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Binding of an ankyrin-1 isoform to obscurin suggests a molecular link between the sarcoplasmic reticulum and myofibrils in striated muscles

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Assembly of specialized membrane domains, both of the plasma membrane and of the ER, is necessary for the physiological activity of striated muscle cells. The mechanisms that mediate the structural organization of the sarcoplasmic reticulum with respect to the myofibrils are, however, not known. We report here that ank1.5, a small splice variant of the *ank1* gene localized on the sarcoplasmic reticulum membrane, is capable of interacting with a sequence of 25 aa located at the COOH terminus of obscurin. Obscurin is a giant sarcomeric protein of ~800 kD that binds to titin and has been proposed to mediate interactions between myofibrils and other cellular structures. The binding sites and the critical aa required in the interaction between ank1.5 and obscurin were characterized using the yeast two-hybrid system, in in vitro pull-down assays and in experiments in heterologous cells. In differentiated skeletal muscle cells, a transfected myc-tagged ank1.5 was found to be selectively restricted near the M line region where it colocalized with endogenous obscurin. The M line localization of ank1.5 required a functional obscurin-binding site, because mutations of this domain resulted in a diffused distribution of the mutant ank1.5 protein in skeletal muscle cells. The interaction between ank1.5 and obscurin represents the first direct evidence of two proteins that may provide a direct link between the sarcoplasmic reticulum and myofibrils.

In keeping with the proposed role of obscurin in mediating an interaction with ankyrins and sarcoplasmic reticulum, we have also found that a sequence with homology to the obscurin-binding site of ank1.5 is present in the ank2.2 isoform, which in striated muscles has been also shown to associate with the sarcoplasmic reticulum. Accordingly, a peptide containing the COOH terminus of ank2.2 fused with GST was found to bind to obscurin. Based on reported evidence showing that the COOH terminus of ank2.2 is necessary for the localization of ryanodine receptors and $InsP_3$ receptors in the sarcoplasmic reticulum, we propose that obscurin, through multiple interactions with ank1.5 and ank2.2 isoforms, may assemble a large protein complex that, in addition to a structural function, may play a role in the organization of specific subdomains in the sarcoplasmic reticulum.

Introduction

The subcellular localization of the endosarcoplasmic reticulum represents a central point in the biology of striated muscle cells (Golovina and Blaustein, 1997; Meldolesi and Pozzan, 1998; Petersen et al., 2001). This issue, which is certainly relevant to the structural organization of all eukaryotic cells, is obviously even more relevant to muscle cells because of the high level of specialization of the sarcoplasmic reticulum in the latter ones (Franzini-Armstrong, 1994; Flucher and Franzini-Armstrong, 1996; Baumann and Walz, 2001). In striated muscles, the sarcoplasmic reticulum, which is specialized in storage, release, and reuptake of Ca^{2+} (Berridge et al., 2000), maintains a highly organized relationship with respect to the contractile apparatus (Epstein and Fischman, 1991; Franzini-Armstrong, 1994). To perform its functions, the sarcoplasmic reticulum forms a sleeve-like structure of intracellular membranes organized around each myofibril. The highly regulated nature of the arrangement of the sarcoplasmic reticulum around myofibrils is such that specific domains of the sarcoplasmic reticulum involved in the mechanisms of Ca^{2+} release and uptake (i.e., terminal cisternae and longitudinal tubules, respectively) are distributed with a regular spacing in correspondence to specific regions of the

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sarcomere (Franzini-Armstrong, 1994). The localization of the sarcoplasmic reticulum in respect to myofibrils is highly relevant to muscle cell physiology, considering that terminal cisternae need to make contact with the t tubule/plasma membrane to contribute to forming specific structures like diads, triads, or peripheral couplings, which underlie the process of excitation–contraction coupling in striated muscles (Franzini-Armstrong, 1994; Flucher and Franzini-Armstrong, 1996). In contrast to the large body of evidence in support of the importance of the intracellular organization of the sarcoplasmic reticulum with respect to myofibrils, the molecular mechanism(s) responsible for the interactions between these two cellular structures is not known.

Ankyrins are a family of proteins involved in the organization of specific membrane domains by mediating the interactions between proteins of the plasma membrane and the subplasma membrane cytoskeleton (Bennett and Baines, 2001). In the past years, specific ankyrin isoforms have been found to associate also with the membrane of intracellular organelles such as the Golgi apparatus and lysosomes (Peters et al., 1995; Devarajan et al., 1996; Beck et al., 1997; Hoock et al., 1997). In striated muscles, ankyrins have been detected at specific sites, including costameres, the postsynaptic membrane, and triads (Pardo et al., 1983; Flucher et al., 1990; Bennett and Baines, 2001). Interestingly, in addition to conventional ankyrins, muscle-specific ankyrin isoforms have also been described. While some of these muscle-specific ankyrin isoforms seem only to interact with the membrane of the sarcoplasmic reticulum (Kordeli et al., 1998; Gagelin et al., 2002), a group of striated muscle–specific isoforms of the *ank1* gene (ank1.5, ank1.6, and ank1.7) are selectively localized on the sarcoplasmic reticulum membrane, with which they are associated through a hydrophobic sequence located at their NH_2-terminal region (Zhou et al., 1997; Birkenmeier et al., 1998; Gallagher and Forget, 1998). Recent studies with *ank2*-deficient mice have indicated that a*nk2* may be important for the localization of proteins involved in Ca^{2+} homeostasis, such as ryanodine receptors and $InsP_3$ receptors at specific domains of the sarcoplasmic reticulum (Tuvia et al., 1999; Mohler et al., 2002). Obscurin is a recently identified muscle protein known to bind to titin (Bang et al., 2001; Young et al., 2001; Russell et al., 2002). Obscurin is an extremely large protein characterized by a modular architecture that contains multiple Ig-like domains, two fibronectin (FN3)-like domains, and a RhoGEF/PH domain. Additional transcripts apparently derived from the obscurin gene have also been detected. These transcripts contain one or two serine-threonine kinase domains (Bang et al., 2001; Russell et al., 2002). Whether the sequence encoding these kinase domains can be associated with the initial obscurin transcript (Young et al., 2001) is, however, not yet clear. Altogether, the modular structure of obscurin makes this protein a very good candidate for mediating multiple interactions between the myofibrils and other cellular structures, including the extramyofibrillar cytoskeleton (Stromer, 1998; Gregorio and Antin, 2000; Bang et al., 2001; Young et al., 2001).

