Lipid Specific Activation of the Murine P_4-ATPase Atp8a1 (ATPase II)†

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ABSTRACT: The asymmetric transbilayer distribution of phosphatidylserine (PS) in the mammalian plasma membrane and secretory vesicles is maintained, in part, by an ATP-dependent transporter. This aminophospholipid "flippase" selectively transports PS to the cytosolic leaflet of the bilayer and is sensitive to vanadate, Ca^{2+}, and modification by sulfhydryl reagents. Although the flippase has not been positively identified, a subfamily of P-type ATPases has been proposed to function as transporters of amphipaths, including PS and other phospholipids. A candidate PS flippase ATP8A1 (ATPase II), originally isolated from bovine secretory vesicles, is a member of this subfamily based on sequence homology to the founding member of the subfamily, the yeast protein Drs2, which has been linked to ribosomal assembly, the formation of Golgi-coated vesicles, and the maintenance of PS asymmetry. To determine if ATP8A1 has biochemical characteristics consistent with a PS flippase, a murine homologue of this enzyme was expressed in insect cells and purified. The purified Atp8a1 is inactive in detergent micelles or in micelles containing phosphatidylcholine, phosphatidic acid, or phosphatidylinositol, is minimally activated by phosphatidylglycerol or phosphatidylethanolamine (PE), and is maximally activated by PS. The selectivity for PS is dependent upon multiple elements of the lipid structure. Similar to the plasma membrane PS transporter, Atp8a1 is activated only by the naturally occurring *sn*-1,2-glycerol isomer of PS and not the *sn*-2,3-glycerol stereoisomer. Both flippase and Atp8a1 activities are insensitive to the stereochemistry of the serine headgroup. Most modifications of the PS headgroup structure decrease recognition by the plasma membrane PS flippase. Activation of Atp8a1 is also reduced by these modifications; phosphatidylserine-*O*-methyl ester, lysophosphatidylserine, glycerophosphoserine, and phosphoserine, which are not transported by the plasma membrane flippase, do not activate Atp8a1. Weakly translocated lipids (PE, phosphatidylhydroxypropionate, and phosphatidylhomoserine) are also weak Atp8a1 activators. However, *N*-methylphosphatidylserine, which is transported by the plasma membrane flippase at a rate equivalent to PS, is incapable of activating Atp8a1 activity. These results indicate that the ATPase activity of the secretory granule Atp8a1 is activated by phospholipids binding to a specific site whose properties (PS selectivity, dependence upon glycerol but not serine, stereochemistry, and vanadate sensitivity) are similar to, but distinct from, the properties of the substrate binding site of the plasma membrane flippase.

Lipids in biological membranes are nonrandomly distributed across the bilayer. The amine-containing phospholipids, phosphatidylethanolamine (PE)[1] and phosphatidylserine (PS), are sequestered on the cytofacial side of the membrane, while the choline-containing phospholipids, phosphatidylcholine (PC) and sphingomyelin (SM), are enriched on the opposite surface. This distribution is maintained by the action of several lipid transport systems: flippases, which catalyze the energy-dependent, cytofacially directed transport of phospholipids; floppases, which catalyze the energy-dependent efflux of phospholipids; and scramblases, which facilitate the energy-independent degradation of transbilayer asymmetry (for recent reviews, see refs *1* and *2*).

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[1] Abbreviations: BCA, 4,4′-dicarboxy-2,2′-biquinone; BSA, bovine serum albumin; $C_{12}E_9$, poly(oxyethylene)-9-lauryl ether; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FBS, fetal bovine serum; gus, β-glucuronidase; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HRP, horseradish peroxidase; MOI, multiplicity of infection; Ni−NTA, nickel−nitrilotriacetic acid; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPhS, 1-palmitoyl-2-oleoylphosphatidylhomoserine; POPP, 1-palmitoyl-2-oleoylphosphatidylhydroxypropionate; POPS, 1-palmitoyl-2-oleoylphosphatidylserine; POPS-*N*-Me, 1-palmitoyl-2-oleoylphosphatidyl-*N*-methylserine; PS, phosphatidylserine; SDS, sodium dodecyl sulfate; Sf21, *Spodoptera frugiperda* cells; TBST, Tris-buffered saline Tween-20; TCA, trichloroacetic acid; TNM-FH, *Trichoplusia ni* medium-formulation Hink; Tris-HCl, tris(hydroxyethyl)aminomethane; POPS-*O*-Me, 1-palmitoyl-2-oleoylphosphatidylserine methyl ester.

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PS sequestration is maintained, in part, by a lipid transporter, the aminophospholipid flippase (*3*−*6*). The biochemical requirements for this activity are well-defined. ATP (*4*, *5*) and Mg^{2+} (*7*, *8*) are required, and PS is transported with a stoichiometry of one PS per ATP molecule hydrolyzed (*9*). While transport is independent of the acyl chain length or degree of saturation (*4*−*6*, *10*−*12*), aminophospholipids with longer acyl chains are transported at a slightly faster rate (*10*, *13*), while lyso lipids are not transported (*4*) and glycerophosphoserine inhibits transport (*14*). Vanadate (*5*, *7*), sulfhydryl reagents (*4*, *15*), alkylating reagents (*16*), bromophenylacylbromide (*17*), and micromolar Ca^{2+} (*4*, *7*, *8*) also inhibit transport.

The substrate functional groups required for aminophospholipid transport have been well-characterized. The flippase prefers PS over PE, transporting PS 10-fold faster than PE (*4*, *5*, *18*) and exhibiting a 34-fold higher affinity for PS (*18*). This result indicates that, while the carboxyl group of serine is not required for lipid recognition, its absence results in decreased transport rates (*18*). Methylation of the PS carboxyl group decreases transport (*15*, *19*), indicating that the headgroup size may be restricted or that, if the carboxylate is present, an essential carboxyl group−protein interaction is necessary. Monomethylation of the amine group of PS (*19*) or PE (*15*) does not alter transport, but increased methylation of the PE amine group significantly reduces PE transport (*15*). *N*-Acylation of PS also inhibits transport (*20*), supporting a requirement for a protonatable amine. Recent evidence indicates that other PS analogues are transported in an ATP-dependent manner but much more slowly (*21*).

