The Leishmania ATP-binding Cassette Protein PGPA Is an Intracellular Metal-Thiol Transporter ATPase*

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Danielle Légaré‡§, Dave Richard‡¶, Rita Mukhopadhyay¶, York-Dieter Stierhof**‡‡, Barry P. Rosen‡, Anass Haimeur‡ §§, Barbara Papadopoulou‡ ¶¶, and Marc Ouellette‡ ¶¶

From the ‡Centre de Recherche en Infectiologie du Centre Hospitalier de l’Université Laval et Division de Microbiologie, Faculté de Médecine, Université Laval, 2705 boulevard Laurier Ste-Foy, Québec G1V 4G2, Canada, the °Department of Biochemistry and Molecular Biology, Wayne State University School of Medicine, Detroit, Michigan 48201, and the **Max-Planck-Institut fur Biologie, D-72076 Tuebingen, Germany

The Leishmania ATP-binding cassette (ABC) transporter PGPA is involved in metal resistance (arsenicals and antimony), although the exact mechanism by which PGPA confers resistance to antimony, the first line drug against Leishmania, is unknown. The results of co-transfection experiments, transport assays, and the use of inhibitors suggest that PGPA recognizes metals conjugated to glutathione or trypanothione, a glutathione-spermidine conjugate present in Leishmania. The HA epitope tag of the influenza hemagglutinin as well as the green fluorescent protein were fused at the COOH terminus of PGPA. Immunofluorescence, confocal, and electron microscopy studies of the fully functional tagged molecules clearly indicated that PGPA is localized in membranes that are close to the flagellar pocket, the site of endocytosis and exocytosis in this parasite. Subcellular fractionation of Leishmania tarentolae PGPA transfectants was performed to further characterize this ABC transporter. The basal PGPA ATPase activity was determined to be 115 nmol/mg/min. Transport experiments using radioactive arsenite-glutathione conjugates clearly showed that PGPA recognizes and actively transports thiol-metal conjugates. Overall, the results are consistent with PGPA being an intracellular ABC transporter that confers arsenite and antimony resistance by sequestration of the metal-thiol conjugates.

Leishmania is a protozoan parasite responsible for a wide variety of human diseases ranging from mild cutaneous infections to fatal visceral diseases. Millions of people are infected worldwide with 400,000 new cases reported each year (1). No effective vaccine is yet available against this parasite, and treatment relies primarily on antimony containing drugs, such as sodium stibogluconate (Pentostam) and N-methylglucamine (Glucantime) (2, 3). However, resistant parasites are now described on a frequent basis in several endemic regions (4–8).

Our in vitro work on metal resistance in Leishmania has led to a model of resistance in which the ABC-binding cassette (ABC) protein PGPA plays a major role (9). ABC proteins are a large family of proteins, several of which are implicated in drug resistance (10). When discovered, PGPA was the most divergent of the eukaryotic ABC protein (11), and when the multidrug resistance protein (MRP-1) was cloned and sequenced, PGPA was found to be its closest homologue (12). Therefore, PGPA is a member of the MRP family, a large family of ABC transporters from which substrates include organic anions and drugs conjugated to glutathione (GSH), glucuronate, or sulfate (for review see Ref. 13). The link between PGPA and metal resistance in Leishmania is clear; the PGPA gene is frequently amplified in Leishmania cells that are selected for resistance to arsenite- or antimony-containing drugs (14–19), and its transfection as well as its disruption prove that PGPA is involved in metal resistance (18, 20–22).

