Characterization of translation products of the polyadenylated RNA of free and membrane-bound polyribosomes of rat forebrain

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Poly(A)⁺ RNA (polyadenylated RNA) isolated from membrane-bound and free polyribosomes was translated in reticulocyte lysates, and the products were analysed by two-dimensional gel electrophoresis. Several translation products were specific to membrane-bound polyribosomal mRNA, including polypeptides of 47 kDa, 35 kDa and 21 kDa, whereas others (e.g. of 37 kDa, 17 kDa and 14 kDa) were specific to free polyribosomal mRNA. Although many products were common to both mRNA species, cross-contamination could be ruled out on the basis of the presence of these and other specific products. The common products included a 68 kDa microtubule-associated protein, tubulin, actin, the brain form of creatine kinase, neuron-specific enolase and protein 14-3-3 and calmodulin, all of which were identified on the basis of two-dimensional gel and peptide analyses. The 35 kDa protein product of membrane-specific mRNA was co-translationally processed *in vitro* by microsomal membranes, resulting in its cleavage to 33 kDa (and partial glycosylation). The 33 kDa processed protein (but not the 35 kDa precursor) was integrated into both dog pancreas and rat brain microsomal membranes. The occurrence of the enzymes and calmodulin as products of membrane-bound polyribosomal mRNA is discussed in the light of their presence on rat brain synaptic plasma membranes [Lim, Hall, Leung, Mahadevan & Whatley (1983) *J. Neurochem.* **41**, 1177–1182] and their existence in a specific component of axonal flow. It is suggested that some of these translation products of the rough endoplasmic reticulum may represent proteins destined for the plasma membrane. However, the identity and location of the 35 kDa membrane-specific product (or its processed form) still remain unestablished.

The brain contains many specialized membrane structures, such as the neuronal synapse and the myelin sheath. Some of the protein components of these membranes are lipoproteins, and others are glycosylated. The endoplasmic reticulum of the brain must play a key role both in the synthesis of membrane proteins and in the series of co- and post-translational events which result in their transfer from the site of synthesis to their eventual destination. In other tissues many membrane and receptor proteins [e.g. Ca²⁺-transport ATPase (Chyn *et al.*, 1979); acetylcholine receptor (Merlie *et al.*, 1981)], secretory proteins [e.g. albumin (Yap *et al.*, 1977)] and proteins destined for subcellular organelles [e.g. cathepsin D (Erickson & Blobel,

1979)] are synthesized by membrane-bound polyribosomes. However, free polyribosomes may also be involved in the synthesis of membrane proteins whose incorporation into the organelle is post-translational [e.g. cytochrome *c* (Korb & Neupert, 1978; Sabatini *et al.*, 1982)].

The poly(A)⁺ RNA populations of free and membrane-bound polyribosomes isolated from the rat forebrain, analysed by translation *in vitro*, have many common components, although certain products are specific to the membrane-bound fraction (Hall & Lim, 1981). Some of these products are developmentally regulated, including the 33 kDa and 21 kDa membrane-specific products (Hall & Lim, 1981). (This 33 kDa product has now been designated 35 kDa on the basis of its migration in higher-resolution SDS/polyacrylamide gels.)

Many membrane-bound proteins are syn-

Abbreviations used: CK, creatine kinase; poly(A)⁺ RNA, polyadenylated RNA; SDS, sodium dodecyl sulphate.

thesized as larger precursors, with an *N*-terminal signal sequence which is responsible for attachment of the polyribosome to the membrane and is subsequently cleaved (Blobel & Dobberstein, 1975a). However, in some membrane or secretory proteins, such hydrophobic regions are part of the sequence of the mature protein [e.g. cytochrome *P*-450 (Bar-Nun *et al.*, 1980)] and may be internal rather than *N*-terminal [e.g. ovalbumin (Palmiter *et al.*, 1978)]. This seems to be the case for the well-characterized brain membrane protein, myelin basic protein. The myelin basic proteins are products of both free and membrane-bound polyribosomal poly(A)⁺ RNA and are not synthesized as larger precursors (Hall *et al.*, 1982).

