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Properties of a GTP sensitive microdomain in rough microsomes

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Stripped rough microsomes (SRM) fuse when incubated with physiological concentrations of GTP and $MgCl_2$. In order to examine further to what extent such fusions are associated with other membrane functions of rough endoplasmic reticulum, we have evaluated the role of cytosolically exposed peptide constituents of SRM in fusion, and the possible relationship of GTP/$MgCl_2$-induced fusion in protein transport across endoplasmic reticulum (ER) membranes, and in ER–Golgi interactions. Controlled proteolytic digestion of SRM led to the loss of fusion capability at 15 μg/ml trypsin – a concentration which maintained the latency of intraluminal mannose-6-phosphatase. Hence, a cytosolically exposed protein(s) regulated fusion. Based on ribonuclease-induced ribosome capping experiments, it was further concluded that the cytosolic oriented protein(s) was sequestered beneath the ribosome. As co-translational cell free translocation of placental lactogen across SRM was similar in control membranes compared to those rendered incapable of fusing, it was concluded that the fusion phenomenon may not be related to translocation. Under conditions promoting homologous fusion of SRM or Golgi membranes, mixtures of the two membranes showed no heterologous membrane fusion as assessed morphologically or by the transport of newly synthesized membrane glycoprotein. These experiments attest to the specificity of cytosolically exposed protein(s) in regulating nucleotide/divalent cation-induced membrane fusion.

Introduction

GTP at physiological concentrations stimulates core glycosylation [1–3], membrane fusion [4–6] and activates membrane permeability changes [7] in rough endoplasmic reticulum membranes from rat liver. These phenomena, however, depend on the prior removal of the majority of ribosomes from the membranes. Since GTP stimulates both glycosylation and permeability changes only when using stripped rough microsomes and not smooth microsomes it has been proposed that GTP plays a role in the vectorial discharge and segregation of protein within the lumina of ER [7]. It has also been suggested that GTP acts at membrane sites located beneath the ribosome [3,6,7]. In the hope of better understanding the effects of GTP on the structure and function of ER membranes we have sought to determine the site of action of this nucleotide and to see whether the principal morphologic and biochemical perturbations induced occur coincidently and within the same membrane domain. Because of the proposed link between GTP and ribosome binding sites we examined

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whether the fusion phenomenon is related to the vectorial translocation of newly synthesized proteins across ER membranes. The last objective of this study was to determine if GTP and $MgCl_2$ could induce functional fusions between membranes derived from the ER and Golgi apparatus.

Materials and Methods

Materials

[^{35}S]Methionine (1100–1200 Ci/mmol) and Enhance were purchased from New England Nuclear (Boston, MA). *Staphylococcus aureus* nuclease, yeast tRNA and bovine pancreatic ribonuclease were obtained from Boehringer Mannheim G.F.R. Oligo dT Cellulose Type III was purchased from Collaborative Research Laboratories (Lexington, MA), trypsin and chymotrypsin for protein translation studies was from Cooper Biomedicals (Malvern, PA), placental ribonuclease inhibitor from Bethesda Research Laboratories (Gaithersburg, MD), guanidine thiocyanate from Fluka AG (Buchs, Switzerland), and XAR-5 X-ray film from Kodak (Rochester, NY). Purified human placental lactogen for use as molecular weight standard was a gift from the National Institute of Health (NIADDK), (Bethesda, MD), antihuman placental lactogen antiserum (anti hPL) was prepared by Dr. Wei Lai, Dept. of Anatomy, McGill University. Unless otherwise stated, all other reagents and chemicals were of reagent grade and obtained from Sigma Chemical Co. (St. Louis, MO).

Preparation of membrane subfractions

Rough microsomes were prepared from rat liver and stripped of their associated RNA using techniques previously described [6]. Stripped rough microsomes (SRM) from dog pancreas were prepared by the method of Shields and Blobel [8]. These were generously supplied by D. Shields (Albert Einstein College of Medicine, NY). Rat liver Golgi apparatus was purified by a one step procedure as described previously [9].

Morphological procedures

Microsomes were fixed with 1.5% ice-cold glutaraldehyde in 50 mM phosphate buffer (pH 7.4). Fractions were recovered by filtration onto Millipore membranes (0.45 μm pore size, Millipore Corp, Bedford, MA) and processed for electron microscopy [5].

Morphometry of membrane surfaces. Morphometry was carried out as previously described [13] using a Zeiss MOP-3 digitizer (Carl Zeiss, Inc., Don Mills, Ontario, Canada). For the ribonuclease experiments quantitation of the amount of smooth surfaced membrane associated with rough microsomes was carried out by taking adjacent non-overlapping micrographs at 20 000-times magnification across the complete thickness of the membrane pellicles. Negatives were printed at a final magnification of $\times$60 000. The prints were fastened onto the translucent measuring tablet of the MOP-3 digitizer and the contour of the membrane profiles was traced manually with the MOP-3 stylus thereby yielding the summed profile perimeter lengths for the microsomes within the micrographs. On the same micrographs vesicle profiles showing distinct regions lacking ribosomal particles (e.g. smooth surface portions of the vesicle profiles) were identified. The smooth surface portions of these vesicle profiles were identified using a felt tip pen and their total summed length was determined by measurement with the MOP-3 stylus. These results were expressed as a percentage of the total membrane length determined above. Vesicle profiles sectioned tangentially were not included in this analysis.

Assay for fusion. Fusion of rough endoplasmic reticulum (RER) membranes has been defined by morphological assay as the formation of large membrane-bounded elements by coalescence of the membranes of small rough microsomes. Formation of large fusion products was demonstrated by thin-section electron microscopy of fixed and embedded microsomes as well as by freeze-fracture electron microscopy of rapidly frozen and cleaved membranes [5]. Since small vesicles fuse to form large vesicles one can make use of the estimate of the size of a population of vesicles as an index of the amount of fusion that has occurred amongst the vesicles. A morphometric assay has been developed and applied to vesicle profiles observed in thin sections [6,13]. The assay consists of measuring vesicle contours (profile perimeter lengths) by stereology. The profile perimeter lengths are accumulated until a total of 1000 μm

of membrane has been measured for each population of vesicle membranes. The profile perimeter lengths are then subdivided into frequency classes thus generating a histogram of membrane lengths. Under conditions of no fusion a greater number of vesicle measurements are required to attain a sum total of 1000 μm of membrane. By contrast fewer vesicle measurements will yield an equivalent sum of membrane lengths in the population of vesicles containing large fusion products. This is because the larger fusion products contribute more membrane to the cumulative sum of membrane lengths. By dividing the total sum of membrane lengths by the number of vesicles employed in the analysis once can obtain the mean or average membrane length for the population of vesicles, this mean can be used reproducibly to compare amount of fusion in a variety of experimental conditions and is particularly useful for studies where subtle effects on membrane fusion must be examined [6,13]. On the other hand if one wishes to document simply whether membrane fusion occurs or not (to examine all or none responses) a less tedious and semiquantitative assay can be used.. This assay was first developed by Creutz [23] for the detection of fusion between chromaffin granules. It classifies fusion in relative terms based on the absence or presence of few or many large membrane fusion products. Large fusion products are vesicles which have profiles larger than the largest unincubated vesicle. For example the largest unincubated rough microsomal vesicle is never larger than 0.5 μm in diameter (the average diameter of unincubated vesicles is 0.16 μm, data not shown).

We have employed the morphometric assay to compare the subtle effects of different concentrations of trypsin on membrane fusion, glycosylation and mannose 6-phosphate latency and we have used the semiquantitative procedure of Creutz [23] in all cases where we studied all or none fusion responses.

Media for membrane fusion

Homologous membrane mixtures. Unless otherwise stated incubations were carried out in standard ER fusion medium consisting of 40 mM Tris-HCl (pH 7.4), 30 mM KCl, 7.5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 1 mM ATP, 10 mM phospho*enol*pyruvate, 25 μg pyruvate kinase plus 0.5 mM GTP [6]. Normally 0.2 ml of microsomes (3.8–7.4 mg/ml) in 4 mM imidazole-HCl buffer (pH 7.4) containing 0.25 M sucrose were added just prior to the start of incubation. Incubations were done at 37°C for varying periods of time as described in Results. The standard Golgi fusion medium was slightly modified from that previously described [10] and consisted of a buffer solution containing 30 mM sodium cacodylate (pH 7), 20 mM $MnCl_2$, and 1 mM ATP. Unless otherwise indicated reactions were started by addition of 0.2 ml of Golgi fraction (60–80 μg protein) and maintained for 2 h at 37°C.

