The Mammalian 43-kD Acetylcholine Receptor-associated Protein (RAPsyn) is Expressed in Some Nonmuscle Cells

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Abstract. Torpedo electric organ and vertebrate neuromuscular junctions contain the receptor-associated protein of the synapse (RAPsyn) (previously referred to as the 43K protein), a nonactin, 43,000-M_r peripheral membrane protein associated with the cytoplasmic face of postsynaptic membranes at areas of high nicotinic acetylcholine receptor (AChR) density. Although not directly demonstrated, several lines of evidence suggest that RAPsyn is involved in the synthesis and/or maintenance of such AChR clusters. Microscopic and biochemical studies had previously indicated that RAPsyn expression is restricted to differentiated, AChR-synthesizing cells. Our recent finding that RAPsyn is also produced in undifferentiated myocytes (Frail, D. E., L. S. Musil, A. Bonanno, and J. P. Merlie. 1989. *Neuron.* 2:1077–1086) led us to examine whether RAPsyn is synthesized in cell types that never express AChR (i.e., cells of other than skeletal muscle origin). Various primary and established rodent cell lines were metabolically labeled with [^{35}S]methionine, and extracts were immunoprecipitated with a monospecific anti-RAPsyn serum. Analysis of these immunoprecipitates by SDS-PAGE revealed detectable RAPsyn synthesis in some (notably fibroblast and Leydig tumor cell lines and primary cardiac cells) but not all (hepatocyte- and lymphocyte-derived) cell types. These results were further substantiated by peptide mapping studies of RAPsyn immunoprecipitated from different cells and quantitation of RAPsyn-encoding mRNA levels in mouse tissues. RAPsyn synthesized in both muscle and nonmuscle cells was shown to be tightly associated with membranes. These findings demonstrate that RAPsyn is not specific to skeletal muscle–derived cells and imply that it may function in a capacity either in addition to or instead of AChR clustering.

The nicotinic acetylcholine receptor (AChR)[1] is concentrated on postsynaptic membranes of Torpedo electric organ and vertebrate neuromuscular junctions in remarkably high density clusters (3). Tightly associated with the cytoplasmic membrane face at these clusters is a nonactin 43,000-M_r peripheral membrane protein first referred to as simply 43K protein (20, 32, 33, 35, 45). Several indirect lines of evidence suggest that 43K protein may be involved in the establishment and/or maintenance of AChR clusters. First, immunofluorescence microscopy studies have demonstrated that 43K protein is detectable only directly under AChR clusters (21, 34) and accumulates at developing nerve–muscle synapses at the same rate as the AChR (10). In addition, 43K protein is in close enough physical proximity to the AChR that it can be chemically cross-linked to its β subunit in isolated Torpedo postsynaptic membranes, raising the possibility that these molecules interact with each other in the intact membrane (11). Extraction of 43K protein from Torpedo electric organ (2, 14, 40) or vertebrate skeletal muscle (5) membranes by treatment with alkaline solutions (pH $\geq$ 11) does not affect the acetylcholine-activated permeability characteristics of the AChR (32), but markedly increases both the lateral (2, 5, 14) and rotational (40) mobility of the receptor in the plane of the plasma membrane. In contrast, removal of other cytoplasmic structural proteins associated with areas of AChR/43K protein clusters, such as vinculin, leaves AChR organization unaltered (6). We have given 43K protein the descriptive name of RAPsyn (receptor-associated protein of the synapse) (18) to reflect the close physical and potential functional relationship between it and the AChR suggested by these findings.

The temporal and spatial coexpression of RAPsyn and the AChR at the neuromuscular junction suggested to us that the synthesis of RAPsyn might be coordinately regulated with that of the AChR and other differentiated muscle-specific proteins (29). We were therefore surprised to find that RAPsyn is synthesized in undifferentiated myoblasts as well (19). Examination of key properties of RAPsyn, including membrane association, degradation rate, and labeling with ^{3}H-myristate, revealed that RAPsyn expression is quantitatively and qualitatively similar in differentiated and undifferentiated

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1. *Abbreviations used in this paper:* AChR, acetylcholine receptor; BTX, bungarotoxin; RAPsyn, receptor-associated protein of the synapse.

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cells (19). The presence of RAPsyn in the absence of AChR production led us to ask whether RAPsyn is also synthesized in cell types that never produce AChR (i.e., in cells of other than skeletal muscle origin). We report here that RAPsyn is synthesized in some, but not all, nonmuscle cells. RAPsyn may therefore serve some function in addition to or instead of AChR clustering.

Materials and Methods

Immunological Reagents

The production and characterization of the rabbit polyclonal anti-RAPsyn serum has been described previously (31). Total AChR α subunit was detected with the rat monoclonal antibody mAb61 which is specific for the α subunit and has been characterized by Tzartos et al. (46) and Merlie and Lindstrom (28).

Cell Culture

The clonal mouse muscle cell lines BC3H-1 (41) and C2 (48) were cultured as described (9, 28). 7-d-old differentiated cultures were used for all experiments. MA-10 cells, a clonal strain of mouse Leydig tumor cells, were grown as described by Ascoli (1). L929 fibroblasts were cultured in DME with 10% FBS. P815 mastocytoma cells and EL4 thymoma cells were maintained in RPMI supplemented with 10% bovine calf serum. All media contained penicillin G and gentamycin. Skeletal muscle cells were dissociated from the forelimbs of 20-d-old embryonic rats and cultured as described (26). Primary heart cell cultures were prepared from 1-d-old rat hearts (atria + ventricles) according to the method of Simpson and Savion and consisted of 75-80% myocytes as defined by beating and morphology (43). After 2 d in culture, the cell medium was switched to DME containing 10% FBS. Heart cells were used 5 d after plating.

