Expression and localization of annexin VII (synexin) in muscle cells

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SUMMARY

Annexin VII (synexin) is a member of the annexin family of proteins, which are characterized by Ca2+-dependent binding to phospholipids. We used PCR to isolate from a λgt11-mouse fibroblast library annexin VII cDNA fragments corresponding to the two isoforms found in both humans and Dictyostelium discoideum. The two isoforms of 47 kDa and 51 kDa differed by 22 amino acids inserted into the proximal third of the hydrophobic N terminus. Annexin VII-specific polypeptides expressed in Escherichia coli were used to generate isoform-specific monoclonal antibodies. Expression of the two isoforms during myogenesis was followed in the myogenic cell lines BC3H1 and L6. Only the 47 kDa isoform was present in undifferentiated L6 or BC3H1 myoblasts. The 51 kDa isoform appeared after myogenesis had been induced and in striated muscle only the 51 kDa isoform was observed. Immunofluorescence showed that annexin VII was located in the cytosol of mononucleated and fused polynucleated cultured cells, whereas in striated muscle, annexin VII was located preferentially at the plasma membrane and the transverse tubules. However, there was also some residual cytosolic staining, which was more abundant in type II (fast twitch) than in type I (slow twitch) fibers. Permeabilization of L6 cells with digitonin in the presence of 5 mM EGTA led to a release of annexin VII from the cells, which paralleled the loss of cytosolic lactate dehydrogenase (LDH) at low detergent concentrations (50 μM). In the presence of 100 μM extracellular Ca2+, annexin VII remained bound to the plasma membrane even in the presence of high digitonin concentrations. Incubation with the Ca2+-specific ionophore A23187 and 100 μM extracellular Ca2+ led to a redistribution of annexin VII from the cytosol to the plasma membrane after 30 minutes of incubation. The results obtained indicate a developmentally and Ca2+-regulated localization and expression of annexin VII and raise the possibility that annexin VII may play a role in excitation-contraction coupling in skeletal muscle.

Key words: annexin VII, myoblasts, skeletal muscle, immunofluorescence, calcium

INTRODUCTION

Proteins of the annexin family are characterized by their ability to bind to phospholipids in the presence of Ca2+. The members of this family have a conserved 70 residue ‘core’ domain, which contains a tetrad repeat in all annexins except annexin VI, which has an octad repeat. Each annexin has a unique N-terminal domain, which varies in both length and amino acid composition. The unique N-terminal domain appears to confer functional diversity, whereas the conserved core domain is responsible for Ca2+ and phospholipid binding (for review see Raynal and Pollard, 1994). The annexin VII N terminus is extraordinarily long and highly hydrophobic (164 amino acids in mouse annexin VII) compared with other annexins. Annexin VII gene sequences have been detected in man (Burns et al., 1989), Dictyostelium discoideum (Döring et al., 1991; Greenwood and Tsang, 1991) and in mouse (Zhang-Keck et al., 1993).

Further functional diversity can be generated in annexins by alternatively spliced exons within the coding sequences. Alternative splicing has been observed for annexin VI (Moss and Crumption, 1990) and annexin VII (synexin). Northern blots in both man and monkey have revealed several tissue-specific polymorphisms, and in humans 2.0 and 2.4 kb annexin VII messages were produced by alternative poly(A) signals. In addition the tissue-specific expression of a cassette exon has been observed in which this exon is inserted into the proximal third of the N-terminal domain at position 145 in the human sequence (Magendzo et al., 1991). This insertion introduces three charged amino acid residues into a domain otherwise completely uncharged, but the functional significance of this change is unclear.

