Identification of the Mouse Muscle 43,000-Dalton Acetylcholine Receptor-associated Protein (RAPsyn) by cDNA Cloning*

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The nicotinic acetylcholine receptor and a receptor-associated protein of 43 kDa are the major proteins present in postsynaptic membranes isolated from Torpedo electric organ. Immunochemical analyses indicated that a protein sharing antigenic determinants with the receptor-associated protein is also present at receptor clusters of muscle cell lines and postsynaptic membranes of vertebrate neuromuscular junctions. We now provide definitive proof that a homolog of the 43-kDa protein exists in mammals. Complementary DNA clones encoding the complete protein sequence have been isolated from the mouse muscle cell line, BC3H1. We heretofore refer to these proteins as nicotinic receptor-associated proteins at synapses or N-RAPsyns. The deduced sequence of mouse RAPsyn has 412 amino acids and a molecular mass of 46,392 daltons. The overall identity with Torpedo RAPsyn is 70%; some regions are extremely well conserved and are therefore postulated to be functionally important. Important domains, including the amino terminus and a cAMP-dependent protein kinase phosphorylation site, are conserved between species. Several structural features are consistent with the proposal that RAPsyn is a peripheral membrane protein that associates with membranes by virtue of covalently bound myristate. Although multiple mRNAs were previously identified in Torpedo electric organ, RNA blot analysis reveals a single polyadenylated RAPsyn mRNA of $\approx$2.0 kilobases in newborn and 4-week-old mouse muscle. Finally, genomic DNA blot analysis indicates that a single N-RAPsyn gene is present in the mouse genome.

The molecular mechanisms involved in the formation and maintenance of synapses are largely unknown. Postsynaptic specialization at the neuromuscular junction is characterized by both morphological and biochemical changes. These changes include a remodeling of the membrane surface such that junctional folds are developed, as well as the preferential localization of nicotinic acetylcholine receptor (AchR), acetylcholinesterase, and a number of other less well characterized proteins (see Merlie and Sanes, 1986 for a review). Because the electric organ of the Torpedo electric fish is a rich source of AchR and postsynaptic membranes, it has facilitated the identification and biochemical characterization of synapse-specific proteins.

The AchR and a nonreceptor protein of 43 kDa are the most abundant proteins present in highly purified preparations of electric organ postsynaptic membranes (Sobel et al., 1977). This nonreceptor protein is of particular interest because it is a peripheral protein on the cytoplasmic surface (St. John et al., 1982; Bridgeman et al., 1987) whose ultrastructural localization has been shown to be coextensive with the AchR in the electric organ (Sealock et al., 1984). To date, this protein has been referred to as the 43-kDa protein based on its apparent molecular weight on sodium dodecyl sulfate-polyacrylamide gels. However, this reference, the 43-kDa protein, is ambiguous and creates confusion because two abundant proteins, creatine kinase and actin, are also present in the electric organ and have similar apparent molecular weights (Gysin et al., 1983). In order to avoid further confusion and to provide an identity for this nonreceptor protein, we refer to it as N-RAPsyn, nicotinic receptor-associated protein at synapses, to reflect its most characteristic features.