Metabolic Labeling of Cells

To label adherent cells with [^{35}S]methionine, cultures were washed twice with warmed DME without methionine. 2 ml of labeling medium (methionine-free DME with 5% dialyzed FCS, 20 μM methionine, and 0.2 mCi of [^{35}S]methionine) was added, and the plate was incubated for the times indicated in the individual experiments. Cell lines grown in suspension culture (P815, EL4) were pelleted by centrifugation at 600 g for 5 min at 4°C and washed twice with methionine-free medium before incubation with labeling medium (6×10^5 cells/ml). To assess the amount of protein synthesized during metabolic labeling of cells, an aliquot of labeled cell lysate was precipitated on Whatman disks with boiling 5% TCA. The disks were washed with 5% TCA at 4°C followed by 95% ethanol, dried, and counted in a scintillation counter.

Preparation of Cell Lysates and Immunoprecipitations

At the end of the labeling period, medium was removed and cultures were rinsed three times with PBS followed by a single wash with extraction buffer (0.05 M NaCl, 0.01 M Hepes, 2.5 mM $MgCl_2$, 0.3 M sucrose, 2 mM PMSF, pH 7.4) (4). The buffer was removed and the monolayers were incubated for $\approx$2 min at room temperature with extraction buffer supplemented with 0.5% SDS, 10 mM N-ethylmaleimide, and protease inhibitors (200 μM leupeptin, 0.2 mg/ml α_2-macroglobulin, 50 μg/ml aprotinin, and 500 μM benzamidine). Cell lysates were removed from the tissue culture plates with an 18-gauge needle attached to a 5-ml syringe and boiled for 3 min. After cooling to room temperature, the lysates were passed three times through a 27-gauge needle to shear DNA released from lysed nuclei. Samples were then diluted with 4 vol of extraction buffer containing 0.5% Triton X-100 and 10 mM N-ethylmaleimide before immunoprecipitation. Immunoprecipitation of RAPsyn and the α subunit of the AChR was conducted as previously described, as was inhibition of RAPsyn immunoprecipitation with a RAPsyn-enriched alkaline extract from Torpedo postsynaptic membranes (pH-11 extract) (31).

Gel Electrophoresis and Fluorography

Immunoprecipitated samples were analyzed on SDS-polyacrylamide gels (24) using the modification of Carr et al. (13) as previously described (31). The gels were processed for fluorography by the method of Bonner and Laskey (8).

Cell Fractionation

To examine the association of RAPsyn with cellular membranes, two 60-mm cultures of MA-10, L929, or BC3H1 cells were incubated with [^{35}S]methionine and/or 2 nM ^{125}I-α bungarotoxin (BTX) for 4 h. The cells were then rinsed four times with PBS, scraped, pelleted by centrifugation at 500 g for 4 min, and swollen by resuspension in 1 ml of hypotonic buffer (10 mM Tris, pH 8.6) containing 10 mM N-ethylmaleimide and a protease inhibitor cocktail (final concentrations of 200 μM leupeptin, 0.2 mg/ml α_2-macroglobulin, 50 μg/ml aprotinin, and 500 μM benzamidine). The cell suspension was then sonicated with a sonifier cell disruptor (model 350; Branson Sonic Power Co., Danbury, CT) with three sets of 10 1-s pulses at an output control of 2 and a 20% duty cycle (7). Nuclei and unbroken cells were removed by sedimentation at 500 g for 10 min at 4°C and the resulting postnuclear supernatant was adjusted to 0.075% BSA (added to reduce nonspecific binding of membranes to centrifuge tubes) $\pm$ 0.2 M NaCl. The postnuclear supernatant was then subjected to centrifugation at 100,000 g for 1 h at 4°C, fractionating it into a soluble supernatant (S100) and a membrane pellet (P100). In some cases, the P100 fraction was further fractionated by flotation through a discontinuous sucrose gradient. The P100 pellet was suspended to 2 ml with 50% wt/wt sucrose and dispersed by 10 strokes in a loose-fitting Dounce (Kontes Glass Co., Vineland, NJ) homogenizer. The fraction was then placed in a siliconized cellulose nitrate tube above 1 ml of 60% (wt/wt) sucrose and overlaid with 1.3 ml of 9% sucrose. All sucrose solutions contained 10 mM Tris, 10 mM N-ethylmaleimide, 0.075% BSA, and protease inhibitors and were adjusted to pH 8.6. The sucrose gradient was centrifuged in an SW60 rotor at 46,000 rpm (215,000 g at R_{av}) for 16 h at 4°C. Material that accumulated at the 9%/50% sucrose interface (crude total membrane fraction) and at the bottom of the tube (large membrane-unassociated aggregates) was collected separately and diluted twice with 10 mM Tris containing 10 mM N-ethylmaleimide and protease inhibitors. For assay of RAPsyn or α subunit content, these fractions were brought to 0.5% Triton X-100 and 0.2% SDS, vortexed briefly, and incubated for 10 min on ice. The samples were then diluted with an equal volume of immunoprecipitation buffer (31) supplemented with 0.5% Triton X-100, 10 mM N-ethylmaleimide, and protease inhibitors before immunoprecipitation with either anti-RAPsyn serum or mAb61 as described above. ^{125}I-α BTX content of the membrane and pellet fractions was determined by gamma counting.

