

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

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Running Title: gp63 secretion requires Sar1 function in *Leishmania*.

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Abstract

Metalloprotease 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 LdSec24¹⁻⁷⁰² to form a prebudding complex. Moreover, LdSec13 specifically interacted with His₆-LdSec31¹⁻⁶⁰³, and LdSec31 bound the prebudding complex via LdSec23. Interestingly, dileucine^{594/595} and valine⁵⁹⁷ residues present in the Ldgp63 C-terminal domain were critical for binding with LdSec24⁷⁰³⁻⁹⁶⁶, and GFP-Ldgp63^{L594A/L595A} or GFP-Ldgp63^{V597S} mutants failed to exit from the ER. Moreover, Ldgp63-containing COPII vesicle budding from the ER was inhibited by LdSar1: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.

Introduction

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 signalling 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 glycosyl-inositol-phospholipids (GIPLs), lipophosphoglycan (LPG), cysteine-protease and metalloprotease gp63 (Ldgp63) have been shown to inactivate macrophage functions and thereby parasite is able to counter the microbicidal activity of macrophages (3,4). Amongst 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 instances, Ldgp63 cleaves complement factor C3b to iC3b to prevent not only 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 signalling for their survival in macrophages (12). Ldgp63 also degrades several transcription factors like NF-

kB, 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 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, while the non GPI anchored protein is directly secreted out (20). However, how Ldgp63 exits from 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 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 '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 GAP activity of Sec23 towards Sar1 resulting in the release of cargo containing COPII coated vesicles from the ER membrane (27-29). However, COPII complex and its role in the secretory pathway in *Leishmania* is not known.

Since Sar1 is the key GTPase for the formation of COPII coated vesicles from ER, therefore, we have 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 dileucine motif of Ldgp63 is required to interact with COPII complex via LdSec24 and GTP form of LdSar1 regulates the exit of Ldgp63 from ER to mediate its secretion via 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

In order to understand the role of Sar1 in the secretion of gp63 in *Leishmania*, we have cloned and expressed Sar1 from *Leishmania donovani*. We used *S.cerevisiae* Sar1 sequence as query and identified putative Sar1 ortholog by BLAST analysis from *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 LdSar1 protein sequence by CLUSTAL W multiple sequence alignment demonstrated that LdSar1 has overall identity of 81% with *Trypanosoma cruzi*, 54% with *Saccharomyces cerevisiae* and 52% with *Homo sapiens* Sar1. Sequence analysis also showed (Fig. 1B) that LdSar1 has highly conserved guanine nucleotide binding regions and 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 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-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 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 GXXXXGKT motif, whereas histidine was substituted for leucine in the DXGHH 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 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 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 COPII complex, we cloned and expressed other COPII proteins (LdSec23, LdSec24, LdSec13 and LdSec31) from *Leishmania* using *S.cerevisiae* COPII protein sequences as query. Using appropriate forward and reverse primers, 2511 bp fragment for LdSec23 (Fig.3A, lane 2), 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 CLUSTAL W 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 characteristic Sec23 helical and β -sheet region; LdSec13 is composed entirely of WD40 repeats and LdSec31 comprised of WD40 repeats at its N-terminal 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 GST/His₆ fusion protein. As we were unable to purify full length LdSec24 and LdSec31, therefore, LdSec24¹⁻⁷⁰², LdSec24⁷⁰³⁻⁹⁶⁶, LdSec31¹⁻⁶⁰³ and LdSec31⁶⁰⁴⁻¹¹⁸⁷ 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 these components of 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 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 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 N-terminus (His₆-LdSec24¹⁻⁷⁰²) of LdSec24 (Fig.4C, Left panel). Our results also showed that GST-LdSec23 binds with His₆-LdSec31 (Fig.4C, Middle panel) but LdSec23 does not interact with LdSec13 (Fig.4C, Right panel). In addition, we found that His₆-LdSec31¹⁻⁶⁰³ interacts with GST-LdSec13 (Fig.4D).

