GTPase Sar1 regulates the trafficking and secretion of the virulence factor gp63 in Leishmania

Received for publication, February 28, 2017, and in revised form, May 30, 2017 Published, Papers in Press, June 2, 2017, DOI 10.1074/jbc.M117.784033

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Metalloprotease gp63 (Leishmania donovani gp63 (Ldgp63)) is a critical virulence factor secreted by Leishmania. However, how newly synthesized Ldgp63 exits the endoplasmic reticulum (ER) and is secreted by this parasite is unknown. Here, we cloned, expressed, and characterized the GTPase LdSar1 and other COPII components like LdSec23, LdSec24, LdSec13, and LdSec31 from Leishmania to understand their role in ER exit of Ldgp63. Using dominant-positive (LdSar1:H74L) and dominant-negative (LdSar1:T34N) mutants of LdSar1, we found that GTP-bound LdSar1 specifically binds to LdSec23, which binds, in turn, with LdSec24 (1–702) to form a prebudding complex. Moreover, LdSec13 specifically interacted with His6–LdSec31 (1–603), and LdSec31 bound the prebudding complex via LdSec23. Interestingly, dileucine 594/595 and valine 597 residues present in the Ldgp63 C-terminal domain were critical for binding with LdSec24 (703–966), and GFP–Ldgp633594A/595A or GFP–Ldgp63V597S mutants failed to exit from the ER. Moreover, Ldgp63-containing COPII vesicle budding from the ER was inhibited by LdSec31:T34N in an in vitro budding assay, indicating that GTP-bound LdSar1 is required for budding of Ldgp63-containing COPII vesicles. To directly demonstrate the function of LdSar1 in Ldgp63 trafficking, we coexpressed RFP–Ldgp63 along with LdSar1:WT–GFP or LdSar1:T34N–GFP and found that LdSar1:T34N overexpression blocks Ldgp63 trafficking and secretion in Leishmania. Finally, we noted significantly compromised survival of LdSar1:T34N–GFP–overexpressing transgenic parasites in macrophages. Taken together, these results indicated that Ldgp63 interacts with the COPII complex via LdSec24 for Ldgp63 ER exit and subsequent secretion.

Leishmania is one of the most deadly parasites, posing a serious threat of debilitating more than 350 million people in 88 countries worldwide (1). Presently, no vaccine is available for the disease, and the drugs used for its chemotherapy are toxic (2). Thus, the major thrust is to understand the biology of the parasite with respect to growth, differentiation, and key intracellular processes, which may help in the identification of new molecular targets for intervention of this disease.

Modulation of host cell signaling by virulence factors secreted by intracellular pathogens is a major strategy used by these pathogens for their survival in the host cells. Interestingly, several Leishmania surface-expressed and secreted molecules like glycosylinositolphospholipids, lipophosphoglycan (LPG),3 cysteine protease, and metalloprotease gp63 (Leishmania donovani gp63 (Ldgp63)) have been shown to inactivate macrophage functions, and thereby parasite is able to counter the microbicidal activity of macrophages (3, 4). Among these, Ldgp63 secreted by Leishmania has been shown to be the major virulence factor required for entry and intracellular survival of the parasites in macrophages (5–8). For instance, Ldgp63 not only cleaves complement factor C3b to iC3b to prevent the complement-mediated lysis of the parasite but also enables the uptake of parasites by complement receptors on macrophages (9, 10). In addition, gp63 of the parasites also binds with macrophage fibronectin receptor to facilitate the internalization of parasite in host cells (11). Moreover, Ldgp63 is shown to modulate the critical serine/threonine kinases to hijack host macrophage signaling for their survival in macrophages (12). Ldgp63 also degrades several transcription factors like NF-κB, STAT1, and AP-1 to alter gene expression in macrophages (13–15). Interestingly, Ldgp63 is found to manipulate the translational system of host cell by cleaving mTOR (16). Thus, it is clear that Ldgp63 secreted by the parasites strongly influences different host cell machineries.

It has been shown that newly synthesized gp63 enters into the ER via its N-terminal signal peptide. Inside the ER, the nascent gp63 undergoes processing via cleavage of the signal peptide, subsequent glycosylation, and the addition of a GPI anchor at a critical asparagine residue at the C terminus (17). The GPI anchor addition is preceded by the removal of the short hydrophobic tail downstream of the site of GPI anchor attachment (18). Another form of gp63, which does not undergo GPI anchor attachment is also processed through ER (19). The GPI-anchored protein anchors to the cell surface via its anchor, whereas the non-GPI-anchored protein is directly secreted out
gp63 secretion requires Sar1 function in Leishmania (20). However, how Ldgp63 exits from the ER and secreted out of the cell is not known.

In the mammalian cells, exit of various cargos from the ER is generally mediated by COPII-coated vesicles. COPII coat formation is initiated with the recruitment of GTPase Sar1 on the ER membrane, which is activated by its guanine nucleotide exchange factor, Sec12 (21, 22). Subsequently, it recruits Sec23/24 heterodimer via specific interactions with Sec23 to form a “prebudding” complex (23, 24) in which cargo is captured by Sec24 (25, 26). Eventually, Sec13/31 heterotetramer is recruited that drives the membrane deformation and stimulates the GTPase-activating protein activity of Sec23 toward Sec24 (27–29). However, the role of COPII complex in the secretory pathway in Leishmania is not known.

Because Sar1 is the key GTPase for the formation of COPII-coated vesicles from the ER, we first characterized the role of LdSar1 and other COPII components to understand the mechanism of exit of Ldgp63 from parasite ER. We have shown that the dileucine motif of Ldgp63 is required to interact with COPII complex via LdSec24, and the GTP form of LdSar1 regulates the exit of Ldgp63 from the ER to mediate its secretion via the conventional secretory pathway. Finally, we have shown that LdSar1-mediated secretion of Ldgp63 is necessary for the survival of Leishmania in macrophages.

