

Signal Recognition Protein Is Required for the Integration of Acetylcholine Receptor δ Subunit, a Transmembrane Glycoprotein, into the Endoplasmic Reticulum Membrane

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ABSTRACT Purified Signal Recognition Protein (SRP) has previously been shown to be required for the translocation of secretory proteins across the microsomal membrane (Walter and Blobel, 1980. *Proc. Natl. Acad. Sci. U. S. A.* 77:7,112-7,116) and to function in the early events of this process (Walter and Blobel, 1981. *J. Cell Biol.* 91:557-561). We demonstrate here that the δ subunit of acetylcholine receptor (AChR- δ), a transmembrane glycoprotein, likewise requires SRP for its asymmetric integration into microsomal membranes. We further demonstrate by partial sequence analysis that AChR- δ is synthesized with a transient NH₂-terminal signal sequence of 21 residues that is cleaved off during integration into microsomal membranes. Integration of AChR- δ into the microsomal membrane vesicles proceeded asymmetrically, yielding a large (44 kdalton) core-glycosylated domain, inaccessible to externally added proteolytic enzymes and a smaller (~16 kdalton) domain exposed on the outside of the vesicles and accessible to externally added proteolytic enzymes. The NH₂ terminus of the molecule is contained in the 44-kdalton domain.

For numerous integral membrane proteins, it appears that the endoplasmic reticulum (ER) is the exclusive site of asymmetric membrane integration and hence the exclusive port of entry into the membranes of all ER-derived organelles. The ER's capacity for asymmetric integration of proteins has long been assumed to be related to its ability to translocate secretory proteins (1, 2). Experimental evidence in support of this conjecture was the observed competition (3) between nascent chains of a secretory protein (bovine preprolactin) and of a viral transmembrane protein (glycoprotein G of vesicular stomatitis virus) for a common translocation "site" in the microsomal membranes (dog pancreas) that were present in a cell-free translation system.

Recently, we have accomplished a partial characterization of the ER's translocation activity. From a 0.5 M salt extract of microsomal membranes we have purified a protein (4) termed Signal Recognition Protein (SRP) that is required to restore translocation competence to the salt-extracted microsomal membrane (5, 6). SRP appears to function in mediating the engagement of polysomes synthesizing secretory proteins with the microsomal membrane (6). Most interestingly, in the absence of microsomal membranes SRP was shown to selectively inhibit translation of mRNA's for secretory proteins (5) by a signal sequence-induced, site-specific arrest of chain elongation (7). This elongation arrest was reversible and was released when salt-extracted microsomal vesicles were added to the cell-free translation system (7).

To investigate whether in fact translocation of secretory proteins and integration of transmembrane proteins share identical initial steps we used the recently accomplished (8) *in vitro* integration into dog pancreas microsomal membranes of the δ subunit of the acetylcholine receptor (AChR- δ) of *Torpedo californica* as a model system.

As in the case of secretory proteins (5, 9) we found that AChR- δ synthesis in a cell-free wheat germ system was inhibited by SRP, that addition of microsomal membranes (salt-extracted) released this inhibition, and that SRP was required to accomplish integration of AChR- δ into such microsomal membranes. We have also demonstrated that AChR- δ is synthesized with an NH₂-terminal signal sequence that was correctly cleaved upon asymmetric integration of AChR- δ into the heterologous microsomal membranes. The NH₂ terminus of the integrated form of AChR- δ was resistant to cleavage by externally added proteases, although this proteolysis removed almost 16 kdaltons from the molecule.

MATERIALS AND METHODS

Materials

[³⁵S]Met (sp act 1,000 Ci/mmol) and Liquifluor scintillator were obtained from New England Nuclear, Boston, MA. [³H]Leu (140 Ci/mmol) was purchased from Amersham Corp., Arlington Heights, IL. Protein A-Sepharose CL-4B was from Pharmacia, Uppsala, Sweden, Trasylol from Mobay Chemical Corp., New York, NY, trypsin from Worthington Biochemical Corp., Freehold, NJ, and chymotrypsin A₄ from Boehringer Mannheim Biochemicals, Indianapolis, IN.

Endo-beta-N-acetylglucosaminidase H (Endo H, EC 3.2.1.96) was the generous gift of Dr. Phillips Robbins, Massachusetts Institute of Technology, Cambridge, MA.

