Transport of Nucleotide Derivatives into Endoplasmic Reticulum and Golgiapparatus Derived Vesicles: a Dissertation

Caroline A. Clairmont
University of Massachusetts Medical School

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Transport of Nucleotide Derivatives into Endoplasmic Reticulum and Golgi Apparatus Derived Vesicles.

A Dissertation Presented
By
Caroline Ann Clairmont

Submitted to the Faculty of the University of Massachusetts Graduate School of Biomedical Sciences, Worcester in partial fulfillment of the requirements for the degree of:
Doctor of Philosophy in Biomedical Sciences
May 1993
Biochemistry and Molecular Biology
TRANSPORT OF NUCLEOTIDE DERIVATIVES INTO ENDOPLASMIC
RETICULUM AND GOLGI DERIVED VESICLES

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Caroline Ann Clairmont

Approved as to Style and Content by:

Reid Gilmore Ph.D., Chairman of Committee

Anthony Carruthers Ph.D., member of Committee

Robert Lahue Ph.D., member of Committee

Cheryl Scheid Ph.D., member of Committee

Ari Helenius Ph.D., member of Committee

Carlos B. Hirschberg Ph.D., Thesis Advisor

Thomas Miller Ph.D., Dean of Graduate School of Biomedical Sciences

Department of Biochemistry and Molecular Biology
May 1993
Dedicated
With Love
To My
Family
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Samuel and Catherine Peraro, my parents. I can never thank them enough for everything they have given me throughout my life. None of my accomplishments would have been possible without their love and support, and I thank them from the bottom of my heart.
In mammals, newly synthesized proteins destined for secretion are translocated cotranslationally into the lumen of the Endoplasmic Reticulum (ER). Once inside, these nascent polypeptide chains are bound by a lumenal ER protein called BiP (Immunoglobulin Binding Protein) or Grp 78 (Glucose Regulated Protein 78). It is hypothesized that this binding is necessary to protect the nascent chains until they are properly folded or assembled with other subunits. When the proteins are folded and assembled, they are released from BiP by a process that is dependent on ATP hydrolysis. Since ATP is synthesized mainly in the mitochondria, we hypothesized that there must be an ATP transporter in the ER which would allow the protein mediated transport of ATP from the cytosol into the ER lumen. We studied the transport of ATP \textit{in vitro} and found that ATP enters the lumen of the ER in a saturable manner with a $K_{\text{mapp}} \sim 3 \mu M$. ATP transport is dependent on time, protein, and vesicle integrity, it is also inhibited by the general anion transport inhibitor, 4,4'-diisothiocyno-2,2'-disulfonic acid stilbene (DIDS). We also found that the transport was inhibited by membrane impermeable protein modifying agents such as N-ethlymaleamide (NEM) and Pronase when added to intact ER vesicles. These results suggest that the transport is mediated by a protein with an active cytoplasmic face. Using monoclonal and polyclonal antibodies to BiP and Grp94 (another resident ER protein) and U.V. crosslinking, we demonstrated
that after transport of ATPβ32P into intact vesicles, radiolabeled BiP and Grp94 could be immunoprecipitated. We also found that labeling of luminal proteins with ATP is dependent on the transport of ATP. Finally using ATP labeled with 35S, we concluded that BiP was able to bind intact ATP and we confirmed earlier work that BiP was thiophosphorylated while Grp94 is not.

The second area of study involves processes that occur further along the secretory pathway in the Golgi apparatus. It was known from previous work that the nucleotide sugar substrates necessary for the synthesis of the linkage region, UDP-xylose (UDP-Xyl), UDP-galactose (UDP-Gal) and UDP-glucuronic acid (UDP-GlcA) were transported into the Golgi apparatus from the cytosol via protein mediated transporters. In order to eventually purify one of these transporter proteins, we wanted to reconstitute their activities. We were able to reconstitute the activities that exhibited kinetic parameters and inhibitor sensitivities very similar to those seen in intact Golgi vesicles. In the case of UDP-xylose it was necessary to prepare the liposomes using endogenous Golgi lipids in order to get transport activity similar to that seen in the intact Golgi vesicles. This suggested a specific lipid requirement for the UDP-xylose transporter. These transporters seem to be antiporters, whereby the nucleotide sugar enters the lumen of the Golgi coupled to the equimolar exit of the corresponding nucleoside monophosphate (Hirschberg, C.B. and Snider, M.D. 1987). We also showed that we could reproduce the hypothesized antiporter system in the reconstituted proteoliposomes by preloading the proteoliposomes with the putative antiporter molecule UMP.
The rationale for developing the reconstituted system is eventually to use this system to purify one of these nucleotide sugar translocators. In the last set of studies, I have shown that this reconstituted system can be used to monitor the purification of the UDP-galactose translocator. Using column chromatography we were able to purify this membrane translocator protein 45,000 fold from a rat liver homogenate.
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List of Abbreviations Used

Apo B: Apolipoprotein B
ATPγS: Adenosine 5'-O-(thio-triphosphate)
bFGF: Basic Fibroblast Growth Factor
BiP: Immunoglobulin Binding Protein
BSA: Bovine Serum Albumin
CAT: Carboxytratectyloside
CHO: Chinese Hamster Ovary
CMP-SA: Cytidine 5'-Monophosphate N-AcetylNeuraminic Acid
Con A: Concanavalin A
DIDS: 4,4'-diisothiocyanato-2,2'-disulfonic acid stilbene
DMP: Dimercaptopropanol
Dol-P: Dolichol Phosphate
DTT: dithiothreitol
GAG: Glycosaminoglycan
GRP: Glucose Regulated Protein
G6PDH: Glucose-6-Phosphate Dehydrogenase
Hepes: N-(2-Hydroxyethyl) piperazine-N'-[2-ethanesulfonic acid]
HMG: 3'-Hydroxy-3-methylglutaryl
HPLC: High Pressure Liquid Chromatography
MDCK: Madin-Darby Canine Kidney
N-CAM: Neural Cell Adhesion Molecule
NEM: N-ethylmaleimide
PM: Plasma Membrane
PAPS: Adenosine 3'-Phosphate, 5'-Phosphosulfate
RER: Rough Endoplasmic Reticulum

$S_i$: Solutes Inside

$S_m$: Solutes in the Medium

$S_o$: Solutes Outside

$S_t$: Total Solutes

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SRP: Signal Recognition Particle

TCR: T Cell Receptor

TGN: Trans Golgi Network

UDP-GlcA: Uridine 5'-Diphosphate Glucuronic Acid

UDP-Xyl: Uridine 5'-Diphosphate Xylose

UMP: Uridine Monophosphate

$V_i$: Volume Inside

$V_o$: Volume Outside

$V_t$: Total Volume

VLDL: Very Low Density Lipoprotein

WGA: Wheat Germ Aggluttin
CHAPTER I
BACKGROUND
A. The Secretory Pathway: A General Overview

The secretory pathway consists of a complex series of events involving multiple cellular organelles, through which newly synthesized nascent polypeptides travel and are sequentially post-translationally modified to yield a mature active protein. Much work has been done to study the intricate steps of this process. In this section, I will briefly summarize some of what is known about this pathway.

Nascent polypeptides are translated from mRNA on cytoplasmic ribosomes. Those polypeptides that are destined to enter the secretory pathway contain a signal sequence (Rapoport, T.A. and Wiedmann, M. 1985). This signal sequence is specific for entry of the polypeptide into the endoplasmic reticulum (ER) and those polypeptides that do not contain this particular signal sequence can have three fates: 1). these polypeptides can be synthesized completely on ribosomes in the cytoplasm where the proteins will remain (Sabatini, D.D. et al 1982), 2). they can be synthesized in the cytosol and contain a specific signal sequence for entry into the mitochondria (Schatz, G., and Mason, T.L. 1974), or the peroxisome (Gould, S.J., et al, 1987), or 3). the polypeptides can be posttranslationally inserted into the ER (Sabatini, D.D. et al 1982).

The signal sequence for entry into the ER is then recognized and bound by the signal recognition particle (SRP) in the cytoplasm (Walter, P., Ibrahimi, I. and Blobel, G. 1981; Anderson, D.M., Mostov,
K.E. and Blobel, G. 1983). SRP is a multisubunit complex containing 6 proteins and one molecule of 7S RNA (Walter, P. and Blobel, G. 1980; Walter, P., and Blobel, G. 1982). The binding of the signal sequence to SRP in the cytoplasm temporarily halts the translation of the message (Walter, P. and Blobel, G. 1981) and the SRP-nascent chain polypeptide-ribosome complex is then brought to the ER membrane. SRP binds to the SRP receptor, located in the ER membrane (Gilmore, R., Blobel, G. and Walter, P. 1982a; Gilmore, R., Blobel, G. and Walter, P. 1982b). In summary, SRP recognizes nascent protein chains that are destined for the secretory pathway, and specifically binds these polypeptides in the cytoplasm and brings the polypeptide-ribosome complex to the ER membrane for translocation into the ER lumen.

One of the proteins in the SRP complex, SRP54, is a GTP binding protein. The binding and hydrolysis of GTP releases the SRP from the signal sequence (Connolly, T. and Gilmore, R. 1989), and, by an unknown mechanism, transfers the ribosome-nascent chain complex to the translocation machinery. Translation of the polypeptide can now continue, and the growing chain is inserted into the lumen of the ER (Gilmore, R. and Blobel, G. 1983). As the signal sequence enters the ER, it is removed by signal peptidase in the lumen (Evans, E., Gilmore, R. and Blobel, G. 1986). As the polypeptide chain continues through the ER membrane into the lumen of the ER, it associates with a luminal protein, BiP (Immunoglobulin Binding Protein) (Haas, I.G., and Wabl, M. 1983). This interaction is thought to protect the nascent chain in order to prevent aggregation or incorrect folding of the
polypeptide. I will discuss the function of BiP in correct assembly of new proteins in greater detail in section B.

In mammals, many proteins that are destined to remain in the ER contain a C-terminal KDEL sequence (Munro, S. and Pelham, H.R.B. 1987). This sequence seems to retain or sort specifically those proteins that are to remain in the ER (Munro, S. and Pelham, H.R.B. 1987). All other proteins, those destined for the Golgi, plasma membrane (PM), lysosomes, or for secretion, are then packaged into small vesicles and transported initially to the Golgi apparatus.

The Golgi apparatus is a complex organelle containing cis, medial and trans stacks. It is flanked by a "pre" tubular membranous compartment where escaped ER resident proteins are captured and sorted back to the ER (Saraste, J. and Kuismanen, E., 1984; Warren, G., 1990; Duden, R. et al, 1991), as well as a "post" tubular membranous compartment called the trans-Golgi network (TGN) (Griffiths, G, and Simons, K., 1986). Proteins move through the Golgi apparatus via vesicle mediated transport (Rothman, J.E. and Orci, L. 1990). Intra Golgi vesicles are encapsulated by a non-clathrin protein coat, and these proteins may play a role in vesicle targeting to the correct Golgi stack (Pryer, N.K. et al, 1992). As proteins move through these stacks from cis to trans, they receive successive modifications, such as glycosylation, sulfation, and phosphorylation, in a stepwise, compartmentalized manner (Farquhar, M.G. 1985; Roth, J. et al 1986; Orci, L. et al 1987). Glycosylation, leads to an array of heterologous oligosaccharides.
These structures play biologically important roles and will be discussed in further detail in section C. The substrates for glycosylation reactions are nucleotide sugars; the sugars are then transferred to acceptors by specific glycosyltransferases (Kornfeld, R. and Kornfeld, S. 1985). Sulfation of proteins (Lee, R.W.H. and Huttner, W.B. 1985), proteoglycans (Young, R.W. 1973; Horwitz, A.L. and Dofman, A. 1986), and oligosaccharides (Fleischer, B. and Zambrano, F. 1974; Green, E.D. et al 1984) utilizes 3’-phosphate 5’-phosphosulfate (PAPS) as the substrate (Robbins, P. and Lipmann, F. 1957) and the sulfate group is transferred to the acceptor by a variety of sulfotransferases. Lumenal phosphorylation of proteins (Bingham, E.W. and Farrell, H.M.Jr. 1974; Bingham, E.W., Farrell, H.M.Jr. and Basch, J.J 1972; Gottlieb, T.A. and Wallace, R.A. 1981) and proteoglycans (Wang, S.-Y. and Williams, D.L. 1982; Oegema, T.R.Jr. et al 1984) occurs via kinases which use ATP as the substrate.

Proteins destined for the plasma membrane, or for secretion, are packaged into non-clathrin coated vesicles from the Golgi apparatus and are transported constitutively to the PM. Proteins destined for the lysosome are found in a compartment called the trans golgi network (TGN). In this compartment lysosomal enzymes are sorted to the lysosome, hormones and neurotransmitters are packaged into secretory vesicles, and receptor-ligand complexes taken in at the plasma membrane are brought to endosomes (Gruenberg, J. and Howell, K.E., 1989; Hubbard, A.L., 1990; Rodman, J.S. et al, 1990).
Proteins travel from one organelle to another via vesicle mediated transport. This process is quite complex and an area of intense study. More is becoming known about this transport, especially through insights gained through yeast genetics. There is increasing evidence concerning how transport vesicles form and bud from the donor organelle, and then travel and fuse with the acceptor. Many yeast genes have been isolated whose gene products are involved in vesicle-mediated sorting and transport (Pryer, N.K. et al 1992). The first yeast genes isolated were the sec mutants from Saccharomyces cerevisiae (Novick, P. et al 1980). When temperature sensitive mutants were grown at the non-permissive temperature a large accumulation of precursor proteins and organelles was seen, demonstrating the consequence of a blockage in the secretory pathway. Another large group of genes identified were the vps genes (vacuolar protein sorting) defective in sorting and transport to the vacuole (Bankaitis, V.A. et al 1986; Rothman, J.H. and Stevens, T.H. 1986; Robinson, J.S. et al 1988; and Rothman, J.H. et al 1989). Many others were subsequently isolated; and with this large collection of different mutants, analysis of double mutants led to the dissection of an order of events along the transport pathway. Biochemical techniques were also used to study this pathway, with the development of a cell free system to study the transport phenomenon (Baker, D. et al 1988; Ruohola, H. et al 1988; and Hicke, L and Schekman, R. 1990). This method showed that transport between organelles was dependent on ATP and also allowed the
purification of the sec 23 gene product (Hicke, L and Schekman, R. 1989).

An insight into how these vesicles could specifically target donor to acceptor compartments came with the discovery of small GTP binding proteins that were involved along the secretory pathway (Pryer, N.K. et al 1992). These GTP binding proteins were found to interact with some of the other known gene products (i.e. the sec genes) along the transport pathway. They are thought to act in one of two ways: first, by amplification or transduction of a signal that is required for vesicular transport, or second, by conferring directionality to the transport vesicle (i.e. mediating vectorial transport).

Protein folding, non-lysosomal degradation and initiation of N-glycosylation take place in the lumenal space of the ER, while terminal N- and O-glycosylation, sulfation and phosphorylation are localized to the lumenal space of the Golgi apparatus. How do the substrates for these reactions, the nucleotide sugars, ATP, and PAPS, get from their sites of synthesis in the cytosol into the lumen of the ER and Golgi? This question will be discussed further in this text, as a major goal of this thesis. In the remainder of Chapter I, I will address steps of the secretory pathway in particular the series of events involved in the correct synthesis of new proteins. I will begin by concentrating on the initial events of protein synthesis that occur in the ER, and then the diverse carbohydrate modifications which occur in the Golgi apparatus.
B. The Endoplasmic Reticulum: The Site of Protein Subunit Assembly, Degradation of Abberant Proteins, and Initiation of N-Glycosylation

Once a protein is translocated into the ER the signal sequence is removed by signal peptidase and, in many cases, the oligosaccharide Glu$_3$Man$_9$GlcNac$_2$ is transferred to the protein by oligosaccharyltransferase. After these initial reactions, there are a series of other reactions that can occur before the proteins are mature and either remain in the ER or are ready to move on to the Golgi apparatus. The main reactions which occur in the lumen of the ER are: 1. correct folding and subunit assembly of the protein, 2. degradation of abberant proteins, and 3. initiation of N-glycosylation of proteins.

Correct folding and subunit assembly of newly synthesized proteins. How do different proteins with different charges pass through a lipid bilayer? The signal sequence targets the proteins to the membrane, as described in Section A, but then each protein must be translocated across the membrane to be modified and ultimately reach its final destination.

Although the components and the exact mechanism of this translocation process are unknown, current evidence suggests that there is a membrane pore which allows translocation of the newly synthesized polypeptide. Electrophysiological techniques have
demonstrated the existence of a protein conducting channel which opens in response to ribosome binding and GTP and closes when ribosomes are released (Simon, S.M., et al 1989; Simon, S.M., and Blobel, G. 1991). In more recent work, it was shown that the protein conducting channel opens in the presence of signal peptides, suggesting that signal peptides are the ligands to open the channel and initiate protein translocation (Simon, S.M., and Blobel, G. 1992).

The polypeptide is translocated co-translationally into the lumen, which requires that the proteins enter unfolded. The ability to separate experimentally the synthesis from the translocation process has added insight into this mechanism (Connolly, T. Gilmore, R. 1986; Hansen, W., Garcia, P.D. and Walter, P. 1986; Perara, E., Rothman, R.E. and Lingappa, V.R. 1986; Waters, M.G. and Blobel, G. 1986). These types of experiments showed that insertion of nascent chains into the membrane had a GTP requirement that was unrelated to protein synthesis (Connolly, T and Gilmore, R. 1986; Wilson, C. et al 1988). It was found that although SRP-ribosome complexes could be targeted to the ER in the absence of GTP, the nascent chain remained accessible to external proteases (i.e. was not translocated) and could be easily extracted by salt washes. This was found to be true for soluble (Connolly, T. and Gilmore, R. 1986) as well as membrane bound proteins (Wilson, C. et al 1988). Translocation was also dependent on ATP hydrolysis which was clearly distinct from the GTP dependent step mentioned above (Hansen, W., Garcia, P.D. and Walter, P. 1986; Waters, M.G. and Blobel, G. 1986; Schlenstedt, G. and
Zimmermann, R. 1987). Translocation of proteins was blocked in the absence of ATP, or when ATP hydrolysis was blocked by using nonhydrolyzable analogs (AMP-PNP).

In yeast, the ATP hydrolysis step seems to be mediated in part by the 72kd heat shock family of proteins (Deshaiies, R.J. et al 1988; Chirico, W.J., Waters, M.G. and Blobel, G. 1988). These cytosolic proteins were found to stimulate translocation. A possible function of these two cytosolic proteins, called Ssa1p and Ssa2p, is to act as protein unfolding enzymes to ensure that nascent polypeptides are unfolded into a translocation competent conformation (Chirico, W.J., Waters, M.G. and Blobel, G. 1988). Another possibility is that Ssa1p/Ssa2p could act to protect or expose hydrophobic sites on the nascent chain depending on whether ATP was bound or not (Chirico, W.J., Waters, M.G. and Blobel, G. 1988). Whatever their exact function, these proteins play an important role in maintaining translocation competence for the newly synthesized polypeptides.

Translocation competence requires the proteins to be in an "unfolded" state; however active proteins have very high degrees of secondary, tertiary, as well as quaternary structure in the case of multisubunit proteins, which must be maintained following the completion of translation into the ER. Therefore, in the lumen of the ER the proteins must be "re-folded" and subunits, if any, must be assembled correctly: How does this occur?

There is a major lumenal protein in the ER called BiP (immunoglobulin binding protein), containing a KDEL sequence which
causes it to be retained in the ER. BiP was so named because it was originally found in very tight association with immunoglobulin heavy chains (Haas, I.G. and Wabl, M. 1983). They found that some of the heavy immunoglobulin chains were non-covalently bound to a 78K protein, distinct from the light chains, which was not associated with completely assembled immunoglobulin chains. It thus appeared that this protein was only associated with the immunoglobulin heavy chain in its "unassembled" state. BiP has now been identified as being identical to glucose regulated protein 78 (GRP 78) (Munro, S. and Pelham, H.R.B. 1986; Hendershot, L.M., Ting, J. and Lee, A.S. 1988). This is an hsp70 related protein which is induced in the presence of stress. Stresses such as glucose starvation, inhibition of glycosylation (Pouyssegur, J., Shiu, R.P.C. and Pastan, I. 1977; Shiu, R.P.C., Pouyssegur, J., and Pastan, I. 1977), as well as viral infection (Stone, K.R., Smith, R.E. and Joklik, W.K. 1974; Isaka, T. et al 1975; Shiu, R.P.C., Pouyssegur, J., and Pastan, I. 1977; Peluso, R.W., Lamb, R.A. and Choppin, P.W. 1978), all lead to an increase in the amount of aberrant proteins in the ER, and an increase in the synthesis of BiP. One can conclude from this fact that one role of BiP may be to associate with aberrant proteins, especially in times of stress, in such a way as to keep them from accumulating, or to keep them from continuing through the secretory pathway in an incomplete state. If BiP is able to bind to unfolded or incorrectly assembled proteins under these conditions, one can infer that BiP's role in a normal cell may be to bind to the newly synthesized proteins which are unfolded
or unassembled and help to correctly process them in the ER. BiP could also act to aid the folding process by increasing the rate of folding. BiP was found to bind to short synthetic peptides (Flynn, G.C., Chappell, T.G. and Rothman, J.E. 1989), these results suggest that BiP is able to bind short peptides, analogous to newly synthesized peptides entering the ER lumen.