^{125}I-α BTX Binding

^{125}I-α BTX was prepared as previously described (30). Cell cultures were incubated in growth medium containing 2 nM ^{125}I-α BTX for 6 h at 37°C. These prelabeled ^{125}I-α BTX surface receptor complexes serve as a marker for the plasma membrane in differentiated muscle cells (44). Nonspecific binding was assessed in the presence of a 250-fold molar excess of unlabeled α BTX.

Peptide Mapping

RAPsyn immunoprecipitated from muscle and nonmuscle cell lines was analyzed by partial proteolytic digestion according to the procedure of Cleveland et al. (16, 17). Cells were metabolically labeled with [^{35}S]methionine for 6 h, lysed, and immunoprecipitated with anti-RAPsyn serum. The immunoprecipitates were then resolved by SDS-PAGE and the portion of the dried gel containing RAPsyn was excised, rehydrated, and loaded into the lanes of a second polyacrylamide gel consisting of a 5% polyacrylamide stacking and 15% polyacrylamide resolving gel. Digestions were conducted within the stacking gel for 30 min at room temperature using 0.2 μg *Staphylococcus aureus* V8 protease (ICN Radiochemicals, Costa Mesa, CA) per sample. The resulting peptide fragments were separated by electrophoresis through the resolving gel and visualized by fluorography.

RNase Protection Assays

Total RNA was isolated by differential precipitation (15). Collected cells or tissues frozen rapidly in liquid nitrogen were homogenized (Tissumizer; Tekmar Co., Cincinnati, OH) in buffered 7.5 M guanidine hydrochloride, and RNA was precipitated with 0.75 vol of ethanol at −20°C. After centrifugation, the pellet was homogenized in 7.5 M guanidine solution and RNA was reprecipitated with 0.5 vol of ethanol. The RNA was reprecipitated a third time, resuspended in 3.75 M guanidine solution, and extracted with

phenol/chloroform (1:1). The RNA was then precipitated with 0.5 vol of ethanol and resuspended in 1.0% SDS. RNA was quantified by absorbance at 260 nm.

The RNase protection protocol was derived from a transcriptional mapping procedure provided by Promega Biotec (Madison, WI). A Pst I fragment of the mouse RAPsyn gene was subcloned into the transcription vector pBS (+) (Stratagene, San Diego, CA). This fragment contains parts of two exons of the mouse RAPsyn cDNA. A radiolabeled antisense probe was synthesized from plasmid linearized with Hind III. A typical 20 μl reaction contained 500 μM each of unlabeled ATP, CTP, and GTP, 12 μM unlabeled UTP, 50 μCi of [α-^{32}P]UTP (3,000 Ci/mM), 0.5 μg of plasmid, and 8 U of T7 polymerase. The newly synthesized probe was extracted with phenol/chloroform (1:1), precipitated with 0.25 vol of 10 M ammonium acetate and 2.5 vol of ethanol and resuspended in hybridization buffer (80% formamide, 40 mM Pipes, pH 6.7, 0.4 M NaCl, and 1 mM EDTA) at 100,000 cpm/μl. Approximately 40% of the radiolabeled nucleotide was incorporated into RNA.

The RNA to be assayed (20 μg) was brought to 200 μl with diethylpyrocarbonate-treated water and precipitated with 50 μl of 10 M ammonium acetate and 625 μl of ethanol. The RNA was resuspended in 26 μl of hybridization buffer and 4 μl of probe was added. The hybridization mix was heated for 5 min at 80°C, briefly centrifuged, and incubated overnight in an inverted position at 45°C in a water bath. After hybridization, 300 μl of ice-cold RNase digestion buffer (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 300 mM NaCl) containing 40 μg/ml of RNase A (Sigma Chemical Co., St. Louis, MO) and 5 μg/ml of RNase T_1 (Sigma Chemical Co.) was added and the digestion mixture was incubated for 60 min at room temperature. The mixture was incubated an additional 15 min at 37°C after the addition of 10 μl of 20% SDS and 50 μg of proteinase K (Sigma Chemical Co.). After one extraction with phenol/chloroform (1:1), the protected RNA was precipitated with 5 μg of carrier tRNA, 0.25 vol of 10 M ammonium acetate and 2.5 vol of ethanol. The precipitated RNA was washed well with 70% ethanol, dried, and resuspended in 5 μl of sample buffer (98% formamide, 20 mM EDTA, 0.1% xylene cylanol, 0.1% bromophenol blue). The sample was incubated at 95°C for 3 min and electrophoresed on a standard 40-cm 6% polyacrylamide/urea sequencing gel. The gel was then incubated in acetic acid/methanol (10% each) to remove the urea, dried, and exposed to Kodak XAR-5 film with an intensifying screen.

