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Rapsyn May Function as a Link between the Acetylcholine Receptor and the Agrin-Binding Dystrophin-Associated Glycoprotein Complex

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Summary

The 43 kDa AChR-associated protein rapsyn is required for the clustering of nicotinic acetylcholine receptors (AChRs) at the developing neuromuscular junction, but the functions of other postsynaptic proteins colocalized with the AChR are less clear. Here we use a fibroblast expression system to investigate the role of the dystrophin-glycoprotein complex (DGC) in AChR clustering. The agrin-binding component of the DGC, dystroglycan, is found evenly distributed across the cell surface when expressed in fibroblasts. However, dystroglycan colocalizes with AChR-rapsyn clusters when these proteins are coexpressed. Furthermore, dystroglycan colocalizes with rapsyn clusters even in the absence of AChR, indicating that rapsyn can cluster dystroglycan and AChR independently. Immunofluorescence staining using a polyclonal antibody to utrophin reveals a lack of staining of clusters, suggesting that the immunoreactive species, like the AChR, does not mediate the observed rapsyn-dystroglycan interaction. Rapsyn may therefore be a molecular link connecting the AChR to the DGC. At the neuromuscular synapse, rapsyn-mediated linkage of the AChR to the cytoskeleton-anchored DGC may underlie AChR cluster stabilization.

Introduction

Efficient signal transmission between neurons and their targets depends on the formation of highly specialized structures both pre- and postsynaptically. At the neuromuscular junction, a particularly striking example of molecular specialization is the clustered distribution of nicotinic acetylcholine receptors (AChRs). Prior to innervation, AChRs are evenly distributed along the muscle membrane. Following contact with the motor nerve ending, the concentration of AChRs increases dramatically and eventually reaches 10,000 molecules/μm^2 in the postsynaptic membrane, while only a few micrometers away the density of AChRs is 1000-fold lower (Fertuck and Salpeter, 1974; Bevan and Steinbach, 1977). Agrin, a nerve-derived extracellular matrix protein, provides at least part of the signal that induces the process of AChR clustering (reviewed in McMahan et al., 1992; Nastuk and Fallon, 1993).

A number of proteins associated with the postsynaptic membrane at the neuromuscular junction have been implicated in the molecular mechanisms underlying formation and maintenance of AChR clusters (reviewed in Apel and Merlie, 1995). Experiments employing extraction of extrinsic membrane proteins from AChR-rich membranes demonstrated that removal of 43, 58, and 87 kDa molecular weight species correlated with increases in the mobility of the receptor (Barrantes et al., 1980; Lo et al., 1980; Cartaud et al., 1981; Rousselet et al., 1982) without affecting its ligand binding or gating properties (Neubig et al., 1979; Elliot et al., 1980). The 43 kDa AChR-associated protein, rapsyn (Frail et al., 1988), is precisely colocalized with AChR (Froehner et al., 1981; Sealock et al., 1984), and accumulating evidence indicates a direct role for rapsyn in AChR clustering (reviewed in Phillips and Merlie, 1992). Rapsyn is identical to the AChR-associated protein referred to as the 43 kDa protein, but we encourage the use of the name rapsyn to distinguish this protein from other 43 kDa components of the postsynaptic membrane. The 58 kDa (recently renamed syntrophin; Adams et al., 1993) and 87 kDa proteins also colocalize with AChR at the neuromuscular junction, but are present extrasynaptically as well (Froehner et al., 1987; Carr et al., 1989). The precise roles of syntrophin and the 87 kDa protein in AChR clustering remain unclear, but biochemical evidence indicates that both proteins associate in a complex with the cytoskeletal protein dystrophin extrajunctionally, and with the dystrophin-related protein utrophin at the synapse (Butler et al., 1992; Matsumura et al., 1992; Wagner et al., 1993; Kramarcy et al., 1994; Suzuki et al., 1994; Ahn and Kunkel, 1995; Suzuki et al., 1995; Yang et al., 1995).

Independent lines of research investigating the role of dystrophin and utrophin in muscle membrane integrity have identified several additional protein components in a complex containing syntrophin and the 87 kDa protein (see Figure 6). This dystrophin–glycoprotein complex (DGC) was first isolated based on its tight association with dystrophin (Ervasti et al., 1990; Yoshida and Ozawa, 1990), but utrophin has subsequently been shown to also associate with the DGC (Matsumura et al., 1992). The DGC is composed of transmembrane proteins of 25, 35, 43 (β-dystroglycan and yet another distinct 43 kDa protein that is less well characterized), and 50 kDa (adhalin); a heavily glycosylated extracellular protein (α-dystroglycan); and syntrophin, an intracellular 59 kDa protein triplet previously characterized as an AChR-associated protein (Ervasti et al., 1990; Yoshida and Ozawa, 1990; Ervasti and Campbell, 1991; Yoshida et al., 1994). An 87 kDa species frequently found in DGC preparations is likely to be the homolog of the Torpedo 87 kDa AChR-associated protein (Ervasti et al., 1990; Yoshida and Ozawa, 1990). Like syntrophin and the 87 kDa protein, other components of the DGC, namely dystroglycan and adhalin, are found colocalized in AChR clusters in cultured muscle cells (Campanelli et al., 1994; Gee et al., 1994). Utrophin is also

closely colocalized with clustered AChRs at the adult and developing neuromuscular junction (Khurana et al., 1991; Ohlendieck et al., 1991a; Bewick et al., 1992; Phillips et al., 1993), and in C2 myotubes utrophin is concentrated mainly at large AChR clusters rather than small ones, implying that utrophin may be involved in the enlargement of AChR clusters rather than in the initial stages of their formation (Phillips et al., 1993). Many other observations have likewise suggested that AChR clusters are stabilized and maintained by interacting with the underlying cytoskeleton (Pumplin and Bloch, 1993). Together, these data indicate an important role for the DGC in AChR clustering. The recent identification of α-dystroglycan as an agrin-binding protein has further focused attention on the role of the DGC in AChR clustering (Bowe et al., 1994; Campanelli et al., 1994; Gee et al., 1994; Sugiyama et al., 1994). Strong biochemical evidence demonstrates that agrin binds to α-dystroglycan, but because experiments to address the functional significance of this binding have produced conflicting results, it remains to be conclusively determined whether binding of agrin to dystroglycan is a necessary requirement for AChR clustering. Regardless, the DGC may play a structural role in AChR clustering, perhaps by providing the cytoskeletal anchor necessary for cluster stabilization and maintenance. Efforts to understand the molecular organization of the DGC, AChR, and associated proteins can therefore advance our understanding of the mechanisms underlying AChR clustering, even if dystroglycan is not the receptor that initiates agrin signal transduction.

