FATZ. The results of the RT-PCR shown in Fig. 2D also confirm that skeletal muscle is the major site of expression of the FATZ protein; however, some expression can be detected in heart, prostate, and testis after 29 cycles of PCR.

Western blot assays using various human and mouse tissues were performed to detect the tissue specificity of the recombinant proteins and to allow an estimation of the molecular weight of the proteins based on electrophoretic mobility. From the results using human tissues (Fig. 3), it can be seen that FATZ is present to a lesser extent in heart and testis than in skeletal muscle tissue even when using higher amounts of total protein (60 μ g) compared with that of skeletal muscle (10 μ g). FATZ has the same pattern of distribution in mouse tissues as in human, which is predominantly found in skeletal muscle and to a lesser extent in heart (data not shown). It may be noted (Fig. 3) that there are two extra bands detected in heart and skeletal muscle (28 and 16 kDa) other than that of the FATZ protein (34 kDa). The molecular weight of FATZ is higher than that estimated from its sequence; this could possibly be due to phosphorylation as FATZ has 11 potential phosphorylation sites predicted by ProSite (24). The extra bands that were detected by the anti-FATZ antibody in heart and skeletal muscle extracts appear to be due to degradation of FATZ. Only the main FATZ protein band (34 kDa) could be detected when muscle tissue or cell extracts were prepared in buffers containing protease inhibitors. Therefore, the FATZ protein seems to be susceptible to protease degradation.

Quantitation—In order to estimate the level of FATZ protein present in muscle tissue, we employed a method (15, 13) based on the intensity of the signal obtained from Western blot analysis to determine the relative amount of a specific protein in tissue extracts. This method only gives an estimate of the amount of protein present in muscle tissue based on two main assumptions as follows: 1) that proteins of the same size, blotted under the same conditions, have the same rate of blotting; 2) that the antibody used has the same affinity for the native and recombinant proteins. However, within these limits it gives a reasonable approximation of the percentage of an unknown recombinant protein present in muscle tissue. This procedure was used to estimate the amount of human FATZ protein (Fig. 4) present in 5 and 10 μ g of total skeletal muscle protein; α -actin was used as a control (13). By densitometric analysis the signal detected from 100 ng of recombinant FATZ protein was equivalent to 10 μ g of total skeletal muscle proteins (Fig. 4), whereas 500 ng of recombinant α -actin was approximately equivalent to 2.5 μ g of total skeletal muscle proteins (data not shown). Therefore, the FATZ protein would appear to be 1% of total muscle protein, and the percentage of α -actin was in agreement with the percentage (19%) previously found for α -actin in rabbit muscle (25). The percentage of 1% for FATZ protein in skeletal muscle is relatively high, 6 times more than expected, since the FATZ mRNA was found with a frequency of 0.2%, although the percentage of protein refers to the mass, whereas the percentage of transcript refers to the number of molecules.