Results

Immunoprecipitation of RAPsyn from Metabolically Labeled Skeletal Muscle and Nonmuscle Cells

To examine whether nonmuscle rodent cells synthesize RAPsyn, various clonal cell lines were labeled with [^{35}S]methionine and detergent lysates of these cells were immunoprecipitated with a rabbit antiserum raised against Torpedo RAPsyn. The monospecificity of this antiserum for RAPsyn and its use in immunoprecipitation of mouse RAPsyn from the muscle cell line BC3H1 have been described previously (31). When confluent cultures of the mouse fibroblast cell line L929 were incubated with [^{35}S]methionine, lysed, and immunoprecipitated with the anti-RAPsyn serum, a prominent labeled species was obtained which migrates on SDS-PAGE as an ≈43,000-M_r band (Fig. 1 *A*, lane *3*). This species comigrates with authentic RAPsyn protein recovered from BC3H1 cells using an identical labeling/immunoprecipitation protocol (Fig. 1 *A*, lane *1*). The anti-RAPsyn serum also recognized a protein of identical electrophoretic mobility from [^{35}S]methionine-labeled MA-10 cells, a clonal line derived from a mouse Leydig cell tumor (1) (Fig. 1 *A*, lane *6*). Several lines of evidence indicate that the ≈43,000-M_r species in L929 and MA-10 cells is recognized by the anti-RAPsyn serum in a specific manner: (*a*) it is not immunoprecipitated when normal rabbit serum (Fig. 1 *A*, lanes *5* and *8*) or preimmune serum (not shown) is substituted for the anti-RAPsyn serum; (*b*) it does not comigrate with actin or other major labeled proteins present in total L929 or

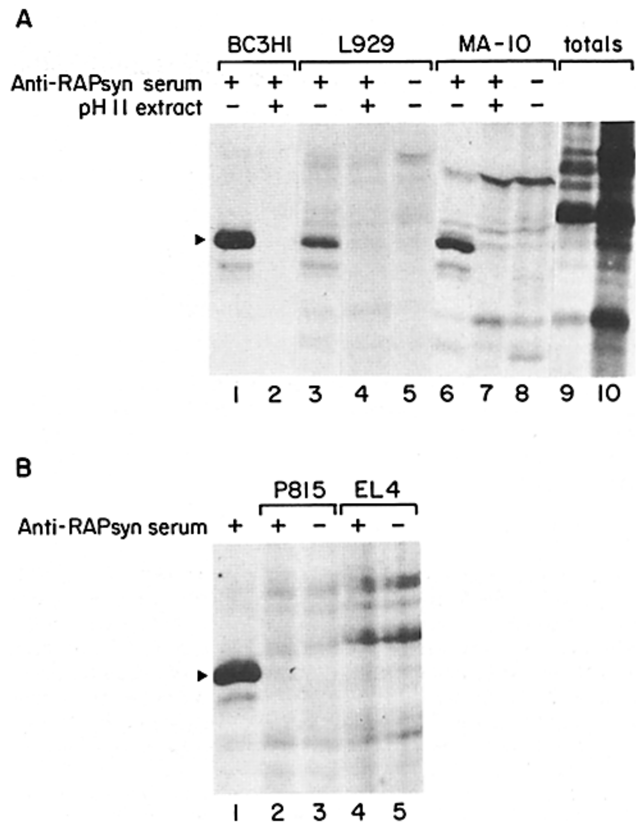

Figure 1. Specificity of immunoprecipitation of [^{35}S]methionine-labeled 43,000-M_r proteins from muscle and nonmuscle cells. Various muscle and nonmuscle cells were labeled for 4 h with [^{35}S]methionine and lysed as described under Materials and Methods before immunoprecipitation or total protein analysis. Equal aliquots of cell lysate were immunoprecipitated with either anti-RAPsyn serum (*A*, lanes *1–4*, *6*, and *7*; *B*, lanes *1*, *2*, and *4*) or normal rabbit serum (*A*, lanes *5* and *8*; *B*, lanes *3* and *5*). Anti-RAPsyn immunoprecipitations were performed in the absence (*A*, lanes *1*, *3*, and *6*; *B*, lanes *1*, *2*, and *4*) or presence (*A*, lanes *2*, *4*, and *7*) of RAPsyn partially purified from AChR-rich Torpedo postsynaptic membranes by pH-11 extraction. (*A*) Immunoprecipitation of lysates from muscle BC3H1 cells (lanes *1* and *2*), fibroblast L929 cells (lanes *3–5*), or Leydig tumor MA-10 cells (lanes *6–8*); total [^{35}S]methionine-labeled protein from L929 (lane *9*) or MA-10 (lane *10*) cell lysates. (*B*) RAPsyn immunoprecipitated from BC3H1 cell lysates (lane *1*); immunoprecipitation of lysates from mastocytoma P815 cells (lanes *2* and *3*) or thymoma EL4 cells (lanes *4* and *5*).

MA-10 cell lysates (Fig. 1 *A*, lanes *9* and *10*); and (*c*) its immunoprecipitation, as that of RAPsyn from BC3H1 cells, is blocked by an alkaline (pH 11) extract of Torpedo postsynaptic membranes consisting of 80% pure RAPsyn protein (Fig. 1 *A*, lanes *2*, *4*, and *7*). Evidence that the pH-11 extract prevents immunoprecipitation of RAPsyn from muscle cells by competitive inhibition has been published previously (31). In addition to the prominent ≈43,000-M_r species, a minor band at ≈40,000 is also specifically immunoprecipitated from L929 and MA-10 cells as well as BC3H1 muscle cells (Fig. 1 *A*, lanes *1*, *3*, and *6*). This species may arise artifactually because of incomplete translation or proteolysis of RAPsyn protein during the isolation procedure, although the possibility that it represents a stable, alternate form of the RAPsyn protein or an immunologically cross-reactive protein has not

