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Functional role of lysine 12 in *Leishmania major* AQP1

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Abstract

Leishmania major aquaglyceroporin (AQP1) is an adventitious metalloid channel that allows the bidirectional movement of arsenite and antimonite. Here we demonstrate that AQP1 is subjected to proteasome-dependent degradation. Treatment of *Leishmania* promastigotes with the proteasome inhibitor MG132 resulted in increased AQP1 accumulation. Site-directed mutagenesis in AQP1 revealed that alteration of lysine 12 to either alanine or arginine improves protein stability. AQP1 expression is stabilized by mitogen-activated protein kinase 2 (MPK2). Cells expressing a dominant-negative MPK2 mutant exhibited severely reduced AQP1 expression, which could be reversed upon addition of MG132. Interestingly, the dominant-negative MPK2 mutant could not destabilize either AQP1_{K12A} or AQP1_{K12R}. While stabilization of AQP1 by MPK2 leads to its relocalization from flagellum to the entire surface of the parasite, altered AQP1_{K12A} or AQP1_{K12R} was restricted to flagellum only. Our data demonstrate that lysine 12 is targeted for proteasomal degradation of AQP1 and plays an integral role in subcellular localization of AQP1 as well as its interaction with MPK2. This work also raises the possibility that a strategy combining antimonial with a proteasome inhibitor may be an effective combination regimen against diverse forms of leishmaniasis.

Keywords

Antimonite; aquaglyceroporin; *Leishmania*; post-translational regulation; ubiquitination; volume regulation

1. Introduction

Leishmaniasis is caused by parasites from the genus *Leishmania* that are transmitted through the bite of infected *Phlebotomus* sandflies in the Old World and *Lutzomyia* in the New World. There are several different forms of leishmaniasis. The most prevalent ones are the cutaneous leishmaniasis, resulting in skin ulcers, mucocutaneous leishmaniasis, affecting the nose, mouth, throat cavities, and visceral leishmaniasis, targeting the internal organs,

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typically spleen, liver, and bone marrow. The first line compounds against all forms of leishmaniasis are the two pentavalent antimony (Sb(V)) containing drugs, sodium stibogluconate (Pentostam®) and meglumine antimoniate (Glucantime®). It is postulated that pentavalent antimonials are reduced to the more active trivalent form (Sb(III)) in the cytosol of host macrophage by a putative Sb(V) reductase. Alternatively, the drug may be taken up by the parasite and reduced to Sb(III) by an endogenous antimony reductase (1).

In order for Sb(III) to be effective as a drug, it needs to enter the parasite.

Aquaglyceroporins (AQPs) have been identified as uptake systems for trivalent metalloids in prokaryotes and eukaryotes (2). The genome of *Leishmania major*, a causative agent of cutaneous leishmaniasis, encodes for five AQPs: AQP1, AQP α , AQP β , AQP γ and AQP δ . Of the five, only AQP1 is involved in the uptake of Sb(III), the activated form of Pentostam® and Glucantime® (3). We have earlier shown that disruption of one of the two *AQP1* alleles in *L. major* conferred a 10-fold increase in Sb(III) resistance (3). AQP1 mRNA levels are significantly less in Sb(III)-resistant *L. major* and *Leishmania tarentolae* cells, indicating that downregulation of AQP1 leads to drug resistance (4). These inferences were corroborated in field isolates from Nepal (5) and India (6). In general, *Leishmania* species responsible for cutaneous leishmaniasis are more sensitive to Sb(III) than those responsible for visceral leishmaniasis. This species-specific sensitivity to metalloid is directly proportional to *AQP1* mRNA levels. The 3'-untranslated region of *AQP1* regulates its mRNA stability, which defines the distinct antimonial sensitivity of each species (7).

Although AQP1 functions as an adventitious drug transporter, its physiological roles are water and solute transport. AQP1 allows the passage of water, glycerol, glyceraldehyde, dihydroxyacetone and sugar alcohols (8). While the structure of *L. major* AQP1 is yet to be elucidated, molecular modeling based on the three-dimensional structure of *Plasmodium falciparum* AQP (PfAQP) (9), along with structure-function correlations of *L. major* AQP1 have defined the residues lining the outer mouth of the channel (Fig. S1). Glutamate 152 on the second extracellular loop near the pore mouth helps the channel differentiate between antimonite and glycerol (10). Mutations in alanine 163 in the same loop altered the permeability to antimonite without any significant loss in water or solute conductivity (11).

