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 InsP3 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 Ca2+ (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 Ca2+ release and uptake (i.e., terminal cisternae and longitudinal tubules, respectively) are distributed with a regular spacing in correspondence to specific regions of the
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 in 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, ankysins 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 NH2-terminal region (Zhou et al., 1997; Birkenmeier et al., 1998; Gallagher and Forget, 1998). Recent studies with ank2-deficient mice have indicated that ank2 may be important for the localization of proteins involved in Ca2+ homeostasis, such as ryanodine receptors and InsP3 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 ank1.6 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 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 ank2 is necessary for the localization of ryanodine receptors and InsP3 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, InsP3 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 × 105 colonies 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.
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. 1B). 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. 1C). 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. 1D). 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 pGBK7 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. 2A). 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. 2A, 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. 2B). Recent work in mice deleted of the ank2 gene revealed that these
experiments revealed that the COOH-terminal region of ank2 (AnkB220) and ank3 (AnkG190) (Mohler et al., 2002). These experiments were performed using chimeric 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 the GST–obscurin fusion protein. Minimal binding was observed in proteins in which only one of the mutants was present (T99P, K101E or R104G, K105E), strongly reduced binding of in vivo–translated obscurin subclone A7 to the GST–obscurin 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. The production of a GST–obscurin fusion protein led to the formation of a complex that contained both fusion proteins and ank1.5. 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. As shown in Fig. 2 E, both full-length ank1.5 and ank1.7 were fused to GST and the resulting GST–ank1.5 and GST–ank1.7 fusion proteins were used in pull-down experiments with GST–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 any of these aa residues (T99P, K101E, and R104G) completely abolished the residual binding observed in proteins containing only one mutated aa (Fig. 2 D).

*Abbreviations used in this paper: HEK, human embryonic kidney; MBP, maltose binding 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 GFP–ObsA7 fusion protein.

(Saito et al., 1984).
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 (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.

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 muscle cells, 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 ank1 isoforms, namely long ank1 isoform ank1.3, ank2.2, and the ank3 isoform AnkG107. A role for ank2 in mediating the assembly of ryanodine and InsP3 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 ank1y 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 Ca2+ release channels (i.e., ryanodine and InsP3 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'-AATGTCAGCTTTGCTACCCAG-CGTTTGGT-3' and 5'-CTGTTTCCCATTCAGGCTGCGCCCGT-3' (ank1.5 and ank1.6) or 5'-GGGTTGGTTGCTGAGTCTGATCTCTTCTT-3' (ank1.5 and ank1.6). 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'-ACTAT-CAATCTTTTAAATTGCAGAC-3' and 5'-CTCATCTTTGCTCTTGCTGCGTC-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 GAL4-2BD 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 × 106 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 5S i-methionine (Amersham Biosciences). The GST fusion proteins were obtained by cloning the cDNA of ank1.5 (aa 22–155), ank1.6 (aa 22–113), 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.). Micromeres 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 agrose 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 micromeres was used in pull-down experiments. The interactions with the fusion proteins (GST for transfected HEK293 and MBP for mouse skeletal muscle micromeres) were performed in PBS, 0.2 mM PMFS, 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 in a humidified incubator at 37°C in 5% CO2. Cultures of skeletal muscle cells were prepared from hind leg muscles of newborn rats. In brief, the muscles were minced and subjected to 4 × 10 min digestion with collagenase, followed by 4 × 10 min trypsin treatment 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 appropriate 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 Na2HPO4, 1.4 mM KH2PO4) 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 Trition buffer (20 mM Hepes, pH 7.4, 300 mM sucrose, 50 mM NaCl, 3 mM MgCl2, 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. Cy3-conjugated anti-mouse secondary antibodies were from Jackson ImmunoResearch Laboratories and were used according to the manufacturer’s instructions. Antibodies against obscurin were from Jackson ImmunoResearch Laboratories and were used according to the manufacturer’s instructions.

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This work was supported by grants from Telethon (GP0168R02), Ministero dell’Istruzione dell’Università e della Ricerca, Agenzia Spaziale Ita-
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