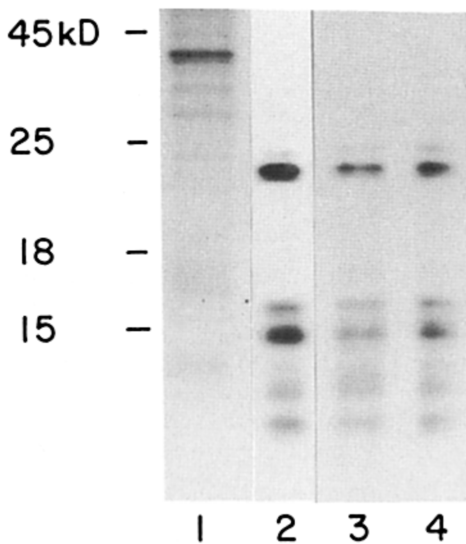

Figure 2. Proteolytic peptide patterns of the 43,000-M_r proteins immunoprecipitated from muscle and nonmuscle cells. Peptide maps of ^{35}S-labeled 43,000-M_r proteins immunoprecipitated with anti-RAPsyn serum were analyzed by the method of Cleveland et al. (17) (see Materials and Methods). (Lanes *2–4*) Digestion patterns of 43,000-M_r protein immunoprecipitated from BC3H1 cells (lane *2*), L929 cells (lane *3*), or MA-10 cells (lane *4*). Lane *1* contains BC3H1 RAPsyn protein incubated in the absence of V8 protease to indicate the position of undigested RAPsyn.

been ruled out. As expected for cells of other than skeletal muscle origin, neither MA-10 nor L929 cells display detectable cell surface binding of a specific ligand for the AChR, ^{125}I–α BTX (data not shown). Furthermore, L929 cells contained no detectable [^{35}S]methionine-labeled AChR α subunit immunoprecipitable by mAb61 (data not shown). We can not be as certain of the absence of α subunit in MA-10 cells, since a faint band with approximately the correct electrophoretic mobility has been observed. However, this material is present in such small amounts that sufficient quantities for peptide mapping have not been obtained. Therefore, we believe that RAPsyn is indeed synthesized in L929 and in MA-10 cells, probably in the absence of expression of AChR subunits, as has been observed previously in undifferentiated C2i myoblasts (19).

When normalized to the amount of [^{35}S]methionine incorporated into total TCA-precipitable cellular protein during a 4-h labeling period, L929 cells and MA-10 cells synthesize ~1/15 and 1/10, respectively, as much immunoprecipitable 43,000-M_r protein as do BC3H1 cells processed identically. In contrast, no reliably detectable 43,000-M_r species can be immunoprecipitated from metabolically labeled mouse T-cell lineage EL4 cells or P815 mastocytoma cells (Fig. 1 *B*). This is true even upon overexposure of the autoradiograph or when the amount of cell lysate used for immunoprecipitation contained five times as much TCA-precipitable protein-associated radioactivity as that required for detection of RAPsyn from L929 cells (not shown). Significant amounts of a 43,000-M_r band were found by immunoprecipitation with anti-RAPsyn in A10 cells, a smooth muscle line derived from embryonic rat thoracic aorta (23), and C3H10T$_{1/2}$ cells, a clonal mouse embryonic fibroblast cell line (38) (data not shown). Thus, a protein electrophoretically and immunologically identical to muscle RAPsyn is synthesized by some, but not all, nonmuscle cell lines.

Peptide Map Analysis of RAPsyn Protein in Skeletal Muscle and Nonmuscle Cells

Additional evidence that the 43,000-M_r species, immunoprecipitated from L929 and MA-10 cells, is identical to RAPsyn from muscle was obtained by peptide mapping. BC3H1, L929, and MA-10 cell cultures were metabolically labeled with [^{35}S]methionine for 6 h and detergent lysates prepared from these cells immunoprecipitated with the anti-RAPsyn serum. The immunoprecipitated material was then isolated by SDS-PAGE and partially digested with *S. aureus* V8 protease using the one-dimensional peptide mapping technique of Cleveland et al. (17). When RAPsyn from mouse muscle BC3H1 cells was subjected to this procedure, a distinctive map consisting of at least five well-resolved peptide fragments was obtained (Fig. 2, lane *2*). A digest pattern indistinguishable from this resulted when material immunoprecipitated from either L929 (Fig. 2, lane *3*) or MA-10 (lane *4*) cells was treated identically. Thus, the 43,000-M_r protein immunoprecipitated from these nonmuscle cells is either identical or very closely related to muscle cell RAPsyn.

Detection of RAPsyn-encoding mRNA in Nonmuscle Cells

To further investigate the synthesis of RAPsyn in nonmuscle cell types, we have used an RNase protection assay to detect and quantify RAPsyn and mRNA in cell lines. The probe used in the assay was a Pst I fragment of the mouse RAPsyn gene that we isolated using mouse RAPsyn cDNA clones (18; our unpublished results). This genomic probe encodes parts of two exons that, when used in the RNase protection assay, were predicted to protect fragments of 313 and 117 bp. A radiolabeled antisense probe derived from the genomic fragment was incubated with total mRNA prepared from L929 cells or differentiated C2 cells. Unhybridized RNA was then enzymatically digested and the protected fragments were analyzed by gel electrophoresis and fluorography. The C2 muscle cell mRNA yielded protected fragments of the predicted size whose intensity was proportional to the amount of C2 mRNA included in the assay (Fig. 3, lanes *1–3*). Protected fragments of identical sizes were obtained with mRNA isolated L929 cells (Fig. 3, lane *4*). The relative amount of RAPsyn mRNA present in the two cell lines was determined by quantifying the autoradiographic signals obtained; there was 5–10 times less RAPsyn mRNA in L929 cells than in C2 cells. Therefore, the combined results of the peptide mapping analysis and the RNase protection assay clearly demonstrate that the material recognized by the anti-RAPsyn serum in nonmuscle cells is indeed RAPsyn.