Factors other than the nature of the nascent polypeptide chain, e.g. a signal-recognition particle (Walter & Blobel, 1982) and putative ribosomal-binding proteins (Kreibich *et al.*, 1978), have also been implicated in polyribosomal attachment to membranes.

In contrast with the myelin basic proteins, other products of brain membrane-bound polyribosomal poly(A)⁺ RNA are synthesized as precursors which are processed during translation *in vitro* when the reticulocyte-lysate system is supplemented with dog pancreas microsomal membranes. The molecular mass of the membrane-specific 35 kDa product is decreased to 33 kDa, and there are modifications of several other translation products, which may include glycosylation as well as cleavage (Hall *et al.*, 1982).

Here we describe the further characterization by two-dimensional electrophoresis of translational products of mRNA from free and membrane-bound polyribosomes of rat brain and the processing *in vitro* of the 35 kDa membrane-specific product by homologous and heterologous membranes.

Materials and methods

Materials

The reticulocyte-lysate translational system was obtained from New England Nuclear, Southampton, U.K., and supplemented with L-[³⁵S]-methionine (9.8 mCi/ml; sp. radioactivity 1164 Ci/mmol), L-[³H]leucine (1 mCi/ml; sp. radioactivity 130 Ci/mmol) and [¹⁴C]methylated protein mixture [molecular-weight markers containing myosin (200 kDa), phosphorylase *b* (92.5 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa) and lysozyme (14.3 kDa)] were obtained from Amersham International, Amersham, Bucks., U.K. Ampholines (pH 3.5–10) were obtained from LKB, Croydon, Surrey, U.K. Calmodulin (bovine brain 3',5'-cyclic nucleotide phosphodiesterase activator)

was purchased from Sigma, Poole, Dorset, U.K. Dog pancreas microsomal membranes were generously given by Dr. Mike Owen, Imperial Cancer Research Fund, Lincoln's Inn Fields, London. Purified creatine kinase, neuron-specific enolase and 14-3-3 from human brain were generously given by Dr. R. J. Thompson (University of Cambridge).

Preparation of brain membrane fractions

(a) *Brain microsomal membranes.* Microsomal membranes were prepared from forebrains of 25-day-old rats essentially as described by Blobel & Dobberstein (1975b). Briefly, a post-mitochondrial supernatant was layered over 1.25 M-sucrose/50 mM-Tris/HCl (pH 7.5)/50 mM-KCl/5 mM-MgCl₂ and centrifuged at 100 000 *g* for 90 min to obtain a microsomal pellet. After resuspension in 0.25 M-sucrose/25 mM-Tris/HCl (pH 7.5)/100 mM-KCl/4 mM-dithiothreitol at a concentration of 120 A₂₆₀ units/ml, the microsomal membranes were stripped of ribosomes by the addition of an equal volume of 15 mM-EDTA, pH 7.2. Microsomal membranes were pelleted through 0.5 M-sucrose/25 mM-Tris/HCl (pH 7.5)/100 mM-KCl/4 mM-dithiothreitol at 100 000 *g* for 1 h and resuspended in 20 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.5, at a concentration of 50 A₂₆₀ units/ml. Small portions were stored frozen at –70°C. This membrane preparation did not contain endogenous mRNA activity when added to reticulocyte lysate.

(b) *Preparation of myelin membrane fraction.* Myelin and the myelin-related SN4 fraction were isolated from forebrains of 25-day-old rats by the method of Waehneltdt *et al.* (1977), and their purity was determined by SDS/polyacrylamide-gel electrophoresis.

Preparation of free and membrane-bound polyribosomes and isolation of poly(A)⁺ RNA

Free and membrane-bound polyribosomes were prepared from rat forebrain by a modification of the method of Ramsey & Steele (1976) as described previously (Hall & Lim, 1981). After phenol extraction of polyribosomal RNA, poly(A)⁺ RNA was isolated by oligo(dT)-cellulose chromatography (Lim *et al.*, 1974; Hall & Lim, 1981).

