Translocation of ATP into the Lumen of Rough Endoplasmic Reticulum-Derived Vesicles and Its Binding to Luminal Proteins Including BiP (GRP 78) and GRP 94*

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

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

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 \( V_{\text{max}} \): 3–7 pmol/min/mg of protein) and protein mediated because treatment of intact vesicles with Pronase, N-ethylmaleimide, or 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid inhibits transport. The entire ATP molecule is being translocated; this was shown by high performance liquid chromatography analysis and the use of a nonhydrolyzable analog. Control experiments rule out that translocation of ATP attributed to rough endoplasmic reticulum-derived vesicles is due to contamination by mitochondria and Golgi vesicles. Following translocation of ATP into the lumen of the vesicles, binding to luminal proteins including BiP (immunoglobulin heavy chain-binding protein-glucose-regulated protein 78) and glucose-regulated protein 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 rough endoplasmic reticulum lumen may serve as a mechanism for making ATP available in proposed energy requiring reactions within the lumen.

BiP\(^1\) (immunoglobulin heavy chain-binding protein) is an abundant luminal RER protein which is part of the group of endoplasmic reticulum resident proteins containing a KDEL retention sequence in their carboxyl terminus (1). BiP, which was found to be identical to glucose-regulated protein 78 (GRP 78) (2, 3), was so named originally because of its tight association with free immunoglobulin heavy chains (4). Since its initial discovery, BiP has been found to bind, in addition to heavy chains, incorrectly assembled multimeric proteins (4–7), proteins with incorrect disulfide bonds (8), mutant proteins (6, 9, 10), as well as aberrantly glycosylated proteins (5, 6, 8, 9). Experiments in vitro and in vivo have suggested that its role may be to promote the correct folding and assembly of secretory proteins (6) and to dissociate protein aggregates in the lumen of the RER and thus prevent secretion of incorrectly assembled or folded proteins (5, 6). 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 (3).

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 (11). One function for this carrier is to make ATP available to the Golgi lumen for subsequent phosphorylation of luminal 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 (3), 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 (12). 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 (13). These results, therefore, imply the existence of an ATP pool inside the lumen of the RER (14). 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 luminal proteins), but not GRP 94, appear to be (thio) phosphorylated.

**Experimental Procedures**

**Materials**

The following radioactive materials were purchased from Du Pont-New England Nuclear: [2,8-\(^3\)H]ATP, 29.6 Ci/mmol; \([\alpha-\(^32\)P\)]ATP, 3000 Ci/mmol; \([\gamma-\(^35\)S\)]ATP, 1273 Ci/mmol; \([\gamma-\(^32\)P\)]ATP, 3000 Ci/mmol; \([\alpha-\(^32\)P\)]GTP, 3000 Ci/mmol; \([\beta-\(^32\)P\)]orthophosphate carrier free; CMP-\(N-\[^9\-\(^3\)H\)]\)acetylneuraminic acid, 13.6 Ci/mmol. Nonradioactive nucleotides and nucleotide derivatives, carboxyatractyloside, NEM (N-ethylmaleimide), and DIDS (4,4'-disothiocyanato-2,2'-disulfonic acid stilbene) were purchased from Sigma. ATP\(^\gamma\)S was purchased from

---

*This work was supported by Grant GM 34396 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\(^1\)The abbreviations used are: BiP, immunoglobulin heavy chain-binding protein; ATP\(^\gamma\)S, adenosine 5'-O-(thio)triphosphate; DIDS 4,4'-disothiocyanostilbene-2,2'-disulfonic acid; RER, rough endoplasmic reticulum; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PAPS, adenosine 3'-phosphate,5'-phosphosulfate; GRP, glucose-regulated protein; NEM, N-ethylmaleimide; HPLC, high performance liquid chromatography.
Boehringer Mannheim. Pronase was purchased from Behring Diagnostics.

