

# Mutagenesis of the 43-kD Postsynaptic Protein Defines Domains Involved in Plasma Membrane Targeting and AChR Clustering

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**Abstract.** The postsynaptic membrane of the neuromuscular junction contains a myristoylated 43-kD protein (43k) that is closely associated with the cytoplasmic face of the nicotinic acetylcholine receptor (AChR)-rich plasma membrane. Previously, we described fibroblast cell lines expressing recombinant AChRs. Transfection of these cell lines with 43k was necessary and sufficient for reorganization of AChR into discrete 43k-rich plasma membrane domains (Phillips, W. D., C. Kopta, P. Blount, P. D. Gardner, J. H. Steinbach, and J. P. Merlie. 1991. *Science (Wash. DC)*. 251:568–570). Here we demonstrate the utility of this expression system for the study of 43k function by site-directed mutagenesis. Substitution of a termination codon for Asp<sup>254</sup> produced a truncated (28-kD) protein that associated poorly with the cell

membrane. The conversion of Gly<sup>2</sup> to Ala<sup>2</sup>, to preclude NH<sub>2</sub>-terminal myristoylation, reduced the frequency with which 43k formed plasma membrane domains by threefold, but did not eliminate the aggregation of AChRs at these domains. Since both NH<sub>2</sub> and COOH-termini seemed important for association of 43k with the plasma membrane, a deletion mutant was constructed in which the codon Gln<sup>15</sup> was fused in-frame to Ile<sup>255</sup> to create a 19-kD protein. This mutated protein formed 43k-rich plasma membrane domains at wild-type frequency, but the domains failed to aggregate AChRs, suggesting that the central part of the 43k polypeptide may be involved in AChR aggregation. Our results suggest that membrane association and AChR interactions are separable functions of the 43k molecule.

**T**HE aggregation of acetylcholine receptors (AChR)<sup>1</sup> and their maintenance at high density in the postsynaptic membrane is an essential feature of the formation of the neuromuscular synapse. Although the mechanisms involved in the aggregation of AChRs remain poorly understood, methods for purifying large amounts of postsynaptic membrane from the *Torpedo* electric organ have permitted some of the likely molecular participants in the process to be identified. In addition to the AChRs themselves, isolated *Torpedo* AChR-rich membrane contains another prominent polypeptide with an apparent molecular mass of 43,000 (16, 45, 66) which is distinct from actin and creatine kinase (21, 28, 46). We will refer to this protein here simply as 43k. 43k is an alkaline-extractable peripheral membrane protein (16, 45) located on the cytoplasmic face of the AChR-rich membrane (46, 53, 68, 74). Several lines of evidence suggest that the AChRs in these membranes are immobilized by a mechanism that involves 43k. After extraction of the membranes at pH 11, which removes 43k and other, less abundant peripheral membrane proteins, AChRs retain their ligand binding and gating properties (16, 45) but become more dispersed in the membrane (2, 12) and more free to rotate about their

transmembrane axis (39, 63). 43k is present in approximately equimolar amounts with the AChRs (7) and therefore could conceivably link AChRs to each other or to the underlying cytoskeleton. Immunolocalization studies have shown a close codistribution between AChR and 43k in the postsynaptic membrane (24, 46, 65). Moreover, 43k can be chemically cross-linked to the  $\beta$  subunit of the AChR in these membranes, indicating a close molecular association (7). However, direct biochemical evidence that 43k interacts with AChRs has proved elusive. The interactions, if they do exist, may be susceptible to the non-ionic detergents used for receptor purification.

The first indication that the mammalian neuromuscular junction contained a protein related to *Torpedo* 43k came from immunofluorescence studies with antibodies to the *Torpedo* protein (24, 53). In cultured muscle cells of various species, anti-43k staining colocalized with AChR clusters that occur spontaneously (6, 36, 51) as well as those induced by neurites (6), agrin (73), positively charged latex beads (51), and electric fields (61), suggesting that 43k may be part of a common mechanism for AChR aggregation.

Peptide sequencing (9) led to the cloning of homologous 43k cDNAs from *Torpedo* (17), mouse (18, 22), and *Xenopus* (1). Although the primary structure of 43k has provided few clues to the mechanism of its colocalization with AChR or its putative function, two unusual features bear further study

1. **Abbreviations used in this paper:** AChR, acetylcholine receptor; 43k, 43-kD AChR-associated protein of the postsynaptic membrane; RSV, Rous sarcoma virus.

(23): a very high content of cysteine residues (9) and an amino-terminal myristoylation site (1, 11, 17, 18, 22, 43). In some proteins myristoylation has been found to be important for association with the cytoplasmic face of the cell membrane (49, 56, 60, 67) but its significance in 43k is unknown.

Evidence for the involvement of 43k in AChR aggregation has been obtained recently by expressing recombinant AChR subunits and 43k in nonmuscle cells (26, 52). Froehner et al. (26) injected synthetic mRNAs for the  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  subunits of the fetal mouse AChR into *Xenopus* oocytes, resulting in the appearance of diffuse AChRs on the cell surface; when mRNA for 43k was included in the injection, anti-43k staining formed into patches on the cell surface at which AChRs also became aggregated. In a separate study, we have transfected a quail fibroblast cell line with expression vectors for either the fetal ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ) or adult ( $\alpha$ ,  $\beta$ ,  $\epsilon$ ,  $\delta$ ) combination of AChR subunits and isolated clones permanently expressing the appropriate acetylcholine gated channel type; in each case the AChRs were dispersed diffusely across the cell surface. Transfection of 43k into these AChR-expressing fibroblasts led to the reorganization of the AChRs into large 43k-rich plasma membrane domains (52). In both frog oocytes and quail fibroblasts 43k aggregated into membrane domains independent of the presence or absence of coexpressed AChR (26, 52). Here, we investigate the ability of recombinant 43k mutants to induce AChR-rich plasma membrane domains in AChR-expressing fibroblasts.

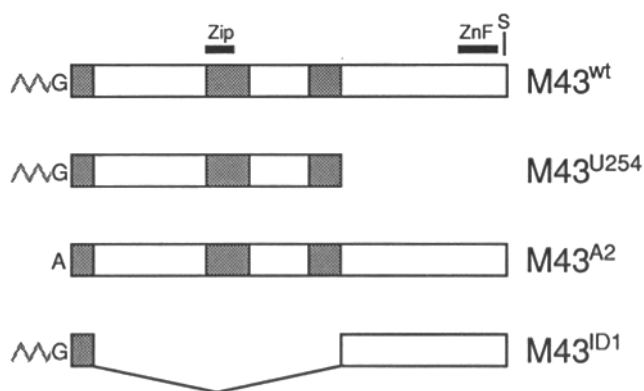
## Materials and Methods

### Mutagenesis of 43k

Expression of wild-type and mutant mouse 43k cDNAs was directed by the Rous sarcoma virus (RSV) long-terminal repeat promoter in the expression construction RSV-M43 described previously (52). The myristoylation-defective mutant (M43<sup>A2</sup>) and the carboxy-terminal truncation mutant (M43<sup>U254</sup>) were generated from a single strand (phagemid) copy of RSV-M43 using an oligonucleotide-directed in vitro mutagenesis system (Amersham International, Arlington Heights, IL). In each case a 27-mer oligodeoxyribonucleotide incorporating two base changes was used to engineer the desired amino acid change and simultaneously alter an adjacent restriction site to serve as a marker, without altering the adjacent amino acid. In this way the M43<sup>A2</sup> mutant clones were screened for the loss of an *Av*II site and the M43<sup>U254</sup> mutant clones were identified by the elimination of the *Eco*RV site. The coding region of each of these mutants was verified by sequencing. The internal deletion mutant, M43<sup>ID1</sup>, was constructed by ligating a *Pvu*II fragment containing the RSV long-terminal repeat promoter and the sequence encoding the mouse 43k NH<sub>2</sub> terminus into the large *Eco*RV fragment of RSV-M43 using standard techniques (40). In this way the coding sequence between the *Pvu*II and *Eco*RV sites of the 43k cDNA was deleted, yielding an in-frame fusion of Gln<sup>15</sup> to Ile<sup>255</sup>. The coding sequence joint was confirmed by sequencing. The three mutants and wild-type 43k are represented in schematic form in Fig. 1.

