Phosphorylation and Purification of Integral Endoplasmic Reticulum Membrane Proteins

by

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© Donna E. Rindress 1989
For Lauren, Daniel, Denis and Brendan for their love, optimism, enthusiasm, support and wisdom.
Felix qui potuit rerum cognoscere causas

(Lucky is he who has been able to understand the causes of things)

Virgil
Saturnalia, 490
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ABSTRACT

Three proteins of stripped rough microsomes (SRM) from dog pancreas or rat liver, with apparent molecular weights of 35, 56 and 90 kDa (pgp35, pp56, pp90), were phosphorylated in vitro by both [γ-32P] GTP and [γ-32P] ATP. Another SRM protein of 15 kDa (pp15) was phosphorylated in vitro only by [γ-32P] GTP. Comparison of the in vitro phosphorylation profile of SRM to those of other well defined subcellular fractions, i.e., plasma membranes, Golgi apparatus, lysosomes, endosomes, mitochondria and smooth microsomes, showed the four major GTP phosphorylated proteins were restricted to the endoplasmic reticulum (ER). Further characterization of these proteins showed them to be phosphorylated on serine residues at the cis face of SRM and to be integral membrane proteins. The 35 kDa protein was glycosylated, with 2 N-linked oligosaccharide sidechains, therefore possessing a luminal domain. Competition and inhibition studies showed that GTP and ATP phosphorylation of the proteins were distinct. Regulation of GTP specific phosphorylation was by adenosine nucleoside. Purification of the 35 and 90 kDa phosphoproteins was achieved, along with another nonphosphorylated glycoprotein of 25 kDa, as a phosphoglycoprotein complex which elutes from Sephacryl S300 chromatography at an apparent molecular weight of about 400,000. Partial sequences from pgp35 CNBr fragments showed identity with the Signal Sequence Receptor (Weidmann et al, 1987) Nature 228: 830-833), suggesting that this phosphoglycoprotein complex may form part of the translocation apparatus of the rough ER. These results suggest a role for phosphorylation of ER membrane proteins in the process of translocation.

Name: Donna E. Rindress
Title: Phosphorylation and purification of integral endoplasmic reticulum membrane proteins
Department: Anatomy
Degree: Doctor of Philosophy
**RESUME**

Trois protéines de microsomes rugueux dénudés (MRD) provenant de pancréas de chien ou de foie de rat, dont les poids moléculaires apparents étaient respectivement de 35, 56 et 90 kDa (pgp35, pp56, pp90), ont été phosphorylées *in vitro* par le (r-32P) GTP et le (r-32P) ATP. Une autre protéine MRD de 15 kDa (pp15) a été phosphorylée *in vitro* uniquement par le (r-32P) GTP. La comparaison du profil de phosphorylation *in vitro* du MRD à celui d'autres fractions intracellulaires bien définies, comme la membrane plasmatique, l'appareil de Golgi, les lysosomes, les endosomes, les mitochondries et les microsomes lisses, a permis de démontrer que les quatre principales protéines soumises à une phosphorylation par le GTP ne se retrouvaient que dans le réticulum endoplasmique (RE). En poussant plus loin la caractérisation de ces protéines, on a pu démontrer qu'elles étaient phosphorylées sur des résidus de sérine à la face cis des MRD et qu'elles se présentaient comme des protéines membranaires intégrales. La protéine de 35 kDa a été glycssylée à l'aide de deux chaînes latérales d'oligosaccharides à liaison N, c'est-à-dire qu'elle est dotée d'un domaine intraluminal. Les études de compétition et d'inhibition ont démontré que les phosphorylations des protéines par GTP et par ATP différaient. La régulation de la phosphorylation spécifique par GTP s'effectue par le biais du nucléoside d'adénosine. On a réalisé la purification de phosphoprotéines de 35 et de 90 kDa de même que celle d'une glycoprotéine non phosphorylée de 25 kDa sous forme d'un complexe de phosphoglycoprotéine élué par chromatographie sur Sephacryl S300 et dont le poids moléculaire apparent est de 400,000. Des séquences partielles provenant de fragments de CNBr (pgp35) semblent identiques aux récepteurs de séquence de signaux (Signal Sequence Receptor) (Weidmann et al (1987) Nature 328: 830-833). D'après ces observations, ce complexe de phosphoglycoprotéine pourrait bien s'inscrire dans un appareil de translocation du RE rugueux. Ces résultats donnent à penser que la phosphorylation des protéines de la membrane du RE intervientrait dans le processus de translocation.

**Nom:** Donna E. Rindress  
**Titre:** Phosphorylation et purification de les protéines membranaire intégrales de le réticulum endoplasmique  
**Département:** Anatomie  
**Diplôme:** Doctorat en Philosophie
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<table>
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<tr>
<td>AMP-PNP</td>
<td>adenyllyl-5'-imidophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ATPγS</td>
<td>adenosine-5'-O(3-thiotriphosphate)</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celcius</td>
</tr>
<tr>
<td>Con A</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>DTT</td>
<td>DL-dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>[ethylene bis (oxyethylenenitriilo)] tetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>xg</td>
<td>number of times greater than gravity</td>
</tr>
<tr>
<td>GMP-PNP</td>
<td>guanylyl-5'-imidodiphosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine-5'-O-(3-thiotriphosphate)</td>
</tr>
<tr>
<td>GTPγS</td>
<td>guanosine 5'-O-(3-thiotriphosphate)</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-(2-Hydroxyethyl)-1-piperazine N'-2-ethane sulfonic acid</td>
</tr>
<tr>
<td>HMG</td>
<td>hydroxymethylglutaryl</td>
</tr>
<tr>
<td>hr</td>
<td>hour</td>
</tr>
<tr>
<td>KIU</td>
<td>kallikrein inhibitor units</td>
</tr>
<tr>
<td>l</td>
<td>liter</td>
</tr>
<tr>
<td>µl</td>
<td>microliter</td>
</tr>
<tr>
<td>µM</td>
<td>micromolar</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>NaF</td>
<td>sodium fluoride</td>
</tr>
<tr>
<td>Na3VO4</td>
<td>sodium orthovanadate</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethylmaleimide</td>
</tr>
<tr>
<td>PAS</td>
<td>periodic acid schiff</td>
</tr>
<tr>
<td>pH</td>
<td>(-\log 10 [H^+]))</td>
</tr>
<tr>
<td>PM</td>
<td>plasma membrane</td>
</tr>
<tr>
<td>RM</td>
<td>rough microsomes</td>
</tr>
<tr>
<td>rER</td>
<td>rough endoplasmic reticulum</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SRM</td>
<td>stripped rough microsomes</td>
</tr>
<tr>
<td>SRP</td>
<td>signal recognition particle</td>
</tr>
<tr>
<td>SRPR</td>
<td>signal recognition particle receptor</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>Tris</td>
<td>2(hydroxymethyl) 2-amino-1,3 propanediol</td>
</tr>
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INTRODUCTION

This work involves the characterization of GTP specific ER membrane fusion, the identification and purification of integral ER membrane phosphorylated proteins, and examination of the hypotheses that (a) the fusion phenomenon is related to the translocation of nascent chains across ER membranes, and (b) the nucleotide requirements of each involve the use of nucleoside triphosphates as phosphate donors in the phosphorylation of one or more constituents responsible for fusion and translocation.

a) **Structure and function of the ER**

The ER is a subcellular organelle with several important functions. The internal membranous ER network was first observed under the electron microscope by Keith Porter (Porter et al, 1945; Porter, 1952). The first definitive morphological and biochemical description of the ER, and of the ER-enriched microsomal fraction, was made by George Palade (Palade, 1956; Palade and Siekevitz, 1956). It has been described morphologically as an extended and continuous membranous network of flattened cisternae and interconnected tubules pervasive throughout most cells (Terasaki et al, 1986). Structurally the ER exhibits dynamic behavior in spread cells in culture, with constant tubular branching and fusion which appears to depend on microtubular function (Lee and Chen, 1988; Terasaki et al, 1986). The dependence of ER structural maintenance on microtubules has been extensively demonstrated, such that antimicrotubular drugs cause disruption and collapse of the ER network (Terasaki et al, 1986; Louvard et al, 1982; Vogl et al, 1983).

The ER carries out several apparently unrelated functions. Table I summarizes the known functions of the ER and the proteins which mediate those functions. Some of these systems involve general biosynthetic functions; others participate in metabolic reactions characteristic of specific cell types. Among those functions are phospholipid and cholesterol synthesis, steroid synthesis and microsomal metabolism, targeting and translocation of nascent secretory and integral membrane proteins, co and post translational modification of those nascent...
proteins, regulation of folding, structure and post ER-targeting, and various other miscellaneous functions.

Phospholipid, cholesterol and steroid synthesis and the enzymes of microsomal metabolism

The ER is the intracellular location of eukaryotic membrane biogenesis, i.e. biosynthesis of membrane phospholipid occurs at the cytosolic face of the ER membrane. Synthesis either takes place in association with the lipid bilayer or in the cytosol followed immediately by incorporation into the bilayer. Most of the enzymes catalyzing the reactions are ER membrane associated and contain large cytosolic active domains. They consist of a series of ATP kinases, phosphatases and acyl transferases, and use cytidine nucleotides as carriers of head alcohol groups or phosphatidic acid. Newly made phospholipid is localized to the cytosolic leaflet of the ER, and is actively translocated to the luminal leaflet. Phospholipid asymmetry is seen in the ER membrane but the mechanism of achieving this asymmetry, and how newly synthesized phospholipid is transported to other cellular membranes is not well understood (Bishop and Bell, 1985; Pagano and Sleight, 1985; Rothman and Lenard, 1977; Imai and Gershengorn, 1987a,b).

The ER is also the site of synthesis of cholesterol, and enzymes of the ER and mitochondria together are responsible for steroid hormone synthesis from cholesterol. Cholesterol biosynthesis is a highly regulated pathway involving a series of enzymes, the rate limiting steps being the ones catalyzed by hydroxymethylglutaryl CoA reductase (HMG CoA reductase) and HMG CoA synthase (Brown et al, 1978). All steroid hormones are derived from either de novo synthesized or internalized cholesterol. The cytochrome P450 reaction cycles in mitochondrial and microsomal membranes which mediate steroid synthesis involve progressive modification as the substrate is transferred back and forth between the two compartments, binding, and hydroxylation (Coon, 1980).

The cytochrome P450s, NADPH cytochrome P450 reductase, cytochrome b5, NADH cytochrome b5 reductase, and NADPH cytochrome c reductase constitute
the "microsomal monooxygenase system" or the "microsomal electron transport chain". They perform a series of hydroxylation, N-dealkylation and O-dealkylation reactions, and besides their involvement in steroid synthesis, they function in the oxidation of numerous endogenous and xenobiotic compounds, both polar and nonpolar. There are many isozymes of P450, all of which are integral to the ER membrane with large globular cytoplasmic active domains (Ortiz de Montellano, 1986; Pompon and Coon, 1984; Black and Coon, 1982; Masaki et al, 1987; Monier et al, 1988; Brown and Black, 1989; Negishi et al, 1975; Gennis, 1977; Kensil and Strittmatter, 1986; White and Coon, 1980).

**Targeting and translocation of nascent peptides**

The ER is also the intracellular site for targeting of nascent secretory, lysosomal and integral membrane proteins and for their translocation across or insertion into the ER membrane. These functions are restricted to the rough or ribosome studded regions of the ER (the rER) (Amar Costesec et al, 1984).

When the nascent chain of a protein destined for the ER emerges from the ribosomal complex, it interacts with the multimeric ribonucleoprotein Signal Recognition Particle (SRP) which is present in substoichiometric amounts with respect to ER bound ribosomes. This SRP complex targets the nascent chain to the ER membrane by binding to its cognate receptor (the SRP receptor). Under physiological conditions, SRP spends about 47% of the time associated with ribosome-nascent chain complexes in the cytosol and another 38% of the time associated with the ER membrane. (Walter and Blobel, 1981, 1983; Siegel and Walter, 1985). The SRP receptor is an integral ER membrane protein also present in substoichiometric amounts with respect to bound ribosomes, indicating transient association between the two. Besides its function in binding SRP nascent chain complexes, it reverses SRP induced elongation arrest and stimulates the release of SRP from the signal sequence, presumably after a stable bond has been formed between the nascent chain ribosomal complex and some other component of the membrane (Gilmore et al, 1982, a,b; Tajima et al, 1986; Lauffer et al, 1986; Connolly and Gilmore, 1989). Recently, a Signal Sequence Receptor
(SSR) has been identified in the ER membrane which binds the signal sequence after SRP and SRP receptor have disengaged, is present in stoichiometric amounts to bound ribosomes, and which is essential for translocation (Wiedmann et al, 1987; Hartmann et al, 1989). The fact that antibodies directed against the SSR can also inhibit SRP receptor function (Hartmann et al, 1989), suggest a close association between the two proteins, at least transiently.

The passage of the nascent chain across the phospholipid bilayer is still poorly understood. An integral ER membrane proteinaceous apparatus functioning as a tunnel through which the chain may pass has been postulated (Blobel and Dobberstein, 1975). More recently, Singer (1987a,b) has elaborated a similar apparatus, hypothesizing an allosteric heteromeric protein, along the lines of the nicotinic acetylcholine receptor, which would facilitate translocation by sequential changes in conformation. The stimulus for conformational change could come from binding of effectors, interaction with other proteins, phosphorylation, or any combination of the three. Recent work has indirectly confirmed the proteinaceous apparatus hypothesis in demonstrating the presence of integral membrane proteins closely interacting with nascent chains during translocation (Connolly et al, 1989) and an aqueous microenvironment for the translocating peptide (Gilmore and Blobel, 1985).

The ribophorins I and II are two transmembrane proteins which are restricted to the rough ER (Marcantonio et al, 1984; Crimaudo et al, 1987). Although the ribophorins have been well characterized, even cloned and sequenced, their function has not yet been established, but there is indirect evidence that they may be involved in nascent chain targeting and/or translocation (Marcantonio, 1984; Crimaudo et al, 1987; Hortsch and Meyer, 1985; Hortsch et al, 1986).

Other proteins have been identified in rough microsomal membranes which may be related to the translocation function but which have not yet been further characterized. Two synthetic signal sequence binding proteins of M₄ 45 kDa which are integral to the ER have been described (Robinson et al, 1987). Two sensitivities (protease and NEM) of ER membranes were described
which did not correlate with signal peptidase, SRP receptor or ribophorins I or II function and/or integrity, but did correlate with protein translocation (Hortsch et al, 1986; Nichitta and Blobel, 1989).

Co and posttranslational modification of nascent peptides

Resident in the ER are several proteins which mediate the several co and posttranslational modifications of newly synthesized polypeptides.

N-linked oligosaccharides play a role in the structure and stability, targeting and recognition of glycoproteins (Gallagher et al, 1988). There are a group of ER enzymes involved in the synthesis of dolichol pyrophosphoryl oligosaccharide for asparagine linked glycosylation of nascent peptides (Kornfeld and Kornfeld, 1980) and an oligosaccharide transferase, which catalyzes the transfer of oligosaccharide chains from dolichol phosphate oligosaccharide to specific asparagine residues of the nascent peptide (Geetha-Habib et al, 1988). Further processing of the oligosaccharide side chains occurs, beginning while still in the ER by glucosidase I and II. These are the first of the oligosaccharide processing enzymes known to be luminal and not integral membrane proteins (Burns and Touster, 1982; Strous et al, 1987).

Many of the proteins targeted to the ER contain on their amino terminal end a short peptide sequence (signal sequence) which is cleaved by the signal peptidase activity which resides at the luminal face. This activity has been purified as part of a glycoprotein complex from rough microsomal membranes (Amar-Costesec et al, 1988; E.Evans et al, 1986; Jackson et al, 1977).

Also within the ER are the activities of protein disulfide isomerase, prolyl hydroxylase, glycosylation site binding protein, and thyroid hormone binding protein, all functional in nascent chain modification. These have all been proposed to reside in a single 57 kDa luminal protein (Geetha-Habib et al, 1988).
Another modification which occurs during or shortly after translation is the covalent addition of lipid. Myristyl and Palmytyl transferase activities are found on the cytosolic face of the ER membrane, while the addition of complex phospholipid tails occurs within the lumen of the ER (Sefton and Buss, 1987).

Regulation of correct folding and structure and targeting

Correct folding and subunit assembly correlates with transport of secretory and membrane proteins from the ER as well as with biological activity (Copeland et al., 1988; Smith et al., 1987). Two glucose regulated proteins which share homology with the heat shock proteins (GRP78/BiP and GRP94/ERp99) are major constituents of normal mammalian ER, and inducible by stimuli which result in nascent peptide malfolding. They have been proposed for a role in assembly and supervision of correct folding (Munro and Pelham, 1986; Kozutsumi et al., 1988; Hendershot et al., 1988; Mazarella and Green, 1987). There is a degradative compartment either part of or closely related to the ER, morphologically and biochemically distinct from the lysosomal compartment, which seems to be involved in the removal of improperly assembled membrane protein complexes from the secretory pathway (Lippincott-Schwartz et al., 1988).

Many of the proteins which reside in the ER lumen are distinguished from those in transit by a carboxy terminal sequence of four amino acids, Lys-Asp-Glu-Leu (KDEL), which is sufficient and necessary for their retention. A KDEL binding protein with ER luminal membrane attachment has been proposed but not yet found (Munro and Pelham, 1987). The protein Egasyn is a permanent ER resident known to retain microsomal beta-glucuronidase via its esterase active site. Only about 10% of Egasyn is occupied by this enzyme, the remaining 90% exists in free form (Medda et al., 1987). This novel form of ER compartmentalization may be extended to other ER proteins.

Exit of secretory and membrane proteins from the ER is the rate limiting step in their passage through the secretory pathway (Lodish et al., 1983; Scheele and Tartakoff, 1985). Nascent proteins are transported initially
to the cis-Golgi compartment, by vesicular intermediates (Lodish et al., 1987), in an energy dependent fashion requiring GTP hydrolysis and Ca2+ (Balch et al., 1987; Beckers and Balch, 1989). There is evidence of a dynamic membrane recycling pathway between the rER and the cis/medial Golgi apparatus, possibly responsible for preservation of total membrane content and surface area of the ER (Lippincott-Schwartz et al., 1988) and the sorting of luminal ER proteins from secreted proteins (Pelham, 1988; Pelham et al., 1988).

Other functions

Glucose-6-phosphatase (G6Pase) is a major protein constituent of the ER, equal in the liver to a quarter to a third of total ER enzyme activity. It catalyzes the final enzymatic step in gluconeogenesis (Hers and Hue, 1983) producing free glucose from glucose-6-phosphate. It also seems to be involved in the regulation of IP3 induced Ca2+ mobilization (Wolf et al., 1986). Because of it's abundance and it's intraluminal location, it is commonly used as a biochemical or cytochemical marker of ER membranes (Beaufay et al., 1974).

Glucuronosyl transferases are involved in both glycosaminoglycan synthesis in the Golgi apparatus and drug detoxification in the ER. There are several different ER glucuronosyl transferase activities, each specific for the particular substrate (e.g., bilirubin, bile acid, testosterone, estrone, 4-nitrophenol) which requires glucuronosylation and excretion via the liver biliary system (Nuwayhid et al., 1986).

About 30-50% of total cellular beta glucuronidase, previously considered only a lysosomal enzyme, has been shown to reside in the lumen of the ER. It is retained via an ester linkage to the ER resident protein Egasyn, and is not a biosynthetic intermediate (Medda et al., 1987). It's specific ER role is not known.

The ER is a high affinity, physiologically relevant site for Ca2+ storage. Found in the ER membrane is a 100 kDa Ca2+ and K+ stimulated (Mg2+ dependent) transport ATPase, which imports Ca2+ into the ER lumen,
probably balanced with the export of ions to maintain electrical balance (Imamura and Schulz, 1985; Kemmer et al, 1987). The ER is implicated as at least one of the intracellular storage sites for calcium, the release of which is regulated by phosphoinositol bisphosphate (IP3) and GTP. Recently the immunocolocalization of IP3 receptors and other ER marker proteins supports this hypothesis (Ross et al, 1989).

**Proteins of unknown function**

Several ER proteins have been described in the literature to which no function has yet been ascribed. A 41 kDa apparently luminally oriented peripheral G protein has been found (Gα) which shares homology with the alfa subunits of several well known G proteins and localizes to the rER (Audigier et al, 1988). Its function is not yet known, but preliminary results suggest that it is not involved in translocation.

Four proteins of Mr 29, 58, 66 and 91 kDa were localized exclusively to the ER by Louvard et al (1982) using immunoperoxidase labelling and antisera against stripped rough ER. These were thought to be good potential immunocytochemical markers for rER membrane, but no attempt was made to determine their role in vivo.

The enzyme nucleoside diphosphatase (NDPase) was localized to the ER (Novikoff and Heus, 1963) and this enzyme activity has since been a popular biochemical and cytochemical marker for the ER. No known in vivo function has been assigned to this enzyme.

