

Clustering of the Acetylcholine Receptor by the 43-kD Protein: Involvement of the Zinc Finger Domain

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Abstract. A postsynaptic membrane-associated protein of M_r 43,000 (43-kD protein) is involved in clustering of the nicotinic acetylcholine receptor (AChR) at the neuromuscular junction. Previous studies have shown that recombinant mouse 43-kD protein forms membrane-associated clusters when expressed in *Xenopus* oocytes. Coexpression with the AChR results in colocalization of the receptor with the 43-kD protein clusters (Froehner, S. C., C. W. Luetje, P. B. Scotland, and J. Patrick, 1990, *Neuron*, 5:403–410). To understand the mechanism of this clustering, we have studied the role of the carboxy-terminal region of the 43-kD protein. The amino acid sequence of this region predicts two tandem zinc finger structures followed by a serine phosphorylation site. Both *Torpedo*

43-kD protein and the carboxy-terminal region of the mouse 43-kD protein bind radioisotopic zinc. Mutation of two histidine residues in this predicted domain greatly attenuates zinc binding, lending support to the proposal that this region forms zinc fingers. When expressed in oocytes, the ability of this mutant 43-kD protein to form clusters is greatly reduced. Its ability to interact with AChR, however, is retained. In contrast, a mutation that eliminates the potential serine phosphorylation site has no effect on clustering of the 43-kD protein or on interaction with the AChR. These findings suggest that protein interactions via the zinc finger domain of the 43-kD protein may be important for AChR clustering at the synapse.

THE postsynaptic membrane of the neuromuscular junction contains a high density of nicotinic acetylcholine receptors (AChRs)¹ ($\sim 10,000$ receptors/ μ^2) (12) that are necessary for efficient neuromuscular communication. Key questions in neuromuscular synaptogenesis concern both the nature of the neuronal signal that directs AChR clustering and the mechanism by which the muscle anchors AChRs at the postsynaptic site (reviewed in 17, 22). The neuronally derived protein agrin, along with other extracellular proteins, may serve as the signal that initiates AChR accumulation (9, 11, 34, 37). A specialized cytoskeletal network of proteins on the postsynaptic membrane may receive and respond to this signal. In concert, components of both the pre- and postsynaptic cells may orchestrate the formation and maintenance of the AChR clusters during neuromuscular synaptogenesis.

The postsynaptic cytoskeleton contains several proteins that may be important for AChR clustering as well as other

structural features of the synapse. These proteins include the AChR-associated 43-kD protein, a unique isoform of β -spectrin, actin, three members of the dystrophin family, and a 58-kD protein associated with the dystrophin family of proteins (reviewed in 17). Of these, the 43-kD protein has received the most attention. Although its function is not known, considerable evidence supports its involvement in AChR accumulation. In *Torpedo* electrolytes and mammalian skeletal muscle, the AChR and 43-kD protein are present in approximately equimolar concentrations (28, 29). Chemical cross-linking studies (7), EM (6, 13, 54) and high-resolution structural analyses (35, 55) support the notion that the 43-kD protein interacts directly with cytoplasmic domains of the AChR. Treatments that remove the 43-kD protein, and other membrane-associated proteins, result in increased lateral and rotational mobility of the AChR (2, 4, 33, 50) while leaving the receptor intact and functional (38). Finally, overexpression of the 43-kD protein in C2 muscle cells disrupts AChR clustering (61).

Coexpression studies have provided the clearest evidence for a role of the 43-kD protein in AChR clustering. Recombinant AChR expressed in *Xenopus laevis* oocytes (19) or quail fibroblasts (42) are distributed diffusely on the cell surface. Coexpression of the mouse 43-kD protein with the AChR results in clustering of the AChR and colocalization with the 43-kD protein. Of particular interest is the observa-

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1. **Abbreviations used in this paper:** AChR, nicotinic acetylcholine receptor; GST, glutathione-S-transferase; PKA, protein kinase A; PKC, protein kinase C.

tion that 43-kD protein expressed alone forms clusters similar to those seen when coexpressed with the AChR (19, 42). Thus, the regulation of 43-kD protein clustering may be a vital step in postsynaptic membrane assembly.

Several conserved structural features of the 43-kD protein may be involved in clustering and/or interaction with AChR (reviewed in 16). The amino terminus of the 43-kD protein is myristylated (10, 36) and could play a pivotal role in membrane targeting or membrane interaction. Although elimination of this myristylation site by site-directed mutagenesis reduces the 43-kD protein's interaction with the membrane, its ability to cluster and to interact with the receptor is retained (43). The central region in the 43-kD protein contains a "leucine zipper" motif which may be involved in protein-protein interactions. Deletion mutants that lack this region still interact with the membrane and form clusters of the 43-kD protein, but are unable to cluster the AChR (43). Near the carboxy terminus is a cysteine-rich region similar in primary structure to the regulatory domain of protein kinase C (PKC) and DNA-binding domains of nuclear proteins (16). Such domains have been shown to bind Zn^{2+} through the formation of zinc finger structures (1, 3, 14). Although it is unlikely that the 43-kD protein is a DNA-binding protein, the 43-kD protein may form zinc finger structures, enabling it to interact with other proteins. Finally, very near the carboxy terminus is a consensus sequence for both PKC and protein kinase A (PKA) phosphorylation. The 43-kD protein in membranes from *Torpedo* electrocytes can be phosphorylated by PKA (24) but the regulatory role of this modification is unknown.

The objective of the studies reported here was to determine by mutagenesis if two domains of the 43-kD protein, the cysteine-rich region (potential zinc finger domain) and the phosphorylation region, are involved in AChR or 43-kD protein clustering. Ideally, these experiments would be performed in innervated muscle cells in which AChR clustering in vivo could be studied. Technical limitations, including the inability to distinguish between endogenous wild-type and mutant 43-kD protein, preclude the use of this system. For this reason, we have expressed the mutated 43-kD protein and AChR in *Xenopus* oocytes. Our studies reveal that both the *Torpedo* 43-kD protein and a recombinant mouse protein containing the cysteine-rich region bind radioisotopic zinc. Mutations in the zinc-binding region reduce the ability of the expressed 43-kD protein to bind radioisotopic zinc and to form clusters of 43-kD protein when expressed in *Xenopus* oocytes. The mutated protein retains its ability to interact with the AChR. Mutations eliminating the phosphorylation site have no effect on clustering or on interaction with the AChR.

Materials and Methods

Materials

^{65}Zn chloride (Carrier free, 100–1,000mCi/mg Zn) was obtained from Amersham Corp. (Arlington Heights, IL). *Torpedo nobiliana* electric organ was purchased from Biofish (Georgetown, MA). *Xenopus laevis* toads were obtained from Nasco (Fort Atkinson, WI). Mouse cDNA clones for the AChR subunits were kindly provided by Jim Boulter (The Salk Institute, La Jolla, CA) and the cDNA clone of mouse 43-kD protein has been described previously (15). pGEX vector-3X was obtained from Pharmacia (Piscataway, NJ). Oligonucleotides were made by DNA Express (Colorado State University, Fort Collins, CO).

