Increased Expression of the 43-kD Protein Disrupts Acetylcholine Receptor Clustering in Myotubes

Corinne M. Yoshihara and Zach W. Hall

Department of Physiology, University of California, San Francisco, California 94143-0444

Abstract. The 43-kD protein is a peripheral membrane protein that is in ~1:1 stoichiometry with the acetylcholine receptor (AChR) in vertebrate muscle cells and colocalizes with it in the postsynaptic membrane. To investigate the role of the 43-kD protein in AChR clustering, we have isolated C2 muscle cell lines in which some cells overexpress the 43-kD protein. We find that myotubes with increased levels of the 43-kD protein have small AChR clusters and that those with the highest levels of expression have a drastically reduced number of clusters. Our results suggest that the 1:1 stoichiometry of AChR and 43-kD protein found in muscle cells is important for AChR cluster formation.

THE most prominent molecular feature of the postsynaptic membrane of the vertebrate neuromuscular junction is the high density of acetylcholine receptors (AChRs)[1] on the peaks of the junctional folds (Fertuck and Salpeter, 1974). The AChRs are directly opposite the presynaptic sites of neurotransmitter release (Heuser et al., 1979), where their high density is essential for efficient neuromuscular transmission. During development of the neuromuscular junction clusters of AChRs form near sites of initial neuromuscular contact (Bevan and Steinbach, 1977; Matthews-Bellinger and Salpeter, 1983), and are the first detectable sign of synaptic differentiation. Experiments in vitro have established that these clusters arise, at least in part, by the lateral migration of existing AChRs in the muscle membrane (Anderson and Cohen, 1977; Ziskind-Conhaim and Hall, 1984), and that clustering can be induced by a basal lamina protein, agrin, which is released by the innervating motor axon (McMahan, 1990; Reist et al., 1992; Cohen and Godfrey, 1992). In addition to components of the basal lamina, proteins in the underlying cytoskeleton may play a role in regulating AChR clustering activity (Froehner, 1991). In particular, recent experiments have implicated a 43-kD subsynaptic protein (Froehner, 1991).

The 43-kD protein is a cytoplasmic, peripheral membrane protein that occurs in ~1:1 stoichiometry with the AChR (LaRochelle and Froehner, 1986) and is exactly colocalized with it in the postsynaptic membrane (Sealock et al., 1984; Toyoshima and Unwin, 1988; Mitra et al., 1989). Although AChR clusters can sometimes occur without detectable expression of the 43-kD protein, as in early *Torpedo* development (Kordeli et al., 1989; LaRochelle et al., 1990; Nghiem et al., 1991), the two proteins are invariably associated in nerve-induced clusters and in clusters that occur spontaneously in cultured myotubes (Froehner, 1991). Removal of the 43-kD protein and other peripheral membrane proteins from purified synaptic membranes by alkaline extraction increases the lateral mobility of the AChR, suggesting that one or more of the proteins plays a role in immobilizing AChRs in clusters (Barrantes et al., 1980; Rousselet et al., 1982). More recently, experiments in which the 43-kD protein and the AChR were expressed in non-muscle cells suggest that the 43-kD protein may be directly responsible for AChR cluster formation. When the 43-kD protein was expressed alone, it formed aggregates or clusters; when expressed with the AChR it caused co-clustering of the AChRs, which were otherwise evenly distributed in the surface membrane (Froehner et al., 1990; Phillips et al., 1991; Brennan et al., 1992).

We have sought to examine the regulatory role of the 43-kD protein in AChR clustering by overexpressing it in muscle cells in which it normally occurs. We find that increased expression of the 43-kD protein interferes with normal AChR cluster formation, and at high levels reduces both the number and size of the clusters that are formed. Our results suggest that the 1:1 stoichiometry of the 43-kD protein and the AChR is important for efficient clustering of the receptor.

Materials and Methods

Cell Culture

C2C12 cells (Yaffe and Saxel, 1977; Blau et al., 1983) were maintained as described (Ralston and Hall, 1989). Myoblasts were grown in growth medium (GM) (Inestrosa et al., 1983) consisting of DME H-16 with 1 g/liter glucose supplemented with 20% FBS, 0.5% chick embryo extract (GIBCO BRL, Gaithersburg, MD), 2 mM L-glutamine, 100 U/ml penicillin. Cultures were made to differentiate upon reaching confluence by changing the medium to fusion medium (FM) (Inestrosa et al., 1983) consisting

1. *Abbreviations used in this paper*: AChR, acetylcholine receptor; CMV, cytomegalovirus; GM, growth medium; ^{125}I-α-BuTx, iodinated α-bungarotoxin; PBS-CMF, PBS-calcium and magnesium free; rBuTx, rhodamine-conjugated α-bungarotoxin.

of DME H-21 supplemented with 5% horse serum and the same concentration of glutamine as in GM. Cells were maintained at 37°C under 8% CO_2 and 100% humidity.

COS cells were grown in DME H-16 supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin. During the transfection the cells were maintained in DME H-16 containing 1% heat-inactivated FBS.

Construction of 43-kD Protein Expression Vector and Introduction of the Epitope Tag

A plasmid containing the mouse 43-kD cDNA (1A15; Froehner, 1989) was digested with EcoRI and HindIII, releasing a 1.489-kb fragment containing the entire coding sequence. The ends of the cDNA were rendered blunt by filling in with Klenow polymerase and nucleotides. BstXI adaptors (Invitrogen, San Diego, CA) were ligated to this fragment, and the 1.505-kb fragment purified and ligated to the BstXI-digested pRc/CMV Neo expression vector (Invitrogen). After sequencing, plasmids with the cDNA in the correct orientation for 43-kD protein expression (pRc/CMV-43K) were chosen.

The 43-kD cDNA, the full-length coding sequence for the mouse protein (Froehner, 1989) minus 5'-untranslated sequences, was subcloned into the expression vector. The T antigen epitope tag was introduced at the COOH terminus of the 43-kD protein by a combination of oligonucleotide site-directed and casette mutagenesis. A single-stranded DNA template corresponding to the sense strand of the 43-kD cDNA was prepared from the pRc/CMV-43K vector by rescuing with M13K07. A ClaI site and a SacII site were introduced at nucleotides 1374–1377 and 1403, respectively, of the mouse 43-kD cDNA by site-directed mutagenesis with a single oligonucleotide CCAAGATGGCCGCGGAAGTCACACAAAGCCCGGCTTCATCGATGAGCGGCGGCA GTT as described in Baldwin et al. (1991). The T antigen epitope tag was introduced by annealing the oligonucleotides CGATGCCCCCGGAGCCCGAGACCTAACTTCCGC and GGAAGTTAGGTCTCGGGCTCCGGGGGCAT and ligating them to the mutated pRc/CMV-43K plasmid digested with ClaI and SacII. These changes caused the amino acids SSMKPGFV (405–412) at the COOH terminus to be replaced by STPPPEPET. The underlined residues are the T antigen epitope tag. The cDNAs in all mutagenesis and subcloning experiments were confirmed by DNA sequencing.

Transfection and Selection of Stably Transfected Cell Lines

Cells were transfected by the standard calcium phosphate precipitation technique (Graham and Van Der Eb, 1973; Wigler et al., 1979). Clones were selected by growing transfected cells in media containing 700 μg/ml (active concentration) G418 (Geneticin; GIBCO BRL). After selection, stable clonal cell lines were maintained with G418 added to the media at a concentration of 100 μg/ml.

Transient Transfection

COS cells were grown in 35-mm dishes and transfected as described previously (Gu et al., 1990) using a modified DEAE-dextran transfection procedure (Seed and Aruffo, 1987). After transfection the cells were replated onto round glass coverslips (12-mm diam).

Immunofluorescence and Histochemistry

Cells were grown on 12-mm-round coverslips coated with 2% gelatin. Indirect immunocytochemical staining was used to stain myotubes for the 43-kD protein and T antigen and COS cells for the AChR alpha subunit. For T antigen detection a modification of the protocol described in Ralston and Hall (1989) was used. Myotubes were incubated with rBuTx at a 1:200 dilution in FM at 37°C for 1 h, washed with PBS, fixed in 2% *p*-formaldehyde in PBS–CMF for 20 min at room temperature, and rinsed with PBS–CMF again. Myotubes were permeabilized by incubation with 1% Triton/PBS–CMF for 10 min and incubated in 10% FBS/4% BSA/PBS for 1 h to inhibit nonspecific binding of antibodies. Myotubes were incubated with primary antibody (4.2 μg/ml; MacArthur and Walter, 1984) in 0.2% Triton/1% BSA/PBS overnight at 4°C, washed with PBS–CMF, incubated for 1 1/2 h with FITC-conjugated second antibody (sheep anti-mouse; Cappel Laboratories, Cochranville, PA) at a 1:500 dilution in 0.2% Triton/1% BSA/PBS–CMF at room temperature and rinsed with PBS–CMF. 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.