**Purification of Subcellular Fractions**

Subcellular fractions from liver were obtained from 150-200 g of Sprague-Dawley male rats. To prepare RER vesicles, rats were killed by decapitation. RER-derived vesicles were isolated as described by Carey and Hirschberg (15) using a modification of the subcellular fractionation procedure originally described by Fleischer and Kervina (16). Protein was determined according to Peterson. (17) RER-derived vesicles were enriched 5-fold over homogenate in sialyltransferase specific activity. (21) 19% yield of total homogenate). Vesicles were at least 90% intact as determined by latency of glucose-6-phosphatase toward mannose 6-phosphate (19). Vesicles were resuspended in 0.06% Triton X-100 in a final concentration of 10 nM [α-32P]ATP (30 pCi) to give a final concentration of 1 μM ATP (50 μCi) for 5 min at 30 °C. Microsomes were then subjected to UV cross-linking for 5 min on ice as described above and vesicles were centrifuged for 25 min at 30,000 g in an airfuge. The vesicle pellet was resuspended in 0.1 ml of 0.5 mM 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 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 radiolabeled with ATP and luminal proteins were extracted as described above.

**ATP Translocation Assay**

Transport was measured using the centrifugation assay previously described (11, 24). Briefly, vesicles from the endoplasmic reticulum, Golgi, or mitochondria (0.5-1.0 mg of protein/ml) were incubated for 2-5 min at 30 °C in a final volume of 1 ml of STMF buffer containing 0.5 mM 2,3-dimercaptopropanol, 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 min at 100,000 × g at 4 °C. The supernatant was removed and analyzed for radioactivity. The luminal proteins (30 μg protein) were then subjected to SDS-PAGE and autoradiography.

**Immunoprecipitation and Western Blotting of Canine Pancreas Microsomal Proteins**

**Immunoprecipitation**—Canine pancreas microsomes (20 μg) were incubated in 100 μl of G buffer at a final concentration of 0.5 μM ATP (0.5 μCi) and subjected to UV cross-linking 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 100,000 g in a Ti-50 Beckman rotor at 4 °C. Soluble proteins in supernatant (0.5 ml) were added to a 3 ml of protein A-Sepharose beads (Pharmacia). Sample buffer (10 ml of 0.5 M 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 Lealavath et al. (20) and were enriched 64-fold over homogenate in sialyltransferase specific activity. (21) 21% yield of total homogenate). At least 90% of the vesicles were sealed, based on the latency of endogenous sialyltransferase activity (21). Golgi-derived vesicles were isolated as described by Fleischer and Kervina (16) and were enriched 7.1-fold in succinate-cytocrome c reductase specific activity (22) (8.3% yield). Canine pancreas microsomes were a generous gift of the R. Gilmore laboratory and were prepared as previously described (23).

**Immunoaffinity Chromatography**

Rat liver RER-derived vesicles (0.2-0.4 mg of protein) were incubated for 3 min at 30 °C in a final volume of 100-200 μl of incubation medium (STMF buffer containing 2,3-dimercaptopropanol). When DIDS inhibition was measured, vesicles were first pretreated with or without DIDS for 10 min on ice (26). The incubation medium contained a final concentration of 10 μM [α-32P]ATP (30 μCi). Following the incubation, the sample was placed in an ice-cold porcelain dish and exposed to UV light for 5 min (3.6 milliwatts/cm²). The sample was then removed and layered onto a 50-μl sucrose cushion, centrifuged in an airfuge at 30,000 g, and further processed as described above.

**Radiolabeling of RER Luminal Proteins**

Rat liver RER-derived vesicles (0.2-0.4 mg of protein) were incubated for 3 min at 30 °C in a final volume of 100-200 μl of incubation medium (STMF buffer containing 2,3-dimercaptopropanol). When DIDS inhibition was measured, vesicles were first pretreated with or without DIDS for 10 min on ice (26). The incubation medium contained a final concentration of 10 μM [α-32P]ATP (30 μCi). Following the incubation, the sample was placed in an ice-cold porcelain dish and exposed to UV light for 5 min (3.6 milliwatts/cm²). The sample was then removed and layered onto a 50-μl sucrose cushion, centrifuged in an airfuge at 30,000 g, and further processed as described above.

