TRANSFER OF PROTEINS ACROSS MEMBRANES

I. Presence of Proteolytically Processed and Unprocessed Nascent Immunoglobulin Light Chains On Membrane-Bound Ribosomes of Murine Myeloma

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ABSTRACT

Fractionation of MOPC 41 DL-1 tumors revealed that the mRNA for the light chain of immunoglobulin is localized exclusively in membrane-bound ribosomes. It was shown that the translation product of isolated light chain mRNA in a heterologous protein-synthesizing system in vitro is larger than the authentic secreted light chain; this confirms similar results from several laboratories. The synthesis in vitro of a precursor protein of the light chain is not an artifact of translation in a heterologous system, because it was shown that detached polysomes, isolated from detergent-treated rough microsomes, not only contain nascent light chains which have already been proteolytically processed in vivo but also contain unprocessed nascent light chains. In vitro completion of these nascent light chains thus resulted in the synthesis of some chains having the same mol wt as the authentic secreted light chains, because of completion of in vivo proteolytically processed chains and of other chains which, due to the completion of unprocessed chains, have the same mol wt as the precursor of the light chain.

In contrast, completion of the nascent light chains contained in rough microsomes resulted in the synthesis of only processed light chains. Taken together, these results indicate that the processing activity is present in isolated rough microsomes, that it is localized in the membrane moiety of rough microsomes, and, therefore, that it was most likely solubilized during detergent treatment used for the isolation of detached polysomes. Furthermore, these results established that processing in vivo takes place before completion of the nascent chain.

The data also indicate that in vitro processing of nascent chains by rough microsomes is dependent on ribosome binding to the membrane. If the latter process is interfered with by aurintricarboxylic acid, rough microsomes also synthesize some unprocessed chains.

The data presented in this paper have been interpreted in the light of a recently proposed hypothesis. This hypothesis, referred to as the signal hypothesis, is described in greater detail in the Discussion section.

Biological membranes present a diffusion barrier for macromolecules such as proteins, but transfer of a large number of specific proteins across membranes is an important physiological activity of virtually all cells. Segregation by a membrane is required not only for secretory proteins but also for lysosomal and peroxysomal proteins and for certain mitochondrial or chloroplast proteins synthesized in the cytoplasm. Transfer of proteins across membranes may even be required for some intramembrane proteins, e.g., if the site of insertion into the membrane were separated from the site of synthesis by the lipid bilayer. The discovery of an abundance of ribosome membrane junctions, particularly in secretory cells in the mid 50's (24, 25) and the demonstration in the mid 60's (1, 26, 27, 29) that nascent chains synthesized on membrane-bound ribosomes are vectorially discharged across the membrane, suggested that the ribosome membrane junction may function in the transfer of proteins across the membrane: by topologically linking the site of synthesis with the site of transfer, the protein would transverse the membrane only in status nascendi in an extended form before assuming its native structure, thus maintaining the membrane's role as a diffusion barrier to proteins.

However, the function of the ribosome membrane junction in the transfer of proteins across the membrane did not explain the cell's ability to determine which proteins can traverse via the ribosome membrane junction. Data accumulated in the late 60's (reviewed in reference 28) indicated that mRNA's for some secretory proteins are translated almost exclusively on membrane-bound ribosomes while mRNA's for some cytosol proteins are translated on free ribosomes. In an attempt to explain this dichotomy, a hypothesis was suggested in 1971 (5). It was postulated that all mRNA's to be translated on bound ribosomes contain a unique sequence of codons to the right of the initiation codon (henceforth referred to as the signal codons); translation of the signal codons results in a unique sequence of amino acid residues on the amino terminal end of the nascent chain (henceforth referred to as the signal sequence); the latter triggers attachment of the ribosome to the membrane. A somewhat more detailed version of this hypothesis, henceforth referred to as the signal hypothesis, is presented in this paper.

Thus far, the most compelling support for the signal hypothesis derives from data reported by several laboratories on the in vitro translation of IgG light chain mRNA isolated from murine myelomas. The translation product of this mRNA is larger than the authentic light chain (15, 19, 23, 32, 34–36). Furthermore, it was established by peptide mapping and partial NH_2-terminal sequence analysis (33) that the in vitro translation product contains an extra sequence of ~20 amino acid residues at the NH_2 terminus. It has been suggested independently that this extra sequence, which is subsequently removed, may function in the binding of the ribosome to the membrane (23). We therefore have chosen the murine myeloma as a model system for these studies. In this paper we present fractionation and in vitro protein synthesis experiments designed to examine some aspects of the signal hypothesis.

METHODS AND MATERIALS

All experiments were carried out with a MOPC 41 murine myeloma obtained from Litton Bionetics, Kensington, Md. The light chain produced by this tumor proved to be ~2,000 daltons smaller than that produced by the original MOPC 41 obtained from Dr. M. Potter of the National Institutes of Health, Bethesda, Md. We assume that this change results from the selection of a deletion mutant clone during our initial transfers, and hereafter we will refer to this myeloma as MOPC 41 DL-1 (see also companion paper).

Fractionation of MOPC 41 DL-1 Tumor

All sucrose solutions used in cell fractionation contained 50 mM triethanolamine·HCl pH 7.4 at 20°C, 50 mM KCl, and 5 mM $MgCl_2$ (TeaKM).[1]

Excised tumors freed of necrotic portions were passed through an ice-cold tissue press and were homogenized in ~3 vol of 0.25 M sucrose with a few strokes in a Potter-Elvehjem homogenizer. All subsequent operations were performed between 1–4°C. The homogenate was centrifuged for 10 min at 10,000 g_{av} in an angle rotor to yield a postmitochondrial supernate. The latter was layered over 2.0 ml of 1.3 M sucrose and centrifuged for 15 min at 105,000 g_{av} in a Spinco no. 40 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The supernate was aspirated with a syringe and subsequently used for the preparation of free ribosomes. The pellet, resuspended by homogenization in 2.3 M sucrose, was used to isolate rough microsomes by the following procedure: 3.5 ml of the suspension was loaded at the

[1] *Abbreviations used in this paper:* AR, autoradiography; ATA, aurintricarboxylic acid; DTT, dithiothreitol; ER, endoplasmic reticulum; L^o, derived large ribosomal subunits; PAGE, polyacrylamide gel electrophoresis; S^N, native small ribosomal subunits; SDS, sodium dodecyl sulfate; TeaKM, 50 mM triethanolamine·HCl pH 7.4 at 20°C, 50 mM KCl, and 5 mM $MgCl_2$.

bottom of a tube fitting the SB 283 rotor of the IEC centrifuge (Damon/IEC Div., Damon Corp., Needham Heights, Mass.) and overlayed with 3-ml aliquots each of 1.75 M, 1.5 M, and 0.25 M sucrose. The discontinuous gradient was centrifuged for 12 h at 190,000 g_{av}. The material banding in the 1.75 M sucrose layer was removed with a syringe, diluted with 1 vol of TeaKM, layered over a 2-ml cushion of 1.3 M sucrose, and centrifuged for 30 min at 105,000 g_{av} in a Spinco no. 40 rotor to yield a pellet consisting essentially of rough microsomes.

To isolate detached ribosomes, such pellets were resuspended in TeaKM; a 10% solution of deoxycholate in water was added to a final concentration of 1%, and the mixture was layered over a 2-ml cushion of 2.0 M sucrose. Centrifugation for 24 h at 105,000 g_{av} in a Spinco no. 40 rotor yielded a pellet of detached ribosomes.

Free ribosomes were prepared from the 30,000 g supernate (see above) which was layered on a discontinuous sucrose gradient containing 2-ml layers each of 2.0 M and 1.75 M sucrose. Centrifugation for 24 h at 105,000 g_{av} in a Spinco no. 40 rotor yielded a pellet of free ribosomes.

Preparations of native small ribosomal subunits (S^N) from rabbit reticulocytes, of derived large ribosomal subunits (L^o) from rat liver free ribosomes by the puromycin-KCl procedure, and of pH 5 enzymes from a high speed supernate of Krebs ascites cells were as previously described (6, 13).

