Acetylcholine Receptor Synthesis from Membrane Polysomes*

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We have established conditions for the fractionation of cytoplasmic and membrane-bound polyribosomes from the clonal mouse cell line BC3H-1. Polyribosome fractions are obtained in good yield and purity. They are active in protein synthesis when incubated with nuclelease-treated rabbit reticulocyte lysates, and we have demonstrated that the cytoplasmic and membrane-bound fractions direct the synthesis of distinctly different sets of proteins. Using immunoprecipitation and sodium dodecyl sulfate gel analysis, we have shown that the membrane-bound but not the cytoplasmic polyribosomes direct the synthesis of two protein species ($M_0 = 39,000$ and 42,000) which are homologous to the native a subunit of acetylcholine receptor. Peptide maps suggest that the two species synthesized in vitro may correspond to the nonglycosylated and glycosylated forms, respectively, of the a subunit.

The importance of the acetylcholine receptor to synapse formation at the vertebrate neuromuscular junction gives special significance to the understanding of mechanisms by which the synthesis of this membrane glycoprotein is regulated (1, 2). Several lines of evidence have led to the view that synthesis of ACh receptor is regulated throughout the life of the muscle fiber, and that regulation is coupled in some way directly to the electrical or mechanical activity of the fiber (3). In most studies effects at the level of synthesis have been inferred from measurements of ACh receptor content (3, 4) and the rate constant for receptor degradation (5). Synthesis in vivo has been measured directly in a few cases (6, 7), confirming the earlier studies. Information concerning the mechanisms of synthesis and its regulation has proven difficult to obtain because the most interesting regulatory phenomena occur in intact tissue in living animals or organ culture and because the ACh receptor represents a very small proportion (less than 0.01%) of the total synthetic activity of such tissue.

Embryonic muscle cells in tissue culture provide some unique advantages for studies of ACh receptor synthesis. Even primary cultures have been shown to be as active as intact tissue for ACh receptor synthesis (8, 9). In addition, at least one clonal mouse cell line has been established which is more active than any known mammalian tissue for ACh receptor synthesis (10). Using such cell culture systems, Devreotes et al. (11) have shown that newly synthesized ACh receptor possessing a-bungarotoxin binding activity first appears in an intracellular precursor pool, and is transferred to the plasma membrane only after 2 h. We have shown recently (12) that newly synthesized ACh receptor subunits require approximately 15-30 min before they acquire a-bungarotoxin binding activity, indicating the existence of a pool of inactive precursors at a stage which precedes the pool defined by the experiments of Devreotes et al. (11).

We hope to be able to define the mechanisms by which ACh receptor synthesis is regulated. As a first step toward this goal, we have established a cell-free system for the synthesis of ACh receptor polypeptides. Using nuclease-treated rabbit reticulocyte lysates and polyribosome fractions prepared from the clonal mouse cell line BC3H-1, we have shown that ACh receptor a subunit is synthesized on membrane-bound polyribosomes.

**EXPERIMENTAL PROCEDURES**

Growth of Cells—The clonal mouse cell line BC3H-1 (obtained from J. Patrick, The Salk Institute) was propagated in 15-cm Lux (Flow Labs, Rockville, MD) tissue culture dishes as described (10, 12).