Although annexins have been characterized well at the structural and biochemical levels, their cellular function is less clear. Annexin VII was discovered by Creutz et al. (1978) during a search for cytosolic proteins that could support Ca2+-dependent membrane fusion, and several functions have been proposed for annexins based on their ability to interact with membrane lipids like inhibitors of phospholipase A2 (Russo-Marie, 1992) and of blood coagulation (Yoshizaki et al., 1992). It has also been suggested that they are involved in inositol phosphate metabolism (Ross et al., 1990), aggregation of
isolated chromaffin granules (Drust and Creutz, 1988), cross-linking function in the cell cortex (Glenney et al., 1987), exocytosis (Ali et al., 1989; Sarafian et al., 1991), endocytosis (Emans et al., 1993), lysosomal targeting (Futter et al., 1993) and the regulation and formation of ion channels (Huber et al., 1990; for review see Moss et al., 1991; Creutz, 1992). Creutz et al. (1978) showed that annexin VII stimulated the aggregation and fusion of isolated chromaffin granula in a Ca²⁺-dependent manner outlined in the 'hydrophobic bridge hypothesis' in which it was suggested that Ca²⁺-induced annexin polymerization initiates granule aggregation (Pollard et al., 1990). In liposome fusion experiments annexin VII exhibited a Ca²⁺-dependent affinity for acidic lipids as they are found on the outer aspect of granula membranes and on the cytosolic layer of plasma membranes (Hong et al., 1982), and in patch-pipet systems it was discovered that annexin VII can form voltage-dependent channels that are highly specific for Ca²⁺ when inserted into phospholipid bilayers (Pollard and Rojas, 1988). The physiological significance of the ion channel activity of annexin VII has yet to be determined. However, these data are supported by crystallographic results obtained for annexin V (Huber et al., 1990), indicating that the common core domain is able to form a channel, even though it lacks an obvious transmembrane domain.

To address the in vivo roles of annexins, especially annexin VII, we have prepared monoclonal antibodies directed against mouse annexin VII and the cassette exon. These reagents have enabled the expression pattern and intracellular distribution of annexin VII isoforms to be studied during myogenesis and, moreover, have shown that, in striated muscle, annexin VII is located at both the plasma membrane and the transverse tubules.

MATERIALS AND METHODS

Cell culture

BC3H1 cells, a nonfusing muscle cell line, originally isolated by Schubert et al. (1974), were obtained from Dr T. Jovin (Max-Plank-Institute of Biophysical Chemistry, Göttingen, FRG). BC3H1 cells were grown on collagen-coated dishes at 37°C in a 10% CO₂-enriched atmosphere in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20% fetal calf serum, 100 i.u./ml penicillin, 100 µg/ml streptomycin. Cells were subcultured to 1:20 at ~80% confluence. L6 rat skeletal muscle myoblasts were obtained from Dr A. Starzinski-Powitz (Institut für Anthropologie und Humangenetik, Frankfurt a. M.). They were grown in Waymouth Medium MB 752/1 supplemented with 15% fetal calf serum. Differentiation was induced by washing the dishes with Hanks’ balanced salt solution (HBSS) and changing the medium to a low mitogenic medium (DMEM supplemented with 3.4 g glucose/L, 1% fetal calf serum). To each 90 mm dish 2 ml medium was added. All media components were obtained from Gibco/BRL (Eggenstein, FRG), insulin and transferrin were obtained from Sigma (Deisenhofen, FRG).