Isolation of Poly (A)-Containing RNA from MOPC 41 DL-1 Rough Microsomes

Pellets containing rough microsomes (3,000–5,000 A_{260} units) were resuspended in 60 ml 150 mM NaCl, 50 mM Tris-HCl pH 8.0, and 5 mM EDTA. A 10% solution of sodium dodecyl sulfate (SDS) was added to a final concentration of 1.5% and RNA was extracted from this mixture with phenol-chloroform-isoamyl alcohol (2) and fractionated on oligo (dT) cellulose as follows. RNA was resuspended in H_2O and subsequently adjusted to 400 mM NaCl, 50 mM Tris·HCl pH 7.5, and 0.2% SDS. The final concentration of RNA was 100–120 A_{260}units/ml. 6 ml of that solution was mixed at room temperature by gentle swirling with 4 ml of packed oligo (dT) cellulose which had been washed several times in 400 mM NaCl, 50 mM Tris·HCl pH 7.5, and 0.2% SDS. The cellulose was then sedimented at 1,000 g, washed twice with 100 mM NaCl, 50 mM Tris·HCl pH 7.5, 0.2% SDS, and transferred to a column. After washing with at least 10 bed volumes of a solution of 100 mM NaCl and 50 mM Tris·HCl pH 7.5, the poly (A)-containing RNA was eluted with a solution of 10 mM Tris·HCl pH 7.5, and 0.1% SDS. The poly (A)-containing RNA was twice precipitated with ethanol, then resuspended in double-distilled water to a concentration of ~10 A_{260}units/ml, and stored at −80°C.

Cell-Free Protein-Synthesizing Systems and Assays

The terms "readout" and "initiation" system have been adopted for the sake of brevity. In the readout system, previously started polypeptide chains are completed, whereas in the initiation system polypeptide chains are synthesized de novo.

INITIATION SYSTEM: The reaction mixture (250 μl) contained: 25 μmol of KCl, 5 μmol of HEPES·KOH (pH 7.3 at 20°C), 0.75 μmol of $MgCl_2$, 0.5 μmol of dithiothreitol (DTT), 0.25 μmol of ATP, 0.05 μmol of GTP, 1.5 μmol of creatine phosphate, a few crystals of creatine phosphokinase, 10 μCi of a reconstituted protein hydrolysate (algal profile) containing 15 ^{14}C-amino acids, 7.5 nmol each of the five amino acids not present in the algal hydrolysate (asparagine, cysteine, glutamine, methionine, and tryptophan) as well as S^N (0.4 A_{260} units), L^o (1.2 A_{260} units), 100 μl pH 5 enzymes and poly (A)-containing RNA (0.05 A_{260} units).

READOUT SYSTEM: The composition of this system was identical to that of the initiation system except that it contained either free or detached ribosomes or rough microsomes instead of S^N, L^o, and mRNA (in one case it also contained S^N).

Incubation in both systems was at 37°C. 10-μl aliquots (unless indicated otherwise in figure legends) were removed at indicated time intervals and spotted on 3M Whatman filter paper disks, which were processed according to Mans and Novelli (21). Radioactivity was determined in toluene-Liquifluor (New England Nuclear Corp., Boston, Mass.) in a Beckman LS 350 liquid scintillation counter at about 75% efficiency.

PROTEOLYSIS OF TRANSLATION PRODUCTS: 25-μl aliquots removed from the two systems described above after incubation (see figure legends), were cooled to 0–2°C in an ice bath, and each treated for 3 h at the same temperature with 3 μl of a solution containing trypsin and chymotrypsin (500 μg of each per ml). Proteolysis was terminated by the addition of 1 vol of 20% TCA, and the ensuing precipitate was prepared for SDS-polyacrylamide gel electrophoresis (PAGE) as described below.

ANALYSIS OF TRANSLATION PRODUCTS BY SDS-PAGE: 25-μl aliquots removed from the two systems either after completion of, or at various times during, incubation were cooled to 0–2°C in an ice bath and treated with an equal volume of ice-cold 20% TCA; after 1 h the ensuing precipitate was collected at 0–4°C by centrifugation in a swinging bucket rotor for 10 min at 2,000 g. The supernate was removed as completely as possible and the precipitate was dissolved by incubation for 20 min at 37°C in 30 μl of a solution containing 15% sucrose, bromophenol blue (serving both as a pH indicator for the sample and as a tracking dye for electrophoresis), 100 mM Tris base and 8 mM DTT (if the solution turned yellow [pH 3], Tris base was added in 1-μl aliquots to restore the blue color [pH 4.5 and

higher]). Solubilization was completed by incubation in a boiling water bath for 2 min. After cooling to room temperature, 2 μl of a 0.5 M solution of α-iodoacetamide was added to each sample, and the mixture was incubated for 1 h at 37°C before a 25-μl aliquot was layered into a slot of a polyacrylamide slab gel.

Rabbit globin, porcine chymotrypsinogen, ovalbumin, and bovine albumin were treated in an identical manner and were used as standards for mol wt determinations.

The slab gel (1 mm thick) consisted of a 10–15% acrylamide gradient serving as a resolving gel and a 5% acrylamide stacking gel, both in SDS and buffers as described by Maizel (20). Electrophoresis was for 20 h and at constant current.

After electrophoresis, the slab gel was stained in a solution containing 0.2% Coomassie Brilliant Blue, 50% methanol, and 10% glacial acetic acid for 2 h and then destained in 50% methanol and 10% acetic acid. After destaining, the gel was soaked in the last solution with 5% glycerol added; this was helpful in preventing the gels from cracking during and after drying on Whatman 3 M paper.

AUTORADIOGRAPHY (AR) OF DRIED POLYACRYLAMIDE GELS AND DENSITOMETRIC ANALYSIS OF BANDS: Dried gels were exposed to medical X-ray film (Cronex 2D, du Pont de Nemours and Co., Inc., Wilmington, Del.), generally for a few days. The films were developed by conventional procedures.

The bands in the developed X-ray films were analyzed using a Joyce-Loebl densitometer (Joyce, Loebl and Co., Inc., Burlington, Mass.). The area under the resulting peaks was integrated and used as a quantitative measure for the radioactivity in the bands.

The validity of this quantitation procedure was verified by analyzing the autoradiographs derived from 10, 20, and 30-μl aliquots of a sample loaded into different slots; it was found that the area under each peak in the densitometry tracing was proportional to the amount loaded into the slot.

Preparation of Labeled Secretion Product from MOPC 41 and MOPC 41 DL-1

Freshly excised tumor freed of necrotic portions was sliced into small pieces using a razor blade. The tumor slices were washed several times in 150 mM NaCl, 20 mM Tris·HCl pH 7.4, and 5 mM $MgCl_2$, using centrifugation in a swinging bucket rotor at 500 *g* and at 4°C. Approximately 1 ml of packed tumor slices were resuspended in 3 ml of a medium containing minimal essential medium balanced salt solution for suspension cultures, vitamins, bicarbonate, and glucose as specified by Eagle (11) and supplemented with 50 μl (= 50 μCi) of 15 ^{14}C-amino acids which were part of a reconstituted protein hydrolysate (see above). The resuspended slices were transferred to a 10-ml Erlenmeyer flask, gassed with 95% O_2-5% CO_2, and incubated for 5 h at 37°C. After incubation the tumor slices were centrifuged into a pellet at 1,000 *g*. The supernate was centrifuged for 1 h at 105,000 *g* in a Spinco no. 40 rotor. The resulting supernate contained secreted, radioactively labeled IgG light chains which were precipitated with 1 vol of ice-cold 20% TCA. The ensuing precipitate was prepared for SDS-PAGE as described above.

Electron microscopy

Pellets were fixed in 2% glutaraldehyde in TeaKM for 1 h at 0°C and postfixed in 2% OsO_4 in TeaKM for 1 h at 0°C. The pellets were stained with 0.5% uranyl acetate in acetate-Veronal buffer before dehydration and Epon embedding (12, 18). Sections were cut on a Porter-Blum MT2-B ultramicrotome (Dupont Instruments, Sorvall Operations, Newtown, Conn.) equipped with a diamond knife (Dupont Instruments, Wilmington, Del.). They were stained with uranyl acetate (38) and lead citrate (37) and viewed with a Siemens Elmiskop 101 at 80 kV.

