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The Role of the Cytoplasmic Domains of Individual Subunits of the Acetylcholine Receptor in 43 kDa Protein–induced Clustering in COS Cells

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The 43 kDa protein, a cytoplasmic peripheral membrane protein, is closely associated with the acetylcholine receptor (AChR) at the neuromuscular junction, where it is thought to anchor the receptor in the postsynaptic membrane. We have used the 43 kDa protein–induced clustering of AChRs that occurs when both proteins are transiently expressed in COS cells to investigate which parts of the AChR might interact with the 43 kDa protein. By constructing chimeric subunits, we showed that the cytoplasmic domains of neither the ϵ nor δ subunits are required for 43 kDa protein–induced clustering. Systematic mutational analysis of the long cytoplasmic loops of the α and β subunits showed that most of the loops can be altered without affecting the ability of the AChR to be clustered; in each case, however, one or more sequences could not be tested, because mutation in these regions prevented AChR assembly. Our results suggest either that these regions are involved in clustering or that the 43 kDa protein can interact with multiple, alternative sites on the cytoplasmic surface of the AChR. Our experiments also show that the postulated sites of tyrosine phosphorylation in the β subunit and of serine phosphorylation in the α subunit can be mutated without affecting 43 kDa protein–induced AChR clustering.

[Key words: ACh receptor, clustering, 43 kDa protein, mutation, COS cell, immunocytochemistry]

The appearance of clusters of acetylcholine receptors (AChRs) in muscle cells near sites of nerve contact is the earliest detectable sign of postsynaptic differentiation during development of the neuromuscular junction (Hall and Sanes, 1993). A cytoplasmic peripheral membrane protein, the 43 kDa protein, is closely associated with the AChR at adult and developing synapses (Froehner et al., 1981; Noakes et al., 1993), and has been proposed to anchor it to the cytoskeleton in the postsynaptic membrane (Froehner, 1991). The 43 kDa protein is present in approximately 1:1 stoichiometry with the AChR (LaRochelle and Froehner, 1987), and is exactly codistributed with it both in the postsynaptic membrane *in vivo* (Sealock et al., 1984; Flucher and Daniels, 1989) and in spontaneous and nerve-induced AChR clusters in cultured muscle cells (Burden, 1985; Bloch and Froehner, 1987; Gordon et al., 1993). Removal of the 43 kDa protein and other peripheral membrane proteins from the membrane by alkaline treatment increases the lateral mobility of the AChR within the cluster (Barrantes et al., 1980; Rousselet et al., 1982), suggesting that one or more of the extracted proteins, such as 43 kDa protein, may attach the AChR to the cytoskeleton.

Recent experiments suggest that the 43 kDa protein not only stabilizes the AChR in clusters, but also induces AChR clusters to form. When AChRs are expressed in nonmuscle cells after transfection or after mRNA injection, they are diffusely distributed on the cell surface; when expressed with the 43 kDa protein, however, the AChRs are found in clusters that are coincident with aggregates of the 43 kDa protein (Froehner et al., 1990; Phillips et al., 1991; Brennan et al., 1992). The 43 kDa protein forms clusters when expressed alone; AChRs thus presumably become clustered via their association with the 43 kDa protein.

Although firm evidence is lacking, several observations suggest that the AChR interacts directly with the 43 kDa protein. A protein that appears to be in direct contact with the AChR in reconstituted images of the postsynaptic electroplax membrane (Toyoshima and Unwin, 1988; Mitra et al., 1989) is likely to be the 43 kDa protein, as it is the only protein in purified *Torpedo* membranes that is present in equimolar amounts with the AChR (LaRochelle and Froehner, 1986, 1987). Also, the 43 kDa protein can be cross-linked to the β subunit of the AChR by treatment of synaptic membranes with a bifunctional cross-linking agent (Burden et al., 1983). The β subunit is of particular interest because agrin, a neurally derived factor that causes AChRs to cluster (McMahan, 1990; Reist et al., 1992), stimulates phosphorylation of a tyrosine residue in this subunit (Wallace et al., 1991).

Each of the subunits of the AChR has a stereotyped structure with a long N-terminal, extracellular domain, four transmembrane domains (M1–M4), and an extracellular C-terminal domain (Karlin, 1980; Changeux, 1981; Claudio, 1989). Either of the two cytoplasmic domains that link the transmembrane segments M1 and M2, or M3 and M4, respectively, provides potential sites for interaction with the 43 kDa protein. To identify the specific subunits and the sites on these subunits that interact with the 43 kDa protein, we have expressed AChRs containing chimeric or mutated subunits in COS cells along with the 43 kDa protein. Our results demonstrate that the cytoplasmic domains of the ϵ and δ subunits and most of the intracellular loops of the α and β subunits are not required for 43 kDa protein–induced AChR clustering. The 43 kDa protein may thus react with specific sequences on the α and β subunits that we were

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unable to test or react with multiple, alternative sites on one or more subunits.

Materials and Methods

Vectors

Full-length cDNAs coding for the α, β, γ, and δ subunits of the AChR were obtained from Dr. J. P. Merlie and N. Davidson (α, Isenberg et al., 1986; β, Buonanno et al., 1986; γ, Yu et al., 1986; δ, Lapolla et al., 1984). The cDNA clone for the mouse ε subunit was isolated as described (Gu et al., 1990). The cDNA coding for CD8 was a generous gift from Dr. D. Littman. Each of the cDNAs was subcloned into the multiple cloning site of pSM, an SV40-based expression vector (Brodsky et al., 1990) that also contains an M13 origin for oligonucleotide-directed mutagenesis.

cDNA for the mouse 43 kDa protein was a generous gift of Dr. S. Froehner (Froehner, 1989). The full-length coding sequence minus the 5′ untranslated sequences was subcloned into the SV40-based expression vector, pcDLSRα296, under the control of an SRα promotor (Takebe et al., 1988). The pSM vector with the 43 kDa protein cDNA insert was cut with EcoRI, and the 43 kDa protein cDNA dropout [1.5 kilobase (kb)] was purified and cloned into the EcoRI-linearized pcDLSRα296 vector.

Mutagenesis

Oligonucleotide-directed mutagenesis was performed according to protocols described previously (Kunkel, 1985; Geisselsoder et al., 1987). Synthetic oligodeoxyribonucleotides for each mutated segment were designed with 10–15 flanking nucleotides on either side of the mutation site and were prepared with an automatic DNA synthesizer. The detailed changes for each mutation are given below. Where appropriate, @ is used to indicate a deleted segment; bases that are altered to produce amino acid changes or to introduce a restriction site are underlined.

Mutations in the cytoplasmic loop of the α subunit

α(Δ299–312). The first 13 amino acids, HHRSPSTHIMPEW, of the long cytoplasmic loop were deleted using the oligonucleotide ATC GTC ATC AAC ACA @ GTG CGC AAG GTT TTT ATC GAC. The base substitution created an FspI site.

*α(*312–322).* The 10 amino acids VRKVFIDTIP were mutated to VRQIFIHKLP, corresponding to the amino acid sequence in the loop of the β subunit, using the oligonucleotide CCC GAG TGG GTG CGG CAG ATT TTT ATC CAC AAG CTT CCA AAC ATC ATG TTT. A HindIII site was created by the change.

*α(*322–332).* The 10 amino acids NIMFFSTMKR were mutated to SLSPRSGWGR, corresponding to the amino acid sequence in the loop of the β subunit, using the oligonucleotide ATC GAC ACT ATC CCA TCC CTC TCG CCT CGC TCC GGA TGG GGA AGA CCA TCC AGA GAT AAA. A BspEI site was created by the change.

*α(*332–339).* The seven amino acids PSRDKQE were mutated to GTDEYFI, corresponding to the amino acid sequence in the loop of the β subunit, using the oligonucleotide TCC ACA ATG AAA AGA GGT ACC GAT GAA TAT TTT ATT AAA AGG ATT TTT ACA. A KpnI site was introduced by the change.

