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Processing *in vitro* of placental peptide hormones by smooth microsomes

(secretory proteins/cell-free protein synthesis/endoplasmic reticulum)

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ABSTRACT **Rough and smooth microsomes were prepared from ascites tumor cells, rat liver, and bovine adrenal cortex. Proteolytic removal of the signal peptide in pre-placental lactogen and asparagine-linked glycosylation of the α subunit of chorionic gonadotropin by these fractions were examined in mRNA-dependent lysates from ascites cells. Both processing steps were performed by smooth microsomes, which was unexpected because it has been presumed that only rough microsomes contain components for ribosomal binding. Thus smooth microsomes are apparently capable of interacting with polysomes bearing secretory nascent chains, and cleavage and asparagine-linked glycosylation activities are present in both rough and smooth endoplasmic reticulum.**

It is well documented that eukaryotic secretory proteins traverse a complex labyrinth of subcellular compartments prior to their exit into the extracellular space. This process begins with synthesis of proteins on ribosomes attached to membranes of the endoplasmic reticulum (ER), and, within minutes after initiation, the nascent polypeptide chains are sequestered into the cisternae. During this translocation across the ER membranes, the newly synthesized chains can undergo one or more processing steps. The "pre-" segment located at the amino terminus of the nascent polypeptide chain is removed during translation by an activity present in microsomal membranes (1, 2). In the case of secretory glycoproteins containing asparagine-linked sugars, mannose-rich oligosaccharide units are transferred from a dolichol-lipid intermediate in the membrane to the nascent chains (3–6). The completed peptide chains are then concentrated in the lumen of the ER and channelled for further transport.

It has been presumed that processing of nascent chains occurs only in the rough ER (RER) and not in the smooth ER (SER). In this connection Kreibich *et al.* (7, 8) have suggested that the major distinction between the RER and SER is the absence in the latter of specific sites for binding polysomes.

For investigating the possible role of smooth microsomes in the processing of presecretory proteins, a variety of smooth and rough microsomal factions were prepared from ascites tumor cells, rat liver, and steer (bovine) adrenal cortex. Each of these tissues has a different proportion of RER to SER as determined by electron microscopy of unfractionated tissue; the majority of the ascites ER is in a smooth configuration (9), the ER of the adrenal cortex is essentially all smooth (10, 11) and that of rat liver contains equivalent proportions of RER and SER.

Unexpectedly, we observed that the smooth microsomal fractions from these tissues effectively sequestered and glycosylated nascent chains of the α subunit of human chorionic gonadotropin (hCG-α) (12). In addition, smooth microsomes also processed the pre- form of the nonglycosylated protein placental lactogen (pre-hPL) (1). These results prove that the above smooth microsomal fractions are capable of interacting with polysomes bearing placental peptide nascent chains and can carry out all the steps involved in processing and sequestration of presecretory proteins.

MATERIALS AND METHODS

Materials. Antiserum generated against highly purified, reduced, and carboxymethylated hCG-α was prepared by S. Birken and R. Canfield as described (12). α-1-Antitrypsin was obtained from Sigma.

Preparation of Subcellular Fractions. Placental RNA, ascites tumor ribosomes, and cell sap (S-100) were prepared as described (1). Assay of protein synthesis, immunoprecipitation of the cell-free products, and their resolution on sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gels were described (5).

The postmitochondrial supernate from ascites tumor cells was prepared as described (1), except that the homogenate was centrifuged at 20,000 × *g* for 10 min (S-20). The S-20 was adjusted to 1.35 M sucrose and the rough and smooth microsomes were isolated from a discontinuous sucrose gradient according to Kruppa and Sabatini (13). Preparation of the postmitochondrial supernate and subcellular fractionation of rat liver were also performed by their procedure.

Cortical tissue of the bovine adrenal gland was separated from the medulla and the resulting cortex was cut into small fragments. Prior to homogenization, sections of the tissue were sampled for examination by electron microscopy. Ten grams of tissue was homogenized in 25 ml of buffer containing 30 mM Tris·HCl (pH 7.5), 120 mM KCl, 5 mM magnesium acetate, and 7 mM 2-mercaptoethanol. Preparation of postmitochondrial supernate and membranes has been described (1).

