Interactions of the Rapsyn RING-H2 Domain with Dystroglycan*

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Rapsyn, a peripheral membrane protein of skeletal muscle, is necessary for the formation of the highly organized structure of the vertebrate neuromuscular junction. For mice lacking rapsyn, there is a failure of postsynaptic specialization characterized by an absence of nicotinic acetylcholine receptors (nAChRs) and other integral and peripheral membrane proteins such as β-dystroglycan and utrophin. Dystroglycan is necessary for the formation of the mature neuromuscular junction and has been shown to interact directly with rapsyn. Previous studies with rapsyn fragments and mutants, expressed in 293T cells along with nAChRs, establish that the rapsyn tetratricopeptide repeat (TPR) domain is involved in self-association and its coiled-coil domain is necessary for nAChR clustering. The function of the rapsyn RING-H2 domain, which is not necessary for rapsyn self-association or nAChR clustering, is unknown. To further characterize these domains, we have used a yeast two-hybrid assay to test for interactions at the plasma membrane between rapsyn domains and a nAChR β-subunit fragment, the β-dystroglycan cytoplasmic domain, or rapsyn domains. The rapsyn coiled-coil domain interacts with the nAChR β-subunit cytoplasmic domain, but not with itself, other rapsyn domains, or β-dystroglycan. The RING-H2 domain interacts only with the β-dystroglycan cytoplasmic domain. Furthermore, when expressed in 293T cells, a rapsyn construct containing as few as two TPRs and the RING-H2 domain self-associates and clusters dystroglycan, but not nAChRs. These results emphasize the modular character of the rapsyn structural domains.

Rapsyn, a 43-kDa peripheral membrane protein expressed in skeletal muscle, plays a critical role in organizing the structure of the nicotinic postsynaptic membrane (for review, see Ref. 1). Rapsyn is colocalized with nicotinic acetylcholine receptors (nAChRs) in the postsynaptic membrane from the earliest stages of innervation (2), and this precise colocalization is a characteristic feature of the adult neuromuscular junction (3). In rapsyn(/−/) mutant mice, which die at birth because of a failure of neuromuscular transmission, nAChRs are expressed by the subsynaptic nuclei and targeted to the postsynaptic plasma membrane, but they are not clustered (4). These mice are characterized by a general disorganization of the postsynaptic membrane, as utrophin (a cytoskeletal protein that is normally colocalized with nAChRs) and β-dystroglycan (the transmembrane protein that binds utrophin) are no longer enriched in the postsynaptic membrane. Biochemical studies using nAChR-rich membranes from Torpedo electric organ indicate that rapsyn can be cross-linked to the nAChR β-subunit (5) and bind to the cytoplasmic domain of β-dystroglycan (6). When expressed in non-muscle cells, rapsyn forms membrane-associated clusters and recruits nAChRs to these clusters (7, 8). The nAChR domain necessary for this interaction has been located within a region of primary structure between the M3 and M4 hydrophobic segments, which is known to be exposed at the cytoplasmic aspect of the nAChR (9, 10). In non-muscle cells, rapsyn can also cluster β-dystroglycan (11). Thus, rapsyn may function as a direct link between nAChRs and the dystrophin/utrophin-associated glycoprotein complex (12, 13) that extends from the extracellular matrix to the cytoskeleton.

Although the three-dimensional structure of rapsyn is not known, its primary structure suggests the presence of distinct structural domains: a myristoylated amino terminus (14), as many as eight tetratricopeptide repeats (TPRs) within rapsyn6–319 (15), a coiled-coil domain (rapsyn298–331) (16), a cysteine-rich domain (rapsyn363–402) predicted to be a RING-H2 domain (17), and a consensus sequence for phosphorylation by protein kinases A and C (rapsyn403–406). Previously, in studies of rapsyn and rapsyn mutants expressed transiently in 293T cells along with nAChRs, we identified the rapsyn structural domains involved in its membrane targeting, self-association, and nAChR-clustering properties (16, 18). Rapsyn plasma membrane targeting requires amino-terminal fatty acylation, whereas rapsyn self-association requires at least two TPRs. Rapsyn clustering of nAChRs depends upon the presence of the putative coiled-coil domain within rapsyn, and a construct containing as few as 3 TPRs and the coiled-coil domain was sufficient for nAChR clustering. Deletion of the rapsyn coiled-coil domain prevented rapsyn clustering of nAChRs. These studies clearly established that neither the rapsyn RING-H2 domain nor the adjacent phosphorylation sites were necessary for membrane targeting, self-association, or nAChR clustering.

