Evidence for *in Situ* and *in Vitro* Association between β-Dystroglycan and the Subsynaptic 43K Rapsyn Protein

CONSEQUENCE FOR ACETYLCHOLINE RECEPTOR CLUSTERING AT THE SYNAPSE*

Annie Cartaud‡, Sébastien Coutant‡, Tamara C. Petrucci§, and Jean Cartaud‡¶

From ‡Biologie Cellulaire des Membranes, Département de Biologie Supramoléculaire et Cellulaire, Institut Jacques Monod, UMR 9922, CNRS et Université Paris VII, 2 Place Jussieu, 75251 Paris Cédex 05, France and §Biologia Cellulare, Istituto Superiore di Sanità, V. le Regina Elena 299, Roma 00161, Italy

The accumulation of dystrophin and associated proteins at the postsynaptic membrane of the neuromuscular junction and their co-distribution with nicotinic acetylcholine receptor (AChR) clusters *in vitro* suggested a role for the dystrophin complex in synaptogenesis. Co-transfection experiments in which α- and β-dystroglycan form a complex with AChR and rapsyn, a peripheral protein required for AChR clustering (Apel, D. A., Roberds, S. L., Campbell, K. P., and Merlie, J. P. (1995) *Neuron* 15, 115–126), suggested that rapsyn functions as a link between AChR and the dystrophin complex. We have investigated the interaction between rapsyn and β-dystroglycan in *Torpedo* AChR-rich membranes using *in situ* and *in vitro* approaches. Cross-linking experiments were carried out to study the topography of postsynaptic membrane polypeptides. A cross-linked product of 90 kDa was labeled by antibodies to rapsyn and β-dystroglycan; this demonstrates that these polypeptides are in close proximity to one another. Affinity chromatography experiments and ligand blot assays using rapsyn solubilized from *Torpedo* AChR-rich membranes and constructs containing β-dystroglycan C-terminal fragments show that a rapsyn-binding site is present in the juxtamembranous region of the cytoplasmic tail of β-dystroglycan. These data point out that rapsyn and dystroglycan interact in the postsynaptic membrane and thus reinforce the notion that dystroglycan could be involved in synaptogenesis.

The accumulation and maintenance of high concentrations of acetylcholine receptors (AChRs)[1] at the postsynaptic membrane of the neuromuscular junction (NMJ) involve several levels of regulatory mechanisms including transcriptional regulation of AChR subunits genes, clustering, and anchoring of the AChR to the cytoskeleton. Clustering of AChR is a complex process involving several partners being triggered by the nerve-derived trophic factor agrin (for a review, see Ref. 1). A major step in the understanding of how agrin works was provided recently by the discovery of MuSK, a synaptic muscle-specific tyrosine kinase (2, 3). Once activated by agrin, MuSK-receptor complex sparks a cascade of events that leads ultimately to the building of the NMJ (4).

In addition, several extrinsic proteins associated with the postsynaptic membrane in muscle or electrocyte have been implicated in the formation and/or the maintenance of AChR clusters. Extraction of these proteins from AChR-rich membrane induces the mobility of the AChR in the plane of the membrane (5–8). These proteins thus participate in the anchoring of AChRs in the membrane. Among them, the 43-kDa AChR-associated protein rapsyn (9) plays a direct role in the clustering of AChR and all other postsynaptic membrane components as deduced from co-transfection experiments (10, 11) and from rapsyn-deficient mice (12). In addition to its structural role, rapsyn is required in an early step of MuSK signaling, *i.e.* AChR phosphorylation (13). From these studies, it could be proposed that rapsyn interacts directly or indirectly with several components of the postsynaptic membrane including MuSK and AChR to fulfill both structural and signaling functions in synaptic differentiation.

Several other peripheral proteins, M_r 58,000 syntrophin(s) (14) and M_r 87,000 dystrobrevin (15), have been identified in *Torpedo* electromotor synapse and co-localize with AChRs at the NMJ. These latter two proteins are part of the dystrophin-glycoprotein complex (DGC) present at the sarcolemma (16) and of the utrophin complex at the synapse (for a review, see Ref. 17). Since dystrophin and dystrophin-associated proteins are found at large AChR clusters in cultured muscle cells as well as at synapses *in vivo*, they may also participate in synaptogenesis (11, 18, 19). Major components of the DGC are dystroglycans. Dystroglycans consist of a 156-kDa extracellular laminin-binding glycosylated protein (α-dystroglycan) and a 46–50-kDa transmembrane glycoprotein (β-dystroglycan) that result from proteolytic cleavage of a single transmembrane precursor (20). Dystroglycans are believed to form a continuous link between the extracellular matrix and the submembranous skeleton at the sarcolemma (for a review, see Ref. 21). α-Dystroglycan has been identified as an agrin-binding site and, as such, was postulated to represent a major actor in synapse formation (for a review, see Ref. 22). However, several observations have weakened the case for α-dystroglycan as the signal-transducing agrin receptor (23–25). Nevertheless, it is likely to play a structural role in AChR clustering, perhaps as part of a diffusion trap for AChRs (18, 19). By using the quail fibroblast expression system, Apel *et al.* (26) reported that upon co-transfection with α- and β-dystroglycan along with rapsyn and AChR subunits cDNAs, dystroglycans co-localize with