Heterologous membrane mixtures. Based on published data using homologous membrane mixtures [6,10] preliminary experiments were carried out to optimize conditions for fusion of both endoplasmic reticulum and Golgi membrane derivatives within the same test tube. We found that GTP and Mn^{2+} could promote fusion of either type of membrane. GTP was active at low concentrations (1 mM) for both types of membrane. Although Mn^{2+} could stimulate fusion of endoplasmic reticulum derivatives at low concentrations (100 μM, results not shown), a minimum of 7 mM Mn^{2+} was required to promote Golgi membrane fusion (data derived from Mn^{2+} concentration curves, not shown). We therefore used an incubation medium containing 30 mM cacodylate (pH 6.6), 10 mM $MnCl_2$, 1 mM GTP and 2 mM ATP to examine heterologous membrane interaction.

Identity of membrane components in heterologous mixtures

Rough microsomes were identified in thin sections of heterologous membrane mixtures by the presence of the morphological marker for the rough endoplasmic reticulum (RER), the ribosome. In order to augment membrane–membrane interaction rough microsomes were treated for the partial release of ribosomes with 0.75 M KCl, 5 mM $MgCl_2$, and 1 mM puromycin. These conditions were similar, but not identical, to those described by Adelman et al. [11] and led to the release of 67% of total RNA [6]. The RNA which remains is easily distinguished morphologically as

ribosomal particles but yet their presence does not hinder fusion.

Golgi components were identified in thin sections of heterologous membrane mixtures by the presence of intraluminal lipoprotein particles [37] and by specific labeling with [^{3}H]neuraminic acid and subsequent detection by radioautography [10]. Radiolabeling of endogenous Golgi constituents was carried out by including 8.6 μCi of the nucleotide sugar CMP-[^{3}H]neuraminic acid, CMP-NeuAc, 18.9 Ci/mmol [10], in the heterologous membrane incubation mixture. Radioautographic detection of labeled glycopeptides in Golgi derivatives was done as previously described [10].

Cytochemistry

Membranes were first incubated in the standard ER fusion medium under various experimental and control conditions and then fixed on ice for 30 min in 1% glutaraldehyde in 100 mM sodium cacodylate (pH 7.5) containing 0.25 M sucrose. The membranes were filtered onto Millipore membranes as described above and the membrane pellicles were washed for at least 30 min in several changes of 100 mM sodium cacodylate buffer (pH 7.5) containing 0.25 M sucrose at room temperature and then incubated for 120 min at 37°C and at pH 7.5 in the cytochemical medium of Novikoff and Goldfischer [14] using either 4 mM thiamin pyrophosphate or inosine diphosphate (IDP) as substrate. Following incubation the membranes were washed in three changes of cacodylate-sucrose buffer, fixed overnight at 0°C with 2.5% glutaraldehyde in 100 mM sodium cacodylate (pH 7) and processed routinely for electron microscopy. In separate experiments membranes were also processed for mannose-6-phosphatase cytochemistry. Pretreated and fixed membranes were incubated for 15, 30 or 60 min at 37°C with 3.6 mM mannose 6-phosphate and 3 mM lead nitrate in 80 mM sodium cacodylate buffer (pH 6.5), washed, and processed for electron microscopy as above.

Enzyme assays

Mannose-6-phosphatase activity and nucleoside diphosphatase were assayed exactly as described by Godelaine et al. [7] in the presence or absence of detergent in order to assess total and free activities, respectively.

Enzyme pretreatments

SRM were treated at 10°C in sucrose-imidazole buffer containing trypsin (0.01–100 μg, type ROTECS Calbiochem-Behring Co., La Jolla, CA). The membrane protein to trypsin ratios varied between 876 : 1 and 13 : 1. Following trypsin treatment the microsomes were incubated at 37°C in standard ER fusion medium plus a 10-fold concentration of soybean trypsin inhibitor (component VI of Rackis, Calbiochem-Behring Co., La Jolla, CA). Control membranes were treated with trypsin as above and subsequently incubated in standard ER fusion medium without inhibitor or they were incubated without pretreatment directly in standard medium at 37°C in the presence or absence of both trypsin and trypsin inhibitor.

Ribonuclease digestion was carried out based on the protocol of Ojakian et al. [12] as follows: 490 μl of rough microsomes (2–3 mg protein/ml) in 0.25 M sucrose and 4 mM imidazole buffer (pH 7.4) were mixed with 10 ml of ribonuclease (0.25–8 μg/ml ribonuclease A type III-A Sigma Chemical Co., MO) in 40 mM Tris-HCl (pH 7.4) and 30 mM KCl. This mixture was incubated for 15 min at 0°C and the microsomes subsequently were washed three times in 0.25 M sucrose-imidazole by repeated centrifugation (40 min at 45 000 rpm using a 60 Ti rotor, Beckman Instruments Inc., Spinco D.L., Palo Alto, CA) and resuspension. Following washing, the microsomes were incubated at 37°C in standard medium. For controls, rough microsomes were treated with ribonuclease as above and subsequently stripped of their associated RNA with pyrophosphate [6] and incubated in standard ER fusion medium. As well, rough microsomes were first stripped with pyrophosphate and then incubated in the same medium containing 8 μg ribonuclease per ml.

Glycosylation

Endogenous glycosylation with rough microsomes was carried out using the procedure previously described [6]. Briefly, SRM (100–500 μg protein) were incubated in the standard ER fusion medium described above to which was added uridine diphosphate-*N*-acetyl-D-[6(n)-^{3}H]glucosamine (UDP-[^{3}H]GlcNAc; 1 μCi, 24 Ci/mmol; New England Nuclear, Boston, MA). Reactions were carried out for varying periods of time and

stopped with ice-cold 5% trichloroacetic acid and carrier albumin (0.1% w/v). The total acid-precipitable products were washed twice with cold 5% trichloroacetic acid, then dissolved in 0.5 ml of Protosol (New England Nuclear) and radioactivity was determined in a Packard model 460 CD spectrometer. Hot trichloroacetic acid-resistant precipitable products were treated as described above except for heating at 90°C for 15 min.

Cell-free protein synthesis

Placental messenger RNA was extracted from human term placenta by a modification of the method of Chirgwin et al. [15] using guanidine thiocyanate and cesium chloride isopycnic centrifugation followed by oligo dT cellulose affinity chromatography [16,17]. RNA was isolated from Vesicular Stomatitis Virus (VSV) infected BHK-21 cells incubated for 4 h with actinomycin D (1 μg/ml), then extracted in guanidinium isothyocyanate essentially by the method of Chirgwin et al. [15].

Prior to in vitro translation dog pancreatic or rat liver SRM were treated as follows: Control membranes were diluted to 1 mg protein per ml in 0.25 M sucrose containing 5 mM imidazole pH 7.4 and GTP and left at 4°C for 1 h. 'Inactivated' membranes were diluted similarly to almost 1 mg/ml protein and incubated at 37°C for 1 h. GTP was then added to bring the concentration to 1 mg/ml protein. GTP-'protected' membranes were diluted in sucrose-imidazole and GTP to 1 mg/ml proteins and incubated for 1 h at 37°C. Each membrane sample (25 μl) was brought to a concentration of 2 U/μl ribonuclease inhibitor [18] and used for in vitro translation. The rest of each pretreated membrane sample was used to assess membrane fusion and in vitro glycosylation as described above.

To assay translocation across microsomal membranes 0.14 μg of protein (rat liver) or 0.08 A_{260} units (dog pancreas) of these membranes were added per μl of in vitro translocation system using micrococcal nuclease treated rabbit reticulocyte lysate (50%) prepared by the method of Jackson and Hunt [19] and programmed with human term placental messenger RNA. The translation mixture also contained 90 mM KCl, 1.2 mM $MgCl_2$, 0.4 U/μl placental ribonuclease inhibitor [18], 0.24 μg/μl yeast transfer RNA and 1 μCi/μl [^{35}S] methionine. Translation mixtures (50 μl) were incubated for 1.5 h at 30°C. They were further incubated for 10 min at 30°C with 100 μg/ml bovine pancreatic nuclease to eliminate [^{35}S] methionyl-tRNA, and then cooled to 4°C. Samples were split into two 25-μl aliquots. One aliquot was treated for 5 min at room temperature with 33 mM tetracaine base [20] in 70 mM KCl, 1 mM $MgCl_2$ (pH 7), followed by an incubation for 45 min at 4°C with 20 μg/ml each of trypsin and chymotrypsin [18] in 70 mM KCl, 1 mM $MgCl_2$ (pH 7). The other aliquot was treated identically except that only 70 mM KCl, 1 mM $MgCl_2$ (pH 7) was added in each incubation. Proteolysis was terminated by the addition of 500 KIU/ml aprotinin. The equivalent of 5 μl original translation mixture was analysed on 12% discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The gel was processed for fluorography with Enhance, dried at 68°C and exposed to X-ray film as previously described [10].