Results
Cloning, expression, and localization of Sar1 homolog from Leishmania

To understand the role of Sar1 in the secretion of gp63 in Leishmania, we have cloned and expressed Sar1 from L. donovani. We used Saccharomyces cerevisiae Sar1 sequence as query and identified putative Sar1 ortholog by BLAST analysis from the L. donovani genome database. Using appropriate forward and reverse primers, Sar1 was amplified from L. donovani cDNA by PCR. Our result showed that respective primers amplified a 588-bp fragment (LdSar1) of Sar1 (Fig. 1A) from Leishmania. The PCR product was cloned, sequenced, and hypothetically translated into amino acid sequence. Comparison of the LdSar1 protein sequence by ClustalW multiple-sequence alignment demonstrated that LdSar1 has overall identity of 81% with Trypanosoma cruzi, 54% with S. cerevisiae, and 52% with Homo sapiens Sar1. Sequence analysis also showed (Fig. 1B) that LdSar1 has highly conserved guanine nucleotide-binding regions and a highly conserved effector loop, as observed in respective mammalian homologs (21). Subsequently, PCR product was cloned into pGEX-4T-2 and pET28a expression vectors to express as GST or His6 fusion proteins, respectively. Specific antibody against LdSar1 protein of Leishmania was made, and Western blot analyses showed that this antibody specifically recognized purified His6–LdSar1 protein as well as endogenous Sar1 from Leishmania cell lysate (Fig. 1C). Immunofluorescence studies using this antibody showed that LdSar1 localizes in a discrete compartment in Leishmania (Fig. 1D).

To identify the LdSar1-positive compartment in Leishmania, we overexpressed LdSar1:WT–GFP protein in Leishmania. Subsequently, these cells were stained with different compartment-specific markers and analyzed by confocal microscopy. No colocalization of LdSar1:WT–GFP was observed with FM4-64-labeled flagellar pocket and 5-min internalized Alexa Fluor-594-conjugated Hb, which primarily labeled early endosomes of Leishmania (Fig. 1E). However, LdSar1:WT–GFP was found to be colocalized with ER Tracker in close proximity to LPG2-labeled Golgi, indicating that it probably localizes in the transitional ER of the parasite (Fig. 1E).

Generation and characterization of LdSar1 mutants

To determine the role of LdSar1 in the exit of gp63-containing vesicles from the ER in Leishmania, dominant-active and dominant-negative mutants of LdSar1 were made based on the similar mutations reported earlier in yeast (30). The LdSar1: T34N mutant was made by substituting threonine for asparagine in the GXXXGKT motif, whereas histidine was substituted for leucine in the DXXGH region in LdSar1:H74L. Our results demonstrated that both LdSar1:WT and LdSar1:H74L bind with [α-32P]GTP, whereas almost no GTP binding was observed with LdSar1:T34N (Fig. 2A). This result demonstrated that LdSar1:T34N mutant has reduced affinity to GTP and thereby acts as a dominant-negative mutant of LdSar1. Analysis of GTPase activities of these mutants revealed that LdSar1:WT protein hydrolyzes GTP to GDP, whereas GTP hydrolysis is significantly inhibited in LdSar1:H74L mutant (Fig. 2B). Taken together, these results indicated that LdSar1 is a functional GTPase.

Subsequently, we determined the localization of LdSar1: H74L–GFP and LdSar1:T34N–GFP in Leishmania. As expected, our results showed that LdSar1:H74L–GFP localizes into a discrete compartment like LdSar1:WT–GFP. In contrast, LdSar1:T34N–GFP, a dominant-negative mutant of LdSar1, failed to localize in the respective compartment, and protein was found to be distributed in the cytoplasm (Fig. 2C).

Cloning, expression, and localization of other COPII proteins in Leishmania

To determine the interaction of LdSar1 with inner and outer coat components of the COPII complex, we cloned and expressed other COPII proteins (LdSec23, LdSec24, LdSec13, and LdSec31) from Leishmania using S. cerevisiae COPII protein sequences as a query. Using appropriate forward and reverse primers, a 2511-bp fragment for LdSec23 (Fig. 3A, lane 1), 2901-bp fragment for LdSec24 (Fig. 3A, lane 3), 1002-bp fragment for LdSec13 (Fig. 3A, lane 4), and 3504-bp fragment for LdSec31 (Fig. 3A, lane 5) were amplified from L. donovani cDNA by PCR. The PCR product was cloned, sequenced, and hypothetically translated into amino acid sequence.

Multiple-sequence alignment by ClustalW revealed that LdSec23 has overall identity of 54% with T. cruzi, 32% with S. cerevisiae, and 32% with H. sapiens; LdSec24 has overall identity of 55% with T. cruzi, 26% with S. cerevisiae, and 29% with H. sapiens; LdSec13 has overall identity of 47% with T. cruzi, 26% with S. cerevisiae, and 31% with H. sapiens; and LdSec31 has overall identity of 49% with T. cruzi, 25% with S. cerevisiae,
and 29% with *H. sapiens* (Fig. 3A). Sequence analysis also demonstrated that LdSec23 and LdSec24 have a characteristic Sec23 helical and β-sheet region, LdSec13 is composed entirely of WD40 repeats, and LdSec31 is composed of WD40 repeats at its N terminus, as observed in their respective mammalian homologs. Subsequently, PCR products of LdSec23 and LdSec13 were cloned into pGEX-4T-2 or pET28a expression vector to express it as a GST/His6 fusion protein. Because we were unable to purify full length LdSec24 and LdSec31, LdSec24(1–702), LdSec24(703–966), LdSec31(1–603), and LdSec31(604–1187) proteins were purified either with His6 or GST tag using appropriate expression vector.