Association of RAPsyn Protein with Membranes in Nonmuscle Cells

One of the most intriguing properties of RAPsyn protein from skeletal muscle and from Torpedo electric organ is its tight association with plasma membranes despite the fact that it is a peripheral rather than integral membrane protein (5, 32, 36). To examine whether RAPsyn is bound to membranes in nonmuscle cells as well, [^{35}S]methionine-labeled

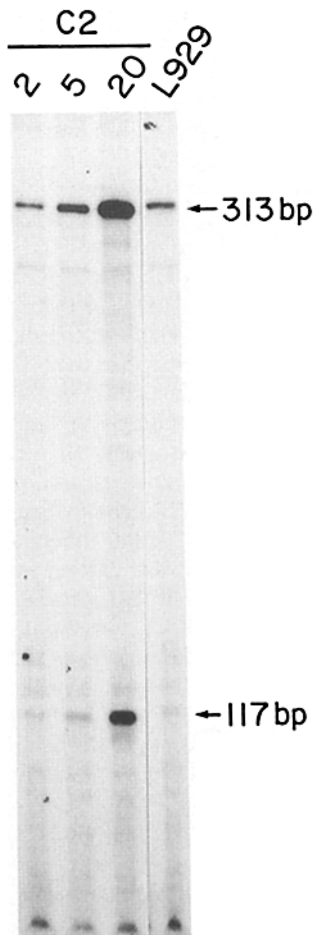

Figure 3. Presence of RAPsyn mRNA in muscle and nonmuscle cells. Total RNA isolated from differentiated C2 myotubes (2, 5, or 20 μg) or L929 fibroblasts (20 μg) was assayed for RAPsyn mRNA by RNase protection and analyzed by electrophoresis and fluorography. The probe used was a fragment of the mouse RAPsyn gene that contained parts of two exons separated by an intron. The sizes of the protected fragments, 313 and 117 bp, were determined by comigration on a sequencing gel and corresponded exactly to the sizes predicted from the cDNA and genomic sequences (data not shown).

L929, MA-10, and BC3H1 cells were disrupted by sonication and fractionated into soluble (S100) and membrane-bound (P100) components. The S100 and P100 fractions as well as a sample of the initial postnuclear supernatant were solubilized and then immunoprecipitated with the anti-RAPsyn serum; the amount of RAPsyn recovered in the three fractions is compared in Table I. When BC3H1 cell sonicates were subjected to this procedure, 80–90% of the RAPsyn was recovered in the P100 fraction. This value is similar to that observed for both the AChR α subunit (98% in the P100 pellet), an integral membrane protein of BC3H1 cells, and surface-labeled ^{125}I–α BTX (93% in the P100 pellet), a plasma membrane marker of differentiated BC3H1 cells. Upon further fractionation of the P100 fraction by flotation in a discontinuous sucrose gradient, virtually all (90–98%) of the RAPsyn, α subunit, and cell surface ^{125}I–α BTX-AChR complexes were recovered in the total membrane fraction. Thus, RAPsyn present in the P100 fraction behaved as if it were truly membrane associated rather than in the form of an insoluble aggregate. When MA-10 and L929 cells were fractionated into P100 and S100, 75–85% of the immunoprecipitated RAPsyn was recovered in the P100 pellet, regardless of whether the salt concentration was kept low (10 mM Tris) or adjusted to 0.2 M NaCl immediately before centrifugation. In contrast, the major actin species present in MA-10, L929, and BC3H1 cell lysates was quantitatively recovered (≈95%) in the S100 fraction in all three cell types, as determined by SDS-PAGE analysis of total labeled proteins present in the postnuclear supernatant P100 and S100 fractions; therefore, soluble proteins partitioned efficiently into the supernatant. RAPsyn thus appears to be tightly membrane associated regardless of cell type, indicating that this association does not require the presence of muscle-specific proteins.

Distribution of RAPsyn in Rodent Tissues

The presence of RAPsyn in MA-10 and L929 cells raised the question as to whether expression of RAPsyn is a property of certain transformed cell lines or whether it occurs in normal rodent cells as well. To address this issue, we prepared total mRNA from various mouse tissues and assayed for the presence of RAPsyn mRNA using the RNase protection assay described above (Fig. 4). As expected, a strong signal was obtained from mRNA prepared from mouse hindlimb (Fig. 4, lane *2*), indicating the synthesis of significant quantities of RAPsyn in adult skeletal muscle (about threefold less than that present in the C2 mouse muscle cell line). Lesser amounts of RAPsyn-encoding mRNA were present in kidney (Fig. 4, lane *5*; ≈20–40-fold less than that present in C2) and in heart (lane *7*; ≈15–25-fold less than that present in C2); the level of expression of RAPsyn in brain, liver, spleen, and uterus was below the limit of detection of the assay (<1/40 of that present in C2 cells); (lanes *3*, *4*, *6*, and *8*). The integrity of the mRNA isolated from the nonexpressing tissues was confirmed by Northern blot analysis with an actin probe (data not shown).