Polyribosome Purification.—We have employed a modification of the method of Ramsey and Steele (13). Five min before harvest, the cell culture medium was made to be 0.36 mM cycloheximide. The 0.36 mM cycloheximide was included in all buffers. Cells were washed with ice-cold phosphate-buffered saline containing 5 mM MgCl2, collected by centrifugation at 700 x g for 5 min, and resuspended in homogenization buffer containing 25 mM Tris-HCl, pH 7.4, 250 mM KCl, 15 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, and 10 mM vanadylribonucleoside complex as an RNase inhibitor (14). The first homogenate was made by using 10 strokes of a tight-fitting Dounce homogenizer. It was centrifuged for 15 min at 131,000 x g, and the pellet was rehomogenized and centrifuged again. The second supernatant was added to the first and this fraction is referred to as the cytoplasmic extract (Table I). The pellet of the second centrifugation was extracted with homogenization buffer, 4% (w/v) Triton X-100, and nuclei were removed by centrifugation at 12,000 x g for 5 min. The resulting supernatant is referred to as the membrane extract. The cytoplasmic and membrane extracts were layered over discontinuous sucrose gradients consisting of 1.5 mL of 2.0 M and 1.5 mL of 1.3 M sucrose in homogenization buffer without RNase inhibitor or cycloheximide. The gradients were centrifuged for 20 h at 178,000 x g. The upper sucrose layers were removed, the tubes were rinsed with buffer containing 10 mM Tris HCl, pH 7.4, 10 mM KCl, 1.5 mM MgCl2, and the pellets were resuspended in the same buffer.

Polyribosomes were analyzed by velocity sedimentation in 10-40% (w/v) sucrose gradients prepared in homogenization buffer. Gradients were centrifuged for 110 min at 28,000 rpm in an SW-28 rotor.

Cell free Protein Synthesis—Protein synthesis was performed with ribonuclease-treated rabbit reticulocyte lysates prepared as described by Pelham and Jackson (15). Resuspended polysomes were incubated with lysate and a reaction mixture containing 150 mM potassium acetate, 2 mM magnesium acetate, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, pH 7.4, and an amino acid mixture.
modified to that of the α subunit of ACh receptor of Torpedo marmorata (16). 0.64 μM L-[35S]methionine (35 Ci/mmol, New England Nuclear) was included for labeling. The K+, Mg2+, and methionine concentrations were determined to be optimal for incorporation stimulated by polyribosomes.

Immunoprecipitation—[35S]methionine-labeled ACh receptor synthesized in vivo was immunoprecipitated by toxin-antitoxin and formalin-fixed, heat-treated Staphylococcus aureus as previously described (12). This method employs an anti-a-bungarotoxin antiserum and precipitates only native ACh receptor molecules to which a-bungarotoxin is bound. For immunoprecipitation of in vitro products an antiserum prepared against sodium dodecyl sulfate-denatured purified fetal bovine muscle ACh receptor (anti-SDS-ACh receptor) was used (12).

Treatment with Endo-β-N-acetylglucosaminidase H—Immunoprecipitates of [35S]-labeled ACh receptor labeled in vivo were treated with 0.2% sodium dodecyl sulfate in distilled H2O at 100 °C to elute labeled proteins. The S. aureus cells were removed by centrifugation and the supernatant was treated with or without 1 milliunit of endo-β-N-acetylglucosaminidase H (Miles Biochemicals, Elkhart, IN) exactly as described by Gahmberg et al. (17).

Polyacrylamide Gel Electrophoresis and Peptide Mapping—One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described previously (18, 12). High resolution two-dimensional gel electrophoresis was performed as described by Garrels (19). Partial proteolytic digests were performed using S. aureus V8 protease (Worthington) (20, 12).

RESULTS AND DISCUSSION

Our first objective was to develop a fractionation procedure for cytoplasmic and membrane-bound polyribosomes from the clonal mouse muscle cell line, BC3H-1. We have employed a modification of the method developed by Ramsey and Steele (13) for rat liver polyribosomes. Since our intention was to use the purified fractions for the cell-free synthesis of ACh receptor, and thereby to determine whether ACh receptor synthesis occurred on cytoplasmic or membrane-bound polysomes, it was essential to obtain the highest quality polyribosomes with respect to integrity, relative and absolute yield, and purity.