We report here that the ank1.5 isoform is capable of interacting with the COOH terminus of obscurin. The interaction between ank1.5 and obscurin is mediated by an aa sequence present in ank1.5, but not in ank 1.6 and ank1.7, that recognizes a specific sequence present in the nonmodular region at the COOH terminus of obscurin. Mutations of specific aa in these regions abolished binding between ank1.5 and obscurin. In addition to in vitro studies, the interaction between ank1.5 and obscurin was also verified in heterologous cells transfected with plasmids encoding ank1.5 and a fusion protein consisting of the COOH terminus of obscurin cloned in frame with GFP. In agreement with in vitro data, transfection of ank1.5 resulted in the association of GFP–obscurin with the ER. Experiments performed in cultured skeletal muscle cells revealed that ank1.5 is present near or at the M line, where it colocalizes with obscurin. Localization of ank1.5 at the M line required the obscurin-binding site because a mutation in this site prevented the localization of ank1.5 at the M line and resulted in a diffuse distribution of the mutated protein. Based on the ability of ank1.5 to specifically interact with a region at the COOH terminus of obscurin, we propose that these two proteins may contribute to hold a stable interaction between the sarcoplasmic reticulum and the myofibrils. In line with the above results, we have also found that, in addition to ank1.5, the ank2.2 isoform can interact with obscurin through a sequence homologous to that present in ank1.5. In light of the evidence that a*nk2* is necessary for the localization of ryanodine receptors and $InsP_3$ receptors (Tuvia et al., 1999; Mohler et al., 2002), our working hypothesis envisions that obscurin plays a role in assembling a scaffold of proteins important to establish an association between the sarcoplasmic reticulum and the cytoskeleton and to redistribute proteins, e.g., ryanodine receptors, $InsP_3$ receptors, and eventually other proteins, at specific domains of the sarcoplasmic reticulum.

Results

ank1.5 binds to a COOH-terminal fragment of obscurin in two-hybrid experiments

To identify potential interaction partners for short isoforms of the *ank1* gene, a human skeletal muscle cDNA library was screened using the yeast two-hybrid technique and the cytoplasmic tail of the ank1.5 cDNA as bait. Screening of a total of 5×10^5 clones resulted in the identification of seven positive colonies (A7, A14, A19, A26, A36, A62, and A38). DNA sequencing revealed that all clones corresponded to overlapping fragments of obscurin, a giant muscle protein associated with the myofibrils (Bang et al., 2001; Young et al., 2001). Obscurin has a modular architecture containing multiple Ig-like domains, two fibronectin (FN3)-like domains, and a RhoGEF/PH domain followed at the COOH terminus by a nonmodular sequence (Fig. 1 A). Five of the clones identified in the two-hybrid screening (A7, A14, A19, A26, and A36) contained totally or partially the RhoGEF/PH and two Ig-like domains, whereas clones A38 and A62 contained only the nonmodular sequence at the COOH terminus (Fig. 1 A). None of these clones contained the kinase domains identified by Bang et al. (2001) and Russell et al. (2002). Because all clones can interact with ank1.5, we concluded that the interaction site was located in the nonmodular sequence at the COOH terminus of obscurin, downstream of aa 6235.

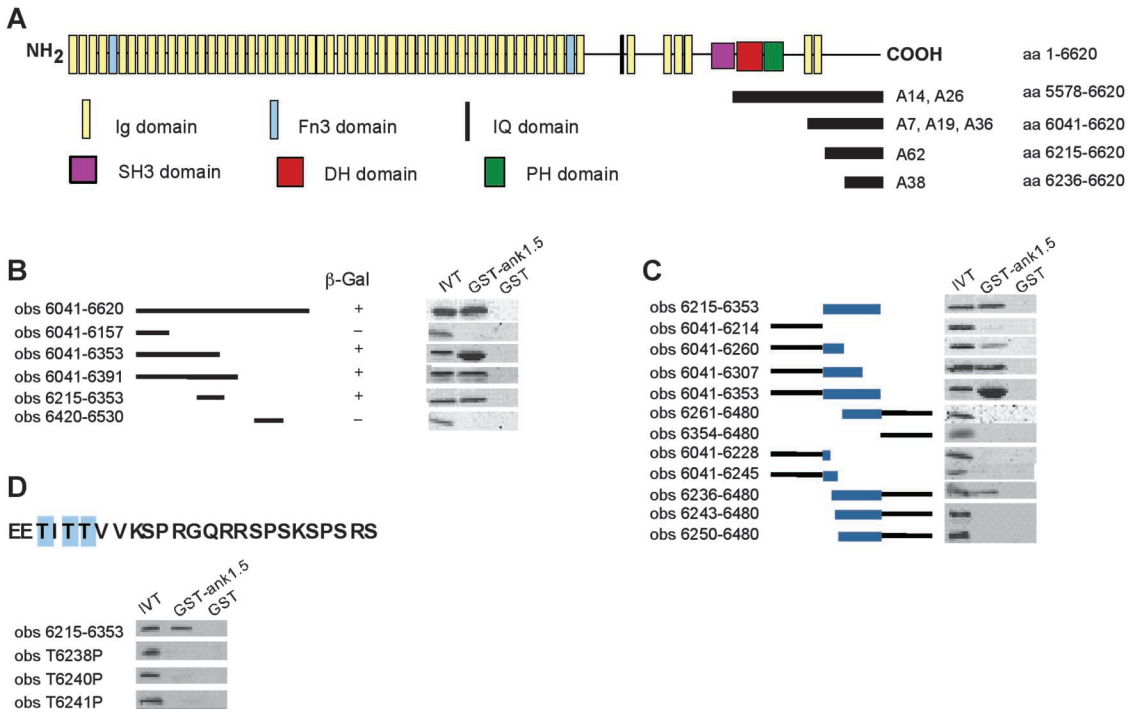

Figure 1. **Association of ank1.5 with the COOH-terminal region of obscurin.** (A) Two-hybrid screening with the cytosolic tail of ank1.5 (aa 22–155) as "bait" identified seven positive clones, corresponding to the COOH terminus of the obscurin protein. (B) Fragments of the obscurin clone A7 were tested for the ability to interact with the bait ank1.5 in the two-hybrid system and with a GST–ank1.5 fusion protein in pull-down experiments. Both experiments demonstrated that the region of obscurin between aa 6236 and 6353 is capable to interact with ank1.5. +, detectable activity; −, no detectable activity. (C) A second series of obscurin fragments was prepared to further restrict the sequence responsible for binding with ank1.5 to 25 aa of obscurin (aa 6236–6260). (D) Mutagenesis of the aa in the COOH terminus of obscurin (aa 6236–6260) capable of mediating binding to ank1.5 was performed and mutant proteins were tested against GST–ank1.5 in in vitro binding assays.