$NaDodSO_4$/polyacrylamide gel electrophoresis of the microsomal fractions was carried out essentially as described by Kreibich *et al.* (7). Rough and smooth microsomal fractions (*ca.* 300 mg of protein) were diluted 1:100 with a buffer containing 500 mM KCl, 50 mM Tris·HCl (pH 7.5), and 10 mM $MgCl_2$. The microsomes were then collected by centrifugation for 20 min at 25,000 rpm in a Spinco SW 60 rotor. The pellets were dissolved in 40 μl of a buffer containing 10 mM Tris·HCl (pH 7.5), 3% $NaDodSO_4$, 10% (vol/vol) glycerol, 0.7 M 2-mercaptoethanol, and 0.002% bromphenol blue and boiled 2–5 min prior to electrophoresis. The gels were stained with Coomassie blue and subsequently photographed.

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Abbreviations: ER, endoplasmic reticulum; RER, rough ER; SER, smooth ER; hCG-α, α subunit of human chorionic gonadotropin; hPL, human placental lactogen; $NaDodSO_4$, sodium dodecyl sulfate.

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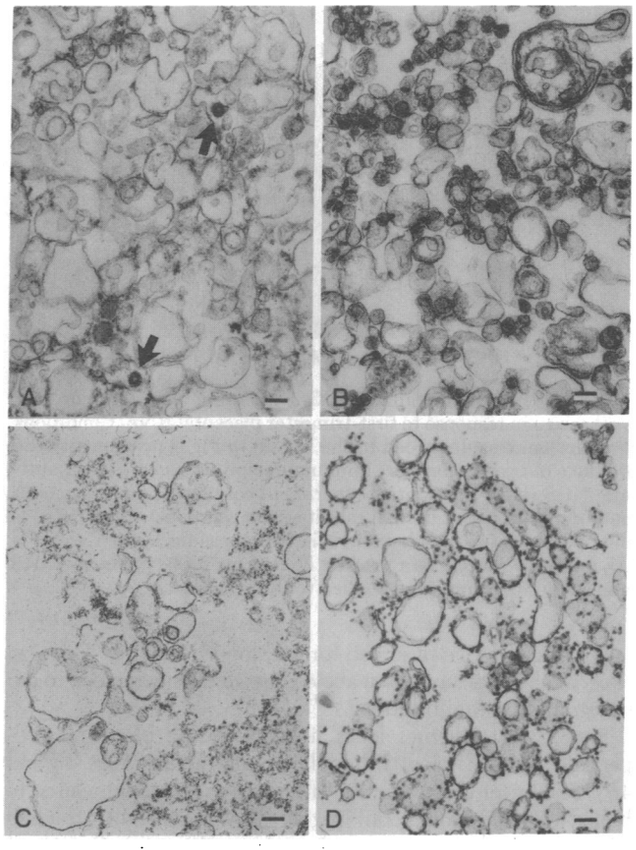

FIG. 1. ER fractions from ascites tumor cells and liver cells. (*A*) SER from ascites cells. Note A-type viral particles (arrows). (*B*) SER from liver cells. (*C* and *D*) Tumor and liver rough fractions, respectively. Sections were double stained with alcoholic uranyl acetate and lead citrate (19). Marker bars represent 0.1 μm.

Phospholipid was determined according to Raheja *et al.* (14), and RNA was measured by the procedure of Munro and Fleck (15). Protein was determined by the method of Lowry *et al.* (16) and NADH-cytochrome *c* reductase activity was assayed as described by Fleischer and Fleischer (17).

Electron Microscopy. Samples were fixed with 2.5% (wt/wt) glutaraldehyde, postfixed in 1% osmium tetroxide, and then embedded in Spurr's low-viscosity resin (18).

Table 1. Characterization of rough and smooth microsomes

Membrane fraction	Phospholipid/ protein	RNA/ protein	NADH-cytochrome *c* reductase, μmol/mg per min	
			+Rotenone	−Rotenone
Ascites				
Smooth	0.338	0.046	0.448	0.651
Rough	0.039	0.470	—	0.03
Rat liver				
Smooth	0.474	0.042	1.187	1.145
Rough	0.249	0.187	0.774	0.751
Adrenal				
Smooth	0.498	0.030	0.509	0.643

Rotenone (4 μg) was added as a 1 mg/ml ethanol solution. Rotenone-sensitive activity presumably indicates the presence of NADH-cytochrome *c* reductase-like activity in mitochondria (13).

RESULTS

The microsomal fractions derived from ascites tumor cells, rat liver, and adrenal cortex were characterized by (*i*) electron microscopy; (*ii*) RNA, protein, and phospholipid composition; and (*iii*) presence of NADH-cytochrome *c* reductase activity.