To determine whether these proteins form a complex in *Leishmania*, an *ex vivo* pull down assay was carried out by incubating immobilized GST-LdSar1 with parasite lysate and bound proteins were detected using specific antibodies against 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 Ldgp63 with COPII complex

To understand how *Leishmania* gp63 exits from ER, we studied the interaction of Ldgp63 with different proteins of COPII complex of *Leishmania*. Our results demonstrated that Ldgp63 specifically binds with LdSec24⁷⁰³⁻⁹⁶⁶ (Fig.5A). This result was further confirmed using immobilized LdSec24⁷⁰³⁻⁹⁶⁶ and *Leishmania* lysate (Fig.5B). Subsequently, efforts were made to determine how Ldgp63 interacts with C-terminal domain of LdSec24. Therefore, we analyzed the sequence of Ldgp63 to identify ER exit motif(s) as it was demonstrated previously in mammalian cells that diacidic, dihydrophobic, valine or few other motifs present in cytoplasmic end of different cargos are critical for their binding to Sec24 (31-34). Interestingly, we found that Ldgp63 contains a valine⁵⁹⁷, dileucine^{594/595} and diaspartate^{548/549} at its cytoplasmic domain. Accordingly, we prepared three mutant proteins of Ldgp63 namely Ldgp63^{V597S}, Ldgp63^{L594A/L595A}, Ldgp63^{E548A/E549A} and determined their binding with LdSec24⁷⁰³⁻⁹⁶⁶. Our results showed (Fig.5C) that LdSec24⁷⁰³⁻⁹⁶⁶ did not bind significantly with Ldgp63^{L594A/L595A} and Ldgp63^{V597S} whereas no inhibition of binding was observed with Ldgp63^{E548A/E549A} in

comparison to Ldgp63:WT. These results indicated that dileucine^{594/595} and valine⁵⁹⁷ residues present in the C-terminal end of Ldgp63 are critical for binding with LdSec24.

Role of LdSar1 in budding of gp63-containing vesicles from 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 if budding of Ldgp63-containing vesicles from ER requires LdSar1, microsomes were prepared from cells overexpressing GFP-Ldgp63. To check the integrity of GFP-Ldgp63 containing microsomes, we treated the microsomes with different concentration of proteinase-K in the presence or absence of detergent. LdSar1 which is peripherally associated with microsomes, was used as control. Our results showed that LdSar1 is digested even at a low concentration of proteinase-K (1μg/ml) in the absence of Triton-X-100 whereas GFP-Ldgp63 is significantly protected even at higher concentrations under similar conditions. However, permeabilization of microsomes with Triton X-100 treatment rendered Ldgp63 sensitive to proteinase K digestion (Fig.6A). 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 ATP regeneration system. Subsequently, the reaction mix was sedimented at 12000 X g, 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 ER is significantly inhibited in the presence of His₆-LdSar1:T34N containing cytosol compared to 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 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 overexpressed parasite. In contrast, the trafficking of RFP-Ldgp63 was completely blocked in cells overexpressing LdSar1:T34N-GFP mutant and all RFP-Ldgp63 was found to be localized in discrete compartment possibly in the transitional ER in the parasite.

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 approximately 20% higher amount of Ldgp63 than untransfected control cells (Fig. 7B). In contrast, approximately 80% reduction in secretion of Ldgp63 was observed in cells overexpressing LdSar1:T34N-GFP in comparison to untransfected control cells (Fig.7B).

To unequivocally prove the requirement of interaction of Ldgp63 with COPII complex via LdSec24 for exit from ER, we overexpressed Ldgp63 and its mutants as GFP-tagged proteins in *L.donovani* promastigotes. Interestingly, we found that GFP-Ldgp63^{L594A/L595A} and GFP-Ldgp63^{V597S} mutants were blocked in discrete compartment possibly in the ER whereas GFP-Ldgp63:WT was distributed in the secretory vesicles as observed earlier (Fig.7C). However, GFP-Ldgp63^{E548A/E549A} trafficking was found to be unaltered compared to 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 overexpressed transgenic parasites and parasite load was determined at indicated times. Our results showed (Fig.8A, 8B) that infection with LdSar1:WT overexpressed transgenic parasites in macrophages is significantly more than control parasites at 0 h. Whereas less infection in

macrophages was observed with LdSar1:T34N overexpressed transgenic parasites compared to control. These results were more pronounced when we analyzed the intracellular survival of the parasites after 48 h of infection. We found about 60 % inhibition of parasite load in macrophages infected with LdSar1:T34N overexpressed transgenic parasites in comparison to infection with control parasites. Interestingly, our results showed that the survival of LdSar1:WT overexpressed transgenic parasites in macrophages is significantly higher than the control cells (Fig.8A, 8B). 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 factor which plays an important role in parasite survival in the host cells (5-7,35). Previous 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 ER and follows the secretory pathway in the parasites is not known. Recently, we have shown that Ldgp63 is trafficked via Rab1 dependent conventional secretory pathway in *Leishmania* (36). Moreover, we have also shown that *Leishmania* has conserved trafficking pathways like higher eukaryotic cells (37-44). Thus, it is tempting to speculate that Ldgp63 might exit from ER by Sar1-dependent COPII mediated process. But, components of COPII complex are not yet identified in *Leishmania*.

