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THE MEMBRANE OF THE ROUGH ENDOPLASMIC RETICULUM CONTAINS CYTOPLASMICALLY EXPOSED HIGH AFFINITY GTP-BINDING SITES

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Binding experiments using [^{14}C]GTP and rat liver rough microsomes gave Scatchard plots with Kd $\simeq$ 0.1 µM and a binding capacity $\simeq$ 40 pmol/mg microsomal protein. Removal of the ribosomes did not modify the binding parameters. GTP was competed out by GTP analogues but not by ATP. Limited proteolysis of rough microsomes decreased the GTP-binding capacity and prevented GTP from suppressing the latency of mannose-6-phosphatase and of the synthesis of dolichol-linked chitobiose. The GTP-binding protein is probably involved in these effects of GTP. Its function could be to act in the association-dissociation of membrane components at the ribosome-membrane junction.

Upon incubation with GTP, the properties of rough microsomes (RM) from rat liver are markedly changed : synthesis of oligosaccharides on endogenous dolichylphosphate (Dol-P) and their subsequent transfer to proteins are considerably enhanced (1-3) ; microsomal vesicles fuse (4) ; luminal enzymes are released or gain free access to substrates present in the surrounding medium (5). These changes are observed at micromolar GTP concentration, only on RM which have been stripped of ribosomes, but neither on intact RM, nor on smooth microsomes (1-5). ITP is weakly active ; other nucleotides, including GTP analogues, are inactive (1, 4, 5). The GTP-induced modifications of RM occur through a common mechanism, still to be elucidated. Possibly, fusion produces leaky vesicles, thereby activating enzymes which are normally hampered by the membrane barrier (5, 6). How RM

Abbreviations : RM, rough microsomes ; Dol-P, dolichylphosphate ; ER, endoplasmic reticulum.

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vesicles are triggered to fuse in the presence of GTP is a still pending question. Here we demonstrate the presence of high affinity GTP-binding sites on the RM membrane. After limited proteolysis of RM, the capacity to bind GTP is lost, in parallel with the effects of this nucleotide. Thus, the endoplasmic reticulum (ER) membrane contains a cytoplasmically-exposed GTP-binding protein, the function of which is discussed.

Materials and Methods

Preparation and stripping of RM

Rat liver microsomes were prepared in 0.25 M sucrose and subfractionated in a sucrose gradient (1). The rough subfraction was washed 3 times and finally resuspended in 0.25 M sucrose. To strip RM, the sucrose solution used for the first two washings contained 3 mM EDTA, or 5 mM Na-pyrophosphate when specified.

Limited proteolysis of RM

Stripped RM, derived from 1 g liver, were incubated for 1 hr at 0°C, in 1 ml of medium consisting of 0,25 M sucrose, 50 mM KCl, 50 mM TRIS-HCl buffer at pH 7.4, and trypsin, pronase, elastase, or subtilisin at concentrations given in the legend of Figures. The proteolytic attack was ended by adding 50 µl of 3 mg/ml soybean trypsin inhibitor for trypsin, 20 mM EGTA for pronase, 20 mg/ml phenylmethylsulfonyl fluoride in 20 % dimethyl sulfoxide for elastase and subtilisin. Before use, pronase was incubated for 1 hr at 50°C in the presence of 10 mM $CaCl_2$ and 0.1 M TRIS-HCl buffer at pH 8.

Incorporation of N-acetylglucosamine into endogenous Dol-P

Stripped RM (0.4 g liver/ml) were incubated with 6 µM UDP-[^{14}C]GlcNAc in medium A, consisting of 0.25 M sucrose, 40 mM TRIS-HCl buffer at pH 7.4, 30 mM KCl, 7.5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 2.5 mM dithiothreitol, 1 mM ATP, 50 µg/ml pyruvate kinase (200 U/mg), and 10 mM phosphoenolpyruvate. After 2 hrs at 37°C, 10 vol of chloroform/methanol (3/2, vol/vol) and 1.6 vol of 4 mM $MgCl_2$ in water were added. The chloroform/methanol extract was analyzed by thin-layer chromatography and the radioactivity running with Dol-PP-$GlcNAc_2$ was counted (1).