Expression during Differentiation—The expression pattern

human	CCCAAAATGCTTCCTTTGGATCTCAGGCTCTAGGTGCAATGTGAAGGGGAGTCACTGGGCAGACTGATCCCTGGCTCAGACAGTTCA	85
human	GTGGGAGAATCCCAAAGGCCCTTTCCCTCTTCTCAGCCTCCGGGCAAGGAGGGAGTCTTGGTTCCAGGGTCTCAGTACCCCCCTGTG	176
human	CCATTTGAGCTGCTTGCCTCATCACTCTCTATTAATAACCAACTTCCTCCCCTGAGGCTGCTGCCACAGCCTGCCAGCTCGTGT	267
human	TCTCCGGTCACAGCAGCTCAGTCTCTCCAAAGCTGCTGGACCCAGGAGAGCTGACCACTGCCGAGCAGCCCGCTGAATCCACCTCCACA	358
mouseG.....G.....C.....CAA·T.....T··TCAG.....C	67
	Met Pro Leu Ser Gly Thr Pro Ala Pro Asn Lys Lys Arg Lys Ser Ser Lys Leu Ile Met Glu Leu Thr	23
human	ATG CCG CTC TCA GGA ACC CCG GCC CCT AAT AAG AAG AGG AAA TCC AGC AAG CTG ATC ATG GAA CTC ACT	427
mouse	··· ··A ···	136
	Arg	23
	Gly Gly Gly Gln Glu Ser Ser Gly Leu Asn Leu Gly Lys Lys Ile Ser Val Pro Arg Asp Val Met Leu	46
human	GGA GGT GGA CAG GAG AGC TCA GGC TTG AAC CTG GGC AAA AAG ATC AGT GTC CCA AGG GAT GTG ATG TTG	496
mouse	··· ··· ··C ··G··· ··· ··· ··· ··· C··· ··· ··· ··· ···G··· ··· ··· ··· ··· ··· ··· ··· ··· ···	205
	Arg	46
	Glu Glu Leu Ser Leu Leu Thr Asn Arg Gly Ser Lys Met Phe Lys Leu Arg Gln Met Arg Val Glu Lys	69
human	GAG GAA CTG TCG CTG CTT ACC AAC CCG GGC TCC AAG ATG TTC AAA CTG CCG CAG ATG AGG GTG GAG AAG	565
mouse	··· ··G ··· ···C ··T··· ··· ··· ··· ···A··· ··· ··· ··· ··· ··· ···G··· ··A··· ··· ··· ···C··· ··· ··· ···A···	274
		69
	Phe Ile Tyr Glu Asn His Pro Asp Val Phe Ser Asp Ser Ser Met Asp His Phe Gln Lys Phe Leu Pro	92
human	TTT ATT TAT GAG AAC CAC CCT GAT GTT TTC TCT GAC AGC TCA ATG GAT CAC TTC CAG AAG TTC CTT CCA	634
mouse	··· ··C ··· ··· ··· ···T··· ··· ···C··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ···T··· ··· ···C···	343
		92
	Thr Val Gly Gly Gln Leu Gly Thr Ala Gly Gln Gly Phe Ser Tyr Ser Lys Ser Asn Gly Arg Gly Gly	115
human	ACA GTG GGG GGA CAG CTG GGC ACA GCT GGT CAG GGA TTC TCA TAC AGC AAG AGC AAC GGC AGA GGC GGC	703
mouse	··· ··· ··A ··· ··· ··· ···AG··· ··· ··· ··· ···C··· ··· ··· ···T···G··C··· ···G···G··G··A·T··G···· ···	409
	Glu	114
	Ser Gln Ala Gly Gly Ser Gly Ser Ala Gly Gln Tyr Gly Ser Asp Gln Gln His His Leu Gly Ser Gly	138
human	AGC CAG GCA GGG GGC AGT GGC TCT GCC GGA CAG TAT GGC TCT GAT CAG CAG CAC CAT CTG GGC TCT GGC	772
mouse	··· ··· ···T···C··A··· ··· ··· ··· ···T···G···· ··· ··· ··· ··· ···C··GT··T··G··G··· ··· ··· ···	472
	Ser	135
	Ser Gly Ala Gly Gly Thr Gly Gly Pro Ala Gly Gln Ala Gly Arg Gly Gly Ala Ala Gly Thr Ala Gly	161
human	TCT GGA GCT GGG GGT ACA GGT GGT CCC GCG GGC CAG GCT GGC AGA GGA GGA GCT GCT GGC ACA GCA GGG	841
mouse	··T·· ··· ··· ··· ··· ···T··· ··· ··· ··· ···T··G···· ··· ··· ··· ··· ···T··G···· ··· ··· ···T··	541
	Phe Ser	158
	Val Gly Glu Thr Gly Ser Gly Asp Gln Ala Gly Gly Glu Gly Lys His Ile Thr Val Phe Lys Thr Tyr	184
human	GTT GGT GAG ACA GGA TCA GGA GAC CAG GCA GGC GGA GAA GGA AAA CAT ATC ACT GTG TTC AAG ACC TAT	910
mouse	C·· ··A ··· C··C··· ··· ··· ···T··· ··· ··· ··· ···T··· ··· ···T··· ··· ··· ···G···· ··· ··· ··· ···T···	610
	Leu Pro	181
	Ile Ser Pro Trp Glu Arg Ala Met Gly Val Asp Pro Gln Gln Lys Met Glu Leu Gly Ile Asp Leu Leu	207
human	ATT TCC CCA TGG GAG CGA GCC ATG GGG GTT GAC CCC CAG CAA AAA ATG GAA CTT GGC ATT GAC CTG CTG	979
mouse	··· ··· ··· ··· ···T···G···· ··· ··· ··· ···T··T··· ··· ··· ··· ···G···· ··· ··· ··· ··· ···A···	679
	Asp	204
	Ala Tyr Gly Ala Lys Ala Glu Leu Pro Lys Tyr Lys Ser Phe Asn Arg Thr Ala Met Pro Tyr Gly Gly	230
human	GCC TAT GGG GCC AAA GCT GAA CTT CCC AAA TAT AAG TCC TTC AAC AGG ACG GCA ATG CCC TAT GGT GGA	1048
mouse	··A··C···T···· ··· ··· ··· ··· ··· ···C··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ···A···· ··· ···C····	748
		227
	Tyr Glu Lys Ala Ser Lys Arg Met Thr Phe Gln Met Pro Lys Phe Asp Leu Gly Pro Leu Leu Ser Glu	253
human	TAT GAG AAG GCC TCC AAA CGC ATG ACC TTC CAA ATG CCC AAG TTT GAC CTG GGG CCC TTG CTG AGT GAA	1117
mouse	··· ···T··C···· ··· ··· ···	817
		250
	Pro Leu Val Leu Tyr Asn Gln Asn Leu Ser Asn Arg Pro Ser Phe Asn Arg Thr Pro Ile Pro Trp Leu	276
human	CCC CTG GTC CTC TAC AAC CAA AAC CTC TCC AAC AGG CCT TCT TTC AAT CGA ACC CCT ATT CCC TGG CTG	1186
mouse	··· ··· ··· ··· ··· ··· ···G···· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ··· ···T···	886
		273
	Ser Ser Gly Glu Pro Val Asp Tyr Asn Val Asp Ile Gly Ile Pro Leu Asp Gly Glu Thr Glu Glu Leu	299
human	AGC TCT GGG GAG CCT GTA GAC TAC AAC GTG GAT ATT GGC ATC CCC TTG GAT GGA GAA ACA GAG GAG CTG	1255
mouse	··· ··· ··· ··· ···A···· ··· ··· ··· ··· ··· ··· ··· ···G···T···· ··· ··· ··· ··· ···G···· ··· ··· ···	955
	His	296
	TGAGGTGTTTCTCTCTGTATTGTCATCAITTTCCCTCTCTGGCTCCAAITTTGGAGAGGAATGCTGAGCAGATAGCCCCATGTGTAATCCA	1348
human	···A···CC····G··C··G···· ··· ··· ··· ···T···· ··· ···T···· ··· ···G···· ···GAA···GAT····A··C····	1048
	GTATCCTTATGGGAATGGAGGAAAAAGGAGAGATC---TACCTTCCATCCTTTACTCCAAGTCCCCTACC-ACGCATCCTTCCCTACCAAC	1436
human	····T···G···C···· ··· ··· ···T···G··T··G··G··CGT··G···· ··· ··· ···C···· ··· ··· ··· ···T··TG···G··A····C···· ···G···	1135
	TCAGAGCTCCCCTTCTACTTGTCTCCATATGGAACCTGCTCGTITATGGAAITTGCTCTGCCACCAGTAACAGTCAATAAAGCTTCAAGGAAAATG	1532
mouse	···· ··· ···A··C··G····TA···· ···T···· ···T···· ··· ··· ··· ···T···· ··· ··· ··· ···G···· ···T····	1229

