*Proc. Natl. Acad. Sci. USA*
Vol. 95, pp. 4007–4012, March 1998
Neurobiology

# Mechanism of nicotinic acetylcholine receptor cluster formation by rapsyn

MANJUNATH K. RAMARAO AND JONATHAN B. COHEN*

Department of Neurobiology, Harvard Medical School, 220 Longwood Avenue, Boston, MA 02115

*Communicated by Gerald D. Fischbach, Harvard Medical School, Boston, MA, January 16, 1998 (received for review December 8, 1997)*

**ABSTRACT** **Rapsyn, a peripheral membrane protein of skeletal muscle, clusters nicotinic acetylcholine receptors (nAChRs) at high density in the postsynaptic membrane. The mechanism of nAChR clustering by rapsyn was analyzed by expressing nAChRs in HEK293T cells with various fragments of mouse rapsyn fused to green fluorescent protein. Membrane targeting of rapsyn is conferred solely by its acylated N terminus, as the myristoylated N-terminal 15 amino acids of rapsyn are sufficient to target green fluorescent protein to the plasma membrane. However, neither N-terminal myristoylation nor the conserved N-terminal amino acid sequence is essential. Membrane targeting, self-association, and nAChR clustering are preserved when the first 10 amino acids of rapsyn were replaced by those of *src,* which also contains a consensus sequence for *N*-myristoylation, or by those of GAP43, which contains a palmitoylation sequence. Rapsyn$_{1–90}$, containing two tetratrichopeptide repeats is sufficient for self-association. Rapsyn$_{1–360}$, lacking the cysteine rich domain, clusters nAChRs, while rapsyn$_{1–287}$, containing seven tetratrichopeptide repeats, does not cluster nAChRs. We identified rapsyn$_{298–331}$ as a potential coiled-coil domain, and established that mutations disrupting coiled-coil propensity prevent nAChR clustering. Thus the structural domains of rapsyn necessary for membrane targeting, self-association, and nAChR clustering are distinct, with nAChR-rapsyn interaction mediated by a previously unrecognized coiled-coil motif.**

At the vertebrate neuromuscular junction the presence of nicotinic acetylcholine receptors (nAChRs) at high density (~10,000 receptors/$\mu m^2$) in the postsynaptic membrane is essential for efficient neuromuscular communication. One molecule that plays a crucial role in the clustering of nAChRs is rapsyn (43 kDa protein), a peripheral membrane protein that is distributed coextensively with nAChRs *in vivo* and in cultured myotubes (1, 2). Mutant mice lacking rapsyn show severe neuromuscular dysfunction with no detectable nAChR clusters on the muscle fiber (3). When expressed alone in nonmuscle cells, nAChRs are distributed diffusely at the cell surface, while rapsyn forms distinct clusters. However, when coexpressed with rapsyn in nonmuscle cells, nAChRs are redistributed into clusters colocalized with rapsyn (4–6).

Although the three-dimensional structure of rapsyn is not known, its primary structure contains motifs that predict the existence of different structural domains, including a consensus sequence for *N*-myristoylation (7), eight tetratrichopeptide repeats (TPRs) (8, 9), a C-terminal cysteine-rich domain that conforms to a RING-H2 motif (10), and a serine phosphorylation site (summarized in Fig. 1*a*). The TPRs are 34-amino acid $\alpha$-helical repeats suggested to be involved in both intramolecular and intermolecular protein-protein interactions. Thus, the putative TPR domains in rapsyn are potentially involved in the intermolecular interactions important for rapsyn self-association or in interactions with other proteins necessary for nAChR clustering. To understand the mechanism by which rapsyn is targeted to the plasma membrane, self-associates, and clusters nAChRs, we created chimeric proteins involving fragments of rapsyn fused at their C termini to green fluorescent protein (GFP) of the jellyfish *Aequoria victoria* (11). The chimeric and wild-type rapsyns were expressed transiently in HEK293T cells along with mouse nAChR ($\alpha_2\beta\varepsilon\delta$) and visualized by fluorescence microscopy 24–36 hr after transfection.

## EXPERIMENTAL PROCEDURES

**Plasmid Construction and Mutagenesis.** To construct pGL-rapsyn$_{1–412}$-GFP and pGL-rapsyn$_{1–360}$-GFP, mouse rapsyn cDNA was first subcloned into the pGreen Lantern vector (pGL, Life Technologies, Gaithersburg, MD) at the *Spe*I and *Not*I sites to form pGL-rapsyn lacking GFP. The stop codon of rapsyn was replaced by a *Sca*I site by PCR and by the use of pBK-CMV (Stratagene) as a shuttle vector. Rapsyn lacking the stop codon was subcloned back into pGL vector, and GFP cDNA was then introduced at the C terminus of rapsyn, resulting in an in-frame fusion of full length rapsyn linked to GFP by an 8-amino acid linker sequence (TSRAAAAT), which is present in all the following chimeric constructs involving rapsyn and GFP. For rapsyn$_{1–360}$-GFP, a *Sca*I site was introduced by PCR after amino acid 360 of rapsyn, and then the cDNA encoding rapsyn$_{1–360}$ was fused in-frame with the GFP cDNA. For pGL rapsyn$_{1–15}$, rapsyn$_{1–90}$, rapsyn$_{1–193}$, or rapsyn$_{1–287}$-GFP, rapsyn cDNA was digested with *Pvu*II (1–15 amino acids), *Bst*UI(1–90 amino acids), *Hin*cII(1–193 amino acids), or *Pml*I (1–287 amino acids), and the fragments encoding the rapsyn N termini were ligated at their C termini in-frame with GFP cDNA in pGL vector.