Characterization of the membrane fractions

Ascites Tumor Cells. As is evident from the morphology, the smooth vesicles were completely devoid of ribosomes (Fig. 1*A*). This fraction also contained the A particles characteristic of murine tumor endoplasmic reticulum (20). Although there were abundant clusters of ribosomes, only a small amount of vesicular material was observed in the rough fraction (Fig. 1*C*).

Consistent with the morphological data, the RNA-to-protein ratio of this smooth fraction was much less than that of the rough microsomes (Table 1). In addition, the former contained significantly more NADH-cytochrome *c* reductase activity than did the latter (Table 1). Significantly, there was a reduction to 1/10th in the weight ratio of phospholipid to protein in the rough as compared to the smooth fraction (Table 1). This was presumably due to the low level of vesicles in the rough fraction.

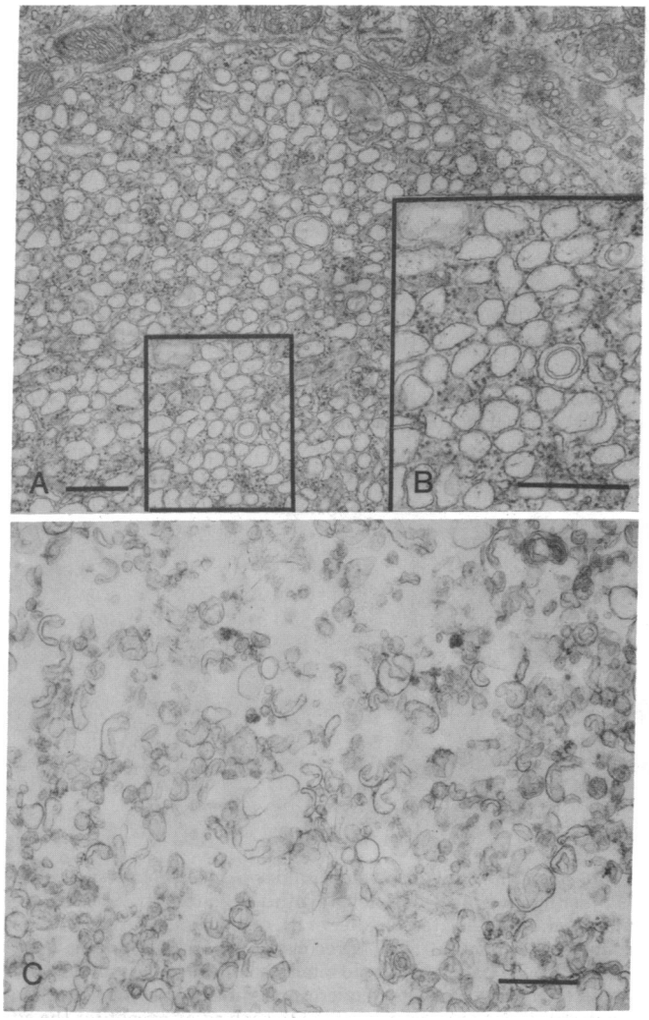

FIG. 2. Electron micrographs of bovine (steer) adrenal cortical tissue (*A*) and the corresponding isolated smooth membrane fraction (*C*). (*B*) Additional magnification of the rectangle in field *A*. Marker bars represent 0.5 μm.

Rat Liver. Liver microsomes were isolated from rats pretreated with phenobarbital (21). This drug enhances extensive proliferation of the endoplasmic reticulum and results in a greater yield of smooth microsomes (21). Analysis of liver microsomal fractions showed the presence of rough (Fig. 1*D*) and highly enriched smooth microsomal vesicles (Fig. 1*B*). The phospholipid-to-protein ratios of the fractions were in agreement with morphological data (Table 1). The specific activity of NADH-cytochrome *c* reductase was less in the rough preparations. This was expected because the rough fraction contains about 30% ribosomal protein, which would reduce the specific activity of this membrane-associated enzyme (Table 1). Thus it was clear that, in contrast to the tumor membranes, the liver rough fraction contained primarily ribosome-studded vesicles.

Adrenal Cortex. It is well documented that steroid-secreting cells are replete with SER (10). The ER of the bovine adrenal cortical cells is essentially smooth and little rough is observed (Fig. 2). Membranes isolated according to Szczesna and Boime (1) were entirely smooth (Fig. 2) and resembled liver and ascites tumor smooth fractions with respect to the ratios of RNA, protein, and phospholipid (Table 1). Moreover, adrenal membranes had levels of NADH-cytochrome *c* reductase activity comparable to those observed for liver ER membranes.