FIG. 1. cDNA and amino acid sequences of human and mouse FATZ. The start and stop codons are in bold, and the polyadenylation sites are underlined. In the coding part of the mouse sequence the conserved nucleotides and amino acids are represented by dots. Dashes indicate gaps that have been inserted to align the two sequences. The sequence data are available from GenBank™/EMBL/DBJ, accession numbers AJ278124 and AJ005620, respectively, for human and mouse FATZ.

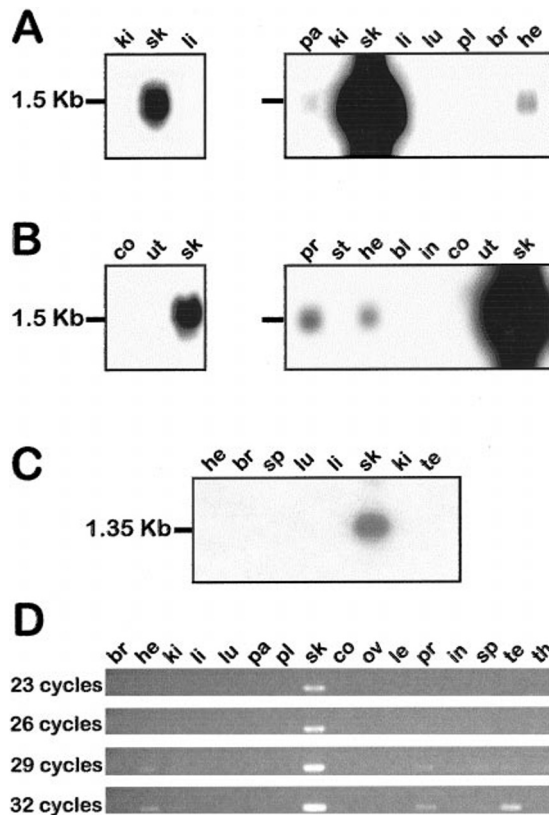


FIG. 2. The pattern of expression of FATZ mRNA in human and mouse tissues by Northern blot and RT-PCR analysis. Blots of mRNA from a variety of human tissues (A) and human muscle tissues (B) were probed with a 3'-untranslated region of FATZ. The panels show exposures of 2 (left) and 24 h (right). C, a blot of mRNA from various mouse tissues probed with a 3'-untranslated region of FATZ, exposed for 24 h. The numbers on the side indicate the size (kb). D, RT-PCR analysis of human tissues at 23, 26, 29, and 32 cycles. The FATZ transcript can be detected in heart, skeletal muscle, prostate, and testis. The abbreviations are as follows: *bl*, bladder; *br*, brain; *co*, colon; *he*, heart; *in*, intestine; *ki*, kidney; *le*, leukocytes; *li*, liver; *lu*, lung; *ov*, ovary; *pa*, pancreas; *pl*, placenta; *pr*, prostate; *sk*, skeletal muscle; *sp*, spleen; *st*, stomach; *ut*, uterus; *te*, testis; and *th*, thymus.