Assay of mannose-6-phosphatase

Mannose-6-phosphatase activity was assayed as previously described (5). Stripped RM were preincubated at 37°C in medium A, with or without GTP, and diluted afterwards with 0.4 vol of 0.25 M sucrose (free activity), or 0.25 M sucrose containing 3 % (w/vol) Na-taurocholate (total activity).

Binding of [^{14}C]GTP

Binding of GTP was determined after incubation of RM (0.1 g liver) in 0.25 ml of medium A, supplemented with [^{14}C]GTP. Equilibrium dialysis was performed in paired cells of 0.25 ml, separated by a Millipore^R filter VMWP 0.05 µm, one of which was loaded with RM. [^{14}C]GTP at concentrations varying between 0.05 and 1 µM was added to both cells. After 24 hrs dialysis at 3°C the radioactivities were determined and the concentrations of free and total ligand were calculated. In other expriments, RM were incubated with 0.025-0.4 µM [^{14}C]GTP for 1 hr at 0°C and

centrifuged for 30 min at 30,000 rpm. Pellets (dissolved in 0.1 ml 1 N NaOH) and supernatants were used to determine bound and free [^{14}C]GTP, respectively. To correct for unspecific binding incubations were run in parallel with 200 µM unlabeled GTP.

Materials

Elastase was purchased from E. Merck AG (Darmstadt, FRG), pronase from Calbiochem (La Jolla, CA). UDP-N-acetyl-[U-^{14}C]glucosamine (247 Ci/mol) and [U-^{14}C]guanosine 5'-triphosphate (476 Ci/mol) were from The Radiochemical Centre (Amersham, UK). GTP-γ-S was from Boehringer Mannheim (FRG). Other biochemical reagents were from the Sigma Chemical Co. (St. Louis, MO).

Results

Evidence for GTP-binding sites at the external surface of stripped RM vesicles was obtained from the results of equilibrium dialysis experiments (Fig. 1). Scatchard analysis gave an apparent affinity constant of 3.4 x 10^6 M^{-1}, and a specific content of 50 pmol/mg microsomal protein. This latter value is somewhat lower than the ribosome load of unstripped RM : 86 pmol/mg protein, as deduced from an RNA content of 0.22 mg/mg protein.

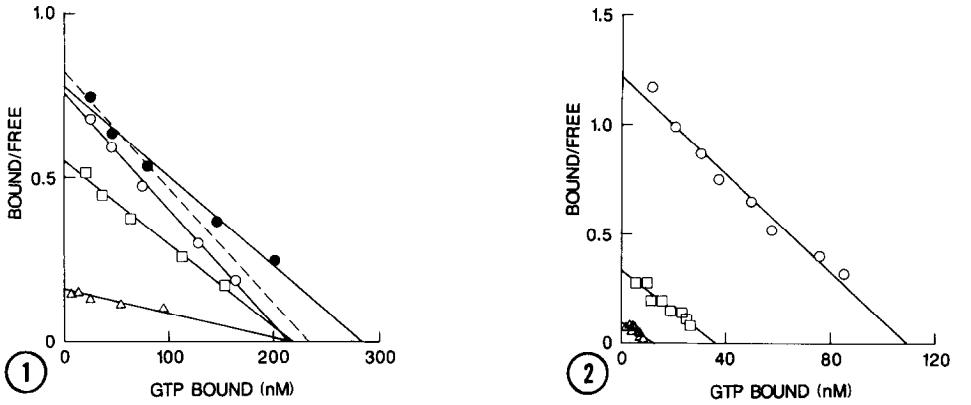

Fig. 1. Binding of GTP to intact and stripped RM. Intact RM (closed circles) and RM stripped with Na-pyrophosphate (open symbols) were dialyzed for 24 hrs (see Materials and Methods) in a medium which contained [^{14}C]GTP at various concentrations (●, ○) and 0.1 µM (□) or 1 µM (△) GTP-γ-S. The dashed line was fitted to the first 3 values of the intact RM preparation. RM contained 10.9 mg protein per g liver.

