Rapsyn, a 43-kDa peripheral membrane protein of skeletal muscle, is essential for clustering nicotinic acetylcholine receptors (nAChR) in the postsynaptic membrane. Previous studies with rapsyn NH2-terminal fragments fused to green fluorescent protein, expressed in 293T cells along with nAChRs, establish the following: Rapsyn-(1–90), containing the myristoylated amino terminus and two tetratricopeptide repeats (TPRs), was sufficient for self-association at the plasma membrane; rapsyn-(1–287), containing seven TPRs, did not cluster nAChRs; whereas rapsyn-(1–360), containing a coiled-coil domain (rapsyn-(298–331)), clustered nAChRs. To further analyze the role of rapsyn structural domains in self-association and nAChR clustering, we have characterized the clustering properties of additional rapsyn mutants containing deletions and substitutions within the TPR and coiled-coil domains. A mutant lacking the coiled-coil domain alone (rapsyn-(A288–348)), failed to cluster nAChRs. Within the coiled-coil domain neutralization of the charged side chains was tolerated, while alanine substitutions of large hydrophobic residues resulted in the loss of nAChR clustering. Rapsyn self-association requires at least two TPRs, as a single TPR (TPR1 or TPR2 alone) was not sufficient. While TPRs 1 and 2 are sufficient for self-association, they are not necessary, as TPRs 3–7 also formed clusters similar to wild-type rapsyn. Fragments containing TPRs co-localized with full-length rapsyn, while the expressed coiled-coil or RING-H2 domain did not. These results are discussed in terms of a homology model of rapsyn, based on the three-dimensional structure of the TPR domain of protein phosphatase 5.

A characteristic and important feature of the vertebrate neuromuscular junction is the clustering of nicotinic acetylcholine receptors (nAChR)1 at high surface density (<10,000/μm2) in the postsynaptic membrane underlying the nerve terminal, with the nAChR surface density decreasing by a factor of 100–1000 within a few microns. Rapsyn, a peripheral membrane protein of muscle, plays a critical role in the clustering of nAChRs and in the organization of the postsynaptic cytoskeletal complex (for a recent review, see Ref. 1). Mutant mice lacking rapsyn show severe neuromuscular abnormality marked by the absence of nAChR clusters in the postsynaptic membrane as well as the absence of cytoskeletal components such as utrophin and dystroglycan (2).

When expressed in nonmuscle cells, rapsyn forms membrane-associated clusters and recruits nAChRs to these clusters (3–6). Furthermore, when coexpressed in fibroblasts rapsyn also clusters β-dystroglycan, the integral membrane protein of the dystrophin complex (7), as well as the agrin receptor, the receptor tyrosine kinase MuSK (8–10). Biochemical studies provide evidence for a direct interaction between rapsyn and the cytoplasmic domain of β-dystroglycan (11).

The three-dimensional structure of rapsyn is not known. However, rapsyn primary structure suggests the presence of distinct structural domains (summarized in Fig. 1). The amino terminus of rapsyn is myristoylated (12, 13). Rapsyn-(6–319) is predicted to form 8 tetratricopeptide repeats (TPRs) (14), while rapsyn-(298–331) contains a putative coiled-coil domain (6). The COOH-terminal cysteine-rich domain of rapsyn between amino acids 363 and 402 is predicted to be a RING-H2 domain (15). Rapsyn amino acids 403–406 contain a consensus sequence for phosphorylation by both protein kinase A and protein kinase C.

We recently provided a first characterization of the rapsyn structural domains involved in membrane targeting, self-association, and nAChR clustering (6). Our results indicated that rapsyn NH2-terminal fatty acylation is both required and sufficient for membrane targeting and that rapsyn-(1–90), containing two TPR domains, is sufficient for self-association. In addition, while rapsyn-(1–360) clustered nAChRs, rapsyn-(1–287) did not, indicating that rapsyn-(287–360) is important for nAChR clustering. Within rapsyn-(287–360) we identified a putative coiled-coil domain, rapsyn-(298–331), and showed that disruption of the coiled-coil propensity by alanine insertions prevented nAChR clustering. These results indicated that the COOH-terminal rapsyn RING-H2 domain and the adjacent phosphorylation sites were not essential for either rapsyn or nAChR clustering.

We report here further mutational analyses of rapsyn to elucidate the role of the coiled-coil domain in nAChR clustering and the role of the TPRs in rapsyn self-association. These rapsyn mutants, fused at their COOH termini to GFP, were expressed transiently in 293T cells along with nAChR subunits and were visualized by fluorescence microscopy 24–36 h after transfection. The results indicate that nAChR clustering depends critically on the structure of the hydrophobic surface of the coiled-coil domain but not on the hydrophilic surface. While TPRs mediate rapsyn self-association, nAChR clustering is retained in mutants lacking as many as four TPRs.

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‡ The abbreviations used are: nAChR, nicotinic acetylcholine receptor; GFP, green fluorescent protein; TPR, tetratricopeptide repeat; PCR, polymerase chain reaction.

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RING-H2 domain, and the consensus sequence for phosphorylation.

EXPERIMENTAL PROCEDURES

Materials and Methods
All restriction enzymes were purchased from New England Biolabs except BsaI (Life Technologies, Inc.). 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 template pGL-rapsyn (mouse rapsyn subcloned into pGreenLantern vector; Life Technologies, Inc.). 50–100 µmol of each primer, 250 µM each dNTP, and 5 units of Pfu DNA polymerase (Stratagene) for 30 cycles at 94 °C for 2 min, 50 °C for 1 min, and 72 °C for 1 min. All constructs were tested both by restriction enzyme analysis and by sequencing across the full length of the inserted fragments.