Translation of poly(A)⁺ RNA in reticulocyte lysates

The reticulocyte-lysate translational system was used as previously described, with the addition of dog pancreas microsomal membranes (4 A₂₆₀ units/ml of lysate) in certain experiments (Hall & Lim, 1981; Hall *et al.*, 1982). Brain microsomal, myelin or SN4 membranes were added to selected translation assays at a final concentration of 4 A₂₆₀ units/ml.

Recovery of heterologous membranes from the reticulocyte-lysate translation system

After translation, dog pancreas or brain microsomal and SN4 membranes were recovered from reticulocyte lysate (100 μ l) by centrifugation (100 000 *g* for 35 min) in a 3 $\times$ 6.5 Ti rotor (MSE) on discontinuous sucrose density gradients (0.8 ml) composed of 0.2M- and 2M-sucrose in 100mM-KCl/5mM-MgCl₂/50mM-Tris/HCl (pH 7.6). In certain cases membranes recovered from the 0.2M/2M interface were washed in 1 ml of 0.2M-sucrose/100mM-KCl/5mM-MgCl₂ (adjusted to pH 11.5 with NaOH) (Mostov *et al.*, 1981) and pelleted by centrifugation at 100 000 *g* for 1 h. Membrane pellets were solubilized in 1% (w/v) Triton X-100 and analysed on SDS/polyacrylamide gels.

Trypsin treatment of processed translation products

Translation products of membrane-bound poly(A)⁺ RNA synthesized in the presence of either rat brain or dog pancreas microsomal membranes were treated with trypsin (0.3 mg/ml) at 4°C for 90 min. Translation products inserted into membranes are trypsin-resistant under these conditions (Sabatini & Blobel, 1970). In control assays, trypsin was added in the presence of 1% Triton X-100 to solubilize the membranes. Sodium deoxycholate was added to selected assays at low concentrations (0.05%), which renders membranes permeable to trypsin without solubilization of the lipid bilayer (Kreibich *et al.*, 1973). Incubations in the presence of trypsin (0.3 mg/ml) and deoxycholate (0.05%) were for 60 min at 4°C. Reactions were terminated by the addition of aprotinin (40 trypsin-inhibitor units), and samples were subjected to SDS/polyacrylamide-gel-electrophoretic analysis.

Polyacrylamide-gel electrophoresis of translation products

One-dimensional SDS/polyacrylamide-gel-electrophoretic analysis of translation products was as described by Laemmli (1970), with a linear acrylamide gradient (10–20%, w/v). Two-dimensional gel electrophoresis was as described by O'Farrell (1975), with the following modifications. In the first-dimension isoelectric-focusing gel, pH 3.5–10 Ampholines (2%, w/v) were used in place of Ampholines pH 5–7 (1.6%) and pH 3.5–10 (0.4%). The lysis buffer also contained pH 3.5–10 Ampholines and SDS (0.5%). This resulted in a wider pH range (approx. 4–9) in the focused gel and better solubilization of protein samples. Isoelectric focusing was at 400 V for 16 h, followed by 1 kV for 6 h. Non-equilibrium pH-gradient electrophoresis was as described by O'Farrell *et al.* (1977), with 2% Ampholines, pH 3.5–10. The second-

dimension SDS/polyacrylamide gel was as described for one-dimensional SDS/polyacrylamide-gel analysis, with vertical slab gels composed of a 3.75%-acrylamide stacking gel over a linear acrylamide-gradient separating gel (10–20%).

Fluorography of gels was as described by Bonner & Laskey (1974) except that acetic acid replaced dimethyl sulphoxide (Burckhardt *et al.*, 1979). Kodak X-S1 X-ray film was used.

Peptide analysis

[³⁵S]Methionine-labelled translation products were co-electrophoresed with purified proteins or with synaptic-plasma-membrane preparations. After surface staining with Coomassie Blue (0.1%, w/v), protein spots were cut from the second-dimension SDS/polyacrylamide gel and analysed by digestion with *Staphylococcus aureus* V8 protease as described by Cleveland *et al.* (1977). Gels containing peptides were silver stained (Bio-Rad silver-staining kit; Bio-Rad, Watford, Herts., U.K.) and photographed. Gels were decolorized with Kodak FX-40 fixative before processing for fluorography.