Originally, only immunoglobulin heavy chains were found to be associated with BiP (Haas, I.G. and Wabl, M. 1983; Bole, D.G., Hendershot, L.M. and Kearney, J.F. 1986; Hendershot, L. et al 1987), but if it is to be a general "folding" enzyme it must bind to many different proteins in the ER. Since its initial discovery, BiP has in fact been found to bind other incomplete or mutant proteins, such as: the nicotinic acetylcholine receptor (Blount, P. and Merlie, J.P. 1991, and hemagglutinin (Gething, M.-J., McCammon, K. and Sambrook, J. 1986). These aberrant proteins remain associated with BiP until they are completely assembled. If they do not become assembled, they remain bound to BiP which retains the proteins in the ER, where they are eventually degraded. How or if BiP is involved in the degradation of these unassembled proteins is not known. BiP was also found associated with proteins containing incorrect disulfide bonds, such as prolactin (Kassenbrock, C.K. et al 1988), or vesicular stomatitis virus G protein (Machamer, C.E.R.W. et al 1990; deSilva, A.M., Balch, W.E. and Helenius, A. 1990), but BiP was not found associated with prolactin containing disulfide bonds at the correct positions (Kassenbrock, C.K. et al 1988).
Unglycosylated or aberrantly glycosylated proteins that have also been found associated with BiP include, invertase (Kassenbrock, C.K. et al 1988), hemagglutinin (Gething, M.-J., McCammon, K. and Sambrook, J. 1986), Factor VIII (Dorner, A.J., Bole, D.G. and Kaufman, R.J. 1987), von Willebrand factor (Dorner, A.J., Bole, D.G. and Kaufman, R.J. 1987), and tissue plasminogen activator (Dorner, A.J., Bole, D.G. and Kaufman, R.J. 1987). In these cases, when the proteins were fully glycosylated their association with BiP was transient and secretion was normal. However when glycosylation was blocked by tunicamycin or when mutagenesis was performed to remove N-linked glycosylation sites on the proteins, then the association with BiP was stable and secretion was decreased dramatically (Dorner, A.J., Bole, D.G. and Kaufman, R.J. 1987). These data suggest strongly that BiP is able to bind to incomplete or unfolded proteins and ensure that these proteins do not leave the ER until they are complete.

The Kar2 gene from yeast was found to be the homolog to BiP (Rose, M.D., Misra, L.M. and Vogel, J.P., 1989). By looking at the phenotype after disruption of this gene Rose, et al found that Kar2 (i.e. BiP) was an essential gene. Mutations in this gene caused an accumulation of secretory preproteins. This clearly shows how important BiP function is along the secretory pathway.

One possible way for BiP to bind to these aberrant proteins is by binding to exposed hydrophobic sequences that are not available in the correctly folded or assembled proteins. Exactly how BiP
functions to bind or to release proteins is not known. However, it is known that BiP does interact with ATP (Munro, S. and Pelham, H.R.B., 1986; Hendershot, L.M., Ting, J. and Lee, A.S., 1988; Kassenbrock, C.K. and Kelly, R.B., 1989) and that ATP is required for the release of proteins that are bound to BiP. When ATP is removed from the cell, proteins remain bound to BiP and are not secreted (Dorner, A.J., Wasley, L.C. and Kaufman, R.J. 1990). It has also been shown that energy (ATP) is required for the correct folding of hemagglutinin (Braakman, I. et al, 1992). In the absence of ATP Braakman et al could detect misfolding of hemagglutinin which could be rescued by the addition of ATP. BiP has a high affinity for ATP as well as an ATPase activity with a low turnover rate suggesting a regulatory role (Kassenbrock, C.K., and Kelly, R.B. 1989). It has been suggested that ATP hydrolysis provides the necessary energy needed to disrupt the BiP-protein complex such that secretion can continue. This is quite similar to the postulated mechanism of action for the cytosolic heat shock proteins Ssa1p/Ssa2p that are involved in protein translocation, as discussed above (Chirico, W.J., Waters, M.G., and Blobel, G., 1988). These, too, are thought to interact with exposed hydrophobic domains and require ATP hydrolysis as an important part of their function. These cytosolic proteins, however, are thought to be involved in the unfolding of nascent polypeptides while BiP is thought to be involved in the folding and assembly of proteins in the ER. It has been proposed that these proteins may act as catalysts in protein translocation and assembly (Flynn, G.C., Chappell, T.G. and
Rothman, J.E., 1989). In a recent study, it was shown that both Ssa1p and BiP were necessary for translocation across the ER membrane in distinct noninterchangeable roles (Brodsky, J.L., *et al.*, 1993).

Another lumenal ER protein of interest in this process is GRP94 (glucose regulated protein 94). GRP94 is a major component of the ER lumen whose synthesis is increased during glucose starvation and other stresses upon the cell (Stone, K.R., Smith, R.E. and Joklik, W.K., 1974; Isaka, T. *et al.*, 1975; Shiu, R.P.C., Pouyssegur, J. and Pastan, I., 1977; Pouyssegur, J., Shiu, R.P.C. and Pastan, I., 1977). Conditions which increase the production of aberrant proteins also stimulate the production of GRP94 in an analogous manner to BiP (Wu, F.S. *et al.*, 1981; Pelham, H.R.B., 1986). A function for GRP94 is not known however, recently, it was found that both GRP78 (BiP) and GRP94 share common regulatory domains and seem to be acted on and regulated by common trans-acting factors (Chang, S.C., Erwin, A.E. and Lee, A.S., 1989). Additionally, chemical crosslinking studies have demonstrated the presence of a ternary complex consisting of unassembled immunoglobulin chains, BiP, and GRP94 (Melnick, J., *et al.*, 1992). This data offers strong evidence that not only does GRP94 act in a manner similar to BiP in binding unassembled immunoglobulin chains, but that GRP94 and BiP may be working together during the folding and assembly of proteins in the ER lumen.

As mentioned earlier, a key component to BiP's function is the hydrolysis of ATP. Without ATP, BiP can not function properly and
remains bound to the proteins and prohibits their secretion from the ER to the Golgi. One major question, then, is how does ATP, which is synthesized primarily in the mitochondria, get from its site of synthesis into the lumen of the ER? This question will be discussed in detail later in the text, as one of the main questions to be addressed in this thesis.

Non-lysosomal degradation route for the removal of aberrant proteins from the secretory pathway. If the multimeric proteins that are synthesized in the lumen of the ER have different rates of synthesis for each of their different subunits, it is possible that individual unassembled subunits could accumulate in the ER. Having a vast accumulation of these unassembled subunits could interfere with the transport of the completed proteins in the secretory pathway. In fact, this phenomenon of varied rates of subunit synthesis does occur, but the cell has found a way to deal with these extra subunits in the ER, through a non-lysosomal, pre-Golgi degradation pathway (Klausner, R.D. and Sitia, R., 1990). The system which has been most highly characterized for this pathway is the synthesis and degradation of the T Cell Receptor (TCR) (Minami, Y. et al, 1987; Lippincott-Schwartz, J. et al, 1988; Bonifacino, J.S. et al, 1989). This cell surface receptor is one of the most highly complex multimeric proteins, consisting of 6 different subunits (α,β,γ,δ,ε,ζ) with a composition of αβγδεζ. Only fully completed heptameric chains are targeted to the cell surface (Sussman, J.J. et al, 1988).
Initially, by developing antibodies to the individual protein subunits, it was found that the five chains, (α, β, γ, δ, and ε) are synthesized in excess (70-90%) over the ζ chain (Minami, J.S. et al, 1987). These five chains formed a pentamer which was rapidly degraded after being transported from the ER, most likely in the lysosome (Minami, Y. et al, 1987). This suggested a very efficient selection system for removing unassembled proteins from the secretory pathway. To investigate the intracellular route used for protein degradation, the individual subunits of the TCR were studied. In one study it was found that if αβ dimers were expressed, that these were rapidly degraded in a pre-Golgi compartment (Lippincott-Schwartz, J. et al, 1989), and this degradation was not inhibited by drugs that block lysosomal proteolysis (Lippincott-Schwartz, J. 1989), while pentamers of αβγδε were transported to the Golgi and degraded in the lysosome. Using ionophores which block ER to Golgi transport, they found that individual α, β, and γ chains were still rapidly degraded: therefore their degradation must be occurring through a pre-lysosomal degradation pathway, most likely in the ER.

How does the ER know which proteins to degrade? As a first approach to answer this question investigators, using the TCR model system, measured the rates of degradation as well as the effect of subunit assembly on degradation rates (Bonifacino, J.S. et al, 1989). These studies utilized several different cell lines: one of which was deficient in the synthesis of δ chains and the others which expressed the individual subunits. In the δ deficient cell line, the other chains
were synthesized but not transported to the Golgi. The fate of these individual chains showed that while the α chains were degraded rapidly, the γ, ζ, and ε chains were more resistant to degradation. This suggested that degradation was dependent on some intrinsic characteristic of the individual subunits. When the subunits were expressed individually, they again found that they were degraded at very different rates, but all in a non-lysosomal compartment. By expressing the chains in combinations, they found that interactions between the chains could stabilize the more sensitive chains, suggesting that assembly into oligomeric structures affects the rates of degradation.

The TCR proteins are not the only proteins found to be degraded by this pathway. Other cell surface proteins that have been studied are the nicotinic acetylcholine receptor (Merlie, J.P. et al, 1982) and the asialoglycoprotein receptor (Amara, J.F., Lederkremer, G. and Lodish, H.F., 1989). In the latter study, a proteolytic intermediate was found consistent with cleavage of the protein at the boundary of the lumenal and transmembrane domains. This suggests strongly that the cleavage event does take place within the ER lumen. A more recent study of the asialoglycoprotein receptor showed that depleting the cells of ATP does not inhibit the initial cleavage to form the intermediate, but it completely inhibits further degradation of this product in the ER (Wikstrom, L. and Lodish, H.F., 1991). Soluble lumenal ER proteins can also be degraded by this pathway, some examples of these are α-globulin (Stoller, T.J.)
This ER degradation pathway also seems to have a regulatory role. Two examples of proteins whose degradation seems to be regulated in the ER is HMG-CoA reductase (Gil, G. et al., 1985), and Apolipoprotein B (Davis, R.A. et al., 1990). HMG-CoA reductase is the rate limiting enzyme for cholesterol biosynthesis. When the cholesterol levels are high the protein is rapidly degraded in the ER, but when the levels of cholesterol are low the protein is much more stable. Apolipoprotein B (ApoB) (Davis, R.A. et al 1990), is a component of lipid metabolism essential for the assembly of very low density lipoproteins (VLDL). This assembly occurs in the ER, and degradation of ApoB in the ER is thought to control the level of VLDL particles secreted, because it has been shown that under different circumstances the amounts of VLDL secreted varies, while the mRNA levels remain constant (Davidson, N.O., et al, 1988; Pullinger, C.R., et al, 1989).

The question of selectivity and regulation of ER degradation has generated more questions than answers, and is a field of great interest at present. Work has shown that there seems to be an ER degradation signal, and that this signal is used to retain proteins destined for degradation in the ER (Klausner, R.D. and Sitia, R., 1990; Wikstrom, L. and Lodish, H.F., 1991). As mentioned above, work by Bonifacino, J.S. et al, (1989) showed that subunit assembly affected the rates of degradation. One possibility, is that assembly of the
subunits masks or protects this degradation signal making the protein no longer recognized for cleavage.

The exact location of the non-lysosomal degradation is not clear, however it is most likely within or very near the ER. This observation was made based on the failure of proteins degraded by this pathway to reach the Golgi (i.e. no Golgi-associated carbohydrates and inability to detect transport out of the ER with subcellular fractionation, fluorescence or electron microscopy (Lippincott-Schwartz, J., 1989)). Two possibilities exist (Klausner, R.D. and Sitia, R, 1990): 1: that the proteins are sorted into a sub-compartments for degradation, or 2: that the proteins containing the degradation signal would be bound to proteolytic complexes found in the ER itself.

ATP is thought to be necessary for the ER degradation pathway, since ATP depletion from cells abolishes the degradation of proteins in the ER (Wikstrom, L. and Lodish, H.F., 1991). Since very little is known about the mechanism of this degradation it is not clear whether ATP is utilized on the inside or the outside of the ER membrane. However, we can speculate based on the location of ATP requiring enzymes. First, ATP depletion inhibits the degradation of the intermediate of the asialoglycoprotein receptor which takes place in a pre-Golgi membrane compartment (Wikstrom, L. and Lodish, H.F., 1991). BiP, which binds to proteins in the ER that are unfolded or unassembled, also resides in the lumen of the ER, and requires ATP for its function. How, or if, BiP is involved in the ER degradation
pathway is not known, but it lends additional support for the idea that ATP is utilized on the inside of the ER membrane. It has recently been shown in yeast that a synthetic peptide is rapidly removed from the ER in a pathway separate from the normal transport system, and that its removal is dependent on ATP (Schekman, R. and Romisch, K., 1992). This then brings us once again to the question of how ATP, which is synthesized primarily in the mitochondria, is found in the lumen of the ER where it is utilized? A likely answer is that there may be an ATP transporter in the ER membrane responsible for the transport of ATP from the cytosol into the lumen of the ER. This is one major topic of this thesis and will be discussed in more detail in Chapter II.

**Biosynthesis of the Glu₂Man₉GlcNac₂ oligosaccharide and initiation of N-glycosylation.** The first major posttranslational modification that occurs on many newly synthesized proteins of the secretory pathway in the ER lumen is the transfer of the oligosaccharide -GlcNac₂Man₉Glu₃ from Dol-P-P-GlcNac₂Man₉Glu₃ by the enzyme oligosaccharyltransferase (Kornfeld, R. and Kornfeld, S. 1985). It has been demonstrated that this oligosaccharide is added to proteins in the lumen of the ER, since the lipid-linked Man₆.₉GlcNac₂ and Glc₁.₃ Man₉GlcNac₂ were found facing the lumen (Snider, M.D., and Robbins, P.W. 1982; Snider, M.D., and Rogers, O.C. 1984). However, not all of the substrates for the oligosaccharide biosynthesis are transported into the ER lumen
(Hirschberg, C.B. and Snider, M.D., 1987). The biosynthesis of this oligosaccharide has a very complex topography; however, much direct evidence has been generated that describes in detail the topography of the biosynthesis of N-linked glycosylation reactions in the ER. Below I will summarize what is known about this topographical question.

The oligosaccharide is assembled bound to the ER membrane via a dolichol (Dol) lipid. The first product synthesized is Dol-P-P-GlcNac$_2$ (N-acetylg glucosamine) which is made by the sequential addition of GlcNac-1-P then GlcNac to Dol-P utilizing UDP-GlcNac as the donor substrate in these reactions (Abeijon, C. and Hirschberg, C.B. 1992). Several pieces of data support the concept that this reaction takes place on the cytoplasmic, and not the lumenal side, of the ER. (Snider, M.D. et al 1980; Hanover, J.A., and Lennarz, W.J. 1982; Adair, W.L. and Cafmeyer, N 1983; Abeijon, C., and Hirschberg, C.B. 1990; Kean, E.L. 1991).

The next intermediate product in the synthesis of this oligosaccharide is formed by the sequential addition of 5 mannose (Man) units to yield Dol-P-P-GlcNac$_2$Man$_{1-5}$. This reaction must also occur on the cytosolic side of the ER since it is known that GDP-Man, which is the donor for this reaction, is not translocated into the lumen of the ER (Hanover, J.A. and Lennarz, W.J. 1982; Perez, M and Hirschberg, C.B. 1986; Hirschberg, C.B. and Snider, M.D. 1987).

Since the late events of the biosynthesis of the oligosaccharide are thought to occur in the lumen (Snider, M.D. and Robbins,
P.W., 1982), this large lipid linked oligosaccharide must be translocated, or "flipped", from the cytoplasmic side of the ER to the lumenal side.

After translocation, the next product synthesized is Dol-P-P-GlcNac₂Man₉. This product is synthesized and found on the lumenal side of the ER. This was shown using the membrane impermeable lectin ConA (Snider, M.D. and Rogers, O.C., 1984). It is also known that the donor for the addition of these mannose residues is not GDP-Man, which cannot be translocated into the ER, but instead the donor is dol-P-Man.

The final stage in the biosynthesis of this dolichol oligosaccharide is glucosylation to yield the product Dol-P-P-GlcNac₂Man₉Glu₃ which can then be added to nascent polypeptide chains in the ER. The glucose residues are added to the oligosaccharide by the donor dol-P-glucose. Although it is not clear where this molecule is synthesized (cytosol or lumen), protease experiments suggest that the synthesis of dol-P-glucose is cytosolic (Snider, M.D. et al., 1980; Hannover, J.A. and Lennarz, W.J., 1982), and therefore must involve a translocation step.

This final product then donates its oligosaccharide portion to nascent polypeptide chains in the ER. This is known to occur in the lumen, based on ConA insensitivity of sealed ER vesicles (Snider, M.D. and Robbins, P.W., 1982; Snider, M.D. Rogers, O.C. 1984). Also, in vitro translation experiments showed that a protein must be translocated across the ER membrane before glycosylation can occur.
(Hirschberg, C.B., and Snider, M.D., 1987). Finally, the enzyme responsible for the transfer of the lipid-linked oligosaccharide to the protein, oligosaccharyltransferase, is a luminal protein (Kelleher, D.J. et al, 1992).

C. The Golgi Apparatus: Diverse Carbohydrate Structure of Glycoproteins and Proteoglycans

Following initial glycosylation, correct folding, and subunit assembly in the ER, proteins arrive at the Golgi apparatus where they are further modified. The complexity in the carbohydrate moiety that can be achieved by the addition of different sugars and the branching of sugar chains is enormous. Complexity is further increased, if we consider that proteins are also phosphorylated and sulfated in the Golgi. In this section I will discuss some of the possible biological roles of the carbohydrate diversity of these proteins, as well as what is known about the topography of the glycosylation and sulfation events that occur in the Golgi.

Roles of carbohydrate diversity of glycoproteins and proteoglycans. Glycosylation of proteins and lipids play many different roles in biology. One of the first known was protein stabilization (Paulson, J.C., 1989). Addition of the N-linked oligosaccharide Glu3Man9GlcNac2- to newly synthesized polypeptides in the ER contributes to correct protein folding (Dube, S. et al, 1988;
Glycosylation is an essential function as shown by the inviability of yeast mutants lacking Dol-P-Man synthase, the enzyme that catalyzes the formation of Dol-P-Man from Dol-P and GDP-Man (Orlean, P. et al., 1988). Glycosylation of proteins has also been shown to be necessary in the prevention of glycoprotein clearance from circulation (Ashwell, G. and Morrell, A., 1974; Ashwell, G. and Hartford, J., 1982) and the prevention of rapid degradation of the LDL receptor when it reaches the cell surface (Kingsley, D.M. et al., 1986). The oligosaccharide moieties of glycoproteins are associated with many biological functions. They can serve as antigenic determinants (Feizi T., 1982; Leffler, H., 1988). N-linked glycosylation of pituitary hormones may act to regulate hormone activity (Berman, M.I. et al., 1985; Sairam, M.R. and Bhargavi, G.N., 1985) and may play a major role in agglutination of erythrocytes and cell adhesion in the glycoproteins laminin, thrombospondin, and von Willebrand's factor (Ginsberg, V. and Roberts, D.D., 1988). Sulfation of N-linked oligosaccharides of certain glycoprotein hormones, in particular, bovine lutropin, modulates its circulatory half-life which in turn regulates its bioactivity (Baenziger, J.U., et al., 1992). Glycoconjugates of viruses such as the Herpes simplex virus (Kuhn, J.E. et al., 1988) are essential to their ability to infect a host. Other examples are the Sendai virus (Markwell, M.A. et al., 1985), and HIV (McDougal, J.S. et al., 1986; Putney, S.D. et al., 1986; Matthews, T.J. et al., 1987, ).
A major class of cell surface and extracellular matrix glycoproteins are proteoglycans. Proteoglycans consist of a protein core and glycosaminoglycan side chains (Fransson, L.-A., 1987). These protein-bound glycosaminoglycans exist in three main forms, all of which contain sulfate: heparin and heparan-sulfate, chondroitin and dermatan sulfate, and keratan sulfate (Ruoslahti, E. and Yamaguchi, Y., 1991). The glycosaminoglycan chains are attached to the protein core through an O-linkage via a tetrasaccharide "linkage region" consisting of Xyl-Gal-Gal-GlcA (Gallagher, J.T., and Steward, W.P., 1986).

An important question is how proteoglycans regulate their diverse biological functions? One possibility could lie in the structure of the glycosaminoglycan side chains. These chains, which are a repeating polymer of -(HexNac-Hexuronic Acid)- (GlcNac/GalNac-GlcA/IduA), are quite extensive on the proteoglycans and could play a role in the function of the protein core. This does in fact seem to be the case: many of the biological roles attributed to proteoglycans are affected by the glycosaminoglycan chains. Glycosaminoglycan chains affect cell adhesion in CHO cell mutants which could not synthesis the glycosaminoglycan chain of heparin/heparan sulfate could not attach to type V collagen whereas wild type CHO cells with no defect in glycosaminoglycan biosynthesis were compentant for attachment (LeBaron, R.G. et al, 1989). Using the same mutant CHO cells, it was shown that the glycosaminoglycan chains were responsible for the binding of heparan sulfate to basic fibroblast growth factor (bFGF)
(Yayon, A. et al, 1991). This function is important for the stability of the bFGF receptor (Gospodarwicz, D. and Chen, J., 1986; Saksela, O et al, 1988), and in modulating the bFGF activity (Thorton, S.C. et al, 1983). Many growth factors other than bFGF interact with proteoglycans, including granulocyte-macrophage colony stimulating factor and interleukin 3 (Roberts, R. et al, 1988). Chondroitin sulfate proteoglycans can also interact and modulate growth factors, such as platelet factor 4 (Rouslahti, E. and Yamaguchi, Y., 1991).