Identification of the ank1.5 binding site in obscurin

Two-hybrid and pull-down experiments performed with small fragments of the COOH terminus of obscurin identified a 118-aa fragment (aa 6236–6353) that was still capable to interact with ank1.5 (Fig. 1 B). Characterization of a second set of obscurin fragments restricted the domain in the COOH-terminal region of obscurin capable of mediating the interaction with ank1.5 to a minimal region of 25 aa (residues 6236–6260) (Fig. 1 C). Site-directed mutagenesis of these 25 aa indicated that the substitution of three threonine residues (T6238, T6240, and T6241) drastically impaired the ability of obscurin to bind to ank1.5 (Fig. 1 D). Mutation of other aa in this region did not significantly affect the binding of the two proteins (unpublished data).

ank1.5, but not ank1.6 and ank1.7, binds to the COOH terminus of obscurin

To identify the sequence in ank1.5 responsible for the interaction with the COOH terminus of obscurin, we performed experiments designed to test the ability of the three small ankyrin isoforms (ank1.5, ank1.6, and ank1.7) to bind to the COOH terminus of obscurin. Two-hybrid experiments were performed using the cytosolic sequences of the ank1.5, ank1.6, and ank1.7 isoforms (cloned in the pGBKT7 vector as a bait) against the obscurin subclone A7 (cloned in the pACT2 vector). Both the ability to grow in selective media lacking histidine and β-galactosidase activity indicated that only ank1.5, not ank1.6 or ank1.7, was capable of binding the COOH terminus of obscurin (Fig. 2 A). From a quantitative point of view, it is worth noting that whereas the interaction of obscurin subclone A7 with ank1.5 yielded a very strong blue reaction in the β-galactosidase assay, interaction with ank1.6 and ank1.7 did not result in any activity at all. In parallel, pull-down experiments were performed using in vitro–translated obscurin subclone A7 and GST fusion proteins of the three ankyrin isoforms. As shown in Fig. 2 A, in agreement with two-hybrid results, binding to the GST–obscurin clone A7 was observed with ank1.5 and not with ank1.6 or ank1.7. The alignment of aa sequences of muscle-specific small ank1 isoforms revealed that ank1.5 contains a stretch of 22 aa (aa 102–123) that is absent in ank1.6 and ank1.7, suggesting that this region is involved in mediating the binding of ank1.5 with the COOH terminus of obscurin.

ank2.2 binds to the COOH terminus of obscurin through a sequence homologous to that of ank1.5

Interestingly, the 22-aa sequence present in ank1.5, but not in ank1.6 and ank1.7, is present also in the long ank1 isoforms and displays a high degree of homology with a region present also in the COOH-terminal region of long ankyrin products of the *ANK2* gene (Bennett and Baines, 2001) and of a recently identified muscle-specific isoform (AnkG107) of the *ANK3* gene (Gagelin et al., 2002) (Fig. 2 B). Recent work in mice deleted of the *ank2* gene revealed that these

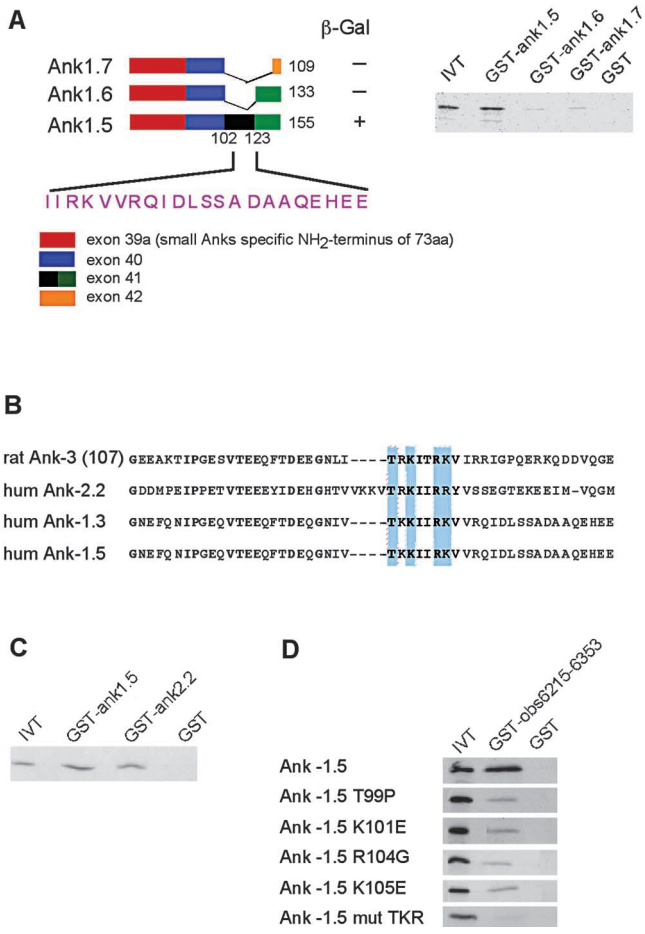

Figure 2. **The site in ank1.5 able to interact with obscurin is located in a sequence highly conserved among ankyrins.** (A) A schematic representation of the structure of the small muscle–specific ankyrin isoforms is presented: a color code identifies the different exons that, by undergoing alternative splicing, generate the different ank1 isoforms. The cytosolic sequences of ank1.5, ank1.6, and ank1.7 were used as baits in two-hybrid system experiments against the obscurin subclone A7. In parallel, in vitro–translated obscurin subclone A7 was allowed to interact with GST–ank1.5, GST–ank1.6, and GST–ank1.7. Both two-hybrid and in vitro binding assays demonstrated that only ank1.5 is capable of binding the obscurin subclone A7. +, detectable activity; −, no detectable activity. (B) The alignment of COOH-terminal sequences of a skeletal muscle–specific ank3 isoform (AnkG107), ank2.2, ank1.3, and ank1.5. Conserved aa residues are in bold. (C) In vitro binding of a GST fusion protein containing the COOH-terminal region of ank2.2 (aa 1758–1872) against in vitro–transcribed and –translated obscurin clone A7. (D) Site-directed mutagenesis of aa 97–123 of ank1.5 was performed and the in vitro–transcribed and –translated mutant proteins were used in binding experiments with the GST–Obs6215–6353 fusion protein. Mutagenesis of any of four aa residues (T99, K101, R104, and K105) of ank1.5 strongly reduced the ability to bind to obscurin.

mice, among other defects, present a displacement of some specific proteins, such as ryanodine receptors and $InsP_3$ receptors, from their normal sites in the sarcoplasmic reticulum in striated muscles (Tuvia et al., 1999). Rescue experiments of *ank2*$^{-/-}$ mice have been performed using chimeric proteins containing different regions of ank2 (AnkB220) and ank3 (AnkG190) (Mohler et al., 2002). These experiments revealed that the COOH-terminal region of ank2 was required for correct localization of ryanodine and $InsP_3$ receptors in the sarcoplasmic reticulum of myocytes from *ank2*$^{-/-}$ mice. Because the COOH terminus of the AnkB220 isoform of ank2, but not that of the AnkG190 isoform of ank3, contains the region of homology with ank1.5, we decided to investigate whether the long isoform ank2.2 could also bind to obscurin. Therefore, the last 115 aa at the COOH terminus of the long isoform ank2.2 (aa 1758–1872), which contains the region of homology with ank1.5, were fused to GST and the resulting GST–ank2.2 fusion protein was used in pull-down experiments with an in vitro–translated fragment of obscurin containing the region between aa 6041–6620 (obscurin subclone A7). As shown in Fig. 2 C, the GST fusion protein containing the COOH terminus of ank2 was found to bind to the in vitro–translated obscurin subclone A7.