Antibodies

mAbs 1234A and 1579A (both anti-43kD) have been described (18, 41). Affinity-purified rabbit anti- α bungarotoxin was generously provided by Robert Sealock (University of North Carolina, Chapel Hill, NC). All other antibodies were purchased from Jackson ImmunoResearch Labs (West Grove, PA).

Preparation of *Torpedo* Membranes and Affinity Purification of *Torpedo* Proteins

Torpedo electrocyte AChR-rich membranes were prepared as previously described (44) through the first sucrose centrifugation with minor modifications. Iodoacetamide and *N*-ethylmaleimide were omitted from all steps. Affinity purification, SDS-gel electrophoresis on no-well 9% acrylamide gels, and immunoblotting of *Torpedo* proteins were all carried out as previously described (8). One region of the gel was stained for protein with Coomassie blue, while the remainder of the gel was electroblotted onto nitrocellulose paper in 25 mM $NaPO_4$, pH 6.5. Nitrocellulose strips were incubated with $^{65}Zn^{2+}$ (see below) or with antibodies according to published procedures (8).

$^{65}Zn^{2+}$ Experiments

$^{65}Zn^{2+}$ blotting experiments were carried out as described by Treich et al. (56) with minor modifications. Proteins transferred to nitrocellulose membranes were incubated in metal binding buffer (100 mM Tris, pH 7.0, 50 mM NaCl, 5 mM DTT) for 1.5 h and then in 6 M guanidine hydrochloride (in metal binding buffer) for 0.5 h to denature the proteins. Samples were quickly but thoroughly washed to remove guanidine hydrochloride and then were incubated in metal binding buffer containing 3 μ Ci $^{65}ZnCl_2$ per ml for 1 h. After incubation, samples were thoroughly washed with metal binding buffer, dried, and exposed to film.

For dot blot analysis, bacterial cell lysates (3 μ l) from cells expressing either GST-43-kD fusion protein or GST alone (as a control) were spotted onto nitrocellulose membranes and dried. To test the ability of divalent cations to compete with $^{65}Zn^{2+}$ binding, several modifications in the above procedure were made. DTT was omitted from all steps after the guanidine hydrochloride incubation. Also, samples were preincubated with 1 mM divalent cation (or control) 10 min before, and throughout incubation with $^{65}Zn^{2+}$. Samples were then thoroughly washed, dried, and exposed to film.

Generation of Glutathione-S-transferase/43-kD Fusion Protein

In-frame gene fusions between the glutathione-s-transferase (GST) cDNA and part of the mouse muscle 43-kD cDNA or the 43-kD_{H-Q} cDNA were prepared by standard procedures. cDNA encoding the carboxy-terminal region of the 43-kD protein (BsaAI/EcoRI restriction fragment containing nucleotides 1204 to 1618) was subcloned into the pGEX-3X vector (cut with restriction enzymes SmaI/EcoRI). This carboxy-terminal region of 43-kD proteins begins at amino acid 349 (see Fig. 2), and when fused to GST, forms a protein of predicted molecular weight \sim 34,500. These constructs were sequenced to verify correct reading frame and to insure that no additional mutations were present (52). Protein induction and cell lysate isolation were performed as described by Pharmacia.

Cell lysates (20 μ l) were resuspended in sample buffer and run on three identical 9% acrylamide minigels. One gel was stained with Coomassie, while the other two were transferred to nitrocellulose membranes (Trans-Blot semi-dry method, 5 V, 0.8 h, 25 mM Tris/192 mM glycine buffer) (Bio-Rad Laboratories, Richmond, CA). On one, the 43-kD protein was detected by Western blot as previously described (8); the other blot was incubated with $^{65}Zn^{2+}$, as described above.

Mutagenesis of Zinc Finger Region and Phosphorylation Site

Mutagenesis of the 43-kD cDNA was accomplished by the polymerase chain reaction as previously described (25). Oligonucleotides used were: 1-(5'-GTAATACGACTACTATAG-3'), 2-(5'-GCGCGGCGGTACTTCGG-3'), 3-(5'-CGCGCCGCATGAGCC-3'), 4-(5'-GCACGTAGTGAGGTTCC-3'), 5-(5'-CTGAGCTGAAAGATCTGGGA-3'), and 6-(CTCCAGATCTTTCAGCTCA-3'). Mutant 43 kD_{H-Q} was made using oligos 1, 4, 5, and 6. Mutant 43 kD_{S-A} was made using oligos 1, 2, 3, and 4. The mutant DNA fragment was digested with SstI and StuI, and used to replace the ap-

appropriate segment of the normal 43-kD cDNA. Both mutants were sequenced (52) to verify that only the appropriate mutations were present.

In mutant 43 kD_{H→Q}, nucleotides 1311 and 1320 have been changed from cytosine and thymidine, respectively, to guanosine. These changes alter codon specificity for amino acids 384 and 387 from histidine to glutamine. Mutant 43 kD_{S→A} changes nucleotides 1372 and 1375 from thymidine to guanosine, resulting in the conversion of codons for amino acids 405 and 406 from serine to alanine.

In Vitro Transcription and Injection into *Xenopus* Oocytes

In vitro transcription and oocyte injections were performed as previously described (5, 19) with minor modifications. RNA encoding the AChR subunits was generated in vitro using the mRNA Transcription Kit (Stratagene Corp., La Jolla, CA) and SP6 RNA polymerase after the vector was linearized by *Hin*DIII (α , β , and δ subunits in vector SP65) or *Eco*RI (γ -subunit in vector SP64). 43 kD, 43 kD_{H→Q}, and 43 kD_{S→A} RNA were all generated in vitro using T3 RNA polymerase from the vector pBS after linearization with *Eco*RI. All RNA was capped with m⁷G(5')ppp(5')G (Pharmacia). 2.5 ng of each RNA was injected into oocytes in a final volume of 40 nl (unless otherwise indicated). Oocytes were incubated for 5 d before analysis.

Oocyte Membrane Preparation

Oocyte membranes were isolated as described by Pradier et al. (46) with minor modifications. 5 d after injection, 30–50 unlabeled, viable oocytes were homogenized thoroughly with 800 μ l ice-cold homogenization buffer (83 mM NaCl, 1 mM MgCl₂, 10 mM Hepes, pH 7.9, and 5 mM EDTA, 5 mM EGTA, and 1 mM PMSF) in a dounce homogenizer. The homogenate was centrifuged twice, 800 g (2,500 rpm in a JA20 rotor [Beckman Instruments, Palo Alto, CA], 10 min at 4°C), and the supernatant was retained. The supernatant was then layered onto 150 μ l 15% sucrose solution and centrifuged 160,000 g (27,000 rpm; SW27 rotor [Beckman Instruments], 1.5 h, 4°C). The membrane pellets were resuspended in 100 mM NaCl, 10 mM MOPS (1 μ l/oocyte).