Glycosaminoglycan chains of proteoglycans are involved in receptor mediated endocytosis of thrombospondin (Murphy-Ullrich, J.E. et al, 1988). Glycosaminoglycans were shown to play a role in development by regulating nephron formation in fetal kidneys depending on the sugar composition and O-sulfation of the chain (Platt, J.L. et al, 1990).

It has recently been shown that the basis for types 1 and 2 macular corneal dystrophy, a progressive disease that leads to "cloudy corneas", visual impairment, and requires corneal transplantation to regain vision, is a defect in GAG biosynthesis (Midura, R.J. et al, 1990). Defects or alterations in GAG synthesis are also thought to be responsible for or to play a role in, aortic dissection (Gutierrez, P.S. et al, 1991). Aortic dissection is a disease of the arterial wall resulting in bleeding into the smooth muscle of this vessel, where a decrease in sulphated GAG was seen in these patients. In brachyolmia, a skeletal dysplasia resulting in dwarfism
(Sewell, A.C. et al, 1991) an increased amount of under sulfated GAG was found as compared to normal patients without this disease.

**Topography of glycosylation and sulfation reactions in the Golgi apparatus.** The addition of the sugar chains to glycoproteins and proteoglycans occurs in the Golgi lumen. The substrates for the glycosylation reactions that take place in the Golgi apparatus are nucleotide sugars. These substrates are all synthesized in the cytosol, with the exception of CMP-sialic acid, which is synthesized in the nucleus (Coates, S.W. et al, 1980). The addition of these sugars to proteins, however, takes place in the Golgi lumen. How then do these nucleotide sugars get inside the Golgi? Much previous work has shown that these nucleotide sugars and PAPS, the donor for sulfation reactions, are transported in a saturable manner to the Golgi lumen via protein-mediated transporters. In proteoglycan glycosaminoglycan synthesis, it has been shown that the formation of the linkage region occurs in the Golgi lumen and that the nucleotide sugar donors (UDP-Xyl, UDP-Gal, and UDP-GlcA) are transported into intact Golgi vesicles (Kimura, J.H. et al, 1984; Nuwayhid, N. et al, 1986; Lohmander, L.S. et al, 1986). The other nucleotide sugars which make up the composition of the GAG (UDP-GlcNac, UDP-GalNac, and UDP-GlcA) have also been shown to be transported into the Golgi (Nuwayhid, N. et al, 1986; Abeijon, C. and Hirschberg, C.B., 1987; Hirschberg, C.B. and Snider, M.D., 1987) as has PAPS (Schwarz, J.K., et al., 1984). These transporters in the Golgi
apparatus are thought to work via an antiporter mechanism, a coupled exchange in which the nucleotide sugar enters coupled with the exit of the corresponding nucleotide monophosphate (Hirschberg, C.B. and Snider, M.D., 1987).

Do these transporters mediate the translocation of individual nucleotide derivatives in a specific manner thereby making each one important for availability of the substrate for glycosylation, or are they simply "pores" in the membrane which will allow any nucleotide sugar to pass through? An insight into the specificity of the transporter proteins came with the discovery of mutant cell lines. These mutants were isolated independently for resistance to wheat germ agglutinin (Stanley, P., et al, 1975; Briles, E.B., et al, 1977). Two CHO (Chinese Hamster Ovary) cell lines called Lec 2 and Lec 8 are specifically deficient in transport of CMP-sialic acid and UDP-Gal, respectively (Deutscher, S.L. et al, 1984; and Deutscher, S.L. and Hirschberg, C.B., 1986). These defects in nucleotide sugar transport correspond to a defect in sialylation and galactosylation of proteins and lipids in these cells. An MDCK (Madin-Darby Canine Kidney) cell line isolated by its resistance to the lectin Ricin called MDCKII-RCA was found to be specifically deficient in UDP-Gal transport, resulting in a dramatic decrease in galactosylation and subsequently sialylation, in both proteins and lipids (Meiss, H.K., et al 1982; Brandli, A.W., et al 1988). In both the CHO and the MDCK cell mutants, transport of other nucleotide sugars was unaffected,
showing that these transporters are physiologically important and specific.

As was mentioned in an earlier section (C1), the great diversity of the GAG side chains of proteoglycans may play a role in the functions of these proteins. Therefore, the study of the biosynthesis and possible regulation of the GAG side chains may answer questions of proteoglycan function, such as how GAG side chains regulate development? How are defects in GAG biosynthesis, which may be responsible for macular corneal dystrophy or aortic dissection, caused and how they can be prevented or treated? The individual translocators may play important roles in the regulation of GAG biosynthesis. To study the function and mechanism of these transporters, we need to first purify them; however, before beginning a purification, reconstitution into artificial membranes will be necessary.
CHAPTER II
INTRODUCTION
The studies described in the following manuscripts and text are designed to study ATP and nucleotide sugar transport into different organelles along the secretory pathway, and, in some cases, to determine how this transport may affect other cellular processes. In the ER and the Golgi apparatus we have studied the transporter proteins of different substrates.

The first question addressed is whether ATP is translocated into the lumen of the ER. The rationale for the study of this transport is that there are a number of ATP dependent processes in the ER or involving the ER. Two examples of these processes are: ATP dependent release of proteins from BiP to continue along the secretory pathway, and ATP dependent degradation pathways. For these functions to occur, ATP must get inside the lumen of the ER. Using an in vitro transport assay, ER vesicles from rat liver and dog pancreas were used to show protein mediated transport of ATP into the lumen. Antibodies against BiP and Grp94 were used to demonstrate one consequence of ATP transport, namely the binding of ATP to these proteins and to demonstrate that binding is dependent on transport.

The remaining studies focus on the nucleotide sugar transporter activities at later stages of the secretory pathway in the Golgi apparatus. The first question addressed is whether one can reconstitute the translocator activities for UDP-xylose, UDP-galactose, and UDP-glucuronic acid, the substrates for the biosynthesis of the
proteoglycan linkage region, into proteoliposomes in a manner similar to intact Golgi vesicles. The rationale for these sets of experiments is two fold. First, we know that these activities exist in intact Golgi based on previous work, but in order to study the translocators themselves we need to reconstitute them from the Golgi membrane into liposomes. Second, in order to purify one of these transporters we need an assay that will allow us to measure transport, but that retains transport activity similar to the intact Golgi vesicle. This reconstituted system could then be used to purify a translocator protein (or proteins). Solubilized Golgi membrane proteins were combined with phosphatidylcholine or endogenous Golgi lipid liposomes and subjected to a freeze/thaw cycle to form proteoliposomes. These proteoliposomes were then assayed for their ability to transport radioactive nucleotide sugars. Temperature sensitivity, inhibitor sensitivity, and substrate affinity were measured and compared to the same parameters seen in intact Golgi vesicles. We showed that we were able to reconstitute the transport activities of UDP-Gal, UDP-Xyl and UDP-GlcA into proteoliposomes in a manner very similar to intact Golgi vesicles.

The final topic deals with the purification of the UDP-galactose transporter protein. Using a modification of the original reconstituted system and conventional chromatography we were able to purify this translocator 45,000 fold from a rat liver homogenate. Radiation inactivation, visualization by protein iodination, and SDS-polyacrylamide gel electrophoresis were used to
monitor the purification and determine tentatively the apparent molecular weight of the UDP-Gal transporter protein (see "Materials, Methods and Results" section for detailed description on the Radiation Inactivation technique).
CHAPTER III
MATERIALS, METHODS, AND RESULTS
A. Translocation of ATP into the Lumen of the Rough Endoplasmic Reticulum-derived Vesicles and Its Binding to Lumenal Proteins Including BiP(GRP78) and GRP94.
TRANSLOCATION OF ATP INTO THE LUMEN OF
ROUGH ENDOPLASMIC RETICULUM DERIVED VESICLES AND
ITS BINDING TO LUMENAL PROTEINS INCLUDING
BIP (GRP78) AND GRP94

Caroline A. Clairmont, Antonio De Maio and Carlos B. Hirschberg

Department of Biochemistry and Molecular Biology
University of Massachusetts Medical Center
Worcester, MA 01655

Running Title: ATP in the RER Lumen
ABSTRACT

Rat liver and canine pancreas rough endoplasmic reticulum derived vesicles, which were sealed and of the same topographical orientation as in vivo, were used in a system in vitro to demonstrate translocation of ATP into their lumen. Translocation of ATP is saturable (apparent $K_m$: 3-4 $\mu$M and $V_{max}$: 3-7 pmol/min/mg protein) and protein mediated because treatment of intact vesicles with pronase, N-ethylmaleimide or DIDS inhibit transport. The entire ATP molecule is being translocated; this was shown by HPLC analysis and the use of a nonhydrolyzable analog. Control experiments rule out that translocation of ATP attributed to RER derived vesicles is due to contamination by mitochondria and Golgi vesicles. Following translocation of ATP into the lumen of the vesicles, binding to lumenal proteins including BiP (immunoglobulin heavy chain binding protein-GRP 78) and GRP 94 was observed. This binding appeared to be specific because similar experiments with GTP were negative. These studies strongly suggest that translocation of ATP into the RER lumen may serve as a mechanism for making ATP available in proposed energy requiring reactions within the lumen.
INTRODUCTION

prevent secretion of incorrectly assembled or folded proteins (Bole, D.G., Hendershot, L.M. and Kearney, J.F., 1986; Gething, M.-J., McCammon, K. and Sambrook, J., 1986). These effects are postulated to be the result of BiP binding to exposed hydrophobic surfaces of nascent protein chains, which are not available in correctly folded or assembled proteins.

Studies have suggested that the release of a completed protein from BiP is ATP dependent. Once the proteins have acquired their correct folding, ATP probably provides energy to disrupt the BiP-protein complex and secretion of these proteins is allowed to proceed (Munro, S. and Pelham, H.R.B., 1986).

An important unanswered question in these studies is how ATP, which is synthesized predominantly in the mitochondria, becomes accessible to the lumen of the endoplasmic reticulum. Recent studies in vitro from our laboratory, have shown that the membrane of rat liver and mammary gland Golgi apparatus contains a specific ATP carrier (Capasso, J.M. et al, 1989). One function for this carrier is to make ATP available to the Golgi lumen for subsequent phosphorylation of lumenal proteins, including caseins and proteoglycans. Demonstration of an ATP carrier in the RER membrane would complement studies in vitro which have shown that BiP-heavy chain complexes are released upon the addition of ATP (Munro, S. and Pelham, H.R.B., 1986), that BiP possesses a high affinity for ATP and an ATPase activity with a low turnover, suggesting a regulatory, as opposed to an enzymatic role.
(Kassenbrock, C.K. and Kelly, R.B., 1989). In addition, studies in vivo have shown that depletion of cellular ATP prevents dissociation of BiP-protein complexes and thereby blocks secretion of these proteins (Dorner, A.J., Wasley, L.C. and Kaufman, R.J., 1990). These results, therefore, imply the existence of an ATP pool inside the lumen of the RER (Pfeffer, S.R. and Rothman, R.J., 1987). We now demonstrate in a system in vitro that rat liver and canine pancreas rough endoplasmic reticulum derived vesicles can translocate intact ATP into their lumen in a protein mediated, saturable manner strongly suggesting the occurrence of a specific membrane carrier for ATP. We further show that following transport of ATP into the lumen it binds to BiP, GRP 94 and other resident RER proteins and that BiP, (as well as other lumenal proteins) but not GRP 94, appear to be (thio) phosphorylated.
EXPERIMENTAL PROCEDURES

Materials- The following radioactive materials were purchased from DuPont-New England Nuclear: [2,8-3H]-ATP 29.6 Ci/mmol; [α-32P]ATP 3000 Ci/mmol; [γ-35S]ATP 1273 Ci/mmol; [γ-32P]ATP 3000 Ci/mmol; [α-32P]GTP 3000 Ci/mmol; [32P]orthophosphate carrier free; CMP-N-[9-3H]acetylneuraminic acid 13.6 Ci/mmol. Non-radioactive nucleotides and nucleotide derivatives, carboxyatractyloside, NEM (N-ethylmaleimide) and DIDS (4,4'diisothiocyanato-2,2'disulfonic acid stilbene) were purchased from Sigma Chemical Company. ATPγS was purchased from Boehringer Mannheim. Pronase was purchased from Calbiochem-Behring.

Purification of Subcellular Fractions- Subcellular fractions from liver were obtained from 150-200 g Sprague-Dawley male rats. To prepare RER vesicles, rats were starved 20-24 hours before decapitation with a guillotine. RER derived vesicles were isolated as described by Carey and Hirschberg (1980) using a modification of the subcellular fractionation procedure originally described by Fleischer and Kervina (1974). Protein was determined according to Peterson (1977). RER derived vesicles were enriched 5 fold over homogenate in glucose-6-phosphatase specific activity (Aaronson, N.N. Jr. and Touster, O., 1974) (19% yield of total homogenate) and contained less than 2% contamination of Golgi membranes and mitochondria (see Table 2). Vesicles were at least 90% intact as determined by latency of glucose-6-phosphatase toward mannose-6-phosphate (Arion, W.J. et al, 1976). Vesicles were resuspended in 0.25 M sucrose, 10 mM
Tris-HCl, pH 7.5, 5 mM MgCl₂, and 10 mM NaF (STMF buffer) and either used immediately or stored at -80°C. Golgi derived vesicles were isolated according to Leelavathi et al. (1970) and were enriched 64 fold over homogenate in sialyltransferase specific activity (Carey, D.J. and Hirschberg, C.B., 1981) (23.1% yield of total homogenate). At least 90% of the vesicles were sealed, based on the latency of endogenous sialyltransferase toward exogenous acceptors (Carey, D.J. and Hirschberg, C.B., 1981). Mitochondria were isolated as described by Fleischer and Kervina (1974) and were enriched 7.1 fold in succinate-cytochrome C reductase specific activity (Sottocasa, G.L. et al., 1967) (8.3% yield). Canine pancreas microsomes were a generous gift of the R. Gilmore laboratory and were prepared as previously described (Walter, P. and Blobel, G., 1983).

**ATP Translocation Assay**—Transport was measured using the centrifugation assay previously described (Capasso, J.M. et al., 1989; Perez, M. and Hirschberg, C.B., 1986). Briefly, vesicles from the endoplasmic reticulum, Golgi or mitochondria (0.5-1.0 mg protein/ml) were incubated for 2-5 minutes at 30°C in a final volume of 1 ml of STMF buffer containing 0.5 mM 2,3-dimercaptopropanol (DMP), 2 µM ATP, or other radioactive substrates. Following incubation, 2 ml of ice cold STMF buffer was added to stop the reaction, and the mixture was immediately centrifuged for 30 minutes (100,000 x g) at 4°C. The supernatant was removed for calculation of [Sₘ] (see below). The surface of each pellet was washed 3 times, each with 1.5 ml of ice cold STMF buffer.
Ice cold water (0.5 ml) was added to each pellet and mixed in a vortex mixer. Perchloric acid (0.5 ml of 0.8%) was added to each sample, transferred to a microfuge tube, and placed on ice for 15 minutes. Following centrifugation in a microfuge for 5 minutes, the supernatant was removed to calculate $S_t$ (see below).

To determine $V_0$ and $V_i$ (see below), $[^3H]$ sodium acetate was used, which shows the same distribution as the standard nonpenetrator, inulin (Carey, D.J., Sommers, L.W. and Hirschberg, C.B. 1986) and 2-deoxy-D-[G-^3H]glucose as a penetrating standard (Carey, D.J., Sommers, L.W. and Hirschberg, C.B., 1986).

**Radiolabeling of RER Lumenal Proteins**—Rat liver RER derived vesicles (0.2-0.4 mg of protein) were incubated for 3 minutes at 30°C in a final volume of 100-200 μl of incubation medium (STMF buffer containing DMP). When DIDS inhibition was measured, vesicles were first pretreated with or without DIDS for 10 minutes on ice (Capasso, J.M. and Hirschberg, C.B., 1984). The incubation medium contained a final concentration of 10 nM [$\alpha$-^32P] ATP (30 μCi). Following the incubation, the sample was placed in an ice cold porcelain dish and exposed to U.V. light for 5 minutes (3.6mW/cm²) (StratalinkerTM U.V. Crosslinker model 2400 from Stratagene). The sample was then removed and layered onto a 50 μl sucrose cushion (0.5 M sucrose, 10 mM Tris-HCl pH 7.5) and centrifuged at 4°C in a Beckman airfuge using the rotor A-100/30 (Beckman Instruments) at 30 psi for 5 minutes (not including the 10 second acceleration time, or the 2 minute deceleration time).
supernatant was removed and discarded. The lumenal proteins were extracted from the pellet with 100 μl of 0.06% Triton X-100 on ice for 5 minutes (Kreibich, G. and Sabatini, D.D., 1975). The sample was again layered onto a 0.5 M sucrose cushion and centrifuged in an airfuge at 30 psi for 5 minutes. The supernatant (including the sucrose cushion), containing the lumenal proteins, was removed and washed 3 times with 2 ml of 10 mM Tris-Cl pH 7.5 through a Centricon 10 filter (Amicon) to give a final volume of 30-80 μl. Protein (100 μg) was then subjected to SDS-PAGE. When broken vesicles were used, RER vesicles were initially pretreated with 0.06% TX-100 in a final volume of 100 μl STMF buffer for 10 minutes on ice. The vesicles were then incubated with ATP at the same final concentration and exposed to U.V. light as described above. Each sample was layered onto 50 μ1 of 0.5 M sucrose cushion, centrifuged and further processed as described above.

Intact canine pancreas microsomes (0.2 mg of protein) in 0.1 ml of G buffer (250 mM sucrose, 50 mM triethanolamine pH 7.5, 1 mM dithiothreitol) were incubated with 0.1 ml of 2 μM [α-32P]ATP (100 μCi) to give a final concentration of 1 μM ATP (50 μCi) for 5 min at 30°C. Microsomes were then subjected to u.v. crosslinking for 5 min on ice as described above and vesicles were centrifuged for 25 min at 30 psi in an airfuge. The vesicle pellet was resuspended in 0.1 ml of 0.06% Triton X-100 in G buffer and incubated on ice for 20 min. Following centrifugation for 5 min at 30 psi, the supernatant, containing the lumenal proteins, was collected and washed three
times, through a Centricon 10, each with 1 ml of 10 mM Tris HCl, pH 7.5. The lumenal proteins (30 µg) were then subjected to SDS-PAGE and autoradiography. For studies involving DIDS inhibition, intact microsomes were first pretreated with 20 µM DIDS on ice for 10 min followed by a 3 min incubation at 30°C. Vesicles were then radiolabelled with ATP and lumenal proteins were extracted as described above.

Immunoprecipitation and Western Blotting of Canine Pancreas Microsomal Proteins

Immunoprecipitation: Canine pancreas microsomes (20 µg) were incubated in 100 µl G buffer at a final concentration of 0.5 µM ATP (0.5 mCi) and subjected to u.v. crosslinking as described above. Samples were then treated with solubilization buffer (50 mM Tris pH 7.5, 150 mM NaCl, and 0.1% Triton X-100) on ice for 10 min. Insoluble aggregates were removed by ultracentrifugation for 30 min at 45,000 rpm in a Ti-50 Beckman rotor. Antibodies against BiP (rat monoclonal, a generous gift of L. Hendershot) or GRP 94 (polyclonal anti-peptide, a generous gift of A. Lee) were added to the supernatant and incubated end-over-end at 4°C for 90 min. Following incubation, 75 µl of a 20% protein A-Sepharose beads slurry was added and incubated end-over-end for an additional 60 min at 4°C. Samples were then centrifuged in a microfuge for 5 min to pellet the protein A-Sepharose beads. The pellet was washed 3 times, each with 1 ml of washing buffer, (50 mM Tris pH 7.5, 0.4 M NaCl and 0.5% Triton X-100). Sample buffer (10% SDS, 0.5 M Tris pH
6.8, 30% glycerol, 0.1 M dithiothreitol, 0.1% bromophenol blue), (75 μl) was added to the protein A-Sepharose pellet and boiled for 5 min. The sample was then subjected to SDS-PAGE and autoradiography.

**Western Blotting** - Canine pancreas lumenal proteins were prepared as described above and subjected to SDS-PAGE. Proteins were then transferred to nitrocellulose sheets for 1 h at 100 V at 10°C. The membrane was incubated with blocking buffer (10 mM Tris pH 8.0, 0.3 M NaCl, 0.1% Tween 20, 5% BSA and 0.01% sodium azide) for 3 h at room temperature followed by incubation end-over-end for 4 h at 4°C with the corresponding antibody at a 1/100 dilution in blocking buffer. The membrane was then washed for 10 min in washing buffer plus detergent (10 mM Tris pH 7.5, 0.9% M NaCl, and 0.05% Tween 20), washed two times, each for 10 min in washing buffer without detergent and once again with washing buffer with detergent. The membrane was then incubated with goat anti-rabbit alkaline phosphate second antibody (Promega) for 2 h at 4°C and was washed with the same washing sequence as above. Immunoreactive bands were visualized with alkaline phosphatase.