Though, there are some isolated reports about the existence of COPII proteins in parasitic protozoa (45-47) but functional significance of these proteins in the secretory pathway of different 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 LdSar1:H74L mutant and our results have shown that this mutant binds GTP but is unable to hydrolyze GTP efficiently. We have also made LdSar1:T34N mutant which 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, 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 these components of 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 the components of COPII proteins fail to localize in discrete compartment when they are co-expressed with RFP-LdSar1:T34N. These results are consistent with previous finding that Sar1 in GTP form is necessary for the recruitment of all subsequent COPII components to ER (49,50).

To understand the mechanism of formation of COPII complex in *Leishmania*, we have studied the interaction between different COPII proteins. Our results have shown that LdSar1 in GTP form specifically binds with LdSec23 but does not interact with any other protein of COPII complex. Thus, the interaction between membrane-bound LdSar1 in 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 N-terminus of LdSec24 to form heterodimer which is subsequently recruited to the ER membrane by interacting with GTP form of LdSar1 to form pre-budding complex. These results are supported by the fact that interaction of Sar1 with Sec23/Sec24 homologues in *Giardia* is critical for maintenance of ERES in parasite (50).

Subsequently, we have determined how LdSec13/31 is recruited to pre-budding complex to form outer layer of the nascent COPII vesicles. We have found that LdSec13 specifically binds with 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). Though all proteins of COPII complex do not interact directly, but, our results have shown that one of the COPII proteins can pull-out all other components from *Leishmania* lysate indicating that they form COPII complex in *Leishmania*. Taken together, our results indicate that LdSec23 plays prominent central role in COPII complex assembly by acting as a bridge between LdSar1 and LdSec24/LdSec31. Therefore, it is tempting to speculate that recruitment of LdSec13-LdSec31 to pre-budding complex initiates membrane curvature and subsequently, triggers the GAP activity of LdSec23 leading to GTP hydrolysis of LdSar1 which releases COPII vesicles from ER (24,28,52).

To determine the functional significance of LdSar1 in the exit of Ldgp63 from 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 ER requires 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 (VSG) in *Trypanosoma* (45). Thus, our results conclusively demonstrated that LdSar1 is critical for the exit of Ldgp63 from 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 ER in *Leishmania*. We have found that Ldgp63 specifically binds with LdSec24. Interestingly, our results have shown that dileucine^{594/595} and valine⁵⁹⁷ 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-Ldgp63^{L594A/L595A} or GFP-Ldgp63^{V597S} mutants in *Leishmania*. Our results have shown that trafficking of GFP-Ldgp63^{L594A/L595A} and GFP-Ldgp63^{V597S} mutants

is blocked possibly in the ER whereas trafficking of GFP-Ldgp63:WT is found to be uninterrupted.

As Ldgp63 is a virulence factor required for intracellular survival of the parasites in macrophages, therefore, 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 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 C-terminal of Ldgp63 with LdSec24 for its exit from 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 dominant negative mutant of LdSar1 blocks the secretion of virulence factor like Ldgp63 and thereby inhibits the parasite survival in the macrophages indicating the possibility of exploiting this pathway in parasite for potential drug targets. As the major focus for design and development of new drugs is the inactivation of key molecules/s needed for parasite survival (53), therefore, the development of appropriate inhibitors or small molecules targeted to parasite COPII proteins could be new therapeutic approach against the parasites.

Experimental Procedures

Materials

Unless otherwise stated, all reagents were procured from Sigma Chemical Co. (St.