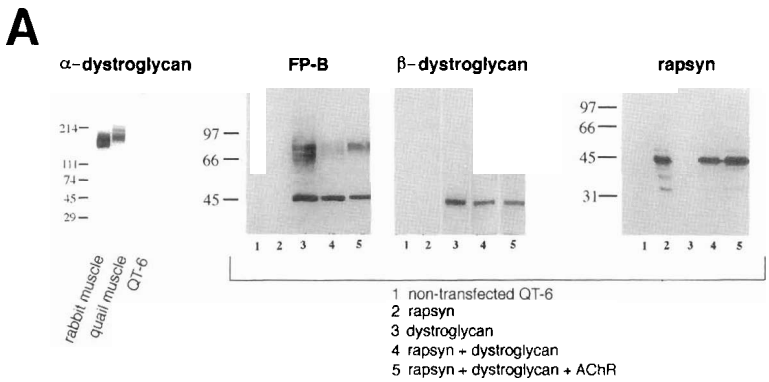
In this paper, we explore the role of the DGC in AChR clustering by examining the molecular interactions between components of the DGC and AChR complex. While research has led to a model for the molecular organization of the DGC and its associated proteins, it remains completely unclear how this network of proteins is linked to the AChR. Here we have expressed recombinant proteins in a fibroblast cell line to investigate the molecular nature of the link between the AChR and the DGC complex. This fibroblast system allows us to reconstitute systematically interactions that normally occur at the neuromuscular synapse in order to study the molecular organization of the postsynaptic apparatus (Phillips et al., 1991a, 1991b; Maimone and Merlie, 1993). Previous studies demonstrated that AChRs expressed in these cells, which do not contain endogenous rapsyn or AChR, were distributed evenly on the cell surface. However, when coexpressed with rapsyn, AChRs became redistributed into large cell surface clusters that colocalized with rapsyn (Phillips et al., 1991a). In this study, we examine the localization of dystroglycan when expressed in fibroblasts by itself and in combination with rapsyn and AChR. Our results suggest that rapsyn can cluster dystroglycan and that the AChR is not needed for the interaction between rapsyn and dystroglycan. Furthermore, a protein recognized by a polyclonal antibody to utrophin does not appear to mediate the rapsyn–dystroglycan interaction. Rapsyn may therefore be the molecular link connecting the AChR to the DGC.

Results

Expression of Dystroglycan in QT-6 cells

Before investigating the role of recombinant dystroglycan in our fibroblast expression system, we wanted to determine whether the quail fibroblast cells (QT-6) contain endogenous dystroglycan. Dystroglycan is expressed in a variety of tissues (Ibraghimov et al., 1992), although other members of the DGC complex appear to be largely muscle specific (Hoffman et al., 1987; Lev et al., 1987; Roberds et al., 1993; Wagner et al., 1993; Yamamoto et al., 1994). We used antibodies directed against the heavily glycosylated 156 kDa extracellular agrin-binding component, α-dystroglycan, and the 43 kDa transmembrane protein, β-dystroglycan. A polyclonal antibody designated FP-B, which recognizes both α- and β-dystroglycan in rabbit skeletal muscle, cross-reacts with the quail muscle β-dystroglycan but not quail α-dystroglycan (data not shown). Western blot analysis of extracts from nontransfected QT-6 cells using FP-B revealed that QT-6 cells contain no endogenous β-dystroglycan (Figure 1A). An anti-α-dystroglycan antibody that recognizes a 156 kDa band in rabbit and quail skeletal muscle likewise failed to detect an immunoreactive species in QT-6 cells (Figure 1A). As this antibody appears to require carbohydrate modifications for recognition of α-dystroglycan (Ervasti and Campbell, 1993), it is possible that an incompletely glycosylated form of α-dystroglycan is found endogenously in QT-6 cells. This possibility is extremely unlikely, however, since α- and β-dystroglycan are derived from a single polypeptide precursor and QT-6 cells contain no endogenous β-dystroglycan.

The absence of immunoreactive dystroglycan in QT-6 cells made it possible to follow the expression of recombinant rabbit dystroglycan introduced by transfection. Western blot analysis revealed robust expression of dystroglycan in cells transfected with a dystroglycan expression construct (Figure 1A). Two bands were detected by the FP-B antibody, which recognizes both α- and β-dystroglycan. The 43 kDa band corresponds to the molecular weight of β-dystroglycan. This 43 kDa band recognized by the dystroglycan antibody is not rapsyn, which also migrates as a 43 kDa protein, since the FP-B antibody showed no cross-reactivity when rapsyn was expressed alone in QT-6 cells (Figure 1A). A β-dystroglycan-specific antibody recognized the 43 kDa band only, and not the higher molecular weight band. This confirmed the identity of the 43 kDa band as β-dystroglycan and further indicated that the higher molecular weight band is not the uncleaved dystroglycan precursor, which would contain the β-dystroglycan epitope, but rather represents α-dystroglycan (Figure 1A). The diffuse α-dystroglycan band around 80 kDa is smaller than native rabbit muscle α-dystroglycan, which migrates at approximately 156 kDa, as shown in Figure 1A. The difference between the QT-6-expressed recombinant form and the muscle form is most likely due to incomplete glycosylation in QT-6 cells of the 57 kDa α-dystroglycan protein core (Ibraghimov et al., 1992; Ervasti and Campbell, 1993).



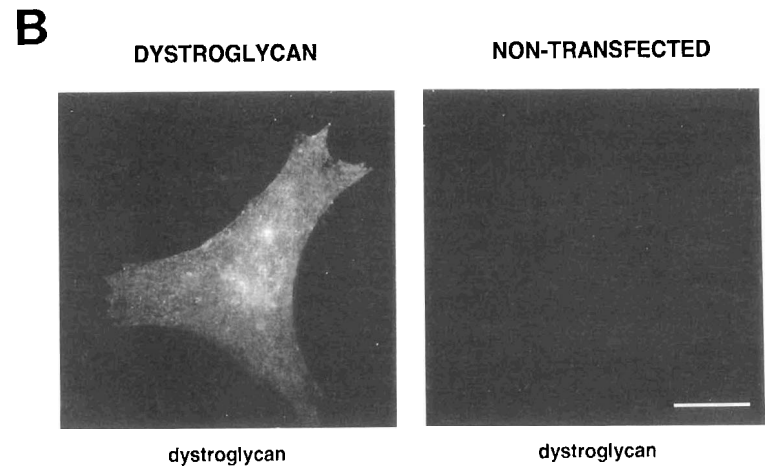


Figure 1. Expression of Dystroglycan in QT-6 Cells

(A) Western blot analysis of rabbit mucle, quail muscle, and quail QT-6 cells (transfected and nontransfected) reveals the absence of endogenous α- and β-dystroglycan in QT-6 fibroblasts and confirms the expression of recombinant α- and β-dystroglycan in QT-6 cells transfected with a dystroglycan expression construct. For anti-α-dystroglycan staining, 100 μg of rabbit muscle crude surface membranes, 250 μg of quail muscle crude surface membranes, or 500 μg of SDS-solubilized whole-cell pellets of QT-6 cell cultures was loaded per lane. For the rest of the blots, different volumes of samples of SDS-solubilized whole-cell pellets from transfected QT-6 cells were loaded to correct for variable transfection efficiency; generally 1 μg of total protein from transfected and nontransfected cells was loaded per lane. Nitrocellulose blots were stained with the antibody indicated above each blot: an antibody to α-dystroglycan, a polyclonal antibody (FP-B) that recognizes both α- and β-dystroglycan, an antibody to β-dystroglycan, and an anti-rapsyn antibody. Molecular weight markers (in kilodaltons) are indicated at left.