Results and discussion

Two-dimensional gel analysis of translation products of membrane-bound and free polyribosomal poly(A)⁺ RNA

A two-dimensional electrophoretic analysis (O'Farrell, 1975) of the translation products of free and membrane-bound polyribosomal poly(A)⁺ RNA is shown in Fig. 1. The 35 kDa membrane-specific band, identified by one-dimensional SDS/polyacrylamide-gel electrophoresis (Hall & Lim, 1981), corresponded to a single major species of pI 5.5–6.0. This polypeptide was virtually absent from the products of free polyribosomal poly(A)⁺ RNA (Fig. 1).

The positions of several rat brain proteins in this two-dimensional system have been established by various methods. Tubulin (α and β), actin, the brain form of CK, neuron-specific enolase, the 68 kDa microtubule-associated protein, the neuron-specific protein 14-3-3 and calmodulin have been identified in analyses of brain proteins by co-migration with the individual purified protein, by limited proteolysis with *S. aureus* V8 protease and/or by tryptic-peptide mapping of iodinated proteins; these proteins are also all components of synaptic plasma membranes (Fig. 2; see Lim *et al.*, 1983, and references therein).

The translation products corresponding to several of these proteins (α - and β -tubulin, actin, CK, 14-3-3, 68 kDa microtubule-associated protein, neuron-specific enolase and calmodulin) were identified on the basis of their exact co-migration

Fig. 1. Two-dimensional polyacrylamide-gel-electrophoretic analysis of translation products of free and membrane-bound polyribosomal poly(A)⁺ RNA

Poly(A)⁺ RNA isolated from free and membrane-bound polyribosomes from 25-day-old animals was translated in reticulocyte lysate containing either [³⁵S]methionine (1.96 mCi/ml) (a) or [³H]leucine (400 µCi/ml) (b). Translation products were analysed by two-dimensional polyacrylamide-gel electrophoresis (see the Materials and methods section; SDS, SDS/polyacrylamide-gel electrophoresis; IEF, isoelectric focusing). Fluorography exposure was for 1 week (a) or 5 weeks (b) at -70°C. The products corresponding to α- and β-tubulin (αT, βT), actin (A), creatine kinase (CK), neuron-specific enolase (E), 68 K microtubule-associated protein (68 K), 14-3-3 and calmodulin (C), as well as the membrane-specific products M1, M2 and 35 kDa protein (35 K), are indicated.

in two-dimensional gels with the individual purified proteins. Since different proteins may have identical co-ordinates in two-dimensional gel analyses, the identity of these translation products was confirmed by mapping with *S. aureus* V8 protease (Cleveland *et al.*, 1977; Fig. 2). In all cases the peptide digests of [³⁵S]methionine-labelled translation products corresponded exactly to the silver-stained peptides generated from the appropriate purified protein (and to the synaptic-plasma-membrane protein). Identical peptide maps were also generated from the products of both free and membrane-bound polyribosomal poly(A)⁺ RNA for actin, CK and protein 14-3-3 (Fig. 2), and for 68 kDa microtubule-associated protein, calmodulin and α- and β-tubulin (results not shown). α- and β-tubulin, actin and CK were major products of both free and membrane-bound poly-

ribosomal poly(A)⁺ RNA (Figs. 1 and 3). Protein 14-3-3 was predominantly a product of free polyribosomal poly(A)⁺ RNA, but was also a product of the membrane-bound polyribosomal fraction, as was calmodulin.

Fig. 3 shows two-dimensional gel-electrophoretic analyses of translation products by non-equilibrium pH-gradient electrophoresis (O'Farrell *et al.*, 1977). The more abundant translation products are clearly distinguished in fluorographs after short exposure times (Fig. 3a), whereas the less abundant, more basic, translation products are detectable only after long exposure (Fig. 3b). There is considerable heterogeneity in the products of both classes of brain polyribosomes (Fig. 3b).