The staining procedure for the 43-kD protein was a modification of the procedure of LaRochelle et al. (1989) and that described above for T antigen staining. The procedures for rBuTx stain, fixation, permeabilization, and block of non-specific binding were the same as described above. Myotubes were incubated with primary antibody (20 nM 1234A, 20 nM 1579A; Peng and Froehner, 1985) overnight at 4°C, washed with PBS–CMF incubated for 1 1/2 h with FITC-conjugated second antibody (sheep anti-mouse; Cappel Laboratories) at a 1:500 dilution in 10% FBS/4% BSA/PBS at room temperature and rinsed with PBS–CMF. Myotubes were incubated with a second FITC-conjugated second antibody (rabbit anti-sheep; Cappel Laboratories) at a 1:500 dilution in 10% FBS/4% BSA/PBS at room temperature for 1 1/2 h, and then rinsed with PBS–CMF. The coverslips were mounted as described above.

For the staining of the AChR alpha subunit on COS cells a modification of a procedure described in LaRochelle et al., 1989 was used (Yu, X., personal communication). COS cells were fixed in 2% *p*-formaldehyde, rinsed with PBS, and blocked in 10% FBS/4% BSA/PBS for 1 h. COS cells were incubated with mAb 210 (215 nM; Ratnam et al., 1986) diluted in 10% FBS/4% BSA/PBS, washed in PBS for 15 min, washed in 10% FBS/4% BSA/PBS for 15 min, incubated in FITC second antibody (goat anti-rat; Cappel Laboratories) at a 1:200 dilution in 10% FBS/4% BSA/PBS at room temperature for 1 h, washed, mounted, and viewed as described above.

Quantitation of AChR Clusters

After cultures of transfected and untransfected muscle cells were stained, randomly chosen fields were examined by fluorescence microscopy using a 40× objective. In the case of staining for the epitope-tagged and -untagged 43-kD protein, myotubes were divided into three groups of strong, moder-

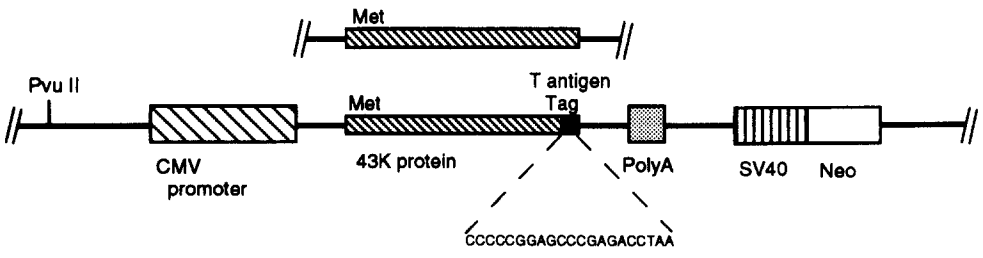

Figure 1. Constructs encoding tagged and untagged 43-kD protein. The full-length coding sequence for mouse 43-kD protein (Froehner, 1989) minus the 5' untranslated sequences was subcloned into a pRc/CMV expression vector containing a cytomegalovirus promoter and a neomycin resistance gene. An SV40 T antigen epitope was introduced at the 3' end of the 43-kD protein to distinguish the introduced 43-kD protein from the endogenous protein. See methods for further details.

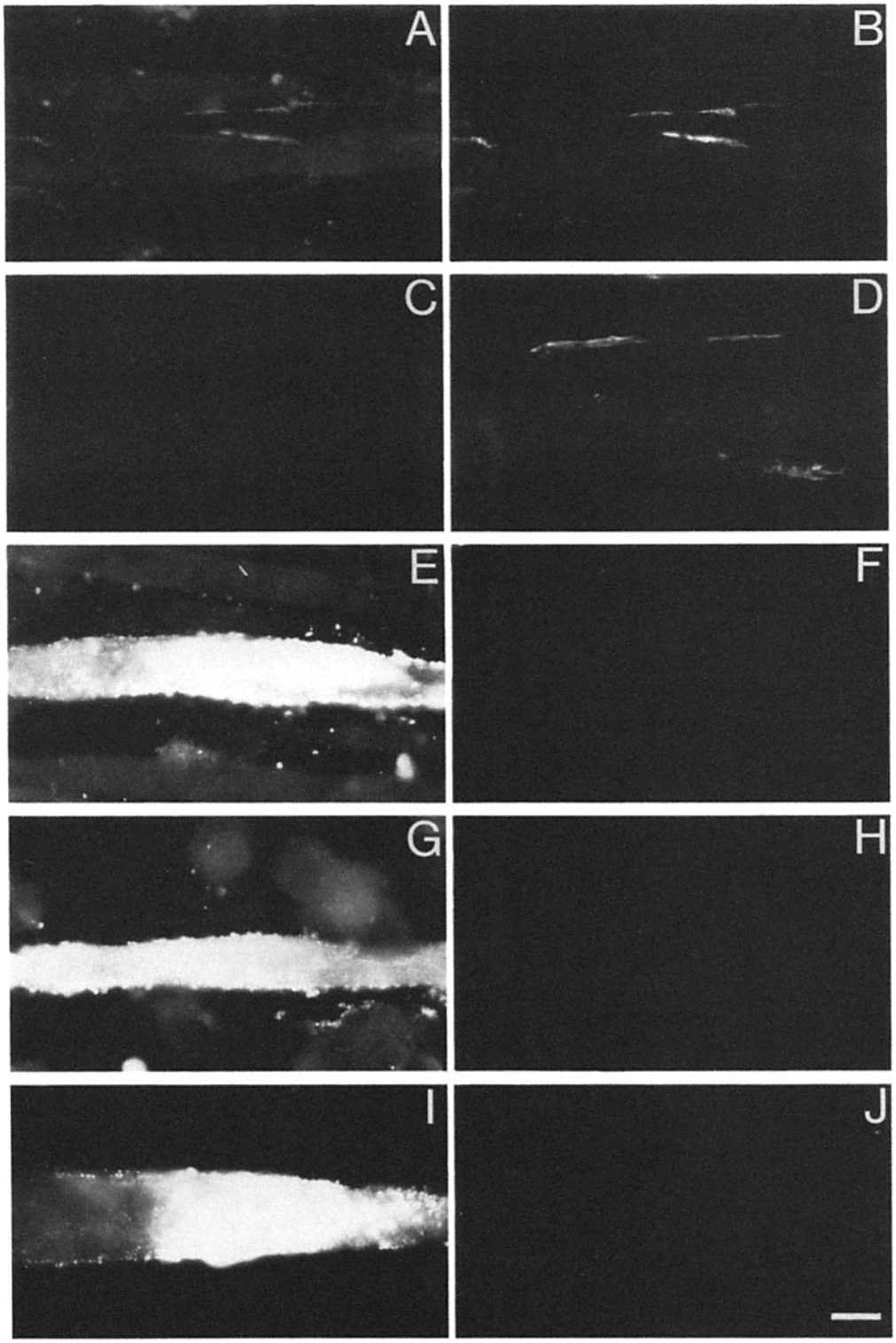

Figure 2. Expression of transfected 43-kD protein and AChR clusters in myotubes. Myotubes from C2 cells and from clonal cell lines expressing untagged (43-1, 43-2) or tagged (43T-1, 43T-2) 43-kD protein were double-stained for AChR and 43-kD protein, or for AChR and Tag. The untagged and tagged 43-kD protein were detected by monoclonal antibodies to the 43-kD protein or to Tag, respectively, followed by FITC-conjugated sheep anti-mouse second antibody. AChRs were labeled with rBuTx. C2 myotubes show colocalized staining of AChR and 43-kD protein and no Tag staining. Myotubes from clonal cell lines expressing high levels of 43-kD protein or tagged 43-kD protein lack AChR staining. (*A, C, E, G, I*): FITC optics; (*B, D, F, H, J*): rhodamine optics. (*A, B*) C2 myotubes stained for 43-kD protein and AChR. (*C, D*) C2 myotubes stained for Tag and AChR. (*E, F*) Myotube with high level of 43-kD protein expression stained for 43-kD protein. (*G, H*) Myotube with high level of tagged 43-kD protein stained for 43-kD protein. (*I, J*) Myotube with high level of tagged 43-kD protein stained for Tag. Bar, 20 μm.

ate, and weak staining according to the intensity and extent of the staining. Myotubes that stained very intensely over most of their surface were classified as staining strongly (see Figs. 3 *A*, 4 *A*, 5 *A*). In these cases, the staining appeared to be relatively uniform at low magnification, but at higher magnification was seen to be punctate. Myotubes were classified as moderately staining when the staining was less intense and mostly punctate (see Fig. 3 *C*); occurred only at the edge of the fiber (see Fig. 5 *C*); and/or when the staining extended less than half the length of the fiber (10–50%) (see Fig. 4 *C*). Myotubes with weak staining included those in which no staining could be detected (in the case of the tagged 43-kD protein) (see Fig. 3 *E*) or in which only occasional staining at the edge of the fiber was seen (see Figs. 4 *E* and 5 *E*). In general, distinction between the three groups was easily made. Those for which it was doubtful were classified as moderate.