[1] The abbreviations used are: AChR, nicotinic acetylcholine receptor; DGC, dystrophin-glycoprotein complex; ECL, enhanced chemiluminescence detection; GST, glutathione S-transferase; LiS, lithium diiodosalicylate; NMJ, neuromuscular junction; PAGE, polyacrylamide gel electrophoresis; SMPB, succinimidyl 4-(*p*-maleimidophenyl) butyrate; mAb, monoclonal antibody.

rapsyn-induced clusters both in the presence or in the absence of co-expressed AChRs. These experiments highlighted a possible role for rapsyn as a link between AChR and the dystroglycans (probably β-dystroglycan).

In order to explore the function of dystroglycans in AChR clustering, we examine *in situ* and *in vitro* the molecular interactions between rapsyn and β-dystroglycan using chemical cross-linking and binding experiments. In cross-linking experiments, these two polypeptides are found in close proximity from one another in the native AChR-rich membrane. Affinity chromatography and ligand blot *in vitro* assays allowed us to identify a rapsyn-binding site in the juxtamembranous region (corresponding to amino acids 787–819) of the cytoplasmic tail of β-dystroglycan. These new data support the notion that rapsyn may function as a molecular link connecting AChRs and DGC at the postsynaptic membrane of the neuromuscular junction. The ability of β-dystroglycan and rapsyn to establish multiple interactions with partners from both inside and outside the cell places these molecules at a key position in the differentiation of the postsynaptic membrane during synaptogenesis.

EXPERIMENTAL PROCEDURES

Materials—The following primary antibodies were used. Anti-rapsyn mouse monoclonal antibody mAb 1234 A was a generous gift from Dr. Froehner (27). Rabbit polyclonal anti-β-dystroglycan antibody was generated against a synthetic peptide of the last 20 amino acids of β-dystroglycan C terminus (28). Anti-GST rabbit polyclonal antibody and glutathione-Sepharose 4B were from Amersham Pharmacia Biotech. Peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG were from Promega, Madison, WI. The protein concentrations were estimated using BCA protein reagent (Pierce). Succinimidyl 4-(*p*-maleimidophenyl) butyrate (SMPB) was purchased from Pierce. All other reagents were from the highest grade available.

Purification of Acetylcholine Receptor-rich Membranes—Acetylcholine receptor-rich membranes were purified according to Saitoh and Changeux (29) from freshly dissected *Torpedo* electric tissue obtained from the Institut de Biologie Marine, Arcachon, France. Proteolysis was prevented by the addition of the protease inhibitors leupeptin (Sigma, 5 μg/ml), pepstatin A (Sigma, 5 μg/ml), EDTA (5 mM), EGTA (5 mM), 4-(2-aminoethyl)benzenesulfonyl fluoride (Calbiochem, 0.2 mM), and *N*-ethylmaleimide (Sigma, 10 mM) in all buffers. Typically 10–20 mg of membrane proteins were obtained from 100–200 g of fresh tissue.

Cross-linking Experiments—Cross-linking experiments were performed according to the protocol described by Burden *et al.* (30) using an heterobifunctional cross-linking reagent that contains *N*-ethylmaleimide and *N*-hydroxysuccinimide as reactive groups. Briefly, AChR-rich membranes were washed with 10 mM sodium phosphate buffer, 1 mM EDTA, 1 mM EGTA, 0.3 mM phenylmethylsulfonyl fluoride, 0.02% sodium azide, pH 7.4, pelleted by centrifugation, and resuspended in 10 mM sodium phosphate buffer, 1 mM EDTA, pH 8.0, at a final concentration of 4 mg of protein/ml. SMPB (0.12 nm between reactive groups) in Me$_2$SO (2% v/v stock solution) was added to the membranes at concentrations of 10^{-7} to 10^{-4} M and incubated for 30 min at room temperature in the dark. Membranes were then pelleted and washed in 10 mM sodium phosphate buffer, 1 mM EDTA, pH 8, before solubilization in SDS-PAGE sample buffer. Forty μg of membrane proteins were loaded in each lane. Analysis of the cross-linked products were subsequently performed by Western blotting followed by ECL detection.

Solubilization of Rapsyn from AChR-rich Membranes by Alkali and LiS—For affinity chromatography, rapsyn was solubilized by alkali treatment for 10 min at 4 °C according to Neubig *et al.* (31). The alkali extract (S11) was neutralized to pH 7.5 with 2 M Tris-HCl, pH 7.0. Stripped membrane fragments and insoluble aggregates were then removed by centrifugation for 40 min at 100,000 × *g*. For two-dimensional SDS-PAGE separation, rapsyn was extracted by lithium diiodosalicylate (LiS) according to Carr *et al.* (32) as follows. AChR-rich membranes were suspended at 15 mg of protein/ml in 10 mM Tris, pH 8.1. A 1/9 volume of 0.1 M LiS in 10 mM Tris, pH 8.1, was added; the suspension was incubated on ice for 60 min; and the membranes were then removed by centrifugation for 40 min at 100,000 × *g*. The final supernatant containing most of the solubilized rapsyn called LiS extract was further processed for two-dimensional analysis.