We report here a further analysis of rapsyn functional domains using a yeast two-hybrid assay as well as additional studies of rapsyn mutants expressed in 293T cells. To test for interactions at the plasma membrane between rapsyn structural domains and rapsyn-associated proteins, we used a yeast two-hybrid assay (19) that depends upon the recruitment of human Son of Sevenless (hSos) to the plasma membrane to rescue Ras-mediated signal transduction. We tested for interactions between rapsyn domains fused to hSos and candidate protein domains anchored in the plasma membrane by fusion with the N-myristoylated v-Src amino terminus. With this assay we looked for interactions within rapsyn and for interac-

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tions of the rapsyn domains with the membrane-anchored cytoplasmic domain of the nAChR β-subunit or of β-dystroglycan.

**EXPERIMENTAL PROCEDURES**

**Materials**

All restriction enzymes were purchased from New England Biolabs. T4 DNA ligase and oligonucleotides were obtained from Life Technologies, Inc. Polymerase chain reactions (PCR) were carried out in 100 µl using 20 ng of the templates described below, 50–100 pmol of each primer, 250 µM each dNTP, and 5 units of T4 DNA polymerase (Stratagene) for 30 cycles at 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min for 30 cycles. All constructs were tested both by restriction enzyme analysis and by sequencing across the full length of the insert fragments. Yeast cdc25H Saccharomyces cerevisiae as well as the vectors pSos and pMyr were purchased from Stratagene. The vector p5 Sos (19) was provided by Dr. A. Aronheim (Technion, Haifa, Israel). The plasmids pCDNA3-dystroglycan (mouse) and pCDNA3-dystroglycan (rabbit) were provided by Dr. S. Carbonetto (McGill University) and K. P. Campbell (University of Iowa), respectively.

**Plasmid Construction**

*Sos-Rapsyn Constructs—* Human Sos was fused at its COOH terminus to full-length rapsyn (Sos-rapsyn1-412), to the rapsyn H2 domain (Sos-rapsyn1-360, 361–412), and to the rapsyn coiled-coil domain (Sos-rapsyn1-287) in a PCR to amplify rapsyn amino acids 361–412. For Sos-rapsyn298–333 the primers GAGGAGACTGACTGCGGCCTC and AGATGGCCGCTCAGAG (underlined are the HindIII and Xmal sites) were used along with the template pCDNA3-dystroglycan (mouse) in a PCR to amplify β-dystroglycan amino acids 776–893. The PCR product was cloned into pGEM T-Easy vector with BstXIII and BglII sites. The primers TCAACCCTGCATGAATCCCAACCGGCGAGCTCCAGCAACCA and GGAACCCCAAGGCGGCCTCGAGG (nucleotides in bold are mismatched to create the underlined NotI site) were used along with the template pGL-rapsyn1-412 in a PCR to amplify rapsyn amino acids 1–412. The PCR product was digested with NotI, and the fragment containing the rapsyn sequence was cloned into pSos vector linearized with NotI. For Sos-rapsyn307–333, the primers GAGGAGACTGCATCTCCACTCGGCTTCAGC and AGATGGCCGCTAGC (HindIII and NotI sites are underlined). For Myr-dystroglycan778–893, primers U (AGCTTAAGCTTTTCCAGCAATGGGGCTCATCTTG) and B (TCGACCCGGGGAAGGGACACACAGAAG) were used along with the template pGEM T-Easy vector with HindIII and XmaI sites. For Myr-dystroglycan778–814 we used the primer U and the primer TCGACCCGGGGAAGGGACACACAGAAG (HindIII site is underlined) and primer B to amplify dystroglycan amino acids 813–893. The PCR products were cloned in pGEM T-Easy vector, the resulting plasmids were digested with HindIII and Xmal, and the fragments containing β-dystroglycan were subcloned into pMyr vector. pGL-rapsyn1-307, 324–331, 331–354, 354–371, and pGL-rapsyn1-298, 331–412 were digested with either BstUI (for 1–354; includes N-Myr-TPR1–12), EcoRV (for 1–254; includes N-Myr-TPR5–12), or PmlI (for 1–287; includes N-Myr-TPR2–17) and NotI. The vector fragment was isolated in each case and ligated with the BsaAI-NotI fragment of rapsyn, which encodes rapsyn 351–411 containing the RING-H2 domain.