For analysis of AChR clusters, myotubes with strong, moderate and weak staining for the epitope-tagged and -untagged 43-kD protein were identified in random fields, and the number of AChR clusters present on them was counted. All AChR clusters, regardless of size, were included. Measurements of the cluster size was made along the long axes of the myotubes.

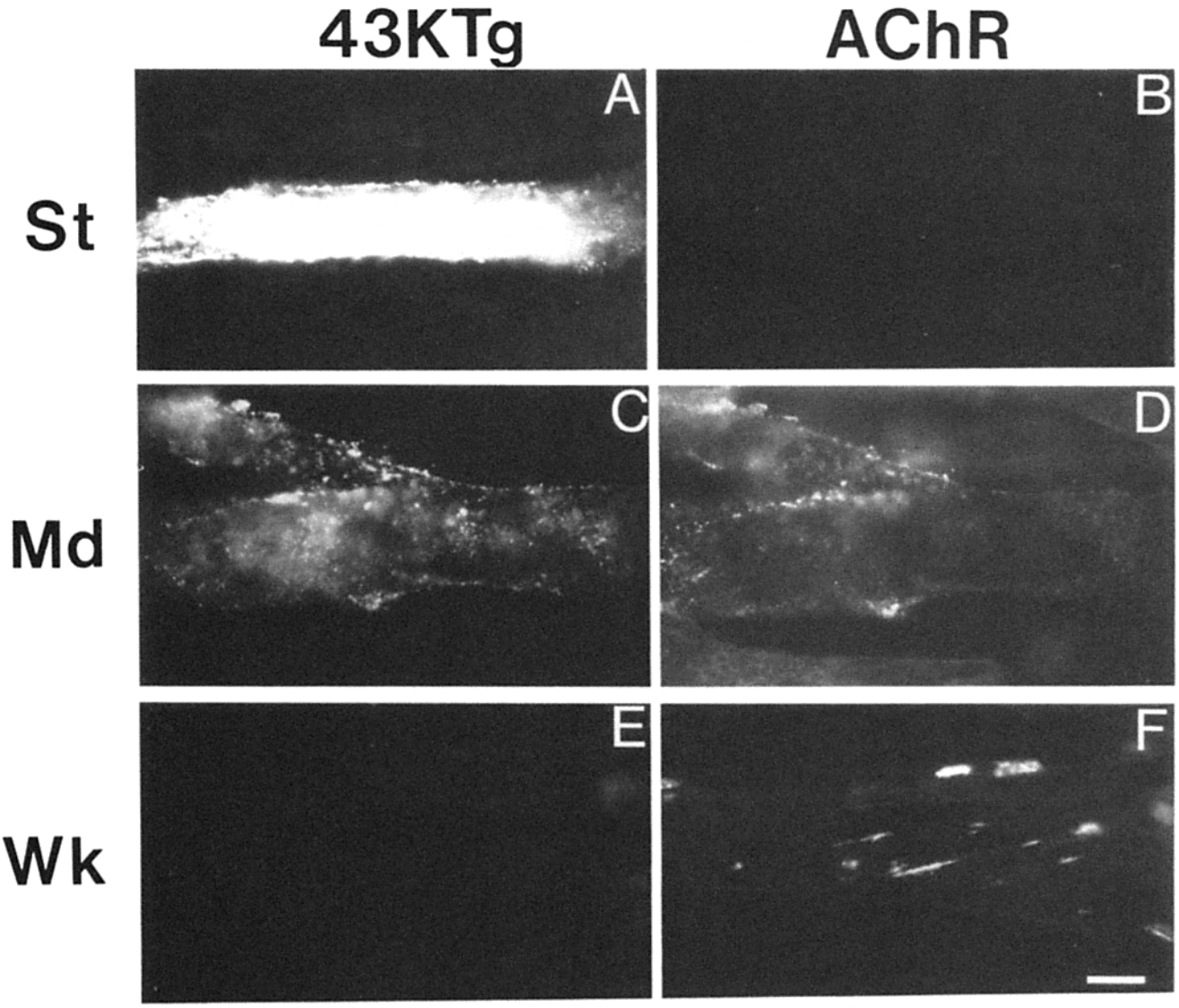

Figure 3. Expression of transfected tagged 43-kD protein and AChR clusters in myotubes. Myotubes from cultures of a clonal cell line expressing tagged 43-kD protein were double-stained with a monoclonal antibody to Tag followed by FITC-conjugated sheep anti-mouse second antibody to detect the tagged 43-kD protein and with rBuTx to detect AChR. (*A*, *C*, *E*): FITC optics; (*B*, *D*, *F*): rhodamine optics. (*A*, *B*) Myotube with high level of tagged 43-kD protein expression; no AChR clusters are visible. (*C*, *D*) Myotube with moderate level of tagged 43-kD protein expression and small AChR clusters. (*E*, *F*) Myotube with low level of tagged 43-kD protein expression and large AChR clusters. Bar, 20 μm.

AChR Surface Expression

Surface AChRs were labeled with ^{125}I-α-BuTx (Amersham Corp., Arlington Heights, IL). Myotube cultures grown in wells of a 24-well dish (15-mm diam) were incubated with 10 nM ^{125}I-α-BuTx in FM for 1/2 h. The cultures were washed three times with FM and solubilized in 0.1 N NaOH. Radioactivity bound to the cells was determined in a Biogamma II counter (Beckman Instruments, Palo Alto, CA) with a 77.7% efficiency for ^{125}I. Protein concentration for cultures was determined by the Bradford method (Bradford, 1976). Protein concentration was determined against an albumin standard.

Autoradiography and Immunohistochemistry

Double labeling was performed on myotubes cultured on glass coverslips. The distribution of AChRs was determined by autoradiography, and T antigen and 43-kD proteins were identified by immunohistochemistry. Myotube cultures were grown on glass coverslips (12-mm round or 22-mm square) and labeled with ^{125}I-α-BuTx as described above. After washing with PBS myotubes were fixed and permeabilized as described for histochemistry. Myotubes were incubated with primary antibody (2.8 μg/ml T antigen antibody or 20 nM 1234A, 20 nM 1579A). Primary antibody binding was detected with HRP-conjugated horse anti-mouse second antibody by incubation with 3,3'-DAB and H_2O_2 substrates. Coverslips were coated with nuclear research emulsion K.2 (Ilford, Knutsford, Chesire, England), exposed for ~2 1/2 d, developed, mounted onto glass slides with permount. Grains were counted with a 100× objective.