GST Fusion Protein Constructs of Dystroglycans—Cloning of rabbit brain dystroglycan and construction of plasmids encoding β-dystroglycan cytoplasmic fragments was achieved as described by Rosa *et al.* (28). The constructs β-C2 and β-C3 corresponding to residues 821–895 and 787–819 of the cytoplasmic tail of dystroglycan and a construct corresponding to residues 467–500 of α-dystroglycan (ectodomain) were expressed as glutathione *S*-transferase (GST) fusion proteins in BL21 strain of *Escherichia coli*. Recombinant proteins were purified following the procedure described by Kennedy *et al.* (33).

Affinity Chromatography—GST and GST fusion proteins were immobilized on glutathione-Sepharose 4B according to the supplier's instructions. Control GST and GST fusion proteins were equilibrated in phosphate-buffered saline and incubated 2.5 h at 4 °C with freshly prepared alkali extract (S11) neutralized to pH 7.5. After incubation, the glutathione-Sepharose 4B beads were washed five times with phosphate-buffered saline by centrifugation. For immunoblot analysis, Laemmli's sample buffer (34) was directly added to Sepharose beads before separation by SDS-PAGE and transfer onto nitrocellulose.

SDS-Gel Electrophoresis and Immunoblotting—8 or 10% SDS-polyacrylamide gel electrophoresis was performed using a Bio-Rad Mini-Protean II slab cell (Bio-Rad). Two-dimensional gel electrophoresis was accomplished essentially as described by O'Farrell (35) on LiS extract following the protocol described by Carr *et al.* (32). Sample buffer was composed of 9.95 M urea, 2% Nonidet P-40, 100 mM dithiothreitol, 0.02% SDS, 0.1% Triton X-100, and 2% Ampholines (1 part pH 3.5–10, 2.5 parts pH 5–8, and 2.5 parts pH7–9, Amersham Pharmacia Biotech, Bromma, Sweden). Isoelectrofocusing gels were made in 0.4% Ampholines pH 3.5–10, in 1% Ampholines pH 5–8, in 1% Ampholines pH 7–9 for 30 min at 150 V, 4 h at 200 V, and 10 min at 800 V in a Bio-Rad Mini-Protean II slab cell, as described by Bollag and Edelstein (36). This method allowed highly reproducible isoelectric focusing and easy comparison of several protein samples. The second dimension was carried out on strips of gels from the first dimension fitted over 10% SDS-PAGE. Proteins separated by one- or two-dimensional gel electrophoresis were electrotransferred to nitrocellulose membranes (Schleicher & Schuell) according to Towbin *et al.* (37) and stained with Ponceau Red. Western blots were performed as described previously (38), revealed using enhanced chemiluminescent detection (ECL, Amersham Pharmacia Biotech) and exposed to Fuji X-Ray films (Fuji, Tokyo, Japan). In some cases, blots were stripped for 1 h at 55 °C in 2% SDS, 0.1 M β-mercaptoethanol, 60 mM Tris, pH 6.7, and reprobed with antibodies as described (38).

Ligand Blot Overlay Assay—β-Dystroglycan-GST fusion protein binding assay was achieved on blots as described by Carr and Scott (39). GST control, dystroglycan constructs β-C2, β-C3, and α-ectodomain (1 μM) were incubated for 2 h on the nitrocellulose strips on which proteins from purified AChR-rich membranes or LiS extract were electrotransferred after separation by one- or two-dimensional SDS-PAGE, respectively. Bound GST fusion proteins were revealed using anti-GST antibody followed by ECL detection.