Fig. 2. Effect of proteolysis on GTP binding to stripped RM. Stripped RM were incubated for 60 min at 0°C with 50 µg/ml elastase (□), or subtilisin (△), or in the absence of protease (○). After proteolysis, they were incubated for 60 min at 0°C with [^{14}C]GTP at various concentrations and centrifuged as described in Materials and Methods.

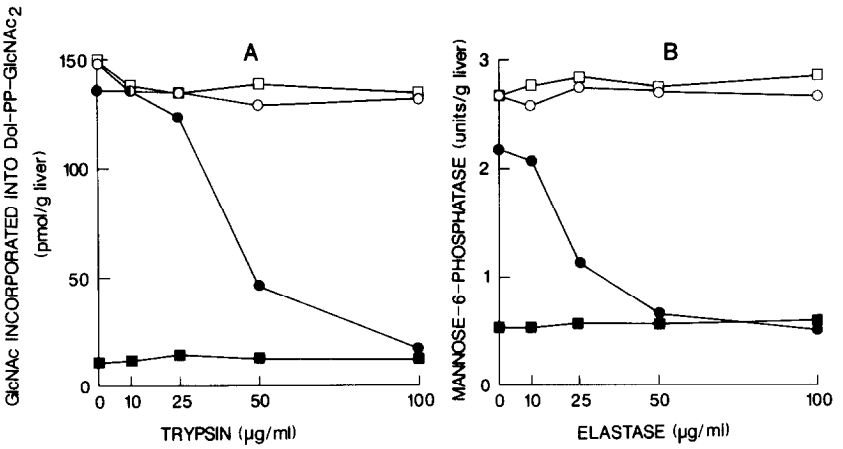

Fig. 3. Effect of proteolysis on the GTP-induced synthesis of Dol-PP-GlcNAc$_2$ (A) and activation of latent mannose-6-phosphatase (B). Stripped RM were incubated at 0°C with the amounts of trypsin (A), or elastase (B) indicated (abscissa). After 60 min, proteolysis was ended as described under Materials and Methods. In A, RM were subsequently incubated with UDP-[^{14}C]GlcNAc for 120 min at 37°C in medium A (■), or in medium A supplemented with 0.5 mM GTP (●), with 2.5 mg/ml Triton X-100 (□), or with 0.5 mM GTP and 2.5 mg/ml Triton X-100 (○). Dol-PP-GlcNAc$_2$ was extracted in chloroform/methanol, isolated by thin-layer chromatography and counted. In B, RM were further incubated for 30 min at 37°C in medium A (□,■), or in medium A supplemented with 0.5 mM GTP (○,●) and subsequently assayed for mannose-6-phosphatase in the absence (■,●) or presence (□,○) of Na-taurocholate.

Since the medium contained 1 mM ATP, these sites have no significant affinity for this nucleotide. In contrast, GTP-γ-S acts as a competitor, with an affinity identical to that of GTP ; GTP was also competed out by GMP-PNP and dGTP (data not shown). Intact RM showed similar GTP-binding properties, but with a significant deviation from linearity, suggestive of unspecific binding. At the low GTP concentrations, RM (dashed line) bound the nucleotide like stripped vesicles.

Centrifugation experiments (Fig. 2) also evidenced GTP-binding sites on stripped RM, with an affinity of 11×10^6 M^{-1} and a specific content of 27 pmol/mg protein. The number of sites was markedly reduced after digestion by subtilisin, elastase (Fig. 2), trypsin, and pronase (data not shown).