Cloning of annexin VII cDNA fragments and expression in Escherichia coli

A mouse fibroblast cDNA library in λgt11 (Clontech Laboratories Inc., CA) was used for amplification of annexin VII fragments by PCR. Three primers were designed according to the human annexin sequence (Burns et al., 1989). One of the 5’ end primers (5’-dCCGATCTCTGCTAGATCCCTTGC-3’) was located upstream of the human cassettes exon at positions 142 to 167, a second 5’ end primer (5’-dCCGAATTCGCCAACTTCTCGATCTAGAAGG-3’) was located in the first core repeat, from position 541 to 565. The 3’ end primer (5’-dCCGAATTCCTCTGCTAGATCCCTTGC-3’) was located in the second core repeat at position 977 to 1002. The resulting products were cloned into the EcoRI restriction site of the ATG-expression vector pT7-7Sen (B. Leiting, unpublished), which carries an epitope derived from the Sendai virus envelope. Recombinant E. coli BL21 cells were identified using a monoclonal antibody to the viral epitope (Einberger et al., 1990). The cells were grown at 37°C to an A₅₉₀ of 0.8. Expression was induced by adding IPTG to a final concentration of 1 mM and the bacterial RNA polymerase was inhibited with 200 µg/ml rifampicin (Sigma). After incubation for 3 hours at 37°C the cells were harvested by low-speed centrifugation (20 minutes; 4,000 g), resuspended in TEDABP buffer (10 mM Tris-HCl, pH 7.8, 1 mM EDTA, 1 mM DTT, 0.02% NaN₃, 1 mM benzamidine, 0.5 mM PMSF), and disrupted by ultrasonication. After centrifugation (20 minutes; 30,000 g) the fusion proteins remained in the pellets, which were treated with TEDABP buffer containing 2, 4 and 6 M urea. The 24 kDa fusion protein containing the cassette exon could be extracted with 4 M urea, the 22 kDa fusion product by the addition of 6 M urea. Both polypeptides were further purified by column chromatography on a DEAE-Sepharose column (DE-52, Whatman Inc., Clifton, NJ) and eluted with a gradient from 0 mM to 400 mM NaCl. The protein containing fractions were pooled, dialysed overnight against TEDABP and concentrated in a Centricon-10 concentrator (Amicon Inc., Beverly, MA) to a final concentration of 1 mg/ml. Samples were stored at −20°C and 0°C and used for immunization.

Production of monoclonal antibodies and immunofluorescence

Balb/c mice were immunized with bacterially expressed partially denatured annexin VII fusion protein containing the N-terminal linked Sendai epitope (Einberger et al., 1990) using Freund’s complete adjuvant or Alugel S (Serva, Heidelberg, FRG). Fusions were performed 7 days after the last boost, essentially as described (Schleier et al., 1984), using 63Ag8-653 and PAIB 3 Ag8I myeloma lines. Hybridomas were screened for their ability to recognize specifically either the annexin VII 47 kDa or 51 kDa isoform in cellular extracts of mouse heart tissue after transfer onto nitrocellulose. The antibodies designated as 217/6, 289/2, 62/6 and 204/10 were used in these studies; the latter two were shown to be specific for the larger isoform.

For antibody staining L6 or BC3H1 cells were grown on coverslips, the medium was removed, the cells were washed once with HBBS, treated where indicated with the ionophore A23187 (Sigma) or the medium was removed, the cells were washed once with HBSS, treated where indicated with the ionophore A23187 (Sigma) or

Fig. 1. cDNA sequence and deduced amino acid sequence of the mouse (ms) and human (hs) annexin VII exon cassette. The positions of the residues within the amino acid sequence are indicated.
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done as described above. Fixed cells or muscle biopsies were washed twice for 5 minutes in PBS. Non-specific binding sites were blocked by incubating the cells twice for 15 minutes in PBG (0.5% BSA, 0.2% gelatine in PBS). Incubation with primary antibody was performed for at least 2 hours. After 6 washes in PBG, coverslips were incubated for 1 hour with 1:2,000 Cy3-conjugated affinity-purified goat anti-mouse IgG (Jackson Immunoresearch Laboratories Inc., Avondale, PA), washed twice in PBG and PBS, rinsed in water, embedded in Gelvatol (Langanger et al., 1983) and examined with a Zeiss Axioshot fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

The monoclonal antibody 10C3 directed against BiP was a gift from Dr S. Fuller (EMBL, Heidelberg, FRG), mAb 10D1 to SERCA1 type Ca$^{2+}$-ATPase (Kaprielian and Fambrough, 1987) was kindly provided by Dr D. Fambrough (Johns Hopkins University, Baltimore, MA) and mAb E5A3 recognizing β-COP (Duden et al., 1991) by Dr T. Kreis (University of Geneva, Switzerland).

