TRANSFER OF PROTEINS ACROSS MEMBRANES

II. Reconstitution of Functional Rough Microsomes from Heterologous Components

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ABSTRACT

The data presented in this paper demonstrate that native small ribosomal subunits from reticulocytes (containing initiation factors) and large ribosomal subunits derived from free polysomes of reticulocytes by the puromycin-KCl procedure can function with stripped microsomes derived from dog pancreas rough microsomes in a protein-synthesizing system in vitro in response to added IgG light chain mRNA so as to segregate the translation product in a proteolysis-resistant space. No such segregation took place for the translation product of globin mRNA. In addition to their ability to segregate the translation product of a specific heterologous mRNA, native dog pancreas rough microsomes as well as derived stripped microsomes were able to proteolytically process the larger, primary translation product in an apparently correct manner, as evidenced by the identical mol wt of the segregated translation product and the authentic secreted light chain. Segregation as well as proteolytic processing by native and stripped microsomes occurred only during ongoing translation but not after completion of translation. Attempts to solubilize the proteolytic processing activity, presumably localized in the microsomal membrane by detergent treatment, and to achieve proteolytic processing of the completed light chain precursor protein failed.

Taken together, these results establish unequivocally that the information for segregation of a translation product is encoded in the mRNA itself, not in the protein-synthesizing apparatus; this provides strong evidence in support of the signal hypothesis.

There are numerous reports in the literature on the in vitro reconstitution of rough microsomes from "stripped" microsomes and either polysomes, ribosomes, or ribosomal subunits (for review see discussion in reference 4). The difficulty inherent in this type of experiment is to distinguish between "nonfunctional" and "functional" ribosome binding to microsomal membranes. The latter would be a very complex process if the sequence of events suggested in the signal hypothesis were proven to be correct; functional binding would be triggered by the signal sequence of the nascent chain emerging from the ribosome (2). On the other hand, nonfunctional binding of ribosomes to membrane proteins could occur in this scheme in the absence of the signal sequence but would not result in transfer of the nascent chain across the membrane. Thus, according to the signal hypothesis functional

binding in vitro would require a stringent set of conditions: it would occur only during protein synthesis and it would result only from the translation of those mRNA's which contain the signal codon sequence. If functional binding took place, then the translation products of these mRNA's should be vectorially discharged and segregated in the intravesicular space, where they should be "processed" and resistant to mild proteolysis. The latter can therefore be employed to assay for functional binding.

The data reported in this paper provide evidence that functional reconstitution of rough microsomes from heterologous components can be achieved in vitro in a manner compatible with the predictions made in the signal hypothesis.

METHODS

Preparation of Rough Microsomes from Dog Pancreas

Fractionation of dog pancreas will be described elsewhere.[1] In brief, a postmitochondrial supernate was loaded over three 5-ml layers of 2.0 M, 1.75 M, and 1.5 M sucrose in 50 mM triethanolamine·HCl pH 7.4 at 20°C, 50 mM KCl, and 5 mM $MgCl_2$ (TeaKM).[2] After centrifugation for 24 h at 140,000 g_{av} in an angle rotor (type A-211 of the IEC centrifuge [Damon/IEC Div., Damon Corp., Needham Heights, Mass.]), the 1.75 M sucrose-TeaKM layer containing the rough microsomes was removed with a syringe, diluted with 1 vol of TeaKM, and layered over 2 ml of 1.3 M sucrose-TeaKM. Centrifugation for 30 min at 100,000 g_{av} yielded a pellet of rough microsomes. Pellets were stored frozen at −80°C for several months without loss of activity in the protein-synthesizing systems.

For protein synthesis in vitro, pellets of rough microsomes were resuspended in 0.25 M sucrose, 100 mM KCl, 20 mM HEPES·KOH pH 7.3 at 20°C, 3 mM $MgCl_2$, and 2 mM dithiothreitol (DTT).

Preparation of Stripped Microsomes

EDTA PROCEDURE: Pancreatic rough microsomes were suspended in ice-cold 50 mM triethanolamine·HCl pH 7.4 at 20°C and 50 mM KCl (TeaK) to a concentration of 100 A_{260} units/ml). A 0.2 M solution of EDTA (pH 7.0) was added to a concentration of 3 μmol EDTA per 10.0 A_{260} units of rough microsomes. 0.5-ml aliquots of this suspension were layered onto 12.5 ml of 10-55% sucrose gradients in TeaK. The gradients were centrifuged for 2 h at 2°C and at 190,000 g_{av} in the swinging bucket rotor SB 283 of the IEC centrifuge. A turbid band comprising EDTA-stripped rough microsomes (RM-EDTA) was visible in the lower third of the gradient at the level of ~40-45% sucrose. In the A_{254} recording, this peak was well separated from the ribosomal subunit peaks in the upper third of the gradient. The RM-EDTA fraction was collected, diluted with 2 vol of TeaK and centrifuged for 30 min at 100,000 g_{av} in a Spinco no. 40 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). 100 A_{260} units of RM yielded 52.0 A_{260} of RM-EDTA. The pellets were stored frozen at −80°C. Before use, the RM-EDTA pellet was resuspended by brief sonication in 0.25 M sucrose, 100 mM KCl, 20 mM HEPES·KOH pH 7.3 at 20°C, 3 mM $MgCl_2$, and 2 mM DTT. In one case, resuspended RM-EDTA was "heat inactivated" (4) by incubation for 15 min at 55°C.