Identification of critical aa in ank1.5 required for interaction with obscurin

Alignment of aa sequences from ank1.3, ank2.2, and the muscle-specific ank3 isoform (AnkG107) with the aa sequence of ank1.5 involved in binding to obscurin reveals the presence of some conserved residue among these proteins. To identify the critical aa that in ank1.5 are required for the interaction with the COOH terminus of obscurin, site-directed mutagenesis of aa 97–123 of ank1.5 was performed, and the in vitro–transcribed and –translated mutant proteins were used in binding experiments with the GST–Obs6215–6353 fusion protein. As shown in Fig. 2 D, these experiments identified four residues (T99, K101, R104, and K105) that, if mutated (T99P, K101E, R104G, and K105E), strongly reduced binding of in vivo–translated obscurin subclone A7 to the GST–ank1.5 fusion protein. Contemporary mutagenesis of three of these aa residues (T99P, K101E, and R104G) completely abolished the residual binding observed in proteins containing only one mutated aa (Fig. 2 D).

Fusion proteins containing the COOH terminus of obscurin can bind ank1.5 from transfected cells and skeletal muscle microsomes

To further expand on the interaction between ank1.5 and the COOH-terminal part of obscurin, human embryonic kidney (HEK)* 293 cells were transfected with myc-tagged plasmids encoding full-length ank1.5 or ank1.7 cDNAs. Microsomes prepared from transfected cells were solubilized and the resulting supernatant was allowed to interact with a fusion protein containing aa 6215–6353 of obscurin in frame with GST. Western blot with anti-myc monoclonal antibodies revealed that ank1.5, but not ank1.7, expressed in HEK293 cells was retained by the GST–obscurin fusion protein (Fig. 3 A). Previous work had shown that ank1.5 is a membrane protein of the sarcoplasmic reticulum (Zhou et al., 1997). To further examine the localization of ank1.5 in the sarcoplasmic reticulum, skeletal muscle microsomes were further fractionated according to established procedures

*Abbreviations used in this paper: HEK, human embryonic kidney; MBP, maltose binding protein.

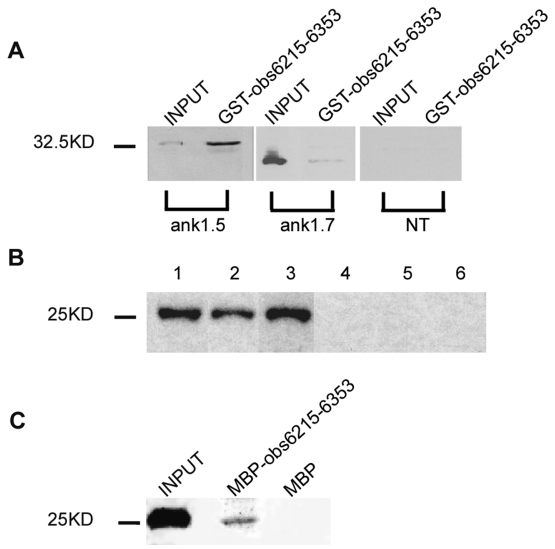

Figure 3. **Exogenous and endogenous ank1.5 interacts with the COOH-terminal region of obscurin.** (A) Microsomes from HEK293 cells transfected with myc-tagged full-length cDNAs of ank1.5 and ank1.7 were used in interaction studies with the GST–Obs6215–6353 fusion protein. (B) ank1.5 is present in the sarcoplasmic reticulum of skeletal muscle. ank1.5 reactivity was detected in the total microsomal fraction (lane 1), in sarcoplasmic reticulum fractions enriched in longitudinal tubules (lane 2), and in terminal cisternae (lane 3). No signal was detected by a preimmune sera when tested against the same preparations of the total microsomal fraction (lane 4), longitudinal tubules (lane 5), and terminal cisternae (lane 5). (C) Microsomes from mouse skeletal muscles were used as a source of endogenous ank1.5 to perform pull-down experiments against the MBP–Obs6215–6353 fusion protein.

(Saito et al., 1984). Selected fractions, enriched in terminal cisternae and longitudinal tubules, were analyzed by Western blot with a rabbit antibody against ank1.5. A major band of an apparent molecular mass of 25 kD, which is compatible with the expected size of ank1.5, was found in fractions of the sarcoplasmic reticulum containing longitudinal tubules and terminal cisternae (Fig. 3 B). Using the ank1.5 antiserum, endogenous ank1.5 solubilized from skeletal muscle sarcoplasmic reticulum was found to bind to the maltose binding protein (MBP)–obscurin (aa 6215–6351) fusion protein, but not to MBP alone (Fig. 3 C).

The COOH terminus of obscurin binds to the ER of heterologous cells expressing ank1.5

To study at the cellular level the interaction between ank1.5 and the COOH-terminal region of obscurin, an expression vector encoding a fusion protein made of GFP followed by the obscurin subclone A7 (GFP–ObsA7) was transfected into NIH 3T3 cells in the absence or in the presence of expression vectors encoding ank1.5, ank1.7, or a mutant ank1.5 unable to bind to obscurin. In transfected cells, ank1.5 or ank1.7 presented a staining corresponding to that of the ER compartment of the cells (Fig. 4, B, E, and H). On the contrary, the GFP–ObsA7 protein, when expressed alone, displayed a diffused cytosolic localization (unpublished data). In cells cotransfected with ank1.5, the GFP–ObsA7 staining was redistributed almost completely to the same sites where ank1.5 was present (Fig. 4, A–C). The specificity of this interaction was confirmed by the lack of colocalization when GFP–ObsA7 was cotransfected with the mutated version of ank1.5, which is unable to interact with obscurin (Fig. 4, D–F). In addition, in agreement with previous results in yeast two-hybrid and pull-down experiments, no colocalization was observed when ank1.7 was transfected with the GFP–ObsA7 plasmid (Fig. 4, G–I).

ank1.5 colocalization with obscurin at the M line region of skeletal muscle cells

The evidence of a specific interaction between ank1.5 and obscurin obtained in in vitro experiments and in heterologous cells opens the question of whether an interaction between these proteins occurs also in skeletal muscle cells. To this end, cultures of differentiating primary skeletal muscle cells were used as an experimental model. To verify the specific localization of the ank1.5 isoform, differentiating skeletal muscle cells were transfected with a plasmid encoding a

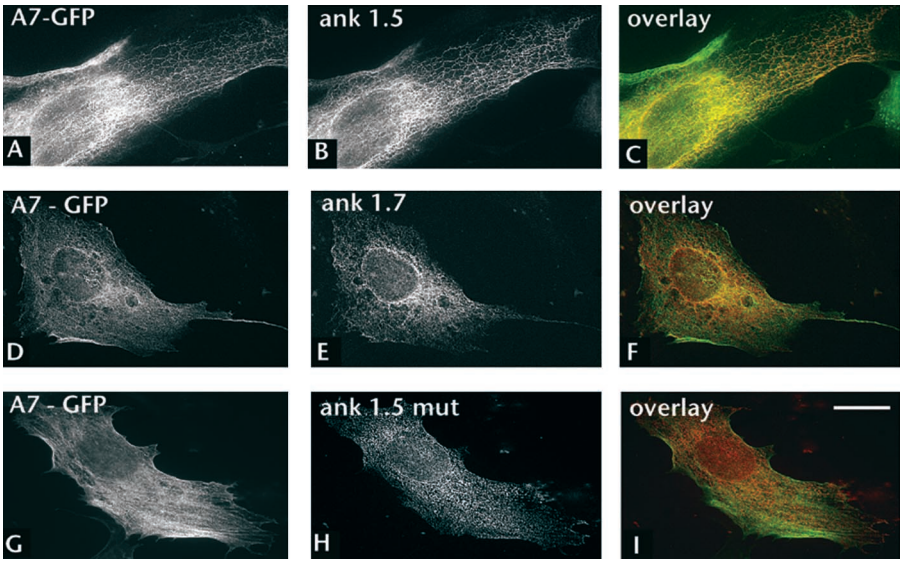

Figure 4. **Effect of ank1.5 on the localization of obscurin in NIH 3T3 cells.** NIH 3T3 cells were transfected with the obscurin subclone A7 joined in frame with GFP, the full-length cDNA of ank1.5, ank1.7, and mutant ank1.5 cloned in pcDNA3 vectors. Cells were decorated with anti-myc monoclonal antibodies and detected by a Cy_3-conjugated anti–mouse antibody (red). Panels show representative sections from the top to bottom of the cells at ~0.25-μm intervals. Bar, 5 μm.