In addition to PGPA amplification, we observed that the levels of trypanothenate (TSH), a biglutathione-spermidine conjugate specific for Trypanosomatidae (23), is increased in all the metal resistant Leishmania parasites that we have studied (18, 19, 24). TSH is increased by the amplification of the GSH1 gene coding for γ-glutamylcysteine synthetase (γ-GCS) (25), the rate-limiting step in glutathione biosynthesis, and by the overexpression of the ornithine decarboxylase gene ODC (26), the rate-limiting step in spermidine biosynthesis. Increased levels of TSH by themselves are not sufficient to observe metal resistance, but the modulation of TSH levels by using specific inhibitors of γ-GCS or ODC will revert the resistance in mutants (26). Co-transfection of the GSH1 or ODC and PGPA genes in partial revertants, but not in wild-type cells, will lead to synergistic levels of resistance (25, 26) strongly suggesting

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† To whom correspondence should be addressed: Center de Recherche en Infectiologie du CHUL, 2705 boul. Laurier Ste-Foy, Quebec G1V 4G2, Canada. Tel.: 418-654-2705; Fax: 418-654-2715; E-mail: marc.Ouellette@crchul.ulaval.ca.

†† Present address: Center of Plant Molecular Biology, D-72076 Tuebingen, Germany.

The abbreviations used are: ABC, ATP-binding cassette; MRP, multidrug resistance protein; GSH, glutathione; TSH, trypanothenate; γ-GCS, γ-glutamylcysteine synthetase; ODC, ornithine decarboxylase; HA, hemagglutinin; PCR, polymerase reaction; GFF, green fluorescent protein; BSO, buthionine-sulfoximine; DFMO, 1–α-difluoromethylornithine; PBS, phosphate-buffered saline; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PGPA, Gp-glycoprotein-like protein A.
that PGPA recognizes metals conjugated to TSH.

PGPA is therefore an important determinant of arsenite and antimonite resistance. However, the mechanism by which this ABC protein confers resistance is unclear. Transport experiments in arsenite resistant *L. tarentolae* mutants indicated the presence of an active efflux system that did not correlate with PGPA gene amplification (27, 28), whereas in *Leishmania mexicana* resistant cells, a perfect correlation between PGPA amplification and efflux was observed (29). An analysis of *L. tarentolae* PGPA transfectants did not show a marked difference in the steady state accumulation of arsenite (21), although a decrease in the uptake of antimony was proposed to explain the resistance in *Leishmania major* PGPA transfectants (30). We have proposed that PGPA is an intracellular ABC transporter that confers resistance by sequestration of metal-thiol conjugates (9). In this study, we have determined the cellular location of PGPA and have studied its transport properties.

MATERIALS AND METHODS

DNA Constructs—The GSH1 plasmid was described elsewhere (25). The *PGPA* constructs are summarized in Fig. 1. A DNA sequence encoding the nine amino acids of the HA epitope (black box) was fused in frame to PGPA by PCR. An EcoRV restriction site was introduced during the PCR strategy to facilitate the in frame cloning of the green fluorescent protein (GFP). The PCR fragment was digested with *Sal*I (a unique restriction site cutting in *PGPA*) and *Xba*I and cloned into pMAC6, which was also digested with *Sal*I (cutting in *PGPA*) and *Xba*I (cutting in the polylinker). This pMACSHA version was subcloned into pSPY-NEO (32) to lead to the PGPAHA version. The absence of unwanted mutations was confirmed by DNA sequencing.

The PGPAHA hybrid was constructed by inserting the coding region of the GFP in the *PGPA* construct in the unique EcoRV site (Fig. 1). A 719-base pair GFP blunt PCR fragment was amplified by the PWO DNA polymerase (Roche Molecular Biochemicals) with the primers GFP-1 (5′-CCA-TGG-TGA-GCA-AAG-CGG-AGG-AAG-AGC-3′) and GFP-2 (5′-ACT-TGT-ACA-GCT-CGT-CCA-TGC-CG-3′) using the phGFP-S65T plasmid (CLONTECH Laboratories) as a template.

**TABLE I**

| Gene(s) transfected | TarIWT | Ase20.3rev |
|---------------------|--------|-----------|
|                     | BSO + DFO | BSO + DFO |
| PSpYNEO             | 1.0     | –         |
| PGPA                | 2.0     | 2.0       |
| PGPAHA              | 2.0     | 2.0       |
| PGPAFFFHA           | 2.2     | 2.1       |
| GSH1                | 1.0     | 1.0       |
| PGPA + GSH1         | 2.0     | 1.5       |
| PGPAHA + GSH1       | 2.2     | 2.0       |
| PGPAFFFHA + GSH1    | 2.1     | 1.1       |

*average of at least three independent growth curves for each transfectant.

