Identification and Partial Purification of PAPS Translocase†

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ABSTRACT: Sulfation of all macromolecules in higher organisms requires the high-energy donor 3′-phosphoadenosine 5′-phosphosulfate (PAPS). PAPS is synthesized via the sequential actions of two cytoplasmic enzymes, ATP sulfurylase and APS kinase, and then must be transferred across the Golgi membrane for utilization by lumenal sulfotransferases. Following the kinetic characterization of the PAPS translocase as a specific transporter that act through an antiport mechanism with PAP as the returning ligand [Ozeran, J. D., Westley, J., Schwartz, N. B. (1996) *Biochemistry 35,* 3685−3694 (accompanying paper)], the present study describes the identification and physical characterization of the PAPS translocase from rat liver Golgi membranes. The following evidence suggests the PAPS translocase is a membrane spanning protein of approximately 230 kDa: isolation by affinity chromatography on β-methylene PAPS matrices of a 230 kDa Golgi membrane protein concomitant with PAPS translocase activity; demonstration that the 230 kDa protein possesses the only PAPS binding site accessible to the cytoplasmic face of intact Golgi membranes, while several other PAPS binding proteins are labeled in solubilized membrane preparations; reduction in size of the 230 kDa membrane protein and loss of PAPS translocase activity following protease treatment; estimation via hydrodynamic analysis of a molecular size of the membrane protein associated with PAPS translocase activity; and correlation of β-methylene PAPS binding and labeling of the 230 kDa Golgi protein with PAPS translocase activity in artificial liposomes. These and the accompanying data have permitted the identification of the first of a potentially large class of Golgi membrane nucleotide-metabolite transporters.

Glycosylation and sulfation are unique among modifications in that they are carried out primarily within subcellular, membrane-bound organelles, and require highly charged donor molecules. This poses certain problems for the mechanisms of synthesis and transport. For example, the universal sulfate donor 3′-phosphoadenosine 5′-phosphosulfate (PAPS) is produced in the cytoplasm by the sequential action of ATP sulfurylase (ATP:sulfate adenylyl transferase, EC 2.7.7.4) and APS kinase (ATP:adenylylsulfate 3′-phosphotransferase, EC 2.7.1.25) (Lyle *et al*., 1994b). Due to the localization of the majority of sulfotransferases within the Golgi lumen (Dorfman, 1970; Vertel *et al*., 1993), a transport system for PAPS was previously proposed (Capasso & Hirschberg, 1984). PAPS transport and nucleotide sugar transport have been described only phenomenologically (Schwarz *et al*., 1984; Milla *et al*., 1992) with no identification of the functional transport proteins. In the previous manuscript we presented the kinetic characterization of the activity responsible for translocation of PAPS from the cytoplasm to the Golgi lumen (Ozeran *et al*., 1996). This characterization has proven instrumental in the identification of the protein responsible for the PAPS translocase activity by allowing us to study PAPS transport both in intact Golgi vesicles and in a reconstituted liposome system.

The present report describes the identification and isolation of the PAPS translocase from rat liver Golgi membranes using a combination of affinity labeling and affinity chromatography based upon the β-methylene PAPS analog (Ng *et al*., 1991). This represents a key step toward the purification and utilization of the PAPS translocase in the dissection and analysis of the sulfate activation and utilization pathway.

MATERIALS AND METHODS

Materials. The radiolabeled compounds, [^{35}S]PAPS (>400 Ci/mmol) γ-[^{32}P]ATP (6000 Ci/mmol), UDP-[^{14}C]galactose (272.8 mCi/mmol), [^{3}H]H_2O (1 mCi/mL), [^{3}H]-2-deoxyglucose (8.0 mCi/mmol, 0.2 mCi/mL), and [^{3}H]methoxyinulin (230 mCi/gram, 0.2 mCi/mL) were purchased from New England Nuclear. Universol liquid scintillation fluid from ICN was used for scintillation counting. Ultrapure sucrose, acrylamide, sodium dodecyl sulfate, and dithiothreitol were purchased from Gibco BRL. Phosphatidylcholine was purchased from Avanti Polar Lipids, Alabaster, AL. Adenosine-3′,5′-bisphosphate (PAP), PAP-agarose, HEPES, and soy trypsin inhibitor (1% solution) were obtained from Sigma Chemical Company. Silica and polyethyleneimine cellulose TLC plates with fluorescent indicator were from Merck. CL-4B Sepharose, Density Marker Beads, and PD10 desalting columns were purchased from Pharmacia. SM-2 Bio-Beads Adsorbent, Bio-Spin-30 chromatography columns, Affi-Gel 10 and 15, 1 × 10 and 1 × 20 cm glass chromatography columns, and "Broad Range" molecular weight standards were from Bio-Rad. Ultrapure "protein grade" NP-40 was from Calbiochem. Octyl thioglucoside and sulfo-SANPAH were from Pierce Chemical. Centricon 30 and Centriprep 30 concentrators, stirred ultrafiltration cells, and CM-30

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[1] Abbreviations: PAPS, 3′-phosphoadenosine 5′-phosphosulfate; APS, adenosine 5′-phosphosulfate; PAP, adenosine 3′,5′-bisphosphate.

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ultrafiltration membranes were from Amicon. Dimethylsulfoxide (DMSO) and reagent grade acetone were from Baker. All other chemicals were reagent grade. Ultrapure PAPS was purchased from Dr. Sanford Singer, University of Dayton, OH. β-Methylene APS, β-methylene PAPS, and 3′-[^{32}P]-β-methylene PAPS analogs were synthesized in this laboratory as described (Callahan *et al*., 1989; Ng *et al*., 1991; J. D. Ozeran, S. Lyle, K. Ng, J. Westley, and N. B. Schwartz, manuscript in preparation). APS kinase from *Penicillium chrysogenum* was a generous gift of Dr. Irwin Segel, University of California, Davis.

Methods. *Golgi Preparation*. Golgi-enriched membrane vesicles were prepared essentially by the method of Leelavathi *et al.* (1979) with modifications based on Geetha-Habib *et al.* (1984). Membranes layering between 21.6% and 40% sucrose, enriched 48-fold in galactosyltransferase activity, were used for further experiments.