While PS flippase activity has been well-characterized in a number of cell types, the protein(s) responsible is still unknown. The biochemical characteristics of PS transport and substrate preferences have been used to identify candidate enzymes. One of these proteins, the bovine chromaffin granule ATP8A1 (ATPase II), was purified and found to be dependent upon PS for ATPase activity (*22*, *23*). The gene encoding this protein was subsequently cloned and sequenced (*24*). The predicted protein sequence is homologous to the product of the yeast *DRS2* gene (*25*), the founding member of the P_4 subfamily of P-type ATPases (*24*). Over 100 P_4-ATPase subfamily members have been identified, including human and mouse homologues of the bovine ATP8A1 (*26*, *27*). Mutations in P_4 subfamily members have been associated with an increased cold sensitivity in plants (*28*), obesity in mice (*29*), defective bile production (*30*), ribosomal assembly (*25*), intracellular vesicle trafficking (*31*−*33*), resistance to alkylphosphocholine drugs (*34*), and precocious PS exposure in spermatozoa (*35*), indicating that P_4-ATPases may play specific roles in the membrane structure or lipid transport. Bovine brain ATP8A1 has been shown to include four isoforms; PS and PE activate all four, but PS activation is less efficient in two shorter isoforms that lack a 15 amino acid insert in a cytoplasmic domain (*36*). Studies with mutants of yeast P_4-ATPases have indicated that subclass members have distinct intracellular locations and may be involved in lipid transport (for a review, see ref *32*). Although these data imply a role for the P_4-ATPases in membrane structure or amphipath transport, the substrates for many of these enzymes have not been positively identified.

The studies presented here test whether the biochemical properties of the chromaffin granule ATPase II (murine Atp8a1) resemble those of the aminophospholipid flippase by measuring the lipid requirements for ATPase activation. The protein was expressed in *Spodoptera frugiperda* (Sf21) insect cells and purified. Similar to the aminophospholipid flippase activity, the ATPase activity of the baculovirus-expressed murine Atp8a1 was selectively and stereospecifically stimulated by PS; however, some lipid recognition requirements differ qualitatively from those of the plasma membrane aminophospholipid flippase.

MATERIALS AND METHODS

Materials. 1-Palmitoyl-2-oleoylphosphatidylserine (PS), 1-palmitoyl-2-oleoylphosphatidylcholine (PC), 1-palmitoyl-2-oleoylphosphatidylglcerol (PG), 1-palmitoyl-2-oleoylphosphatidylethanolamine (PE), 1-palmitoyl-2-oleoylphosphatidic acid (PA), and brain phosphatidylinositol (PI) were purchased from Avanti Polar Lipids (Alabaster, AL). 1-Palmitoyl-2-oleoylphosphatidylhydroxypropionate (POPP), 1-palmitoyl-2-oleoylphosphatidyl-*N*-methylserine (POPS-*N*-Me), 1-palmitoyl-2-oleoylphosphatidylserine methyl ester (POPS-*O*-Me) and 1-palmitoyl-2-oleoylphosphatidylhomoserine (POPhS) were synthesized by phospholipase D catalyzed headgroup exchange from 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) and the respective alcohol and purified as described (*37*, *38*). Sf21 cells were purchased from Invitrogen (Carlsbad, CA). *Trichoplusia ni* medium-formulation Hink (TNM-FH) medium (Hink's medium) and ExCell medium were purchased from SAFC Bioscience (Lenexa, KS). Fetal bovine serum (FBS) was purchased from Intergen (Purchase, NY) or Atlas Biologicals (Fort Collins, CO). Trypan blue, ouabain, 4,4′-dicarboxy-2,2′-biquinone (BCA), penicillin, and streptomycin were purchased from Sigma (St. Louis, MO). Bovine serum albumin (BSA), Tween-20, INDIA-HisProbe-horseradish peroxidase (HRP), and SuperSignal Working Solution were purchased from Pierce (Rockford, IL). Poly(oxyethylene)-9-lauryl ether ($C_{12}E_9$), E-64, and α_2-macroglobulin were purchased from Calbiochem (San Diego, CA). Nickel−nitrilotriacetic acid (Ni−NTA) resin was purchased from Qiagen (Valencia, CA). Sterox was purchased from Bacharach, Inc. (Pittsburgh, PA). Citrate was purchased from Electron Microscopy Sciences (Gibbstown, NJ). DH_{10}Bac-competent cells and β-glucuronidase (gus) control DNA were purchased from Invitrogen (Carlsbad, CA). Bacto tryptone, yeast extract, and Bacto agar were purchased from Difco (Detroit, MI). All other chemicals were reagent-grade.

Preparation of Baculovirus Stocks. A baculovirus stock containing the murine Atp8a1 gene was prepared using the Invitrogen BAC-TO-BAC system (Invitrogen, Carlsbad, CA). The murine Atp8a1 gene (∼4.7 kb), in a pBS-SK^- plasmid (∼3.0 kb) (*24*), was excised and placed into the pFastBacHT plasmid, adding an N-terminal His_6 tag (MSYY**HHHHHH**-DYDIPTTENLYFQGAMDP) to the protein. In parallel studies, a pFastBacHT vector containing the β-glucuronidase gene was prepared as a control. Baculoviral DNA was prepared following the BAC-TO-BAC system protocol.

In separate studies, transposed *Escherichia coli* were amplified in culture and the recombinant bacmid DNA was isolated by a minipreparation procedure (Qiagen, Valencia, CA). Sequencing of the DNA was done using the dideoxy method (*39*, *40*) according to the procedure supplied with the Sequenase 2.0 kit (Amersham Life Sciences, Piscataway, NJ).

Bacmid DNA [5 μL in ExCell medium (200 μL)] containing Cellfectin reagent (6 μL, Invitrogen) was added to Sf21 cells (9×10^5 cells) pretreated with ExCell medium containing antibiotics (50 IU/mL penicillin and 50 μg/mL streptomycin at 27 °C for 1 h) and incubated (27 °C, 5 h). The transfection mixture was removed, and the ExCell medium with antibiotics (2 mL) was added (27 °C, 72−100 h). Cells and supernatants were collected and centrifuged (500*g*, 5 min), and the virus-containing supernatant was collected and stored at 4 °C.

Cell Culture and Infection. Sf21 cells were grown in monolayer cultures at 27 °C in TNM-FH medium (Grace's medium) supplemented with 10% FBS. For infection with baculovirus, cells were plated at 70% confluency in ExCell serum-free medium and the virus was added at a multiplicity of infection (MOI) of 5. A total of 6 h after inoculation, TNM-FH medium with 10% heat-inactivated FBS was added to the cells. Infected cells were harvested 48 h after inoculation by centrifugation (500*g*, 5 min). The virus-containing supernatant was removed and stored at 4 °C, while the cell pellet was stored at −70 °C. Cells were counted with a hemocytometer, and viability was determined by trypan blue exclusion.