Louis, MO). RT-PCR kit, Platinum HiFidelity *Taq* polymerase, TRIzol, M199 medium, Bicinchoninic acid (BCA) reagent and Gentamicin were purchased from Life Technologies, Thermo Fischer Scientific Inc. (Waltham, MA). Luria-Bertani (LB) broth and LB-Agar were supplied by Difco Laboratories (MD, USA). Fetal Calf Serum (FCS) was procured from Biological Industries, Israel. pGEX-4T-2 expression vector, Glutathione sepharose beads, Anti-His₆ antibody and ECL reagents were procured from GE Healthcare (Buckinghamshire, UK). Anti-GST antibody was procured from Santa Cruz Biotechnology Inc. (Dallas, Texas). Restriction enzymes were obtained from Promega Life Science (Madison, WI). Complete and Incomplete Freund's adjuvants were procured from Becton Dickinson and Company (Franklin Lakes, NJ). 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 (Eugene, OR). Geneticin and Blasticidin were procured from Gibco BRL (Gaithersburg, MD) and Calbiochem respectively. [α -³²P] GTP (800 Ci/mmol) was procured from Perkin-Elmer Life Sciences Inc. USA. All other reagents used were of analytical grade.

Cells

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

Human acute monocytic leukemia-derived cells (THP-1) cells were obtained from American Type Culture Collection (ATCC, Cat. No. TIB-202) Manassas, Virginia. Cells were routinely cultured in complete RPMI (RPMI-1640 containing 10% FCS and 50 µg/ml gentamycin) 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 (100ng/ml) for 24h. Cells were washed and incubated for another 24

h in complete RPMI without PMA and used for experimental purpose.

Cloning of COPII proteins from Leishmania donovani

In order to clone Sar1, Sec23, Sec24, Sec13 and Sec31 from *L. donovani*, the respective *S. cerevisiae* protein sequences were used as query and putative homologs of these proteins were identified from *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 sequences, LdSar1, LdSec23, LdSec24, LdSec13 and LdSec31 gene products were further subcloned into *Bam*HI/*Eco*RI sites of the pGEX-4T-2 and pET28a expression vectors and transformed into the BL21 strain of *Escherichia coli*. Primer sequences used in PCR are indicated below.

LdSar1: Forward 5'-ATGGGCTGGTTTAGC TGGTTTGGGAC-3' and Reverse 5'-CTACG AGCTCTTCAGATACTGTGAGAGCCAG-3'; LdSec23: Forward 5'-ATGAGCGGCG ATTAC GTCTACGGAACTACG-3' and Reverse 5'-CTACTGTGCCACCGCCAGCTTCTTGAG-3'; LdSec31: Forward 5'-ATGCGACTGA AGAACACGGCGCTATGCTG-3' and Reverse 5'-TCACAGATAGGAAAGGTTTCGCGAAGG CC-3'; LdSec24: Forward 5'-ATGATGTACT CCGCCGGTGCTATGTACG-3' and Reverse 5'-CTACTTGCCGACGCTCACAGCGTCC-3'; LdSec13: Forward 5'-ATGGTGCCACACCT GGCAAATGGCGC-3' and Reverse 5'-CTAC TGCTGGAAGTCCTCAAAGGAAGTTTAA TCCAC-3'.

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 by PCR mediated site directed mutagenesis using appropriately designed mutant primers as described previously (36). Briefly, megaprimer for H74L was generated using WT Sar1 forward primer and mutated reverse primer (5'-GCCTGCTGAAGGCCACCCATATC-3') by PCR and subsequently full length mutated product was generated using this megaprimer as forward primer with WT reverse primer. Similarly, megaprimer for T34N was generated using WT Sar1 forward primer and mutated reverse primer (5'-CCAAGAAGGGTGTGTTTT GCCGGCGTTG-3') by PCR and subsequently full length mutated product was generated using this megaprimer as forward primer with WT reverse primer. Finally, these PCR products were cloned into *Bam*HI/*Eco*RI sites of pGEX-4T-2 or pET28a expression vector and transformed into the BL21 strain of *Escherichia coli*.