PUROMYCIN-KCL PROCEDURE: This procedure was essentially that described by Adelman et al. (1). Pellets of pancreatic RM were resuspended in 500 mM KCl, 50 mM triethanolamine·HCl pH 7.4, 2 mM $MgCl_2$, and 1 mM DTT (HSB) containing 1 mM puromycin (pH 7.0) to a concentration of 100 A_{260} units of rough microsomes per ml. After incubation for 10 min at 37°C, 0.5-ml aliquots of this suspension were layered onto 12.5 ml of 10-40% sucrose gradients in HSB. The gradients were centrifuged in an SB 283 rotor of the IEC centrifuge for 1.5 h at 20°C and at 190,000 g_{av}. A turbid band, more diffuse than in the EDTA procedure, was visible in the lower half of the gradient, but was well separated from the ribosomal subunits seen in the A_{254} recording in the upper half of the gradient. The turbid band of stripped microsomes was collected, diluted with 2 vol of HSB, and layered over 2 ml of 0.7 M sucrose in TeaKM. Centrifugation for 30 min at 105,000 g_{av} in a Spinco no. 40 rotor yielded a pellet of puromycin-KCl-stripped rough microsomes (RM-PURO). 100 A_{260} units of rough microsomes yielded 28.0 A_{260} units of RM-PURO. The lower A_{260} yield in the puromycin-KCl procedure is due to the more complete removal of ribosomal subunits, while in the EDTA procedure a significant amount of large ribosomal subunits remained bound to the membrane (7). Before use, RM-PURO pellets were resuspended by brief sonication as described above for EDTA-stripped microsomes.

All other procedures were detailed in the preceding paper (2).

RESULTS AND DISCUSSION

The choice of components for the in vitro reconstitution experiments described in this paper was

[1] Scheele, G., and G. Blobel. Manuscript in preparation.

[2] *Abbreviations used in this paper:* AR, autoradiography; DTT, dithiothreitol; HSB, 500 mM KCl, 50 mM triethanolamine·HCl pH 7.4, 2 mM $MgCl_2$, and 1 mM DTT; L^0, derived large ribosomal subunits; PAGE, polyacrylamide gel electrophoresis; RM-PURO, puromycin-KCl-stripped rough microsomes; S^N, native small ribosomal subunits; SDS, sodium dodecyl sulfate; TeaK, 50 mM triethanolamine·HCl pH 7.4 at 20°C and 50 mM KCl; TeaKM, 50 mM triethanolamine·HCl pH 7.4 at 20°C, 50 mM KCl, and 5 mM $MgCl_2$.

based on the predictions made in the signal hypothesis (2, 3). As representatives of mRNA's postulated to contain or to lack the signal codon sequence, we chose the mRNA for the light chain of IgG, isolated from rough microsomes of murine myeloma MOPC 41 or MOPC 41 DL-1 and the mRNA's for the two globin chains, isolated from free polysomes of rabbit reticulocytes, respectively. Since it is postulated that free and bound ribosomes are interchangeable, we deliberately employed ribosomal subunits derived from free ribosomes of rat liver or rabbit reticulocytes. Rough microsomes and derived stripped microsomes were isolated from dog pancreas. Thus, not only were the components derived from cells of different types, but also from different species, in an attempt to demonstrate the widespread equivalence of the sites involved in reconstitution.

Our first attempt was aimed only at a "partial" reconstitution. We were interested in finding out whether rough microsomes of dog pancreas could be utilized in vitro for the translation of heterologous mRNA's such as globin and light chain mRNA's. Rough microsomes were therefore incubated in a "readout" system containing light chain mRNA and the small amount of initiation factors present in the pH 5 enzymes. Both the time-course as well as the final level of polypeptide synthesis (Fig. 1) were similar in the presence or absence of light chain mRNA. For comparison, the time-course of translation in an "initiation" system in the absence of rough microsomes is also shown in Fig. 1. The stimulation by mRNA is much less in this experiment than that observed previously (2) where large ribosomal subunits from rat liver free ribosomes rather than from reticulocyte ribosomes (in this experiment) were used in the initiation system. It was found (G. Blobel and B. Dobberstein, unpublished observations) that large subunits prepared from polysomes of a variety of cells contain significant amounts of mRNA, which are translated in the initiation system. In the present experiment the use of large ribosomal subunits derived from rabbit reticulocyte polysomes introduced significant amounts of globin mRNA. The latter apparently competed more efficiently in our initiation system with the added light chain mRNA than the liver mRNA's introduced in the previous experiments (2) with the large ribosomal subunits from liver ribosomes. The result is a greater stimulation of polypeptide synthesis by light chain mRNA in the initiation system containing large ribosomal subunits from rat liver rather than from rabbit reticulocytes. Nevertheless, light chain mRNA is translated in both cases. Again, the translation product of light chain mRNA (Fig. 2, slot B) is larger by ~4,000 in mol wt than the secreted light chain (Fig. 2, slot S). Most striking, however, were the results of translation of light chain mRNA in the presence of pancreatic rough microsomes. Its translation product was not found in the position of the light chain precursor (compare slot B with slots C and D); instead, it was found to have the same size as the secreted light chain (compare slots C and S), indicating that the light chain precursor had been proteolytically processed when pancreatic rough microsomes were present. The fact that the proteolytically processed precursor protein had the same mol wt as the authentic secreted light chain suggested that pancreatic rough microsomes were able to carry out correctly the proteolytic processing of a heterologous precursor protein.