The positions of aldolase and pyruvate kinase in this non-equilibrium two-dimensional gel-electrophoretic system were determined by co-migration

Fig. 2. *S. aureus* V8 protease digests of [³⁵S]methionine-labelled translation products of free and membrane-bound polyribosomal poly(A)⁺ RNA were electrophoresed in two-dimensional gels together with either purified proteins (rat α - and β -tubulin, 68 kDa microtubule-associated protein, actin and CK, bovine calmodulin) or with synaptosomal membrane preparations. Protein spots cut from the two-dimensional gels were analysed by partial proteolytic digestion (see the Materials and methods section). The silver-stained peptides of purified proteins (P) and identified synaptic-plasma-membrane proteins (SPM) are shown alongside fluorographs of the peptide digests of the co-migrating [³⁵S]methionine-labelled translation products of either free (F*) or membrane-bound (M*) polyribosomal poly(A)⁺ RNA. Proteins analysed were β -tubulin, 68 kDa microtubule-associated protein (68 K MAP), actin, CK, neuron-specific enolase protein (NSE), 14-3-3 [large (L) and small (S) subunits] and calmodulin. For purified NSE (rat) and purified human 14-3-3, the peptide analyses shown for comparison with the digests of [³⁵S]methionine-labelled translation products were performed in separate gels. Abbreviation: MW, molecular-weight markers, 30 kDa and 14 kDa.

Fig. 3. Non-equilibrium pH-gradient two-dimensional-gel-electrophoretic analysis of translation products of free and membrane-bound polyribosomal poly(A)⁺ RNA

[³⁵S]Methionine-labelled translation products of free and membrane-bound polyribosomal poly(A)⁺ RNA were analysed by non-equilibrium pH-gradient electrophoresis ('pH') followed by SDS/polyacrylamide-gel electrophoresis ('SDS') (O'Farrell *et al.*, 1977) as described in the Materials and methods section. Fluorography exposure was for 3 days (a) or 30 days (b) at -70°C. A schematic comparison of translation products of free and membrane-bound polyribosomal poly(A)⁺ RNA is shown in (c): M1-M10, membrane-bound polyribosomal poly(A)⁺ RNA specific products; 1-10, polypeptides either specific to or highly enriched in translation products of free polyribosomal poly(A)⁺ RNA. The positions of putative pyruvate kinase (PK) and aldolase (Ald) are indicated.

and tryptic-peptide mapping (T. Leung, C. Hall & L. Lim, unpublished work). The translation products which exactly co-migrated with these two proteins were predominantly synthesized in response to free polyribosomal poly(A)⁺ RNA.

In addition to the major 35 kDa membrane-

specific product, several other unidentified translation products were specific to the membrane-bound polyribosomal fraction [Fig. 1b, M1 (21 kDa) and M2 (47 kDa); Fig. 3, products M1-M10]; some of these, e.g. M6, M9 and M10, were detected on non-equilibrium pH-gradient gels only

Fig. 4. Processing *in vitro* of translation products of membrane-bound poly(A)⁺ RNA

(a) Poly(A)⁺ RNA isolated from membrane-bound polyribosomes was translated in reticulocyte lysate containing [³⁵S]methionine in the presence or absence of different membrane preparations, and the products were analysed by SDS/polyacrylamide-gel electrophoresis. Lanes 1 and 7, translation products synthesized in the absence of membranes; translation products synthesized in the presence of dog pancreas microsomal membranes (lanes 2 and 5), of purified myelin (lane 3), of SN4 membranes (lane 4) and of brain microsomal membranes (lane 6). (b) Two-dimensional-gel analysis of translation products of membrane-bound polyribosomal poly(A)⁺ RNA synthesized in the presence of dog pancreas microsomal membranes. Isoelectric focusing (IEF) was as described in the Materials and methods section. The positions of the 35kDa uncleaved and 33kDa processed products are indicated. Abbreviations: MW, molecular-weight markers; SDS, SDS/polyacrylamide-gel electrophoresis.

after long exposure. Others were either predominantly or exclusively products of the free polyribosome fraction (e.g. Fig. 3, 1–10).

