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Expression of RNA transcripts for the postsynaptic 43 kDa protein in innervated and denervated rat skeletal muscle

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A cDNA clone encoding the mouse muscle postsynaptic 43 kDa protein was isolated and sequenced. The amino acid sequence of this protein, which is closely associated with nicotinic acetylcholine receptors at *Torpedo* electrocyte and vertebrate skeletal muscle synapses, is very similar in different species. A cysteine-rich region homologous to part of the regulatory domain of protein kinase C may be important in interactions of this protein with the lipid bilayer. RNA transcripts for the 43 kDa protein increase only 2–3-fold after denervation of rat skeletal muscle, in sharp contrast to the α-subunit of the muscle nicotinic receptor which increases more than 30-fold. Thus, the expression of these two proteins is regulated by different mechanisms.

Postsynaptic membrane; Protein, 43 kDa; cDNA; Denervation; (Rat muscle)

1. INTRODUCTION

A peripheral membrane protein of 43 kDa is closely associated with the nicotinic acetylcholine receptor (AChR) in the postsynaptic membrane of *Torpedo* electrocytes [1–4], at vertebrate neuromuscular junctions [5] and in receptor clusters on cultured muscle cells [6–8]. The 43 kDa protein is not required for ligand-gated ion flux through the receptor channel [9]. However, its removal from both *Torpedo* [10,11] and rat postsynaptic membranes [8] by alkaline extraction is accompanied by increased mobility and rearrangement of the receptors. Thus, although its function is not known, the 43 kDa protein is thought to play some role in anchoring or stabilizing AChR at synaptic sites (review [13]). The 43 kDa protein may link the receptor to the underlying postsynaptic cytoskeleton, possibly by direct association with actin [14] or spectrin [15].

In both *Torpedo* electric organ [16] and differentiated mouse skeletal muscle cells [17], the AChR and the 43 kDa protein are present in approximately equimolar concentrations. Furthermore, muscle cell lines that are genetically deficient in AChR also express reduced amounts of the 43 kDa protein [18]. It is possible that the coordinate expression of these two proteins is regulated at the level of transcription of the corresponding genes. As an initial approach to this question, I have isolated a cDNA clone encoding the mouse 43 kDa protein and compared the expression of this gene with that encoding the AChR α-subunit. Denervation of rat skeletal muscle causes a dramatic increase in mRNA for the receptor α-subunit, but only a small increase in mRNA for 43 kDa protein. These results indicate that expression of 43 kDa protein and AChR α-subunit is regulated by different mechanisms.

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2. MATERIALS AND METHODS

2.1. *Isolation and sequencing of a cDNA clone for mouse muscle 43 kDa protein*

cDNA encoding the *Torpedo* 43 kDa protein (clone T43K.1, kindly provided by Dr John Merlie [19]) was used to screen a λgt10 library constructed from poly(A^+) RNA isolated from

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BC3H-1 mouse muscle cells [20]. Hybridization with the random-primer labeled cDNA probe was performed in 50 mM Tris (pH 7.4), 1 M NaCl, 1% SDS, 100 μg/ml salmon sperm DNA, 1 × Denhardt's solution at 65°C for approx. 15 h. Filters were then washed with 2 × SSPE (SSPE: 180 mM NaCl, 1 mM EDTA, 10 mM sodium phosphate; pH 7.4), first at room temperature and then at 65°C, and exposed to X-ray film overnight. From screening of approx. 10^6 phage, 20 positive plaques were selected. The 1.6 kb *Eco*RI insert of clone M43K.1 was subcloned into M13mp19. Both strands of the insert were sequenced by the dideoxynucleotide method [21] using subclones generated by the progressive deletion method [22] and a Sequenase kit (US Biochem. Corp., Cleveland, OH).

2.2. *Northern blot analysis*

Isolation of RNA from normal and denervated rat muscle and Northern blot analysis were performed as described by Goldman et al. [23]. Restriction fragments encoding the mouse muscle AChR α-subunit [24] and human actin [25] were kindly provided by Sylvia Evans (Salk Institute) and William Rigby (Dartmouth Medical School), respectively.

3. RESULTS AND DISCUSSION

Previous studies utilizing immunoaffinity purification and Western blotting [17] and metabolic labeling [27] have identified a protein in BC3H-1 mouse muscle cells with characteristics similar to those of the Torpedo 43 kDa protein. To determine the primary structure of this protein, a cDNA clone, M43K.1, was isolated from a BC3H-1 λgt10cDNA library by hybridization with cDNA encoding the *Torpedo* 43 kDa protein. The nucleotide sequence of M43K.1 contains a continuous open reading frame of 1236 nucleotides beginning with ATG at position 160 and ending with a TGA stop codon. The amino acid sequence of the protein encoded by this cDNA is very similar to the *Torpedo* 43 kDa protein.