*α(*339–349).* The 10 amino acids KRIFTQDIDI were mutated to RPPPSDFLFP, corresponding to the sequence of the β subunit, using the oligonucleotide AGA GAT AAA CAA GAG AGG CCT CCT CCT TCA GAT TTC CTA TTT CCA. A StuI site was introduced by the change.

α(Δ349–359). The 10 amino acids SDISGKPGPP were deleted using the oligonucleotide GAA GAC ATA GAT ATC @ CCT ATG GGC TTT CAC. An EcoRV site was introduced without any amino acid change.

α(Δ359–369). The 10 amino acids PMGFHSPLIK were deleted using the oligonucleotide TCT GGG AAG CCG GGC CCT CCA @ CAC CCT GAG GTG AAA AGC. An ApaI site was introduced without any amino acid change.

*α(*367–382).* The 15 amino acids IKHPEVKSAIEGVKY in the amphipathic helix were also mutated to GLPQELREVISSISY, corresponding to the sequence of the β subunit, using the oligonucleotide TTT CAC TCT CCG CTG GGC CTG CCC CAG GAG CTG AGA GAG GTC ATC TCA TCG ATC AGC TAC ATT GCA GAG ACC ATG. A ClaI site was introduced by the change.

Mutations in the cytoplasmic loop of the β subunit

β(Δ338–353). The 15 amino acids KRPKPERDQLPEPHH in the β loop were deleted using the oligonucleotide CCT CCA TAC CTA GGC CTG @ TCT CTT TCT CCA. A StuI site was created without amino acid change.

*β(*343–353).* The six charged amino acids were neutralized to hydrophobic amino acids over a window of 10 amino acids to mutate ERDQLPEPHH to VLVQLPVPLL using the oligonucleotide CCC AAA CCC GTA CTA GTC CAA CTC CCT GTA CCA CTT CTC TCT CTT TCT. A SpeI site was introduced by the change.

*β(*353–363).* The 10 amino acids SLSPRSGWGR were mutated to NIMFFSTMKR, corresponding to the sequence in the loop of the α subunit, using the oligonucleotide CCT GAA CCA CAT CAC AAT ATT ATG TTT TTT AGT ACC ATG AAA AGA GGA ACT GAT GAA TAT. A SspI site was introduced.

*β(*363–370).* The seven amino acids GTDEYFI, including the tyrosine phosphorylation site, were mutated to PSRDKQE, corresponding to the sequence in the loop of the α subunit, using the oligonucleotide AGT GGC TGG GGC AGG CCT TCT CGT GAT AAA CAA GAA CGG AGG CCT CCA AGT. A StuI site was created.

*β(*370–380).* The 10 amino acids RKPPSDFLFP were mutated to KRIFTQDIDI, corresponding to the sequence in the loop of the α subunit, using the oligonucleotide GAT GAA TAT TTC ATC AAG AGG ATT TTT ACT CAA GAT ATC GAC ATT AAA CTT AAC AGG TTT. An EcoRV site was created.

*β(*380–390).* The 10 amino acids KLNRFQPESS were mutated to SDISGKPGPP, corresponding to the sequence in the loop of the α subunit, using the oligonucleotide GAT TTT CTT TTC CCT TCA GAT ATC TCG GGT AAG CCT GGA CCA CCT GCC CCG GAC CTG CAG. An EcoRV site was created.

β(Δ390–405). The 15 amino acids APDLRRFIDGPTRAV were deleted using the oligonucleotide CAG CCT GAA TCA TCT @ GGT CTG CCT CAG GAG. The endogenous SmaI in the position of amino acid sequence TRA was also deleted.

*β(*420–430).* The 10 amino acids MARQLQEQED were mutated to IAETMKSDQE, corresponding to the sequence in the loop of the α subunit, using the oligonucleotide TCC TCA ATC AGC TAC ATT GCC GAA ACG ATG AAG TCT GAT CAG GAA CAC GAC GCA CTG AAG. A BclI site was created.

*β(*405–420).* The 15 amino acids in the amphipathic helix of the loop of the β subunit, GLPQELREVISSISY, were also mutated to QASPAIQACVDACNL, corresponding to the sequence of the amphipathic helix of the γ subunit, using the oligonucleotide CCA ACC CGG GCT GTA CAG GCC TCT CCG GCG ATT CAA GCG TGC GTT GAC GCA TGC AAC CTC ATG GCC CGA CAG CTT. A StuI site was created.

Cell culture and transient transfection

COS cells were maintained in growth medium [DMEM H21 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin-streptomycin]. COS cells were transiently transfected as described previously (Gu et al., 1990), using a modified DEAE-dextran transfection procedure (Seed and Aruffo, 1987). About 18 hr before transfection, COS cells were trypsinized and 2×10^5 cells were plated into one well in a 12-well plate. The transfection medium was DMEM H21 supplemented with 2 mM L-glutamine mixed with 1% heat-inactivated FBS, 0.15 mM chloroquine diphosphate, 0.6 mg/ml DEAE-dextran, and routine amounts of plasmid cDNAs for AChR subunits and the 43 kDa protein. The routine cDNA concentrations for the AChR subunits and the 43 kDa protein were empirically determined for maximum response (α, 0.66 μg/ml; β, 0.33 μg/ml; δ, 0.13 μg/ml; ε, 0.5 μg/ml; 43 kDa, 0.2 μg/ml). COS cells were incubated in the transfection medium for about 4 hr in a 37°C incubator with 8% CO_2 and 100% humidity. In all transfections, the transfection mixtures were removed after about 4 hr, and the cells were treated in 10% dimethyl sulfoxide in PBS for 2 min at room temperature, and washed once with PBS before being returned to 37°C in 2 ml of growth medium. Twenty-four hours later, the transfected cells were trypsinized and distributed into two wells in a 24-well plate containing round glass coverslips (12 mm in diameter). The staining procedures were carried out the next day. Transfections for coexpression of the α subunit alone with the 43 kDa protein, or for the β subunit alone with the 43 kDa protein, or for the CD8 protein with the 43 kDa protein, were performed in the same way

as above except that the cDNA concentrations for the α, β subunits and for CD8 were 1 μg/ml.

For the assay of surface AChRs, transfections were carried out in almost the same way as above except that the size of the dishes and the amounts of cells and plasmids were different: 7×10^5 cells were plated into one 60 mm dish; 3 ml of transfection mixture was added to each dish containing the routine amounts of cDNAs for the AChR subunits (α, 1.32 μg; β, 0.66 μg; δ, 0.26 μg; ϵ, 1.0 μg). The transfected cells in one 60 mm dish were split into three wells of a 24-well plate for triplicate determinations. Assay for surface AChRs was performed 24 hr later.

Immunofluorescence and histochemistry

Immunocytochemical staining for the surface AChRs was carried out on COS cells grown on 12 mm round coverslips and transfected with cDNAs of the AChR subunits with or without cDNA for the 43 kDa protein. The transfected COS cells were fixed in 2% paraformaldehyde in Ca^{2+}/Mg^{2+} free PBS for 20 min, and then rinsed with PBS, blocked with blocking buffer (10% FBS/4% BSA/PBS) for 1 hr to inhibit nonspecific binding of the antibodies, and incubated for 2 hr with a monoclonal antibody, MAb210, that recognizes a site on the N-terminus of the α subunit (Ratnam et al., 1986), at a concentration of 215 nM diluted in the blocking buffer. The COS cells were washed twice with PBS and once with the blocking buffer for 30 min, and then incubated for 1 hr with the secondary antibody FITC-conjugated goat anti-rat IgG, at a 1:200 dilution in the blocking buffer. After washing three times with PBS, the coverslips were mounted on glass slides with *p*-phenylenediamine in glycerol (Platt and Michael, 1983). The slides were viewed with a Leitz Orthoplan II microscope under epifluorescence illumination equipped with a Vario-Orthomat camera system. Staining of COS cells expressing CD8 with or without the 43 kDa protein was carried out in a similar manner, except that the first antibody was OKT8 (Hoffman et al., 1980) against the CD8 protein and the second antibody was FITC-conjugated sheep anti-mouse.