Subsequently, we determined the localization of other COPII proteins of *Leishmania* by co-expressing RFP–LdSar1:WT with LdSec23–GFP, LdSec24–GFP, GFP–LdSec13, or GFP–LdSec31. We found that LdSec23–GFP, LdSec24–GFP, GFP–LdSec13, and GFP–LdSec31 are co-localized with RFP–LdSar1:WT, indicating that all of these components of the COPII complex reside along with LdSar1 in the parasite (Fig. 3B). Interestingly, these proteins failed to localize in the discrete compartment when the cells were co-expressed with RFP–LdSar1:T34N, a GTP binding-deficient mutant of LdSar1 (Fig. 3C).

**Interaction of LdSar1 with other COPII proteins in Leishmania**

The interaction of LdSar1 with other COPII proteins was determined by protein–protein interaction. Accordingly, binding experiments were carried out using one protein (GST/His6-tagged) of the COPII complex immobilized on glutathione/Ni-NTA beads as bait and incubated with equimolar amount of
other target protein (His6/GST-tagged) of the COPII complex. The binding was determined using the antibody against the target protein. Our results demonstrated that His6–LdSar1:WT and His6–LdSar1:H74L bind with GST–LdSec23, whereas no binding was observed with His6–LdSar1:T34N, indicating that the GTP form of LdSar1 specifically binds with LdSec23 (Fig. 4A). In contrast, we found that GST–LdSar1 does not interact with His6–LdSec24, His6–LdSec31, and His6–LdSec13 (Fig. 4B). Subsequently, GST–LdSec23 was found to specifically bind with the N terminus (His6–LdSec24(1–702)) of LdSec24 (Fig. 4C, left). Our results also showed that GST–LdSec23 binds with His6–LdSec31 (Fig. 4C, middle), but LdSec23 does not interact with LdSec13 (Fig. 4C, right). In addition, we found that His6–LdSec31(1–603) interacts with GST–LdSec13 (Fig. 4D).

To determine whether these proteins form a complex in Leishmania, an ex vivo pulldown assay was carried out by incubating immobilized GST–LdSar1 with parasite lysate, and bound proteins were detected using specific antibodies against the indicated proteins (Fig. 4E). Our results showed that immobilized LdSar1 pulled out LdSec23, LdSec24, LdSec13, and LdSec31 from Leishmania lysate (Fig. 4F).

**Identification and characterization of binding of Ldg63 with COPII complex**

To understand how Leishmania gp63 exits from the ER, we studied the interaction of Ldg63 with different proteins of the COPII complex of Leishmania. Our results demonstrated that Ldg63 specifically binds with LdSec24(703–966) (Fig. 5A). This result was further confirmed using immobilized LdSec24(703–966) and Leishmania lysate (Fig. 5B). Subsequently, efforts were made to determine how Ldg63 interacts with C-terminal domain of LdSec24. Therefore, we analyzed the sequence of Ldg63 to identify ER exit motif(s), because it was demonstrated previously in mammalian cells that diacidic, dihydrophobic, valine, or a few other motifs present in the cytoplasmic end of different cargos are critical for their binding to Sec24 (31–34). Interestingly, we found that Ldg63 contains a valine 597, dileucine 594/595, and diasparsate 548/549 at its cytoplasmic domain. Accordingly, we prepared three mutant proteins of Ldg63, namely Ldg63V597S, Ldg63L594A/L595A, and Ldg63E548A/E549A, and determined their binding with LdSec24(703–966). Our results showed (Fig. 5C) that LdSec24(703–966) did not bind significantly with Ldg63V597S and Ldg63E548A/E549A, whereas no inhibition of binding was observed with Ldg63L594A/L595A in comparison with Ldg63:WT. These results indicated that dileucine 594/595 and valine 597 residues present in the C-terminal end of Ldg63 are critical for binding with LdSec24.

**Role of LdSar1 in budding of gp63-containing vesicles from the ER in Leishmania**

In vitro vesicle budding assays have served as an important tool to analyze the packaging and exit of cargo in COPII-coated vesicles. To decipher whether budding of Ldg63-containing vesicles from the ER requires LdSar1, microsomes were prepared from cells overexpressing GFP–Ldg63. To check the integrity of GFP–Ldg63–containing microsomes, we treated the microsomes with different concentrations of proteinase K in the presence or absence of detergent. LdSar1, which is peripherally associated with microsomes, was used as a control. Our results showed that LdSar1 is digested even at a low concentration of proteinase K (1 μg/ml) in the absence of Triton X-100, whereas GFP–Ldg63 is significantly protected even at higher concentrations under similar conditions. However, permeabilization of microsomes with Triton X-100 treatment ren-
**Figure 3. Identification and localization of other COPII proteins in Leishmania.**

A. Amplification of a 2511-bp fragment of LdSec23 (lane 2; submitted to the GenBank™ database under accession number KY484913), a 2901-bp fragment of LdSec24 (lane 3; submitted to the GenBank™ database under accession number KY484914), a 1002-bp fragment of LdSec13 (lane 4; submitted to the GenBank™ database under accession number KY484912), and a 3504-bp fragment of LdSec31 (lane 5; submitted to the GenBank™ database under accession number KY484915) from *L. donovani* cDNA by PCR using appropriate forward and reverse primers, as described under “Experimental procedures.” Lane 1, DNA ladder. Hypothetical translation of obtained sequences of LdSec23, LdSec24, LdSec13, and LdSec31 into protein sequences shows significant identities with different domains of respective sequences from *T. cruzi*, *S. cerevisiae*, and *H. sapiens*. Percentage identities of respective *Leishmania* proteins with other organisms are shown below in each protein.