RESULTS

Cross-linking of Rapsyn and β-Dystroglycan in Native AChR-rich Membranes by Succinimidyl 4-(p-Maleimidophenyl) Butyrate (SMPB)—To study the topography of postsynaptic membrane polypeptides toward rapsyn, we have used the cross-linking protocol developed by Burden *et al.* (30) for the study of the association between AChR subunits and rapsyn. These authors have taken advantage of the fact that rapsyn contains most (up to 90%) of the available free sulfhydryls in AChR-rich membranes. Accordingly, they selected a heterobifunctional cross-linker, SMPB, containing *N*-ethylmaleimide and *N*-hydroxysuccinimide as reactive groups, to selectively tether rapsyn to neighboring polypeptides. This reagent thus allows the exploration of the close environment of rapsyn in the membrane. In these conditions, most cross-linked products included rapsyn and extensive cross-linking among other membrane polypeptides was minimized. As the reagent was not cleavable, the composition of the polypeptide bands that appear following cross-linking was analyzed by Western blotting using antibodies against rapsyn and β-dystroglycan. At SMPB concentrations ranging from 10^{-6} to 10^{-5} M, a few cross-linked products containing rapsyn did appear in immunoblots of purified AChR-rich membranes (Fig. 1). A major band at 110 kDa was previously identified as the rapsyn/AChR-β-subunit cross-
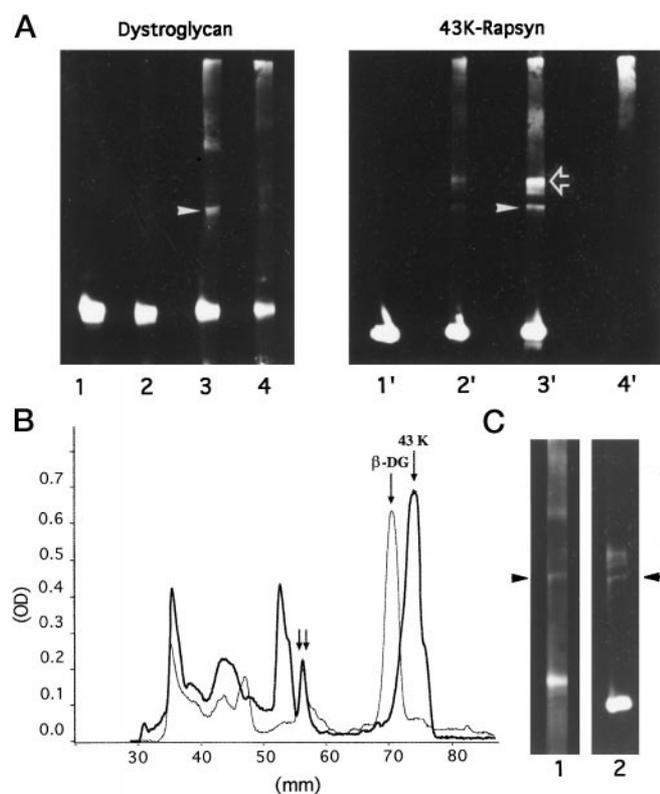
FIG. 1. *A*, rapsyn and β-dystroglycan can be cross-linked in AChR-rich membranes with SMPB. *Lanes 1–4* and *1'* to *4'* represent blots from SDS-PAGE of membranes cross-linked with 0 M and 7×10^{-6}, 4.2×10^{-5}, and 2.5×10^{-4} M SMPB, respectively. *Left panel* was probed with anti-β-dystroglycan antibodies. *Right panel* was probed with anti-rapsyn mAb 1234A antibody. Immunoblots were revealed with chemiluminescent (ECL) detection and printed directly from x-ray films. These experiments show that the cross-linked product at 90 kDa (*arrowheads* in *lane 3* and *3'*) is composed of β-dystroglycan and rapsyn. The *open arrow* in the *right panel* points to the position of the 110-kDa cross-linked product containing rapsyn and the β-subunit of the AChR, as previously reported by Burden *et al.* (30). Note that all of the rapsyn was cross-linked at high SMPB concentration and was recovered at the top of the resolving gel (*lane 4'*), whereas only part of dystroglycan was cross-linked under the same conditions (*lane 4*). *B*, densitometric scans of *lane 3* and *3'* from immunoblots shown in *A* demonstrate the coincidence (*double arrow*) of labelings of the 90-kDa cross-linked product, probed for β-dystroglycan (*β-DG, light line*) and rapsyn (*heavy line*). *C*, membrane proteins cross-linked with 4.2×10^{-5} M SMPB were blotted and probed with anti-dystroglycan antibody (*lane 1*) and stripped and reprobed with anti-rapsyn mAb antibody 1234 A (*lane 2*). The position of the 90-kDa cross-linked product labeled with both antibodies is indicated by *arrowheads*.