Source of Materials

Oligo (dT) cellulose T-2 from Collaborative Research, Inc., Waltham, Mass. ATP, disodium salt; GTP, sodium salt; creatine phosphate, disodium salt; and creatine phosphokinase, salt-free powder from Sigma Chemical Co., St. Louis, Mo. α-iodoacetamide from Calbiochem, San Diego, Calif. DTT from R. S. A. Corp., Ardsley, N. Y. Coomassie Brilliant Blue and reconstituted protein hydrolysate (^{14}C), algal profile (1 mCi/1 ml) from Schwarz/Mann Div., Becton, Dickinson and Co., Orangeburg, N.Y., bovine pancreatic trypsin, 2× crystallized (185 U/mg), and bovine pancreatic α-chymotrypsin, 3× crystallized (49 U/mg), from Worthington Biochemical Corp., Freehold, N. J.

RESULTS

Cell Fractionation of MOPC 41 DL-1 Tumors

Among the characteristic ultrastructural features of murine myelomas are greatly dilated endoplastic reticulum (ER) cisternae and the occurrence of intracisternal A particles budding from the ER membranes. These features may have contributed to the unusually high density of rough microsomes, the bulk of which were found to band isopynically at 1.75 M sucrose. Some rough microsomes sedimented even through a 2.0 M sucrose cushion (conventionally used in the fractionation of liver cells (3) as a cutoff concentration for preventing sedimentation of rough microsomes) and therefore were present as contaminants in the free ribosome fraction (see Fig. 2). Rough microsomes were further fractionated by detergent treatment to isolate a fraction referred to as

detached ribosomes. Sedimentation profiles of free as well as detached ribosomes in sucrose gradients are shown in Fig. 1. Many of the ribosomes, particularly in the case of detached ribosomes, were in the form of polysomes indicating that RNase action during cell fractionation was minimal. Characteristic for the profile of detached ribosomes (Fig. 1, BR) is the presence of significant amounts of large ribosomal subunits (designated L), whereas the profile of free ribosomes (Fig. 1, FR) is distinguished by the presence of a large amount of monosomes and a small but significant amount of S^N; large ribosomal subunits may be present but may not be resolved from the monosome peak. Furthermore, some material contained in the free-ribosome preparation had sedimented to the bottom of the sucrose gradient tube. This pellet was fixed, stained, sectioned, and inspected by electron microscopy. It showed both free ribosomes as well as rough microsomes (Fig. 2). A fraction collected from the sucrose gradient (Fig. 1, FR) comprising the monosome peak as well as the polysome region was sedimented by centrifugation and prepared for electron microscopy. It showed only free ribosomes without contamination by rough microsomes (electron micrograph not shown). Thus, for purification of free ribosomes, sucrose gradient centrifugation can be used to eliminate rough microsome contamination. Electron microscopy of the rough microsomal fraction showed the characteristic ribosome-studded vesicles (Fig. 3). Occasionally a few lysosomes were seen. Frequently the ER showed a thickening and there were also intracisternal particles characteristic for murine myelomas.

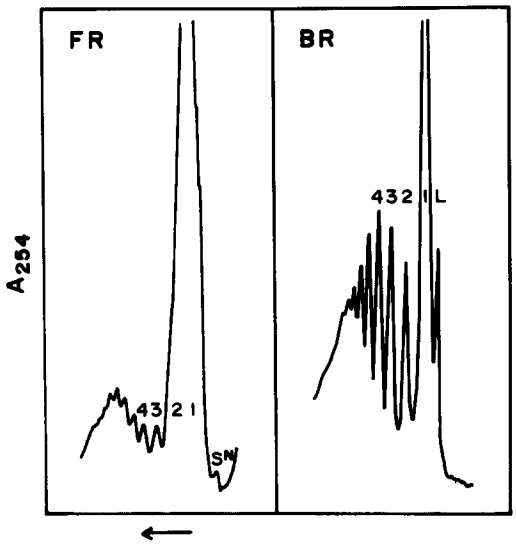

FIGURE 1 Sedimentation profile of free (FR) and detached (BR) ribosomes from MOPC 41 DL-1. Pellets of free and detached ribosomes were resuspended in ice-cold double-distilled water. 0.2-ml aliquots containing 3.0 A_{260} units were layered on 12.5 ml of 10–40% sucrose gradients in 100 mM KCl, 20 mM triethanolamine·HCl pH 7.4 at 20°C, and 3 mM $MgCl_2$. The gradients were centrifuged at 4°C in an SB 283 rotor of an IEC centrifuge for 100 min at 190,000 g_{av}. Fractionation of the sucrose gradients and recording of the optical density were as described (6). Arrow indicates direction of sedimentation. The native small ribosomal subunit peak is designated as S^N, the large ribosomal subunit peak as L, the mono-, di-, tri-, and tetrasome peaks, as 1, 2, 3, and 4, respectively.

In Vitro Translation of mRNA for the Light Chain of IgG

The crude mRNA prepared from rough microsomes as described under Materials and Methods was translated in a heterologous system, developed in this laboratory (13) (henceforth re-

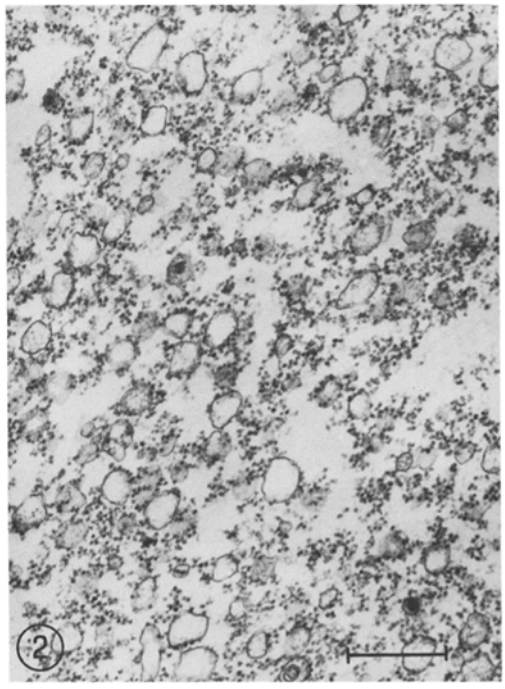

FIGURE 2 Presence of rough microsomes in the free ribosome fraction. Pellet resulting from sucrose gradient centrifugation of free ribosomes (see Fig. 1) was prepared for electron microscopy (see Materials and Methods). × 25,500. The bar denotes 0.5 μm.

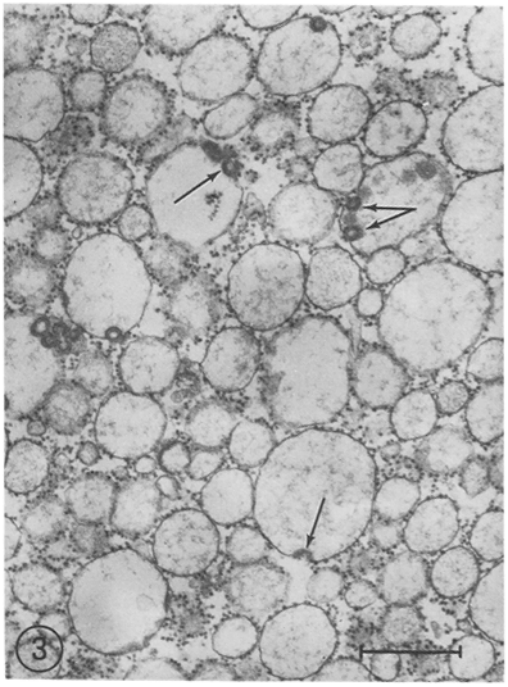

FIGURE 3 Rough microsome fraction was isolated and prepared for electron microscopy as described in Materials and Methods. Arrows point to local thickenings of the ER membrane and to intracisternal A particles. × 25,500. The bar denotes 0.5 μm.

ferred to as initiation system) consisting of S^N from rabbit reticulocytes (as a source of small ribosomal subunits as well as initiation factors), L^O prepared by the puromycin KCl procedure (6) from rat liver free ribosomes, and pH 5 enzymes from Krebs ascites cells. The time-course of polypeptide synthesis in this system in the presence or absence of mRNA is shown in Fig. 4. There was a more than twofold stimulation of polypeptide synthesis in the presence of mRNA. There was inhibition of polypeptide synthesis in the presence of aurintricarboxylic acid (ATA) at a concentration (10^{-4} M) which has been reported to inhibit initiation but not elongation in polypeptide synthesis (17). The extent of inhibition was similar in the absence (data not shown) and in the presence of mRNA.