### Maintenance and Transfection of Cells

Quail QT-6 fibroblasts (42) were maintained as previously described (5). The QT-6-derived fetal AChR-expressing line, Q-F18, was maintained similarly but with the addition of 300  $\mu$ M G418 to the growth medium. For the immunofluorescence studies described in Fig. 4, cells were transfected by the standard calcium phosphate technique (40). The following day the cells were trypsin treated and replated onto glass coverslips in 24-well trays at a density of  $3 \times 10^5$  cells per well (52). Cells were reincubated for one day more in medium supplemented with 10 mM sodium butyrate. Coverslips were rinsed in warm serum-free medium and fixed for 20 min in PBS containing 1% paraformaldehyde, 100 mM L-lysine, 10 mM sodium m-periodate, and 0.1% saponin. After rinsing in PBS, cells were permeabilized for



**Figure 1.** Schematic diagram of wild-type and mutant forms of 43k. Horizontal bars show the relative lengths of the mutant polypeptides. Shaded portions indicate regions of particularly high ( $\geq 86\%$ ) amino acid conservation between *Torpedo* (17), mouse (18, 22), and *Xenopus* (1). *G*'s represent Glycine<sup>2</sup>, the target for myristoylation, and *A* indicates the substitution of alanine to prevent myristoylation. The zig zag line indicates the myristate moiety. Bars at the top show the locations of potential leucine zipper (*Zip*), zinc finger (*ZnF*), and serine phosphorylation (*S*) (23).

10 min in 1% Triton X-100 in PBS (36), washed three times in PBS, and stored overnight in 1% normal goat serum in PBS. For cell fractionation studies and the immunofluorescence experiments depicted in Fig. 3, cells were transfected by the method of Chen and Okayama (14) modified as indicated below. Cells were grown to subconfluence on a 60-mm plate. 1 h before transfection, the growth medium was changed to DME containing 5% FCS and 1% DMSO. Plasmid DNA (20  $\mu$ g) was mixed with 25  $\mu$ l of 2.5 M CaCl<sub>2</sub>, and H<sub>2</sub>O was added to a final volume of 250  $\mu$ l. An equal volume of buffer [50 mM *N,N*-bis(2-hydroxyethyl)-2-aminoethane-sulfonic acid, 280 mM NaCl, and 1.5 mM Na<sub>2</sub>HPO<sub>4</sub>] pH 6.95 was added, and the mixture was incubated for 10–20 min at room temperature. The DNA solution (0.5 ml) was added dropwise to the plate of cells which were then incubated for 15–24 h at 36°C under 3% CO<sub>2</sub>. The cells were washed twice with complete 199 Earle's medium, fed with the same medium, and incubated overnight at 36°C under 5% CO<sub>2</sub> before harvesting for subcellular fractionation.

### Subcellular Fractionation

For subcellular fractionation, one 60-mm plate of cells (QT-6 or Q-F18) transfected as above was rinsed two times with PBS containing 300  $\mu$ M PMSF. The cells were scraped into a microfuge tube, pelleted at 500 *g* for 4 min, and swollen by resuspension in 1 ml of hypotonic buffer (10 mM Tris, pH 8.6, 10 mM *N*-ethylmaleimide, 25 mM sucrose, and protease inhibitors at final concentrations of 200  $\mu$ M leupeptin, 0.2 U/ml  $\alpha$ -2-macroglobulin, 50  $\mu$ g/ml aprotinin, and 500  $\mu$ M benzamide). The cells were lysed with 50 strokes in a 1-ml Dounce homogenizer, and the homogenate was divided into two 400- $\mu$ l aliquots for fractionation and a 100- $\mu$ l aliquot which was retained as unfractionated total cell lysate. One 400- $\mu$ l aliquot was made 0.075% BSA and 0.2 M NaCl, and then centrifuged in an SW60 rotor (Beckman Instruments, Inc., Palo Alto, CA) at 100,000 *g* for 1 h at 4°C. The supernatant (S100) was removed and the pellet (P100) was resuspended in 400  $\mu$ l of hypotonic buffer. Both fractions were brought to 0.5% Triton X-100, 0.2% SDS and vortexed. The other 400- $\mu$ l aliquot was mixed with 2 ml of 50% sucrose (wt/wt) in an ultraclear centrifuge tube (Beckman Instruments, Inc.), and overlaid with 2 ml of 5% sucrose. The sucrose solutions contained 10 mM Tris, pH 8.6, 10 mM *N*-ethylmaleimide, 0.075% BSA, and protease inhibitors at the concentrations listed above. The discontinuous sucrose gradient was centrifuged in an SW60 rotor at 215,000 *g* for 16–20 h at 4°C. The material that accumulated at the 5%/50% sucrose interface (crude membranes) was collected and made 0.5% Triton X-100 and 0.2% SDS. The pellet was resuspended in 400  $\mu$ l of hypotonic buffer containing 0.5% Triton X-100 and 0.2% SDS. The isopycnic flotation of plasma membrane at the 5%/50% interface was confirmed using <sup>125</sup>I- $\alpha$ -bungarotoxin-labeled cell surface AChRs (Q-F18 cells) as a surface membrane marker. The distribution of specifically bound <sup>125</sup>I- $\alpha$ -bungarotoxin between the

various cell fractions was approximately the same whether cells were transfected with wild-type 43k (M43<sup>wt</sup>), M43<sup>A2</sup>, M43<sup>U254</sup>, or M43<sup>ID1</sup>. For the discontinuous sucrose gradient, 53–60% of the specific counts were found in the 5%/50% interface fraction, 34–37% in the 50% sucrose fraction, 2–3% in the 5% sucrose fraction, and 5–7% in the sucrose pellet. For the 100,000-g fractions, the P100 contained 80–95% of specific counts while the S100 contained 5–20%.

### Gel Electrophoresis and Immunoblotting

Subcellular fractionation samples were subjected to electrophoresis on SDS–12.5% polyacrylamide gels (37) and transferred to nitrocellulose (0.45  $\mu$ m; Schleicher & Schuell, Inc., Keene, NH). Transfer was carried out in 96 mM glycine, 12.5 mM Tris containing 20% (vol/vol) methanol, for 75 min at 400 mA. Replicas were incubated at room temperature for several hours in TS (10 mM Tris, 150 mM NaCl, pH 8.0) containing 5% nonfat dry milk powder to block nonspecific binding. Replicas were then incubated overnight at 4°C with a 1:1,000 dilution of affinity-purified rabbit anti-43k (for M43<sup>wt</sup>, M43<sup>A2</sup>, and M43<sup>ID1</sup>) or a 1:10 dilution of mAb 1234A hybridoma supernatant (for M43<sup>U254</sup>) in TS containing 2.5% nonfat milk powder. After washing three times in TS–0.05% Tween 20, filters were incubated for 1.5 h at room temperature in affinity-purified goat antibodies to rabbit (or mouse) IgG conjugated to alkaline phosphatase (Boehringer Mannheim Corp., Indianapolis, IN). Antibodies were diluted 1:2,000 in TS containing 2.5% nonfat milk powder. Filters were then washed twice in TS–0.05% Tween 20, washed once in TS, and developed for 10 min in a color reaction solution containing 100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM MgCl<sub>2</sub>, 0.3 mg/ml nitro blue tetrazolium, and 0.15 mg/ml bromo 4-chloro 3-indoyl phosphate.