Finally, microsomal esterase is another ER marker enzyme (Beaufay et al, 1974) which has not yet found an in vivo function.
**TABLE I**

Proteins of the Endoplasmic Reticulum

**Enzymes of lipid, cholesterol and steroid synthesis and microsomal metabolism**

- Enzymes involved with cholesterol biosynthesis, e.g., hydroxymethylglutaryl CoA synthetase and reductase.  
  (Kochevar and Anderson, 1987)

- Enzymes of steroid synthesis  
  (Coon, 1980; Gill, 1987)

- Enzymes of phospholipid synthesis e.g., Phosphatidylinositol synthase (CDP-diacylglycerol inositol phosphatidytransferase)  
  (Greenberg, 1968)  
  (Imai and Gershengorn, 1987a,b)

- Cytochromes P450 and NADPH and cytochrome P450 reductase  
  (Brown and Black, 1989)  
  (Monier et al, 1988)  
  (Masaki et al, 1987)

- NADPH cytochrome c reductase and NADH cytochrome c reductase  
  (Negishi et al, 1975)  
  (Amar-Costesec et al 1984)

- Cytochrome b5 and NADH and cytochrome b5 reductase  
  (Gennis, 1977)  
  (Kensil and Strittmatter, 1986)

- Glucuronosyl transferase  
  (Nuwayhid et al, 1986)

**Proteins involved in targeting and translocation of nascent peptides**

- Signal recognition particle (SRP)  
  (Walter and Blobel, 1981, 1983b)  
  (Siegel and Walter, 1985)

- SRP receptor  
  (Gimore et al, 1982a,b)  
  (Tajima et al, 1986)  
  (Lauffer et al, 1985)

- Signal sequence receptor (SSR)  
  (Wiedmann et al, 1987)  
  (Hartmann et al, 1989)

- Ribophorin I and II  
  (Kreibich et al, 1983)  
  (Marcantonio et al, 1984)  
  (Harnik-Ort et al, 1987)  
  (Crimaudo et al, 1987)  
  (Amar-Costesec et al, 1988)
Two 45 kDa signal sequence binding proteins detected by in vitro binding of synthetic signal sequences (Robinson et al, 1987)

Two unidentified proteinaceous components of ER membrane necessary for translocation (Hortsh et al, 1986)

Enzymes involved in co and post-translational modification of nascent peptides

A group of enzymes involved in synthesis of dolichol pyrophosphoryl oligosaccharide for asparagine linked glycosylation of nascent peptides (Kornfeld and Kornfeld, 1980)

Oligosaccharide transferase (Geetha-Habib et al, 1988)


Prolyl-4-hydroxylase (Kivirikko and Myllyla, 1982)

Glucosidase I and II (Strous et al, 1987) (Burns and Touster, 1982)

Alpha-mannosidase (Bischoff and Kornfeld, 1983)


A group of enzymes which covalently modify nascent proteins with lipid e.g., palmityl and myristyl transferase (Sefton and Buss, 1987)

Proteins involved in posttranslocation supervision of folding, structure and targeting

A group of proteins implicated in the regulation of protein folding and quaternary structure

a) BIP/GRP78 (Ig heavy chain binding protein/glucose regulated protein) (Hendershot et al, 1987, 1988) (Munro and Pelham, 1986)

-Egasyn (Medda et al, 1987)
- Unidentified KDEL receptor (Pelham, 1988)

**Proteins with other function**

- Glucose-6-phosphatase (Amar-Costesec 1984, 1988)
- (Ca2+ and K+ stimulated) Mg2+ dependent Transport ATPase of ER (Imamura and Schulz, 1985)
- InsP3 receptor (Ross et al, 1989)
- Beta-glucuronidase (Medda et al, 1987)

**Proteins of unknown function**

- G_{rer} (Audigier et al, 1988)
- 29, 58, 66, and 91 kDa (four proteins detected by immunocytochemical electron microscopy) (Louvard et al, 1982)
- An 83 kDa protein to which no function has yet been attributed (Sharma et al, 1978)
- Nucleoside diphosphatase (Novikoff, 1963)
- Unidentified GTP binding protein(s) of SRM which mediate fusion and stimulate glycosylation (Paiement et al, 1987) (Godelaine and Beaufay, 1987)
- 19, 20, 22, 24 kDa (four GTP binding proteins of unknown function) (Comerford and Dawson, 1989)
- Microsomal esterase (Amar-Costesec, 1984)
b) **Known roles for nucleotides in the ER**

All of the major functions of the ER require nucleotides, as does structural maintenance (Terasaki et al., 1986; Louvard et al., 1982; Vogl et al., 1983), and it appears that the dynamic structure is probably also dependent on the hydrolysis of nucleotides (Lee and Chen, 1988; Terasaki et al., 1986) since both are dependent on microtubular assembly/disassembly, a GTP dependent process (vide infra: The tubulins). Table II summarizes known roles for nucleoside phosphates in ER functions.

**ATP dependent functions**

ATP is utilized as phosphate donor in the catalysis of many of the reactions of ER enzymes. It is required for activation of the head groups in the synthesis of phospholipid (Catt, 1987), for the stimulation of HMG-CoA reductase in cholesterol synthesis (Ingebritsen and Cohen, 1983), and for stimulation and regulation of steroid biosynthesis. Dolichol pyrophosphoryl oligosaccharide, cholesterol and steroid synthesis are regulated by external stimuli via a cAMP phosphorylation cascade and have been shown to be controlled by the activity of cAMP dependent protein kinase (Banerjee, 1987; Gill, 1987). "TP hydrolysis is necessary for translocation of nascent chains across the ER membrane but the molecular basis for this requirement has not yet been elucidated (Waters and Blobel, 1986; Hansen et al., 1986; Rothblatt and Meyer, 1986). Again, ATP hydrolysis is necessary to maintain a "relaxed" conformation of certain small, usually hydrophobic peptides, to maintain their ability to insert into the ER membrane (Muller and Zimmerman, 1988). The translocation of phospholipid from the cytosolic to the luminal leaflet of the ER bilayer is ATP dependent (Zachowski et al., 1985). Calcium uptake into the ER occurs via the ATP requiring (Ca²⁺ and K⁺ stimulated) Mg²⁺-dependent ATPase (Imamura and Schultz, 1985). Mannosylphosphodolichol synthase activity is stimulated by ATP dependent phosphorylation (Banerjee et al., 1987) and the binding of the luminal ER protein BiP to various nascent peptides is regulated by ATP phosphorylation (Munro and Pelham, 1987). There are many other proteins of the ER which are phosphorylated in vitro with ATP as phosphate donor (Rindress et al., 1989; Behar Banellier et al.,...
1980). Whether these same proteins are phosphorylated in vivo, and what role they play in ER function remains to be elucidated.

GTP dependent functions

The binding of GTP can selectively inhibit the mannosyl transferase of the multienzyme system which carries out the synthesis of dolichol pyrophosphoryl oligosaccharide for N-glycosylation (Spiro and Spiro, 1986).

It has also been demonstrated that the formation of a functional ribosomal complex - membrane junction (Connolly and Gilmore, 1986), translocation of nascent chains across ER membranes (Hansen et al, 1986) insertion of integral proteins into ER membranes (Wilson et al, 1988; Hoffman and Gilmore, 1988) and the displacement of SRP from the signal sequence after the formation of a stable interaction with the ER membrane (Connolly and Gilmore, 1989), are GTP dependent. Robinson and Austin (1987) have further identified a 22 kDa G protein which enhances nascent chain translocation.

GTP dependent Ca\(^{2+}\) efflux from and fusion of rough microsomal vesicles has been described by Gill et al (1986), Dawson et al (1986, 1987) and Lukacs et al (1987) and recently this has been elaborated upon by Ghosh et al (1989); GTP appears to be stimulating in vivo the transfer from a GTP sensitive Ca\(^{2+}\) pool to the phosphoinositol bisphosphate (IP3) Ca\(^{2+}\) pool in the ER.

GTP hydrolysis has also recently been identified as one of the requirements for reconstitution of vesicular transport between the ER and the cis-golgi apparatus (Beckers and Balch, 1989).

In vitro incubation of stripped rough microsomal membranes (SRM) with physiological concentrations of GTP and Mg\(^{2+}\) has resulted in specific morphological and biochemical phenomena. Homologous fusion of SRM vesicles, enhanced glycosylation of endogenous protein content and changes in membrane permeability are all specific to GTP and stripped, rough
microsomal membranes (Paiement et al, 1980; Godelaine et al, 1983; Paiement and Bergeron, 1983; Paiement et al, 1987). Godelaine and Beaufay (1987) have identified a GTP binding protein in the ER which appears to mediate these GTP specific phenomena. Because in vitro GTP specific fusion occurs at physiological concentrations of GTP and divalent cation, is absolutely restricted to stripped rough ER membranes (Paiement et al, 1987), and is seen in all tissue and species tested (J. Paiement, personal communication), a corresponding in vivo event seems likely.

Another G protein has been discovered in the ER but no function has yet been identified for it; preliminary results suggest that it is not related to translocation of nascent peptides (Audigier et al, 1988). Comerford and Dawson (1989) have found four microsomal proteins which bind GTP when immobilized on nitrocellulose, and have suggested that one or two of them are involved in GTP specific fusion of ER membranes, but have proposed no function for the others.

As with ATP, in vitro incubation of microsomal membranes in the presence of GTP leads to the phosphorylation of a limited number of peripheral and integral membrane proteins (Rindress et al, 1989; Lukacs et al, 1987; Behar-Bannelier et al, 1980; Dawson et al, 1986). Discovery of the in vivo role, if indeed there is one, of most of these proteins requires further work. One of these GTP phosphorylated proteins however has recently been identified as the signal sequence receptor, a constituent of the ER translocation apparatus (Wada et al, 1989).

**Other nucleotide dependent functions**

Addison (1988) and Mueckler and Lodish (1986a) showed the need for nucleotides in translocation and insertion of nascent chains at the ER membrane, but did not determine nucleotide specificity. The synthesis of phospholipids at the ER membrane, besides requiring ATP as phosphate donor also uses CTP extensively as a carrier for the head alcohol groups or for phosphatidic acid (Imai and Gershengorn, 1987; Coleman and Bell, 1978; Bishop and Bell, 1985).
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<td><strong>Nucleotide effects on structure and/or function in the endoplasmic reticulum</strong></td>
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**ATP:**
- ATP is involved in phospholipid, cholesterol, steroid synthesis, and many reactions of the microsomal redox system  
  (Foulkes et al., 1983)  
  (Ingebritsen et al., 1983a,b)  
  (Ingebritsen and Cohen, 1983)
- Stimulation of mannosphosphodolichol synthase activity  
  (Banerjee et al., 1987)
- Co and post translational translocation  
  (Waters and Blobel, 1986)  
  (Hansen et al., 1986)  
  (Rothblatt and Meyer, 1986)
- Maintenance of correct conformation of small peptides for membrane insertion  
  (Muller and Zimmerman, 1988)
- Ca\(^{2+}\) uptake in ER Ca\(^{2+}\) pools  
  (Wolf et al., 1986)  
  (Imamura and Schulz, 1985)
- Modulation of BiP  
  (Munro and Pelham, 1987)
- Phosphorylation of a set of peripheral and membrane proteins  
  (Rindress et al., 1989)  
  (Behar-Banellier et al., 1980)

**GTP:**
- Selectively inhibits mannosyl transferase of multienzyme system carrying out N-glycosylation  
  (Spiro and Spiro, 1986)
- Insertion of integral proteins into ER membrane  
  (Wilson et al., 1988)  
  (Hoffman and Gilmore, 1988)
- Nascent chain translocation  
  (Waters and Blobel, 1986)  
  (Hansen et al., 1986)
- Formation of functional ribosome-membrane junction  
  (Connolly and Gilmore, 1986)
- GTP binding to alfa subunit of SRP receptor displaces SRP from signal sequence  
  (Connolly and Gilmore, 1989)
- 22 kDa G protein enhances translocation (Robinson and Austin, 1987)

- Ca2+ efflux and fusion of microsomal vesicles (Dawson et al., 1987)
  (Lukacs et al., 1987)

- Ca2+ transport into an InsP3 sensitive Ca2+ pool in the ER (Ghosh et al., 1989)

- ER/cis Golgi transport (Beckers and Balch, 1989)

- Stimulation of in vitro asn-linked glycosylation, fusion of ER membranes and changes in ER membrane permeability
  (Godelaine et al., 1983)
  (Paiement and Bergeron, 1983)
  (Paiement et al., 1987)
  (Paiement et al., 1980)
  (Godelaine and Beaufay, 1987)

- Four GTP binding proteins (19, 20, 22, 24 kDa) (Comerford and Dawson, 1989)
- 41 kDa G protein (G_{rer}) - function unknown (Audigier et al., 1988)

- Phosphorylation of a set of peripheral and membrane proteins
  (Rindress et al., 1989)
  (Lukacs et al., 1987)
  (Behar-Bannelier et al., 1980)
  (Dawson et al., 1986)

Other nucleotides:

- Co and posttranslational translocation at a step subsequent to binding of the polysomal complex to ER membrane (NTP) (Addison, 1988)

- Insertion of integral protein into the ER membrane (NTP) (Mueckler and Lodish, 1986)

- UMP used in exchange reaction for UDP-glucose import into the ER lumen (Perez and Hirschberg, 1986)

- CTP used extensively as a carrier in the synthesis of phospholipid (Pagano and Sleight, 1985)
  (Rothman and Lenard, 1977)
c) Nucleotides and cellular regulation

i) Phosphorylation

The phosphorylation of proteins is a ubiquitous phenomenon used to regulate intracellular events, via an integrated network of pathways, and the cell's primary mechanism of posttranslational modification of protein function. Addition of a charged phosphate group can activate or inhibit protein function, produce conformational changes, stimulate or prevent intermolecular interactions, or in some cases apparently effect no change. Multiple phosphorylations on a single protein can produce cooperative or antagonistic results, and is often used to more finely modulate enzyme activity (Browning et al, 1985; Cohen, 1989; Cohen, 1982).

Phosphorylation-dephosphorylation as a regulatory mechanism was first recognized when it was found that the state of phosphorylation of the several enzymes of that pathway mediated glycogen metabolism in skeletal muscle (Krebs and Fischer, 1956; Krebs et al, 1959; Friedman and Larner, 1963). Since then the number of proven and putative protein kinases has risen dramatically (Hunter, 1987). The vast majority of phosphorylation occurs at serine and threonine residues on the phosphorylated protein (>99%) while a very small proportion (about 0.03%) occurs at tyrosine residues (Cohen, 1982).

The three major molecular components of phosphorylation are the protein kinase, the protein phosphatase and substrate.
Protein Kinases

All currently known kinases show a high degree of homology in their catalytic domains (Hunter, 1987). In vitro, most protein kinases have multiple substrate specificities, although for certain protein kinases (e.g. Rhodopsin kinase and rhodopsin) only one substrate has been found (Browning, 1985). Substrates themselves are often phosphorylated by more than one protein kinase, at the same or different sites. A large number of protein kinases have been found, in all tissue and cell types examined, both in the cytosol and associated with cellular membranes (Browning, 1985; Cohen, 1982; Hunter 1987). The major mammalian protein kinases include the cAMP dependent and cGMP dependent protein kinases, several distinct calmodulin regulated protein kinases, the diglyceride stimulated protein kinase C's, the protein tyrosine kinases (including the src, abl, fps gene families and the EGF, insulin and PDGF receptor families) as well as numerous other serine and threonine kinases (Browning et al, 1985; Hunter, 1987; Sibley, 1987; Cohen, 1989).

Protein Phosphatases

For phosphorylation to be an efficient regulatory device, it must be reversible, and several protein phosphatases, with their own substrate specificities and regulatory factors, have been shown to be active in regulation of cellular events. Generally phosphatases exhibit preference for either serine/threonine phosphate or tyrosine phosphate, although there is among some phosphatases an overlap in phosphoamino acid specificity (Lau et al, 1989). Not as numerous as the kinases, protein
phosphatases are as ubiquitous, being found in all cell and tissue types examined, and distributed throughout the cell (Lau, 1989; Browning, 1985).

Serine phosphatases are categorized into four groups based on their substrate specificity, the regulatory factors to which they respond, their ionic requirements for activity, and their subunit structure (Ingebritsen and Cohen, 1983; Cohen, 1989; Lau, 1989). The protein phosphatases are regulated in many different ways. Second messengers cAMP and calcium/calmodulin, divalent cations such as Mn$^{2+}$, Mg$^{2+}$ and Ca$^{2+}$, zinc, fluoride and vanadate have been shown to variously modulate the behaviour of phosphoserine and phosphotyrosine phosphatases (Lau, 1989; Cohen, 1989). Other forms of protein phosphatase regulation include (a) structural alteration of their substrate, (b) modification of their enzyme activity by association with specific inhibitors or activators or (c) changing their intracellular location (i.e. translocation between membrane association and cytosol) (Ingebritsen and Cohen, 1983). While not as well characterized, at least some tyrosine phosphatases are thought also to be regulated by association with specific inhibitors and activators and by translocation between intracellular sites (Lau, 1989; Ingebritsen et al, 1988).

**Regulatory proteins and phosphorylation**

Several proteins which are neither kinases nor phosphatases are known to regulate phosphorylation reactions. Generally they do so by direct interaction with one or more of the proteins involved in the
phosphorylation/dephosphorylation pathway, modulating the activity of that protein.

Two protein inhibitors (inhibitor I and inhibitor II) of type I serine phosphatases, when phosphorylated, associate with that enzyme inhibiting phosphatase activity (I) and causing intracellular relocation (II) (Cohen, 1985). Two similar proteins in bovine brain have been discovered which interact with certain phosphotyrosyl phosphatases in a like manner (Ingebritsen et al, 1988).

Dopamine and cyclic AMP regulated phosphoprotein (DARPP-32), a phosphoprotein which is present exclusively in neurons, when phosphorylated is a specific and potent inhibitor of serine protein phosphatase I. This protein shows a high degree of homology to the Inhibitor I protein discussed above (Browning et al, 1985).

Synapsin I is another endogenous neuronal phosphoprotein involved in a regulatory event. Unphosphorylated it is found in association with the membrane of synaptic vesicles; when phosphorylation occurs it dissociates, stimulating fusion of synaptic vesicles with the plasma membrane and the release of neurotransmitter (Browning et al, 1985).

Another protein, arrestin, while not itself phosphorylated, binds only to phosphorylated rhodopsin, preventing the binding and subsequent activation of transducin, thus inhibiting the phosphorylation cascade stimulated by cGMP phosphodiesterase (Wilden et al, 1986).
A thiol dependent protein named "the deinhibitor" found in hepatic microsomes prevents inactivation of protein phosphatase I by its inhibitors (I and II) and is inactivated by phosphorylation (Defreyn et al, 1977).

Finally, very basic proteins such as histone H1 and polyamines have been shown to stimulate greatly the activity of the type 2 protein phosphatases (Cohen, 1989).

**Cellular events regulated by phosphorylation**

Hunter has described the phosphorylation-dephosphorylation of protein kinases and phosphatases as part of a permanent regulatory circuit analagous to the electrical amplification and feedback regulation of transistors; kinases being used either as on/off switches or amplifiers or modulators of activity; phosphatases generally providing feedback circuitry (Hunter, 1987).

nuclear proteins, ribosomal protein S6, other ribosomal proteins, eIF-2) (Gurley et al., 1981; Hook et al., 1981; Johnson, 1982; Langan, 1969; Roberts, 1982; Thomas, 1982), cholesterol synthesis (HMG-CoA reductase) (Ingebritsen and Cohen, 1983), fatty acid synthesis and aromatic amino acid breakdown (acyetyl CoA carboxylase, ATP citrate lyase, phenylalanine hydroxylase) (Ingebritsen, 1983a,b; Foulkes, 1983), glycolysis and gluconeogenesis (6-phosphofructo-2-kinase/fructose2,6 bisphosphatase, pyruvate kinase, 6-phosphofructo-1-kinase, fructose 1,6 bisphosphatase). Phosphorylation mechanisms are also used to effect maintenance and changes of cytoskeletal organization (Map-2, Tau, Neurofilaments, myosin light chains, actin, tubulin) (Nestler and Greengard, 1984; Hathaway and Traugh, 1979; DeMaille and Pechere, 1983, Goldenring et al., 1982); for neurotransmission (sodium, potassium and calcium channels; nicotinic acetylcholine, muscarinic acetylcholine, beta-adrenergic and GABA-modulin receptors) (Costa and Catterall, 1984a,b; Alkon et al., 1983; DeRiemer et al., 1985; Huganir et al., 1984; Burgoyne, 1983, Stadel et al., 1983; Wise et al., 1983), and to regulate the many other proteins involved in transmembrane signalling (G proteins, their many receptors and effectors, many polypeptide growth factor receptors) (Browning, 1985, Sibley et al., 1987; Cohen, 1989).

Nucleotide specificity

Until recently, investigation of protein kinases has focussed primarily on ATP as phosphate donor. At present, only one protein kinase is known to use GTP specifically as phosphate donor, phosphoenolpyruvate carboxykinase (PEP-CK), an enzyme of the gluconeogenic pathway in
mitochondria. It converts oxaloacetate to phoshoenolpyruvate with the release of CO2 and GDP (Erecinska and Wilson, 1982). Another recently characterized enzyme, Casein Kinase II, which is implicated in the regulation of cytosolic heat shock protein hsp90 (Lees-Miller and Anderson, 1989) and appears to be involved in microtubule assembly (Diaz-Nido et al, 1988), exhibits a low km for both ATP and GTP as phosphate donor (Dahmus, 1981; Hathaway and Traugh, 1979; Thornburg and Lindell, 1977). Again, with few exceptions (e.g., Behar-Bannelier and Murray, 1980; Smith and Wells, 1983a,b; Rindress et al, 1989) examination of phosphorylated substrates has focussed primarily on ATP phosphorylation events. It is possible that other nucleoside triphosphates, notably GTP, are important substrates as phosphate donors. With the increasing interest in GTP binding proteins with respect to control of many general cellular processes, perhaps new GTP (or other NTP) kinases may come to light.

ii) GTP binding proteins

GTP binding proteins known at present comprise several different families, including "G proteins", the tubulins, ras proteins, several factors involved in protein synthesis, unidentified proteins participating in secretion and endocytosis, and various other small (20-25 kDa) GTP/GDP exchange proteins (Matsui et al, 1988). The general principle behind the functioning of all these proteins involves GTP binding and hydrolysis to control switching between two different protein conformations. Generally, the GTP bound form is active, and activity is turned off by GTP hydrolysis (Bourne, 1988). Another catalytic activity is often required to effect
the replacement of GDP with GTP in a nucleotide exchange reaction. This on/off switch can be used to propagate and amplify regulatory signals (G-proteins), to cause a reaction to proceed in a single direction (protein synthesis), to mediate vectorial transport of vesicles (secretion and endocytosis) or to stimulate or effect cellular proliferation (microtubular assembly, ras proteins).

"G proteins"

These proteins exist as a family of structurally and functionally homologous membrane associated guanine nucleotide binding regulatory proteins that mediate many hormonal and sensory transduction processes in eukaryotes. They carry signals from stimulated membrane glycoprotein receptors to effector enzymes and channels. The stimulatory and inhibitory G proteins of the adenylate cyclase system (Gs and Gi respectively), are activated by multiple membrane receptors, e.g., the beta-adrenergic (Ross and Gilman, 1980) and serotonin receptors (Siegelbaum et al, 1982). The phosphoinositide cascade which controls intracellular calcium concentration and various calcium sensitive processes such as secretion, is regulated by Gplc, another G protein, again activated by more than one type of receptor, e.g., the vasopressin (Pobiner et al, 1985), Mast cell IgE (Smith et al, 1985; Nakamura and Ui, 1985) ADP, thrombin and epinephrine (Haslam and Davidson, 1984) and chemotactic receptors (Krause et al, 1985). Potassium channels in heart pacemaker cells are opened in response to Gk, which is activated by the muscarinic acetylcholine receptor (Pfaffinger et al, 1985; Breitwieser and Szabo, 1985). Transducin, a G-protein expressed in rod and cone cells of
vertebrate retina and in invertebrate visual organs, mediates visual transduction by stimulating a cGMP phosphodiesterase cascade when activated by photoexcited rhodopsin (Stryer, 1986); a G-like stimulatory G-protein is apparently involved in olfactory sensory transduction (Chen et al, 1986; Pace et al 1985).