(B) Recombinant rabbit dystroglycan expressed in QT-6 fibroblasts is evenly distributed across the cell surface. QT-6 cells were transfected with an expression construct for dystroglycan (left) or mock-transfected with an equal amount of pSK DNA (right). Permeabilized cells were stained with a polyclonal antibody (FP-B) that recognizes rabbit α- and β-dystroglycan. Nontransfected cells stained for dystroglycan exhibited no staining above background, confirming the specificity of dystroglycan staining and the lack of an endogenous immunoreactive dystroglycan. Cells that were not permeabilized before fixation also showed a diffuse pattern of staining (data not shown), confirming that the recombinant dystroglycan is correctly processed in QT-6 cells to yield cell surface α-dystroglycan accessible to extracellular staining. Bar, 10 μm.

Indeed, α-dystroglycan-specific antibodies that have carbohydrate moieties as part of their epitope did not recognize the 80 kDa band (data not shown).

Recombinant dystroglycan expressed in QT-6 cells was found evenly distributed across the cell as assessed by immunofluorescence microscopy (Figure 1B). Both permeabilized and nonpermeabilized (data not shown) transfected cells showed specific staining with an antibody that recognizes both α- and β-dystroglycan. The dystroglycan expressed must therefore be exposed on the cell surface, available for staining. Thus, our results indicate that recombinant dystroglycan expressed in QT-6 cells is correctly processed to yield extracellular α-dystroglycan and membrane-spanning β-dystroglycan. The even distribution of dystroglycan across the cell surface demonstrates that, unlike rapsyn, dystroglycan expressed alone in QT-6 cells does not form clusters.

Dystroglycan Colocalizes with AChR-Rapsyn Clusters

Dystroglycan is concentrated at the sites of agrin-induced AChR clusters in C2 cells and is found both at the neuromuscular junction and throughout the sarcolemmal membrane in skeletal muscle (Ervasti and Campbell, 1991; Campanelli et al., 1994; Gee et al., 1994). To determine whether dystroglycan associates with clusters formed in QT-6 fibroblasts, which should not express many muscle-specific proteins, we coexpressed rapsyn, AChR, and dystroglycan in QT-6 cells and determined the distribution of each protein using immunofluorescence microscopy. Dystroglycan was found colocalized with AChR and rapsyn in AChR–rapsyn clusters (Figure 2A). The codistribution was remarkably precise; dystroglycan staining exactly duplicated the rapsyn or AChR staining of clusters. The size and shape of clusters varied considerably, yet all clusters

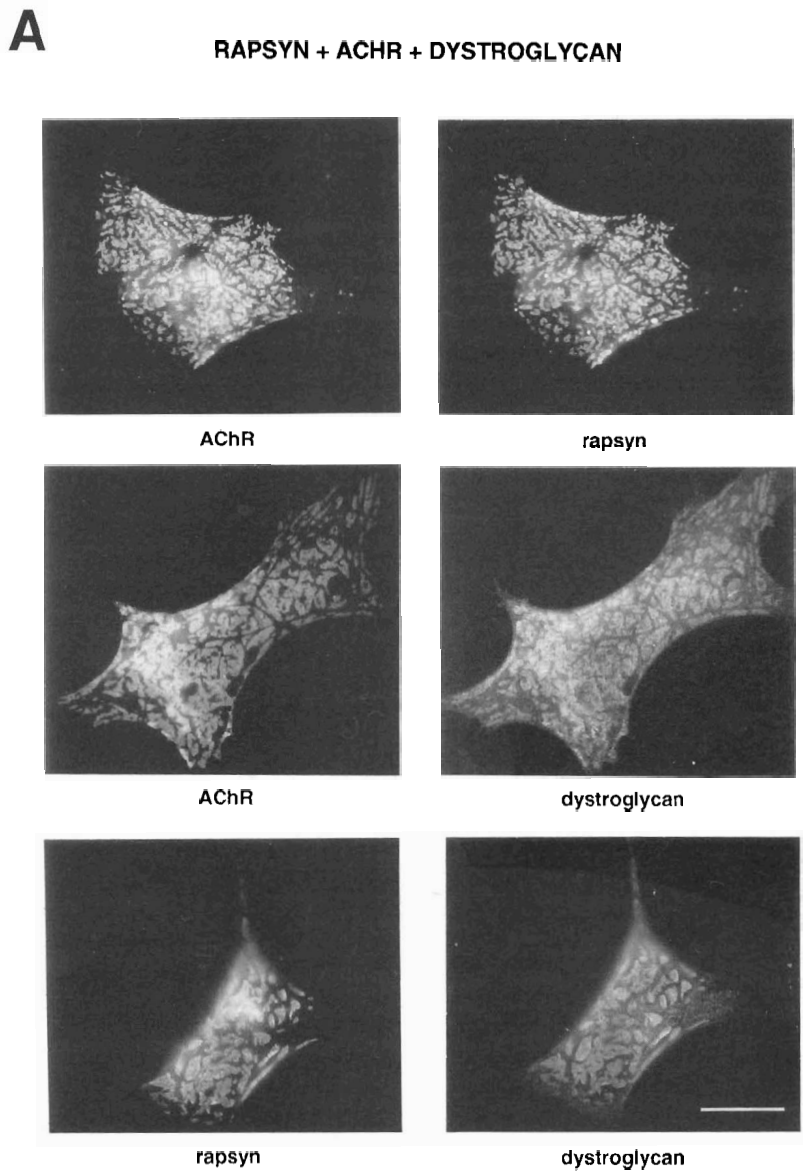


Figure 2. Dystroglycan Colocalizes with AChR/43K Clusters in Transfected QT-6 Cells

(A) QT-6 cells transfected with rapsyn, AChR, and dystroglycan expression constructs were permeabilized and double stained pairwise with anti-rapsyn, anti-AChR, and anti-dystroglycan antibodies as indicated beneath each panel, followed by appropriate affinity-purified, species-specific secondary antibodies. Dystroglycan staining was found precisely colocalized with AChR and rapsyn staining of AChR–rapsyn clusters. Appropriate controls for fluorescent cross-bleed and antibody specificity were carried out in these and all subsequent immunofluorescence experiments (see Experimental Procedures). Bar, 10 μm.

(B) QT-6 cells transfected with AChR and dystroglycan were stained as in (A) to visualize AChR and dystroglycan staining. In the absence of rapsyn, both AChR and dystroglycan staining was evenly distributed across the cell. Bar, 10 μm.