AQP1 is expressed exclusively in the flagellum of promastigotes (extracellular form in sand fly vector); whereas in amastigotes (intracellular form in vertebrate host) it is localized to the flagellar pocket, rudimentary flagellum and contractile vacuoles. The promastigote flagellum is necessary for parasite migration and attachment within the sand fly gut, while the rudimentary amastigote flagellum is proposed to be involved in sensory perception and cellular organization, thus playing a critical role for intracellular survival of *Leishmania* within the vector and vertebrate host (12). Consequent to its flagellar localization, AQP1 is critical in the movement of *Leishmania* towards an osmotic gradient. AQP1 also plays an important role in the regulation of cellular volume of the parasite following an osmotic stress (8).

AQP1 activity is post-translationally modulated by mitogen-activated protein kinase 2 (MPK2). MPK2 phosphorylates AQP1 at threonine 197 that reduces AQP1 protein turnover and improves AQP1 stability. While AQP1 is localized to the flagellum of promastigotes,

upon phosphorylation, it is relocalized to the entire surface of the parasite. Lysine 42 of MPK2 is critical for its kinase activity. Expression of *MPK2*_{K42A} exerts a dominant-negative effect on AQP1 expression by reducing its stability (13). In this study we show that the dominant-negative effect is due to enhanced proteasomal degradation of AQP1. Our results show that proteasome inhibition rapidly induces expression of *AQP1*, inhibits degradation of AQP1 protein, and enhances parasite sensitivity to Sb(III).

2. Materials and methods

2.1. Cells and transfectants

The *Leishmania donovani* LdBob cell line was kindly provided by Dr. Stephen Beverley (14). LdBob cells were grown as promastigotes as described earlier (8, 13). While an *AQP1* null-mutant in *L. major* has only recently been developed at Ouellette laboratory (15), the LdBob cell line was chosen based on a logical extension of our previous studies (7, 8, 10, 11, 13). Also, LdBob cells have the lowest *AQP1* mRNA levels, are highly resistant to Sb(III), show lowest Sb(III) accumulation, and exhibit poor osmoregulatory activity (7), which makes LdBob as good as an *AQP1* knockdown strain.

Altered *AQP1* and *MPK2* constructs were transfected into LdBob cells as described earlier (13). All transfectants were maintained in the presence of 300 µg ml⁻¹ hygromycin B (Life Technologies) and 60 µg ml⁻¹ geneticin (Life Technologies). Plasmids and transfectants used in this study are described in Table S1.

2.2. Oligonucleotide-directed mutagenesis

Mutations in *L. major AQP1* gene were introduced by site-directed mutagenesis using QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies). The mutagenic oligonucleotides used for both strands and the respective changes introduced (underlined) are as follows: K12A, 5'-GAGGACCAACACGAGAGTGCGCGAAACTTCATGTCTGCAG-3' and 5'-CTGCGACATGAAGTTTCGCGCACTCTCGTGTGGTCCTC-3'; K12R, 5'-GACCAACACGAGAGTAGGCGAAACTTCATGTCTGC-3' and 5'-CGACATGAAGTTTCGCCTACTCTCGTGTGGTC-3'. Each mutation was confirmed by DNA sequencing (Eton Bioscience). Altered *AQP1* was digested with HindIII and XbaI and inserted into the HindIII-XbaI sites of pSP72ahygro.

2.3. Antimonite sensitivity assays

Antimonite sensitivity was determined by diluting log phase promastigote transfectants to 10⁶ cells ml⁻¹ in a culture medium containing various concentrations of Sb(III) in the form of potassium antimonyl tartrate (Sigma-Aldrich). Following 72 h incubation, cell growth was monitored from the absorbance at 600 nm using a Spectramax 340 microplate reader (Molecular Devices). Percentage survival was plotted against Sb(III) concentrations and EC₅₀ was determined using SigmaPlot 12.0. Each assay was performed at least three times in triplicates. Error bars were calculated from the mean ± SE.