Recently it has been shown that several neural receptors coupled to phospholipase C hydrolysis of inositol lipids have mitogenic potential and a role has been proposed for these G protein mediated systems in cell development and proliferation (Julius et al, 1989; Jackson et al, 1988; Ashkenazi et al, 1989).

G-proteins are heterotrimeric, consisting of α subunits which bind and hydrolyze guanine nucleotide, and two smaller subunits (β and γ). αβγ complexes are detected when GDP is bound to Ga. When the α subunit replaces GDP with GTP, the α and β-γ subunits dissociate, and both appear to be involved in various effector activations (Bourne, 1989). GTP is then hydrolyzed and Ga-GDP reassociates with high affinity to the β γ subunit. In the absence of excited receptor, this exchange cycle is slow; in the presence of excited receptor the rate of exchange of GTP for bound GDP is accelerated, and subsequently the activation of the effector enzyme or channel.

There are at least nine distinct α subunits which show a high degree of homology (Dever et al, 1987) and are in some cases interchangeable. Functions can be assigned to several regions of the α chains, such as those implicated in guanine nucleotide binding, interaction with the β γ
subunit, the effector protein and the excited receptor, as well as the sites modified by cholera and pertussis toxin. Only two distinct β subunits and two or three distinct γ subunits have been identified.

Besides the inhibitory effect of Gi, there are other regulatory controls over G protein activity. As receptor activation stimulates Ga binding to it, Ga binding to the receptor results in a reduction of receptor activity. For at least two G protein receptors (Rhodopsin and the β adrenergic receptor), there appears to be a phosphorylation turn off mechanism, where phosphorylation of the activated receptor (Benovic et al, 1986; Kuhn, 1980; Stryer, 1986) results in quenching of its activity. This may also occur at the level of the G protein itself (Katada et al, 1985). There are also proteins which inhibit the binding of Ga to its receptor (Wilden et al, 1986; Lefkowitz, 1987).

The adenylate cyclase system is one of the most well characterized of the G-protein transmembrane signalling systems. Because the production of cAMP is controlled by external signals, the two distinct G protein transducing systems respond to their respective stimulatory and inhibitory receptors. The receptors are highly hydrophobic, containing several membrane spanning domains, and their ligand binding sites are found in hydrophobic sequences within the bilayer (Levitzki, 1988). They interact with Ga and Gi on the cytosolic face of the pm. The ligand bound receptor induces a conformational change in Ga-GDP which facilitates the nucleotide exchange reaction, the rate limiting step in the activation of Ga. Ga, G1 α and G1 α are highly homologous, and the β α subunits of Ga and G1 are functionally interchangeable and probably identical in sequence (Levitzki,
Using purified $G_s$ and $G_i$, it has been demonstrated that the activated $\alpha$ subunit ($\alpha$-GTP) dissociates from the $\beta\gamma$ unit and can by itself affect adenylate cyclase activity (May et al, 1985; Cerione et al, 1985). The $\beta\gamma$ subunit acts as membrane anchor for the $\alpha$ subunit as well as playing a role in the inhibition of adenylate cyclase by $G_i$, both directly interacting with the enzyme and by complexing with $G_s$-$\alpha$ (Katada et al, 1984a, b, 1986). The ligand bound receptor activates several $G_s$ proteins which in turn activate adenylate cyclase at a much amplified rate.

There are several criteria used to determine whether $G$ proteins are involved in a particular transduction process: a) GTP and non hydrolyzable GTP analogs decrease the binding of ligand to receptor, b) GTP binding and hydrolysis are stimulated by stimulation of the receptor, c) the activity which is putatively effected is stimulated by the presence of GTP and stimulated receptor, d) either cholera and/or pertussis toxin alters this effect, e) the addition of nonhydrolyzable GTP analog GTP$\gamma$S can mimic the effect of stimulated receptor, and finally, f) functional reconstitution of the transduction process in vitro (Stryer and Bourne, 1986).

The tubulins

The tubulins ($\alpha$ and $\beta$) are the cytosolic building blocks of microtubules. Because multiple copies of both $\alpha$ and $\beta$ genes exist and the gene products are subject to differential posttranslational modifications in many organisms there are several different $\alpha$ and $\beta$ subtypes (Gull et al, 1986; Bulinski et al, 1988; Joshi et al, 1987). In most eukaryotic cells,
microtubules form a variety of subcellular structures. In non mitotic cells they form a complex cytoskeletal network where they participate in the maintenance of shape and structure (Maccioni, 1986); during mitosis they restructure themselves into the spindle apparatus and are involved in the translocation of chromosomes during chromosomal segregation and may also play a role in the appropriate apportioning of membranous organelles and cytoskeletal proteins to daughter cells during cell division (Masuda et al, 1988; Armas-Portela et al, 1988; Gorbsky et al, 1988; Kamech et al, 1988); they are involved in both particular and vesicular ATP dependent transport (Hyams et al, 1987; Vale et al, 1987; Crawford et al, 1988), and the ATP powered generation of dynamic behavior in cells (Lee et al, 1988).

In the presence of GTP at 37° C they spontaneously assemble in vitro (in the absence of free Ca\textsuperscript{2+}) in a unidirectional manner. Each α & dimer binds two molecules of GTP, one of which is hydrolyzed to GDP when the dimer is incorporated into a polymerizing microtubule (Lin et al, 1987; Barton et al, 1987; Carlier et al, 1987; O'Brien et al, 1987; Flynn et al, 1987). Evidence suggests that microtubules are normally in a steady state of assembly disassembly, and are constantly in need of GTP. The reversibility of assembly-disassembly of microtubules has been demonstrated in vivo (Cassimeris et al, 1987; Keates et al, 1988; Martin et al, 1987).

Other microtubule proteins (MT) and microtubule associated proteins (MAPs) interact with the tubulins, the microtubule associated proteins (MAPs), and function in the assembly and regulation of microtubular structure. Many are phosphorylated, and it appears that phosphorylation and dephosphorylation are used to regulate microtubule assembly and function.

The ras proteins

The ras proteins are guanine nucleotide binding proteins associated with the cytosolic surface of the plasma membrane which possess weak GTP hydrolytic activity (Willingham et al, 1980; Shih et al, 1980, 1982; Sweet et al, 1984; McGrath et al, 1984; Gibbs et al 1984). At least three different mammalian ras genes code for similar 21 kDa proteins, H-ras, K-ras and N-ras (Shimizu et al, 1983). Single missense mutations in the ras gene which reduce the GTPase activity of the encoded proteins or overexpression of normal proteins have produced several oncogenic mutants (Sweet et al, 1984; McGrath et al, 1984; Gibbs et al, 1984) and have been linked to altered proliferation and/or differentiation (Santos and Nebreda, 1989). Homologous genes to those found in mammals have been found in every organism investigated, in all cell types and at all developmental stages (Shilo and Weinberg, 1981; DeFeo-Jones et al, 1983; Powers et al, 1984; Reymond et al, 1984; Newman-Silberberg et al, 1984; Mueller et al, 1982, 1983). It is hypothesized that they normally exist in an equilibrium between the active GTP bound and inactive GDP bound forms, but mammalian cellular targets are not yet known.
Because of their similarity to G proteins in structure, location and biochemistry, a role has been proposed for them in signal transduction. In yeast ras proteins have been shown to activate adenylate cyclase (Toda et al, 1985; Broek et al, 1985); they stimulate maturation of Xenopus oocytes via a non-adenylate cyclase mediated pathway (Birchmeier et al, 1985); in several other lower eukaryotes they play a role in maturation, growth, and differentiation (Santos and Nebreda, 1989). Although still poorly understood in mammals, transformation by ras indirectly effects changes in phospholipase A2 and phospholipase C metabolism (Yu et al, 1988; Alonso et al, 1988), stimulates the serine/threonine kinase activity of Raf-1 (Morrison et al, 1988), activates transcriptional factors (Wasylyk et al, 1988), and induces terminal differentiation (Andres et al, 1988).

Certain cellular proteins have been identified which interact directly with ras proteins. The GAPs (GTPase activating proteins) bind specifically to ras proteins stimulating the GTPase activity greater than 100-fold. At least two forms exist and are expressed at different stages of differentiation (McCormick, 1989). GAP activity is regulated by its lipid microenvironment and is inhibited by various phospholipids (e.g., phosphatidic acid, the inositol phosphates, arachidonic acid). It appears to interact with many different ras and ras like proteins. As well, the yeast CDC25 gene product regulates the nucleotide exchange reaction through direct, transient interaction with ras proteins, and will also interact with mammalian ras (Tamanoi, 1988; Powers et al, 1989). GAPs, Mg$^{2+}$ concentration, ras specific kinases, CDC25 like proteins are all known to regulate ras function (Santos and Nebreda, 1989).
Several proteins involved in the synthesis of proteins bind and hydrolyze GTP. These are polypeptide chain biosynthesis factors initiation factor 2 (IF2) (Groner and Revel, 1971), elongation factor Tu (EF Tu) (Gordon, 1968), elongation factor G (EF G) (Bodley et al, 1969) and release factor (RF) (Beaudet and Caskey, 1971), and the signal recognition particle receptor (Connolly and Gilmore, 1989).

IF2 forms a ternary complex with GTP and methionyl-tRNA\textsubscript{met} which binds to mRNA and the small ribosomal subunit along with other initiation factors. The GTP is hydrolyzed once the large ribosomal subunit binds, and all initiation factors are released to repeat the cycle. EF Tu promotes the binding of aa-tRNA to ribosomes during polypeptide chain elongation. It interacts sequentially with GTP, aa-tRNA and ribosomes, hydrolyzes the GTP and then the EF Tu-GDP is reactivated by EF Ts, another elongation factor, by catalyzing the exchange of GTP for GDP. EF G is another GTP binding protein which provides the energy for peptidyl transfer on the ribosome by GTP hydrolysis. And finally, RF binds and hydrolyzes GTP during the release of the completed peptide (Lake, 1981; Moldave, 1985). More recently, the gene for the 54kDa subunit of signal recognition particle has been shown to contain a region of homology with GTP binding proteins, although no GTP binding/hydrolyzing function has yet been identified (Romisch et al, 1989; Bernstein et al, 1989); and the signal recognition particle receptor has a GTP binding and hydrolysis requirement to effect the dissociation of SRP from the signal sequence (Connolly and Gilmore, 1989).
The common factor in the function of all of these GTP binding proteins which act during protein synthesis is the use of GTP binding and hydrolysis to effect the vectorial translocation of a molecule from one binding site to another.

Other GTP binding proteins

The rapidly growing family of GTP binding proteins also includes proteins involved in secretion (Melancon et al, 1987; Segev et al, 1988; Schmitt et al, 1988; Goud et al, 1988; Beckers and Balch, 1989) and endocytosis (Mayorga et al, 1989), as well as several other small molecular weight GTP binding proteins with no identified function, e.g., smg-p25A, B, and C proteins (Kikuchi et al, 1988; Matsui et al, 1988), the ADP-ribosylation factor (Kahn and Gilman, 1986), and mammalian Gp (Waldo et al, 1987).

The YPT1 yeast gene (Segev et al, 1988; Schmitt et al, 1988) encodes a GTP binding protein apparently resident in the Golgi apparatus that facilitates the transport of secretory proteins through that compartment.

The SEC4 yeast gene product (Goud et al, 1988) is involved at a stage of transport between the Golgi apparatus and the plasma membrane and has been immunolocalized at the cytosolic surface of these membranes. No specific biochemical function has been identified for these proteins. Both Melancon (Melancon et al, 1988) and Beckers (Beckers and Balch, 1989) while not identifying any specific protein, have shown that GTP binding protein(s) are involved in secretory protein transport between Golgi saccules (Melancon et al, 1988) and from the ER to the Golgi Apparatus.
GTP binding proteins have also been implicated functionally in the transport of molecules along the endocytic pathway (Mayorga et al, 1989). The mechanism of action of these GTP proteins is not yet known but analogies have been made with both the G-protein signal transducing mechanism and the EF Tu type energy dependent unidirectional recycling mechanism (Bourne, 1988). Presumably they serve as regulatory components coupling the energy of GTP hydrolysis to the process of transport.

d) Rationale for the work

ATP and GTP hydrolysis have been implicated directly in the structure and functions of the eukaryotic endoplasmic reticulum. The transport of nascent chains of secretory and membrane proteins into and across the ER membrane has been demonstrated to be dependent on ATP and GTP (Hansen et al, 1986; Mueckler and Lodish, 1986; Rothblatt and Meyer, 1986; Hoffman and Gilmore, 1988). As well, physiological concentrations of GTP and MgCl₂ promote the fusion of isolated rough microsomes after ribosome removal (Paiement et al, 1980; Paiement and Bergeron, 1983; Paiement et al, 1987). Furthermore, ribosome capping experiments and controlled protease digestion experiments indicate that a cytosolically exposed protein of the ER located at the ribosome membrane junction is involved in the GTP specific fusion phenomenon (Paiement et al, 1987).

Fusion requires GTP hydrolysis (Paiement et al, 1980) whereas protein translocation requires ATP hydrolysis and has an additional GTP requirement (Hansen et al, 1986; Mueckler and Lodish, 1986; Connolly and
Gilmore, 1986; Pain and Blobel, 1987; Wilson et al, 1988). The nonhydrolyzable analog, GMP-PNP has been shown to substitute for GTP during protein translocation providing that the nascent chain remains associated with the ribosome (Connolly and Gilmore, 1986; Wilson et al, 1988). Recently, Connolly and Gilmore (1989) have shown that the SRP receptor is a GTP binding protein which uses GTP or the non-hydrolyzable analog GMP-PNP to effect the release of SRP from the signal sequence. However, the effect of GMP-PNP on translocation is augmented by GTP itself (Hoffman and Gilmore, 1988), contrary to expectations for a G protein. This indicates an additional need for GTP hydrolysis and suggests that either this is a novel type of G protein, or GTP may serve another role, e.g. as phosphate donor in a phosphotransferase reaction.

Molecular mechanisms have been put forward tentatively to explain the two nucleotide specific phenomena described above (Connolly and Gilmore, 1989; Singer et al, 1987a,b; Paiement and Bergeron, 1989). One model proposes that GTP specific ER membrane fusion and GTP hydrolysis requirements for nascent chain translocation are related and both may involve the phosphorylation of a specific group of integral membrane proteins of the ER (Paiement and Bergeron, 1989), i.e., the putative translocation apparatus. Therefore, we have characterized further the GTP specific fusion reaction of ER membranes, and attempted to correlate it directly with translocation. We have further used translocation competent rough microsomes in order to identify integral membrane proteins which act as substrates for \textit{in vitro} phosphorylation with [\textit{r}-32P] ATP or [\textit{r}-32P] GTP, and having identified them, to purify these proteins and determine their function \textit{in vivo}. 
EXPERIMENTAL PROCEDURES

a) Materials

[35S] methionine (1100-1200 Ci/mmol), [γ-32P] GTP (s.a. 30-42 Ci/mmol), [γ-32P] ATP (s.a. 3000 Ci/mmol) and Enhance were purchased from New England Nuclear Research Division, DuPont Canada (Toronto, Ontario). Staphylococcus aureus nuclease, yeast tRNA and bovine pancreatic ribonuclease were obtained from Boehringer Mannheim (Montreal, Quebec). Oligo dT Cellulose Type III was purchased from Collaborative Research Laboratories (Lexington, Massachusetts). Trypsin and chymotrypsin for protein translation and controlled proteolysis studies were from Cooper Biomedicals (Malvern, Pennsylvania). Placental ribonuclease inhibitor was from Bethesda Research Laboratories (Gaithersburg, Maryland), guanidine thiocyanate from Fluka AG (Buchs, Switzerland) and Kodak XAR-5 OMAT x-ray film from Picker International Canada Inc. (Montreal, Quebec). Purified human placental lactogen (hPL) for use as a molecular weight standard was a gift from the National Institute of Health (NIADDK, Bethesda, Maryland), anti human placental lactogen antiserum (anti hPL) was prepared by Dr. Wei Lai, McGill University. N-Glycanase (250 U/ul) was purchased from Genzyme (Boston, Massachusetts). DEAE Bio-Gel A, Hydroxyapatite BioGel HT and reagents for SDS-PAGE and protein determination were from BioRad Laboratories (Mississauga, Ontario). Whatman DE52 Ion Exchange Resin was obtained from Fisher Scientific (Montreal, Quebec) and Sephacryl S300 HR and Concanavalin A Sepharose were from Pharmacia LKB Biotechnology (Montreal, Quebec). Centricon-10 and Centriprep-10 concentrators were purchased from Amicon Canada (Mississauga, Ontario).

Unless otherwise stated, all other reagents and chemicals were of reagent grade and obtained from Sigma Chemical Company (St. Louis, Missouri), Anachemia Canada Inc. (Lachine, Quebec) and Boehringer Mannheim (Montreal, Quebec).

b) Animals

Sprague Dawley rats were obtained from Charles River Laboratory (St.
Constant, Quebec). Dog pancreas were obtained from the laboratories of Dr. M. Levy (Royal Victoria Hospital, Montreal, Quebec) and Dr. B. Sasyniuk (McGill University, Montreal, Quebec). New Zealand White rabbits were obtained from Lapro Laboratories (Stokley, Quebec) for use in the preparation of rabbit reticulocyte lysate and in the preparation of antisera.

c) Subcellular fractionation

Rat liver rough microsomes were prepared and stripped as previously described (Paiement and Bergeron, 1983). Translocation competent dog pancreas stripped rough microsomes were prepared according to Walter and Blobel (1983a). Membranes were stored (10-12 mg/ml) in aliquots frozen at -70°C in 20 mM HEPES-NaOH, pH 7.4/50% glycerol/1 mM DTT and thawed only once. For use in localization, rough and smooth microsomes from dog pancreas were prepared according to a modified protocol of Tartakoff and Jamieson (1974). This procedure differed from the original in that the homogenization and centrifugation steps were carried out in STKH buffer (0.3 M sucrose/50 mM Tris-HCl, pH 7.4/100 mM KCl/5 mM MgCl₂) containing 1 mM DTT and the protease inhibitors PMSF (1 mM) and Aprotinin (100 KIU/ml). The resulting smooth and rough microsomes were recovered by centrifugation through a cushion of 0.5 M sucrose containing 50 mM Tris-HCl, pH 7.4/100 mM KCl/1 mM DTT at 150,000 g for 60 min to remove protease inhibitors. Pellets were resuspended in 20 mM HEPES-NaOH, pH 7.4/50% glycerol/1 mM DTT and used immediately for in vitro phosphorylation.

Golgi fractions (GF1, GF2, GF3, Golgi light, intermediate and heavy), small vesicle fractions and the load zone fractions were prepared by discontinuous sucrose gradient centrifugation of rat liver microsomes as described by Bergeron (1979) and Bergeron et al (1982). Liver endosomal fractions designated as Li and Gi fractions were prepared from parent light-mitochondrial (L) and microsomal (P) fractions respectively by the flotation method of Khan et al (1986). Purified rat liver plasma membrane fractions were prepared according to Hubbard et al (1983) as modified by Kay et al (1986). Secondary lysosomes were isolated exactly as described by Wattiaux et al (1978), and mitochondrial fractions were prepared
freshly by Dr. G. Shore as previously described (Shore et al, 1983).

d) **Homologous membrane fusion**

Incubations were carried out in standard ER fusion medium consisting of 40 mM Tris-HCl (pH 7.4), 30 mM KCl, 7.5 mM MgCl₂, 2.5 mM MnCl₂, 1 mM ATP, 10 mM phosphoenolpyruvate, 25 μg pyruvate kinase plus 0.5 mM GTP. (It had previously been determined that GTP could induce fusion at concentrations as low as 0.1 μM (Paiement and Bergeron, 1983), but 0.5 mM GTP concentration was regularly used because of the extreme lability of this nucleotide.) 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 generally for 60 min. Reactions were started by addition of the membrane.

Fusion of rER has been defined by morphological assay as the formation of large membrane bounded elements by coalescence of the membranes of small rough microsomes. Morphological procedures used, morphometry of membrane surfaces and assays for fusion are as described in Paiement et al (1987).

**Assay for glycosylation and mannose-6-phosphatase latency.** Endogenous glycosylation with rough microsomes was carried out using the procedure described by Paiement and Bergeron (1983). SRM (100-500 μg protein) were incubated in the standard ER fusion medium described above to which was added 1 μCi uridine diphosphate-N-acetyl-D-[6(n)-3H]glucosamine (UDP-[3H]NAG, 24 Ci/mmol). Reactions were carried out for varying periods of time and stopped with ice cold 5% TCA and 0.1% (w/v) albumin. The total acid precipitable products were washed twice with cold 5% TCA and dissolved in 0.5 ml of Protosol. Radioactivity was determined in a Packard model 460 CD spectrometer. Hot TCA resistant precipitable products were treated as described above except they were heated at 90°C for 15 minutes. Mannose-6-phosphatase activity was assayed exactly as described by Godelaine et al (1983) in the presence or absence of detergent to assess total and free activities respectively.
e) Proteolysis of rER membranes.

The effect of proteolysis on fusion. SRM were treated at 10°C in sucrose-imidazole buffer containing trypsin (0.01-100 μg) for 10 min. Following trypsin treatment the microsomes were incubated at 37°C in standard ER fusion medium plus a 10 fold concentration of soybean trypsin inhibitor. 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.

Protease digestion of translation product. After the incubations were finished, samples were split into two 25 μl aliquots. One aliquot was treated for 5 min at room temperature with 33 mM tetracaine base (Scheele et al, 1980) in 70 mM KCl, 1 mM MgCl₂, pH 7, followed by a 45 min incubation at 4°C with 20 μg/ml each of trypsin and chymotrypsin (Amar-Costesec et al, 1984) also in 70 mM KCl, 1 mM MgCl₂, pH 7. The other aliquot was treated identically except that only 70 mM KCl, 1 mM MgCl₂, pH 7 was added in the second incubation. Proteolysis was terminated by the addition of 500 KIU/ml aprotinin. The equivalent of 5 μl of original translation mixture was analyzed on 12% discontinuous SDS-PAGE. Gels were processed for fluorography with Enhance, dried at 68°C and exposed to X-ray film as described below.