**Identification of Radioactive Solutes Within Vesicles and Incubation Medium** - Perchloric acid was first removed from the solutes within the vesicles as previously described (Wice, B.M. et al, 1985). Briefly, 0.75 ml of the S fraction (see below) was combined with 1.5 ml of the extraction mixture (2.2 ml trioctylamine, 5.3 ml 1,1,2-trichlorotrifluoroethane) and centrifuged for 1 min at 800 rpm. The top layer was removed and diluted with an equal volume
of water and loaded onto the HPLC column. Samples from the reaction medium were filtered and directly loaded onto the HPLC column. Adenosine, AMP, ADP, ATP and ATPγS were separated by HPLC using a Synchronmax AX 100 column as previously described (Capasso, J.M. et al, 1989). The following linear gradient was run: 0.05 M KH₂PO₄, pH 3.35 from 0-5 min; the concentration was then increased to 0.06 M KH₂PO₄, pH 3.35 and a linear gradient was run from min 5 to min 10 to 0.75 M KH₂PO₄, pH 3.35 followed by isocratic elution at this concentration until 25 min. The flow rate was 1.5 ml/min. The retention times were the following: adenosine, 1.5 min; phosphosulfate, 4.0 min; AMP, 4.61 min; ADP, 9.52 min; ATP, 15.98 min; ATP γS, 20.03 min.

SDS-Polyacrylamide Gel Electrophoresis-SDS-PAGE was done with the buffer system described by Laemmli (1970) using 1.5 mm slab gels. The concentration of acrylamide in the stacking gel was 2% and that of the running gel was 10%. Samples were run for 18-20 hours at 18 mA with a cooling bath at 10°C. Protein was visualized with Comma his blue staining. Gels were autoradiographed for 18-24 hours at -80°C with Kodak X-Omat film.

Calculations Used to Determine ATP Translocation-The detailed calculations used in these experiments have been described before (Perez, M. and Hirschberg, C.B., 1987) and an example is given in Table 1.
\[ [S_m] = \text{concentration of solutes in the incubation medium} \]
\[ (\mu M) = \frac{\text{cpm/ml of solutes in the supernatant}}{\text{specific activity of solute expressed as cpm/nmol}}. \]

\[ S_t = \text{total solutes in the pellet expressed in pmol/mg of protein} = \frac{\text{total soluble radioactivity associated with the pellet, expressed as cpm/mg of protein}}{\text{specific activity of solutes, expressed as cpm/pmol}}. \]

\[ V_t = \text{total pellet volume (\mu l/mg of protein)} = \text{volume outside (in between) } + \text{ inside vesicles} = \frac{\text{cpm/mg of protein in the pellet for deoxyglucose}}{\text{cpm/\mu l of supernatant for deoxyglucose}}. \]

\[ V_o = \text{pellet volume which is outside (in between) vesicles (\mu l/mg of protein)} = \frac{\text{cpm/mg of protein in pellet for acetate}}{\text{cpm/\mu l of supernatant for acetate}}. \]

\[ V_i = \text{pellet volume which is inside vesicles (\mu l/mg of protein)} = V_t - V_o. \]

\[ S_o = \text{solute which are outside (in between) vesicles in the pellet} \]
\[ (\text{pmol/mg of protein}) = V_o \times [S_m] \times \frac{\text{cpm/mg of protein}}{\mu l}. \]

\[ S_i = \text{solute which are inside vesicles in the pellet} \]
\[ (\text{pmol/mg of protein}) = S_t - S_o. \]

\[ [S_i] = \text{concentration of solutes inside vesicles in the pellet (\mu M)} = \frac{S_i}{V_i \times \text{cpm/\mu l of protein}}. \]
Results

Translocation of ATP into rat liver RER derived vesicles: accumulation of radioactive solutes within vesicles. We were interested in determining whether ATP could be translocated, from the cytosol, to the lumen of the RER where it might serve as an energy source. For this purpose, highly purified vesicles from rat liver RER, which were sealed and of the same membrane topographical orientation as in vivo (Arion, W.J. et al., 1976), were incubated with [γ-32P]ATP. Following centrifugation of the vesicles and further processing as described in "Experimental Procedures" it was determined that the vesicle pellet contained 10.8 pmol/mg of protein of total radioactive solutes, $S_t$ (Experiment 1, Table 1). These solutes have previously been shown to be the sum of solutes within vesicles in the pellet, $S_i$, and those in between (outside) the vesicles of the pellet, $S_o$ (Perez, M. and Hirschberg, C.B., 1987). Detailed calculations are described in "Experimental Procedures" and in the legend of Table 1. To determine the amount of solutes which are in between (outside) the vesicles in the pellet, $S_o$, the concentration of ATP in the incubation medium (in pmol/μl) is multiplied by the volume which is in between (outside) the vesicles in the pellet, $V_o$, (in μl/mg protein)). This latter value is obtained from the volume accessible to sodium acetate which shows the same distribution in the pellets as the nonpenetrator inulin (Carey, D.J., Sommers, L.W. and Hirschberg, C.B., 1980). Table 1 shows that only a
Table 1

RER-derived vesicles (0.5 mg of protein) were incubated for 5 minutes at 30°C with [γ-32P]ATP (2 μM, 0.48 μCi) or [γ-35S]ATP (2 μM, 0.1 μCi) in a total volume of 1 ml of STMF buffer containing 0.5 mM 2,3-dimercaptopropanol (DMP). Translocation of ATP was determined as described under "Experimental Procedures". The various parameters were measured as described below. Following incubations in duplicate, it was determined that there were $5.7 \pm 0.6 \times 10^3$ dpm/mg protein for [γ-32P]ATP and $1.8 \pm 0.06 \times 10^3$ dpm/mg protein for [γ-35S]ATP of radioactive solutes in the pellet. In addition, it was determined that there were $2.08 \pm 0.02 \times 10^6$ dpm/ml for [γ-32P]ATP and $3.0 \pm 0.02 \times 10^5$ dpm/ml for [γ-35S]ATP in the incubation medium. The concentration of radioactive solutes in the incubation medium, [Sm], was calculated to be $2.0 \pm 0.1 \mu M$ for [γ-32P]ATP, and $1.65 \pm 0.07 \mu M$ for [γ-35S]ATP. Using the same RER preparations, [3H] sodium acetate (7 μM, $1.6 \times 10^6$ dpm) was incubated under the same conditions as described above. These incubations resulted in $1624 \pm 20$ dpm/μl in the supernatant solution and $1646 \pm 15$ dpm/mg protein in the pellet. From these values, the volume outside vesicles in the pellet, $V_0$, was calculated to be $1.1 \pm 0.01 \mu l/mg$ protein. Another set of duplicate incubations was done using [3H]deoxyglucose (0.07 μM, $1.3 \times 10^6$ dpm). These incubations resulted in $1859 \pm 54$ dpm/mg protein in the pellet and $1277 \pm 14$ dpm/μl in the supernatant. From these values, the total volume of the vesicle pellet, $V_t$, was calculated to be $1.46 \pm 0.04 \mu l/mg$ protein. The volume inside the vesicles in the pellet, $V_i$, was the difference between $V_t$ and $V_0$ and was therefore $0.36 \pm 0.05 \mu l/mg$ protein. In a parallel experiment, the same amount of RER proteins was incubated in duplicate with 2 μM [32P]orthophosphate (0.41 μCi). The pellet contained $1.68 \pm 0.02 \times 10^3$ dpm/mg protein. $S_t$ was calculated to be $3.7 \pm 0.1$ pmol/mg protein and $S_0$ was $2.0 \pm 0.1$ pmol/mg protein. $S_i$ was calculated to be $1.7 \pm 0.1$ pmol/mg protein.
Table 1

Translocation of [γ-\(^{32}\)P]ATP and [γ-\(^{35}\)S]ATP into RER Derived Vesicles: Concentration of Radioactive Solutes Within Vesicles Following a 5 minute Incubation.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>[(\gamma-^{32})P]ATP</th>
<th>[(\gamma-^{35})S]ATP</th>
<th>[(^{32})P]Pi</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[(S_m)]</td>
<td>[(S_i)]</td>
<td>[(S_i)]</td>
</tr>
<tr>
<td></td>
<td>µM</td>
<td>µM</td>
<td>µM</td>
</tr>
<tr>
<td>Experiment 1</td>
<td>2.0±0.1</td>
<td>10.8±1.2</td>
<td>2.0±0.1</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>1.6±0</td>
<td>24.1±2.4</td>
<td>1.8±0.08</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>2.0±0.1</td>
<td>3.7±0.1</td>
<td>2.0±0.1</td>
</tr>
</tbody>
</table>
small amount of total solutes in the pellet were in between (outside) the vesicles, \( S_0 \), and that approximately 82% of the total radioactive solutes in the pellet were within vesicles, \( S_i \). The concentration of radioactive solutes within the vesicles in the pellet, \([S_i]\) was obtained by dividing the solutes within the vesicles, \( S_i \), by the internal volume of the vesicles, \( V_i \). This latter value is calculated by subtracting the volume in between (outside) the vesicles in the pellet, \( V_0 \), from the total volume of the vesicle pellet, \( V_t \). This value was obtained by determining the volume accessible to a standard penetrator, deoxyglucose (Carey, D.J., Sommers, L.W. and Hirschberg, C.B., 1980).

Table 1 also shows that there was a 12-fold concentration of radioactive solutes, derived from \([\gamma^{\text{32P}}]ATP\), within vesicles compared to the concentration of ATP in the incubation medium. Transport was dependent on intact vesicles and was not observed with permeabilized ones.

It was important to determine whether the radioactive solutes detected within the lumen of the vesicles incubated with \([\gamma^{\text{32P}}]ATP\) could have arisen from breakdown in the incubation medium of ATP to \([^{32}\text{P}]\)orthophosphate and subsequent entry of this solute into the lumen of the vesicles. When RER vesicles were incubated with the same concentration of \([^{32}\text{P}]\)orthophosphate as previously used for \([\gamma^{\text{32P}}]ATP\), less than 2 pmol/mg of protein of radiolabelled solutes were within the lumen of the vesicles (Experiment 3, Table 1). These solutes were enriched only 2-fold over the concentration of
[32P]orthophosphate in the incubation medium, strongly suggesting, as a first approximation, that [γ-32P]ATP was entering the vesicles.

**Intact ATP enters the lumen of rat liver RER derived vesicles.** Because ATP can be metabolized we wanted to know whether it entered the lumen of RER vesicles intact and what its fate was following entry. Intact RER vesicles were therefore incubated with [3H]ATP for 5 min so that the adenosine derived species could be determined in the incubation medium and within vesicles following incubation. We determined by HPLC that the radioactive solutes in the incubation medium were 44% ATP, 18% ADP, 9% AMP and 29% adenosine while in the lumen of the vesicles they were 13% ATP, 29% ADP, 15% AMP and 43% adenosine. This showed that significant amounts of ATP appeared to enter the lumen intact, even though considerable metabolism had occurred (see below). Because of this breakdown, [γ-35S]ATP was used in some incubations; this ATP analog was only degraded, 11% in the reaction medium following a 5 min incubation, and not at all in the lumen of the vesicles. We therefore determined whether [γ-35S]ATP could be transported into the lumen of RER vesicles. As shown in Table 1, Experiment 2, almost 3-fold higher amounts of radioactive solutes were detected in the lumen of the vesicles compared to incubations with [γ-32P]ATP even though the [γ-35S]ATP concentration in the incubation medium was 20% lower than when [γ-32P]ATP was used (experiment 1). It can also be seen that incubations with [γ-35S]ATP, resulted in a 37-
fold concentration of radioactive solutes in the lumen of the vesicles over that of the incubation medium. As mentioned above, HPLC analyses showed that [γ-35S]ATP was the only radiolabelled solute within the vesicles. We also established that [γ-35S]ATP enters the lumen of Golgi vesicles with an affinity and a rate very similar to that previously described for [γ-32P]ATP (Capasso, J.M. et al, 1989); with [γ-35S]ATP the K_m was 0.3 μM and the V_max 67 pmol/mg protein/5 min (Figure 1) while with [γ-32P]ATP the K_m was 0.9 μM and the V_max was 58 pmol/mg protein/5 min (Capasso, J.M. et al, 1989). We found less degradation of [γ-35S]ATP in the incubation medium when Golgi vesicles (5% degradation) were used instead of RER (11% degradation). We also determined that orthophosphate enters the lumen of RER vesicles in a very slow, nonsaturable manner. This further strengthens our initial hypothesis that intact ATP is entering the lumen of RER vesicles.

Translocation of ATP into rat liver RER derived vesicles is not the result of contamination with mitochondria or Golgi vesicles. We have previously shown that ATP can be transported into the lumen of Golgi vesicles (Capasso, J.M. et al, 1989). Because of this and the known transport of ATP into mitochondria, it was important to rule out that transport of ATP into either of these organelles was responsible for the major transport signal being attributed to RER vesicles. To determine whether the transport attributed to RER vesicles was due to transport into a few
Figure 1: *Rate of ATPγS transport into golgi vesicles versus concentration.* Golgi vesicles (1 mg), were incubated with ATPγ35S at a constant specific activity (1000 cpm/pmol) for 5 minutes at 30°C. Transport was measured as described in "Experimental Procedures". Km and Vmax were calculated by linear regression analysis.
mitochondria contaminating the RER vesicle preparation, we determined the amount of ATP transport which was inhibited by carboxyatractyloside, a known inhibitor of mitochondrial ATP transport. As shown in Table 2, experiment 1, 95% of the transport of ATP into mitochondria was inhibited by carboxyatractyloside; however, the same concentration of carboxyatractyloside only inhibited 15% of the RER transport. The same conclusion was reached when ATP transport was measured with a mixture of mitochondria and RER derived vesicles Table 2, experiment 2. This result and the very low specific activity of mitochondrial marker enzyme, succinate-cytochrome C reductase (approximately 2% of protein), strongly argue against a significant mitochondrial contamination in the RER vesicle preparation.

In order to determine whether the transport measured into RER vesicles could be attributed to Golgi vesicles contaminating the RER vesicle preparation, transport of ATP and of CMP-sialic acid were measured into both vesicle preparations. This latter solute is known to be a specific substrate only for Golgi vesicles. As seen in Table 2, experiment 3, the RER vesicle preparation contained virtually no detectable CMP-sialic acid transport activity strongly suggesting that the transport attributed to RER vesicles could not have been the result of contamination by Golgi vesicles.
Table 2

In experiment #1, RER vesicles (0.5 mg of protein) and mitochondria (0.5 mg protein) were incubated with $[\gamma^{35}S]$ATP (2 μM; 0.18 μCi/nmol) in STMF buffer with DMP for 5 minutes at 30°C and transport was measured as described under "Experimental Procedures". When the effect of carboxyatractyloside was measured, RER vesicles and mitochondria were first incubated with buffer containing the glycoside for 5 minutes on ice. Results are the average of three independent determinations. In experiment #2 the incubations were carried out as in experiment #1 except that a mixture of RER vesicles (0.5 mg) and mitochondria (0.5 mg) was used in the last condition. From this differential sensitivity, it can be calculated that there is less than 2% contamination by mitochondria.

In experiment #3, RER (1.0 mg of protein) or Golgi (0.4 mg of protein) -derived vesicles were incubated with $[^3H]$ATP (2 μM; 0.7 μCi/nmol) or CMP-$[^3H]$SA (2 μM; 0.1 μCi/nmol) in STMF buffer for 3 minutes at 30°C. Translocation was measured as described under "Experimental Procedures". Results are the average of two independent determinations.
Table 2

Differential sensitivity toward carboxyatractyloside (CAT) of ATP transport into rat liver RER vesicles and mitochondria (experiments 1 and 2) and translocation of CMP-sialic acid into rat liver RER and Golgi vesicles (experiment 3).

<table>
<thead>
<tr>
<th>Incubation Medium</th>
<th>Si (pmol/mg protein)</th>
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<tr>
<td></td>
<td>RER</td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
</tr>
<tr>
<td>2μM ATP[γ&lt;sup&gt;35&lt;/sup&gt;S]</td>
<td>42.2±2.8</td>
</tr>
<tr>
<td>2μM ATP[γ&lt;sup&gt;35&lt;/sup&gt;S]+ 2μM CAT</td>
<td>36.1±2.3</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
</tr>
<tr>
<td>2μM ATP[γ&lt;sup&gt;35&lt;/sup&gt;S]</td>
<td>36.5±1.6</td>
</tr>
<tr>
<td>2μM ATP [γ&lt;sup&gt;35&lt;/sup&gt;S]+ 2μM CAT</td>
<td>28.0±2.3</td>
</tr>
<tr>
<td>Incubation Medium</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Experiment #3</td>
<td></td>
</tr>
<tr>
<td>2μM [γ&lt;sup&gt;35&lt;/sup&gt;S]ATP</td>
<td>22.3±1.6</td>
</tr>
<tr>
<td>2μM CMP-[&lt;sup&gt;3&lt;/sup&gt;H]SA</td>
<td>2.4±1.0</td>
</tr>
</tbody>
</table>
Translocation of ATP into the lumen of RER derived vesicles is saturable, protein mediated and inhibited by stilbene derivatives. The accumulation of solutes derived from \([\gamma^{-35}S]ATP\) in the lumen of rat liver RER derived vesicles was linear with protein up to 0.7 mg (30°C, 2μM ATP and 5 minutes) and with time up to 5 minutes (30°C, 2μM ATP and 0.5mg of protein) Figure 2A and B). As shown in Figure 3, the accumulation of solutes within the lumen of RER vesicles was saturable with an apparent $K_m$ of 4 μM and a $V_{max}$ of 33 pmol/5min/mg protein.

The above experiment suggested that ATP is translocated into the RER lumen in a carrier mediated manner. To obtain preliminary evidence whether such carrier is a protein with a cytoplasmic domain in the RER membrane, vesicles were incubated with Pronase and NEM under conditions where the activity of a lumenal marker, glucose-6-phosphatase, was not affected. As seen in Table 3, these impermeable effectors significantly reduced ATP translocation, suggesting that ATP transport is mediated by a protein with a cytosolic domain. This is analogous to translocation of nucleotide sugars and ATP into the Golgi lumen (Perez, M. and Hirschberg, C.B., 1986; Pfeffer, S.R. and Rothman, R.J., 1987).

We had previously determined that DIDS was an inhibitor of transport of nucleotide sugars, PAPS and ATP into the lumen of Golgi-derived vesicles (Capasso, J.M. and Hirschberg, C.B., 1984). As shown in Figure 4, DIDS also inhibited the transport of ATP into rat liver microsomes.
Figure 2: Transport of ATPγS into RER Vesicles is Linear with Protein and Time. Panel A; RER vesicles (0-2 mg) were incubated with ATPγ35S (2 μM, 1000 cpm/pmol) for 5 minutes at 30°C. Transport was measured as described. Panel B; RER vesicles (0.8 mg) were incubated with ATPγ35S (2 μM, 1000 cpm/pmol) for various times (0-10 minutes) at 30°C. Transport was measured as described.
Figure 3. Rate of solute accumulation within RER vesicles versus ATP concentration in the incubation medium; translocation of ATP into RER vesicles is saturable. RER vesicles (0.5 mg protein) were incubated at 30°C for 5 minutes with different concentrations of [γ-35S]ATP (0.091 μCi/nmol, at constant specific activity). Translocation was measured as described under "Experimental Procedures". Inset: The points of the double reciprocal plot were fitted by linear regression analysis (r²=0.99) to give a K_m of 4 μM and a V_max of 33 pmol/5min/mg of protein. Units of 1/Si are (pmol/5min/mg protein)^{-1}. 
Table 3

Inhibition of ATP Translocation into Rat Liver RER Vesicles by Pronase and N-Ethylmaleimide

<table>
<thead>
<tr>
<th>[γ-35S]ATP</th>
<th>Inhibitor</th>
<th>Inhibition %</th>
<th>Percent of Glucose-6-Phosphatase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>μM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>NONE</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>0.2</td>
<td>Pronase 1:10</td>
<td>60.4±1.9</td>
<td>99.2</td>
</tr>
<tr>
<td>0.2</td>
<td>Pronase 1:10</td>
<td>90.0±1.1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>+0.1% TX-100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>NONE</td>
<td>0</td>
<td>N.Da</td>
</tr>
<tr>
<td>0.2</td>
<td>NEM 5mM</td>
<td>37.0±9.9</td>
<td>N.D.</td>
</tr>
<tr>
<td>0.2</td>
<td>NEM 10mM</td>
<td>31.5±2.1</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

aND, not determined.

Rat liver RER vesicles (0.5 mg of protein) were pretreated without or with 5 or 10 mM N-ethylmaleimide at 30°C for 5 min or Pronase (1:10) on ice for 10 min. Vesicles were then incubated with 0.2 μM [γ-35S]ATP for 5 min at 30°C. Transport was stopped by dilution in cold buffer and tubes were placed on ice. Transport of solutes and glucose-6-phosphatase activity towards mannose-6-phosphate was then measured as described in "Experimental Procedures". S_i for the control in the pronase experiments was 3.2 ± 0.35 pmol/mg/min and for the NEM experiments was 3.5 ± 0.14. The control for glucose-6-phosphatase activity was 38 nmol/mg/min.
Figure 4: Inhibition by DIDS of ATP translocation into Rat Liver. Microsomes (0.5 mg of protein) were incubated in 0.1 ml STMF buffer, containing DMP, for 10 min on ice with or without DIDS and then immediately used for transport assays with 10 nM [α-32P]ATP(10 μCi) as described under "Experimental Procedures".
Translocation of ATP into the lumen of RER derived vesicles is a pre-requisite for its binding and (thio) phosphorylation of lumenal proteins. As mentioned in the Introduction, one of the principal reasons to study whether ATP can enter the lumen of the RER vesicles, is to determine whether it can serve, as postulated, as a potential energy source to dissociate the BiP-protein complexes in the lumen of this organelle (Dorner, A.J., Wasley, L.C., and Kaufman, R.J. 1990). We therefore incubated rat liver RER vesicles with [α-32P]ATP, followed by exposure to u.v. light to allow cross-linking of ATP to luminal proteins. These were then extracted, and subjected to SDS-PAGE and autoradiography. Figure 5 shows a commassie stained gel of the extracted luminal proteins (lane 2) and the membrane pellet after extraction (lane 3). As shown in Figure 6, lane 1, several luminal proteins became radiolabelled after crosslinking with [α-32P]ATP.