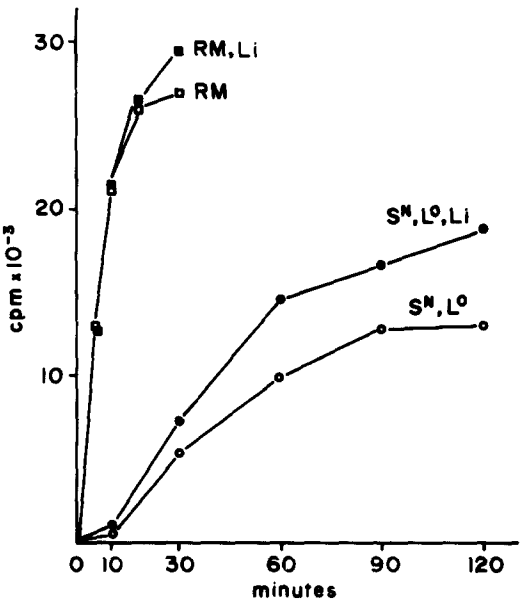

FIGURE 1 Time-course of polypeptide synthesis in an *initiation* system in the presence (S^N, L^o, Li) or absence (S^N, L^o) of light chain mRNA or in a readout system containing pancreatic rough microsomes and no added mRNA (RM) or added light chain mRNA (RM, Li).

These results suggested that the light chain mRNA employed a functional ribosome-membrane junction for the transfer of its nascent chain into the intracisternal space while it was being

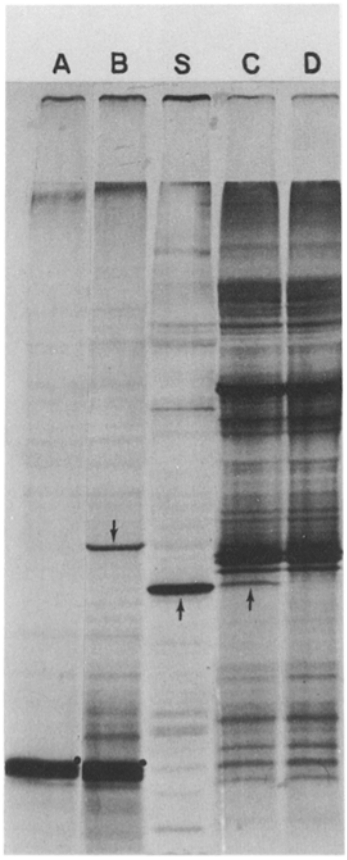

FIGURE 2 Analysis by SDS-PAGE and AR of the products synthesized as described in Fig. 1. Shown are labeled products synthesized either in an initiation system (at the 120-min time point) without added mRNA (slot A) or with added light chain mRNA (slot B) and in a readout system (at the 30-min time point) containing pancreatic rough microsomes without added mRNA (slot D) or with added light chain mRNA (slot C). For comparison the labeled secreted light chain of IgG is shown in slot S. The latter as well as the isolated light chain mRNA were isolated from MOPC 41 tumor. Downward pointing arrow indicates the unprocessed precursor of the light chain of IgG while upward pointing arrows indicate both the secreted light chain (slot S) as well as the processed precursor of the light chain (slot C). Dots designate the globin chains.

translated. Thus, the light chain mRNA may have engaged those microsomal ribosomes which were nonfunctionally bound to the membrane during cell fractionation in low salt concentrations and at 0°C but were readily detached from the membrane during incubation at the higher salt concentrations and the higher temperature (37°C) required for in vitro protein synthesis. Recruitment of these ribosomes would have been followed by the establishment of a functional ribosome-membrane junction (see discussion of companion paper) and therefore to a reconstitution of rough microsomes from ribosomes and membranes homologous to each other but triggered by a heterologous mRNA.

Before presenting further reconstitution experiments resting on this interpretation, it is necessary to deal with a mutation in the MOPC 41 line. The preceding experiment was performed with light chain mRNA isolated from rough microsomes of the original MOPC 41 tumor. However, mRNA isolated from MOPC tumor after several transfers (see Materials and Methods section of preceding paper) and translated under conditions as de-

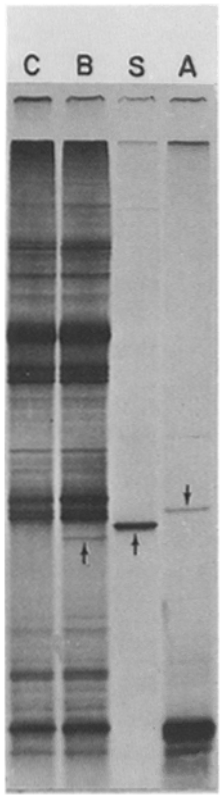

FIGURE 3 Analysis by SDS-PAGE and AR either of products synthesized in a readout system containing pancreatic rough microsomes and no added mRNA (slot C) or light chain mRNA (slot B) or of products synthesized in an initiation system containing light chain mRNA (slot A). Light chain mRNA was isolated from MOPC 41 DL-1 tumor while labeled secreted light chain (slot S) was from MOPC 41 tumor.