Cell Culture, Transfections, and Growth Curves—Promastigotes of *L. tarentolae* TarIWT cell line were cultured at 28 °C in SDM-79 medium containing 5% fetal bovine serum. The *L. tarentolae* PGPA null mutant and the arsenite revertant mutant Ase20.3rev have been described previously (15, 22). Parasites were transfected by electroporation using a single discharge at 450 V and 500 microfarads. In experiments where tunicamycin (Sigma) was used, promastigote cultures were subdivided and grown without drug or with tunicamycin at 1 mg and 5 mg/ml. Resistance of resistance with 5 mg 1-thionine-(SR)-sulfonamide (BWO), a specific inhibitor of γ-GCS (33), or 5 mg 1,2-difluoromethyl-ornithine (DFMO), a specific inhibitor of ODC (34), was done as described previously (25, 26).

Immunofluorescence and Electron Microscopy—For immunofluorescence imaging, cells were prepared as described previously (35). For detection of PGPAHA transfectants, cells were spotted into wells of coated slides, allowed to air dry, permeabilized at −20 °C with methanol for 15 min, washed with PBS, and blocked with 5% bovine serum albumin in PBS for 1 h. Incubations with primary and secondary antibodies (in the dark) were performed at room temperature for 1 h in a humidified chamber, each followed by three 5-min washes in PBS containing 1% bovine serum albumin. The polyclonal rabbit antibody HA.11 (Babco, Berkeley, CA) against the HA tag was used at 1:50 dilution in PBS + 1% bovine serum albumin. The secondary antibody, fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) was used at 1:200 dilution in the same buffer. In some experiments, PGPAHA transfectant cells were also revealed directly using the fluorescein isothiocyanate-labeled-anti-HA antibody (Babco). Permeabilized fixed cells were examined by using a Nikon LaboPHo microscope equipped with a fluorescein isothiocyanate filter. To visualize the nucleus and kinetoplast DNA, Rhase-pretreated cells were stained with propidium iodide. Evans’ blue was used as a counterstain. Paraformaldehyde fixed cells were also examined by laser confocal microscopy as described previously (35). For immunoelectron microscopy, cells were fixed with 4% or 2% formaldehyde, 0.05% glutaraldehyde in 0.1 M Hepes, pH 7.0, for 60 min on ice. Fixed *Leishmania* cells were processed for ultrathin cryosection immunogold labeling as described previously (36). Thawed frozen sections were reacted with rabbit anti-GFP antibodies (CLONTECH) or a monoclonal mouse anti-HA antibody (HA.11, Babco, Richmond, VA) for 60 min each. Reactions were revealed using protein A-gold (6 nm) or goat anti-rabbit-IgG-Nanogold (Nanoprobes, Stony Brook, NY). A silver enhancement (silver lactate, gum arabic) of Nanogold was performed as described previously (36). Uranyl acetate staining and final embedding in 1.8% methylcellulose was done according to previously published methods (38).
Cell Fractionation and Immunoblots—Protein electrophoresis, protein transfer, and their reactions with polyclonal anti-PGPA (18) or anti-HA antibodies were performed following standard procedures (39). Cells were collected, washed, resuspended in an isosmotic buffer (4.4% (w/v) solution containing 10 mM Tricine-NaOH, pH 7.8), and broken by abrasion using acid-washed glass beads (150–212 μm diameter, Sigma). More than 95% of the cells were disrupted as estimated by light microscopy. The homogenate was resuspended in sorbitol buffer containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.15 mg/ml trypsin inhibitor). Unbroken cells and glass beads were sedimented, and the final supernatant (≈ 9 ml) was carefully layered onto the top of discontinuous (40:28:18:5%) iodixanol (Optiprep™) gradients. The gradients were centrifuged in a swinging bucket (SW41, Beckman) at 40,000 rpm for 90 min at 4 °C, and fractions (500 μl) were collected from the bottom. Each fraction was then diluted with sorbitol buffer and spin at 60,000 rpm for 1 h (TY70.1, Beckman). Pellets were washed 3 times with buffer C (75 mM Hepes-KOH, 0.15 M KCl, 5 mM MgCl₂, pH 7.4) and resuspended in ~300 μl of the same buffer and homogenized 3 times in a glass potter. Protein concentration was determined by the Amido Black method.