The staining procedure for the 43 kDa protein was a modification of the procedure of LaRochelle et al. (1989). Transfected COS cells were fixed in 2% paraformaldehyde, rinsed once with PBS, permeabilized by incubation with 1% Triton/PBS for 10 min, blocked with the blocking buffer for 1 hr, and incubated for 2 hr with a mixture of three monoclonal antibodies against the 43 kDa protein (1234A, 19 nM; 1201C, 5 nM; 1579A, 11 nM) (Peng and Froehner, 1985). The cells were then washed twice with PBS and once with the blocking buffer for 30 min, and incubated for 1 hr with the second antibody FITC-conjugated sheep anti-mouse IgG, at a 1:200 dilution in the blocking buffer. The coverslips were washed three times with PBS, mounted with *p*-phenylenediamine in glycerol, and visualized in fluorescence microscopy.

Double-labeling experiments were performed with a protocol modified from Ralston and Hall (1989). COS cells cotransfected with cDNAs of the AChR and the 43 kDa protein were incubated with rhodamine–α-bungarotoxin (RH–α-BTX) at a 1:200 dilution in growth medium at 37°C for 1 hr, rinsed with PBS, fixed in 2% paraformaldehyde in PBS for 20 min, rinsed once with PBS, and then permeabilized with 1% Triton in PBS for 10 min, washed with PBS once, and incubated with the blocking buffer for 1 hr. COS cells were then incubated for 2 hr with a mixture of the first antibody, a polyclonal rabbit anti–α-BTX at 1:100 dilution, and three monoclonal mouse antibodies against 43 kDa protein at the same concentrations as above. COS cells were washed twice with PBS and once with the blocking buffer, incubated for 1 hr with a mixture of the second antibodies FITC-conjugated sheep anti-mouse IgG and RH-conjugated goat anti-rabbit IgG at 1:200 dilutions. The coverslips were washed three times with PBS, mounted, and viewed as described above. The same COS cells expressing both RH-AChR and FITC–43 kDa protein signals were photographed.

Staining protocols for COS cells expressing either the α or β subunit with the 43 kDa protein were as follows: COS cells transfected with cDNAs for either the α or β subunit and the 43 kDa protein were fixed for 20 min with 2% paraformaldehyde, rinsed once with PBS, and then permeabilized for 10 min with 1% Triton-PBS, rinsed once with PBS, and blocked for 1 hr with the blocking buffer. COS cells in one well were then incubated for 2 hr with the first antibody, either MAb210 for the α subunit or MAb124 for the β subunit, respectively, and then washed twice with PBS and once with the blocking buffer for 1 hr. The COS cells were then incubated for 1 hr with the second antibody FITC-conjugated goat anti-rat IgG, washed three times with PBS, mounted, and visualized under fluorescence microscopy. COS cells in a neighboring well were stained for the 43 kDa protein using the 43 kDa protein staining procedure described above.

All the staining procedures were carried out at room temperature unless otherwise stated.

AChR surface expression

Surface expression of α-BTX binding sites was measured by incubating intact transfected COS cells with 10 nM ^{125}I–α-BTX (Amersham, Arlington Heights, IL) for 1.5 hr at 37°C. Sham transfections were used to determine nonspecific binding. Unbound toxin was then removed by washing the cells twice with PBS. The cells were then solubilized with 0.1 M NaOH and the radioactivity was measured in a gamma counter.

Results

Coexpression of the 43 kDa protein with the AChR in COS cells induces AChR clustering

When cDNAs for the four subunits of the adult mouse AChR ($\alpha\beta\delta\epsilon$) are transfected into COS cells, AChRs appear on the surface that resemble those seen at adult mouse end-plates (Gu et al., 1990). The distribution of the AChRs on the surface of the transfected COS cells was examined by staining them either with RH–α-BTX (results not shown), or with a monoclonal antibody to an epitope on the extracellular N-terminal domain of the α subunit (Fig. 1*A*), followed by FITC-conjugated second antibody. Only about 20–30% of the cells expressed the AChR at high enough levels to be detected. In virtually all of these cells, however, the receptors were diffusely distributed on the cell surface (Fig. 1*A*, Table 1). When a cDNA for the 43 kDa protein was included in the transfection mixture, a different pattern of staining was observed in which the AChRs appeared in small clusters on the surface of the COS cells (Fig. 1*B*). This pattern of staining was seen in approximately half the number of the cells that showed strong staining for the AChR (Table 1). When the 43 kDa protein was expressed in COS cells in the absence of the AChR, and the cells were permeabilized and stained using antibodies to the 43 kDa protein, a similar pattern of staining was seen (Fig. 1*C*). The 43 kDa protein appeared to be associated with the surface membrane in small aggregates or clusters that resemble those seen with antibodies to the AChR when both proteins were expressed. Forty-three kilodalton protein aggregates were also seen in the cytoplasm and diffuse staining was observed in the nucleus. When ϵ subunit cDNA was replaced by γ subunit cDNA in the transfection mixture so that the embryonic form of the AChR was expressed (Gu et al., 1990), similar results were obtained (not shown). To test whether AChRs were specifically clustered, we transfected COS cells with a cDNA for CD8, an integral membrane protein expressed on the surface of cytotoxic lymphocytes (Littman, 1987), with and without the cDNA for the 43 kDa protein. Whether expressed with (Fig. 1*D*) or without (not shown) the 43 kDa protein, CD8 was always seen to be diffusely distributed on the cell surface (Table 1).

Forty-three kilodalton protein–induced AChR clustering was also investigated in double-labeling experiments. cDNAs for the γ or ϵ subunits and for the α, β, and δ subunits were cotransfected with or without the 43 kDa protein cDNA into COS cells. The AChRs were labeled with RH–α–BTX, followed by rabbit anti–α-BTX and RH-conjugated goat anti-rabbit IgG; the 43 kDa protein was labeled with a mixture of mouse monoclonal antibodies against the 43 kDa protein, followed by FITC-conjugated sheep anti-mouse IgG. In the case of both the ϵ-AChR (Fig. 2*A,B*) and the γ-AChR (not shown), the receptor and the