Plasmid Construction and Mutagenesis

Rapsyn-(1–41)-GFP—This construct encodes rapsyn amino acid sequence 1–41 containing the consensus sequence for N-myristylation and TPR1. Primers GACACTATAGAAGGTACG and GCAAGTACTGCGTGTGGATGAAGCAGCTGGAGAAGGGCTCT and GCCATCCAGTCTCAGTCTAGCAGTCTACGACTCCGCTATGAGC were used along with pGL-rapsyn-(1–412)-GFP (6) as a template in a PCR to amplify rapsyn amino acids 1–41. The PCR product with SpeI and SalI ends was subcloned into pBCKMV vector (Stratagene). The resulting plasmid was digested with SpeI and NotI, and the fragment containing the rapsyn sequence was subcloned into pGL vector lacking the GFP insert (made by digesting pGreenLantern with NotI, removing the GFP insert, and religating the vector fragment). GFP cDNA was then inserted at the NotI site to result in pGL-rapsyn-(1–41)-GFP.

Rapsyn-(1–412)-GFP—This construct encodes rapsyn amino acid sequence 1–41 containing the consensus sequence for N-myristylation fused in-frame to amino acids 33–90 (TPR2). Primers GTGTTGATAGAAGGTAGGAGAAGGGCTCT and GCCATCCAGTCTCAGTCTAGCAGTCTACGACTCCGCTATGAGC were used along with pGL-rapsyn-(1–412)-GFP (6) in a PCR to amplify rapsyn amino acids 1–41. The PCR product with SpeI and SalI ends was subcloned into pBCKMV vector (Stratagene). The resulting plasmid was digested with SpeI and NotI, and the fragment containing the rapsyn sequence was subcloned into pGL vector lacking the GFP insert (made by digesting pGreenLantern with NotI, removing the GFP insert, and religating the vector fragment). GFP cDNA was then inserted at the NotI site to result in pGL-rapsyn-(1–412)-GFP.

Rapsyn-(1–412)-GFP—This construct encodes full-length rapsyn fused with GFP. Primers AGATATAGAATTACCTGGCCACATCGACGCTGTGTTGCTGGTGTTGCAATGGCTGGGATGATGGCCTGCAGTTCCAGAAGGTAGGAGAAGGGCTCT and GCCATCCAGTCTCAGTCTAGCAGTCTACGACTCCGCTATGAGC were used along with pGL-rapsyn-(1–360)-GFP (6), which includes these 20 amino acids. Primers GACACTATAGAAGGTACG and GCAAGTACTGCCTGTTCCAGAAGGGCTCT and GCCATCCAGTCTCAGTCTAGCAGTCTACGACTCCGCTATGAGC were used along with pGL-rapsyn as a template in a PCR to amplify rapsyn cDNA sequence from 859 to 1020. The resulting PCR product was digested with SpeI and XbaI and then inserted into pGL vector.

Rapsyn-(298–331)-GFP—This construct encodes for rapsyn amino acids 298–331, which was obtained by digesting pGL-rapsyn with PvuII and NotI. GFP cDNA was then inserted at the NotI site to result in pGL-rapsyn-(298–331)-GFP.

Rapsyn-(1–412)-GFP—This construct encodes full-length rapsyn with a deletion of amino acids 16–90 (TPRs 1 and 2). The cDNA fragment of mouse rapsyn from NotI (43) to BstUI (269) was deleted. Following digestion of pGL-rapsyn-(1–412)-GFP with PvuII, the vector and insert fragments were isolated. The insert fragment consisting of rapsyn sequence was further digested with BstUI and NotI. The vector fragment was digested with NotI. The vector fragment with PvuII-NotI ends was then ligated with the insert fragment with BstUI-NotI ends, resulting in pGL-rapsyn-(1–412)-GFP. GFP cDNA was then inserted at the NotI site to generate pGL-rapsyn-(1–412)-GFP.

Rapsyn-(351–411)-GFP—This construct encodes full-length rapsyn with a deletion of amino acids 91–254 (TPRs 3–6). The cDNA fragment of mouse rapsyn from NotI (22) to EcoRV (760) was deleted. Following digestion of pGL-rapsyn-(1–340)-GFP with SpeI and EcoRV, the vector and insert fragments were isolated. The insert fragment consisting of rapsyn sequence was further digested with EcoRI and ligated with the vector fragment with SpeI-EcoRV ends, resulting in pGL-rapsyn-(351–411)-GFP.

Rapsyn-(1–340)-GFP—This construct encodes rapsyn amino acids 1–340, terminates immediately after the coiled-coil domain and lacks the RING-H2 domain as well as the 20 amino acids between the coiled-coil and the RING-H2 domains. This construct differs from rapsyn-(1–360)-GFP (6), which includes these 20 amino acids. Primers TGAGGCCGCAGCCGCCTCTTGAAGCTCCTTTTGCAGGTAGATGTGCTTCCACT and TACGACTCCGCTATGAGC were used along with pGL-rapsyn as a template in a PCR to amplify rapsyn cDNA sequence from 1045 to 1236. The underlined letters denote the introduced XbaI site. The resulting PCR product was digested with PmlI and XbaI, and it was substituted in place of the PmlI-XbaI fragment of pGL-rapsyn-GFP, resulting in pGL-rapsyn-(1–340)-GFP.

Rapsyn-(1–91)-GFP—This construct encodes a rapsyn mutant that lacks TPRs 1 and 2, the coiled-coil domain, and the RING-H2 domain. The NH2-terminal sequence of pGL-rapsyn-(1–287)-GFP (6) between SpeI and EcoRV was removed and replaced with that from pGL-rapsyn-(1–91)-GFP.