### Anti-43k Antibodies

Rabbit antiserum to recombinant mouse 43k fusion protein was kindly provided by Dr. Don Frail (Abbot Laboratories, North Chicago, IL). An *Escherichia coli* expression system (62) was used to produce a fusion protein containing the full-length 43k sequence. Rabbits were immunized with the insoluble fusion protein. Crude antiserum was immunoaffinity purified over Affigel (Bio-Rad Laboratories, Richmond, CA) derivitized with a crude preparation of the fusion protein and was adsorbed to Affigel derivitized with a similar preparation containing full-length *Torpedo* 43k fusion protein to remove cross-reacting antibodies. Mouse monoclonal anti-43k antibodies, mAb 1234A (51) and mAb 1579A (35) were generously provided by Dr. S. C. Froehner.

### Immunofluorescent Staining

Coverslips were inverted (cell side down) on 50- $\mu$ l drops of primary antibody and were incubated for 1 h at room temperature. Affinity-purified rabbit anti-43k and/or rat anti-AChR monoclonal antibody, mAb 35 (72), were diluted (1:50 and 1:200, respectively) in PBS containing 1% normal goat serum. Coverslips were washed three times in PBS and were incubated with affinity-purified TRITC-goat anti-rabbit IgG (1:100) and/or FITC-goat anti-rat IgG (1:100) in PBS containing 1% normal goat serum as above. After washing as above the coverslips were rinsed briefly in distilled water and mounted for fluorescent microscopy using an antifading mountant (30). The carboxyl-terminal mutant M43<sup>U254</sup>, which was not recognized by the polyclonal anti-43k antibody, was stained instead with the mouse monoclonal anti-43k mAb 1234A (51) hybridoma supernatant diluted 1:8 as previously described (52). To control for fluorescent crossbleed and secondary antibody specificity, parallel coverslips were stained in the absence of either anti-AChR or anti-43k primary antibody.

## Results

### Subcellular Distribution of Recombinant Wild-Type 43k

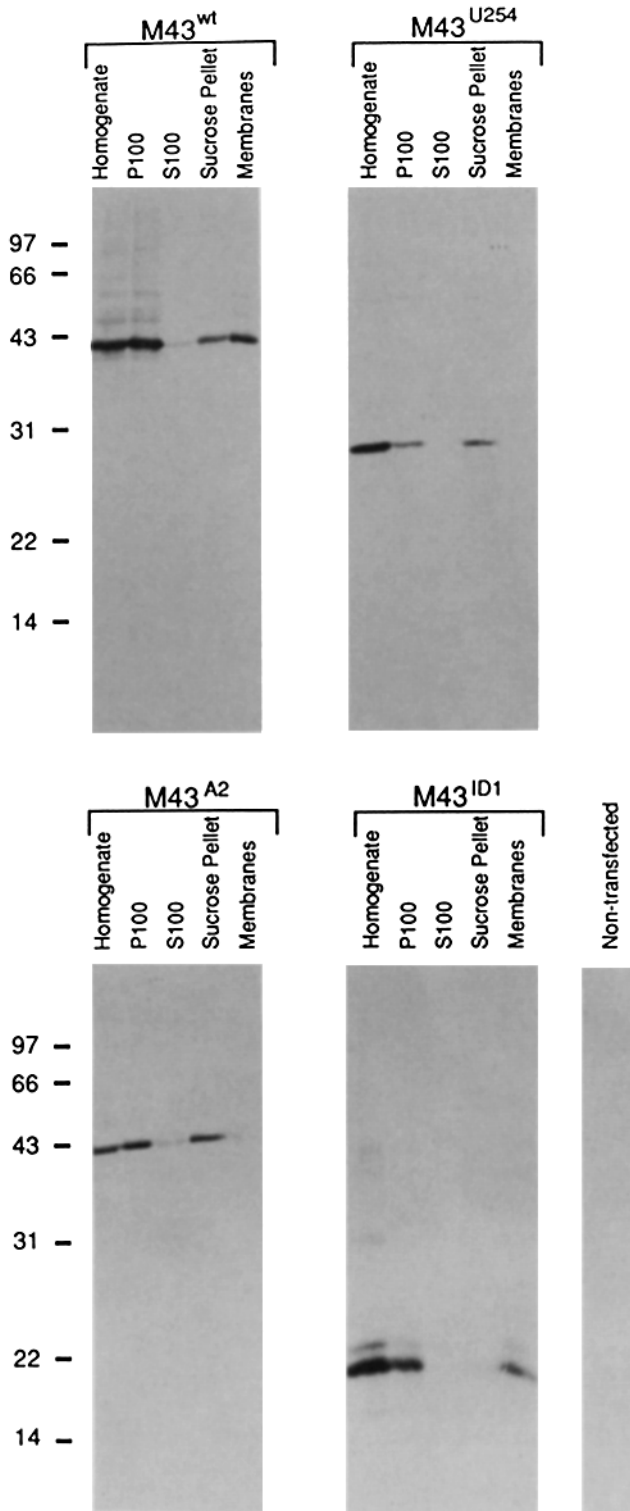
Recombinant mouse 43k was expressed transiently in the quail fibroblast cell line, QT-6, by the acid CaPO<sub>4</sub> transfection method (14) and was examined by immunoblot. Homogenates of cells transfected with M43<sup>wt</sup> revealed a major anti-43k-stained band at ~43 kD, which was absent in nontransfected QT-6 fibroblasts (Fig. 2). Homogenates fractionated

by centrifugation at 100,000 g showed only a small proportion of the 43k in the soluble (S100) fraction. The majority was pelleted with membranes, nuclei, and other organelles (P100) (Fig. 2). To investigate the association of 43k with membranes, homogenates were fractionated in a discontinuous sucrose gradient under centrifugation at 215,000 g. The resulting 5%/50% sucrose interface (membrane fraction) yielded a strong anti-43k-stained band (Fig. 2), providing evidence that transfected 43k associates with membranes when expressed in these cells that are devoid of detectable endogenous AChR (5). A lesser amount of 43k was found in the sucrose pellet (Fig. 2) suggesting that 43k may also associate with nuclei or some other dense intracellular structures.