PCR was used to replace the N-terminal 10 amino acids of rapsyn (MGQDQTKQQI) in pGL-rapsyn-GFP by those of mouse *src* (MGSNKSKPKD), which contains a consensus sequence for *N*-myristoylation (12, 13) (pGL-*src*$_{1–10}$-rapsyn$_{11–412}$-GFP) or by those of murine GAP43 (MLCCMRRTKQ), which contains a consensus sequence for *S*-palmitoylation (14, 15) (pGL-GAP43$_{1–10}$-rapsyn$_{11–412}$-GFP).

The one construct not tagged by GFP, pBK-$\beta$-gal$_{1–25}$-rapsyn was made by fusing a cDNA encoding the N-terminal 25 amino acids of $\beta$-galactosidase ($\beta$-gal) (MTLITPSSKLTLTKGNKSWSSRACR) in-frame to the rapsyn cDNA with a five amino acid linker (STSAT).

All PCRs were carried out in 100 $\mu$l with 20 ng of template pGL-rapsyn, 50–100 pmol of each primer, 2.5 mM of 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.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.



Abbreviations: nAChR, nicotinic acetylcholine receptor; $\alpha$BgTx, $\alpha$-bungarotoxin; GFP, green fluorescent protein; TPR, tetratrichopeptide repeat; $\beta$-gal, $\beta$-galactosidase.

A commentary on this article begins on page 3341.

*To whom reprint requests should be addressed. e-mail: jbcohen@warren.med.harvard.edu.

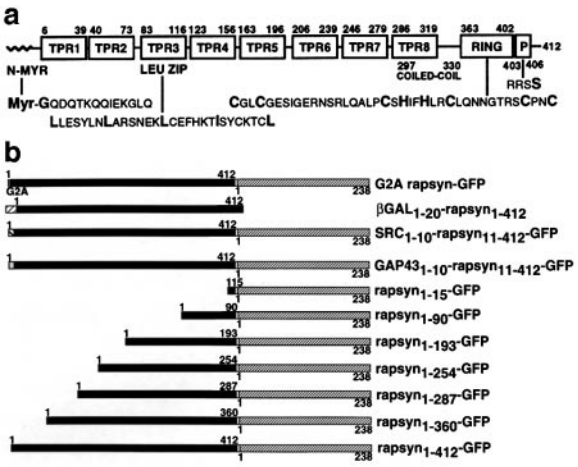


FIG. 1. (*a*) Structural domains of rapsyn. The N terminus of rapsyn is myristoylated (N-Myr). Indicated are the N-terminal 10 amino acids absolutely conserved across species as well as rapsyn$_{83–116}$, a leucine zipper motif (Leu zip), and rapsyn$_{363–402}$, the cysteine rich (RING-H2) domain. Rapsyn$_{403–406}$ is a consensus sequence for sites of both PKA and PKC phosphorylation. Also indicated are the borders of the eight putative TPRs and the borders of the predicted coiled-coil domain. (*b*) Chimeric proteins consisting of rapsyn, N-terminal modifications of full length rapsyn, or C-terminal deletions of rapsyn, each fused at its C terminus via an 8-amino acid linker with GFP, were constructed as described in *Experimental Procedures.*

All constructs containing PCR fragments were sequenced across the full length of the fragments.

**Transfection and Cell Staining.** Human embryonic kidney cells constitutively expressing simian virus 40 TAg (HEK293T, also known as tsA201) were a gift from Richard Horn (16). Cells were maintained at 37°C in DMEM supplemented with 10% fetal calf serum. Cells were grown on glass coverslips coated with collagen (4 mg/ml) placed in 24-well plates. Twenty-four hours after plating the cells, transfections were carried out by the calcium phosphate method. Mouse nAChR subunits in the vector pSM were transfected in the ratio 2:1:1:1 for $\alpha$ (0.5 $\mu$g), $\beta$, $\varepsilon$, and $\delta$, respectively, along with rapsyn constructs (0.5 $\mu$g).

For most experiments, distributions of surface nAChRs and rapsyn-GFP were visualized in unfixed, nonpermeabilized HEK293T cells 24–36 hr posttransfection. Cells were incubated with 100nM $\alpha$-bungarotoxin ($\alpha$BgTx) for 1 hr at room temperature, rinsed with PBS, and then incubated for one hour with affinity-purified rabbit anti-$\alpha$BgTx antibody [2 nM in blocking buffer (PBS with 10% calf serum, 4% BSA)], followed by incubation with rhodamine-labeled goat anti-rabbit secondary antibody (10 $\mu$g/ml in blocking buffer). Cells were visualized with a Nikon Optiphot-2 microscope with a 100× PlanApo oil objective (NA1.4). Rapsyn and its fragments were visualized by the fluorescence of GFP through fluorescein isothiocyanate optics (Ex: 465–495 nm, Em: 515–555 nm), and nAChRs through rhodamine optics (Ex: 535–565 nm, Em: long pass >590 nm). $\beta$-Gal-rapsyn, which was not fused to GFP, was visualized in cells fixed with 2% paraformaldehyde and permeabilized with 1% Triton X-100. $\alpha$BgTx was added to the cells after fixation, and then after permeabilization cells were incubated with anti-$\alpha$BgTx and with a mouse mAb (19F4a) against rapsyn (17). The cells were then incubated with fluorescein-conjugated goat anti-mouse antibody (10 $\mu$g/ml) for $\beta$-gal rapsyn and rhodamine-conjugated goat anti-rabbit for nAChRs. Photographs were taken with Kodak 160T film, and figures were prepared from the digitized images with Adobe PHOTOSHOP 4.0.

