Phosphorylation of secretory and integral membrane proteins and of proteoglycans also occurs in the lumen of the Golgi apparatus. ATP, the phosphate donor in these reactions, must first cross the Golgi membrane before it can serve as substrate. The existence of a specific ATP transporter in the Golgi membrane has been previously demonstrated in vitro using intact Golgi membrane vesicles from rat liver and mammary gland.

We have now identified and purified the rat liver Golgi membrane ATP transporter. The transporter was purified to apparent homogeneity by a combination of conventional ion exchange, dye color, and affinity chromatography. An ~70,000-fold purification (2% yield) was achieved starting from crude rat liver Golgi membranes. A protein with an apparent molecular mass of 60 kDa was identified as the putative transporter by a combination of column chromatography, photoaffinity labeling with an analog of ATP, and native functional size determination on a glycerol gradient. The purified transporter appears to exist as a homodimer within the Golgi membrane, and when reconstituted into phosphatidylcholine liposomes, was active in ATP but not nucleotide sugar or adenosine 3′-phosphate 5′-phosphosulfate transport. The transport activity was saturable with an apparent $K_m$ very similar to that of intact Golgi vesicles.

Post-translational modifications of proteins, occurring in the lumen of the Golgi apparatus, include glycosylation, sulfation, and phosphorylation. Although the significance and general mechanistic features of the first two reactions are well understood, very little is known about the latter one. Secreted proteins such as caseins (1, 2) and vitellogenin (3, 4) and integral Golgi membrane proteins (5) have been shown to undergo phosphorylation. Although it has been suggested that phosphorylation can contribute to maintaining the stability of the protein by protecting it against proteolytic degradation in situ (1, 2) or, in the case of proteoglycans, as a specific targeting signal (7), the significance of these post-translational modifications remains to be determined.

ATP, the phosphate donor in the above phosphorylation reactions, is synthesized mainly in the mitochondrial matrix. To be accessible to the lumen of the Golgi apparatus, where these reactions occur, it must first cross the mitochondrial membranes through a specific and well-characterized transporter and then the Golgi membrane. The existence of a specific transporter in the latter membrane has been demonstrated in vitro using intact rat liver and mammary gland Golgi membrane vesicles (5). Transport was found to be temperature-dependent, saturable at micromolar concentrations of ATP and appears to be via an antiporter mechanism (9, 10), with AMP being the most likely antiporter (5). Casein phosphorylation was found to occur subsequent to transport of ATP into the lumen of rat mammary gland Golgi vesicles (5). ATP transport has also been shown to occur across the membrane of the rough endoplasmic reticulum (ER) (11), where it is involved in energy-requiring reactions (reviewed in Ref. 12) and phosphorylation reactions such as of BiP (11, 13). The former include also dissociation of complexes between chaperones and correctly folded and assembled proteins in the lumen of the ER, disulfide bond formation, and protein polymerization (reviewed in Ref. 14). Recently, it has also been shown that ATP is required for protein translocation into the ER of yeast and mammalian cells (reviewed in Refs. 15 and 16).

To understand the importance of phosphorylation as a post-translational Golgi luminal event and to analyze the possibility that regulation of ATP transport into the Golgi lumen can affect the biosynthesis and function of macromolecules being synthesized in the Golgi apparatus, we need initial knowledge of the amino acid and nucleic acid sequences of such transporter. Moreover, the existence of specific ATP transporters located in the membranes of three different intracellular organelles, mitochondria, Golgi apparatus, and ER, raises the question of how these proteins with the same function are localized in different organelles and whether or not they share common structural features.

Here we used a reconstituted phosphatidylcholine proteoliposome system (17) to monitor the purification of the ATP transport activity from a rat liver Golgi membrane preparation. Column chromatography and photoaffinity radiolabeling followed by SDS-PAGE electrophoresis were used to identify a 60-kDa protein as the ATP transporter. Proteoliposomes containing this protein were active in ATP but not in nucleotide sugars or PAPS transport; a similar apparent $K_m$ of ATP transport than previously reported for intact Golgi vesicles was

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### Supporting Information

1 The abbreviations used are: ER, endoplasmic reticulum; PAPS, adenosine 3′-phosphate 5′-phosphosulfate; PAGE, polyacrylamide gel electrophoresis.
Purification of the Golgi Membrane ATP Transporter