Speculation concerning the function of RAPsyn has centered on the colocalization of RAPsyn with AchR. RAPsyn does not play an obvious role in AchR function since the removal of RAPsyn from postsynaptic membrane vesicles does not alter receptor properties, including the kinetics of binding of acetylcholine, the binding of local anesthetic, and the stimulated efflux of $^{22}$Na (Neubig et al., 1979). However, RAPsyn may play a role in the formation and maintenance of the postsynaptic membrane. It has been proposed that RAPsyn is a cytoskeletal protein that is involved in the localization of the AchR at the postsynaptic membrane (Froehner, 1986) since the rotational movement of the receptor is increased in the absence of RAPsyn (Rousselet et al., 1982). Although there is no evidence to suggest that RAPsyn and AchR are covalently linked, cross-linking studies indicate that RAPsyn is in close proximity to the $\beta$ subunit of the AchR (Burden et al., 1983). RAPsyn has also been reported to be an actin-binding protein (Walker et al., 1984) and a protein kinase (Gorden et al., 1983; Gorden and Milfay, 1986). Anti-Torpedo RAPsyn antibodies have been used to identify a cross-reacting epitope(s) that localizes with AchR at postsynaptic membrane of rat neuromuscular junctions (Froehner et al., 1981; Froehner, 1984). The colocalization of this RAPsyn epitope(s) with the acetylcholine receptor in both Torpedo electric organ and vertebrate neuromuscular junctions indicates that RAPsyn-like proteins have an important function at cholinergic postsynaptic membranes. We recently isolated and characterized RAPsyn cDNAs from Torpedo electric organ (Frail et al., 1987) that encoded the chemically determined Torpedo RAPsyn protein sequence (Carr et al.,...
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Results from an unamplified library were screened with a 1.0-kilobase fragment of the mouse RAPsyn gene. This fragment was previously identified and isolated from a mouse genomic library by low-stringency hybridization with a Torpedo RAPsyn cDNA (data not shown). Thirty-seven putative RAPsyn cDNA clones were identified. Five of these clones were plaque-purified, and the inserts were subcloned into either M13mp19 or Bluescribe (Stratagene) vectors and sequenced. Structural maps of three of the inserts are shown in Fig. 1. One insert, M19, contained the complete coding sequence of mouse RAPsyn. We have previously characterized two RAPsyn mRNAs from Torpedo electric organ that encoded two forms of the RAPsyn protein, one form being 23 amino acids longer than the other at the carboxyl terminus (Frail et al., 1987). All five mouse RAPsyn cDNAs that we have sequenced encode a protein that is homologous with the long form of Torpedo RAPsyn.

The sequence of the RAPsyn mRNA is shown in Fig. 2. The mRNA contains 106 nucleotides of 5'-noncoding sequence, 1236 coding nucleotides, and 214 nucleotides of 3'-noncoding sequence. The sequence terminates in a short poly(A) tail that is 15 nucleotides downstream of a polyadenylation signal (Proudfoot and Brownlee, 1976). The entire 5'-noncoding sequence is also present in a mouse RAPsyn genomic clone thus indicating that this sequence does encode mouse RAPsyn mRNA sequences and is not the result of a cloning artifact (data not shown). The nucleotide conservation between Torpedo and mouse RAPsyn coding regions is 71%.

The Protein Sequence of Mouse RAPsyn—The deduced protein sequence of mouse RAPsyn, shown in Fig. 2, corresponds to the long form of Torpedo RAPsyn. The protein contains 412 amino acids, including the initiator methionine, and has a molecular mass of 46,392 daltons. The alignment of the mouse and Torpedo RAPsyn proteins, shown in Fig. 3, indicates that the RAPsyn protein sequence has been well conserved through evolution. The overall sequence identity of the two proteins is 70%. This high degree of homology between the mouse and Torpedo RAPsyn proteins is similar to the homologies between the mouse and Torpedo α, β, γ, and δ subunits of the AchR (80, 59, 54, and 59%, respectively) (Boulter et al., 1985; Buonanno et al., 1986; Boulter et al., 1986; LaPolla et al., 1984). Some regions of the protein are extremely well conserved, indicating that they may be functionally important. In particular, the first 18 amino acids are identical and two large regions are highly conserved (starting

![Fig. 1. Structural map of RAPsyn mRNAs and related cDNAs.](image-url)