Gel Electrophoresis. Polyacrylamide gels were prepared according to the method of Laemmli (1970). Gels of 6% acrylamide were prepared with a 3.5% stacking gel and run in a Bio-Rad Mini-Protean II apparatus for 40−45 min at 200 V. As a modification when working with membrane proteins (Ragan, 1986), SDS−PAGE sample buffer contained 4 M urea to aid in the complete denaturation and dissociation of polypeptides, and samples were gently heated to 37 °C for 10 min instead of boiling. Gels were stained with Coomassie Brilliant Blue (0.01%) in 25% methanol (v/v) containing 10% glacial acetic acid and destained in the same solution. Stained gels were dried between sheets of cellophane on a Hoeffer "Easy Breeze" gel dryer, and radiolabeled gels were exposed for autoradiography at −70 °C using Reflection film from DuPont/NEN.

Protein Assays. Protein determinations were made based on the method of Smith *et al.* (1985), using bovine serum albumin as the standard and Pierce's BCA reagent for color development. The Pierce standard or microtiter plate protocols were used for all samples.

Sulfotransferase Assays. Sulfotransferase activity was assayed according to the method of Schwartz *et al*. (1978). Sulfated glycosaminoglycans were isolated by cetylpyridinium chloride/NaCl precipitation as described by Dorfman and Ho (1970), and incorporated [^{35}S] was determined by liquid scintillation counting.

Trypsin Digestion of Golgi Membranes. Golgi membranes (4 mg of protein/mL) were suspended in digestion buffer (50 mM HEPES, pH 7.2, 90 mM KCl) containing 4 μg of trypsin/mL. After 0, 5, 10, 15, and 20 min at 25 °C, digestion was stopped by the addition of 100 μL of a 1% solution of soybean trypsin inhibitor. Control samples (no trypsin) had soybean trypsin inhibitor added at the specified intervals. Following termination, PAPS translocase activity was assayed as described for intact Golgi membranes using 275 μL of the digestion reaction mixture to provide ∼1 mg of Golgi protein. Soybean trypsin inhibitor had no effect on transport activity when added to control incubations.

PAPS Transport into Intact Golgi Vesicles. Transport of solutes into intact Golgi vesicles was assayed as described in the accompanying manuscript (Ozeran *et al*., 1996).

Solubilization and Reconstitution of Golgi Membrane Proteins. Golgi membrane proteins were solubilized and reconstituted as described in the accompanying manuscript (Ozeran *et al*., 1996). Reconstituted Golgi protein samples were assayed immediately for PAPS translocase activity.

PAPS Transport into Reconstituted Liposomes. PAPS transport in reconstituted vesicles was measured as described in the accompanying manuscript (Ozeran *et al*., 1996). Reconstituted Golgi protein samples were assayed immediately for PAPS translocase activity.

Kinetic Data Analysis. All kinetic data were analyzed using the Microsoft QuickBASIC program "MacEnzkin" on an Apple Macintosh IIci computer. This program fits the initial velocity measurements as a function of substrate concentration to the best rectangular hyperbola by an iterative least-squares function.

Cross-Linking of 3′-[^{32}P]-β-Methylene PAPS to Golgi Proteins. Photoaffinity cross-linking with β-methylene-PAPS was carried out using the heterobifunctional cross-linker sulfosuccinimidyl 6-[4′-azido-2′-nitrophenylamino]-hexanoate (sulfo-SANPAH) (Ji & Ji, 1989). A sulfo-SANPAH−3′-[^{32}P]-β-methylene PAPS conjugate was synthesized according to manufacturer's instructions. Briefly, 3 μg of sulfo-SANPAH (from a stock with 60 μg/μL of DMSO) in BBS was combined with ∼0.3 nmol of 3′-[^{32}P]-β-methylene-PAPS in a total volume of 500 μL and incubated at room temperature, protected from light. After 1 h, 10 μL of 1 M Tris, pH 8, was added to scavenge unreacted sulfosuccinimidyl functional groups. After an additional 15 min, 5 μL of this solution was added to Golgi membrane or soluble protein samples containing ∼50 μg of protein. These were incubated in a dry block at 37 °C and protected from light for 15 min and then irradiated with long-wavelength ultraviolet light (∼366 nm) at a distance of ∼1 cm for 10 min at room temperature. Following irradiation, electrophoresis sample buffer containing 180 mM β-mercaptoethanol was added and samples were heated to 37 °C for 15 min prior to separation on 6% polyacrylamide gels. No labeling occurred in the absence of light. The labeling pattern did not change after alkali (0.1 M Na_2CO_3) extraction of Golgi membranes.

Sucrose Density Centrifugation. Golgi proteins solubilized in 0.5% octyl thioglucoside (10 mg/mL) in 0.5 mL were layered in 13-mL polyallomer tubes on preformed gradients of 5−20% sucrose in homogenizing buffer containing 0.5% octyl thioglucoside made with H_2O and D_2O. After centrifugation in a Beckman SW 41 Ti rotor at 40 000 rpm for 48 h, 0.5-mL fractions were collected from the top of the tube. Aliquots were reconstituted and assayed for PAPS translocase activity as described above, and refractive indices were measured with a Fisher refractometer.

RESULTS

Localization of PAPS Transport. To confirm that the PAPS translocase protein is an integral membrane protein, Golgi membranes were exposed to conditions which would be expected to cause the dissociation of peripheral proteins or the loss of lumenal contents. Golgi vesicles were first incubated in a high-salt (0.5 M KCl) buffer to dissociate peripheral proteins. Although this treatment did not significantly change PAPS translocase activity (7.99 ± 0.70 vs 7.76 ± 0.86 pmol/min/mg of protein), it did cause the loss of residual, membrane-associated ATP sulfurylase/APS kinase activity (0.062 vs 0.477 pmol/h/mg of protein), a phenomenon previously observed by Geller (1987). These results are consistent with the suggestion that the PAPS translocase activity is not the result of a factor peripheral to or weakly