In Vitro Synthesis of AChR- δ Subunit

The preparation of *Torpedo californica* RNA, its translation in a micrococcal nuclease-digested wheat germ cell-free protein synthesizing system, and subsequent immunoprecipitation of the δ subunit of AChR were as previously described (8), except that in some cases [3 H]Leu was used rather than [35 S]Met as a source of label. Translation reaction mixtures were denatured by heating at 100°C for 3–5 min in the presence of 1% SDS, before addition of Triton X-100 and, subsequently, of antibody. Dog pancreas rough microsomal membranes (RM), salt-washed RM (K-RM) and purified SRP were prepared as described (4). Conditions for posttranslational proteolysis are described in the figure legends.

Endoglycosidase H Digestion

After thorough washing of Protein A-Sepharose beads containing immunoprecipitated translation products, the beads (typically 20 μ l) were eluted with 1 vol of 1 mM Tris-HCl, pH 7.4, 50 mM DTT and 1% SDS at 100°C for 3 min. This elution yielded 50–60% of the total bound material; for purposes of sequencing, three additional elutions were performed to recover the remainder. Eluates were subsequently diluted with an equal volume of 0.3 M Na Citrate, pH 5.5, containing 100 U/ml Trasylol and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) as protease inhibitors. 0.05 vol of an endoglycosidase H solution was added so that the final enzyme concentration was 0.033 U/ml, and the samples were incubated at 37°C for 16 h (Pre-AChR- δ which is nonglycosylated suffered no degradation under these conditions.) The final solubilization for electrophoresis was performed by adding 0.2 vol of a solution containing 1.0 M sucrose, 1.0 M Tris-HCl, pH 8.8, 10 mM EDTA, 15% SDS, 0.05% Bromophenol Blue, and 100 mM DTT followed by heating for 3 min at 100°C. Alkylation was performed by adding 0.1 vol of a solution containing 1.0 M iodoacetamide and incubating at 37°C for 45 min.

Preparation of Samples for Radiosequencing

After electrophoresis of immunoprecipitated translation products on 7.5–15% linear gradient SDS polyacrylamide gels, radioactive polypeptides were identified by autoradiography. In cases where [3 H]Leu was the source of label, a parallel sample labeled with [35 S]Met was run as a marker. Elution of proteins from the unfixed, dried gels was performed by shaking into 10 mM NH_4HCO_3 , pH 8.8, 1 mM EDTA, 0.1% SDS for 16 h at room temperature. 1 mg of ovalbumin and 2 mg of sperm whale apomyoglobin were added as carriers. Two sequential elutions were performed and the eluates pooled. Subsequent steps in the sample preparation were as described (3).

RESULTS

Cotranslational Integration of AChR- δ into Microsomal Membranes Requires SRP

As previously demonstrated (8), translation of total *Torpedo* electroplax RNA in a cell-free wheat germ system followed by immunoprecipitation with monospecific antibodies against AChR- δ yielded, upon analysis of the immunoprecipitate by SDS PAGE and autoradiography, a single 59-kdalton band (Fig. 1, lane 2). Supplementation of the cell-free wheat germ system with dog pancreas rough microsomal membranes (RM) yielded three additional slower moving bands (Fig. 2, lane 3), the major and slowest moving one having an apparent M_r of 64,000. Using Con A affinity chromatography we have previously shown that this major additional band represents a core-glycosylated form of AChR- δ (8). Since incubation with endo H resulted in conversion of all the multiple bands into a single band (Fig. 1, lane 4) it is clear that they represent fully (64 kdalton) and partially glycosylated forms of AChR- δ . From the staggered mobility differences we estimate that the major 64-kdalton form contains three core sugars and the two minor bands contain two and one core sugar(s), respectively. (The number of high mannose- and complex-oligosaccharide units