ATPase Assay. ATPase activity was measured as described by Zimmerman et al. (*41*). Briefly, samples were incubated with *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; 62.5 mM), $MgCl_2$ (12.5 mM), ethylene glycol bis-(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA; 1.25 mM), ouabain (250 μM), $C_{12}E_9$ (0.125%), and the specified lipid (250 μM) at the indicated concentration. In some experiments, vanadate (100 μM) was added. The sample was incubated on ice for 15 min. ATP (20 μL, 12.5 mM) was added, and the sample was incubated at 37 °C. The reaction was stopped by adding a solution of Malachite green (0.033%), 4 N HCl ammonium molybdate (1.1%), and Sterox (2%). After 10 s, citrate (100 μL, 34%) was added. The optical density of samples at a wavelength of 660 nm was measured. The amount of phosphate released was determined by a comparison to inorganic phosphate standards.

Protein Assay. For protein amounts greater than 1 μg/μL, the micro BCA assay (Pierce, Rockford, IL) was used, with BSA as the reference protein. When protein amounts were less than 1 μg/μL, a modified dot blot method was used (*42*, *43*). A precut nitrocellulose membrane (Invitrogen, Carlsbad, CA) was soaked in ddH_2O for 1 h and then in 0.6% trichloroacetic acid (TCA) for 1 h. A sodium dodecyl sulfate (SDS) buffer (250 μL of 0.15 M tris(hydroxyethyl)aminomethane (Tris-HCl) and 0.4% SDS at pH 7 and room temperature) and 60% TCA (50 μL) were added to the samples. The samples were incubated (15 min, room temperature) and then placed onto the nitrocellulose membrane using a HYBRI-DOT 96-well Filtration Manifold (Gibco, Carlsbad, CA). The wells were washed with 0.6% TCA (250 μL × 2) and ddH_2O (250 μL). The membrane was dried for 15 min in a 100 °C oven and stained with 0.1% amido black in 10% acetic acid/25% 2-propanol for 10 min. The membrane was destained with 10% acetic acid/50% methanol (3 × 5 min) and rinsed with ddH_2O. The membrane was dried in a 100 °C oven for 15 min and analyzed by reflectance densitometry at 300 dpi resolution. The public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/) was used to quantify the density of the protein stain. The protein concentration was determined by a comparison with images of spots containing known concentrations of BSA.

Microsomal Membrane Preparation. Membranes were prepared by a modification of the method of Heinemann and Ozols (*44*). Sf21 cells (7×10^7) infected with murine Atp8a1 virus (48 h postinoculation) were resuspended in microsomal lysis buffer [0.01 M Tris, 1 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 10 μM E-64, and 10 μg/mL α_2-macroglobulin at pH 7.4 and 4 °C; 1 mL/10^7 cells]. Cells were incubated on ice for 20 min, homogenized by 15 passes with a tight dounce homogenizer, and centrifuged (600*g*, 10 min, 4 °C) to remove nuclei and unbroken cells. The postnuclear supernatants were pooled and centrifuged (10000*g*, 20 min, 4 °C) to remove mitochondria. Postmitochondrial supernatants were pooled and centrifuged (105000*g*, 45 min, 4 °C) to isolate microsomal membranes. The microsomal pellets were resuspended in 10 mM HEPES and 0.3 M sucrose at pH 7.3 with protease inhibitors (10 μM E-64 and 10 μg/mL α_2-macroglobulin). The presence of Atp8a1 in the microsomal fraction was confirmed by Western blot detection as described below.

Murine Atp8a1 Purification. Sf21 cells were resuspended in lysis buffer (1 mL/ 10^7 cells; 50 mM Tris, 100 mM KCl, 1% $C_{12}E_9$, 10 μM E-64, and 10 μg/mL α_2-macroglobulin at pH 8.5 and 4 °C), incubated on ice (15 min), sonicated with a Fisher Scientific Sonic Dismembrator 550 (3 × 10 s), and homogenized with 20 passes of a tight dounce homogenizer. The lysate was centrifuged (500*g*, 5 min, 4 °C), and the resulting supernatant was added to Ni−NTA resin (Qiagen, Valencia, CA; 0.1 mL of a 50% slurry for every 2×10^6 cells), which had been prewashed with 20 mM imidazole, 20 mM Tris, 500 mM KCl, 10% glycerol, 0.1% $C_{12}E_9$, and 5 mM β-mercaptoethanol at pH 8.5 and 4 °C. After 45 min of incubation at 4 °C with end-over-end mixing, the slurry was placed in a 10 mL disposable column (BioRad, Hercules, CA) and washed with 300 column volumes of 30 mM imidazole in 20 mM Tris, 500 mM KCl, 10% glycerol, 0.1% $C_{12}E_9$, and 5 mM β-mercaptoethanol at pH 8.5 and 4 °C. Atp8a1 was eluted with 10 mL of 100 mM imidazole in 20 mM Tris, 100 mM KCl, 10% glycerol, 0.1% $C_{12}E_9$, and 5 mM β-mercaptoethanol at pH 8.5 and 4 °C. The final pH was adjusted to 7.4 by adding an appropriate amount of 20 mM Tris, 10% glycerol, 0.1% $C_{12}E_9$, and 5 mM β-mercaptoethanol at pH 7.0 and 4 °C.

SDS−PAGE. To determine the purity of the murine Atp8a1, proteins were separated on a 7.5% Laemmli polyacrylamide gel (*45*) at a constant current and proteins were detected by silver stain (*46*).

Western Blotting. Proteins separated on a 10% Laemmli polyacrylamide gel (*45*) were transferred to a nitrocellulose membrane (Osmonics, Livermore, CA) using a semi-dry blotting apparatus (Pharmacia, Piscataway, NJ). The membrane was blocked with Tris-buffered saline Tween-20 (TBST; 25 mM Tris, 0.15 M NaCl, and 0.05% Tween-20 at pH 7.6) containing BSA (2.5 mg/mL) for 1 h at room temperature, washed with TBST (2 × 10 min), and incubated with INDIA-HisProbeHRP (1:5000 dilution in TBST, Pierce, Rockford, IL) for 2 h at room temperature. The membrane was washed with TBST (4 × 10 min) and exposed to

SuperSignal Substrate Working Solution (Luminol/Enhancer solution with stable peroxide buffer, Pierce, Rockford, IL). The membrane was placed in a clear film protector and exposed to imaging film (X-OMAT, Eastman Kodak Co., Rochester, NY) for various times for 30 s−2 h.

Trypsinolysis/Mass Spectrometry Analysis. In-gel trypsin digestion of the 120 kDa band followed by mass spectrometry analysis was performed following the method of Beardsley et al. (*47*). Briefly, the gel band was cut from the gel, reduced with DTT (1.54 g/L), treated with iodoacetamide (10 g/L), and incubated with trypsin (12.5 ng/μL, 37 °C, 12−16 h). The peptides were extracted from the gel with increasing concentrations of acetonitrile (0−80%) and processed with MALDI−TOF mass spectrometry.