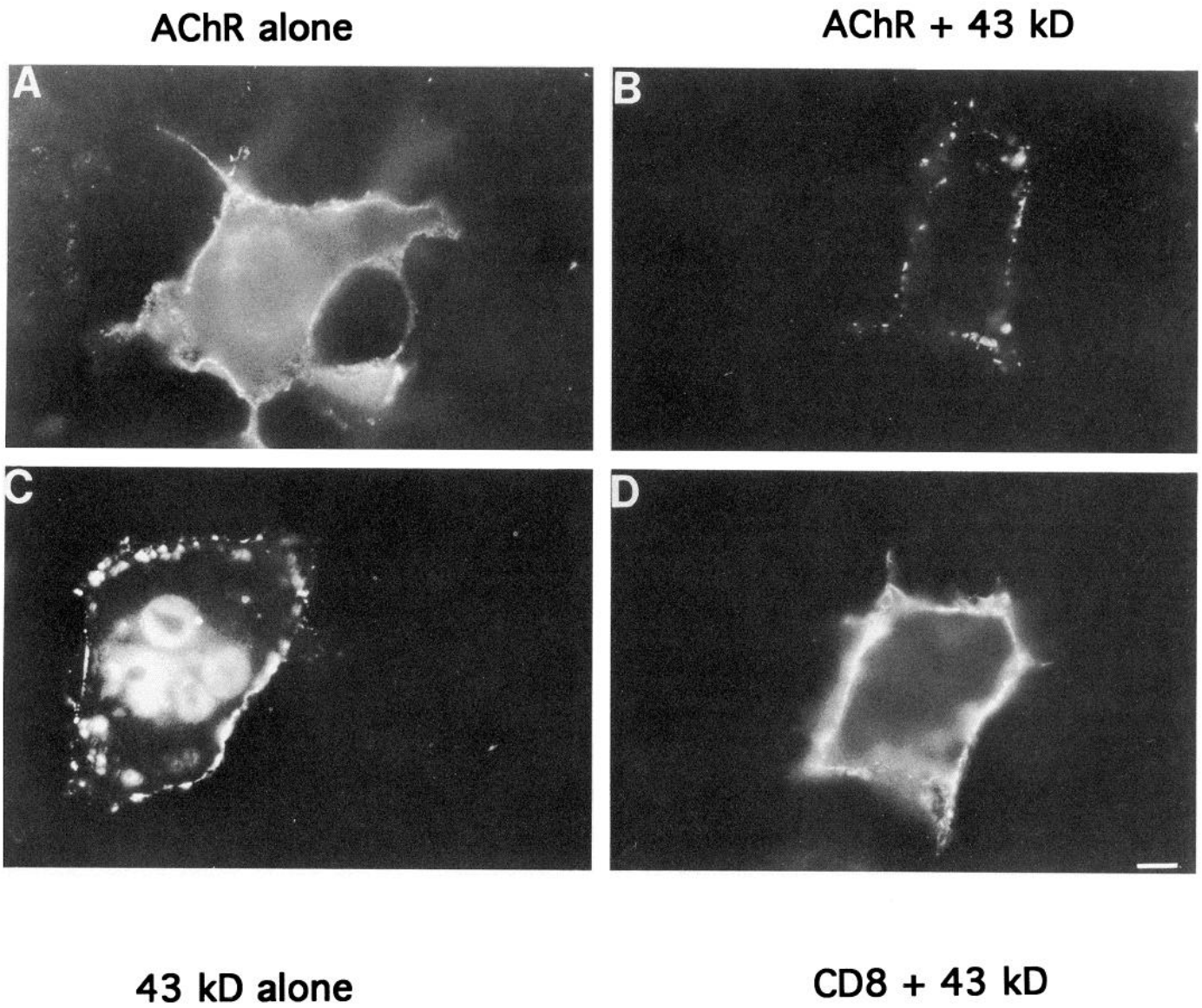

Figure 1. Clustering of the AChR and the 43 kDa protein in transfected COS cells. *A* and *B*, COS cells were transfected with cDNAs for the α, β, δ, and ϵ subunits, minus (*A*) or plus (*B*) 43 kDa protein cDNA. AChRs on the surface (*A* and *B*) were stained with MAb210 against the α subunit, followed by FITC-conjugated goat anti-rat IgG, and visualized with fluorescence microscopy. *C*, COS cells were transfected with the 43 kDa protein cDNA alone, permeabilized, and then stained with a mixture of three monoclonal antibodies against the 43 kDa protein, followed by FITC-conjugated sheep anti-mouse IgG, and visualized with fluorescence microscopy. Further details are given in Materials and Methods. *D*, COS cells were transfected with cDNAs for the 43 kDa protein and CD8 peptide, permeabilized, and then stained with a monoclonal antibody against CD8 protein, followed by FITC-conjugated sheep anti-mouse IgG, and visualized with fluorescence microscopy. Scale bar, 10 μm.

43 kDa protein were colocalized on the surface. Our results in COS cells, which are consistent with those found by others in nonmuscle cells (Froehner et al., 1990; Phillips et al., 1991; Brennan et al., 1992), thus suggest that the 43 kDa protein alone forms aggregates at the surface membrane and that the AChR is induced to aggregate by its association with the 43 kDa protein. The results with CD8 suggest that the AChR clustering is a specific effect that does not occur with other membrane proteins.

AChR clustering does not require the cytoplasmic loops of either the γ or δ subunits

If the 43 kDa protein induces AChR clustering by direct interaction with one or more of the AChR subunits, the interaction is likely to be mediated by their cytoplasmic loops. To test whether individual subunits interact with the 43 kDa protein, we constructed chimeric subunits in which the N- and C-terminal domains were derived from one subunit and the intervening sequence from another subunit (Yu and Hall, 1991). In many cases, the resulting chimeric subunits did not support AChR assembly when expressed in COS cells and no surface AChR was obtained. When a cDNA of the chimeric subunit ϵ_β, containing the N- and C-terminal domains of the ϵ subunit and the transmembrane and cytoplasmic domains of the β subunit, was cotransfected into COS cells with the cDNAs of the α, β, and δ subunits, surface AChRs were detected (Yu and Hall, 1991). These AChRs were diffusely distributed on the cell surface (Fig. 3*A*); when coexpressed with the 43 kDa protein, however, clusters of the AChR were readily observed (Fig. 3*B*). The clusters occurred in approximately the same proportion of AChR-expressing cells as seen with the native AChR (Table 1). Similar results were obtained when the corresponding chimera for the