Rapsyn-(1–287)-GFP—This construct encodes the coiled-coil domain of rapsyn in fusion with GFP. Primers AGATATAGAATTACCTGGCCACATCGACGCTGTGTTGCTGGTGTTGCAATGGCTGGGATGATGGCCTGCAGTTCCAGAAGGTAGGAGAAGGGCTCT and GCCATCCAGTCTCAGTCTAGCAGTCTACGACTCCGCTATGAGC were used along with pGL-rapsyn-(1–287)-GFP as a template in a PCR to amplify rapsyn cDNA sequence from 859 to 1020. The resulting PCR product was digested with SpeI and XbaI and then inserted into pGL vector.

Rapsyn-(298–331)-GFP—This construct encodes for rapsyn amino acids 298–331, which was obtained by digesting pGL-rapsyn-(1–360)-GFP (described previously (6)) with SpeI and EcoRV. The NH2-terminal sequence (SpeI-EcoRV) of rapsyn was swapped with that of rapsynn4 resulting in pGL-rapsyn-(298–331)-GFP.

Rapsyn-(351–411)-GFP—This construct encodes the rapsyn domain of rapsyn in fusion with GFP. Primers AGATATAGAATTACCTGTTGGCCACATCGACGCTGTGTTGCTGGTGTTGCAATGGCTGGGATGATGGCCTGCAGTTCCAGAAGGTAGGAGAAGGGCTCT and GCCATCCAGTCTCAGTCTAGCAGTCTACGACTCCGCTATGAGC were used along with pGL-rapsyn-(351–411)-GFP as a template in a PCR to amplify rapsyn cDNA sequence from 1045 to 1236. The underlined letters denote the introduced SpeI site. The resulting PCR product was digested with SpeI and XbaI, and then inserted into pGL vector resulting in pGL-rapsyn-(351–411)-GFP.

Rapsyn-(298–331)-GFP—This construct encodes full-length rapsyn with a deletion of amino acids 16–90 (TPRs 1 and 2). The cDNA fragment of mouse rapsyn from NotI (43) to BstUI (269) was deleted. Following digestion of pGL-rapsyn-(1–412)-GFP with PvuII, the vector and insert fragments were isolated. The insert fragment consisting of rapsyn sequence was further digested with BstUI and NotI. The vector fragment was digested with NotI. The vector fragment with PvuII-NotI ends was then ligated with the insert fragment with BstUI-NotI ends, resulting in pGL-rapsyn-(298–331)-GFP. GFP cDNA was then inserted at the NotI site to generate pGL-rapsyn-(298–331)-GFP.

Rapsyn-(1–412)-GFP—This construct encodes full-length rapsyn with a deletion of amino acids 194–254 (TPR 6). The cDNA fragment of mouse rapsyn from HindIII (577) to EcoRV (760) was deleted. Following digestion of pGL-rapsyn-(1–412)-GFP with SpeI and EcoRV, the vector and insert fragments were isolated. The insert fragment consisting of rapsyn sequence was further digested with HindIII and ligated with the vector fragment with SpeI-EcoRV ends, resulting in pGL-rapsyn-(194–254)-GFP.

Rapsyn-(1–287)-GFP—This construct encodes full-length rapsyn with a deletion of amino acids 255–287 (TPR 7). The cDNA fragment of mouse rapsyn from EcoRV (760) to PmlI (859) is deleted. pGL-rapsyn-(1–412)-GFP was digested with EcoRV and PmlI. The vector fragment consisting of rapsyn sequence was isolated and religated, resulting in pGL-rapsyn-(255–287)-GFP.

Rapsyn-(1–287)-GFP—This construct encodes full-length rapsyn with a deletion of amino acids 288–348 which contain the coiled-coil domain (6). The cDNA fragment of mouse rapsyn between BsaAI sites (6) was amplified with full-length of the inserted fragments.

Rapid Prototyping and Coiled-coil Domains

FIG. 1. Schematic diagram of the structural domains of rapsyn. Indicated are the myristoylated NH2 terminals (N-myr) and the borders of the seven putative TPRs, the coiled-coil domain, the RING-H2 domain, and the consensus sequence for phosphorylation.
Expression of Rapsyn, Rapsyn Mutants, and nAChR in 293T Cells

Transfection of 293T cells by the calcium phosphate method, cell staining, and immunofluorescence experiments were done as described (6), except that the staining was done on cells fixed for 20 min with 2% paraformaldehyde followed by an incubation with a buffer containing PBS, 10% calf serum, 4% bovine serum albumin, and 100 mM L-lysine for 1 h. Under these conditions, α-bungarotoxin binding to surface nAChRs was similar to that of unfixed cells, and the distribution of nAChRs on the cell surface was generally uniform with submicron granularity. Most of the rapsyn clusters ranged from 1 to 5 μm in size with occasional larger plaques of 5–10 μm. Cells were visualized using a Nikon Eclipse E600 epifluorescence microscope with a Nikon 100X Plan Fluor objective (NA1.2). Green and red fluorescence were visualized through Nikon 91617 (excitation, 480 nm) and Nikon 91617 (excitation, 540 nm) filters (excitation, 540 nm) through Nikon 91617 (excitation, 480 nm) and Nikon 91617 (excitation, 540 nm) filters, respectively. In some experiments photographs were taken with Kodak 160T film and digitized. Most of the images were acquired with a Micromax CH250 CCD camera (Princeton Instruments) using MetaMorph software. Figures were prepared from the digitized images using Adobe Photoshop.