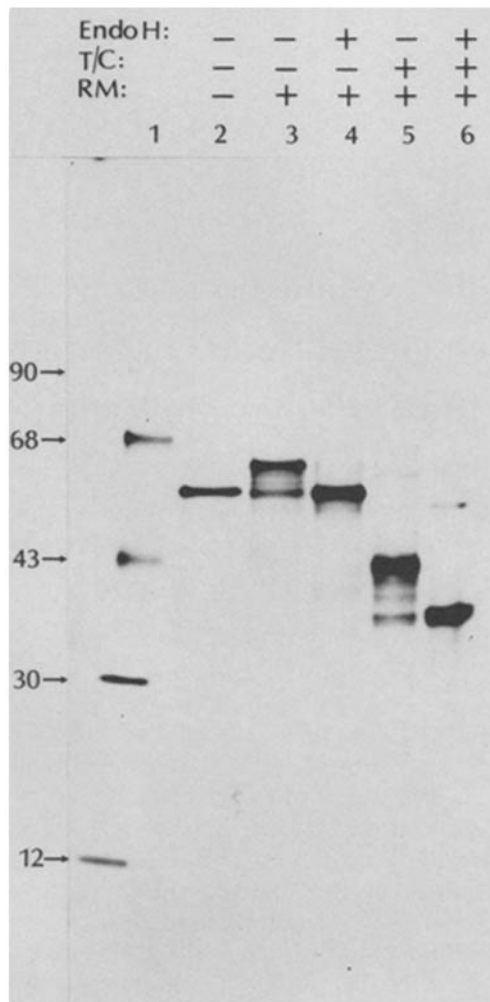


FIGURE 1 Glycosylation variants of the δ subunit of AChR of *Torpedo californica* which were asymmetrically integrated in vitro into dog pancreas microsomal membranes. *Torpedo californica* total cellular RNA was translated in a wheat germ cell-free system (50 μ l), AChR- δ was purified by immunoprecipitation and displayed by SDS PAGE and subsequent autoradiography (8). Endo H+ indicates that immunoprecipitates were digested with Endo H before electrophoresis, as described in Materials and Methods. Endo H- indicates that samples were incubated in parallel without enzyme. T/C indicates posttranslational incubation with 300 μ g/ml each trypsin and chymotrypsin, for 1 h at 25°C, before SDS solubilization and immunoprecipitation. Proteolysis was terminated by addition of 1,000 U/ml Trasylol. RM indicates that dog pancreas rough microsomes were included in the translation at 2 A_{280} U/ml (4) final concentration. The minor, high molecular weight band in lane 5 is a proteolytic intermediate which has an Endo H-sensitive counterpart in lane 6. Lane 1, 14 C-labeled molecular weight markers. Note that the smallest protease-resistant fragment of AChR- δ in lane 5 comigrates with the deglycosylated material in lane 6 and is therefore probably derived from a nonglycosylated, membrane-integrated form of AChR- δ .

in mature AChR- δ has not yet been determined.) The fastest moving species contains no sugar but is integrated into the membrane. A corresponding heterogeneity in the glycosylation of AChR- δ was reflected in the products that were generated after posttranslational incubation of microsomal vesicles with proteolytic enzymes. We detected several membrane-protected fragments (Fig. 1, lane 5) which were converted to a single band upon subsequent incubation with endo H (Fig. 1, lane 6). Thus, it is likely that the major 44-kdalton fragment (lane 5)

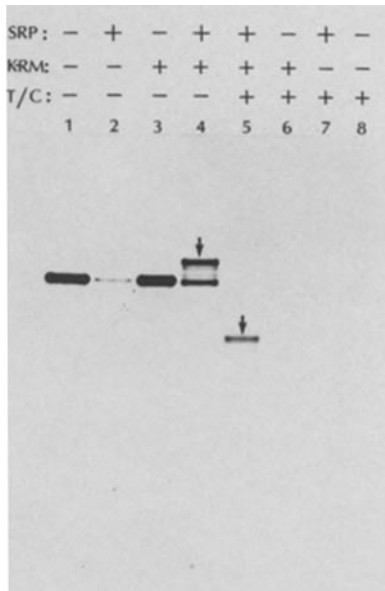


FIGURE 2 Asymmetric integration of the δ subunit of AChR of *Torpedo californica* into dog pancreas microsomal membranes is dependent on SRP. Translation and subsequent analyses were as in Fig. 1. SRP+ and K-RM+ indicate that Signal Recognition Protein (220 U/ml) and/or 0.5 M KOAc-extracted RM (equivalent (4) of 2 A_{280} U/ml) were present during the translation incubation, respectively. T/C+ indicates that posttranslational proteolysis, with 300 μ g/ml each trypsin and chymotrypsin, was carried out, for 45 min at 26°C in the presence of 5 mM $CaCl_2$. Digestions were terminated by the addition of 1,000 U/ml Trasylol, 0.4 mM PMSF, and 10 mM EDTA. The arrows in lanes 4 and 5 indicate the fully glycosylated form of AChR- δ and its major, membrane-protected glycosylated fragment, respectively. The partially glycosylated membrane-protected fragments of AChR- δ (see also Fig. 1, lane 5) are present in this experiment but require longer autoradiographic exposures to be visualized.