2.4. Antimonite accumulation assays

Log phase LdBob promastigotes were washed twice with PBS, pH 7.4 (Invitrogen) and suspended in PBS at a density of 10^8 cells ml^{-1} . Promastigotes were then incubated with 10 μM Sb(III) for 10 min at room temperature. A 200 μl portion was filtered through a 0.2- μm nitrocellulose filter (EMD Millipore) and the filter washed once with 5 ml of ice-cold PBS. The filters were digested with 0.4 ml of concentrated HNO_3 (69–70%) (EM Science) for 1 h at 70°C , allowed to cool to room temperature, and diluted with high-pressure liquid chromatography grade water (Sigma-Aldrich) to produce a final concentration of HNO_3 of approximately 3%, and then analyzed by a SCIEX ELAN® DRC-e inductively coupled plasma mass spectrometer (PerkinElmer). Standard solutions were made in the range of 0.5–10 ppb in 3% HNO_3 using antimony standards (Ultra Scientific). Each transport experiment was repeated at least three times with duplicate samples. Error bars were calculated from the mean $\pm$ SE.

2.5. Cell volume measurements

Log phase LdBob promastigotes were washed twice in PBS and re-suspended at a density of 10^9 cells ml^{-1} . One hundred microliter portions of the cell suspension were transferred to a microtiter plate. Hypo-osmotic shock was induced by dilution of the isotonic cell suspension with an equal volume of deionized water to obtain a final osmolarity of 150 mOsm and was confirmed with an osmometer (Precision Systems). The absorbance at 550 nm was recorded every 15 s for 3 min in a Spectramax 340 microplate reader. A decrease in absorbance corresponds to an increase in cell volume. Isosmotic control experiments consisted of dilution of cell suspensions with appropriate volumes of isosmotic buffer. Each experiment was repeated at least three times with triplicate samples. Error bars were calculated from the mean $\pm$ SE.

2.6. MG132 treatment of LdBob transfectants

MG132 is a specific, potent, and cell-permeable proteasome inhibitor (16). To examine the effect of MG132 (EMD Millipore) on *AQP1* expression, LdBob transfectants were pretreated with or without 10 μM MG132 for 48 h. After the treatment cells were lysed and plasma membrane-enriched fraction isolated as described earlier (8). The total protein content of plasma membrane-enriched fraction was determined by a filter assay method (17). Fifty microgram protein sample was separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) on 12% acrylamide gels and transferred to a Schleicher & Schuell Protran® nitrocellulose transfer membrane (PerkinElmer). Western blot analysis was performed according to the manufacturer's directions using a Western Lightning Ultra kit (PerkinElmer) and an affinity-purified AQP1 antiserum (8).

Antimonite sensitivity of LdBob transfectants following MG132 treatment was determined by diluting 2×10^6 cells ml^{-1} in a fresh culture medium containing 10 μM MG132 and increasing concentrations of Sb(III) in the form of potassium antimonyl tartrate (Sigma-Aldrich). EC_{50} was estimated through manual cell counting with a hemocytometer. To estimate Sb(III) accumulation, MG132 treated cells were washed once with PBS containing 10 μM MG132, and resuspended in PBS at a density of 10^8 cells ml^{-1} . Cells were then incubated with 10 μM Sb(III) for 10 min at room temperature. Sb(III) accumulation was

measured as described above. Experiments were repeated twice with triplicate samples each time. Error bars were calculated from the mean $\pm$ SE.

2.7. Western blot analysis

The flagellar and pellicular plasma membrane fractions from LdBob transfectants were isolated as described earlier (8, 13). The purity of flagellar preparation was monitored by phase-contrast microscopy (~99% pure by microscopic count). Either 25 μ g of flagellar or 50 μ g of pellicular membrane preparations were electrophoresed into 12% SDS-PAGE and probed with an affinity-purified AQP1 antiserum (8, 13).

Mid-log phase LdBob promastigotes were treated in the presence or absence of protein synthesis inhibitor cycloheximide (70 μ g ml⁻¹; Sigma-Aldrich) and harvested at different time points (13). Flagellar membrane fraction was isolated and *AQP1* expression analyzed by Western blotting (13). For AQP1_{WT} + MPK2_{K42A} cells treated with cycloheximide, 70 μ g of flagellar membrane preparation was loaded onto 12% SDS-PAGE. For all other cell types described in Fig. 4, 25 μ g of membrane preparation was loaded in each lane. The experiment was repeated twice and relative AQP1 levels were evaluated by densitometric analysis using ImageJ (<http://imagej.nih.gov/ij/>).