The distribution of rapsyn-GFP constructs within the HEK293T cells shifted as a function of time after transfection. For rapsyn-GFP, 6–8 hr posttransfection, diffuse fluorescence was visible clearly without evident clusters. After transfection (15–18 hr), clusters were visible at the cell surface, and their number and size increased between 20–24 hr. Beyond 24 hr there was a progressive redistribution of rapsyn-GFP from the surface to intracellular aggregates, while surface expression of nAChRs increased between 20 and 40 hr posttransfection. We found 30 hr posttransfection to be optimal for viewing surface clusters of rapsyn, its fragments, and nAChRs. By 50 hr posttransfection, there was considerable shrinkage of the cells as well as significant cell death. For quantification, cells were identified expressing both nAChRs and rapsyn-GFP. In cells expressing nAChRs alone, rhodamine fluorescence was distributed diffusely on the cell surface, with an occasional granular appearance characterized by smaller size ($\approx$0.5 $\mu$m) and fluorescence intensity than seen for nAChR clusters associated with rapsyn-GFP. These microclusters are probably due to antibody induced redistribution of nAChRs, as they were not seen in cells fixed before addition of anti-$\alpha$BgTx antibody. In cells cotransfected with rapsyn-GFP, such micro clusters, distributed randomly on the surface with respect to rapsyn-GFP clusters, were seen in 5–10% of cells. Despite this complexity, studies were carried out with unfixed cells, because prior fixation of cells with 2% paraformaldehyde reduced binding of $\alpha$-BgTx and therefore surface nAChR visualization.

## RESULTS

In initial experiments we compared rapsyn-GFP and full-length rapsyn in terms of membrane targeting and clustering. When coexpressed with nAChRs, GFP, a soluble protein, remained diffusely distributed within the cytoplasm (Fig. 2*a*) and did not interact with nAChRs that were distributed uniformly in the plasma membrane (Fig. 2*b*). Thus, GFP did not interfere with the plasma membrane targeting of nAChRs. Furthermore, GFP, when present as a fusion protein at the C terminus of rapsyn (rapsyn-GFP), did not interfere with the ability of rapsyn to form clusters (Fig. 2*c*) or to cluster nAChRs

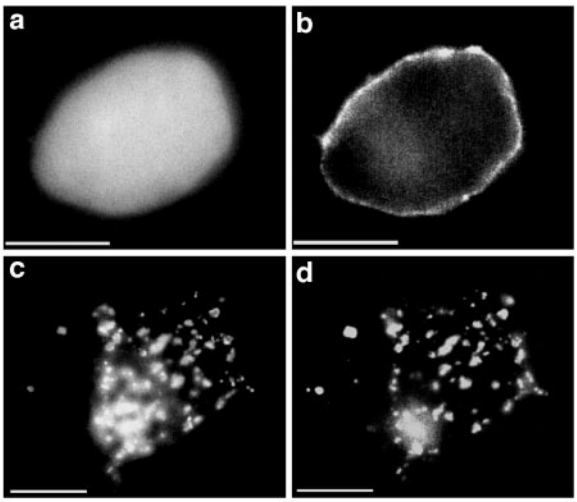


FIG. 2. Rapsyn-GFP, but not GFP, clusters nAChRs. cDNAs encoding nAChR subunits and GFP (*a* and *b*) or rapsyn-GFP (*c* and *d*) were transfected into HEK293T cells. Surface nAChRs in unfixed, nonpermeabilized cells were labeled sequentially with $\alpha$BgTx, rabbit anti-$\alpha$BgTx, and rhodamine-conjugated goat-anti-rabbit IgG. GFP or rapsyn-GFP was visualized with fluorescein isothiocyanate-optics (*a* and *c*) and nAChRs with rhodamine-optics (*b* and *d*). GFP, a cytosolic protein, was distributed throughout the cytoplasm (*a*) and did not interact with nAChRs distributed diffusely at the cell surface (*b*). Tagging of rapsyn with GFP did not interfere with the ability of rapsyn to form clusters (*c*) or to cluster nAChRs (*d*) that were colocalized with the rapsyn-GFP clusters (seen in all cells that expressed both proteins). (Bar = 10 $\mu$m.)

(Fig. 2*d*). The dimensions of these clusters were typically 1–2 $\mu$m, a size similar to the rapsyn-induced nAChR clusters in *Xenopus* oocytes (4) or COS cells (6), but smaller than the 10–20 $\mu$m nAChR clusters induced by rapsyn in QT6 quail fibroblasts (5). Because incorporation of GFP at the C terminus of rapsyn did not interfere with rapsyn clustering of nAChRs, we then constructed a series of chimeric proteins containing rapsyn with N-terminal substitutions or C-terminal deletions fused to GFP (Fig. 1*b*).

**Rapsyn Membrane Targeting.** Rapsyn is myristoylated at the N-terminal glycine (18) and its N-terminal 10 amino acids are absolutely conserved across species from *Torpedo* to human. This conservation of primary structure is greater than necessary for *N*-myristoylation (19) and suggests that the N terminus may be involved in protein interactions. As seen previously with rapsyn expressed in QT6 cells (20), mutation of this glycine to alanine, which prevents myristoylation, resulted in targeting of rapsyn$_{G2A}$-GFP to the nucleus in ≈80–90% of transfected cells with nAChRs remaining distributed diffusely at the cell surface (Fig. 3 *a* and *b*). Rapsyn$_{G2A}$-GFP was distributed diffusely in the cytoplasm as well in the remaining 10–20% of cells. Similarly, when the *N*-myristoylation site was masked by addition of the N-terminal 25 amino acid residues of $\beta$-galactosidase to the N terminus of rapsyn, this mutant protein was also targeted to the nucleus in 80–90% of the cells (Fig. 3 *c* and *d*), with additional cytoplasmic distribution seen in the remaining cells. These results indicated that N-terminal fatty acid modification is essential for targeting of rapsyn to the plasma membrane. The role of the conserved N-terminal decapeptide of rapsyn in targeting was further tested by replacing it by the N-terminal decapeptide from mouse *src*, which is also *N*-myristoylated (12, 13) but contains a different amino acid sequence. In addition, to investigate the specific requirement of *N*-myristoylation, the N-terminal 10 amino acids of rapsyn were replaced by those of mouse growth-associated protein 43 (GAP43), which contains two cysteine residues that are palmitoylated (14, 15). *src*$_{1–10}$-rapsyn$_{11–412}$-GFP and GAP43$_{1–10}$-rapsyn$_{11–412}$-GFP were each targeted to the plasma membrane, formed distinct membrane-associated clusters, and furthermore produced nAChR clusters similar in size to wild-type rapsyn-GFP (Fig. 3 *e–h*).