B. Localization of different COPII components in *Leishmania* was determined by co-expressing RFP–LdSar1 with LdSec23–GFP, LdSec24–GFP, GFP–LdSec13, or GFP–LdSec31, as described under “Experimental procedures.” To determine whether the GTP form of LdSar1 is required for the localization of other components of the COPII complex in *Leishmania* in ER, LdSec23–GFP, LdSec24–GFP, GFP–LdSec13, or GFP–LdSec31 was co-expressed with RFP–LdSar1:T34N, as described under “Experimental procedures.” Finally, cells were visualized under a confocal microscope. Yellow indicates the colocalization of indicated markers/proteins in one plane after z-stack analysis by confocal microscopy. Results are representative of three independent observations.
Ldgp63 secretion requires Sar1 function in Leishmania

These results indicated that Ldgp63 is present within microsomes. Subsequently, these microsomes were then treated with urea to remove peripheral proteins and then incubated with Leishmania cytosol containing His6–LdSar1:WT or His6–LdSar1:T34N in the presence of the ATP regeneration system. Subsequently, the reaction mix was sedimented at 12,000 × g for 4 min to pellet the donor microsomes, and COPII vesicles budded from microsomes were recovered in the supernatant. Finally, budding of GFP–gp63–containing COPII vesicles was determined by Western blot analysis using anti-GFP antibody. Our results showed that in vitro budding of Ldgp63-containing COPII vesicles from the ER is significantly inhibited in the presence of His6–LdSar1:T34N–containing cytosol compared with His6–LdSar1:WT and control cytosol (Fig. 6B). These results indicated that LdSar1 in GTP-bound form is required for the exit of Ldgp63-containing COPII vesicles from the ER.

Role of LdSar1 in the trafficking and secretion of Ldgp63 in Leishmania

To determine the role of LdSar1 in the trafficking of Ldgp63 in Leishmania, we compared the trafficking of Ldgp63 in LdSar1 and its dominant-negative mutant overexpressing Leishmania. Accordingly, RFP–Ldgp63 was coexpressed with LdSar1:WT–GFP or LdSar1:T34N–GFP in Leishmania. Our results showed (Fig. 7A) that RFP–Ldgp63 was localized into discrete punctate structures, possibly the secretory vesicles in Leishmania, when it was overexpressed alone. Similar distribution of RFP–Ldgp63 was observed in LdSar1:WT–GFP–overexpressing parasite. In contrast, the trafficking of RFP–Ldgp63 was completely blocked in cells overexpressing LdSar1:
gp63 secretion requires Sar1 function in Leishmania

Consequently, we determined the amount of endogenous gp63 released into Leishmania culture medium by LdSar1 and its mutant-overexpressing cells. We found that LdSar1:WT–GFP–overexpressing parasites secreted an ~20% higher amount of Ldgp63 than untransfected control cells (Fig. 7B). In contrast, ~80% reduction in secretion of Ldgp63 was observed in cells overexpressing LdSar1:T34N–GFP in comparison with untransfected control cells (Fig. 7B).

To unequivocally prove the requirement of interaction of Ldgp63 with COPII complex via LdSec24 for exit from the ER, we overexpressed Ldgp63 with COPII complex via LdSec24 in L. donovani promastigotes. Interestingly, we found that Ldgp63 mutants with COPII complex via LdSec24 for exit from the ER, whereas GFP–Ldgp63:WT was distributed in the secretory vesicles as

Figure 5. Ldgp63 specifically binds with LdSec24 of the COPII complex. A, to identify the cargo binding protein of the COPII complex in Leishmania, the indicated His6 fusion proteins of the COPII complex were immobilized on Ni-NTA beads and incubated with GST–Ldgp63, as described under “Experimental procedures.” Binding of the respective COPII protein with GST–Ldgp63 was detected by Western blot analysis using anti-Ldgp63 antibody. Ni-NTA beads incubated with the same amount of GST–Ldgp63 were used as a control. B, similar experiments were carried out by incubating immobilized His6–LdSec24(1–702) or His6–LdSec24(703–966) with Leishmania cytosol. C, to determine critical residues of Ldgp63 required for binding with LdSec24, GST–Ldgp63:WT, GST–Ldgp63V597S, or GST–Ldgp63L594A/L595A was immobilized on beads and incubated with His6–LdSec24(703–966). Binding of LdSec24 with Ldgp63 was determined by Western blot analysis using anti-His6 antibody. GST immobilized on glutathione beads was used as a control. All results are representative of three independent experiments.
observed earlier (Fig. 7C). However, GFP–Ldgp63E548A/E549A trafficking was found to be unaltered compared with the control cells.

**LdSar1-mediated secretion of Ldgp63 is necessary for the survival of Leishmania in macrophages**

To determine whether LdSar1–mediated secretion of Ldgp63 in Leishmania is required for intracellular survival of parasites in macrophages, THP-1 differentiated macrophages were infected with LdSar1:WT- or LdSar1:T34N-overexpressing transgenic parasites, and parasite load was determined at the indicated times. Our results showed (Fig. 8, A and B) that infection with LdSar1:WT-overexpressing transgenic parasites in macrophages is significantly greater than with control parasites at 0 h. Whereas less infection in macrophages was observed with LdSar1:T34N-overexpressing transgenic parasites compared with control. These results were more pronounced when we analyzed the intracellular survival of the parasites after 48 h of infection. We found ~60% inhibition of parasite load in macrophages infected with LdSar1:T34N-overexpressing transgenic parasites in comparison with infection with control parasites. Interestingly, our results showed that the survival of LdSar1:WT-overexpressing transgenic parasites in macrophages is significantly higher than in the control cells (Fig. 8, A and B). These results demonstrated that LdSar1-mediated efficient secretion of Ldgp63 is required for the intracellular survival of the parasites.

**Discussion**

A 63-kDa metalloprotease of *Leishmania* (Ldgp63) is one of the well-characterized virulence factors that plays an important role in parasite survival in the host cells (5–7, 35). Previous
gp63 secretion requires Sar1 function in Leishmania

A. 

![Graph showing number of parasites in infected macrophages]

B. 