**TABLE I**

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein</th>
<th>Specific activity</th>
<th>Activity</th>
<th>Fold purification</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triton extract</td>
<td>25,400</td>
<td>134</td>
<td>3,400,000</td>
<td>15</td>
<td>100</td>
</tr>
<tr>
<td>1st DEAE-Sephael</td>
<td>2,040</td>
<td>1,176</td>
<td>2,400,000</td>
<td>130</td>
<td>72</td>
</tr>
<tr>
<td>Blue-Sephael</td>
<td>540</td>
<td>3,703</td>
<td>2,000,000</td>
<td>390</td>
<td>59</td>
</tr>
<tr>
<td>2nd DEAE-Sephael</td>
<td>19,528</td>
<td>26,923</td>
<td>1,400,000</td>
<td>2,700</td>
<td>41</td>
</tr>
<tr>
<td>Carboxymethylcellulose</td>
<td>3</td>
<td>303,333</td>
<td>910,000</td>
<td>30,000</td>
<td>27</td>
</tr>
<tr>
<td>3'-5'-ADP-agarose</td>
<td>0.5</td>
<td>566,000</td>
<td>283,000</td>
<td>60,000</td>
<td>8</td>
</tr>
<tr>
<td>Glycerol gradient</td>
<td>0.1</td>
<td>656,000</td>
<td>65,656</td>
<td>70,200</td>
<td>2</td>
</tr>
</tbody>
</table>

Frozen rat livers were purchased from Pel-Freez Biologicals. [2,8-\[^3^H\]ATP (15–30 Ci/mmole) was purchased from American Radiolabeled Chemicals, Inc. Carboxymethylcellulose 3 (Sigma; 5 cm × 5 cm each) equilibrated in buffer A containing 0.2 M NaCl was purchased from Amersham Pharmacia Biotech, and 8-azido-[\(^{32}\)P]ATP (2–15 Ci/mmole) was purchased from ICN Pharmaceuticals, Inc. Extradi-Gel G was purchased from Pierce. All other chemicals were obtained from Sigma.

**Methods**

**Purification of the Rat Liver Golgi Membrane ATP Transporter**

All the operations described below were performed at 4 °C. **Step 1: Detergent Extraction**—A crude Golgi fraction was prepared from 6 kg of frozen rat livers according to the procedure of Leelavathi et al. (18). This fraction was resuspended in 10 mM Tris-HCl, pH 7.2, 1 mM MgCl\(_2\), 1 mM dithiothreitol, 0.3 mM sucrose, 20% glycerol (v/v), 0.3% Triton X-100 (v/v) with protease inhibitors (0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.1 mM antipain, 0.1 mM aprotinin, and 1 mM leupeptin, and 0.1 mM pepstatin A). The suspension was stirred for 45 min at 4 °C and centrifuged at 80,000 g for 1 min in a Sorvall 2:2 rotor at 40,000 rpm for 40 h at 4 °C. Fractions of 0.35 ml were collected. g-Allen (200 μl), 400 μl, 1 mM dehydrogenase (150 μl), d-galactosidase (120 μl), phosphohexose B (100 μl), tumor necrosis factor a-converter (80 μl), and bovine serum albumin (66 μl) were used as internal molecular markers.

**Photoaffinity Radiolabeling with 8-Azido-[\(^{32}\)P]ATP**

All the following experiments were performed in a dark room in the presence of a filtered safe-light. Fractions to be photolabeled were incubated with 8-azido-[\(^{32}\)P]ATP (0.2 μM final concentration) at 0 °C for 1 min in 25 μl of buffer A. The mixture was irradiated on ice for 1 min in a Stratalinker UV 2400 oven (Stratagene; 5 cm of distance, maximum energy), and the reaction was stopped by the addition of loading buffer. Samples were then immediately subjected to 10% SDS/PAGE, and the autoradiography of the dried gel was done at −80 °C on Kodak film.

**Isolation and Topography of Rat Liver Golgi Vesicles**

For the characterization of the ATP transporter, rat liver Golgi vesicles were isolated as described (18) and resuspended in cryoprotective buffer (19). Sialyltransferase activity was enriched ~50-fold over crude homogenate. Approximately 90% of the vesicles were sealed and of the same membrane topographical orientation as in vivo (20).

**Transport Assay**

Transport of solutes into intact rat liver Golgi vesicles was assayed as described before (21). To follow the transporter purification, the ATP transport activity was reconstituted in phosphatidylcholine liposomes (17, 22, 23) and incubated in the presence of [\(^3^H\)]ATP (4 μM; 400 cpm/pmol) for 5 min at 30 °C. The reaction mixture was then applied to a 3-ml Dowex 1 × 2–100 column (Sigma) as described previously (22, 23). Fractions of 300 μl were collected, and the radioactivity was determined by liquid scintillation spectrometry.