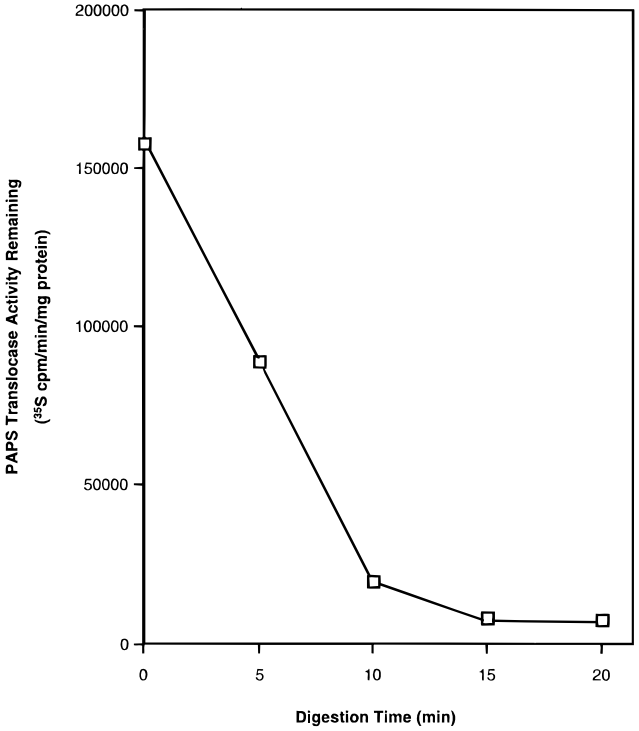

FIGURE 1: Effect of protease digestion on PAPS translocase activity. Initial velocities of PAPS translocase activity were determined following trypsin digestion of Golgi vesicles as described in Materials and Methods. Controls contained no trypsin, but had soybean trypsin inhibitor added at the specified intervals. Aliquots of the digestion mixture (containing ~1 mg of starting Golgi protein) were assayed for uptake of [^{35}S]PAPS and PAPS transport was calculated as described in Materials and Methods.

associated with the Golgi membrane. Hypo-osmotic shock of vesicles was performed to cause lysis and resealing of vesicles with concomitant loss of vesicle contents. This procedure also did not cause significant change in the PAPS translocase activity (8.77 ± 0.16 vs 7.76 ± 0.45 pmol/min/mg of protein). Assays of sulfotransferase activity of Golgi membranes before and after hypo-osmotic shock showed only slight differences (461 ± 43 vs 424 ± 32 pmol/h/mg of protein); however, significant amounts of sulfotransferase activity were released from the lumenal contents and recovered in the "shocked supernatant" (198 ± 55 pmol/h/mg of protein), suggesting that while a portion of Golgi sulfotransferases are soluble enzymes, the PAPS translocase is tightly associated with the Golgi membrane itself.

To confirm the protein nature of the transporter entity and to assess its cytoplasmic accessibility, intact Golgi membranes were digested with trypsin as described and then assayed for residual PAPS translocase activity. After 5 min of incubation, only 55% of the PAPS translocase activity was recovered (Figure 1). By 15 min of digestion, less than 1% of the original PAPS translocase activity was detectable. This acute sensitivity to protease digestion strongly suggests that PAPS translocase is indeed active within the Golgi membrane. It also suggests possible methods to further characterize the structure and orientation of PAPS translocase by partial proteolysis and peptide isolation.

Partial Purification of PAPS Translocase. The availability of the β-methylene APS and β-methylene PAPS analogs provided unique tools for the analysis of the PAPS translocase. Because affinity chromatography is a highly specific method for the purification of proteins, affinity matrices were

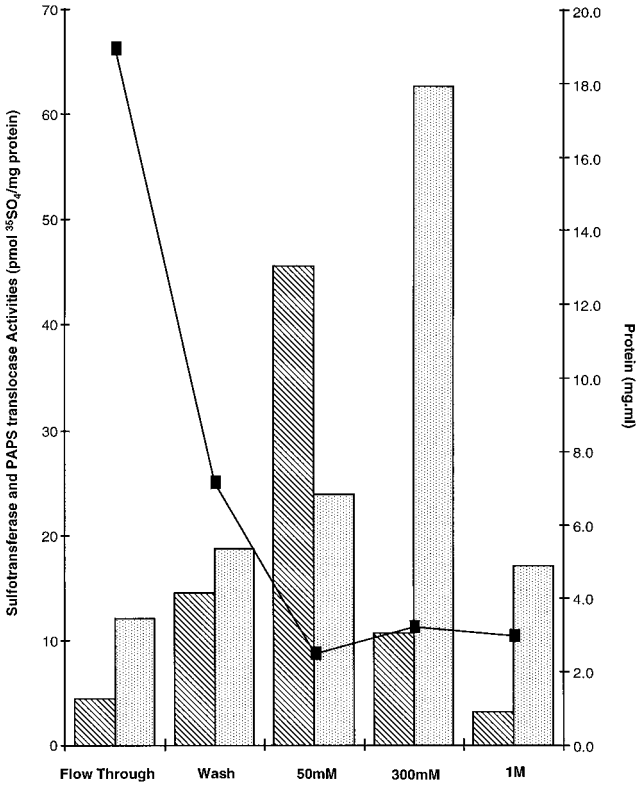

FIGURE 2: Partial purification of PAPS translocase on β-methylene PAPS agarose. Golgi membrane proteins (~30 mg) were solubilized as described in the accompanying manuscript and loaded onto a 5 mL column of β-methylene PAPS agarose equilibrated with PG buffer containing 0.1% NP-40. After washing with 15 mL of the same buffer, the column was eluted using a KCl step gradient of 0, 50, 300 mM, and 1.0 M KCl in PG buffer containing 0.1% NP-40 and collected as 15 mL fractions. Protein in individual fractions was concentrated using Centriprep-30 concentrators and aliquots were reconstituted and assayed for PAPS translocase activity, or assayed for sulfotransferase activity as described in Materials and Methods. Relative PAPS translocase (hatched lines) specific activities of the reconstituted column fractions, and sulfotransferase (gray shading) specific activities of the soluble material are shown.

synthesized by coupling β-methylene PAPS analog to CNBr-activated Sepharose CL-4B, Affi-Gel 10, and Affi-Gel 15. Coupling was preceeded by converting the β-methylene PAPS analog to its 6-aminohexyl derivative based on previously described methodology (Guilford *et al*., 1972; Brodelius *et al*., 1974; Trayer *et al*., 1984). Affi-Gel-based matrices were synthesized according to the manufacturers' directions. Additionally, because of its efficacy as an inhibitor, affinity matrices consisting of 3′,5′-ADP were also tested.