Results

Isolation and Characterization of Muscle Cell Lines That Express Elevated Levels of the 43-kD Protein

To obtain muscle cells that express high levels of the 43-kD protein, C2C12 cells were transfected with an expression vector encoding the mouse 43-kD protein under the control of a human cytomegalovirus (CMV) promoter and enhancer

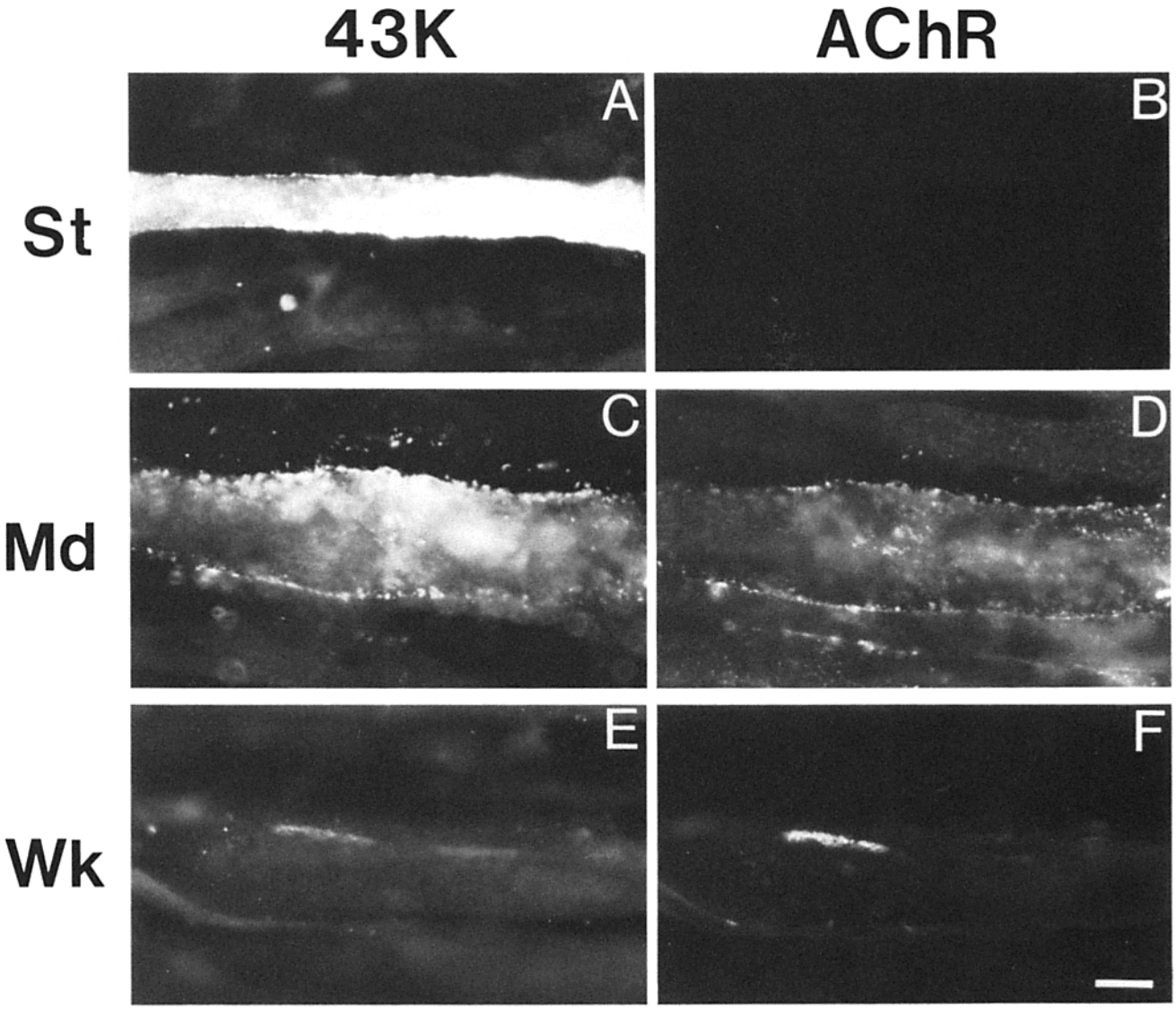

Figure 4. Expression of 43-kD protein and AChR clusters in myotubes transfected with the tagged 43-kD protein. Myotubes from cultures of a clonal cell line expressing tagged 43-kD protein were double-stained with monoclonal antibodies to 43-kD protein followed by FITC-conjugated sheep anti-mouse and rabbit anti-sheep second antibodies to detect the 43-kD protein. (*A*, *C*, *E*): FITC optics; (*B*, *D*, *F*): rhodamine optics. (*A*, *B*) Myotube with high level of 43-kD protein expression; no AChR clusters are visible. (*C*, *D*) Myotube with moderate level of 43-kD protein expression and small AChR clusters. (*E*, *F*) Myotube with low level of 43-kD protein expression and large AChR clusters. Bar, 20 μm.

(see Materials and Methods) (Fig. 1). Two forms of the vector were used in parallel experiments. In one case, we introduced an epitope tag into the 43-kD protein so that the protein encoded by the vector could be distinguished from the endogenously expressed 43-kD protein. To tag the protein, six amino acids corresponding to the COOH terminus of the SV-40 T antigen (MacArthur and Walter, 1984) were substituted for the last five amino acids of the 43-kD protein. In the other case the vector encoded the unmodified 43-kD protein. In both cases, the plasmid also contained a neomycin resistance gene controlled by the SV-40 early promoter.

To maximize the number of cells that take up the DNA, low-density cultures of actively dividing myoblasts were transfected. Transfected cells were selected by growth in Geneticin (G418) and individual clones screened for high levels of expression of the 43-kD protein either by a mAb to the T antigen (Tag) epitope (MacArthur and Walter, 1984) and/or to the 43-kD protein (Peng and Froehner, 1985). For both sets of transfectants, 90–100% of G418-resistant clones expressed the protein introduced via the plasmid DNA. In each case, two lines of transfectants expressing either the tagged (cell lines 43T-1 and 43T-2) or the untagged (43-1 and 43-2) form of the protein were selected for further study.

Myoblasts from the cell lines expressing either tagged or untagged 43-kD protein fused and formed myotubes. Myotube cultures of the transfected cell lines showed occasional spontaneous contractions as do normal C2 myotube cultures. Although the rate of fusion for some of the cell lines appeared to be slightly lower than that of the parental line, these differences probably represent the normal variation seen with subclones of the C2 cell line (Gordon and Hall, 1989). The AChR expressed on the surface of normal and