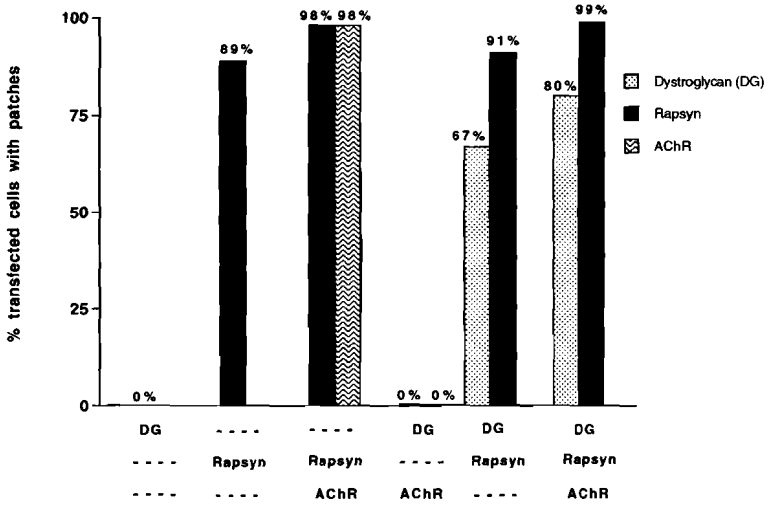


Figure 3. Frequency of Staining Patterns

QT-6 cells were transfected with expression constructs for rapsyn, dystroglycan, and AChR, in various combinations or each alone, as indicated on the X axis. Stained cells were viewed by immunofluorescence microscopy. Coverslips were scanned systematically, and successfully transfected cells, as judged by staining above background for each introduced protein, were examined for the presence of clusters. Bars represent the percentage of transfected cells exhibiting clusters containing the particular protein being tested. Dystroglycan colocalization with AChR–rapsyn and rapsyn clusters is a very frequent phenotype. Some cells with AChR–rapsyn or rapsyn clusters did not show corresponding dystroglycan staining of clusters, but all dystroglycan-stained clusters also stained with anti-rapsyn or anti-AChR antibodies. Representative data are presented from one of two independent transfection experiments for each experimental condition. At least 200 transfection-positive cells were scored for each condition per experiment.

had sharply defined boundaries and were usually present on all surfaces of the cell. Dystroglycan-containing clusters were indistinguishable from clusters formed with rapsyn and AChR (Maimone and Merlie, 1993).

AChR–rapsyn clusters form in QT-6 cells without the addition of agrin (data not shown) (Phillips et al., 1991a). Surprisingly, dystroglycan association with AChR–rapsyn clusters in QT-6 cells also occurred in the absence of agrin. Furthermore, the addition of agrin did not cause any detectable changes in dystroglycan-containing cluster frequency or morphology. We were unable to determine whether the recombinant dystroglycan expressed in QT-6 cells successfully bound agrin in these experiments. Nonetheless, the absence of an agrin requirement for cluster formation in QT-6 cells suggests that at least some of the molecular events leading to cluster formation occur independently of agrin stimulation, or that pathways regulating clustering may be constitutively activated in QT-6 cells.

Dystroglycan colocalization with AChR–rapsyn clusters was a very frequent phenotype; 81% of the cells with AChR–rapsyn clusters also exhibited dystroglycan staining at clusters (Figure 3). The remaining 19% of the cells, which exhibited no detectable dystroglycan staining at AChR–rapsyn clusters, generally had only very weakly fluorescent clusters when viewed for AChR staining; it is likely that the dystroglycan staining of these clusters was merely beneath the limits of detection. When dystroglycan and AChR were expressed in the absence of rapsyn, both proteins were found to be evenly distributed, suggesting that rapsyn is clearly necessary for the formation of clusters containing AChR and dystroglycan (see Figure 2B).

Dystroglycan Interacts with Rapsyn Clusters Independently of AChR and Utrophin or Dystrophin

We next wanted to determine whether AChR is necessary for dystroglycan association with AChR–rapsyn clusters. As demonstrated previously, rapsyn was organized into clusters in cells transfected with rapsyn alone, suggesting that rapsyn aggregation may be the primary event in AChR cluster formation (Figure 4A). When cells were transfected with rapsyn and dystroglycan without AChR, dystroglycan was found colocalized with rapsyn clusters (Figure 4B). This colocalization was not due to cross-reactivity of the dystroglycan primary or secondary antibody, as indicated by the lack of specific staining for dystroglycan in cells transfected with rapsyn only (Figure 4A). Numerous other membrane proteins, including N-cadherin, concanavilin-A- and wheat germ agglutinin–binding glycoproteins, glucose transporters, CD8, and a potassium channel, have previously been shown not to be clustered by rapsyn, indicating the specificity of AChR and dystroglycan clustering by rapsyn (Froehner et al., 1990; Maimone and Merlie, 1993; Yu and Hall, 1994; M. M. Maimone, W. D. Phillips, and J. P. M., unpublished data). Furthermore, the colocalization of dystroglycan with rapsyn clusters was a frequently observed phenomenon, as 74% of the cells with rapsyn clusters also showed positive staining for dystroglycan in clusters (see Figure 3). These results indicate that dystroglycan can associate with rapsyn clusters in the absence of AChR and further support a central role for rapsyn in assembly of the postsynaptic apparatus.

The observed association of rapsyn with dystroglycan could be a direct interaction between the two proteins, or the interaction could be mediated by one or more additional proteins. Our results show that AChR is not necessary for the rapsyn–dystroglycan association, but one or more endogenous proteins in the QT-6 fibroblasts could play a role in the interaction. For example, QT-6 cells may express AChR-associated or DGC proteins that could potentially mediate rapsyn binding to dystroglycan. We were unable to determine whether most of these putative mediators are expressed in QT-6 fibroblasts owing to the lack