We were unable to express full length LdSec24 and LdSec31 in *E. coli* as observed previously (54). Therefore, LdSec31¹⁻⁶⁰³ and LdSec31⁶⁰⁴⁻¹¹⁸⁷ truncations were PCR amplified from LdSec31-pGEM-T Easy construct using specific primers (LdSec31¹⁻⁶⁰³: WT Forward primer and Reverse 5'-CTACAGCGC GTCACCGAGCAGATTG3'; LdSec31⁶⁰⁴⁻¹¹⁸⁷: Forward 5'-ATGCGCGGCCAGCAGAACT ATG-3' and WT Reverse primer). Similarly, LdSec24¹⁻⁷⁰² and LdSec24⁷⁰³⁻⁹⁶⁶ truncations were PCR amplified from LdSec24-pGEM-T Easy construct using specific primers (LdSec24¹⁻⁷⁰²: WT Forward primer and Reverse 5'-CTACGAGTTGATGACCGGTCCAATGG TGGAAG-3'; LdSec24⁷⁰³⁻⁹⁶⁶: Forward 5'-ATGATCAACTCCCTCGGCATGTCGTGC-3' and WT Reverse primer). These truncated fragments were cloned into *Bam*HI/*Hind*III restriction sites (LdSec24) or *Bam*HI/*Eco*RI restriction sites (LdSec31) of pGEX-4T-2 or pET28a expression vector. The truncated constructs were transformed into *E. coli* (BL21 strain) to express as GST/His₆ tagged fusion proteins.

Respective cells containing indicated construct were grown separately in LB and induced with 0.5 mM isopropyl 1-thio-D-galactopyranoside for 3 h at 37 °C for the expression and purification of respective GST or His₆ 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, LdSec31⁶⁰⁴⁻¹¹⁸⁷, LdSec24⁷⁰³⁻⁹⁶⁶ or LdSec13 His₆-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 GTP overlay assay (36). Briefly, 2 µg of GST-LdSar1:WT or its mutants was blotted onto nitrocellulose membrane and membrane was incubated with 1 µCi/ml of [α -³²P] GTP in 50 mM phosphate buffer, pH 7.5 containing 5 mM MgCl₂, 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 MgCl₂, 1 mM NaH₂PO₄ and 10 mM β -mercaptoethanol) for 20 min at 25°C and bound nucleotide was eluted with 1 M guanidine-HCl. Immobilized nucleotide free protein was then loaded with 2 pmol [α -³²P] GTP (800 Ci/mmol) in 20 µl buffer A for 10 min at 0°C. Subsequently, 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.

Overexpression of COPII complex proteins in *Leishmania*

To overexpress COPII complex proteins as GFP fusion proteins in *Leishmania* promastigotes, respective clones were subcloned into the *NotI/BamHI* sites of the pXG-GFP+2 vector (LdSec31 and LdSec13) to

express the proteins as N-terminus GFP tag and *BamHI/EcoRV* sites of pXG-GFP+ vector (LdSar1, LdSec23 and LdSec24) to express the proteins as C-terminus GFP tag. Subsequently, *Leishmania* promastigotes were transfected with respective constructs by electroporation as described previously (36,40). Finally, positive clones were selected in the presence of G418 antibiotic at an initial concentration of 10 µg/ml and thereafter stably maintained at antibiotic concentration of 50 µg/ml. Overexpression of COPII proteins was confirmed by confocal microscopy.

Subcellular localization of COPII complex proteins in *Leishmania*

To identify the LdSar1-positive compartment, LdSar1:WT-GFP overexpressing *Leishmania* were labeled with markers for different intracellular compartments as described earlier (36,40). The flagellar pocket was labeled with FM4-64 (30 µM) for 5 min at 4°C. Similarly, 5 min internalization of Alexa Fluor-594 labeled hemoglobin (Alexa 594-Hb) was used to mark the early endocytic compartment. ER of *Leishmania* promastigotes was visualized by staining with 50 nM of ER tracker red in PBS on ice for 5 min. Golgi complex was visualized by overexpressing the cells with LPG2-HA followed by immunostaining with Alexa 546-labeled rabbit anti-HA antibody (1:500). To determine the localization of different COPII proteins in *Leishmania*, RFP-LdSar1 cells were coexpressed with LdSec23-GFP, LdSec24-GFP, GFP-LdSec31 or GFP-LdSec13. The positive co-transfectants were selected in the presence of both G418 (50 µg/ml) and Blasticidin (15 µg/ml) and co-expression was examined by confocal microscopy.