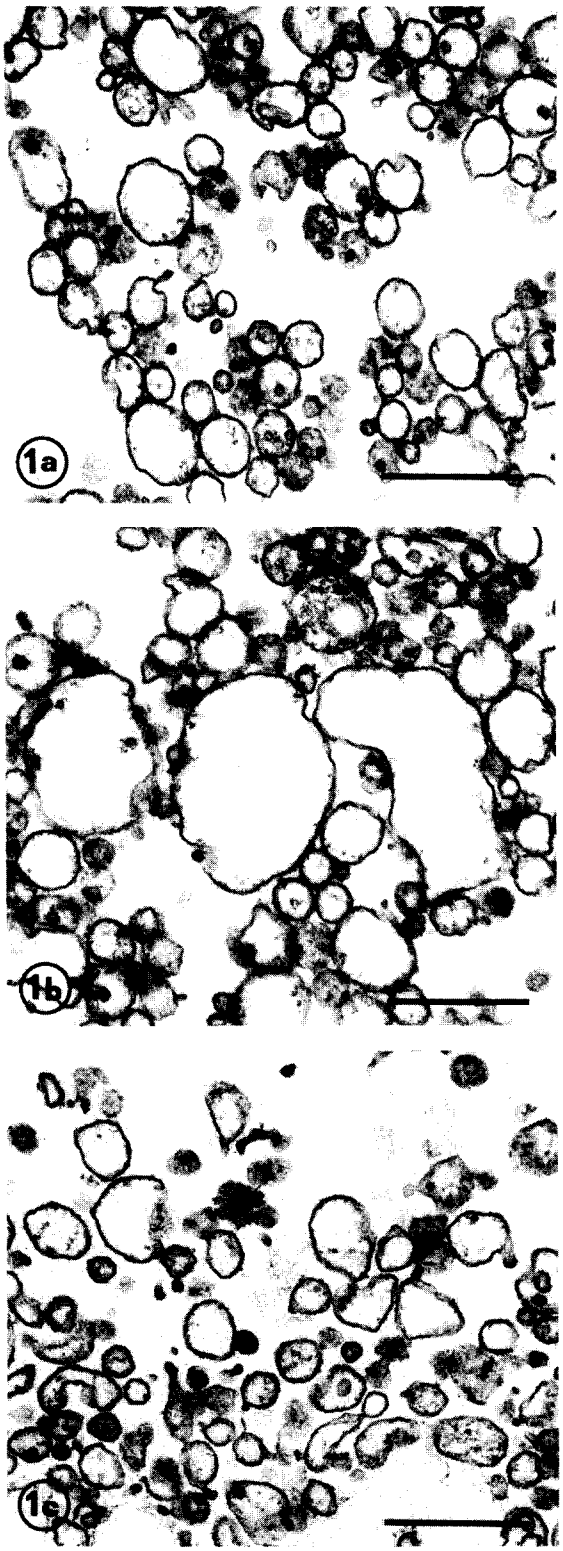
Posttranslational incubations with Golgi membranes

After translation of VSV RNA with dog pancreatic SRM (40 μg protein/100 μl assay) for 60 min at 30°C, the membranes were recovered by centrifugation (Beckman airfuge, 3 min, 20 lb/inch^2) then resuspended in 30 mM sodium cacodylate (pH 7.4), containing 0.25 M sucrose, 1 mM $MnCl_2$, 1 mM $MgCl_2$ and 0.5 mM GTP. After a 5 min preincubation at 37°C, additional buffered GTP and $MnCl_2$ were added to bring their concentrations to 1.5 mM and 10 mM, respectively. 10 μM each of UDP-GlcNAc, uridine diphosphate galactose (UDP-Gal) and CMP-NeuAc and freshly prepared Golgi membranes were added to the microsomes and incubated for 1 h at 37°C. Samples were either diluted with 1 volume of 0.1 M Tris-HCl (pH 6.9), 5% SDS, 1.5 M β-mercaptoethanol and 20% glycerol and subjected to SDS-PAGE or they were incubated for 16 h at 37°C with or without 30 μg endoglycosidase H and subjected to SDS–PAGE.

Results

Effect of preincubation of stripped rough microsomes with GTP

Preincubation of SRM from rat liver at 37°C



for 30 min in the absence of GTP led to membrane inactivation. These membranes did not fuse to form large fusion products in a subsequent postincubation at 37°C in the standard ER fusion medium (Fig. 1a). However, the addition of GTP to SRM during preincubation prevented inactivation. These membranes responded to a postincubation by undergoing membrane coalescence and forming large fusion products (Fig. 1b). Membranes preincubated in the presence of GTP alone without postincubation were unfused (i.e., vesicles were no larger than unincubated vesicles, Fig. 1c). Fusion was examined by quantitation. Since inactivated vesicles had a similar mean perimeter (535 nm) to unincubated vesicles (525 nm) it was concluded that the former were unable to fuse in the standard ER fusion medium. GTP-protected vesicles, however, were able to fuse under similar conditions as demonstrated by the fact that they had undergone an increase in average perimeter length (860 nm). Vesicles incubated with GTP alone did not undergo a size change and had a mean perimeter length (490 nm) i.e. close to that

Fig. 1. Electron micrographs of rat liver SRM following various preincubation and postincubation schemes (a–c).
(a) SRM after preincubation at 37°C for 30 min in the presence of buffered sucrose and following postincubation at 37°C for 60 min in standard ER fusion medium containing 0.5 mM GTP. The vesicle profiles are devoid of ribosomes, many are tightly aggregated but none have fused as judged by the absence of fusion products larger than 0.5 μm and by morphometric measurement of 1808 vesicle profiles (the mean vesicle perimeter was 535 nm, close to 525 nm calculated for unfused vesicles previously reported [6]).
(b) SRM after preincubation at 37°C for 30 min in the presence of buffered sucrose plus 0.5 mM GTP and following postincubation in standard ER fusion medium containing 0.5 mM GTP for 30 min. The vesicle profiles are devoid of ribosomes, many are aggregated and highly variable in size. Coalescence of the normal-sized vesicles has led to the formation of large membrane-bounded fusion products several of which have diameters larger than 0.5 μm. Morphometric measurement of 1126 profiles gave a mean vesicle perimeter of 860 nm indicating a significant increase in size of the vesicle population due to intervesicle fusion.
(c) SRM after incubation at 37°C for 60 min in buffered sucrose containing 0.5 mM GTP. The vesicle profiles are loosely aggregated, and are unfused. No vesicle profiles have diameters larger than 0.5 μm and morphometric measurement of 1937 vesicles gave a mean perimeter of 490 nm, close to that of the unfused vesicles in (a). Bars, 0.5 μm.

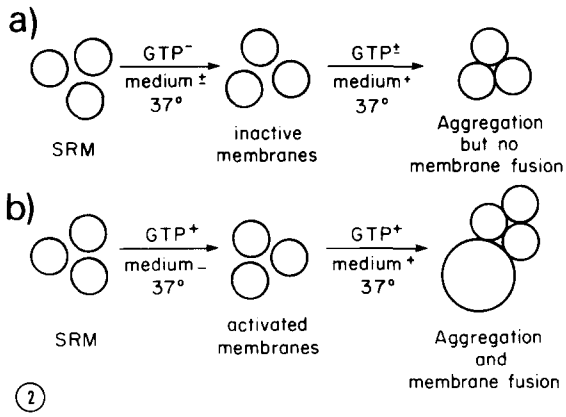


Fig. 2. Summary of incubation protocols that allow detection of membrane activation by GTP. Membrane inactivation occurs when SRM are incubated at 37°C in the absence of GTP plus or minus standard ER fusion medium (a). When such membranes are postincubated at 37°C in standard ER fusion medium plus or minus GTP they aggregate but do not fuse (a). Membrane activation occurs when SRM are incubated in the presence of GTP and without standard ER fusion medium at 37°C (b). Activated membranes will then respond in a subsequent postincubation in standard ER fusion medium plus GTP by undergoing membrane fusion.

of unincubated vesicles. These experiments defined an activated state of SRM (Fig. 2) and indicated that both a specific nucleotide (GTP) and specified cations (Mg^{2+} and/or Mn^{2+}) were required for membrane fusion. Similar results were found for SRM isolated from dog pancreas (data not shown).