Protease digestion of SRM prior or subsequent to phosphorylation. Proteolysis of membrane fractions (4 mg/ml) was carried out by incubating 200 μg of SRM with trypsin and chymotrypsin (20 μg/ml each at 0°C for 30 min) either before or after in vitro phosphorylation with [γ-32P] GTP. Protease activity was terminated by the addition of 30 μg/ml Aprotinin. We have previously shown (Paiement et al, 1987) that under similar conditions of protease treatment, newly translocated proteins within the lumina of these membranes were protected from digestion.

f) Cell free protein synthesis and translocation assay

Placental messenger RNA was extracted from human term placenta by a modification of the method of Chirgwin et al (1979) using guanidine thiocyanate and cesium chloride isopycnic centrifugation followed by oligo

**Rabbit reticulocyte lysate** was prepared by the method of Jackson and Hunt (1983) and used at 50% final concentration.

**In vitro translation** reaction mixture contained 90 mM KCl, 1.2 mM MgCl₂, 0.4 U/μl placental ribonuclease inhibitor (Amar-Costeseck et al, 1984; Scheele and Blackburn, 1979), 0.24 μg/μl yeast transfer RNA, 1 μg/μl [35S] methionine (1100-1200 Ci/mmol) and 50% rabbit reticulocyte lysate. Translation mixtures (50 μl) were incubated for 1.5 hr at 30°C. They were further incubated for 10 min at 30°C with 100 μg/ml bovine pancreatic nuclease to eliminate [35S] methionyl-tRNA, and then cooled to 4°C.

**Coupled in vitro translation/translocation assays** were performed on both rat liver and dog pancreas stripped rough microsomes. Prior to incubation they received the following treatment: "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 hr. "Inactivated" membranes were diluted similarly to almost 1 mg/ml protein and incubated at 37°C for 1 hr. 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 protein concentration and incubated for 1 hr at 37°C. Each membrane sample (25 μl) was brought to a concentration of 2 U/μl ribonuclease inhibitor (Amar-Costeseck et al, 1984) and then used directly for **in vitro** translation/translocation assays at concentrations of 0.14 μg protein (rat liver) or 0.08 A260 units (dog pancreas) per μl of total reaction mixture.

**Immunoprecipitation of translation product** was done according to Anderson and Blobel (1983). To 50 μl of translation product, 900 μl of 1% Triton X-100/0.02% NaN₃/20 mM methionine/10 mM EDTA/PBS was added. These solubilized samples were centrifuged at 45k rpm for 45 min at 4°C in a Beckman TL100.2 rotor to eliminate ribosomes. To the supernatant 0.4 ml of 4M NaCl, CT3399 rabbit anti-hPl antiserum (12 mg/ml IgG, 0.3 mg/tube) at a 1:100 dilution and 0.13 ml of Pansorbin (2.3 mg IgG binding
capacity/ml) was added per tube. Samples were rotated at 4°C for 2 h, the immunoprecipitated pellets centrifuged down and prepared for SDS-PAGE.

g) In Vitro Phosphorylation

Membranes were incubated with 6-8.5 μM of [γ-32P] ATP or [γ-32P] GTP (s.a. 30-62 Ci/mmol) in a buffer containing 40 mM Tris-HCl (pH 7.4)/2.5 mM MnCl₂/7.5 mM MgCl₂/30 mM KCl in a final volume of 100 μl. Phosphorylations were carried out for 30 min at 4°C. Control experiments showed that in vitro phosphorylation of the proteins of interest was stronger at this temperature rather than at room temperature or 37°C. The reaction was terminated by the addition of 11 μl of a ten-fold concentrated stop solution to yield a final concentration of 1 mM GTP/2 mM Na₃VO₄/12.5 mM Tris-HCl, pH 7.4. SDS-PAGE of phosphorylated samples was performed on a discontinuous gel system.

h) Phospholipid extraction and analysis

Phospholipids were extracted as described by Schacht (1981) and thin layer chromatography was carried out on Merck silica gel 60 plates using a solvent of chloroform/methanol/3.3 M ammonium hydroxide (43:38:12). Phospholipid standards were visualized by spraying with Rhodamine (0.05% in 95% MeOH). Radiolabelled spots were visualized by radioautography after exposure at -70°C for 12 hr. Radioactivity was quantified by scraping the radioactive areas indicated by the X-ray film into scintillation vials and radiolabel determined using Cerenkov counting.

i) Phosphoprotein Examination

N-Glycanase Digestion. After Triton X-114 extraction of [γ-32P] ATP or [γ-32P] GTP phosphorylated membranes (about 500 μg), the detergent phase proteins were recovered by precipitation with ethanol/hexanes (4:1). The pellet was solubilized with 100 μl of 1% SDS/50 mM sodium acetate (pH 5.5) and diluted with 0.9 ml of 1% Triton X-100/50 mM sodium acetate (pH 5.5). The reaction was initiated by the addition of 1 or 2 μl of N-glycanase (250 U/μl), incubated for 16 hr at 37°C and was terminated by the addition
of 100 µl of 100% trichloroacetic acid. After centrifugation, the pellet was washed with ethanol/hexanes (4:1) and analyzed by SDS-PAGE.

**Con A Sepharose chromatography.**
After Triton X-114 extraction of [γ-32P] GTP phosphorylated membranes (about 500 µg) the detergent phase was diluted with 1 ml of 0.15 M NaCl/0.2% Tween 20/50 mM potassium phosphate, pH 7.5/5% glycerol and loaded onto Con A Sepharose (100 µl) equilibrated with the above buffer. The gel was washed with 10 ml of 1N NaCl/0.1% Tween 20/50 mM potassium phosphate buffer (pH 7.5). The glycoprotein bound to Con A Sepharose was eluted with 5 ml of 0.5 M α-methyl D-mannoside/0.2% Tween 20/50 mM potassium phosphate buffer, pH 7.5 and 200 µl of 1% SDS/50 mM Tris-HCl, pH 7.5.

**Phosphoamino acid analysis.**
After Triton X-114 extraction (Bordier, 1981) of [γ-32P] GTP phosphorylated membranes (1 mg), the protein recovered in the detergent phase was analyzed by SDS-PAGE. The gel was incubated in 70% methanol/2.5% glycerol for 30 min at room temperature and dried for 30 min at 60°C under vacuum. The radioactive bands were detected by radioautography and excised. The excised pieces were homogenized in 1 ml of 0.1% SDS/50 mM DTT/50 mM Tris-HCl, pH 7.5. The protein was extracted by rotation for 3 h at room temperature and concentrated with an amicon concentricon-10 concentrator. The extracted protein was precipitated by adding ethanol/hexanes (4:1). Partial acid hydrolysis of the precipitated protein was carried out at 110°C for 70 min. The phosphoamino acids were analyzed using thin layer electrophoresis according to the method of Cooper et al (1983). The radioactive bands were detected by radioautography with enhancing screens.

j) The phosphorylation reaction

**Triton X-114 Detergent Extraction of Membrane Proteins.**
Precondensation of the Triton X-114 detergent to remove the more hydrophilic Triton detergent molecules, and Triton X-114 detergent extraction of amphiphilic integral membrane proteins of SRM was carried
out exactly according to the method of Bordier (1981).

**Ribonuclease digestion of phosphorylated membranes.**

Ribonuclease digestion of SRM (4 mg/ml) was carried out by incubating 100 μg of SRM with ribonuclease (100 μg/ml) for 10 min at 20°C before or 30°C after *in vitro* phosphorylation with [γ-32P] GTP and samples processed directly for SDS-PAGE and radioautography.

**NEM pretreatment.**

Stripped rough microsomes (2 mg/ml) in 20 mM HEPES-NaOH (pH 7.4)/8.33% glycerol/0.167 mM Dithiothreitol were incubated at 25°C for 10 min with 0-3.4 mM N-ethyl maleimide after which *in vitro* phosphorylation with [γ-32P] ATP or [γ-32P] GTP was carried out at 4°C for 30 min as described above.

**Triton X-100 detergent solubilization.**

Stripped rough microsomes (4 mg/ml) in 20 mM HEPES-NaOH, pH 7.4/8.33% glycerol/0.167 mM DTT were incubated for 15 min at 4°C with variable volumes of 4% Triton X-100 detergent solution, such that final concentrations ranged from between 0 and 2% detergent. This was followed by *in vitro* phosphorylation with [γ-32P] ATP or [γ-32P] GTP under standard phosphorylating conditions.

**Glycerol Kinase assay**

Dog pancreas SRM (4 mg/ml) in 20 mM HEPES-NaOH, pH 7.4/8.33% glycerol/0.167 mM DTT were phosphorylated with [γ-32P] ATP or [γ-32P] GTP in the presence of glycerokinase (either 2 or 4 μg/100 μl reaction mixture). The glycerol already present in the membranes accounted for a concentration of 1 M glycerol. To test whether this high concentration of glycerol might have affected the results (the optimal glycerol concentration for glycerol kinase is 10 mM), SRM were spun down at 55,000 rpm for 1 h at 4°C in a Beckman TLA100 rotor, washed and resuspended in the same buffer but containing only 10 mM glycerol. After phosphorylation and radioautography, the results were identical to those with 1 M glycerol (not shown).
Specificity and inhibition studies

SRM were incubated under standard phosphorylation conditions as described above with either [r-32P] ATP or [r-32P] GTP, except for the addition of one of several nonradiolabeled nucleoside phosphates (ATP, ADP, AMP, cAMP, GTP, GDP, CMP, cGMP), nonhydrolyzable nucleotide analogs (AMP-PNP, ATPγS, GMP-PNP, GTPγS) or the purine nucleosides adenosine and guanosine. Unlabelled ATP and GTP were used in ten fold increments (0.1 to 1000 μM) in inhibition assays of phosphorylation with radiolabeled GTP and ATP. All the others were used at equimolar (7 μM) and excess (1 mM) concentration with radiolabeled GTP and ATP to examine specificity of regulation. The other nucleotides, CTP and UTP and the xanthosine ITP were also tested at equimolar and excess concentrations for their effects on phosphorylation. Direct testing of phosphorylation with [r-32P] radiolabelled GTP, UTP and ITP was precluded by the prohibitive cost of their preparation.

k) Purification of gp25, gp35, and pp90

Scaled up phosphorylation reactions were carried out with 160-320 mg of dog pancreatic stripped rough microsomes, 10% of which was radiolabeled with [r-32P] GTP (6-8.5 μM, 30-42 Ci/mmol) and 90% phosphorylated separately with the same concentration of unlabelled GTP. The reaction was terminated as described above and subjected to Triton X-114 extraction (Bordier, 1981), modified by the omission of a sucrose cushion during centrifugation.

The Triton X-114 detergent phase was diluted (1:25) with buffer A (20 mM Tris-HCl, pH 7.5/0.2% Tween 20). To this was added 2.5 mM sodium β-glycerophosphate and 5% glycerol. The above mixture was then added to 30-60 ml of Whatman DE52 equilibrated with buffer A and rotated for 60 min at 4°C. After filtration through Whatman #1 filter paper, the DE52 resin was washed in 350 ml of 0.01 M NaCl in buffer A, then transferred to a 30 mm x 200 mm column. The column was further washed with 750 ml of 0.01 M NaCl in buffer A. The bound protein was eluted using a salt gradient (0.01 M - 0.25 M NaCl) in buffer A containing 2.5 mM β-glycerophosphate
and 5% glycerol). One hundred fractions of 5 ml were collected. Samples were electrophoresed on 5-15% acrylamide gradient SDS-PAGE minigels using a small aliquot (10 μl) of every fifth fraction. Gels were stained with Coomassie Brilliant Blue, destained, dried under vacuum and exposed to Kodak X-OMAT AR-5 film for 12 h. From the radioautographic pattern the tubes containing the peak amounts of pgp35 were pooled. Pgp35 eluted between 0.23-0.25 M NaCl.

The fraction eluted from DE52 (55-85 ml) was then diluted three fold. To this was added 2 mM EDTA, 1 mM EGTA and 2.5 mM sodium β-glycerophosphate, mixed well and incubated for 30 min at 4°C. The sample was then applied to a column (20 mm x 95 mm) containing 20 m of DEAE Bio-Gel A equilibrated with 0.01 M NaCl/2.5 mM sodium β-glycerophosphate in buffer A. The loaded sample was washed with 2.5 times its bed volume of 0.05 M NaCl in buffer A containing 2.5 mM sodium β-glycerophosphate and 5% glycerol and the protein was eluted with a 0.1-0.4 M NaCl gradient containing glycerol and sodium β-glycerolphosphate in buffer A. One hundred 2 ml fractions of eluted sample were collected. A small aliquot from every fifth fraction was taken, precipitated with ethanol/hexanes (4:1), electrophoresed and the gel exposed to X-ray film for 12 hr. The peak fractions containing pgp35 (which eluted between 0.22-0.27 M NaCl) were pooled.

To the pooled sample (20-35 ml) was added MgCl₂ to a final concentration of 1 mM. The sample was concentrated in Centriprep-10 and Centricon-10 concentrators to a final volume of 500 μl. This concentrated sample was centrifuged at maximum speed in a Brinkmann Eppendorf centrifuge for 5 min at 4°C. The supernatant was applied to a Sephacryl S300 column (1.5cm x 100cm) that had previously been equilibrated with S300 column buffer (20 mM Tris-HCl, pH 7.5/1 mM sodium pyrophosphate/0.15 M NaCl/0.1% Tween 20 and 5% glycerol). Two ml fractions were collected and a 15 μl aliquot of every second or third tube was electrophoresed on a 5-15% gradient SDS-PAGE minigel and exposed to X-ray film for 18 hr. Peak fractions containing pgp35 were pooled.

For hydroxyapatite chromatography, the sample eluted from Sephacryl S300 (ca. 15 ml) was dialyzed for 30 min at 4°C in 2 l of 10 mM Tris-HCl, pH
7.5/0.2% Tween 20/0.2 mM Na$_3$VO$_4$. The dialyzed sample was centrifuged at 6000xg for 20 min at 4°C. The supernatant was applied to a column containing two ml of hydroxyapatite Bio-Gel HT that was equilibrated with 10 mM Tris-HCl, pH 7.5/0.2% Tween 20/0.2 mM Na$_3$VO$_4$ buffer. One hundred 1.5 ml fractions were collected of eluted material and analyzed by SDS-PAGE and radioautography for the peak fractions. The protein pgp35 eluted between 0.04 and 0.07 M potassium phosphate.

Final purification of pgp35 was effected by SDS-PAGE. After SDS-PAGE of purified samples eluted from Sephacryl S300 or hydroxyapatite, the pgp35 band was identified by radioautography of the dried gel. The band was excised and eluted with 20 mM HEPES-NaOH, pH 7.5/50 mM DTT/0.1% SDS and an aliquot reelectrophoresed.

1) Identification of rER phosphoproteins

Partial purification of the signal peptidase complex.
Partial purification of the signal peptidase complex (SPC) was accomplished exactly as described in Evans et al (1986), except using [r-32P] GTP phosphorylated SRM as the source of membrane. Phosphorylated SRM was solubilized in a stepwise manner through a salt step gradient and chromatographed over a QAE Sephadex A25 column. The Coomassie Brilliant Blue and radioautographic profiles obtained from samples of each step in the gradient and the flowthrough and eluant from the QAE Sephadex column allowed us to follow the progressive purification of the SPC and locate the SRM phosphoproteins.

Immunoprecipitation of phosphorylated SRM proteins with antisera to the SRP receptor.

SRM (1 mg/ml) were incubated under standard phosphorylation conditions with [r-32P] ATP and [r-32P] GTP, and the reactions stopped with 10 mM ATP and GTP/2 mM Na$_3$VO$_4$/2mM NaF/20 mM β-glycerophosphate. 60 µl (54 µg) each of stopped reaction mixture was incubated with anti-SRα (epitope A) mouse monoclonal (1/200 dilution), anti SRβ rabbit polyclonal serum (1/40 dilution) or anti-mp30 rabbit polyclonal IgG (1/1000 dilution) or with
corresponding control antibodies or antisera (mouse IgG, normal rabbit serum and rabbit anti-mouse IgG respectively) at the same dilutions. Each tube was adjusted to 1xRIPA (0.3 N NaCl/0.1 M Tris Cl, pH 7.4/2% Triton X-100/2% sodium deoxycholate/0.2% SDS/0.01 M EDTA) concentration. Samples were rotated for 1 h at 4°C. Pansorbin was added at 10 times the volume of antisera or antibody and samples were rotated again for 1 h at 4°C. They were layered over a 1 M sucrose cushion (in 0.2 M HEPES, pH 7.4) and centrifuged for 5 min in an Eppendorf tabletop microfuge. Pellets were washed several times, immunoprecipitated protein released by boiling in SDS-PAGE sample preparation buffer and electrophoresed on SDS-PAGE.

m) Qualification and quantification of samples

Protein content of subcellular fractions was determined by the method of Bradford (1976) and of eluted fractions following chromatography by the measurement of the optical density at 280 nm.

A modified Laemmli (1970) procedure was used for processing on SDS-PAGE. Samples were dissolved in 1.5% SDS/10% 2-mercaptoethanol/10% glycerol/0.1% Bromophenol Blue/62.5 mM Tris-HCl, pH 6.8, and heated for 15 min at 65°C or boiled for 5 min, then applied to discontinuous SDS polyacrylamide gels containing 4% acrylamide in the stacking gel and a 5-15% gradient of acrylamide in the resolving gel. Approximately 20 µg of cell fraction protein was applied to each lane.

After SDS-PAGE gels were processed for PAS staining as described by Neville and Glossman (1974) or were fixed (stained using a solution of 10% acetic acid, 50% methanol in distilled water containing 0.5% Coomassie Brilliant Blue for two hours at room temperature with gentle agitation. Destaining was accomplished using a solution of 7% acetic acid, 30% methanol in distilled water.

Gels were soaked in the same destaining solution containing 2% glycerol for 20 min prior to drying at 68°C under vacuum for 1.5 hours. Dried gels were then subjected to radioautography at -70°C with enhancing screens. Radioactive bands were detected by radioautography using Kodak X-OMAT AR-
5 film. Relative intensities of radiolabeled proteins were determined by scanning densitometric analysis of developed X-ray films using the Zeineh soft laser scanning densitometer (model SL-504-XL) Bio-med Instruments Inc. (Fullerton, Ca.). Radioactivity was also evaluated by liquid scintillation counting and Cerenkov counting (Packard Tricarb 460 C).
RESULTS

This section is divided into two parts. In Part One, the GTP specific in vitro phenomena previously described in rER membranes (see below) and mentioned in the previous section with respect to known effects of nucleotides in the ER, are introduced. This brief section of Results centers on further characterization of these phenomena and our efforts to show their physiological relevance. In Part Two, our goals broaden to include an examination of \([\tau\text{-}32\text{P}]\) GTP and \([\tau\text{-}32\text{P}]\) ATP phosphorylation in stripped rough microsomal vesicles. Work includes characterization of phosphorylated substrates, examination of the specificity and regulation of the phosphorylation reaction, and localization of the four major membrane phosphoproteins identified in this work. Subsequent purification of these proteins was successful in isolating a complex of three ER membrane proteins, of which two were phosphorylated, and two glycosylated. Finally, we attempted to identify these proteins and to demonstrate a physiological role for integral rER membrane phosphoproteins in nascent chain translocation.
PART ONE:
IN VITRO GTP SPECIFIC PHENOMENA IN THE RER

The GTP specific fusion of homologous ER membranes was first described by Paiement (Paiement et al, 1980; Paiement and Bergeron, 1983; Paiement, 1987). It is an in vitro morphological phenomenon which occurs coincidentally with two biochemically measurable phenomena, enhanced addition of asparagine linked oligosaccharides to endogenous nascent peptides (Godelaine et al, 1977; Godelaine et al, 1979a,b) and increased permeability of microsomal vesicles (Godelaine et al, 1977; Godelaine et al, 1983; Paiement et al, 1987). Both biochemical phenomena have been colocalized to fused stripped microsomal membranes (Paiement and Bergeron, 1983; Paiement et al, 1987), suggesting that all three phenomena are manifestations of the same molecular event. Certain conditions characterize these three GTP specific phenomena (the "GTP effect"). The rER membranes must be stripped of the majority of their ribosomes, there must be physiological concentrations of both GTP and Mn$^{2+}$ or Mg$^{2+}$ and only hydrolyzable GTP is effective, GMP PNP will not work (Paiement et al, 1980).

a) Characterization of GTP specific phenomena in the rER

In the hope that a better understanding of the molecular basis for these phenomena would elucidate their physiological role, we attempted further characterization. Fig. 1 illustrates the in vitro conditions with which we were able to dissect the GTP specific fusion of SRM. Once stripped of ribosomes, GTP must be present before membranes are incubated at temperatures greater than 25-30°C or membrane "inactivation" with respect to fusion occurs, and subsequent addition of GTP will cause membrane aggregation but not fusion (Fig. 1a); if GTP is present in the incubation, but not divalent cation, the membranes may be warmed to temperatures greater than 25-30°C and remain fusion competent or "activated" with respect to fusion, but fusion will only occur upon the further addition of Mn$^{2+}$ or Mg$^{2+}$ (Fig. 1b). Hence, the nucleotide and cation effects of these GTP specific phenomena can be uncoupled.
Figure 1

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 (Paiement et al, 1987).
a) SRM → GTP⁻, medium ±, 37° → inactive membranes
   → GTP⁺, medium ±, 37° → Aggregation but no membrane fusion

b) SRM → GTP⁺, medium ±, 37° → activated membranes
   → GTP⁺, medium ±, 37° → Aggregation and membrane fusion
Examination of fusion between many types of phospholipid membranes has revealed that when certain conditions are met, fusion between two membranes can take place in the absence of proteins (Duzgunes, 1985). It has also been shown in some instances to be mediated by fusogenic proteins (Duzgunes, 1985). To determine whether or not there was proteinaceous involvement in the "GTP effect" controlled proteolysis of SRM was done with increasing concentrations of trypsin, followed by assay for fusion, enhanced glycosylation and increased permeability of SRM (Fig. 2). The hydrolysis of mannose-6-phosphate (m-6-p) by the luminally disposed ER enzyme glucose-6-phosphatase is a well characterized test of the integrity of the microsomal membrane (Beaufay et al, 1974). Because there is no specific transporter for m-6-p in ER membranes, in the absence of detergent the degree of hydrolysis of m-6-p is a quantitative indicator of the leakiness of the microsomal vesicles. Assay in the presence of detergent determines total mannose-6-phosphatase activity possible.