**Rapsyn Self-Association.** To identify the structural requirements for rapsyn self-association and/or nAChR clustering, we created chimeric proteins beginning at the N terminus of rapsyn and containing an increasing number of TPRs. A chimeric protein composed of amino acids 1–15 of rapsyn fused to GFP, though targeted to the plasma membrane, did not form clusters (Fig. 4 *a* and *b*). However, rapsyn$_{1–90}$-GFP did form distinct clusters at the cell surface, though it could not cluster nAChRs (Fig. 4 *c* and *d*). Similarly, rapsyn$_{1–193}$-GFP as well as rapsyn$_{1–287}$-GFP formed distinct clusters but did not cluster nAChRs (Fig. 4 *e–h*). Because amino acids 1–15 of rapsyn are insufficient to form a TPR, while amino acids 1–90 of rapsyn contain two TPRs, this suggests that their presence is sufficient for the clustering of rapsyn itself.

**nAChR Clustering.** Because rapsyn$_{1–287}$-GFP did not cluster nAChRs, while rapsyn$_{1–412}$ did, we then examined rapsyn$_{1–360}$-GFP, a construct containing TPRs 1–8 but lacking the cysteine-rich domain (RING-H2 domain, rapsyn$_{363–402}$). nAChRs were clustered at the cell surface and colocalized with rapsyn$_{1–360}$-GFP in 99/100 cells expressing both proteins (Fig. 4 *i–l*). The sizes of the clusters were similar to those seen for wild-type rapsyn-GFP. This result shows that rapsyn$_{287–360}$ is important for nAChR clustering, while the RING-H2 domain is not required either for rapsyn self-association or the recruitment of nAChRs to the clusters.

Rapsyn$_{287–360}$ contains TPR8 (rapsyn$_{286–319}$), but we also determined that, in addition, it contains a region, rapsyn$_{298–331}$, which has a high probability to be organized as an alternative $\alpha$-helical motif, a coiled-coil structure (21). By use of the algorithms of Lupas (22), based on sequence profiles, or Berger *et al.* (23), based on pairwise residue correlation, the