Western Blots of Oocyte Membranes

Oocyte membrane preparations were diluted with SDS sample buffer containing DTT (final concentration 5 mM) and volumes equivalent to five oocytes (see Fig. 5) or 15 oocytes (see Fig. 9) were boiled 5 min and loaded onto a 17-well (see Fig. 5) or 6-well (see Fig. 9) 9% acrylamide minigel. Proteins were transferred to immobilon membranes (Trans-Blot™ semi-dry method, 15 V, 0.5 h, 25 mM Tris/192 mM glycine buffer) (Bio-Rad Laboratories) (see Fig. 5) or nitrocellulose membranes (25 mM NaPO₄ buffer, pH 6.5, 250 mA, 2 h) (see Fig. 9). Detection of proteins was accomplished using mAb 1234A (25 nM) followed by alkaline phosphatase-conjugated goat anti-mouse IgG (1/5,000) as previously described (8).

Immunofluorescence Labeling of Oocytes and Microscopy

Oocytes were immunostained for the AChR and 43-kD protein as previously described (19) with one modification of AChR staining. AChRs were initially labeled with unconjugated α -bungarotoxin (32 nM) instead of rhodamine-conjugated α -bungarotoxin, fixed, permeabilized, and then incubated with rabbit anti- α bungarotoxin antibody (2 μ g/ml) and rhodamine-conjugated anti-rabbit IgG (1/200) (Jackson ImmunoResearch Labs). Oocytes were examined under a Zeiss Axioskop microscope with a 63 \times planapo objective (see Figs. 6 and 7) or a Zeiss Universal microscope with a 63 \times planeofluor objective (see Fig. 10) (Carl Zeiss, Oberkochen, Germany).

Confocal Microscopy/Computer Analysis of Clusters

A Bio-Rad MRC-600 laser scanner mounted on a reverse Nikon microscope with a Nikon 60X/1.4 objective was used to acquire the images of AChR clustering (Nikon Corp., Melville, NJ). The images were obtained from the average of four frames under the identical parameters. Object measurement was performed with an *Image-1* computer program. The image was first filtered by a low pass filter (both horizontal and vertical size = 6) to smooth the edges of the clusters. Then, only clusters with a shape factor ($4\pi A/p^2$, where A is area, and the p is the length of an edge) ≥ 0.4 were measured, eliminating most objects that were composed of multiple clusters. Data

sheets of measurements were transformed to *Sigmaplot* to construct the size frequency histograms.

Results

The 43-kD Protein is a Zinc-binding Protein

The cysteine-rich region of the 43-kD protein shows sequence similarity to other known zinc-binding proteins. To determine if the 43-kD protein binds Zn²⁺, purified postsynaptic membranes from *Torpedo* electric organ were separated by SDS gel electrophoresis, and nitrocellulose replicas were incubated with ⁶⁵Zn²⁺. As previously described (44), the most abundant proteins in these membrane preparations are the subunits of the AChR and the 43-kD protein (Fig. 1 A, lane 1). Only a single protein band bound ⁶⁵Zn²⁺ (Fig. 1 A, lane 3). This protein had the same mobility as the 43-kD protein, as shown by immunoblotting with a mAb to the 43-kD protein (Fig. 1 A, lane 2). To verify that this protein was indeed the 43-kD protein and not another protein of similar size, the 43-kD protein was affinity purified from *Torpedo* membranes. SDS-gel electrophoresis revealed a single band by Coomassie staining (Fig. 1 B, lane 1) that is recognized by anti-43-kD protein mAb (Fig. 1 B, lane 2). Affinity-purified 43-kD protein retains the ability to bind

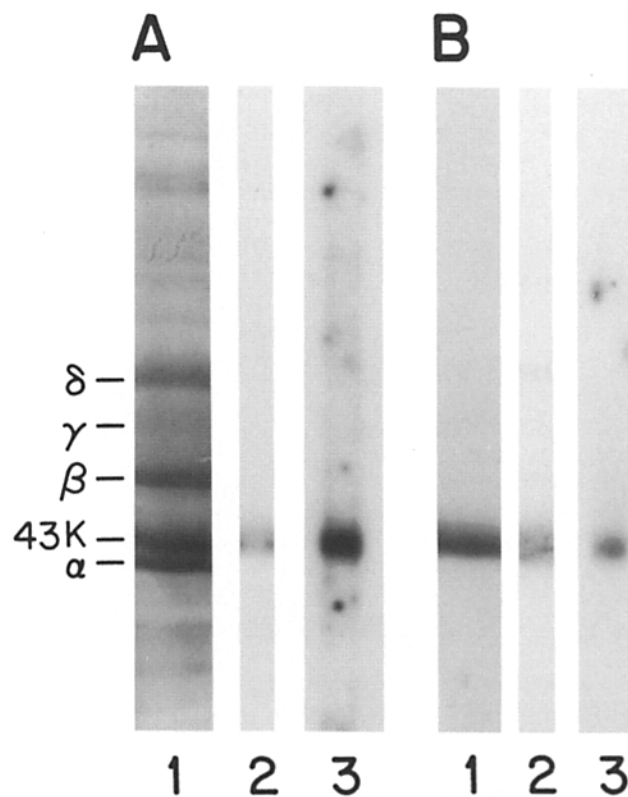


Figure 1. ⁶⁵Zn²⁺ binding to *Torpedo* 43-kD protein. (A) Postsynaptic membranes isolated from *Torpedo* electric organ. (B) Affinity-purified *Torpedo* 43-kD protein. In both cases, (lane 1) Coomassie-stained gel; (lane 2) Western blot with anti-43-kD mAb (10 nM mAb 1234A); (lane 3) autoradiogram of blot incubated with ⁶⁵Zn²⁺. (A) Lane 3 was exposed for 44 h. (B) Lane 3 was exposed for 75 h. The positions of the 43-kD protein and the AChR subunits (Greek letters) are indicated on the left.

$^{65}\text{Zn}^{2+}$ (Fig. 1 B, lane 3). Purified AChR shows no $^{65}\text{Zn}^{2+}$ -binding activity (data not shown). Thus, Zn^{2+} binding to the 43-kD protein is specific.