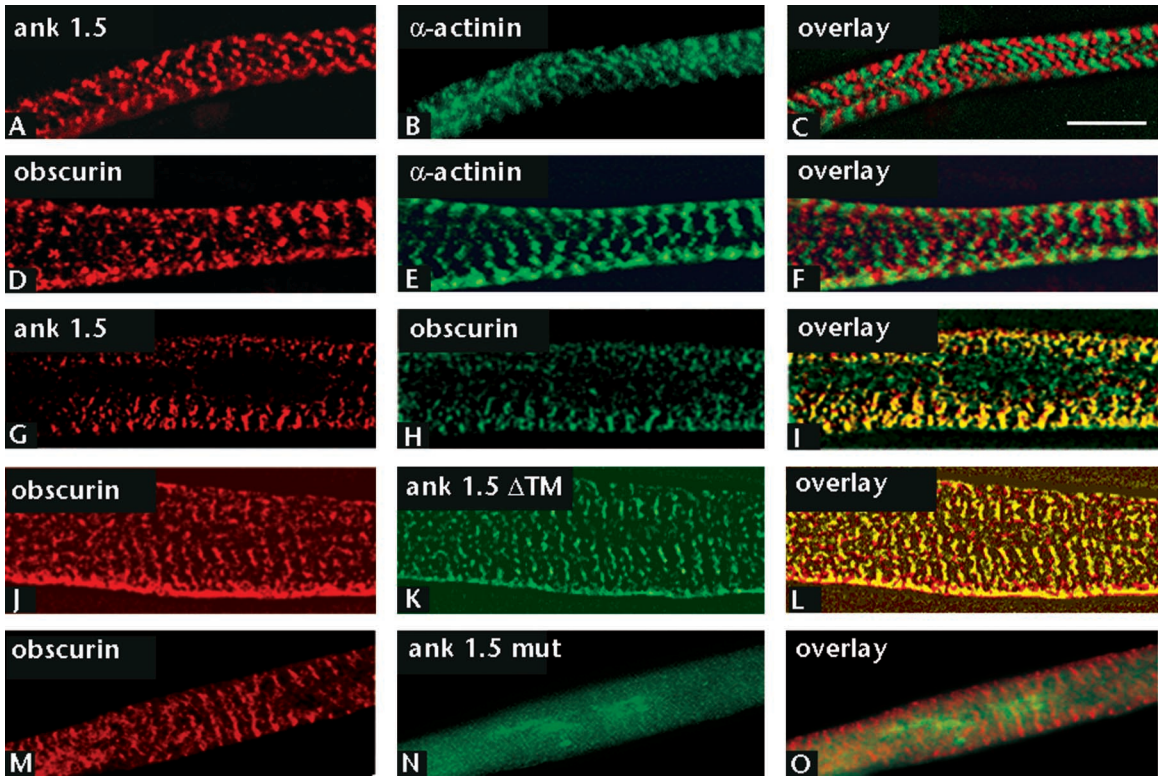

Figure 5. **Localization of ank1.5 and obscurin in differentiating skeletal muscle cells.** Differentiated skeletal muscle cells were stained with rabbit antibodies against the myc epitope to recognize the myc-tagged ank1.5 protein (A) and with monoclonal antibodies against α-actinin, which stain the Z line (B). The alternating pattern of ank1.5 and α-actinin is compatible with the localization of ank1.5 near or at the M line. Cells were stained with rabbit antibodies against the COOH-terminal region of obscurin (D) and with monoclonal antibodies against α-actinin (E). Again, the obscurin staining is compatible with localization near or at the M line. Staining of the cells with antibodies against ank1.5 (G) and against obscurin (H) indicates that the two proteins colocalize. Soluble ank1.5 (ank1.5 ΔTM) missing the transmembrane domain (K) is still able to colocalize with obscurin at the M line (L). A myc-tagged mutant ank1.5, unable to bind to obscurin, presents a diffuse signal (N) distinct from that of obscurin (M). Bar, 5 μm.

myc-tagged ank1.5 cDNA. Immunofluorescence studies using anti-myc antibodies and antibodies against α-actinin yielded two distinct alternating signals, which are compatible with the localization of ank1.5 near or at the M line (Fig. 5, A–C). In the original report on the localization of small muscle–specific ank1 isoforms (Zhou et al., 1997), a signal specific for the endogenous small ank1 isoforms was observed at the Z line and M line in skeletal muscle sections. This apparent discrepancy could be due to the transfection strategy and the use of primary cell cultures in our experimental approach. Alternatively, it could reflect a specific localization of ank1.5 with respect to other small ank1 isoforms, considering that Zhou et al. (1997) used a polyclonal antibody raised against an aa sequence that is present in ank1.5 but also in other small ank isoforms.

To localize endogenous obscurin protein, differentiated skeletal muscle cells were immunostained with rabbit antibodies raised against the COOH-terminal region of obscurin. The immunostaining obtained with antibodies against obscurin was found to alternate with that of α-actinin, which is compatible with the localization of obscurin at or near the M line region (Fig. 5, D–F). Obscurin has been shown to have a complex localization pattern in striated muscle cells, where it appears to be located at the M line, although Z line localization has also been observed (Bang et al., 2001; Young et al., 2001). We have occasionally observed an additional obscurin staining compatible with the Z line region, but this is far less frequent and reproducible than the M line region signal (unpublished data). On the other hand, it should be stated that labeling of obscurin with respect to the Z line and M line might vary depending on the localization of the epitope recognized by the antibodies (Young et al., 2001; Bang et al., 2001). At the same time, alternative splicing of obscurin could also affect the recognition by different antibodies (Bang et al., 2001; Russell et al., 2002). Double staining of skeletal muscle cells with antibodies against the myc-tagged ank1.5 and against endogenous obscurin indicated that the two proteins colocalize (Fig. 5, G–I). The colocalization of ank1.5 with obscurin was also observed by transfection of a plasmid encoding a soluble ank1.5 protein lacking the transmembrane domain (Fig. 5, J–L), which suggests that the colocalization of ank1.5 with obscurin does not require sarcoplasmic reticulum localization of the former protein.