The distribution of transfected M43<sup>wt</sup> protein in QT-6 cells was further investigated by immunofluorescent staining of permeabilized cells. As previously reported (52), anti-43k staining was frequently observed to be concentrated into discrete plasma membrane domains of various sizes (Fig. 3 *a*, *broad arrows*). In addition, many transfected cells showed anti-43k staining of small granule-like structures scattered throughout the cell and large aggregates of what appeared to be membrane vesicles in the central portion of the cell (Fig. 3 *a*, *small arrow*). We do not know whether the latter structures, which were usually adjacent to the nucleus, were pelleted with the nuclei and other dense organelles through the 50% sucrose (such an association might explain the fractionation of 43k found in the sucrose pellet [Fig. 2]). To permit a quantitative comparison of M43<sup>wt</sup> protein with the mutant 43k's, coverslips were systematically scanned and anti-43k-positive cells were scored for the presence of staining in the plasma membrane, nucleus, and other structures. Of those QT-6 cells expressing transfected M43<sup>wt</sup> protein, 92% displayed anti-43k staining of discrete plasma membrane domains, 50% showed staining of central aggregates of membranes, and 27% had peripheral granule-like structures stained (Table I).

### Subcellular Distribution of Mutant 43k's

Mutations of the mouse 43k cDNA were constructed to investigate their effects on the association of 43k with the plasma membrane and with AChRs. The amino terminus of mouse 43k is normally modified by the addition of an *N*-linked myristate moiety at Gly<sup>2</sup> (11, 19, 43). Myristoylation has been found to be necessary for the association of some proteins with the plasma membrane (49, 56, 60, 67), whereas some myristoylated proteins do not appear to associate with the plasma membrane at all (70). The *N*-myristoyl transferase is absolutely specific for proteins with glycine at position 2 (69) (after the removal of the initiator methionine); thus myristoylation of a protein can be completely prevented by a single glycine to alanine substitution at this site (49, 70). Oligonucleotide directed mutagenesis of M43<sup>wt</sup> was used to convert Gly<sup>2</sup> to Ala<sup>2</sup> leaving the remainder of the polypeptide sequence unaltered (M43<sup>A2</sup>). As expected, immunoblotting of homogenates of M43<sup>A2</sup>-transfected cells showed a band similar in mobility to that of M43<sup>wt</sup> protein (Fig. 2). After fractionation at 100,000 g, the majority of the M43<sup>A2</sup> protein was found in the P100 and little was found in the S100 (Fig. 2). However, when centrifuged through 50% sucrose, little of the M43<sup>A2</sup> protein was found in the membrane fraction (Fig. 2). Instead, most of the M43<sup>A2</sup> pelleted (Fig. 2), suggesting that without the myristoyl-glycine modification



**Figure 2.** Immunoblot analysis of the subcellular distribution of transfected 43k. QT-6 fibroblasts were transfected with expression constructions M43<sup>wt</sup>, M43<sup>U254</sup>, M43<sup>A2</sup>, or M43<sup>ID1</sup>, and were harvested 2 d later. Whole cell homogenate (including nuclei) and equivalent amounts of P100 and S100 fractions were electrophoresed in SDS-polyacrylamide gels containing 12.5% acrylamide. A membrane fraction was prepared from whole homogenate by floating membranes through 50% sucrose under isopycnic centrifugation at 215,000 g. Membranes were collected at the 5%/50% sucrose interface and the pellet fraction, containing nuclei (sucrose pellet), was loaded for comparison. Nontransfected QT-6 cell ho-

**Table I. Localization of Anti-43k Immunostaining in QT-6 and (Q-F18) Cells\***

Mutant	Percentage of cells with anti-43k staining localized in				
	Membrane domains	Aggregated vesicles	Scattered granules	Nucleoplasm	Diffuse†
M43 <sup>wt</sup>	92 (83)	50 (40)	27 (64)	1 (<1)	2 (<1)
M43 <sup>U254</sup>	1 (2)	76 (81)	89 (60)	<1 (<1)	3 (21)
M43 <sup>A2</sup>	10 (17)	33 (50)	3 (11)	78 (81)	43 (59)
M43 <sup>ID1</sup>	68 (75)	62 (75)	41 (51)	1 (<1)	9 (<1)

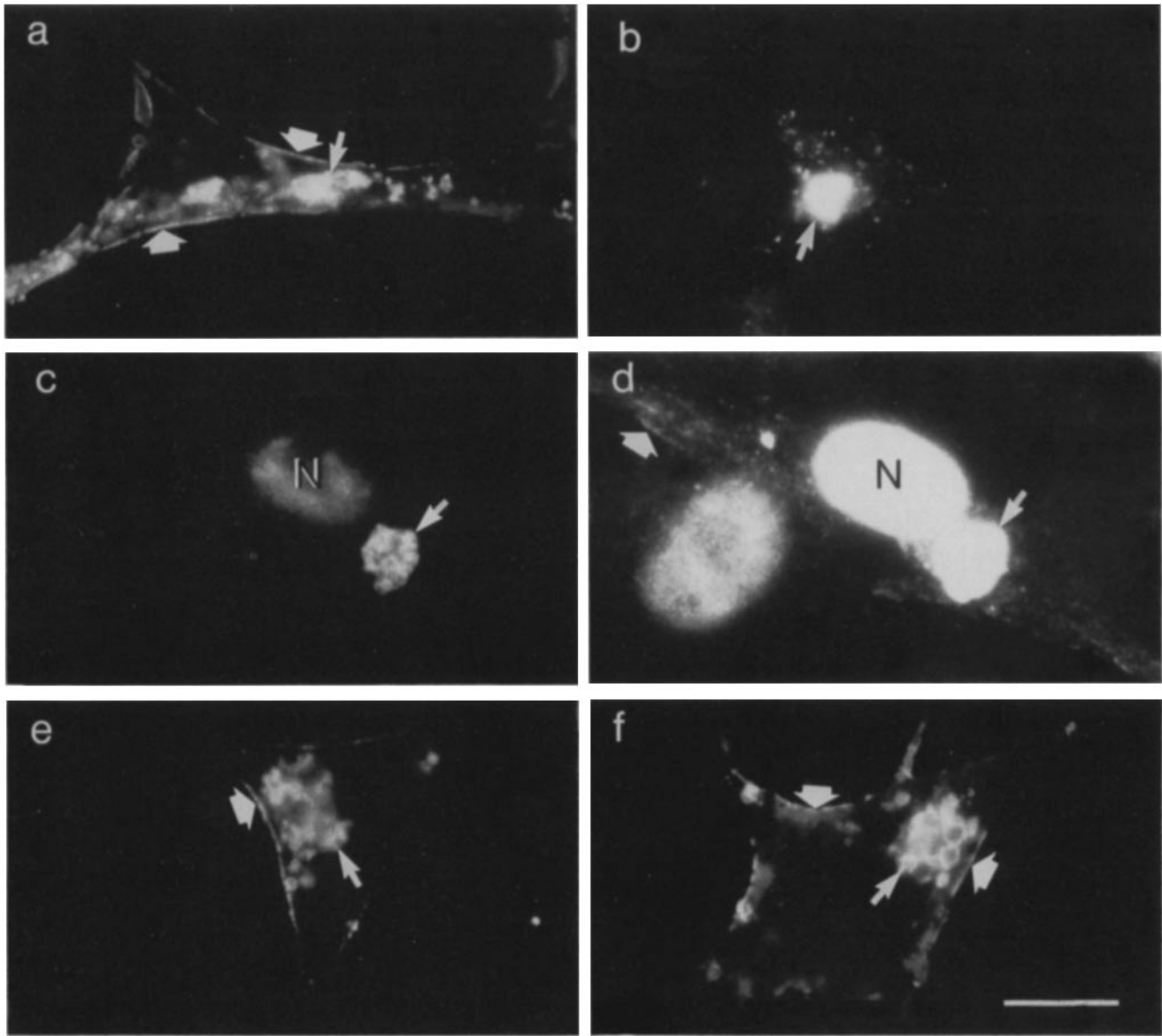
\* Percentages represent at least 100 anti-43k-positive cells from each of 2-4 independent transfection experiments. Numbers in parentheses are results for Q-F18 cells.