Analysis of the products by SDS-PAGE and AR (Fig. 5) showed that a prominent radioactive band (slot B) was synthesized by the initiation system in the presence of the crude mRNA fraction (supposed to contain mostly light chain mRNA). This polypeptide has an estimated mol wt of 25,000 and is therefore larger in mol wt by ~4,000 than the secreted light chain of IgG of MOPC 41 DL-1 (slot S), which has a mol wt of 21,000. It was tentatively identified as the "precursor" of the light chain on the basis of work by other laboratories (15, 19, 23, 32, 34–36) showing that the primary translation product of mRNA for the light chain of IgG is longer than the secreted light chain.

In Vitro Translation of mRNA's Contained in Free Ribosomes, Bound Ribosomes, and Rough Microsomes

The time-course of polypeptide synthesis in a readout system, containing either free or detached ribosomes and pH 5 enzymes, is shown in Fig. 6 and is compared to the time-course of mRNA translation in an initiation system (see above). Because pH 5 enzymes contain only small amounts of initiation factors, polypeptide synthesis is essentially completed in the readout system after a 40-min incubation, whereas in the initiation system translation continues for more than 120 min, although at a slower initial rate.

Analysis of the products by SDS-PAGE and AR (Fig. 7) showed that two major products were synthesized by detached ribosomes. One of them (slot B–, upward pointing arrow) is a polypeptide of the same mol wt as the authentic secreted light

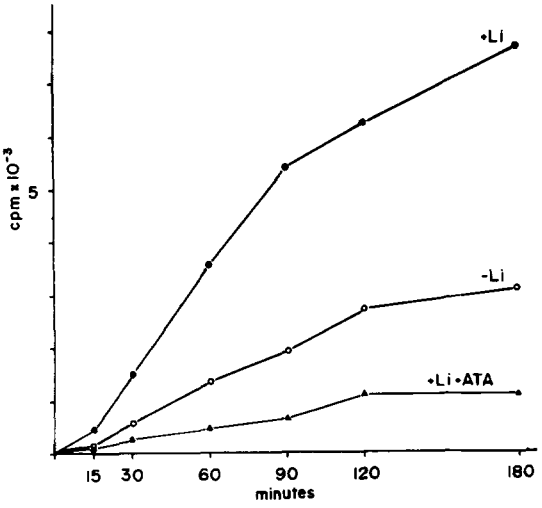

FIGURE 4 Time-course of polypeptide synthesis in an *initiation* system in the presence of mRNA for the light chain of IgG (+Li), in the presence of light chain mRNA and of 10^{-4} M ATA (+Li + ATA), and in the absence of mRNA (−Li). For details, see Materials and Methods.

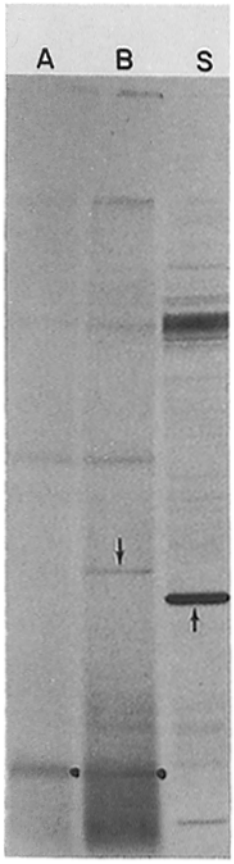

FIGURE 5 Analysis by SDS-PAGE and AR of an experiment of the type described in Fig. 4. Shown are the labeled products synthesized at the 180-min time point in the absence of mRNA (slot A) or in the presence of mRNA for the light chain of IgG (slot B). For comparison the labeled secreted light chain of IgG is shown in slot S (upward pointing arrow). Downward pointing arrow (slot B) indicates precursor of the light chain of IgG. Dots indicate the globin chains.

chain (shown in slot S); the other one (slot B−, downward pointing arrow) has the same mol wt as the precursor of the light chain synthesized in the initiation system (slot A). The products synthesized by crude *free* ribosomes (slot F−) also contained a band of a mol wt identical to that of the light chain of IgG. However, this band was shown to be due to the presence of rough microsomes in the crude free-ribosome fraction, since it was absent when purified free ribosomes collected from a sucrose gradient (see above) were tested in the same manner (data not shown).

It was shown recently that nascent polypeptides synthesized on free ribosomes from rat liver are sensitive to mild proteolysis, except for a fragment of ~40 amino acid residues on the carboxyl terminal which is thought to be protected because of its localization within the large ribosomal subunit (4). Products synthesized by rough microsomes of rat liver, on the other hand, were shown to be largely resistant to proteolytic attack since they are protected by the surrounding ER membrane (29). In agreement with these findings are the results shown in Fig. 7 slots B and F. The two major translation products of detached ribosomes were sensitive to mild proteolysis (slot B+); however, more than 60% (estimated from densitometric analysis) of the band in the position of the authentic light chain of IgG, apparently synthesized by rough microsomes present in the crude free-ribosome fraction, was resistant to proteolytic attack (slot F+; see also Fig. 14 slots A+ and A−).

The synthesis by detached ribosomes of two major polypeptides, one of them with the same mol

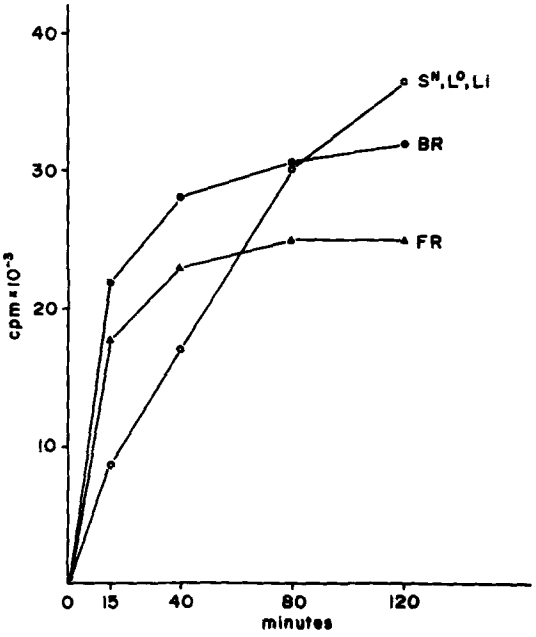

FIGURE 6 Time-course of polypeptide synthesis in a readout system containing 9.8 A_{260} units of crude free (FR) or 5.4 A_{260} units of detached (BR) ribosomes. For comparison the time-course of polypeptide synthesis in an initiation system containing mRNA for the light chain of IgG (S^N, L^O, Li) was included. In the latter case, 50-μl aliquots were counted, whereas in the former 10-μl aliquots were counted.