Interestingly, the localization of ank1.5 near the M line was only observed in fully differentiated skeletal muscle cells; in nondifferentiated cells, transfected ank1.5 appeared to have a diffuse distribution, while obscurin-specific signal could not be detected (unpublished data). Thus, the regular pattern of ank1.5 observed in differentiated skeletal muscle cells might

depend on the expression of either obscurin or another protein regularly spaced near the M line. To more directly establish whether the observed colocalization of ank1.5 and obscurin is mediated by a direct interaction between the two proteins, a plasmid encoding full-length ank1.5 containing three mutated aa residues in the domain required for in vitro interaction with obscurin (ank1.5 mutTKR; Fig. 2) was transfected in differentiating skeletal muscle cells. As shown in Fig. 5 (M–O), mutation of the obscurin-binding site of ank1.5 resulted in a diffuse distribution of ank1.5 upon transfection in skeletal muscle cells while obscurin signal maintained its regular sarcomeric arrangement. Consequently, colocalization of ank1.5 with obscurin at the M line required a functional obscurin-binding site.

Discussion

Striated muscle cells contain a highly developed and robust system of protein–protein interactions, which guarantees that the overall structure and integrity of muscle cells and the subcellular localization of the various organelles is maintained independently of the morphological changes associated with contraction and relaxation cycles (Stromer, 1998; Baumann and Walz, 2001; Bellin et al., 2001). In this context, a central role is played by the dystrophin complex and associated molecules that provide a robust scaffold that holds the myofibrils, cytoskeleton, plasma membrane, and extracellular matrix together (Blake et al., 2002). Among the many structures that require a solid anchor in the dynamic world of striated muscle cells are the highly sophisticated membrane systems, which include the t tubule on the plasma membrane and the different regions of the sarcoplasmic reticulum, which need to maintain contact among them and with the myofilaments in order to allow the precise transmission of signals that underlies the mechanism of excitation–contraction (Flucher et al., 1993; Franzini-Armstrong, 1994). The existence of proteins necessary for the organization of these structures has been postulated and heavily investigated for years (Stromer, 1998; Baumann and Walz, 2001; Salanova et al., 2002). Significant developments in this field have been obtained recently with the discovery of proteins like the junctophilins (Takeshima et al., 2000; Ito et al., 2001) and MG29 (Nishi et al., 1999; Pan et al., 2002), which represent interesting candidates for mediating the interaction between the t-tubule/plasma membrane and the sarcoplasmic reticulum. In addition, more recently, an interaction between the minK subunit of the delayed rectifier potassium current channel located on the t tubule and the sarcomeric protein T-cap has also been reported (Furukawa et al., 2001). However, as of today, nothing is known about the identity of specific proteins responsible for the interaction between the sarcoplasmic reticulum and the myofibrils.

Here we report that ank1.5, a muscle-specific ank1 isoform, which is an integral protein of the sarcoplasmic reticulum of striated muscles, contains a specific domain capable to selectively interact with the COOH-terminal part of obscurin, a component of myofibrils. In agreement with data on the characterization of the interaction between ank1.5 and obscurin obtained in vitro, we present evidence that ank1.5 colocalizes with obscurin at the M line in differentiated skeletal muscle cells. This localization appears to depend on the integrity of the obscurin-binding site in ank1.5, as shown by the lack of M line localization of a mutant ank1.5 lacking the aa required for binding to obscurin. These data therefore provide the first experimental evidence of two proteins capable of mediating interactions between the sarcoplasmic reticulum and the myofibrils. Considering the complexity of the organization of the sarcoplasmic reticulum network in striated muscle cells, several other proteins are likely to participate in mediating the subcellular localization/organization of the sarcoplasmic reticulum in muscle cells. In this context, it is interesting to note that a sequence with homology to the obscurin-binding site of ank1.5 is also present in other ankyrin isoforms, namely the long ank1 isoform ank1.3, ank2.2, and the ank3 isoform AnkG107. A role for ank2 in mediating the assembly of ryanodine and $InsP_3$ receptors at specific domains of the sarcoplasmic reticulum of striated muscle cells has been reported (Tuvia et al., 1999). The region of the ank2.2 protein necessary for the localization of calcium release channels in the sarcoplasmic reticulum has been restricted to a small region at the COOH terminus of ank2.2 (Mohler et al., 2002). We have found that the COOH terminus of ank2.2 is capable of binding to obscurin through a binding site homologous to that of ank1.5. On this basis, we propose a model where the interaction of obscurin with different ankyrin isoforms may promote the assembly of a large protein complex, which, in addition to supporting the association of the sarcoplasmic reticulum with the myofibrils, may participate in the mechanisms underlying the localization of Ca^{2+} release channels (i.e., ryanodine and $InsP_3$ receptors), and eventually other proteins, to specific domains of the sarcoplasmic reticulum. Obviously more work is required for better defining the complexity of these interactions.

Materials and methods

Construct preparations

The cDNAs of ank1.5, ank1.6, ank1.7 (GenBank/EMBL/DDBJ accession nos. NM020478, NM020479, and NM020480) were amplified from a human skeletal muscle cDNA library (CLONTECH Laboratories, Inc.) by PCR, using the following primers: 5′-AATGTGGACTTTCGTCACCCAGCTGTTGGT-3′ and 5′-CTGTTTCCCCCTTTTCAGGCTGGCCCGCTT-3′ (ank1.5 and ank1.6) or 5′-GGGGTTGGGTGTCGAGGTGTGATCCTTCTT-3′ (ank1.7). The cDNA corresponding to bp 5269–5616 of ank2 (sequence data available from GenBank/EMBL/DDBJ under accession no. NM020977) was amplified using the following primer pair: 5′-ACTATCATTTATGAAGGAGCCAAAATCCAC-3′ and 5′-CTCATTGTTGTCCTCTGACTGCTCGGTGTC-3′. The PCR products were inserted into pGEMTeasy (Promega) and pcR2.1 (Invitrogen) cloning vectors. Expression vectors for yeast, mammalian, and bacterial cells were pGBKT7 (Matchmaker system III; CLONTECH Laboratories, Inc.), pcDNA3.1 (Invitrogen), pEGFP-C3 (CLONTECH Laboratories, Inc.), pGEX4T (Amersham Biosciences), and pMAL-c2X (New England Biolabs, Inc.).

Yeast two-hybrid studies

The cDNA of ank1.5 (coding for aa 22–155) inserted into the pGBKT7 vector (Matchmaker system III) in fusion with GAL4-BD was used as bait to screen a human skeletal muscle cDNA library fused with GAL4-AD in pACT2 vector (CLONTECH Laboratories, Inc.). The *Saccharomyces cerevisiae* strain Y153 was cotransformed with the bait vector and the library-derived plasmid as described by the manufacturer (Matchmaker system III). 5×10^5 independent clones from a skeletal muscle cDNA library were screened with the recombinant bait. The DNA of colonies positive for HIS3 activation and in β-galactosidase assays was extracted and electroporated

in *Escherichia coli* to select and isolate the library plasmids. All the clones isolated were retransformed in yeast, and HIS3 activation and β-galactosidase assays were performed to confirm the interaction with the bait. Interactions were further confirmed in two-hybrid assays by exchanging the bait DNA-BD vector with the library-derived DNA-AD vector and vice versa.