scribed in the previous experiment yielded a translation product (Fig. 3, slot A) which was smaller by about 2,000 mol wt. Moreover the size of the processed translation product (Fig. 3, slot B) was also smaller by about 2,000 mol wt than the secreted light chain (Fig. 3, slot S), casting doubt on our conclusion from the preceding experiment, that pancreas rough microsomes correctly process a foreign nascent chain. However, the secreted light chains used for comparison in Fig. 3 (slot S) were prepared from the original MOPC 41 line. If proteolytic processing by pancreatic rough microsomes were indeed correct, then it should follow that the secreted light chain of MOPC 41, after several transfers, would also be shorter by a mol wt of 2,000. Comparison of the secretion product of original MOPC 41 and MOPC 41 after several transfers (Fig. 4 slots A and B) confirmed this postulate. We can tentatively conclude that at some generation we selected a mutant clone of MOPC 41 which had suffered a deletion of amino acid residues amounting to a mol wt of ~2,000. This mutant line is referred to as MOPC 41 DL-1. Since both precursor as well as secreted light chain are smaller by ~2,000 mol wt, the signal sequence was apparently not affected by this deletion. Light chain mRNA as well as secreted light chain from MOPC 41 DL-1 were utilized in all experiments described in the remaining part of the paper.

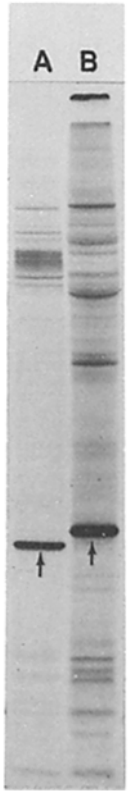

FIGURE 4 Analysis by SDS-PAGE and AR of products secreted by MOPC 41 (slot B) and MOPC 41 DL-1 (slot A) tumors.

The following experiments were designed to test whether the interpretation given to the observed proteolytic processing in vitro of the light chain precursor protein could be further corroborated. Translation of light chain mRNA in the previous experiments was not very efficient since it depended on the small amounts of initiation factors present in the pH 5 enzymes. A much more efficient translation could be expected in an initiation system, i.e. in the presence of native small ribosomal subunits (S^N) and derived large ribosomal subunits L^o from reticulocytes, even if pancreatic rough micorsomes were also present. Furthermore, translation of light chain mRNA on ribosomal subunits from reticulocytes would challenge the capacity of dog pancreas rough microsomes for establishing a ribosome-membrane junction in vitro with heterologous ribosomes. If this were impossible, we would expect synthesis largely of precursor protein, since proteolytic processing, i.e. removal of the signal sequence, presumably occurs in the membrane compartment only if a functional ribosome-membrane junction has been established and only after the signal sequence, penetrating the membrane, has been intracisternally segregated. From previous work (6) we knew that it was possible to assay directly for segregation, in that only segregated chains are largely resistant to proteolytic enzymes. In order to increase our confidence in the expected resistance of the segregated and processed light chain to added proteolytic enzymes, we also carried out translation of globin mRNA under identical conditions. If the information for segregation were encoded entirely in the mRNA, as predicted by the signal hypothesis, we would expect that the synthesized globin chains would not be segregated and would therefore be sensitive to added proteolytic enzymes.

From the data shown in the preceding paper we knew that detached polysomes from MOPC 41 DL-1 responded to the presence of reticulocyte initiation factors by initiating the synthesis of new chains. A similar response could be expected from

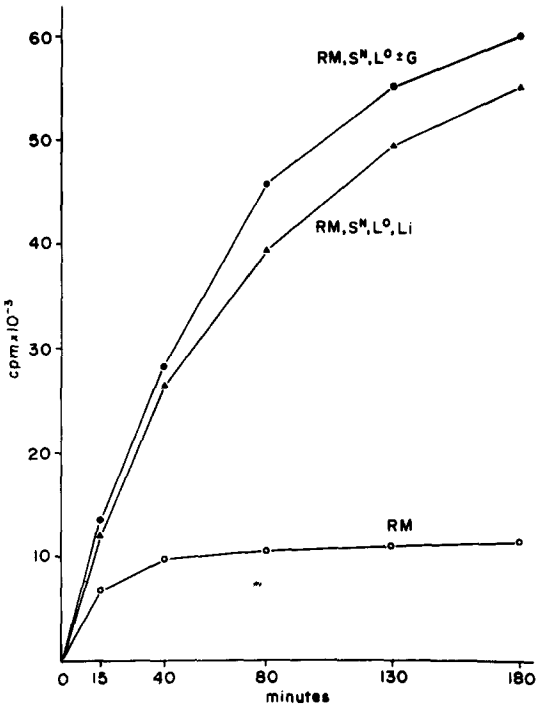

FIGURE 5 Time-course of polypeptide synthesis either in a readout system containing pancreatic rough microsomes (RM) or in an initiation system containing pancreatic rough microsomes and no added or added globin mRNP (RM, S^N, $L^O \pm G$) or added light chain mRNA (RM, S^N, L^O, Li) isolated from MOPC 41 DL-1 tumor.

rough microsomes. In order to assess the extent of stimulation, we compared polypeptide synthesis by dog pancreas rough microsomes in an initiation system to those in a readout system. It can be seen from Fig. 5 that polypeptide synthesis in the initiation system occurred not only at higher initial rates but it continued for at least 180 min, resulting at that point in a fivefold stimulation when compared to translation in a readout system (curve RM). There was no difference in the kinetics of polypeptide synthesis in the initiation system in the presence or absence of globin mRNA. The use of the large ribosomal subunit from reticulocytes apparently introduced an already saturating amount of globin mRNA. The presence of light chain mRNA in the initiation system caused a slight decrease of stimulation.