ATPase Activity—The basal Mg²⁺-ATPase activity of the PGPAHA variant and the PGPA null mutant was determined by measuring the release of inorganic phosphate from ATP using a colorimetric method at 850 nm (40) as described by Shapiro and Ling (41) for the measurement of NTPase activities of mammalian ABC transporters. To inhibit the contribution of other contaminating ATPases, the ATPase assay was performed in the presence of sodium azide to inhibit F₁F₀-type ATPase activity and ouabain to inhibit Na/K-type ATPases. These inhibitors do not interfere with ABC-dependent drug transport (42). Protein fractions (10 μg) were incubated at room temperature in a final volume of 45 μl of buffer assay (final concentrations: 60 mM Tris, pH 7.3, 50 mM KCl, 10 mM sodium azide, 2 mM ouabain). ATPase reaction was started by the addition of 5 μl of MgATP (7 mM MgSO₄, 5 mM ATP) for 20 min and stopped by the addition of 50 μl of 12% SDS. In preliminary attempts to stimulate the ATPase activity, we used arsenite or antimonials conjugated or not conjugated with glutathione or trypanothione (28) in con...
and thiol 

man), and washed with 5 ml of cold buffer D. The filters were dried, and protein of 175 kDa (Fig. 1, B

rated into the COOH terminus (Fig. 1, C).

experiments (data not shown). For that reason, PGPA was detected in the membrane fraction corresponding to the cytosol (Fig. 1B, lane 4), and PGPAHA was found exclusively in the membrane fraction (Fig. 1B, lane 5).

The transfected PGPA-tagged versions led to proteins of the expected size as determined by Western blot analysis. Both tagged proteins gave the same resistance level to metals as the transfected untagged PGPA version (Table I), demonstrating that the epitope-tagged proteins are still functional. We have shown previously that co-transfection of GSH1 and PGPA in the revertant As20.3 rev but not in wild-type cells will confer metal resistance in a synergistic manner (25). Similarly, the co-transfection with GSH1 of either tagged versions of PGPA produced higher resistance levels than the individual contribution of each determinant (Table I).

We have shown previously that the combination of the γ-GCS inhibitor BSO and the ODC inhibitor DFMO reversed resistance in arsenite or antimonite resistant mutants (19, 26). On the basis of co-transfection experiments, we have proposed that PGPA is a thiol-X pump (25), but the combination BSO + DFMO was never used before on PGPA transfected cells. This combination indeed decreased PGPA-mediated resistance either in a wild-type or revertant background (Table I), and the tagged PGPA versions had similar properties. The combination BSO + DFMO was more effective in reversing resistance than BSO or DFMO alone (data not shown). Overall, these results suggest that the PGPAHA and PGPAGFPHA fusions confer the same phenotype as the overexpressed wild-type PGPA protein.

PGPA-HA did not react with HA-fluorescein isothiocyanate-labeled antibody (Fig. 2A). However, when cells were permeabilized, a strong signal was observed upon reaction with the antibody. Indeed, a discrete bright fluorescent spot was observed within the cell or close to the flagellar pocket (Fig. 2B). The flagellar pocket is located at the base of the flagellum and is considered to be the sole site for endocytosis and exocytosis in Kinetoplastidae (43, 44).