An indication of the integrity of the fractionated polyribosomes can be gotten from the distribution of absorbance at 260 nm and [3H]-labeled RNA in analytical sucrose gradients shown in Fig. 1. This experiment was done using cells which had been labeled with [3H]uridine so that we could determine quantitatively the distribution of RNA in the different fractions. Table I represents a summary of another such experiment. Table I shows that: 1) approximately 50% of the polyribosomes present in the initial extracts were recovered in the purified fractions for both cytoplasmic and membrane fractions. 2) The distribution of polysomes between the cytoplasmic and membrane fractions is 9:1. 3) Approximately 85% of the ribosomes in the purified polysome fractions were distributed equally between small and large polysomes. These data indicate that our fractionation procedure does not lead to any selective losses from either cytoplasmic or membrane polyribosome fractions. Purified polysome fractions contain less monosomes than the initial extracts because monosomes are lost during the discontinuous sucrose gradient step. The relatively high value for monosomes in the cytoplasmic extract is not unusual for cells in stationary phase (21), it is approximately twice that reported for primary cultures of quail myotubes (22). The occurrence of monosomes in the purified membrane polysome fraction was variable (compare data from separate experiments in Fig. 1 and Table I) and probably due to polysome runoff or breakage during fractionation.

A further indication of the integrity of the isolated polyribosome fractions was obtained from experiments which assayed their capacity to stimulate incorporation of L-[35S]methionine by nuclease-treated rabbit reticulocyte lysates (15). Fig. 2 shows that incorporation of [35S]methionine by nuclease-treated lysates was absolutely dependent upon addition of polyribosomes. Incorporation stimulated by polyribosome continued for longer times at greater rates than the incorporation due to endogenous mRNA in non-nuclease-treated lysates. Cytoplasmic and membrane-bound polyribosomes were equally active when activity was expressed in terms of radioactivity incorporated per unit of absorbance at 260 nm in a 90-min incubation. We have determined that the nuclease treated rabbit reticulocyte lysates used for these experiments were completely inactive when purified functional BC3H-poly(A) + RNA or pure rabbit globin mRNA was added.
Therefore, the incorporation of \(^{35}\text{S}\) methionine stimulated by BC3H-1 polyribosomes shown in Fig. 2, as well as in those figures to follow, was most likely due to "runoff" or elongation, and not to re-initiation in vitro.

The purity or degree of fractionation of the two polyribosome classes was estimated by comparing the protein products synthesized in reticulocyte lysates. The products were analyzed by high resolution two-dimensional polyacrylamide gel electrophoresis (19). Fig. 3 shows portions of two such gels, with A and B containing cytoplasmic and membrane polyribosome products, respectively. The actual identities of the proteins are not important, although it is possible to identify tentatively many of the abundant structural proteins. The important aspect of the comparison of the two gels is that there is a different set of proteins enriched in the two fractions. In the case of the cytoplasmic polyribosome fraction, we have indicated 4 (of many) abundant species which are synthesized in greatly reduced quantities by the membrane-bound fraction; we estimate an enrichment of about 5-fold. There are fewer abundant proteins which appear to be unique to the membrane-bound fraction; we have indicated 3. On the basis

**Fig. 2.** Time course of \(^{35}\text{S}\) methionine incorporation by nuclease-treated rabbit reticulocyte lysates. 13.8 \(A_{260}\) units of cytoplasmic (circle) or 8.6 \(A_{260}\) units of membrane-bound (triangle) polyribosomes were added to 0.45-ml incubations containing 10 \(\mu\)Ci of \(^{35}\text{S}\) methionine as described under "Experimental Procedures." One incubation contained nuclease-treated lysate but no polyribosomes (square). Another contained lysate which had not been nuclease-treated and no polyribosomes (triangle). At the times indicated, 2-\(\mu\)l aliquots were removed, precipitated with hot trichloroacetic acid, and counted.