**Identification of Radioactive Solutes within Vesicles and Incubation Medium**

Percoll acid was first removed from the solutions within the vesicles as previously described (28). Briefly, 0.75 ml of the 50% Percoll fraction (see below) was combined with 1.5 ml of the extraction mixture (22 ml of trichloroacetic acid, 5.3 ml of 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. Adequate ATP, AMP, ADP, ATP, and ATP-S were separated by HPLC using a Synchronax AX-100 column as previously described (11). The following linear gradient was run: 0.6 M KH₂PO₄, pH 3.35, from 0 to 5 min; the concentration was then increased to 0.66 M KH₂PO₄, pH 3.35, and a linear gradient was run from 0 to 10 min 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-S, 20.03 min.
**SDS-Polyacrylamide Gel Electrophoresis**

SDS-PAGE was done with the buffer system described by Laemmli (29) 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 h at 18 mA with a cooling bath at 10 °C. Protein was visualized with Coomassie Blue staining. Gels were autoradiographed for 18–24 h 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 (30) and an example is given in Table I. Briefly, $[S_o]$ = concentration of solutes in the incubation medium (micromolar) = (counts/min/ml of solutes in the supernatant)/(specific activity of solute expressed as counts/min/nmol). $S_t$ = total solutes in the pellet expressed in picomole/mg of protein = (total soluble radioactivity associated with the pellet, expressed as counts/min/mg of protein)/(specific activity of solutes, expressed as counts/min/pmol). $V_i$ = total pellet volume (microliter/mg of protein) = volume outside (in between) + inside vesicles = (counts/min/mg of protein in the pellet for deoxyglucose)/(counts/min/μl of supernatant for deoxyglucose). $V_o$ = pellet volume which is inside (between) vesicles (microliter/mg of protein) = (counts/min/mg of protein in pellet for acetate)/(counts/min/μl of supernatant for acetate). $V_v$ = pellet volume which is outside (between) vesicles in the pellet (picomole/mg of protein) = $V_o$ (microliter/mg of protein) × $[S_o]$ (picomole/μl). $S_t$ = solutes inside vesicles in the pellet (picomole/mg of protein) = $S_v$ = $S_o$ − $S_t$. $[S_i]$ = concentration of solutes inside vesicles in the pellet (micromolar) = $S_v$ (picomole/mg of protein)/$V_i$ (microliter/mg 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 (19), were incubated with $[^{32}P]$ATP. Following centrifugation of the vesicles and further processing as described under “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 I). These solutes have previously been shown to be the sum of solutes within vesicles in the pellet, $S_v$, and those in between (outside) the vesicles of the pellet, $S_o$ (30). Detailed calculations are described under “Experimental Procedures” and in the legend of Table I. 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 picomole/μl) is multiplied by the volume which is in between (outside) the vesicles in the pellet, $V_o$ (in microliter/mg of protein). This latter value is obtained from the volume accessible to sodium acetate which shows the same distribution as the nonpenetrator inulin (25). Table I shows that only a small amount of total solutes in the pellet were in between (outside) the vesicles, $S_o$, and that approximately 82% of the total radioactive solutes in the pellet were within vesicles, $S_v$. The concentration of radioactive solutes within the vesicles in the pellet, $[S_i]$, was obtained by dividing the solutes within the vesicles, $S_v$, 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, $V_o$, from the total volume of the vesicle pellet, $V_v$. This value was obtained by determining the volume accessible to a standard penetrator, deoxyglucose (25). Table I also shows that there was a 12-fold concentration of radioactive solutes, derived from $[^{32}P]$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 (not shown).