The degree of similarity in the composition of translation products of free and membrane-bound polyribosomal poly(A)⁺ RNA is noteworthy. This was not due to cross-contamination, since certain products were specific to one or other polyribosomal fraction (Hall & Lim, 1981; Figs. 1 and 3). The introduction of an additional salt wash (0.25M-KCl) of the membrane fraction during the preparation of membrane-bound polyribosomes did not substantially alter the composition of the translation products of membrane-bound poly(A)⁺ RNA (results not shown). This salt concentration is reported to remove polyribosomes that are either adventitiously associated with or loosely bound to membranes, leaving only those that are attached via nascent polypeptide chains (Adelman *et al.*, 1973).

The presence of tubulin and actin as products of both free and membrane-bound polyribosomal fractions may reflect their subcellular distribution. These proteins are ubiquitous membrane proteins in the brain (Strocchi *et al.*, 1981; Lim *et al.*, 1983). Tubulin and actin are also present in detergent-resistant synaptic-junction preparations (Kelly & Cotman, 1978). Detailed enzymic and structural analysis of brain membrane fractions has revealed that other proteins whose distribution was primarily cytosolic (including neuron-specific enolase, CK, pyruvate kinase and 14-3-3) are in fact also substantial constituents of synaptic plasma membranes (Lim *et al.*, 1983; T. Leung, C. Hall & L. Lim, unpublished work). CK, neuron-specific enolase and protein 14-3-3 were identified as products of membrane-bound as well as of free polyribosomes. The synthesis of other proteins, including the glial-cell markers myelin basic protein, S-100 and carbonic anhydrase, has also been reported to occur on both membrane-bound and free polyribosomes in the brain (Hall *et al.*, 1982; Cosgrove *et al.*, 1983; Sapirstein *et al.*, 1983).

Processing in vitro of the specific 35kDa product of membrane-bound poly(A)⁺ RNA

Cleavage and integration into membranes. In contrast with these proteins, the major 35kDa translation product is exclusive to the membrane-bound polyribosomal fraction and is synthesized as a larger precursor (Hall *et al.*, 1982). This suggests that the protein is synthesized with an *N*-terminal leader sequence which attaches to the rough endoplasmic reticulum and is subsequently cleaved in accordance with the signal hypothesis of Blobel & Dobberstein (1975a). The presence of either dog pancreas or rat brain microsomal membranes (see the Materials and methods section) during translation *in vitro* resulted in a decrease in molecular mass of approx. 2kDa and in pI of this protein

(Figs. 4a and 4b). There was also an increase in the relative synthesis of this product in the presence of membranes. In the presence of the brain microsomal membranes, the processing was incomplete (Fig. 4a). This could be the result of the loss of processing activity during membrane isolation, or alternatively due to the heterogeneity of brain microsomal membranes.

There was no processing of the 35 kDa translation product when other brain membrane fractions, including myelin and the myelin-like fraction SN4 (Waehneldt *et al.*, 1977), were present during translation *in vitro* (Fig. 4a). The unprocessed protein appears to be lipophilic; the 35 kDa product was recovered with the SN4 fraction after translation *in vitro* and was bound to membranes whether these were present during translation or added post-translationally (Fig. 5a).

The decrease in size of the 35 kDa product takes place co-translationally, since the post-translational addition of dog pancreas membranes to the lysate and further incubation in the presence of cycloheximide did not result in the cleavage of the 35 kDa precursor (Fig. 5b, lanes 8 and 9).