**Fig. 3.** Two-dimensional gel electrophoresis of the total \(^{35}\text{S}\) labeled products of cell-free synthesis stimulated by isolated polyribosomes. Aliquots containing 10\(^3\) cpm of \(^{35}\text{S}\) labeled protein were taken from 90-min incubations of cytoplasmic (A) and membrane-bound (B) polyribosomes. The samples were prepared and subjected to two-dimensional electrophoresis as described by Garrels (19). The first dimension gel was prepared with pH 5–7 amphotolines (basic ends to the right), the second dimension was a sodium dodecyl sulfate-10% polyacrylamide gel. The gels were processed for fluorography (23) and exposed to x-ray film for 3 weeks. Only a portion of each gel is presented. Open and filled arrowheads indicate spots which are enriched in cytoplasmic and membrane-bound fractions, respectively. As a reference, the 3 intense spots in the middle of A are actins (19).

**Fig. 4.** Polyacrylamide gel analysis of immunoprecipitates of ACh receptor synthesized in vivo and in vitro. \(^{35}\text{S}\) labeled ACh receptor was immunoprecipitated from a Triton X-100 extract of one 10-cm dish of BC3H-1 labeled 10 min with 1 mCi of \(^{35}\text{S}\) methionine as described (12). \(^{35}\text{S}\) labeled proteins were treated without or with endoglycosidase H, and were separated by electrophoresis (lanes A and B, respectively). Approximately 5 \(\times\) 10\(^4\) cpm were applied for each. The in vitro products were immunoprecipitated with anti-SDS-ACh receptor (12) from 0.45-ml reaction mixtures containing 7.44 \(A_{260}\) units of cytoplasmic (lane C) or membrane-bound (lane D) polyribosome fractions and 20 \(\mu\)Ci of \(^{35}\text{S}\) methionine. Incubations were for 90 min. The final immunoprecipitates contained approximately 4 \(\times\) 10\(^5\) cpm in each case. The samples were analyzed by electrophoresis on sodium dodecyl sulfate-10% polyacrylamide gels (18). The gels were dried and exposed to x-ray film by autoradiography for 7 days. The migration positions of the \(M_r = 42,000\) native \(\alpha\) subunit and its \(M_r = 39,000\) endoglycosidase H derivative are shown in the left margin. The migration position of actin (A) is indicated in the right margin.
jected to partial proteolysis, and the resulting peptides were resolved in a sodium dodecyl sulfate-15% polyacrylamide gel (20, 12). Lanes A and C–F were prepared from bands cut from the gel shown in Fig. 4. They are: A, the M, = 42,000 native α subunit from Fig. 4A; C, the M, = 39,000 endoglycosidase H digestion product of the α subunit from Fig. 4B; D, the M, = 39,000 band of the in vitro product of membrane-bound polyribosomes, Fig. 4D; E, the M, = 42,000 band from Fig. 4D; and F, the actin band cut from Fig. 4C. Lane B shows the peptide map of the α subunit synthesized in vitro and precipitated with anti-SDS-ACh receptor. We have placed asterisks in lanes D and E to indicate the position of two faint bands. The numbers in the right margin are molecular weight values (× 10^3) for standard proteins (12).

of these 7 proteins we conclude that our fractionation procedure does yield polyribosome fractions which are enriched for different mRNAs. Finally, the fact that both fractions produce discrete products is a good indication of polyribosome integrity.

We have demonstrated that the membrane-bound polyribosome fraction directs the synthesis in vitro of ACh receptor α subunit. This has been done by immunoprecipitation of the in vitro products with rabbit anti-SDS-ACh receptor and sodium dodecyl sulfate-polyacrylamide gel analysis. Fig. 4 shows a comparison of in vitro products of the membrane-bound fraction, lane D, and the cytoplasmic fraction, lane C. We have shown previously that ACh receptor labeled in vivo with [35S]methionine and immunoprecipitated with α-bungarotoxin and anti-α-bungarotoxin is composed of 4 distinct subunits: α, the most abundant species, apparent M, = 42,000; β, M, = 46,000; γ, M, = 48,000; and δ, M, = 60,000 (12). An example of such a preparation is shown in Fig. 4, lane A. The most abundant single band immunoprecipitated from the in vitro products synthesized by membrane-bound polyribosomes (Fig. 4, lane D) co-migrates exactly with the α subunit. This band is completely absent from the immunoprecipitates of the most abundant species, apparent M, = 39,000 band from Fig. 4B; D, the M, = 39,000 band of the in vitro product of membrane-bound polyribosomes, Fig. 4D; E, the M, = 42,000 band from Fig. 4D; and F, the actin band cut from Fig. 4C. Lane B shows the peptide map of the α subunit synthesized in vitro and precipitated with anti-SDS-ACh receptor. We have placed asterisks in lanes D and E to indicate the position of two faint bands. The numbers in the right margin are molecular weight values (× 10^3) for standard proteins (12).