was derived from the fully glycosylated 64-kdalton band (lane 3) and, correspondingly, that each of the other three membrane-protected fragments (lane 5) were derived from both partially glycosylated and nonglycosylated (but membrane-integrated) counterparts (lane 3).

It was demonstrated in earlier studies (4) that extraction of rough microsomes (RM) with 0.5 M KOAc yielded vesicles (K-RM) that were incapable of translocating nascent secretory proteins. Purified SRP restored the translocation competence of K-RM. A similar extraction and reconstitution effect was observed for the integration of AChR- δ . Thus, K-RM were unable to produce either core glycosylation (absence of glycosylated forms in Fig. 2, lane 3) or membrane integration (absence of protected fragment following posttranslational proteolysis in lane 6) of AChR- δ . Addition of SRP together with K-RM yielded the familiar (compare with lane 3 of Fig. 1) fully and partially glycosylated or nonglycosylated forms of AChR- δ (Fig. 2 lane 4) that were correctly integrated into microsomal membranes when probed by posttranslational proteolysis (lane 5). (All the partially glycosylated tryptic fragments were also obtained, but these were prominent only after longer autoradiographic exposures.) Recently, it has been shown (7) that SRP, when present in the cell-free wheat germ system (not supplemented with microsomal membranes), selectively inhibited translation of mRNAs for secretory proteins but not of those for cytosolic proteins (5, 9). As shown in Fig. 2, lane 2, the synthesis of AChR- δ was likewise strongly

inhibited by SRP (compare to the amount of AChR- δ synthesized in the absence of SRP in lane 1). Moreover, as was the case for cell-free synthesis of secretory proteins (7), the inhibitory effect of SRP on AChR- δ synthesis was abolished when K-RM were present in the cell-free system (lane 4).

Partial NH₂-Terminal Sequence Analysis of Various In Vitro Synthesized Forms of AChR- δ

The objectives of our partial NH₂-terminal sequence analysis of various in vitro synthesized forms of AChR- δ were to investigate (a) whether AChR- δ was synthesized with a transient NH₂-terminal signal sequence, (b) whether integration into the membrane was accompanied by cleavage of such a transient signal sequence, and (c) whether the membrane protected-fragment was generated by cleavage from the NH₂ or COOH terminus.

To meet the first objective, AChR- δ was synthesized (without added microsomal membranes [see Fig. 1, lane 1]) in the presence of [³H]Leu and [³⁵S]Met and subjected to 20 cycles of automated Edman degradation (Fig. 3a). Another separately synthesized sample was labeled only with [³H]Leu and subjected to 45 cycles of automated Edman degradation (Fig. 3b). There was Met at position 1. However, in both runs we observed a one-residue broadening of the Leu peaks compatible with a frame shift. Therefore, our interpretation of these data (Fig. 3c) is that we were sequencing two species of "pre-AChR- δ " present in approximately equal amounts, one having lost its NH₂-terminal Met (pre-AChR- δ') and the other one having retained it (pre-AChR- δ). On the basis of this interpretation (Fig. 3c) we assigned Leu to positions 9, 10, 14, 28, 32, and 33. Alignment of this sequence with the known sequence of authentic AChR- δ (10) (Fig. 5) indicates that Leu 28, 32, and 33 of pre-AChR- δ corresponds to Leu 7, 11, and 12 of authentic AChR- δ and therefore suggests that pre-AChR- δ contains a 21-residue-long signal sequence.