The PAPS transport activity was bound to the various affinity matricies and therefore partially purified. Solubilized Golgi protein (10 mg/mL) was loaded onto a column (5 mL) of β-methylene-PAPS agarose and eluted with a discontinuous salt gradient of PNG buffer containing 50 mM KCl (15 mL), 300 mM KCl (15 mL), and 1 M KCl (15 mL). Fractions were assayed for protein content, sulfotransferase activity, and PAPS translocase activity; a typical activity elution profile is depicted in Figure 2. While the majority of the protein was recovered in the flow-through fraction, only low activities of both PAPS translocase and sulfotransferase were detected. The second (50 mM KCl) fraction contained substantial PAPS translocase activity (~70%) but only a modest amount (18%) of the sulfotransferase activity.

In contrast, the 300 mM KCl fraction contained the bulk of the sulfotransferase activity (75%) but only 15% of the PAPS translocase activity, indicating that significant separation of sulfotransferase and translocase was achieved by the differential salt elution. Overall the β-methylene-PAPS agarose affinity chromatography system provided an 8.7-fold purification of PAPS translocase activity versus solubilized Golgi protein. Although this represents a significant purification in a single step, it is recognized that the translocase is present in trace quantities in the Golgi, thus requiring additional procedures which are under development.

When affinity chromatography is used, it is generally assumed that the ligand is bound by the protein(s) of interest with a high affinity. This is clearly the case for binding of β-methylene-PAPS by sulfotransferases, as they do not elute until the ionic strength is significantly higher than physiological concentrations, while the appearance of PAPS translocase elution in as low as 50 mM KCl indicates a lower affinity of translocase to ligand. Therefore this system exhibits the effects of differential affinity for a single ligand by two (or more) proteins. Thus, the PAPS translocase is in competition with the sulfotransferases for the available β-methylene-PAPS binding sites of the matrix, but binds with a lower affinity and thus is primarily excluded from the majority of binding sites. The sulfotransferase, on the other hand, binds tightly to the β-methylene-PAPS analog. Thus, the competition between the PAPS translocase and the sulfotransferases is at least partially responsible for the early appearance of the PAPS translocase in the elution. The limited availability of the β-methylene PAPS analog for synthesis of this affinity matrix thus results in a fairly low capacity for PAPS translocase in the presence of significant amounts of sulfotransferase contamination. This technique, however, remains a useful tool in the purification of PAPS translocase. Due to the ability to remove the majority of "contaminating" sulfotransferase by allowing it to bind to the β-methylene PAPS analog. PAPS translocase which is significantly enriched over the starting material can thus be obtained for further analyses.

Adenosine 3′,5′-bisphosphate (PAP) agarose was also used as an affinity matrix. Separation of Golgi proteins on PAP-agarose was performed using the same buffers and gradient composition as for β-methylene-PAPS agarose, and sulfotransferase activity and PAPS translocase activity were assayed as above (Figure 3). A similar pattern of separation was seen between the PAPS translocase and sulfotransferase activities with the PAP-agarose yielding a 6.8-fold purification of PAPS translocase over the solubilized Golgi membrane protein. The similarity between the purification of the translocase on β-methylene-PAPS agarose and PAP-agarose supports the suggestion that ligand specificity of PAPS translocase is based primarily on the phosphate of the 3′-position (Ozeran *et al*., 1996).

Identification of Functional PAPS Translocase. In order to identify an entity responsible for the PAPS translocase activity, correlation of some method of detection or visualization with the recoverable activity is necessary (Lefkowitz, 1978; Flier, 1981; Kinouchi *et al*., 1991), and thus several lines of experimentation were carried out. The availability of β-methylene APS and β-methylene PAPS analogs again provided the critical tools for this identification. Having shown that the β-methylene PAPS analog can bind to PAPS translocase in intact Golgi membranes as a competitive

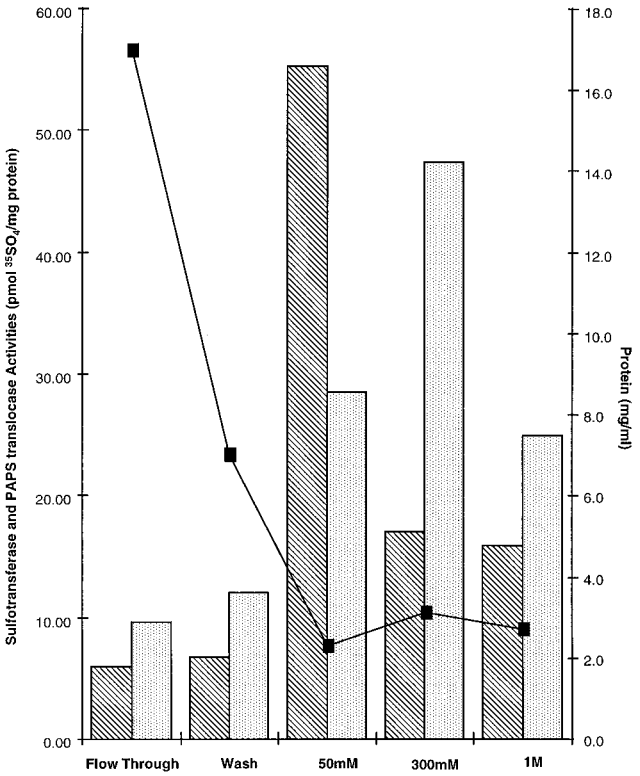

FIGURE 3: Partial purification of PAPS translocase on adenosine 3′,5′ bisphosphate agarose. Golgi membrane proteins (~30 mg) were solubilized as described and loaded onto a 5 mL column of adenosine 3′,5′-bisphosphate agarose which was equilibrated, eluted, and analyzed for PAPS translocase and sulfotransferase activities as described in Figure 2. Relative PAPS translocase (hatched lines) specific activities of the reconstituted column fractions, and sulfotransferase (gray shading) specific activities of the soluble material are shown.

inhibitor of transport (Ozeran *et al*., 1996), a radiolabeled form of the β-methylene PAPS analog was synthesized (Ozeran *et al*., manuscript in preparation) and used as an affinity label for PAPS binding sites at the cytoplasmic surface of the Golgi membrane. Because the sulfo-SANPAH−3′-[^{32}P]-β-methylene PAPS conjugate was not transported across Golgi membranes (data not shown), labeling of solubilized Golgi membrane proteins was also used to differentiate between cytoplasmic and lumenal oriented PAPS binding sites. This labeling was then correlated with reconstituted PAPS translocase activity from partially purified Golgi membrane proteins.