**Figure 1.** Structural map of RAPsyn mRNAs and related cDNAs. The alignment of several cDNA inserts (thin lines) and the mouse RAPsyn mRNA (thick line) is shown. The numbers above the mRNA refer to nucleotides; the adenosine in the initiator methionine is +1. The numbers below the cDNA inserts refer to the first and last nucleotides of RAPsyn sequence present in the inserts. Insert 105 contains additional sequences ligated at one end that are unrelated to RAPsyn sequences (denoted by *X*). The arrows denote nucleotide sequences obtained using RAPsyn-specific synthetic oligonucleotides (→) or a universal (vector-specific) oligonucleotide (←).
et al., concentrated at the surfaces of proteins (Kuntz, 1972), and phosphatases. Finally, mouse RAPsyn, like reverse turn structure (data not shown). Reverse turns are natural features of the mouse RAPsyn protein. Purified RAPsyn protein readily associates with lipid vesicles of var-

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1  ATG GGC CAG GAC CAG ACA AAG CAA CAG ATT GAA AAA GGC CAG CAG TAG CAC CAG TAC CAG TCC ACC ACG ACA GAG AAG GCA CNG  
2  Met Gty Gin Asp Gin Thr Lys Gin Gin Ile Gin Lys Gly Leu Gin Leu Tyr Gin Ser Asn Gin Thr Glu Lys Ala Leu  
9  Gln GYP TCG TTT AAG AAG GTC GAG AAG GGC TCC GAC CTC GYG GCC TCC TCG CAG GAG GGC TCC TGG GTA ACA GCT  
27  Gln Val Thr Met Lys Val Leu GLy Ser Asp Gin Leu Val Gly Arg Phe Arg Val Leu Gly Cys Leu Val Thr Ala  
157  CAC TGY GAG GGC GGC CTC TAC AAA GAG ATG TTT GCC GTG CAG CAT ACT GCT GCG OCT GCG GTC CAG CAC TTC TAC GCG GTC  
198  His Ser Glu Met Gty Arg Tyr Lys Glu Met Leu Lys Phe Val Thr Ala Val Gin Ile Asp Thr Ala Gin Leu Arg Lys  
235  GCT GAC TTC TCT GGA AAC TAC CTT CNG CCG GGC AGC AAT GAG AAG CAA TTC GCG TTC CCG GGA GAA CAG GAT  
272  Ala Asp Phe Leu Leu Gin Ser Tyr Leu Asn Leu Leu Arg Ser Asn Gin Lys Lys Gly Phe His Lys Thr Ile Ser  
313  TTC TAC ACC TGC CTC GCG TCG CCT GCC ACC AGC GCT GCT GAC CAG TCT GCG GTC CAT CAT GCG GCC ACC AGC  
349  Tyr Cys Lys Thr Cys Leu Gly Val Phe Thr Arg Ala Arg Gly Leu Gin Ser Val Ser Met Gly  
391  AAT GCT TGC CTT CTC ACC TTC TTC CAG AGG GCC CTT GAG AGG GCC CTT GAC ACC AAC TCT  
428  Asn Ala Gin Cys GGC ACC CTC TTC TTC CAG AAG GGC TTC GAG AGG GCC TTC TAC ACC AAC TCT  
465  AAT GYG ACC ATC TGT GCG TCT GCC TTC TGC TGC AGC GCG ACT GCC GTC ACT TAC GCC CAG GAC TTT GAG AAA GCC  
502  Asp Thr Gin ACC ATG TGT GCC TAC GCC CTA CGC TTC TGC TGC AGC GCG ACT GCC CAG GAC TTT GAG AAA GCC  