Quantification of Cells with Rapsyn or nAChR Clusters

In general, within each cell expressing both rapsyn and nAChR, not all rapsyn clusters are associated with nAChRs. This is expected since only the surface nAChRs are labeled with α-bungarotoxin, while rapsyn-GFP is distributed at the plasma membrane and also intracellularly. For experiments involving expression of rapsyn-GFP (or mutants) and nAChRs in 293T cells, quantification of nAChR clusters relative to rapsyn clusters was done as follows. In each experiment, 100 cells positive for both rapsyn and nAChR expression were identified. For these we quantified the number of cells with nAChRs that co-localized with rapsyn clusters and the number of cells with nAChRs distributed diffusely. In experiments involving rapsyn mutants with TPR deletions, in some cells nAChRs were seen both clustered with rapsyn and also distributed diffusely on the surface. For quantification, these cells were classified as containing clustered nAChRs. Each experiment was repeated at least four times, cells were scored as above, and the results are presented as the % of cells with nAChR clusters (mean and S.D.).

Molecular Modeling of Rapsyn

All molecular modeling studies were done with Insight II, Version 98 (MSI, San Diego, CA) on a Silicon Graphics IRIS work station. The mouse rapsyn TPR domain was homology modeled based upon the three-dimensional crystal structure of the TPR domain of protein phosphatase 5 (17). The coordinates of this domain, containing 3 TPRs, were obtained from the Brookhaven Protein Data Base (PDB number 1A17). The sequences of the rapsyn TPRs 1–7 (14) were used sequentially to create the model. TPRs 1–3 and 4–6 were each modeled individually, and then they were combined in register. TPR 7, initially modeled with TPR 8, was then added. For rapsyn, the intervening sequences between TPRs 2 and 7 each contain 6–9 amino acids, while in protein phosphatase 5 there are no residues between TPRs. For rapsyn random loops were generated for these sequences, and these loops were then fused with the TPR structures. Rapsyn amino acids 286–331 were modeled as an energy minimized, single long helix, based on our previous prediction that rapsyn 288–331 has a high propensity to be organized as an α-helical coiled-coil (6). The orientation of the coiled-coil domain with respect to the TPRs was done arbitrarily and represents only one of the possibilities. The entire rapsyn model was then energy minimized using the Discover module, first with steepest descent algorithm and then by conjugate gradient algorithm until the energy of the entire molecule was low.

RESULTS

Rapsyn Coiled-coil Domain Is Essential for Clustering nAChRs—Previously we had shown (6) that rapsyn-(1–287)-GFP could self-associate but not cluster nAChRs, while rapsyn-(1–360)-GFP could cluster nAChRs. Furthermore, rapsyn amino acids 286–331 were predicted to have a high propensity to form an α-helical coiled-coil, and alanine insertions within this region that disrupted coiled-coil propensity also disrupted rapsyn’s ability to cluster nAChRs without affecting rapsyn self-association. To further address the importance of the rapsyn coiled-coil domain in nAChR clustering, we made two additional constructs: (i) rapsyn-(1–340)-GFP, with the rapsyn sequence terminated immediately after the coiled-coil domain; and (ii) rapsyn-(1–340)-GFP, with full-length rapsyn with the coiled-coil domain deleted (Fig. 2a). When expressed in 293T cells, rapsyn-(1–340)-GFP formed distinct membrane-associated clusters in all transfected cells and clustered nAChRs at the cell surface (Fig. 2, b and c) in 95 ± 5% of cells expressing
both proteins. The appearance of rapsyn/nAChR clusters was indistinguishable from that of wild-type rapsyn. In contrast, the construct lacking the coiled-coil domain, rapsyn-(1–15, 91–340)-GFP, formed membrane-associated clusters similar to wild type but did not cluster nAChRs (Fig. 2, d and e). When nAChR distribution was quantified in six separate transfection experiments, nAChRs were clustered with rapsyn in only 8 ± 2% of cells. These results establish that the coiled-coil domain is essential for nAChR clustering, while the adjacent amino acids 340–360 of rapsyn are not necessary.

The Structure of Hydrophobic Face of the Rapsyn Coiled-coil Domain Is Important for nAChR Clustering—Rapsyn-(298–331) has a high propensity to organize as a coiled-coil domain, since it can form a 10-turn amphipathic α-helix with a hydrophobic moment of 0.6 (6) (Fig. 3a). When modeled as a right-handed α-helix, the amphipathic character of the helix is clearly visualized with a hydrophobic face (Fig. 3b, left) and a hydrophilic face (Fig. 3b, right). Striking features of the hydrophilic surface are the presence of a strip of six acidic side chains forming a continuous surface over four helix turns and the presence of 5 lysine residues spaced to form a lysine ladder. These lysines are conserved in rapsyn from different species. The hydrophilic surface, which extends over 10 turns, consists of 5 alanine residues in the first four helical turns and nine bulky side chains (Leu, Ile, and Val).

To analyze the importance of the particular amino acids that contribute to the hydrophobic and hydrophilic helix surfaces, three constructs with point mutations were tested (Fig. 4a): (i) rapsyn-(298–331)-ED → QN-GFP, full-length rapsyn fused to GFP with the 3 Glu and 2 Asp within amino acids 298–331 mutated to Gln and Asn, respectively; (ii) rapsyn-(298–322)-KR → Q-GFP, with the 4 Lys and 1 Arg within amino acids 298–322 all replaced by Gln; (iii) rapsyn-(298–331)-LIV → A-GFP, with the 6 Leu, 1 Ile, and 2 Val within amino acids 298–331 all replaced by alanine. None of these substitutions altered the coiled-coil propensity as calculated by the COILS program (18) (data not shown). When expressed in 293T cells, rapsyn-(298–331)-ED → QN-GFP formed distinct clusters in all transfected cells. nAChRs were clustered with rapsyn at the surface (Fig. 4, b–d) in 72 ± 7% cells expressing both proteins and were diffusely distributed in the other cells. Similarly, rapsyn-(298–322)-KR → Q-GFP also formed clusters in all transfected cells, and nAChRs were associated with these clusters (Fig. 4, e–g) in 81 ± 11% cells expressing both proteins. In contrast, while rapsyn-(298–331)-LIV → A-GFP did form clusters at the cell surface in all the cells, only in 17 ± 8% of these cells were nAChRs associated with any of these clusters. In a representative cell (Fig. 4, h–j) nAChRs are not associated with rapsyn clusters although there is a microgranular distribution of nAChRs in some parts of the cell surface (see also Ref. 6). These results establish that despite the conservation of the charged residues in rapsyn-(298–331), their presence is not necessary for nAChR clustering. In contrast, alteration of the structure of the hydrophobic surface results in the lack of nAChR clustering.