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One-dimensional peptide maps of partial proteolytic digests of α subunit [35S]-labeled in vivo and the M, = 42,000 band from the in vitro reaction are very similar (Fig. 5, lanes A, B, and E), confirming the identity of the α subunit synthesized in vitro. However, we expected that the in vitro product might differ from native α in its migration on gels and perhaps its peptide map due to incomplete processing of prepeptides and/or oligosaccharide side chains (24). In particular, we have evidence that the α subunit synthesized in vivo is glycosylated with at least one "high mannose" or "simple" N-linked oligosaccharide side chain. One characteristic of this type of glycoprotein is that the oligosaccharide chain is sensitive to endoglycosidase H (25). Treatment of the native α subunit with endoglycosidase H results in a reduction in the apparent molecular weight (M, = 42,000 to M, = 39,000) (Fig. 4, lane B). A polypeptide with very similar apparent molecular weight is immunoprecipitated from the in vitro products of the membrane-bound polyribosomes. The peptide map of the in vitro M, = 39,000 species identifies it as an α-related polypeptide. Furthermore, the peptide maps suggest that the M, = 39,000 in vitro product is the nonglycosylated form of the α subunit and the M, = 42,000 in vitro product is a glycosylated form. There is a clear difference in a single peptide between the in vivo native α and the in vivo native α treated with endoglycosidase H (Fig. 5, lanes A and C). We can detect this same difference in the M, = 39,000 and 42,000 in vitro products. These peptides are clearly visible on the x-ray films, but are poorly reproduced in prins. We have placed asterisks to indicate their position. More direct chemical analyses of the peptides are necessary to confirm this suggestion. The other bands specific to the membrane-bound fraction (Fig. 4, lane D) have not yet been analyzed.

If the major α subunit-like product of our in vitro reactions is glycosylated, it could be so only because the nascent chains of the α subunit polyribosomes are glycosylated in vivo before isolation. This is completely consistent with the accepted mechanism of N-linked glycosylation of glycoproteins (24). Furthermore, if the M, = 39,000 polypeptide represents the nonglycosylated form, the relative amounts of the M, = 42,000 and 39,000 polypeptides indicate that the site of glycosylation must be very close to the NH2 terminus of the protein.

Finally, both the M, = 39,000 and 42,000 in vitro products appear to be contaminated with actin, since their peptide maps show a faint band corresponding to the major actin peptide (Fig. 5, lane F). This would seem to be an indication of: 1) the relative degree of contamination of membrane polyribosomes with cytoplasmic polyribosomes, and 2) of the degree of premature chain termination in the cell-free protein synthesis reaction.

These experiments demonstrate that the ACh receptor α subunit is synthesized in vivo on membrane-bound polyribosomes, as was predicted by the current version of the signal hypothesis (26). That integral plasma membrane proteins of eukaryotes are synthesized on membrane-bound polyribosomes has been demonstrated for only a few other proteins (23, 27, 28). In addition to establishing this important aspect of ACh receptor synthesis, our results provide us with an effective step in the purification of BC3H-1 mRNA specific for ACh receptor polypeptides. The 5-fold enrichment of the membrane-bound polyribosomes obtained by fractionation should increase the abundance of ACh receptor mRNA to approximately 0.5% of the total membrane-associated mRNA. We think these experiments represent an important first step in our attempts to study mechanisms involved in the synthesis of ACh receptor.

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