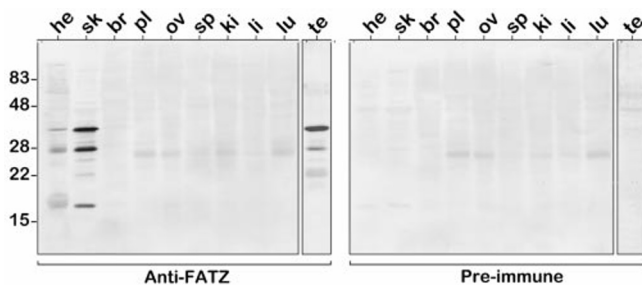


FIG. 3. Tissue distribution of the FATZ protein. Protein extracts from human skeletal muscle (10 μ g) as well as from other human tissues (60 μ g) were run on a 15% SDS-polyacrylamide gel and then blotted. The membrane was probed with mouse polyclonal antibody specific for the FATZ protein and preimmune mouse sera; both were used at 1/200 dilution. Sigma color molecular weight marker was used in this experiment. The FATZ protein is present primarily in skeletal muscle and to a lesser extent in heart and testis.

of the FATZ protein during differentiation was studied both by Western blotting (Fig. 5A) and immunofluorescence (Fig. 5B). Cell lysates were prepared from both human and mouse muscle cells at different times after the addition of differentiation medium. As can be seen in Fig. 5, very little FATZ protein could be detected in both human and mouse undifferentiated cell lysates, whereas the FATZ protein (34 kDa) was clearly visible in differentiated cells and increased with time of differentia-

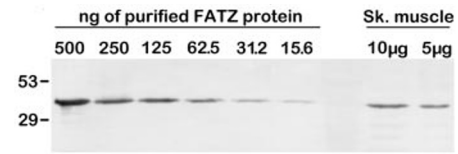


FIG. 4. Western blot analysis of known amounts of recombinant FATZ protein (left) and known amounts of total protein (10 and 5 μ g) from skeletal muscle tissue (right). The recombinant protein has an additional 12-amino acid tag at the NH₂-terminal end of the FATZ sequence; therefore, it has a slightly higher molecular weight than the native muscle protein. Mouse polyclonal antibody to FATZ was used at 1/200 dilution. The FATZ protein present in skeletal muscle tissue was calculated by densitometric analysis; 100 ng of FATZ recombinant protein is equivalent to 10 μ g of total skeletal muscle protein.

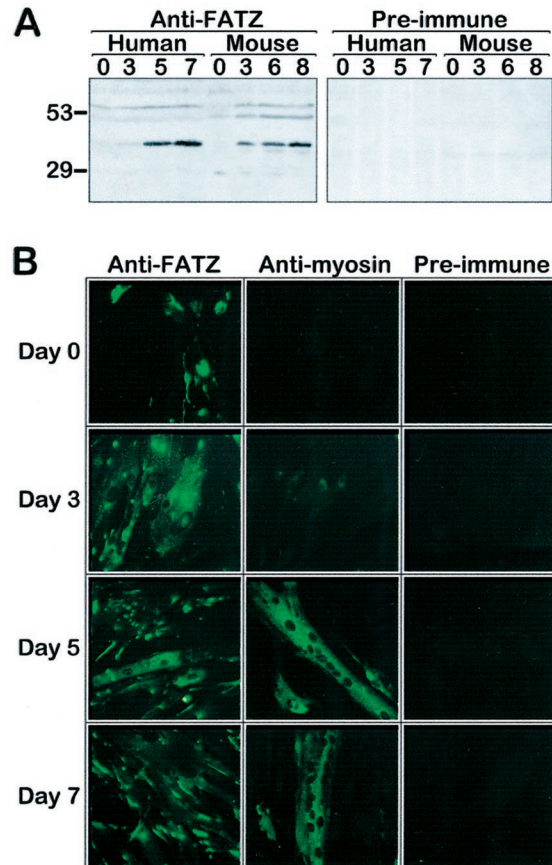


FIG. 5. Protein expression during differentiation was monitored in primary human and mouse muscle cells by Western blot analysis (A) and indirect immunofluorescence (human myoblasts) (B). The anti-FATZ antibody and mouse preimmune sera were used at dilutions of 1/200 for Western blotting, 1/50 for immunofluorescence, and 1/200 for myosin.

tion. In addition to the 34-kDa band of FATZ protein a lower band (28 kDa) and some faint higher (52 and 56 kDa) bands could be detected. It is possible that some proteins in muscle cells have epitopes similar to that of the FATZ protein; however, the lower band would appear to be due to degradation.