RESULTS

Members of the P_4-type ATPase class have been proposed to be aminophospholipid transporters (*24*), implying that the ATPase activity of these enzymes will be stimulated by the aminophospholipids, PS and PE. Therefore, we investigated whether the ATPase activity of a purified protein from this class, Atp8a1, is stimulated by aminophospholipids and whether this stimulation resembles the biochemical and lipid requirements of the plasma membrane aminophospholipid flippase activity.

Protein Expression. Sf21 cells were infected with the Atp8a1-containing baculovirus at an MOI of 5 and harvested 48 h postinoculation. Separate samples were infected with gus-containing baculovirus to control for nonspecific effects of infection or were treated with virus-free media (uninfected). Infection with either viral construct increased the total protein content (2.0 ± 0.1 ng/cell) and decreased cell viability (80% viable after 48 h) compared to uninfected cells (0.4 ± 0.1 ng/cell and 95% viability, respectively). To confirm the expression of Atp8a1, cellular lysates of uninfected, gus-infected, and Atp8a1-infected cells were separated by SDS−PAGE and transferred to a nitrocellulose membrane. The membrane was treated with INDIA-HisProbeHRP, which is specific for the His_6 tag attached to Atp8a1. Only lysates from murine Atp8a1-infected cells contained a HisProbe-reactive protein at 120 kDa (data not shown). Atp8a1-infected cellular lysate was separated by SDS−PAGE and visualized with Coomassie blue. The 120 kDa band was excised and subjected to trypsinolysis, and the resulting peptides were analyzed by MALDI−TOF/MS. This procedure resulted in 21 nontrypsin peaks, 16 of which had an *m*/*z* within 50 ppm of the predicted weights of peptides, resulting from a theoretical digestion of His_6-tagged Atp8a1 (data not shown).

ATPase Activity of Infected Sf21 Cells. Uninfected, Atp8a1-infected, and gus-infected Sf21 cells were solubilized with detergent (1% $C_{12}E_9$), and vanadate-sensitive ATPase activity was assessed in the presence of additional PS (Figure 1A). Infection of Sf21 cells with a baculovirus containing the Atp8a1 gene resulted in a 3.5-fold increase in PS-stimulated, vanadate-sensitive ATPase activity (4.9 ± 0.2 nmol min^{-1} mg^{-1}), compared to uninfected cells (1.4 ± 0.1 nmol min^{-1} mg^{-1}). This increase in ATPase activity was the result of the presence of the Atp8a1 gene and was not due to infection alone; ATPase activity of cells infected with the gus-containing virus was similar to that of uninfected cells (1.2 ± 0.2 nmol min^{-1} mg^{-1}).

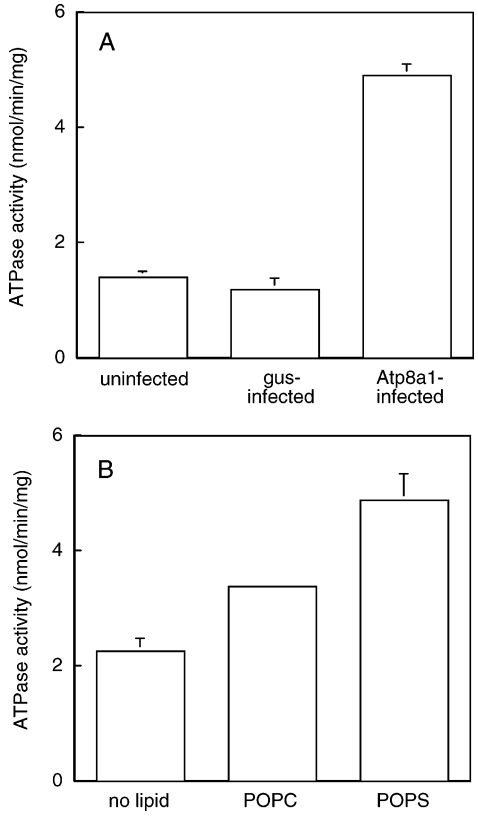

FIGURE 1: PS-stimulated, vanadate-sensitive ATPase activity of Sf21 cells. (A) Uninfected ($n = 3$), gus-infected ($n = 3$), and murine Atp8a1-infected ($n = 6$) Sf21 cells were detergent-solubilized. The cell lysates were incubated with additional POPS (250 μM) and ATP (2.5 mM) in the presence or absence of vanadate. After 1 h of incubation, the production of phosphate was measured. ATPase activity was defined as the difference in phosphate production for samples without vanadate and those containing vanadate. (B) Murine Atp8a1-infected cell lysates were incubated (1 h, 37 °C) without additional lipid ($n = 3$), with additional POPC (250 μM; $n = 3$), or with additional POPS (250 μM; $n = 6$) in the presence or absence of vanadate, and phosphate production was measured.

To determine if the expressed Atp8a1 was specifically activated by lipids, the ATPase activity of cell lysates obtained from Sf21 cells infected with Atp8a1-containing virus was measured in the presence of additional PS, additional PC, or no additional lipid (Figure 1B). PS increased ATPase activity 2.1-fold, from 2.3 ± 0.2 nmol min^{-1} mg^{-1} in the absence of lipid to 4.9 ± 0.5 nmol min^{-1} mg^{-1}. PC also produced an increase in ATPase activity (3.4 ± 0.5 nmol min^{-1} mg^{-1}) but to a lesser degree (1.5-fold) than PS. These data indicate that infection of Sf21 cells with Atp8a1-containing baculovirus resulted in an increase in lipid-selective ATPase activity and support the hypothesis that the Atp8a1 is a lipid-stimulated, vanadate-sensitive ATPase.

Specific Activity of Microsomal Fractions. The vanadate-sensitive ATPase activity of microsomal preparations of Atp8a1-infected and gus-infected cells was measured in the presence of PS (250 μM) and ATP (2.5 mM). Microsomes containing Atp8a1 had a specific activity of 4.4 ± 0.8 nmol min^{-1} mg^{-1}, while microsomes from gus-infected cells had a lower specific activity of 1.0 ± 0.5 nmol min^{-1} mg^{-1} (Figure 2). Solubilization of microsomes with detergent did not reveal cryptic ATPase activity, indicating that the

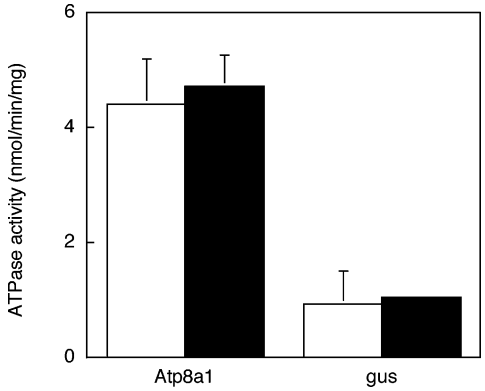

FIGURE 2: PS-stimulated, vanadate-sensitive ATPase activity of Sf21 microsomal membranes. Microsomes from gus-infected and murine Atp8a1-infected Sf21 cells were prepared as described in the text. POPS (250 μM) and ATP (2.5 mM) were added to intact (open bars; $n = 4$) or detergent-solubilized (filled bars; $n = 4$) microsomes, and the samples were incubated (1 h, 37 °C). The production of phosphate was measured, and the specific activity was defined as the difference between samples without vanadate and those containing vanadate.