539  Asp Thr Gin ACC ATG TGT GCC TAC GCC CTA CGC TTC TGC TGC AGC GCG ACT GCC CAG GAC TTT GAG AAA GCC  
576  Asp Thr Gin ACC ATG TGT GCC TAC GCC CTA CGC TTC TGC TGC AGC GCG ACT GCC CAG GAC TTT GAG AAA GCC  
613  Asp Thr Gin ACC ATG TGT GCC TAC GCC CTA CGC TTC TGC TGC AGC GCG ACT GCC CAG GAC TTT GAG AAA GCC  
649  GAT GGC ACC ATC TGT GCG TCT GCC TTC TGC TGC AGC GCG ACT GCC GTC ACT TAC GCC CAG GAC TTT GAG AAA GCC  
686  CAG TAC CTC ATG TGT GCC TAC GCC CTA CGC TTC TGC TGC AGC GCG ACT GCC GTC ACT TAC GCC CAG GAC TTT GAG AAA GCC  
723  CAG TAC CTC ATG TGT GCC TAC GCC CTA CGC TTC TGC TGC AGC GCG ACT GCC GTC ACT TAC GCC CAG GAC TTT GAG AAA GCC  
760  CAG TAC CTC ATG TGT GCC TAC GCC CTA CGC TTC TGC TGC AGC GCG ACT GCC GTC ACT TAC GCC CAG GAC TTT GAG AAA GCC  
797  CAG TAC CTC ATG TGT GCC TAC GCC CTA CGC TTC TGC TGC AGC GCG ACT GCC GTC ACT TAC GCC CAG GAC TTT GAG AAA GCC  
834  CAG TAC CTC ATG TGT GCC TAC GCC CTA CGC TTC TGC TGC AGC GCG ACT GCC GTC ACT TAC GCC CAG GAC TTT GAG AAA GCC  
871  CAG TAC CTC ATG TGT GCC TAC GCC CTA CGC TTC TGC TGC AGC GCG ACT GCC GTC ACT TAC GCC CAG GAC TTT GAG AAA GCC  
908  CAG TAC CTC ATG TGT GCC TAC GCC CTA CGC TTC TGC TGC AGC GCG ACT GCC GTC ACT TAC GCC CAG GAC TTT GAG AAA GCC  
945  CAG TAC CTC ATG TGT GCC TAC GCC CTA CGC TTC TGC TGC AGC GCG ACT GCC GTC ACT TAC GCC CAG GAC TTT GAG AAA GCC  
982  CAG TAC CTC ATG TGT GCC TAC GCC CTA CGC TTC TGC TGC AGC GCG ACT GCC GTC ACT TAC GCC CAG GAC TTT GAG AAA GCC  
1019  CAG TAC CTC ATG TGT GCC TAC GCC CTA CGC TTC TGC TGC AGC GCG ACT GCC GTC ACT TAC GCC CAG GAC TTT GAG AAA GCC  
1056  CAG TAC CTC ATG TGT GCC TAC GCC CTA CGC TTC TGC TGC AGC GCG ACT GCC GTC ACT TAC GCC CAG GAC TTT GAG AAA GCC  
1093  CAC TTC TGT GAC TCC GGT GAC GAG AAG AAC ACC CGG CAG CCG GCC TCC TCG ACC ACC TTT CAC ACC TCA GCG  
1130  His Val Leu Gin Arg Leu Arg Thr His Val Val Arg Phe His Glu Cys Val Glu Glu Thr Glu Thr Cys Gly  
1167  CTC TGT GAC TCC GGT GAC GAG AAG AAC ACC CGG CAG CCG GCC TCC TCG ACC ACC TTT CAC ACC TCA GCG  
1204  Leu Cys Gly Glu Ser Ile Gly Arg Arg Ser Arg Gin Leu Leu Arg Ser Gin His Cys Leu Ser Glu Ser Tyr Arg Ser  
1241  CTC TGT GAC TCC GGT GAC GAG AAG AAC ACC CGG CAG CCG GCC TCC TCG ACC ACC TTT CAC ACC TCA GCG  
1278  CTC TGT GAC TCC GGT GAC GAG AAG AAC ACC CGG CAG CCG GCC TCC TCG ACC ACC TTT CAC ACC TCA GCG  
1315  Leu Cys AAA AAT GGC ACT AGG AGC TGG CCC ACC TGC CAC CTC TGC ACC ACC TTT CTA CAG CCG GCC TCG TGA  
1352  Leu Gin Asn Asn Gly Leu Arg Thr His Pro Ser Asn Arg Ser Arg Ser Met Lys Pro Gly Phe Val  