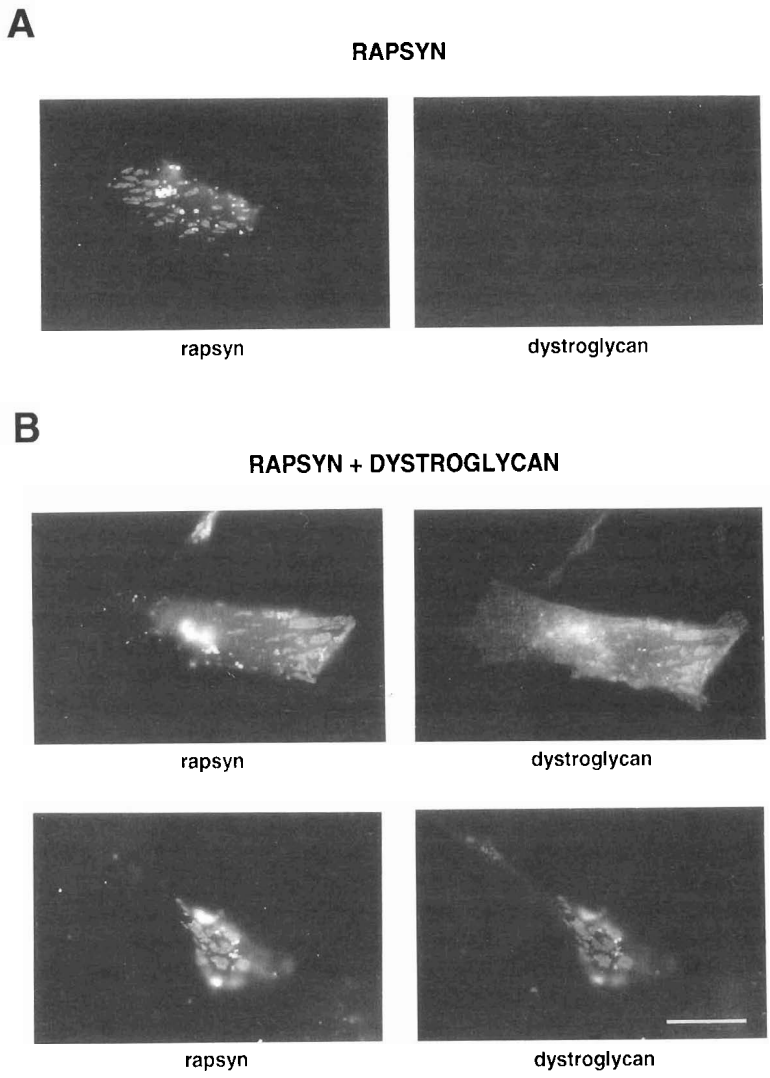


Figure 4. Dystroglycan Associates with Rapsyn Clusters in the Absence of AChR

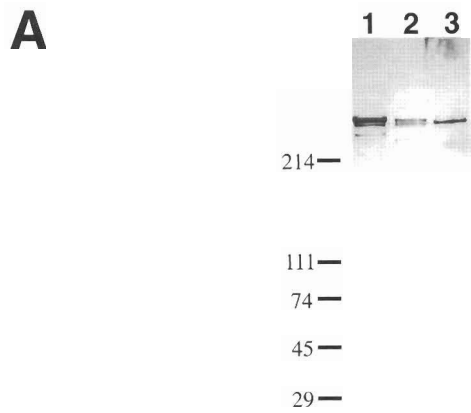
(A) QT-6 cells transfected with rapsyn only were permeabilized and double labeled with anti-rapsyn and anti-dystroglycan antibodies as indicated beneath each panel. Rapsyn staining demonstrated that rapsyn was organized into clusters when expressed alone in QT-6 cells. The absence of staining for dystroglycan confirms the lack of fluorescent cross-bleed between the two fluorescence channels, and the absence of cross-reactivity of the anti-dystroglycan primary or secondary antibody with rapsyn or the anti-rapsyn primary antibody, respectively.

(B) Permeabilized cells transfected with rapsyn and dystroglycan were stained as in (A). Dystroglycan staining was found colocalized with rapsyn clusters, indicating that dystroglycan can associate with rapsyn clusters in the absence of AChR.

Bar, 10 μm.

of species cross-reactivity of available antibodies with the quail proteins (data not shown). Using Western blot analysis, we tested available antibodies for their ability to recognize immunoreactive species in rabbit muscle, quail muscle, and QT-6 fibroblasts. While antibodies to the 87 kDa protein, the syntrophin triplet, the 35 kDa DGC component, and dystrophin positively identified bands of the correct molecular weight in rabbit muscle extracts, these antibodies failed to cross-react with any species in quail muscle or QT-6 cells. We cannot, therefore, definitively rule out the participation of endogenous forms of these molecules in rapsyn-mediated clustering of dystroglycan. However, antibodies against adhalin recognized a 50 kDa species in quail muscle but not in QT-6 cells (data not shown), making it possible to rule out this DGC protein as a mediator of the rapsyn–dystroglycan interaction. In addition, an antibody designated BH11, raised against a C-terminal fragment of utrophin (Khurana et al., 1991), recognized a single species in QT-6 cells (Figure 5A). Western blot analysis further revealed that this antibody recognized two bands in rabbit and quail muscle. Owing to the high degree of homology between dystrophin and utrophin, it is possible that BH11 cross-reacts with both proteins. At present, it is difficult to determine which of the discrete bands in quail muscle corresponds to utrophin or dystrophin; it is therefore unclear which of the two proteins represents the immunoreactive species in QT-6 cells. In the remainder of the paper, we will refer to the protein recognized by BH11 as utrophin/dystrophin. However, as full-length dystrophin is expressed primarily in muscle, with lower levels in brain, it is likely that the immunoreactive species in QT-6 fibroblasts represents utrophin (Hoffman et al., 1987; Lev et al., 1987; Chamberlain et al., 1988; Nudel et al., 1988).

To determine whether the endogenous utrophin/dystrophin in QT-6 cells mediates the rapsyn–dystroglycan interaction, transfected cells were stained with the BH11 antibody. This antibody stains the neuromuscular junction in mouse and quail muscle sections (Khurana et al., 1991; P. G. Noakes, W.-X. A. Guo, and J. P. M., unpublished data) and would therefore be expected to recognize endogenous utrophin/dystrophin during immunostaining of the quail fibroblasts. Immunofluorescence staining pat-



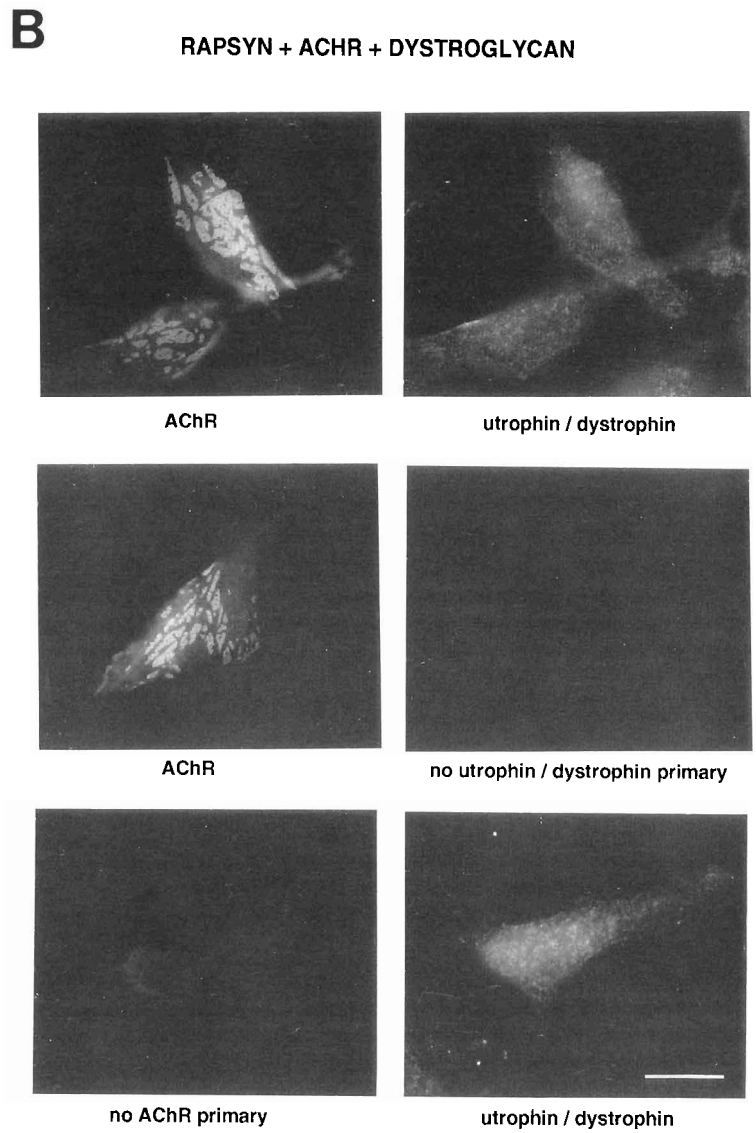


Figure 5. Utrophin/Dystrophin Does Not Mediate the Rapsyn–Dystroglycan Interaction

(A) A total of 100 μg of rabbit muscle crude surface membranes (lane 1), 250 μg of quail muscle crude surface membranes (lane 2), or 500 μg of SDS-solubilized whole-cell pellets of QT-6 cell cultures (lane 3) was loaded per lane on a 3%–12% gradient gel, and blots were stained with a rabbit polyclonal antibody (BH11) raised against a C-terminal fragment of utrophin. Molecular weight markers (in kilodaltons) are indicated at left.