**Protein Visualization**

The purity of the various fractions active and inactive in ATP transport activity was determined by SDS/PAGE. Visualization was done by Coomassie Blue/silver nitrate staining (OWL Separation System) or by labeling proteins with 300 μCi of Na\(^{125}\)I and chloramphenicol T (22, 23). Protein was quantified using the BCA protein assay kit (Pierce).

**RESULTS**

**Purification of the ATP Transporter**—The rat liver Golgi membrane ATP transporter was purified ~70,000 over the crude Golgi membrane preparation with a yield of 2% (Table I). To monitor the purification through the different purification steps, membrane determined. Finally, native functional size determination on a glycerol gradient suggested that the ATP transporter exists as a homodimer in the membrane of the Golgi apparatus.
proteins were reconstituted into phosphatidylcholine liposomes by freeze-thawing and then assayed for their ability to translocate radiolabeled ATP in vitro. The purity of the ATP transporter during the purification was determined by SDS/PAGE (Fig. 1). We began the purification with a crude Golgi membrane preparation; the ATP transport activity was extracted after a two-step solubilization with Triton X-100. In the first step, Golgi membranes were treated with a low concentration of Triton X-100 (0.3%, v/v). These conditions removed the peripheral membrane proteins and did not result in a significant loss of transport activity or in its extraction from the membrane. In the second step, a higher concentration of Triton X-100 was used (1.1%, v/v) to almost completely solubilize the membrane proteins. Approximately 85% of the total ATP transporter activity from the Golgi membrane preparation could be solubilized under these conditions, with a 15-fold purification over the crude Golgi membrane preparation.

The Triton X-100 extract was then applied to a combination of conventional ion exchange, dye color, and affinity chromatography columns. Details of the different chromatographic steps are given under “Experimental Procedures,” and the results of each step are given in Table I.

The Triton X-100 extract (Fig. 1, lane 1) was loaded onto a first DEAE-Sephacel column followed by elution with 0.7 M NaCl in buffer A. 72% of the transport activity was recovered with a 130-fold purification over the crude Golgi preparation (Fig. 1, lane 2). In the next two chromatographic steps, the Blue-Sepharose and the second DEAE-Sephacel column, the transport activity was found in the flow-through (negative columns). These two negative columns combined together, resulted in the binding of most of the applied proteins but not the ATP transport activity (Fig. 1, lanes 3 and 4). 41% of the initial activity was recovered after these two steps with a 2,700-fold overall purification. The active fraction obtained from the second DEAE-Sepharose was then loaded onto a carboxymethylcellulose column and eluted with a linear gradient of 0.1–1.5 M NaCl and 20–1% glycerol (Fig. 2) with a 30,000-fold overall purification (Fig. 1, lanes 5 and 6). In the next step we used a 3’-5’-ADP-agarose column, which provided an important and substantial purification (Fig. 1, lanes 7 and 8). The transport activity was eluted with a linear gradient of 0–1.5 M NaCl and 10–1% glycerol (Fig. 3). This strategy resulted in a 60,000-fold overall purification with a recovery of 8% of the initial transport activity. In order not to use high volumes of this active fraction, small aliquots of the sample were subjected to radioiodination with chloramine T before electrophoresis and visualization by autoradiography. The SDS-gel profile of the active fraction (Fig. 1, lane 7) showed two protein bands of 60- and 58-kDa. These were not visualized in fractions inactive for ATP transport activity (Fig. 1, lane 8).

**Glycerol Gradient—**A glycerol gradient was used as a last step of purification and to estimate the functional size of the ATP transporter. The rationale for this was based on the fact that other Golgi nucleotide sugar and nucleotide sulfate transporters appear to be homodimers in the membrane (9, 10) and, when solubilized in the presence of 0.5% Triton X-100, also behave as dimers (22, 23). The pooled active fraction from the 3’-5’-ADP-agarose column was loaded on top of a 8–30% glycerol gradient and centrifuged for 40 h, as described under “Experimental Procedures.” Fig. 4A shows the profile of the transporter activity throughout the gradient; a peak in the 120-kDa area, corresponding to the native protein, was observed with a single protein band of 60 kDa in the denaturing gel (Fig. 1, lane 9, and Fig. 4B), which correlated with the ATP transport activity.
transport activity (Fig. 4). This latter strategy gave a 70,000-fold overall purification and a recovery of 2% of the initial transport activity. Photoaffinity Radiolabeling with 8-Azido-[γ-32P]ATP—Photoaffinity radiolabeling was used as an independent criterion to demonstrate that the transport activity is a protein of 60 kDa. We reconstituted into proteoliposomes active fractions from the glycerol gradient (see Fig. 1, lane 9) and inactive fractions from the carboxymethylcellulose (see Fig. 1, lane 6); aliquots of active and inactive fractions were radioiodinated before SDS-PAGE and then subjected to autoradiography.