To meet our second and third objectives we synthesized AChR- δ with added microsomal membranes (RM) in the presence of [³H]Leu. Half of the material was subjected to posttranslational proteolysis and, subsequent to immunoprecipitation, to Endo H incubation to yield a membrane-protected deglycosylated fragment of AChR- δ (Fig. 1, lane 6); the results of automated Edman degradation (21 cycles) of this fragment are shown in Fig. 4b. The other half was subjected to Endo H treatment alone to yield deglycosylated but otherwise intact AChR- δ (Fig. 1, lane 4); the results of automated Edman degradation (20 cycles) of this polypeptide are shown in Fig. 4a. In both cases, Leu was found in positions 7, 11, and 12, in perfect alignment with the NH₂-terminal sequence of authentic AChR- δ (Fig. 5). These partial sequence data then suggest that integration of AChR- δ into dog pancreas microsomal membranes was accompanied by cleavage of an NH₂-terminal signal sequence at the correct site to yield mature AChR- δ and, moreover, that the membrane-protected fragment of AChR- δ is generated by cleavage exclusively at the COOH-terminal end, whereas an NH₂-terminal core-glycosylated portion is protected presumably as a result of its translocation into the vesicle lumen.

DISCUSSION

SRP was originally purified (4) based on its requirement for in vitro translocation of a secretory protein (bovine prolactin) across the microsomal membrane. Here, we have demonstrated

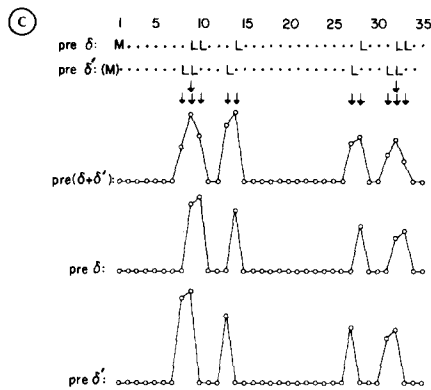
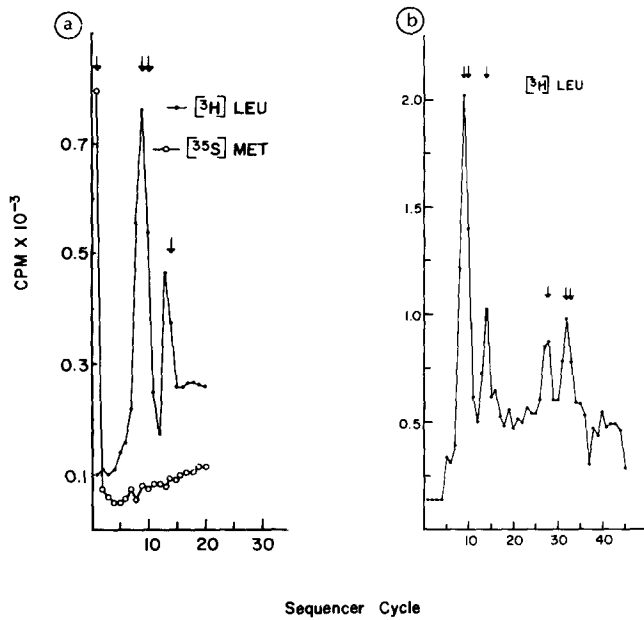


FIGURE 3 Partial NH₂-terminal sequence analysis of pre-AChR- δ . 0.3-ml translation reactions containing (a) [³H]Leu (350 μ Ci) and [³⁵S]Met (350 μ Ci) were carried out (8) except that citrate synthetase (50 U/ml) and oxaloacetate (0.25 mM) were included to prevent NH₂-terminal acetylation (11). Sample preparation was as described in Materials and Methods. The sample in (a) was run on a Beckman 890C sequencer running on a DMAA program, whereas that in (b) was run on a different Beckman 890C sequencer running on a Quadrol program (Beckman Instruments, Inc., Fullerton, CA). Both runs yielded a broadening in the first peak that is characteristic of a frame-shift. Since the shift was obtained on two different machines, it must be due to an inherent characteristic of the sample and not to poor cleavage. The most likely explanation is that the sequence represents a linear combination of peaks from two populations of pre-AChR- δ molecules. One of these (pre- δ') has had its initiator Met cleaved and is therefore one residue shorter than the intact pre-AChR- δ (see [c]). Cell-free systems are known to contain enzymes that remove the initiator Met (12). Furthermore, the frame-shift is not seen in sequences of AChR- δ (see Fig. 4a, b) where the signal sequence (and therefore the initiator Met) has been removed from all the molecules. Sequence assignments are indicated by arrows, and the leading part of the peaks (due to contribution of counts from pre-AChR- δ') is ignored. Input radioactivity was: (a) [³⁵S]Met, 25,000 cpm; [³H]Leu, 50,000 cpm; (b) [³H]Leu, 75,000 cpm. (c): Diagram illustrating how the observed sequence [Pre($\delta + \delta'$)] may be accounted for by the linear combination of sequences from two populations of pre-AChR- δ molecules, one of which (pre- δ') lacks its NH₂-terminal Met. Each arrow represents the contribution to the total counts in a peak of radioactivity from one [³H]Leu residue.