Effect of membrane fusion on loss of latency

Latency of mannose-6-phosphatase activity has been used as a quantitative index of the structural integrity of microsomes [21]. Latency was therefore assessed for SRM under fusogenic conditions. Before incubation approx. 85% to 90% of mannose-6-phosphatase enzyme activity was latent, demonstrating low permeability to mannose 6-phosphate (Fig. 3, 0 min preincubations). Similar results were obtained when membranes were preincubated 30 min in the absence of medium plus or minus GTP and when they were preincubated 30 min in the presence of medium minus GTP (Fig. 3). Membrane fusion did not occur following incubation using any of these conditions (data not shown). Following incubation under conditions that created fusion the membranes lost appreciable (56%) latency for mannose-6-phosphatase (Fig.

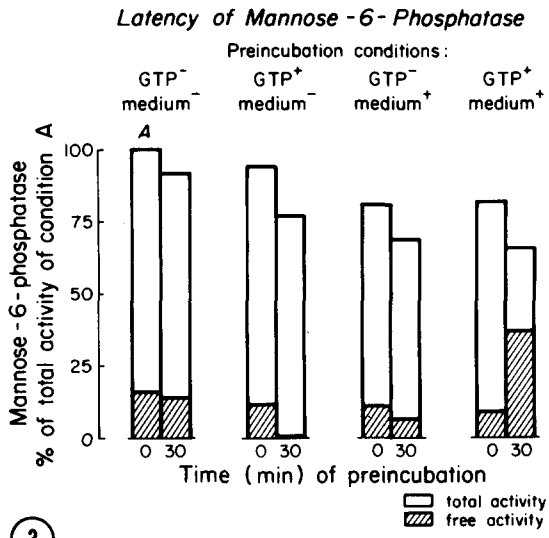


Fig. 3. Latency of mannose-6-phosphatase. SRM were incubated for 0 or 30 min in the presence or absence of buffered GTP with or without additional medium (see Materials and Methods). At appropriate times total or free mannose-6-phosphatase activity was assayed as described by Godelaine et al. [7]. Appreciable loss of latency is revealed only when stripped rough microsomes are incubated with GTP and other medium constituents (i.e., under conditions which yielded fused membranes). None of the other conditions (e.g., GTP^- $medium^-$, GTP^+ $medium^-$, GTP^- $medium^+$) resulted in fused membranes (data not shown). The data represent the average of two experiments.

3) indicating a considerable increase in membrane permeability. Similar results were obtained by assessing for nucleoside diphosphatase activity (data not shown).

Localization of the site of increased permeability

Attempts to localize the sites of GTP-induced permeability changes were carried out by using electron microscope cytochemistry on preincubated microsomes. SRM preincubated in standard ER fusion medium and subsequently processed for nucleoside diphosphatase cytochemistry showed heavy deposits of reaction product, most of which was associated with the luminal aspect of the membrane of large fusion products (Fig. 4a). SRM preincubated in medium lacking GTP showed no fused membranes and spotty deposits of reaction product (Fig. 4b). Similar results were obtained using mannose-6-phosphatase cytochemistry (not shown).

Relationship of membrane proteins to the site of action of GTP

Controlled proteolytic digestion was carried out in order to assess the role of membrane proteins on GTP dependent fusion and core glycosylation. Preincubation of SRM with differing concentration of trypsin led to parallel inhibitions of membrane fusion and GTP-enhanced incorporation of

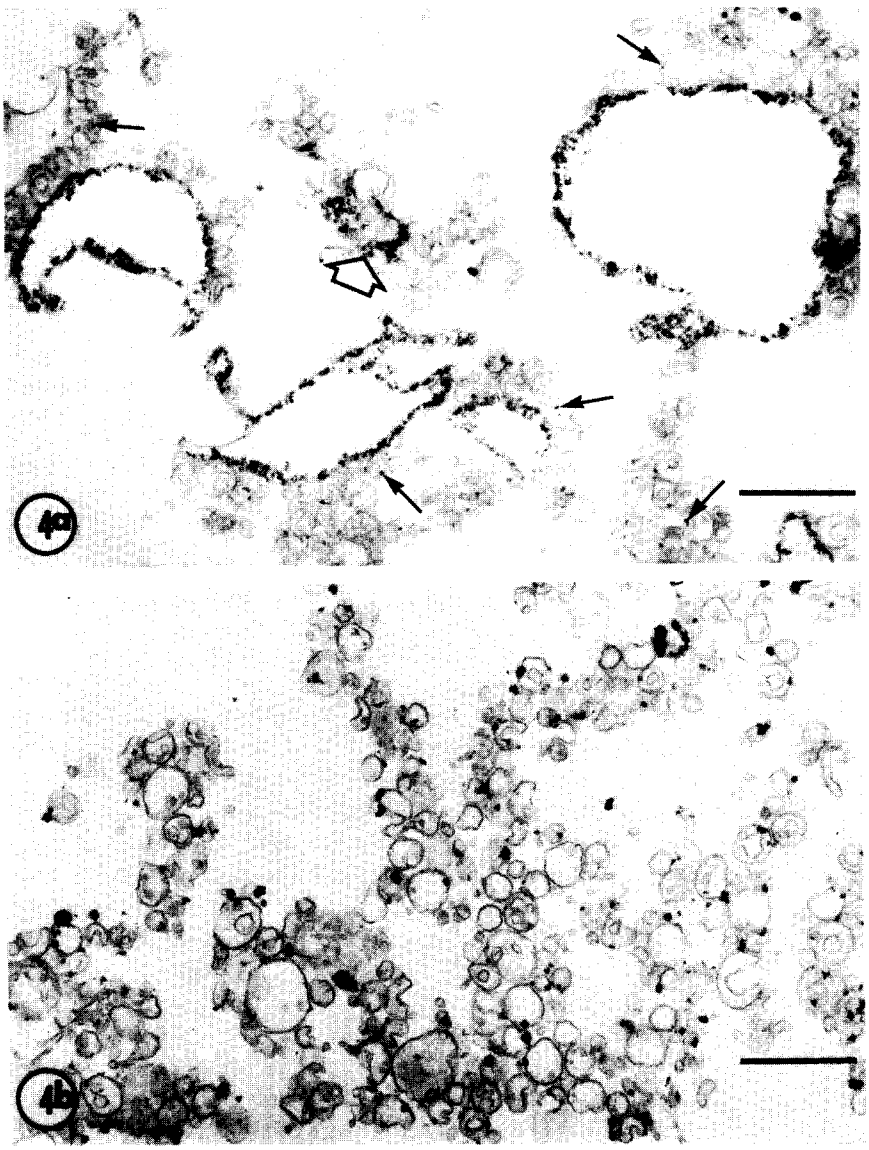
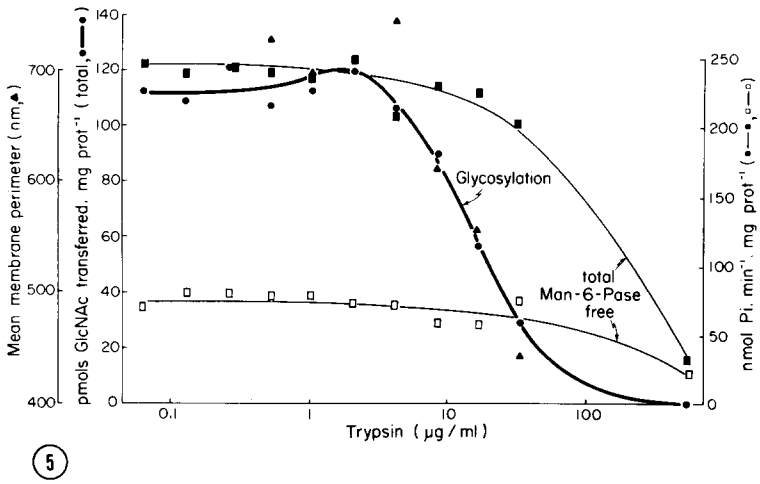


Fig. 4. Electron micrographs of SRM following incubation in standard ER fusion medium plus GTP (a) and minus GTP (b) at 37°C for 60 min and subsequent processing for nucleoside diphosphatase cytochemistry with TPP as substrate as described in Materials and Methods. In (a) enzyme reaction product is particularly evident as dense aggregates along the lumenal border of the membranes of large fusion products. The fusion products products are larger than 0.5 μm in diameter and have numerous small unfused vesicles tightly bound to their periphery. Enzyme reaction product is less evident in the unfused vesicles and appears as discrete electron dense deposits within their lumina (arrows). The open arrow points to a large fusion product cut tangentially and showing a high concentration of enzyme reaction product. In (b) the vesicle profiles are small, none have fused to form fusion products and consequently all vesicles are smaller than 0.5 μm in diameter. Enzyme reaction product appears as discrete electron dense deposits in the lumina of most vesicles. Bars, 0.5 μm.