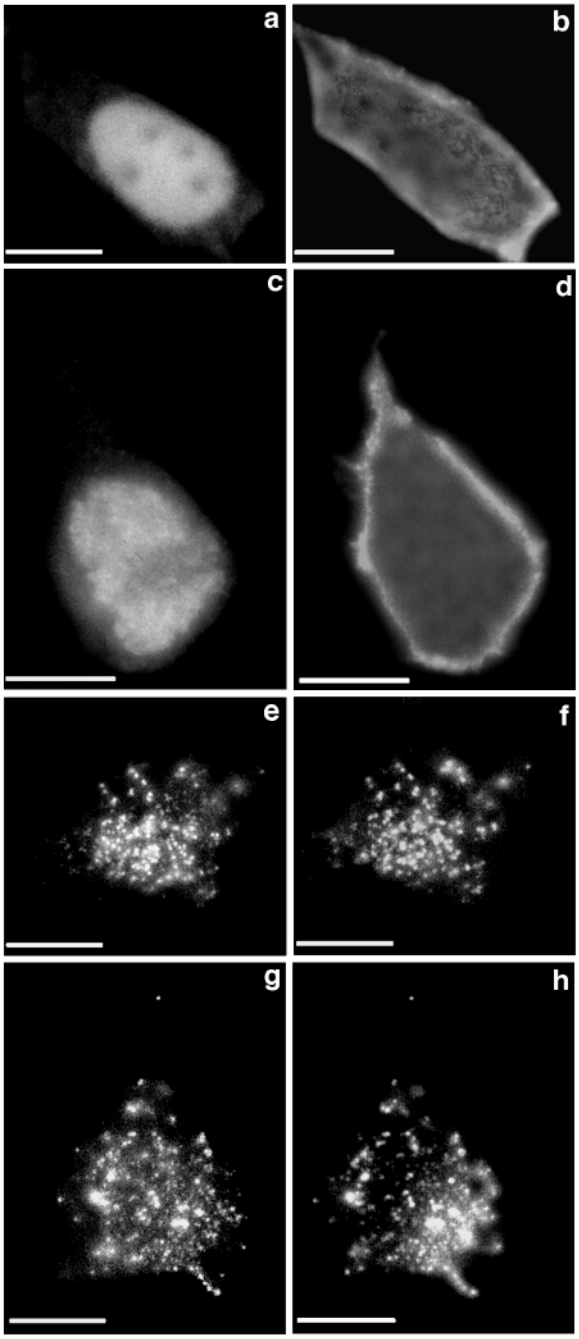


FIG. 3. N-terminal fatty acid modification of rapsyn is essential for targeting of rapsyn-GFP to the plasma membrane. HEK293T cells were cotransfected with cDNAs for nAChR subunits and N-terminal mutants of rapsyn: rapsyn$_{G2A}$-GFP (*a* and *b*); $\beta$-gal$_{1–25}$-rapsyn (*c* and *d*); *src*$_{1–10}$-rapsyn$_{11–412}$-GFP (*e* and *f*); GAP43$_{1–10}$-rapsyn$_{11–412}$-GFP (*g* and *h*). Distributions of rapsyn (*Left*) and nAChRs (*Right*) were visualized with fluorescein isothiocyanate and rhodamine optics, respectively. Mutation of Gly-2 to Ala (*a* and *b*) or addition of 25 amino acid residues of $\beta$-gal to the N terminus of rapsyn (*c* and *d*), which prevents *N*-myristoylation, resulted in targeting of mutant rapsyn-GFP or $\beta$-gal-rapsyn to the nucleus without affecting the surface expression of nAChRs. Substitution of the N-terminal 10 amino acids of rapsyn-GFP by that of the mouse *src* sequence (*e* and *f*), which also contains consensus sequence for *N*-myristoylation but a different amino acid sequence, or by that of GAP43 (*g* and *h*), which has a consensus sequence for *S*-palmitoylation, did not affect the plasma membrane targeting and self-association of mutant rapsyn-GFP or its ability to cluster nAChRs. (Bar = 10 $\mu$m.)

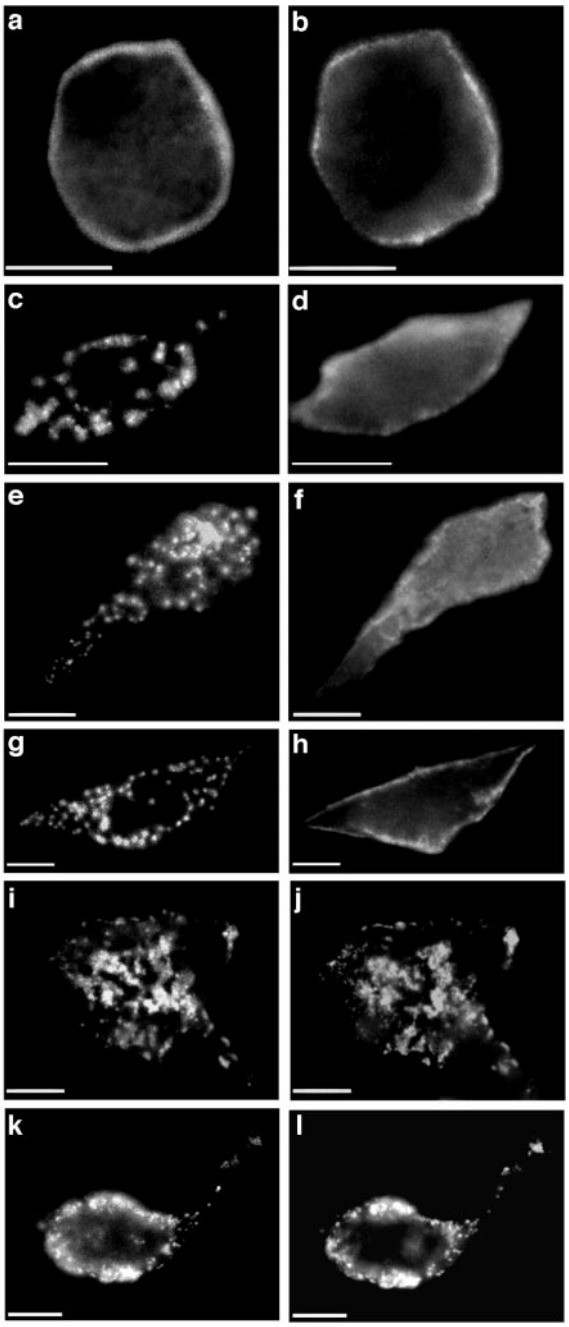


FIG. 4. Nonoverlapping structural domains within rapsyn are involved in self-association and nAChR clustering. The distributions of rapsyn (*Left*) and nAChRs (*Right*) were visualized in cells coexpressing nAChRs and N-terminal fragments of rapsyn tagged by GFP. (*a* and *b*) Rapsyn$_{1–15}$-GFP was targeted to the plasma membrane but failed to self-associate or cluster nAChRs. (*c* and *d*) Rapsyn$_{1–90}$-GFP was targeted to the plasma membrane and formed distinct clusters similar to wild-type rapsyn-GFP but did not cluster nAChRs. Rapsyn$_{1–90}$-GFP was clustered in 82/100 cells positive both for GFP and nAChRs, while nAChRs were not clustered in any cells. (*e* and *f*) Rapsyn$_{1–193}$-GFP and (*g* and *h*) rapsyn$_{1–287}$-GFP also formed distinct clusters at the cell surface but failed to cluster nAChRs. Rapsyn$_{1–287}$-GFP was clustered in 100/100 cells positive both for GFP and nAChRs, while nAChRs were distributed diffusely in 90/100 cells. nAChR clusters were colocalized with rapsyn$_{1–287}$-GFP in 3/100 cells, while in 7/100 cells there was a granular distribution of nAChRs not colocalized with rapsyn$_{1–287}$-GFP. (*i–l*) Rapsyn$_{1–360}$-GFP formed distinct clusters at the cell surface (*i, k*, two representative cells) and formed nAChR clusters (*j* and *l*) similar in size to those formed by wild-type rapsyn-GFP. nAChRs were colocalized with rapsyn$_{1–360}$-GFP clusters in 99/100 cells positive for both GFP and nAChR. In 1–2 cells there was a granular distribution of nAChRs not colocalized with rapsyn$_{1–360}$-GFP. (Bar = 10 $\mu$m.)