linked product (Ref. 30 and Fig. 1*A*). Moreover, an additional 90-kDa band appeared following cross-linking on Western blots probed with anti-rapsyn antibody and revealed by ECL. This band was also immunolabeled by antibodies to β-dystroglycan and, therefore, represents a rapsyn/β-dystroglycan cross-linked product (Fig. 1*A, lanes 3* and *3'* and Fig. 1*B*). Interestingly, the apparent mass of this cross-linked product (=90 kDa) corresponds almost exactly to the sum of individual polypeptide masses of rapsyn and β-dystroglycan (43 and 46–50 kDa, respectively). The specificity of the immunodetections in these experiments was attested by the fact that the major cross-linked product at 110 kDa did not cross-react with the anti-β-dystroglycan antibodies and that only one additional cross-linked product at about 140 kDa was observed upon probing with anti-β-dystroglycan. This 140-kDa product did not cross-react with anti-rapsyn antibodies (Fig. 1*A, lanes 3* and *3'*). The 140-kDa product is presently still unidentified. To ascertain that the 90-kDa product indeed resulted from cross-linking
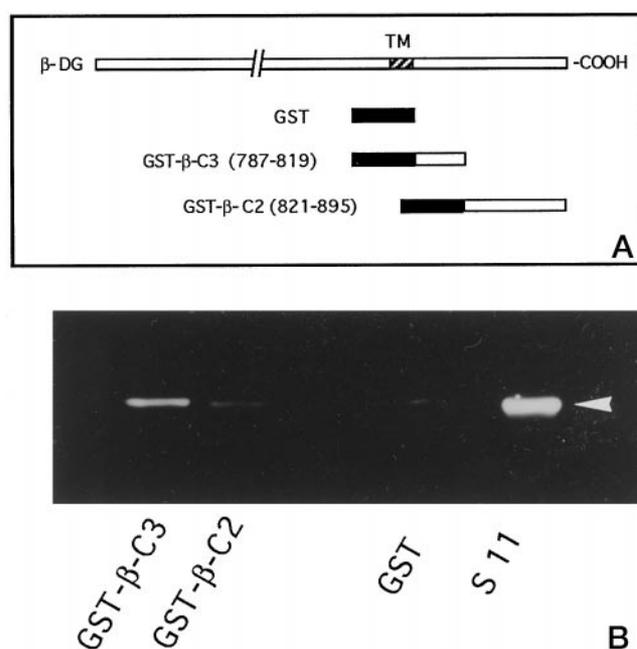
FIG. 2. *A*, schematic representation of β-dystroglycan and constructs containing β-dystroglycan C-terminal fragments. Full-length β-dystroglycan is represented by *open box*. The transmembrane sequence is indicated as *hatched*. The two GST fusion proteins (β-C3 corresponding to amino acids 787–819 and β-C2 corresponding to amino acids 821–895) covering almost the entire cytoplasmic domain of β-dystroglycan are represented in phase with full-length β-dystroglycan. *Filled boxes* indicate GST sequences. *B*, rapsyn binds to GST-β-C3 construct in affinity chromatography experiments. Rapsyn solubilized from *Torpedo* AChR-rich membranes was subjected to affinity columns including GST-β-C3, GST-β-C2, or GST control. After washing, samples were resolved on 10% SDS-PAGE, electrotransferred to nitrocellulose paper, and immunoreacted with anti-rapsyn mAb 1234 A, followed by ECL detection. The position of rapsyn (=43 kDa) in immunoblots is indicated using the starting pH 11 supernatant (*S11, arrowhead*). Rapsyn was recovered almost exclusively from GST-β-C3-conjugated Sepharose columns.

between rapsyn and β-dystroglycan, the blots previously probed with the anti-β-dystroglycan antibody were stripped and reprobed with the anti-rapsyn antibody (Fig. 1*C*). This experiment demonstrated that the 90-kDa product cross-reacted with both antibodies. Cross-linking with higher SMPB concentrations, *i.e.* with 10^{-4} M, results in the complete disappearance of rapsyn at its usual position in SDS-PAGE, whereas there is a corresponding increase in a high molecular weight material at the top of the resolving and stacking gels (Fig. 1*A, lane 4'*). In the same conditions only part of the β-dystroglycan was cross-linked (Fig. 1*A, lane 4*). This indicates that only a fraction of dystroglycan undergoes cross-linking with rapsyn. Although cross-linking experiments do not provide the demonstration that the two proteins are indeed interacting, these experiments provide support for a close proximity between rapsyn and β-dystroglycan in the postsynaptic membrane.

Rapsyn Binds to the Juxtamembranous Domain of the Cytoplasmic Tail of β-Dystroglycan in Vitro—In an attempt to investigate *in vitro* the interaction between rapsyn and β-dystroglycan, we developed an affinity chromatography assay. Rabbit brain dystroglycan cDNA which showed high homology with *Torpedo* sequences (40) was digested, and two constructs containing β-dystroglycan C-terminal fragments corresponding to the juxtamembranous and distal cytoplasmic tail domains have been generated. The schematic representation of these two β-dystroglycan constructs (β-C2 and β-C3) is shown in Fig. 2*A*. The two constructs cover almost the entire β-dystroglycan tail without redundancy. The purified fusion proteins have already

Rapsyn-β-Dystroglycan Interaction

been checked by SDS-PAGE and their molecular weights corresponded to that expected (see Ref. 28). A few lower size bands were observed for each fusion protein. These products might result from incomplete translation or from a proteolytic cleavage occurring near the C terminus, even though fusion proteins have been produced in protease-deficient *E. coli* strain (see Ref. 28 for discussion). Binding of rapsyn to β-C2, β-C3 constructs and GST control was carried out using freshly solubilized rapsyn. The rapsyn from AChR-rich membranes was solubilized by an alkali treatment that efficiently dissociates rapsyn from the membrane as well as other components of the submembranous cytoskeleton (31). Following adjustment at pH 7.6 and centrifugation, the alkali extract (S11) was incubated for 2.5 h at 4 °C with the two constructs and with GST control immobilized on glutathione-Sepharose 4B column. After extensive washings, the bound rapsyn was monitored by immunoblotting using mAb 1234 A anti-rapsyn antibody. As shown in Fig. 2*B*, rapsyn was only recovered with the GST-β-C3-conjugated column. At variance, very low rapsyn recovery was obtained with GST-β-C2 or GST columns. However, as revealed by silver staining, several polypeptides were trapped together with rapsyn on the GST-β-C3 column (data not shown). This could eventually be attributed to the aggregation of alkali-extracted polypeptides with rapsyn as a consequence of adjustment to pH 7.6 before application to the column.