Localization of FATZ Protein in Human Muscle Cells—Immunofluorescence experiments were undertaken to obtain an indication of the cellular localization of FATZ in muscle cells (Fig. 5B) at various stages of differentiation as well as in skeletal (Fig. 6A) and heart muscle tissue sections (data not shown). Antibodies to muscle α -actin and myosin were used as controls. Although the FATZ protein can be detected in undifferentiated muscle cells (Fig. 5A), it was mainly present in differentiated cells (Fig. 5B). In undifferentiated cells incu-

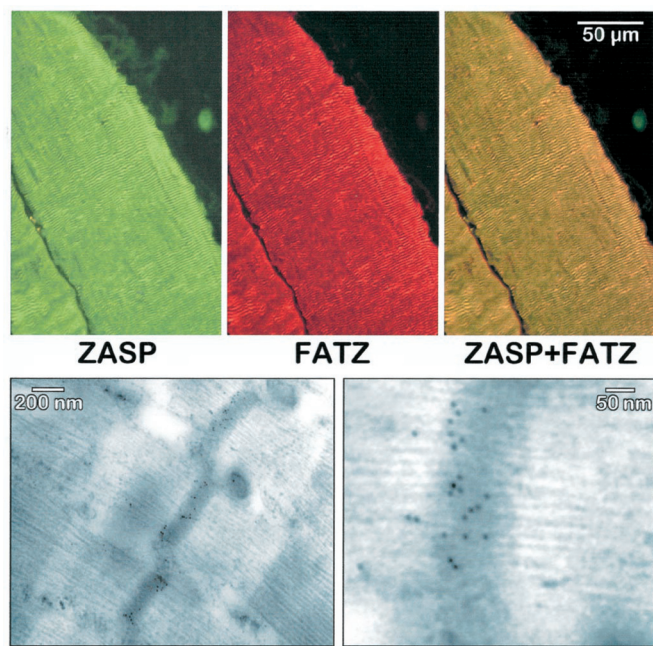


FIG. 6. *A*, indirect immunofluorescence of skeletal muscle tissue sections using mouse polyclonal antibody to FATZ (1/30 dilution) and fluorescein isothiocyanate direct labeled anti-ZASP antibody (1/4 dilution). Tetramethylrhodamine isothiocyanate-labeled goat anti-mouse Ig (red) was used as the second antibody. The bar shown in the figure is 50 μm in length. *B*, localization of the FATZ protein in human skeletal muscle by immunoelectron microscopy. The anti-FATZ mouse polyclonal antibody was used at 1/50 dilution and the second antibody anti-mouse IgG conjugated with 5-nm gold particles (Sigma G7527) at 1/20 dilution. *B*, a section of skeletal muscle at low magnification (*left*) and (*C*) high magnification (*right*), the bars are 200 and 50 nm, respectively. The FATZ protein is seen throughout the Z-disc.

bated with antibodies to FATZ a fluorescent signal can be seen localized near the nucleus and pseudopodia, whereas in the multinucleated differentiated cells a strong fluorescent signal can be detected in a more extensive area of the myotubes (Fig. 5*B*). Only a very weak fluorescent signal can be detected in undifferentiated and differentiated cells incubated with preimmune serum (Fig. 5*B*) and in undifferentiated cells incubated with myosin (Fig. 5*B*). In contrast, in differentiated cells incubated with myosin (Fig. 5*B*), a very strong fluorescent signal can be detected throughout the myotubes.

Sections of human heart and muscle when stained with the anti-FATZ antibody show a characteristic cross-striated pattern, which is typically seen with sarcomeric proteins (Fig. 6*A*). In double fluorescence experiments an alternate banding pattern could be detected using antibodies to FATZ (red) and ZASP (green), and these signals seem to be coincident (Fig. 6*A*). As ZASP is known to be located in the Z-disc of skeletal muscle (13), this would suggest that also FATZ could be located there.

When dealing with structural proteins immunogold electron microscopy is a useful tool to pinpoint the exact localization of the protein in the cell; therefore, we used this technique to confirm the localization of the FATZ protein in the Z-disc of heart and skeletal muscle (Fig. 6*B*).

Interaction of FATZ Protein with Other Muscle Proteins—Since the different techniques used to study protein-protein interactions have advantages and disadvantages, it is useful to use more than one method. Immunoprecipitation experiments of *in vitro* translated proteins from human heart and skeletal muscle confirmed the result of the RT-PCR and Northern blot assays, showing that there is more FATZ protein in skeletal muscle than in heart, but did not produce a clear result about possible proteins co-immunoprecipitating with FATZ (data not shown).