† Some cells displayed an overall diffuse anti-43k staining in addition to the more intense staining of the structures indicated in the left-hand columns. Since the fibroblasts were flat, it was not possible to determine with confidence whether the diffuse staining was associated with the plasma membrane or was indeed cytoplasmic.

43k associates poorly with the plasma membrane. When examined by immunofluorescence, M43<sup>A2</sup>-transfected cells showed relatively little plasma membrane staining. As with M43<sup>wt</sup>, they showed anti-43k staining of large, centrally located membrane aggregates. Surprisingly, M43<sup>A2</sup> protein-expressing cells also exhibited strong but diffuse staining in the nucleoplasm (Fig. 3 c, Table I). Localization within the nuclei and perinuclear membranes may explain why most of the M43<sup>A2</sup> protein pellets through 50% sucrose (Fig. 2). The reason for the anomalous targeting of M43<sup>A2</sup> protein to the nucleoplasm is unknown (but see Discussion). Nuclear staining was only rarely observed in transfections with the other 43k mutants or the wild-type (Table I). Anti-43k-stained plasma membrane domains were less common on cells transfected with M43<sup>A2</sup> than with M43<sup>wt</sup> (Table I), and usually were stained only weakly, requiring longer photographic exposures (Fig. 3 d, broad arrow).

To determine the role of the carboxyl terminus of 43k, the Asp<sup>254</sup> codon was mutagenized to cause early termination (M43<sup>U254</sup>, Fig. 1). The polyclonal anti-43k antibody and monoclonal antibody mAb 1579A (35) both failed to bind to the resulting carboxyl-truncated protein on immunoblots (data not shown), but mAb 1234A (51) readily detected the expected 28-kD band in homogenates of M43<sup>U254</sup>-transfected cells (Fig. 2), suggesting that the former two antibodies recognize epitopes on the deleted COOH terminus. As with the M43<sup>wt</sup>, most of the M43<sup>U254</sup> protein was found in the P100 fraction of cell homogenates rather than the S100 (Fig. 2). However, when applied to a discontinuous sucrose gradient, M43<sup>U254</sup> protein did not float through 50% sucrose with the membrane fraction as did M43<sup>wt</sup> protein, but was instead found in the sucrose pellet (Fig. 2). By immunofluorescence, M43<sup>U254</sup>-transfected cells showed intense anti-43k staining associated with centrally located membrane aggregates (Fig. 3 b, arrow) and with scattered granule-like structures. Consistent with the cell fractionation results, anti-43k staining of plasma membrane was rarely observed in

mogenate was loaded as a control. After electroblotting, nitrocellulose blots were stained with rabbit anti-43k antibody (M43<sup>wt</sup>, M43<sup>A2</sup>, M43<sup>ID1</sup>) or a mouse monoclonal anti-43k, mAb 1234A (51), (M43<sup>U254</sup>) followed by appropriate alkaline phosphatase-conjugated secondary antibody. Numbers at left indicate size markers.

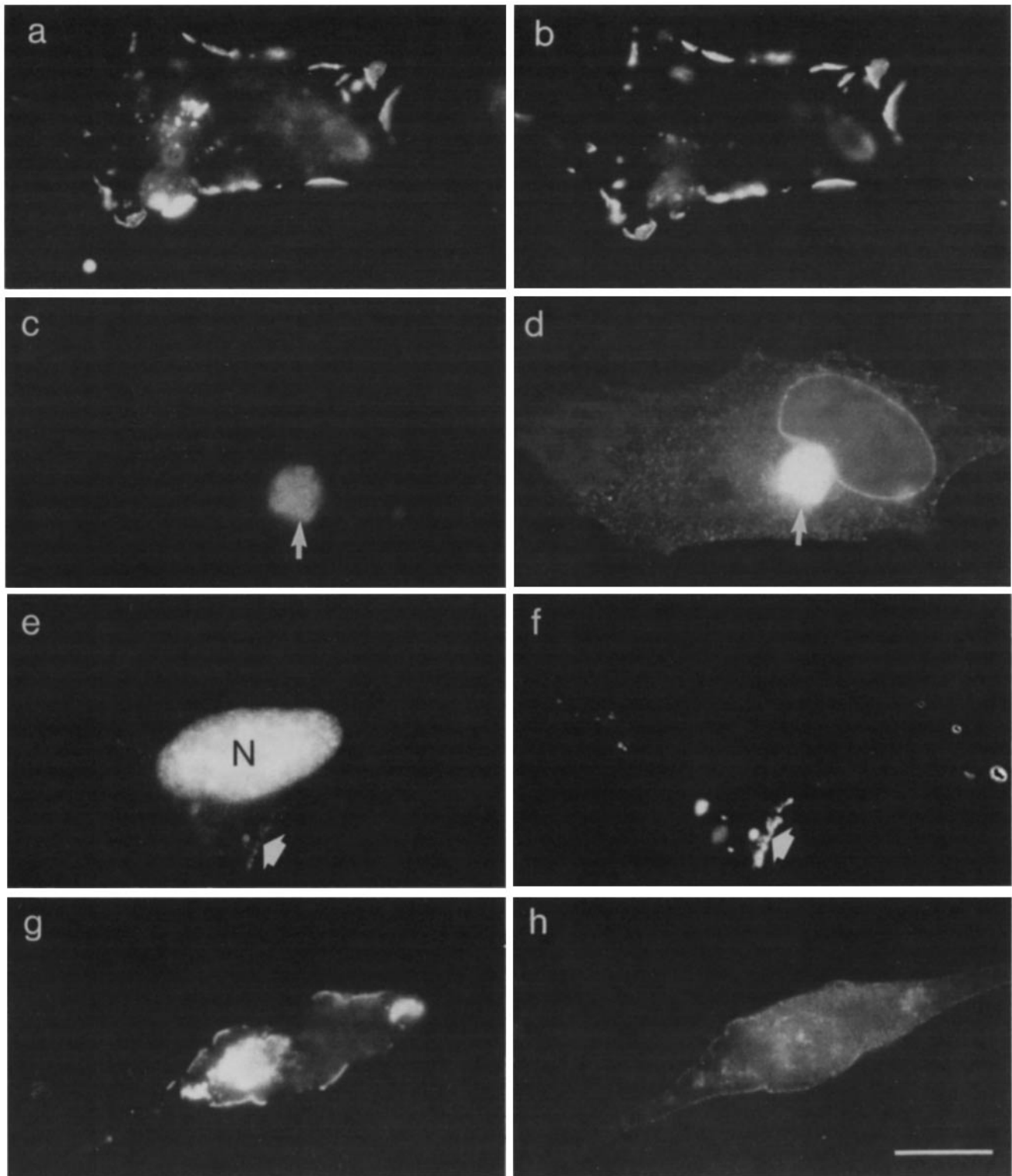


**Figure 3.** Immunofluorescent localization of 43k in transfected fibroblasts. QT-6 fibroblasts transfected with (a) M43<sup>wt</sup>, (b) M43<sup>U254</sup>, (c and d) M43<sup>A2</sup>, or (e and f) M43<sup>ID1</sup> were fixed, permeabilized, and stained with rabbit anti-43k antibody (a and c-f) or monoclonal anti-43k, mAb 1234A (b), followed by FITC-conjugated, affinity-purified secondary antibody (*Materials and Methods*). A cell transfected with M43<sup>A2</sup> (c) shows strong anti-43k staining of the nucleus (N) and of aggregated vesicles (small arrow). d is a longer photographic exposure of the field in c revealing a less intensely stained membrane domain (broad arrow). The anti-43k-stained nucleus of a second cell is also visible with this longer exposure (lower left of d). Broad arrows in a and d-f indicate anti-43k-stained plasma membrane domains which were common in M43<sup>wt</sup>- and M43<sup>ID1</sup>-transfected cells. Cells transfected with any of the four constructions frequently displayed aggregates of vesicle-like structures (small arrows). Bar, 10  $\mu$ m.