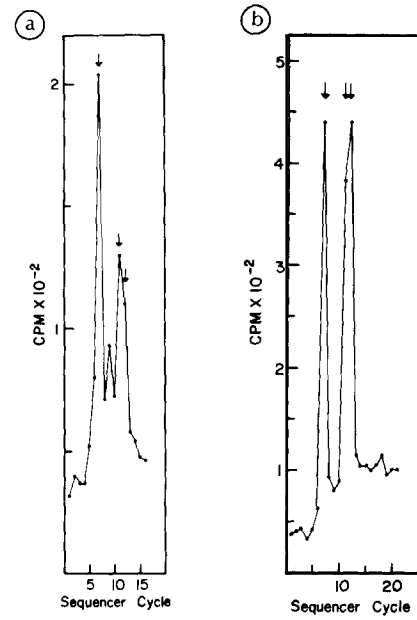


FIGURE 4 Partial NH₂-terminal sequence analysis of membrane-integrated (processed) AChR- δ (a) and of its proteolysis-resistant, membrane-protected fragment (b). 0.6-ml translation reaction mixtures containing 700 μ Ci of [³H]Leu, 2 A₂₈₀ U/ml of RM and ca. 100 U (5) of SRP were performed as in Fig. 2. In the case of (a), microsomal membranes were reisolated following translation, by centrifugation through a cushion of 0.5 M sucrose. This step was performed to eliminate residual, nonintegrated pre-AChR- δ molecules that were not resolved from deglycosylated, integrated AChR- δ by this gel system. (Proteolytically processed, deglycosylated AChR- δ is smaller by only 2,000 daltons than pre-AChR- δ ; this difference is not distinguishable for proteins in the M_r 60,000 range on a 7.5–15% gel.) Posttranslational incubations for (a) and (b) were as described in Fig. 1, lanes 4 and 6, respectively. Sample preparation for sequencing was as described in Materials and Methods. Input radioactivity was (a) 8,400 cpm and (b) 14,700 cpm of [³H]Leu. The small peak in position 9 of (a) did not fall on the repetitive yield curve.

	1	5	10	15	20	25	30
A) PRE- δ	M	LL	L	L
B) PROCESSED δ						L
C) "PROTECTED" δ						L
D) AUTHENTIC δ						V	N E E E R L I N D L L I V

FIGURE 5 Summary of partial sequence data of AChR- δ . (A) Pre-AChR- δ (see Fig. 3); (B) "processed" AChR- δ (see Fig. 4 a); (C) protected AChR- δ fragment (see Fig. 4 b); and (D) complete NH₂-terminal sequence for authentic AChR- δ (10). Underlined residues indicate basis for alignment of pre-AChR- δ with authentic AChR- δ . Arrow indicates site of signal peptidase cleavage.

that this same purified protein is required for the integration of a transmembrane glycoprotein, the δ subunit of the acetylcholine receptor. Our results provide the first direct identification of specific shared steps in signal sequence-dependent translocation and integration processes. Such shared steps had been postulated previously (1, 2) but were supported by circumstantial (13) and indirect (3) evidence. The fact that SRP is required for signal sequence-dependent membrane integration clearly contradicts the claim (14, 15) that insertion of nascent polypeptides into the ER membrane proceeds without the participation of protein mediators.

The role of SRP in the integration of a transmembrane glycoprotein appears to be indistinguishable from its role in the translocation of a secretory protein. As in the case of cell-free synthesis of secretory proteins (5, 9), SRP, in the absence of microsomal membranes, inhibited the synthesis of the transmembrane glycoprotein AChR- δ . Likewise, this translation-inhibitory effect was reversible and abolished by the addition of microsomal membranes. We presume that the translation-inhibitory effect of SRP was due—as in the case of a secretory protein (7)—to a signal sequence-induced, site-specific elongation arrest. So far, however, we have not succeeded, as we did for a secretory protein (7), in demonstrating the existence of a discrete SRP-arrested peptide for AChR- δ . A most likely explanation is that such an arrested peptide might not be immunoprecipitable by our anti-AChR- δ antibodies.