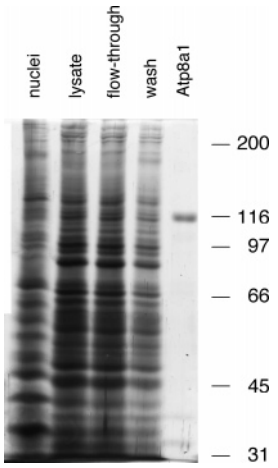

FIGURE 3: Purification of His_6-tagged murine Atp8a1. SDS−PAGE (7.5% acrylamide) of the murine Atp8a1 purification from Sf21 cells. Sf21 cells expressing murine Atp8a1 were detergent-solubilized, homogenized, and centrifuged to remove nuclei (nuclei), and the remaining sample (lysate) was incubated with Ni−NTA resin (4 °C). After 45 min, the lysate−resin slurry was added to a column. Proteins that did not bind to the column appeared in the flow-through. The column was washed with a 30 mM imidazole buffer (wash), and Atp8a1 was eluted with a 100 mM imidazole buffer (Atp8a1). The migration of protein molecular-weight markers is indicated (in kilodaltons).

catalytic ATPase domain was exposed on the cytoplasmic surface of the microsome.

Purification of Murine Atp8a1. Utilizing a genetically incorporated His_6 tag, Atp8a1 was purified from Sf21 cells by Ni−NTA affinity chromatography. Only one protein (120 kDa) eluted in the 100 mM imidazole fraction (Figure 3). Typically, 2 mg of Atp8a1 protein was produced per 1 L of Atp8a1-infected Sf21 cells. The transfer of this protein to a nitrocellulose membrane followed by exposure to INDIA-HisProbeHRP confirmed that the 120 kDa protein was a His-tagged protein (data not shown).

Lipid-Stimulated Activity of Detergent-Solubilized Atp8a1. Lipid-stimulated, vanadate-sensitive ATPase activity was measured for the purified, detergent-solubilized Atp8a1 (Figures 4 and 5). No activity was observed in the absence

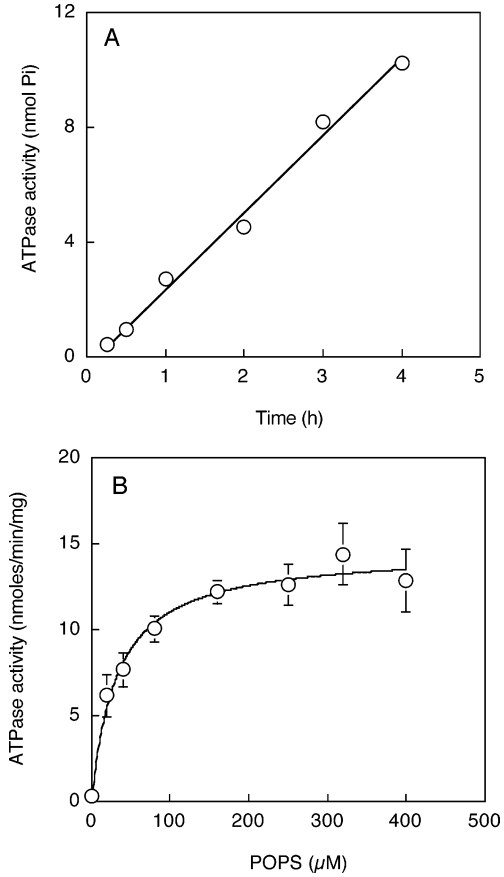

FIGURE 4: Activation of Atp8a1 ATPase activity by PS. (A) POPS (250 μM) was added to the detergent-solubilized Atp8a1 in the presence or absence of vanadate and incubated for the indicated times at 37 °C in the presence of ATP ($n = 1$). (B) POPS, at the indicated concentrations, was added to the detergent-solubilized Atp8a1 in the presence or absence of vanadate and incubated for 3 h at 37 °C in the presence of ATP. ATPase activity was defined as the difference in activity of samples without vanadate and those with vanadate ($n = 3$).

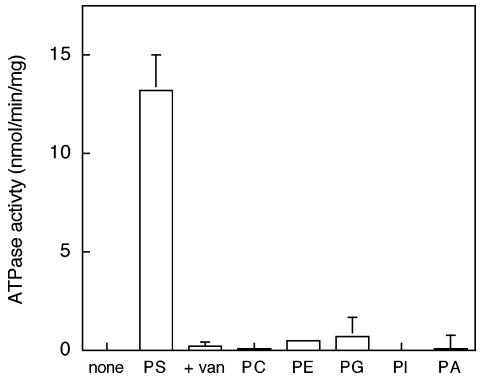

FIGURE 5: Lipid-stimulated, vanadate-sensitive activity of detergent-solubilized Atp8a1. No lipid (none, $n = 3$), PS ($n = 53$), PS + van ($n = 4$), PC ($n = 7$), PE ($n = 5$), PG ($n = 3$), PI ($n = 4$), or PA ($n = 3$) was added (250 μM) to the purified, detergent-solubilized Atp8a1 in the presence or absence of vanadate and incubated for 3 h at 37 °C in the presence of ATP. ATPase activity was defined as the difference in activity of samples without vanadate and those with vanadate.

of lipid (0.1 ± 0.1 nmol min^{-1} mg^{-1}), while the addition of PS (250 μM) activated the enzyme significantly (13.2 ± 1.8 nmol min^{-1} mg^{-1}). Phosphate release was linear for at least 4 h (Figure 4A), and activation of the ATPase by PS resulted in a V_{max} of 14.6 ± 0.5 nmol min^{-1} mg^{-1} and an apparent