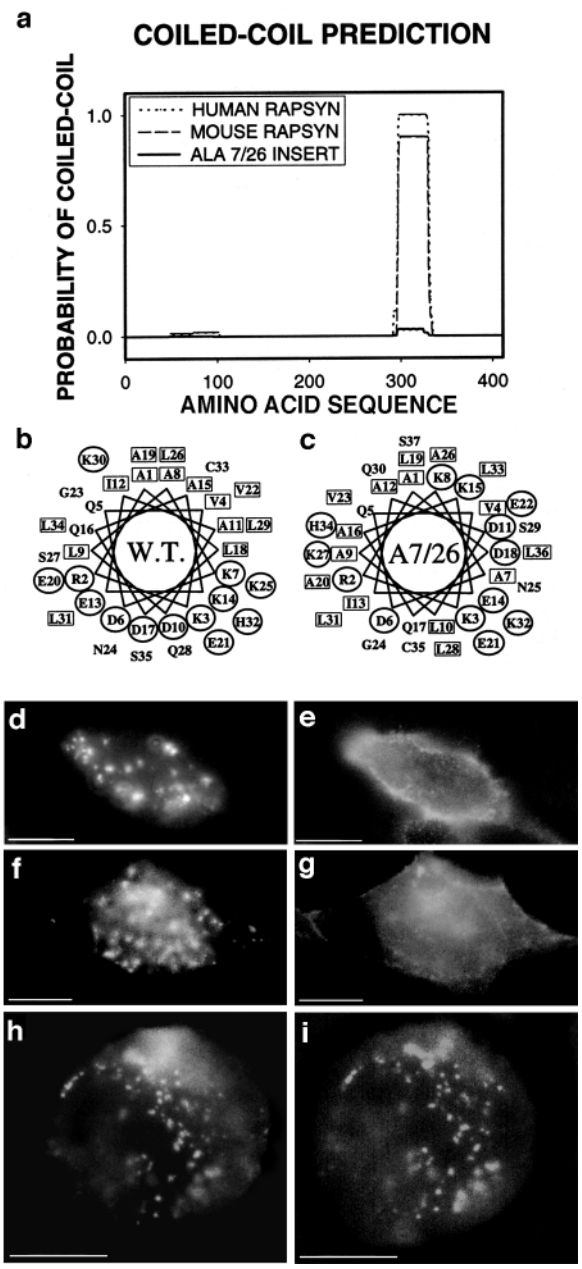





FIG. 5. Disruption of the predicted coiled-coil motif of rapsyn by alanine insertions results in the loss of nAChR clustering by rapsyn without affecting its self-association. (*a*) The probability of $\alpha$-helical coiled-coil structure in human, mouse, and the Ala-7/26 mutant of mouse rapsyn as predicted by the algorithm of Lupas (22) (COILS 2.2, 28 amino acid window, no position weighting). The *x* axis represents the amino acid number of rapsyn. Only amino acids 298–331 of mouse and human rapsyn have a high probability of forming a coiled-coil structure. Insertions of alanine after positions 303 (Ala-7) and 321 (Ala-26) of mouse rapsyn abolishes the coiled-coil propensity of this region. (*b* and *c*) Helical wheel representations of mouse rapsyn$_{298–331}$ (*b*) and rapsyn$_{298–331}$ with alanine insertions (*c*). Charged residues are circled and hydrophobic residues are boxed. The hydrophobic moment ($\mu$H) for mouse rapsyn$_{298–331}$ is 0.55, while for Ala-7/26, $\mu$H = 0.1. (*d–i*) The distributions of rapsyn (*d, f*, and *h*) and nAChRs (*e, g*, and *i*) were visualized in cells expressing nAChRs and rapsyn$_{Ala\text{-}7/26}$-GFP (*d–g*) or rapsyn$_{Ala\text{-}244}$-GFP (*h* and *i*). Insertion of Ala-7/26 resulted in disruption of nAChR clustering by rapsyn in 189/209 cells positive for both GFP and nAChR, while in 20/209 cells nAChR clusters were colocalized with rapsyn$_{Ala\text{-}7/26}$-GFP clusters. For rapsyn$_{Ala\text{-}244}$-GFP, containing an alanine insertion after rapsyn amino acid 243 (between TPRs 6 and 7), nAChRs were coclustered in 97/100 cells positive for both GFP and nAChRs, while in three cells nAChRs were distributed diffusely at the surface. (Bar = 10 $\mu$m.)

only region of mouse or human rapsyn with high probability to exist as a coiled-coil structure is contained within amino acids 298–331, a sequence overlapping but not coextensive with TPR8 (Fig. 5*a*). These 34 amino acids would form an amphipathic $\alpha$-helix with a hydrophobic moment of 0.55 (Fig. 5*b*). The predicted coiled-coil structure is also conserved in rapsyns of *Torpedo* and *Xenopus*, even though the level of amino acid conservation (38% identity over 34 amino acids) is much lower than the level of conservation over the entire rapsyn sequence (61% identity over 412 amino acids).