It was important to determine whether the radioactive solutes detected within the lumen of the vesicles incubated with $[^{32}P]$ATP could have arisen from breakdown in the incubation medium of ATP to $[^{32}P]$orthophosphate and subsequent entry of this solute into the lumen of the vesicles. When RER vesicles were incubated with the same concentration of $[^{32}P]$orthophosphate as previously used for $[^{32}P]$ATP, less than 2 pmol/mg of protein of radiolabeled solutes were within the lumen of the vesicles (experiment 3, Table I). These solutes were enriched only 2-fold over the concentration of $[^{32}P]$orthophosphate in the incubation medium, strongly suggesting, as a first approximation, that $[^{32}P]$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 the lumen of RER vesicles intact and what its fate was following entry. Intact RER vesicles were therefore incubated with $[^{3}H]$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, $[^{32}P]$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 $[^{32}P]$ATP could be transported into the lumen of RER vesicles. As shown in Table I, experiment 2, almost 3-fold higher amounts of radioactive solutes were detected in the lumen of the vesicles compared to incubations with $[^{32}P]$ATP even though the $[^{32}P]$ATP concentration in the incubation medium was 20% lower than when $[^{3}P]$ATP was used (experiment 1). It can also be seen that incubations with $[^{32}P]$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 $[^{32}P]$ATP was the only radiolabeled solute within the vesicles. In experiments not shown, we also established that $[^{32}P]$ATP enters the lumen of Golgi vesicles with an affinity and a rate very similar to that previously described for $[^{32}P]$ATP (11); with $[^{3}S]$ATP the $K_{m}$ was 0.3 μM and the $V_{max}$ was 67 pmol/mg of protein/5 min while with $[^{32}P]$ATP the $K_{m}$ was 0.9 μM and the $V_{max}$ was 58 pmol/mg of protein/5 min. We found less degradation of $[^{32}P]$ATP in the incubation medium when Golgi vesicles were used instead of RER. We also determined that orthophosphate enters the lumen of RER vesicles in a very slow nonsaturable manner (not shown). 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 (11). 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 mitochondria contaminating the RER vesicle prep-
Translocation of [γ-32P]ATP and [γ-35S]ATP into RER-derived vesicles: concentration of radioactive solutes within vesicles following a 5-min incubation

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.0 ± 0.1</td>
<td>8.8 ± 1.0</td>
</tr>
<tr>
<td>2</td>
<td>1.6 ± 0</td>
<td>22.3 ± 1.6</td>
</tr>
<tr>
<td>3</td>
<td>2.0 ± 0.1</td>
<td>1.7 ± 0.1</td>
</tr>
</tbody>
</table>

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 luminal marker, glucose-6-phosphatase, was not affected. As seen in Table III, these impermeable effectors significantly reduced ATP translocation, suggesting that it is mediated by a protein with a cytosolic domain. This is analogous to translocation of nucleotide sugars and ATP into the Golgi lumen (14, 24).

We had previously determined that DIDS was an inhibitor of transport of nucleotide sugars, PAPS, and ATP into the lumen of Golgi-derived vesicles (26). As shown in Fig. 2, DIDS also inhibited the transport of ATP into rat liver microsomes.

Translocation of ATP into the Lumen of RER-derived Vesicles Is a Prerequisite for Its Binding and (Thio) Phosphorylating Luminal 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. We therefore incubated rat liver RER vesicles with [α-32P]ATP, followed by exposure to UV light to allow cross-linking of ATP to luminal proteins. These were then extracted, and subjected to SDS-PAGE and autoradiography. As shown in Fig. 3, lane 1, several luminal proteins became radioiodinated.

We wanted to determine whether inhibition of ATP transport into RER vesicles also resulted in decreased ATP cross-linking to luminal proteins. For this purpose, the previously described radiolabeled ATP cross-linking protocol was repeated, with the difference that 20 and 100 μM DIDS were first added for 10 min to the vesicles on ice. As shown in Fig. 3, lane 2, 20 μM DIDS produced approximately 40% inhibition of radiolabeling of bulk luminal proteins while ATP translocation was inhibited by 35%. 100 μM DIDS pretreatment (lane...
relative to the incubation medium, occurring as a consequence of transport (Table I).