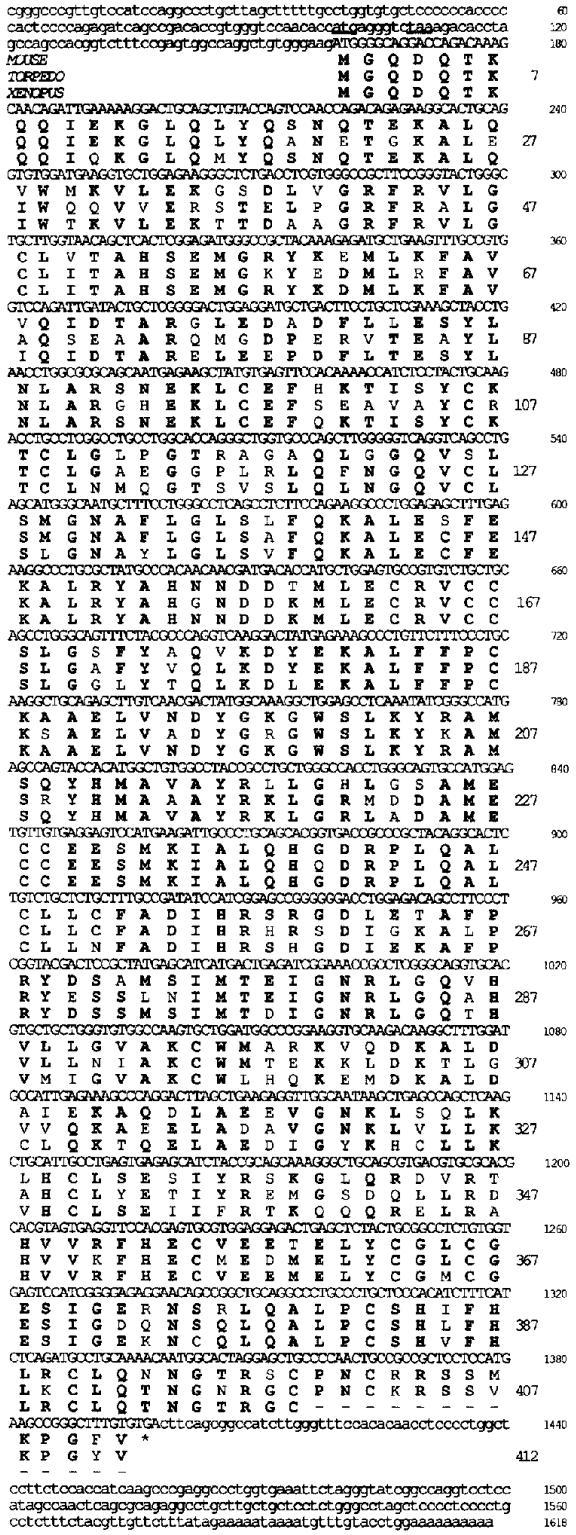

Fig.1. Comparison of the primary structure of 43 kDa protein from mouse muscle, *Torpedo* electric organ, and *Xenopus* muscle. The nucleotide sequence for mouse BC3H-1 clone M43K.1 is shown (open reading frame encoding the 43 kDa protein is shown in capital letters). Potential start and stop codons upstream of the initiator methionine codon for 43 kDa protein are underlined. The number of the last nucleotide or amino acid in each row is indicated on the right. Amino acid identities at each position are shown in bold-face characters. Nucleotide 936 is either G or C. When compared to the mouse muscle clone described by Frail et al. [26] M43K.1 extends 53 bases further in the 5'-direction and encodes a Val at position 345 rather than Leu. Amino acid sequence for *Torpedo* 43 kDa protein is a composite taken from [19,28,29]. Residue 394 is either Asn [19] or Asp [28]. Partial amino acid sequence for *Xenopus* 43 kDa protein is from [30].

Comparison of the primary structure of *Torpedo* 43 kDa protein [19,28,29] with the amino acid sequence deduced from M43K.1 and a partial cDNA encoding the *Xenopus* 43 kDa protein [30] demonstrates that the 43 kDa protein is highly conserved across species (fig.1). 70% of the *Torpedo* residues, and 80% of those of *Xenopus* are identical in the mouse protein. Two highly conserved regions, one encompassing the N-terminus and the other near the C-terminus, may be especially important for interaction of the 43 kDa protein with other components of the postsynaptic membrane.