Determination of interactions between different COPII proteins

To determine the interactions amongst different COPII proteins, *in vitro* binding experiments were carried out using one GST/His₆ tagged protein (1µg) of 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 for 1 h at 4°C in PBS, pH 7.2. Subsequently, beads were washed 3 times with ice-cold PBS containing 1% Triton X-100 followed by 3 washes with ice-cold PBS to remove nonspecific interaction. Finally,

protein bound to the beads was separated by SDS-PAGE and analyzed by Western blot analysis using the antibody against the target protein. Similar assay was carried out using immobilized indicated His₆-tagged COPII protein with GST-Ldgp63 or its mutants to determine the cargo binding component of COPII complex in *Leishmania*.

To determine the existence of COPII proteins as a complex in *Leishmania*, 5 µg of GST-LdSar1 was immobilized on glutathione sepharose beads and incubated with 3 mg of *Leishmania* lysate for 2 h at 4°C in PBS, pH 7.2. Subsequently, beads were washed 6 times with ice-cold PBS to remove nonspecific interactions. Finally, proteins bound to beads were analyzed by Western blot using antibodies against respective COPII proteins.

Expression and purification of recombinant Ldgp63 and mutants

To purify Ldgp63 as GST tagged fusion protein, gene specific primers were designed against Ldgp63 sequence (Forward 5'-ATGTCCGTCGAC AGCAGCAGCAC-3' and Reverse 5'-CTAGAGCGCCACGGCCAG CAG-3') and its ORF was amplified from Ldgp63-pNUS-mRFPnD construct (36) by PCR using High Fidelity *Taq* polymerase. Subsequently, the PCR product was cloned in *Bam*H1/*Eco*R1 restriction sites of pGEX-4T-2 expression vector to express it as GST tagged fusion protein as described previously. It was also sub-cloned into *Bam*H1/*Eco*R1 restriction sites of pXG-GFP+2 vector for expression as GFP fusion protein in *Leishmania* promastigotes.

Ldgp63 point mutants (Ldgp63^{V597S} and Ldgp63^{L594A/L595A}) were PCR amplified using similar conditions used for Ldgp63-pGEX-4T-2 amplification by appropriate mutant primers (Ldgp63^{V597S}: WT Forward primer and Reverse 5'-CTAGAGCGCCGAGGCCAGCAG-3'; Ldgp63^{L594A/L595A}: WT Forward primer and Reverse 5'-CTAGAGCGCCACGGCCGCCGC -3'). Ldgp63^{E548A/E549A} mutant was PCR amplified using a megaprimer based approach. Briefly, a forward primer was designed comprising of the respective mutations (5'-AGCGCCTTCGCGGCGGGCGGCTAC-3') and was used along with wild type reverse primer to amplify 140bp megaprimer from Ldgp63-pGEX-4T-2 construct. Subsequently, this megaprimer was used as a reverse primer along with wild type forward primer to amplify

full length mutant Ldgp63. All the mutant proteins were subsequently cloned in *Bam*H1/*Eco*R1 sites in pGEX-4T-2 vector and expressed as GST fusion protein as described previously. These Ldgp63 mutants were also sub-cloned in *Not*I/*Bam*H1 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-Ldgp63 overexpressing parasites (6×10^9) were washed with PBS and resuspended in 10 ml of HEPES buffer (100 mM HEPES, pH 7.4, 60 mM KCl, 10 mM MnCl₂, 10 mM MgCl₂ with protease inhibitor) containing 10% glycerol. The cells were disrupted in a Parr nitrogen cavitation bomb after equilibration at 1,500 psi for 25 min at 4°C. The unbroken cells and nucleus were removed by centrifugation at 500 X g for 7 min. The post nuclear supernatant containing microsomes was centrifuged at 100,000 X g for 1 h at 4°C in 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 X g for 1 h at 4°C. The enriched microsomes were finally resuspended in 1 ml (15-17 mg/ml protein) of the HEPES buffer without glycerol and used for *in vitro* budding assay. To check the integrity of GFP-Ldgp63 containing microsomes, microsomes (30 µg) were incubated with indicated concentrations of proteinase K in 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 cocktail and SDS loading buffer and samples were analyzed by Western blot analysis using anti-GFP antibody.

Finally, *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 U/ml creatine phosphokinase, 0.1mM 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, rest of the reaction mixture (50 µl) was centrifuged at 12,000 X g for 4 min to separate the COPII budded vesicles in the supernatant from the donor membranes in the pellet. Presence of GFP-Ldgp63 in the budded vesicles was determined by western blot using anti-GFP antibody.