Analysis of the translation products of this experiment by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography (AR) is shown in Fig. 6. The observed stimulation of polypeptide synthesis by rough microsomes in an initiation system over that in a readout system is in part reflected in an increased density of the characteristic pancreatic bands and in part by a massive synthesis of globin chains (Fig. 6, slot A−, versus slots B−, C−, and D−). A densitometric analysis (Table I) of some of the pancreatic bands (marked 54, 26, 25, 24 in Fig. 6) of globin and of the processed light chain was performed in order to permit a more quantitative analysis of this stimulation. It can be seen from Table I that the synthesis of the lower mol wt pancreatic bands (26, 25, and 24) was stimulated two- to threefold. The presence of either globin or light chain mRNA did not significantly alter the degree of stimulation in the synthesis of pancreatic bands 54, 26, 25, and 24. Furthermore, the synthesis of globin in the initiation system (due to the presence of globin mRNA in the large ribosomal subunit fraction) was similar in the absence or presence of either added globin or light chain mRNA (Table I). Most striking, however, there was again synthesis of only processed light chain when rough microsomes were present in an initiation system for the translation of light chain mRNA (Fig. 6, slot D−). Apparently no precursor of the light chain was synthesized under these conditions; although the position of the precursor of the light chain coincides with that of pancreatic polypeptide 25 (compare slot F− with slot A− in Fig. 6), there was no increased synthesis of this band (see Table I) which could be expected if unprocessed precursor had been synthesized.

The translation products shown in Fig. 6 in the slots marked (−) were treated with proteolytic enzymes, and the results of the treatment, analyzed by SDS-PAGE and AR, are shown in Fig. 6 in the slots marked (+). It can be seen from slots B+, C+, and D+ that a large fraction of the globin present in slots B−, C−, and D− was degraded. In contrast, the characteristic pancreatic bands, as well as the processed light chain, were largely protected. Quantitation of these results by densitometric analysis of the autoradiograph (Fig. 6) is shown in Table II. It can be seen that more than 95% of the globin was degraded while between 60 and 70% of the pancreatic bands and of the processed light chain was protected. The protection which was afforded to the processed light chain is not due to its resistance to proteolysis per se since it was shown in the preceding paper (2)

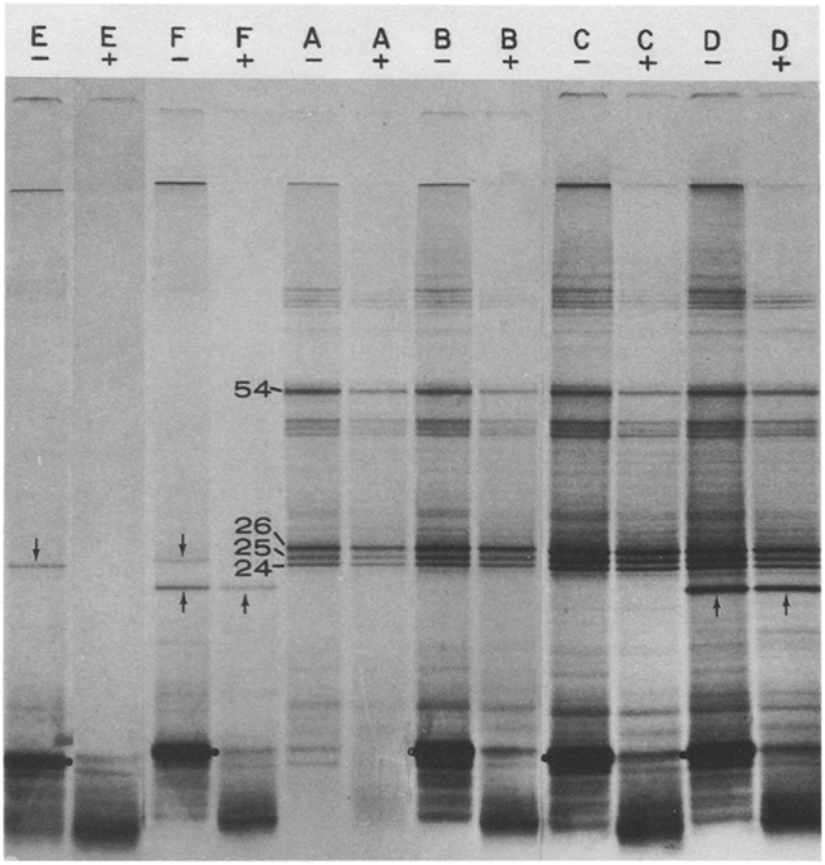

FIGURE 6 Analysis by SDS-PAGE and AR of products (slots A-D) synthesized at the 180-min time point as described in Fig. 5, and of products synthesized in an initiation system containing light chain mRNA from MOPC 41 DL-1 tumor in the absence (slot E−) or presence (slot F−) of EDTA-stripped pancreatic microsomes. All synthesized products were subsequently incubated in the absence (−) or presence (+) of proteolytic enzymes (see Materials and Methods section of companion paper). Products of pancreatic rough microsomes in a readout system are shown in slot A; of pancreatic rough microsomes in an initiation system containing no added mRNA (slot B) or containing globin mRNP (slot C) or containing light chain mRNA (slot D). Designation by dots and arrows as in Fig. 2. Numbers 54, 26, 25, 24 to the left of column A− refer to some characteristic pancreatic polypeptides with mol wt of 54,000, 26,000, 25,000, and 24,000, respectively. Slots E, C, and D were from a separate slab gel.

that processed light chains synthesized by detached ribosomes from MOPC 41 DL-1 were degraded. It should be noted, however, that the extent of protection of pancreatic bands and of the processed light chain was not complete, probably due to the leakiness of some membrane vesicles.