We wanted to determine whether inhibition of ATP transport into RER vesicles also resulted in decreased ATP crosslinking to luminal proteins. For this purpose, the previously described radiolabelled ATP cross-linking protocol was repeated, with the difference that 20 μM and 100 μM DIDS were first added for 10 min to the vesicles on ice. As shown in Figure 6, lane 2, 20 μM DIDS produced approximately 40% inhibition of radiolabelling of bulk luminal proteins while ATP translocation was inhibited by 35%. 100 μM DIDS pretreatment (lane 3) resulted in a 60% inhibition of ATP translocation and 75% inhibition of ATP binding to bulk luminal
Figure 5: Protein profile of extracted lumenal proteins from RER. RER vesicles were pretreated with 0.06% TX-100 on ice for 10 minutes. Lumenal proteins were extracted as described. Lumenal proteins (lane 2) and the membrane pellet after extraction (lane 3) were run on SDS-PAGE, and stained with commasie. Lane 3 are molecular weight standards.
Figure 6. *ATP* and *GTP* Translocation into rat liver RER vesicles and subsequent binding to luminal proteins: Composite Electropherogram-Autoradiography. Intact RER vesicles (lanes 1-3) or permeabilized ones (lanes 4-6) were pretreated without (lanes 1 and 4) or with 20 μM (lanes 2 and 5), or 100 μM (lanes 3 and 6) DIDS. Vesicles were then incubated with 30 μCi of [α-32P]ATP and luminal proteins extracted from the intact vesicles as described in "Experimental Procedures." Following SDS-PAGE, the samples were subjected to autoradiography. In lanes 7 and 8, intact or permeabilized vesicles were labelled with 30 μCi of [γ-32P]GTP as described in "Experimental Procedures" and the same subsequent work up was followed as described for the ATP.
proteins. This experiment suggests, as a first approximation, that inhibition of ATP transport results in an inhibition of ATP binding to lumenal proteins; therefore, ATP transport is a prerequisite for this binding.

It was important in the above experiment to rule out the possibility that the effect by DIDS on ATP transport and subsequent binding to lumenal proteins was due to a direct inhibition by DIDS on binding of ATP to these lumenal proteins. We therefore subjected permeabilized vesicles to the previous crosslinking protocol in the presence or absence of DIDS. As shown in Figure 6, lanes 4-6, one can see that the inhibition by DIDS of ATP radiolabelling of lumenal proteins was not seen with permeabilized vesicles. The same result was obtained with vesicles permeabilized with octylglucoside (Figure 7). Together with the above results, this strongly suggests that DIDS inhibited transport of ATP into the lumen but not binding per se to lumenal proteins. It can also be seen that the intensity of radiolabelled lumenal proteins in the absence of DIDS was significantly higher in intact than in permeabilized vesicles (Figure 6 lanes 1 versus 4). This is most likely due to the concentrating of ATP, in the lumen of the intact vesicles relative to the incubation medium, occurring as a consequence of transport (Table 1).

Preliminary studies had shown that GTP could not enter the lumen of RER vesicles (at 2 μM, S1 for GTP transport into RER is 0.5 pmol/mg/5 min.). When intact rat liver RER derived vesicles were incubated with [α-32P]GTP and then subjected to u.v. crosslinking as
Figure 7: RER derived vesicles permeabilized with TX-100 or octylglucoside: Composite electropherogram-Autoradiography. RER derived vesicles were permeabilized with TX-100 (0.1%) (lanes 1 and 2) or octylglucoside (OCTYL.) (0.1%) (lane 3) and pre-incubated in the presence (lanes 2 and 3) or absence (lane 1) of 20 µM DIDS. The permeabilized vesicles were then incubated with 30 µCi of [α-32P]ATP for 5 minutes and crosslinked as described. Following SDS-PAGE, the samples were subjected to autoradiography.
described above, no radiolabelling of lumenal proteins was observed consistent with no transport of GTP into the lumen. Upon incubation of permeabilized vesicles with GTP, no distinct protein bands were radiolabelled (Figure 6, lane 8) and the pattern was different from radiolabelling with ATP.

We wanted next to determine whether ATP was bound to specific lumenal ER proteins such as BiP and GRP 94. However, antibodies available against BiP react with the corresponding canine pancreas but not rat liver proteins. Therefore, we incubated canine pancreas microsomes with ATP and determined that these vesicles transported this nucleotide in a saturable manner with a $K_{\text{mapp}}$ of 3 $\mu$M and a $V_{\text{max}}$ of 14 pmol/mg protein/5 min. We also determined that DIDS inhibited transport of ATP into the lumen of these microsomes: at 20 $\mu$M DIDS the inhibition was 35 percent while at 100 $\mu$M it was 40 percent.

Canine pancreas microsomes were then incubated with radiolabelled ATP and subjected to crosslinking as described above. Figure 8, lane 1 shows that lumenal proteins, including those with apparent mobility of 78 kD and 94 kD, were radiolabelled. This radiolabeling was inhibited by preincubating microsomes with 20 $\mu$M DIDS (Fig 8, lane 2). This stilbene derivative does not inhibit crosslinking of a protein of apparent mobility of 78 kD per se as shown with permeabilized microsomes. However, because ATP was not concentrated within these microsomes, other proteins were not radiolabelled as intensely as with intact vesicles (Fig 8, lanes 3 and
Figure 8. ATP translocation into dog pancreas microsomes and subsequent binding to BiP, Grp94 and other luminal proteins. Composite electropherogram-autoradiography of microsomal luminal proteins, and identification of BiP and Grp94 by Western blotting. Intact (lanes 1 and 2) or permeabilized (lanes 3 and 4) dog pancreas microsomal vesicles were preincubated with 20 µM DIDS (lanes 2 and 4), or without DIDS (lanes 1 and 3) as described under "Experimental Procedures." The microsomes were incubated with [α-32P]ATP, subjected to U.V. crosslinking and luminal proteins extracted. Following SDS-PAGE the samples were subjected to autoradiography. The arrows mark the positions of protein with mobilities of 78 kD and 94 kD. Luminal microsomal protein were transferred to nitrocellulose as described and blotted with a non-immune serum (lane 5), or antibodies against Bip (lane 6) or Grp94 (lane 7). Bands were visualized with alkaline phosphatase reaction.
Further proof that the radiolabelled proteins were indeed GRP 78 and GRP 94 was obtained by Western blots with antibodies against BiP and GRP 94 (Fig 8, lanes 5 and 6) and by immunoprecipitation of the [α-32P]-ATP-labeled lumenal proteins with the corresponding antibodies (Fig 9, lanes 2 and 3). Labeling of GRP94 with ATP is much less than for GRP78, however, we do believe that this is specific binding based on the previous figure showing the crosslinking and the phosphorylation data below. We can see a small amount of high molecular weight labeled bands at the top of the gels in figures 6 and 8. This is most likely due to a small amount of aggregation as a result of the crosslinking or the boiling of the samples before running the gel. We know that the crosslinking is not causing a large amount of aggregation since communase gels of proteins with or without crosslinking show no difference.

Previous studies had shown that while BiP is phosphorylated (Hendershot, L.M., Ting, J. and Lee, A.S., 1988), GRP is not (Lee, A.S., Bell, J. and Ting, J., 1984). To obtain preliminary evidence that this phosphorylation was a consequence of transport of ATP into the lumen of intact ER vesicles, canine pancreas microsomes were incubated with [γ-35S]ATP with or without u.v. crosslinking as described previously. As shown in Fig 10, lane 1, proteins of mobilities of 78 kD and 94 kD become radiolabeled upon crosslinking, but only BiP is labeled in the absence of u.v., suggesting that solely the latter is (thio)phosphorylated.
Figure 9. ATP transport into canine pancreas microsomes and subsequent binding to BiP and Grp94. Composite electropherogram-autoradiography of immunoprecipitates with anti-BiP and anti-Grp94 antibodies. Canine pancreas microsomes were labeled with ATP as described in "Experimental Procedures." The labeled microsomes were then immunoprecipitated with a non-immune serum (lane 1), or antibodies against BiP (lane 2), or Grp94 (lane 3), and subjected to autoradiography. The arrows mark the position of BiP (78 kD) and Grp94 (94 kD). Lanes 1 and 2 were exposed for 6 days, while lane 3 was exposed for 28 days.
Figure 10 Transport and Interaction of [γ-35S]ATP with Lumenal Microsomal Proteins with and without U.V. Crosslinking: Composite Electropherogram-Autoradiography of Microsomal Lumenal Proteins. Intact canine pancreas microsomes (200 μg) were incubated with [γ-35S]ATP (1 μM, 500 μCi) as described. The sample was then exposed to U.V. (lane 1) on ice for 5 min or placed on ice directly (lane 2) with no U.V. for 5 min. Lumenal proteins were extracted as described, run on SDS-PAGE and exposed to autoradiography.
DISCUSSION

This study has shown that, in a system in vitro, rough endoplasmic reticulum derived vesicles can translocate intact ATP from the incubation medium into their lumen. Translocation of ATP was found to be saturable with an apparent $K_m$ of 3-4 $\mu$M and inhibited by inhibitors of anion transport such as DIDS. Because transport was inhibited by proteases and N-ethyl maleimide under conditions where a lumenal marker enzyme activity such as glucose-6-phosphatase was not affected, we infer that the transport is mediated by a protein. The absence of transport of GTP under the above conditions strongly suggests that the transporter for ATP is specific. Following transport of ATP, the nucleoside triphosphate was concentrated in the lumen approximately 30-fold. Preliminary experiments suggest that the mechanism of translocation is via an antiporter, AMP or ADP. This is similar to the mechanism of translocation of ATP in the Golgi apparatus (Capasso, J.M. et al, 1989) and that of nucleotide sugars and nucleotide sulfate in the Golgi apparatus and rough endoplasmic reticulum (Perez, M. and Hirschberg, C.B., 1986).

Important controls in this study were the demonstration that the transport of ATP could not be attributed to a few contaminating mitochondria or Golgi vesicles in the RER preparation. The most conclusive experiment regarding mitochondrial contamination was the demonstration that the transport of ATP into RER vesicles was
not significantly inhibited by carboxyatractyloside while at the same time this glycoside was highly inhibitory of transport of ATP into mitochondria. This effect was also seen when using a mixture of mitochondria and RER derived vesicles.

We believe that one possible function for ATP translocation into the lumen of the RER may be to serve as an energy source in the disruption of the nascent polypeptide chain-BiP complexes (Munro, S. and Pelham, H.R.B., 1986; Kassenbrock, C.K. and Kelly, R.B., 1989; Dorner, A.J., Wasley, L.C. and Kaufman, R.J., 1990). Although direct evidence for such reactions has not been provided, we find, particularly striking, the fact that the binding of ATP to BiP and GRP 94 was significantly higher when intact microsomes were used as opposed to permeabilized ones (Fig. 6, lanes 1 vs 4 and Fig. 8, lanes 1 vs 3); this enabled ATP to be concentrated in the lumen and resulted in a higher concentration for binding.

We were surprised by the binding of ATP to GRP 94; it is possible that this glucose regulated protein may also have a role, together with BiP in protein assembly in the lumen of the rough endoplasmic reticulum (Shiu, R.P.C., Pouyssegur, J. and Pastan, I., 1977). Although a role for GRP 94 is unknown, its synthesis is increased during glucose starvation (Shiu, R.P.C., Pouyssegur, J. and Pastan, I., 1977; Pelham, H.R.B. 1986) as well as by conditions that cause an increase in aberrant proteins, in a manner similar to that of BiP/GRP 78 (Pelham, H.R.B., 1986; Wu, F.-S. et al, 1981). However no enzymatic activity has been associated with GRP 94.
BiP binds ATP with approximately 10-fold higher affinity than ADP and 1000 fold higher than AMP (Kassenbrock, C.K. and Kelly, R.B., 1989). Because the crosslinking experiments were done with the radiolabelled nucleoside triphosphate in the alpha position, we cannot rule out that some of the observed binding was also due to ADP and AMP. It is, however, very unlikely, in view of the above described binding affinities that ATP was not the predominant crosslinked nucleotide. The u.v. crosslinking experiments with [γ-35S]ATP clearly show that GRP 94 was bound to intact ATP because no thiophosphorylation was observed.

Recently a yeast gene KAR2 from Saccharomyces cerevisiae was isolated and found to be the homologue of the mammalian BiP/GRP 78 gene (Rose, M.D., Misra, L.M. and Vogel, J.P., 1989). It was found that this gene is essential for cell viability. This result shows that BiP/GRP 78 function is required, and strongly suggests therefore that translocation of ATP, which probably is a prerequisite for BiP function in yeast, must also be an essential step in the assembly of proteins and cell viability. The physiologic role of ATP-dependent phosphorylation of BiP is not known. Other proteins, such as the core protein of proteoglycans are also phosphorylated, although it is unclear whether this occurs in the lumen of the ER or in the Golgi apparatus where phosphorylation of casein (Capasso, J.M. et al, 1989) and vitellogenin have been demonstrated.

Future studies should lead to an understanding of the structural relationships between ATP translocators in mitochondria,
RER and Golgi membranes and of functions that this translocation event may be relevant for in the RER lumen.
ACKNOWLEDGEMENT

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B. Reconstitution into Proteoliposomes and Partial Purification of the Golgi Apparatus Membrane UDP-Galactose, UDP-Xylose, and UDP- Glucuronic Acid Transport Activities.

Work contributed by Caroline Clairmont:

3. Studies on the antiporter mechanism involving the UDP-Gal and UDP-Xyl transporters.

Work contributed by Marcos Milla:

3. Antiporter mechanism involving the UDP-GlcA transporter.
4. Initial work suggesting a lipid specificity for the UDP-Xyl transporter.
RECONSTITUTION INTO PROTEOLIPOSOemes AND PARTIAL
PURIFICATION OF THE GOLGI APPARATUS MEMBRANE
UDP-GALACTOSE, UDP-XYLOSE AND
UDP-GLUCURONIC ACID TRANSPORT ACTIVITIES

Marcos E. Milla, Caroline A. Clairmont and Carlos B. Hirschberg

Department of Biochemistry and Molecular Biology
University of Massachusetts Medical Center
Worcester, MA 01655

Running Title: Proteoglycan Linkage Oligosaccharides
Abstract

Previous studies in vitro on proteoglycan biosynthesis from our laboratory have shown that nucleotide sugar precursors of all the sugars of the linkage oligosaccharides (xylose, galactose and glucuronic acid) and of the glycosaminoglycans (N-acetylglucosamine, N-galactosamine and glucuronic acid) are transported by specific carriers into the lumen of Golgi vesicles. More recently, we also reported the reconstitution in phosphatidylcholine liposomes of detergent solubilized Golgi membrane proteins containing transport activities of CMP-sialic acid and PAPS. We have now completed the successful reconstitution into liposomes of the Golgi membrane transport activities of UDP-galactose, UDP-xylose and UDP-glucuronic acid. Transport of these nucleotide sugars into Golgi protein proteoliposomes occurred with the same affinity, temperature dependence and sensitivity to inhibitors as observed with intact Golgi vesicles. Preloading of proteoliposomes with UMP, the putative antiporter for Golgi vesicle transport of these nucleotide sugars, stimulated transport of the nucleotide sugars by two to three fold. Transport of UDP-xylose into Golgi protein proteoliposomes was dependent on the presence of endogenous Golgi membrane lipids while that of UDP-galactose and UDP-glucuronic acid was not. This suggests a possible stabilizing or regulatory role for Golgi lipids on the UDP-xylose translocator. Finally, we have also shown that detergent solubilized Golgi membrane translocator proteins can be
partially purified by an ion exchange chromatographic step before successful reconstitution into liposomes, demonstrating that this reconstitution approach can be used for the biochemical purification of these transporters.
Introduction

Proteoglycans have a protein core and glycosaminoglycan side chains (Fransson, L.-A., 1987). Among their many diverse and important biological functions are: control of permeability in the basement membrane of kidney (Kanwar, Y.S., Linker, A. and Farquhar, M.G., 1980), anticoagulation in endothelial cells (Bourin, M.-C. et al, 1986), anchorage receptors for enzymes (Williams, M.P. et al, 1983; Brandan, E. et al, 1985), and modulators of growth factor activities and extracellular matrix components in cell-cell interactions (i.e. receptors for fibronectin and N-CAM (Saunders, S., and Bernfield, M., 1988; Edelman, G.M., 1986)). Previous studies had shown that in proteoglycans, the protein core is linked via a tetrasaccharide (Xyl-Gal-Gal-GlcA) to a glycosaminoglycan polymer (Gallagher, J.T. and Steward, W.P., 1986).

Studies from our laboratory (Nuwayhid, N. et al, 1986) and from Kimura et al, (1984) and Lohmander et al, (1986) have shown that the formation of the peptide core O-xylose linkage occurs in the lumen of the Golgi apparatus (Kimura, J.H., Lohmander, L.S. and Hascall, V.C., 1984; Nuwayhid, N. et al, 1986; Lohmander, V.C. et al, 1986). We have also shown in studies in vitro that nucleotide sugar precursors of all the sugars of the linkage oligosaccharides and of glycosaminoglycans (N-acetylg glucosamine, N-acetylgalactosamine and glucuronic acid) are transported by specific carrier activities into the lumen of Golgi vesicles (Nuwayhid, N. et al, 1986; Abeijon, C. and
Hirschberg, C.B., 1987; Hirschberg, C.B. and Snider, M.D., 1987). The same was found for PAPS, the sulfate donor for glycosaminoglycan N and O sulfation (Hirschberg, C.B. and Snider, M.D., 1987). These transporters appear to be antiporters in which the nucleotide sugar or nucleotide sulfate enter the lumen of the Golgi in a reaction coupled to the equimolar exit of the corresponding nucleoside monophosphate (Hirschberg, C.B. and Snider, M.D., 1987). The description of mutant cell lines deficient in specific transport of UDP-Gal and CMP-sialic acid into Golgi vesicles coupled with the corresponding defect in galactosylation and sialylation of proteins and lipids, strongly suggest that these transporters are of physiologic relevance and that there are individual uridine nucleotide sugar transporters in the Golgi membrane as opposed to a common one (Deutscher, S.L. et al, 1984; Deutscher, S.L. and Hirschberg, C.B., 1986). The specific glycosyl and sulfotransferases have also been found to be highly enriched in membranes from the Golgi apparatus.

The glycosaminoglycan region of proteoglycans has been shown to play important roles, among others, in (a) cell-cell interaction and cell adhesion (LeBaron, R.G. et al, 1989; Arruffo, A. et al, 1990); (b) modulation of growth factors (Yayon, A. et al, 1991; Rouslahti, E. and Yamaguchi, Y., 1991); (c) receptor mediated endocytosis (Murphy-Ullrich, J.E. et al, 1988), (d) development (Platt, J.L. et al, 1990) and (e) coagulation and anticoagulation (Bourin, M.C. et al, 1986; Jackman, R.W. et al, 1986). Recently, it has also been shown that the molecular
basis of types 1 and 2 macular corneal dystrophy is a defect in glycosaminoglycan biosynthesis (Midura, R.J. et al, 1990).

An important question is how proteoglycans perform such diverse functions as described above. To answer this, one must evaluate their biosynthesis and how it is regulated. Because the nucleotide sugar and PAPS transporters may have important regulatory roles in the biosynthesis of the GAG chains, we have begun the purification and characterization of the nucleotide sugar transporters involved in the biosynthesis of the linkage tetrasaccharides of proteoglycans.

Recently we reported the reconstitution into phosphatidylcholine liposomes of detergent solubilized Golgi membrane proteins containing transport activities of CMP-sialic acid and PAPS (Milla, M.E. and Hirschberg, C.B., 1989). These Golgi protein proteoliposomes had essentially the same solute specificity, affinity, temperature dependence and sensitivity to transport inhibitors of these nucleotide derivatives as intact Golgi vesicles.

We have now successfully reconstituted into liposomes the Golgi membrane transport activities of UDP-galactose, UDP-xylose and UDP-glucuronic acid. The transport characteristics of these nucleotide sugars into proteoliposomes were very similar to those into intact Golgi vesicles with respect to $K_{mapp}$, sensitivity towards inhibitors and stimulation by UMP, the putative transport antiporter. We have used this reconstituted system to partially purify the Golgi membrane UDP-Gal and UDP-GlcA translocators.
EXPERIMENTAL PROCEDURES

Materials- The following radioactive substrates were purchased from DuPont, New England Nuclear: UDP-[1-3H(N)]Xylose (8.9 Ci/mmol); UDP-[4,5-3H(N)] Galactose (43.3 Ci/mmol); UDP-[14C(U)]Glucuronic acid (303 mCi/mmol). All other materials were purchased from Sigma Chemical Company. Frozen male rat livers were purchased from Pel-Freez., Inc.