Fig. 3 illustrates the loss of sensitivity to GTP in stripped RM after proteolysis. Before proteolysis, the synthesis of Dol-PP-GlcNAC$_2$ from endogenous Dol-P was considerably enhanced by GTP, Triton X-100, or the combination of both (Fig. 3A). After digestion with trypsin, RM preparations could still produce nor-

mal amounts of Dol-PP-GlcNAc$_2$ in the presence of Triton X-100, but not in the presence of GTP alone. Similar results (not shown) were obtained with pronase, elastase, and subtilisin. Before proteolysis, mannose-6-phosphatase, a latent activity of the enzyme glucose-6-phosphatase, became fully active on incubation of stripped RM with GTP, but addition of a detergent became gradually necessary to manifest the full activity after treatment with increasing amounts of elastase (Fig. 3B), or other proteases (data not shown).

Discussion

Our results demonstrate high affinity GTP-binding sites in rat liver RM. Removing the ribosomes did not noticeably change their concentration, indicating that they belong to the membrane, not to the ribosomes, and that they are accessible even when quiescent ribosomes are bound to the membrane. Their estimated number is approximately half that of the ribosomes. They show a sensitivity to proteases in sharp contrast with the resistance of mannose-6-phosphatase and glycosylation activities, which argues for a disposition at the cytoplasmic surface and makes it possible that their concentration *in situ* is higher than in the isolated RM.

As the capacity of GTP to relieve the latency of mannose-6-phosphatase and of Dol-PP-GlcNAc$_2$ synthesis is lost almost in parallel with GTP binding in proteolyzed vesicles, the GTP-binding protein of the ER is most probably involved in the effects of GTP on stripped RM. Although this nucleotide binds to intact RM, the need of detaching the ribosomes for GTP to act on the membrane does not rule out that conclusion. Indeed, in addition to GTP binding, membrane modification may be conditioned by another event which depends on the removal of ribosomes. The stripped RM aggregate in the reaction medium, whereas the intact vesicles do not (4). Conceivably, aggregation of the vesicles and the resulting tight contact between membranes may be a prerequisite for fusion to occur in the presence of GTP. This, or another non-physiological aspect of the means used to strip RM, probably explains that the GTP-dependent system of the ER can manifest itself *in vitro* by effects without direct physiological relevance (5).

The presence of a GTP-binding protein at the cytoplasmic surface of the rough ER raises the question of its physiological role. Because the sensitivity to GTP is restricted to RM, we have suggested that a membrane function in protein synthesis by bound polysomes depends on GTP (5). Recently, Perara et al. (7) were able to show that, in dog pancreas RM, posttranslational translocation of polypeptides still in a complex with ribosomes requires energy substrates. Considering distinct steps of this process, Connolly and Gilmore (8) showed that GTP is needed to form a stable and functional ribosome-membrane junction, but that nascent chain transport, per se, does not depend on ribonucleotide hydrolysis. A possible function for the GTP-binding protein of the ER membrane demonstrated here may thus be to form the active ribosome-membrane association. In agreement with this view, non-hydrolyzable analogues of GTP promote functional ribosome attachment to the membrane (8) and compete with GTP in binding experiments (Fig. 1). However, these analogues do not induce fusion (4) and the related modifications of the stripped RM vesicles (1, 5). Therefore, the physiological function behind these modifications most likely involves GTP hydrolysis. GTP could be cleaved at a later stage of polypeptide translocation, possibly after termination of protein synthesis. It has been postulated that membrane constituents assemble into a transient polypeptide translocation apparatus which dissociates when the ribosome is functionally released (see 9). Some data are compatible with this hypothesis. In particular, stripped microsomes rebind 80S ribosomes with high affinity in the absence of protein synthesis (10, 11); the sites for ribosome rebinding and the ribosomes originally bound are present in nearly identical number and show the same distribution between microsomal subfractions (11). Thus, native microsomes apparently carry only occupied binding sites, as expected if the ribosome-binding structure dissociates upon termination of the polypeptide in the cell. GTP hydrolysis at this stage could facilitate the dissociation of the polypeptide translocation apparatus. Conceivably, a similar rearrangement of membrane components could be induced by GTP hydrolysis in stripped RM and result in fusion of vesicles where membranes are in close contact.

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