Rapsyn Self-association Requires Two TPRs—Previous results had shown that rapsyn-(1–90) containing the myristoylated amino terminus and the first two TPRs was sufficient for rapsyn self-association (6). To identify the minimal structural requirements for rapsyn self-association, we also created chimeric proteins consisting of rapsyn-(1–41), encoding N-Myr and TPR1, or rapsyn-(1–15, 91–330) encoded N-Myr and TPR2, each fused at its COOH terminus to GFP. Furthermore, to analyze the specific requirements of TPRs 1 and 2 in rapsyn self-association, we created rapsyn-(16–90)-GFP, which encodes a full-length rapsyn with TPRs 1 and 2 deleted, as well as rapsyn-(1–15, 91–287)-GFP, encoding TPRs 3–7, and rapsyn-(1–15, 91–340)-GFP, containing TPRs 3–7 and the coiled-coil domain (Fig. 5a). When expressed in 293T cells, rapsyn-(1–90)-GFP formed distinct clusters (Fig. 5b). However, constructs containing myristoylated TPR1 (rapsyn-(1–41)-GFP) or TPR2 (rapsyn-(1–15, 33–90)-GFP), although at least in part targeted to the plasma membrane, failed to form any clusters in more than 98% of transfected cells (Fig. 5, c and d, respectively). In all the cells expressing rapsyn-(1–41)-GFP or rapsyn-(1–15,
a granular distribution of nAChRs clearly not co-localized with rapsyn—
with nAChRs co-clustered in only 17% cells. Formed clusters in all the cells, and surface nAChRs were co-localized in 81% cells. For this cell there was a granular distribution of nAChRs clearly not co-localized with rapsyn—
33–90)-GFP, in addition to fluorescence at the plasma membrane, a fraction was from within the cell. Rapsyn-(1–90)-GFP, which lacked TPRs 1 and 2 but contained TPRs 3–7 and the coiled-coil and RING-H2 domains, formed distinct clusters similar to wild-type rapsyn in all cells (Fig. 4e). Rapsyn self-association was clearly a property of the TPR domain, since similar clustering was also seen for rapsyn-(1–15, 91–287)-GFP (Fig. 5f) and for rapsyn-(1–15, 91–340)-GFP (Fig. 5g).

The Interaction of Rapsyn Domains with Wild-type Rapsyn—We also characterized the capacity of constructs encoding rapsyn domains to associate with wild-type, full-length rapsyn at the cell surface. The distribution of rapsyn domains fused to GFP was visualized by fluorescein isothiocyanate optics, while wild-type rapsyn was visualized by the binding of rhodamine-conjugated goat anti-mouse antibody to mAb 19F4A, a mouse monoclonal that recognizes rapsyn of many species (16). Based upon epitope mapping studies with Torpedo rapsyn, mAb194A recognizes a COOH-terminal epitope (rapsyn-(396–411)),3 and based upon immunofluorescence it did not recognize rapsyn-(1–360) or other smaller fragments expressed in 293T cells (data not shown). When coexpressed with full-length rapsyn, rapsyn-(1–15)-GFP was distributed diffusely at the cell surface, but it was not associated with the rapsyn clusters (Fig. 6a and b). Rapsyn-(1–90)-GFP, containing TPRs 1 and 2, formed clusters, as previously shown (Fig. 5 and Ref. 6), and these clusters were co-localized with the clusters of full-length rapsyn (Fig. 6a and c). Rapsyn-(1–90)-GFP, which lacks the myristylation consensus sequence and the RING-H2 domain but contains TPRs 1–7 and the coiled-coil domain, formed clusters co-localized with the rapsyn clusters (Fig. 6a and d). Rapsyn-(287–340)-GFP, comprising the coiled-coil domain, was distributed diffusely at the cell surface, but it was not associated with the rapsyn clusters (Fig. 6a and d). Rapsyn-(1–90)-GFP, which lacks the myristylation consensus sequence and the RING-H2 domain but contains TPRs 1–7 and the coiled-coil domain, formed clusters co-localized with the rapsyn clusters (Fig. 6a and d). Rapsyn-(287–340)-GFP, comprising the coiled-coil domain, was distributed diffusely at the cell surface, but it was not associated with the rapsyn clusters (Fig. 6a and d). Rapsyn-(1–15, 91–287)-GFP, which lacked TPRs 1 and 2, and the RING-H2 domain, formed clusters similar to wild-type rapsyn (f and g, respectively). Bar = 10 µm.