These results therefore demonstrate that isolated dog pancreas rough microsomes responded to reticulocyte initiation factors by an increased translation of their homologous mRNA's. Furthermore, rough microsomes retained their ability to segregate these products. Most remarkably, however, they were able to discriminate between the translation products of heterologous mRNA's, segregating only that of the light chain mRNA but not that of globin mRNA.

The translation of light chain mRNA in an initiation system containing dog pancreas rough microsomes as well as reticulocyte subunits does not prove that the latter have been utilized for translation and for the segregation of translation products. It is entirely possible that, if given the choice, the light chain mRNA would engage exclusively pancreatic microsomal ribosomes (see

TABLE I

Levels of Polypeptide Synthesis in a Readout System Containing Pancreatic Rough Microsomes Only (a) and Stimulation of Polypeptide Synthesis in an Initiation System Containing Pancreatic Rough Microsomes and No Added mRNA (b), or Either Globin (c), or Light Chain mRNA (d)

Assay	54	26	25	24	Light chain of IgG	Globin chains
(*a*) RM	3.2	3.9	2.2	2.4	—	—
(*b*) RM + S^N + L^O	4.3	10.3	8.0	7.3	—	26.8
(*c*) RM + S^N + L^O + G	3.9	9.6	7.0	6.0	—	25.8
(*d*) RM + S^N + L^O + Li	3.8	9.2	7.0	6.3	9.2	25.4

Data were obtained from a densitometric analysis of autoradiograph shown in Fig. 6 and represent arbitrary units of density. Pancreatic chains 54, 26, 25, and 24 (see Fig. 6) represent polypeptides of 54,000, 26,000, 25,000, and 24,000 mol wt, respectively.

TABLE II

Percent Resistance to Proteolysis (6) of Pancreatic Polypeptides, the Light Chain of IgG, and the Globin Chains

Polypeptides		% resistance
Pancreatic Polypeptides	54	46 ± 12
	26	71 ± 9
	25	62 ± 9
	24	67 ± 9
Light chain of IgG		65 ± 10
Globin chains		Less than 5%

Percent resistance was calculated from densitometric analysis of data in Fig. 6 and represent means of four determinations ± 1 SD.

above) using only the initiation factors supplied by the S^N fraction. It was therefore decided to challenge the capacity of the microsomal membrane to establish a ribosome-membrane junction with heterologous free ribosomal subunits more directly. With this intent we prepared "stripped" microsomes by using EDTA treatment of dog pancreas rough microsomes and subsequent centrifugation in sucrose gradients to separate stripped microsomes from the dissociated ribosomal subunits (see Materials and Methods). This procedure has been shown (7) to unfold and inactivate ribosomal subunits and to result in a complete dissociation of the small-large subunit junction but only in a partial dissociation of the large subunit-membrane junction. A significant amount of large ribosomal subunits therefore remain attached to the stripped membranes. However, since the remaining large ribosomal subunits were unfolded by this treatment, they are not expected to participate in the translation of mRNA. The presence of stripped microsomes in an initiation system containing light chain mRNA had a slight inhibitory effect on the final incorporation level when compared to that obtained in their absence (data not shown). Furthermore, polypeptide synthesis was entirely dependent on the presence of the large ribosomal subunits of reticulocytes. Their omission from the initiation system gave no polypeptide synthesis (data not shown) demonstrating that the unfolded large ribosomal subunits remaining on the stripped membranes were inactive.

Analysis by SDS-PAGE and AR (Fig. 6, slot F−) of the product made in the initiation system in the presence of stripped microsomes and light chain mRNA revealed the following newly synthesized components: (*a*) globin (due to the presence of globin mRNA introduced by large ribosomal subunits of reticulocytes, see above), (*b*) a band in the position of the processed light chain, and (*c*) a faint band in the position of the unprocessed light chain. Furthermore, analysis by SDS-PAGE and AR after proteolysis of the translation products (Fig. 6, slot F+) showed that the processed light chain is largely protected from proteolytic attack, while both the globin as well as the unprocessed light chains were degraded.

These data establish that stripped microsomes are able to segregate (and to process) the bulk of the light chain mRNA translation product even if heterologous free ribosomal subunits are used for translation. Although these results strongly suggest that a heterologous ribosome-membrane junc-

tion had been established in vitro to accomplish segregation, they do not prove it. However, proof that these heterologous ribosomes become bound to the microsomal membrane in a "salt-resistant" linkage (1) only during translation of light chain mRNA or mRNA's for other secretory proteins (e.g., secretory protein of dog pancreas) but not of globin mRNA will be provided in another report.[3]

Like rough microsomes, stripped microsomes also discriminated against segregation of the translation product of globin mRNA (Fig. 6 F+). The fact that a small amount of the translation product of light chain mRNA was not segregated and was present as unprocessed precursor suggested that segregation is required for proteolytic processing and is therefore compatible with the sequence of events suggested in the signal hypothesis.