Cysteine-rich Carboxy-Terminal Region of the Mouse 43-kD Protein Binds Zn^{2+}

To test the hypothesis that the cysteine-rich region of 43-kD protein contains the zinc-binding activity, we generated an in-frame gene fusion between the GST cDNA and cDNA encoding the carboxy-terminal region of mouse 43-kD protein. This region of the 43-kD protein begins at amino acid 349 (14 amino acids amino-terminal of the presumed zinc finger domain), and includes the entire carboxy terminus, comprising ~15% of the protein (Fig. 2). Lysates from bacterial cells transformed with this cDNA construct, called GST-43 kD, contained large amounts of a protein of the appropriate size (~34,500 D), while the cells expressing GST protein alone contain a smaller protein (27,500 D) (Fig. 3 A, lanes 1 and 2). Western blots of these cell lysates reveal that GST-43 kD, but not GST alone, is recognized by anti-43-kD mAb 1579A (Fig. 3 B, lanes 1 and 2). Neither protein is recognized by control mouse IgG (data not shown). Incubation of blots with $^{65}\text{Zn}^{2+}$ demonstrates that GST-43 kD binds Zn^{2+} (Fig. 3 C, lane 2). Cell lysates from bacterial cells expressing GST protein show no $^{65}\text{Zn}^{2+}$ binding (Fig. 3 C, lane 1). These results indicate that not only is the mouse 43-kD protein able to bind Zn^{2+} like *Torpedo* 43-kD protein, but more importantly, that the cysteine-rich, carboxy-terminal region alone is sufficient for Zn^{2+} binding.

We examined the ability of several divalent cations to inhibit binding of $^{65}\text{Zn}^{2+}$ to GST-43 kD by dot blot analysis. Mg^{2+} , Ca^{2+} , and Mn^{2+} (all at 1 mM) have no effect on $^{65}\text{Zn}^{2+}$ binding (Fig. 4). At the same concentrations, Co^{2+} partially reduces $^{65}\text{Zn}^{2+}$ binding, while Cu^{2+} , Cd^{2+} , and Zn^{2+} completely eliminate binding. These results are consistent with observations reported for other zinc-binding proteins (56, 59).

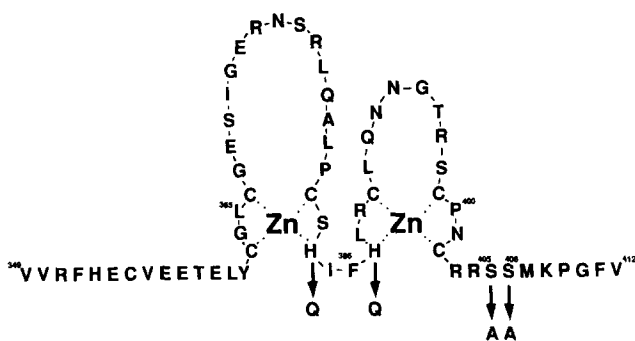


Figure 2. Hypothetical zinc finger structure for the carboxy-terminal domain of the 43-kD protein. Shown is the mouse 43-kD protein sequence beginning with valine 349 and ending with valine 412 (COOH-terminal residue of the 43-kD protein). Mutant 43 kD_{H→Q} changes amino acids 384 and 387 from histidine to glutamine. Mutant 43 kD_{S→A} changes amino acids 405 and 406 from serine to alanine. The sequence represents the entire coding region of the mouse 43-kD cDNA that was fused to GST cDNA to produce the GST-43-kD fusion protein.

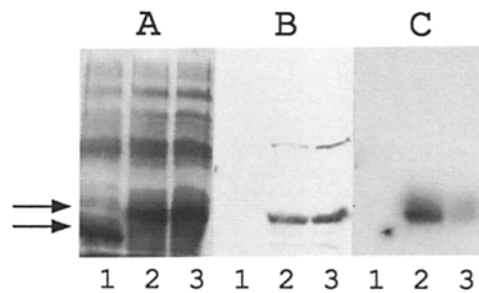


Figure 3. $^{65}\text{Zn}^{2+}$ binding to the carboxy-terminal region of the normal and mutant mouse 43-kD protein. (A) Coomassie-stained gel. (B) Western blot with anti-43-kD mAb (25 nM mAb 1579A). (C) Autoradiogram of blot incubated with $^{65}\text{Zn}^{2+}$ (20-h exposure). In all panels (lane 1) GST protein (control); (lane 2) GST-43-kD protein; (lane 3) GST-43 kD_{H→Q}. Lower arrow on the left indicates the position of GST protein alone; the upper arrow indicates the position of GST-43-kD protein or GST-43-kD_{H→Q} protein.

Mutation of the 43-kD Protein in the Cysteine-rich Region Attenuates Zn^{2+} Binding

Further evidence that the proposed region of the 43-kD protein is a zinc-binding domain comes from mutagenesis studies. Mutant 43-kD_{H→Q} changes amino acids at positions 384 and 387 from histidine to glutamine (see Fig. 2). These two histidines should be important for formation of the proposed zinc finger structure (Fig. 2). As with GST-43 kD, the carboxy-terminal region of 43-kD_{H→Q} cDNA was fused, in frame, to the GST cDNA, and expressed in bacterial cells. Results presented in Fig. 3 (lane 3, A and B) demonstrate that GST-43-kD_{H→Q} protein is expressed in amounts equal to GST-43 kD, and is also recognized by mAb 1579A. $^{65}\text{Zn}^{2+}$, however, is significantly reduced (Fig. 3 C, lane 3). Histidines 384 and 387 are therefore critical for the Zn^{2+} -binding activity of the 43-kD protein.

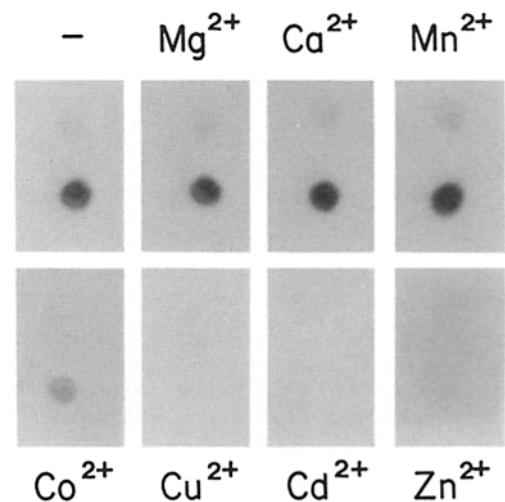


Figure 4. Competition of $^{65}\text{Zn}^{2+}$ binding to the 43-kD fusion protein by divalent cations. Bacterial cell lysates (3 μl s) from cells expressing GST protein alone (upper spot) or GST-43K fusion protein (lower spot) were applied to nitrocellulose membranes, dried, and treated as described. The competition experiment was carried out in the presence of 1 mM divalent cations, as indicated above or below blot. Upper left blot was incubated in the absence of competing divalent cations. Autoradiogram was exposed 3 hours.