Processing activity of membrane preparations

We then analyzed these microsomal preparations for processing activity in ascites tumor cell-free lysates.

In this system term and first trimester human placental RNA directed the synthesis of the pre- form of hPL and hCG-α, respectively (Figs. 3*A* and 4*A*; refs. 1 and 4). Addition of total ascites microsomes resulted in the cleavage of pre-hPL (Fig. 3*A*; ref. 1). Surprisingly, the smooth microsomes also cleaved pre-hPL to a smaller protein that comigrated with authentic hPL (Fig. 3).

In the presence of smooth microsomes, the glycosylated, cleaved form of hCG-α was observed (Fig. 4*A*). This protein was immunologically identical to the glycosylated hCG-α when unfractionated ascites membranes were added to these lysates (5). Thus a nonproteolytic processing step—i.e., asparagine-linked core glycosylation—was also associated with the smooth microsomes. Processing by the ascites rough microsomes was much less than that observed for the smooth fraction. This was expected because, as shown above, few vesicles were seen in the rough microsomal fraction.

Smooth microsomes from liver processed placental prepeptides more efficiently than the rough fraction (Figs. 3*B* and 4*B*). The lower activity in the latter was presumably related to blocking of ribosomal binding sites by membrane-attached ribosome–nascent chain complexes formed *in vivo*. Consistent with this was the observation that stripping ribosomes from the rough fraction with EDTA enhanced processing (Fig. 5).

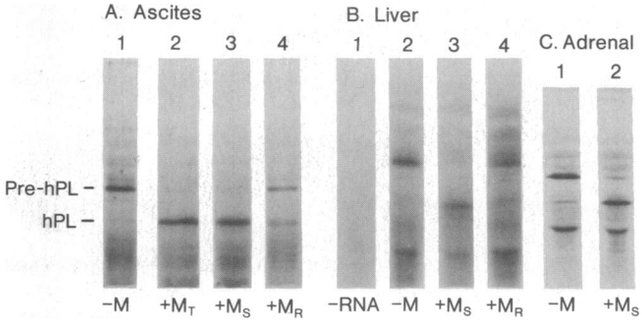

FIG. 3. NaDodSO$_4$/polyacrylamide gel electrophoresis of total [^{35}S]methionine-labeled proteins synthesized in the presence of term placental RNA. Ascites (*A*), liver (*B*), or adrenal cortex (*C*) microsomes were added to 180 μl of reconstituted ascites tumor cell-free lysates. M$_T$, M$_S$, and M$_R$ refer to total, smooth, and rough microsomes, respectively, and M refers to microsomes of any type. The phospholipid equivalent of 30 μg was added to each assay except for the addition of 6 μg of the ascites rough preparation. The marker hPL (M_r 22,600) is shown. Equivalent amounts of radioactivity (about 60,000 cpm) were added to each lane except for the minus RNA lane, which contained 20,000 cpm.

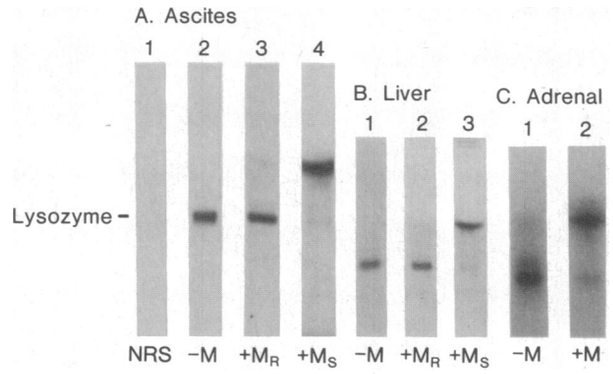

FIG. 4. Autoradiograph of hCG-α immunoprecipitated protein synthesized in response to first-trimester placental RNA. Conditions were the same as described in the legend to Fig. 3, except that the products of translation were immunoprecipitated with hCG-α antisera. NRS refers to a control experiment containing ascites smooth membranes in which normal rabbit serum was substituted for subunit-specific antisera. Other abbreviations are as for Fig. 3. Lysozyme (M_r 14,000) was used as a marker. Except for 2000 cpm in the NRS lane, 6000–8000 cpm were applied to the other slots.