2.8. Immunofluorescence analysis

Late-log-phase LdBob promastigotes (~5 x 10⁵ cells) were fixed in 4% paraformaldehyde/PBS overnight at 4°C. The cells were washed twice with PBS, transferred to teflon coated immunofluorescence slides (Polysciences), and permeabilized with 0.1% Triton X-100, 50 mM Na₂PO₄, 50 mM glycine, pH 7.2, for 2 min. After blocking with 1% bovine serum albumin (BSA) in PBS for 30 min, cells were incubated for 1 h at room temperature with AQP1 antiserum (1:300 in 1% BSA). The primary antibody was removed by washing three times with 1% BSA followed by incubation with the monoclonal anti- α -tubulin antibody (Sigma-Aldrich) 1:500 for another hour. After three washes with 1% BSA, the slides were incubated with Alexa Fluor® 488-conjugated goat anti-rabbit IgG (Life Technologies) (1:1,000 in 1% BSA) and Alexa Fluor® 594-conjugated goat anti-mouse IgG (Life Technologies) (1:2,000 in 1% BSA) for 1 h. Finally, the cell nuclei were stained with DAPI (0.1 mg ml⁻¹ final concentration) (Sigma-Aldrich) for 10 min, the slides washed three times with 1% BSA, and once with sterile water. The slides were mounted in Vectashield® Antifade Mounting Medium (Vector Laboratories) and examined by a DeltaVision™ Elite Microscopy System (GE Healthcare Bio-Sciences) with a 60x oil immersion objective.

3. Results

3.1. Enhancement of AQP1 expression upon proteasome inhibition

The effect of a cell-permeable proteasome inhibitor MG132 on *AQP1* expression was examined. LdBob cells transfected with *AQP1*_{WT} were incubated with or without 10 μ M MG132 for forty eight hours. Treatment with MG132 led to morphological changes. Although cell viability was not compromised, cells became small and round with severely stunted flagella (Fig. S2). Changes in cell shape upon MG132 treatment have been reported in mammalian cells (18, 19). In the absence of MG132, expression of *AQP1* is too low in

total membrane fractions to be detected by immunoblotting (8). In contrast, *AQP1* expression was noted in MG132 treated cells (Fig. 1A) indicating that, MG132-induced inhibition of the ubiquitin-proteasome pathway leads to an increased accumulation of AQP1. Consistent with an increase in *AQP1* expression, MG132 treated cells were three times more sensitive to Sb(III) (Fig. 1B), and accumulated twice as much metalloid than untreated cells (Fig. 1C).

AQP1 expression is positively regulated by mitogen activated protein kinase, MPK2. MPK2-mediated phosphorylation of AQP1 occurs on residue Thr197, and such posttranslational modification serves to stabilize AQP1 half-life (13). Lys42 of MPK2 is critical for its kinase activity. Expression of a dominant-negative *MPK2_{K42A}* mutant in LdBob cells showed a significant reduction in AQP1 expression (13). However, addition of MG132 in cells co-expressing *AQP1_{WT}* and *MPK2_{K42A}* restored *AQP1* expression (Fig. 1A) suggesting that, the reduction in AQP1 protein level was caused by enhanced proteasomal degradation. Consequent to restoration of *AQP1* expression, a 10-fold increase in hypersensitivity to Sb(III) (Fig. 1B) and a 4-fold increase in metalloid accumulation (Fig. 1C) was noted in cells treated with MG132 versus untreated cells.