FIG. 4. Mutational analysis of the rapsyn coiled-coil domain. a, schematic representation indicating the substitutions made of hydrophobic and charged side chains in the coiled-coil domain. b-j, 293T cells were transfected with cDNAs encoding rapsyn mutants tagged with GFP, along with nAChR subunits. Distributions of rapsyn (left) and surface nAChRs (center) were visualized in cells expressing both proteins, and overlaps of these two images are shown in the right panel (rapsyn (green), nAChRs (red)). b-d, rapsyn-(298–331)-ED → QN formed clusters in all the cells, and surface nAChRs were co-localized with these clusters in 72 ± 7% cells. e-g, rapsyn-(298–322)-KR → Q formed clusters in all the cells with surface nAChRs co-localized in 81 ± 11% cells. h-j, rapsyn-(298–331)-LIV → A formed clusters in all the cells with nAChRs co-clustered in only 17 ± 8% cells. For this cell there was a granular distribution of nAChRs clearly not co-localized with rapsyn-(298–331)-LIV → A (bar = 10 µm.)

FIG. 5. Rapsyn self-association requires at least two TPRs. a, schematic representation of the constructs. b-g, cDNAs encoding rapsyn-(1–90)-GFP (b), rapsyn-(1–41)-GFP (c), rapsyn-(1–15, 33–90)-GFP (d), rapsyn-(1–15, 91–287)-GFP (e), rapsyn-(1–15, 91–287)-GFP (f), or rapsyn-(1–15, 91–340)-GFP (g) were transiently transfected into 293T cells. GFP containing proteins were visualized with fluorescein isothiocyanate optics. Rapsyn-(1–90)-GFP with two TPR motifs formed membrane-associated clusters (b), while rapsyn-(1–41)-GFP (c), or rapsyn-(1–15, 33–90) (d), which consists of either TPR1 or TPR2, respectively, did not form clusters. Rapsyn-(16–90)-GFP, lacking TPRs 1 and 2, formed distinct membrane-associated clusters (e), similar to rapsyn-(1–90)-GFP. Similarly, rapsyn-(1–15, 91–287)-GFP, which lacked TPRs 1 and 2, the coiled-coil domain, and the RING-H2 domain, or rapsyn-(1–15, 91–340)-GFP, which lacked TPRs 1 and 2, and the RING-H2 domain, formed clusters similar to wild-type rapsyn (f and g, respectively). Bar = 10 µm.

3 J. Cohen, unpublished observations.
specific TPRs were created and expressed in 293T cells along with nAChRs (Fig. 7a). nAChRs were clustered by rapsyn-(1–15)-GFP, the construct lacking the first two TPRs, in 62 ± 6% of cells expressing both proteins, with nAChRs distributed uniformly in other cells. Fig. 7, b and c, show representative images of a cell containing clustered nAChRs, and Fig. 7, d and e, show a cell where nAChRs, distributed on the surface with some granularity, are not associated with the rapsyn clusters. Similarly, rapsyn-(16–90)-GFP, a construct lacking TPRs 3–6, formed distinct clusters in all the cells, with nAChRs co-clustered (Fig. 7, f and g) in 44 ± 18% of cells. Other rapsyn mutants lacking either TPR6 (rapsyn-(194–254)-GFP; Fig. 7, h and i) or TPR7 (rapsyn-(255–287)-GFP; Fig. 7, j and k) were clustered in all the cells but clustered nAChRs in 52 ± 11 and 41 ± 15%, respectively. These results indicate that a mutant rapsyn containing as few as three TPRs (TPRs 1, 2, and 7) is able to cluster nAChRs but that neither TPR 1, 2, or 7 is required. However, for these TPR deletion mutants there were two populations of cells, those with nAChR clusters and those without. Further studies are required to identify the differences between these two groups.

**Nuclear Localization of Non-myristoylated Rapsyn—** Rapsyn-(G2A)-GFP, with the amino-terminal glycine mutated to alanine to prevent N-myristoylation, is preferentially targeted to the nucleus (6, 20). To determine whether it is the COOH-terminal region of rapsyn containing the RING-H2 domain that contributes to the nuclear retention of rapsyn-(G2A)-GFP, we examined the distribution in 293T cells of rapsyn-(G2A-360)-GFP that contained the G2A mutation and lacked the RING-H2 domain (amino acids 361–412; Fig. 8a). When coexpressed with nAChRs in 293T cells, as expected, rapsyn-(G2A)-GFP was targeted to the nucleus, while nAChRs were distributed diffusely on the plasma membrane (Fig. 8, b and c). In contrast, rapsyn-(G2A-360)-GFP was distributed in the cytoplasm and excluded from the nucleus, with occasional enrichment at the surface. Most of the nAChRs appeared uniformly distributed on the cell surface (Fig. 8, d and e).

**DISCUSSION**

The results presented here extend our understanding of the role of the rapsyn coiled-coil and TPR domains in nAChR clus-
The nuclear localization of G2A-rapsyn requires the presence of the rapsyn RING-H2 domain. a, schematic representation of the constructs. b–e, 293T cells were co-transfected with cDNAs encoding rapsyn mutants, each fused to GFP at their COOH terminus, and the nAChR subunits. Distributions of rapsyn (left) and surface nAChRs (right) were visualized in cells expressing both proteins. Rapsyn-(G2A)-GFP (b) was targeted to the nucleus, while nAChRs (c) were distributed diffusely in the plasma membrane. G2A-rapsyn-(1–293)-GFP (d) was distributed as intracellular aggregates but was excluded from the nucleus, while nAChRs (e) were distributed diffusely in the plasma membrane. Bar = 10 μm.