(B) Permeabilized QT-6 cells transfected with rapsyn, AChR, and dystroglycan were double labeled with an anti-AChR antibody and a polyclonal antibody raised against the C-terminal fragment of utrophin, as indicated beneath each panel. As depicted in the top two panels, utrophin/dystrophin staining was not detected at AChR–rapsyn clusters. To ensure that the observed diffuse staining pattern represented specific staining above background, parallel coverslips were stained in the absence of either the anti-AChR or BH11 primary antibody as indicated beneath the corresponding panel. Such cells showed AChR staining of patches or diffuse utrophin/dystrophin staining similar to that shown in the top two panels, but no flurorescent signal on the second channel. Bar, 10 μm.

terns in QT-6 cells indicated an even distribution of utrophin/dystrophin throughout the cell, with no observed concentration of the immunoreactive species at dystroglycan-containing clusters (Figure 5B). Utrophin/dystrophin does not therefore appear to mediate the rapsyn–dystroglycan interaction. Together, these data indicate that rapsyn interacts independently with dystroglycan and the AChR, and therefore could serve as a molecular link be-

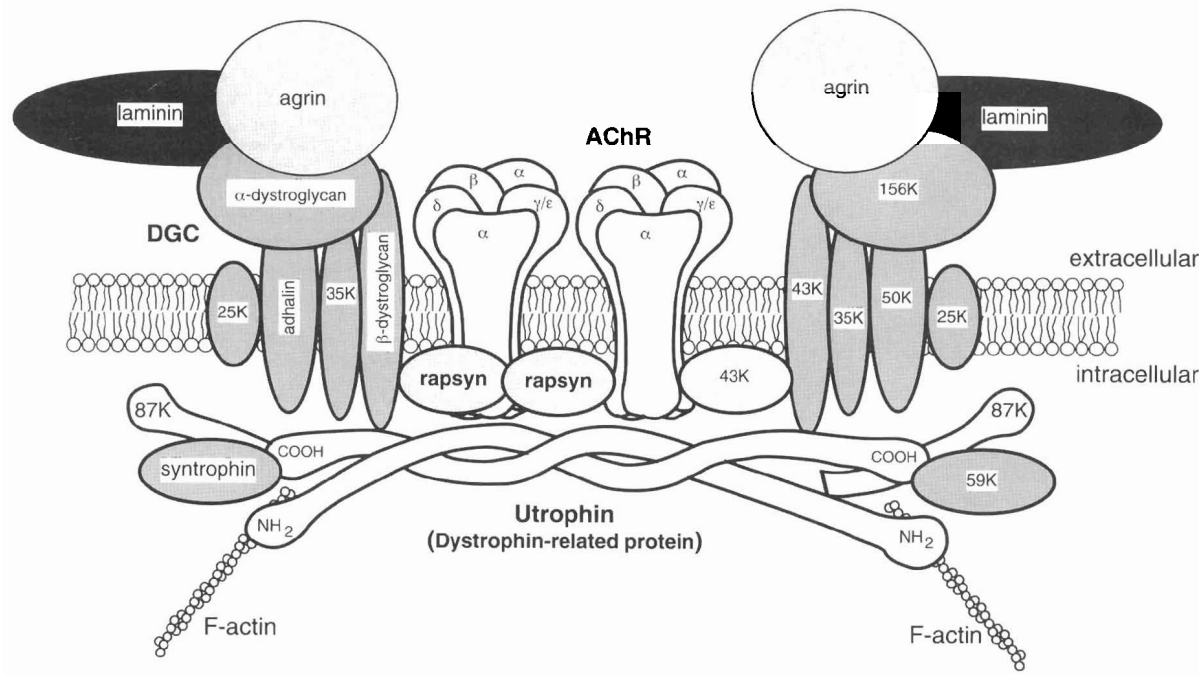


Figure 6. Schematic Model of the Hypothetical Molecular Organization of the Postsynaptic Apparatus at the Neuromuscular Junction

Shown are the molecules proposed to play a role in AChR clustering at the neuromuscular junction, arranged with predicted interactions depicted. Apparent molecular weights that originally defined each depicted protein are indicated at the right; on the left, alternative names are shown. We encourage the use of the names rapsyn and β-dystroglycan to differentiate these 43 kDa proteins. This is especially important in light of the recent distinction of yet another 43 kDa DGC component as a unique species. We propose that rapsyn links the AChR to the DGC, thereby facilitating the association of AChR clusters with the cytoskeleton. Biochemical analysis of Torpedo electric organ postsynaptic membranes suggests a 1:1 stoichiometry of AChR and rapsyn (LaRochelle and Froehner, 1986), but other components appear to be less abundant, consistent with our proposal that the formation of mature clusters involves the linkage of many AChR–rapsyn small clusters to each dystroglycan molecule (see text for further explanation).

tween the AChR and the DGC (Figure 6). Furthermore, utrophin/dystrophin does not appear to play a necessary role in this linkage.

Discussion

We have investigated the interaction between the AChR and the DGC using the QT-6 heterologous cell system. QT-6 cells lack rapsyn and AChR, as well as any detectable dystroglycan; yet recombinant rapsyn and AChR readily form clusters when coexpressed by transfection in these fibroblasts, thereby making this an ideal system for reconstitution experiments. The introduction of one component of the DGC, dystroglycan, into QT-6 cells coexpressing AChR and rapsyn resulted in the codistribution of dystroglycan to AChR–rapsyn clusters. Dystroglycan expressed by itself, however, was found evenly distributed across the cell. This is in contrast to rapsyn, which forms clusters when expressed alone in QT-6 cells. Dystroglycan association with clusters, therefore, is likely to occur as a secondary event and is not needed to initiate cluster formation; rather, the role of dystroglycan in cluster formation in native muscle may be to provide an anchor point within the cytoskeleton-associated DGC complex to which clusters bind and are thereby stably linked to the cytoskeleton.