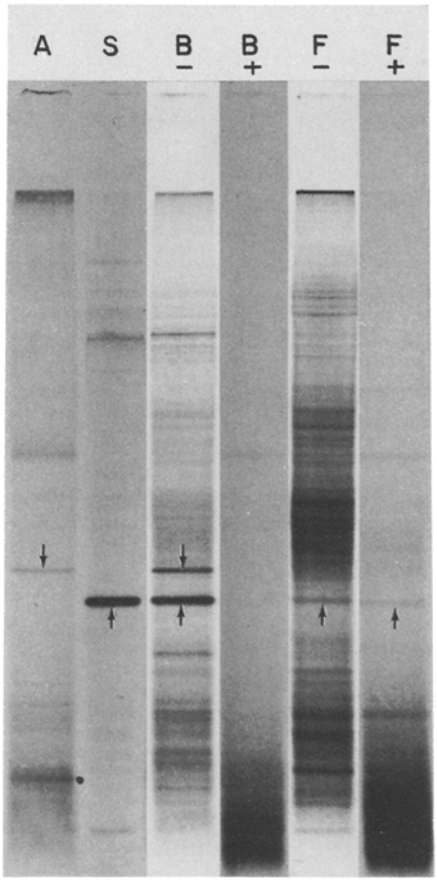

FIGURE 7 Analysis by SDS-PAGE and AR of products synthesized at the 120-min time point as described in Fig. 6 after incubation in the absence or presence of proteolytic enzymes. Shown are the labeled products synthesized in an initiation system in the presence of light chain mRNA (slot A), the labeled secreted light chain (slot S), and the products synthesized in a readout system containing detached ribosomes (slot B-) subsequently subjected to proteolysis (slot B+) or containing crude free ribosomes (slot F−), subsequently subjected to proteolysis (slot F+). Symbols used were as in Fig. 5.

wt as the precursor and the other with the same mol wt as the secreted light chain, suggested that isolated detached ribosomes are heterogeneous with respect to their content of processed and unprocessed nascent light chains. On the basis of the predictions made in the signal hypothesis, one could reason that those ribosomes located near the 5′ end of mRNA should contain unprocessed nascent light chains that still have their signal sequence, while those near the 3′ end should contain already proteolytically processed nascent light chains.

These assumptions were borne out by data obtained from a time-course experiment using detached ribosomes in a readout system; translation was stopped at various time points and the products were analyzed by SDS-PAGE and AR. To insure that there was no initiation in the readout system, a condition which was not met in the previous experiment, ATA was added in a concentration which has been reported (17) to inhibit initiation, but not elongation or release of nascent chains. That such conditions were achieved is demonstrated by the data shown in Fig. 4 and by the lack of inhibition observed in the readout system in the presence of ATA and detached ribosomes (data not shown). From the autoradiograph shown in Fig. 8 it is evident (slots 1 and 6) that at the earlier time points of readout, only processed chains were synthesized, apparently as a result of completion of chains by ribosomes near the 3′ end of mRNA. Only at later time points when ribosomes located further to the left on the mRNA have completed their readout were unprocessed chains synthesized (slots 9, 18, 25, and 50). The data of a quantitative analysis (see Materials and Methods) of this experiment are summarized in Fig. 9. It can be seen that synthesis of already processed chains was essentially completed after a 10-min incubation when there was only a barely detectable synthesis of unprocessed chains; the latter were synthesized only in the following 30 min. no significant synthesis of either chain was observed after a 50-min incubation (data not shown).

While in the preceding experiment initiation had to be ruled out in order to substantiate the conclusion that detached ribosomes contain both processed and unprocessed chains, the following experiment was performed under conditions in which initiation could take place. Such conditions were achieved by adding to the readout system, containing detached ribosomes, increasing amounts of S^N from rabbit reticulocytes as a source of initiation factors (13). Up to twofold stimulation in polypeptide synthesis was observed as a result of the addition of increasing amounts of S^N (Fig. 10). This stimulation could have been the result of the presence of small amounts of globin mRNA present in the S^N fraction. However, this was ruled out by product analysis using SDS-

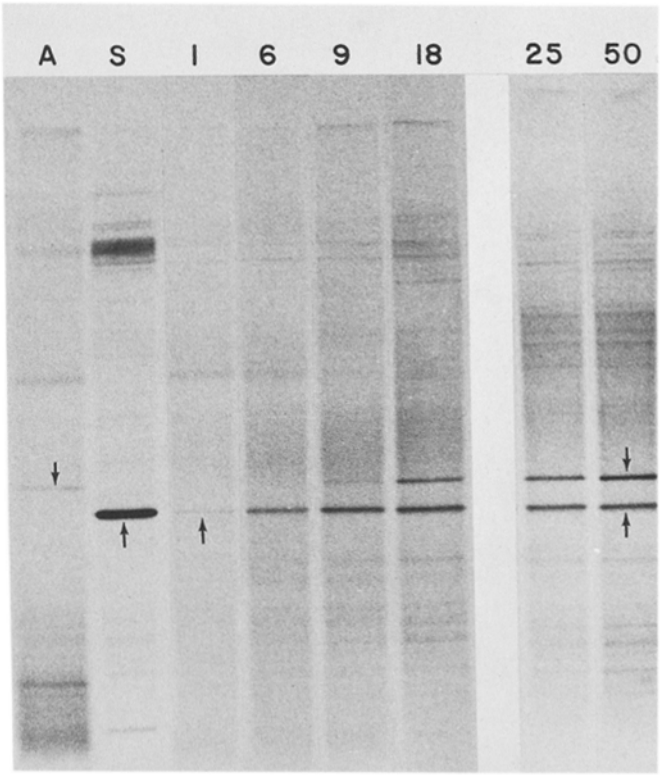

FIGURE 8 Analysis by SDS-PAGE and AR of the products synthesized by detached ribosomes (1.8 A_{260} units) during the course of readout in the presence of 10^{-4} M ATA. Readout was terminated at 1, 6, 9, 18, 25, and 50 min (slots 1, 6, 9, 18, 25, 50, respectively) by cooling 25-μl aliquots to 0°C and adding 25 μl 20% TCA. For comparison, the precursor of the light chain (slot A) synthesized in an initiation system (see Fig. 5) and the labeled secreted light chain (slot S) were included. Symbols used were as in Fig. 5. Slots 25 and 50 were from a separate slab gel.

PAGE and AR (Fig. 11). Only small amounts of globin were synthesized in response to increasing amounts of added S^N. Quantitative analysis (Fig. 12) of the radioactivity in the bands corresponding to the processed and unprocessed light chain of IgG revealed that the stimulation by increasing amounts of S^N can be accounted for by a proportional stimulation in the synthesis of unprocessed chains while there was no stimulation in the synthesis of processed chains.

Finally, readout experiments using isolated rough microsomes were performed. Fig. 13 shows the time-course of polypeptide synthesis in rough microsomes in the absence and presence of ATA, which produced only a slight inhibition of amino acid incorporation. Analysis of the products by SDS-PAGE and AR (Fig. 14) revealed the synthesis of a polypeptide corresponding to the mol wt of the processed light chain of IgG (slot A−). In contrast to the results obtained with detached ribosomes (see above), newly synthesized unprocessed chains were not detected. However, unprocessed light chains were synthesized when in vitro readout of rough microsomes took place in the presence of 10^{-4} M ATA (Fig. 14, slot B−).

The products synthesized in the readout experiment with rough microsomes were subjected to mild proteolysis and subsequently analyzed by SDS-PAGE and AR. As expected, the processed chains, presumably inside the microsomal vesicles, were largely protected from proteolytic attack. Densitometric analysis revealed that ~60% was resistant to proteolysis in agreement with the results shown in Fig. 7, slot F+. However, the

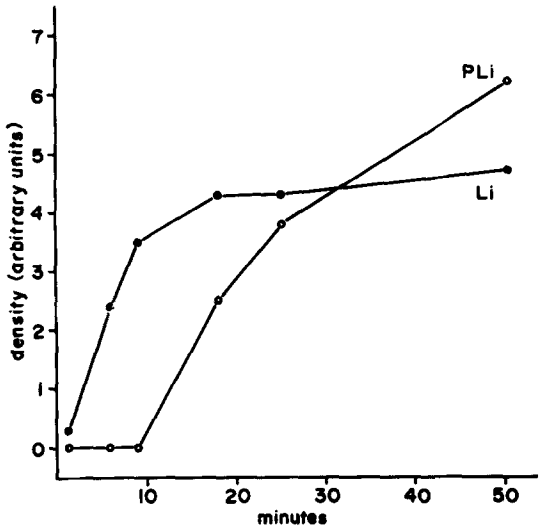

FIGURE 9 Quantitation by densitometry of the autoradiograph shown in Fig. 8. PLi and Li designate the unprocessed (downward pointing arrow in Fig. 8) and the processed light chains of IgG (upward pointing arrow in Fig. 8), respectively.

unprocessed chains synthesized on rough microsomes in the presence of ATA were degraded (Fig. 14, slot B+), supporting the interpretation that these chains were not segregated in the intravesicular space.