A parallel loss of both fusion and glycosylation was seen such that inhibition of both occurred in a concentration range of trypsin where membrane latency was unaffected (Fig.2). Therefore both fusion and enhanced glycosylation appeared to be mediated by an rER protein. Because enhanced permeability was observed at much higher trypsin concentrations with loss of latency only beginning at a concentration of protease where both fusion and glycosylation were almost completely abolished, this protein(s) must be cytosolically oriented.

b) GTP specific rER phenomena and nascent peptide translocation

Previous work has shown that GTP specific fusion, enhanced glycosylation and permeability changes in homologous ER membranes occur only in stripped rough and not smooth microsomal membranes (Godelaine et al, 1983). The fusion event has also been shown to occur at a site located beneath the ribosome on the membrane (Paiement et al, 1987). It has been suggested therefore that GTP plays a role in the vectorial discharge and segregation of nascent peptides within the ER lumen (Godelaine et al, 1983; Paiement et al, 1987).
Figure 2

Effect of trypsin on GTP-stimulated glycosylation (*), fusion (•), and mannose-6-phosphatase latency (••).

Microsomes were pretreated with varying concentrations of trypsin (0.1-100 μg/ml) and then incubated in standard ER fusion medium containing GTP at 37°C for 60 min. Glycosylation and mannose-6-phosphatase activity were measured as described in Paiement et al (1987). Incorporation of [3H] GlcNAc into cold TCA 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 TCA 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 (*).
We examined the possibility that there was a direct correlation between the GTP specific phenomena and translocation across the ER membrane exploiting our ability to distinguish GTP sensitive (activated) and insensitive (inactivated) membranes. In Fig. 3 we examined whether GTP could influence cotranslational vectorial translocation in dog pancreas and rat liver stripped rough microsomes.

Towards this end, it was necessary to establish a working in vitro translation/translocation assay. Total messenger RNA was purified from full term human placental tissue as described in Experimental Procedures. This tissue was chosen because greater than 50% of total mRNA codes for human placental lactogen, a small secretory protein with a cleaved signal sequence (T. Evans et al, 1986), the cleavage of which is a good indication of translocation across the rER membrane (Jackson and Blobel, 1980). Rabbit reticulocyte lysate was prepared according to Jackson and Hunt (1983), and the rest of the constituents required for cell free translation prepared as described in Experimental Procedures. Translocation competent dog pancreas microsomal membranes were prepared according to Walter and Blobel (1983a), and rat liver microsomal membranes according to Paiement and Bergeron (1983). The mRNA, rabbit reticulocyte lysate and microsomal membranes required concentration-response testing for optimal translation and translocation of this particular mRNA (not shown).

In Fig. 3 control SRM from rat liver or dog pancreas translocated human preplacental lactogen and converted a variable (dog > rat) proportion of preplacental lactogen (A,B, lanes 2) to a peptide of mobility identical to placental lactogen (hPL)(compare lanes 3, A and B). Trypsin digestion showed variable (dog > rat) protection of the mature hPL band but not the precursor form (compare lanes 4, A and B). However, membrane dependent cleavage of preplacental lactogen to hPL was also observed in SRM rendered resistant to GTP/Mg$^{2+}$ by prior heat inactivation (1 h, 37°C)(lanes 5, A and B) as well as in GTP protected membranes (lanes 7, A and B). Subsequent trypsin proteolysis revealed protection by dog pancreatic SRM with less observed in rat liver SRM (lanes 6 and 8,A and B). Parallel experiments (Fig. 4) followed by immunoprecipitation of translation
Radioautograph showing cell free translation and membrane dependent translocation of human placental lactogen 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) and a $M_r$ of 29,500 was seen for pre-hPL i.e. 3200 greater in mol. wt. than a comigrating standard of hPL stained by Coomassie brilliant blue and having an $M_r = 26,300$. 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 $M_r$ 26,300 (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 $M_r$ 29,500 but variable retention of the band comigrating with hPL. 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 with anti-hPL antiserum as shown in Figure 4.
A. Rat Liver SRM

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B. Dog Pancreas SRM

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Radioautograph showing immunoprecipitation with anti human placental lactogen antiserum of products of cell free translation and membrane dependent translocation of human placental lactogen using dog pancreas stripped rough microsomal membranes (mb).

Results are identical to those seen in Figure 3B. (a) Untreated membranes, (b) Control membranes which were incubated at 4°C for 1 hr in the absence of GTP, (c) Inactivated membranes which were incubated at 37°C for 1 hr in the absence of GTP, and (d) Protected membranes which had GTP present during a 1 hr preincubation at 37°C. The positions of pre-hPL (29.5 kDa) and hPL (26.3 kDa) are indicated at the right. On the left are shown positions of molecular weight standards in kilodaltons.
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20.1–

1 2 3 4 5 6 7 8 9 10 11 12
products 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.

Direct testing of our hypothesis proved inconclusive. Approaching the question from another position we hypothesized the involvement of GTP as phosphate donor in the phosphorylation of ER membrane proteins (Paiement and Bergeron, 1989).
PART TWO:
IN VITRO PHOSPHORYLATION WITH RADIOLABELED ATP AND GTP

Direct evaluation of the GTP sensitivity of translocation competent microsomal membranes showed no correlation between the ability of these membranes to fuse and to translocate. However, because indirect evidence strongly suggested a relationship between these two events (vide supra) and because nonhydrolyzable GTP analogs were unable to stimulate the GTP specific phenomena (Paiement et al, 1983) we redirected our approach. Several molecular mechanisms have been proposed to explain the requirement for GTP hydrolysis and the nucleotide specific phenomena which occur in the ER (Connolly and Gilmore, 1989; Singer et al, 1987; Paiement and Bergeron, 1989). One model suggests that phosphorylation of a specific group of integral membrane proteins may be involved in one of the major functions of rough ER membranes, i.e., translocation of nascent proteins (Paiement and Bergeron, 1989).

a) In vitro phosphorylation of stripped rough microsomes with radiolabeled nucleotides and analysis of phosphorylated components of SRM

To this end, translocation competent stripped rough microsomes were phosphorylated in vitro with [$\gamma$-32P] GTP, and the radioautographic profile of phosphorylated proteins after SDS-PAGE examined (Fig. 5). Because most of the previous GTP specific fusion work was performed on rat liver SRM, these membranes were incubated under conditions which optimized fusion (40 mM Tris, 30 mM KCl, 7.5 mM MgCl$_2$, 2.5 mM MnCl$_2$, 500 $\mu$M GTP) varying certain conditions of the standard fusion reaction (temperature, time, ATP regenerating system and phosphatase inhibitors). To achieve 500 $\mu$M GTP concentration it was necessary to dilute the [$\gamma$-32P] GTP (29 Ci/mmol, 0.06 $\mu$mol/ml) 8-fold with nonradiolabeled GTP, weakening the radioautographic profile. It can be seen that where an ATP regenerating system is present (lanes 1,3,5,7) only two major bands of radioactivity are seen at about 53 and 148 kDa. With no ATP regenerating system (lanes 2,4,6,8,9), another pattern is seen, most clearly when no phosphatase inhibitors are present (lane 9). In this latter case, a series of GTP phosphorylated proteins with apparent molecular weights of approximately 13, 15, 25, 35, 53, 90 and 148 kDa are seen.
Radioautograph of [*-32P] GTP phosphorylation of rat liver stripped rough microsomes.

Translocation competent rat liver stripped rough microsomes (1 \( \mu \)g/ul) were incubated with standard fusion medium (40 mM Tris, 30 mM KCl, 7.5 mM MgCl\(_2\), 2.5 mM MnCl\(_2\), 0.5 mM GTP) while varying the phosphorylation reaction conditions. Reactions were performed at 0°C (lanes 1-4) or 30°C (lanes 5-9) for 5 min (lanes 1,2,5,6) or 30 min (lanes 3,4,7,8,9) in the presence or absence of an ATP regenerating system. In all lanes except lane 9 phosphatase inhibitors were included (50 mM fluoride, 10 \( \mu \)M vanadate. 40 \( \mu \)g of phosphorylated protein from each reaction was electrophoresed on a 5-15% SDS-polyacrylamide gel, the gel dried and exposed to KODAK XAR-5 OMAT film for 24 hours. On the right apparent molecular weights in kDa are indicated for GTP phosphorylated proteins.
<table>
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<th>Temp. (°C)</th>
<th>Time (min)</th>
<th>ATP regen. system</th>
<th>Phosphatase inhibitors</th>
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<td>+ - + - + + - -</td>
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Because earlier work had indicated that dog pancreas SRM produced better results for both GTP specific fusion quantitation (personal communication, J. Paiement) and in cell free translation/translocation assays (Fig. 3) than rat liver SRM, and because they were more easily isolated in large quantities and were more stable, the two membranes were compared after in vitro phosphorylation (Fig. 6, RL and DP). As well, because two very different patterns were seen in the presence and absence of an ATP regenerating system (Fig. 5), the membranes were incubated with \( [\gamma-32P] \) GTP (G) and \( [\gamma-32P] \) ATP (A) to compare the two different phosphorylation profiles. For stronger radiolabeling, \( [\gamma-32P] \) GTP was not diluted with unlabeled nucleotide, and a final concentration of 7 \( \mu M \) \( [\gamma-32P] \) GTP was used for phosphorylation, a concentration approaching the lower limit for GTP specific fusion. This change of concentration of GTP and specific activity of the label are thought to account for the few differences in the radioautographic pattern seen here compared to Fig. 5. As with results for fusion and translocation, dog pancreas SRM showed much stronger phosphorylation than rat liver. However, the two GTP phosphorylation patterns were similar (lanes 2,4). The dog membranes were therefore used in all phosphorylation reactions thence on. By contrast, ATP (lanes 1,3) and GTP (lanes 2,4) phosphorylation patterns were very different from each other for both rat liver and dog pancreas. Four prominent GTP phosphorylated proteins are seen for RL and DP, with apparent molecular weights of 15, 35, 56 and 90 kDa. Of these, only the 15 kDa phosphoprotein was phosphorylated primarily by GTP.

The phosphorylation reaction was optimized with respect to temperature and pH (Fig. 7). In panel A dog pancreas SRM was incubated with radiolabeled ATP or GTP at 0°C or 30°C under "standard" phosphorylation conditions outlined in Experimental Procedures. Of the four main phosphorylated proteins of interest (indicated at the right), the 15 kDa phosphorylated protein appeared to be more strongly phosphorylated at 0°C. Little temperature effect was seen on the phosphorylation of the 35, 56 or 90 kDa bands. In panel B, the change of pH from 5 to 7.5 had no effect on the phosphorylation profile. Above pH 8 phosphorylation of the 15 and 35 kDa bands decreased, while the 56 and 90 kDa phosphoproteins remained the same
In vitro phosphorylation of stripped rough microsomes from dog pancreas and rat liver.

Phosphorylation of translocation competent stripped rough microsomal membranes (SRM) with $[\tau-32P]$ ATP (A) or $[\tau-32P]$ GTP (G) isolated from dog pancreas (DP) or rat liver (RL). Cell fractions were phosphorylated as described in Experimental Procedures, processed for SDS-PAGE and radioautography. Approximately 40 $\mu$g cell fraction protein was applied to each lane. Mobilities of $[\tau-32P]$ GTP phosphorylated peptides of 15, 35, 56 and 90 kDa are indicated by arrows at the right.
Optimization of \([\tau-32P]\) GTP phosphorylation of stripped rough microsomes.

Panel A. Temperature effect on phosphorylation. SRM were incubated under standard phosphorylation conditions with \([\tau-32P]\) GTP (lanes 1, 2) and \([\tau-32P]\) ATP (lanes 3, 4) at 0°C or 30°C, run in a 5-15% SDS-polyacrylamide gel and processed for radioautography. Positions of the 90, 35 and 15 kDa phosphorylated proteins are shown at the right.

Panel B. pH effect on phosphorylation. Stripped rough microsomes were incubated with \([\tau-32P]\) GTP under standard fusion conditions while varying pH from 5 to 9 (lanes 2 to 9). Samples were electrophoresed on 5-15% SDS polyacrylamide gel and processed for radioautography. The 90, 35 and 15 kDa phosphoproteins are indicated at the right.
across the whole pH 5 to pH 9 range. All future phosphorylations were performed at pH 7.5 and 0°C to optimize phosphorylation of all the proteins.

The ER is the sole site of phospholipid synthesis in the cell and nucleotides are used extensively in that synthesis. We were interested in seeing whether [\(\tau\)-32P] from ATP or GTP was incorporated into phospholipid. When translocation competent SRM from dog pancreas were incubated with radiolabeled ATP or GTP, 75% and 87% respectively of the incorporated radioactivity was found in protein and the remainder in phospholipid (Table III). Thin layer chromatography of phospholipid extracts of radiolabeled phosphorylated SRM demonstrated that the majority of the radioactivity incorporated into phospholipid was found in a spot of mobility equivalent to that of the phosphatidyl serine standard (61% for [\(\tau\)-32P] ATP and 83% for [\(\tau\)-32P] GTP) (Fig. 8, Table IV).

Because the majority of radiolabeling took place on protein constituents of SRM with both [\(\tau\)-32P] ATP and [\(\tau\)-32P] CTP, and because the hypothesis which we were pursuing involved phosphorylation of SRM proteins, the next step after their identification was characterization of the GTP phosphorylated proteins.
Radiolabeled phospholipid component of [r-32P] ATP and [r-32P] GTP phosphorylated SRM.

To visualize the distribution of 32P-radiolabeled components of phosphorylated SRM, after standard phosphorylation of SRM with [r-32P] GTP and [r-32P]ATP lipid was extracted using the method of Schact (1981).

Panel A shows the radioautograph of a 5-15% SDS-polyacrylamide gel of a standard phosphorylation reaction. Positions of 90, 35 and 15 kDa phosphorylated proteins are indicated at right. An arrowhead indicated radiolabeled phospholipid running at the electrophoretic front.

In Panel B aliquots of the same phosphorylation reactions were lipid extracted and run on ascending thin layer chromatography inert silica plates. The position of migration of phospholipid standards are shown at right. The unlabeled bar indicates the position of an unidentified radiolabeled phospholipid.
TABLE III

Percent incorporation of radiolabel from \([r-32P]\) ATP or \([r-32P]\) GTP into protein and phospholipid after phosphorylation of dog pancreas SRM

<table>
<thead>
<tr>
<th>Nucleotide Donor</th>
<th>([r-32P]) ATP</th>
<th>([r-32P]) GTP</th>
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<tr>
<td>Protein:</td>
<td>75 ± 22*</td>
<td>87 ± 7</td>
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<td>Phospholipid:</td>
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</table>

*Average of three experiments ± SD

After standard phosphorylations of dog pancreas SRM with \([r-32P]\) ATP or \([r-32P]\) GTP membranes were washed and samples of each reaction were incubated with 10% TCA under conditions which precipitate both lipid and protein associated radioactivity (30 min on ice) or radiolabeled protein only (30 min on ice followed by 30 min at 90°C). After washing, radioactivity in the pellets was quantitated by Cerenkov counting. Radioactivity in phospholipid was determined by subtraction of counts in the hot TCA pellet from those in the cold pellet.
TABLE IV

Percent recovery of \([r-32P]\) ATP or \([r-32P]\) GTP radio-labeled phospholipid after ascending thin layer chromatography of \textit{in vitro} phosphorylated dog pancreas SRM

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>\textbf{Percent total radiolabel}</th>
<th>\textbf{([r-32P]) ATP}</th>
<th>\textbf{([r-32P]) GTP}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>((78 \pm 6^*))**</td>
<td>((94 \pm 8))</td>
<td></td>
</tr>
<tr>
<td>IP3</td>
<td>2 ± 1</td>
<td>1 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>IP2</td>
<td>2 ± 1</td>
<td>2 ± 1</td>
<td></td>
</tr>
<tr>
<td>PA</td>
<td>31 ± 8</td>
<td>4 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>X</td>
<td>2 ± 1</td>
<td>2 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>PI</td>
<td>nd***</td>
<td>2 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>PS</td>
<td>61 + 2</td>
<td>83 ± 9</td>
<td></td>
</tr>
<tr>
<td>PE</td>
<td>3 + 0.1</td>
<td>7 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>

* standard deviation from average of three experiments
** percent of radiolabel recovered from origin
*** no detectable radiolabel

IP3 = phosphatidylinositol 3,4-bisphosphate; IP2 = phosphatidylinositol 4-phosphate; PA = phosphatidic acid; X = unidentified phospholipid; PI = phosphatidylinositol; PS = phosphatidylserine; PE = phosphatidylethanolamine

Dog pancreas SRM were incubated with \([r-32P]\) ATP or \([r-32P]\) GTP under standard phosphorylation conditions, the phospholipid component extracted in chloroform/methanol (1:2 v/v) according to Schact (1981) and chromatographed on Merck silica gel 60 plates in chloroform/methanol/3.3 M ammonium hydroxide \((43/38/12)\). The plates were processed for radioautography to visualize the radiolabeled phospholipids, and sprayed with Rhodamine \((0.05\% \text{ in } 95\% \text{ methanol})\) to visualize phospholipid standards. Radiolabelled spots were scraped from the plates and radioactivity quantitated by Cerenkov counting.
b) Membrane orientation of ER phosphoproteins

Phosphoproteins of the SRM were examined. Extraction of integral membrane proteins by the Triton X-114 protocol of Bordier (Fig. 9A) demonstrated that the majority of proteins phosphorylated by [γ-32P] ATP were peripheral proteins. However, the major integral membrane phosphoproteins were similar for both ATP and GTP phosphorylation, except for the 15 kDa polypeptide which was almost exclusively GTP phosphorylated.

The orientation of phosphorylation of the phosphopeptides was assessed by controlled protease digestion (Fig. 9B). When mild protease treatment of membranes was carried out before in vitro phosphorylation with [γ-32P] GTP, radiolabeling was reduced generally. The bands corresponding to pp15 and ppg35 disappeared and a new band ca. 33-34 kDa was observed (Fig. 9B, lane 1) presumably representing a proteolytic fragment of ppg35. A 70% reduction in ppg35 labeling was seen with the label migrating at a mobility consistent with a reduction in size of 1-2 kDa. When the identical protease treatment was carried out after in vitro phosphorylation, negligible radiolabeling of any of the phosphoproteins was observed (Fig. 9B, lane 2).

Two major complexes involved in the synthesis and targeting of nascent peptides to the rER membrane require ribonucleic acid in their functioning, i.e. the ribosomal complex and the signal recognition particle (vide supra). To determine whether there was RNA involvement with any of the phosphoproteins we were examining or in their phosphorylation, SRM were ribonuclease treated either before (pre) or after (post) incubation with [γ-32P] GTP (Fig. 10). No change was seen in either pattern of phosphorylation.

N-linked glycosylation occurs at the luminal face of the rER membrane (Perez and Hirschberg, 1986). Thus an integral ER membrane protein which is N-glycosylated must possess a luminal domain. The glycoprotein nature of these phosphoproteins was evaluated by N-glycopeptidase F (N Glycanase in Fig. 11) treatment and Concanavalin A chromatography (Fig. 11). After in vitro phosphorylation using [γ-32P] ATP or [γ-32P] GTP, N-
Membrane association and protease sensitivity of ER proteins phosphorylated by ATP or GTP in vitro.

Panel A. Triton X-114 extraction of integral membrane phosphoproteins of ER. In vitro phosphorylation of SRM was carried out with \(^{32}P\) ATP or \(^{32}P\) GTP and separated into the detergent (DET, lanes 3, 6) and aqueous phases (AQ, lanes 2, 5) according to the procedure of Bordier (1981). Samples were then processed on 5-15% SDS-PAGE. 50 µg of phosphorylated protein (Total) were loaded in lanes 1 and 4. Positions of molecular weight markers are shown on the left in kDa and the mobilities of pp15, pp56 and pp90 are indicated by the arrows on the right.

Panel B. Mild protease treatment of SRM prior to (pre) and after (post) in vitro phosphorylation with \(^{32}P\) GTP. Comparison is made with the intensity of the radiolabeled phosphoproteins in control incubations. The positions of the 15, 35, 56 and 90 kDa phosphoproteins are shown on the right by horizontal arrows. The mobility of the radiolabeled protein in lane 1 (upward arrow) corresponds to an apparent molecular mass of 33 kDa with 28% of the radioactivity of the band at 35 kDa in lane 3 (horizontal arrow). Positions of molecular weight markers are indicated in kDa on the left.
Figure 10

Ribonuclease sensitivity of [r32P] GTP phosphorylated stripped rough microsomal membranes.

To assess the role of ribonucleic acid in GTP phosphorylation, stripped rough microsomal membranes were incubated in the presence of 100 µg/ml ribonuclease before (pre) or after (post) phosphorylation with [r-32P] GTP and compared to standard GTP (G) and ATP (A) phosphorylation reactions. No ribonuclease effect is seen. The positions of pp15, pgp35 and pp90 are indicated at the right.
**Figure 11**

**N-glycanase sensitivity and Concanavalin A binding of phosphorylated proteins.**

Panel A. N-glycanase (Glycopeptidase F) treatment of SRM after phosphorylation with [γ32P] ATP (A) or [γ32P] GTP (G). The Triton X-114 detergent extract (det) was subjected to digestion by increasing concentrations of N-glycanase (N-GLYC). Only the band corresponding to pgp35 shows a dose dependent reduction concurring with the appearance of two bands of apparent molecular mass of 32 kDa and 29 kDa (indicated by arrows).

Panel B. Binding of pgp35 to Concanavalin A. After in vitro phosphorylation of SRM with [γ32P] GTP, the Triton X-114 extract was chromatographed through Con A Sepharose in the presence (FT + α-mm) or absence (FT+ α-mm) of α-methyl D-mannoside. The phosphoglycoprotein (pgp35) was not eluted by the α-methyl D-mannoside alone but could only be removed from the column with the addition of SDS (Eluate + SDS) as described by Poliquin and Shore (1980). The mobilities of pgp35 and pp15 are indicated at the right.
(A) N-Glycanase

(B) Con A

Diagram showing results of N-Glycanase and Con A treatments on samples labeled A and G, indicating changes in molecular weight markers.
glycopeptidase F treatment was carried out on Triton X-114 extracted proteins (Panel A). The results demonstrated that only the radiolabeled band at 35 kDa was sensitive to this enzyme. With increasing doses of N-glycopeptidase F the progressive appearance of two bands at 32 and 29 kDa was observed (Panel A, lanes 7,8). Triton X-114 extracted \([\tau-32P]\) GTP phosphorylated SRM were incubated with Con A Sepharose (Fig. 11, Panel B). When no competing saccharides were present in the sample, only the 35 kDa phosphoprotein was retained (Panel B, lane 2). To remove specifically bound hydrophobic proteins from Con A Sepharose columns requires SDS (Poliquin and Shore, 1980). When an SDS elution was performed after sample loading and extensive washing of the Con A sepharose column, only the 35 kDa protein was detected (Panel B, lane 4).

c) Examination of the phosphorylation reaction

GTP specific fusion is a well defined event with several specific characteristics (Paiement and Bergeron, 1989; vide supra). Our original goal was a better understanding of the molecular basis of GTP specific fusion. Having found that GTP is able to act as phosphate donor to specific rER membrane proteins, we attempted to correlate fusion and phosphorylation.