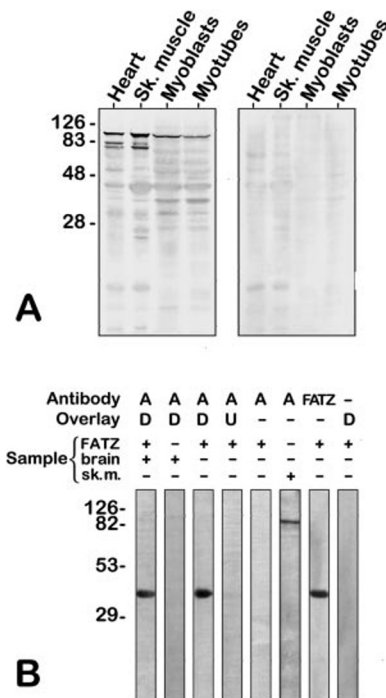


FIG. 7. **Overlay assays demonstrating that α -actinin 2 binds to the FATZ protein.** *A*, a GST overlay assay. Human heart and skeletal muscle tissues (60 μg) and cell lysates of human skeletal muscle myoblasts and myotubes (60 μg) were run on a 15% SDS-PAGE and blotted. The membranes were incubated with GST-FATZ (*left*) and GST (*right*) protein (6 μg) and then washed and incubated with goat anti-GST antibody (dilution 1/1000) and then with anti-goat antibody conjugated to AP (1/20,000). *B*, overlay assay using undifferentiated (*U*) and differentiated (*D*) human muscle cells (60 μg). Various combinations of human brain tissue (10 μg) and His-tagged FATZ protein (1 μg) as well as human skeletal muscle protein (10 μg) as a control were separated by SDS-PAGE and then blotted onto Immobilon P. The membrane strips were incubated with cell lysate or buffer, washed, and incubated with the appropriate antibody, designated A for anti- α -actinin (1/500 dilution) and FATZ for anti-FATZ (1/200 dilution) antibody.

Further experiments were done using different techniques to discover if any muscle proteins could bind FATZ. Recombinant FATZ protein tagged with GST was used in an overlay assay (Fig. 7*A*) to determine if any proteins from human heart or skeletal muscle tissue or cell extracts were able to bind FATZ. In fact, as can be seen in Fig. 7*A*, the GST-FATZ protein was able to bind three proteins in the range of 80–110 kDa present in heart and skeletal muscle, the highest of which can be detected also in cultured muscle cells. Based on the molecular weight of these unknown binding proteins and the fact that they were present in skeletal muscle, it was decided to check if the highest band could be due to α -actinin 2 (approximately 104 kDa). To verify this hypothesis, the His-tagged FATZ protein was blotted onto Immobilon P membrane, which was incubated with extracts of differentiated human muscle cells. As can be seen in Fig. 7*B*, by using antibodies against α -actinin 2, a specific interaction with FATZ can be detected. To prove that the interaction was specific, the His-FATZ protein (1 μg) was mixed with other proteins (brain extract, 10 μg) without altering the binding.

The GST pulldown assay (Fig. 8) was also used to detect protein interaction. By using GST-FATZ and radiolabeled differentiated muscle cell extracts, a band of approximately 300 kDa can be detected that may correspond to γ -filamin/ABP (as supported by the yeast two-hybrid experiments described below). Two strong bands can be seen, one at approximately 100 kDa that may correspond to α -actinin 2 and one that is also present in the control of approximately 43 kDa that could be

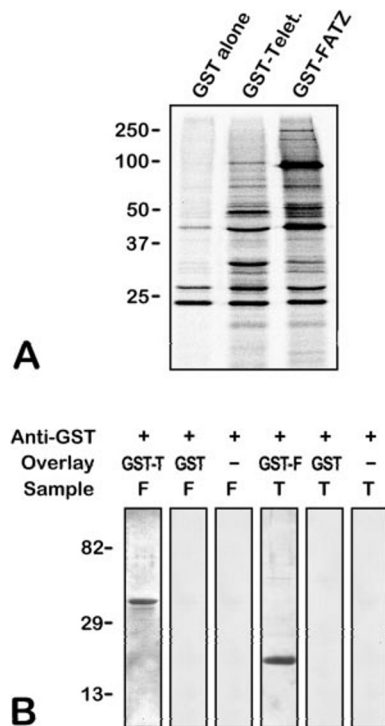


FIG. 8. GST-pulldown and overlay assays. *A*, 2 μ g of GST alone, GST-teletonin, and GST-FATZ attached to glutathione-Sepharose 4B were mixed with lysates of radiolabeled C2C12 differentiated cells, washed, and then separated on a 15% SDS-polyacrylamide gel (low bisacrylamide 0.4%). The gel was fixed, dried, and exposed to SR Packard phosphorscreen. *B*, GST overlay assay. His-tagged FATZ (*F*) or teletonin (*T*) proteins (1 μ g) were separated by SDS-PAGE, blotted, and then incubated with buffer (–) or fusion proteins (6 μ g), GST-teletonin, GST-FATZ, and GST alone. The membranes were then incubated with goat anti-GST antibody (1/1000 dilution) followed by anti-goat antibody conjugated to AP (1/20,000 dilution).

α -actin. Several other bands of various molecular masses can also be detected as follows: a single band of approximately 68 kDa and then four close together ranging from 49 to 57 kDa. The pattern of bands seen in the GST-teletonin pulldown (Fig. 8*A*) is quite different from that of GST-FATZ; there is a band of 34 kDa that may be FATZ and a band of 53 kDa. There are some bands detected in both GST-FATZ and GST-teletonin that are also present in the control, GST alone. Teletonin would appear to be another binding partner of FATZ as can be seen in the overlay assays in Fig. 8*B*. Recombinant His-tagged FATZ and teletonin were capable of specifically binding GST-teletonin and GST-FATZ, respectively, but neither could bind GST protein alone. The binding of the proteins was detected using anti-GST antibody.