**Yeast Transformation and Interaction Testing**

All media were made as described (19) with reagents from Bio 101. The yeast strain cdc25H was cotransfected with 1 µg of each plasmid using the alkali-cation yeast kit from Bio 101. Cells were resuspended in 200 µl of SOC medium and plated on glucose minimal medium lacking leucine and uracil. To test for protein interactions, yeast cells were picked and spotted onto plates containing glucose or galactose and incubated for 4 days at 25 or 37 °C. Plates were scanned to obtain digitized images. For colonies where no growth on galactose was seen after 4 days at 37 °C, no growth was observed after incubation for several days longer.

**Expression of Rapsyn, Rapsyn Mutants, and Dystroglycan in 293T Cells**

**Transfection of 293T cells by the calcium phosphate method, cell staining, and immunofluorescence experiments were done as described (18), except that after fixation, the cells were usually permeabilized with 1% Triton X-100 for 10 min and blocked with blocking buffer for 30 min before incubation with the primary antibody. Cells were visualized using a Nikon Eclipse E800 epifluorescence microscope with a Nikon 100X Plan Fluor objective (NA1.3). Images were acquired with a Micromax CH250 CCD camera (Princeton Instruments) using MetaMorph software.

Rapsyn or its fragments were visualized by the binding of either anti-rapsyn (1-16), an affinity-purified rabbit polyclonal antibody directed against a synthetic peptide corresponding to the NH2-terminal 16 amino acids of rapsyn (non-myristoylated) (at 2 µg/ml), or mouse monoclonal mAb 22F10 (20) (1:1000 dilution of ascites), followed by the appropriate ALEXA™488-conjugated secondary antibody (1:300 dilution, Molecular Probes). mAb 22F10 was prepared by using Torpedo rapsyn as immunongen. Based upon epitope-mapping studies mAb 22F10 recognizes N-myristoylated rapsyn amino acids 1–11 with >1000-fold greater affinity than the non-myristoylated peptide.3

The distribution of dystroglycan was visualized by binding to one of the antibodies listed below followed by the appropriate ALEXA™594- or Cy3-conjugated secondary antibody. 1) Affinity-purified sheep polyclonal FP-B directed against a bacterial expressed fusion protein containing amino acids 307–388 of rabbit α1(II)-dystroglycan (Ref. 21; a gift from Dr. K. P. Campbell) was used at a dilution of 1:10 to visualize transplanted dystroglycan in 293T cells that were fixed and permeabi-
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RESULTS

Quantification of Cells with Rapsyn or Dystroglycan Clusters—For experiments involving expression of rapsyn or its mutants and dystroglycan in 293T cells, quantification of dystroglycan clusters relative to rapsyn clusters was done as follows. In each experiment, 100 cells positive for both rapsyn and dystroglycan expression were identified. For these we counted the number of cells with dystroglycan that colocalized with rapsyn clusters. Each experiment was repeated at least three times, cells were scored as above, and the results are presented as the % of cells with (or without) clustered dystroglycan.

To provide a further analysis of the properties of the rapsyn structural domains, we wanted to identify the binding properties of the individual domains, and we were particularly interested in identifying proteins interacting with the RING-H2 domain. However, we were concerned that RING domains often have a strong tendency to aggregate (24–26), and some self-activate when tested in transcription factor yeast two-hybrid assays (27). As an alternative, we tested for the interactions of rapsyn domains with potential partners by use of a yeast two-hybrid system. We tested for the interactions of rapsyn domains with potential binding partners by use of a yeast two-hybrid assay that identifies protein interactions at the plasma membrane (19). The assay takes advantage of a yeast strain that is temperature-sensitive for the Ras exchange factor cdc25. At the permissive temperature of 25 °C, the temperature-sensitive mutant cdc25H is functional, and the yeast grow well, but at 37 °C, cdc25H is nonfunctional, and the yeast fail to grow. The expression of hSos, the mammalian homologue of cdc25, is able to complement the cdc25 mutation only if it can be localized to the plasma membrane. hSos membrane localization can be accomplished if it is expressed as a hybrid protein fused to a bait protein that interacts with a partner which is localized at the plasma membrane. Localization of the partner to the plasma membrane is achieved by fusing it to the myristoylated amino terminus of v-Src. The expression of the partner is driven by the Gal1 promoter, which is induced by galactose. When the bait and the partner are both expressed and interact, the Ras-signaling pathway is rescued, allowing the growth of yeast at 37 °C on plates containing galactose.