3.2. Putative ubiquitination site of AQP1

Proteasome mediated degradation and/or endocytosis of membrane proteins including aquaporins takes place by ubiquitination at the critical lysine exposed to cytosol (20, 21). AQP1 shares 32% identity and 50% similarity with *Plasmodium falciparum* aquaglyceroporin (PfAQP). A topology prediction of AQP1 based on the crystal structure of PfAQP (9) predicted the AQP1 N- and C-termini to be intracellular (Fig. S1) (10). Similarly, the TMHMM Server v. 2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>) predicts a cytosolic location for AQP1 N- and C-termini. AQP1 has six lysine residues located at position 12, 114, 138, 146, 157 and 203 of its primary sequence (Fig. S3). Only Lys12 was predicted by UbPred: Predictor of Protein Ubiquitination Sites program (<http://www.ubpred.org/>) as the sole ubiquitination site with high confidence. Consequently, to examine its role in ubiquitin-mediated degradation, Lys12 was altered to alanine and arginine by site-directed mutagenesis.

3.3. Role of Lys12 in AQP1 stability

The expression levels of wild type and altered *AQP1* in LdBob cells was estimated by immunoblot analysis using anti-AQP1 antibody. Either *AQP1_{K12A}* or *AQP1_{K12R}* could be detected at slightly higher levels ($P=0.03$ and $P<0.001$, respectively) than wild type in the flagellar membrane indicating that alteration of Lys12 to either alanine or arginine increased AQP1 expression (Fig. 2).

When coexpressed with *MPK2*, wild type AQP1 is detected in both flagellar and pellicular membrane (13). However, coexpression of *AQP1_{K12A}* (or *AQP1_{K12R}*) with wild type *MPK2* showed elevated AQP1 amounts in the flagellar but none in the pellicular membrane fraction (Fig. 2). As described previously (13), coexpression with altered *MPK2_{K42A}* led to a significant reduction in AQP1 protein levels (Fig. 2). Interestingly, a near complete rescue of *AQP1* expression was noted when altered AQP1 (*AQP1_{K12A}* or *AQP1_{K12R}*) was

coexpressed with *MPK2*_{K42A} (Fig. 2). This suggests that alteration of Lys12 of AQP1, a putative ubiquitylation target, prevents *MPK2*_{K42A} mediated destabilization.

Immunofluorescence of *Leishmania* promastigotes also indicated that AQP1 was present in the flagellum only (Fig. 3A). AQP1 is phosphorylated by *MPK2* and undergoes shuttling to both the flagellum and pellicular membrane of the parasite (Fig. 3B). Congruent with immunoblot analysis (Fig. 2), AQP1 is hardly detected in cells coexpressing *MPK2*_{K42A} (Fig. 3C). Altered AQP1 (*AQP1*_{K12A} or *AQP1*_{K12R}) is similarly located as the wild type on the flagella of promastigotes (Fig. 3D and G). Interestingly, when coexpressed with *MPK2*_{WT}, altered AQP1 was observed on the flagellum only but not the pellicular membrane of promastigotes (Fig. 3E and H). We interpret this observation as Lys12 being an essential element in the relocation of AQP1 between different cellular compartments. In contrast to coexpression of wild type *AQP1* and *MPK2*_{K42A} where AQP1 is hardly detectable (Fig. 3C), coexpression of altered *AQP1* with *MPK2*_{K42A} showed the localization of *AQP1*_{K12A} or *AQP1*_{K12R} to flagellum alone (Fig. 3F and I). These observations again suggest that alteration of Lys12 prevents proteasome-mediated degradation.

The stability of AQP1 was estimated from cycloheximide inhibition of protein synthesis. Cycloheximide inhibits protein synthesis in eukaryotes by blocking translational elongation. After cycloheximide treatment, *AQP1*_{WT} protein levels gradually dropped with about half of the protein degrading within 48 hours (Fig. 4). Coexpression with *MPK2*_{WT} increased the stability of *AQP1*_{WT} considerably with nearly no degradation during the experimental period. Coexpression of *MPK2*_{K42A} decreased the half-life of *AQP1*_{WT} to < 4 h. In contrast, coexpression of either *AQP1*_{K12A} or *AQP1*_{K12R} with *MPK2*_{K42A} leads to stabilization of altered AQP1 (Fig. 4). These results indicate that alteration of Lys12 confers AQP1 stability.