![Images showing localization of LdSar1 in parasites]

studies have shown that newly synthesized Ldgp63 is targeted to the ER through ER-signaling sequence present at the N terminus of the protein, and finally the protein is secreted out by the parasites (18–20). However, how Ldgp63 exits from the ER and follows the secretory pathway in the parasites is not well characterized. Among the different components of the COPII complex, we have first cloned and characterized the Sar1 homolog from Leishmania. We have found that Leishmania has a quite conserved homolog of Sar1 like higher eukaryotic cells. Like any other small GTP-binding protein, the generation of GTP-locked constitutively active mutant and GDP-locked dominant-negative mutant is a useful tool to determine the function of respective GTPase (30). Therefore, we have made a LdSar1:H74L mutant, and our results have shown that this mutant binds GTP but is unable to hydrolyze GTP efficiently. We have also made a LdSar1:T34N mutant that shows reduced affinity to GTP.

To determine the localization of LdSar1 in Leishmania, we have overexpressed LdSar1 or its mutant proteins in Leishmania as GFP or RFP fusion proteins. LdSar1:WT–GFP is found to be predominantly localized in ER labeled with ER Tracker and in close proximity to LPG2-labeled Golgi. As expected, RFP–LdSar1:T34N, the GDP-locked dominant-negative mutant of LdSar1, is distributed throughout cytosol. Subsequently, we have found that LdSec23–GFP, LdSec24–GFP, GFP–LdSec13, and GFP–LdSec31 are co-localized with RFP–LdSar1:WT, indicating that all of these components of the COPII complex are also present in LdSar1-positive ER. Correspondingly, it has been shown that PfSec13 localizes in transitional ER in Plasmodium (48). In contrast, all of the components of COPII proteins fail to localize in discrete compartments when they are co-expressed with RFP–LdSar1:T34N. These results are consistent with the previous finding that Sar1 in the GTP form is necessary for the recruitment of all subsequent COPII components to the ER (49, 50).

To understand the mechanism of formation of the COPII complex in Leishmania, we have studied the interaction between different COPII proteins. Our results have shown that LdSar1 in the GTP form specifically binds with LdSec23 but does not interact with any other protein of the COPII complex. Thus, the interaction between membrane-bound LdSar1 in the GTP form with cytosolic LdSec23 acts as a first step for the formation of COPII vesicles. Subsequently, we have found that LdSec23 specifically binds with the N terminus of LdSec24 to form a heterodimer that is subsequently recruited to the ER membrane by interacting with GTP form of LdSar1 to form the prebudding complex. These results are supported by the fact that interaction of Sar1 with Sec23/Sec24 homologues in Giardia is critical for maintenance of endoplasmic reticulum exit sites in parasites (50).

Subsequently, we have determined how LdSec13–LdSec31 is recruited to the prebudding complex to form the outer layer of the nascent COPII vesicles. We have found that LdSec13 specifically binds with the N terminus of LdSec31, which in turn interacts with LdSec23. These results are consistent with the previous observations that central region of Sec31 interacts with Sec23, whereas N-terminal of Sec31 specifically binds with Sec13 in yeast (51). Although all proteins of the COPII complex do not interact directly, our results have shown that one of the COPII proteins can pull out all other components from Leishmania lysate, indicating that they form the COPII complex in Leishmania. Taken together, our results indicate that LdSec23
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plays a prominent central role in COPII complex assembly by acting as a bridge between LdSec1 and LdSec24–LdSec31. Therefore, it is tempting to speculate that recruitment of LdSec13–LdSec31 to the prebudding complex initiates membrane curvature and, subsequently, triggers the GTPase-activating protein activity of LdSec23, leading to GTP hydrolysis of LdSar1, which releases COPII vesicles from the ER (24, 28, 52).

To determine the functional significance of LdSar1 in the exit of Ldgp63 from the ER in Leishmania, we have analyzed the budding, trafficking, and secretion of Ldgp63 in cells overexpressing dominant-negative mutant (LdSar1: T34N) of LdSar1. Interestingly, we have found that budding of Ldgp63-containing COPII vesicles from the ER requires the GTP form of LdSar1. Consequently, our in vivo results have shown that overexpression of LdSar1: T34N–GFP blocks the trafficking and secretion of Ldgp63 by the parasites. This is consistent with the fact that the conditional expression of dominant-negative form of TbSar1 blocks the trafficking of variant surface glycoprotein in Trypanosoma (45). Thus, our results conclusively demonstrated that LdSar1 is critical for the exit of Ldgp63 from the ER and its subsequent secretion in Leishmania.

Finally, we have tried to understand the mechanism of how Ldgp63 interacts with COPII complex for its exit from the ER in Leishmania. We have found that Ldgp63 specifically binds with LdSec24. Interestingly, our results have shown that dileucine 594/595 and valine 597 motifs present in the C-terminal of Ldgp63 is critical for binding with C terminus of LdSec24. Subsequently, to determine the significance of such interaction in the trafficking and secretion of Ldgp63 by Leishmania in vivo, we have overexpressed GFP–Ldgp63:WT, GFP–Ldgp63L594A/L595A, or GFP–Ldgp63V597S mutants in Leishmania. Our results have shown that trafficking of GFP–Ldgp63L594A/L595A and GFP–Ldgp63V597S mutants is blocked, possibly in the ER, whereas trafficking of GFP–Ldgp63:WT is found to be uninterrupted.

Because Ldgp63 is a virulence factor required for intracellular survival of the parasites in macrophages, we have checked the survival of LdSar1: T34N-overexpressing transgenic parasites in macrophages. In correlation with the fact that LdSar1: T34N overexpressing transgenic parasites are severely compromised in the secretion of Ldgp63, we have found that these parasites are unable to grow optimally in macrophages. These results further confirm that LdSar1-mediated secretion of Ldgp63 is critical for intracellular survival of Leishmania in macrophages.