When dystroglycan and rapsyn were coexpressed in the absence of AChR, dystroglycan was found colocalized with rapsyn clusters. AChR is therefore not necessary for the observed interaction between dystroglycan and clusters. Since adhalin was undetectable in QT-6 cells, this transmembrane DGC component does not mediate the rapsyn–dystroglycan interaction. Utrophin/dystrophin also does not appear to mediate this interaction, since immunofluorescence staining of an endogenous species recognized by an anti-utrophin antibody demonstrated no colocalization with dystroglycan-containing clusters. The lack of species cross-reactive antibodies made it impossible for us to determine whether other AChR-associated and DGC proteins are present in rapsyn–dystroglycan clusters. However, it is improbable that dystrophin, the DGC transmembrane 35 kDa protein, or the 87 kDa protein mediate the rapsyn–dystroglycan interaction: these proteins are not likely to be present in QT-6 fibroblasts, as their expression appears to be restricted to muscle and the nervous system (Hoffman et al., 1987; Lev et al., 1987; Chamberlain et al., 1988; Nudel et al., 1988; Matsumura et al., 1993; Wagner et al., 1993; Yamamoto et al., 1994). Isolation of cDNAs for syntrophin has revealed a multigene family of syntrophin proteins (Adams et al., 1993; Ahn et al., 1994; Yang et al., 1994), and although one or more of these isoforms may be present in QT-6 cells, in vitro binding assays indicate that syntrophin does not interact directly with dystroglycan (Yang et al., 1995). Furthermore,

coimmunoprecipitation and coimmunoaffinity purification experiments demonstrated that rapsyn was not present in isolated complexes containing syntrophin or the 87 kDa protein, again arguing against the possibility that syntrophin or the 87 kDa protein mediate the rapsyn–dystroglycan interaction (Butler et al., 1992; Wagner et al., 1993). Future biochemical approaches will clearly be necessary to determine whether one or more additional proteins mediate the observed association of rapsyn with dystroglycan, or whether the interaction may be direct.

Multiple lines of evidence implicate the DGC in AChR clustering. The DGC is an oligomeric transmembrane complex that binds to utrophin at the synapse, and utrophin in turn associates with actin to anchor the DGC to the submembrane cytoskeleton (see Figure 6). The recent discovery that agrin binds to α-dystroglycan suggests that the DGC may serve as a link between the extracellular binding of agrin and the intracellular events that mediate formation of AChR clusters (Bowe et al., 1994; Campanelli et al., 1994; Gee et al., 1994; Sugiyama et al., 1994). Previous indications of the importance of the DGC in AChR clustering came from the increasing emphasis on the colocalization of utrophin at AChR clusters (Khurana et al., 1991; Ohlendieck et al., 1991a; Bewick et al., 1992; Phillips et al., 1993; Campanelli et al., 1994; Gee et al., 1994), as well as the realization that a 59 kDa DGC-associated protein is identical to the AChR-associated protein syntrophin (Adams et al., 1993; Yang et al., 1994). Furthermore, DGC components are enriched at the synapse, in addition to being found in extrasynaptic sarcolemmal membranes (Matsumura et al., 1992). Although it remains to be determined whether agrin binding to α-dystroglycan plays a part in AChR clustering, a structural role for the DGC in AChR clustering is strongly implied. One function of the DGC may therefore be to provide a scaffolding through which AChRs may stably associate with the cytoskeleton, thereby promoting the formation or maintenance of high density clusters. Our finding that rapsyn mediates the interaction between the AChR and the DGC provides molecular evidence to connect these two halves of the postsynaptic apparatus (see Figure 6). Rapsyn may therefore be a necessary link in the chain of proteins connecting the AChR to the cytoskeleton.

Our observation that endogenous utrophin/dystrophin does not colocalize with clusters formed in QT-6 fibroblasts is surprising in light of the well-established interactions between dystrophin and utrophin and the DGC, and of the proposed role of utrophin in cluster formation (Ervasti et al., 1990; Yoshida and Ozawa, 1990; Ervasti and Campbell, 1991; Khurana et al., 1991; Ohlendieck et al., 1991a; Bewick et al., 1992; Matsumura et al., 1992; Phillips et al., 1993; Campanelli et al., 1994; Gee et al., 1994; Suzuki et al., 1994). Experiments with C2 muscle cells demonstrated that, while dystrophin was undetected in these cells, utrophin was found at large clusters but not small clusters, implying a role for utrophin in the maturation of clusters rather than in their initial formation (Phillips et al., 1993). Similarly, it is possible that clusters found in QT-6 cells represent an immature form of clusters that have not stably associated with the cytoskeleton. The regulatory factors that allow maturing clusters to connect to the cytoskeleton may be missing or inactive in QT-6 cells. Alternatively, perhaps the rabbit dystroglycan expressed in QT-6 cells does not bind to the endogenous quail utrophin or dystrophin because of species differences. Binding of utrophin to β-dystroglycan has yet to be demonstrated, but the homology between utrophin and dystrophin, which does bind directly to β-dystroglycan (Suzuki et al., 1994), suggests that such interactions are likely. Finally, it is possible that clusters may interact with other cytoskeletal proteins besides utrophin or dystrophin, as suggested by the observation that AChR–rapsyn clusters in QT-6 cells are partially resistant to extraction with Triton X-100 (Phillips et al., 1993). Regardless of why clusters in QT-6 cells have no associated endogenous utrophin/dystrophin, our results demonstrate that at the molecular level utrophin/dystrophin is not required for rapsyn to interact with β-dystroglycan; instead, rapsyn can directly link the AChR with the transmembrane DGC components, independently of the involvement of this cytoskeletal protein.

Our results indicating that rapsyn links the AChR to the DGC provide a critical advance in our understanding of the molecular architecture of the postsynaptic apparatus (see Figure 6). However, a number of uncertainties concerning the organization and function of other relevant AChR-associated and DGC proteins still remain. For example, if rapsyn is the molecular link between the AChR and the cytoskeleton-associated DGC, what are the functions of syntrophin and the 87 kDa protein, which were previously proposed to anchor the AChR to the cytoskeleton (Cartaud et al., 1981; Bloch et al., 1991; Froehner, 1991; Adams et al., 1993; Wagner et al., 1993)? We hypothesize that syntrophin and the 87 kDa protein are not necessary for linking the AChR to the DGC, but rather may play a structural or regulatory role in linking the DGC to the cytoskeleton. In addition, many of the molecules thought to play a role in agrin-induced AChR clustering are found throughout the sarcolemmal membrane and are not restricted to the synaptic membrane as one might have expected. How then is the participation of these proteins in events underlying AChR clustering restricted to the synapse where AChRs are clustered? The specificity must derive from associations between these proteins and molecules unique to the synapse; extrajunctionally, they may interact with different molecules to carry out functions unrelated to AChR clustering. For example, evidence from both normal and dystrophic muscle indicates that the DGC plays a critical role in muscle membrane structure and function throughout the fiber, and stabilizing interactions between the DGC and dystrophin appear to be necessary for this function (reviewed in Matsumura and Campbell, 1994). At the synapse, however, the DGC interacts with synaptically localized utrophin and through this spatially restricted interaction may play a specialized role in AChR clustering (Khurana et al., 1991; Ohlendieck et al., 1991a; Bewick et al., 1992; Matsumura et al., 1992; Phillips et al., 1993). Rapsyn and one of the syntrophin isoforms are also unique to the synapse (Froehner et al., 1981; Sealock et al., 1984; Peters et al., 1994), and the participation of the DGC in the events that underlie AChR clustering may

be restricted to the neuromuscular junction through an interaction with these synaptically localized proteins.

To understand the function of the DGC and associated proteins in AChR clustering, it is essential to identify all the proteins with which the DGC and AChR complex components associate. Our results demonstrate that rapsyn can interact independently with dystroglycan and AChR, and thereby may be a molecular link connecting the AChR to the DGC. Our results further suggest that rapsyn can link the AChR directly to the DGC through interactions with dystroglycan, and not through association with the DGC-associated cytoskeletal anchor utrophin/dystrophin. By linking the AChR to one member of the cytoskeleton-anchored DGC, dystroglycan, rapsyn may mediate the association of the AChR with the cytoskeleton.