**Detergent extraction**

Detergent extraction using increasing amounts (0.1 µM to 100 µM) of the detergent digitonin was carried out at room temperature. After a brief wash with HBSS, cells were treated for 10 minutes with digitonin containing HBSS in the absence or presence of 100 µM Ca$^{2+}$. The supernatant was removed and the cells were either subjected to immunofluorescence studies or resuspended in 1 ml of HBSS per plate. The supernatant and the resuspended cells were centrifuged (10 minutes; 800 g) to get rid of detached cells and to pellet the extracted cells, respectively. Samples of the supernatant were then submitted to SDS-PAGE directly, whereas the cell pellet had to be lysed before by passing the cells 10 times through a 21G-needle.

**Image processing**

Light micrographs were digitised at intervals corresponding to roughly 0.1 µm and analysed using the methods commonly employed for electron micrographs (reviewed by Stewart, 1988). Both Fourier and cross-correlation methods were employed. Average images were produced by correcting for both shear and lattice disorder as described for regular bacterial surface layers (Stewart et al., 1986).

**Fig. 2.** Specificity of annexin-VII-specific mAbs. Total cellular extracts of mouse heart tissue (A) and undifferentiated BC3 H1 cells (B) were separated by SDS-PAGE (10% acrylamide). Immunoblots were prepared by using the indicated primary antibodies and horseradish-peroxidase-labeled goat anti-mouse IgG as secondary antibody. Polypeptides of 47 and/or 51 kDa are specifically recognized. In the control lane the reaction of the secondary antibody is shown (control). It reacts with endogenous immunoglobulin molecules present in mouse heart tissue.

**Fig. 3.** Differential expression of annexin VII during myogenesis. Total cellular extracts (20 µg of total protein per lane) of differentiating BC3 H1 (A and B) and L6 myoblasts (C) were analysed in SDS-PAGE and western blots using mAb 217/6 (A, C and D) and the annexin VII isoform-specific mAb 62/6 (B). Differentiation was induced by changing the medium to a low mitogenic medium, and samples were taken from day 0 to 6. In D the differential expression of annexin VII isoforms in mouse striated muscle (1), rabbit striated muscle (2), undifferentiated (3) and 10 day differentiated L6 cells (4) is shown.
Miscellaneous methods
DNA manipulations were done according to Sambrook et al. (1989). Lactate dehydrogenase activity was determined according to the spectrophotometric procedure of Wroblewski and LaDue (1955). SDS-PAGE was performed according to Laemmli (1970), and western blotting using the method of Towbin et al. (1979). Protein was determined according to the method of Bradford (1976) using BSA as a standard. The fiber type distribution in human muscle biopsies was confirmed by Na⁺/K⁺-ATPase reaction at pH 9.4 (Round et al., 1980).

RESULTS
Expression of two mouse annexin VII isoforms
We obtained two cDNA clones encoding part of the mouse annexin VII from a mouse fibroblast library using PCR with a set of primers derived from the human annexin VII cDNA sequence (Burns et al., 1989). One clone was a 463 bp cDNA fragment that was identical to the subsequently reported mouse sequence (Zhang-Keck et al., 1993), whereas the other 583 bp fragment contained an additional 66 bp cassette exon (Fig. 1). This exon was inserted into the proximal one third of the N-terminal domain and was in the same position as the analogous insert found in the human sequence (Magendzo et al., 1991). The 22 amino acids encoded by the 66 bp alternatively spliced exon introduce three acidic residues into an otherwise almost uncharged and hydrophobic domain. The amino acid sequences of the exons from human and mouse annexin VII differ in four positions (Fig. 1), but the charge of the exon was not altered by these substitutions. All substitutions resulted from C/T transitions.