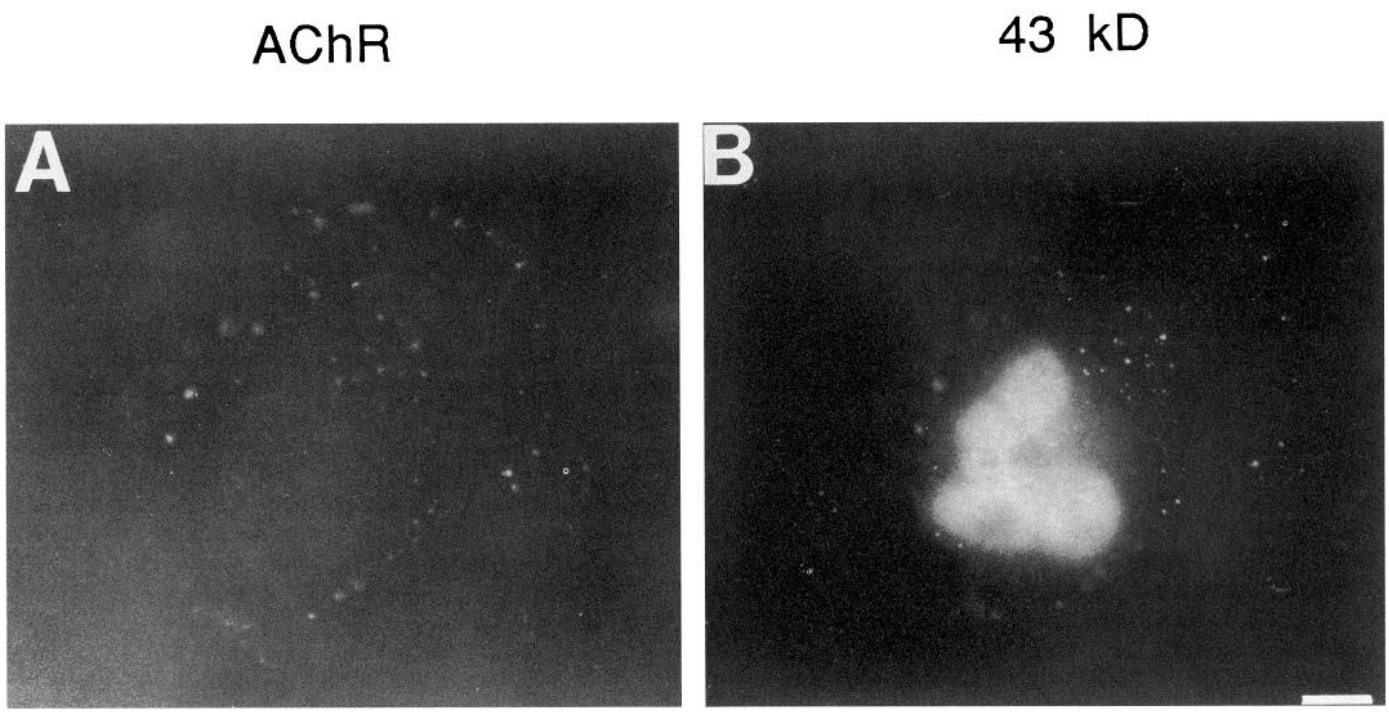

Figure 2. Colocalization of the surface AChR and the 43 kDa protein. COS cells were transfected with cDNA for the 43 kDa protein and with cDNAs for the α, β, δ, and ϵ subunits. Surface AChR was labeled by RH–α-BTX, followed by rabbit anti–α-BTX, and RH-conjugated goat anti-rabbit IgG. The 43 kDa protein was stained with a mixture of the three monoclonal antibodies against the 43 kDa protein, followed by FITC-conjugated sheep anti-mouse IgG. The same cells expressing both the AChR and the 43 kDa protein were photographed in the fluorescence microscope. *A*, RH optics; *B*, FITC optics. Scale bar, 10 μm.

δ subunit, δ_β, was expressed with α, β, and ϵ subunits. As with the ϵ_β receptor, receptors containing the δ_β subunit were diffusely distributed on the surface, but became clustered when coexpressed with the 43 kDa protein (Fig. 3*C,D*). Moreover, even when both ϵ_β and δ_β subunits replaced their normal counterparts, the AChR expressed on the surface could be induced to form clusters by coexpression with the 43 kDa protein (Fig. 3*E,F*). In the case of each chimera, the toxin binding activity expressed on the cell surface was sensitive to *d*-tubocurarine, and sedimented in a sucrose gradient at a position identical to that of the authentic AChR (Yu and Hall, 1991, unpublished observations). We also observed colocalization of the receptors containing chimeric subunits and of the 43 kDa proteins in double-labeling experiments (not shown). Since 43 kDa protein–induced clustering of the assembled receptor required the cytoplasmic regions of neither the ϵ nor δ subunits, and occurred in the absence of both, we conclude that interaction of the 43 kDa protein with these regions is not necessary for clustering to occur.

Table 1. Forty-three kilodalton protein-induced clustering of AChRs with chimeric subunits and of CD8

Membrane protein	43 kDa protein	(Cells with AChR clusters)/(AChR-positive cells)
AChR	+	48/107
	–	3/106
δ_β-AChR	+	55/105
	–	4/103
ϵ_β-AChR	+	57/102
	–	2/105
δ_β, ϵ_β-AChR	+	64/100
	–	3/104
CD8	+	12/200
	–	0/200

cDNAs of the AChR subunits or CD8 protein shown in the table were transfected with or without the cDNA of the 43 kDa protein into COS cells. AChRs on the surface were stained with MAb210, followed by FITC-conjugated goat anti-rat IgG, and visualized with fluorescence microscopy. CD8 protein on the surface was stained with OKT8 antibody against CD8 protein, followed by FITC-conjugated sheep anti-mouse IgG, and visualized with fluorescence microscopy. The number of AChR- or CD8 protein–positive cells was counted, as well as the fraction containing clusters.

To investigate whether the cytoplasmic domains of α and β subunits are required for clustering, we also constructed chimeras in which either the entire region between the extracellular N- and C-terminal domains or simply the long cytoplasmic loop of each of the subunits was replaced by the corresponding region from other subunits. Cotransfection of these chimeric cDNAs with the other required subunit cDNAs did not result in surface expression of AChRs.

Expression of neither the α nor the β long cytoplasmic loop exerts a dominant negative effect on 43 kDa protein–induced clustering of the AChR

If the long cytoplasmic loop of either the α or the β subunit interacted directly with the 43 kDa protein, then expression of an altered subunit that contained the loop but was not incorporated into the AChR might exert a dominant negative effect on clustering by competing with the intact AChR for complex formation with the 43 kDa protein. Increasing the concentration of either the α or the β subunit cDNA in the transfection mixture by up to fivefold, however, gave no significant change either in the amount of surface AChR (not shown) or in its ability to be

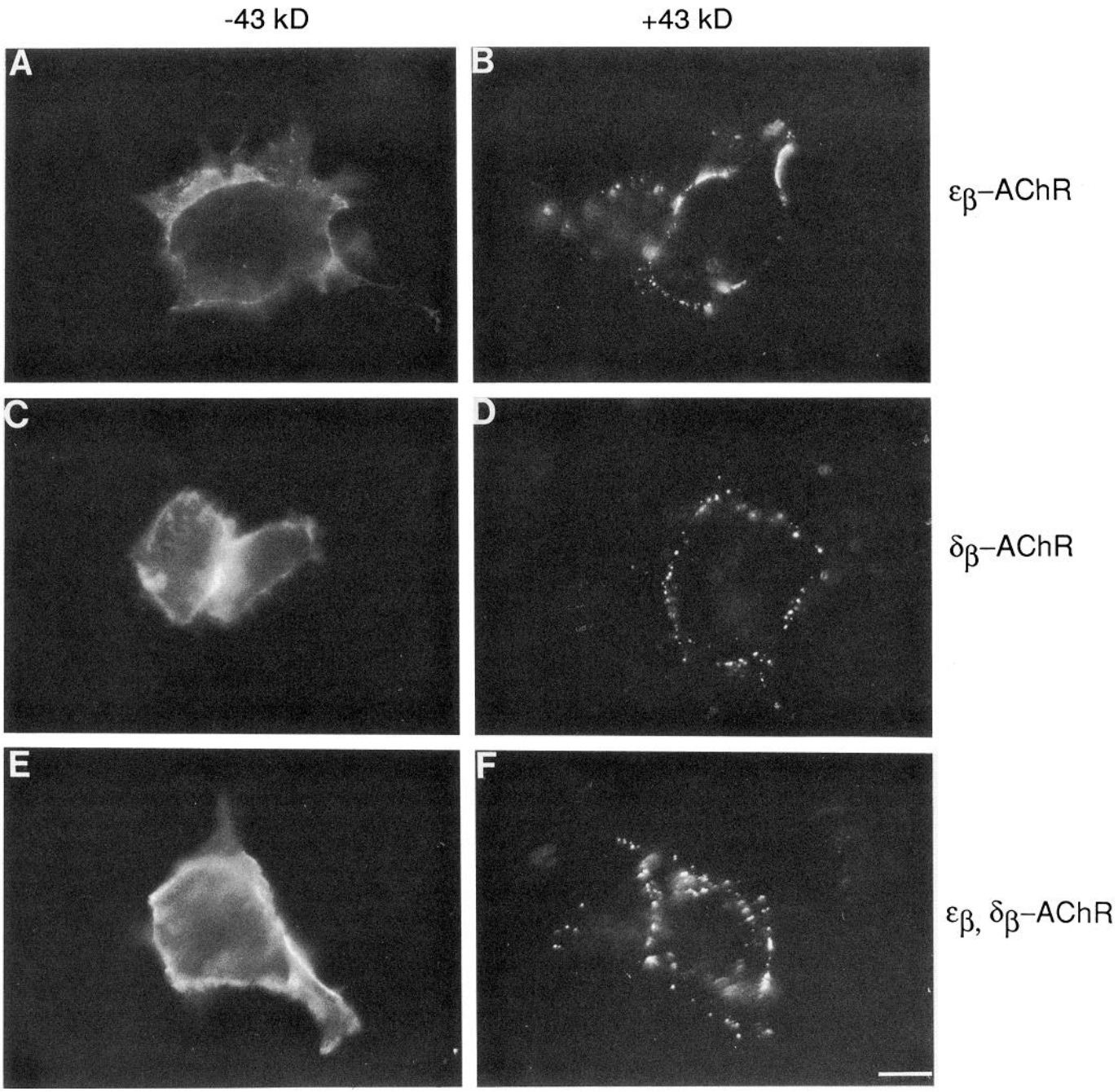

Figure 3. Forty-three kilodalton protein–induced clustering of AChRs containing chimeric subunits in the absence (*A, C,* and *E*) and presence (*B, D,* and *F*) of the 43 kDa proteins. COS cells cotransfected with cDNAs for AChRs containing either chimeric ϵ_β (*A, B*) or δ_β (*C, D*), or both subunits (*E, F*), and the distribution of the AChRs was examined by immunocytochemistry. In all cases, the surface AChRs were stained with MAb210, followed by FITC-conjugated goat anti-rat IgG, and visualized under fluorescence microscopy. More information is given in Materials and Methods. Scale bar, 16 μm.

clustered by the 43 kDa protein (Fig. 4*A,B*). In each case, immunoblotting experiments established that the amount of subunit expressed was increased in proportion to the increase in cDNA (data not shown).