Characterization of the ATP Transporter—To characterize the ATP transporter, we reconstituted the highly purified 3′-5′-ADP-agarose fraction (Fig. 1, lane 7) into phosphatidylcholine liposomes. Transport of ATP into proteoliposomes was saturable with an apparent $K_m$ of 3.3 μM (Fig. 6), very similar to that of intact Golgi vesicles (1.3 μM; Fig. 6, inset). The same fraction was inactive in transport of PAPS, CMP-sialic acid, and UDP-N-acetylgalactosamine (Table II).

DISCUSSION

We have identified, purified, and characterized the ATP transport activity from rat liver Golgi membranes. The transporter showed an apparent molecular mass of 60 kDa, and its identity was confirmed by functional reconstitution of the purified protein into liposomes as well as photoaffinity labeling.

To purify the ATP transporter protein by column chromatography to apparent homogeneity, a 70,000-fold purification was required. This fold of purification was expected because a similar apparent fold was required for other low-abundance Golgi membrane proteins such as the UDP-GalNAc transporter.
the PAPS transporter (22), and the heparan sulfate N-deacetylase/N-sulfotransferase (24).

After glycerol gradient ultracentrifugation, the transporter migrated in the 120-kDa area, twice its apparent molecular mass as determined by reducing gel electrophoresis, suggesting that the ATP transporter is functional as a homodimer in Golgi membranes. These results are consistent with previous reports showing that some nucleotide derivative transporters are arranged in the Golgi membrane as homodimers (9, 10) and with analogous results obtained by us with the PAPS (22) and UDP-GalNAc transporters (23). Under the exact conditions used in this work, the PAPS transporter, a 75-kDa protein that has been shown to oligomerize as a homodimer (22), migrated in the 150-kDa area of a glycerol gradient, whereas the UDP-GalNAc transporter, a 43-kDa protein (23), migrated in the 80–90-kDa area (23).

In addition to the results obtained after column chromatography and glycerol gradient ultracentrifugation (see Figs. 1 and 4), independent evidence suggesting that the 60 kDa is indeed the ATP transporter was obtained by functional reconstitution of the transporter into proteoliposomes and photoaffinity radiolabeling using 8-azido[γ-32P]ATP, an azido anilide derivative of ATP. When reconstituted into proteoliposomes, the highly purified transporter was active in ATP transport, and the transport activity was saturable with an apparent $K_m$ very similar to that of intact Golgi vesicles, suggesting that the two activities are identical.

The possibility that the 60-kDa protein we purified as the Golgi membrane ATP transporter is instead the ATP transporter from a different membrane, i.e. mitochondria or the ER, is very unlikely because the mitochondrial ATP transporter has a different molecular mass, ~35 kDa (25), and the one from the ER is not functional when reconstituted into proteoliposomes by the freeze-thaw procedure as used in this work (Ref. 26; see “Experimental Procedures”).

Although it has been clearly shown that phosphorylation is one of the post-translational modifications that both secreted and integral Golgi membrane proteins and proteoglycans undergo during their transit through the Golgi apparatus (1–8), its functional importance is largely unknown. If indeed, as it has been suggested, it contributes to maintaining the stability of the protein backbone from proteolytic degradation in situ (1, 2) or serves as a specific targeting signal, as in the case of proteoglycans (7), remains to be determined. Understanding the importance of phosphorylation as a post-translational event and evaluation of how the possible regulation of ATP transport into the Golgi lumen can affect the biosynthesis/modification and function of macromolecules represents a major biological question. The purification of the ATP transporter constitutes an important step toward this direction. It will enable us to obtain the peptide sequence of the transporter, and from this, it will allow us to proceed toward its cloning. This in turn will enable us to study how the transporter is arranged in the membrane, if it is structurally related to the ATP transporter from mitochondria and ER, and whether its expression can regulate the post-translational modifications of the above macromolecules.

REFERENCES