Cells transfected with GFP alone displayed fluorescence throughout the cell (Fig. 2C), whereas cells transfected with the PGPAGFPHPA fusion revealed a discrete fluorescent spot located at a position similar to the PGPAHA protein at or close to the flagellar pocket (Fig. 2D). The same localization of PGPA was observed in L. tarentolae wild-type or As20.3rev or in Leishmania donovani cells transfected with PGPAGFPHPA (data not shown). The location of the PGPAGFPHPA protein was also observed by confocal microscopy. The nuclei and kinetoplast DNA were labeled with propidium iodide, whereas the autofluorescence of GFP was used to locate the PGPAGFPHPA protein. Interestingly, this fusion was located just below the kinetoplast (Fig. 2E) at a position close to or at the flagellar pocket. By confocal microscopy, a second region of fluorescence between the nucleus and the kinetoplast has been detected in

centations ranging from 100 nM to 1 mM. Transport Studies—Cellular fractions of the PGPAHA transfectant, TnHWT cell line, and PGPA null mutant were isolated on iodixanol gradients as described above. Transport studies using 73AsO2 and thiol complexes 73As(GS)2 were carried essentially as described previously (28). Frozen cellular fractions were thawed rapidly and stored on ice until use. The transport assay contained 5 mM MgCl2 and 0.1 mM 73As(GS)2 or 73AsO2 in buffer D (75 mM Hepes-KOH, pH 7.0, 0.15 mM KCl). Cellular fractions were added at 0.5 mg of protein/ml, and the mixture was incubated at 23 °C for 3 min, at which time the reaction was initiated by the addition of ATP to 10 mM. The total reaction volume was 0.3 ml. At the indicated intervals, samples (50 μl) were removed, filtered on wet nitrocellulose filters (0.22 μM pore size, Whatman), and washed with 5 ml of cold buffer D. The filters were dried, and radioactivity was measured in a scintillation counter.

RESULTS

PGPA-Tagged Versions Are Functional—Existing polyclonal antibodies against PGPA are useful for detecting this ABC protein in Western blots (18) but not for immunolocalization experiments (data not shown). For that reason, PGPA was tagged at the COOH terminus (Fig. 1A) with the HA epitope (YPYDVPDYA) derived from the influenza hemagglutinin protein, for which commercial antibodies are available. We also generated a PGPAGFPHPA construct by fusing the coding portion of GFP in frame with PGPA (Fig. 1A). Both the PGPAHA and PGPAGFPHPA constructs were independently electroporated into Leishmania cells. The HA.11 antibody recognized a protein of 175 kDa (Fig. 1B, lane 2) in the PGPAHA transfectant, identical in size to wild-type PGPA (18). In the PGPAGFPHPA transfectant, a protein of ~200 kDa reacted with the same commercial antibody, a size that commensurates with the presence of the 27 kDa of GFP protein in the fusion protein (Fig. 1B, lane 3). To demonstrate that PGPA is a membrane protein, we have fractionated PGPAHA-transfected cells by differential centrifugation and reacted the soluble and the membrane fractions with the HA.11 antibody. No PGPA was found in the supernatant fraction corresponding to the cytosol (Fig. 1B, lane 4), and PGPAHA was found exclusively in the membrane fraction (Fig. 1B, lane 5).

PGPA Is Localized in Intracellular Membranes—PGPA versions tagged with HA or GFP were localized within the cells using HA antibodies or GFP fluorescence. The anti-HA antibody was used to detect the PGPAHA protein by immunofluorescence. Non-permeabilized Leishmania cells expressing PGPAHA did not react with HA-fluorescein isothiocyanate-labeled antibody (Fig. 2A). However, when cells were permeabilized, a strong signal was observed upon reaction with the antibody. Indeed, a discrete bright fluorescent spot was observed within the cell at or close to the flagellar pocket (Fig. 2B). The flagellar pocket is located at the base of the flagellum and is considered to be the sole site for endocytosis and exocytosis in Kinetoplastidae (43, 44).

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parasites highly overexpressing PGPA. This location possibly corresponds to the Golgi apparatus. Therefore, it is possible that PGPA passes through the Golgi before reaching a membrane close to the flagellar pocket. However, preliminary experiments using subinhibitory to partially inhibitory concentrations of the N-glycosylation inhibitor tunicamycin failed to demonstrate that PGPA was N-glycosylated (data not shown).