Interaction Partners for Rapsyn Structural Domains—Fig. 1 presents the results obtained using individual domains of rapsyn fused with hSos as bait and full-length rapsyn or the dystroglycan cytoplasmic domain of the nAChR β-subunit as target. As negative controls each construct tested was cotransfected with either a myristoylated transcription factor (Myr-MafB) or hSos alone, and no growth at 37 °C was seen with any construct. Based upon the growth seen under selective conditions, Myr-rapsyn1–412 interacted with Sos-rapsyn1–412, rapsyn1–360-Sos, and rapsyn1–287-Sos but not with Sos-rapsyn1–287 or Sos-rapsyn361–412 (Fig. 1, first row). These data indicate that the rapsyn TPR domain (rapsyn1–287) can associate with full-length rapsyn, whereas the coiled-coil domain or the RING-H2 domain cannot. Although the RING-H2 and coiled-coil domains did not interact with rapsyn, they were each able to interact with domains of other proteins (see below).
As with full-length rapsyn (Sos-rapsyn 5–412) but not with the amino acids. For both antibodies, rapsyn or with rapsyn 1–360-Sos, but when expressed with the distal domain (Fig. 2B). We were surprised to find, however, that rapsyn—dystroglycan antibodies, and dystroglycan813–893, encoding the membrane distal part of dystroglycan. To further define the region within the β-dystroglycan cytoplasmic domain that was involved in binding the rapsyn RING-H2 domain, we tested for interactions between rapsyn domains fused with Sos and Myr-β-dystroglycan778–814, which encodes the membrane proximal domain, or Myr-β-dystroglycan813–893, encoding the membrane distal domain (Fig. 2B). Myr-dystroglycan813–893 did not interact with full-length rapsyn or with rapsyn1–360-Sos, but when expressed with the Sos-RING-H2 domain there was evidence of limited yeast growth at 37 °C.

Rapsyn Clustering of Dystroglycan in 293T Cells—When expressed in QT-6 fibroblasts, dystroglycan is distributed uniformly at the cell surface, but when co-expressed with rapsyn, dystroglycan is colocalized with clustered rapsyn (11). We wanted to identify the rapsyn domains required to recruit dystroglycan to rapsyn clusters. We initially planned to express rapsyn-GFP constructs (16, 18) with dystroglycan in 293T cells and visualize the expressed rapsyn domains by the fluorescence of GFP and dystroglycan by the binding of appropriate antibodies. We were surprised to find, however, that rapsyn-GFP was not able to cluster dystroglycan (data not shown) and that even with full-length rapsyn, the clustering of dystroglycan could be seen only with one of the five dystroglycan antibodies tested, the FP-B polyclonal antibody that had been used in previous studies of dystroglycan clustering (11). For cells coexpressing rapsyn and dystroglycan, β-dystroglycan appeared diffusely distributed at the cell surface and not associated with rapsyn clusters when visualized by a monoclonal antibody (Fig. 3, a and b) or polyclonal antibody (Fig. 3, c and d) directed against the β-dystroglycan COOH-terminal 15 or 20 amino acids. For both antibodies, β-dystroglycan was detected only in transfected cells, and antibody binding was not seen in fixed, nonpermeabilized cells (data not shown). Similarly, when α-dystroglycan was visualized either by mAb IIH6 (Fig. 3, e and f) or mAb 1B7 (Fig. 3, g and h), α-dystroglycan was distributed diffusely at the surface and was not associated with rapsyn clusters. For these antibodies, staining was also seen in nonpermeabilized, transfected cells (data not shown). For these four antibodies, dystroglycan immunostaining associated with rapsyn clusters was seen in less than 1% of cells expressing both proteins.