To determine if the RAPsyn mRNA present in nonmuscle tissues was actually translated, the synthesis of RAPsyn in cells derived from several tissues was examined. Cells were pulse labeled with [^{35}S]methionine and assayed for RAPsyn synthesis by immunoprecipitation. Both mouse and rat cells were used since the anti-RAPsyn serum recognizes RAPsyn

Table I. Distribution of ^{35}S-labeled RAPsyn among Subcellular Fractions Prepared from Muscle and Nonmuscle Cells

Cell line	Fraction	^{35}S-RAPsyn, % of total recovered	^{35}S-actin, % of total recovered	^{35}S-α, % of total recovered	^{125}I-BTX, % of total recovered
BC3H1	S100	15	97	2	7
	P100	85	3	98	93
	9%/50% sucrose	95		96	90
MA10	S100	17	95		
	P100	83	5		
L929	S100	24	95		
	P100	76	5		

Association of RAPsyn with membranes in muscle and nonmuscle cells. BC3H1, MA-10, and L929 cells were labeled for 4 h with [^{35}S]methionine and, in the case of BC3H1 cells, 2 nM ^{125}I-α BTX. The cells were then rinsed, scraped, and broken by sonication. A postnuclear supernatant was prepared and fractionated into a crude membrane pellet (P100) and a soluble fraction (S100) by centrifugation for 1 h at 100,000 *g*. Between 80 and 100% of the RAPsyn protein, actin, α subunit, and ^{125}I-BTX-labeled AChR present in the postnuclear supernatant was recovered in the S100 and P100 fractions. The P100 fraction from BC3H1 cells was further fractionated by flotation in a discontinuous sucrose gradient. Recovery of RAPsyn, α subunit, and bound ^{125}I-BTX in the 9%/50% sucrose interface was 80–90% of that loaded onto the gradient, with the remainder distributed throughout the gradient. All values represent the average of at least two separate experiments.

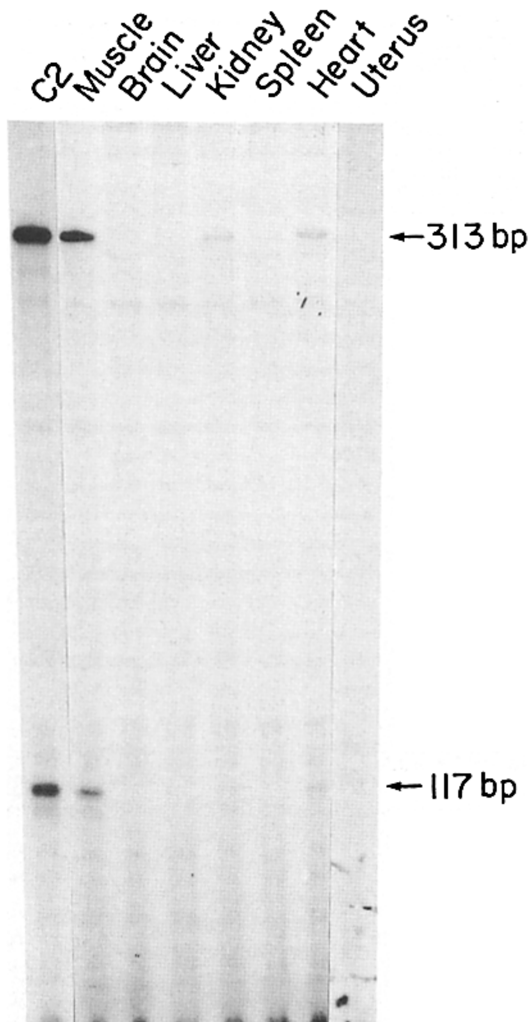

Figure 4. Distribution of RAPsyn RNA expression in mouse tissues. Total RNA (20 μg) was isolated from differentiated C2 myotubes and mouse muscle, brain, liver, kidney, spleen, heart, and uterus, and assayed for RAPsyn mRNA by RNase protection. The protected fragments were analyzed by electrophoresis and fluorography. In all cases in which the 313-bp fragment was observed, the 117-bp fragment was also observed. Although the 117-bp fragment is not visible in the lane labeled Kidney, it is visible in a long exposure of the original x-ray film.

synthesized by embryonic rat myotubes in primary culture (Fig. 5 *B*, lane *5*). A specific signal was also obtained from 5-d-old cultures of embryonic rat cardiac myocytes, demonstrating the synthesis of RAPsyn in normal, untransformed cells of nonskeletal muscle origin (Fig. 5 *B*, lanes *2–4*). In contrast, no 43,000-M_r material was reliably immunoprecipitated from the rat hepatoma cell line H-4-II-E, indicating RAPsyn synthesis in these cells was below the limit detectable in the immunoprecipitation assay (<1/5 the RAPsyn produced by an equivalent number of L929 cells) (Fig. 5, lanes *7* and *8*).

Discussion

Initially described as a component of Torpedo electric organ, RAPsyn (43K protein) was detected by immunostaining in vertebrate skeletal muscle nearly a decade ago (21). The distribution of RAPsyn in both tissues was found to be coextensive with postsynaptic clusters of AChRs (34, 42), suggesting that RAPsyn expression was restricted to AChR-containing cells of skeletal muscle origin. Here we provide immunological evidence, as well as evidence from peptide mapping and RNase protection studies, that RAPsyn is synthesized in certain cell types unrelated to skeletal muscle. This unexpected result raises interesting questions regarding the function of RAPsyn and its relationship with the AChR.