DISCUSSION

The results of cell fractionation reported here are in agreement with those of Cioli and Lennox (10). Although our cell fractionation was performed on solid MOPC 41 DL-1 tumors, a conventionally prepared free-ribosome fraction also was found to be contaminated by rough microsomes (see Fig. 2). This contamination was eliminated by isokinetic sucrose gradient centrifugation. Purified free ribosomes did not contain any detectable light chain mRNA activity, although it cannot be ruled out that some light chain mRNA was present and resulted in the synthesis of unprocessed light chains, which overlapped with the presence of other bands in the autoradiograph (see Fig. 7, slot F−).

Upon in vitro translation in an initiation system (see Materials and Methods) of a crude mRNA fraction containing the mRNA for the light chain of IgG, a product was synthesized which was larger by ~4,000 mol wt than the authentic secreted light chain. Similar results have been reported by several laboratories (15, 19, 23, 32, 34–36).

The majority of the data presented in this paper were obtained from in vitro translation of the light chain mRNA contained in the isolated rough microsome and detached ribosome fractions of MOPC 41 DL-1. It was shown here that both fractions contain unprocessed light chains (i.e., chains still containing the signal sequence) together with processed light chains, demonstrating that removal of the signal sequence in vivo takes place well before the nascent chain is completed. However, the difference between these two fractions is that in vitro only rough microsomes retain their ability for proteolytic removal of the signal sequence. Thus, in vitro completion of their nascent chains also results in concomitant proteolytic removal of the signal sequence from their unprocessed chains, and thus in the synthesis of only processed chains (see Fig. 14, slot A−). In contrast, detached ribosomes, having lost their ability

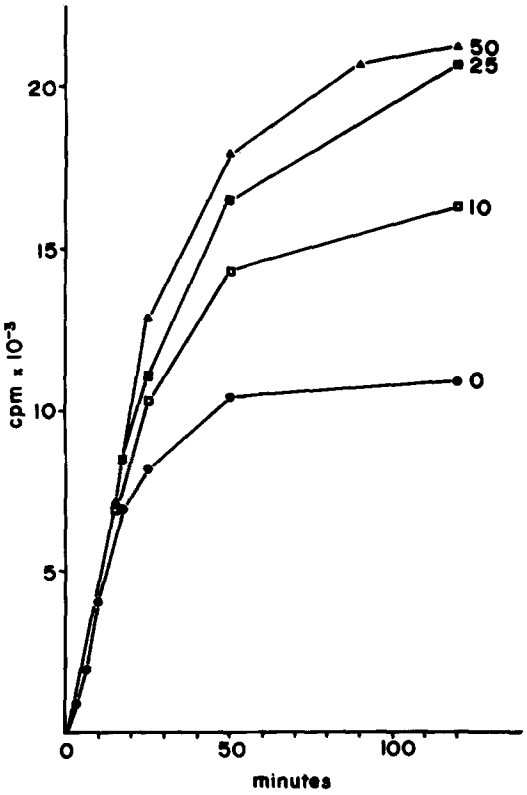

FIGURE 10 Time-course of polypeptide synthesis in a readout system containing detached ribosomes (1.8 A_{260} units) and S^N from reticulocytes as a source for initiation factors. Numbers next to curves designate the amount (in microliters) of S^N (10.5 A_{260} units/ml) present in the 250-μl assay.

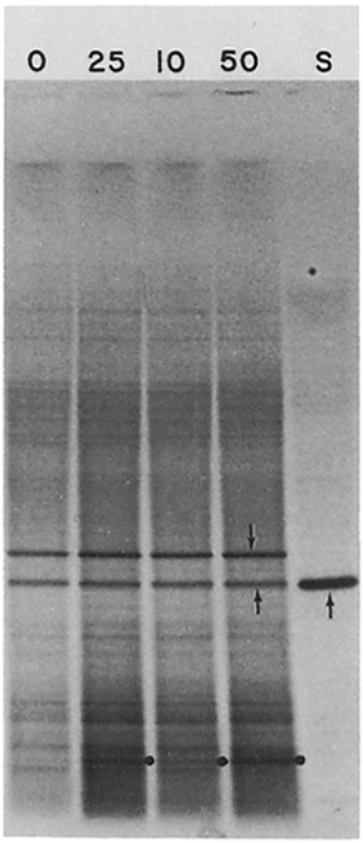

FIGURE 11 Analysis by SDS-PAGE and AR of the products synthesized at 120-min time point as described in Fig. 10. Slot numbers refer to microliters of S^N present in the readout system with detached ribosomes (see Fig. 10). For comparison, the labeled secreted light chain of IgG is included in slot S. Symbols used were as in Fig. 5

for in vitro processing, yielded both unprocessed as well as processed light chains upon completion of their nascent chains in vitro. This result can be rationalized by assuming that the processing activity is part of the membrane and was lost during the preparation of detached ribosomes by detergent solubilization of rough microsomes. Alternatively, the processing enzyme(s) may still be present in isolated detached ribosomes but in an inactivated form or may become inactivated rapidly during incubation in the readout system.

The data presented here also indicated that those polysomal ribosomes containing unprocessed chains are located on the mRNA to the left of those containing already processed chains (Figs. 8 and 9). Furthermore, the distribution of radioactivity between unprocessed and processed chains (more than one half in the former, see Fig. 9) indicated that probably more than one of the polysomal ribosomes contain unprocessed chains. This suggested that removal of the signal sequence occurs only after the ribosome has already translated a considerable portion of the mRNA. If removal of the signal sequence is an endoproteolytic event and if the processing activity is localized in the membrane *trans* rather than *cis* with respect to the ribosome-membrane junction, then the entire signal sequence would be required to have traversed the 70-Å distance which comprises the thickness of the ER membrane before processing could take place. Assuming that the signal sequence comprises ~20 amino acid residues, and adding to these ~19 and ~39 residues which comprise the portions of the nascent chain (3.6 Å per residue in the extended configuration) in the membrane and in the ribosome (4), respectively, then a total of ~78 amino acid residues have to be polymerized before processing can take place. Since the interribosomal distance on a ribosome-saturated polysome amounts to 90 nucleotides, or 30 codons, it is possible for the mRNA to accommodate two to three ribosomes

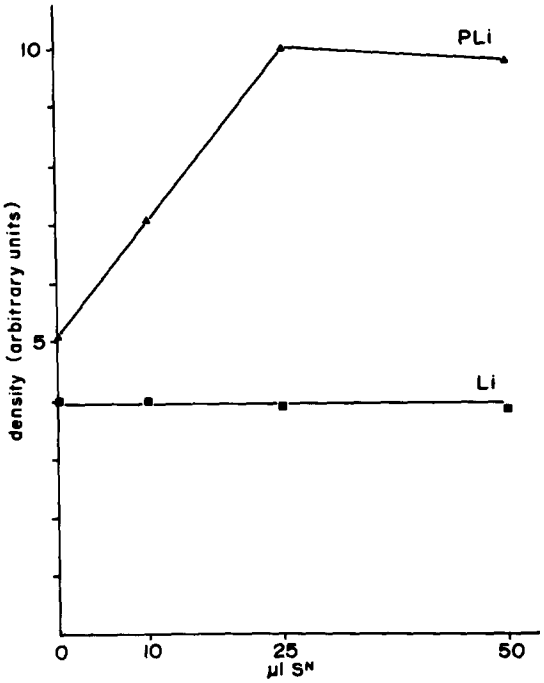

FIGURE 12 Quantitation by densitometry of autoradiograph shown in Fig. 11. PLi and Li designate the unprocessed and processed chains of IgG, respectively.