3.4. Role of Lys12 in AQP1 activity

Loss of AQP1 confers resistance due to decreased Sb(III) accumulation in *Leishmania* promastigotes and conversely increased expression can reverse resistance (3, 4). Cells expressing wild type *AQP1* were 130-fold more sensitive to Sb(III) than vector control (Table 1). Consistent with the slightly enhanced expression of altered *AQP1* (Fig. 2), cells expressing *AQP1*_{K12A} and *AQP1*_{K12R} showed slightly higher Sb(III) accumulation, and 1.5- and 2-fold sensitivity to Sb(III), respectively, than wild type (Table 1). Coexpression of wild type *AQP1* and *MPK2* leads to an elevation of AQP1 protein levels (Fig. 2), a 10-fold increase in metalloid accumulation, and a 1,300-fold increase in Sb(III) sensitivity than vector control (Table 1). Coexpression of *MPK2*_{K42A} resulted in depletion of AQP1 (Fig. 2). Cells showed near similar Sb(III) accumulation and metalloid sensitivity as the vector control (Table 1). In contrast, cells coexpressing altered *AQP1* (*AQP1*_{K12A} or *AQP1*_{K12R}) and *MPK2*_{K42A} had restored AQP1 levels (Fig. 2) and exhibited similar accumulation and sensitivity to metalloid as those expressing wild type *AQP1* and *MPK2* (Table 1). No changes were noted in *AQP1* mRNA levels in any of the transfectants (Table S2). Thus an increase in AQP1 stability is directly proportional to enhanced Sb(III) accumulation and sensitivity.

When LdBob cells are exposed to media with reduced osmolarity, they initially swell, but subsequently undergo compensatory shrinking referred to as regulatory volume decrease.

This regulatory volume decrease ability of LdBob cells is directly proportional to its AQP1 protein level (7, 8, 13). LdBob cells were subjected to a 50% reduction in osmolarity (from 300 to 150 mOsm) and the changes in cell volume after the hypo-osmotic shock were monitored by following the absorbance at 550 nm. After a hypo-osmotic shock, cells expressing wild-type AQP1 showed a drop in absorbance indicating cell swelling, followed by a steady rise in absorbance to near original levels, signifying volume recovery. Cells expressing wild-type AQP1 exhibited a volume recovery of 56 seconds whereas the vector control needed 180 seconds (Fig. 5 and S4). Consistent with their slightly increased expression, cells expressing either *AQP1*_{K12A} or *AQP1*_{K12R} exhibited a complete volume recovery in 43 seconds and 30 seconds, respectively (Fig. 5 and S4). When cells coexpressing wild-type *AQP1* and *MPK2* were subjected to a similar hypo-osmotic shock, the swelling and recovery process was essentially complete within the dead time of the spectrophotometer (Fig. 5 and S4). Cells coexpressing *AQP1*_{WT} and *MPK2*_{K42A} exhibited near negligible expression of *AQP1* and took nearly 150 seconds for volume recovery (Fig. 5 and S4). In contrast, cells coexpressing *AQP1*_{K12A} (or *AQP1*_{K12R}) and either the wild type or altered *MPK2* exhibited an identical volume recovery as cells transfected with wild type *AQP1* and *MPK2* (Fig. 5 and S4). Taken together, these results demonstrate that reduced AQP1 ubiquitination following alteration of Lys12 leads to increased protein stability and increased levels of AQP1 expression (Fig. 4), which provides improved osmoregulation in promastigotes (Fig. 5 and S4).

4. Discussion

In this study we show that *L. major* AQP1 could be stabilized in the presence of the proteasome inhibitor MG132, suggesting that AQP1 is degraded by the proteasome pathway. Alteration of Lys12 to either alanine or arginine led to slightly higher expression of *AQP1*. Cells expressing either *AQP1*_{K12A} or *AQP1*_{K12R} exhibited increased metalloid sensitivity and accumulation than those expressing wild type *AQP1*. Taken together, our data demonstrate that Lys12 is targeted for ubiquitination and degradation of AQP1 and plays an integral role in regulating its activity. Unfortunately, despite repeated attempts, we were unable to show a direct ubiquitination event when immunoprecipitation experiments were performed with anti-ubiquitin antibody (Abcam). In addition to non-specific binding of anti-ubiquitin antibody with other ubiquitinated proteins in the flagellar membrane, both the polyclonal anti-ubiquitin and anti-AQP1 antibodies cross-reacted with each other, resulting in a high background.