(5)

Fig. 5. Effect of trypsin on GTP-stimulated glycosylation (●), fusion (▲) and mannose-6-phosphatase latency (□,■). Microsomes were pretreated with varying concentrations of trypsin (see Materials and Methods) and then incubated in the standard ER fusion medium containing GTP at 37°C for 60 min. Glycosylation and mannose-6-phosphatase activity were measured as described in Materials and Methods. Incorporation of [^{3}H]GlcNAc into cold trichloroacetic acid -precipitable products (●) began to decrease after pretreatment of membranes with 5 μg trypsin per ml. Incorporation was greater than 75% diminished for membranes pretreated with 32 μg of trypsin per ml. However, the latency of stripped rough microsomes was maintained following treatment over this concentration range of trypsin (as judged by the fact that total mannose-6-phosphatase activity was unaffected by such treatment). Quantitative analysis of membrane fusion by morphometry (the triangles represent mean perimeters of the vesicle populations, greater than 1000 μm of membrane was measured in each case) showed that fusion was affected by trypsin in a similar manner to that seen for the incorporation of sugar into cold trichloroacetic acid-precipitable products. Linear regression analysis gave a correlation coefficient of $r = 0.988$ which was significant ($P < 0.001$) when membrane fusion (▲) was compared to incorporation of sugar into cold trichloroacetic acid-precipitable products (●).

[^{3}H]GlcNAc into total acceptors with 50% inhibition of both phenomena occurring at 15 μg/ml trypsin (Fig. 5). This constituted a ratio of trypsin to membrane protein of about 1 : 100. In contrast, latency of mannose-6-phosphatase activity was maintained at trypsin concentrations of greater than 50 μg/ml (Fig. 5).

The influence of ribosome distribution on fusion and glycosylation was evaluated by ribonuclease treatment. While control microsomes had ribosomes randomly distributed along their surfaces (Fig. 6a) microsomes treated with low concentrations of ribonuclease (0.5–1 μg/ml) had visible aggregates of ribosomes and bare membrane patches (Fig. 6b). High concentrations of ribonuclease caused aggregation of ribosomes and visible damage to ribosome structure (Fig. 6c). Comparison of the relative amounts of bare membrane areas after treatment with ribonuclease revealed a ribonuclease dependent increase in the amount of such smooth surface regions (Table I). However, these microsomes did not undergo membrane fusion or readily glycosylate endogenous acceptors when incubated in standard ER fusion medium (Table I). Control microsomes pretreated with ribonuclease and then stripped of associated ribosomes by pyrophosphate did fuse following incubation in standard ER fusion medium (not shown). Furthermore SRM incubated in standard ER fusion medium containing ribonuclease (8 μg/ml) also fused (Fig. 6d) and revealed enhanced glycosylation (Table I).

Influence of GTP on cell-free translocation of secretory proteins across ER membranes

The ability to distinguish GTP-sensitive and insensitive membranes was exploited. Hence, we examined whether GTP could influence co-translational vectorial translocation in rat liver and dog pancreas microsomes. Control SRM from rat liver or dog pancreas translocated human preplacental lactogen and converted a variable (dog > rat SRM) proportion of preplacental lactogen (Fig. 7A, lane 2; Fig. 7B, lane 2) to a peptide of mobility identi-

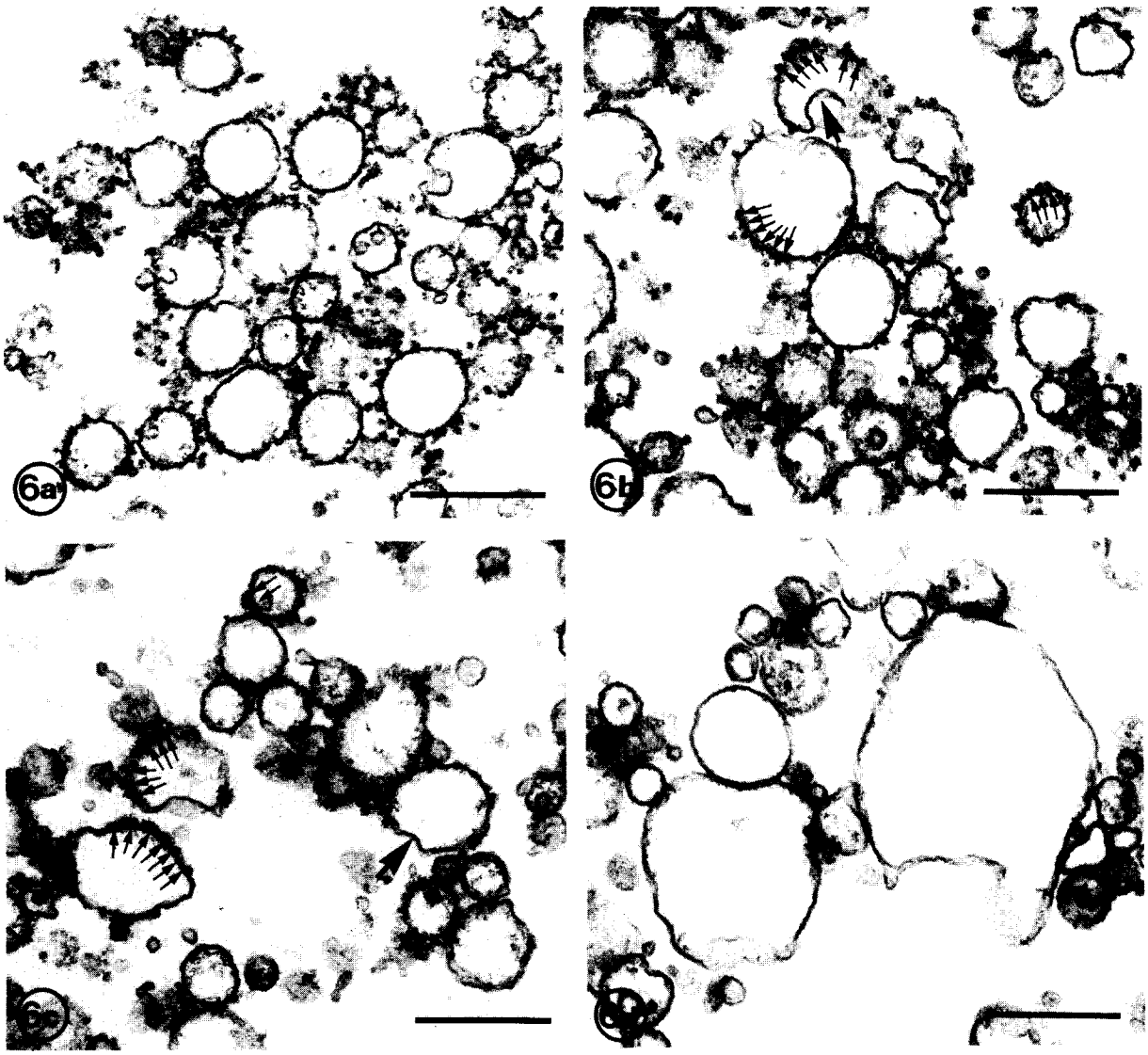


Fig. 6. Effect of ribonuclease digestion on ribosome distribution along the surface of rat liver rough microsomes and on the fusion of SRM. Rough microsomes were pretreated without ribonuclease (a) or with 0.5 μg/ml ribonuclease (b) or 8 μg/ml ribonuclease (c) and incubated in GTP with standard ER fusion medium for 60 min at 30°C as described in Materials and Methods. Control SRM incubated in the presence of 8 μg/ml ribonuclease is also shown (d). Ribonuclease digestion led to marked aggregation of ribosomes along the surfaces of rough microsomal membranes (arrows in (b) and (c)) but did not promote fusion of vesicles as judged by the presence of vesicle-profiles which had a normal size-distribution (i.e., none are greater than 0.5 μm in diameter). Despite the presence of 8 μg/ml ribonuclease, control SRM underwent fusion as indicated by the presence of large fusion products with diameters greater than 0.5 μm (d). This indicates that ribonuclease even at high concentrations will not prevent GTP-stimulated fusion amongst membranes from which ribosomes have been previously removed. There is one noteworthy difference in the ribosome 'capping' we have observed and that reported by Ojakian et al. [12]. We rarely observed the capping of ribosomes into invaginated profiles as reported by these authors. Rather, membrane invaginations (large arrows) were ribosome free i.e. similar to the micrographs of Ojakian et al. [12] where anti 60S ribosomal protein antibodies induced capping (see Fig. 12 of Ref. 12). The reason for this difference may be due to the slightly different conditions employed (lack of high speed supernatant to 'stop' the RNAase treatment) or differences in the preparation of rough microsomes. Bars, 0.5 μm.