To localize PGPAGFPPHI more precisely, electron microscopy with immunogold labeling was employed. The fusion protein was detected using an anti-GFP antibody with silver-enhanced Nanogold (Fig. 3A) or 6-nm gold particles (Fig. 3, B and C). Clearly, the flagellar pocket membrane was not labeled (Fig. 3). Instead, the antibody reacted with vesicular and tubular membranes (Fig. 3, B and C) very close to the L. tarentolae flagellar pocket membrane, which appear to be part of the recently described tubular clusters (36). The same location was detected in L. tarentolae and L. donovani (data not shown), and HA labeling for detecting PGPAGFPHA gave similar results, although the label density was lower (data not shown). Both the anti-GFP and anti-HA antibodies did not give a signal in non-transfected cells (data not shown). Finally, in heavily labeled cells, the region where the Golgi is normally located was also labeled, which is consistent with the observation made using confocal microscopy (Fig. 2E). Thus, PGPA is localized in membranes close to the flagellar pocket. Further work will be required, however, to confirm the identity of this organelle.

PGPA Has Basal ATPase Activity and Can Transport Metal-Glutathione Conjugates—PGPA-containing fractions were isolated by subcellular fractionation using iodixanol gradients. Aliquots derived from a PGPAGFPHAI transfectant and from a PGPA null mutant were collected from the bottom of the gradient. These proteins in those fractions were separated on acrylamide SDS gels, transferred, and reacted with the anti-HA antibody. Most of the PGPAGFPHA was found in fractions 8–11 (Fig. 4A). Fractions 5 and 14 exhibited ATPase activity, but similar activity was found in fractions derived from either the PGPA transfectants or the PGPA null mutant (Fig. 4B). In contrast, ATPase activity was present in fractions 8–11 only in the PGPAGFPHAI transfectant (Fig. 4B). By subtracting the ATPase activity of the various fractions derived from the two cell lines, ATPase activity that correlated specifically with the presence of the PGPAGFPHAI protein could be identified (Fig. 4, compare A with C). Under our experimental conditions, the basal ATPase activity of PGPA is 115 nmol/mg/min. The same basal activity was observed in membranes permeabilized with 0.01% Triton X-100, suggesting that the ATP binding and catalytic sites were readily accessible in the cellular fractions. Preliminary attempts to stimulate the basal ATPase activity of PGPA using metals or metal-thiol conjugates were unsuccessful.

The transport of $^{73}$As(III) in PGPA-enriched vesicles showing ATPase activity was examined. Vesicles isolated on iodixanol gradients from L. tarentolae wild-type cells, from the PGPA null mutant, and from the PGPAGFPHA transfectant were compared by SDS-polyacrylamide gel electrophoresis stained with Coomassie Blue or silver nitrate and were also reacted with a monoclonal antibody directed against the kinetoplastid membrane protein 11 (KMP11, Cedarlane Laboratories). The pro-
protein profiles and antibody reactions looked similar for the three vesicle preparations (data not shown). Vesicles containing PGPA showed increased ATP-dependent As(GS)$_3$ transport (Fig. 5). No transport was observed with the unconjugated form of the metal (Fig. 5). Vesicles derived from wild-type cells also transported As(GS)$_3$ at lower rates than vesicles from the transfectant. Vesicles from a PGPA null mutant had low initial rates of uptake, although some transport was observed at longer times (Fig. 5). This may indicate the presence of a low activity non-PGPA transporter.

**DISCUSSION**

To understand the mechanism of metal resistance in *Leishmania* mediated by the ABC transporter PGPA, we developed tools to detect PGPA using either versions of PGPA tagged with the HA epitope or PGPA fused to GFP. GFP is widely used to localize proteins (45) and was recently shown to be useful in *Leishmania* (35, 46). These tagged versions conferred resistance (Table I), suggesting that the cellular location of the tagged PGPA versions is probably the same as the wild-type PGPA. The results of co-transfection experiments of PGPA with either GSH1 (25) or ODC (26) suggested that PGPA recognizes metals that are conjugated to TSH. The data from experiments with BSO, a specific inhibitor of glutathione biosynthesis, and DFMO, a specific inhibitor of spermidine biosynthesis, are consistent with this hypothesis (Table I).