We have earlier shown that AQP1 is a substrate of MPK2 (13). Thr197 in the cytosolic D-loop of AQP1 is phosphorylated by MPK2, and phosphorylation prolonged the half-life of AQP1. In contrast, alteration of Lys42 of MPK2 to alanine lowered the half-life of AQP1 considerably, indicating a direct correlation between phosphorylation and increased AQP1 stability. The dominant-negative effect on *AQP1* expression can be reversed upon addition of MG132, implying a positive effect of MPK2 on the stability of AQP1 by inhibiting its ubiquitination and subsequent proteasomal degradation. The protein disorder prediction server (22), PrDOS (<http://prdos.hgc.jp/>), predicts the 20 residue N-terminal region facing the cytosol to be highly dynamic. The disordered cytosolic N-terminus is often involved in accelerated degradation of proteins by ubiquitination (23, 24). It is possible that

phosphorylation of AQP1 confers stability to its N-terminal disordered region and occludes Lys12 from ubiquitin-mediated proteasomal degradation. Surprisingly, coexpression of *MPK2_{K42A}* with either *AQP1_{K12A}* or *AQP1_{K12R}* did not affect the stability of altered AQP1. This suggests that alteration at Lys12 propagates a conformational change at the N-terminus region that affects interaction of AQP1 with MPK2 and/or components of the ubiquitin/proteasome pathway. Further studies are required to elucidate the mechanism by which AQP1 activity is regulated by MPK2. Nevertheless, our analyses demonstrate that MPK2 is associated with the regulation of AQP1 stability and Lys12 of AQP1 is critical for MPK2-mediated regulation.

While AQP1 is localized to the flagella of promastigotes, upon phosphorylation, it is distributed over the entire surface of the parasite. Absence of phosphorylation of AQP1 restricted its localization to flagellum only (13). Interestingly, while MPK2 upregulated the expression of either *AQP1_{K12A}* or *AQP1_{K12R}*, the altered protein was localized to flagellum only. We interpret this observation as Lys12 being critical to pellicular localization of AQP1. The mechanisms for targeting proteins to flagella are now just being defined and are crucial to our understanding of flagellar function in kinetoplastids. In trypanosomes, a consensus tripeptide motif histidine-leucine-alanine (HLA) contained within two cytoskeletal flagellar proteins, paraflagellar rod A and actin-related protein, represents an essential part of a flagellar targeting machinery (25). However, such a feature is not universal. For example, the *Leishmania enriettii* glucose transporter, ISO1, resides primarily in the flagellar membrane, whereas the ISO2 glucose transporter is located in the pellicular plasma membrane. Deletion analyses suggest that the flagellar targeting information for ISO1 does not constitute a simple linear sequence of amino acids but a minimum of two separate sequences in tandem (26). While no such sequence has been identified in *Leishmania* AQP1, our current observation leads us to speculate that, the N-terminus cytosolic region along with residues in loop D (Fig. S1) may be involved in targeting AQP1 between various cellular membranes. Further studies including deletion analyses of AQP1 N-terminal region is currently in progress. In the absence of transcriptional control, such a post-translational regulatory mechanism provides a novel way for *Leishmania* to survive during its digenetic life cycle. It is tempting to speculate that MPK2-mediated upregulation of AQP1 may counter the osmotic stress, which is critical for survival and transmission of the parasite between two environments, the vector and the vertebrate host. On the other hand, the intrinsically disordered N-terminal domain containing Lys12 may serve as an on-off switch for proteasomal-dependent degradation, especially during drug exposure, thereby conferring resistance.

In summary, our results indicate Lys12 to have a tripartite role in AQP1 activity: (i) a target for ubiquitin-proteasome-mediated degradation; (ii) regulating interaction with MPK2; and (iii) protein flux between the flagellum and pellicular membrane of the parasite. Since AQP1 is degraded through ubiquitin-proteasome pathways, drugs that block proteasome functions will upregulate AQP1 expression in *Leishmania*, which will lead to increased Sb(III) uptake and sensitivity. A combination drug regimen consisting of an antimony containing drug such as Pentostam® or Glucantime® along with a proteasome inhibitor such as bortezomib may therefore be an effective treatment against drug-resistant leishmaniasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Sb(III)	Antimonite
AQP1	<i>Leishmania major</i> aquaglyceroporin
MPK2	<i>Leishmania major</i> mitogen-activated protein kinase 2