cal to placental lactogen (hPL, compare Fig. 7A, lane 3 with Fig. 7B, lane 3). Trypsin digestion showed variable (dog > rat SRM) protection of the band of mobility identical to hPL but not the preform (compare Fig. 7A, B, lanes 4). However, membrane-dependent cleavage of preplacental lactogen to hPl was also observed in SRM ren-

TABLE I

EFFECT OF RIBONUCLEASE ON THE DISTRIBUTION OF MEMBRANE-ASSOCIATED RIBOSOMES, MEMBRANE FUSION AND CORE GLYCOSYLATION IN ROUGH MICROSOMES

RNAase [a] (μg/ml)	% membrane length devoid of ribosomes [b]		Fusion [c]	Incorporation of [^{3}H]GlcNAc into TCA-precipitable products [d]	
	RM	SRM		Total	Acid resistant
0	30 ± 5 (32 ± 4) [e]		–	10.4 ± 0.1	2 ± 1
0.25	30 ± 2 (36 ± 3)		–	13 ± 3	3 ± 2
0.50	43 ± 6 (46 ± 4)		–	15 ± 6	2 ± 0.3
1.00	54 ± 10 (57 ± 7)		–	19 ± 2	4 ± 2
0		100	+	65 ± 14	9 ± 4
8.00		100	+	85 ± 12	14 ± 1

[a] Rough microsomes were treated with different concentrations of ribonuclease then washed as described in the Materials and Methods.

[b] Following incubation in standard ER fusion medium containing GTP for 60 min at 30°C rough microsomes were fixed and processed for electron microscopy. The % membrane length devoid of ribosomes was calculated and represents an analysis of greater than 200 μm of membrane at each concentration. Microsomes treated with greater than 0.25 μg/ml ribonuclease and then incubated in complete medium had % length values significantly greater ($P < 0.001$) than controls as judged by statistical comparisons of means.

[c] Membrane fusion was considered to have occurred whenever incubations yielded a population of vesicles which had a diameter greater than 0.5 μm in thin sections.

[d] For biochemistry rough microsomes were treated identically with or without ribonuclease and then incubated in complete medium containing GTP and UDP-[^{3}H]GlcNAc. Following incubation duplicate samples were removed and assayed for total incorporation into ice-cold trichloroacetic acid precipitates as well as incorporation into hot acid-resistant precipitates as described in Materials and Methods. Incorporation of *N*-acetyl[^{3}H]glucosamine into endogenous acceptors (pmol/mg protein) represents mean values ± S.D. obtained from three separate experiments each carried out in duplicate.

[e] % membrane length values in parentheses represent results of a separate experiment.

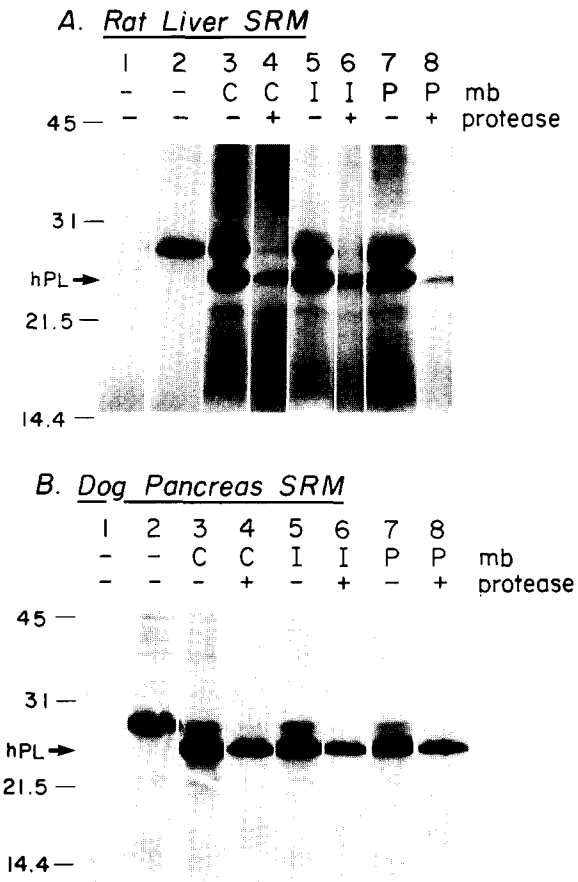


Fig. 7. Radioautograph showing cell-free translation and membrane dependent translocation of human placental lactogen mRNA using membranes (mb) from rat liver (A) or dog pancreas (B). Cell-free translation was carried out in the absence of membranes (A, B, lanes 2 with an M_r of 29500 noted for pre-hPL i.e. 3200 greater in mol. wt. than a comigrating standard of hPL stained by Coomassie brilliant blue and having an M_r 26300). Addition of control SRM (C) or SRM which had been rendered insensitive to GTP/Mg^{2+} by prior incubation at 37°C for 1 h (I) or SRM protected from heat inactivation by GTP (P) led to the generation of a new band of M_r 26300 (A, B, lanes 3, 5, 7) i.e. identical to that of comigrating hPL standard (hPL). Posttranslational addition of trypsin/chymotrypsin (protease) led to loss of the band of M_r 29500 but variable retention of the band comigrating with hPL i.e. M_r 26300. Control experiments where translation was carried out in the absence of SRM or placental mRNA revealed no translocated product. Identification of translation products as pre-hPL and hPL was verified by specific immunoprecipitation [22] with hPL antiserum followed by SDS-PAGE and radioautography (not shown).

dered resistant to GTP/Mg^{2+} by prior heat inactivation (1 h, 37°C) as well as in GTP-protected membranes (Fig. 7A, B, lanes 5, 7). Subsequent trypsin proteolysis revealed protection by dog pancreatic SRM with less observed in rat liver SRM (Fig. 7A, B, lanes 6, 8). Parallel experiments with specific antiserum to human placental lactogen verified the identity of the radiolabeled bands as preplacental lactogen and placental lactogen as evaluated by SDS–PAGE of immunoprecipitates (data not shown).

Effect of incubation conditions which promote homologous membrane fusion on the interaction between rough microsomes and Golgi membranes

We next examined the possibility that GTP divalent cation induced fusion may be relevant to interorganelle transport of protein. We first determined whether common nucleotide and cation factors could promote both ER-ER membrane fusion and Golgi-Golgi membrane fusion. A systematic evaluation of the nucleotide and divalent cation requirements for homologous membrane fusion revealed that ER membranes and Golgi membranes could be induced to fuse by the same factors, that is in the presence of Mn^{2+} and GTP (Table II). We then asked the question can rough microsomes interact with Golgi membranes using incubation mixtures which contain the above two important fusion factors? We looked for evidence of interaction using both morphological and biochemical approaches. Morphologically, we looked for the formation of hybrid membrane fusion products recognized by the presence of ribosomes (indicative of ER origin) and an association with radioactive label as detected following incorporation of [^{3}H]neuraminic acid and processing for radioautography (indicative of Golgi origin). Despite repeated efforts we were unable to detect hybrid membrane fusion products (i.e., products with associated ribosomes and radioautographic grains). The fusion products (identified by their size, i.e., greater than 0.5 μm diameter) that were observed in such mixtures were either exclusively of ER or Golgi origin (Fig. 8). Following incubation the ER derivatives (as identified by associated ribosomes) were mainly aggregated together (Fig. 8a) and separated apart from Golgi derivatives (as identified by lipoprotein content, Fig. 8b, or radioautographic label, not shown) which were also found in aggregates (Fig. 8b). On infrequent occasions ER derivatives were found near Golgi elements (Fig. 8c). [^{3}H]Neuraminic acid label (radioautographic grains) was rarely observed associated with ribosome-studded vesicles.