Isolation of Golgi Vesicles and Preparation of a Golgi Protein Extract- Livers from six male Sprague-Dawley rats (150-200g) (approximately 50-80 g wet weight) were removed and Golgi vesicles were isolated by the method of Leelavathi et al (1970). Protein was assayed by the method of Peterson (1977). The obtained Golgi vesicles (40-50 mg) were enriched 65 fold over homogenate in sialyltransferase specific activity (Carey, D.J. and Hirschberg, C.B., 1981; 23% yield of total homogenate). At least 90% of the vesicles were sealed, and of the same topography as in vivo, based on the latency of sialyltransferase toward exogenous acceptors (Carey, D.J. and Hirschberg, C.B., 1981). A protein extract was prepared from these vesicles as described by Milla and Hirschberg (1989) Briefly, purified Golgi vesicles in buffer A (10 mM Tris-HCl pH 7.0; 3.0 M sucrose; 1 mM MgCl2; 1 mM dithiothreitol; 20% (vol:vol) glycerol) were solubilized with Triton X-100 at a final concentration of 0.5%. After high speed centrifugation of the protein-detergent
mixture, the detergent was removed from the supernatant fraction by extraction at 4°C with Bio-Beads SM-2 (0.3 g/ml of supernatant; Bio-Rad). This suspension was then concentrated by ultrafiltration and stored in aliquots at -80°C.

**Extraction of Endogenous Golgi Lipids**- Endogenous Golgi lipids were extracted from frozen rat livers as described previously (Folch, J, Lees, M. and Sloane Stanely, G.H., 1957; Keenan, T.W. and Morre, D.J., 1970). Briefly, purified Golgi vesicles (30-40 mg of protein) obtained as described above, were resuspended in 3 ml of buffer C (0.3 M sucrose; 10 mM Tris-HCl pH 7.0; 1 mM MgCl₂) and extracted 2 times with 4 volumes of chloroform:methanol (2:1), and once with 4 volumes of chloroform alone. The organic solvent fractions were combined and dried under nitrogen. Dried lipids were resuspended in 5 ml of chloroform and extracted once with 1 ml of 10 mM Tris HCl pH 7.0. The chloroform layer was then dried under nitrogen to a volume of 1-2 ml. The sample was then transferred to a preweighed 10 ml glass ampule and completely dried under nitrogen. The lipids were weighed, redissolved in 0.3 ml of hexane, dried under nitrogen and lyophilized overnight.

**Preparation of Liposomes**- Egg yolk phosphatidylcholine (30 mg) or endogenous Golgi lipids (15-20 mg) in hexane were dried on the walls of a 10 ml glass ampule under nitrogen. The dried lipids were then resuspended in 1 ml of buffer B (10 mM Tris-HCl, pH 7.0; 0.3 M sucrose; 1 mM MgCl₂; 1 mM dithiothreitol; 1% (vol/vol) glycerol). In some cases, UMP was present in this buffer at a concentration of 100
μM. The resulting emulsion was sonicated (Heat systems Ultrasonics model W-225 with cup horn device and continuous water circulation, 28-30% of maximum output) at 30°C until a clear suspension was obtained (approximately 30 min).

Reconstitution of Golgi Protein into Liposomes- The freeze-thaw method of Kasahara and Hinkle was used for the reconstitution (Kasahara, M. and Hinkle, P.C., 1976). One ml of liposomes, prepared as above, were mixed by gently stirring with 1.0 mg of Golgi protein extract in buffer B. The mixture was then quickly frozen in a dry ice/acetone bath and allowed to thaw slowly at room temperature (15-20 min). This freeze/thaw cycle was repeated for a total of 5 times. After the fifth thawing, the mixture was sonicated for 10 sec and applied to a Sephadex G-50 column (20x1 cm; fine) and eluted with buffer B. Fractions of 1.5 ml were collected. Proteoliposomes eluted in the void volume and were used directly for transport assays. This step was done in order to remove free UMP present in some cases.

Transport Assay- Transport of solutes into intact Golgi vesicles was assayed as described by Perez and Hirschberg (1986). Transport into Golgi protein proteoliposomes was done as described by Milla and Hirschberg (1989); briefly, proteoliposomes (0.4 ml) were mixed with 0.1 ml of buffer B containing the radiolabeled solute whose transport was being assayed. For competitive inhibition studies, inhibitors were added together with the radiolabeled substrate in buffer B, while in the case of non-competitive inhibition studies, the
proteoliposomes were first preincubated with the inhibitor on ice for 10 minutes and then radiolabeled solutes were added. After 5 minutes at 30°C, the mixture was applied to a Sephadex G-50 column (20 x 1cm) and eluted with buffer B. Fractions of 1.5 ml were collected and their radioactivity was determined by liquid scintillation spectrometry. Solute within proteoliposomes eluted in the void volume while free solutes eluted later. Protein incorporated into the liposomes was determined by the method of Schaffner and Weissman (1973).

Partial Purification of the UDP-Galactose and UDP-Glucuronic Acid Translocators- Golgi vesicles (30 mg of protein) were solubilized in buffer B + 0.5% Triton X-100. The sample was centrifuged at 100,000 x g for 60 min and the supernatant was applied to a 6 ml DEAE-Sephacel column equilibrated with 5 column volumes of buffer B + 0.5% Triton X-100. The column was washed with 2 column volumes of buffer B + 0.5% Triton X-100 (fraction WI), followed by 4 column volumes of buffer B + 0.5% Triton X-100 + 50 mM NaCl (fraction WII). The column was then eluted with 2 column volumes of buffer B + 0.5% Triton X-100 + 300 mM NaCl (fraction EI), and finally with 2 column volumes of buffer B + 0.5% Triton X-100 + 1 M NaCl (Fraction EII). Approximately 0.5 mg of protein from each fraction were applied to a 1 ml Extract Gel-D (Pierce) column to remove the detergent. The column was equilibrated with buffer B and pretreated with hemoglobin to block non-specific binding, and approximately 98% of the TX-100 was removed from the sample.
These fractions were then reconstituted into phosphatidylcholine vesicles; UDP-galactose and UDP-glucuronic acid transport activity was assayed as described above. All fractions were stored at 4°C during the purification.

RESULTS

Transport of UDP-Galactose into Golgi protein proteoliposomes is saturable and temperature dependent. Proteoliposomes were prepared from phosphatidylcholine liposomes and a mixture of bulk Golgi membrane proteins as described in "Experimental Procedures." Briefly, solubilized Golgi membrane proteins were then added to the phosphatidylcholine liposomes and subjected to freeze/thaw cycles. The proteoliposomes, consisting of a fairly homogenous vesicle population with an average diameter of 200 nm, have been previously shown to be approximately one-half "right side out" vesicles and one-half "inside out" vesicles (based on their latency of sialyltransferase and N-heparan sulfate sulfotransferase activities) (Milla, M.E. and Hirschberg, C.B., 1989).

Upon incubation of the proteoliposomes with different concentrations of UDP-Gal, transport of the nucleotide sugar into the vesicles was determined by measuring the amount of radiolabelled nucleotide sugar which eluted in the void volume of a Sephadex G-50 column. As seen in Figure 11, transport was saturable with an
Figure 11: Rate of UDP-Gal transport into Golgi proteoliposomes versus concentration. Proteoliposomes (preloaded with 100 μM UMP) were incubated with different concentrations of UDP-[3H]Gal at a constant specific activity (1000 cpm/pmol) for 5 min at 30°C. Transport was measured as described under "Experimental Procedures". The values were corrected for non-specific binding or diffusion of labelled substrates by subtracting the signal obtained with a mixture of protein and liposomes which had not been freeze-thawed. These values were less than 25% of those obtained with proteoliposomes until 10 μM UDP-Gal (at 2 μM UDP-Gal the signal for freeze-thawed proteoliposomes was 8.8 pmol/mg while without freeze-thawing it was 2 pmol/mg). Inset: saturation curve for UDP-Gal transport into intact Golgi vesicles. K_{app} and V_{max} were calculated using a double reciprocal plot fit by linear regression analysis.
apparent $K_m$ of 4.2 µM. This value was very similar to the apparent $K_m$ of UDP-Gal transport into intact Golgi vesicles (Figure 11, inset: $K_m$ app=2.4 µM). A control was also done for non-specific adsorption of nucleotide sugar to vesicles. This was measured as the amount of radiolabelled nucleotide sugar bound to a mixture of Golgi proteins and liposomes, which had not been subjected to freeze/thaw cycling, and that eluted in the void volume of Sephadex G-50 column. The value for these controls was always less than 25% of that obtained with proteoliposomes until 10µM.

Transport of UDP-Gal into proteoliposomes was linear with time between 1 and 6 min and with protein up to 1 mg (Figure 12A and B). Transport was also dependent on temperature with that at 0°C being 33% of that at 30°C (Table 4). This value was similar to that observed with intact Golgi vesicles where transport at 0°C was 42% of that at 30°C (Table 4). As seen in Figure 11, insert, the $V_{max}$ obtained with Golgi vesicles was approximately 8 fold higher than into proteoliposomes. Several reasons may account for this difference: (a) the population of Golgi vesicles is virtually 100% "right side out" while only 50% of the translocator incorporated into proteoliposomes is of this orientation; (b) in the proteoliposomes there is no transfer of galactose to endogenous membrane acceptors, which provide a "sink" for the transport reaction in intact vesicles; (c) as previously shown, only approximately 15-20% of the Golgi membrane proteins were incorporated into proteoliposomes during the reconstitution step.
Figure 12: Transport of UDP-Galactose into Golgi proteoliposomes is linear with time and protein. Panel A: Golgi proteoliposomes were incubated with UDP-[\(^3\)H]Galactose (2 μM, 1000 cpm/pmol) for the indicated times at 30°C. Transport was stopped by placing on ice, and was assayed as described. Panel B: The indicated amounts of Golgi solubilized protein were added to liposomes and subjected to freeze/thaw to make proteoliposomes. Transport was then assayed as described with 2 μM UDP-[\(^3\)H]Galactose at 1000 cpm/pmol.
Table 4

Transport of UDP-galactose into Golgi vesicles and Golgi proteoliposomes is temperature-dependent.

<table>
<thead>
<tr>
<th>TEMPERATURE</th>
<th>Golgi proteoliposomes pmol/mg protein/5 min</th>
<th>Proteoliposomes mg protein/5 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>30°C</td>
<td>44.6</td>
<td>10.0</td>
</tr>
<tr>
<td>0°C</td>
<td>18.5</td>
<td>3.0</td>
</tr>
<tr>
<td>30°C (not reconstituted)</td>
<td>NDa</td>
<td>2.2</td>
</tr>
</tbody>
</table>

*aND= not determined

Intact Golgi vesicles (0.5 mg of protein) or Golgi protein proteoliposomes were incubated with 2 μM UDP-[3H]Gal as described in Experimental Procedures. Results are average of two independent determinations.
**Characterization of UDP-Galactose transport into Golgi protein proteoliposomes.** We had previously shown that transport of nucleotide sugars into Golgi vesicles occurred via an equimolar exchange with the corresponding (lumenal) nucleoside monophosphate. To further characterize the transport of UDP-Gal into proteoliposomes, we determined whether or not it was stimulated by the presence of lumenal UMP, the substrate generated in the lumen after transfer. As shown in Table 5, transport of UDP-Gal was stimulated approximately 3 fold when proteoliposomes had been preloaded with 100 μM UMP as compared to buffer alone.

In previous studies we had shown that DIDS and halogenated deoxynucleoside monophosphates inhibited the transport of nucleotide sugars into Golgi vesicles competitively. To determine whether or not this also occurred with UDP-Gal, we compared the effect of DIDS and 5'-I-dUMP on UDP-Gal transport into Golgi vesicles and Golgi proteoliposomes. As shown in Table 6, 5'-I-dUMP produced a 90% inhibition of transport into Golgi vesicles and a 60% inhibition into Golgi proteoliposomes. DIDS inhibited UDP-Gal transport by 10% into Golgi vesicles and 27% into proteoliposomes.

Together the above results suggest that the transport characteristics of UDP-Gal into proteoliposomes are similar to those into Golgi vesicles and that the reconstitution assay can be used to begin to monitor the purification of the Golgi membrane UDP-Gal
Table 5

Transport of UDP-galactose, UDP-glucuronic acid, and UDP-Xylose into Golgi protein proteoliposomes is stimulated by preloading proteoliposomes with UMP.

<table>
<thead>
<tr>
<th>Solute</th>
<th>Transport</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-UMP</td>
</tr>
<tr>
<td></td>
<td>pmol/mg protein/5 min</td>
</tr>
<tr>
<td>UDP-Galactose</td>
<td>3.0</td>
</tr>
<tr>
<td>UDP-Glucuronic acid</td>
<td>13.4</td>
</tr>
<tr>
<td>UDP-Xylose</td>
<td>4.5</td>
</tr>
</tbody>
</table>

Liposomes (phosphatidylcholine for UDP-Gal and UDP-GlcA and Golgi lipids for UDP-Xyl) were prepared in the presence or absence of 100 μM UMP. Golgi protein proteoliposomes were obtained as described under Experimental Procedures and were incubated in buffer containing 2 μM of the radioactive solute for 5 min at 30°C. Results are an average of two independent determinations.
Table 6

Effect of Inhibitors of UDP-Galactose transport into Golgi vesicles and Golgi protein proteoliposomes.

| Condition   | Vesicles      | Transport % inhibition
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/mg</td>
<td>protein/5 min</td>
</tr>
<tr>
<td>Control</td>
<td>Proteoliposomes</td>
<td>5.0</td>
</tr>
<tr>
<td>20 µM DIDS</td>
<td>Proteoliposomes</td>
<td>3.3</td>
</tr>
<tr>
<td>200 µM</td>
<td>Proteoliposomes</td>
<td>1.9</td>
</tr>
<tr>
<td>5' I-dUMP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Golgi</td>
<td>44.6</td>
</tr>
<tr>
<td>20 µM DIDS</td>
<td>Golgi</td>
<td>41.6</td>
</tr>
<tr>
<td>200µM 5' I-dUMP</td>
<td>Golgi</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Golgi vesicles (0.5 mg of protein) or Golgi protein proteoliposomes were incubated with various inhibitors. In the case of DIDS, Golgi vesicles or proteoliposomes were preincubated with DIDS for 3 minutes at 30°C followed by addition of 2 µM UDP-[³H]Gal. Transport was assayed after 5 min. In the case of 5' I-dUMP the inhibitor was added to the incubation mixtures containing the radiolabelled substrate at time zero. Results are an average of two independent determinations.
transporter. We will present evidence below demonstrating that this is so.

**Translocation of UDP-Xylose into Golgi protein proteoliposomes is saturable and requires endogenous Golgi lipids.** Xylose is the sugar which links the protein core with the other sugars of the proteoglycan linkage region. We wanted to determine, therefore, whether the nucleotide donor of xylose, UDP-Xyl, could be transported into phosphatidylcholine Golgi proteoliposomes. In preliminary experiments we found that the temperature dependence of UDP-Xyl transport into Golgi phosphatidylcholine proteoliposomes at 0°C was 80% of that at 30°C (0°C; 5.0 pmol/mg, 30°C; 7.7 pmol/mg). In addition, we found that transport at 30°C was virtually non-inhibited by 20 μM DIDS (30°C; 7.7 pmol/mg, 30°C + 20 μM DIDS; 5.1 pmol/mg). Because these characteristics did not resemble UDP-Xyl transport into Golgi vesicles, we decided to substitute phosphatidylcholine liposomes for liposomes made of endogenous rat liver Golgi lipids. Using liposomes made from a crude Golgi fraction, the reconstitution was slightly improved with transport at 0°C being 50% of that at 30°C however, we could only get transport activities similar to the intact Golgi vesicles if the liposomes were made from pure Golgi lipids. As shown in Table 7, the transport of UDP-Xyl into these pure Golgi proteoliposomes was highly dependent on temperature, the value at 30°C being approximately 8 fold higher than that at 0°C.
Table 7

Transport of UDP-xylose, and UDP-glucuronic acid (UDP-GlucA) into Golgi protein proteoliposomes: temperature dependence, lipid requirements, and effect by inhibitors.

<table>
<thead>
<tr>
<th>Solutes</th>
<th>Conditions</th>
<th>Proteoliposomes</th>
<th>Transport pmol/mg protein/5min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDP-GlucA</td>
<td>30°C</td>
<td>Phosphatidylcholine</td>
<td>20.5</td>
</tr>
<tr>
<td>UDP-GlucA</td>
<td>0°C</td>
<td>Phosphatidylcholine</td>
<td>1.7</td>
</tr>
<tr>
<td>UDP-GlucA</td>
<td>30°C, DIDS</td>
<td>Phosphatidylcholine</td>
<td>9.4</td>
</tr>
<tr>
<td>UDP-Xylose</td>
<td>30°C</td>
<td>Golgi Lipids</td>
<td>6.5</td>
</tr>
<tr>
<td>UDP-Xylose</td>
<td>0°C</td>
<td>Golgi Lipids</td>
<td>0.8</td>
</tr>
<tr>
<td>UDP-Xylose</td>
<td>30°C, DIDS</td>
<td>Golgi Lipids</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Golgi protein proteoliposomes were incubated with 2 μM UDP-[3H]Xyl or 2 μM UDP-[14C]GlcA for 5 min at 0°C or 30°C. For DIDS inhibition, proteoliposomes were preincubated with 20 μM DIDS for 3 min at 30°C. Results are an average of two independent determinations.
In addition, 20 μM DIDS inhibited transport into proteoliposomes by approximately 50%. Transport of UDP-Xyl into these Golgi proteoliposomes was also saturable with an apparent $K_m$ of 2.6 μM (Figure 13). This value is very similar to that seen in intact Golgi vesicles (Figure 13, inset, $K_m$ app=4.1 μM). As shown in Table 5, transport of UDP-Xyl was stimulated 2.5 fold by preloading the proteoliposomes with 100 μM UMP compared to buffer, consistent with UMP being the antiporter molecule.

UDP-Glucuronic Acid is translocated into Golgi protein proteoliposomes in a manner similar to that of Golgi vesicles. Glucuronic acid together with galactose and xylose, is part of the oligosaccharide linkage region of proteoglycans. The nucleotide donor of glucuronic acid, UDP-glucuronic acid, was transported into phosphatidylcholine Golgi proteoliposomes in a temperature dependent manner with the value at 30°C being approximately 12 fold higher than at 0°C (Table 7). In addition, this transport into proteoliposomes was inhibited approximately 60% by 20 μM DIDS (Table 7) a value very similar to the inhibition seen in Golgi vesicles (44%, 225 pmol/mg protein/5 min in controls and 99 pmol/mg protein/5 min in DIDS treated samples). Transport of UDP-GlcA into proteoliposomes was also found to be saturable (Figure 14), with an apparent $K_m$ of 3.3 μM, a value very similar to that observed with Golgi vesicles (Figure 14, inset, $K_m$ app=4.9 μM). Preloading these proteoliposomes with 100 μM UMP resulted in a 2 fold
Figure 13: Rate of UDP-Xyl transport into Golgi protein proteoliposomes versus concentration. Proteoliposomes, prepared from endogenous Golgi lipids, and preloaded with 100 μM UMP, were incubated with UDP-[3H]Xyl at a constant specific activity (1500 cpm/pmol) for 5 min at 30°C. Non-specific binding and Km_{app} and V_{max} were determined as described in the legend of Figure 1 and fit by linear regression analysis.
Figure 14: Rate of UDP-GlcA transport into Golgi protein proteoliposomes versus concentration. Proteoliposomes preloaded with 100 μM UMP were incubated with UDP[14C]GlcA at a constant specific activity (670 cpm/pmol) for 5 min at 30°C. The values were corrected for non-specific binding and diffusion of the radiolabelled substrate by subtracting the amount of radiolabelled UDP-GlcA associated with the proteoliposomes in incubations containing 100 μM DIDS. This method gives, in this case, comparable values for non-specific binding as the non-freeze-thaw method (approximately 20% binding at 2 μM UDP-GlcA). $K_m$ and $V_{max}$ were calculated by the method of Hofstee.
stimulation of UDP-GlucA transport compared to buffer alone (Table 5).