Mutation in the Zinc Finger Region Attenuates Clustering of the 43-kD Protein

We have used the oocyte expression system to examine the ability of 43 kD_{H→Q} to form membrane-associated clusters. First, to determine if the mutated protein is expressed in oocytes at levels comparable to the nonmutated 43-kD protein (hereafter called 43-kD protein), we performed Western blotting on membranes isolated from oocytes expressing 43-kD protein or 43 kD_{H→Q}. To preclude any effects of mutations on antibody binding, we used mAb 1234A which recognizes an epitope in the amino terminal end of the 43-kD protein (C. Carr and J.B. Cohen, personal communication). Membranes from oocytes injected with 43 kD or 43-kD_{H→Q} RNA contain a protein of *M_r* 43,000 recognized by mAb 1234A that is absent from uninjected control oocytes (Fig. 5). When equal concentrations of RNA were injected into oocytes, approximately equivalent protein expression was observed for 43-kD protein and 43 kD_{H→Q} (Fig. 5, lanes 1 and 2). Oocytes injected with a five-fold higher concentration of 43-kD_{H→Q} RNA showed a higher level of protein expression (Fig. 5, lane 3).

The ability of 43 kD_{H→Q} to form clusters was examined by immunofluorescence microscopy. Oocytes injected with RNA encoding 43 kD_{H→Q} express clusters of heterogeneous sizes: most are very small (essentially indistinguishable from a diffuse distribution) while a small number are similar in size to those on oocytes injected with the 43-kD RNA (Fig. 6, compare A to B). The average size of 43-kD_{H→Q} clusters appears much smaller than 43-kD clusters. Oocytes expressing higher levels of 43-kD_{H→Q} protein also have smaller clusters, while oocytes injected with 1/2 the usual concentration of nonmutated 43-kD RNA exhibit normal size clusters (data not shown).

Mutation in the Zinc Finger Region of 43-kD Protein Does Not Affect Interaction with AChR

Coexpression studies were performed to determine if the zinc-finger mutant 43 kD_{H→Q} is able to associate with the AChR. Oocytes were injected with RNA encoding AChR alone, AChR and 43-kD protein, or AChR and 43-kD_{H→Q}

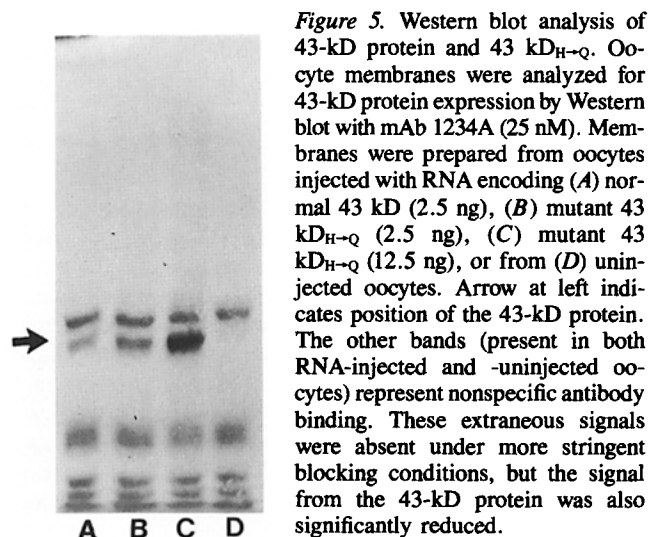


Figure 5. Western blot analysis of 43-kD protein and 43 kD_{H→Q}. Oocyte membranes were analyzed for 43-kD protein expression by Western blot with mAb 1234A (25 nM). Membranes were prepared from oocytes injected with RNA encoding (A) normal 43 kD (2.5 ng), (B) mutant 43 kD_{H→Q} (2.5 ng), (C) mutant 43 kD_{H→Q} (12.5 ng), or from (D) uninjected oocytes. Arrow at left indicates position of the 43-kD protein. The other bands (present in both RNA-injected and -uninjected oocytes) represent nonspecific antibody binding. These extraneous signals were absent under more stringent blocking conditions, but the signal from the 43-kD protein was also significantly reduced.

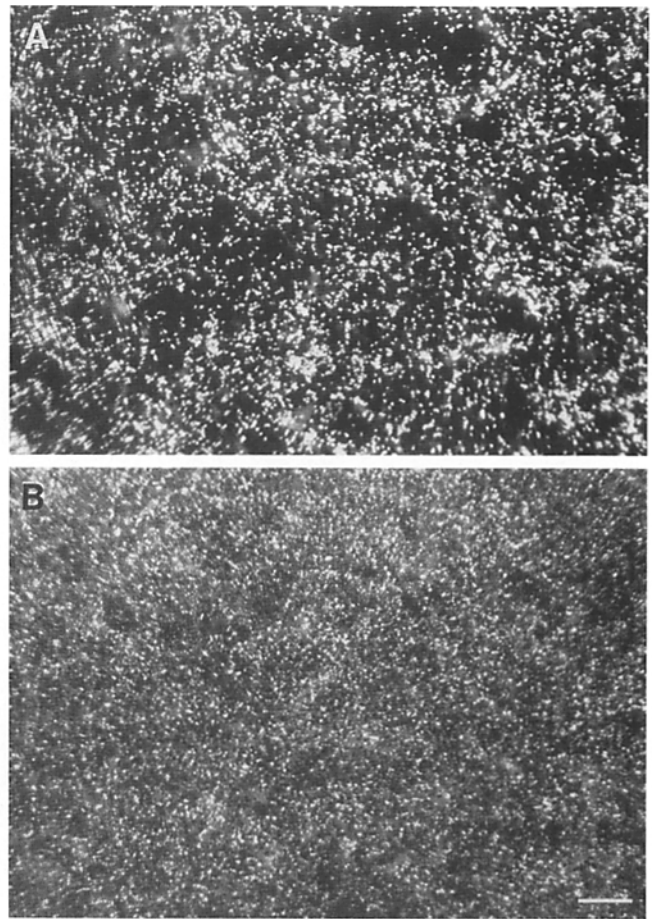


Figure 6. Immunofluorescence analysis of 43-kD protein and 43-kD_{H→Q} distribution. Photographs show an en face membrane view of a region of an oocyte expressing either normal 43-kD protein (A) or mutant 43-kD_{H→Q} (B). Oocytes were labeled for the 43-kD protein with mAb 1234A. The data above is representative of four independent experiments comprising 22 oocytes (A) and 33 oocytes (B), all of which had distributions similar to those shown. Bar, 10 μ m.

protein. 5 d later, the distribution of AChR and the 43-kD protein was examined by immunofluorescence microscopy. Clusters of AChR and 43-kD_{H→Q} protein (Fig. 7, E and F) are smaller than those formed by AChR and normal 43-kD protein (Fig. 7, C and D).

In spite of the smaller cluster sizes, AChR and 43 kD_{H→Q} still show co-localization (compare Fig. 7, E and F). Furthermore, there is a distinct difference between the receptor distribution in oocytes expressing AChR and 43 kD_{H→Q}, and the distribution seen in oocytes expressing only the AChR (compare Fig. 7, A and E). These observations suggest that 43 kD_{H→Q} alters the distribution of AChR and thus must retain the ability to associate with the receptor.