Dystroglycan clustering by rapsyn was readily apparent in >95% of cells expressing both proteins when the transfected dystroglycan was detected using the FP-B antibody under permeabilized conditions (Fig. 3, i (rapsyn) and j (dystroglycan)). For 293T cells transfected with dystroglycan in the absence of rapsyn, dystroglycan was distributed diffusely at the cell surface as seen with the other α- or β-dystroglycan antibodies, and for cells transfected with rapsyn only but incubated after permeabilization with FP-B antibody and fluorescent secondary antibody, no fluorescence above background was seen (data not shown).

In additional experiments, when rapsyn and dystroglycan were coexpressed in QT-6 fibroblasts and dystroglycan was visualized by the FP-B antibody, it was clustered with rapsyn. When dystroglycan was visualized by the other antibodies, the...
staining was seen to be distributed diffusely (data not shown). In the following experiments to identify the rapsyn domains involved in dystroglycan clustering, we used the FP-B antibody to visualize transfected dystroglycan.

**Rapsyn Domains Necessary for Dystroglycan Clustering**—As a first test of the requirement of the RING-H2 domain for the clustering of dystroglycan, we attempted to coexpress dystroglycan with rapsyn<sub>1–360</sub>, lacking the RING-H2 domain. Unfortunately, in contrast to rapsyn<sub>1–412</sub> or rapsyn<sub>1–360-GFP</sub> (16), based on immunofluorescence there was no evidence of expression of rapsyn<sub>1–360</sub> (or clustering of dystroglycan). To identify the rapsyn domains required in conjunction with the RING-H2 domain for dystroglycan clustering, we expressed three rapsyn constructs with varying numbers of TPRs fused to the RING-H2 domain (Fig. 4a). Rapsyn<sub>1–287</sub>, consisting of seven TPRs fused to the RING-H2 domain but lacking the coiled-coil domain, formed clusters similar to wild-type rapsyn and clustered dystroglycan in all the cells expressing both proteins (Fig. 4, b and c), as did rapsyn<sub>1–254 + 351–412</sub> with six TPRs and the RING-H2 domain (Fig. 4, d and e). Rapsyn<sub>1–90 + 351–412</sub>, with only TPRs 1 and 2 and the RING-H2 domain, also formed distinct clusters at the plasma membrane and clustered dystroglycan in all cells expressing both proteins (Fig. 4, f and g). No clustering of nAChRs was seen for any of these three rapsyn constructs lacking the coiled-coil domain (Fig. 4, h–m).

**DISCUSSION**

The results presented here extend our understanding of the functional roles of the rapsyn TPR and coiled-coil domains and identify for the first time a binding partner, β-dystroglycan, for the rapsyn RING-H2 domain. Previous studies based upon the properties of rapsyn domains expressed in 293T cells (16, 18) established that the rapsyn TPR domain is involved in self-association and that neither the coiled-coil domain nor the RING-H2 domain, when expressed alone, was able to associate with full-length, wild-type rapsyn. Furthermore, the studies showed that the presence of the coiled-coil domain was required for nAChR clustering. In the 293T expression system, however, nAChR interactions with rapsyn could be identified only in the context of rapsyn clustering, and it was not possible to determine whether the rapsyn coiled-coil domain interacted with an nAChR domain in the absence of the TPR domain.

As an alternative approach to studying the functional properties of individual rapsyn domains, we used the yeast two-hybrid assay based on hSos recruitment (19) to test for interactions between rapsyn domains and potential interacting domains from rapsyn, the nAChR β-subunit, or β-dystroglycan. Our results establish that the rapsyn domains can each interact in an independent and selective manner with other protein domains. The TPR domain is involved in self-association, and it did not interact with other rapsyn domains or with the cytoplasmic domain of the nAChR β-subunit or of β-dystroglycan.
The rapsyn coiled-coil domain, even in the absence of the rapsyn TPR domain, can interact with the nAChR β-subunit cytoplasmic domain but not with the rapsyn RING-H2 or TPR domain or with the β-dystroglycan cytoplasmic domain. Rapsyn and the rapsyn coiled-coil domain can also interact with the cytoplasmic domains of other nAChR subunits, but studies of these interactions will be described in a later report.