Role of LdSar1 in Ldgp63 trafficking in Leishmania

To determine the role of LdSar1 in the trafficking of Ldgp63, *Leishmania* promastigotes overexpressing LdSar1:WT-GFP or LdSar1:T34N-GFP was transfected with RFP-Ldgp63 using the same protocol as described previously (36). Co-transfected cells were selected in the presence of G418 (50 µg/ml) and blasticidin (15µg/ml). Localization of RFP-Ldgp63 in LdSar1:WT or LdSar1:T34N overexpressed cells was determined by confocal microscopy.

Detection of Secreted Ldgp63 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 x g for 10 min at 4 °C) and amount of Ldgp63 secreted by respective cells in cultures medium was detected by acetone precipitation followed by Western blot analysis using anti-Ldgp63 antibody as described previously (36). The respective cell pellets were also analyzed by Western blot 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 (100ng/ml). Cells (2.5×10^5) 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 an MOI of 40 for 3 h in FCS free RPMI-1640 medium. Cells were washed thrice to remove uninternalized parasites and incubated for indicated periods of times at 37°C in RPMI-1640 medium containing 10% FCS. At respective time point, 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 of 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 less than 0.05 was considered to be significant for all analyses.

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions

AM conceived and coordinated the study and wrote the paper. SP performed all experiments and analyzed results.

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Abbreviations

The abbreviations used are: Ld, *Leishmania donovani*; COPII, Coat protein complex II; Ldgp63, *Leishmania* metalloprotease gp63; ER, Endoplasmic reticulum; GFP, Green Fluorescent Protein; RFP, Red Fluorescent Protein; GAP, GTPase activating protein.

Figure legends

FIGURE 1. Cloning and localization of Sar1 in *Leishmania donovani*. A. A 588 bp fragment of LdSar1 was amplified from *L. donovani* cDNA by PCR using appropriate forward and reverse primers as described in “Experimental Procedures”. Lane 1 represents DNA ladder. The sequence of LdSar1 has been submitted to GenBank data base under accession number KY484911. B. Hypothetical translation of obtained sequence of LdSar1 into protein sequence shows significant identities with Sar1 homologs from *Trypanosoma cruzi*, *Saccharomyces cerevisiae*, and *Homo sapiens*. Residues implicated in GTP/GDP binding (G1-G5) are marked. C. Antibody against LdSar1 was raised in mice as described in “Experimental Procedures”. The specificity of anti-LdSar1 antibody was checked using cell lysate as well as purified His₆-LdSar1, His₆-LdRab5 and His₆-LdRab1. D. Permeabilized cells were probed with anti-LdSar1, washed and visualized using Alexa Fluor 594-labelled goat anti-mouse secondary antibody. Green, localization of LdSar1; Red, Nucleus (Nu) stained with Syto-Red. Cells were examined under oil immersion objective of LSM 510 Meta confocal microscope. All results are representative of three independent preparations. E. To identify the LdSar1 positive compartment in *Leishmania*, cells overexpressing LdSar1:WT-GFP were stained with various compartment specific markers; like FM4-64 for flagellar pocket, 5 min internalized Alexa fluor-594 Hb for early endosome, LPG2-HA for Golgi and ER tracker Red for ER as described in “Experimental Procedures”. Finally, cells were visualized under 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.

FIGURE 2. Characterization of LdSar1 and its mutants. A. GTP binding of purified LdSar1:WT and its mutants was detected using [α -³²P] GTP overlay assay. LdRab5:WT and GST proteins were used as control. B. GTPase activity of LdSar1 and its mutants was determined as described in “Experimental Procedures”. C. To determine the localization of LdSar1 mutants in *Leishmania*, cells were transfected with indicated construct to overexpress the respective protein in *Leishmania* as GFP fusion protein. Finally, cells were visualized under confocal microscope. Results are representative of three independent observations.

FIGURE 3. Identification and localization of other COPII proteins in *Leishmania*. A. Amplification of 2511 bp fragment of LdSec23 (lane 2, submitted to GenBank data base under accession number KY484913), 2901 bp fragment of LdSec24 (lane 3, submitted to GenBank database under accession number KY484914), 1002 bp fragment of LdSec13 (lane 4, submitted to GenBank database under accession number KY484912) and 3504 bp fragment of LdSec31 (lane 5, submitted to GenBank database under accession number KY484915) from *L. donovani* cDNA by PCR using appropriate forward and reverse primers as described in “Experimental Procedures”. Lane 1 represents 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* protein 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 in “Experimental Procedures”. C. To determine whether GTP form of LdSar1 is required for the localization of other components of COPII complex in *Leishmania* in ER, LdSec23-GFP, LdSec24-GFP, GFP-LdSec13 or GFP-LdSec31 were co-expressed with RFP-LdSar1:T34N as described in “Experimental Procedures”. Finally, cells were visualized under 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.