We then tested whether expression of α and β subunits in which the N-terminal domains were deleted would exert a dominant negative effect on 43 kDa protein–induced AChR clustering. Such deletions should prevent incorporation of the mutated subunits into the AChR (Yu and Hall, 1991; Verrall and Hall, 1992), but leave the cytoplasmic portions of the subunits intact. Although analysis by immunoblotting showed that both truncated proteins were expressed at levels that were comparable to those of the corresponding intact subunit, neither decreased the frequency of AChR clustering (data not shown).

Finally, we sought evidence for intracellular association between the 43 kDa protein and the α or the β subunit by expressing them in COS cells in the absence of other subunits and examining their distribution by immunofluorescence. The α (Fig. 5*A*) and β subunits (Fig. 5*B*), identified by staining with specific monoclonal antibodies, were diffusely distributed throughout the cell, in a pattern characteristic of endoplasmic reticulum staining (Gu et al., 1989). No aggregates were seen. In contrast,

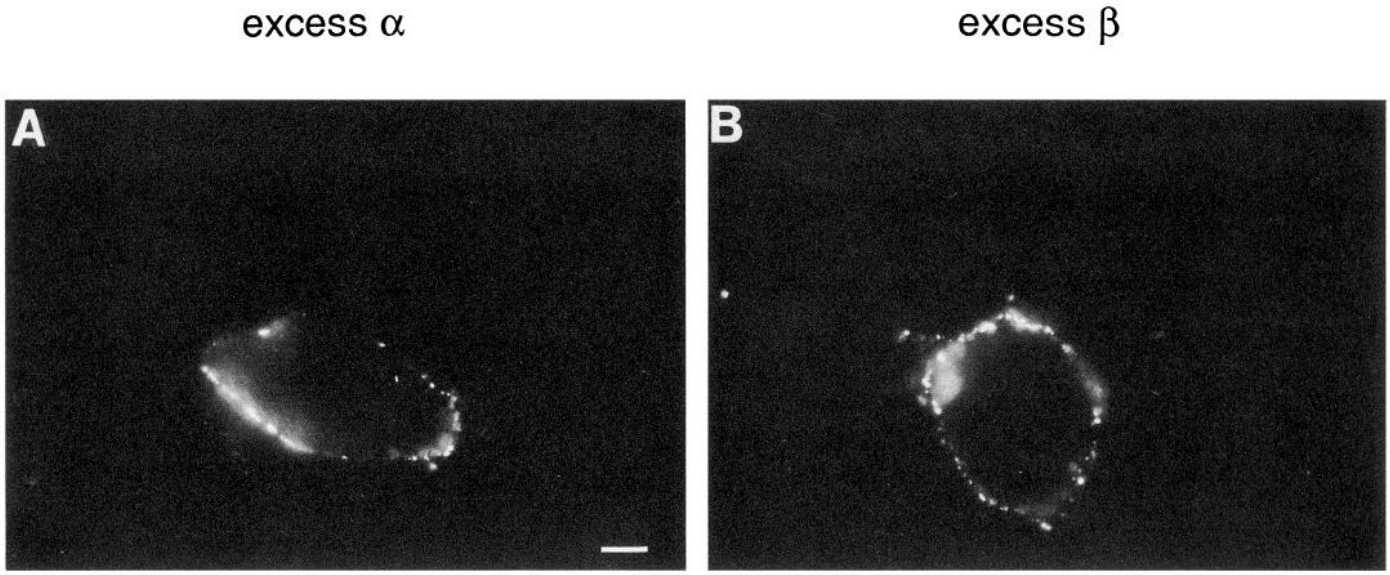

Figure 4. Forty-three kilodalton protein–induced clustering of the surface AChRs was not inhibited by excess expression of the α or β subunit. COS cells were transfected with standard concentrations of the cDNAs for the α, β, δ, and ε subunits and for the 43 kDa protein plus a twofold excess of the α subunit cDNA (*A*), or with a threefold excess of the β subunit cDNA (*B*). The surface AChRs were stained with MAb210, followed by FITC-conjugated goat anti-rat IgG, and photographed with fluorescence microscopy. Scale bar, 10 μm.

the 43 kDa protein, stained with a combination of specific monoclonal antibodies, appeared to be concentrated in aggregates on the cell surface and in the cytoplasm (Fig. 5*C,D*). The clearly different patterns of distribution give no evidence for intracellular association between the α or β subunit and the 43 kDa protein, and suggest that these subunits do not associate with the 43 kDa protein before their incorporation into the AChR.

Mutational analysis of the long cytoplasmic loops of the α and β subunits identifies small regions that may be required for clustering

If a unique segment of either the α or β subunit was required for AChR clustering, then its removal or replacement by a corresponding segment from the other subunit might be expected to block AChR clustering. We thus used conventional *in vitro* mutagenesis to make a systematic analysis of the long cytoplasmic loops of both the α and β subunits by deleting successive segments of the loops or by replacing the segments with corresponding sequences from the other subunit (Figs. 6, 7). Because the loops are poorly conserved, the changes made by the replacements were in each case significant (see Materials and Methods). For each deletion or replacement, the construct was tested with the other three subunits, with and without the 43 kDa protein, in the COS cell expression system. Surface expression of the AChR was monitored by ^{125}I–α-BTX binding and the distribution of the AChR containing the mutated α or β subunit by staining with MAb210. The results are summarized in Figures 6 and 7. In all cases in which expression of the AChR on the surface was obtained, clustering induced by the 43 kDa protein was seen. For the α subunit, substitution or deletion in 7–15 amino acid segments through a length of 83 amino acids, extending from α299 to α382, did not affect the ability of the AChR to be clustered. Altered α subunits with deletions and substitutions within a region of 26 amino acids (α382–α408) at the C-terminal end of the loop, however, did not become assembled into AChR expressed on the surface. The possible participation of this segment in clustering could thus not be eliminated.

For the β subunit, deletions or substitutions over a sequence extending from β338 to β430 did not prevent AChRs containing these subunits from being clustered. Deletion of the segment from β405 to β420 disrupted AChR assembly, as did substitution of the corresponding α sequence. When the corresponding γ sequence for this segment was substituted into the β subunit, however, surface AChR expression did occur, and the resulting AChR was clustered when coexpressed with the 43 kDa protein. Mutation of specific nonconserved amino acids within this sequence did not affect 43 kDa–induced AChR clustering (Fig. 7). Two regions at each end of the loop, β310–β338 and β430–β448, could not be mutated or deleted without disrupting AChR assembly. Thus either of these segments could participate in AChR clustering.