Experimental Procedures

Antibodies

The following primary antibodies were used: anti-rapsyn rabbit polyclonal antibody 5943 (Phillips et al., 1991b), anti-AChR rat monoclonal antibody mAb61 (Tzartos et al., 1981), anti-dystroglycan sheep polyclonal antibody FP-B (Ibraghimov et al., 1992), anti-β-dystroglycan rabbit polyclonal antibody previously designated "43 C-terminal" (Ibraghimov et al., 1992), anti-α-dystroglycan mouse monoclonal IIH6 (Ibraghimov et al., 1992), and anti-utrophin rabbit polyclonal antibody BH11 (Khurana et al., 1991). Secondary antibodies used in immunofluorescence staining included TRITC–goat anti–rabbit IgG (Boehringer Mannheim, Indianapolis, IN), TRITC–rabbit anti–rat IgG (Sigma Chemical, St. Louis, MO), Cy3–donkey anti–rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), FITC–donkey anti–sheep IgG (Sigma), and FITC–goat anti–rat IgG (Boehringer Mannheim). Secondary antibodies used in Western blotting included peroxidase-conjugated goat anti–rabbit IgG (Boehringer Mannheim), goat anti–mouse IgM (Boehringer Mannheim), and donkey anti–sheep IgG (Sigma).

Western Blot Analysis

Rabbit and quail muscle crude surface membranes were prepared as described previously (Ohlendieck et al., 1991b). Whole-cell pellets of QT-6 cell cultures were solubilized in modified Laemmli buffer (Laemmli, 1970). For blots probed with antibodies IIH6 and BH11, samples were separated on 3%–12% gradient SDS–polyacrylamide gels, transferred to nitrocellulose, and stained as previously described (Ohlendieck et al., 1991b). IIH6 was used at a concentration of 3 μg/ml, and BH11 was diluted 1:2000 before use. For all other blots, QT-6 samples from nontransfected and transfected cells were separated on 7.5% or 10% polyacrylamide gels and transferred to nitrocellulose (0.45 μm; Schleicher & Schuell, Inc., Keene, NH). Transfer was carried out in 96 mM glycine, 12.5 mM Tris, 20% (v/v) methanol for 75 min at 400 mA. Nitrocellulose transfers were blocked overnight at 4°C in PBS/Tween-20 (phosphate-buffered saline with 0.5% Tween-20) containing 5% nonfat dry milk, followed by incubation with primary antibody for 2 hr at room temperature. Primary antibodies were diluted into PBS/Tween-20 containing 2.5% nonfat dry milk and used at dilutions of 1:500 (antibody 5943), 1:25 (FP-B), and 1:2000 (β-dystroglycan-specific antibody). After washing once for 15 min, then four times for 5 min each in PBS/Tween-20, filters were incubated for 1 hr with secondary antibodies diluted 1:2000 in PBS/Tween-20 containing 2.5% nonfat dry milk. Filters were washed again once for 15 min and four times for 5 min, incubated for 1 min in chemiluminescence reagents according to the manufacturer's instructions (DuPont NEN, Boston, MA), and exposed to film (X-Omat, Kodak) for varying amounts of time.

Expression Constructs

The cDNAs for mouse rapsyn, mouse AChR subunits (α, β, γ, and δ), and rabbit dystroglycan were each expressed from similar constructs consisting of a pSKII(+) plasmid backbone (Stratagene, San Diego, CA) and the specified cDNA under the control of the Rous sarcoma virus long terminal repeat. The rapsyn and AChR expression constructs have been described previously (Phillips et al., 1991a; Maimone and Merlie, 1993). To construct the dystroglycan expression plasmid, the rapsyn coding region was removed from the rapsyn expression vector and replaced by a portion of the rabbit dystroglycan cDNA (Ibraghimov et al., 1992). Specifically, a 3.4 kb BglII–EcoRV fragment containing the entire coding region of the rabbit dystroglycan cDNA was ligated with a 3.7 kb XmaI–NcoI fragment from the rapsyn expression construct after both fragments were made blunt with Klenow. The latter contained the intact Rous sarcoma virus long terminal repeat directly 5′ of the insertion site and the AChR α subunit polyadenylation signal 3′ of the NcoI site. The ligation junctions were sequenced to confirm correct insertion and maintenance of the correct reading frame.

Cell Culture and Transfection

The quail fibroblast cell line, QT-6 (Moscovici et al., 1977), was maintained as previously described (Blount and Merlie, 1988). For immunofluorescence staining studies, QT-6 cells were plated onto glass coverslips (13 mm diameter) in 6 cm tissue culture dishes and transfected 1 day later when cells were 25%–50% confluent. For Western blot analysis, QT-6 cells were plated directly onto 6 cm tissue culture dishes and transfected 1–2 days later when cells reached 60%–90% confluency. Cells were transfected using the calcium phosphate precipitation method of Chen and Okayama (1987) modified as previously described (Phillips et al., 1991b). A total of 4 μg of each plasmid was used, and pSKII(+) was added as necessary to bring the total amount of plasmid transfected per 6 cm dish to 24 μg. Transfection efficiencies typically ranged from 40% to 60%.

Immunofluorescence Staining

QT-6 cells on glass coverslips were washed with PBS and then fixed for 20 min in PBS containing 1% paraformaldehyde, 100 mM L-lysine, 10 mM sodium meta-periodate, and 1% saponin. After rinsing with PBS, cell were permeabilized for 10 min in 1% Triton X-100 in PBS, washed three times in PBS, and stored overnight in 1% fetal calf serum in PBS. Coverslips were inverted (cell side down) onto 50 μl drops of primary antibody and incubated for 2 hr at room temperature. Cells were frequently incubated with two antibodies simultaneously to facilitate visualization of potential colocalization. Primary antibodies were diluted 1:50 (antibody 5943), 1:250 (FP-B), 1:1000 (BH11), and 1:200 (mAb61) in PBS containing 1% fetal calf serum. Coverslips were washed three times with PBS, inverted onto 50 μl drops of secondary antibody diluted in PBS with 1% fetal calf serum, and incubated for 1 hr at room temperature. Secondary antibodies were used at dilutions of 1:500 (TRITC–rabbit anti–rat), 1:25 (FITC–donkey anti–sheep), 1:5000 (Cy3–donkey anti–rabbit), and 1:200 (TRITC–goat anti–rabbit and FITC–goat anti–rat). After washing three times in PBS, coverslips were finally rinsed briefly in distilled water and mounted for fluorescence microscopy onto an antifading mountant (Johnson and Nogueira Araujo, 1981). To visualize staining of extracellular epitopes, cells were fixed with 1% paraformaldehyde and were not permeabilized, but were otherwise processed as described above. To control for fluorescent cross-bleed and antibody specificity in double-labeling experiments, parallel coverslips were stained in the absence of either of the two primary antibodies. The specificity of each primary antibody was confirmed using nontransfected cells.

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