The cDNAs were expressed in *E. coli* and the resultant polypeptides used to produce annexin-VII-specific antibodies (Fig. 2). Monoclonal antibodies 217/6 and 289/2 recognized specifically a 47 kDa protein corresponding to the smaller 47 kDa annexin VII isoform in mouse liver, kidney and lung (data not shown), and a 51 kDa protein in skeletal muscle (Fig. 3D, lanes 1 and 2). Both isoforms were found in brain and heart tissue. In brain, the 51 kDa and 47 kDa isoforms were present in a ratio of approximately 4:1, whereas in heart the ratio was

Fig. 4. Localization of annexin VII in L6 myoblasts. Undifferentiated (A and B) and differentiated cells (C-E) were grown on coverslips, fixed and labeled with mAB 217/6 specific for annexin VII (A and C). In E the ER was visualized by staining with mAB 10C3 against BiP. Detection of the monoclonal antibodies was done using Cy3-conjugated goat anti-mouse IgG. In B and D the corresponding phase-contrast images are shown. Bar, 10 µm.
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1:1. mAbs 217/6 and 289/2 also reacted with annexin VII in chicken, rabbit and human tissue. We also obtained two antibodies (62/6 and 204/10) that specifically recognized the mouse cassette exon in western blots (Fig. 2). However, they did not crossreact with the 51 kDa isoform present in bovine, rabbit or human skeletal muscle tissue.

Annexin VII is differentially expressed during myogenesis

Western blots showed that undifferentiated L6 myoblasts or
BC3H1 cells expressed only the 47 kDa isoform (Fig. 3D, lane 3). Differentiation was induced by changing to a low mitogenic medium and produced distinctive changes in morphology. BC3H1 cells, which do not fuse, changed their fibroblast-like shape to a more torpedo-like form, whereas L6 cells began to fuse. After 5 days of differentiation, the 51 kDa isoform appeared in BC3H1 cells (Fig. 3A and B). In L6 cells, this isoform appeared after 3 days (Fig. 3C). In differentiated BC3H1 cells, the 51 kDa isoform became dominant and, after 6 days of differentiation, the ratio of 51 kDa to 47 kDa isoform was about 7:1. L6 cells showed equal amounts of the 47 kDa and 51 kDa isoforms, even after prolonged differentiation. In contrast, when muscle tissue from mouse, rabbit or human was analysed by western blotting, only the 51 kDa isoform was observed (Fig. 3D, lanes 1 and 2). Compared to undifferentiated cells, differentiated cells expressed two to three times more annexin VII protein; this was apparent both in immunoblots (Fig. 3A) and in immunofluorescence studies (Fig. 4C).

Annexin VII is located in the cytosol in undifferentiated and differentiated myoblasts

Immunofluorescence using a range of annexin-VII-specific antibodies was employed to determine the distribution of annexin VII in both undifferentiated and differentiated L6 and BC3H1 cells. All antibodies gave clear cytoplasmic staining (Fig. 4A and C). We used as a control an antibody to the ER-protein BiP (Kassenbrock et al., 1988), which showed the location of the endoplasmic reticulum (Fig. 4E). Detergent extraction assays further supported a cytoplasmic location of annexin VII. For these studies we used the mild detergent digitonin, which forms pore-like complexes with the cholesterol in membranes (Fukami and Flatmark, 1986). Lactate dehydrogenase (LDH) activity (Schliwa et al., 1987) was used as a marker of the cytosolic fraction. In both undifferentiated and differentiated cells, annexin VII and the LDH were released after 10 minutes of incubation at digitonin concentrations of 50 µM (shown for annexin VII in Fig. 5A and C). At concentrations lower than 50 µM digitonin, neither annexin VII nor LDH was released into the supernatant. Enzymes specific for peroxisomes or mitochondria are released at about 200 µM digitonin and 1 mM, respectively (Fukami and Flatmark, 1986). Since annexins exhibit Ca2+-regulated lipid binding activity, the same experiments were performed in the presence of Ca2+. When 100 µM Ca2+ was added to the cells, LDH was released as well as β-COP (Fig. 5E), a 110 kDa protein, which is present on vesicles as a coat and also in a large cytosolic complex (Waters et al., 1991). Annexin VII was retained under these conditions and was not released even in the presence of 100 µM diginton (Fig. 5B). Immunofluorescence studies of cells after digitonin treatment showed that digitonin concentrations higher than 50 µM abolished annexin VII staining inside the cell in the presence of EGTA (Fig. 6C). In the presence of 100 µM Ca2+ during digitonin treatment, annexin VII staining was observed at the plasma membrane, which appeared to be ruptured by the detergent treatment (Fig. 6E). The additional staining of the nuclei appeared to be non-specific and was not seen with other annexin-VII-specific antibodies. The differentiation state of cultured cells did not influence the localization of annexin VII as seen by these methods.