M43<sup>U254</sup>-transfected cells (Table I). Thus, either the 43k COOH terminus is required for plasma membrane targeting or loss of the COOH terminus results in misfolding and sequestration of 43k in perinuclear aggregates and granules, leaving no 43k available for plasma membrane binding.

The results described above suggest that part or all of the carboxy-terminal sequence (Ile<sup>255</sup> to Val<sup>412</sup>), as well as the myristoylated amino terminus, are important for the formation of 43k-rich plasma membrane domains. To determine whether these two regions alone were sufficient for the formation of plasma membrane domains, a mutant lacking the central portion of the protein was constructed. We took ad-

vantage of Pvu II and Eco RV restriction sites in the cDNA to fuse codon 15 to codon 255, while maintaining the reading frame. This deletion mutant (M43<sup>ID1</sup>, Fig. 1), when transfected into QT-6 cells, yielded a broad band of staining on immunoblots corresponding to the predicted molecular mass of 19 kD (Fig. 2). As with M43<sup>wt</sup> protein and the other mutants, M43<sup>ID1</sup> protein was more concentrated in the P100 fraction than in the S100 fraction (Fig. 2). In contrast to the other mutants, M43<sup>ID1</sup> protein migrated with the membrane fraction through the discontinuous sucrose gradient (Fig. 2). Immunofluorescence confirmed the presence of M43<sup>ID1</sup> protein in the plasma membrane (Fig. 3, e and f). Furthermore,



**Figure 4.** Immunofluorescent double labeling of 43k and AChR in transfected Q-F18 fibroblasts. Q-F18 cells were transfected with (a and b) M43<sup>wt</sup>, (c and d) M43<sup>U254</sup>, (e and f) M43<sup>A2</sup>, or (g and h) M43<sup>ID1</sup>. After fixation and permeabilization, cells were labeled by indirect immunofluorescence with anti-43k and anti-AChR antibodies followed by appropriate affinity-purified, species-specific secondary antibodies. Cells were examined first for anti-43k staining with rhodamine-conjugated secondary antibodies (a, c, e, and g), and then for anti-AChR staining with FITC-conjugated secondary antibody (b, d, f, and h). Cells transfected with M43<sup>U254</sup> displayed intense anti-43k staining of what appeared to be aggregates of vesicles (c, arrow). FITC anti-AChR staining remained diffuse in M43<sup>U254</sup> transfected cells, requiring longer photographic exposures to display the unclustered AChR. Under these conditions the intense rhodamine anti-43k staining of intracellular structures (c) penetrated into the FITC (anti-AChR) channel (d, arrow). The broad arrow in e shows an example of an anti-43k-stained membrane domain in an M43<sup>A2</sup>-transfected cell at which AChR became localized (f, arrow). N, the anti-43k-stained nucleus of the cell in e. Bar, 10  $\mu$ m.

despite deletion of more than half of the wild-type polypeptide sequence, M43<sup>ID1</sup> protein was able to aggregate into plasma membrane domains similar in size (Fig. 3, *e* and *f*, *broad arrows*), and with a similar frequency (Table I) to those formed by transfection of the wild-type protein. Immunofluorescent staining also revealed M43<sup>ID1</sup> protein in perinuclear membrane aggregates (Fig. 3, *e* and *f*, *small arrows*) and scattered granule-like structures (Table I).

### Coexpression of AChR and Mutant 43k

To investigate the ability of mutant 43k to aggregate AChRs, the 43k cDNAs were transfected into the fibroblast cell line Q-F18, a derivative of QT-6 which stably expresses the fetal form of the skeletal muscle AChR (52). Before studying the effects of 43k on the distribution of AChRs in these cells we first examined the possibility that the presence of AChRs in the cells might itself alter the subcellular distribution of the introduced 43k. Homogenates of cells transfected with M43<sup>wt</sup>, M43<sup>U254</sup>, M43<sup>A2</sup>, or M43<sup>ID1</sup> were fractionated as described for QT-6 (above). In each case the transfected protein fractionated the same way for Q-F18 as shown for QT-6 in Fig. 2 (data not shown). Additionally, examination of transfected cells by immunofluorescence confirmed that for each of the mutants the subcellular distribution of anti-43k staining in Q-F18 cells was similar to that seen previously in QT-6 cells (Table I, numbers in brackets are results for Q-F18 cells). Thus, the presence or absence of AChRs did not appear to affect the subcellular localization of 43k.

The effect of 43k on the spatial distribution of AChRs was studied by double immunolabeling with anti-43k antibody together with the anti-AChR antibody, mAb 35, which recognizes the main immunogenic region of the  $\alpha$  subunit (72). Since immunostaining was performed after permeabilization of the cells, the anti-AChR images frequently reveal AChRs associated with intracellular membranes (note ring of staining around the nucleus in Fig. 4 *d*) as well as cell surface AChRs. As previously reported (52), transfection of M43<sup>wt</sup> into Q-F18 fibroblasts resulted in the colocalization of AChRs with 43k-rich plasma membrane domains, suggesting that M43<sup>wt</sup> protein induces the aggregation of AChRs (Fig. 4, *a* and *b*). The ability of M43<sup>wt</sup> protein to induce AChR-rich membrane patches was quantitated to permit comparison with the mutant 43k's. Coverslips were scanned systematically and anti-43k-positive cells were scored for the presence of AChR-rich plasma membrane domains. Approximately 80% of total 43k-positive cells displayed 43k-rich membrane patches. Of these ~79% had corresponding AChR aggregates (Fig. 5). Why some cells with 43k-rich membrane domains did not show corresponding AChR aggregates remains uncertain. However, the intensity of anti-AChR staining varied widely among individual Q-F18 cells within a culture, with some cells showing no staining above background (data not shown). Hence, the small proportion of cells showing no colocalized AChRs may simply represent the fraction of cells expressing no detectable AChRs.