Noteworthy is the observation here that, although salt-excreted vesicles lost a significant complement of their peripheral membrane protein (4), their ability to core-glycosylate remained unperturbed, as the only component of the salt extract added back to this system was purified SRP. However, we cannot rule out the possibility that SRP plays some direct role in the core-glycosylation process.

To make this study of AChR- δ integration fully comparable to those of other ER-directed membrane proteins, it was necessary to demonstrate that AChR- δ has a signal sequence by a method independent of the SRP-requirement assay. Our partial sequence analysis of pre-AChR- δ indicates that it is synthesized with a transient NH₂-terminal signal peptide whose length has been estimated at 21 residues. (The position of the Leu residues must be considered tentative, however, due to the problem of frame-shift.) Taken together with the demonstration that SRP is required for the asymmetric transmembrane insertion of AChR- δ , the results of the present study imply that any membrane protein that requires SRP for its integration into microsomal membranes will contain the functional equivalent of a signal sequence, regardless of whether cleavage of this sequence occurs.

Besides the general implications of our model system, studied here for the process of signal sequence-dependent asymmetric integration of proteins into membranes, our present data provide information intrinsically relevant for the biosynthesis and topology of the δ subunit of the acetylcholine receptor. We found the signal sequence of AChR- δ to be correctly cleaved upon integration into dog pancreas microsomal membranes. The partially determined NH₂-terminal sequence of this microsomal membrane-integrated form of AChR- δ was identical to the known (10) NH₂-terminal sequence of AChR- δ isolated from the assembled acetylcholine receptor in the plasma membrane. Since the *in vitro* integrated form of AChR- δ most likely corresponds to an early *in vivo* biosynthetic counterpart in the ER, our data suggest that AChR- δ does not undergo further cleavages at the NH₂ terminus in its subsequent assembly and pathway from the ER to the plasma membrane.

Evidence has been presented for incomplete glycosylation of some of the AChR- δ molecules integrated *in vitro* (Fig. 1). Since glycosylation of proteins in microsomal membranes proceeds by quantal addition of "core" oligosaccharide groups (16), it seems most likely that the glycosylation variants observed for AChR- δ differ in the number of these core groups which they contain. The uppermost band (Fig. 1, lane 3) would in that case contain three core sugar groups, while the three lower bands would contain two, one, and none, respectively. It is not known whether these nonglycosylated and partially

glycosylated forms of AChR- δ have *in vivo* counterparts, or whether they are due to the limiting glycosylation capacity of the dog pancreas microsomes used *in vitro*. In any case, this incomplete glycosylation did not affect or alter the integration of the AChR- δ polypeptides into the membrane. A similar conclusion has been drawn from studies on the G protein of vesicular stomatitis virus (17).

Partial sequence analysis of the membrane-protected fragment of AChR- δ that was inaccessible to digestion with trypsin and chymotrypsin indicated that it contained the NH₂ terminus (Figs. 4b and 5), in addition to the core sugars (Fig. 1, lane 6). In principle, the NH₂ terminus could be inaccessible to proteolytic enzymes because it is located either fully within the lipid bilayer, as part of an integrated domain, or else in the ectoplasmic space on a fully translocated domain. Electron diffraction analysis of mature membrane-bound AChR indicates that the bulk of the protein mass is located in the extracellular space (18). It is therefore likely that this large extracellular domain contains the NH₂ terminus. A similar topology has been proposed (19, 20) based on trypsinization studies of mature AChR- δ in alkaline-extracted *Torpedo* plasma membrane vesicles. However, it remains to be resolved whether AChR- δ contains a single transbilayer segment connecting two large water-soluble domains on opposite sides of the membrane (a translocated 44-kdalton domain, core-glycosylated and containing the NH₂ terminus, and an untranslocated 16-kdalton domain, unglycosylated and containing the COOH terminus) or whether segments of these domains are further integrated into the hydrophobic core of the lipid bilayer.

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