Site-directed mutagenesis

To map the residues involved in ankyrin–obscurin interaction, ank1.5 (aa 22–155) and a portion of obscurin (aa 6215–6353) were used as templates to perform site-directed mutagenesis using the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions.

Protein expression

All in vitro transcription and translation experiments were performed using the TnT Quick Coupled Reticulocyte Lysate System as described by the manufacturer (Promega). To label the peptides, the reactions were performed in the presence of ^{35}S L-methionine (Amersham Biosciences). The GST fusion proteins were obtained by cloning the cDNA of ank1.5 (aa 22–155), ank1.6 (aa 22–133), ank1.7 (aa 22–109), ank2.2 (aa 1758–1872), and obscurin (aa 6215–6353) into pGEX4T expression vector (Amersham Biosciences). The MBP fusion protein was obtained by cloning ank1.5 (aa 22–155) into pMAL-c2X expression vector (New England Biolabs, Inc.). Microsomes from mouse and rabbit skeletal muscles as well as from HEK293 cells (transfected with ank1.5, ank1.7, and ank1.5 mut) were obtained as previously described (Saito et al., 1984).

In vitro interaction studies and pull-down assay

The GST and MBP fusion proteins were expressed in XL1blue cells. The cell lysates (PBS, 20 mM EDTA, 1% Triton X-100) were incubated for 20 min at 4°C with the resin to immobilize the fusion proteins; glutathione–sepharose 4B beads (Amersham Biosciences) and maltose agarose beads (New England Biolabs, Inc.) for GST and MBP, respectively. For binding experiments, 5 μl of TnT protein extract was incubated with 15 μg of fusion protein (50 μl of beads, 50% slurry) in interaction buffer (PBS, 1% Triton X-100, 1% BSA) for 1 h at 4°C. A total of 250 μg of protein from microsomes was used in pull-down experiments. The interactions with the fusion proteins (GST for transfected HEK293 and MBP for mouse skeletal muscle microsomes) were performed in PBS, 0.2 mM PMSF, 1% Triton X-100 for 2 h at 4°C. Beads were washed in PBS, 1% Triton X-100. Proteins eluted in SDS, β-mercaptoethanol sample buffer were separated by SDS-PAGE.

Cell cultures, DNA transfection, and immunofluorescence staining

HEK 293 and NIH 3T3 cells were maintained in α-MEM medium supplemented with 2 mM glutamine (Bio-Whittaker), 100 μg/ml streptomycin, 100 U/ml penicillin (Bio-Whittaker), 1 mM sodium pyruvate (Bio-Whittaker), 10% heat-inactivated fetal calf serum (Bio-Whittaker) at 37°C under 5% CO_2. Cultures of skeletal muscle cells were prepared from hind leg muscles of newborn rats. In brief, the muscles were minced and subjected to three successive treatments with 0.125% trypsin. Cells were resuspended in DME plus 10% heat-inactivated fetal calf serum and plated onto gelatin-coated coverslips. After 2 d, cells were transfected with appropriated constructs and then switched to differentiating media (i.e., DME supplemented with 2% horse serum) for 3–6 d. DNA transfections were performed using the Lipofectamine-Plus method (Invitrogen), following the manufacturer's instructions. Cells were fixed with 3% paraformaldehyde, 2% sucrose in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4) for 10 min at room temperature, washed, and incubated for 15 min in 5% goat serum in PBS. After washing, cells were permeabilized with Hepes Triton buffer (20 mM Hepes, pH 7.4, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 0.5% Triton X-100). For ankyrin detection, cells were incubated for 1 h at room temperature with an anti-myc monoclonal antibody at a 1:1,000 dilution. Cy_3-conjugated anti–mouse secondary antibodies were from Jackson ImmunoResearch Laboratories and were used according to the manufacturer's instructions. Antibodies against obscurin were prepared by injection of rabbits with a fusion protein containing aa 6215–6353 of obscurin in frame with GST as previously described (Giannini et al., 1995). Images were collected with an epifluorescence Axioplan 2 imaging microscope (Carl Zeiss MicroImaging, Inc.) equipped with a MicroMAX digital CCD camera (Princeton Instruments), digitized, stored, and subsequently processed with Meta Imaging Series 4.5 software (Universal Imaging Corp.).

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References

Bang, M.L., T. Centner, F. Fornoff, A.J. Geach, M. Gotthardt, M. McNabb, C.C. Witt, D. Labeit, C.C. Gregorio, H. Granzier, and S. Labeit. 2001. The complete gene sequence of titin, expression of an unusual approximately 700-kDa titin isoform, and its interaction with obscurin identify a novel Z-line to I-band linking system. *Circ. Res.* 89:1065–1072.

Baumann, O., and B. Walz. 2001. Endoplasmic reticulum of animal cells and its organization into structural and functional domains. *Int. Rev. Cytol.* 205: 149–214.

Beck, K.A., J.A. Buchanan, and W.J. Nelson. 1997. Golgi membrane skeleton: identification, localization and oligomerization of a 195 kDa ankyrin isoform associated with the Golgi complex. *J. Cell Sci.* 110:1239–1249.

Bellin, R.B., T.W. Huiatt, D.R. Critchley, and R.M. Robson. 2001. Synemin may function to directly link muscle cell intermediate filaments to both myofibrillar Z-lines and costameres. *J. Biol. Chem.* 276:32330–32337.

Bennett, V., and A.J. Baines. 2001. Spectrin and ankyrin-based pathways: metazoan invenctions for integrating cells into tissues. *Physiol. Rev.* 81:1353–1392.

Berridge, M.J., P. Lipp, and M.D. Bootman. 2000. The versatility and universality of calcium signalling. *Nat. Rev. Mol. Cell Biol.* 1:11–21.

Birkenmeier, C.S., J.J. Sharp, E.J. Gifford, S.A. Deveau, and J.E. Barker. 1998. An alternative first exon in the distal end of the erythroid ankyrin gene leads to production of a small isoform containing an NH_2-terminal membrane anchor. *Genomics.* 50:79–88.

Blake, D.J., A. Weir, S.E. Newey, and K.E. Davies. 2002. Function and genetics of dystrophin and dystrophin-related proteins in muscle. *Physiol. Rev.* 82:291–329.

Devarajan, P., P.R. Stabach, A.S. Mann, T. Ardito, M. Kashgarian, and J.S. Morrow. 1996. Identification of a small cytoplasmic ankyrin (Ank_{G119}) in the kidney and muscle that binds βIΣ spectrin and associates with the Golgi apparatus. *J. Cell Biol.* 133:819–830.

Epstein, H.F., and D.A. Fischman. 1991. Molecular analysis of assembly in muscle development. *Science.* 251:1039–1044.

Flucher, B.E., and C. Franzini-Armstrong. 1996. Formation of junctions involved in excitation-contraction coupling in skeletal and cardiac muscle. *Proc. Natl. Acad. Sci. USA.* 93:8101–8106.