Fig. 2. The nucleotide and protein sequences of mouse muscle RAPsyn. The nucleotide sequence is a composite of sequences obtained from three inserts, M27, M19, and M5, shown in Fig. 1. The majority of the sequence was obtained from both strands of insert M27, whereas the sequences of the 5' and 3' ends were obtained from both strands of inserts M19 and M5, respectively. The first nucleotide of the initiator methionine codon is +1.

at Leu$^{127}$, 40/44, 91%; starting at Ala$^{205}$, 32/33, 96%). Also considered is a consensus sequence for cAMP-dependent protein kinase phosphorylation near the carboxyl terminus (Fry et al., 1986). The predicted secondary structure of this region (Garnier et al., 1978) is distinguished by a large amount of reverse turn structure (data not shown). Reverse turns are concentrated at the surfaces of proteins (Kuntz, 1972), and so the consensus sequence for cAMP-dependent phosphorylation would presumably be accessible to protein kinases and phosphatases. Finally, mouse RAPsyn, like Torpedo RAPsyn, has a very high cysteine content (21/412, 4.9%).

We have used available algorithms to predict some structural features of the mouse RAPsyn protein. Purified Torpedo RAPsyn protein readily associates with lipid vesicles of various compositions, and this association is disrupted by alkali (Porter and Froehner, 1985). We have, therefore, analyzed the hydrophobic character of mouse RAPsyn using the hydrophathy scale devised by Kyte and Doolittle (1982). This analysis, shown in Fig. 4, is useful for identifying membrane-spanning regions and short hydrophobic and hydrophilic regions of proteins. As expected, RAPsyn does not have any predicted membrane-spanning regions (segments of at least 19 residues with an average hydrophathy value of greater than 1.6). In fact, the hydrophathy profile is rather unremarkable in that there are no obvious hydrophobic regions that might be responsible for membrane interactions. We also used helical wheel diagrams (Schiffer and Edmundson, 1967) to detect possible amphipathic helices, but none were found. However,
served substitutions are defined as: pairs of amino acids considered to be conserved substitutions. Conserved substitutions are defined as: pairs of identical amino acids have been aligned. Protein sequences

The mouse and *Torpedo* RAPsyn protein sequences have been aligned. No gaps were introduced to obtain the alignment. Pairs of identical amino acids are boxed in, and stippled boxes denote pairs of amino acids considered to be conserved substitutions. Conserved substitutions are defined as: S, T, P, A, and G; N, D, E, and Q; H, R, and K; M, I, L, and V; F, Y, and W (Dayhoff et al., 1976).

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**Fig. 3.** The alignment of mouse and *Torpedo* RAPsyn protein sequences. The mouse and *Torpedo* RAPsyn protein sequences have been aligned. No gaps were introduced to obtain the alignment. Pairs of identical amino acids are boxed in, and stippled boxes denote pairs of amino acids considered to be conserved substitutions. Conserved substitutions are defined as: S, T, P, A, and G; N, D, E, and Q; H, R, and K; M, I, L, and V; F, Y, and W (Dayhoff et al., 1976).

**Fig. 4.** The hydropathy profile for mouse RAPsyn. The average hydrophobicity values for each amino acid (window = 7) was determined using the algorithm of Kyte and Dolittle (1982); hydrophobic and hydrophilic regions were plotted above or below the neutral hydrophobicity line, respectively.

mouse RAPsyn contains covalently bound myristate, and myristate has been shown to be involved in the membrane association of several other proteins, including pp

**Fig. 5.** RNA blot hybridization analysis of mouse RAPsyn. Poly(A)" mRNA (5 µg) was fractionated by electrophoresis, transferred to nylon membrane, and hybridized with a radiolabeled mouse RAPsyn probe. The samples are from differentiated BC3H1 cells (BC3H-1) and newborn and 4-week-old mouse muscle (Nb, 4 wk, respectively). The size of the hybridizing mRNAs (≈2.0 kilobases) were determined from the relative migration of mRNA standards of known size (data not shown).