One of the substitutions that we made is of particular interest in view of the observation that agrin, a neurally derived, extracellular matrix protein that induces clustering in muscle cells, stimulates the phosphorylation of the β subunit of the AChR in chick (Wallace et al., 1991). When the seven amino acids GTDEYFI containing the site of tyrosine phosphorylation on the β cytoplasmic loop were mutated to those of the α subunit PSRDKQE (containing no tyrosine), AChR clustering induced by the 43 kDa protein was not affected [Fig. 7; see subunit β(*363–370)]. Thus tyrosine phosphorylation of the β subunit is not required for the coclustering of AChRs with the 43 kDa protein that is seen in COS cells. Moreover, when seven amino acids, PSRDKQE, including a serine in the loop of the α subunit, which is phosphorylated by protein kinase C (Huganir and Miles, 1989), were mutated to GTDEYFI, no effects on clustering of the AChR were seen [Fig. 6; see subunit α(*332–339)]. Thus, phosphorylation at these two sites is not required for 43 kDa protein–induced AChR clustering.

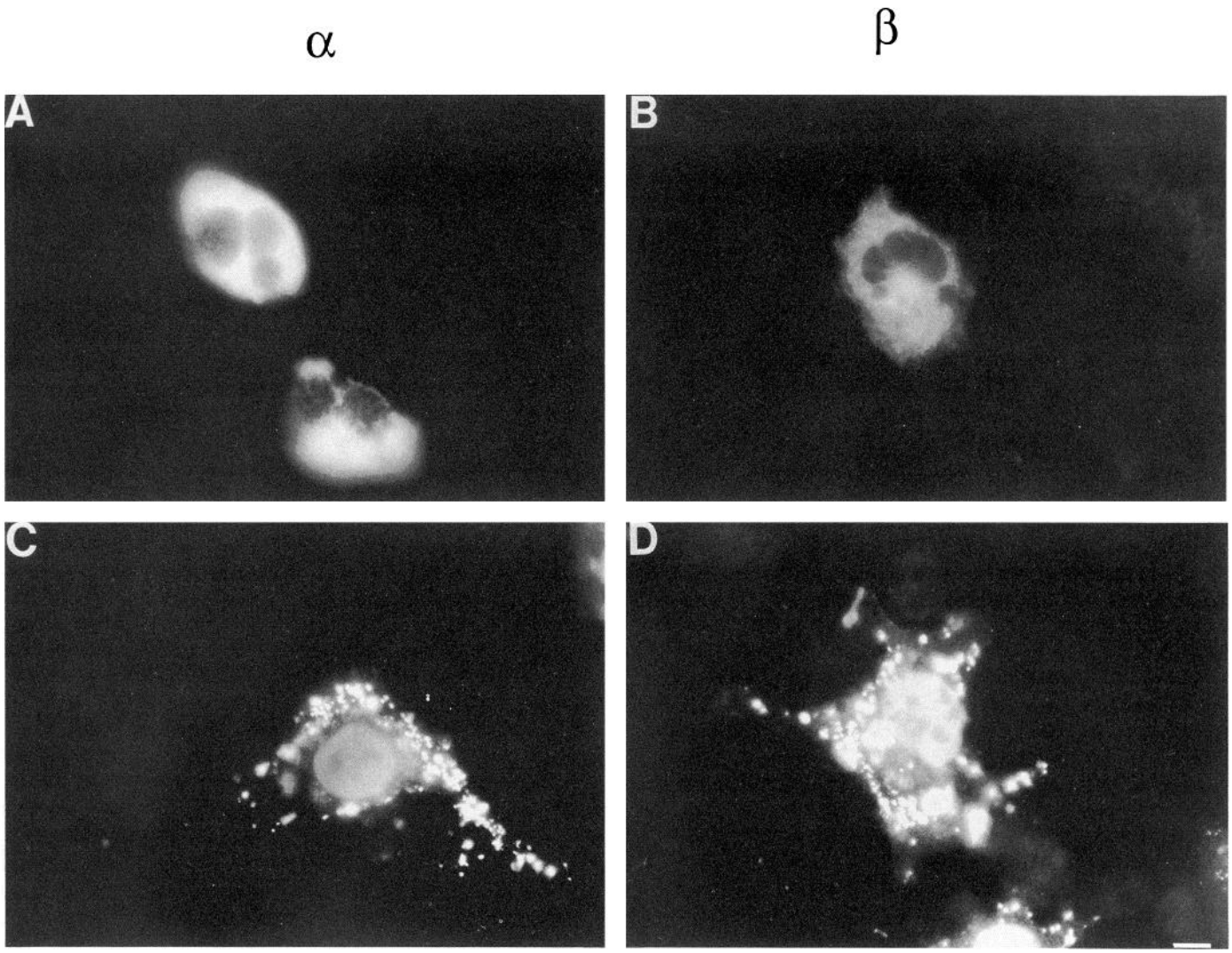

Figure 5. Different distributions of the α or β subunit and the 43 kDa protein were observed when both were coexpressed in COS cells. *A,* Distribution of the α subunit. The α subunit was coexpressed in COS cells with the 43 kDa protein, and the cells stained with MAb210 against the N-terminus of the α subunit, followed by FITC-conjugated goat anti-rat IgG, and visualized in fluorescence microscopy. *B,* Distribution of the β subunit. The β subunit was coexpressed in COS cells with 43 kDa protein, and the cells stained with MAb124 against the loop of the β subunit, followed by FITC-conjugated goat anti-rat IgG, and visualized in fluorescence microscopy. *C,* Distribution of the 43 kDa protein. The 43 kDa protein was coexpressed in COS cells with the α subunit, and the cells stained with the monoclonal antibodies to the 43 kDa protein (see Materials and Methods), followed by FITC-conjugated sheep anti-mouse IgG, and photographed in fluorescence microscopy. *D,* Distribution of the 43 kDa protein. The 43 kDa protein was coexpressed in COS cells with the β subunit, stained with the monoclonal antibodies followed by FITC-conjugated sheep anti-mouse IgG, and photographed in fluorescence microscopy. Scale bar, 10 μm.

Discussion

Our results show that in COS cells, as in other nonmuscle cells, AChRs expressed alone are diffusely distributed on the cell surface, but become clustered when coexpressed with the 43 kDa protein. Because the 43 kDa protein forms aggregates when expressed alone, and because the AChR clusters are coextensive with the 43 kDa protein aggregates when the two are expressed together, AChR clustering is likely to arise from direct interaction with the 43 kDa protein. This interaction is probably specific, as CD8, another membrane protein, does not become coclustered with the AChR when the two are expressed together.

Previous experiments have used *in vitro* mutagenesis to define the domains of the 43 kDa protein that are required for clustering of the AChR (Phillips et al., 1991). We have attempted to carry out similar experiments for the subunits of the AChR. Because the 43 kDa protein is a peripheral membrane protein on the cytoplasmic side of the membrane (Froehner, 1991), we have focused largely on the cytoplasmic domains of the AChR subunits, particularly those connecting M3 and M4.