The Rapsyn Coiled-coil Domain and nAChR Clustering—

Mutational analyses of the hydrophobic and hydrophilic surfaces of the rapsyn coiled-coil domain establish that it is the specific structure of the hydrophobic surface that is crucial for rapsyn to cluster nAChRs (Fig. 4). A classical coiled-coil domain is characterized by a heptad repeat sequence \((a \ b \ c \ d \ e \ f \ g)\), in which amino acids at positions \(a\) and \(d\) are typically hydrophobic, residues at \(e\) and \(g\) are often charged, and those at the solvent-exposed \(b\), \(c\), and \(f\) positions are predominantly polar. In the rapsyn coiled-coil domain there is a continuous hydrophobic surface (Fig. 3b) defined by side chains at position \(d\) (Ala\(^1\), Ala\(^6\), Ala\(^8\), Val\(^{22}\), and Leu\(^{29}\)) and position \(a\) (Ile\(^{12}\), Ala\(^{19}\), and Leu\(^{26}\)), along with Val\(^{3}\) and Leu\(^{4}\) (position \(g\)) and Leu\(^{5}\) (position \(e\)).

Finally, it was surprising that either the charge-neutralizing substitutions of the highly conserved positively charged residues (lysine ladder) or the negatively charged residues had no significant effect on nAChR clustering. While our results suggest clearly that charges are not required at these positions, it is possible that the size, hydrophilicity, or hydrogen bonds forming capabilities of the side chains at these positions are important. However, the high degree of charge conservation at these positions in rapsyn from species ranging from human to Caenorhabditis elegans suggests that these side chains may be involved in ionic interactions contributing to the stability of a helix bundle as seen in other homo-oligomeric or hetero-oligomeric coiled-coil structures (22, 23). Significantly, rapsyn-(287–340)-GFP, which contains only the coiled-coil domain, did not interact with full-length rapsyn. This result indicates that the coiled-coil domain is unlikely to self-associate, and it is consistent with the hypothesis that the rapsyn coiled-coil domain associates with a coiled-coil domain in the nAChR.

There is growing evidence for direct interaction between rapsyn and nAChRs. Early chemical cross-linking studies and freeze fracture immunoelectron microscopy both supported the view that rapsyn is in close proximity to the cytoplasmic domains of nAChR (24, 25) (for a review, see Ref. 26). The 4.6-A structure recently determined by electron microscopy of tubular crystals of Torpedo postsynaptic membranes included on the cytoplasmic surface electron dense regions immediately underneath the nAChR which were attributed to rapsyn (27). nAChR subunits expressed individually in nonmuscle cells can be clustered by rapsyn (28), and recent studies indicate that this clustering is mediated by the large cytoplasmic loop between the M3 and M4 transmembrane regions of the nAChR α subunit (29). In addition, overlay experiments with \(^{125}\)I-rapsyn suggest that rapsyn binds to itself with high affinity and to full-length nAChR α, γ, or ε subunit, but not to truncated β or δ subunit lacking the cytoplasmic loop (30).

Multiple Determinants in the TPR Domain Mediate Rapsyn Self-association—Analysis of the clustering properties of rapsyn mutants containing deletions within the predicted TPR domain as well as the clustering properties of other GFP-tagged rapsyn domains strengthens the hypothesis that rapsyn does contain TPRs that mediate self-association. Furthermore, rapsyn self-association requires at least two TPRs. While rapsyn-(1–90) was sufficient for self-association, rapsyn constructs consisting of N-myristoylated TPR1 (rapsyn-(1–41)) or TPR2 (rapsyn-(1–15, 33–90)) alone were insufficient for self-association. Since rapsyn-(1–15, 91–287)-GFP, containing only TPRs 3–7, was also capable of clustering, it is clear that other TPRs can substitute for TPRs 1 and 2. The results concerning the interactions of rapsyn structural domains with wild-type rapsyn provide further evidence that the TPR motifs mediate rapsyn self-association. Rapsyn-(1–15)-GFP, containing the consensus sequence for N-myristoylation, was targeted to the plasma membrane but it did not associate with clustered, full-length rapsyn. However, rapsyn-(1–90)-GFP was co-localized with clustered full-length rapsyn. In contrast, rapsyn-(287–340)-GFP (coiled-coil domain) and rapsyn-(351–411)-GFP (RING-H2 domain) each failed to associate with rapsyn clusters.

TPR domains have been widely recognized as protein interaction domains (reviewed in Ref. 31 and 32). Identified TPR binding motifs include short peptide sequences or helix bundles. Within protein chaperone complexes, the EEVD sequences at the COOH termini of Hsp70 and Hsp90 bind to distinct TPRs in the NL\(_2\) and COOH-terminal domains of Hop (33). In a yeast transcription repressors complex, TPRs 1–3 within SSN6 can bind to a four helix bundle at the NH\(_2\) terminus of Tup1 (34) or to a tripeptide sequence within the homeo domain of a cell type regulator, α2 (35). However, a TPR domain can also be involved in more distributed binding interactions. Amino acid side chains distributed in 7 TPRs in the α-subunit of protein prenyltransferase make extensive contacts with the β-subunit side chains (36), and based upon yeast two-hybrid and biochemical assays, the TPR domain of the serine/threonine phosphatase protein 5 is involved in binding...
to the TPR domains of the CDC16 or CDC27 subunits of the anaphase promoting complex (37), but not to PEX5, an unrelated TPR protein. Since there are no previous reports of TPR domains mediating protein self-association, it will be of particular interest to define the structure of the rapsyn-(1–90), the minimal self-association domain, and also to determine whether the rapsyn TPR domain is involved in rapsyn binding to other proteins.

**Rapsyn TPRs and nAChR Clustering**—Analysis of rapsyn mutants containing TPR deletions revealed that nAChR clustering was still seen for mutant rapsyns lacking TPRs 3–6 and, furthermore, that deletions of TPRs 1 and 2 or TPR 7 (proximal to the coiled-coil domain) were tolerated. Changes in the relative orientation of rapsyn’s coiled-coil domain or in the distance between the membrane anchoring site (N-Myr) and the coiled-coil domain of rapsyn due to internal TPR deletions may affect the ability of rapsyn to interact with nAChRs efficiently. Alternatively, it may be that for these deletion mutants the ability to cluster nAChRs might be particularly sensitive to the levels of protein expression or turnover.