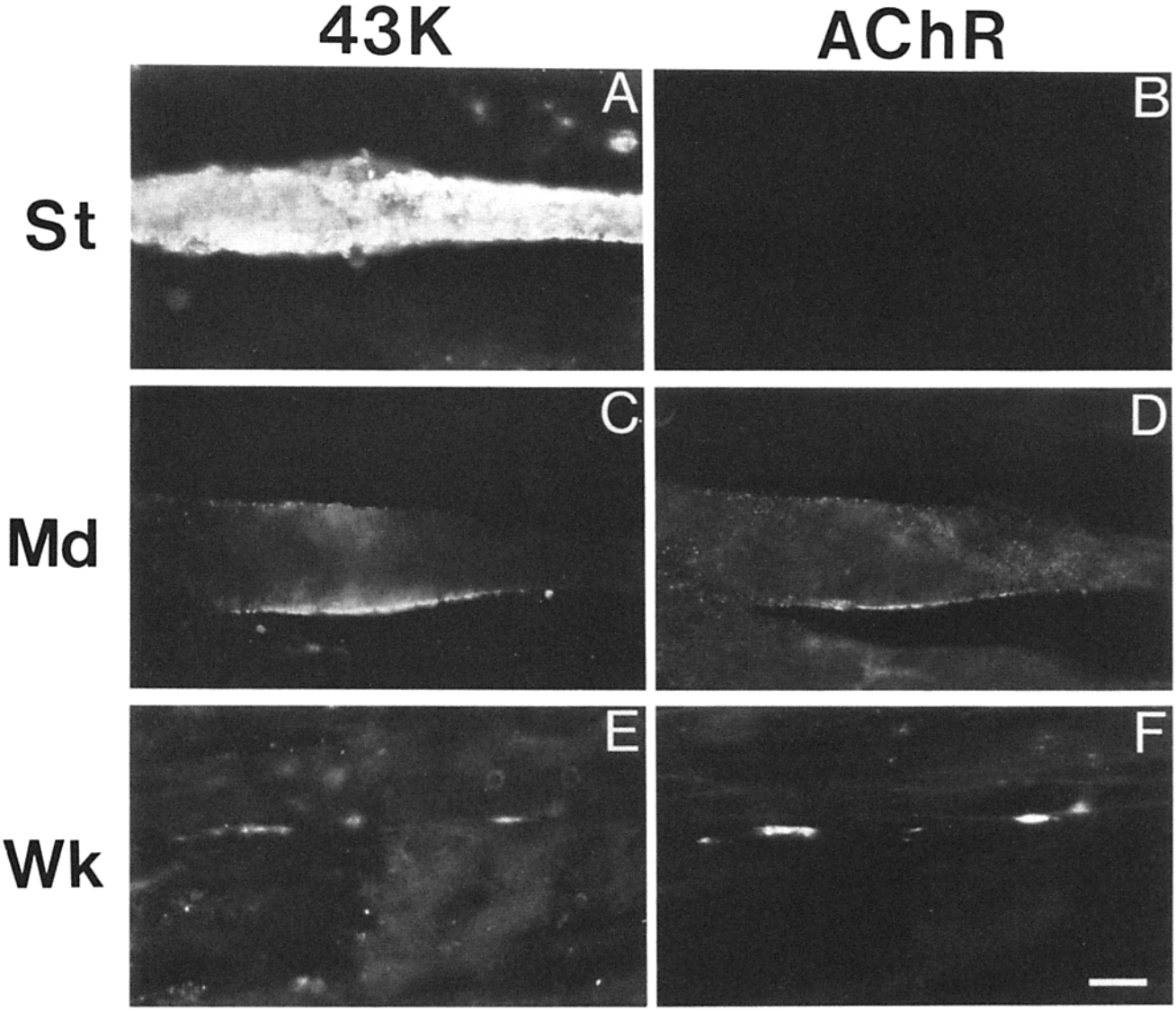

Figure 5. Expression of 43-kD protein and AChR clusters in myotubes transfected with the 43-kD protein. Myotubes from cultures of a clonal cell line expressing untagged 43-kD protein were double-stained with monoclonal antibodies to 43-kD protein followed by FITC-conjugated sheep anti-mouse second antibody to detect 43-kD protein and rBuTx to detect AChR. (*A*, *C*, *E*): FITC optics; (*B*, *D*, *F*): rhodamine optics. (*A*, *B*) Myotube with high level of untagged 43-kD protein expression; no AChR clusters are visible. (*C*, *D*) Myotube with moderate level of untagged 43-kD protein expression and small AChR clusters. (*E*, *F*) Myotube with low level of untagged 43-kD protein expression and large AChR clusters. Bar, 20 μm.

transfected myotubes was measured by the binding of ^{125}I-α-bungarotoxin (^{125}I-α-BuTx) to intact myotube cultures. The levels seen in the transfected cell lines were variable, but well within the normal range for C2 cells (see Table II).

Immunofluorescence staining of both myoblast and myotube cultures of the transfected cell lines showed that the expression of the 43-kD protein in individual cells was highly variable in all cases. When cells expressing the tagged 43-kD protein (43T-1 and 43T-2) were stained with the Tag mAb, most showed no detectable staining, whereas a small minority (ca. 10%) showed staining of variable intensity and pattern (Fig. 2 *I*; and Fig. 3, *A*, *C*, and *E*). Myotubes with high levels of expression (<5%), showed bright and relatively uniform levels of staining over the entire surface; when examined at high magnification, the staining was seen to be punctate. Myotubes with moderate levels of expression (5–10%) showed a staining pattern that was more consistently punctate, and often only some areas of the cell surface were stained. Finally, the remaining myotubes showed no detectable staining (see figures for examples). The expression of the tagged 43-kD protein was also visualized by staining with anti-43-kD mAbs which gave a pattern of staining seen with the Tag mAb (Fig. 4). A pattern of high, moderate, and low staining was seen when cells transfected with the 43-kD cDNA were stained with mAbs to the 43-kD protein (Fig. 2 *E*; and Fig. 5, *A*, *C*, and *E*). In these lines (43-1 and 43-2) cells with low overall staining (Fig. 5 *E*) showed patches of 43-kD protein that presumably represent endogenous protein.

Elevated Levels of 43-kD Protein Expression Lead to Fewer, Smaller AChR Clusters

When myotube cultures of the transfected C2 cell lines were stained with rhodamine-conjugated α-bungarotoxin (rBuTx),

Table I. Abundance and Size of AChR Clusters

	Cells analyzed	AChR clusters	Clusters per cell	Cluster size (μm)
A. Tagged 43-kD protein staining				
strong	189	88	0.47 ± 0.1571	0.75 ± 0.1142 (0.63–1.05)
moderate	122	2,437	19.98 ± 2.713	0.93 ± 0.0111 (0.63–10.5)
weak	97	1,084	11.18 ± 1.2128	5.53 ± 0.1186 (0.63–58.8)
B. Untagged 43-kD protein staining				
strong	68	75	1.10 ± 0.5523	1.06 ± 0.0615 (0.63–2.1)
moderate	90	1,952	21.69 ± 2.4128	1.06 ± 0.0231 (0.63–25.2)
weak	217	1,372	9.73 ± 0.5662	8.62 ± 0.2331 (0.63–56.7)
C2	42	343	8.17 ± 0.8100	9.73 ± 0.2225 (0.63–63.0)

Myotube cultures of clonal cell lines expressing tagged (43T-1 and 43T-2) or untagged (43-1 and 43-2) 43-kD protein were stained with rBuTx for detection of AChR clusters and by fluorescence immunohistochemistry for detection of Tag or 43-kD protein. Values represent the mean ± SE.

the size and number of AChR clusters was found to be smaller than with normal C2 cells. AChR clusters in untransfected C2 myotubes (343 clusters in 42 cells) were large (average cluster size 9.7 ± 0.2 μm) and abundant (8.0 ± 0.8 clusters/cell), whereas those in the clones were more variable in size (Table I) and occurred with a variable frequency (0–22 clusters/cell).

In double-label experiments, we examined separately the frequency and size of clusters on myotubes with strong, moderate or weak staining for the 43-kD protein. We found that in myotubes expressing both the tagged and untagged 43-kD proteins, the average size of AChR clusters in cells showing strong staining was drastically reduced (Table I). Rather than the large clusters that are characteristic of C2 myotubes, these cells had smaller AChR clusters that resembled those seen in non-muscle cells in which the 43-kD protein and the AChR are co-expressed (Froehner et al., 1990; Phillips et al., 1991; Brennan et al., 1992; Fig. 6). In contrast, myotubes in the same cultures that stained weakly for the 43-kD protein had AChR clusters that were similar in size to those seen in normal C2 cells. AChR clusters in myotubes with moderate staining had a reduced average size, but also showed large variability, ranging from the small clusters similar to those seen in myotubes with strong 43-kD protein expression to large clusters of normal size (Table I).

When the number of AChR clusters per myotube was examined, those with strong staining had ~10% the number of clusters seen in myotubes with weak staining or in normal

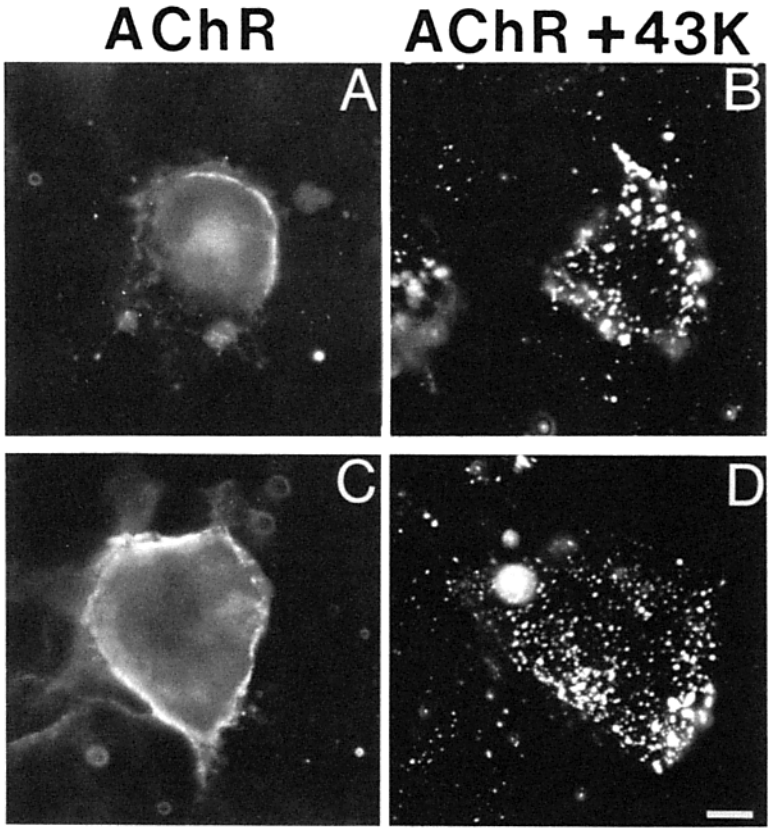

Figure 6. Co-expression of 43-kD protein and AChR subunits in COS cells induces AChR clusters. COS cells were transiently transfected with cDNAs to AChR subunits, either with or without the 43-kD protein, or the tagged 43-kD protein. Cells were stained with a monoclonal antibody to the extracellular domain of the AChR alpha subunit followed by FITC-conjugated goat anti-rat second antibody. AChRs are clustered when co-expressed with untagged 43-kD protein (*B*) and when co-expressed with tagged 43-kD protein (*D*) but are not clustered when expressed alone (*A*, *C*). Bar, 10 μm.