The Rapsyn RING-H2 Domain and Dystroglycan—Biochemical studies (6) provided the first evidence, based upon a gel “overlay assay,” that dystroglycan778–819, the membrane proximal portion of the cytoplasmic domain, can bind to rapsyn. Our results now establish that it is the RING-H2 domain of rapsyn that interacts with that region of β-dystroglycan and that this domain cannot interact with any of the domains within full-length rapsyn or with the nAChR domain. The interaction between the RING-H2 domain and the β-dystroglycan cytoplasmic domain can occur in the absence of the rapsyn TPR or coiled-coil domain, and the RING-H2 domain can function as an independent binding domain. Furthermore, based upon the 293T assay, a rapsyn construct containing the minimal domain structure necessary for self-association at the plasma membrane (Myr-TPRs1+2) and the RING-H2 domain causes dystroglycan to be clustered with rapsyn.

The Stability of Expressed Rapsyn Domains—In the hSos recruitment assay, one of the interacting proteins is anchored at the plasma membrane by fusion with the NH2-terminal v-Src membrane-targeting sequence, and the other protein can be expressed either as an NH2-terminal or COOH-terminal hSos fusion protein. In our yeast assay we did not systematically test both fusion orientations. Rather, we were guided by previous results obtained in the 293T expression system (16). Thus, we expressed rapsyn1–297-GFP and rapsyn1–360-GFP because those rapsyn fragments, when fused at their COOH termini to GFP, expressed well. Interestingly, when we coexpressed Sos-rapsyn1–360 with Myr-rapsyn5–412, there was no yeast growth under selective conditions, and based upon immunofluorescence, we found no evidence of the expression of rapsyn1–360 protein in the 293T assay. In contrast, Sos-rapsyn1–360-GFP was expressed and interacted with Myr-rapsyn5–412. Thus, it appears that expressed rapsyn COOH-terminal truncation mutants can be unstable if the normal COOH-terminal RING-H2 domain is removed, but the expressed proteins can be stabilized by either GFP or hSos at the COOH terminus. The rapsyn coiled-coil and RING-H2 domains were readily expressed when fused at the COOH terminus of hSos, and the coiled coil domain was also expressed when fused at the COOH terminus of the v-Src membrane targeting sequence (not shown).

Rapsyn Clustering of Dystroglycan in 293T Cells—We were surprised that in the 293T cell assay, dystroglycan clustering by rapsyn was seen only when dystroglycan was visualized by the FP-B antibody and not when visualized by two monoclonals specific for α-dystroglycan or by a monoclonal or a polyclonal antibody specific for the COOH terminus of β-dystroglycan. However, the clustering of dystroglycan by rapsyn, as visualized by the FP-B antibody, was unambiguous and seen in essentially all cells that expressed both proteins. Based upon immunoblots, the affinity-purified FP-B antiserum recognizes determinants in α- and β-dystroglycan (21). Since we do not detect dystroglycan clustering with either antibody directed against α-dystroglycan, we suspect that the majority of α-dystroglycan does not remain associated with β-dystroglycan on the 293T cell surface. When β-dystroglycan was visualized with antibodies directed against the COOH-terminal 20 amino acids, there was also no evidence of β-dystroglycan clustering by rapsyn. One possible explanation for this is that the antibody (primary or secondary) cannot bind when β-dystroglycan is in association with rapsyn. The fact that rapsyn1–412 but not rapsyn1–412-GFP can cluster dystroglycan demonstrates that modifications at the COOH terminus of rapsyn also can interfere with dystroglycan clustering. It also may be relevant that Tyr-890, the fourth amino acid before the COOH terminus of dystroglycan, is a site of tyrosine phosphorylation that regulates dystrophin binding to β-dystroglycan (28). It remains to be determined whether β-dystroglycan clustered with rapsyn is phosphorylated at Tyr-890 and whether the antipeptide antibodies directed against the β-dystroglycan COOH terminus recognize the epitope when phosphorylated.

Mapping the Interactions between Rapsyn RING-H2 Domain and β-Dystroglycan—Sos-rapsyn interacted with Myr-dystroglycan778–814 but not with Myr-dystroglycan813–893. For the RING-H2 domain in isolation (Sos-rapsyn361–412) the growth of yeast under selective conditions was also seen in the presence of Myr-dystroglycan813–893 although growth was considerably slower than in the presence of the Myr-dystroglycan778–814. Further studies are required to determine whether these differences in growth reflect differences in the affinity of interaction of the RING-H2 domain for determinants in the two β-dystroglycan subdomains. Since Sos-rapsyn1–412 interacted only with the membrane proximal subdomain, it is that interaction that appears more biologically relevant.