The presence of dog pancreas or brain microsomal membranes during translation *in vitro* resulted in the 33 kDa processed product being resistant to trypsin digestion (Fig. 5b, lanes 4 and 6). However, the unprocessed 35 kDa product, which was also present together with the 33 kDa product when brain microsomal membranes were included in the translation assay, was not trypsin-resistant (Fig. 5b, lanes 1 and 4). Trypsin treatment in the presence of 1% Triton X-100 led to the disappearance of both 35 kDa and 33 kDa products. These results suggest that the 33 kDa processed protein (but not the 35 kDa protein) is either integrated into the microsomal membranes during translation or transported into the lumen of the microsomal vesicle, rendering it trypsin-resistant unless membranes were solubilized. The 33 kDa protein was trypsin-sensitive in the presence of 0.05% deoxycholate (Fig. 5c), suggesting that it is at least partially exposed on the luminal side of the vesicle (Kreibich *et al.*, 1973). Resistance to alkaline extraction (pH 11.5) has also been used as a criterion of membrane integration (Shanahan & Czech, 1977; Mostov *et al.*, 1981). Brain and pancreas microsomal membranes were recovered from the reticulocyte lysates after translation *in vitro* by centrifugation through discontinuous sucrose gradients, and the alkali-washed membranes were analysed by SDS/polyacrylamide-gel electrophoresis (Fig. 5d). The 33 kDa translation product was the major component in both heterologous and homologous membrane fractions (Fig. 5d), compatible with its being integrated into membranes. Other translation products of membrane-bound

poly(A)⁺ RNA, e.g. a 64 kDa species, were also integrated into membranes (Fig. 5d).

The trypsin resistance of the 33 kDa processed product, but not the 35 kDa precursor, when membranes are present during translation, the alkali-resistant membrane association of the 33 kDa product, and the absence of processing when membranes are added post-translationally, suggest that the synthesis of the 35 kDa precursor by membrane polyribosomes, its cleavage to a 33 kDa product and its integration into rough endoplasmic reticulum are linked events.

The 35 kDa protein is clearly a major translation product of membrane-bound polyribosomal poly(A)⁺ RNA (Figs. 1 and 3), although this may not accurately reflect the relative concentration of the specific mRNA, since individual mRNA species may have differing translational efficiencies in reticulocyte lysate (Asselbergs *et al.*, 1980). However, assuming that the translational efficiencies are approximately equivalent, this mRNA appears to be present in amounts comparable with those of the mRNA species for the major structural proteins, tubulin and actin. Extensive two-dimensional gel analyses of both soluble proteins and the proteins of brain membranes (including those isolated from rough and smooth endoplasmic reticulum, synaptic membranes and mitochondria) has so far failed to reveal a major membrane protein corresponding directly in molecular weight, pI and peptide map to this major primary translation product (results not shown). The reasons for this may lie either in difficulties in completely solubilizing certain membrane glycoproteins or lipoproteins for electrophoretic analysis or in the possibility that the mRNA codes for a protein which undergoes rapid turnover. Alternatively the primary translation product may undergo further processing, possibly involving glycosylation.

Glycosylation

The presence of dog pancreas microsomal membranes during translation *in vitro* resulted in the partial glycosylation of the 33 kDa (and to a lesser extent of the 35 kDa) protein, as determined by its specific binding to concanavalin A-agarose (results not shown). *In vivo*, glycosylation of this primary translation product may be more extensive, and the protein may be further glycosylated in the Golgi.

Calmodulin also occurs as a membrane component. To determine whether translation products destined for the membrane interacted with calmodulin, the translation products were subjected to affinity chromatography. The primary 35 kDa translation product was found to bind