Therefore, the specificity of binding of rapsyn to β-C3 remained to be demonstrated. This was carried out by ligand blot assays in order to ascertain the specificity of the interaction between rapsyn and dystroglycan. Ligand blot overlay assays were first performed with proteins from AChR-rich membrane separated by one-dimensional SDS-PAGE. Blots were then probed for dystroglycan binding using the β-C2 and β-C3 constructs as well as a construct corresponding to the C-terminal domain of α-dystroglycan (ectodomain) and GST control (see "Experimental Procedures"). After incubation with the different constructs at 1 μM, the binding was revealed using anti-GST antibodies. A unique band corresponding to a protein with molecular weight very close to that of rapsyn was observed with the GST-β-C3 construct (Fig. 3*A*). No signal was detected with the other constructs. When lower concentrations of β-C3 (*i.e.* 0.1 μM) were used in the assays, no signal was detected. Several proteins of the AChR-rich membrane are likely to display a molecular weight close to that of rapsyn in one-dimensional SDS-PAGE. The demonstration that β-dystroglycan indeed binds to rapsyn was obtained with a two-dimensional ligand blot assay. As previously shown (41–43), rapsyn comprises several (three to four) isoelectric variants (p*I* = 6.9–7.5) displaying identical peptide maps and possibly resulting from phosphorylation (see Ref. 44 for discussion). Fig. 3*B* confirms that rapsyn extracted by LiS from AChR-rich membranes exhibits discrete isoelectric spots after two-dimensional separation, cross-reacting with the anti-rapsyn mAb 1234 A. The ligand blot assay performed with GST-β-C3, GST-β-C2, GST-ectodomain, and GST control (1 μM) demonstrates that GST-β-C3 binds to all rapsyn variants (Fig. 3*B*). Again, no signal was obtained with the GST-Ecto and GST control constructs, attesting to the specificity of the binding. When considered with the results of the affinity chromatography experiments (see Fig. 2*B*), a weak binding was also observed with GST-β-C2. We estimated this binding at 5–10% that obtained with GST-β-C3.

<center>DISCUSSION</center>

In this work, we have shown that rapsyn is localized close to β-dystroglycan at the cytoplasmic aspect of the postsynaptic membrane of *Torpedo* electrocytes *in situ* and that it binds to β-dystroglycan *in vitro*. The cross-linker selected for this study
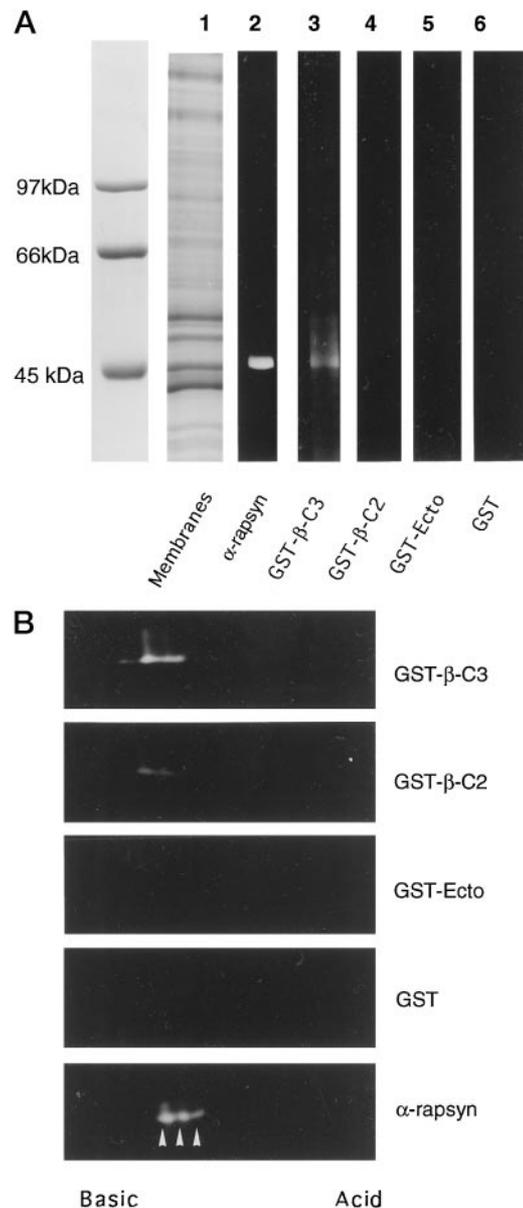
FIG. 3. **The juxtamembranous domain of β- dystroglycan binds to rapsyn in ligand blot assays.** *A*, the ligand blot assay was performed on polypeptides of the AChR-rich membranes separated by one-dimensional 8% SDS-PAGE and electrotransferred onto nitrocellulose. One μM of GST-β-C3, GST-β-C2, GST-ectodomain, or GST control fusion proteins were overlaid on nitrocellulose strips. The binding of the fusion proteins was detected with an anti-GST antibody followed by ECL detection. *Lane 1* shows the polypeptides of AChR-rich membranes (Coomassie Blue staining). *Lane 2* indicates the position of rapsyn (=43 kDa) in the same preparation by Western blotting using anti-rapsyn mAb 1234A. *Lanes 3–6* correspond to ligand blot assays with GST-β-C3, GST-β-C2, GST-Ecto, or GST control fusion proteins, respectively. GST-β-C3 binding was restricted to the 43-kDa region of the blot. *B*, ligand blot assays were performed on peripheral polypeptides of AChR-rich membranes extracted by LiS and separated on two-dimensional gels. Strips corresponding approximately to pH 6–8 and 35–60 kDa were overlaid with 1 μM GST-β-C3, GST-β-C2, GST-Ecto, or GST control fusion proteins and processed as indicated above. All the rapsyn variants reacted strongly with GST-β-C3 as compared with other constructs. However, a faint signal was detected with GST-β-C2. The positions of the rapsyn variants were indicated (*arrowheads*) on a Western blot.