Raising intracellular Ca2+ changes annexin VII localization

The influence of intracellular Ca2+ concentration on the activation of annexin VII was investigated in patch-pipet assays by Pollard and Rojas (1988). These authors showed that in the presence of 50 mM Ca2+ annexin VII incorporated into acidic
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Kuijpers et al. (1992) performed localization studies and found that annexin VII can only bind to isolated chromaffin granules in the presence of 1 mM Ca$^{2+}$. We show here that annexin VII changes its subcellular localization in living cells in response to the addition of Ca$^{2+}$. Intracellular Ca$^{2+}$ was manipulated by adding the specific ionophore A23187 in the presence of 100 µM extracellular Ca$^{2+}$. At different incubation times, L6 or BC3H1 cells were fixed and examined by immunofluorescence studies. After a period of 30 minutes both annexin VII isoforms were localized at the plasma membrane (Fig. 7B and C), and the staining of the cytosol as compared to untreated control cells was reduced (Fig. 7A). In contrast to the results obtained by treatment with high digitonin concentrations, the plasma membrane did not appear to be damaged by A23187 treatment. An extended incubation time led to a complete relocalization of annexin VII from the plasma membrane to the nuclear membrane. After 90 minutes an ER-like staining was observed and later on annexin VII was associated with large vesicles. If the amount of ionophore was reduced from 10 µM to 1 µM, the changes in localization of annexin VII also took place, although the reaction was slower (data not shown). The staining of the nuclei in Fig. 7B is non-specific as described above.

Localization of annexin VII in skeletal muscle

In contrast to the generalized cytosolic localization of annexin VII in differentiated and undifferentiated L6 cells or BC3H1 cells, transverse sections of striated muscle tissue showed dis-
Distinctive plasma membrane staining (Fig. 8A-C) together with a subcellular polygonal staining pattern (Fig. 8B). Longitudinal sections labelled with annexin VII antibodies (Fig. 8D) showed a pattern of punctate striations, with strong staining at the junction between the A- and I-bands. Image processing of such micrographs (Fig. 9A) showed that the pattern was remarkably well ordered and computed Fourier transforms showed distinctive spots corresponding to both the longitudinal 2.2 µm sarcomere repeat and a roughly 0.8 µm transverse repeat. The spots in the transform were, however, rather broad and smeared somewhat into lines because of the underlying disorder of the pattern. We therefore used real-space correlation methods to correct partially for this disorder and to obtain an averaged image of the pattern (Fig. 9B), which showed that the dots formed two lines separated by about 0.85 µm axially, with the dots in each line separated by about 0.8 µm. Such a pattern is characteristic of the transverse tubule (t-tubule) system of vertebrate skeletal muscle (see, for example, Franzini-Armstrong and Jorgensen, 1994; Jorgensen et al., 1990). In addition to the pattern of two punctate lines, there was also some residual staining of the cytosol, which varied between fiber types. Fast twitch fibers (type II), which were positive for SERCA1 type Ca$^{2+}$-ATPase (Fig. 10B) or Na/K-ATPase (Fig. 10C), showed stronger annexin VII staining of the cytosol than did slow (type I) twitch fibers (Fig. 10A). This staining pattern was observed with annexin-VII-specific antibodies 217/6 and 62/6, antibody 289/2, stained only the plasma membrane (Fig. 8C).

**DISCUSSION**

We have produced specific monoclonal antibodies against two mouse annexin VII isoforms and used them to examine the pattern of isoform expression during muscle differentiation and location in vertebrate skeletal muscle tissue.