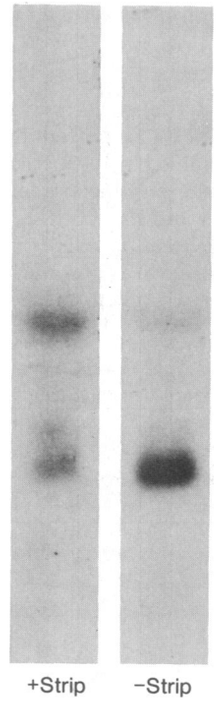

FIG. 5. Cleavage and glycosylation of hCG-α by liver rough microsomal membranes pretreated with 30 mM EDTA to strip off attached ribosomes. First-trimester placental RNA was translated in the presence of stripped (27 μg of phospholipid) or unstripped (30 μg of phospholipid) rough microsomes. The membranes were treated according to Blobel and Dobberstein (2) except that centrifugation was over a 0.5 M sucrose cushion for 1 hr at 100,000 × *g*.

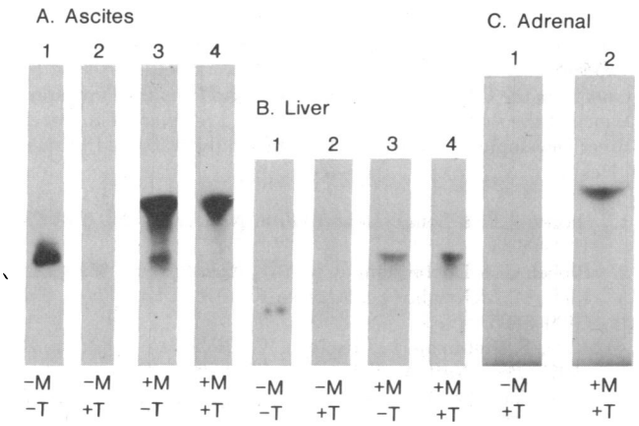

FIG. 6. Effect of trypsin on the distribution of hCG-α synthesized in the absence and presence of smooth microsomal membranes (M) derived from various tissues. Where indicated, trypsin (T) was added (5 μg/180 μl of reaction mixture) after 60 min of incubation and the incubation was continued an additional 30 min. Then 50 μg of α-1-antitrypsin was added and the labeled proteins were immunoprecipitated. Equivalent amounts of protein were applied to the gels.

Adrenal smooth membranes processed pre-hPL and pre-hCG-α with the same efficiency as ascites tumor membranes (Figs. 3*C* and 4*C*). Thus smooth membranes isolated from three different tissues were capable of processing nascent secretory peptide chains.

To determine if the processed proteins were sequestered within vesicles, the ability of smooth membranes to protect glycosylated hCG-α against added exogenous protease was examined (2). Only the processed form was protected from trypsin digestion (Fig. 6). These data demonstrated that processing activity in the smooth fractions was coupled to insertion into the vesicles.

Kreibich *et al.* (7, 8) have proposed that two integral membrane proteins designated ribophorins I and II (M_r 65,000 and 63,000, respectively) confer specificity for polysome binding to the endoplasmic reticulum. These proteins were found in both rough membranes and in rough membranes stripped of ribosomes. They were apparently not present in smooth microsomes.

The distribution of proteins in the RER and SER fractions from rat liver, previously demonstrated to contain cleavage and glycosylation activity, were compared by $NaDodSO_4$ gel electrophoresis (Fig. 7) to assess whether they contained the ribophorins. The distribution of proteins in both preparations was very similar to that previously reported by Kreibich *et al.* (7, 8). In particular, two proteins in the RER preparation with M_r of 64,000 and 65,000 (arrows) were seen. They were apparently present in equimolar amounts, and corresponded in size to the ribophorins. It was noteworthy that these proteins were barely detectable in the smooth fraction. Of further interest, there was another protein ($M_r > 100,000$) present in the rough but not in the smooth fraction. This further demonstrated that these preparations, as isolated, represented two distinct populations.

DISCUSSION

The processing and sequestration of precursors to secretory proteins in membranes of the ER is postulated to involve (*i*) binding of the nascent chain to sites in the membrane, (*ii*) movement of the growing nascent chain across the membrane, (*iii*) proteolytic removal of the signal (pre-) peptide, and (*iv*) core glycosylation of the nascent chains of secretory glycoproteins.