When intact Golgi membranes were labeled, only a single high molecular weight protein (~230 kDa) was labeled by the sulfo-SANPAH−3′-[^{32}P]-β-methylene PAPS conjugate (Figure 4, lane 4). The specificity of this labeling can be seen in the protection from labeling in the presence of β-methylene-PAPS or adenosine 3′,5′-bisphosphate, which compete with the photoaffinity label for access to the PAPS translocase binding site (Figure 4, lanes 5 and 6). The radioactivity seen at the top of the gel (interface between the stacking gel and the 6% resolving gel) may result from precipitation of membrane proteins (Ragan, 1986) in the presence of SDS or maybe due to nonspecific reactive intermediates generated during the photolysis step of the photolabeling which form large polymers of cross-linked proteins (Bayley & Knowles, 1977; Ji *et al*., 1989; Fan *et al*., 1991). The net result is that nonspecifically labeled material may be found trapped at the tops of gels, and the

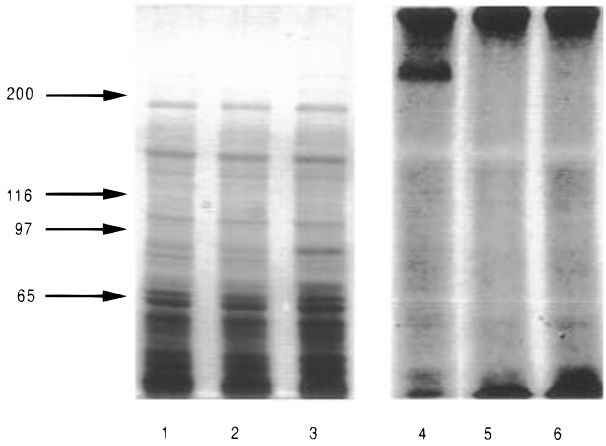

FIGURE 4: Cross-linking of 3′-[^{32}P]-β-methylene-PAPS to intact Golgi membranes. Intact Golgi vesicles were labeled with the sulfo-SANPAH conjugate of 3′-[^{32}P]-β-methylene-PAPS as described in Materials and Methods and proteins were separated on 6% SDS−PAGE gels, dried, and exposed for autoradiography for 72 h. Lanes 1−3, Coomassie-stained protein; lanes 4−6, autoradiograph. Lanes 1 and 4 contained Golgi vesicles labeled in the absence of inhibitors. Lanes 2 and 5 were labeled in the presence of 100 μM β-methylene PAPS. Lanes 3 and 6 were labeled in the presence of 1 mM adenosine 3′,5′-bisphosphate. Molecular weight standards were rabbit muscle myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase B (97 (kDa), and bovine serum albumin (66 kDa).

roughly equal amounts of labeled material which appear at the tops of each lane (Figure 4, lanes 4−6) suggests this to be the case since nonspecific labeling is equal among these samples regardless of the specific labeling of PAPS translocase (lane 4) or the inhibition of specific labeling by β-methylene-PAPS or adenosine 3′,5′-bisphosphate (lanes 5 and 6).

In contrast, when sulfo-SANPAH−3′-[^{32}P]-β-methylene-PAPS was cross-linked with detergent-solubilized Golgi proteins, several proteins became radiolabeled (Figure 5, lane 4). These include bands which might represent the ~110 kDa *N*-heparan sulfate sulfotransferase (Eriksson *et al*., 1994) and the ~32 kDa liver phenol-sulfating phenol sulfotransferase (Falany *et al*., 1990). Although the prior solubilization of Golgi proteins and the presence of additional detergents aid in alleviating the total accumulation of material at the stacking gel interface, material labeled nonspecifically and polymerized by the cross-linker remains. Altogether, these data suggest that the ~230 kDa protein represents the only protein with an accessible PAPS binding site on the cytoplasmic surface. The specificity of this labeled ~230 kDa band, as representing a protein with the only apparent PAPS binding site on the cytoplasmic face of the Golgi membrane, leads to the suggestion that it is the PAPS translocase protein.

As shown previously (Figure 1), PAPS translocase activity was lost upon digestion of Golgi membranes with trypsin. When trypsin digested Golgi membranes were labeled with 3′-[^{32}P]-β-methylene-PAPS using sulfo-SANPAH, a reduction in the size of the putative translocase was seen (Figure 6). If Golgi membranes were first labeled and then digested with trypsin, a similar reduction was found. These results suggest that, although the PAPS binding site itself is protected from digestion, a portion (~50 kDa) of the translocase which is necessary for activity is sensitive to the protease treatment. This phenomenon of small change in size and in labeling, accompanied by a loss of activity, is also observed in the case of the human erythrocyte nucleoside transporter (Kwong *et al*., 1993).

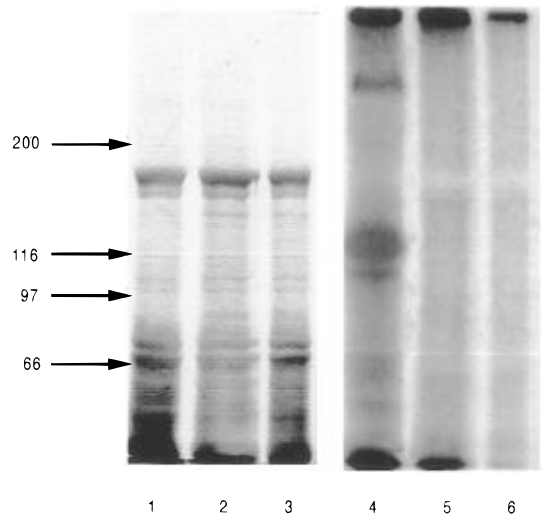

FIGURE 5: Cross-linking of 3′-[^{32}P]-β-methylene-PAPS to solubilized Golgi membrane proteins. Golgi membrane proteins which had been solubilized with 0.5% NP-40 (~50 μg of protein) were labeled with the sulfo-SANPAH−3′-[^{32}P]-β-methylene-PAPS conjugate in the absence or presence of 100 μM β-methylene-PAPS or 1 mM adenosine 3′,5′-bisphosphate as described. Lanes 1−3, Coomassie-stained protein; lanes 4−6, autoradiograph. Lanes 1 and 4 contained Golgi vesicles labeled in the absence of inhibitors. Lanes 2 and 5 were labeled in the presence of 100 μM β-methylene PAPS. Lanes 3 and 6 were labeled in the presence of 1 mM adenosine 3′,5′-bisphosphate. Molecular weight standards were rabbit muscle myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase B (97 (kDa), and bovine serum albumin (66 kDa).