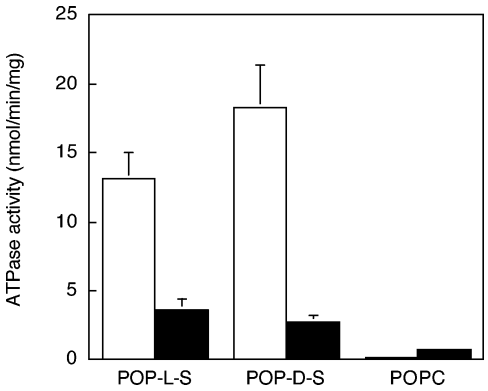

FIGURE 6: Lipid stereospecificity of Atp8a1. Various PS and PC stereoisomers [250 μM; *sn*-1,2 POP-L-S ($n = 53$), *sn*-2,3 POP-L-S ($n = 4$), *sn*-1,2 POP-D-S ($n = 3$), *sn*-2,3 POP-D-S ($n = 4$), *sn*-1,2 POPC ($n = 3$), and *sn*-2,3 POPC ($n = 3$)] were added to the purified, detergent-solubilized Atp8a1. ATPase activity was measured for 3 h at 37 °C in the presence or absence of vanadate (125 μM) and was defined as the difference between these samples. Open bars represent the *sn*-1,2 lipids, and filled bars represent *sn*-2,3 lipids.

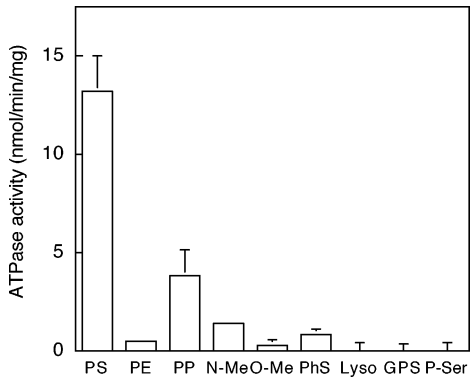

FIGURE 7: PS structural requirements of murine Atp8a1. PS analogues (250 μM) were added to the purified, detergent-solubilized Atp8a1, and ATPase activity was measured for 3 h at 37 °C in the presence or absence of vanadate (125 μM). Activity was defined as the difference between samples without vanadate and those with vanadate. PS, POPS ($n = 53$); PE, POPE ($n = 5$); N-Me, POPS-*N*-Me ($n = 3$); O-Me, POPS-*O*-Me ($n = 3$); PhS, POPhS ($n = 3$); Lyso, lyso-oleoyl-PS ($n = 1$); GPS, glycerophosphorylserine ($n = 3$); and P-Ser, phosphoserine ($n = 6$).

K_M of 32 ± 5 μM (Figure 4B). The presence of vanadate (100 μM) inhibited activation by PS (0.2 ± 0.2 nmol min^{-1} mg^{-1}; Figure 5). Less activity was obtained in the presence of PE (0.5 ± 0.1 nmol min^{-1} mg^{-1}) and PG (0.8 ± 0.9 nmol min^{-1} mg^{-1}). PC, PA, and PI did not activate the ATPase. Purification of proteins, as described above, from Sf21 cells infected with a control virus lacking the Atp8a1 gene, resulted in a preparation that had no detectable ATPase activity (data not shown).

Because the plasma membrane aminophospholipid flippase is activated only by the *sn*-1,2-stereoisomer of PS, the stereoselectivity of Atp8a1 for PS was determined (Figure 6). Maximal activation occurred in the presence of *sn*-1,2-glycerol lipids, regardless of serine stereochemistry. Incubation with *sn*-1,2-P-L-S yielded an activity of 13.2 ± 1.8 nmol min^{-1} mg^{-1}, while *sn*-1,2 P-D-S yielded an activity of 18.3 ± 3 nmol min^{-1} mg^{-1}. Altering the glycerol stereochemistry to *sn*-2,3 glycerol led to a decrease in specific activity, again independent of serine stereochemistry. Incubation with *sn*-2,3-P-L-S produced an activity of 3.6 ± 0.8 nmol min^{-1} mg^{-1}, while incubation with *sn*-2,3-P-D-S led to an activity of 2.7 ± 0.5 nmol min^{-1} mg^{-1}. Regardless of glycerol stereochemistry, PC did not activate the enzyme; *sn*-1,2-PC and *sn*-2,3-PC stimulated ATPase activity similarly (0.1 ± 0.1 and 0.8 ± 0.2 nmol min^{-1} mg^{-1}, respectively). Maximum activation of Atp8a1 in the presence of PS and the ability of the enzyme to discriminate between various PS stereoisomers indicate that the activation results from the association of PS with a specific binding site on the enzyme.

The functional group requirements for PS recognition by Atp8a1 were determined (Figure 7). Maximal activation occurred in the presence of POPS (13.0 ± 1.5 nmol min^{-1} mg^{-1}). Removal of the amine group (POPP) activated the enzyme to ~33% of the activity observed in the presence of PS (3.9 ± 1.0 nmol min^{-1} mg^{-1}), while removal of the carboxyl group (PE) limited activation of the enzyme. Methylation of the amine (POPS-*N*-Me) or carboxyl (POPS-*O*-Me) group as well as the addition of a methylene group (POPhS) yielded minimal activation of the enzyme. Neither lyso PS, L-α-glycerophosphorylserine (GPS), nor phosphoserine (P-ser) activated the enzyme in a vanadate-sensitive manner, indicating that both acyl chains and the glycerol backbone are required for enzyme activation, a trait shared by the flippase activity.

DISCUSSION

The P_4 subfamily of ATPases has been implicated in a variety of membrane-related functions, most of which focus on amphipath transport (for reviews, see refs *2*, *48*, *49*). Defects in P_4-ATPase subfamily members have been linked to cold sensitivity in plants (*28*), familial intrahepatic cholestasis (*30*), ribosomal assembly (*25*), and clathrin-dependent vesicle trafficking (*31*−*33*). Although a substantial amount of indirect evidence for the function of these enzymes exists, few studies have addressed the properties of these proteins in their purified form. This paper addressed the question of whether Atp8a1 and, by homology, class 1 P_4-ATPases (including Drs2p) are aminophospholipid-specific ATPases. A mouse homologue of the bovine ATPase II protein, which shares 97.6% homology with the bovine chromaffin granule ATP8A1, was overexpressed using the baculovirus expression system and purified, and the biochemical requirements for ATPase activity were determined in a detergent−lipid−protein mixed micelle system.