The interaction of FATZ with α -actinin 2 and γ -filamin/ABP-L was confirmed by the yeast two-hybrid assay that was used to identify proteins interacting with the FATZ *in vivo*, by screening human skeletal muscle cDNA fusion libraries, as described below.

Yeast Two-hybrid Analysis—The screening for FATZ-binding proteins was done using 3.75 million yeast colonies transformed with both a human skeletal muscle cDNA library fused to the B42 transcriptional activation domain and a bait construct encoding the FATZ protein fused to the LexA DNA binding domain. Seventy four plasmids were isolated and sequenced as follows: seventy two of them coded for α -actinin 2, one for α -actinin 3, and one for γ -filamin/ABP-L. All of the α -actinin 2 clones contained a complete COOH terminus, and most were full-length, and the shortest started at residue 393 (Fig. 9*A*). The specificity of the interaction between the FATZ

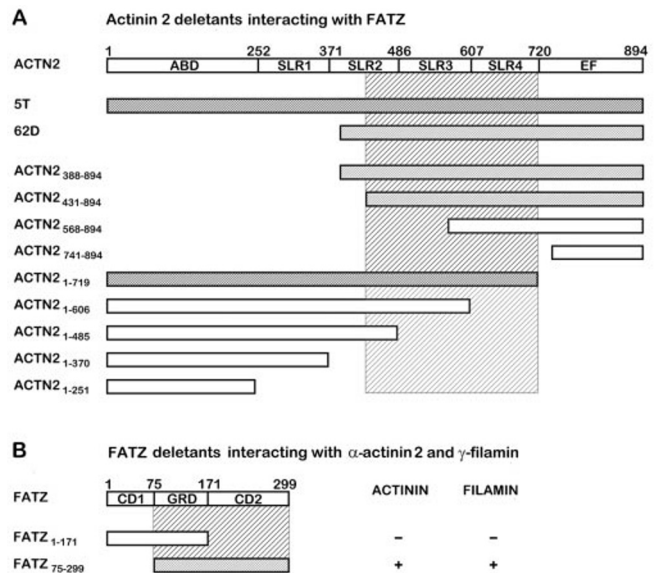


FIG. 9. Protein-protein interactions detected by yeast two-hybrid analysis. *A*, α -actinin 2 deletants interacting with FATZ as bait, the clones 5T and 62D are, respectively, the longest and shortest of the positive clones of α -actinin 2. The clones designated ACTN are NH₂- and COOH-terminal deletions produced in order to define the interaction domain between FATZ and α -actinin 2. The regions common to the interacting clones are indicated by stripes. *B*, FATZ deletants tested for interaction with α -actinin 2 and γ -filamin/ABP-L. FATZ can be divided into three regions, the NH₂-terminal (CD1), the glycine-rich domain (GRD), and the COOH-terminal (CD2), respectively, amino acid residues 1–75, 75–171, and 171–299.

protein and α -actinin 2 was confirmed by measuring the β -galactosidase activity in a separate co-transformation experiment. The two-hybrid method was also employed to identify the region of α -actinin 2 interacting with the FATZ protein. Four NH₂-terminal and five COOH-terminal truncation mutants fused to GAL4 and B42, respectively, were co-transformed with the FATZ bait in the L40 yeast strain, and their interactions were quantified using the β -galactosidase assay. As a result the FATZ-binding site was mapped to the region that spans the SR3 and SR4 spectrin-like repeats of α -actinin 2 (amino acids 431–719).

To confirm the single positive clone of γ -filamin/ABP-L, the library plasmid of clone 60-D was isolated and retested with FATZ using pHybLex/Zeo-Laminin and pHybLex/Zeo as negative controls. Transactivation of the β -galactosidase reporter was only detected with the FATZ bait. Clone 60-D was sequenced, and the insert sequence was used to search for homology in the GenBankTM data base. The best match was between 60-D and the 3'-portion of the muscle γ -filamin/ABP-L (GenBankTM accession number AJ012737), precisely in the region between residues 2276 and 2692.

In order to investigate further the exact sites of interaction between FATZ and its binding partners (α -actinin 2 and γ -filamin/ABP-L), two deletion clones of FATZ (FATZ-(1–171), FATZ-(75–299)) were assayed for interaction with the 5-T and 60-D clones found by screening the library. These deletion mutants were constructed as a result of an alignment with other putative FATZ homologues as described under "Discussion," which allowed the identification of distinct domains within the FATZ sequence as indicated in Fig. 9*B*. The clone lacking the last 128 amino acids did not interact with α -actinin 2 and γ -filamin/ABP-L. However, when co-transformed with FATZ-(75–299), both α -actinin 2 and γ -filamin/ABP-L turned on the β -galactosidase reporter. Although the yeast two-hybrid assay confirmed the binding of FATZ to α -actinin 2 the identity

of the other two binding proteins detected by GST overlay assay remains unsolved. However, using the yeast two-hybrid assay, FATZ has been shown to bind γ -filamin/ABP-L and possibly α -actinin 3.