If amino acids 298–331 of rapsyn do form a coiled-coil structure important for nAChR clustering, then insertion of alanine residues can be used to disrupt coiled-coil propensity and thus to disrupt the ability of rapsyn to cluster nAChRs. To test this hypothesis, we used calculations of coiled-coil probability to identify two positions, one after amino acid 303 and the other after 321, at which insertions would disrupt coiled-coil propensity and amphipathic character (Fig. 5 *a* and *c*). With rapsyn$_{\text{Ala-298}}$ referred to as position one, we refer to these insertions as Ala-7 and Ala-26, respectively. When expressed in HEK293T cells, rapsyn$_{\text{Ala-7/26}}$-GFP formed distinct clusters at the cell surface but was unable to cluster nAChRs (Fig. 5 *d–g*). Out of 209 transfected cells positive for both GFP and nAChR, rapsyn$_{\text{Ala-7/26}}$-GFP was clustered in all cells, while in 90% of these (189/209) there were no nAChR clusters. In 20 cells nAChRs were colocalized with rapsyn$_{\text{Ala-7/26}}$-GFP. Insertion of a single alanine after rapsyn amino acid 243, between TPR6 and TPR7, did not prevent the clustering of nAChRs by rapsyn$_{\text{Ala-244}}$-GFP (Fig. 5 *h* and *i*). Insertion of single alanine within the putative coiled-coil domain either at position 7 (within TPR8) or 26 (after TPR8) resulted in an intermediate pattern of nAChR distribution. For both constructs, in 60–65% of cells, there were no nAChR clusters, while in 30–35%, nAChRs were clustered with rapsyn (data not shown). In rapsyn$_{\text{Ala-7}}$ the region of probable coiled-coil formation is restricted to rapsyn$_{303–331}$, while in rapsyn$_{\text{Ala-26}}$, it is limited to rapsyn$_{298–319}$. The fact that the Ala-7/26 double insertion disrupted nAChR but not rapsyn clustering supports the hypothesis that rapsyn$_{298–331}$ is involved in nAChR clustering by coiled-coil interactions. Because nAChR clustering was still seen in some cells expressing rapsyn$_{\text{Ala-7}}$ or rapsyn$_{\text{Ala-26}}$, it also may be that short coiled-coil structures in this region are sufficient for nAChR clustering in this model system.

## DISCUSSION

The results presented here establish that distinct structural motifs within rapsyn are involved in its membrane targeting, self-association, and clustering of nAChRs. Though the N-terminal acylation of rapsyn is required for membrane targeting, *N*-myristate itself is not essential because it can be replaced by *S*-palmitate. Moreover, the conserved N-terminal 10 amino acids of rapsyn are clearly not essential for rapsyn membrane association, self-association, or nAChR clustering. Thus, it is unlikely that rapsyn's conserved N terminus is involved in selective recognition by a specific membrane receptor, in contrast to p60$^{\text{v-src}}$ for which the myristoylated N terminus has been implicated in binding to a membrane bound 32-kDa protein (24).

Rapsyn when expressed alone in nonmuscle cells self-associates to form distinct clusters (4–6). Our results establish that rapsyn$_{1–90,}$ containing the first two putative TPRs, is sufficient for rapsyn self-association, while further studies will be required to determine whether a smaller construct containing a single TPR is sufficient. TPRs are predicted $\alpha$-helical motifs present in many structurally unrelated proteins that are found in multiprotein complexes (8). However, as yet no structures have been reported for proteins containing TPRs. While the TPRs in rapsyn may play an important role in rapsyn self-association, there is no direct evidence that this structural motif exists within rapsyn. Interestingly, while rapsyn$_{298–331}$ has the highest probability of $\alpha$-helical coiled-coil formation, in addition, rapsyn$_{49–76}$ is the only other region with significant coiled-coil propensity [based on the algorithm of Lupas (22) with a 28-amino acid window and weighting factor of 2.5 for a and d positions].

Rapsyn clusters nAChRs both *in vivo* and in cell culture. Though rapsyn$_{1–90}$ is sufficient for self-association, the presence of neither the leucine zipper motif within TPR3 nor the seven TPRs within rapsyn$_{1–287}$-GFP are sufficient for nAChR clustering. Further, the cysteine-rich domain within rapsyn is not required for rapsyn self-association or nAChR clustering. This conclusion is consistent with the observation that a mutant rapsyn containing substitutions disrupting zinc coordination in the RING-H2 domain still clustered when expressed in *Xenopus* oocytes and clustered nAChRs, although clusters were substantially smaller than seen for wild-type rapsyn (25).

Amino acids contained within rapsyn$_{287–360}$ are essential for nAChR clustering. There is no evidence that TPR8 (rapsyn$_{286–319}$) is the motif mediating clustering. Rather, our results indicate that rapsyn$_{298–331}$, a predicted coiled-coil domain, is crucial for nAChR clustering. While additional constructs will be required to determine whether amino acids within rapsyn$_{332–360}$ are involved, insertion of alanine at positions predicted to disrupt the coiled-coil formation disrupted nAChR clustering without affecting rapsyn self-association. It will be important to determine whether rapsyn$_{298–331}$ is involved in direct interactions with nAChRs. Inspection of rapsyn$_{298–331}$ modeled as an $\alpha$-helix reveals the presence of four lysines (Lys-300, -304, -311, and -322) aligned one above the other in the form of a ladder on the same face of the helix as well as four acidic side chains (Asp-303, -307, -314, and Glu-318). While the hydrophobic surfaces of $\alpha$-helices are usually involved in the packing of the helices in coiled-coils (21), these charges, which are conserved throughout vertebrate rapsyns, may play an important role in nAChR clustering. It is noteworthy that within the regions of nAChR subunit primary structure exposed at the cytoplasmic surface, in each subunit there are stretches of 14–20 amino acids predicted to exist as amphipathic $\alpha$-helices (26) with a possibility of coiled-coil structure. Involvement of lysines in the mechanism of nAChR clustering would be consistent with the observation that exposure of nicotinic postsynaptic membranes to pH 11 is required (27) to release rapsyn from the membrane.