Activation of Atp8a1 by Phospholipids Is Similar, although Not Identical, to the Plasma Membrane Aminophospholipid Flippase Substrate Requirements. Atp8a1, like aminophospholipid flippase activity, is a vanadate-, Ca^{2+}-, and sulfhydryl reagent-sensitive ATPase (*22*). However, the aminophospholipid-specific transporter is identified by its ability to discriminate between PS, PE, and PC. In the present study, we explored in detail whether a similar specificity can be demonstrated in a purified enzyme from this subfamily, including both stereochemical and structural features. The enzyme was completely inactive in the absence of added lipid and was activated maximally by the addition of PS. Other lipids failed to activate the enzyme substantially. Atp8a1 was not activated by PC, which is not a flippase substrate, nor is the enzyme activated by PE, which is a weak flippase substrate. The negatively charged lipids, PA and PI, did not activate the ATPase activity of Atp8a1, while PG was a weak

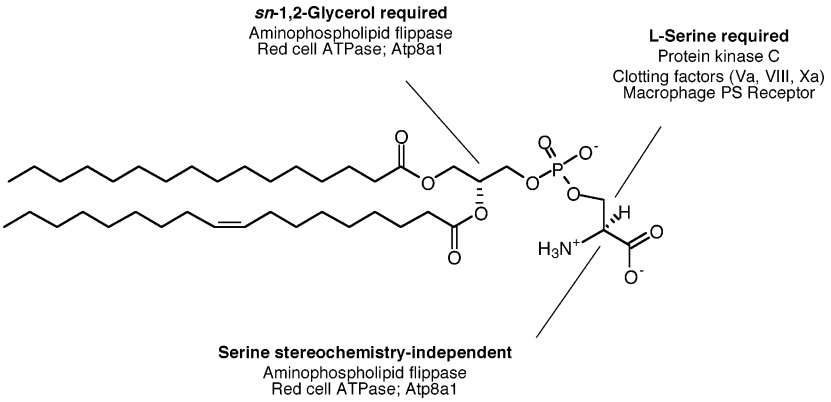

FIGURE 8: PS structural elements required for recognition by PS-binding proteins. PS-binding proteins fall into two major classes: those that selectively recognize the L-serine stereosisomer (protein kinase C, clotting factors Va, VIII, and Xa, and the macrophage PS receptor) and those that selectively recognize the *sn*-1,2-glycero stereoisomer (aminophospholipid flippase, red blood cell ATPase, and Atp8a1).

activator, indicating that PS recognition is not due to the net negative charge on PS. Enzyme activation also required the presence of both fatty acyl groups. The elimination of one (lyso PS) or both (GPS) fatty acyl groups, prevented ATPase activation. With the notable exception of the lack of activation by PE, these properties are in general agreement with the substrate specificity of the aminophospholipid flippase.

Stereochemical Requirements for PS Recognition by Murine Atp8a1 Are Identical to the Aminophospholipid Flippase. A hallmark of the specificity of the aminophospholipid flippase for the transport of PS is the strict requirement for the *sn*-1,2-glycerol configuration but no specificity for the headgroup serine stereochemistry (*14*, *19*, *50*). Inversion of the PS serine α-carbon stereochemistry from the naturally occurring L-serine to the nonnatural D-serine did not alter ATPase activity of Atp8a1, indicating that both stereoisomers are recognized by this enzyme. Inversion of the C2 carbon of the glycerol backbone from the naturally occurring *sn*-1,2 glycerol conformation to the *sn*-2,3-glycerol isomer eliminated activation by PS, regardless of the stereochemistry of the serine headgroup. These stereochemical requirements are identical to those for the aminophospholipid flippase, indicating that both enzymes recognize the lipid by making specific contacts with the glycerol backbone but that binding to the headgroup functional groups is flexible.

Functional Group Requirements for PS Recognition by Atp8a1 Are Similar but Not Identical to the Aminophospholipid Flippase. To further investigate the contacts of the enzyme with PS, ATPase activity was measured in the presence of various PS analogues. Deletion of the amine group (PP) allowed for ATPase activation, although at only 33% of the level attained in the presence of PS. This activation was specific for PP and not the result of the increased negative charge on this lipid; other negatively charged lipids (PG, PA, and PI) did not activate ATPase activity. The activation of Atp8a1 in the presence of PP but not PE indicates that the carboxyl group is required for recognition of PS by Atp8a1 and that the amine group is preferred but not required for recognition. In comparison, the aminophospholipid flippase prefers phospholipids with a primary amine group (PE and PS) but can also transport PP, although at a reduced rate (Smriti and D. Daleke, unpublished observations). These data indicate that, in both enzymes, two contacts are made between the lipid and the enzyme but only one is required for enzyme activation. An additional contact, by either the primary amine or the carboxyl group, further stimulates activity. These data are also consistent with the lack of a stereochemical preference for the serine headgroup and indicate that the binding of these potentially charged groups to the enzyme is flexible.

The results also indicate that there are limits to the size of the lipid-binding site. Phosphatidylhomoserine was incapable of activating Atp8a1 and is not a strong flippase substrate. Further, the addition of a methyl group to either the amine or the carboxyl group eliminates Atp8a1 activation, which is consistent with either a size constraint or the disruption of an important contact with the protein. In contrast, flippase activity tolerates methylation of the amine group (PS-*N*-Me) well, but methyl esterification of the carboxyl group significantly reduces the transport activity (ref *15* and Smriti, E. Nemergut, and D. Daleke, unpublished observations).

Lipid Requirements for ATPase Activity of Atp8a1 Differ from Other PS-Dependent Proteins. The ATPase activity of Atp8a1 is only stimulated by PS and not by PE or PS-*N*-Me, all three of which are substrates of the aminophospholipid flippase. Both are stimulated by PS regardless of the stereochemistry of the serine α carbon but have a strict requirement for the C2 glycerol backbone stereochemistry. This stereospecificity of the interaction of these enzymes with PS is unique among PS-binding proteins (Figure 8). Other PS-selective proteins are either selective for the L-serine isomer [protein kinase C (*51*−*53*), clotting factors Va, VIII, and Xa (*54*−*56*), and the macrophage PS receptor (*57*)] or will tolerate the *sn*-2,3-glycerol isomer [protein kinase C (*51*−*53*)]. One reason for the difference may be that Atp8a1 and the aminophospholipid flippase are integral membrane proteins and interact with PS laterally, in the plane of the membrane, whereas the aforementioned PS-binding proteins all interact from the aqueous surface. Thus, the PS-binding sites of Atp8a1 and the aminophospholipid flippase share a similar structure. However, the differences in lipid specificity, particularly in regard to PE and PS-*N*-Me, suggest that, if Atp8a1 is a PS flippase, the PS-binding motifs differ between the plasma membrane PS flippase and Atp8a1 and, thus, that Atp8a1 may not be the enzyme responsible for aminophospholipid flippase activity at the plasma membrane. Alternatively, if the vesicle and plasma membrane translocases are

one and the same, the specificity of ATPase activation differs from the specificity of substrates transported.