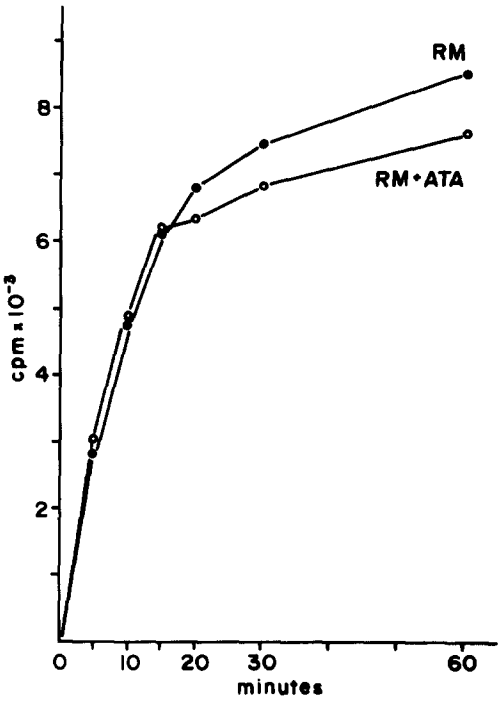

FIGURE 13 Time-course of polypeptide synthesis in the absence of ATA (RM) or presence (RM+ATA) of 10^{-4} M ATA in a readout system containing rough microsomes (4.3 A_{260} units).

still containing the signal terminal of their nascent chain, i.e., containing unprocessed chains.

The synthesis by detached ribosomes of unprocessed chains of the same mol wt as the translation product of light chain mRNA in a heterologous reconstituted system suggests that the latter is not an in vitro artifact. It could have been argued, otherwise, that translation of the light chain mRNA, which has been performed so far in all cases in heterologous systems, resulted in artifactual initiation at some point to the left of the initiation codon and therefore caused the synthesis of a larger precursor protein, while in vivo such a precursor protein would not have been synthesized.

By using ATA to inhibit initiation but not elongation and release of the nascent chain, it was clearly ruled out that the synthesis of unprocessed chains by detached ribosomes reflected in vitro initiation rather than completion of unprocessed chains. Conversely, the observed twofold stimulation of polypeptide synthesis by detached ribosomes, in a readout system which was supplemented by initiation factors, was shown to be entirely the result of increased synthesis of unprocessed chains, while the level of synthesis of processed chains was not affected. This result also demonstrated that initiation factors from rabbit reticulocytes which contain predominantly free ribosomes were able to perform in the translation of the light chain mRNA contained in bound ribosomes, supporting the contention that identical initiation factors are used for translation on free and bound ribosomes.

It should be noted that the presence of unprocessed chains in rough microsomes was detected only if completion of their nascent chains in vitro occurred in the presence of ATA. This result is of particular interest since Borgese et al. (8) have recently shown that ribosomes will not bind to stripped membranes in vitro in the presence of ATA. This result can therefore be rationalized as

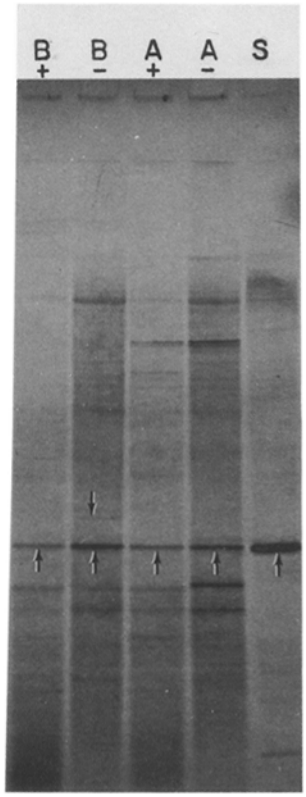

FIGURE 14 Analysis by SDS-PAGE and AR of the products synthesized at the 60-min time point as described in Fig. 13 and subsequently incubated in the absence or presence of proteolytic enzymes. Products synthesized in the absence or presence of 10^{-4} M ATA are shown in slots A and B, respectively. Products not subsequently incubated with proteolytic enzymes (see Materials and Methods) are shown in slots marked (−), those incubated in slots marked (+). For comparison the labeled secreted light chain of IgG is shown in slot S.

follows: Those ribosomes located farthest to the left on the mRNA in a rough microsome are not yet attached to the membrane. In the absence of ATA, attachment of these ribosomes as well as subsequent processing of their nascent chains can occur in vitro during chain completion, resulting in the synthesis of processed chains. In the presence of ATA, however, these ribosomes will not be able to attach. This in turn will deprive their nascent chains of access to the processing activity, but will not interfere with their completion in vitro. Alternatively, it is also possible that the synthesis of unprocessed chains in the presence of ATA does not result only from an interference in establishing the ribosome-membrane junction but is due to a direct inhibition of the processing activity by ATA.

Finally, it was demonstrated here that the microsomal membrane in the rough microsome fraction provides protection for the completed and released chain against proteolysis. Although protection of newly synthesized chains in microsomes (isolated from rat liver) has been demonstrated previously (29), it was assayed not by product analysis but by measuring the percentage of acid-insoluble radioactivity remaining after proteolysis. However, since this protection was observed to be significantly less than 100%, this type of assay could not distinguish between protection resulting from partial hydrolysis of all chains or resulting from a combination of complete resistance of some chains and complete hydrolysis of others. Using product analysis by SDS-PAGE and AR after proteolysis, it was established here (see also companion paper) that the protection of the majority of the product was complete in that there was no reduction in its mol wt. This constitutes important information since resistance to proteolysis (29) is at present the only rigorous assay for vectorially discharged chains. The original centrifugation assay (showing that newly made chains were sedimented with the membranes or were solubilized by detergent treatment) which was used to demonstrate vectorial discharge (26, 27) has subsequently been shown to be inadequate (8, 9, 31).

It should be emphasized that most of our conclusions remain to be confirmed by further characterization of the in vitro translation products, in particular of the band which we have assumed to be the unprocessed precursor of the light chain of IgG (on the basis of its mol wt). However, preliminary data[2] obtained by sequence analysis of 50 amino terminal residues of this putative precursor protein support our conclusions.

[2] Devillers-Thiery, A., G. Blobel, and T. J. Kindt. Manuscript in preparation.

An Hypothesis for the Transfer of Proteins across Membranes

Most of the data which led to the formulation of an hypothesis for the transfer of proteins across membranes, referred to henceforth as the signal hypothesis, have been reviewed previously (5) and therefore will not be discussed here. Alternative hypotheses have been summarized previously (30) and will be omitted here. Instead, a more detailed version of the signal hypothesis than that presented previously (5), based on theoretical considerations as well as recent data from this and other laboratories, will be outlined.

As mentioned above, the essential feature of the signal hypothesis (illustrated in Fig. 15) is the occurrence of a unique sequence of codons, located immediately to the right of the initiation codon, which is present only in those mRNA's whose translation products are to be transferred across a membrane. No other mRNA's contain this unique sequence. Translation of the signal codons results in a unique sequence of amino acid residues on the amino terminal of the nascent chain. Emergence of this signal sequence of the nascent chain from within a space in the large ribosomal subunit triggers attachment of the ribosome to the membrane, thus providing the topological conditions for the transfer of the nascent chain across the membrane.

Following is an attempt to formulate this sequence of events in greater detail. It is suggested that translation of mRNA's containing signal codons begins on a free ribosome. Thus, initiation of translation of all mRNA's whether or not they contain signal codons proceeds by the same mechanism, eliminating the need for specialized ribosomes or initiation factors. Similarly, elongation will proceed on a free ribosome for both categories of mRNA's until anywhere from 10 to 40 amino acid residues of the nascent chain have emerged from the ribosome. Only at this point is the membrane able to distinguish between amino terminals of nascent chains as containing or not containing the unique signal sequence. If they lack the signal sequence, attachment of the ribosome to the membrane will not occur. If they contain the signal sequence, attachment may occur, but does not necessarily follow. It is conceivable, for example, that the availability of ribosome binding sites

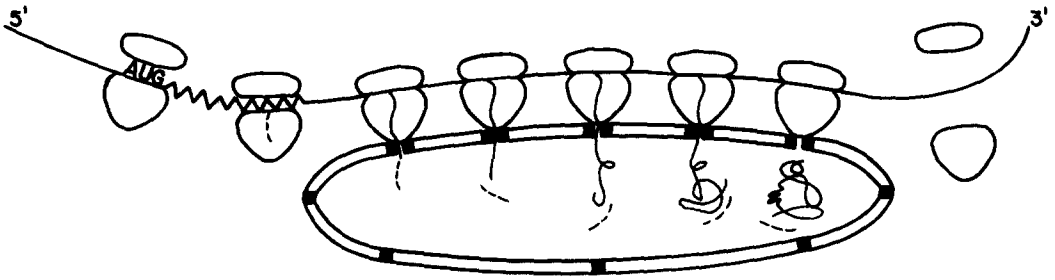

FIGURE 15 Illustration of the essential features of the signal hypothesis for the transfer of proteins across membranes. Signal codons after the initiation codon AUG are indicated by a zig-zag region in the mRNA. The signal sequence region of the nascent chain is indicated by a dashed line. Endoproteolytic removal of the signal sequence before chain completion is indicated by the presence of signal peptides (indicated by short dashed lines) within the intracisternal space. For details see text.