Preliminary studies had shown that GTP could not enter vesicles and then immediately used for transport assays with 10 μM [α-

Fig. 3. Reciprocal plots of ATP transport into 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-32P]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-phosphatase was then measured as described under "Experimental Procedures." It is important to rule out the possibility that the effect of DIDS on ATP transport and subsequent binding to luminal proteins was due to a direct inhibition by DIDS on binding of ATP to these luminal proteins. We therefore subjected permeabilized vesicles to the previous cross-linking protocol in the presence or absence of DIDS. As shown in Fig. 3, lanes 4–6, one can see that the inhibition by DIDS of ATP radiolabeling of luminal proteins was not seen with permeabilized vesicles. The same result was obtained with vesicles permeabilized with octylglucoside (not shown). Together with the above results, this strongly suggests that DIDS inhibited transport of ATP into the lumen but not binding per se to luminal proteins. It can also be seen that the intensity of radiolabeled luminal proteins in the absence of DIDS was significantly higher in intact than in permeabilized vesicles (lane 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 I).

Preliminary studies had shown that GTP could not enter the lumen of RER vesicles (not shown). When intact rat liver
RER-derived vesicles were incubated with \([\alpha^{32}\text{P}]\text{GTP}\) and then subjected to UV cross-linking as described above, no radiolabeling of luminal proteins was observed consistent with no transport of GTP into the lumen. Upon incubation of permeabilized vesicles with GTP, no distinct protein bands were radiolabeled (Fig. 3, lane 8) and the pattern was different from radiolabeling with ATP.

We wanted next to determine whether ATP was bound to specific luminal 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 \(K_{\text{max}}\) of 3 \(\mu\text{M}\) and a \(V_{\text{max}}\) of 14 pmol/mg of protein/5 min. We also determined that DIDS inhibited transport of ATP into the lumen of these microsomes: at 20 \(\mu\text{M}\) DIDS, the inhibition was 35% while at 100 \(\mu\text{M}\) it was 40%.

Canine pancreas microsomes were then incubated with radiolabeled ATP and subjected to cross-linking as described above. Fig. 4, lane 1, shows that luminal proteins, including those with apparent mobility of 78 and 94 kDa, were radiolabeled. This radiolabeling was inhibited by preincubating microsomes with 20 \(\mu\text{M}\) DIDS (Fig. 4, lane 2). This stilbene derivative does not inhibit cross-linking of a protein of apparent mobility of 78 kDa per se as shown with permeabilized microsomes. However, because ATP was not concentrated within these microsomes, other proteins were not radiolabeled as intensely as with intact vesicles (Fig. 4, lanes 3 and 4).

Further proof that the radiolabeled proteins were indeed GRP 78 and GRP 94 was obtained by Western blots with antibodies against BiP and GRP 94 (Fig. 4, lanes 5 and 6) and by immunoprecipitation of the \([\alpha^{32}\text{P}]\text{ATP}\)-labeled luminal proteins with the corresponding antibodies (Fig. 5, lanes 2 and 3).

Previous studies had shown that while BiP is phosphorylated (2), GRP 94 is not (31). 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 \([\gamma^{32}\text{S}]\text{ATP}\) with or without UV cross-linking as described previously. As shown in Fig. 6, lane 1, proteins of mobilities of 78 and 94 kDa become radiolabeled upon cross-linking, but only BiP is labeled in the absence of UV, suggesting that only the latter is (thio)phosphorylated.

**DISCUSSION**

This study has shown that, in a system in vitro, rough endoplasmic reticulum-derived vesicles can translocate intact

---

**Fig. 3.** ATP and GTP translocation into rat liver RER vesicles and subsequent binding to luminal proteins: composite electropherogram-autoradiogram. Intact RER vesicles (lanes 1–3) or permeabilized ones (lanes 4–6) were pretreated without (lanes 1 and 4) or with 20 (lanes 2 and 5) or 100 \(\mu\text{M}\) (lanes 3 and 6) DIDS. Vesicles were then incubated with 30 \(\mu\text{Ci}\) of \([\alpha^{32}\text{P}]\text{ATP}\) and luminal proteins extracted from the intact vesicles as described under "Experimental Procedures." Following SDS-PAGE, the samples were subjected to autoradiography. In lanes 7 and 8, intact or permeabilized vesicles were labeled with 30 \(\mu\text{Ci}\) of \([\gamma^{32}\text{P}]\text{GTP}\) as described under "Experimental Procedures." The microsomes were incubated with \([\alpha^{32}\text{P}]\text{ATP}\) and luminal proteins extracted. Following SDS-PAGE the samples were subjected to autoradiography. The arrows mark the positions of protein with mobilities of 78 and 94 kDa. Luminal microsomal proteins were transferred to nitrocellulose as described and blotted with a non-immune serum (lane 5), or antibodies against BiP (lane 6) or GRP 94 (lane 7). Bands were visualized with alkaline phosphatase reaction.