We have quantitatively compared the sizes of AChR clusters induced by 43 kD and 43 kD_{H→Q}. Oocytes were viewed by confocal microscopy and images were subjected to computer analysis. The parameters for shape determination were set to minimize inclusion of irregularly shaped aggregates that were composed of multiple clusters (Fig. 7 C, arrow). Although it was impossible to eliminate all of these ag-

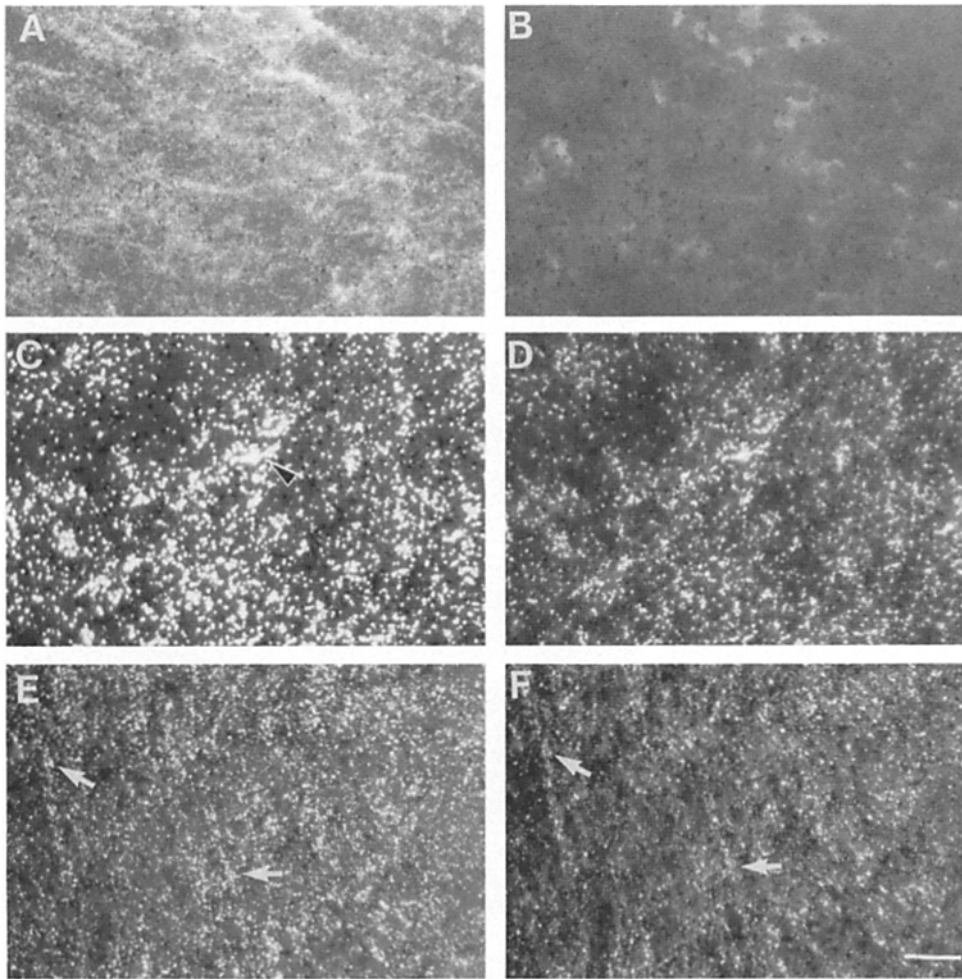


Figure 7. Coexpression of AChR and 43-kD_{H→Q} protein. Oocytes expressing AChR alone (A and B), AChR and normal 43-kD (C and D), or AChR and 43 kD_{H→Q} (E and F) were labeled for immunofluorescence for both the AChR (A, C, and E) and 43-kD protein (B, D, and F). A and B, C and D, E and F represent paired views of the same region of an oocyte under rhodamine (AChR) and fluorescein (43 kD) optics, respectively. In 10 independent experiments, all of 67 oocytes expressing AChR alone showed diffuse receptor distribution. In eight experiments, 68 of 69 oocytes expressing AChR and normal 43-kD protein showed clustered AChR distribution. In five experiments, 36 of 38 oocytes expressing AChR and 43 kD_{H→Q} showed the AChR distribution seen above. The three oocytes lacking clusters all showed a diffuse distribution of the AChR. Arrow in C represents a cluster not included in the size analysis in Fig. 8. Arrows in E and F indicate codistribution of AChR and 43 kD_{H→Q}. Bar, 10 μm.

gregates, only a few remained and the number remaining was similar in the two groups of oocytes. Unlike clusters of AChR and normal 43 kD, most of the AChRs clustered by 43 kD_{H→Q} could not be analyzed because they were too small. Therefore, these results represent a conservative difference between the sizes of the clusters of these two groups. Nevertheless, the size frequency histograms show a distinct difference between AChR clusters induced by normal and mutant 43-kD protein (Fig. 8). The size of clusters formed by normal 43-kD protein seems to follow a Gaussian distribution with a mean of 121, although there was some asymmetry due to aggregates of clusters. The size of clusters induced by 43 kD_{H→Q} had a skewed distribution with the majority of clusters falling into the bin from 0 to 20. Thus, the visual impression that clusters made by 43 kD_{H→Q} are smaller is confirmed by this quantitative assessment.

Phosphorylation of 43-kD Protein at Ser-405 or Ser-406 Is Not Essential for Clustering

We have mutated the amino acids that comprise the putative phosphorylation site which lies just adjacent to the zinc finger region (see Fig. 2). The mutated 43-kD cDNA, called 43 kD_{S→A}, changes two serines to alanines at amino acid positions 405 and 406, and thereby prevents phosphorylation at these sites. Western blotting of membranes demonstrates

that expression of 43-kD_{S→A} protein is comparable to that of 43-kD protein (Fig. 9). The distribution of 43 kD_{S→A} is indistinguishable from that of 43-kD protein, as revealed by immunofluorescence microscopy (data not shown). Furthermore, co-expression of 43 kD_{S→A} with AChR results in the formation of clusters (Fig. 10, panel B), identical to those seen with 43-kD protein (Fig. 10, A). These data demonstrated that phosphorylation of the 43-kD protein at ser-405 or ser-406 is not essential for clustering of the 43-kD protein itself, or for clustering the AChR, in *Xenopus* oocytes.