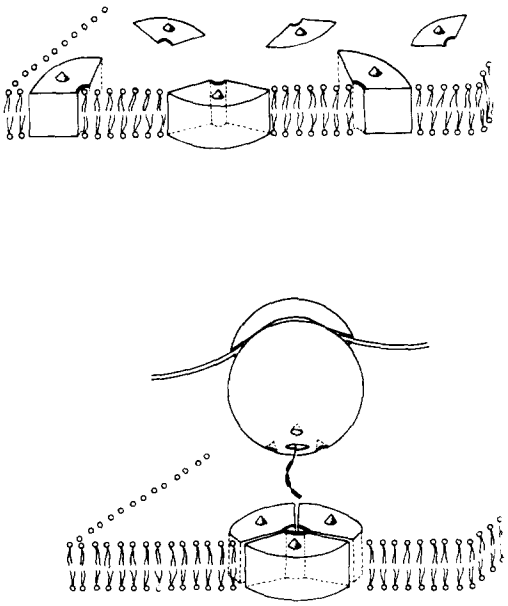

FIGURE 16 Hypothetical model for the formation of a transient tunnel in the membrane through which the nascent chain would be transferred. Specific regions of the signal sequence (indicated by line thickenings) of the nascent chain emerged from the large ribosomal subunit tunnel are recognized by one site each (indicated by a line thickening) on three membrane proteins. Recognition results in loose association of these proteins, subsequently "cross-linked" by interaction of sites on the large ribosomal subunit (indicated by notches around the tunnel exit on the large ribosomal subunit) with one site each on the three membrane proteins (indicated by cones). For details see text.

in the membrane may be limiting, allowing translation of signal sequence-containing peptides to continue to completion on nonattached ribosomes. In this event, chains containing the signal sequence would be released into the "soluble" compartment. These chains may be rapidly degraded, since they may be unable to assume their distinct native structure through the enzymatic modifications which are confined to the intracisternal space (e.g., proteolytic removal of the signal sequence or glycosylation). If, on the other hand, ribosome attachment occurs, translation will continue on a bound ribosome until the nascent chain is released and vectorially discharged. It is suggested that after discharge of the completed chain, the ribosome is detached from the membrane (evidence for the existence of a detachment factor will be presented elsewhere[3]). The released ribosome may again start translation of any mRNA, independent of whether the latter does or does not contain signal codons.

Ribosome attachment to, as well as detachment from, the membrane are likely to involve a complex sequence of events. A number of theoretical considerations form the basis for proposing the following model (illustrated in Fig. 16). The signal sequence of the nascent chain emerging from within a tunnel in the large ribosomal subunit may dissociate one or several proteins which have been found to be associated with the large ribosomal subunit of free ribosomes (7, 14, 22). Dissociation of these proteins may in turn uncover binding sites on the large ribosomal subunit. At the same time the emerging signal sequence also recruits two or more membrane receptor proteins and causes their loose association so as to form a tunnel in the membrane (see Fig. 16). This association is stabi-

[3] Blobel, G. Manuscript in preparation.

lized by each of these membrane receptor proteins interacting with the exposed sites on the large ribosomal subunit, with the latter playing the role of a cross-linking agent. Binding of the ribosome would link the tunnel in the large ribosomal subunit with the newly formed tunnel in the membrane in continuity with the transmembrane space. After release of the nascent chain into the transmembrane space, ribosome detachment from the membrane would eliminate the crosslinking effect of the ribosome on the membrane receptor proteins. The latter would be free again to diffuse as individual proteins in the plane of the membrane. As a result of their disaggregation, the tunnel would be eliminated. The tunnel, therefore, would not constitute a permanent structure in the membrane.

Recognition of the signal sequence by membrane receptor proteins may require precise synchronization with translation. Thus if, after emergence of the signal sequence from within the ribosome, translation were to continue without concomitant ribosome attachment, folding of the signal sequence with contiguous sequences of the nascent chain might effectively prevent subsequent ribosome membrane attachment. For this reason we postulate confluence of the large subunit and membrane tunnel, rather than other conceivable arrangements in order to provide topological conditions which would prevent the formation of secondary structures of the nascent chain at the ribosome membrane junction.

The model described above thus provides for a binding of the ribosome to the membrane which is functional, specific, and transient, that is limited in time as well as in space. Functional binding of the ribosome is coupled to tunnel formation in the membrane. By definition, then, nonfunctional binding does not involve tunnel formation and therefore does not provide the topology for the transfer of the nascent chain across the membrane. Functional binding also is specific in that it is limited only to those ribosomes which carry nascent chains containing the signal sequence. Finally, it is limited in time, in that it is linked to ongoing translation and it is limited in space, in that it occurs only on those membranes which contain ribosome binding sites and which possess sufficient fluidity to permit specific aggregation of these sites.

It should be noted that the signal hypothesis does not call for a direct attachment of mRNA to the membrane. In fact, it predicts that if initiation of protein synthesis were blocked, while elongation and release were to proceed, mRNA containing signal codons would be found in the soluble compartment of the cell rather than on rough microsomes. Cell fractionation then would recover these mRNA's in the free ribosome fraction, most likely in the form of mRNP's or bound to a free ribosome.

The original version of the signal hypothesis did not deal with the fate of the signal sequence. However, since the amino terminal sequence is different among the authentic secretory proteins, removal of the signal sequence before actual secretion was implied. It has been suggested (23) that the extra sequence in the amino terminal of the light chain precursor is removed by a membrane-associated activity. Data relevant to the proteolytic processing of the nascent chain are presented in this and in the following paper.

As already mentioned, the sequence of events suggested in the signal hypothesis may not be restricted to secretory proteins but may apply to the synthesis of all proteins which have to be transferred across a membrane. Thus, the mRNA's for lysosomal and peroxysomal proteins may contain identical signal codons as the mRNA's for secretory proteins, if the ribosome-ER junction is utilized for their transfer across the ER membrane. The same reasoning may apply to the synthesis of certain mitochondrial proteins which are synthesized in the cytoplasm. A junction of cytoplasmic ribosomes with the outer mitochondrial membrane has been described (16) and could be utilized for the transfer of these proteins across the outer mitochondrial membrane, presumably involving specific signal sequences and membrane recognition sites which are different from those of secretory proteins and the ER membrane, respectively. Finally, the synthesis of some membrane proteins may require transfer through the membrane before the protein could be inserted into the membrane. Depending on what particular membrane proteins would be required to be transferred for subsequent insertion, signal sequences and corresponding membrane recognition sites utilized for secretory and mitochondrial proteins could be involved. Other membrane proteins, in particular in those instances in which the site of synthesis is not separated from the site of insertion by the lipid bilayer, may be synthesized on free ribosomes.

These considerations make it evident that differ-

ent signal sequences may exist. Moreover, the signal sequence for one group of proteins, e.g. for secretory proteins, may not be identical in all cases. Phylogenetic as well as ontogenetic variability may exist. Only after the sequence of a sufficient number of "signals" has been established will it be possible to recognize those sequence features which are essential for their postulated function, namely recognition by membrane binding sites. In addition, the signal sequence should contain information for its correct removal by the processing enzyme(s). One could therefore envision altered signal sequences which will not be recognized by membrane binding sites, with the result that transfer across the membrane could not take place. Likewise, an altered sequence may still be recognized by the membrane and result in transfer of the protein across the membrane but it may not serve as a substrate for the processing enzyme. The latter condition may be physiological for some proteins (for instance those proteins which need to retain the signal sequence for their proper function), pathological for others. The former condition, however, may be entirely pathological, since it would not lead to transfer across the membrane.

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