DISCUSSION

The three proteins that interact with FATZ are all located in the Z-band, and by understanding their known interactions we may obtain some indication of the function of FATZ. Filamin is an actin-binding protein that is found as different isoforms. Filamin-1 is a non-muscle filamin found in a wide variety of cell types (26), whereas γ -filamin/ABP-L (27) is expressed primarily in heart and skeletal muscle (28, 29). γ -Filamin/ABP-L is also called filamin-2 and has been shown to bind γ - and δ -sarcoglycans, which are members of the dystrophin-glycoprotein complex (30). Recent studies have shown that γ -filamin/ABP-L is up-regulated on differentiation of cultured muscle cells, leading to the hypothesis that γ -filamin/ABP-L might be involved in Z-disc assembly as it is present in the initial stages of Z-disc formation (31). Our results indicate that FATZ undergoes a similar up-regulation (Fig. 5); therefore, for the same reasons, FATZ could also play an important role in Z-disc assembly.

Another protein that interacts with FATZ is telethonin, which is present mainly in striated and cardiac muscle (15) and is another protein of the Z-disc (4). Telethonin is one of the substrates of the serine kinase domain of titin (5), and titin phosphorylates the COOH-terminal domain of telethonin in early differentiating myocytes (32). Recently, telethonin has been found to be responsible for a form of autosomal recessive limb-girdle muscular dystrophy (AR LGMD) type 2G (33). The LGMDs are a genetically heterogeneous group of disorders that affect mainly the proximal musculature, and telethonin is the first sarcomeric protein found to cause an AR LGMD. Further studies will be necessary to elucidate the pathogenesis of this form of muscular dystrophy that may possibly involve titin and FATZ since they both interact with telethonin.

Although α -actinin has an important role in the function of muscle by stabilizing the contractile machinery, not much is known about the way in which α -actinin-binding proteins can assist in this process. In the past few years several α -actinin-binding proteins have been discovered either by projects that had the specific aim of identifying them or as a result of characterization of new muscle genes obtained from systematic sequencing projects of muscle. α -Actinin 2 can be divided into three domains each having distinct functions, and these are the NH₂-terminal actin binding domain, the spectrin-like repeat domain that has 4 repeats required for dimer formation (34), and the COOH-terminal domain containing a EF-hand putative calcium-binding site (8). Various muscle proteins have been shown to bind different domains of α -actinin 2; these proteins can be arbitrarily classified into groups based on the domain of α -actinin 2 involved in the interaction. Actin binds to the NH₂-terminal region; ALP binds to the spectrin-like repeat region (35), and ZASP/Cypher binds to the EF-hand region (13, 36). A special case is titin that binds via its NH₂-terminal Z repeats zr1 and zr5 to the COOH-terminal region of α -actinin 2 (2, 3, 37) and also via its Zq-Z4 region to the SR2-SR3 spectrin-like repeats of α -actinin 2 (3).

Thus, there are a variety of actinin-binding proteins in the Z-disc, and it is possible that they may be involved in competition with FATZ for binding to α -actinin 2. However, those proteins binding to domains other than the spectrin-like regions of α -actinin 2 probably do not exert a direct effect; actin, zyxin, CRP1, vinculin, and ZASP are in this category since they have been shown to bind to the globular NH₂-terminal actin binding domain or to the COOH-terminal calmodulin-like domain of α -actinin 2 (13, 38–41). Other proteins, however, such

as myotilin, titin, and ALP have been shown to interact with the spectrin-like regions 2–4 (3, 35, 42), the same region that we demonstrate as being the binding site for FATZ. Further studies are necessary to define better the overlapping binding sites of this series of proteins within the spectrin-like motifs and to study how the exchange or competition of different binding partners could modulate assembly and functioning of the Z-disc. Interactions of specific spectrin-like domains of α -actinin have been demonstrated to mediate the ordered formation of the functional protein dimer (12). In view of this, the different spectrin-like domains or their particular sub-domains could also be the selective target for specific interaction with the various members of the family of actinin-binding proteins.

Finally, we want to predict that FATZ could be just one member of a small family of novel proteins. In fact, by screening our specific muscle EST data base and the public data bases with the FATZ sequence, we found a cluster of four ESTs and a single EST that share a high degree of similarity to FATZ. The sequences of these two transcripts have been completed (data not shown), and their functional characterization is under way. The amino acid alignment of these proteins with FATZ shows a high similarity in the NH₂ terminus (75 residues) as well as in the COOH terminus (approximately 120 residues), which are indicated as CD1 and CD2, respectively, in Fig. 9B. The central part of all three proteins (approximately 100 residues in FATZ) is less conserved, and in the case of FATZ is particularly rich in glycine (GR region in Fig. 9). This sequence conservation would suggest the presence of two different and novel functional domains in FATZ that could explain the multiple binding capacity of this protein of the Z-disc as demonstrated in our work. Further studies are now necessary to resolve at a finer level the specific role of the two domains in the complex network of interactions that FATZ seems to promote in the Z-band of the muscle sarcomere.

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