Discussion

The postsynaptic 43-kD protein is intimately associated with the nicotinic AChR and may play a key role in clustering receptors at the neuromuscular junction (16). Current models of AChR clustering predict that the neuronal signal, carried by such molecules as agrin, initiates a series of events in muscle cells that culminate in clustering (22). One possibility is that the 43-kD protein is directly affected by the agrin-stimulated pathway and participates in the earliest events in cluster formation. Developmental studies of the rat neuromuscular junction show that 43-kD protein is present at the earliest stages of AChR cluster formation, supporting this theory (39). Alternatively, initial clustering may occur

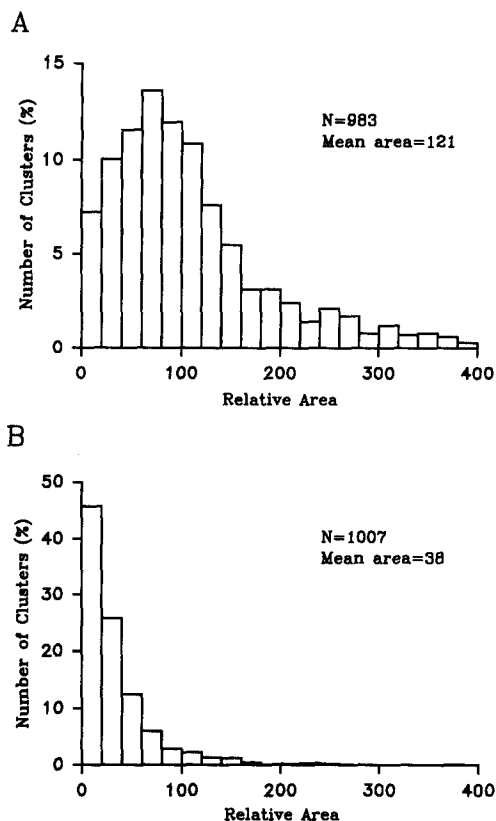


Figure 8. Size frequency histogram of AChR clusters. Size distribution of AChR clusters from oocytes coexpressing AChR and either normal 43-kD protein (A) or mutant 43 kD_{H→Q} (B). In both cases, seven fields from two different oocytes were analyzed. Note different vertical scales in A and B.

by a different mechanism, such that 43-kD protein becomes associated with AChR clusters at a later stage, possibly serving a stabilization function. Consistent with this idea, the 43-kD protein is absent from initial AChR clusters formed before innervation in developing *Torpedo* electrocytes (27, 31). In either case, the 43-kD protein is likely to have an important role in clustering and/or stabilization of AChR clusters.

In native postsynaptic membranes, 43-kD proteins reside in close proximity to each other (45), and are likely to interact. Thus, identification of the sites of interaction in the 43-kD protein will be important, no matter what the role of this protein in AChR clustering. The ability of the 43-kD protein to cluster in the absence of AChR (19, 42) may be

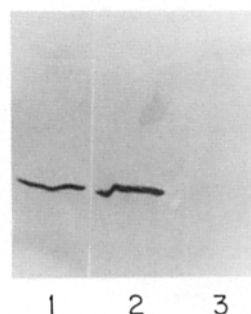


Figure 9. Western blot analysis of 43-kD protein and 43 kD_{S→A}. Oocyte membranes were analyzed for 43-kD protein expression by Western blot with mAb 1234A (25 nM). Membranes were prepared from oocytes injected with RNA encoding normal 43-kD protein (lane 1), 43 kD_{S→A} (lane 2), or from uninjected cells (lane 3).

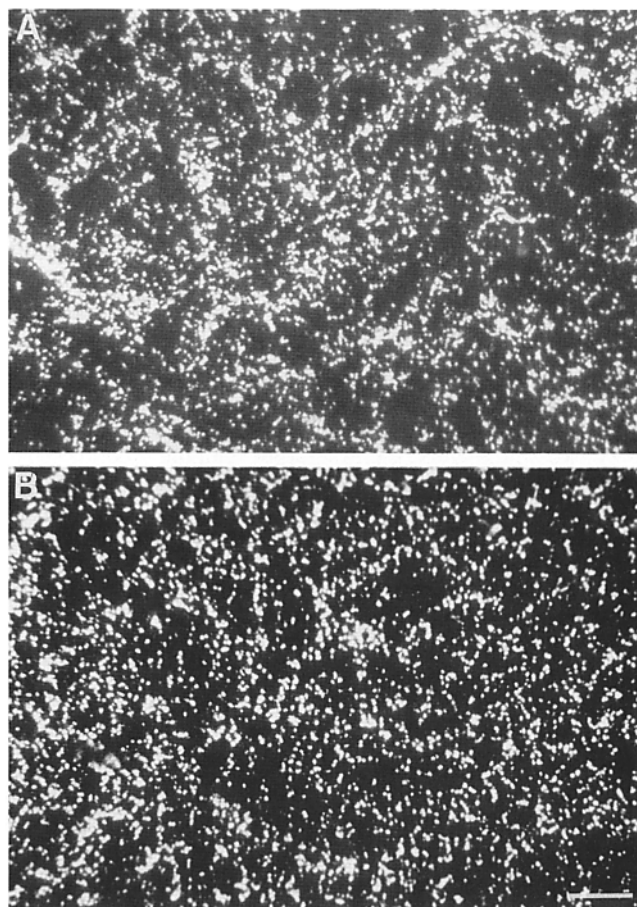


Figure 10. Coexpression of AChR and 43-kD_{S→A} protein. Oocytes expressing AChR and either normal 43-kD protein (A) or 43 kD_{S→A} (B) were labeled for immunofluorescence for AChR. In three independent experiments, 16 of 17 oocytes expressing AChR and 43 kD_{S→A} revealed clustered AChR distribution similar to that seen on oocytes expressing AChR and normal 43-kD protein. One oocyte exhibited diffuse AChR distribution. Bar, 10 μ m.

a consequence of these interactions. This phenomenon has only been observed with recombinant 43-kD protein expressed in nonmuscle cells, however. Furthermore, there are examples of muscle cells which express 43-kD protein that is not organized into clusters. Genetic variants of C2 cells defective in glycosaminoglycan biosynthesis or lacking AChR, as well as BC3H1 myocytes, express 43-kD protein but do not cluster it (21, 29, 30). Thus, there may be important differences in the regulation of 43-kD protein clustering in muscle and nonmuscle cells.

We have demonstrated that a zinc-binding domain is important for interactions leading to 43-kD clustering. This domain resides near the carboxy terminus of the mouse 43-kD protein, and presumably also in the *Torpedo* protein, in a region predicted to contain two zinc finger structures (16). Since no other major protein in membrane preparations binds Zn²⁺, the association with 43-kD protein appears to be specific. Mutagenesis of two highly conserved histidines, thought to be intimately involved in the formation of zinc finger structures of other proteins (14), markedly reduces zinc binding, supporting our proposal that the carboxy-

terminal domain of the 43-kD protein forms a zinc fingerlike structure.