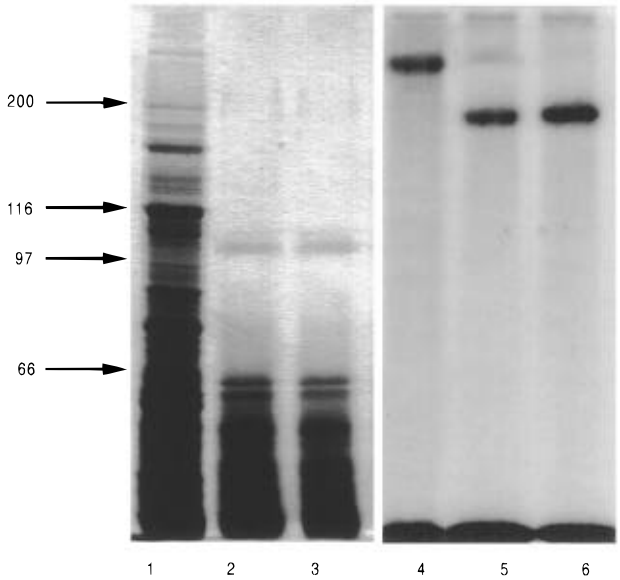

FIGURE 6: 3′-[^{32}P]-β-Methylene-PAPS labeling of trypsin-digested Golgi membrane vesicles. Intact Golgi vesicles were labeled with the sulfo-SANPAH conjugate of 3′-[^{32}P] β-methylene-PAPS, following or preceding digestion by trypsin as described in Materials and Methods. Proteins were separated on 6% SDS−PAGE gels, dried and exposed for autoradiography for 72 h. Lanes 1−3, Coomassie-stained protein; lanes 4−6, autoradiograph. Lanes 1 and 4, undigested Golgi vesicles. Lanes 2 and 5 were labeled following trypsin digestion. Lanes 3 and 6 were labeled prior to trypsin digestion. Molecular weight standards were rabbit muscle myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase B (97 (kDa), and bovine serum albumin (66 kDa).

Labeling and Reconstitution of Affinity-Purified Protein. Labeling of protein fractions from β-methylene-PAPS affinity