In conclusion, we have delineated the mechanism of secretion of Ldgp63, a critical virulence factor of Leishmania. This is the first demonstration that LdSar1 plays a critical role in exit of Ldgp63 from the ER and in its trafficking and subsequent secretion by the parasites. We have also shown that Leishmania has conserved COPII proteins like higher eukaryotic cells, and these proteins interact with each other through appropriate domains to make a functional COPII complex in the parasite. Most importantly, our results have demonstrated that Ldgp63 is recruited into COPII complex through the interaction of dileucine motif present in the C terminus of Ldgp63 with LdSec24 for its exit from the ER. Finally, we have shown that intracellular survival of Leishmania in macrophages requires Sar1-mediated secretion of Ldgp63. Our findings that disruption of ER-to-Golgi trafficking via a dominant-negative mutant of LdSar1 blocks the secretion of virulence factors like Ldgp63 and thereby inhibits the parasite survival in the macrophages, indicating the possibility of exploiting this pathway in parasites for potential drug targets. Because the major focus for design and development of new drugs is the inactivation of key molecule(s) needed for parasite survival (53), the development of appropriate inhibitors or small molecules targeted to parasite COPII proteins could be a new therapeutic approach against the parasites.

Experimental procedures

Materials

Unless otherwise stated, all reagents were procured from Sigma. The RT-PCR kit, Platinum HiFidelity Taq polymerase, TRizol, M199 medium, bicinechonic acid (BCA) reagent, and gentamicin were purchased from Life Technologies, Inc. Luria-Bertani (LB) broth and LB-agar were supplied by Difco. FCS was procured from Biological Industries (Beit-Haemek, Israel). pGEX-4T-2 expression vector, glutathione-Sepharose beads, anti-His antigen, and ECL reagents were procured from GE Healthcare (Buckinghamshire, UK). Anti-GST antibody was procured from Santa Cruz Biotechnology, Inc. (Dallas, TX). Restriction enzymes were obtained from Promega (Madison, WI). Complete and incomplete Freund’s adjuvants were procured from BD Biosciences. The Leishmania expression vectors, pXG-GFP2 and pNUS-mRFP-nD were kindly provided by Dr. S. M. Beverley (Washington University, St. Louis, MO) and Dr. Jean-Paul di Rago (Institut de Biochimie et Génétique Cellulaires, Bordeaux, France), respectively. Alexa Fluor-594 succinimidyl ester, FM4-64, and LysoTracker Red were obtained from Molecular Probes, Inc. (Eugene, OR). Geneticin and blasticidin were procured from Gibco and Calbiochem, respectively. [α-32P]GTP (800 Ci/mmol) was procured from PerkinElmer Life Sciences. All other reagents used were of analytical grade.

Cells

Promastigotes of L. donovani (LdBob) were kindly provided by Dr. R. Madhubala (Jawaharlal Nehru University, New Delhi, India). Cells were routinely grown at 23 °C in sterile liquid M199 medium buffered with HEPES (40 mM) containing gentamicin (50 μg/ml) and supplemented with 10% FCS.

Human acute monocytic leukemia-derived (THP-1) cells were obtained from the ATCC (Manassas, VA) (catalog no. TIB-202). Cells were routinely cultured in complete RPMI (RPMI 1640 containing 10% FCS and 50 μg/ml gentamicin) at 37 °C in a humidified incubator with 5% CO2. THP-1 cells were differentiated into macrophages in the presence of phorbol 12-myristate 13-acetate (100 ng/ml) for 24 h. Cells were washed and incubated for another 24 h in complete RPMI without PMA and used for experimental purposes.
Cloning of COPII proteins from *L. donovani*

To clone Sar1, Sec23, Sec24, Sec13, and Sec31 from *L. donovani*, the respective *S. cerevisiae* protein sequences were used as queries and putative homologs of these proteins were identified from the *L. donovani* genome database. The appropriate gene-specific primers were designed, and the ORF of respective genes was amplified from *L. donovani* cDNA using these primers by RT-PCR. Briefly, PCR was performed in a PerkinElmer Life Sciences thermocycler for 30 cycles (denaturation at 94 °C for 30 s, annealing at 62 °C (LdSar1 and LdSec13) or 64 °C (LdSec23, LdSec24, and LdSec31) for 30 s, and extension at 68 °C for 1.5 min (LdSar1 and LdSec13) or 4 min (LdSec23, LdSec24, and LdSec31) using High Fidelity Taq polymerase. The PCR product was cloned into pGEM-T Easy vector (Promega Life Science) as per the manufacturer’s protocol and sequenced using M13 universal primers. After confirming the sequence, the respective products were further subcloned into BamHI/EcoRI sites of the pGEX-4T-2 and pET28a expression vectors. The truncated constructs were transformed into BL21 strain of *Escherichia coli*. Primer sequences used in PCR were as follows: LdSar1, 5′-ATGGGCTGTGTTTAGTCTGGTGGGAC-3′ (forward) and 5′-CTACAGGACTCTCCATCTGAGAGCGCAG-3′ (reverse); LdSec23, 5′-ATGAGCCGGATTACGTCTACG-GAAACTACG-3′ (forward) and 5′-CTACTGCTGGAAGTCCTTCAAA-3′ (reverse); LdSec24, 5′-ATGGGTTCCACACCGCAGCCACGTTCTTGAG-3′ (forward) and 5′-ATGGCGACTG-3′ (reverse); LdSec24, 5′-ATGGCGACTG-3′ (reverse); LdSec31, 5′-ATGGGTTCCACACCGCAGCCACGTTCTTGAG-3′ (forward) and 5′-ATGGCGACTG-3′ (reverse); LdSec31, 5′-ATGGCGACTG-3′ (reverse).