Transport studies showing that whole cells of a PGPA null mutant is capable of $^{75}$As extrusion indicates that the *L. tarentolae* PGPA protein is not a plasma membrane efflux pump (27, 28). Instead, we have proposed that PGPA was located within an intracellular organelle (9). Localization studies using tagged versions of PGPA confirm that it is located intracellularly at a position between the kinetoplast and the flagellar pocket (Fig. 2). Electron microscopy clearly demonstrated that PGPA is located in membranes close to but not linked to the flagellar pocket (Fig. 3). A similar location was also found in *L. donovani*. Further work will be required to characterize these membranes, but they are similar to the vesicular and tubular clusters close to the flagellar pocket that was recently described (36). These tubulovesicular elements were proposed to be potentially part of both the endocytic and exocytic pathways (36). The availability of preparations enriched in these membranes (Fig. 4) will facilitate the characterization of these vesicles.

PGPA confers resistance to metals most probably by sequestration of the conjugated metals within these organelles. These vesicles could be exocytosed via the nearby flagellar pocket, the only known site of exocytosis in this and related kinetoplastid parasites (36, 44). Because there were no major changes in the steady state accumulation of $^{75}$As in intact cells of PGPA transfectants (21), there is most probably a steady state in which the rate of drug entry and exocytosis are similar. Resistance by sequestration appears to be a frequent mechanism used by cells for resisting metals (for review, see Ref. 47). A GS-X pump accumulating glutathione S-transferase conjugates in intracellular vesicles was proposed to be one mechanism by which mammalian cells resist cisplatin (48). The ABC transporter Hmt1p confers cadmium tolerance by sequestering phytochelatin (a glutathione-like molecule) cadmium complexes in the fission yeast vacuole (49). The yeast ABC transporter Ycf1p, a homologue of PGPA (50), confers cadmium, arsenite, and antimonite resistance by mediating the vacuolar accumulation of metal-glutathione complexes (51–53).

We show in this study that PGPA is indeed a transporter that does not transport free AsIII or SbIII as does the bacterial arsAB ATPase (54) but instead transports metal-thiol conjugates (Fig. 5). This is an important confirmation of predictions made from co-transfection experiments and of drug reversal studies using TSH biosynthesis inhibitors (25, 26). At least two types of metal-thiol pumps appear present in *Leishmania*. One is present in plasma membrane vesicles (28), whereas the other is PGPA, which has only approximately 10% of the activity of the plasma membrane transporter. PGPA is part of a large gene family (55); perhaps another of these is the plasma membrane thiol transporter. Vesicles of wild-type cells containing PGPA do transport As(GS)$_3$ (Fig. 5). It is not clear, however, whether this basal activity is mediated solely by PGPA, as PGPA null mutants did transport the metal-thiol conjugates but at a lower level (Fig. 5). Although vesicles of the various mutants were similar, minor contamination with plasma membrane vesicles that could contribute to metal-thiol transport cannot be excluded. PGPA has a basal ATPase activity (Fig. 4C). Preliminary attempts to observe the stimulation of this activity using either arsenite or antimonite either conjugated or not conjugated with thiols (see under “Materials and Methods”) so far have not been successful. ATPase activity of the human MRP-1 is only slightly stimulated by substrates (56). Possibly other methods such as vanadate trapping (57) may be required to show stimulation of the PGPA ATPase activity by its substrate.
In conclusion, PGPA is shown in this study to be an intracellular ABC protein that transports metals conjugated to glutathione. In vivo, the physiological thiol is probably TSH, the major thiol species in Leishmania. It will be of interest to determine the identity of the organelle containing PGPA. PGPA is part of a complex resistance mechanism requiring at least three mutations: increased thiol levels, an increased amount of transporter, and another elusive mutation that may correspond to an increase in a trypanothione S-transferase activity. Further studies are warranted as parasites, yeast, plant, and mammalian cells seem to use similar strategies for detoxification. As antimony is the drug of choice against Leishmania, it will be of great interest to determine whether drug resistant field strains adopt similar strategies to resist chemotherapeutic interventions.

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