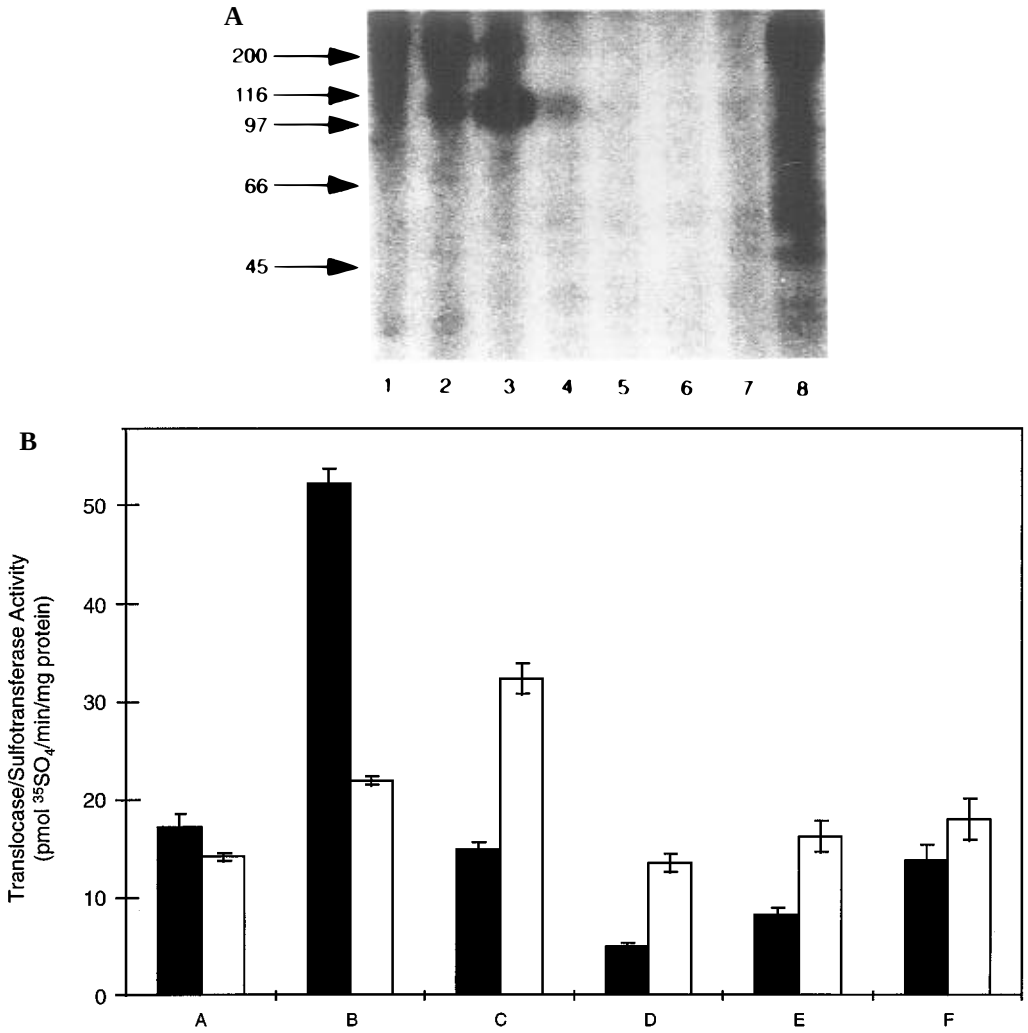

FIGURE 7: Correlation of 3′-[^{32}P]-β-methylene PAPS labeling with partially purified reconstituted PAPS translocase activity. Golgi membrane proteins (~30 mg) were solubilized and purified by β-methylene PAPS agarose-chromatography as described for experiment in Figure 2. Aliquots were either labeled with the sulfo-SANPAH conjugate of 3′-[^{32}P]-β-methylene PAPS as described, reconstituted and assayed for PAPS translocase activity or assayed for sulfotransferase activity. (A) Labeled fractions separated by SDS−PAGE on a 6% polyacrylamide gel. Lane 1, column flow through and wash. Lane 2, 50 mM KCl wash fraction. Lane 3, 300 mM KCl wash fraction. Lane 4, 1.0 M KCl wash fraction. Lane 5 is molecular weight standards. Lanes 6 and 7 are unlabeled controls. Lane 8, intact Golgi membranes labeled with the sulfo-SANPAH conjugate of 3′-[^{32}P]-β-methylene PAPS as described. Molecular weight standards were rabbit muscle myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase B (97 kDa), and bovine serum albumin (66 kDa). (B) Relative PAPS translocase (■) specific activities of the reconstituted column fractions and sulfotransferase (□) specific activities of the soluble material are shown.A, column flow through and wash. B, 50 mM KCl wash fraction. C, 300 mM KCl wash fraction. D, 1.0 M KCl wash fraction. E, solubilized Golgi membrane proteins. F, intact Golgi.

columns allows the identification via visual detection of PAPS binding proteins in these fractions. Additionally, as activity can be reconstituted from these same fractions, 3′-[^{32}P]-β-methylene-PAPS labeling of a protein can be correlated with the presence or absence of translocase activity. For reconstitution, column fractions were concentrated and equal amounts of protein (0.50 mg/mL) were incorporated into liposomes. PAPS translocase activity in these vesicles was assayed as described. Protein with PAPS translocase activity recovered from the 50 mM KCl fraction from the β-methylene-PAPS agarose column corresponds with the presence of a labeled band at ~230 kDa in that lane (Figure 7A, lane 2). The double band labeled at ~110 kDa also correlates with the recovered sulfotransferase activity (Figure 7A, lane 3). Labeling of fractions with high translocase activity versus those with high sulfotransferase activity shows a correlation with the appearance of the ~230 kDa band with the peak of translocase activity (Figure 7A, lane 2, and 7B, 50 mM KCl; and 7A, lane 3, and 7B, 300 mM KCl). This result, in conjunction with the presence of the ~230 kDa protein as the only band labeled in intact membranes, allows the putative identification of PAPS translocase as a 230 MW integral protein of the Golgi membrane.

To confirm that the PAPS translocase is a large (>200 kDa) protein, hydrodynamic studies were carried out using linear gradients of 5%−20% sucrose according to the method described by Schwartz and Rodén (1974). Analytical methods described by Clark (1976), Sadler (1979), and Griffith (1986) allowed the determination of the sedimentation coefficient (s_{20}) and the partial specific volume ($\bar{v}$) for the solubilized translocase. Aliquots of gradient fractions were reconstituted and assayed for PAPS translocase activity, and refractive indices were measured with a Fisher refractometer (Figure 8). Using the method described by Griffith (1986) for the rotor size, angular velocity and time of centrifugation, sedimentation coefficients of 5.5 S and 4.15

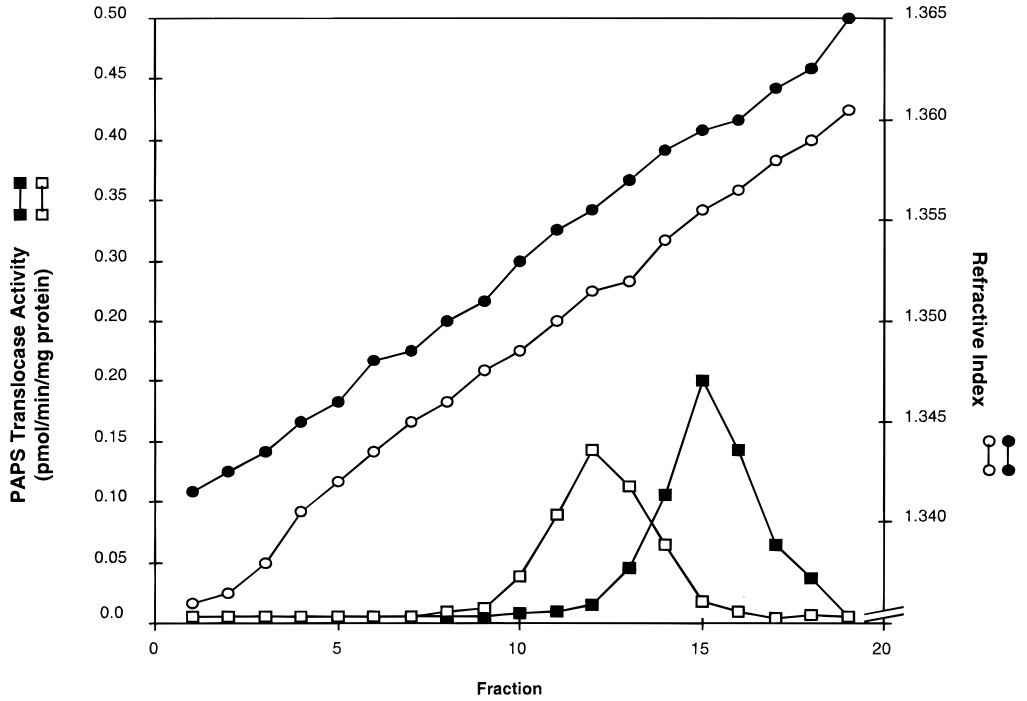

FIGURE 8: Estimation of molecular size of PAPS translocase by sedimentation analysis. Golgi membrane vesicles (10 mg of protein/mL) were solubilized with 0.5% octyl thioglucoside and subjected to sucrose density centrifugation as described in Materials and Methods. Following centrifugation, 0.5 mL fractions were reconstituted and assayed for PAPS translocase activity (H_2O gradient, ■; D_2O gradient, □) as described, and refractive indices (H_2O gradient, ●; D_2O gradient, ○) were measured with a Fisher refractometer.

S were calculated for the PAPS translocase in the H_2O and D_2O gradients, as determined by the peak position of PAPS translocase activity within each gradient.