The 43 kDa protein is tightly associated with the postsynaptic membrane and can be removed only by rather harsh treatments [1,9]. At least part of its association with the membrane involves direct interaction with the lipid bilayer without the participation of other proteins [31]. The first ten amino acids at the N-terminus, which are identical in 43 kDa protein from all three species, include a sequence appropriate for myristylation. The 43 kDa protein from *Torpedo* electric organ [29] and that from BC3H-1 muscle cells [27] have been shown to be myristylated, a feature often involved in the association of proteins with membranes (review [32]). However, not all myristylated proteins are membrane bound and myristylation in some cases is not sufficient for association with the lipid bilayer [32]. In addition, structural studies of the *N*-myristylated picornavirus capsid protein VP4 have shown that the fatty acid resides in a hydrophobic pocket formed by amino acid side chains [33]. Since alkaline extraction readily removes the 43 kDa protein from the membrane without modification of the myristylated N-terminus [28], Carr et al. [29] have suggested that the fatty acid does not anchor 43 kDa protein to the membrane by imbedding in the lipid bilayer, but may instead be important in interactions between the 43 kDa protein and the AChR. Thus, the high degree of conservation of the N-terminal end of the 43 kDa protein may reflect not only the structure required for recognition by fatty acyl transferase but also the properties needed for interaction with the receptor or other postsynaptic proteins.

The second feature of potential importance in postsynaptic membrane interactions is a pattern of 5 cysteine residues near the carboxy-terminus, beginning with Cys-363 and ending with Cys-399. This pattern, which also appears as a tandem repeat in the N-terminal regulatory domain of all members of the protein kinase C family [35–37] and in phospholipase A_2 [34], has been proposed as a structural element involved in interaction of proteins with phospholipid [34]. Homology with the protein kinase C gene in this region is not limited to the cysteines, especially for the sequence spanned by Cys-382 and Cys-390 (the third and fourth cysteines in this pattern) (fig.2). A hydrophobic residue at position 385, a His at residue 387, and a basic amino acid at position 389 are features common to protein kinase C. In phospholipase A_2, this region is part of the active site [34]. However, except for the cysteine pattern, 43 kDa protein and phospholipase A_2 exhibit no significant homology. Features common to the catalytic sites of protein kinases are also absent from 43 kDa protein [26]. The similarity between 43 kDa protein and the

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                  *  *                *        *        *
Mouse    43K      CGLCGESI-GERNSRLQALPCSHIFHLRCLQNNGTRSC    399
Torpedo  43K      CGLCGESI-GDQNSQLQALPCSHLFHLRCLQTNGNRGC    399
Xenopus  43K      CGMCGESI-GEKNCQLQALPCSHVFHLRCLQTNGTRGC    399

Rabbit PKC α      CSHCTDFIWGFGKQGFQCQVCCFVVHKRCHEF-VTFSC     85
Rabbit PKC β      CSHCTKFIWGFGKQGFQCQVCCFVVHKRCHEF-VTFSC     85
Rabbit PKC γ      CSHCTDFIWGFGKQGFQCQVCCFVVHKRCHEF-VTFSC     85
Rabbit PKC δ      CSHCTDFIWGIGIQGLQCQVCSFVVHRRCHEF-VTFEC     85
Bovine PKC α      CSHCTDFIWGFGKQGFQCQVCCFVVHKRCHEF-VTFSC     86
Human  PKC β      CSHCTDFIWGFGKQGFQCQVCCFVVHKRCHEF-VTFSC     86
Human  PKC γ      CSHCTDFIWGIGKQGLQCQVCSFVVHRRCHEF-VTFEC     85
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Fig.2. Similarities in primary structure of 43 kDa protein and protein kinase C. Amino acid identities and conservative substitutions are shown in bold-face type. Positions of cysteine residues are indicated by the asterisks at the top. Numbers on the right indicate the position in the sequence of the last cysteine in the sequence shown. Nomenclature and sequences for protein kinase C were taken from the following sources: rabbit [35], bovine [36], human [36].

regulatory region of protein kinase C, along with the observation that sulfhydryl alkyltion alters the extractability of the 43 kDa protein by alkaline pH [38], suggest a role for this structural element in membrane association.