The mouse RAPsyn gene was characterized by blot analysis of genomic DNA ("Southern" blotting) (Fig. 6). RAPsyn cDNA insert M19 (Fig. 1), which encodes the RAPsyn protein, was hybridized to restricted mouse genomic DNA at high stringency; two (BamHI) or three (EcoRI and HindIII) genomic fragments were identified. However, a RAPsyn probe that encodes only the amino terminus of the protein hybridized to one of the genomic fragments in each of the three digests, indicating that the M19 insert hybridized to multiple fragments derived from a single RAPsyn gene and not from two different genes. The restriction map of a RAPsyn mouse genomic clone is consistent with this conclusion (data not shown). We have not been able to identify additional hybridizing fragments under conditions of low stringency.

**DISCUSSION**

**RAPsyn Is a Novel, Well Conserved Protein**—RAPsyn was first described as a peripheral membrane protein that associated with nicotinic acetylcholine receptors at postsynaptic membranes of *Torpedo* electric organ (St. John et al., 1982;
exons, was hybridized and radiolabeled insert from the migration of HindIII-cut fragments of genomic blot, containing restricted, fractionated DNA from genomic blot, similar to that shown in restriction enzymes, BamHI, EcoRI, and HindIII (denoted Arg''). Since the function of RAPsyn is not known, we can although there are some primary sequence requirements for amino acids at the amino terminus specify the substrate for degree of conservation at the amino terminus because (i) homologies are even more impressive, including the first 18 amino acids towards the carboxyl terminus of the first, includes several hydrophilic residues followed by an aspartate that may be involved in hydrophobic bonding to the ATP molecule (Fry et al., 1986). Bairach and Claverie (1988) derived model degenerate sequences for these two sequence patterns; these sequences, (LIV)GXXG(FY)GXX(LIV) and (LIV)(HY)XD(FILMVY)XXXnX(FILMV)(FILMV), successfully identified protein kinases from sequence databanks. Others have considered crystallographic structures of known ATP-binding proteins in order to determine patterns of secondary structure around the ATP-binding domain. Models of secondary structure suggest that 1) the GXXXXG sequence is at or near a strand of $\beta$ sheet and is immediately followed by $\alpha$ helix and 2) the aspartate terminates a strand of $\beta$ sheet (Fry et al., 1986; Bradley et al., 1987).

With these sequence considerations in mind, we have not been able to identify RAPsyn as a protein kinase. Mouse RAPsyn does not contain any sequences that satisfy the patterns of Bairach and Claverie (1988). RAPsyn does have two GXXXXG sequences, and we have analyzed these further. The first sequence, GXXXXG, is not conserved between Torpedo and mouse and the predicted secondary structure preceding and following this sequence (data not shown) is not $\beta$ sheet or $\alpha$ helix, respectively, as suggested by Fry et al. (1986) and Bradley et al. (1987). The predicted secondary structure surrounding the GXXXXG sequence (data not shown) does conform to these suggested models, but there are other primary sequence features shared among protein kinases that are not present in this region (Fry et al., 1986). Therefore, if mouse RAPsyn is a protein kinase, then it does not conform to the predicted primary and secondary sequence requirements established for most other protein kinases.

**Concluding Remarks**—The results of both RNA and DNA blot hybridization analysis are consistent with a single mouse N-RAPsyn gene. We do not yet know if the expression of this RAPsyn gene is restricted to muscle or if RAPsyn is associated with nicotinic cholinergic synapses in the brain. It is tempting to speculate that RAPsyn-like molecules are associated with neuronal synapses. Recently, a peripheral membrane protein
of M, 93,000 has been shown to be associated with the cytoplasmic domains of the postsynaptic glycine receptor complex (Schmitt et al., 1987). The structural relationship between this protein and RAPsyn is not known, although we have not been able to detect genes that are related to RAPsyn using low stringency hybridization conditions. The function of RAPsyn remains a mystery; however, we will now be able to use RAPsyn cDNAs to express the protein in environments where its biochemistry and function can be investigated.

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