This involved the in vitro phosphorylation of microsomal membranes while varying one of the conditions known to inhibit fusion (Fig.12). GTP specific fusion occurs only in rough microsomal membranes which have been stripped of the majority of their ribosomes (Paiement et al, 1987). In Panel A, both stripped (SRM) and unstripped rough microsomes (RM) were incubated with \([\tau-32P]\) ATP and \([\tau-32P]\) GTP and their radioautographic profiles compared. Only phosphorylation of pp90 was somewhat inhibited in rough microsomes vs. stripped rough microsomes. In fact, the rough membranes showed more bands of phosphorylated protein.

In Panel B, SRM were preincubated for an hour at 37°C, a condition which renders SRM unable to fuse (Paiement and Bergeron, 1983). Although ATP phosphorylation was not affected by this treatment, GTP phosphorylation was almost completely abolished.
The effects of the presence of ribosomes, "heat inactivation" and the absence of divalent cations on phosphorylation of SRM with $[\gamma-32P]$ GTP and $[\gamma-32P]$ ATP.

Panel A. Stripped rough microsomes (SRM) and unstripped rough microsomes (RM) were incubated under standard phosphorylation conditions with $[\gamma-32P]$ ATP and $[\gamma-32P]$ GTP, aliquots electrophoresed on 5-15% SDS-polyacrylamide gel and processed for radioautography.

Panel B. SRM were incubated at 37°C for 1 h in the absence of GTP under conditions in which they are "inactivated" for fusion. They were then incubated under standard phosphorylation conditions with $[\gamma-32P]$ ATP and $[\gamma-32P]$ GTP. Lane 1 shows a typical ATP phosphorylation reaction; lane 2 shows almost complete inhibition of GTP phosphorylation after "heat inactivation".

Panel C. SRM were incubated under standard phosphorylation conditions in the presence and absence of divalent cation (Me$^{2+}$). In lanes 1 and 3, where both Me$^{2+}$ and Mn$^{2+}$ are present, normal ATP and GTP phosphorylation is seen. In lanes 2 and 4 where no divalent cations are present, neither ATP nor GTP phosphorylation occurs.
Divalent cations are necessary for fusion to occur (Mg$^{2+}$ and/or Mn$^{2+}$) (Paiement et al., 1980). When neither Mg$^{2+}$ nor Mn$^{2+}$ were present (Panel C), neither ATP nor GTP phosphorylation took place.

Although heat pretreatment was shown to abolish GTP and not ATP phosphorylation, the presence of ribosomes on the membrane did not, and the absence of divalent cations was inhibitory to both ATP and GTP phosphorylation. Using these indirect correlatory methods we were unable to show consistently any relationship between fusion and phosphorylation.

The identification of integral rER membrane phosphoproteins, related or not to GTP specific fusion, is novel. The use of GTP as well as ATP as phosphate donor is unusual and not previously described in the ER. Therefore the phosphorylation reaction itself was examined further.

Calcium has been implicated in the functioning of several GTP-binding proteins and many kinases (Sibley et al., 1987; Browning et al., 1985). In Fig. 13, Panel A, GTP phosphorylation was conducted in the presence of increasing concentrations of EGTA, a calcium specific chelating agent. No inhibitory or stimulatory effect was seen, suggesting that calcium is not involved in regulation of these phosphorylation reactions. Nor was any effect seen in other experiments where Ca$^{2+}$ was added directly to the phosphorylation reaction (not shown). Also heparin, a specific inhibitor of Casein Kinase II activity, (the only enzyme which has been shown to exhibit low Km for both ATP and GTP; vide supra) did not affect either ATP or GTP phosphorylation (not shown).

RM must be isolated in the presence of reducing agents to maintain their translocation competence (Walter and Blobel, 1983a). To see whether redox state was important to GTP phosphorylation, SRM were incubated with [$\gamma$-32P] GTP and increasing concentrations of DTT (Fig. 13, Panel B). At concentrations as high as 50 mM no effect at all was seen.

Many proteins possess in an active site or functional domain a superreactive cysteine residue. SRM were incubated under phosphorylating
Figure 13

The effects of EGTA and DTT on phosphorylation of SRM with [γ-32P] GTP and [γ-32P] ATP.

Panel A. To determine whether calcium was involved in the GTP phosphorylation reaction, increasing concentrations of EGTA, a chelating agent specific for Ca²⁺, were included in standard GTP reactions. Positions of pp15, pgp35 and pp90 are shown at right.

Panel B. To determine whether disulfide bonding played any role in GTP phosphorylation, increasing concentrations of DTT were used in standard GTP reactions. Lanes 1 to 4 show the radioautographic profile of GTP phosphorylation as DTT increased from 0 to 50 mM DTT. Pp15, pgp35 and pp90 are indicated at right.
conditions with the sulfhydryl-blocking agent NEM at concentrations from 0-3.4 mM (Fig. 14). While some GTP phosphorylated proteins appeared to be unaffected by high concentrations of NEM (notably pp56 and pp90), the phosphorylation of most was inhibited in a dose dependent manner such that by 1 mM NEM there were few bands to be seen. ATP phosphorylation on the other hand was only moderately reduced at 3.4 mM NEM, except for one phosphoprotein (indicated by downward arrows in lane 1 and 2) which disappeared by 1 mM NEM. The phosphorylation of SRM appears to be mediated by two different activities, one which uses ATP primarily and is NEM insensitive, and one which uses GTP primarily and is sensitive to NEM. The different sensitivities of ATP and GTP phosphorylation of pgp35 to NEM is illustrated in Panel B. It will be noted that even the 0 mM NEM control profiles are not identical to other control phosphorylations (compare with Fig. 6). We believe this is explained by the NEM pretreatment of SRM, which occurs at 25°C for 10 minutes prior to addition of GTP and appears to change the phosphorylation pattern even in the absence of NEM.

Until now phosphorylation of SRM was done using intact membranes. When SRM were first treated with increasing concentrations of the detergent Triton X-100 and then incubated for phosphorylation, similar results to those seen for NEM were obtained (Fig. 15). After [γ-32P] GTP phosphorylation, the 15 and 35 kDa phosphoproteins disappeared quickly by 0.5% detergent concentration; phosphorylation of the 56 and 90 kDa phosphoproteins was not affected. After [γ-32P] ATP phosphorylation, the pgp35 band decreased gradually in intensity and was still easily visible at 1% Triton X-100. No other effects were seen on ATP phosphorylation. Again, these results suggest that the phosphorylation of SRM is mediated by at least two activities, one which can use GTP as phosphate donor and phosphorylates mainly membrane proteins (see Fig.9), the other which appears to use only ATP and phosphorylates primarily peripheral, but also membrane proteins.

In Fig. 5 rat liver SRM was phosphorylated in the presence and absence of the phosphatase inhibitors sodium fluoride and sodium vanadate (Antoniw and Cohen, 1976; Paris and Pouyssegur, 1987; Paris et al, 1987). When these inhibitors were not present (lane 9) a slightly cleaner GTP
Figure 14


Panel A. SRM containing 0.17 mM DTT was treated with 0 to 3.4 mM N-ethyl maleimide at 25°C for 10 min followed by in vitro phosphorylation with [γ32P] ATP or [γ32P] GTP as described in the Experimental Procedures. Samples were electrophoresed on SDS-PAGE and exposed to X-ray film. As seen in lanes 1 and 7 warming the membranes to 25°C for the NEM reaction affects the in vitro phosphorylation pattern (compare with Fig. 6) diminishing phosphorylation of pgp35. Furthermore, a new band of 38 kDa (indicated by vertical arrows) is ATP phosphorylated. The positions of pgp35 and pp15 are indicated by horizontal arrows on the right.

Panel B. Quantitation of radiolabel in pgp35 by densitometry using data taken from lanes 1 to 6 and lanes 7 to 12 for [γ32P] ATP and [γ32P] GTP respectively.
C

A

<table>
<thead>
<tr>
<th>ATP</th>
<th>GTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.4</td>
</tr>
<tr>
<td>0</td>
<td>0.4</td>
</tr>
</tbody>
</table>

1 2 3 4 5 6 7 8 9 10 11 12

B

Percent control

pgp35 ATP

pgp35 GTP

NEM concentration (mM)
Figure 15

The effect of Triton X-100 membrane solubilization on in vitro phosphorylation with [γ32P] ATP and [γ32P] GTP.

Panel A. In vitro phosphorylation of SRM with [γ32P] ATP or [γ32P] GTP proceeded in the presence of increasing concentrations of Triton X-100 detergent. In lanes 1 to 5 radiolabeled ATP phosphorylation of pgp35 decreases dramatically at detergent concentrations between 0.5 and 1%. After phosphorylation with radiolabeled GTP (lanes 6 to 10) it can be seen that both pgp35 and ppl5 phosphorylation are inhibited in parallel and decrease dramatically at detergent concentrations between 0.1 and 0.5%. The mobilities of ppl5 and pgp35 are indicated by horizontal arrows at the right.

Panel B. Densitometry of pgp35 after in vitro phosphorylation of SRM with [γ32P] GTP (●) and [γ32P] ATP (○) as a function of increasing Triton X-100 concentrations in the incubation mixture. Also shown is the effect of the detergent on phosphorylation of ppl5 by [γ32P] GTP (●). Fifty percent inhibition of ATP phosphorylated pgp35 is seen to be around 0.7% Triton X-100 while that for GTP phosphorylated pgp35 and ppl5 around 0.33%.
phosphorylation profile was seen, and some of the phosphorylated bands were stronger. In Fig. 16, this experiment was repeated on dog pancreas SRM for both ATP and GTP phosphorylation. No difference was seen between phosphorylation with ATP or GTP alone and their respective phosphorylations with sodium vanadate. When sodium fluoride was included in the reactions, however, the 135 kDa band which is seen almost exclusively in ATP phosphorylations was more prominent in ATP phosphorylation (lanes 2,3) and was equally prominent in GTP phosphorylation (lanes 5,6). Also, when sodium fluoride alone was added to either ATP or GTP incubations, there was increased incorporation of radiolabel in the region above the 135 kDa band.

d) Specificity and regulation of in vitro phosphorylation.

The kinase activities which we have identified above are unlike any previously described in ER. We analyzed them further in the hope of understanding the basis for the differences in nucleotide and substrate specificity.

Glycerol kinase in the presence of glycerol specifically hydrolyzes ATP and not GTP and effectively removes it from solution by the phosphorylation of glycerol (Hayashi and Lin, 1967). To assess whether phosphorylation with [\( \tau \)-32P] GTP was due to its interconversion to ATP, glycerol kinase and glycerol were added to the in vitro phosphorylation incubation mixture (Fig. 17). As illustrated in Panel A, glycerol kinase prevented the phosphorylation of ER proteins by [\( \tau \)-32P] ATP in a dose dependent fashion but had no effect on in vitro phosphorylation with [\( \tau \)-32P] GTP.

A dose response inhibition study with increasing concentrations of unlabeled ATP or GTP was carried out in order to estimate the relative affinities of ATP or GTP for their respective phosphorylation apparatus (i.e., kinase(s), regulatory proteins and substrates). Fifty percent inhibition of transfer of radiolabel from [\( \tau \)-32P] GTP to pp35 or pp15 was observed at a concentration of ca. 20 \( \mu \)M unlabeled GTP (Fig. 17B). However, phosphorylation with [\( \tau \)-32P] GTP was even more sensitive to
The effect of phosphatase inhibitors on in vitro phosphorylation of SRM.

Stripped rough microsomes were incubated under standard phosphorylation reaction conditions with \([\tau-32P]\) ATP and \([\tau-32P]\) GTP in the presence and absence of phosphatase inhibitors. In lanes 1, 2 and 3 of both the ATP and GTP panels reactions included 0.2 mM sodium vanadate, 0.2 mM sodium fluoride, and 0.2 mM sodium vanadate and 0.2 mM sodium fluoride. Lanes marked A and G are untreated phosphorylations for use as reference. The positions of the 15, 35 and 90 kDa phosphoproteins are indicated on either side.
**Figure 17**

**Nucleotide specificity of phosphorylation.**

Panel A. Effect of glycerol kinase. SRM were phosphorylated with [r32P] GTP or [r32P] ATP in the presence of 1M glycerol, 0.15 mM DTT. In vitro phosphorylation proceeded in the absence (control lanes 1, 2) or presence of 2 μg glycerol kinase (a, lanes 3, 4) or 4 μg glycerol kinase (b, lanes 5, 6). After standard phosphorylation samples were processed for SDS-PAGE and radioautography.

Panel B. Densitometry of pgp35 or ppl5 radioautograph of phosphorylation of SRM with [r32P] GTP in the presence of increasing concentrations of unlabeled ATP (○, ●) or unlabeled GTP (▲, ▲). Values are expressed as a percent of control phosphorylation (7 μM [r-32P] GTP, s.a. 36 Ci/mmol, no unlabeled nucleotide present).

Panel C. Densitometry of pgp35 and ppl5 radioautograph after phosphorylation of SRM with [r32P] GTP (7 μM) in the absence (control) or presence of 1 mM excess unlabeled nucleoside, nucleoside phosphates or non-hydrlyzable analogs.
unlabeled ATP with 50% inhibition seen at 0.1 μM ATP. The opposite was not observed, i.e., unlabeled GTP at a concentration of 1 mM had no effect on phosphorylation of pgp35 by [γ-32P] ATP or on any of the phosphopeptides phosphorylated by [γ-32P] ATP (Fig. 19).

Further information about the specificity of the phosphorylation reaction was obtained from the inhibitory effects of analogs on pgp35 and pp15 phosphorylation by [γ-32P] GTP (Fig. 17C). The phosphorylation of these two proteins was inhibited by GTP, GDP and the non-hydrolyzable analogs of GTP (GTPγS and GMP-PNP) at 1 mM. Considerably less inhibition was observed by GMP, cGMP and guanosine. Surprisingly, all the adenine nucleoside phosphates (ATP, ADP, AMP, cAMP), non-hydrolyzable ATP analogs (ATPγS and AMP-PNP), and adenosine itself completely inhibited phosphorylation of pgp35 and pp15 by [γ-32P] GTP. However, no inhibition of [γ-32P] ATP phosphorylation of pgp35 was seen by 1 mM GTP, GDP, GMP, cGMP, GTPγS, GMP-PNP, or guanosine (not shown).

Further studies (Fig. 18) using inhibitors at equimolar (7 μM) as well as excess (1 mM) concentrations, with respect to concentration of radiolabeled nucleotide, indicated that adenine nucleosides (Panel B, lanes 1 to 5) and non-hydrolyzable analogs (Panel B, lanes 6, 7) even at 7 μM concentration were good inhibitors of GTP phosphorylation of pp15, 35, 56 and 90, better in fact than their guanine equivalents (Panel A, lanes 1 to 7). At this concentration of unlabeled ATP (lane 1), the GTP phosphorylation pattern resembles much more closely that of ATP, suggestive of an alteration of substrate specificity. This pattern change is seen in the presence of equimolar concentrations of non-hydrolyzable analogs as well. Of note also is the newly visible band at 135 kDa (also seen in Fig. 19, Panel A, lane 1). No inhibition of [γ-32P] ATP phosphorylation of SRM was seen in the presence of equimolar or excess guanine nucleoside phosphates or nonhydrolyzable analogs (not shown).

The contrast in regulation of GTP and ATP phosphorylation was evaluated further by including various unlabeled nucleotides during in vitro phosphorylation of SRM (Fig. 19). The striking effect and specificity of 10 μM ATP on the inhibition of 32P incorporation from 10 μM GTP was
**Figure 18**

Radiosautograph showing the effects of guanine and adenine nucleoside phosphates and nonhydrolyzable analogs on $[^{-32}P] \text{GTP}$ in vitro phosphorylation.

Panel A. SRM was incubated with $[^{-32}P] \text{GTP}$ under standard phosphorylation conditions in the presence of equimolar (7 μM) or excess (1 mM) unlabeled GTP (lanes 1, 8), GDP (lanes 2, 9), GMP (lanes 3, 10), cGMP (lanes 4, 11), guanosine (lanes 5, 12), GTP$_r$S (lanes 6, 13) and GMP-PNP (lanes 7, 14). Samples were electrophoresed on SDS-PAGE and processed for radioautography. Mobilities of the four major GTP phosphorylated integral ER membrane proteins are indicated at the left.

Panel B. All conditions correspond to those in Panel A, with the addition of the corresponding adenine nucleosides and analogs.
Figure 19


SRM was phosphorylated with $[^{32}P]GTP$ or $[^{32}P]ATP$ as described in Experimental Procedures, in the presence of 10 $\mu$M or 1 mM unlabeled ATP (A), GTP (G), ITP (I), CTP (C) and UTP (U). Phosphorylation reactions with $[^{32}P]GTP$ are shown in Panel A, those with $[^{32}P]ATP$ in Panel B. Control GTP and ATP phosphorylations in the absence of unlabeled nucleotide are shown in lanes 11 for A and B. The positions of the 15, 35, 56 and 90 kDa bands have been indicated by arrowheads to the left and right.
confirmed (GTP, lane 1) with no effect noted by other unlabeled nucleotides at this concentration (GTP, lanes 2-5). Again it is notable that the band at $M_r$ 135 kDa (Fig. 19, GTP, lane 1) showed enhanced phosphorylation by [$\tau$-32P] GTP in the presence of 10 $\mu$M unlabeled ATP. This peptide corresponds in molecular weight to the major peptide phosphorylated by [$\tau$-32P] ATP (Fig. 19, ATP, lane 10). Excess (1 mM) ATP, GTP, GTP and UTP abolished 32P labeling (GTP, lanes 6,7,9,10). ITP abolished 32P incorporation into all peptides except pp35 and pp15 (GTP, lane 8). By contrast, 32P labeling of [$\tau$-32P] ATP phosphorylated SRM was inhibited by equimolar unlabeled ATP (ATP, lane 1) and abolished at high concentration (ATP, lane 6). One mM UTP, but not GTP or ITP also inhibited radiolabeling and CTP produced an intermediate amount of inhibition.

e) Localization of four major [$\tau$-32P] GTP phosphorylated integral membrane proteins to the ER

The relevance of phosphorylated integral rER membrane proteins with respect to their involvement in important ER limited functions, depends on their restriction to that organelle. Using well characterized subcellular fractionation procedures, we determined the subcellular location of pp15, pgp35, pp56 and pp90.

Separation of dog pancreatic microsomes into rough and smooth fractions (Fig. 20) revealed a higher level of in vitro phosphorylation activity in smooth vs. rough microsomes with ATP as phosphate donor. The opposite was observed with GTP. Of note was the absence of the 15, 35, 56 and 90 kDa bands when in vitro phosphorylation of smooth microsomes was carried out with [$\tau$-32P] GTP (lane 2).

A more detailed analysis of the subcellular distribution of in vitro phosphorylation activity was carried out with rat liver membranes isolated according to previously characterized protocols (Figs. 21 and 22). The phosphorylation activity of SRM (lanes 1) was compared to GF1, GF2 and GF3 (lanes 2,3,4), i.e., Golgi light, intermediate and heavy subfractions as well as the small vesicle fraction (SV, lanes 5) and the residual load
Figure 20

In vitro phosphorylation with ATP (A) or GTP (G) of smooth and rough microsomal subfractions isolated from dog pancreas homogenates.

A comparison of phosphorylation patterns for rough (RM) and smooth (SM) microsomal subfractions separated by a modification of the method of Tartakoff and Jamieson (1974) reveals the restriction of the majority of the 15, 35, 56 and 90 kDa proteins phosphorylated by \([\gamma^{32P}]\) GTP to RM. Positions of molecular weight markers are indicated on the left. Arrows on the right show the mobilities of the 15, 35, 56 and 90 kDa bands.
**Figure 21**

**Subcellular distribution of in vitro phosphorylation activity of defined rat liver subcellular fractions.**

Panel A. The phosphopeptide profile of dog pancreatic SRM phosphorylated with \([r-32P]\) GTP (lane 1) was compared to that of freshly prepared Golgi fractions (GF1, GF2, GF3, lanes 2,3,4), small vesicle fraction (SV, lane 5), and the residual load zone (LZ, lane 6) prepared from rat liver microsomes. In separate experiments Li and G1 (late endosomes, lane 7; Golgi endosomes, lane 8) fractions were purified from parent L and P fractions phosphorylated with GTP. The results of separate experiments with liver plasma membrane fractions (PM, lane 9), liver lysosomes (lane 10) or rat heart mitochondrial fractions (MIT, lane 11) is also shown. The arrowheads to the right of each series of experiments denote the positions of the 15, 35, 56 and 90 kDa phosphoproteins as determined from concurrently electrophoresed dog pancreatic SRM. Vertical arrows in lane 7 denote phosphopeptides of 30, 35, 52 kDa which may be unique to endosomes.

Panel B. Con A binding and elution of phosphoproteins of dog pancreatic SRM (lane 12) or rat liver Golgi fractions (lanes 13-15), SV (lane 16), LZ (lane 17), as well as the Li endosomal fraction (lane 18) and the Golgi-endosomal fraction (lane 19) were carried out after *in vitro* phosphorylation with \([r-32P]\) GTP and solubilization as described in Methods. The mobility of the major glycoprophosphoprotein (pgp35) of SRM is shown in lane 12, and by arrowheads to the right of lanes 17 and 19 as they were seen on concurrently run lanes in separate experiments.
GTP Phosphorylation

A

SRM GF1 GF2 GF3 SV LZ Li Gi Pm Lys Mit

B

SRM GF1 GF2 GF3 SV LZ Li Gi

CON A

1 2 3 4 5 6 7 8 9 10 11

12 13 14 15 16 17 18 19
Figure 22

ATP phosphorylation of rat tissue subcellular fractions.