Partial purification of the UDP-Galactose and UDP-Glucuronic Acid translocators. The above results demonstrate that Golgi membrane nucleotide sugar translocators can be reconstituted into proteoliposomes, with transport characteristics that are very similar to intact Golgi vesicles. This reconstitution can therefore be used as an assay to monitor the purification of nucleotide sugar translocators. We have begun to attempt the purification of the UDP-Gal and UDP-GlcA translocators. As shown in Table 8, a Triton X-100 extract of purified Golgi membranes (purified 55 fold from a rat liver homogenate) was applied to a DEAE-Sephacel column followed by elution of the column with buffer containing two salt concentrations. Fractions eluted from the column were then reconstituted into phosphatidylcholine liposomes and assayed for UDP-Gal and UDP-GlcA transport activities. One can see that the UDP-Gal and UDP-GlcA transport activities are highly enriched (3000 and 660 fold over homogenate respectively) in the fraction eluted with buffer containing 1 M NaCl, demonstrating that one can specifically separate the UDP-Gal and UDP-GlcA transport activity from the bulk of the Golgi proteins as a step towards the purification of these transporters. The recovery yield of both transport activities was approximately 300 percent compared to the applied total Golgi membrane detergent extract. We are currently determining whether this is due to true activation or removal of an inhibitor.
Table 8

Partial purification of Golgi membrane UDP-galactose and UDP-Glucuronic acid transporters

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg)</th>
<th>Specific Act. (pmol/mg/5 min.)</th>
<th>Fold Purification (Purification)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>30.0</td>
<td>1.14</td>
<td>[1]</td>
</tr>
<tr>
<td>FT</td>
<td>6.2</td>
<td>4.2</td>
<td>3.6</td>
</tr>
<tr>
<td>WI</td>
<td>5.1</td>
<td>10.6</td>
<td>9.3</td>
</tr>
<tr>
<td>WII</td>
<td>6.2</td>
<td>9.4</td>
<td>8.2</td>
</tr>
<tr>
<td>EI</td>
<td>10.0</td>
<td>0.55</td>
<td>0.5</td>
</tr>
<tr>
<td>EII</td>
<td>2.4</td>
<td>54.7</td>
<td>48.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg)</th>
<th>Specific Act. (pmol/mg/5 min.)</th>
<th>Fold Purification (Purification)</th>
</tr>
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<tbody>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FT</td>
<td>6.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WI</td>
<td>5.1</td>
<td></td>
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<tr>
<td>WII</td>
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</tr>
<tr>
<td>EI</td>
<td>10.0</td>
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<td></td>
</tr>
<tr>
<td>EII</td>
<td>2.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Golgi vesicles (20-30 mg of protein) were solubilized with 0.5% Triton X-100. The soluble proteins were applied to a DEAE-Sephacel column and eluted as described under "Experimental Procedures". The UDP-Gal and UDP-GlcA transport activities were assayed using the reconstituted proteoliposome system. T, total Triton X-100 extract of purified Golgi vesicles; FT, flow-through; WI, wash with buffer B + 0.5% Triton X-100; WII, wash with buffer B + 0.5% Triton X-100 + 50 mM NaCl; EI, elution with buffer B + 0.5% Triton X-100 + 300 mM NaCl; EII, elution with buffer B + 0.5% Triton X-100 + 1 M NaCl.
DISCUSSION

We have shown that the transport activities for UDP-Gal, UDP-Xyl and UDP-GlcA, which are donors of sugars that are part of the proteoglycan linkage region, can be successfully reconstituted into Golgi protein proteoliposomes. Transport of these nucleotide sugars into proteoliposomes was very similar compared to Golgi vesicles with regard to $K_{\text{mapp}}$, temperature dependence, and inhibitor characteristics. In addition, specific proteins are required to be part of the proteoliposomes for individual nucleotide or nucleotide sugar transport to occur, as shown by control experiment in this (Table 8) and previous studies (Milla, M.E. and Hirschberg, C.B., 1989).

Liposomes alone do not show transport activity and neither does mixing proteins with liposomes without proper integration of the former in the bilayer. This behavior coupled to the fact that the rate of transport was stimulated by preloading the proteoliposomes with UMP, strongly suggests that these proteoliposomes can be used in an assay to monitor the purification of transporter proteins. As shown in Table 8, the above prediction was correct (see below).

A surprising result was the observation that the transport of UDP-xylose required liposomes made of Golgi membrane lipids and not solely phosphatidylcholine. So far, this nucleotide sugar is the only one specifically requiring endogenous Golgi lipids for an assay in vitro as opposed to solely phosphatidylcholine which can be used to
measure transport of UDP-Gal, UDP-GlcA, CMP-sialic acid and PAPS. While a definitive answer for the reason of this specificity will have to await the purification of the UDP-xylose transporter, several possibilities come to mind: (a) this particular transporter may be dependent on specific endogenous Golgi lipids for its catalytic activity; (b) the transporter can only partition into a specific lipid environment; and (c) the conformation or stability of the transporter may be dependent on specific Golgi membrane lipids.

As we mentioned previously, transport into proteoliposomes is stimulated by preloading with UMP; the question arises of why proteoliposomes devoid of UMP can translocate these nucleotide sugars at all? We have observed that under these conditions, transport of nucleotide sugars into proteoliposomes occurs only up to solute equilibration but does not result in solute accumulation. This accumulation only occurs when proteoliposomes contain UMP. This nucleoside monophosphate (in the lumen of preloaded proteoliposomes) is a competitive inhibitor of transport of uridine nucleotide sugars. It likely prevents exit of these nucleotide sugars from the proteoliposome lumen following their entry and in addition serves as an antiporter for uridine nucleotide sugar entry.

We had originally decided to attempt to reconstitute these Golgi transport activities into proteoliposomes as an essential initial step towards the purification and characterization of nucleotide sugar transporter proteins and determination of their possible role in the regulation of glycosaminoglycan biosynthesis. The results shown
above strongly suggest that this strategy is correct and that further ion exchange and affinity chromatographic approaches together with a filtration assay which is being developed will enable the purification to homogeneity of these transporter proteins.

Acknowledgment- We thank Karen Welch for excellent typing.
C. Purification of the UDP-Galactose Translocator Protein from Rat Liver.
ABSTRACT

Previous work from our lab has shown that the UDP-Galactose transporter protein can be reconstituted into liposomes with activity characteristics similar to intact Golgi vesicles (Chapter III, Section B). Using a modified version of this reconstitution method as an assay system, we have purified the UDP-Galactose transporter 45,000 fold over the initial rat liver homogenate. Visualization of proteins by iodination has shown that we have achieved a high degree of purification. Using radiation inactivation, we have preliminary results suggesting tentatively that the UDP-Galactose transporter has a MW of ~100-120 Kd in vivo.
EXPERIMENTAL PROCEDURES

Materials and methods - Uridine Diphospho-D-[6-3H] Galactose (18.9Ci/mmol), and 125Iodine (100 mCi/ml) was purchased from Amersham. Extracti-Gel D was from Pierce. Dowex 1X2 50-100 strongly basic anion exchange resin was purchased from BioRad. WGA-Agarose was purchased from EY laboratories. All other reagents used were from Sigma Chemical Company. Frozen male rat livers were obtained from Pel-Freeze.

Isolation of a crude Golgi fraction and preparation of a soluble protein extract - A crude Golgi fraction was isolated from 300 g frozen male rat livers. The fractionation was based on a partial Leelavathi method for obtaining pure Golgi (Leelavathi, D.E. et al, 1970). This partial fractionation resulted in a crude Golgi fraction which was approximately 15-20X purified over the homogenate by measuring the activity of a Golgi marker enzyme sialytransferase (Carey, D.J. and Hirschberg, C.B., 1981). A soluble protein extract was obtained using a modification of the method of Milla, M.E., and Hirschberg, C.B., 1989. Briefly, the crude Golgi vesicles were diluted to a final concentration of 2 mg/ml in buffer A (10 mM Tris-HCl pH 7.0, 3.0 M sucrose, 1 mM MgCl2, 1 mM DTT, 10 mM NaF, 10% glycerol) and homogenized by hand in a glass dounce (Pestel A) for 10 strokes on ice. The homogenate was then brought to a final TX-100 concentration of 0.5% and vortexed for 2 minutes. This solubilized protein extract was centrifuged at 45,000 rpm (Beckman
Ti50 rotor) for 45 minutes, and the supernatant collected to be used for subsequent purification steps.

**DEAE-sephacel chromatography** - The above supernatant was brought to 0.3 M NaCl and added to a 20 ml DEAE-Sephacel column equilibrated with buffer A with a final concentration of 0.3 M NaCl and 0.5% TX-100. The column was loaded at 1 ml/min, and a flowthrough fraction was collected. The column was washed with Buffer A + 0.3 M NaCl + 0.5% Tx-100 (5 volumes), eluted with a 80 ml linear gradient from 0.3 M NaCl to 1.2 M NaCl, and 20 fractions of 4 ml each were collected. Transport of UDP-galactose was assayed in each fraction, and in all subsequent column fractions, by reconstitution (see below).

**UDP-hexanolamine chromatography I** - Active fractions from the DEAE-Sephacel column were pooled, and the NaCl was removed by repeated washes in buffer A over a Centricon 30 filter. The desalted sample was then applied to 5 ml of UDP-hexanolamine agarose equilibrated in Buffer A + 0.5% TX-100 on an end-over-end mixer for 1 hour at 4°C. The agarose was poured into a column and a flowthrough fraction collected. The column was washed with 2 column volumes of Buffer A + 0.5% TX-100 and 2 column volumes of Buffer A + 0.3 M NaCl + 0.5% TX-100. The column was then eluted in batch with one column volume of Buffer A + 1.0 M NaCl + 0.5% TX-100 (Fraction EII). Column flow rate was 1 ml/min.
WGA-agarose chromatography- The active EII fraction was treated to remove the NaCl as described above. The desalted fraction was then added to 5 ml of WGA-Agarose, equilibrated in Buffer A + 0.5% TX-100, on an end-over-end mixer for 3 hours at 4°C. The resin was poured into a column, a flowthrough fraction collected, and the column washed with 2 column volumes of Buffer A + 0.5% TX-100. The column was then batch eluted with 1 column volume of Buffer A + 0.5 M N-Acetylglucosamine + 0.5% TX-100 (Fraction B). Column flow rate was 1 ml/min.

UDP-hexanolamine chromatography II- Fraction B obtained from the WGA-agarose chromatography was desalted as described above and applied to 5 ml of fresh UDP-hexanolamine agarose equilibrated in Buffer A + 0.5% TX-100 on an end-over-end mixer for 1 hour at 4°C. The resin was poured into a column and a flowthrough fraction was collected. The column was washed with 2 column volumes of Buffer A + 0.5% TX-100, eluted with a 10 ml linear NaCl gradient from 0-1.2 M NaCl, and 10, 1 ml fractions were collected. The column flow rate was 0.5 ml/min.

Reconstitution of column fractions- All fractions, including the total solubilized Golgi membrane proteins used as the starting material, were treated in the following manner for reconstitution. Individual samples to be reconstituted were exhaustively desalted by washing with Buffer A over a Centricon 30 filter. The desalted samples were then treated to remove the TX-100 by passage over
Extracti-Gel columns (TX-100 binding resin). The Extracti-Gel was initially pre-treated with hemoglobin to block all non-specific protein binding sites. Pre-treated Extracti-Gel columns (0.2 ml) were poured in 1 ml syringes, at room temperature, and washed 2 times with Buffer A. The desalted samples were then added to the Extracti-Gel columns. The columns were washed once with 100 μl of Buffer A and both the flow through and the wash fractions (~300 μl) were collected and put on ice. The protein solution without detergent was then added immediately to 200 μl of prepared liposomes (preparation of liposomes as described in Milla, M.E. et al, 1989), quickly frozen in dry ice/acetone and allowed to thaw slowly at room temperature. This freezing and thawing cycle was repeated 5 times. After the fifth cycle, the completed proteoliposomes could then be used to assay UDP-galactose transport.

Transport assay for reconstituted fractions- 200 μl of the completed proteoliposomes were combined with 50 μl of incubation medium (Buffer A containing radiolabeled UDP-galactose to give a final concentration of: 2 μM UDP-Gal at 2000 cpm/pmol), and incubated for 5 minutes at 30°C. The samples were then placed on ice to stop the reaction, and added to 2.5 ml Dowex columns. The Dowex columns are poured at room temperature in 3 ml syringes and washed 3 times with water. After addition of the sample, six 300 μl fractions are collected (using water to elute). Proteoliposomes containing transported UDP-[3H]galactose elute in fractions 2-4, while free UDP-[3H]galactose is retained on the resin. Background
radioactivity is corrected for by assaying transport in mock reconstitutions (i.e. liposomes alone).

**Protein iodination**- A 100 μl sample (~1 μg of protein) was combined with 500 μCi $^{125}$I and 60 μg Chloramine T for 10 minutes at room temperature. The reaction was quenched with 60 μg of sodium metabisulfite and 500 μg of KI at room temperature (final volume 130 μl), and placed on a microdialyzer (Pierce) with a 8000 MW cutoff dialysis membrane. The sample was dialysed against 8 liters of 10 mM Tris-HCl pH 7.0, 0.1% TX-100 overnight at a flow rate of 5 ml/min. The samples were collected, and run on SDS-PAGE. The completed gel was fixed in 40% methanol, 10% acetic acid for 1 hour, washed in 10% ethanol, 5% acetic acid for 1 hour, dried onto two cellophane sheets and exposed to X-ray film for 7-10 hours.

**Radiation inactivation**- Highly purified Golgi vesicles were prepared (Leelavathi, D.E. *et al*, 1970) and equilibrated in cryoprotectant buffer (14% (vol/vol) glycerol, 1.4% (wt/vol) D-sorbitol, 150 mM KCl, and 5 mM Hepes-Tris pH 7.5). Aliquots of this vesicle preparation (0.5 ml of a 20 mg/ml stock) were frozen in dry ice in 2 ml glass ampules (Type 12012, Kimble), and sealed with an oxygen torch. The sealed ampules were then kept at -80°C, and transported in dry ice. The vesicles were irradiated at -135°C with a beam of 13 MeV electrons produced by a linear accelerator. After the irradiation, the vesicles were kept at -80°C, until transport was assayed. The ampules were opened and purged with nitrogen and
the samples were allowed to thaw slowly at room temperature. Transport of UDP-galactose was then assayed as described (Perez, M., and Hirschberg, C.B., 1987). Transport activity (A) at each radiation dose was assessed relative to the control unirradiated transport activity (A₀). Results were expressed in a semi-log plot of ln(A/A₀) versus Dose (Mrads). A more detailed description of the radiation inactivation method and theory will be given in the next section.

Glucose 6-phosphate dehydrogenase assay- Exogenous glucose 6-phosphate dehydrogenase (400 µg) from *Leuconostoc mesenteroides* (Olive, C., and Levy, H.R., 1967), was added to each ampule before radiation treatment as an internal control for the radiation inactivation method (McIntyre, J.O., and Churchill, P. 1985). Briefly, 1.2 ml of 70 mM Tris-HCl pH 7.8, 4.3 mM MgCl₂ was combined with 0.15 ml of 2 mM NADP and 0.15 ml 33 mM Glucose 6-Phosphate in a 1.5 ml U.V. cuvette. The reaction is started with the addition of 0.3-0.6 µg of glucose 6-phosphate dehydrogenase enzyme. The change in absorbance at 340 nm over time (3 minutes) is recorded. Units of enzyme activity are: change in absorbance/min/mg protein/µmole of NADP.
RADIATION INACTIVATION:

Radiation inactivation is used to measure the molecular size of an enzyme in situ. This technique relies on the loss of biological activity in molecules that are exposed to ionizing radiation (reviewed in Kempner, E.S., 1988). Since the loss of a specific biological activity is measured as a function of the radiation dose, purified samples are not needed. The independent destruction of individual species in a sample allows you to assay the loss of activity of a number of enzymes, thus determining their molecular masses, in a single sample. This technique can be applied to any sample that can withstand freezing and thawing without a loss in activity. I will briefly describe the theoretical basis of target inactivation.

THEORETICAL BASIS: (reviewed in Kempner, E.S., 1988)

Gamma rays and high energy electrons cause random ionizations through the target mass. These primary ionizations occur with the electrons of the outer orbitals in the atoms of the macromolecules. Energy from the primary ionization is transferred to the macromolecule and is absorbed by the target through excitation, ionization and free radical formation. This energy transfer results in irreversible damage to the macromolecule by covalent bond breakage or conformational changes. A direct hit by the radiation causes the destruction of that molecules biological activity. Radiation exposure also causes the formation of OH⁻ and H₂O₂ from its interaction with water. In a liquid sample these could very easily
diffuse and cause loss of activity to the macromolecule, however by using frozen samples, these secondary products do not diffuse and therefore any damage done to the macromolecules is due directly to the interaction of the radiation on the target mass. The basis for target theory has three major assumptions: 1. the only significant effects are caused by direct action of the radiation, 2. all molecules suffering a primary ionization (i.e. a direct hit) become completely devoid of biological activity, and 3. all molecules that escape a primary ionization remain fully active. Therefore, there are two distinct populations of molecules, hit and unhit. Although these assumptions seem quite large, this technique has been used successfully for the determination of the molecular size, in situ, of many enzymes (Kempner, E.S., et al 1980; Hymel, L. et al, 1985; Beliveau, R., et al, 1988; Stevens, B.R., et al, 1990; Elsner, R.H., and Ziegler, K. 1992).

**CALCULATIONS:**

\[ A = A_0 e^{-qmd} \]

- Poisson equation

\[ A = \text{Surviving activity, } A_0 = \text{Original activity, } q = \text{constant, } m = \text{mass, and } d = \text{dose.} \]

\[ \ln(A/A_0) = -qmd \]

- Basis for the inactivation curve resulting in a straight line with a slope, \( k = -qm \)

\[ m = -k/q = 6.4 \times 10^{11} \]
RESULTS

Initial purification of the UDP-Gal, and UDP-GlcA transporters from rat liver. Our first attempt at the purification of these transporters was aimed at answering the question of whether we could take a fraction from a column, reconstitute it into liposomes and recover activity. The first column tried was a DEAE-Sephacel column with a batch elution that was shown in the previous section (Table 8). We know however, that there are several UDP-nucleotide sugar transporters in the Golgi membrane, so we needed to know where the others, specifically UDP-GlcA and UDP-GlcNAc would elute. We found that all three different transporter activities eluted in the same EII fraction. Therefore, we next wanted to separate these three UDP-transporters from each other. To do this we used a NaCl gradient as opposed to a batch elution. Using the original reconstitution assay system from Milla, M.E. et al, 1989, I could in fact separate the activities of UDP-GlcA and UDP-GlcNAc from the UDP-Gal transporter activity (Figure 15). At this point I decided to concentrate on the purification of the UDP-Gal transporter alone for the following reasons: first, from the above DEAE-Sephacel profile, I was able to separate this activity from the others. Second, as mentioned previously, the UDP-Xyl translocator could only be reconstituted into liposomes made from endogenous Golgi lipids which were more difficult and time consuming to obtain than using
Figure 15: Profile of the UDP-Gal, UDP-GlacNac and UDP-GlcA transport activities from the DEAE-Sephasel chromatography. Fractions from a DEAE-Sephasel column eluted with a linear NaCl gradient from 0.3 M to 1.2 M were collected. Every three fractions were pooled to make a "fraction pool". The fractions were then dialyzed to remove the NaCl and run over Extracti-gel to remove the detergent, and reconstituted into phosphatidylcholine liposomes. Each fraction was then assayed for UDP-Gal (open circles), UDP-GlcA (closed triangles) and UDP-GlcNAc (closed squares) transport activity by the method described by Milla, M.E. et al, 1989.
phosphatidylcholine liposomes for UDP-Gal reconstitution. Finally the cost of the radiolabeled UDP-GlcA was prohibitively expensive to be used on a large scale that would be necessary for a purification.

Before beginning the purification, I needed to redesign the reconstituted assay system to make it practical for use on a purification. The original assay took more than 40 minutes to assay a single sample, and required a large amount of protein to see a signal. For these reasons, a new method was developed as described in the Methods section, that takes only 1-2 minutes per sample and requires a much smaller amount of protein. This new method involves the use of a small Dowex column instead of a sizing column to separate the free radiolabeled nucleotide sugar from the radiolabel that was transported inside the liposome. The free UDP-[3H]galactose would be bound to the Dowex, while the transported UDP-[3H]galactose would be inside the liposomes and would come out in the flow through. This method was specific: it could distinguish between liposomes containing protein and a blank containing lipids alone (Figure 16). Also using Golgi proteoliposomes it could distinguish between a Golgi substrate (UDP-Gal) and one that was not (GDP-Man) (Figure 16). Using this new reconstitution assay we began the purification of the UDP-galactose translocator protein from rat liver.
Figure 16: Specificity of the new reconstitution assay system; differentiation between UDP-Gal, and GDP-Mann transport into Golgi proteoliposomes and transport of UDP-Gal into lipids alone. Golgi proteoliposomes were incubated with 2 μM UDP-[3H]Gal (open squares), or 2 μM GDP-[3H]Man (closed circles) for 5 minutes at 30°C. Transport was measured as described in the Methods section. Lipids alone (closed triangles) were incubated with 2 μM UDP-[3H]Gal for 5 minutes at 30°C, and transport measured as described.
Purification of the UDP-Galactose transporter (Table 9). A crude Golgi fraction was obtained from rat liver using a modified Leelavathi method for the isolation of pure Golgi vesicles (Leelavathi, D.E., et al, 1970). This fraction was enriched approximately 15-20 fold over the initial rat liver homogenate based on the activity of a Golgi marker enzyme sialyltransferase. To this crude Golgi fraction, 0.5% TX-100 was added, vortexed, and the mixture was spun at 100,000Xg for 45 minutes. This extract was then added to a DEAE-Sephacel column and eluted with a linear NaCl gradient from 0.3 M to 1.2 M. As can be seen in figure 17A, the UDP-galactose transport activity is eluted between fractions 8-12, with a purification fold of 60 and an overall purification of 1190 fold over the homogenate. These fractions were pooled and applied to a UDP-hexanolamine agarose affinity column. This column contains a UDP moiety attached to an agarose solid support; the rationale for using this column was that the UDP-Gal transporter will bind the UDP specifically. This column was eluted in batch with 1.0 M NaCl, yielding a purification fold of 389 or 7778 fold purification over the homogenate. The active fraction was added to a WGA-Agarose affinity column. Since we began the purification with a crude Golgi fraction as opposed to a pure one, we have, in addition to Golgi proteins, a large amount of RER proteins, among which are UDP binding proteins. In order to remove these we chose to use the WGA column. WGA is a lectin that binds Golgi-specific carbohydrates.
Table 9

Purification of the UDP-Galactose Golgi membrane transporter protein from rat liver

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg)</th>
<th>Activity (pmol)</th>
<th>Spec. Act. (pmol/mg/5min)</th>
<th>Yield (%)</th>
<th>Purification Fold</th>
<th>Final Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>TX-100 Extract</td>
<td>800</td>
<td>2,435</td>
<td>3.0</td>
<td>100</td>
<td>1</td>
<td>1-20</td>
</tr>
<tr>
<td>DEAE-Sephacel</td>
<td>10</td>
<td>1,786</td>
<td>179</td>
<td>73</td>
<td>60</td>
<td>60-1,200</td>
</tr>
<tr>
<td>UDP-Hexanolamine I</td>
<td>0.5</td>
<td>583</td>
<td>1,167</td>
<td>24</td>
<td>389</td>
<td>389-7,780</td>
</tr>
<tr>
<td>WGA-Agarose</td>
<td>0.11</td>
<td>306</td>
<td>2,781</td>
<td>13</td>
<td>927</td>
<td>927-18,540</td>
</tr>
<tr>
<td>UDP-Hexanolamine II</td>
<td>0.008</td>
<td>54</td>
<td>6,750</td>
<td>2</td>
<td>2,250</td>
<td>2,250-45,000</td>
</tr>
</tbody>
</table>

A crude Golgi preparation (1000-2000 mg) was solubilized in 0.5% TX-100. The soluble proteins were subjected to a series of chromatography steps and eluted under various conditions as described in the methods section. The UDP-Gal transport activites were measured using the modified reconstitution system described. TX-100 extract, total TX-100 extract of the crude Golgi preparation that was added to the DEAE Sephacel column: DEAE-Sephacel, the active fractions from the NaCl gradient elution (8-12) pooled and added to the UDP-HEx I column: UDP-Hex I, active fraction which is eluted after a series of washes with 1 M NaCl: WGA-Agarose, active fraction eluted specifically with 0.5 M N-acetylglucosamine: UDP-Hex II, active fractions from NaCl gradient elution (3-5).
Figure 17: Activity and protein profiles from the DEAE-Sephacel Chromatography. A crude Golgi fraction (~1000 mg) was solubilized and added to a DEAE-Sephacel column as described in the methods section. The column was eluted with a linear NaCl gradient from 0.3 M-1.2 M. UDP-Gal transport activity was assayed from each fraction as described in the methods. Panel A shows a typical activity profile from this column, with the UDP-Gal transport activity eluting between fractions 8-14. Panel B shows the protein profile from the active peak. Samples from the total soluble extract (lane 2), the flowthrough (lane 3), the wash (lane 4) and the active fractions 6-12 (lanes 6-12) were run on SDS-PAGE and stained with silver.
Therefore proteins from the RER should not bind to this column, allowing separation of our Golgi protein from the contaminants. This column was eluted specifically with 500 mM N-acetylglucosamine, yielding a purification of 930 fold and an overall purification of 18,500 fold over the homogenate. The active fraction was then applied to a final UDP-hexanolamine column, and eluted with a linear NaCl gradient from 0-1.2 M NaCl. As can be seen in figure 19A, the UDP-galactose transport activity is eluted between fractions 3-6, yielding a final purification fold of 45,000 over the homogenate.