**Generation and purification of different mutants of COPII proteins**

To understand the functions of *Leishmania* Sar1 more precisely, two mutants (viz. GTP-locked (H74L) and GDP-locked (T34N)) were generated using appropriately designed mutant primers, as described previously (36). Briefly, megaprimer for H74L was generated using this megaprimer as the forward primer with a WT reverse primer. Similarly, megaprimer for T34N was generated using WT Sar1 forward primer and mutated reverse primer (5′-GCCCTGCTAAGGCGCCACCATATC-3′) by PCR, and subsequently full-length mutated product was generated using this megaprimer as the forward primer with a WT reverse primer. Similarly, megaprimer for T34N was generated using the WT Sar1 forward primer and mutated reverse primer (5′-CCAGAGAAGGTTTTTTGCGGCTGTG-3′) by PCR, and subsequently full-length mutated product was generated using this megaprimer as the forward primer with a WT reverse primer. Finally, these PCR products were cloned into BamHI/EcoRI sites of pGEX-4T-2 or pET28a expression vector and subsequently full-length mutated product was generated using this megaprimer as the forward primer with a WT reverse primer. These truncated constructs were transformed into E. coli (BL21 strain) to express as GST/His$_6$-tagged fusion proteins.

Respective cells containing the indicated construct were grown separately in LB and induced with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside for 3 h at 37 °C for the expression and purification of respective GST or His$_6$ fusion proteins using reduced glutathione or Ni-NTA beads, respectively, by standard procedures (36).

**Generation of antibodies against different COPII proteins**

10 μg of LdSar1, LdSec23, LdSec31(604–1187), LdSec24(703–966), or LdSec13 His$_6$ fusion protein was immunized in BALB/c mice to raise antibodies by a standard method, as described previously (40). The specificity of the antibodies against the respective proteins was determined by Western blot analysis using the respective purified proteins.

**GTP overlay assay**

GTP binding activity of purified LdSar1:WT and its mutants was detected by a GTP overlay assay (36). Briefly, 2 μg of GST–LdSar1:WT or its mutants was blotted onto a nitrocellulose membrane, and the membrane was incubated with 1 μCi/ml [α-32P]GTP in 50 mM phosphate buffer, pH 7.5, containing 5 mM MgCl$_2$, 1 mM EGTA, and 0.3% Tween 20 for 3 h at 24 °C. Finally, the unbound radioactivity was removed by extensive washing and visualized by autoradiography.

**GTPase assay**

The GTP hydrolysis activities of LdSar1:WT and its mutants were determined as described previously (36). Briefly, 5 μg of immobilized protein on glutathione beads was incubated with buffer A (20 mM Tris–HCl, pH 7.8, 100 mM NaCl, 5 mM MgCl$_2$, 1 mM Na$_2$HPO$_4$, and 10 mM β-mercaptoethanol) for 20 min at 25 °C, and bound nucleotide was eluted with 1 mM guanidine–HCl. Immobilized nucleotide-free protein was then loaded with 2 pmol of [α-32P]GTP (800 Ci/mmol) in 20 μl of buffer B for 10 min at 0 °C. Subsequently, the beads were washed and incubated for 1 h at 23 °C to allow the hydrolysis of bound GTP. Subsequently, the beads were washed, incubated in 8 μl of buffer B (0.2% SDS, 2 mM EDTA, 10 mM GDP, 10 mM GTP, pH 7.5), and heated at 70 °C for 2 min to elute the nucleotide from the protein. An aliquot was analyzed using thin-layer chromatography and visualized by autoradiography.
gp63 secretion requires Sar1 function in Leishmania

Overexpression of the COPII complex proteins in Leishmania

To overexpress COPII complex proteins as GFP fusion proteins in *Leishmania* promastigotes, the respective clones were subcloned into the NotI/BamHI sites of the pXG-GFP+2 vector (LdSec31 and LdSec13) to express the proteins as N-terminal GFP tag and BamHI/EcoRV sites of pXG-GFP+ vector (LdSar1, LdSec23, and LdSec24) to express the proteins as C-terminal GFP tag. Subsequently, *Leishmania* promastigotes were transfected with the respective constructs by electroporation, as described previously (36, 40). Finally, positive clones were selected in the presence of G418 (50 μg/ml) for 5–7 days. GFP, GFP–LdSec31, and GFP–LdSec13. The positive co-transfectants were selected with an equimolar amount of another target protein (His6/GST-tagged) of the COPII complex for 1 h at 4°C in PBS, pH 7.2. Subsequently, beads were washed with PBS and resuspended in 1 ml (15–17 mg/ml of protein) of the HEPES buffer without glycerol and used for an in vitro budding assay. To check the integrity of GFP–Ldg63–containing microsomes, microsomes (30 μg) were incubated with the indicated concentra-

Expression and purification of recombinant Ldg63 and mutants

To purify Ldg63 as GST-tagged fusion protein, gene-specific primers were designed against the Ldg63 sequence (forward, 5’-ATGTCCGTCAGACGAGCAGAC-3’; reverse, 5’-CTAGAGCGCCACGGCCAGCAG-3’), and its ORF was amplified from Ldg63-pNUS-mRFpNDS construct (36) by PCR using High Fidelity Taq polymerase. Subsequently, the PCR product was cloned in BamHI/EcoRI restriction sites of pXG-GFP+2 vector for expression as GFP fusion protein in *Leishmania* promastigotes.

Ldg63 point mutants (Ldg63V597S and Ldg63L594A/L595A) were PCR-amplified using similar conditions used for Ldg63-pGEX-4T-2 amplification by appropriate mutant primers (Ldg63V597S, WT forward primer and reverse 5’-CTAGAGCGCCAGGCGCAGAC-3’, Ldg63L594A/L595A, WT forward primer and reverse 5’-CTAGAGCGCCAGCGCCAGC-3’). Ldg63V597S and Ldg63L594A/L595A mutant was PCR-amplified using a megaprimer-based approach. Briefly, a forward primer was designed comprising the respective mutations (5’-AGGCCCTTGGGCCGCGC-3’) and was used along with wild-type reverse primer to amplify a 140-bp megaprimer from the Ldg63-pGEX-4T-2 construct. Subsequently, this megaprimer was used as a reverse primer along with wild-type forward primer to amplify full-length mutant Ldg63p. All of the mutant proteins were subsequently cloned in BamHI/EcoRI sites in pGEX-4T-2 vector and expressed as GST fusion protein, as described previously. These Ldg63 mutants were also subcloned in NotI/BamHI restriction sites of pXG-GFP+2 vector for expression as GFP fusion protein in *Leishmania* promastigotes.