Except for phosphorylation with \( [\tau-32P] \) ATP instead of \( [\tau-32P] \) GTP all conditions were the same as those described in Figure 21.
zone fractions (LZ, lanes 7) from the discontinuous sucrose gradient used to generate the Golgi fractions. Also evaluated were a late endosome fraction (Li, lanes 7) floated from parent light mitochondrial fractions as well as a combined Golgi-endosome fraction (Gi, lane 8) floated from P fractions. Furthermore, liver parenchymal plasma membrane (PM, lanes 9) and highly purified lysosomes (LYS, lanes 10) were assessed as well as highly purified rat heart mitochondria (MIT, lanes 11). Each fraction demonstrated a unique pattern of in vitro phosphorylated peptides. Of noteworthy significance was the simple pattern of phosphorylation of highly purified late endosomes (lanes 7) and lysosomes (lanes 10). As well, controlled proteolysis (not shown) of phosphorylated mitochondrial fractions indicated the presence of five proteins phosphorylated by ATP and/or GTP which are protease sensitive (indicated in lanes 11).

Comparison of the phosphopeptide mobilities with those in dog pancreatic SRM (lanes 1) revealed no 15 or 90 kDa bands. However, the 35 kDa band migrated close to unique phosphopeptides in Golgi and endosome fractions (i.e., lanes 2-4, 7,8). This was resolved when the fractions were solubilized and chromatographed with Concanavalin A Sepharose. Whereas the 35 kDa band of SRM was bound and eluted from SRM (lanes 12) no equivalent phosphopeptide was observed in Golgi or endosome fractions (lanes 13-19) for either GTP (Fig. 21) or ATP (Fig. 22) in vitro phosphorylation.

All four major GTP phosphorylated ER lumenal proteins, pp15, pp35, pp56 and pp90, were detected only in well defined dog pancreas and rat liver subcellular fractions which were highly enriched in rER.

In our progression towards the understanding of a physiological role for these phosphoproteins, we required a handle with which to analyze them and our first step involved their purification.
Purification of a phosphoglycoprotein complex from [γ-32P] GTP phosphorylated SRM

The more restricted phosphorylation of SRM membrane proteins after in vitro phosphorylation with [γ-32P] GTP was exploited in an attempt to purify these phosphoproteins.

After in vitro phosphorylation with [γ-32P] GTP and extraction of integral membrane proteins with Triton X-114, [γ-32P]-labeled phosphopeptides were subjected to two sequential anion exchange chromatography steps with DEAE columns followed by gel permeation chromatography using Sephacryl S300 followed by chromatography over hydroxyapatite. As outlined in Experimental Procedures, glycerol (5%) was required for the replacement of Triton X-114 with Tween 20, the detergent used to maintain solubility of the integral membrane phosphoproteins during the chromatographic steps. Each stage of the purification was monitored by SDS-PAGE of eluted fractions, Coomassie Brilliant Blue and PAS staining of protein and glycoprotein and radioautography of radiolabeled peptides.

Peak fractions containing proteins of interest were then concentrated and applied to subsequent columns. A reproducible purification of the phosphopeptide pgp35 was effected (Fig. 23). The phosphoprotein pp90 appeared to copurify with pgp35 during the purification steps. Unfortunately, ppl5 and pp56 were lost at early steps in the purification (Panel B, lane 3) and their isolation was left for a later time. No further purification of pgp35 and pp90 was observed after application of the Sephacryl S300 eluted phosphoproteins to hydroxyapatite (lanes 5,6). Excision of the bands corresponding to pgp35 and pp90 in lane 6 and reelectrophoresis led to single bands of Coomassie Brilliant Blue stained protein and radiolabel (lanes 7,8). Staining of SDS-PAGE gels by the PAS procedure of Neville and Glossman revealed two reactive bands. One corresponded as expected to pgp35, the other to a band of 25 kDa which was not phosphorylated, and which also copurified with pgp35. These PAS positive bands are indicated by downward and upward arrows in lanes 5 to 7 (Fig. 23A).
Figure 23

Purification of pgp35.

Panel A. Coomassie Brilliant Blue stained protein profiles of peak fractions containing pgp35 purified by the steps outlined for Panel B.

Panel B. X-ray radioautograph of phosphorylated proteins after in vitro phosphorylation of SRM with [r32P] GTP (lane 1); after extraction of integral membrane proteins with Triton X-114 (lane 2); after DE52 chromatography (lane 3); after DEAE Bio-Gel A cellulose chromatography (lane 4); after Sephacryl S300 chromatography (lane 5); and hydroxyapatite chromatography (lane 6). Lanes 7, 8 indicate the final purification of pgp35 and pp90 by excision of the bands from SDS-PAGE (from lane 6), elution and reelectrophoresis on SDS-PAGE. Approximately 40 µg protein was loaded onto lane 1. The upward arrows in lanes 5, 6 indicate a non-phosphorylated PAS positive band of 25 kDa. The downward arrows indicate the only other PAS positive band in these lanes, i.e. pgp35.
Fig. 24 illustrates the Sephacryl S300 step in the purification of [\(\tau\)-32P]GTP phosphorylated pgp35. After this point, only the 35 and 90 kDa phosphoproteins are seen (Fig. 23B, lane 5) and no further separation of pp90 and pgp35 from other integral ER membrane proteins occurs (Fig. 23A, lane 5). In fact, as can be seen from Fig. 24, these proteins elute as a single peak from Sephacryl S-300 at an apparent M\(_r\) of 400 kDa. Samples were evaluated for protein content by absorbance at 280 nm (upper panel) and 32P radioactivity by Cerenkov counting (lower panel). SDS-PAGE and radioautography (inset) indicated that the first radioactive peak, which eluted about M\(_r\) 400,000, (fractions 32-39), contained pp90 and pgp35. The second peak (fractions 40-45) did not contain macromolecular radioactivity as assessed by SDS-PAGE. A similar peptide profile eluting as a single peak was observed after Triton X-114 extraction, anion exchange chromatography and lentil lectin chromatography (not shown).

Kinase specificity is defined initially by the amino acid on which phosphate is added, i.e., serine, threonine or tyrosine. In the ongoing characterization of rER membrane phosphoproteins and analysis of the kinase reactions we had observed, purified pp90 and pgp35 were analyzed to determine amino acid sites of phosphorylation. After phosphorylation of SRM with [\(\tau\)-32P]GTP followed by Triton X-114 extraction and SDS-PAGE, the bands corresponding to pp90 and pgp35 were excised from the gel, and subjected to acid hydrolysis and two dimensional electrophoresis. As seen in Fig. 25, only radiolabeled phosphoserine was detected.

Four phosphorylated ER membrane proteins have been identified and purification achieved of what is apparently an integral rER membrane phosphoglycoprotein complex. No function however has yet been discovered for any of these proteins, although their restricted locale and transmembrane nature is suggestive of a possible role in translocation. Because of this and similarities in size of some of these proteins to proteins of the signal peptidase complex (Evans et al, 1987) and the signal recognition particle receptor (Gilmore et al, 1982a,b), an attempt was made to determine whether any of these proteins were associated, or indeed the same.
**Figure 24**

**Sephacryl S300 chromatography of phosphoproteins.**

After *in vitro* phosphorylation of SRM with [\(\gamma^{32}P\)] GTP as described in Experimental Procedures, integral membrane proteins were extracted with Triton X-114 and chromatographed sequentially on DE52 and DEAE Bio-Gel A. After ion exchange chromatography, peak fractions were collected and concentrated, then applied to a 1.5 x 100 cm column containing Sephacryl S300 and eluted with 20 mM Tris-HCl, pH 7.5/1 mM sodium pyrophosphate/0.15 M NaCl/0.2% Tween 20/5% glycerol. Samples were evaluated for protein content by absorbance at 280 nm and 32P radioactivity by Cerenkov counting. SDS-PAGE and radioautography (inset) indicated that the first radioactive peak (fractions 32-39) contained pp90 and pgp35. The second peak (fractions 40-45) did not contain macromolecular radioactivity as assessed by SDS-PAGE. The positions of the molecular weight markers are indicated by the downward pointing arrows corresponding to aldolase (Mr 158,000), bovine serine albumin (Mr 67,000), ovalbumin (Mr 43,000) and chymotrypsinogen A (Mr 25,000).
Identification of phosphoamino acids in pgp35 and pp90.

After in vitro phosphorylation of SRM with [*32P] GTP, proteins were extracted with Triton X-114 and resolved by SDS-PAGE. Gel segments corresponding to pgp35 and pp90 were excised and extracted as described in Experimental Procedures, followed by partial acid hydrolysis with 6N HCl for 70 min at 110°C. 32P phosphoamino acids (2300 cpm of pgp35 and 1200 cpm of pp90) were then developed by two-dimensional thin layer electrophoresis in the presence of unlabeled phosphoamino acid markers. The first (vertical direction) electrophoresis was carried out at pH 1.9 and the second (horizontal direction) electrophoresis at pH 3.5, at 500 V for 50 min for each dimension. PS, phosphoserine; PT, phosphothreonine; PY, phosphotyrosine; Pi, phosphoric acid. X-ray film was exposed for three days at -70°C.
Identification of phosphoamino acids in pgp35 and pp90.

After *in vitro* phosphorylation of SRM with \([\gamma^{32}P]\) GTP, proteins were extracted with Triton X-114 and resolved by SDS-PAGE. Gel segments corresponding to pgp35 and pp90 were excised and extracted as described in Experimental Procedures, followed by partial acid hydrolysis with 6N HCl for 70 min at 110°C. 32P phosphoamino acids (2300 cpm of pgp35 and 1200 cpm of pp90) were then developed by two-dimensional thin layer electrophoresis in the presence of unlabeled phosphoamino acid markers. The first (vertical direction) electrophoresis was carried out at pH 1.9 and the second (horizontal direction) electrophoresis at pH 3.5, at 500 V for 50 min for each dimension. PS, phosphoserine; PT, phosphothreonine; PY, phosphotyrosine; Pi, phosphoric acid. X-ray film was exposed for three days at -70°C.
Identification of \( \tau^{-32P} \) GTP phosphorylated integral ER membrane proteins

At least two of the integral ER membrane proteins being studied (pp15 and gp25) were of molecular weights similar to the group of ER membrane proteins recently purified and called the Signal Peptidase Complex (SPC) (Evans et al., 1986). To determine whether these proteins were related we partially purified this complex from \( \tau^{-32P} \) GTP phosphorylated SRM following the procedures of Evans (1986) (Fig. 26). Positions of the four major SPC proteins, easily recognizable at the high salt solubilization and QAE Sephadex sievortive chromatography steps (lanes 3, 5), are indicated at the right in Panels A and B. Mobilities of the four major GTP phosphorylated proteins, shown at the left on the same panels, can be seen to be at different positions when compared with the proteins of the SPC.

Antisera were obtained to the \( \alpha \) and \( \beta \) subunits of the signal recognition particle (SRP) receptor and to its associated membrane protein mp30 (from P. Walter). With these \( \tau^{-32P} \) GTP and \( \tau^{-32P} \) ATP phosphorylated SRM were probed (Fig. 27). After immunoprecipitation with these three antisera and subsequent electrophoresis of immunoprecipitated pelets, none of the four major phosphorylated ER membrane proteins were detected, a strong indication that they share no antigenic determinants with the SRP receptor proteins, nor are there any interactions between the two groups of proteins strong enough to result in coprecipitation. Of note however are three \( \tau^{-32P} \) ATP phosphorylated proteins (Fig. 28, ATP panel, lanes 1, 2, 3) in the region between pp15 and pgp35, as indicated by small asterisks to the left of the panel, which may be of some relevance to the function of the SRP receptor.
Partial purified signal peptidase complex proteins do not correspond to any of the found major GTP phosphorylated integral ER membrane proteins.

Panel A. Coomassie Brilliant Blue stained polyacrylamide gel showing partial purification of the signal peptidase complex (SPC) according to the procedure of Evans et al (1986). Lanes labeled A and G are standard SRM phosphorylations with ATP and GTP respectively. [γ-32P] GTP phosphorylated SRM solubilized according to Evans et al is seen in lane 1 (Sol. Load). After centrifugation through a step gradient, aliquots were removed from three regions, low salt (lane 2), high salt where the SPC concentrates (lane 3) and the pellet (lane 4). The region of high salt concentration was then chromatographed over a QAE Sephadex A25 column (QAE) and an aliquot taken of the pooled flowthrough peak fractions (lane 5). Positions of the four major SPC proteins easily recognizable at this step of SPC purification are shown by arrows at the right, and are labeled in kDa. Mobilities of our four major GTP phosphorylated proteins are indicated at the left.

Panel B. Radioautograph corresponding to the Coomassie stained SDS-polyacrylamide gel in Panel A. Each lane represents the same step in SPC purification as in Panel A. None of the major GTP phosphorylated integral membrane proteins (indicated at left) corresponds to any of the known proteins of the SPC (shown at right).
Figure 27

The four major GTP phosphorylated integral ER membrane proteins are not recognized by antisera to either subunit of the SRP receptor or the associated protein mp30.

Both [r-32P] GTP and [r-32P] ATP phosphorylated SRM were incubated with antisera to the two subunits of the SRP receptor and to an SRP receptor associated protein mp30, as well as with control antisera. Lanes 1, 2 and 3 of both GTP and ATP panels show no specifically immunoprecipitated phosphoproteins using anti-SRa (epitope A) mouse monoclonal (1/200 dilution), anti-SRß rabbit polyclonal (1/40 dilution) and anti-mp30 rabbit polyclonal IgG (1/1000 dilution) respectively. Lanes 4, 5 and 6 of both GTP and ATP panels show control immunoprecipitations for lanes 1, 2 and 3 using unrelated mouse immunoglobulin, normal rabbit serum and rabbit anti-mouse IgG respectively (same dilutions). Lanes labeled G and A are untreated phosphorylation reactions for use as reference. The positions of phosphoproteins of 15, 35, 56 and 90 kDa are indicated at the right. Three [r-32P] ATP phosphorylated proteins in the molecular weight region between 15 and 35 kDa are indicated by small asterisks to the left of the ATP panel.
DISCUSSION

Summary

The initial goal of the work described here was to characterize further GTP specific in vitro phenomena in rER hoping to better understand the molecular basis for these events, and to elucidate the physiological role(s) of which these events are likely to be an in vitro manifestation. We examined directly the involvement of proteins in these GTP specific rER events and attempted to correlate them with nascent peptide vectorial translocation across rER. When direct correlation proved inconclusive, we postulated a role for GTP as phosphate donor in some rER restricted phosphorylation event and showed that a limited number of rER restricted membrane phosphoproteins are indeed phosphorylated in vitro with GTP as phosphate donor. In comparing GTP and ATP phosphorylation we found considerable overlap in substrate specificity. With one exception, i.e., ppl5, which was significantly phosphorylated only when GTP was available, GTP phosphorylated proteins were primarily an integral membrane protein subset of the ATP phosphorylated proteins. However, ATP and GTP phosphorylation appear to occur via two distinct kinase activities which are differently regulated. We purified a phosphoglycoprotein complex of rER membrane proteins, containing two rER restricted integral membrane phosphoproteins and two N-glycosylated integral membrane proteins. Finally, we showed that this complex of proteins, and indeed all four major rER membrane phosphoproteins do not correspond to either of the complexes of proteins already known to participate in targeting and translocation of nascent peptides in rER membranes.

a) Fusion and translocation

The physiological relevance of the GTP specific fusion of rER membranes is strongly supported by the concentrations of GTP (10 μM) and MgCl₂ (as low as 100 μM) required to elicit SRM fusion in vitro (Paiement, 1987), which fall well within the physiological range (Clifford et al, 1972; Vanden Berghe et al, 1977; Corkey et al, 1986; Schramm, 1982). Results indicate that GTP induces a primary event within the microsomal membrane
which by itself does not result in morphological or biochemical changes, but in the presence of Mn\(^{2+}\) or Mg\(^{2+}\) stimulates fusion, enhanced glycosylation and membrane leakiness, thus showing that the nucleotide and cation effects can be uncoupled. Quantitative comparison revealed coincident inhibition of ER membrane fusion and glycosylation following trypsin treatment. Since the concentrations of trypsin required to inhibit maximally membrane fusion and glycosylation barely affected mannose-6-phosphatase activity, an intraluminally oriented enzyme, it was concluded that a cytosolically oriented protein constituent of SRM may serve as an intermediate in the events which lead to fusion, permeability changes and glycosylation.

The location of this protein(s) beneath the ribosome (Godelaine et al, 1983; Paiement et al, 1987), and the restriction of this phenomenon to stripped rough microsomes (Paiement et al, 1980) suggested a possible role for GTP hydrolysis in nascent chain translocation. Taking advantage of our ability to distinguish GTP sensitive and insensitive membranes this hypothesis was tested directly by in vitro coupled translation/translocation using preincubated SRM. No correlation was seen between fusion competence and translocation competence. The design of our experiments however did not rule out the hypothesis. We did not take into account the possibility that a proportion of the translocation apparatus present in purified SRM fractions might already be in an activated state equivalent to our in vitro "GTP activation" and ready to translocate. The subsequent in vitro fusion in the presence of divalent cation may correspond in vivo to the interaction of an activated hydrophobic or amphipathic protein with another membrane protein, a situation seen before
in GTP binding protein systems (Sibley et al, 1987). Because ATP and GTP have been implicated in the translocation of newly synthesized proteins across the ER membranes by several investigators (vide supra) and because of the strong suggestion of a relationship between translocation and GTP-hydrolysis specific fusion, the problem was approached from another angle. One possible model which explains both of these nucleotide mediated events is that GTP is being used as a phosphate donor in the phosphorylation of an integral membrane protein(s). This hypothesis was again tested directly by phosphorylating, under fusion conditions, translocation competent dog pancreas SRM with [γ-32P] ATP and [γ-32P] GTP and examining the respective phosphorylated substrates.

b) In vitro phosphorylation, specificity and regulation

In further studies we have described the in vitro phosphorylation of integral membrane proteins and phospholipids of ER membranes by [γ-32P] GTP and [γ-32P] ATP. For both ATP and GTP phosphorylation a small proportion of 32-P was incorporated into phospholipid, 25 and 13% of the total radioactivity respectively. Of this, phosphatidyl serine was the only phospholipid with a substantial incorporation of 32P in GTP phosphorylation. Only phosphatidyl serine and phosphatidic acid showed significant radiolabeling in ATP phosphorylation. The ATP incorporation of γ-32P into phospholipid might occur directly by phosphorylation of serine alcoholic head groups during their activation or of glycerol to glycerol-3-phosphate, but GTP γ-32P incorporation would most likely be indirect, perhaps via nucleoside diphosphokinase transfer of the gamma phosphate to ADP (Ginther and Ingraham, 1974). How phospholipid synthesis occurs at 4°C in isolated SRM which should be free of the cytosolic
constituents needed for the process (e.g., free serine, enzymes for the synthesis of hydrophilic precursors) is not understood. Possibly there is a continual turnover of the serine head groups on phosphatidyl serine at the cytosolic face of ER membranes and these are phosphorylated by membrane associated proteins.

A remarkable difference in protein phosphorylation with the two labeled nucleotides was observed. The integral membrane protein designated as pp15 was almost exclusively phosphorylated by \([\tau-32P] \text{GTP}\). Although the three other major integral membrane proteins (designated as pp90, pp56 and pp35) were phosphorylated by both nucleotides, GTP reproducibly phosphorylated pp35 more heavily under standard phosphorylation conditions. Furthermore, after Triton X-114 extraction of the integral membrane proteins it was clear that ATP preferentially phosphorylated peripheral proteins with a 135 kDa polypeptide as the major substrate; this protein showed little to no phosphorylation by \([\tau-32P] \text{GTP}\) under normal phosphorylation conditions, but was strongly phosphorylated when equimolar concentrations of adenosine nucleosides or fluoride ion was present. The 148 kDa phosphorylated band seen in Fig. 5 also may be this 135 kDa phosphoprotein running aberrantly on that gel.

GTP protein phosphorylation was not explained by transphosphorylation to ATP via microsomal nucleoside diphosphokinase since the ATP specific glycerol kinase abolished radiolabeling by \([\tau-32P] \text{ATP}\) with no effect on the specificity or extent of phosphorylation of the substrates phosphorylated by \([\tau-32P] \text{GTP}\). This finding was confirmed when the presence of radiolabelled GTP and ATP in the reaction mixtures during and
after phosphorylation was assessed using thin layer chromatography (not shown). No radiolabeled ATP was detected at any point in GTP phosphorylation reactions.

Regulation of in vitro phosphorylation was also nucleotide specific. The unique pattern of ATP phosphorylation of SRM was unaffected by other unlabeled nucleoside phosphates or nucleotide analogs including GMP-PNP. This contrasted with the phosphorylation of SRM protein substrates by \([\tau-32P]\) GTP. An exquisite regulation by adenine nucleotides was revealed. These included the non-hydrolysable analogs of ATP and adenosine itself. Concentrations as low as 0.1 \(\mu\)M unlabeled ATP altered the pattern of in vitro phosphorylation by \([\nu32P]\) GTP (not shown). The usual pattern of preferential radiolabeling of integral membrane protein substrates was altered to that of labeling of the peripheral protein substrates (e.g., the 135 kDa protein) usually preferred by \([\tau-32P]\) ATP. Such observations also were not explained by transphosphorylation, i.e., transfer of label from \([\tau-32P]\) GTP to ATP since the addition of non-hydrolyzable ATP\(_pS\) at 10 \(\mu\)M to a reaction containing 10 \(\mu\)M \([\tau-32P]\) GTP also led to the same shift in the pattern of protein substrate phosphorylation. Taken together, these data therefore indicate that distinctly regulated protein kinase activities are responsible for the phosphorylation of SRM proteins using GTP versus ATP as phosphate donor. Differential phosphorylation of the same substrates by distinct ATP and GTP specific kinase activities has been reported previously with respect to microtubule proteins (Diaz-Nido et al, 1989). However, regulation of our phosphorylation reactions are not comparable to those reported by Diaz-Nido.
In our examination of kinase activity, we found GTP and ATP phosphorylation differentially sensitive to NEM alkylation. Again, after membrane solubilization with Triton X-100, GTP and ATP phosphorylation diminished at quite different detergent concentrations.

It is striking that the GTP phosphorylated proteins appear to be a membrane associated subset of the ATP phosphorylated proteins. One possible explanation of the differences seen in substrate specificity for the two nucleotides may be intracellular translocation of a kinase, its location dependant on regulatory conditions, and its substrate specificity on its location, in the same way that certain type 1 protein phosphatases are regulated in part by relocation within the cell (Cohen, 1989), that protein kinase A is translocated between the cytosol and the nucleus (Nigg, 1985), or the β adrenergic receptor kinase is translocated between the cytosol and the plasma membrane (Strasser, 1986). One result of SRP receptor activation with GTP might be relocation of the kinase responsible for "GTP phosphorylation" to a specific region of SRM membrane. NEM alkylation, which inhibits GTP phosphorylation so much more than it does ATP phosphorylation, might occur at a site of interaction between the kinase and the membrane.