The apparent coupling between segregation and proteolytic processing is of more than theoretical interest. Testing for proteolytic processing could provide another useful and reliable assay, specifically for the segregating capacity of the ribosome membrane junction and more generally for functional reconstitution of rough microsomes from components.

The small amount of synthesis of unprocessed light chain sensitive to proteolytic attack (Fig. 6, slot F− and F+) was apparently synthesized by ribosomes which did not bind to the stripped membranes during translation. The failure to attach could have been due to a limitation in the amount of available active binding sites on the stripped membrane. Inactivation of some of these sites may have resulted from the manipulations for the preparation of stripped membranes (EDTA treatment of rough microsomes, fractionation on sucrose gradients, sedimentation of the stripped membrane fraction, and sonication for resuspension; see Materials and Methods). Alternatively, some of the derived large ribosomal subunits may have suffered a loss of competence for binding (e.g., during the puromycin-KCl dissociation), but may have retained their activity in translation. The evidence obtained from an experiment in which increasing amounts of stripped microsomes were used in an attempt to provide more active membrane binding sites is not clear-cut, although it tends to support the first interpretation. The ambiguity introduced by this type of experiment was evident from the time-course of polypeptide synthesis (data not shown). Addition of increasing amounts of stripped microsomes to the initiation system containing light chain mRNA caused an increasing inhibition of polypeptide synthesis. Thus, although it was observed (Fig. 7 and Table III) that increasing amounts of stripped mem-

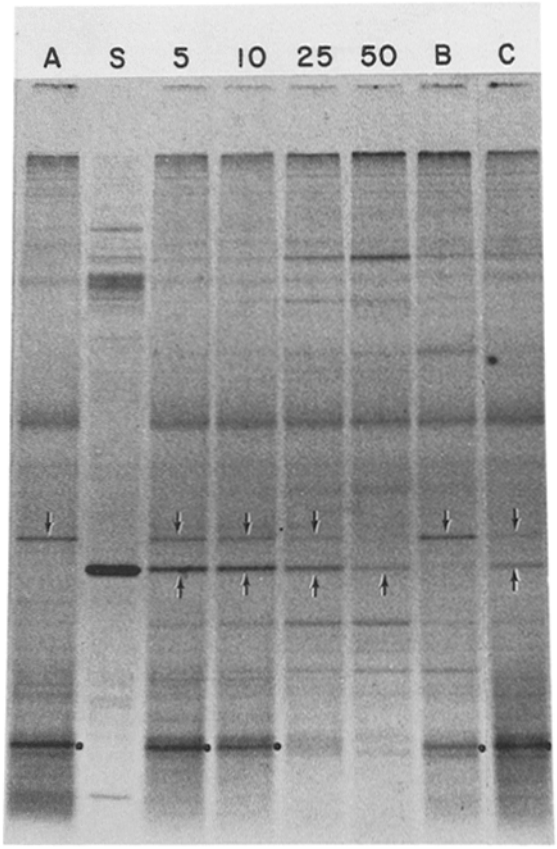

FIGURE 7 Analysis by SDS-PAGE and AR of products synthesized in an initiation system containing light chain mRNA from MOPC 41 DL-1 in the absence (slot A) or in the presence of either increasing amounts of EDTA-stripped pancreatic microsomes (slots 5, 10, 25, and 50), or heat-inactivated, EDTA-stripped pancreatic microsomes (slot B) or puromycin-KCl-stripped pancreatic microsomes (slot C). For comparison the labeled secreted light chain of IgG from MOPC 41 DL-1 tumor is shown in slot S. Slots 5, 10, 25, 50 refer to microliters of EDTA-stripped microsomes (76.0 A_{260} units/ml) present in initiation system. 25 μl of heat-inactivated, EDTA-stripped microsomes (slot B) and 25 μl (33.4 A_{260} units/ml) of puromycin-KCl-stripped microsomes (slot C) were present in the initiation system. Designations by dots and arrows as in Fig. 2.

[3] Blobel, G., B. Dobberstein, and G. Scheele. Manuscript in preparation.

TABLE III

Synthesis of Processed (Li) and Nonprocessed (PLi) Light Chains of IgG in an Initiation System Containing Light Chain mRNA and Either No Added EDTA-Stripped Microsomes (0 μl RM-EDTA) Increasing Amounts of RM-EDTA (5, 10, 25, 50 μl) or Heat-Inactivated RM-EDTA (25 μl)

RM-EDTA	Li	PLi
μl		
0*	0.0	3.4
5	4.9	0.8
10	4.8	0.9
25	2.8	0.4
50	0.8	0.0
25 (55°C)	0.0	3.2

* 25-μl aliquots of incubation mixture were used for SDS-PAGE while others (not marked with an asterisk) were derived from 50-μl aliquots of the incubation mixture. Data were obtained from densitometric analysis of autoradiograph shown in Fig. 7.

branes resulted in a decreased synthesis of unprocessed chain, there was also a decreased synthesis of processed light chain. Therefore, small amounts of unprocessed chain may have been synthesized, even in the presence of high concentrations of stripped microsomes, but may have escaped detection because of the lower levels of polypeptide synthesis.