In mouse, as well as in human and monkey (Magendzo et al., 1991), alternative splicing gives rise to two annexin VII isoforms. The amino acid sequence of the additional cassette exon found in the larger annexin VII isoform in human and mouse differs in only four positions, and these substitutions do not alter its charge (Fig. 1). The mouse annexin VII isoform-specific antibodies that we have generated in this study did not crossreact with the 51 kDa isoform carrying the cassette exon in human, bovine or rabbit tissue, indicating some variation of the amino acid sequence in this region during evolution in different organisms. These data imply that the charge of the cassette exon is important for any proposed specific function of the 51 kDa annexin VII isoform, rather than the precise amino acid sequence that distinguishes each isoform.

Our isoform-specific antibodies have demonstrated a distinctive pattern of annexin VII expression during differentiation of muscle cells. The only data available to date on the intracellular distribution of annexin VII are those of Kuijpers and coworkers (1992), who performed semiquantitative immunolocalization studies in adrenal chromaffin granules and chromaffin cells. Using a monoclonal antibody against annexin VII these authors found annexin VII to be present in the nucleus, the cytosol and mainly around the granule membrane. Most tissues in human (Magendzo et al., 1991) or mouse (this study), such as liver, lung and kidney, cultured fibroblasts and myoblasts, expressed only the 47 kDa isoform (Fig. 3). The 51
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A 47 kDa isoform seems to be restricted to innervated tissue such as brain, heart and skeletal muscle (Fig. 3). We have also demonstrated that a change in the expression pattern from the 47 kDa to the 51 kDa isoform can be seen in cell culture after changing the medium to a low mitogenic medium (Fig. 3). In contrast to muscle tissue, the 47 kDa annexin VII isoform persisted, albeit at reduced levels, in BC3H1 cells. The 47 kDa isoform could well reflect the presence of a cell population that did not differentiate. Alternatively, the residual 47 kDa isoform might be due to an arrested state of differentiation in the cultured cells, which cannot be overcome without extracellular signals such as, for example, nerve impulses. In addition to the differing expression pattern of annexin VII isoforms and the increasing amount of annexin VII protein during myogenesis, its subcellular localization also changed. In culture, both immunofluorescence microscopy and detergent extraction showed that both annexin VII isoforms were localized in the cytosol of either myoblasts or polynucleated myotubes (Figs 4C and 6C). The distribution of annexin VII also depended on the cellular Ca\textsuperscript{2+} level. Annexin VII attached at the ruptured plasma membrane in the presence of digitonin and 100 \textmu M extracellular Ca\textsuperscript{2+} (Fig. 6E), consistent with other studies that showed Ca\textsuperscript{2+}-dependent binding of annexins to membranes (Pollard et al., 1990; Chander and Wu, 1991). Comparing the two methods used for manipulating intracellular Ca\textsuperscript{2+}, digitonin and A23187, the latter is much more gentle and specific. Although both treatments led to a change in the localization of annexin VII from the cytosol to the plasma membrane, differences were observed that could be due to the fact that digitonin-treated cells may lose putative annexin-VII-associated cytosolic proteins through the permeabilized plasma membrane. During digitonin permeabilization annexin VII showed either a cytosolic or a plasma membrane localization, and no other subcellular organelles were stained during the permeabilization process. In contrast, cells permeabilized with A23187 showed a plasma membrane localization of annexin VII after 30 minutes of incubation, but during further incubation for periods up to 4 hours, the localization of annexin VII changed successively from the plasma membrane to the nuclear membrane, endoplasmic reticulum, and vesicles (data not shown). This phenomenon, which was not observed in digitonin-extracted cells, indicated that annexin VII has the potential not only to bind to the plasma membrane but also to other membranes as the nuclear membrane or membranes of the endoplasmic

![Image](image_url)