Torpedo 43 kDa protein contains potential sites of phosphorylation, on both serine and tyrosine [28]. The serine phosphorylation consensus sequence RRSS (*Torpedo* residues 403–406) is conserved in mouse; the *Xenopus* cDNA clone is not full-length and ends prior to this region. Three tyrosine residues were identified in *Torpedo* 43 kDa protein as possible sites of phosphorylation [28]. One of these, Tyr-332, is not conserved in either mouse or *Xenopus* 43 kDa protein, while Tyr-105 and Tyr-196 are found in all three species. Tyr-196 is preceded by a basic residue (Lys-188) with two acidic residues (Glu-191 and Asp-195) in the intervening space, a structure particularly suited for recognition by tyrosine kinases [39]. Since phosphorylation of 43 kDa protein has not been demonstrated in any species, the functional significance of these sites is unknown.

Although extensive sequence identity is found throughout the 43 kDa protein from mouse, *Torpedo*, and *Xenopus*, two regions exhibit significantly lower conservation. At least one non-conservative amino acid substitution occurs at 11 positions between residues 69 and 118 (22%), while the sequence 299–347 contains 19 non-conservative changes (40%). All other 50-residue stretches contain 5 or fewer non-conservative substitutions. These variable regions may subdivide the protein into functional domains.

Denervation of skeletal muscle causes a substantial increase in synthesis of AChR [40], due in large part to increases in the abundance of mRNA for AChR subunits [23,41–43]. To compare the regulation of expression of receptor and 43 kDa protein, poly(A^+) RNA was purified from innervated rat leg muscle and from leg muscle 5 days after transection of the sciatic nerve. Northern blot analysis showed a large increase (at least 30-fold) in RNA for the AChR α-subunit (fig.3), in agreement with results from other laboratories [23,41–43]. In contrast, only a small increase (2–3-fold) in RNA for the 43 kDa protein was observed. No detectable change in the level of actin RNA was seen when the same blot was hybridized with a human β-actin DNA probe.

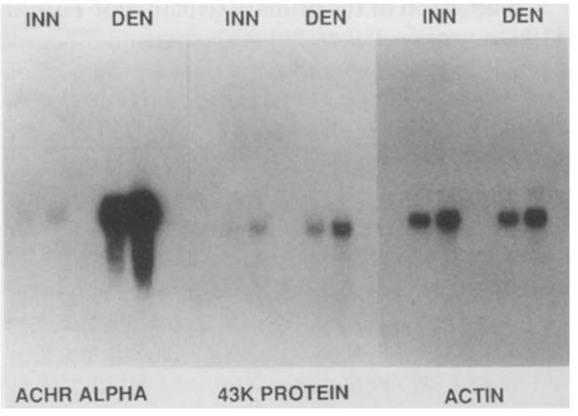

Fig.3. Northern blot analysis of 43 kDa protein RNA transcripts in innervated and 5 day-denervated rat leg muscle. Poly(A^+) RNA (2 and 4 μg) was hybridized with cDNA clone BMA 407 (mouse AChR α-subunit), with cDNA clone M43K.1 (mouse 43 kDa protein) (4 and 8 μg), or with a cDNA clone encoding human actin (4 and 8 μg).

These results are similar to those reported for denervated mouse skeletal muscle [44]. In contrast, Baldwin et al. [30] have reported a large increase in 43 kDa mRNA, comparable to that of the receptor α-subunit (30–35-fold) after denervation of *Xenopus* muscle. In neither rodent nor toad muscle has the change in 43 kDa protein levels after denervation been measured directly. However, it seems unlikely that the amount of 43 kDa protein rises in concert with the receptor after denervation in one species, but not in another. The species differences may reflect the relative importance of transcriptional and post-transcriptional regulatory mechanisms in governing the expression of this protein.

One possible mechanism for regulating the levels of 43 kDa protein involves assembly of the postsynaptic apparatus. Genetic variants of the C2 mouse muscle cell line deficient in the expression of AChR also exhibit reduced levels of 43 kDa protein [18]. RNA transcripts for 43 kDa protein are present in these variants in amounts comparable to those for the wild type. To account for this finding, we proposed that the 43 kDa protein may be stabilized by association with the receptor, a regulatory mechanism that may also be important in denervated muscle.

Regulation of the 43 kDa protein at the translational level is also possible. The 5′-untranslated region of the mouse 43 kDa protein cDNA predicts

an AUG initiator codon 60 nucleotides upstream from the correct start site, a relatively rare occurrence in eukaryotic mRNA. This AUG is preceded by an A three positions upstream, thus providing the proper context for a relatively strong initiator codon [45]. The upstream AUG is followed by codons for arginine, valine and a termination codon. Multiple short open reading frames have been found in the 5′-leader of yeast GCN4 mRNA where they either attenuate or stimulate expression, depending on the growth conditions [46]. Similar mechanisms may also regulate 43 kDa protein expression at the translational level in rodent muscle.

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