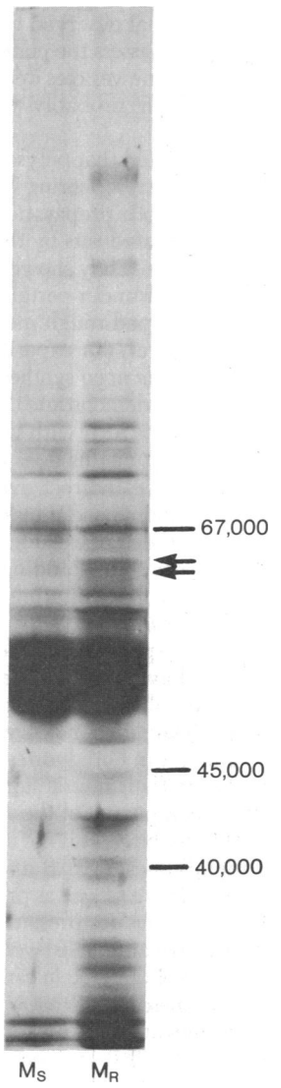

FIG. 7. $NaDodSO_4$/polyacrylamide gel pattern of proteins in rough microsomes (M_R) and smooth microsomes (M_S) derived from rat liver. Samples (300 μg of protein for each) were prepared and analyzed in a linear $NaDodSO_4$/polyacrylamide gradient gel (8–12%). Numbers on the right represent M_r derived from the mobility of marker proteins run in a parallel lane: bovine serum albumin (67,000), ovalbumin (45,000), and creatine kinase (40,000).

Smooth microsomal membranes are by definition devoid of ribosomes; the results presented here show that this fraction, as isolated from ascites tumor cells, rat liver, and adrenal cortex, will effectively translocate and process nascent pre- forms of hPL and hCG-α. Thus the data show that SER can interact with nascent chains and that RER and SER cannot be distinguished on the basis of the above activities.

One crucial question that arises is whether the processing activities that we observed resulted from a population of smooth microsomes that arose from stripping of rough microsomes during preparation. Two lines of evidence argue against this point. First, because stripped liver rough membranes contain the ribophorins (7, 8), their absence in the liver smooth membranes shows that these membranes were not contaminated with detectable quantities of stripped rough membranes. Second, the cells of the bovine adrenal cortex contain primarily SER and very little RER. Smooth membranes isolated from the adrenal completely processed pre-hPL and pre-hCG-α. Significantly, the level of adrenal membranes required to achieve

these effects was the same as that observed for liver or tumor membranes. We cannot as yet assess the competency to bind polysomes by smooth membrane vesicles derived from Golgi or plasma membranes, which probably contaminate our preparation.

Kreibich *et al.* (7, 8) postulated that polysome binding sites of RER were determined by the ribophorins. Their studies were performed on rough and smooth preparations isolated from livers of fed phenobarbital-treated rats by the same fractionation procedures reported here. They showed that, in the absence of protein synthesis, and under certain salt conditions, ribosomes were bound to stripped rough membranes but not to smooth membranes. However, our experiments, which involved a functional assay (i.e., *de novo* synthesis and processing of placental secretory proteins), demonstrate that the interaction of membranes derived from the SER with polysomes bearing placental pre- peptide nascent chains does not require ribophorins. Thus the data provide indirect evidence that ribophorins are not involved in binding and translocation of nascent peptides. However, the results do not exclude a possible role for ribophorins in the *in vitro* ribosomal binding occurring in the absence of nascent chains, or in facilitating the anchoring of ribosomes to ER membranes *in vivo*.

Although the translation experiments were performed with ascites tumor lysates, we have also observed that all of the smooth fractions described above process pre-hPL and pre-hCG-α in a recticulocyte lysate (data not shown). Nevertheless, it is conceivable that a factor in both lysates was bound to the membranes and resulted in their interaction with polysomes.

Although the SER is a component of the secretory network, its functional relationship to the RER is not clear. Dallner *et al.* (22) suggested that membranes of RER and SER are closely related. The data presented here as well as previous studies (23), showing that the same enzymes are present in RER and SER, support this hypothesis. Because it is well established that cleavage of the pre- portion of the protein can occur only during protein synthesis, it seems inescapable that when cleavage takes place on SER, the ribosomes and SER must, at least transiently, be bound together—i.e., the SER has become RER *pro tem*. Perhaps the distinction between RER and SER has been overemphasized. Mechler and Vassalli (24, 25) presented evidence that a significant proportion of membrane-bound ribosomes can be released after translation and that they can enter the free ribosome pool. Is it possible that *in vivo* the RER that is observed morphologically represents a population of ribosome-bearing nascent chains that are still attached to the ER, and that upon termination not only ribosomes but other components as well, such as factors involved in ribosomal binding, are removed?

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