As indicated above, 43k-rich plasma membrane domains occurred at lower frequency on Q-F18 cells transfected with M43<sup>A2</sup> than with M43<sup>wt</sup>. However, AChRs became localized with a substantial proportion of these (Fig. 4, *e* and *f*; *arrows*). In four separate transfection experiments with M43<sup>A2</sup>, 29  $\pm$  10% of cells which displayed 43k-rich membrane patches showed AChR colocalized with the 43k

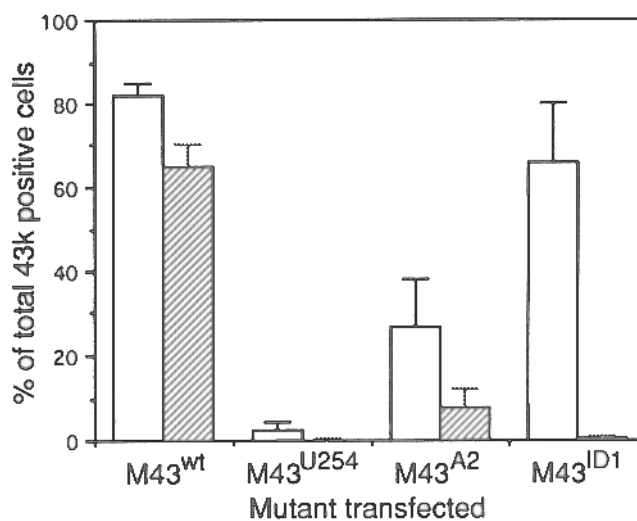


Figure 5. Frequency of formation of 43k-rich and AChR-rich membrane domains by wild-type 43k and 43k mutants. Bars represent the percentage of anti-43k-positive cells which displayed anti-43k-stained (*open bars*) and anti-AChR-stained (*shaded bars*) membrane domains after transfection of M43<sup>wt</sup>, M43<sup>U254</sup>, M43<sup>A2</sup>, or M43<sup>ID1</sup>. Error bars indicate the standard deviation for three (M43<sup>wt</sup>, M43<sup>U254</sup>, M43<sup>ID1</sup>) or four (M43<sup>A2</sup>) independent transfection experiments. The total number of cells examined were 438, 547, 816, and 522 respectively.

patches (Fig. 5). Thus, elimination of the myristoyl-glycine modification affected the number of 43k-rich plasma membrane patches but did not eliminate the propensity of those patches to aggregate AChRs.

When Q-F18 cells were transfected with M43<sup>U254</sup>, the majority of anti-43k-positive cells showed no 43k-rich membrane patches. The truncated 43k was localized mainly in intracellular compartments as described for QT-6 cells above (in Fig. 4 *c*, the arrow indicates what appeared to be an aggregate of vesicles). The rhodamine anti-43k staining of these structures was so intense that it penetrated the FITC optical channel (Fig. 4 *d*, *arrow*). Nevertheless, staining with anti-AChR antibody showed that cell surface AChRs remained dispersed in the plasma membrane of these cells as is evident from the diffuse FITC staining in Fig. 4 *d*. Anti-43k-stained membrane patches appeared on only 12 out of 547 expressing cells. Of these 12 cells, only 1 showed anti-AChR staining colocalized with anti-43k-stained patches (Fig. 5).

M43<sup>ID1</sup> protein expressed in Q-F18 cells was able to form anti-43k-stained membrane patches similar in size, and with a similar frequency to those formed by M43<sup>wt</sup> protein (Figs. 4 *g* and 5). However, in contrast to the results with M43<sup>wt</sup> and M43<sup>A2</sup>, the 43k-rich domains formed by M43<sup>ID1</sup> protein only rarely displayed colocalized AChRs. Of the 342 cells that displayed discrete anti-43k-stained plasma membrane domains, only 4 cells showed anti-AChR staining colocalized at any of these domains (Fig. 5). Instead, anti-AChR staining remained diffusely distributed across the plasma membrane (Fig. 4 *h*). Taken together, our results suggest that the portion of 43k deleted from the M43<sup>ID1</sup> protein may be specifically involved in AChR aggregation, while the COOH terminus and myristoylated NH<sub>2</sub> terminus may be involved in targeting 43k to the plasma membrane.

## Discussion

There is now strong evidence that 43k can induce AChR aggregation. Transfection of 43k into fibroblasts already expressing recombinant AChR led to the reorganization of the AChR into 43k-rich plasma membrane domains (52). Froehner et al. (26) have obtained similar results when recombinant AChR and 43k mRNAs were coinjected into *Xenopus* oocytes. Given the close spatial relationship between 43k and AChR clusters at synaptic contacts on muscle cells (6, 24, 53) it seems likely that 43k is central to at least one mechanism that may be used for clustering and maintaining high densities of AChRs in the postsynaptic membrane of the neuromuscular junction. From observations with M43<sup>wt</sup> it was possible to predict that 43k-induced AChR aggregation may involve three distinct and sequentially occurring steps. (a) Association with the plasma membrane. Purified *Torpedo* 43k has been shown to bind to synthetic phospholipid vesicles even in the presence of high salt (54). Hence the first step presumably involves association of newly formed 43k with the plasma membrane. (b) Aggregation in the plasma membrane. When expressed in nonmuscle cells recombinant 43k not only associated with membrane but was able to aggregate into membrane domains in the absence of AChRs or other muscle-specific proteins (26, 52). It remains unclear whether domain formation relies on direct 43k-43k interaction or is achieved indirectly by linkage to elements of the cytoskeleton. (c) Clustering of AChRs. By whatever means they were formed, these 43k-rich membrane domains were capable of aggregating coexpressed AChRs (26, 52).

To learn more about the molecular events involved in 43k-induced AChR aggregation, we have introduced mutations into 43k and investigated the ability of the mutated proteins to aggregate AChR in our fibroblast expression system. After transfection into fibroblasts, M43<sup>wt</sup> protein was found mainly in the membrane and particulate fractions of cells and only a small proportion was found in the cytosol, as previously reported for endogenous 43k (19, 21, 44). Immunofluorescent staining also revealed 43k in aggregates of membrane near the nucleus and in small granule-like structures throughout the cytoplasm of some cells (Figs. 3 a and 4 a). Similar intracellular structures become sites of localization for the membrane-associated tyrosine kinase p60<sup>v-src</sup> when it is expressed at high levels in cells (32, 58). Hence, these may be general sites of accumulation of overexpressed peripheral membrane proteins. Results with mutant 43k's suggest that both the COOH-terminus and the NH<sub>2</sub>-terminal *N*-myristoyl-glycine modification are necessary to ensure efficient association with the plasma membrane and formation of 43k-rich plasma membrane domains. A mutant protein in which 239 amino acids were deleted from the center of the polypeptide (M43<sup>ΔD1</sup> protein) retained the ability to form 43k-rich membrane domains at the normal frequency, but lacked the ability to aggregate AChRs into those domains. Hence the COOH and NH<sub>2</sub> termini of 43k seem important for membrane association while the center of the molecule may be involved in AChR aggregation. The present mutants do not allow us to separate the association of 43k with the plasma membrane from the formation of 43k-rich plasma membrane domains. Other observations suggest that these may indeed be separable functions of 43k. For example, the mouse cell lines BC3H1 and C2 express similar amounts of endogenous 43k

in their membranes (19, 35) but unlike C2 myotubes, the 43k in BC3H1 cells rarely aggregates to form membrane domains (35).

Substitution or elimination of the NH<sub>2</sub>-terminal glycine can be used to prevent myristoylation of proteins that normally contain this modification (49, 69). Substitution of Gly<sup>2</sup> with Ala<sup>2</sup> resulted in the redistribution of 43k from the plasma membrane fraction into the denser 50% sucrose pellet fraction. This was accompanied by a marked reduction in the number of 43k-rich plasma membrane domains observed by immunofluorescence. Similar experiments with the Rous sarcoma virus tyrosine kinase, p60<sup>v-src</sup>, and other myristoyl proteins, support the idea that the myristoylated NH<sub>2</sub> terminus in some cases may serve to target a protein to the plasma membrane (49, 56, 60, 67). With some (50) but not all (32) cytosolic proteins, fusion to a peptide consisting of the first 14 amino acids of p60<sup>v-src</sup> redirected the protein into the membrane fraction of the cell. Furthermore, recent evidence suggests that membrane localization is mediated by a receptor localized in the plasma membrane and specific for the myristoylated NH<sub>2</sub>-terminal peptide of p60<sup>v-src</sup> (57, 59). The almost perfect conservation of the first 18 residues of 43k (one conservative substitution in *Xenopus*) goes well beyond the limited requirement for myristoylation (31, 69) and suggests itself as a potential recognition sequence for such a myristoyl peptide receptor.