In all cases examined, we have found RAPsyn to be tightly membrane associated in both muscle and nonmuscle cells. Binding of RAPsyn to membranes therefore appears to be an intrinsic property of RAPsyn and is not dependent on the presence of AChR or other muscle-specific proteins. A similar conclusion had previously been drawn by Porter and Froehner from cell-free reconstitution studies demonstrating high affinity binding of isolated Torpedo RAPsyn to pure (protein-free) liposomes (36). The basis for the inherent lipophilicity of RAPsyn has not been established. Our recent finding that RAPsyn in mouse muscle BC3H1 cells is myristoylated (31) suggests, however, that RAPsyn may be anchored to the lipid bilayer via its myristate moiety, as are certain other peripheral membrane proteins (12, 37, 39). Consistent with this possibility is our finding that RAPsyn can be metabolically labeled with ^{3}H-myristate to the same extent in MA-10 and L929 cells as in BC3H1 cells (data not shown). Definitive proof of an essential role for myristate in the association of RAPsyn with membranes will, however, require both direct chemical identification of myristic acid with RAPsyn from nonmuscle cells and demonstration that abolishment of RAPsyn myristoylation by site-directed mutagenesis renders the protein incapable of associating with membranes.

The presence of RAPsyn in nonmuscle cells provides

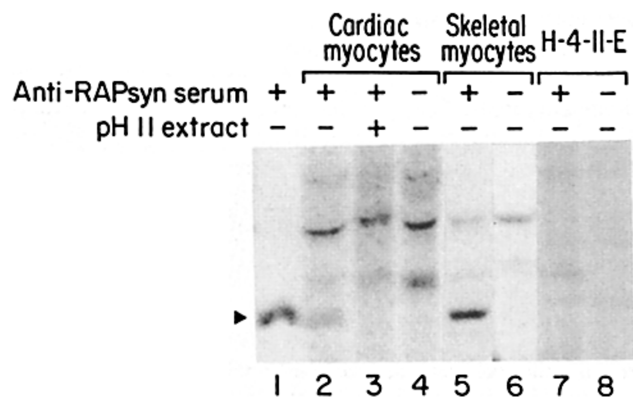

Figure 5. Synthesis of RAPsyn in other rodent cells. Monolayers of the rat hepatoma cell line H-4-II-E (lanes *7* and *8*), primary cultures of embryonic rat heart cells (lanes *2–4*), or skeletal muscle cells (lanes *5* and *6*) were labeled with [^{35}S]methionine for 4 h, lysed, and aliquots of cell lysate were immunoprecipitated with either anti-RAPsyn serum (lanes *2*, *3*, *5*, and *7*) or normal rabbit serum (lanes *4*, *6*, and *8*). In lane *3*, immunoprecipitation was performed in the presence of RAPsyn partially purified from AChR-rich Torpedo postsynaptic membranes by pH-11 extraction. (Lane *1*) Positive control: RAPsyn immunoprecipitated from BC3H1 cells.

strong, albeit indirect, evidence that RAPsyn carries out some function other than or in addition to AChR clustering. One possibility is that RAPsyn is involved in other processes requiring the aggregation of proteins on the plasma membrane, such as receptor-mediated endocytosis (27) or cell surface antigen patching. In the later instance, the lack of detectable RAPsyn synthesis in EL4 and P815 cells suggests that RAPsyn does not participate in cap formation in lymphoid cells. Alternatively, RAPsyn could function in a capacity not directly related to protein aggregation. In this regard it is of interest to note that RAPsyn from Torpedo electric organ has been reported to be an actin-binding protein (47) and a protein kinase (22), although evidence for either function in intact cells is lacking. Careful examination of whether RAPsyn codistributes with clustered plasma membrane proteins in nonmuscle cells might be useful in elucidating the role of RAPsyn in AChR-negative tissues.

There are several potential reasons why RAPsyn has not previously been described in nonmuscle cells. Using a two-site immunological assay, LaRochelle and Froehner detected RAPsyn in Torpedo electric organ and skeletal muscle but not in Torpedo liver, brain, spleen, or heart (25). These results differ from ours since we have found mouse heart to be positive for RAPsyn expression at both the mRNA and protein level. Although species differences cannot be ruled out, the most probable explanation for this discrepancy lies in the inability of the two-site assay to detect levels of RAPsyn <25% of that present in skeletal muscle (25). Since RAPsyn-encoding mRNA is ≈5–8-fold less abundant in mouse heart as in skeletal muscle, it is likely that the amount of RAPsyn present in Torpedo heart is below the limit of detection of the two-site ELISA. Low expression levels may also account for the lack of detection of RAPsyn in nonmuscle cells by immunofluorescence microscopy (5, 34). A more important factor may, however, be the localization of RAPsyn in nonmuscle cells: if the majority of RAPsyn is distributed along the membrane diffusely rather than focally (as on the postsynaptic membrane), it may not be concentrated enough to be visualized by immunofluorescence. A similar situation is likely to exist in undifferentiated skeletal muscle cells and prevent the detection of RAPsyn in these cells by light microscopy, despite our finding that RAPsyn expression is quantitatively and qualitatively similar in differentiated and undifferentiated muscle cells (19).

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