Fig. 5. Co-translational processing, membrane affinity and insertion of the 33 kDa product into microsomal membranes (a) SN4 myelin-like membranes were added to the reticulocyte lysate containing membrane-bound polyribosomal poly(A)⁺ RNA either during translation (37°C for 40 min) (lanes 1, 3 and 5) or after translation was terminated by the addition of cycloheximide (100 µg/ml) (lanes 2, 4 and 6). After further incubation, SN4 membranes were recovered and translation products from isolated membranes (lanes 1 and 2), supernatant fractions (lanes 5 and 6) and total translations (lanes 3 and 4) were analysed on SDS/polyacrylamide gels. (b) Brain microsomal membranes (lanes 1, 3 and 4) or dog pancreas microsomal membranes (lanes 5 and 6, 8 and 9) were added to reticulocyte lysate containing membrane-bound polyribosomal poly(A)⁺ RNA during translation (lanes 1, 3, 4, 5, 6 and 8) or post-translationally after the addition of cycloheximide (lane 9), and the translation products were analysed by SDS/polyacrylamide-gel electrophoresis. Portions of the translation mixture (10 µl) were treated with trypsin (lanes 3–6) in the presence (lanes 3 and 5) or absence (lanes 4 and 6) of 1% Triton X-100 as described in the Materials and methods section. Translation products of membrane-bound poly(A)⁺ RNA synthesized in the absence of membranes were electrophoresed in lanes 2 and 7. (c) Translation products synthesized in the presence of rat brain microsomal membranes (lanes 1 and 2) or dog pancreas microsomal membranes (lanes 3 and 4) were treated with trypsin in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of 0.05% deoxycholate as described in the Materials and methods section. (d) After translation in the presence of rat brain microsomal membranes (1) or dog pancreas microsomal membranes (2), membranes were recovered as described in the Materials and methods section and translation products that were membrane-associated after alkaline treatment were analysed on SDS/polyacrylamide gels. Key: ←, processed 33 kDa product; ↔, unprocessed 35 kDa product.

quantitatively to calmodulin-Sepharose in a Ca²⁺-dependent manner (results not shown).

Conclusion

Two findings emerge from this study: (1) membrane polyribosomes contain a major mRNA

coding for a 35 kDa protein which *in vitro* is subject to further processing by appropriate membranes, and (2) free and membrane-bound polyribosomes contain in common mRNA species for certain proteins, including enzymes classically considered as being cytosolic (see Figs. 1–3). Certain of these

enzymes, including neuron-specific enolase, CK and pyruvate kinase, were found to be present not only in the cytosol but, surprisingly, also in the synaptic plasma membranes (Lim *et al.*, 1983). Another relevant finding, which was reported after completion of our work on synaptic plasma membranes, is that one of the most abundant non-receptor constituents of acetylcholine-receptor-rich membranes isolated from *Torpedo* electric tissue is in fact the brain form of CK (Barrantes *et al.*, 1983).

Neuron-specific enolase, CK, calmodulin and actin are also all major radioactive constituents of the slow component (SCb) of axonally transported proteins, as is pyruvate kinase (Brady & Lasek, 1981; Brady *et al.*, 1981). [Tubulin is present in SCa, the other slow component of axonal flow (Brady & Lasek, 1981).] It was suggested that these axonally transported enzymes travel with actin as discrete packages in SCb, although it was considered enigmatic that apparently 'cytosolic' enzymes should travel together with a structural protein such as actin. The presence of these enzymes on the synaptic plasma membranes now provides a possible explanation. Clathrin is also a part of the SCb complex (Garner & Lasek, 1981); thus it is conceivable that this complex could represent constituents of packaged membrane assembly units which are axonally transported from their site of synthesis on rough endoplasmic reticulum to their site of assembly on the plasma membrane. Clathrin-coated vesicles have been implicated in the intracellular transport of newly synthesized membrane components (Pearse, 1978, 1980), including the vesicular-stomatitis-virus envelope protein (Rothman & Fine, 1980) and lymphocyte membrane glycoproteins (Kinnon & Owen, 1983). However, in nervous tissue, newly synthesized membrane glycoproteins are reported to be conveyed from the Golgi to cell terminals via the separate fast component of axonal transport (Rambourg & Droz, 1980; Hammerschlag *et al.*, 1982).

The identity of the 35 kDa translation product of the membrane-bound mRNA (or its processed form) remains unestablished. The definitive identification and functional analysis of the product of this abundant membrane polyribosomal mRNA *in vivo* will ultimately depend on the use of recombinant-DNA technology. It is nevertheless gratifying that detailed analysis of the abundant translation products of free and membrane-bound polyribosomal mRNA has led to the discovery of 'cryptic' enzymes on synaptic plasma membranes (Lim *et al.*, 1983) which are involved in the generation of ATP, so vital to synaptic function.

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