Comparison with Other P-Type ATPases. Conclusions about the lipid specificity of P-type ATPases have been drawn primarily from comparisons of the interaction of naturally occurring lipids with these enzymes, without the type of detailed structural analysis presented here. Almost all subfamilies of P-type ATPases are mono- or divalent cation transporters. Although some of these subfamilies display some degree of phospholipid specificity to support ATPase and transport activity, none show specificity for PS. The plasma membrane Ca^{2+}-ATPase (*58*) and the Na^+,K^+-ATPase (*59*) both show a preference for acidic phospholipids to express maximal activity but show no preference for aminophospholipids. In addition, although the P_4-ATPases share overall sequence similarity with the cation ATPases, suggesting that they also share a similar structure, key sequence differences imply transport of very different molecules. When the sequence of Atp8a1 is threaded onto the structure of the sarcoplasmic reticulum Ca^{2+}-ATPase, the model produced is consistent with a role in the transport of more bulky substrates by the P_4-ATPases (*60*). Four of the eight consensus motifs that define the P_4 subfamily are clustered at the membrane interface and contain nonpolar residues that could provide access for amphipaths to the membrane interior. In addition, the membrane-buried amino acid residues required for cation transport in the Ca^{2+}-ATPase are substituted with uncharged or nonpolar residues in the P_4 subfamily (*24*, *61*), suggesting transport of more complex, perhaps amphipathic, molecules.

Several P_4-ATPases have been implicated in the transbilayer transport of phospholipids. The most significant of these are the founding member of this class, the yeast Drs2p, and related yeast isoforms, Dnf1−3p and Neo1p (for a review, see ref *32*). Studies with yeast mutants have shown that Dnf1p and Dnf2p are required for the uptake of fluorescent analogues of PC, PE, and PS across the yeast plasma membrane (*62*). Similar studies have shown that Drs2p plays a limited role in the transport of fluorescent lipid analogues at the plasma membrane, although it may be capable of PS and PE transport (*24*, *63*, 64). Using isolated yeast membrane vesicles, Natarajan et al. report that functional Drs2p is required for the transport of fluorescent PS across the Golgi membrane (*65*). It has also been reported that a plant member of the P_4-ATPase subfamily, ALA1, rescues the cold-sensitive phenotype of *drs2* cells and transports PS and PE (*28*). These results suggest that not all members of the P_4-ATPase subfamily are specific for aminophospholipids but that relatives of Drs2 and ALA1 may prefer this class of phospholipids.

In addition to Drs2p, the closest relatives of the murine Atp8a1 that have been characterized biochemically include the bovine chromaffin granule ATPase II, four bovine brain isoforms of the same enzyme, and ATP8B1. The bovine chromaffin granule ATPase II is not activated by PC, PE, or negatively charged lipids, with the exception of partial activation (∼25%) by cardiolipin and lyso PS (*23*). Ding et al. (*36*) described four bovine brain isoforms of ATP8A1 that differed from one another by the presence (α) or absence (β) of a 15 amino acid insert in the second cytoplasmic loop and the presence of one of two alternative 20 amino acid sequences in the first cytoplasmic loop. Both PS and PE activated the ATPase activities of all of these isoforms, but the α isoform exhibited the greatest stimulation by PS. The murine Atp8a1 studied here is most similar in sequence to the β_1 bovine brain isoform. However, the response to PS and PE was more consistent with that observed by the α_2 isoforms. Other sequence variations may play a role in these differences. Mutations in a related family member, ATP8B1, are associated with familial intrahepatic cholestasis, which is characterized by the poor export of bile. Transient transfection of CHOK1 cells with ATP8B1 enhances PS transport (*66*), but the transport activity of other lipids was not described. In summary, the activation of the purified Atp8a1 by lipid is similar to that reported for its closest relatives in the subfamily but is distinct from other P-type ATPases.

The magnitude of the ATPase activity exhibited by the purified Atp8a1 (∼15 nmol min^{-1} mg^{-1}) is much lower than that of other P-type ATPase subfamilies, which are typically in the range of ∼1 μmol min^{-1} mg^{-1}. However, the activity of the preparation described here is comparable to that of the bovine brain β_1 isoform. The bovine brain isoforms reported by Ding et al. were purified in two stages, and the initial stage of purification (Ni−NTA chromatography) was similar to that described in this paper. At this level of purification, the isoform (β_1) yielded an ATPase activity of 89 nmol min^{-1} mg^{-1}, approximately 6-fold greater than the activity reported here for Atp8a1. Further purification of the bovine brain enzymes (hydroxylapaptite chromatography and glycerol-gradient density centrifugation) yielded an increase in specific activity ($\beta_1 \sim 2.5$ μmol min^{-1} mg^{-1}) (*36*). The differences between the activity of the murine and bovine isoforms may indicate either that Atp8a1 is not properly assembled by the insect cell expression system or that the correct environment for full activation of ATP has not been provided. Two possible explanations are that (1) the native substrate for the Atp8a1 enzyme is not PS and the activity observed here is only basal PS-stimulated activity or (2) that another protein component(s) is required for full activation of the ATPase activity. The recent discovery that members of the Cdc50p/Lem3p family in yeast, a group of small membrane proteins structurally unrelated to ATPases, associate with yeast P_4-ATPases and are required for their proper localization (*67*) lends credence to the latter possibility. Exploration of these hypotheses is the subject of ongoing work. Regardless, the present data indicate that Atp8a1 and perhaps other P_4-ATPases are unique in their recognition and activation by PS.

Conclusive evidence that P_4-ATPases transport amphipaths awaits successful reconstitution of the enzyme and demonstration of ATP-dependent lipid transport. This lack of evidence, considering the number of P_4-ATPases that have been characterized, likely reflects the challenges of measuring transbilayer lipid transport in reconstituted liposomes and providing the proper environment to support activity. The yeast P_4-ATPase data indicate that other protein partners may be required for full activity or proper reconstitution (*67*). In addition, lipid transport activity in a reconstituted proteoliposome may create membrane perturbations, such as a bilayer imbalance resulting from transbilayer lipid transport, that would limit activity. Overcoming these limitations and successfully reconstituting P_4-ATPases is the focus of future studies.

CONCLUSION

The ATPase activity of murine Atp8a1 (mATPAse II) is PS-selective. Lipid recognition is not strictly dependent upon molecular charge, because other negatively charged lipids do not activate the enzyme, but is strongly dependent upon lipid stereochemistry. Atp8a1 recognizes both serine α-carbon isomers but only recognizes the *sn*-1,2-glycerol isomer. PS stimulation requires the serine carboxyl group, is maximal when the serine amine group is present, and is restricted by the headgroup size. Although this pattern is similar to that seen for the aminophospholipid translocase, it is distinct and clear qualitative differences exist between the substrate requirements for aminophospholipid flippase activity and Atp8a1 activation.

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