We also looked for evidence of ER–Golgi interaction using a biochemical approach. RNA was prepared from cells infected with VSV and used for cell-free protein synthesis in the presence of SRM. These microsomes were then incubated in the presence of Golgi membranes from rat liver. Assuming ER-Golgi fusion it was predicted that

TABLE II

COMPARISON OF CATION AND NUCLEOTIDE SPECIFICITIES FOR MEMBRANE FUSION IN ROUGH ENDOPLASMIC RETICULUM MEMBRANES AND GOLGI MEMBRANES

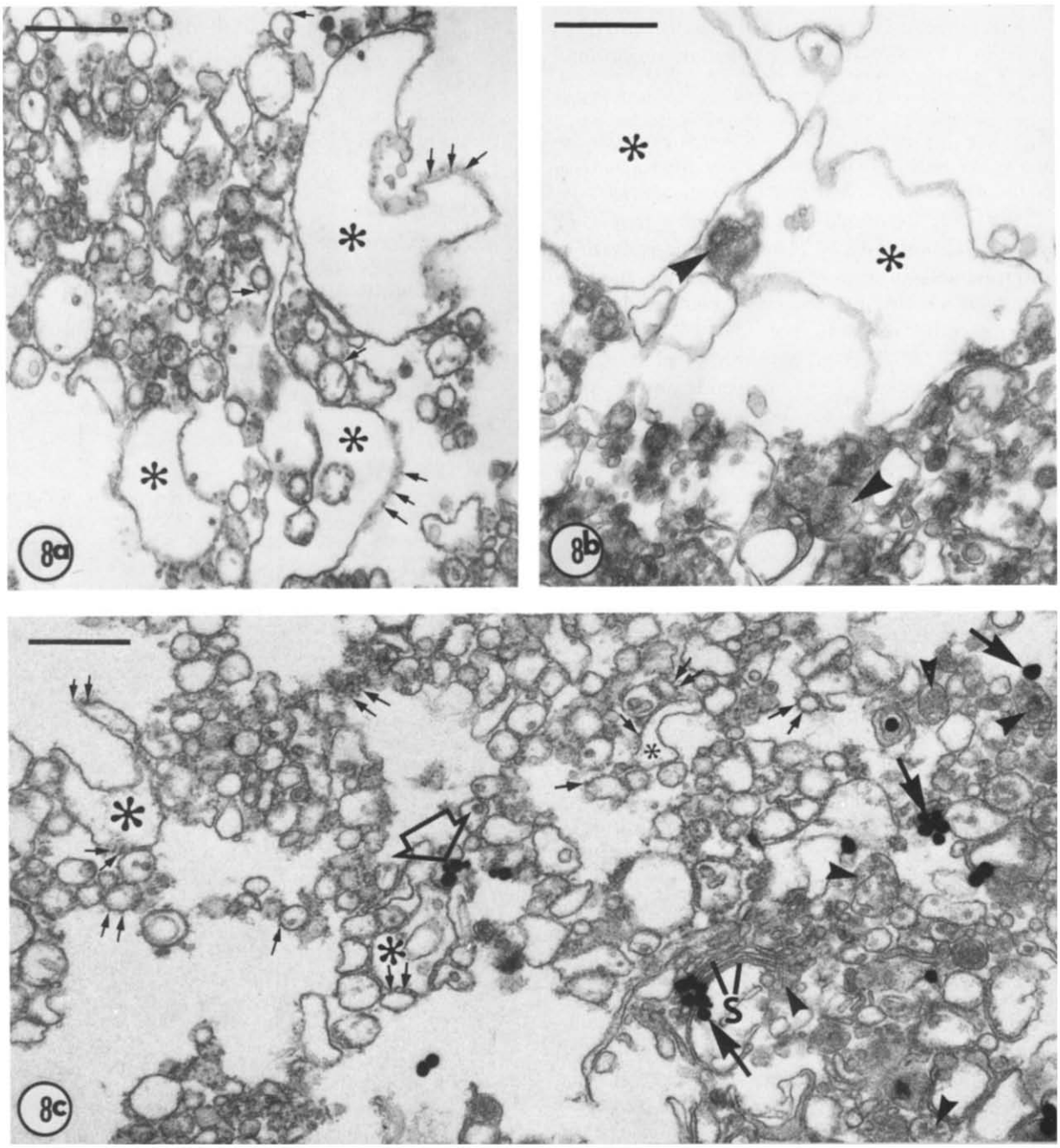
Cation or nucleotide	Amount of fusion [b]	
	RER [a]	Golgi [a]
Mg^{2+}	+ +	−
Mn^{2+}	+ + +	+ + +
Ca^{2+}	+	−
Zn^{2+}	−	−
Cu^{2+}	−	−
Cd^{2+}	−	N.D. [c]
ATP	−	+ + +
CTP	−	+ + +
GTP	+ + +	+ + +
ITP	−	+ + +
UTP	−	+ + +

[a] ER membranes were incubated in standard ER fusion medium containing 0.5 mM GTP plus 2 mM of the test cation or in the presence of standard ER fusion medium containing 7.5 mM Mg^{2+} and 2 mM Mn^{2+} plus 1 mM of the test nucleotide. Incubations were done at 37°C for 60 min. Golgi membranes were incubated in standard Golgi medium containing 2 mM ATP plus 20 mM of the test cation or in the presence of standard Golgi medium containing 20 mM Mn^{2+} plus 2 mM of the test nucleotide. Golgi incubations were carried out at 37°C for 60 min.

[b] Degree of fusion was assayed as previously suggested by Creutz [23] and as described in Materials and Methods. This involved the semiquantitative determination of the amount of formation of large vesicles as seen in the electron microscope: + + +, extensive fusion, many large vesicles evident; + +, intermediate fusion, large vesicles distinct but smaller; +, limited fusion, very few vesicles two or three times the size of the average population of vesicles; −, no fusion, all vesicles the same size as unincubated vesicles.

[c] N.D., not determined.

the VSV G protein (which is normally endoglycosidase H digestion sensitive in the ER) would become endo H insensitive due to terminal glycosylation following fusion of ER membranes with Golgi membranes. In fact SDS-PAGE analysis revealed no alteration in the electrophoretic mobility (expected if the G protein had been terminally glycosylated) or endo H sensitivity of ER containing core glycosylated G protein following incubation with Golgi membranes under fusion condi-



tions (Fig. 9). Similarly, SRM containing core glycosylated G when incubated with Golgi membranes did not become sensitive to neuraminidase whereas mature G isolated in vivo was sensitive (data not shown). Furthermore, radiolabeled G after incubation of SRM with Golgi membranes under fusion conditions supplemented with terminal sugar nucleotides for terminal glycosylation did not bind lectins from *Arbus precatorius, Brandeiraea simplicifolia, Ricinus communis* or soybean, all of which have affinity for galactose and/or *N*-acetylgalactosamine.

Fig. 8a. Electron micrograph of puromycin-treated rough microsomes (see Materials and Methods) following incubation at 37 °C for 120 min in the presence of Golgi membranes and a medium containing 30 mM sodium cacodylate pH 6.6, 10 mM $MnCl_2$, 1 mM GTP and 2 mM ATP. This field shows only rough microsomes. The vesicle profiles are aggregated and exhibit a heterogeneity in size because many vesicles have undergone vesicle fusion in the presence of GTP and Mn^{2+} (three large fusion products with diameters greater than 0.5 μm are evident, asterisks). Ribosomal remnants, representing about one third of the original RNA associated with the microsomes are observed along the periphery of both fused and unfused structures (arrows). Bar, 0.5 μm.

Fig. 8b. Electron micrograph of Golgi elements following incubation in the presence of puromycin-treated rough microsomes exactly as in Fig. 8a. The vesicle profiles are aggregated and exhibit a heterogeneity in size because many vesicles have undergone membrane fusion in the presence of Mn^{2+} and the nucleotides GTP and ATP (large fusion products with diameters greater than 0.5 μm are evident, asterisks). Small unfused vesicles can be seen at the periphery of fused elements and contain electron dense lipoprotein particle content(arrowheads). The morphology of the lipoprotein content has undergone change due to in vitro incubation and is similar to that previously observed [10,13]. Bar, 0.5 μm.