Table II. Surface Binding of ^{125}I-α-BuTx to Clonal Cell Lines Stably Transfected with Tagged (43T) or Untagged (43) 43-kD Protein

Cell line		pmols α-BuTx/μg protein
C2	(10)*	1.40 ± 0.05
43T-1	(6)	0.84 ± 0.10
43T-2	(7)	1.10 ± 0.07
43-1	(5)	2.10 ± 0.21
43-2	(5)	1.60 ± 0.21

Each value of toxin-binding activity represents the mean ± SE. Results are expressed as pmol of toxin bound and are normalized to total protein for each culture. C2 refers to the untransfected C2 muscle cell line. 43T-1 and 43T-2 are clonal cell lines expressing tagged 43-kD protein. 43-1 and 43-2 are clonal cell lines expressing untagged 43-kD protein.
* Number of cultures.

C2 myotubes (Table I). Myotubes with moderate staining for the 43-kD protein, however, had a larger than normal number of AChR clusters. Thus, on average, cells with moderately increased levels of the 43-kD protein had smaller, more frequent AChR clusters than did normal C2 myotubes (Table I; Figs. 3–5).

Overexpressed 43-kD Protein Does Not Reduce AChR Levels

One explanation for the ability of the 43-kD protein to reduce the size and frequency of AChR clusters in C2 myotubes when expressed at high levels is that 43-kD protein expression in some way inhibits the surface expression of the AChR, and that at lower density AChRs form fewer and smaller clusters. Although mass cultures showed normal expression of surface AChRs (Table II), the myotubes in which 43-kD protein expression was highest (and in which cluster formation was most strongly inhibited) formed only a small proportion of those in the culture; reduction of surface AChR expression in these myotubes might thus go undetected. To test this notion, myotube cultures of normal and transfected cell lines were labeled with ^{125}I-α-BuTx, stained for 43-kD protein using HRP-conjugated second antibody, and subjected to autoradiography (Fig. 7). Grain densities were determined separately for myotubes with strong, moderate and weak 43-kD protein expression. Examination of the parental cell line and four transfected cell lines, two expressing tagged 43-kD protein and the other two the untagged 43-kD protein, revealed no significant difference between the different classes of myotubes (Table III), nor between myotubes of transfected and untransfected cells. Thus, expression of the 43-kD protein at high levels affects the distribution of AChRs, but not their density.

The 43-kD Protein Is Functionally Active

A second possibility for the inverse correlation is that the 43-kD protein introduced into C2 muscle cells is defective and exerts a dominant-negative effect. This hypothesis is particularly plausible for the epitope-tagged 43-kD protein which may retain only partial function. Several observations, however, suggest that the expressed 43-kD protein is functional. First, immunofluorescence shows that the epitope-tagged 43-kD protein can co-cluster with AChRs (Fig. 3). This is best seen in myotubes with moderate expression in which the 43-kD protein staining is not continuous, and in which some

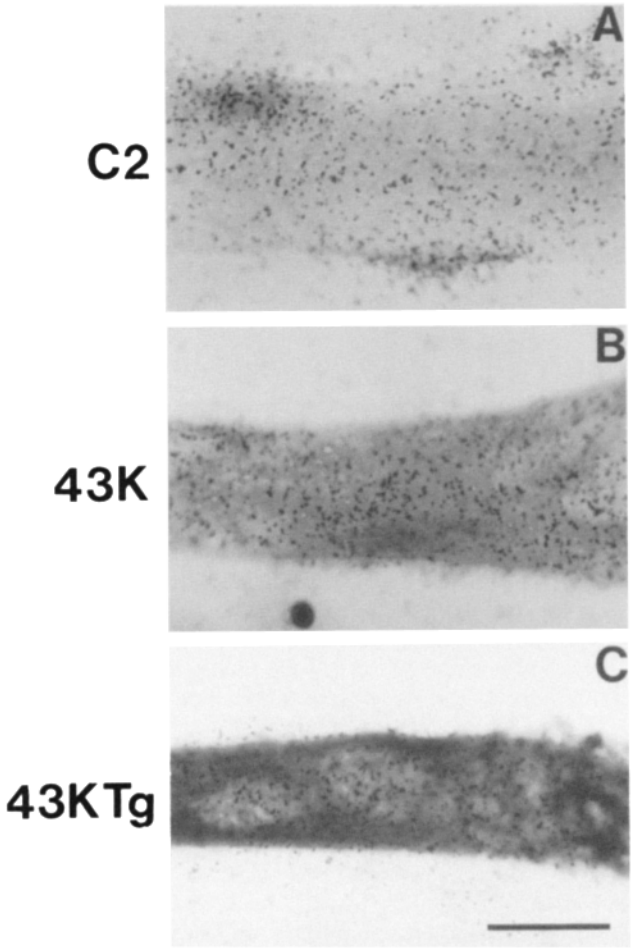

Figure 7. Combined autoradiographic localization of AChRs and immunohistochemical localization of tagged or untagged 43-kD protein on myotubes. Myotubes from cultures of clonal cell lines were labeled with ^{125}I-α-BuTx for detection of surface AChRs and stained with monoclonal antibodies to 43-kD protein or to Tag, followed by HRP-conjugated horse anti-mouse second antibody staining. Autoradiography and immunocytochemical staining for HRP were carried out as described in Methods. (*A*) AChR on C2 myotube. Staining for 43-kD protein colocalizes with AChR clusters. (*B*) Myotube from a clonal cell line expressing 43-kD protein stained for 43-kD protein. (*C*) Myotube from a clonal cell line expressing tagged 43-kD protein stained for Tag. Bar = 20 μ.

AChR clusters still form. Second, when the epitope-tagged 43-kD protein was transiently expressed in COS cells with the subunits of the AChR, it was able to induce clustering of AChRs like that seen with the normal 43-kD protein (Fig. 6). Our results thus suggest that it is the abnormally high levels of 43-kD protein and not its defective function that is responsible for the inhibition of AChR clustering.

Discussion

To study the role of the 43-kD protein in clustering of the AChR in the muscle cell, we have created stably transfected muscle cell lines that overexpress the protein. Our principal finding is that elevated levels of the 43-kD protein disrupt the formation of the large clusters normally observed in cultures of C2 muscle cells. At moderate levels of overexpression of

Table III. AChR Density on C2 Muscle Cells

Tag/43-kD protein staining	Transfectant	Grains/μm^2
Strong	Tagged	0.547 ± 0.055
	Untagged	0.374 ± 0.083
Moderate	Tagged	0.564 ± 0.057
	Untagged	0.565 ± 0.127
Weak	Tagged	0.546 ± 0.040
	Untagged	0.572 ± 0.065
Untransfected		0.414 ± 0.041

Myotubes were labeled with ^{125}I-α-BuTx for detection of surface AChRs by autoradiography and with HRP-conjugated second antibody for immunohistochemical detection of Tag or 43-kD protein. Tagged transfectants are clonal cell lines 43T-1 and 43T-2; untagged transfectants are clonal cell lines 43-1 and 43-2. *t* test and analysis of variance *F* test were performed on the data.

the 43-kD protein, smaller, more numerous clusters are seen, whereas at high levels of overexpression, the number of clusters is drastically reduced. Our results suggest that stoichiometric levels of expression of the 43-kD protein and AChR may be necessary for normal AChR clustering activity in C2 muscle cells.

We created cell lines expressing either the normal 43-kD protein or a modified protein tagged with an SV-40 T antigen epitope. No difference was observed at any stage between lines expressing the tagged and untagged proteins. In each of the transfected cell lines expression of the 43-kD protein was variable, with only a small minority of the myotubes expressing high levels of the protein. The reason for the variable expression by different cells is unknown. In some cases, particularly among cells that showed moderate expression, we observed that different regions of the myotube stained with different intensity for the 43-kD protein. The variegated pattern of staining suggested that different nuclei, even within the same myotube, expressed the 43-kD protein to variable extents. Variability in expression of endogenous proteins by different nuclei within the same myotube has been reported (Harris et al., 1989). The restricted distribution of the 43-kD protein within some myotubes suggests not only that there are local differences in expression of the protein, but also that the protein is locally anchored so that it does not diffuse throughout the myotube (Pavlath et al., 1989; Hall and Ralston, 1989; Gordon et al., 1992).