43k contains an unusually high cysteine content, a feature that has hindered biochemical studies (54). In particular, the COOH-terminal end of 43k, truncated from M43<sup>U254</sup>, contains a region consisting of ~10% cysteine (9). As previously noted (9, 22), the spacing of the five cysteines after Cys<sup>363</sup> bears some similarity to that of the repeated pattern in the putative phospholipid, diacylglycerol, and phorbol ester binding domain of the protein kinase C family. Some of the intervening, noncysteine residues also align with the kinase C consensus (22). However, 43k lacks one of two cysteines that may be crucial for phorbol ester binding to the kinase C sequence (47). The 40 residues starting at Cys<sup>363</sup> fit quite closely (23) to a recently reported variant of the zinc finger motif (20). Although generally associated with protein-DNA binding (3), zinc finger-like structures in other contexts may be important in protein-protein interactions (48). In any event, it will be of interest to examine the effect of more subtle deletions or substitutions in this domain on the association of 43k with the plasma membrane. The truncated COOH terminus also contains a potential site for phosphorylation by either cAMP-dependent protein kinase (1, 9, 18) or protein kinase C (23) although it is not clear whether this site is normally phosphorylated. Truncation of the COOH-terminal 158 residues, which eliminates both of these motifs (M43<sup>U254</sup>) virtually abolished association of the transfected protein with the plasma membrane (Figs. 2, 3 b, and 4 c). It remains uncertain whether the failure of M43<sup>U254</sup> to associate with the plasma membrane is due to elimination of a membrane binding domain or is the result of improper folding of the truncated protein. On the other hand, deletion of a larger portion of the protein, between residues 15 and 255 (M43<sup>ΔD1</sup>), resulted in a protein that associated with the membrane and formed 43k-rich plasma membrane domains typical of the M43<sup>wt</sup>. Hence the COOH-terminal 158 residues, in conjunction with the 15 amino acids of the myristoylated NH<sub>2</sub> terminus comprise se-



quences necessary and sufficient for formation of 43k-rich membrane domains in fibroblasts.

The plasma membrane domains formed by transfection of M43<sup>IDI</sup> rarely showed colocalized AChRs, suggesting that residues 16–254, which were deleted in this mutant, may contain a binding domain for AChRs. We cannot exclude the alternative possibility that misfolding of the M43<sup>IDI</sup> protein was responsible for the loss of AChR aggregation function. However, such misfolding, if it occurred, did not affect the ability of the protein to target to the plasma membrane or to aggregate into plasma membrane domains. Two large regions with particularly high amino acid conservation are present in the deleted sequence. The first of these (residues 127–170) contains 86% amino acid identity across 43k sequences from *Torpedo*, mouse, and *Xenopus* (1, 18, 22). This region contains conserved leucine residues at positions 136, 143, and 150 with no prolines that would disrupt an  $\alpha$  helix. This spacing of leucines at every seventh position is the hallmark of the leucine zipper, in which leucine residues are aligned along one side of an  $\alpha$  helix to permit interaction with a second, adjacent leucine zipper helix, forming a coiled coil structure (38, 55). The leucine zipper motif has been shown to be important for dimer formation by the transcription factor proteins fos and jun (34, 64). Heptad leucine repeats are also present in a variety of cytoplasmic and transmembrane proteins including intermediate filament proteins (8), and voltage-gated ion channels (41). *Xenopus* 43k has a fourth heptad-spaced leucine at position 129, while both *Torpedo* and mouse have methionine, an allowable substitution (38), at this site. Thus, 43k may contain eight turns of leucine zipper  $\alpha$  helix (23). The average hydrophobicity and hydrophobic moment in this stretch of 43k suggests membrane-associated  $\alpha$  helix (9), raising the question of whether it interacts with another membrane protein. The  $\alpha$  subunit of the AChR includes an imperfectly conserved four-heptad repeat extending from M4 into the putative extracellular tail but no other potential leucine zipper was evident in the cytoplasmic or transmembrane domains of any of the subunits. Therefore, it seems unlikely that 43k associates with the AChR by leucine zipper interactions. The leucine zipper of 43k might, alternatively, mediate homodimerization or interactions with other proteins known to be concentrated on the cytoplasmic face of the postsynaptic membrane (4, 10, 25, 75).

The second highly conserved sequence includes residues 225–257 of 43k and retains 93% identity across the three species (1, 18, 22). All but the last three amino acids of this conserved region were deleted in the M43<sup>IDI</sup> protein. The region contains a short stretch of hydrophobic residues that, like those in the first conserved region, might conceivably form nonionic interactions with the AChR subunits (9), irrespective of any potential leucine zipper interactions.

We cannot at present explain the anomalous localization of M43<sup>A2</sup> protein in the nucleoplasm (Figs. 3 c and 4 e). This was not a general property of overexpressed wild-type or mutant 43k's. Significant nuclear staining was only seen in a small minority of cells after transfection with M43<sup>wt</sup> or M43<sup>IDI</sup> (Table I). It is possible that the myristoyl-glycine-mediated membrane association may normally be dominant over some cryptic "nucleophilic" feature of the 43k polypeptide. M43<sup>U254</sup> protein, which also associated poorly with the plasma membrane was, in contrast to M43<sup>A2</sup> protein, rarely

localized in the nucleoplasm, suggesting that the putative nucleophilic domain may be located on the COOH terminus. Alternatively, M43<sup>U254</sup> protein may be sequestered in the cell as an improperly folded protein. It is tempting to consider the possibility that the cryptic nucleophilic property of 43k may hint at some hitherto unpredicted function of 43k. The nuclei located beneath the postsynaptic membrane are thought to regulate transcription of AChR genes in a manner quite different from that of nuclei elsewhere within the muscle fiber; the transcription factors responsible and the way in which they are locally controlled are unknown (13, 33, 64a). One intriguing possibility is that 43k clustered at high density on the postsynaptic membrane might serve as a cytoplasmic anchor (29), binding nuclear proteins responsible for transcriptional control of the AChR, analogous to the way the cytoplasmic protein I- $\kappa$ B regulates entry into the nucleus of the transcription factor NF- $\kappa$ B (27). M43<sup>A2</sup> protein, which lacks myristoyl-glycine-mediated membrane association, might thus be carried into the nucleus as part of a transcription factor-M43<sup>A2</sup> protein complex. Alternatively, 43k itself may be capable of binding to DNA through its zinc finger domain, and passively accumulating in the nucleus (15) when not tightly associated with the plasma membrane.

The primary structure of 43k is well conserved throughout its length, marking it difficult to delimit likely functional domains (1, 18, 22). The mutants described in this paper represent a first attempt to dissect functionally important peptide sequence elements within 43k. Identification of the residues involved in membrane association and AChR aggregation will require a study of smaller deletions. It will also be necessary to determine whether other membrane-associated or cytoskeletal proteins are involved. The present results indicate the likely involvement of the COOH terminus and the *N*-myristoylated NH<sub>2</sub> terminus in the formation of 43k-rich membrane domains and suggest that the central portion of the molecule may include an AChR binding domain.

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