Rapsyn also is likely to interact with proteins other than the nAChR in the nicotinic postsynaptic membrane. Proteins of the dystrophin/utrophin complex as well as receptors for neuregulins, nerve-derived factors important for nAChR transcriptional regulation, are normally concentrated with nAChRs but are absent from the neuromuscular junction of rapsyn-deficient mice (3, 28). When expressed in nonmuscle cells, rapsyn clusters and colocalizes with $\beta$-dystroglycan (29) suggesting that rapsyn may serve as a link between nAChRs and the proteins of the dystrophin complex. Further, when expressed in nonmuscle cells, rapsyn clusters and activates the synapse-specific receptor tyrosine kinase MuSK, which plays an important role in the agrin-induced clustering of the nAChRs (30, 31). It remains to be determined whether the rapsyn cysteine-rich domain or the TPRs are involved in interactions with MuSK or $\beta$-dystroglycan.

Our results establish that membrane targeting of rapsyn, its self-association, and its clustering of nAChRs are separable functions mediated by distinct domains. The myristoylated N-terminal 15 amino acids are sufficient for membrane targeting, but not essential, because within full-length rapsyn it can be replaced by other fatty acylated N termini. In the presence of the acylated N terminus, TPRs 1 and 2 are sufficient for self-association, while even rapsyn$_{1–287}$ is insufficient for nAChR clustering. Inclusion of rapsyn$_{298–331}$, a

putative coiled-coil domain, is essential for nAChR clustering, and mutations disrupting the coiled-coil propensity prevent nAChR clustering.

We would like to thank Drs. C. Weitz and G. Fischbach for valuable comments concerning this manuscript, Dr. R. Sealock for the anti-αBgTx antibody, and Drs. Z. Hall and the late J. Merlie for nAChR and rapsyn plasmids. This work was supported by U.S. Public Health Service Grant NS 19522.

1. Froehner, S. C. (1993) *Annu. Rev. Neurosci.* **16,** 347–368.
2. Sanes, J. R. (1997) *Curr. Opin. Neurobiol.* **7,** 93–100.
3. Gautam, M., Noakes, P. G., Mudd, J., Nichol, M., Chu, G. C., Sanes, J. R. & Merlie J. P. (1995) *Nature (London)* **377,** 232–236.
4. Froehner, S. C., Luetje, C. W., Scotland, P. B., Patrick, J. (1990) *Neuron* **5,** 403–410.
5. Phillips, W., Kopta, C., Blount, P., Gardner, P., Steinbach, J.-H, & Merlie, J. (1991) *Science* **251,** 568–570.
6. Yu, X. M. & Hall, Z. W. (1994) *J. Neurosci.* **14,** 785–795.
7. Frail, D. E., McLaughlin, L. L., Mudd, J. & Merlie, J. P. (1988) *J. Biol. Chem.* **263,** 15602–15607.
8. Lamb, J. R., Tugendreich, S. & Hieter, P. (1995) *Trends Biochem. Sci.* **20,** 257–260.
9. Ponting, C. C. P. & Phillips, C. (1996) *Biochem. J.* **314,** 1053–1056.
10. Freemont, P. S. (1993) *Ann. N.Y. Acad. Sci.* **684,** 174–192.
11. Cubitt, A. B., Heim, R., Adams, S. R., Boyd, A. E., Gross, L. A. & Tsien, R. Y. (1995) *Trends Biochem. Sci.* **20,** 448–455.
12. Pellman, D., Garber, E. A., Cross, F. R. & Hanafusa, H. (1985) *Nature (London)* **314,** 374–377.
13. Schultz, A. M., Henderson, L. E. & Oroszlan, S. (1985) *Science* **227,** 427–429.
14. Zuber, M. X., Strittmatter, S. M. & Fishman, M. C. (1989) *Nature (London)* **341,** 345–348.
15. Seykora, J. T., Myat, M. M., Allen, L. A. H., Ravetch, J. V. & Aderem, A. (1996) *J. Biol. Chem.* **271,** 18797–18802.
16. Margolskee, R. F., McHendry-Rinde, B. & Horn, R. (1993) *BioTechniques* **15,** 906–911.
17. Carr, C., Fischbach, G. D. & Cohen, J. B. (1989) *J. Cell. Biol.* **109,** 1753–1764.
18. Carr, C., Tyler, A. N. & Cohen, J. B. (1989) *FEBS Lett.* **243,** 65–69.
19. Towler, D. A., Gordon, J. I., Adams, S. P. & Glaser, L. (1988) *Annu. Rev. Biochem.* **57,** 69–99.
20. Phillips, W. D., Maimone, M. M. & Merlie, J. P. (1991) *J. Cell Biol.* **115,** 1713–1723.
21. Lupas, A. (1996) *Trends Biochem. Sci.* **21,** 375–382.
22. Lupas, A. (1996) *Methods Enzymol.* **266,** 513–525.
23. Berger, B., Wilson, D. B., Wolf, E., Tonchev, T., Milla, M. & Kim, P. S. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 8259–8263.
24. Resh, M. D. & Ling, H. (1990) *Nature (London)* **346,** 84–86.
25. Scotland, P. B., Colledge, M., Melnikova, I., Dai, Z. & Froehner, S. C. (1993) *J. Cell Biol.* **123,** 719–728.
26. Finer-Moore, J. & Stroud, R. M. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 155–159.
27. Neubig, R. R., Krodel, E. K., Boyd, N. D. & Cohen, J. B. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 690–694.
28. Moscoso, L. M., Chu, G. C., Gautam, M., Noakes, P. G., Merlie, J. P. & Sanes, J. R. (1995) *Dev. Biol.* **172,** 158–169.
29. Apel, E. D., Roberds, S. L., Campbell, K. P. & Merlie, J. P. (1995) *Neuron* **15,** 115–126.
30. Apel, E. D., Glass, D. J., Moscoso, L. M., Yancopoulos, G. D. & Sanes, J. R. (1997) *Neuron* **18,** 623–635.
31. Gillespie, S. K. H., Balasubramanian, S., Fung, E. T. & Huganir, R. L. (1996) *Neuron* **16,** 953–962.