The 43-kD protein expressed in C2 myotubes appeared to be functional. First, when expressed in myotubes, the epitope-tagged 43-kD protein formed co-clusters with AChRs. Second, both the tagged and untagged proteins formed small clusters with the AChR when the two proteins were transiently co-expressed in COS cells, as seen by others (Froehner et al., 1990; Phillips et al., 1991; Brennan et al., 1992). These observations suggest that the inhibition of cluster formation by high levels of the 43-kD protein in muscle cells is not due to a dominant negative effect.

Overexpression of the 43-kD protein also had little effect on AChR expression. The reduction in size and number of clusters caused by high levels of the 43-kD protein could be explained if the number of AChRs available for cluster formation were reduced. No evidence was found for such a reduction, however. The number of surface AChRs in cultures of the transfected cell lines was within the range of values seen with normal C2 cells (Table II). More importantly, when levels of surface AChR expression in individual myotubes were examined by autoradiography, no difference was found between myotubes that expressed high levels of the 43-kD protein and those that expressed low levels. Both values were similar to those seen in myotubes of the parental cell line (Table III).

The most likely explanation of our results is that a stoichiometric ratio of AChR and the 43-kD protein is important for the formation of the large AChR clusters that muscle cells form. Immunoassay experiments on cultured muscle cells have suggested that the ratio between the two protein is ~1:1 (LaRochelle and Froehner, 1986), and x-ray diffraction studies have identified a cytoplasmic peripheral protein associated with each AChR that is probably the 43-kD protein (Toyoshima and Unwin, 1988; Mitra et al., 1989). The 43-kD protein is thought to immobilize the AChR and to promote its association into clusters (Froehner et al., 1991). Although the mechanisms by which it acts are unknown, the 43-kD protein could promote AChR clustering either by cross-linking AChRs, by self-association (Porter and Froehner, 1985), or by association with a linking protein or cytoskeletal structure. In any of these cases, an excess of 43-kD protein molecules over AChR oligomers could result in a competition between "free" 43-kD proteins and 43-kD proteins that are associated with AChRs, causing aggregation of the 43-kD protein, and thus disrupting cluster formation. As the amount of 43-kD protein is increased, AChR clusters may diminish in size until they no longer are detectable as clusters, as seen in myotubes with very elevated levels of the 43-kD protein.

An intriguing observation in our experiments is that in myotubes expressing moderately increased levels of 43-kD protein, the large AChR clusters appear to be replaced by multiple small clusters, thus increasing the total number of clusters (Table I). The small AChR clusters, which are also seen in myotubes with strong staining for the 43-kD protein, resemble in their appearance the clusters of AChR and 43-kD protein that appear in non-muscle cells transiently expressing both proteins (Froehner et al., 1990; Phillips et al., 1991; Brennan et al., 1992). This resemblance may be seen by comparing the AChR clusters in Figs. 3–5 with those in Fig. 6. These small AChR clusters thus appear to be characteristic of cells in which the 43-kD protein is overexpressed.

One interpretation of the small AChR clusters is that they represent a precursor from which the larger clusters are formed. The overexpression of the 43-kD protein in our experiments could thus be regarded as inhibiting the coalescence of small AChR clusters to form large ones. The increase in small AChR clusters according to this view might be analogous to the accumulation of a reaction intermediate.

Alternatively, large and small AChR clusters may form by separate, and competing, mechanisms. According to this view, the formation of small AChR clusters may be driven by the self-association of the 43-kD protein. The 43-kD protein spontaneously aggregates when expressed alone in non-muscle cells (Froehner et al., 1990; Phillips et al., 1991), consistent with its properties in vitro (Porter and Froehner, 1985). When expressed at high levels, either in muscle or in non-muscle cells, aggregation of the 43-kD protein could thus result in the formation of clusters of the AChR with which it is associated.

In normal muscle cells in which the expression of the 43-

kD protein is low, small clusters of the AChR and the 43-kD protein, such as those seen in transfected cells, are infrequently observed. Interestingly, in two genetic variants of C2 myotubes which have approximately normal amounts of the 43-kD protein but do not cluster AChRs, the 43-kD protein is not aggregated (LaRochelle et al., 1989; Gordon et al., 1993). One of these variants does not form AChR clusters because it has virtually no AChR (Black et al., 1987); the other, which is defective in proteoglycan synthesis, has normal levels of the AChR and 43-kD protein (Gordon et al., 1993). Although myotubes formed by this variant do not form spontaneous AChR clusters as normal C2 myotubes do, they can be induced to do so by some forms of exogenously added agrin (Ferns et al., 1992; unpublished observations). The results from these mutant cells suggest that in muscle cells expression of the 43-kD protein and the AChR is not alone sufficient to produce AChR clustering. Thus, the formation of large AChR clusters in muscle cells may be regulated by other factors, that could act either by modifying the 43-kD protein or by a separate mechanism. Under conditions in which the 43-kD protein is expressed at high levels, however, its self-aggregation into small clusters may override the normal process.

Further experiments will be required to investigate the role of the 43-kD protein in AChR clustering. The results presented here, however, provide clear evidence that high levels of the 43-kD protein inhibit normal cluster formation by muscle cells, and suggest that stoichiometric matching of levels of the 43-kD protein to the AChR in muscle cells may be critical to the mechanisms of cluster formation.

We thank Drs. S. Froehner, J. Lindstrom, I. Clark, and B. Schwer for antibodies and plasmids; Dr. T. Baldwin for advice on mutagenesis, reagents, and comments on the manuscript; Dr. E. Ralston and S. Strickland for suggestions on autoradiography; Drs. J. LaVail and K. Topp for allowing use of their equipment and help with data analyses; X. M. Yu for advice concerning COS cell staining; Drs. L. Reichardt, L. Jan, and members of the Hall Lab for insightful comments on the manuscript; C. Yoshihara expresses her gratitude to Drs. J. LaVail, F. Solomon, and T. Baldwin for encouragement and advice during the course of this project.

This work was supported by grants from the National Institutes of Health and the Muscular Dystrophy Association. C. M. Yoshihara was a postdoctoral fellow of the University of California President's Fellowship Award.

Received for publication 30 December 1992 and in revised form 12 April 1993.

References

Anderson, M. J., and M. W. Cohen. 1977. Nerve-induced and spontaneous redistribution of acetylcholine receptors on cultured muscle cells. *J. Physiol. (Lond.).* 268:757–773.

Baldwin, T. J., M.-L. Tsaur, G. A. Lopez, Y. N. Jan, and L. Y. Jan. 1991. Characterization of a mammalian cDNA for an inactivating voltage-sensitive K^+ channel. *Neuron.* 7:471–483.

Barrantes, F. J., D. Ch. Neugebauer, and H. P. Zingsheim. 1980. Peptide extraction by alkaline treatment is accompanied by rearrangement of the membrane-bound acetylcholine receptor from *Torpedo marmorata*. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 112:73–78.

Bevan, S., and J. H. Steinbach. 1977. The distribution of α-bungarotoxin binding sites on mammalian skeletal muscle developing in vivo. *J. Physiol. (Lond.).* 267:195–213.

Black, R., D. Goldman, S. Hochschwender, J. Lindstrom, and Z. W. Hall. 1987. Genetic variants of C2 muscle cells that are defective in synthesis of the α-subunit of the acetylcholine receptor. *J. Cell Biol.* 105:1329–1336.

Blau, H. M., C.-P. Chiu, and C. Webster. 1983. Cytoplasmic activation of human nuclear genes in stable heterocaryons. *Cell.* 32:1171–1180.

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. *Anal. Biochem.* 72:248–254.

Brennan, C., P. B. Scotland, S. C. Froehner, and L. P. Henderson. 1992. Functional properties of acetylcholine receptors coexpressed with the 43K protein in heterologous cell systems. *Dev. Biol.* 149:100–111.

Cohen, M. W., and E. W. Godfrey. 1992. Early appearance of and neuronal contribution to agrin-like molecules at embryonic frog nerve-muscle synapses formed in culture. *J. Neurosci.* 12:2982–2992.

Ferns, M., W. Hoch, J. T. Campanelli, F. Rupp, Z. W. Hall, and R. H. Scheller. 1992. RNA splicing regulates agrin-mediated acetylcholine receptor clustering activity on cultured myotubes. *Neuron.* 8:1079–1086.