Borgese et al. (4) reported recently that ribosomes do not bind to heat-inactivated stripped microsomes. Translation of light chain mRNA in an initiation system containing heat-inactivated, stripped microsomes should therefore result in the synthesis only of unprocessed, proteolysis-sensitive chains, if processing is dependent on ribosome binding. This was indeed observed as can be seen from the results shown in slot B of Fig. 7. The unprocessed chains synthesized in this experiment were not segregated since they were sensitive to proteolysis (data not shown). However, since heat inactivation of the stripped membranes most likely also affected their processing activity, this result does not prove that ribosome attachment and processing of the nascent chain are coupled.

An alternative procedure for removal of the ribosomes from rough microsomes using puromycin and high concentrations of KCl was recently described (1). It was therefore of interest to assay the capacity of these stripped microsomes in comparison to the EDTA-stripped membranes. No differences were observed. Similarly as in the EDTA-stripped microsomes, there was inhibition of polypeptide synthesis in the presence of increasing amounts of puromycin-KCl-stripped microsomes in the initiation system (data not shown). Furthermore, product analysis by SDS-PAGE and AR again showed the synthesis of a proteolysis-resistant, processed chain (Fig. 7, slot C) and of a small amount of proteolysis-sensitive, unprocessed chain.

The fact (established in the preceding paper) that proteolytic processing takes place in vivo on the nascent chain, i.e. before completion of the nascent chain, did not rule out that in vitro processing can take place on a completed and ribosome-released precursor protein. It could be argued that the ~40% or so of the proteolytically processed chains which were sensitive to proteolytic enzymes were attacked not because of leaky vesicles but because they were not segregated in spite of the fact that they were proteolytically processed. Furthermore, it could be argued that segregation and processing of the light chain mRNA product was not a consequence of the scheme suggested in the signal hypothesis. Segregation (and/or proteolytic processing) could instead occur by virtue of the physicochemical properties of the completed and folded proteins recognized by the membrane. This possibility, however, was clearly ruled out by an experiment in which either rough microsomes or stripped microsomes were incubated with light chain precursor. The results shown in Fig. 8, slot B demonstrate that no proteolytic processing took place. Furthermore, no segregation took place since the light chain precursor was completely degraded when proteolytic enzymes were subsequently added (data not shown). All attempts to obtain in vitro proteolytic processing by incubating detergent-solubilized stripped microsomes with the light chain precursor failed (see Fig. 8, slots C, D, and E). The rationale for using detergent solubilization by Triton X-100 (slot C) or deoxycholate (DOC) (slot D) was to solubilize the processing activity presumably localized in the microsomal membranes or in the intravesicular space and therefore to establish accessibility to the substrate. SDS in low concentrations was used in case there was a requirement for an unfolding of the unprocessed chain.

The negative results can be interpreted in a

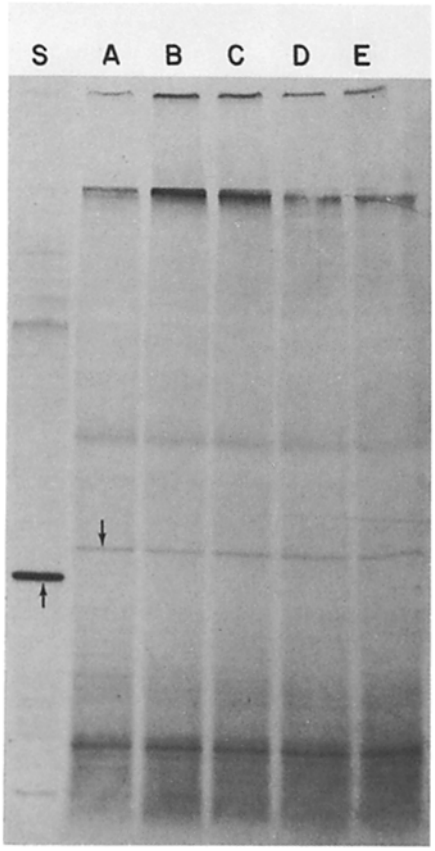

FIGURE 8 Analysis of SDS-PAGE and AR of products synthesized in an initiation system containing light chain mRNA from MOPC 41 DL-1 tumor (slot A) and subsequently incubated for 1 h at 37°C with EDTA-stripped pancreatic microsomes alone (slot B) or additional Triton X-100, final concentration 1% (slot C) or DOC, final concentration 1% (slot D), or SDS, final concentration 0.1% (slot E). For comparison the labeled secreted light chain of IgG is shown in slot S.

variety of ways. It is possible that the ribosome-released chains have achieved a sufficient degree of folding so that their signal sequence (in particular if hydrophobic) is buried within the molecule; SDS in the concentrations used may have been insufficient to cause unfolding or may have inactivated the processing enzyme. Similar reasoning can be applied to the results with Triton X-100 and DOC: the processing activity may have been inactivated or the completed and folded chain may not be the correct substrate.

It should be noted here that processing of the translation product of light chain mRNA in vitro has been reported previously by Milstein et al. (5). It was observed that it occurred only when translation took place in an ascites S 30, but not if it took place in a reticulocyte lysate. It was proposed that the ascites S 30 system presumably contained membraneous material which was responsible for processing, while the absence of membranes in the reticulocyte lysate system prevented in vitro processing (5). Although this suggestion was not supported by direct experimental evidence, our data presented in this paper indicate that the interpretation advanced by Milstein et al. was probably correct.

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