which reacts both with available free sulfhydryls, essentially on rapsyn, and with primary amines on neighboring polypeptides is therefore particularly suitable for the exploration of the close environment of rapsyn in the postsynaptic membrane *in*

situ. The observation that only one product (the 90-kDa) among a very few cross-linked products cross-reacted with both anti-rapsyn and anti-β-dystroglycan antibodies strongly favors the hypothesis that rapsyn and dystroglycan are intimately associated within the membrane. In good agreement with this *in situ* approach, *in vitro* binding experiments pointed out a domain of β-dystroglycan (the juxtamembranous cytoplasmic 787–819 residues) which specifically binds to rapsyn at micromolar concentration. Interestingly, as deduced from its primary sequence (see Ref. 21 and references therein), the juxtamembranous domain of β-dystroglycan contains two clusters of lysine residues that likely account for the efficient cross-linking to rapsyn by SMPB. Our experiments thus suggests that the interaction between rapsyn and dystroglycan observed *in vitro* also occurs *in situ*. This is in full agreement with the co-distribution of the two proteins at the cytoplasmic side of the postsynaptic membrane in *Torpedo* electrocyte (19).

At variance with rapsyn which is totally cross-linked at a high concentration of SMPB, only part of dystroglycan can be cross-linked in the same conditions. This may indicate that only a fraction of dystroglycan molecules are engaged in interaction with rapsyn. Very few cross-linking products containing β-dystroglycan were observed in the resolving gels. Thus, our cross-linking experiments do not allow us to assess whether α-dystroglycan, dystrophin, or Grb2 were cross-linked with β-dystroglycan. In fact, the cross-linking agent used in this study was not designed to explore the environment of β-dystroglycan but rather that of rapsyn.

Taken together, our results raise the possibility that distinct dystroglycan complexes may exist in the postsynaptic membrane. Moreover, since rapsyn is absent from extrasynaptic sarcolemma, one could postulate that several dystroglycan complexes, differing with their association with various partners, are confined to different domains of the sarcolemma. At the synapse, the rapsyn dystroglycan interaction could likely be involved in the clustering of AChR.

Various Functions for Distinct Dystroglycan Complexes at the Sarcolemma—The ability of dystroglycans to establish multiple interactions with partners from both inside and outside the cell places these molecules at a central position in sarcolemmal organization. Dystroglycans directly contribute to the connection of the extracellular matrix with the actin-based cytoskeleton. Several signal transducing molecules, such as Grb2 (45) or nitric-oxide synthase (46), have been reported to associate with the DGC. However neither Grb2 nor NOS are part of the DGC *sensu stricto*. The present data further emphasize the property of the DGC to associate weakly and/or transiently with a variety of physiological partners in order to mediate various signals. In the synaptic membrane, the present experiments show that DGC associates with AChR via rapsyn. However, these two proteins do not co-purify with DGC after detergent extraction (38, 47), perhaps pointing to a loose association. Thus, the DGC is likely to play many more functions than previously thought.