In vitro phosphorylation of pp90 and pgp35 was exclusively on serine residues and at the cytosolically exposed surfaces of the proteins. This would localize the activity of the serine kinase(s) to the cytosolic surface of the SRM.
It is noteworthy that pp15 and pgp35 share certain characteristics of phosphorylation not seen with the other proteins. They both exhibit parallel sensitivity to alterations of pH, Triton X-100 membrane solubilization and NEM alkylation of the SRM membranes prior to phosphorylation, and in the presence of 1 mM ITP, a concentration at which GTP phosphorylation is abolished in the presence of all other unlabelled nucleotides tried, they are the only two remaining phosphorylated proteins. Although no clear explanation for these phenomena presents itself, they indicate that the two proteins share a common kinase, and although they do not copurify, perhaps a common physiological role.

No convincing correlation was made between GTP specific fusion of SRM and phosphorylation. While pretreatment of SRM at 37°C to render the membranes fusion incompetent also made them phosphorylation incompetent, the presence of ribosomes did not inhibit or significantly change the GTP phosphorylation pattern, and both ATP and GTP phosphorylation showed absolute dependence on divalent cations (Mn²⁺ and/or Mg²⁺), a very common requirement for many kinase activities. Only one of the GTP phosphorylated membrane proteins appeared to use only GTP as phosphate donor. This does not necessarily eliminate the others as candidates for the GTP specific fusion protein(s). Phosphorylation can occur at more than one site, and multiple site phosphorylation, by the same or different kinases is often used to effect more than one function, or to regulate or modulate biological response (Cohen, 1982).
c) The rER phosphoproteins

Further examination of the integral membrane protein substrates after in vitro phosphorylation showed that the sites of phosphorylation were cytosolically exposed. This was concluded from mild protease digestion experiments under conditions where we and others (Paiement et al, 1987; Amar-Costesec et al, 1984) have demonstrated previously that cotranslationally translocated nascent chains were protected from digestion. Interestingly, slightly different results were found when proteolysis was carried out prior to or after phosphorylation, suggesting that in vitro phosphorylation at 4°C caused some alteration in the conformation of the cytosolic domain of the pgp35 protein substrate.

Only one of the protein substrates (pgp35) was N-glycosylated as based on binding to Con A and sensitivity to N-glycopeptidase F. Increasing concentrations of the enzyme led to the sequential appearance of bands at 33 kDa and 29 kDa suggesting that pgp35 was most probably N-glycosylated at 2 sites (Lewis et al, 1985).

d) Localization to rER of four major membrane phosphoproteins

The subcellular distribution of in vitro phosphorylation activity was surveyed among well characterized subcellular fractions. For dog pancreas, the integral membrane proteins identified by [γ-32P] GTP phosphorylation in SRM were phosphorylated by rough microsomes with little to no phosphorylation in smooth microsomes. Indeed, even the major 135 kDa peripheral protein phosphorylated by [γ-32P] ATP was restricted to rough microsomes. The smooth microsome fraction from pancreatic homogenates has been characterized as mainly Golgi apparatus and plasma
membrane fragments (Tartakoff and Jamieson, 1974) although other small smooth membrane components such as endosomes and small vesicular carriers would also be expected to be found in the fraction. A more detailed subcellular fractionation study was realized with rat liver homogenates. Distinct fractions corresponding to various components of the Golgi apparatus (Bergeron et al, 1982), endosomes (Khan et al, 1986), plasma membranes (Hubbard et al, 1983) and lysosomes (Wattiaux et al, 1978) showed no phosphorylation of bands corresponding to pp15 or pp90 with [γ-32P] GTP as phosphate donor. However, the major phosphoglycoprotein of SRM, i.e., pgp35, migrated close to several 32P labeled phosphoproteins in Golgi and endosome fractions. Con A chromatography indicated that these bands did not correspond to pgp35 since the latter bound to Con A and the former did not. As well, highly purified mitochondria showed no in vitro phosphorylation of bands corresponding to pp15, pgp35 or pp90. Finally, the fraction designated as LZ (lanes 6 of Figs. 21, 22), the load zone for flotation of Golgi fractions, would be expected to contain fragments of the smooth ER as well as plasma membrane (Tartakoff and Jamieson, 1974). Little to no phosphorylation of pp15, pgp35 or pp90 was found in this fraction. Hence, in vitro phosphorylation of these integral membrane proteins was restricted to rough ER.

The unique in vitro phosphorylation patterns of each subcellular fraction examined should also be of considerable use in the identification of novel markers or organelles, especially for those for which none has yet been found (e.g., endosomes).
e) Purification of a phosphoglycoprotein complex

By the strategy of in vitro phosphorylation of integral membrane proteins of SRM, we have identified and purified a complex of phosphoglycoproteins of the ER. The integral membrane phosphoglycoprotein pgp35 was phosphorylated by both $[\gamma-32P]$ GTP and $[\gamma-32P]$ ATP with a distinct regulation of phosphorylation observed for each nucleotide. The use of $[\gamma-32P]$ GTP as phosphate donor increased the proportion of phosphorylation into integral membrane proteins, thereby enhancing the signal for subsequent purification. Extraction of phosphorylated SRM into Triton X-114 followed by sequential anion exchange chromatography and gel permeation chromatography resulted in the final purification of 3 major proteins by preparative SDS-PAGE, two which were phosphorylated (pp90, pgp35) and two which were glycosylated (pgp35, gp25). In work subsequent to that presented here, the same proteins were purified by lentil lectin chromatography instead of DEAE Bio-Gel A and Sephacryl S-300 chromatography, thereby enhancing the rapidity and ease of purification (Wada et al, 1989). Final purification for both protocols was by preparative SDS-PAGE.

Subsequent to the purification of the three proteins of the complex purified above, antisera were raised against each of pp90, pgp35 and gp25 which uniquely recognized their respective protein antigens. Immunoprecipitation of solubilized SRM with each respective antiserum led to the coprecipitation of all three integral membrane proteins (Wada et al, 1989). After purification and chromatography on Sephacryl S-300, a reproducible association of pp90, pgp35 and gp25 was observed at an apparent molecular weight of $400 \times 10^3$. It is unlikely that this
association was nonspecific because (i) protein aggregates would be expected to elute with the void volume and (ii) the proportions of pp90, pgp35 and gp25 were consistently similar after Sephacryl S-300 chromatography (pp90 stained much more intensely with Coomassie Blue than pgp35 or gp25, which were stained to a similar degree). Taken together, the data from gel permeation chromatography, lentil lectin chromatography and immunoprecipitation were consistent and we conclude that pp90, pgp35 and gp25 formed a complex.

f) Identification of rER phosphoproteins

At least one of the rER phosphoproteins identified was in the same range of molecular weight as some of the proteins of the recently purified signal peptidase complex (Evans et al, 1986). Partial purification of that complex from phosphorylated SRM and comparison of the Coomassie stained and radioautographic profiles led us to conclude that none of the phosphoproteins we had identified corresponded to those identified by Evans as the signal peptidase complex. That is not to say the two groups may not interact, but they do not associate under these purification conditions. After antisera were prepared to the proteins of the purified phosphoglycoprotein complex (I. Wada), immunoblotting of dog pancreas rough microsomal membranes with anti-gp25 antiserum and with three antisera to the SP22/23 glycoprotein of the Signal Peptidase Complex showed no cross recognition between the antisera (personal communication from G. Blobel). In fact, there was a 2 to 3 kDa difference between the mobilities of gp25 and SP22/23.
We also probed any possible association between the rER membrane phosphoproteins and the signal recognition particle receptor. Immunoprecipitation of \([\gamma-32P] ATP\) and \([\gamma-32P] GTP\) phosphorylated SRM using antisera or IgG directed against the \(\alpha\) and \(\beta\) subunits of the SRP receptor and the 30 kDa associated membrane protein mp30 (from P. Walter), showed no recognition of our four major membrane phosphoproteins. Of interest however are four small \([\gamma-32P] ATP\) phosphorylated proteins of approximate molecular weights 16, 17.5, 27 and 28 kDa which are recognized by the antisera to mp30 and the \(\beta\) subunit of the SRP receptor. These may be involved in the regulation of SRP receptor function and deserve further investigation.

The purification protocol was designed to purify pgp35. Recently, Hartman et al (1989) have purified a major 34 kDa integral membrane glycoprotein from dog pancreatic microsomes. Antibodies directed against the 34 kDa protein inhibited in vitro protein translocation; therefore the authors have proposed a role for this protein in translocation. More recently the full length sequence of the protein has been deduced from cDNA clones (Prehn et al, 1989). This protein has been further identified as the 34 kDa protein which was cross linked to the signal sequence of prolactin during cotranslational translocation and therefore was termed the signal sequence receptor (Weidman et al, 1987 & 1989). Partial sequence analysis of CNBr fragments of the protein we have identified as pgp35 confirmed the identity of our protein with the 34 kDa glycoprotein Signal Sequence Receptor (Fig. 28)(Wada et al, 1989; Hartmann et al, 1989; Prehn et al, 1989; Weidmann et al, 1989). Both proteins are N-glycosylated at 2 sites and copurify with the 25 kDa glycoprotein described above (unpublished
data). Although the biochemical significance of the complex identified remains to be determined, identification of pgp35 as the signal sequence receptor and phosphorylation of pgp35 and pp90 strongly suggest a role in translocation which may be regulated by phosphorylation.
Personal communication from T. Rapoport (Akademie der Wissenschaften der DDR, Zentralinstitut für Molekularbiologie, Berlin-Buch, DDR) comparing his cDNA sequence for the Signal Sequence Receptor (Wiedmann et al, 1987) to partial sequences of pgp35 determined using CNBr fragments.
SSR SEQUENZ

5 10 15 20 25 30
1 M R V L P R L L L L L L A F P A V L L R G G P G G S L V

31 A A Q D L T E D E E T V E D S I I E D E D D E D E A E V E E D E

61 P T D L A E D K E E E D V S G E P E A S P S A D T T I L F V

81 G E D F P A N N I V K F L V G F T N K G T E D F I V E S L

121 D A S F R Y P O D Y O F Y ! O N F T A L P L N T V V P P O R

151 Q A T F E Y S F I P A E P M G G R P F G L V I N L N Y K D L
181 N G N V F O D A V F N Q T V T I I E R E D G L D G E T I F M
211 Y M F L A G L G L L V V V G L H G L L E S R K R K R P I Q K
271 R L P R K R A Q X R S V G S D E
Overview and implications

In conclusion, the studies reported here identify for the first time integral membrane proteins of the ER which are phosphorylated by membrane associated GTP and ATP dependent kinase activities. One of the integral membrane phosphoproteins, pgp35 corresponds to the putative signal sequence receptor (Weidmann et al, 1987, 1989) on the basis of close identity from the amino acid sequences of CNBr fragments of the protein. Furthermore, pgp35 was purified as a complex with the phosphoprotein identified here as pp90 and a nonphosphorylated glycoprotein of molecular mass 25 kDa, which we speculate may be constituents of the translocation apparatus of the rough ER.

The phosphorylation of integral membrane proteins of the rough ER by GTP may be of relevance to the phenomenon of nascent chain translocation, as well as to other GTP related phenomena in the ER. GTP, but not non-hydrolysable analogs, causes profound and nucleotide specific permeability changes in rough microsomes. This is true during GTP dependent fusion of stripped rough microsomes in vitro (Paiement et al, 1987) as well as after the in vitro fusion of rough microsomes with planar lipid bilayers (Simon et al, 1989). In both instances, it has been speculated that these permeability changes may reflect the regulated opening of the postulated aqueous pore (Gilmore and Blobel, 1985) of the translocation apparatus (Paiement et al, 1987; Paiement and Bergeron, 1989).

Several groups have identified GTP binding proteins in the ER (Connolly and Gilmore, 1989; Godelaine and Beaufay, 1987; Robinson and Austen, 1987; Audigier et al, 1988; Comerford and Dawson, 1989; Lanoix et al, 1989).
Some of these proteins may serve to regulate the phosphorylation events reported here either directly as kinases themselves, or as regulators of the phosphorylation we have identified or other ER functions. If phosphorylation of the putative translocation apparatus can be shown to regulate nascent chain transport, then the specificity and regulation of nucleotide as phosphate donor may be related to sequential conformational changes in the proteins which make up the aqueous pore (Gilmore and Blobel, 1985; Connolly and Gilmore, 1989) as domains of the nascent chain are translocated across the membrane.

Our original goal was to determine the physiological role for GTP hydrolysis specific fusion in SRM. We have shown that GTP can indeed be used as a phosphate donor in vitro in SRM, and that certain proteins which have been implicated in nascent peptide translocation are phosphorylated in vitro with GTP. We have not shown conclusively a relationship between fusion and phosphorylation, or between fusion and translocation. If indeed GTP specific fusion is related to translocation, perhaps it is only as an in vitro artifact of ribosomal stripping. When ribosomes are not present and a certain membrane protein(s) is phosphorylated, hydrophobic sequences are exposed which lead to membrane fusion. When ribosomes are present however, this phosphorylation may constitute a primary step in a sequence of events leading to translocation.

Three functionally distinct apparatuses appear to work in concert in directing newly synthesized proteins to the ER membrane, translocating them across and modifying the segregated protein intraluminally. This machinery must recognize the highly hydrophobic and variable signal
sequences of these proteins prior to executing its specific function. Several constituents seem to be required at each step. The signal recognition particle, a complex of 6 polypeptides and an RNA molecule, and its receptor are needed for the first stage (Walter et al, 1984) and the signal peptidase complex for the final stage (Evans et al, 1986). The protein complex we have identified containing the signal sequence receptor would be expected to be functionally coupled with the other complexes in translocation, perhaps as Gilmore and Blobel's aqueous pore (1985).
A model for the mechanism of phosphorylation in regulation of translocation.

Using the G-protein pathway of activation as the model, the following scenario may help to elucidate the mechanism of translocation of nascent peptides across the ER membrane. This analogy is a synthesis of current knowledge about translocation, G-protein signal transduction, cell regulation by protein phosphorylation, the data included in this work and the model of Singer et al (1987). It can be broken down into several testable points of conjecture.

Signal recognition particle (SRP) binds to the signal recognition particle receptor (SRPR) (Walter et al, 1984). The SRPR moves into proximity with the signal sequence receptor (SSR) and the signal sequence (SS) of the nascent chain binds to the SSR (Weidmann et al, 1987). The SSR is activated (like the β adrenergic receptor (BAR) after binding epinephrine [Sibley and Lefkowitz, 1985]), a transient interaction is formed between the SSR and the SRPR which enhances the binding of GTP to the SRPR (like BAR activation enhancing the binding of G, and the exchange of GTP for GDP [Cassel and Selinger, 1978; Lad et al, 1980]). SRP is released when GTP binds (Connolly and Gilmore, 1989), perhaps due to a change of conformation (like the dissociation of the α and β-gamma subunits of G, [Northup et al, 1983a, b; Codina et al, 1984]). GTP bound SRPR stimulates an effector and several phosphorylation events result (like G* -α stimulates adenylate cyclase and the phosphorylation cascade following enhances production of cAMP [Gilman, 1984a, b; Stryer and Bourne, 1986]), at least one of which requires GTP as phosphate donor (vide supra; Hoffman and Gilmore, 1988; Hansen et al, 1986). Four major integral ER membrane
proteins are phosphorylated with GTP and three of these also with ATP, but with distinct characteristics of phosphorylation (vide supra). One of the membrane phosphoproteins is the SSR (vide supra) and another is part of a heteromeric protein complex which includes the SSR (vide supra) and is likely part of the "translocation apparatus" (Blobel and Dobberstein, 1975; Connolly and Gilmore, 1989). Binding of the SRPR to the SSR changes the affinity of the SSR for its cognate ligand, the signal sequence (as in the negative heterotropic effect of Gβ binding to the BAR and changing its affinity for epinephrine [Lad et al, 1980]). Vectorial transfer of the signal sequence domain takes place in a luminal direction, thus initiating the first step in the sequential transfer of nascent chain through the membrane (Wessels and Spiess, 1988; reviewed in Singer et al, 1987). Phosphorylation of the activated SSR decreases affinity between itself and the SRPR and the two dissociate (like phosphorylation of the BAR quenches its ability to stimulate Gα by changing its affinity of association [Stadel et al, 1983, 1986; Sibley et al, 1986]). The nascent chain has now formed a stable interaction with the translocation apparatus (Connolly and Gilmore, 1986) and is sequestered in an aqueous environment (Gilmore and Blobel, 1985) in a proteinaceous compartment beneath the ribosome and within the lipid bilayer (Connolly et al, 1989). The SRPR (like Gα) has stimulated its target effector(s), hydrolyzed GTP and is available in its GDP bound (inactive) state to participate in another targeting (signal transducing) event. Meanwhile a series of events has been initiated (like the many metabolic events initiated by cAMP dependent protein kinase activation) and is being propagated by the sequential phosphorylation of components of the translocation apparatus and of regulatory proteins such as phosphatases and other modulator proteins
[Cohen, 1989]. Phosphorylation at alternate sites on the apparatus, or alternate positions on individual proteins in the apparatus, might cause allostERIC changes (as described by Wesseling-Resnick et al, 1987) which effect the sequential vectorial transfer of the nascent chain domain by domain through the membrane (Singer et al, 1987). One or more kinase activities are implicated, which have low Km’s for GTP and ATP, are regulated by the binding but not hydrolysis of one of the adenosine nucleoside phosphates (like adenosine regulates the activity of adenylate cyclase [Cooper and Rodbell, 1979]) at the cytosolic face of the rER, and do not respond in either a stimulatory or inhibitory manner to guanine nucleoside phosphates, Ca²⁺ or heparin. (vide supra).
Contribution to the existing body of knowledge

The work described in this thesis has added several new pieces of information to the existing body of scientific knowledge.

With respect to the mechanism of action of GTP on rER membranes, we have shown that the GTP specific fusion event in SRM is mediated by cytosolically exposed protein constituent(s) of those membranes, and that the nucleotide and cation effects of GTP specific fusion can be uncoupled.

We have identified a limited number of integral membrane phosphorylated proteins which are restricted to rER membranes and which can be phosphorylated using ATP and/or GTP as phosphate donor.

We have purified two of those integral rER membrane phosphoproteins, one of which was subsequently identified as the signal sequence receptor of Weidman (1987, 1989). In purification of these two phosphoproteins, we have detected a transmembrane phosphoglycoprotein complex of approximate $M_r$ 400,000 in rER membranes which may be involved in the translocation of nascent chains across the ER bilayer.

To our knowledge, this is the first work describing differential phosphorylation of integral rER membrane proteins with GTP and ATP as phosphate donors; and the first work describing regulation and modulation of a GTP specific kinase activity by adenosine.
Future Work

Although it has provided us with several answers, this work has more importantly formed the basis for many more questions. Future work should follow up on several of those questions which were not answered in the course of this work, and should investigate further those many areas where we have just begun to understand.

We need to determine the relationship of fusion to phosphorylation and to translocation. Efforts thus far have not been able to show either relationship conclusively. In vitro reconstitution studies of all three in liposomes or artificial membranes may allow clearer examination of corresponding molecular events. If a relationship can be demonstrated, fusion may offer a good system with which to examine the hydrophobic interactions between proteins which mediate translocation. If not, identification of the molecules involved in fusion may yet point to the primary event stimulated by GTP and another physiological role in the rER (e.g., the release of Ca\textsuperscript{2+} from a GTP sensitive Ca\textsuperscript{2+} pool in the ER [Dawson et al, 1987; Lukacs et al, 1987; Ghosh et al, 1989]).

We need to identify, purify and reconstitute the kinase(s), phosphatase(s), and other regulatory proteins involved in GTP and ATP phosphorylation of the four major phosphoproteins of SRM membrane. This will be necessary for elucidation of the complex regulation which we have described.

It would be a boon also to the study of the mechanism of translocation if we could reconstitute in vitro the function of the SSR complex of proteins
using liposomes or artificial membranes, and it appears that at least some
of the kinase(s), phosphatase(s) and other proteins mentioned above may
be necessary.

The two integral rER membrane phosphoproteins, pp15 and pp56 which did
not copurify with pgp35 should also be purified and studied. Antisera
to pp15 and 56 should be prepared and they should also be sequenced and
cloned. There are many rER functions (vide supra) which are likely to be
regulated by phosphorylation.

The distinctive patterns of phosphorylation with ATP and GTP of several
of the subcellular fractions have not been described before and
examination of these proteins may, for example, help explain the mysteries
of targeting and transport between various organelles. They may also
serve as specific markers for certain organelles, especially those for
which none have yet been found (e.g. endosomes).

The determination and comparison of the exact sites of phosphorylation on
the four major membrane ATP and GTP phosphoproteins will address two
issues. Are there any truly GTP specific sites of phosphorylation and if
so, are they responsible for GTP specific fusion? Does this occur in vivo
and if so, what is the regulatory/modulatory role that dual
phosphorylation might play?

It is necessary to show the in vivo relevance of the phosphorylation of
pgp35 (and other phosphoproteins). Several methods might be used: the
introduction of antibodies to the phosphorylated proteins into living
cells or semipermeabilized cells; in vitro phosphorylation in the presence of signal sequence; in vitro translocation in the presence of radiolabelled ATP and GTP and examination of phosphorylated substrates; stimulation of secretion in cell culture or temperature sensitive secretion mutants and examination of differences in phosphorylation. Genetic approaches now used to study translocation (Ferro-Novick, 1985) also might be applied to examination of the importance of these membrane phosphoproteins.
This figure illustrates what have been ascertained thus far about the proteinaceous constituents of the rough endoplasmic reticulum which appear to be involved in translocation of nascent proteins across the rough ER membrane. The double headed arrow (←→) represents transient interaction between proteins or groups of proteins.

Four complexes of proteins have been characterized. The Signal Recognition Particle complex, the Signal Recognition Particle Receptor complex, the Signal Peptidase complex and the Signal Sequence Receptor complex. The numbers on each protein indicate relative molecular mass, and those proteins which are known to be phosphorylated are flagged with a P. S-P indicates that phosphorylation is known to occur on serine residues. CHO represents carbohydrate modification at the luminal face of the membrane. The 15 and 56 kD proteins are also shown, although no direct connection has yet been made with translocation.
REFERENCES