Fertuck, H. C., and M. M. Salpeter. 1974. Localization of acetylcholine receptor by ^{125}I-α-bungarotoxin binding at mouse motor endplates. *Proc. Natl. Acad. Sci. USA.* 71:1376–1378.

Froehner, S. C. 1989. Expression of RNA transcripts for the postsynaptic 43 kDa protein in innervated and denervated rat skeletal muscle. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 249:229–233.

Froehner, S. C. 1991. The submembrane machinery for nicotinic acetylcholine receptor clustering. *J. Cell Biol.* 114:1–7.

Froehner, S. C., C. W. Luetje, P. B. Scotland, and J. Patrick. 1990. The postsynaptic 43K protein clusters muscle nicotinic acetylcholine receptors in *Xenopus* oocytes. *Neuron.* 5:403–410.

Gordon, H., and Z. W. Hall. 1989. Glycosaminoglycan variants in the C2 muscle cell line. *Dev. Biol.* 135:1–11.

Gordon, H., E. Ralston, and Z. W. Hall. 1992. Cooperation between the products of different nuclei in hybrid myotubes produces acetylcholine receptor clusters. *Proc. Natl. Acad. Sci. USA.* 89:6595–6598.

Gordon, H., M. Lupa, D. Bowen, and Z. W. Hall. 1993. A muscle cell variant defective in glycosaminoglycan biosynthesis forms nerve-induced but not spontaneous clusters of the acetylcholine receptor and the 43 kDa protein. *J. Neurosci.* 13:586–595.

Graham, F. L., and A. J. Van Der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology.* 52:456–467.

Gu, Y., A. Franco, Jr., P. D. Gardner, J. B. Lansman, J. R. Forsayeth, and Z. W. Hall. 1990. Properties of embryonic and adult muscle acetylcholine receptors transiently expressed in COS cells. *Neuron.* 5:147–157.

Hall, Z. W., and E. Ralston. 1989. Nuclear domains in muscle cells. *Cell.* 59:771–772.

Harris, D. A., D. L. Falls, and G. D. Fischbach. 1989. Differential activation of myotube nuclei following exposure to an acetylcholine receptor-inducing factor. *Nature (Lond.).* 337:173–176.

Heuser, J. E., T. S. Reese, M. Dennis, Y. N. Jan, L. Jan, and L. Evans. 1979. Synaptic vesicle exocytosis captured by quick freezing and correlated with quantal transmitter release. *J. Cell Biol.* 81:275–300.

Inestrosa, N. C., J. B. Miller, L. Silberstein, L. Ziskind-Conhaim, and Z. W. Hall. 1983. Developmental regulation of 16S acetylcholinesterase and acetylcholine receptors in a mouse muscle cell line. *Exp. Cell Res.* 147: 393–405.

Kordeli, E., J. Cartaud, H.-O. Nghiem, A. Devillers-Thiery, and J. P. Changeux. 1989. Asynchronous assembly of the acetylcholine receptor and of the 43-kD v_1 protein in the postsynaptic membrane of developing *Torpedo Marmorata* electrocyte. *J. Cell Biol.* 108:127–139.

LaRochelle, W. J., and S. C. Froehner. 1986. Determination of the tissue distributions and relative concentrations of the postsynaptic 43 kDa protein and the acetylcholine receptor in *Torpedo*. *J. Biol. Chem.* 261:5270–5274.

LaRochelle, W. J., E. Ralston, J. R. Forsayeth, S. C. Froehner, and Z. W. Hall. 1989. Clusters of 43-kDa protein are absent from genetic variants of C2 muscle cells with reduced acetylcholine receptor expression. *Dev. Biol.* 132:130–138.

LaRochelle, W. J., V. Witzemann, W. Fiedler, and S. C. Froehner. 1990. Developmental expression of the 43K and 58K postsynaptic membrane proteins and nicotinic acetylcholine receptors in *Torpedo* electrocytes. *J. Neurosci.* 10:3460–3467.

MacArthur, H., and G. Walter. 1984. Monoclonal antibodies specific for the carboxy terminus of simian virus 40 large T antigen. *J. Virol.* 52:483–491.

Matthews-Bellinger, J. A., and M. M. Salpeter. 1983. Fine structural distribution of acetylcholine receptors at developing mouse neuromuscular junctions. *J. Neurosci.* 3:644–657.

McMahan, U. J. 1990. The agrin hypothesis. *In* Cold Spring Harbor Symposia on Quantitative Biology LV. Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, NY. 407–418.

Mitra, A. K., M. P. McCarthy, and R. M. Stroud. 1989. Three-dimensional structure of the nicotinic acetylcholine receptor and location of the major associated 43-kD cytoskeletal protein, determined at 22 A by low dose electron microscopy and X-ray diffraction to 12.5 A. *J. Cell Biol.* 109:755–774.

Nghiem, H.-O., J. Hill, and J.-P. Changeux. 1991. Developmental changes in the subcellular distribution of the 43K (v_1) polypeptides in *Torpedo marmorata* electrocyte: support for a role in acetylcholine receptor stabilization. *Development.* 113:1059–1067.

Pavlath, G. K., K. Rich, S. G. Webster, and H. M. Blau. 1989. Localization of muscle gene products in nuclear domains. *Nature (Lond.).* 337:570–573.

Peng, H. B., and S. C. Froehner. 1985. Association of the postsynaptic 43K protein with newly formed acetylcholine receptor clusters in cultured muscle cells. *J. Cell Biol.* 100:1698–1705.

Phillips, W. D., C. Kopta, P. Blount, P. D. Gardner, J. H. Steinbach, and J. P. Merlie. 1991. ACh receptor-rich membrane domains organized in fibroblasts by recombinant 43-kildalton protein. *Science (Wash. DC).* 251:568–570.

Platt, J. L., and A. F. Michael. 1983. Retardation of fading and enhancement of intensity of immunofluorescence by p-phenylenediamine. *J. Histochem. Cytochem.* 31:840–842.

Porter, S., and S. C. Froehner. 1985. Interaction of the 43K protein with components of *Torpedo* postsynaptic membranes. *Biochemistry.* 24:425–432.

Ralston, E., and Z. W. Hall. 1989. Transfer of a protein encoded by a single nucleus to nearby nuclei in multinucleated myotubes. *Science (Wash. DC).* 244:1066–1069.

Ratnam, M., P. B. Sargent, V. Sarin, J. L. Fox, D. L. Nguyen, J. Rivier, M. Criado, and J. Lindstrom. 1986. Location of antigenic determinants on primary sequences of subunits of nicotinic acetylcholine receptor by peptide mapping. *Biochemistry.* 25:2621–2632.

Reist, N. E., M. J. Werle, and U. J. McMahan. 1992. Agrin released by motor neurons induces the aggregation of acetylcholine receptors at neuromuscular junctions. *Neuron.* 8:865–868.

Rousselet, A., J. Cartaud, P. F. Devaux, and J. P. Changeux. 1982. The rotational diffusion of the acetylcholine receptor in *Torpedo marmorata* membrane fragments studied with a spin-labelled α-toxin: importance of the 43,000 protein(s). *EMBO (Eur. Mol. Biol. Organ.) J.* 1:439–445.

Sealock, R., B. E. Wray, and S. C. Froehner. 1984. Ultrastructural localization of the M_r 43,000 protein and the acetylcholine receptor in *Torpedo* postsynaptic membranes using monoclonal antibodies. *J. Cell Biol.* 98: 2239–2244.

Seed, B., and A. Aruffo. 1987. Molecular cloning of the CD2 antigen, the T-cell erythrocyte receptor, by a rapid immunoselection procedure. *Proc. Natl. Acad. Sci. USA.* 84:3365–3369.

Toyoshima, C., and N. Unwin. 1988. Ion channel of acetylcholine receptor reconstructed from images of postsynaptic membranes. *Nature (Lond.).* 336:247–250.

Wigler, M., A. Pellicer, S. Silverstein, R. Axel, G. Urlaub, and L. Chasin. 1979. DNA mediated transfer of the adenine phospho-ribosyltransferase locus into mammalian cells. *Proc. Natl. Acad. Sci. USA.* 76:1373–1376.

Ziskind-Conhaim, L., I. Geffen, and Z. W. Hall. 1984. Redistribution of acetylcholine receptors on developing rat myotubes. *J. Neurosci.* 4:2346–2349.

Yaffe, D., and O. Saxel. 1977. Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature (Lond.).* 270:725–727.