Our experimental approach is based on the hypothesis that the 43 kDa protein interacts with the AChR through a specific region of a particular subunit, so that removal or alteration of this region would abolish the ability of the AChR to be clustered by coexpression with the 43 kDa protein. We first made receptors lacking the cytoplasmic domains of the ϵ and δ subunits and found that AChRs containing these mutated subunits could still be clustered by the 43 kDa protein. Thus, neither the cytoplasmic nor transmembrane domains of the ϵ or δ subunits are required for clustering. Similar results have been obtained in experiments in which the AChR and 43 kDa protein were

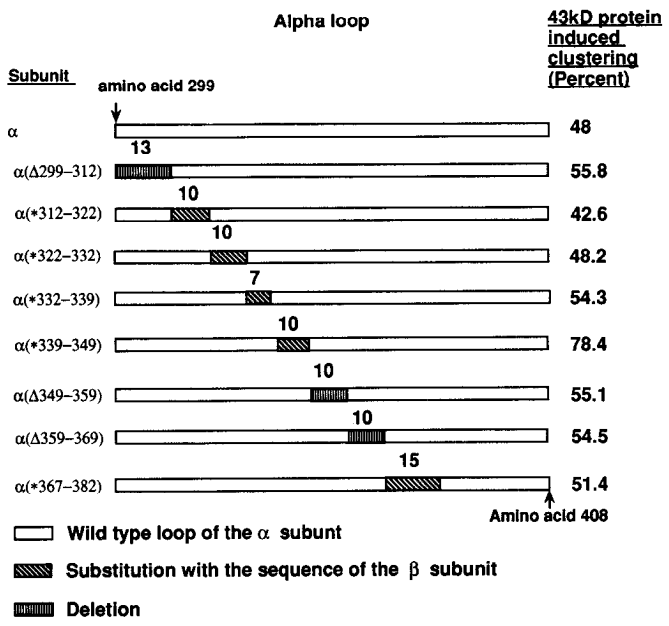

Figure 6. Forty-three kilodalton protein–induced clustering of AChRs containing mutated α subunits. A schematic diagram of the mutated α subunits is shown along with the fraction of cells expressing AChRs that have clustered AChRs when the mutated subunit is expressed along with other AChR subunits and with the 43 kDa protein. Each mutated α subunit was constructed by *in vitro* mutagenesis (see Materials and Methods). cDNAs of the β, δ, and ϵ subunits plus cDNA of each mutated α subunit were cotransfected with or without the cDNA of the 43 kDa protein into COS cells. AChRs on the surface were stained and AChR-positive cells were counted in the same way as described in Table 1.

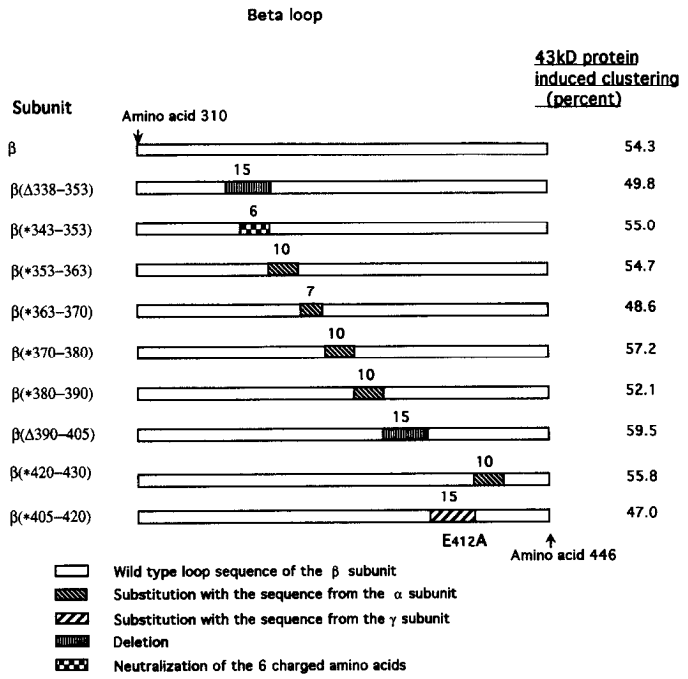

Figure 7. Forty-three kilodalton protein–induced clustering of AChRs containing mutated β subunits. A schematic diagram of the mutated β subunits is shown along with the fraction of cells expressing AChRs that have clustered AChRs when the mutated subunit is expressed along with other AChR subunits and with the 43 kDa protein. Each mutated β subunit was constructed by *in vitro* mutagenesis (see Materials and Methods). cDNAs of the β, δ, and ϵ subunits plus cDNA of each mutated β subunit were cotransfected with or without the cDNA of the 43 kDa protein into COS cells. AChRs on the surface were stained and AChR-positive cells were counted in the same way as described in Table 1.

expressed in *Xenopus* oocytes (P. Scotland and S. Froehner, personal communication).

Because cytoplasmic regions of the ϵ and δ subunits were not required, we then examined the α and β subunits, focusing particularly on their long cytoplasmic loops. Expression of chimeric subunits in which the loops were exchanged between α and β subunits did not support AChR assembly, so we were unable to rule out interactions of the 43 kDa protein with either. Because of the potential importance of the cytoplasmic loops, we then made a systematic series of deletions or substitutions in them. In each case, deletions or substitutions throughout most of their lengths did not alter the ability of the AChR to be clustered in the presence of the 43 kDa protein. For the α subunit, deletions from α299 to α382 had no effect on clustering; for the β subunit, deletions or substitutions from β338 to β430 were also without effect. For each subunit, however, we were unable to make conclusions about a sequence of 16–26 amino acids that occurs near the junction of the cytoplasmic loop and M4. Removal or alteration of these sequences prevented AChR assembly. In other experiments, we have suggested that this region is required for steps in the assembly pathway subsequent to heterodimer formation (Yu and Hall, unpublished observations). Also in the case of the β subunit, deletions and substitutions in a short region near the N-terminus of the loop (amino acids 310–338) failed to support surface expression.

Our experiments thus are unable to identify a specific sequence that is required for 43 kDa protein–induced AChR clustering. These results can be interpreted in two ways. First, one or more of the sequences that we were unable to test could mediate the interactions required for clustering. Second, there may not be a specific sequence that is required for clustering. In this case, the 43 kDa protein or other proteins could interact with multiple or alternate sites on the receptor subunits, such that removal of a single sequence would not abolish clustering. The interactions leading to clustering thus might not depend on any single sequence. The proposed location of the 43 kDa protein beneath the AChR (Toyoshima and Unwin, 1988; Mitra et al., 1989) would be consistent with multiple sites of interaction.

One clear result of our experiments is to show that phosphorylation of the tyrosine residue on the cytoplasmic loop of the β subunit is not required for 43 kDa protein–induced clustering. This tyrosine, which can be removed without affecting clustering, is postulated to be the site at which the β subunit is phosphorylated in response to the neurally released clustering factor agrin (Wallace et al., 1991). We also show that a serine residue (α333) in the cytoplasmic loop of the α subunit of the AChR can be removed without affecting clustering.

In our experiments, we made several unsuccessful attempts to detect interactions between individual subunits and the 43 kDa protein. Thus overexpression of neither α nor β subunits detectably decreased AChR clustering as might be expected if the isolated subunits competed with intact AChRs for the 43 kDa protein. Also, no relation was found between the intracellular distribution of the 43 kDa protein and the β subunit. These

observations suggest that these subunits may not interact with the 43 kDa protein before their incorporation into the AChR.

An unanswered question is the relation between the clustering of AChRs induced by the 43 kDa protein in nonmuscle cells and the AChR clustering seen in muscle cells. Although the 43 kDa protein is found in aggregates when expressed alone in nonmuscle cells, its distribution in muscle cells appears to be regulated in a different way. Thus, the 43 kDa protein is not aggregated or clustered in variants of the C2 muscle cell line that do not form AChR clusters, either because they lack AChRs (LaRochelle et al., 1989) or because they make a defective extracellular matrix (Gordon et al., 1993). Moreover, muscle cells in which the 43 kDa protein is overexpressed show a decrease in the number of large clusters that are normally seen and an increased number of small clusters similar to those seen in COS cells (Yoshihara and Hall, 1993). These experiments raise the possibility that the 43 kDa–induced clustering seen in nonmuscle cells may occur by a mechanism that is different from that seen in muscle cells. Alternatively, the mechanism of 43 kDa protein–induced clustering seen in nonmuscle cells may resemble that seen in muscle cells, but may only be part of the more complex system that regulates AChR clustering in muscle cells. In either case, expression in nonmuscle cells provides a simplified system in which interactions between the AChR and the 43 kDa protein can be mapped in experiments such as those presented here.

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