Several tertiary structures, in addition to the classic zinc finger structure, have been proposed and/or proven for zinc-binding proteins (3, 58). These include a "Zn(II)₂Cys₆ binuclear cluster" found in the yeast transcription factor, GAL4 (40, 47), and the glucocorticoid receptor "zinc twist" (53, 58). In some cases, zinc-binding domains have been described as ring fingers and B boxes, depending upon the number and position of the histidines, cysteines, and supporting amino acids (48). In the absence of structural data, we do not know the structure formed by the 43-kD protein. However, the zinc-binding motif in the 43-kD protein is most similar to one found in a group of transcription factors, characterized by the PML protein (20, 26). These proteins have two zinc fingers, the first larger than the second, separated by only two residues, which are also characteristic of the two proposed zinc fingers in the 43-kD protein. Furthermore, several amino acid residues are conserved in 14 members of the PML family and in the 43-kD protein zinc finger motif, including a hydrophobic residue at position 365, an aromatic residue at position 386, and an invariant proline at position 400 (numbers refer to residues in the 43-kD protein; see Fig. 2).

In addition to the 43-kD protein, we know of only two other membrane-associated cytoskeletal protein that contain zinc fingers. Two adhesion plaque proteins, zyxin, and an associated 23-kD protein, contain LIM domains (51). LIM domains have been identified in several proteins involved in gene regulation and contain a motif very similar to zinc binding domains. Together, these results and ours suggest that zinc fingers may be a more common structural motif for protein-protein interactions at the plasma membrane than previously has been recognized.

In addition to the potential zinc fingers, the 43-kD protein sequence predicts a leucine zipper, another structural motif common to DNA-binding factors. Although the 43-kD protein is not known to have DNA-binding activity, it is interesting to note that it occasionally appears in the nucleus when expressed in COS cells (5), or when mutations reduce its interaction with the membrane (43). Whether this phenomenon is artifactual or indicative of a previously unrecognized function for the 43-kD protein is unknown.

We have not identified the metal ion associated in vivo with the 43-kD protein. As with other zinc finger proteins, the binding of radioisotopic Zn²⁺ to the 43-kD protein is inhibited by cadmium, copper, and cobalt, but not by calcium, magnesium, and manganese (56). Thus, it remains possible that a metal ion other than zinc is associated with the 43-kD protein. Purification of *Torpedo* postsynaptic membranes in the presence of ZnCl₂ increases the retention of proteinaceous material on the cytoplasmic side of the membrane, which in part must be comprised of 43-kD protein (32); R. Sealock, personal communication). Furthermore, purification of membranes under conditions that would be expected to release Zn²⁺ from a cysteine-rich-binding site (inclusion of iodoacetamide and chelating agents in buffers) promotes loss of the 43-kD protein, such that the ratio with AChR is less than the expected equimolar amount (28). Thus, coordination of Zn²⁺ or some other metal ion by the 43-kD protein may be important in assembling and maintaining the postsynaptic cytoskeletal apparatus.

Mutation of two potentially critical histidines in the 43-kD protein zinc finger motif substantially reduces zinc binding but does not completely eliminate it. We suggest two possible explanations for the partial retention of this activity. First, the zinc finger structure that we propose may be incorrect. In addition to possibilities discussed above, residues upstream of the proposed structure may be involved (histidine 353 and cysteine 355, for example). The mutation of histidines 384 and/or 387 might then alter Zn²⁺ binding through a more general, indirect effect on the structure of the zinc finger domain. The second possibility is that the mutated protein adopts a structure that binds Zn²⁺ with lower affinity than the native protein. For example, elimination of histidines involved in metal ion coordination might allow other residues, such as cysteines or even glutamines (recently shown to coordinate Zn²⁺; [57]) to form an alternative, less stable structure. In either case, one or both of the histidines replaced in 43 kD_{H→Q} are likely to be critical residues in the zinc-binding motif.

Mutant 43 kD_{H→Q}, when expressed in oocytes, forms smaller membrane-associated clusters than the normal 43-kD protein, with a more diffuse distribution than the wild-type protein. Wild-type size clusters are seen infrequently. Thus, like the binding of Zn²⁺, mutation of two histidine residues reduces but does not eliminate cluster formation of the 43-kD protein. This attenuation of clustering is not a consequence of reduced expression of the mutant protein. Western blot analysis of oocyte membrane preparations revealed similar levels of expression of the wild-type and mutant 43-kD protein. These preparations presumably contained membranes derived from intracellular organelles as well as surface membranes. Technical limitations prevented a quantitative assessment of the content of 43-kD protein specific to the plasma membrane. Qualitative estimates judged by immunofluorescence intensity indicate, however, that the mutations do not create trafficking defects that greatly reduce the amount of 43-kD protein that reaches the surface membrane. We conclude, therefore, that the reduced clustering ability of 43 kD_{H→Q} is not a result of reduced protein expression, but must be a consequence of dysfunction of the protein itself.

The mechanism of AChR clustering in muscle cells is undoubtedly more complex than in oocytes, since the postsynaptic membrane contains numerous cytoskeletal proteins not present in the oocyte clusters. On the other hand, the oocyte system allows us to examine the interaction of specific components of the neuromuscular junction, in the absence of other junction-specific proteins. Thus, the simplicity of the oocyte system has the potential advantage of revealing steps in the clustering pathway that cannot be detected in developing muscle. Recently, a multistep model for clustering of AChR by the 43-kD protein has been proposed (23). According to this model, AChR first spontaneously aggregate into small clusters (<0.1-μm diam), or "crowds," and subsequently to intermediate sized (0.5–2 μm) clusters, which eventually coalesce into mature clusters. Clusters induced by wild-type 43-kD protein resemble the intermediate clusters, while those formed by 43 kD_{H→Q} are more like crowds. One explanation for our results is that the 43-kD zinc fingers, and the interactions that they mediate, are critical for the transition from "crowds" to intermediate clusters.

The 43-kD zinc finger mutant appears to retain its ability

to interact with the AChR since, in double label immunofluorescence experiments, the distribution of 43 kD_{H-Q} and AChR were indistinguishable. Thus, the site of interaction between 43 kD and AChR appears to be outside the zinc finger domain, a conclusion consistent with deletion studies (43).

The carboxy-terminal domain of the 43-kD protein contains a second conserved feature that may be important for regulating its activity. A consensus motif for both cyclic AMP-dependent and PKC-mediated phosphorylation (ser 405/406) is located immediately downstream of the zinc finger domain. The 43-kD protein in *Torpedo* membranes is phosphorylated on serine by cAMP-dependent kinases, but the site of phosphorylation has not been defined (24). The full activity of the mutant lacking serines 405 and 406 indicates that phosphorylation at this site is not necessary for clustering, at least in the oocyte expression system. Since we have not determined if the 43-kD protein expressed in oocytes is phosphorylated under basal conditions, it remains possible that phosphorylation at this or some other site may modulate clustering. The close proximity of the phosphorylation site to the zinc fingers suggests a regulatory role for kinases in the clustering of 43-kD protein. Agents that stimulate PKC activity inhibit agrin-induced and spontaneous cluster formation in chick myotubes (49, 60). In neither case, however, is the identity of the protein substrate for PKC known. Experiments to examine the involvement of the 43-kD protein in this process are in progress.

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