Fig. 8c. Electron microscope radioautograph of puromycin-treated rough microsomes and Golgi membranes incubated as in Figure 8a except that the medium also contained CMP-[^{3}H]NeuAc. In this field both rough microsomes and Golgi elements are present. The vesicle profiles representing the rough microsomes can be seen on the left and upper portions of the radioautograph. They are aggregated and heterogeneous in size due to fusion in the presence of GTP and Mn^{2+}. Both fused vesicles (larger than 0.5 μm, asterisks) and unfused vesicles display ribosomal remnants at their periphery (small arrows). The vesicle and saccule profiles (S) representing Golgi elements can be seen on the right hand portion of the radioautograph. They are aggregated, contain lipoprotein particle content (arrowheads) and have associated with them radioautographic silver grains (large arrows) which indicate that they are the sites of location of neuraminic acid label. Silver grains observed over fusion products which have ribosomal remnants at their periphery (open arrow) are thought to originate from closely applied and heavily labeled Golgi elements. Bar, 0.5 μm.

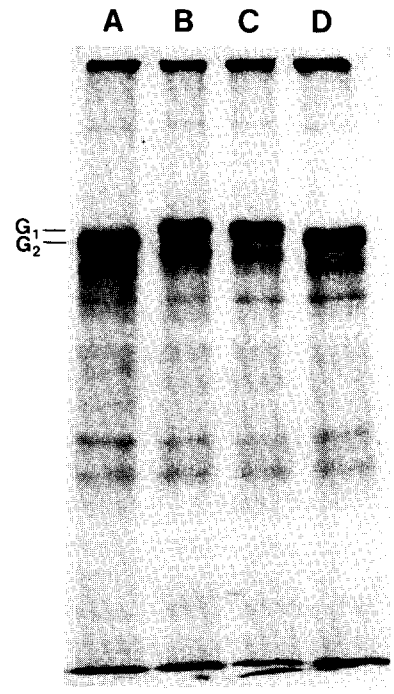


Fig. 9. Posttranslational incubation of dog pancreatic SRM with liver Golgi membranes. RNA from VSV infected cells was translated in the presence of SRM as described in Materials and Methods then incubated with (lane A) or without (lane B) endo H for 16 h at 37°C and subjected to SDS-PAGE. Identical samples of SRM after translation were incubated with Golgi membranes under fusogenic conditions as described in Materials and Methods and then incubated without (lane C) or with endo H (lane D). The order of addition of constituents is indicated beneath each gel lane. G_1, core glycosylated glycoprotein 'G' of molecular weights approx. 66000. G_2, endo H digested derivative of G_1 of approx. 63000 mol. wt. Molecular weights were estimated from corresponding virion proteins and molecular weight markers (not shown).

Discussion

The physiological relevance of the phenomena under study is strongly supported by the con-

centrations of GTP (10 μM) and $MgCl_2$ (as low as 100 μM, unpublished observations) required to elicit fusion of SRM in vitro. Such concentrations are well within the physiological range [24–27]. Hence, a complete description of the membrane events which are regulated by GTP/Mg^{2+} and the identification of the molecules involved in fusion should point to the true physiological role of the phenomena in vivo.

In the present study a number of new facts have been obtained which are relevant to the mechanism of action of GTP on RER membranes. Furthermore, several hypotheses attempting to explain why GTP acts on ER membranes to enhance glycosylation and membrane fusion have been tested. These points are considered in further detail.

Nucleotide and cation effects on membrane fusion can be uncoupled

When SRM were preincubated at 37°C in the absence of GTP they become inactivated and did not respond by fusion in a subsequent postincubation at 37°C in the presence of standard medium containing GTP and the specific cations Mg^{2+} and Mn^{2+}. The simple addition of a physiological concentration of GTP to SRM in sucrose solution and preincubation at 37°C prevented inactivation and the membranes responded to a postincubation in standard ER fusion medium by undergoing structural (fusion) and biochemical (permeability and core glycosylation) changes. These results indicated that GTP induced a primary event within the microsomal membrane which in itself did not affect precocious morphological and biochemical changes. In order for such changes to occur the membranes required subsequent incubation in the presence of the cation $MgCl_2$ and $MnCl_2$. Thus nucleotide and cation effects could be uncoupled. The identity of the primary event stimulated by GTP remains to be determined.

GTP stimulated permeability changes occur in association with the membranes of large fusion products

The addition of GTP along with divalent cations ($MgCl_2$ or $MnCl_2$) to SRM led to loss of latency of mannose-6-phosphatase and nucleoside diphosphatase as previously documented by Godelaine et al. [7]. We show here that permeability changes occurred coincident with membrane fusion. Furthermore, the electron microscopy cytochemical data indicated that the heaviest and most uniform deposits of reaction product were associated with large membrane fusion products. This subset of membranes was previously shown by combined electron microscope morphometry and radioautography to constitute the main site of GTP/$MgCl_2$ stimulated endogenous glycosylation [6]. In contrast to the conclusions of Godelaine et al. [7], our data indicate that the GTP/$MgCl_2$ induced loss of latency and hence enhanced glycosylation occurred coincidentally within the same fused SRM. Loss of latency and enhanced glycosylation can be explained as increased leakiness of ER membranes induced by fusion. Other in vitro model fusion systems have been observed to express leakiness [23,28–32]. In the case of ER fusion and associated leakiness we do not understand why they should go together. Perhaps a search for in vivo equivalents [33] may enlighten us on these points.

GTP activation of ER membranes occurs via a cytosolically exposed trypsin-sensitive constituent

A quantitative comparison (Fig. 5) revealed coincident inhibition of ER membrane fusion and glycosylation following trypsin treatment. Since the concentrations of trypsin required to maximally inhibit membrane fusion and glycosylation hardly affected mannose-6-phosphatase activity, an intraluminally oriented enzyme, it was concluded that a cytosolically oriented protein constituent(s) of SRM may serve as an intermediate in the cascade of events which eventually lead to fusion, permeability changes and glycosylation. As guanylyl imidodiphosphate would not stimulate fusion [5] then phosphorylation of a cytosolically oriented protein is a plausible but speculative primary event stimulated by GTP [5].

GTP acts at a membrane site near or in association with ribosome binding regions

RNAse induced ribosome capping led to increased smooth membrane surface areas associated with rough microsomes. As much as 50 percent of vesicle surface area could be exposed by such treatment and yet membrane fusion did not

occur during incubation in the standard ER fusion medium. Only ribosome removal elicited GTP/$MgCl_2$ dependent fusion of SRM and associated enhanced glycosylation (Table I). Ojakian et al. [12] concluded that the majority of intramembrane particles and consequently membrane proteins, do not co-distribute with ribosomes under analogous conditions of RNAase induced ribosome capping. The results point to a selective location of the GTP-sensitive protein(s), near or beneath the ribosome in ER membranes. They also rule out steric hindrance as the reason for needing to remove ribosomes in order to see fusion.

The involvement of GTP in vectorial protein translocation and interorganelle transport

The hypothesis that GTP may play a role in co-translational translocation [7] has been tested. The ability of SRM to respond to GTP/$MgCl_2$ and undergo fusion did not correlate with the capacity of SRM to translocate newly synthesized proteins through the same membranes. Hence, SRM preincubated at 37°C in the absence of GTP were unable to fuse when challenged with GTP/$MgCl_2$. These membranes were fully competent to translocate prehuman placental lactogen co-translationally into the lumina of the vesicles as evaluated by conversion of pre-hPL to hPL and trypsin resistance of the latter but not the former. The same result was obtained with membranes preincubated at 37° but 'protected' by GTP or control membranes. It is nevertheless noteworthy that nucleotides (ATP and GTP have been implicated in the translocation of newly synthesized peptides across ER membranes [34–36]). The design of our experiments did not exclude the possible participitation of hydrophobic or amphipathic phosphopeptides in the transport of nascent chains or in the GTP/$MgCl_2$ stimulated fusion phenomenon.

We also assessed the possible role of GTP in heterologous membrane–membrane interactions (ER–Golgi). The morphological studies revealed no unequivocal formation of an ER (marked by ribosome remnants) - Golgi apparatus (sialic acid radiolabeled) hybrid despite considerable homologous membrane fusion i.e. ER-ER and Golgi-Golgi. Furthermore, no evidence for transport of a specific membrane glycoprotein (i.e. VSV G) from ER to Golgi in vitro was found under conditions of optimal GTP-dependent membrane fusion. These studies demonstrate the remarkably exquisite specificity of in vitro fusions and point to the existence of membrane recognition factors at the surface of SRM and Golgi membranes which act to promote homologous membrane interaction but seemingly exclude heterologous fusion.

The testing of the role of GTP in translocation and cell-free transport of membrane proteins yielded negative findings. Future studies must incorporate an experimental design which clarifies (a) the mechanism of action of GTP and (b) the physiological relevance of the phenomena stimulated by this nucleotide.

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