Flucher, B.E., M.E. Morton, S.C. Froehner, and M.P. Daniels. 1990. Localization of the α_1 and α_2 subunits of the dihydropyridine receptor and ankyrin in skeletal muscle triads. *Neuron.* 5:339–351.

Flucher, B.E., H. Takekura, and C. Franzini-Armstrong. 1993. Development of the excitation-contraction coupling apparatus in skeletal muscle: association of sarcoplasmic reticulum and transverse tubules with myofibrils. *Dev. Biol.* 160:135–147.

Franzini-Armstrong, C. 1994. The sarcoplasmic reticulum and the transverse tubules. *In* Myology. A.E. Engel and C. Franzini-Armstrong, editors. McGraw-Hill Inc., New York. 176–199.

Furukawa, T., Y. Ono, H. Tsuchiya, Y. Katayama, M.L. Bang, D. Labeit, S. Labeit, N. Inagaki, and C.C. Gregorio. 2001. Specific interaction of the potassium channel β-subunit minK with the sarcomeric protein T-cap suggests a T-tubule-myofibril linking system. *J. Mol. Biol.* 313:775–784.

Gagelin, C., B. Constantin, C. Deprette, M.A. Ludosky, M. Recouvreur, J. Cartaud, C. Cognard, G. Raymond, and E. Kordeli. 2002. Identification of AnkG107, a muscle-specific ankyrinG isoform. *J. Biol. Chem.* 277:12978–12987.

Gallagher, P.G., and B.G. Forget. 1998. An alternate promoter directs expression of a truncated, muscle-specific isoform of the human ankyrin 1 gene. *J. Biol. Chem.* 273:1339–1348.

Giannini, G., A. Conti, S. Mammarella, M. Scrobogna, and V. Sorrentino. 1995. The ryanodine receptor/calcium channel genes are widely and differentially expressed in murine brain and peripheral tissues. *J. Cell Biol.* 128:893–904.

Golovina, V.A., and M.P. Blaustein. 1997. Spatially and functionally distinct Ca^{2+} stores in sarcoplasmic and endoplasmic reticulum. *Science.* 275:1643–1648.

Gregorio, C.C., and P.B. Antin. 2000. To the heart of myofibril assembly. *Trends Cell Biol.* 10:355–362.

Hoock, T.C., L.L. Peters, and S.E. Lux. 1997. Isoforms of Ankyrin-3 that lack the NH_2-terminal repeats associate with mouse macrophage lysosomes. *J. Cell Biol.* 136:1059–1070.

Ito, K., S. Komazaki, K. Sasamoto, M. Yoshida, M. Nishi, K. Kitamura, and H. Takeshima. 2001. Deficiency of triad junction and contraction in mutant skeletal muscle Junctophilin type 1. *J. Cell Biol.* 154:1059–1067.

Kordeli, E., M.A. Ludosky, C. Deprette, T. Frappier, and J. Cartaud. 1998. AnkyrinG is associated with the postsynaptic membrane and the sarcoplasmatic reticulum in the skeletal muscle fiber. *J. Cell Sci.* 111:2197–2207.

Meldolesi, J., and T. Pozzan. 1998. The heterogeneity of ER Ca^{2+} stores has a key role in nonmuscle cell signaling and function. *J. Cell Biol.* 142:1395–1398.

Mohler, P.J., A.O. Gramolini, and V. Bennett. 2002. The ankyrin-B C-terminal domain determines activity of ankyrin-B/G chimeras in rescue of abnormal inositol 1,4,5-trisphosphate and ryanodine receptor distribution in ankyrin-B (−/−) neonatal cardiomyocytes. *J. Biol. Chem.* 277:10599–10607.

Nishi, M., S. Komazaki, N. Kurebayashi, Y. Ogawa, T. Noda, M. Iino, and H. Takeshima. 1999. Abnormal features in skeletal muscle from mice lacking Mitsugumin29. *J. Cell Biol.* 147:1473–1480.

Pan, Z., D. Yang, R.Y. Nagaraj, T.A. Nosek, M. Nishi, H. Takeshima, H. Cheng, and J. Ma. 2002. Dysfunction of store-operated calcium channel in muscle cells lacking mg29. *Nat. Cell Biol.* 4:379–383.

Pardo, J.V., J.D. Siliciano, and S.W. Craig. 1983. A vinculin-containing cortical lattice in skeletal muscle: transverse lattice elements ("costamers") mark sites of attachment between myofibrils and sarcolemma. *Proc. Natl. Acad. Sci. USA.* 80:1008–1012.

Peters, L.L., K.M. John, F.M. Lu, E.M. Eicher, A. Higgins, M. Yialamas, L.C. Turtzo, A.J. Otsuka, and S.E. Lux. 1995. *Ank3* (epithelial ankyrin), a widely distributed new member of the ankyrin gene family and the major ankyrin in kidney, is expressed in alternatively spliced forms, including forms that lack the repeat domain. *J. Cell Biol.* 130:313–330.

Petersen, O.P., A. Tepikin, and M.K. Park. 2001. The endoplasmic reticulum: one continuous or several separate Ca^{2+} stores? *Trends Neurosci.* 24:271–276.

Russell, M.W., M.O. Raeker, K.A. Korytkowski, and K.J. Sonneman. 2002. Identification, tissue expression and chromosomal localization of human Obscurin-MLCK, a member of the titin and Dbl families of myosin light chain kinases. *Gene.* 282:237–246.

Saito, A., S. Seiler, A. Chu, and S. Fleischer. 1984. Preparation and morphology of sarcoplasmic reticulum terminal cisternae from rabbit skeletal muscle. *J. Cell Biol.* 99:875–885.

Salanova, M., G. Priori, V. Barone, E. Intravaia, B. Flucher, F. Ciruela, R.A.J. McIlhinney, J.B. Parys, K. Mikoshiba, and V. Sorrentino. 2002. Homer proteins and $InsP_3$ receptors co-localize in the longitudinal sarcoplasmic reticulum of skeletal muscle fibers. *Cell Calcium.* 32:193–200.

Stromer, M.H. 1998. The cytoskeleton in skeletal, cardiac and smooth muscle cells. *Histol. Histopathol.* 13:283–291.

Takeshima, H., S. Komazaki, M. Nishi, M. Iino, and K. Kangawa. 2000. Junctophilins: A novel family of junctional membrane complex proteins. *Mol. Cell.* 6:11–22.

Tuvia, S., M. Buhusi, L. Davis, M. Reedy, and V. Bennett. 1999. Ankyrin-B is required for intracellular sorting of structurally diverse Ca^{2+} homeostasis proteins. *J. Cell Biol.* 147:995–1007.

Young, P., E. Ehler, and M. Gautel. 2001. Obscurin, a giant sarcomeric Rho guanine nucleotide exchange factor protein involved in sarcomere assembly. *J. Cell Biol.* 154:123–136.

Zhou, D., C.S. Birkenmeier, M.W. Williams, J.J. Sharp, J.E. Barker, and R.J. Bloch. 1997. Small, membrane-bound, alternatively spliced forms of ankyrin 1 associated with the sarcoplasmatic reticulum of mammalian skeletal muscle. *J. Cell Biol.* 136:621–631.