Because the difference in density between the two solutions (H_2O versus D_2O) at a given sucrose concentration affects sedimentation rates only according to their partial specific volumes (Clarke & Smigel, 1989), simultaneous determination of s_{20} in sucrose gradients made in H_2O and D_2O allows the unambiguous determination of the partial specific volume. From these gradients, the partial specific volume of the translocase particle in solution was calculated to be 0.761 cm^3/g. By combining the sedimentation coefficient values with a diffusion coefficient, estimated as described by Tinoco *et al.* (1978), and the partial specific volume for the PAPS translocase/octyl thioglucoside micelles, an estimation of a molecular weight for the solublized PAPS translocase particle of 280 ± 40 kDa was calculated (Cantor & Schimmel, 1980). This correlates well with the molecular size determined by SDS−PAGE considering the probable presence of detergent and possibly lipid in the micellar structure (Sadler, 1979).

DISCUSSION

The physiological separation of the PAPS synthetic enzymes from the sulfotransferases responsible for sulfate ester formation by subcellular compartmentalization is part of a common theme in eukaryotes. Because of this separation (Lyle *et al*., 1994a) and the energy difficulties in transferring the charged PAPS through the Golgi membrane (Parsegian, 1969), the production of sulfated macromolecules requires the existence of a mechanism (transporter function) to allow entry of PAPS into the Golgi lumen. Most commonly the process of identifying a receptor or transporter relies on the correlation of the identification of the entity, through antibody or ligand binding (Williams & Lefkowitz, 1976; Lefkowitz, 1978; Tolliver *et al*., 1981; Schreiber *et al*., 1982), with one or more biological effects (Flier, 1981). The present studies detail the identification of the Golgi membrane protein (PAPS translocase) responsible for transfer of PAPS from cytosol to Golgi lumen.

Separation of PAPS translocase activity from ATP sulfurylase/APS kinase by washing of membranes, and from some of the major sulfotransferases within the Golgi lumen through the use of the β-methylene-PAPS affinity chromatography system, has allowed us to correlate the presence of the ~230 kDa protein with PAPS translocase activity exclusively. That the 230 kDa Golgi membrane protein is the translocase is strengthened by the following additional data: the uniqueness of the ~230 kDa protein as possessing the only PAPS binding site accessible to the cytoplasmic face of the Golgi membrane; the loss of PAPS translocase activity upon protease digestion associated with the reduction in the size of this protein only; and the estimation by an independent hydrodynamic analysis of a molecular size for the protein associated with PAPS translocase activity commensurate with estimates by SDS−PAGE. These data all suggest that the PAPS translocase is a membrane-spanning protein of approximately 230 kDa.

Curiously, our conclusions are not in agreement with a recently published report which identified the PAPS translocase as a 75 kDa protein which exists in its active form in the membrane as a 160 kDa dimer (Mandon *et al*., 1994). However, several factors in the analysis of the presented data lead to questions regarding these conclusions: difficulties in reliability and interpretation often associated with the method used to estimate size (Kempner, 1993; Erickson *et al*., 1994); photoaffinity labeling performed only on solubilized protein samples, which provides no information on the orientation of the protein nor its accessibility from the cytoplasmic face of the Golgi membrane; labeling and

detection of the partially purified preparations in a nonspecific manner using chloramine-T and ^{125}I; presentation of PAP-labeled material on SDS−PAGE gels cut off above 100 kDa; and kinetic values obtained for PAPS affinity which are not in accord with our data nor with their earlier work on the subject (Schwarz *et al*., 1984). Lastly, the activity used to track the putative 75 kDa PAPS translocase does not exhibit *trans* acceleration, the classic hallmark of transport (Stein, 1986), suggesting that the behavior being measured may be that of a simple receptor or enzyme binding a ligand/substrate, rather than that of a transporter. Interestingly, Habuchi *et al*. (1993) used similar methods to identify and purify a chondroitin 6-sulfotransferase which was characterized as a 160 kDa homodimer of 75 kDa subunits. Our more rigorous criteria for the identification and characterization of PAPS translocase from Golgi membrane using sulfo-SANPAH−3′-[^{32}P]-β-methylene-PAPS failed to label any proteins in the 75 kDa range. Thus the experimental characterization of the PAPS translocating activity in the present manuscript, together with the thorough kinetic analysis which clearly demonstrates *trans* acceleration behavior for the 230 kDa protein presented in the accompanying manuscript, support our conclusions which identify the PAPS translocase as a ∼230 kDa Golgi membrane protein.

The correlation of β-methylene-PAPS binding and labeling of the 230 kDa protein of Golgi membranes with the reconstitution of PAPS translocase activity permitted the identification of the first of a potentially large class of Golgi membrane nucleotide-metabolite transporters. In light of the functional subcellular separation of the synthesis of activated nucleotide sugars and nucleotide sulfate from the sites of glycosylation and sulfation, it also becomes clear that much remains unknown about the systems involved in the transport of these activated donor molecules into the Golgi and/or endoplasmic reticulum. The transport of PAPS from the cytosol to the Golgi lumen is presumably necessary for normal growth and development. Although there are no known mutants of PAPS transport, mutants have been identified for transporters of sugar nucleotides in Chinese hamster ovary cells by treatment of cells in culture with lectins (Deutscher *et al*., 1984; Stanley *et al*., 1991). While these mutations provide cellular resistance to lectins, it is not at all clear how these mutations might affect the viability of an organism. It is anticipated that the identification and characterization of the PAPS translocase will be instrumental in gaining a better understanding of these pathways as well as the processes of sulfation and glycosylation in general.

Exploration of coupling and possible channeling of PAPS translocase with the activities of the sulfurylase/kinase system and/or sulfotransferases is a serious consideration for future studies. On the basis of our previous work on the ATP sulfurylase/APS kinase (Lyle *et al*., 1994b, 1995) and more recently elucidation of the complete cDNA sequence for this bifunctional enzyme (Li et al., 1995), coupled with the availability of a purified PAPS translocase, a hybrid reconstituted system provides a unique opportunity to study the overall activity of a system that produces transported PAPS from ATP and free sulfate. Consideration of this system as a whole will aid in examination of defects such as those which produce the brachymorphic mouse phenotype (Lyle *et al*., 1995) and diastrophic dysplasia (Shapiro, 1992; Hästbacka *et al*., 1994). New information about the PAPS translocase and the intact system of sulfate activation and utilization also provides a model for examination of alterations in pathways involving transport in a multitude of other systems.

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