RING zinc finger domains mediate protein-protein interactions involved in the formation of large molecular scaffolds (25, 26). Some RING domains self-associate or bind to other RING domains, whereas at least three other RING binding domains recognize a proline-rich sequence defined by PXBXPXP, where B and J are Leu/Val and Ala/Ser, respectively. In the complex between the c-Cbl proto-oncogene, a RING domain ubiquitin-protein ligase, and a ubiquitin conjugating enzyme, side chains on one of the surfaces of the c-Cbl RING domain (including cysteines also involved in zinc coordination) interact with amino acids in loops on the surface of the conjugating enzyme, whereas a second surface of the RING domain interacts with another domain within the ligase (29). Our results establish that the rapsyn RING-H2 domain is clearly not involved in self-association and that it binds to a protein lacking a RING domain. Although the membrane proximal β-dystroglycan subdomain (dystroglycan778–813) that binds to rapsyn does contain a proline triplet (Pro-807–809), it is the membrane distal cytoplasmic domain (dystroglycan813–893) that does not interact with rapsyn that actually contains 22 prolines including prolines 887–889, which are directly involved in binding to the dystrophin WW domain (30, 31) and to the adaptor protein Grb2 (32). Further studies are required to determine the surface of the rapsyn RING-H2 domain involved in dystroglycan binding and to identify the determinants within dystroglycan778–813 important for rapsyn binding.

Rapsyn RING-H2 Domain, Dystroglycan, and nAChR Clustering—Our studies demonstrate that the rapsyn-RING-H2 domain interacts with the β-dystroglycan cytoplasmic domain in non-muscle cells, and there is every reason to expect that this interaction occurs in muscle and is important for the organization of nicotinic postsynaptic membrane. At the adult neuromuscular junction dystroglycan is enriched in the postsynaptic membrane, interacting with utrophin that is colocalized with nAChRs and rapsyn in the primary gutter and with dystrophin in the secondary folds (33). In addition, in experiments with cultured C2 myotubes, utrophin was found associated primarily with the large, but not the small, nAChRs clusters, which suggested that interactions between rapsyn/nAChR and proteins of the dystrophin complex may be involved in the growth
of nAChR clusters (34). Recently, it has been shown (35) that for myotubes differentiated from dystroglycan −/− embryonic stem cells and for neuromuscular junctions of dystroglycan-deficient chimeric mice, the organization of nAChR clusters is more fragmented than in wild-type muscle. In addition, in the absence α-dystrobrevin, a dystrophin homolog that also binds to β-dystroglycan and is enriched with nAChRs in the postsynaptic membrane, there is a similar failure of neuromuscular junction maturation (36). These results emphasize that αβ-dystroglycan and the link it forms between the extracellular matrix and the cytoskeleton are not required for the initial formation of nAChR clusters but are important for the maturation of the nAChR clusters into large, compact arrays.

Several studies provided indirect evidence that the rapsyn RING-H2 domain might be involved in interactions with β-dystroglycan or other dystrophin/utrophin-associated proteins. When coexpressed with nAChRs in Xenopus oocytes, a mutant rapsyn containing substitutions within the RING-H2 domain failed to form nAChR clusters (37). Furthermore, injection of a fusion protein containing the rapsyn RING-H2 domain into rat myotubes caused a fragmentation of nAChR clusters (38) without any evidence that the RING-H2 domain itself was interacting with rapsyn or nAChRs. Although it remains to be determined whether the rapsyn RING-H2 domain binds to proteins in addition to β-dystroglycan, these results in conjunction with our data indicate that the interaction between the rapsyn RING-H2 domain and β-dystroglycan is crucial for the maturation of the nAChR clusters. However, the size of the rapsyn clusters in 293T does not increase in the presence of transfected dystroglycan, and there may be other, as yet unidentified, proteins that interact with rapsyn and are important for nAChR clustering. We are currently using the hSos recruitment assay to look for interactions between rapsyn and other proteins such as MuSK, the receptor tyrosine kinase that binds agrin (39), that are known to be localized with nAChRs in the nicotinic post-synaptic membrane and to screen libraries from skeletal muscle to identify other proteins interacting with rapsyn.

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