**Properties of the RING-H2 Domain**—Although nonmyristoylated rapsyn (20) or rapsyn-GFP (6) is targeted to the nucleus, the primary structure of rapsyn does not contain any known consensus sequence for nuclear localization. However, the cysteine-rich domain of rapsyn is a RING-H2 domain, a structural motif found in many transcription factors localized in the nucleus (15). Hence, we reasoned that in the absence of a dominant membrane-targeting signal such as N-Myr, the RING-H2 domain of rapsyn might contribute to its nuclear retention. Consistent with this, rapsyn-(G2A-360), which lacks the RING-H2 domain, was found in the cytosol and was excluded from the nucleus. Thus, with the myristoylated NH2-terminal domain of rapsyn acting as dominant membrane targeting signal, the RING-H2 domain can potentially interact with other proteins at the cell surface. In the absence of the membrane-targeting signal, rapsyn’s RING-H2 domain may interact with proteins that are normally localized to the nucleus and by virtue of this interaction be targeted to the nucleus.

**An Homology Model of the Rapsyn TPR Domain**—To provide a structural rationale for rapsyn activity, we constructed a model of rapsyn (Fig. 9A) based upon the three-dimensional structure of the 3 TPRs in the serine/threonine protein phosphatase 5 (17). Rapsyn amino acid sequence 6–279 was modeled as having 7 TPRs (14). Rapsyn amino acids 298–331 was modeled as a single α-helix, based on our previous results (6) indicating that rapsyn-(298–331) formed a helix. TPRs 1–7 (rapsyn-(1–287)) form a super-helix extending ~70 Å in length. The concave (inner) surface of the superhelix has a diameter of ~25 Å, while that of the external surface is ~40 Å. In this context, it is interesting to note that in the Torpedo postsynaptic membrane, the diameter of the cytoplasmic projection is ~65 Å (38). In our model, helix B of TPR 7 ends at rapsyn-(279), and rapsyn-(286–331) is modeled as a single helix beginning with three turns (rapsyn-(286–297)) packed along helix B of TPR7 that precede the predicted coiled-coil domain (rapsyn-(298–331)). With this packing the helix extends ~70 Å beyond the TPR domain with the hydrophobic surface of the coiled-coil domain oriented toward the concave surface of the TPR superhelix. We should emphasize that we have no experimental data that constrain the orientation of the coiled-coil domain relative to the TPR domain. An interesting alternative packing would be for the coiled-coil domain of rapsyn (potentially in association with a coiled-coil domain from nAChR subunit(s)) to be actually encompassed within the TPR superhelix. Such a structure has been proposed for a yeast transcription repressor complex, where a four-helix bundle at the NH2 terminus of Tup1 is bound within the superhelix groove of TPRs 1–3 of SSN6 (34).

Fig. 9A is a surface representation of rapsyn-(1–340) with acidic side chains in blue and basic in red. The two images are rotated 180° to emphasize the surface structure, and the arrows point to the interior (concave) surface of the superhelix at the level of TPR2 (left) and TPR7 (right). The numbers refer to the corresponding TPR and the coiled-coil domain is denoted by cc. **B**, an enlarged view of the rapsyn TPRs 1–3. The CPK representation of the lysines in TPR1 is shown in red. Lysine at positions 6, 11, 23, 30, and 34 are oriented on the same face of the TPR1 helices and can potentially interact with the acidic phospholipid head groups.
outer surface or in the loops connecting the TPR domains, often as ion pairs. Among these charges, it is striking that there are five noncontiguous lysines at positions 6, 11, 23, 30, and 34 in TPR1 on the external surface (Fig. 9b). Contiguous stretches of positively charged residues have been shown to be required for the membrane association of other myristoylated (src and MARCKS) or palmitoylated (GAP43) proteins (reviewed in Ref. 39). Because rapsyn primary structure does not have such a contiguous stretch of positively charged residues, it is possible that the three-dimensional alignment of these lysine residues close to the myristoylated amino terminus plays the equivalent role in rapsyn’s plasma membrane association.

Based on the primary structure it has been suggested (40) that rapsyn-(82–110), which contains a heptad repeat of Leu/Ile residues, would be organized as an α helix and function as a leucine zipper coiled-coil motif potentially important for nAChR clustering (20). However, in our model, this sequence is within TPR3, with the leucines distributed on the two helices and contributing to the helix packing.

Our data clearly demonstrate that rapsyn self-association is mediated by the TPR domain. It remains to be determined whether this self-association is mediated by interactions involving the loops between the TPRs or either the external or the internal surfaces of the superhelix. While there has been no evidence of self-association in the known TPR structures, early studies of the TPR domain of the yeast nuc 2+ protein, a truncated polypeptide with nine tandem TPRs, provided evidence that aggregates of as many as several hundred molecules could be formed (41). The crystal structure of the armadillo (arm) repeat domain of the nuclear import factor karyopherin α, consisting of 10 repeats of a three-helix bundle, reveals a homodimer with extensive contacts within the groove (42).

In the present study, we have provided evidence that the rapsyn coiled-coil domain is essential for nAChR clustering and that at least two TPR repeats are necessary and sufficient for rapsyn self-association. nAChR clustering is retained for rapsyn constructs that contained the coiled-coil domain and as few as three TPRs. In the future, it will be of interest to identify the nAChR domain(s) involved in interaction with rapsyn and to determine the structure of the rapsyn coiled-coil domain in association with the nAChR domains.

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