Furthermore, dedicated regions of the cytoplasmic tail of β-dystroglycan are respectively engaged in the binding of dystrophin/apodystrophin (amino acids 880–895, see Refs. 28 and 48), Grb2 (proline-rich domains, see Ref. 45), and rapsyn (amino acids 787–819, this work, Fig. 4). In addition, α-dystroglycan binds merosin or agrin depending of its cellular localization (see Ref. 21 and references therein). As such, dystroglycan appears as the pivotal element in the DGC. In skeletal muscle fibers, the interaction of DGC with rapsyn only takes place at the postsynaptic membrane, whereas dystroglycan is distributed in extrasynaptic regions as well. A plausible hypothesis is that neural cues, *i.e.* agrin signaling, may locally alter the
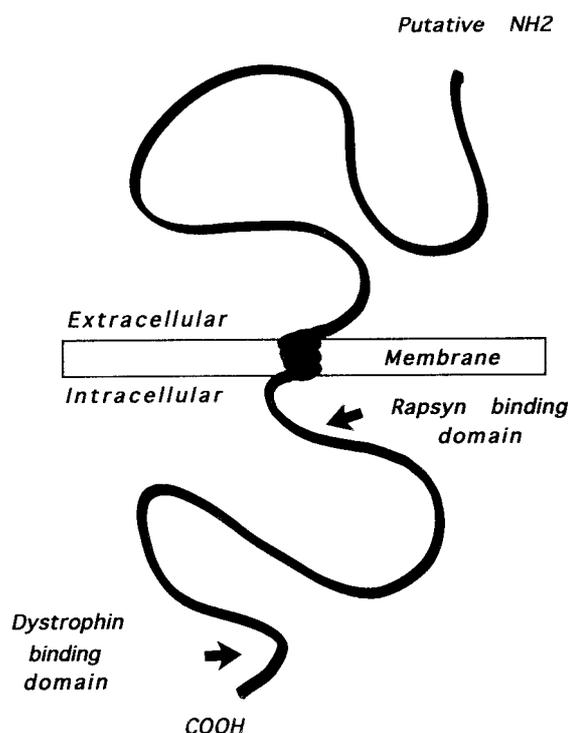
FIG. 4. **Schematic representation of structural and binding domains of β-dystroglycan.** Putative extracellular N-terminal, transmembrane, and cytoplasmic C-terminal domains are represented. The *arrows* point to rapsyn and dystrophin binding domains in the cytoplasmic tail (adapted from Jung *et al.* (48)).

associations between dystroglycan and rapsyn, thus leading to the differentiation of the postsynaptic membrane domain. Tyrosine phosphorylation on proteins are known to be involved in the signaling cascade triggered by the nerve ultimately leading to AChR clustering (49, 50). Interestingly, β-dystroglycan possesses several potential tyrosine phosphorylation sites (21, 45), suggesting that tyrosine phosphorylation may modulate the interaction with rapsyn.

Functional Implication for Rapsyn-Dystroglycan Interaction in Synaptogenesis—Several lines of evidence point to a role of the DGC in AChR clustering. Dystrophin/utrophin and components of the DGC, in particular α/β dystroglycan, co-distribute with AChR clusters both *in vivo* and *in vitro*. In co-cultured nerve and muscle cells derived from *Xenopus* embryos, Cohen *et al.* (18) reported that dystroglycan associated with synaptic as well as nonsynaptic AChR clusters and that it underwent the same nerve-induced changes in distribution as AChR. Also, during early *Torpedo* embryonic development, we observed that AChR accumulates concomitantly with dystrophin and dystroglycan in the ventral plasma membrane domain of developing electrocytes (19). Finally, co-transfection experiments in quail fibroblasts with recombinant AChR, rapsyn, and dystroglycan show that these proteins form stable complexes in the membrane and that rapsyn can cluster AChR and dystroglycan independently (26). However, utrophin-dystrophin-deficient mice that display severe progressive muscular dystrophy have moderately affected neuromuscular junctions (51, 52). Thus, synaptic differentiation could occur in the absence of dystrophin and utrophin. In these double knock-out mice, as reported by Deconinck *et al.* (51), dystrophin-associated proteins β_2-syntrophin and β-dystroglycan are retained at the NMJ, suggesting that these components might be required for AChR clustering. In this context, the present data support the notion that rapsyn-β-dystroglycan interaction is involved in synaptic differentiation.

The agrin signaling pathway at the NMJ has been the focus of intensive investigation in the past few years. Agrin has been shown to bind to α-dystroglycan with high affinity (for a review, see Ref. 53), raising the possibility that dystroglycan plays a critical role in agrin signaling at the neuromuscular junction. This hypothesis is inconsistent in light of the observation that the domain of agrin involved in binding to α-dystroglycan is distinct from the part of agrin molecule that induces AChR clustering (24, 25). At variance, the MuSK receptor complex is likely to represent at least part of the signaling agrin receptor involved in AChR clustering via a cascade of phosphorylations. The recent observations of Apel *et al.* (13) point to a model in which rapsyn has multiple interactions with MuSK and that rapsyn, in addition to its structural role, is required in an early step in MuSK signaling leading to AChR clustering. Our present data raise the attractive hypothesis that dystroglycan is part of the MuSK signaling pathway via its interaction with rapsyn.

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Evidence for *in Situ* and *in Vitro* Association between β-Dystroglycan and the Subsynaptic 43K Rapsyn Protein: CONSEQUENCE FOR ACETYLCHOLINE RECEPTOR CLUSTERING AT THE SYNAPSE

Annie Cartaud, Sébastien Coutant, Tamara C. Petrucci and Jean Cartaud