**Fig. 4.** ATP translocation into dog pancreas microsomes and subsequent binding to BiP, GRP 94, and other luminal proteins: composite electropherogram-autoradiography of microsomal luminal proteins, and identification of BiP and GRP 94 by Western blotting. Intact (lanes 1 and 2) or permeabilized (lanes 3 and 4) dog pancreas microsomal vesicles were preincubated with 20 \(\mu\text{M}\) DIDS (lanes 2 and 4) or without DIDS (lanes 1 and 3) as described under "Experimental Procedures." The microsomes were incubated with \([\alpha^{32}\text{P}]\text{ATP}\) and luminal proteins extracted. Following SDS-PAGE the samples were subjected to autoradiography. The arrows mark the positions of protein with mobilities of 78 and 94 kDa. Luminal microsomal proteins were transferred to nitrocellulose as described and blotted with a non-immune serum (lane 5), or antibodies against BiP (lane 6) or GRP 94 (lane 7). Bands were visualized with alkaline phosphatase reaction.

**Fig. 5.** ATP transport into canine pancreas microsomes and subsequent binding to BiP and GRP 94: composite electropherogram-autoradiography of immunoprecipitates with anti-BiP and anti-GRP 94 antibodies. Canine pancreas microsomes were labeled with ATP as described under "Experimental Procedures." The labeled microsomes were then immunoprecipitated with a non-immune serum (lane 1), or antibodies against BiP (lane 2) or GRP 94 (lane 3), and subjected to autoradiography. The arrows mark the position of BiP (78 kDa) and GRP 94 (94 kDa). Lanes 1 and 2 were exposed for 6 days, while lane 3 was exposed for 28 days.
ATP from the incubation medium into their lumen. Translocation of ATP was found to be saturable with an apparent $K_v$ of 3–4 $\mu$M and inhibited by inhibitors of anion transport such as DIDS. Because transport was inhibited by proteases and N-ethylmaleimide under conditions where a luminal 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 suggested that the mechanism of translocation is via an antipporter, AMP or ADP. This is similar to the mechanism of translocation of ATP in the Golgi apparatus (11) and that of nucleotide sugars and nucleotide sulfate in the Golgi apparatus and rough endoplasmic reticulum (24).

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 carboxyatractyslactoside 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 mitochondrial 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 (3, 12, 13). 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. 3, lanes 1 versus 4 and Fig. 4, lanes 1 versus 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 (32). Although a role for GRP 94 is unknown, its synthesis is increased during glucose starvation (32, 33), as well as by conditions that cause an increase in aberrant proteins in a manner similar to that of BiP/GRP 78 (33, 34). However, no enzymatic activity has been associated with GRP 94.

ATP binds BiP with approximately 10-fold higher affinity than ADP and 1000-fold higher than AMP (12). Because the cross-linking experiments were done with the radiolabeled 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 cross-linked nucleotide. The UV cross-linking experiments with [$\gamma$-${}^{32}$S]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 (35). It was found that this gene is essential for cell viability. This result shows that BiP/GRP 78 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 (11) and vitellogenin has 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.

Acknowledgments—We thank Reid Gilmore, Paula Collins, and Peter Rapejeiko for canine dog microsomes, Linda Hendershot and Amy Lee for anti-BiP and GRP-94 antibodies, Aravinda de Silva for very helpful discussions, and Karen Welch and Annette Stratton for excellent typing.

Note Added in Proof—Additional recent publications on the putative functions of BiP and GRP 94 have appeared (36–38).

REFERENCES
ATP in the RER Lumen