In vitro budding of COPII vesicles from microsomes

To carry out in vitro budding of COPII vesicles from *Leishmania*, microsomes were prepared as described previously (55). Briefly, GFP–Ldg63–overexpressing parasites (6 × 10⁹) were washed with PBS and resuspended in 10 ml of HEPES buffer (100 mM HEPES, pH 7.4, 60 mM KCl, 10 mM MgCl₂ with protease inhibitor) containing 10% glycerol. The cells were disrupted in a Parr nitrogen cavitation bomb after equilibration at 1,500 p.s.i. for 25 min at 4°C. The unbroken cells and nuclei were removed by centrifugation at 500 × g for 7 min. The postnuclear supernatant–containing microsomes were centrifuged at 100,000 × g for 1 h at 4°C in an MLA-80 rotor (Beckman Optima Max-XP). The resulting pellet–containing microsomes was resuspended in 10 ml of HEPES buffer without glycerol and centrifuged again at 100,000 × g for 1 h at 4°C. The enriched microsomes were finally resuspended in 1 ml (15–17 mg/ml of protein) of the HEPES buffer without glycerol and used for an in vitro budding assay. To check the integrity of GFP–Ldg63–containing microsomes, microsomes (30 μg) were incubated with the indicated concentra-
tions of proteinase K in the presence or absence of 0.5% Triton X-100 in 30 µl of HEPES buffer for 10 min at 4 °C. The reaction was stopped by adding protease inhibitor mixture and SDS loading buffer, and samples were analyzed by Western blot analysis using anti-GFP antibody.

Finally, an in vitro budding assay was carried out as described previously (56). Briefly, 100 µg of microsomes were resuspended in 100 µl of budding buffer (HEPES buffer containing 1 mM ATP, 8 mM creatine phosphate, 31 units/ml creatine phosphokinase, 0.1 mM GTP) supplemented with gel-filtered cytosol (400 µg) containing 2 µg of His6–LdSar1 or His6–LdSar1:T34N protein. The reaction mixture was incubated at 23 °C for 30 min, and the reaction was stopped by incubating on ice for 5 min. 50 µl of the reaction mixture was removed and used as input. Subsequently, the rest of the reaction mixture (50 µl) was centrifuged at 12,000 g for 4 min to separate the COP II budded vesicles from the supernatant from the donor membranes in the pellet. The presence of GFP–Ldg63 in the budded vesicles was determined by Western blotting using anti-GFP antibody.

**Role of LdSar1 in Ldg63 trafficking in Leishmania**

To determine the role of LdSar1 in the trafficking of Ldg63, Leishmania promastigotes overexpressing LdSar1:WT–GFP or LdSar1:T34N–GFP were transfected with RFP–Ldg63 using the same protocol as described previously (36). Co-transfected cells were selected in the presence of G418 (50 µg/ml) and blasticidin (15 µg/ml). Localization of RFP–Ldg63 in LdSar1: WT- or LdSar1:T34N-overexpressing cells was determined by confocal microscopy.

**Detection of secreted Ldg63 in spent medium**

To determine the amount of gp63 secreted by LdSar1:WT- or LdSar1:T34N-overexpressing Leishmania, 1 × 10^7 parasites were grown in 1 ml of FCS-free M199 medium for 24 h at 23 °C. Subsequently, cells were pelleted by centrifugation (1500 × g for 10 min at 4 °C), and the amount of Ldg63 secreted by the respective cells in culture medium was detected by acetone precipitation followed by Western blot analysis using anti-Ldg63 antibody, as described previously (36). The respective cell pellets were also analyzed by Western blotting using specific antibody.

**Quantification of intracellular growth of Leishmania in macrophages**

THP1 cells were harvested and resuspended in RPMI 1640 medium containing 10% FCS and PMA (100 ng/ml). Cells (2.5 × 10^6) were seeded on sterile glass coverslips placed in a 6-well plate and incubated for 24 h at 37 °C to differentiate into macrophages. Cells were washed and incubated for another 24 h in PMA-free RPMI 1640 medium containing 10% FCS at 37 °C. Subsequently, differentiated macrophages were infected with respective Leishmania promastigotes at a multiplicity of infection of 40 for 3 h in FCS-free RPMI 1640 medium. Cells were washed three times to remove uninternalized parasites and incubated for the indicated periods of time at 37 °C in RPMI 1640 medium containing 10% FCS. At the respective time points, coverslips containing infected cells were washed three times in PBS and fixed with methanol for 10 min at 24 °C. Coverslips were air-dried and permeabilized with 0.4% saponin in PBS containing 0.1 mg/ml RNase A for 1 h at 37 °C. Samples were washed and stained for 5 min with 50 mg/ml propidium iodide, followed by three washes with PBS. Coverslips were mounted in ProLong gold antifade reagent (Molecular Probes) and viewed in a LSM510 confocal microscope (Zeiss) using an oil immersion objective. Numbers of parasites present per macrophage were microscopically estimated, and results are expressed as numbers of parasites present in 100 macrophages.

**Statistical analysis**

Statistical analysis was performed using Sigma Plot version 12. Student’s two-tailed paired t test was used to determine differences between control and test groups with 95% confidence intervals. p values < 0.05 were considered to be significant for all analyses.

**References**


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**gp63 secretion requires Sar1 function in Leishmania**

Author contributions—A. M. conceived and coordinated the study and wrote the paper. S. P. performed all experiments and analyzed results.

Acknowledgments—We thank Dr. S. M. Beverley (Washington University, St. Louis, MO) and Dr. Jean-Paul di Rago (Institut de Biochimie, Bordeaux, France) for providing plasmids. We also thank Dr. Ruchir Rastogi for critically reviewing the manuscript.
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