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Fig. 1. AQP1 is subjected to proteasome-mediated degradation

(A) LdBob transfectants were incubated in the absence () or presence (+) of 10 μM MG132 and the plasma membrane fractions prepared as described in Materials and methods. Each lane was loaded with 50 μg of total membrane protein and tested for the presence of AQP1 by Western blotting using an anti-AQP1 antibody; (B) Sb(III) sensitivity (EC₅₀) of LdBob transfectants ± MG132; (C) Sb(III) uptake in LdBob transfectants ± MG132. Data were expressed as mean ± SE of three independent experiments in triplicate.

Fig. 2. Cellular localization of AQP1

Immunoblot analysis of flagellar and pellicular membrane fraction of LdBob transfectants probed with anti-AQP1 antibody. Details on each transfectant are described in Table S1. Twenty five microgram of flagellar and 50 µg of pellicular protein was loaded in each lane. The experiments were repeated three times with similar results.

Fig. 3. Immunofluorescence microscopy of LdBob transfectants

Cells were stained for AQP1 (green), DNA (blue), and α -tubulin (red). The three images were finally merged. Details of each transfectant are described in Table S1. Each experiment was repeated three times with similar results. Approximately fifty microscopic fields were examined with similar findings.

Fig. 4. Turnover of AQP1

Mid-log phase LdBob transfectants were treated with 70 $\mu\text{g ml}^{-1}$ cycloheximide (CHX). Cells were harvested at the indicated times following CHX addition. The flagellar membrane fraction was isolated as described in Materials and methods. All lanes were loaded with 25 μg of flagellar protein except AQP1_{WT} + MPK2_{K42A} which was loaded with 70 μg . AQP1 expression was analyzed by Western blotting using anti-AQP1 antibody.

Fig. 5. Volume regulation in LdBob transfectants

LdBob promastigotes expressing the vector (●); *AQP1*_{WT} (○); *AQP1*_{K12A} (▼); *AQP1*_{WT} + *MPK2*_{WT} (△); *AQP1*_{WT} + *MPK2*_{K42A} (■); *AQP1*_{K12A} + *MPK2*_{WT} (◆); and *AQP1*_{K12A} + *MPK2*_{K42A} (□) were subjected to a hypo-osmotic shock, and the relative changes in cell volume were followed by monitoring the absorbance at 550 nm, as described in Materials and methods. Error bars were calculated from the mean $\pm$ SE of three independent experiments in triplicate.

Table 1Antimonite accumulation and sensitivity of LdBob cells expressing *AQP1* and/or *MPK2*

Strain	Sb(III) accumulation (nmol/10 ⁹ cells)	EC ₅₀ [Sb(III)] μ M
Vector	2 $\pm$ 0.2	130 $\pm$ 5
AQP1 _{WT}	9 $\pm$ 0.6	1 $\pm$ 0.01
AQP1 _{K12A}	11 $\pm$ 0.4	0.7 $\pm$ 0.09
AQP1 _{K12R}	12 $\pm$ 0.7	0.5 $\pm$ 0.02
AQP1 _{WT} + MPK2 _{WT}	21 $\pm$ 0.4	0.1 $\pm$ 0.002
AQP1 _{WT} + MPK2 _{K42A}	3 $\pm$ 0.3	80 $\pm$ 3
AQP1 _{K12A} + MPK2 _{WT}	19 $\pm$ 0.7	0.1 $\pm$ 0.002
AQP1 _{K12A} + MPK2 _{K42A}	21 $\pm$ 0.7	0.1 $\pm$ 0.002
AQP1 _{K12R} + MPK2 _{WT}	19 $\pm$ 0.6	0.1 $\pm$ 0.002
AQP1 _{K12R} + MPK2 _{K42A}	19 $\pm$ 0.9	0.1 $\pm$ 0.002
MPK2 _{WT}	2 $\pm$ 0.2	105 $\pm$ 5
MPK2 _{K42A}	2 $\pm$ 0.2	105 $\pm$ 5

Data are presented as mean $\pm$ SE of three independent experiments in triplicate.