**Fig. 10.** Distribution of annexin VII in fast-twitch and slow-twitch muscle fibers. Transverse sections of human striated muscle were subjected to immunofluorescence studies, using annexin-VII-specific mAb 217/6 (A) and mAb 10D1 specific for fast-twitch fiber-specific Ca\textsuperscript{2+}-ATPase (B). C shows the same section after histochemical Na\textsuperscript{+}/K\textsuperscript{+}-ATPase staining and (D) the corresponding phase-contrast image. Arrowheads mark identical cells. A, B and D, ×200; C, ×400.
reticulum or vesicles. A binding specificity of annexin VII concerning lipids has been reported by Hong et al. (1982). In liposome fusion assays it was shown that annexin VII has a Ca\textsuperscript{2+}-dependent affinity for acidic lipids as they are found on the outer aspect of granula membranes and on the cytosolic layer of plasma membranes. Moreover, in contrast to the results obtained with cultured myoblasts, sections of skeletal muscle showed that in this tissue annexin VII was found preferentially at the plasma membrane (Fig. 8A-C) and in subcellular structures (Fig. 8B and D), indicating that the change of localization of annexin VII observed by digitonin and ionophore treatment was not artificial, but may occur also in vivo.

There are indications that the distribution of annexin VII is well regulated, since the ratio of cytosolic to membrane-associated annexin VII is different in type I and type II muscle fibers (Fig. 10A). Type-specific differences have been reported for other Ca\textsuperscript{2+}-binding proteins, e.g. parvalbumin (Gillis et al., 1982) and proteins involved in Ca\textsuperscript{2+} regulation like the Ca\textsuperscript{2+}-ATPase (Kaprielian and Fambrough, 1987). Since the Ca\textsuperscript{2+} flux between the sarcoplasmic reticulum and the cytosol is different in these two types of fibers (Lamb and Walsh, 1987), it is possible that the cytosolic steady state Ca\textsuperscript{2+} concentrations in both fibers differ within the physiological range, which then might influence the localization of annexin VII in each fiber type. This regulation might occur either directly (since annexin VII has been found to bind calcium ions on its outer surface) or indirectly via annexin-VII-associated proteins.

The characterization of annexin VII expression and distribution during myogenesis has not given support to the hypothesis that annexin VII is involved in membrane fusion processes, as proposed by Creutz and coworkers (1978). Annexin VII was not associated with vesicles or concentrated specifically at regions of the plasma membrane where cell fusion takes place during myogenesis. Rather it seems, as recently proposed by Döring (1993), that annexin VII is involved in Ca\textsuperscript{2+} homeostasis of cells.

Our immunofluorescence studies on mouse skeletal muscle showed a striking localization of annexin VII to the plasma membrane and also to the transverse (t-)tubule system (Figs 8 and 9), where its distribution resembles that of the 1,4-dihydropyridine receptor (Franzini-Armstrong and Jorgensen; 1994; Jorgensen et al., 1989). Although annexin VII has not been localized previously, localization data are available for annexin VI (Hazarika et al., 1991), which is thought to be involved in the regulation of the sarcoplasmic reticulum Ca\textsuperscript{2+}-release channel. In contrast to annexin VII, annexin VI is located over the entire sarcoplasmic reticulum and shows a quite different striation pattern in longitudinal sections of chick muscle.

The location of annexin VII to the t-tubules of skeletal muscle is intriguing in the context of the mechanism of excitation-contraction coupling. The t-tubule system carries the electrical depolarization signal from the plasma membrane into the muscle fiber and thereby induces Ca\textsuperscript{2+} release from the sarcoplasmic reticulum (reviewed by Schneider, 1994). At the junction between t-tubules and sarcoplasmic reticulum, the depolarization signal is transduced to a chemical (Ca\textsuperscript{2+}) signal that, in turn, causes Ca\textsuperscript{2+} release from the sarcoplasmic reticulum and thus contraction. The two important components of this transduction system are the ryanodine receptor as Ca\textsuperscript{2+} channel and the dihydropyridine receptor as voltage sensor. It may be that additional components are also required (see Meissner, 1994; Schneider, 1994; Franzini-Armstrong and Jorgensen, 1994, for reviews) that facilitate the interaction between the two receptors or amplify or modulate the signal. In this context the Ca\textsuperscript{2+}-binding ability of annexin VII may be important, especially in the context of the observation that it can form voltage-dependent Ca\textsuperscript{2+} channels when inserted into phospholipid bilayers (Pollard and Rojas, 1988).

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