**Protein Visualization.** Using silver stain (figure 17B) or iodination (figures 18B and 19), we can see that we are achieving purification from the starting TX-100 extract. Figure 17B is a silver stain of fractions off the DEAE-Sephacel column beginning with the loaded sample (TX-100 extract) (lane 2), the flow through (lane 3), the wash fraction (lane 4), and the fractions through the activity peak (lanes 6-12). We used iodination to visualize the protein profiles from the last three columns. Because of the very small amount of proteins in the fractions we were unable to see any bands using comassie or silver stain. There is a small possibility that our protein of interest will not be iodinated, however the same is true for all of the staining methods (i.e. not all proteins can be labeled with silver). Figure 18 is an iodination of the flowthrough and active fractions (lanes 1, and 2) from the UDP-hexanolamine I column, and active fraction (lane 3) from the WGA column. Figure 19B is an
iodination of protein samples through the activity peak of the UDP-
hexanolamine II column. Although we can not positively identify a
particular protein band at this point as being the UDP-Gal
translocator, based on the activity profiles and the radiation
inactivation data (described below), we can suggest some possible
candidates, which I will describe below and in the discussion section.
Figure 18: Protein profile from UDP-Hexanolamine I and WGA columns visualized by Iodination and Autoradiography. The active fractions from the DEAE column were pooled and applied to the UDP-hexanolamine column as described. The active fraction was then applied to a WGA-agarose column and eluted as described in the methods. Protein (1 ug) was iodinated (0.5 mCi) as described and run on SDS-PAGE. The gel was dried in cellophane sheets and exposed to X-ray film. The runthrough and active fractions from the UDP-hexanolamine I column (lanes 1 and 2), and the active fraction from the WGA-agarose column (lane 3) are shown.
Figure 19: Activity and protein profiles from the UDP-Hexanolamine II Chromatography. The active fraction from the WGA-agarose column was applied to the UDP-hexanolamine II column and eluted as described with a linear NaCl gradient from 0 M-1.2 M. Panel A shows the UDP-Gal transport activity. Panel B shows the protein profile across the activity peak. Samples (0.1 ug) were iodinated (0.5 mCi) as described and run on SDS-PAGE. The gels were dried in cellophane sheets and exposed to X-ray film. Lanes 2-8 of the gel correspond to fractions 2-8 off the UDP-hexanolamine II column.
Radiation inactivation. Pure Golgi vesicles were exposed to varying doses of ionizing radiation as described in the Methods section. The irradiated samples were then assayed (~0.8 mg), in quadruplicate, for UDP-galactose transport activity. The resulting inactivation curve is shown in Figure 20, showing a loss of activity with increasing amounts of radiation. There are two different interpretations for the data. First if we assume the data fit a straight line then using the calculations described, we can tentitively estimate the molecular weight of the UDP-Gal translocator in the membrane at 100-120 Kd. The second interpretation is more complicated. If we assume that the data fall on a two part curve, then we can draw two straight lines. Using the calculations described, this would show two components responsible for the transport we are measuring, one at 100Kd, and the other at 300Kd. Before this conclusion can be reached we need to repeat this experiment 4-6 times. These results are very tentative and based only on two seperate experiments.

As a control for the radiation procedure itself, an internal standard (glucose-6-phosphate dehydrogenase (G6PD)), was added to the Golgi vesicle preparation at each dose. This enzyme was chosen since its molecular weight is known, and it was shown to be a reliable internal standard for radiation inactivation studies (McIntyre, J.O., and Churchill, P. (1985). G6PD activity (Olive, C., and Levy, H.R., 1967) was measured in triplicate for each dose and its inactivation curve is shown in Figure 21. Using the same calculations we obtain a target size of 106Kd, which is very close to its known.
Figure 20: Radiation Inactivation Curve for the UDP-Galactose Transporter. Pure Golgi vesicles (12 mg) were irradiated with different amounts of ionizing radiation and the effect on the transport of UDP-galactose was measured as described in the methods. The ratio of the remaining activity to the original activity was plotted in a semi log vs. dosage in Mrads.
Figure 21: Radiation Inactivation curve for the control enzyme Glucose-6-phosphate Dehydrogenase. Control enzyme (400 ug) was added exogenously to the pure Golgi vesicles before irradiation. At each dose, the activity of this enzyme was assayed as described in the methods. A semi log plot of remaining activity vs. dosage was prepared.
molecular size of 104Kd. This demonstrates that the radiation procedure itself is correct and any data we measure for the UDP-Gal transporter is due to this unique transporter and not due to the radiation technique
CHAPTER IV

DISCUSSION
A. Summary.

As proteins mature along the secretory pathway, they undergo many different posttranslational modifications. These modifications are acquired within the organelles of this pathway: the endoplasmic reticulum (ER) and the Golgi apparatus. Protein folding, subunit assembly, and degradation take place in the lumen of the ER, and they all require ATP. Proteins then travel to the Golgi apparatus where they undergo terminal N- and O-glycosylation, sulfation and phosphorylation which take place in the lumen.

Since ATP and nucleotide sugars are not synthesized in the lumen of these organelles, how are they brought into the lumen of these organelles where they are utilized? This question has been the main focus of this thesis research. In the case of ATP, we have shown that there is an ATP transporter in the ER membrane. For the case of the nucleotide sugars we knew that transporters existed (in the Golgi apparatus) for the linkage region sugars of proteoglycans (UDP-Gal, UDP-Xyl and UDP-GlcA). Through reconstitution, we were able to develop a system where these proteins could be removed from the Golgi vesicles, yet retain all of the functional characteristics we observed in the intact Golgi. We then used a modification of this system to purify the UDP-Galactose transporter 45,000 fold over a rat liver homogenate.
B. Transport of ATP into the Lumen of the Endoplasmic Reticulum.

This work has demonstrated the existence of an ATP transport activity in the ER membrane. Transport is saturable and dependent on vesicle integrity, with a $K_m$ of $4 \mu M$, and a $V_{max}$ of $33$ pmol/5min/mg protein. It is also inhibited by the membrane impermeable substrates DIDS, with $50\%$ inhibition occurring at $\sim 50 \mu M$ DIDS, and NEM and pronase, under conditions which did not disrupt the vesicle integrity (i.e. the activity of a lumenal enzyme remained unchanged). This data strongly suggests that this is a protein mediated transport with an active site facing the cytosol. Alternatively, it is possible that NEM and pronase do not directly interact with the active site, but could effect a different site which indirectly inhibits the transport (i.e. allosterically). Since it was known that ATP translocators exist in the Golgi apparatus and mitochondria, one possibility is that the signal we were measuring in the ER fraction was really due to contamination of our ER preparation with Golgi or mitochondria membranes. However, we know that this transport signal was an independent transporter of the ER, and not due to contamination of these other organelles, based on two criteria. First, the transport activity showed differential sensitivity to carboxyatractyloside (CAT); CAT strongly inhibits the mitochondria transporter ($95\%$ inhibition at $2 \mu M$ CAT) but has little or no effect on the transport signal we measure in ER ($10\%$ inhibition at $2 \mu M$...
Second, by assaying marker enzymes in each vesicle preparation (cytochrome c reductase for mitochondria, and sialyltransferase for the Golgi apparatus) as well as a specific Golgi nucleotide sugar transporter for CMP-SA, we showed that there was very little (<2%) contamination by mitochondrial or Golgi membranes in our ER preparation.

There are several ATP dependent processes which occur in the lumen of the ER, and one unanswered question to date has been how does ATP get inside the ER? With this work we have shown that ATP is able to transport across the ER membrane by this translocator protein. Very recently, an ATP transport activity was also found in the endoplasmic reticulum of yeast (Mayinger, P., and Meyer, D. 1993).

By examining the binding of ATP to BiP, we could study a consequence of ATP transport. Photoaffinity labelling demonstrated that once ATP was transported into the ER it could be bound to lumenal ER proteins. This binding was specific for ATP, since we did not see the same phenomena with GTP. By taking advantage of the ATP transport inhibitor DIDS, we could show that the binding of ATP was dependent on its ability to be transported. When the transport of ATP was inhibited by DIDS, we saw a corresponding decrease in the amount of ATP bound to lumenal proteins. When ER vesicles were incubated with 20 μM DIDS, we saw a 40% decrease in the ATP radiolabelling of bulk lumenal proteins (by scanning desitometry), and a 35% decrease in ATP transport.
To show ATP binding specifically to BiP, we obtained a monoclonal antibody and, by immunoprecipitation and Western analysis, showed that BiP, a known lumenal protein, could be labeled with ATP when [α-32P] ATP was added to the outside of intact vesicles. This demonstrated that ATP was being transported inside the ER and that once inside it could specifically bind to BiP. As was discussed in the "Background" section, BiP may play a very important role in the folding and correct assembly of proteins in the ER, with ATP hydrolysis being a key step in its function (Dorner, A.J. et al, 1990; Braakman, I. et al, 1992). With our data we have shown how ATP becomes available to BiP in the lumen of the ER, that once inside the ER it does in fact bind to BiP, and that this binding is dependent on the transport.

Another protein of interest in the lumen of the ER is GRP94. Although not much is known about its function, it has been suggested that GRP94 and BiP may work together or in a similar manner, as catalysts in protein folding (Shiu, R.P.C. et al, 1977; Chang, S.C. et al, 1989). For this reason we looked at the binding of ATP to GRP94. By immunoprecipitation and Western analysis we were able to show for the first time a direct interaction of ATP with GRP94. Until this work there was no direct evidence that GRP94 was able to bind ATP, and because we used [α-32P]ATP we were able to look only at binding reactions. Although the binding of ATP to GRP94 was less intense than the binding of ATP to BiP by immunoprecipitation, we feel that it is specific based on the crosslinking data. This data lends
additional support to the hypothesis that GRP94 and BiP may work by similar mechanisms to control protein folding and subunit assembly in the lumen of the ER. Additionally, with the recent work by Melnick, J., et al, 1992, showing the presence of a complex containing both BiP and GRP94 together with unassembled immunoglobulin chains, it is becoming clear that BiP and GRP94 do work together in protein folding and assembly.

C. Reconstitution of UDP-galactose, UDP-xylose and UDP-glucuronic acid Transport Activities into Golgi Proteoliposomes.

Proteoglycans are a large class of glycoproteins with many diverse cellular processes. These proteins contain a very large and diverse carbohydrate region called the glycosaminoglycan (GAG) attached to the protein core via a linkage region made up of Xyl-Gal-Gal-GlucA. Many functions of the proteoglycan molecules have been attributed to the glycosaminoglycan chains themselves (Lebaron, R.G. et al, 1989; Yanyon, A. et al, 1991; Murphy-Ullrich, J.E. et al, 1988). As a first step toward understanding the diverse function of these proteins, we decided to study the biosynthesis of the proteoglycan linkage region. The synthesis takes place in the Golgi lumen, although the substrates are found in the cytosol. It was known from previous work that there were specific nucleotide sugar transporters for the substrates UDP-Gal, UDP-Xyl, and UDP-GluA in the Golgi
membrane (Nuwayhid, N. et al, 1986; Kimura, J.H. et al, 1984; Lohmander, L.S. et al, 1986). To ultimately purify a transporter protein(s) and study if or how they are regulated, we needed to reconstitute them into liposomes.

This work has shown the successful reconstitution of the transport activities for UDP-Gal, UDP-Xyl and UDP-GluA into Golgi proteoliposomes. We judge a successful reconstitution by three main criteria: the first is the affinity for substrate. In all cases we could reconstitute the activity of the individual transporters with the same apparent $K_m$ for substrate as that seen in the intact Golgi vesicles, and transport remained saturable at low ($\mu$M) concentrations. The second criterion is temperature sensitivity. In the intact Golgi vesicle, these transporters are very sensitive to temperature; they transport substrate at a much lower rate at 0°C than at 30°C (i.e. transport at 0°C is 10-25% of the transport value seen at 30°C). Once again, in each case we could reproduce this effect in the reconstituted system. The third criterion we use is inhibitor sensitivity. All of these transporters in the intact Golgi vesicle are affected to different degrees by a variety of inhibitors, both competitive and non-competitive. When we assayed these effects in the reconstituted state, we found that the inhibitor sensitivity was quite similar to that seen in the intact Golgi vesicles.

In every case, the rate ($V_{max}$) for transport into the reconstituted proteoliposomes was always lower than the intact Golgi vesicles (e.g. for UDP-galactose; the $V_{max}$ for transport into intact
Golgi vesicles is 150 pmol/mg/5min, while the $V_{\text{max}}$ for transport into proteoliposomes is only 15 pmol/mg/5min). There are a few possible explanations for this result. The first is that in the proteoliposomes only ~50% of the transporters are in the correct orientation. Also the solubilized Golgi membrane proteins must go through centrifugation, concentration and removal of detergent before they are added to the liposomes. These manipulations most likely will cause some loss of activity. However, one of the main reasons that we see a lower $V_{\text{max}}$ in the proteoliposomes is most likely due to the uncoupling of the transport/transfer system that occurs in the intact Golgi vesicles. In the proteoliposomes there is no transfer to acceptors on the inside of the liposomes. For this reason there is no "sink" to drive the substrate in at a faster rate.

One possible mechanism for how these transporter proteins function is via an antiporter mechanism (Hirschberg, C.B. and Snider, M.D., 1987). This hypothesis states that the entry of one nucleotide sugar molecule would be coupled to the exit of one corresponding monophosphate. We wanted to see if we could reproduce this effect in the reconstituted system. If the antiporter mechanism could be established in the reconstituted system, we should be able to increase the rate of transport into the proteoliposomes by preloading the proteoliposomes with the corresponding nucleotide monophosphate, the putative exchange substrate. In this case we would be supplying the energy, or a necessary component, needed for the transporter to pump against its concentration gradient. In
our studies we are looking at the transport of three different UDP-nucleotide sugars yielding the reaction product UMP. After preloading the liposomes with UMP, we were able to see an increase in the transport signal of 1.5-3 fold for each transporter.

One possible question that arose is that if UMP is necessary to provide energy to the transporter, how were we able to see transport in the proteoliposome in the absence of UMP? If we think of the proteoliposome as being "empty" (i.e. no UMP or UDP-sugar inside) then what we are seeing in the absence of UMP is transport following its concentration gradient from outside to inside, and then "stopping" at equilibrium. This is another reason, that the transport rate we see in the reconstituted system is much lower than in the intact vesicle: the proteoliposomes are not able to concentrate the substrate against its concentration gradient. When we provide the antiporter substrate on the inside, transport can proceed beyond equilibrium and we see an increase in the transport signal. Another explanation is to think of the transporter as having 2 binding sites, one on the outside for nucleotide sugar, and another on the inside for the corresponding nucleotide monophosphate. In the absence of substrate on the inside, the transporter is much slower or more inefficient in its ability to "flip" back to the outside once one molecule of nucleotide sugar is brought inside, thereby lowering the rate of substrate entry. In the presence of the substrate on the inside, however, the transporter protein now has this site filled and will quickly "flip" back to the outside to release the monophosphate.
It can then receive another nucleotide sugar, thereby increasing the rate at which the nucleotide-sugar binding site is exposed on the outside and subsequently increasing the rate of substrate entry. These explanations however, are only hypotheses which may be tested in a more rigorous manner using the reconstituted sytem with a purified protein.

When we initially began to study these transporter proteins by reconstitution, we chose to use phosphatidylcholine as the lipid source for the liposomes. One very interesting finding that we saw was that while both UDP-Gal and UDP-GluA transporters from this study (as well as CMP-SA and PAPS transporters (Milla, M.E. and Hirschberg, C.B., 1989)), could be reconstituted into phosphatidylcholine liposomes in a manner very similar to intact Golgi vesicles, the transport of UDP-Xyl could not. The only way that we could satisfy the reconstitution criteria for UDP-Xyl was to reconstitute the solubilized Golgi membrane proteins into liposomes made from extracted endogenous Golgi lipids. When we assayed UDP-Xyl transport under these conditions, we could observe transport characteristics that were similar to intact Golgi vesicles. To date, this is the only transporter protein we have studied that will not reconstitute well into phosphatidylcholine vesicles. This dependence on endogenous Golgi lipids was quite specific. When we made liposomes out of extracted endogenous lipids from a crude Golgi preparation as opposed to a pure Golgi preparation, we could measure a transport rate that was better than that seen with
phosphatidylcholine liposomes alone, but worse than the transport rate seen with pure endogenous Golgi lipid liposomes. These results suggested that some necessary Golgi component was being diluted as the preparation became more crude. This finding suggested a role for a specific Golgi lipid environment for the correct function of the UDP-Xyl transporter protein.

D. Purification of the UDP-galactose Transporter Protein from Rat Liver.

Using a modified version of the reconstitution system described above, I was able to purify the UDP-galactose translocator protein 45,000 fold from a rat liver homogenate. The original reconstitution system, although very accurate and reproducible, was not practical to monitor the purification of a protein. By substituting a Dowex anion exchange column for the Sephadex sizing column, I could remove the untransported UDP-Gal from the proteoliposomes containing UDP-Gal very efficiently.

The transporter protein binds very tightly to DEAE resin and is eluted with high salt. Because of this property, we see a very high purification with this column. The next series of columns are affinity purification steps. UDP-hexanolamine specifically binds UDP binding proteins, while the WGA column binds Golgi specific carbohydrates which allows us to narrow the field of proteins to only those that bind UDP and have Golgi specific carbohydrates. Finally, by running
the UDP-hexanolamine column again, this time using a gradient elution, I hoped that any remaining proteins that bound UDP would bind with different affinities and that they could be resolved in this way.

Using the technique of radiation inactivation we were able to get an accurate and reproducible calculation of the molecular weight of the UDP-Gal transporter protein in situ. This method gave a molecular weight of 100-120 Kd. From the iodinated samples off the UDP-hexanolamine II column (figure 16B) we can see a few bands that correlate with the activity profile in figure 16 A. One band is approximately 65Kd and the other is approximately 50Kd. These two bands are very good candidates for the UDP-Gal transporter protein, and the results from the radiation inactivation studies suggest that the UDP-Gal translocator in the membrane could be either a homodimer of either band or a heterodimer. The possibility that the UDP-galactose transporter protein is a dimer is supported by the data that other known antiporters such as the ADP/ATP carrier from mitochondria (Hackenberg, H., and Klingenberg, M. 1980; Klingenburg, M. 1981) is a dimer, the Na⁺/K⁺ ATPase (Esmann, M., et al 1980), and the Na⁺-H⁺ antiporter in brush border membranes (Beliveau, R., et al 1988) are multimers. However, this data is very tentative and we must keep in mind the alternative explanation of a two component system which will be more difficult to solve.

Overall, this study shows that we can use this reconstituted system as a means to monitor the purification of the UDP-galactose
translocator protein. We suggest, therefore, that this system could be used to purify any transporter protein that can be reconstituted into proteoliposomes while retaining activity in a manner similar to the intact system. Having a pure transporter protein is the first step in studying their mechanism. We would like, in the future, to be able to address the questions of localization of the UDP-Gal translocator within the Golgi apparatus as well as the transporter's role in proteoglycan glycosaminoglycan biosynthesis.
CHAPTER V

BIBLIOGRAPHY
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