FIGURE 4. Determination of interactions amongst various COPII proteins in *Leishmania*. A. To detect direct binding of LdSar1 and its mutants with LdSec23, His₆-LdSar1 or its mutants was immobilized on Ni-NTA beads and incubated with GST-LdSec23 as described in “Experimental Procedures”. Binding of LdSar1 with GST-LdSec23 was detected by Western blot analysis using anti-GST antibody. Ni-NTA beads incubated with same amount of GST-LdSec23 were used as control. 10% of the GST-LdSec23 was used as an input. B. Similar binding assays were carried out using immobilized GST-LdSar1 with His₆-LdSec24¹⁻⁷⁰² or His₆-LdSec24⁷⁰³⁻⁹⁶⁶ (left panel), His₆-LdSec31¹⁻⁶⁰³ or His₆-LdSec31⁶⁰⁴⁻¹¹⁸⁷ (middle panel) or His₆-LdSec13 (right panel). C. Similar binding assays were carried out using immobilized GST-LdSec23 with His₆-LdSec24¹⁻⁷⁰² or His₆-LdSec24⁷⁰³⁻⁹⁶⁶ (left panel), His₆-LdSec31¹⁻⁶⁰³ or His₆-LdSec31⁶⁰⁴⁻¹¹⁸⁷ (middle panel) or His₆-LdSec13 (right panel). D. Similar binding assay was carried out using GST-LdSec13 with His₆-LdSec31. E. Antibodies against respective COPII proteins were raised in mice as described in “Experimental Procedures”. Specificity of these antibodies was determined using indicated proteins. F. To determine the existence of these proteins as a complex in *Leishmania*, GST-LdSar1 was immobilized on beads and incubated with *Leishmania* lysate as described in “Experimental Procedures”. Binding of indicated COPII proteins from *Leishmania* lysate with immobilized LdSar1 was detected by Western blot using antibodies against respective COPII proteins. All results are representative of three independent experiments.

FIGURE 5. Ldgp63 specifically binds with LdSec24 of COPII complex. A. To identify the cargo binding protein of COPII complex in *Leishmania*, indicated His₆-fusion proteins of COPII complex were immobilized on Ni-NTA beads and incubated with GST-Ldgp63 as described in “Experimental Procedures”. Binding of respective COPII protein with GST-Ldgp63 was detected by Western blot analysis using anti-Ldgp63 antibody. Ni-NTA beads incubated with same amount of GST-Ldgp63 were used as a control. B. Similar experiments were carried out by incubating immobilized His₆-LdSec24¹⁻⁷⁰² or His₆-LdSec24⁷⁰³⁻⁹⁶⁶ with *Leishmania* cytosol. C. To determine critical residues of Ldgp63 required for binding with LdSec24; GST-Ldgp63:WT, GST-Ldgp63^{V597S}, GST-Ldgp63^{L594A/L595A} or GST-Ldgp63^{E548A/E549A} was immobilized on beads and incubated with His₆-LdSec24⁷⁰³⁻⁹⁶⁶. 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.

FIGURE 6. Role of LdSar1 in the budding of Ldgp63 containing vesicles from *Leishmania* Microsomes. A. To determine the role of LdSar1 in the budding of Ldgp63 containing vesicles, GFP-Ldgp63 containing microsomes were prepared as described in “Experimental Procedures”. To check the integrity of GFP-Ldgp63 containing microsomes, microsomes were treated with indicated concentrations of proteinase K in presence or absence of Triton X-100. Results were analyzed by Western blot using anti-GFP antibody. LdSar1 was used as a control. B. *In vitro* budding of Ldgp63 containing COPII vesicles was carried out by incubating the microsomes in budding buffer supplemented with gel filtered cytosol containing His₆-LdSar1 or His₆-LdSar1:T34N protein. Budded vesicles were separated from microsomes by centrifugation as described in “Experimental Procedures”. Cytosol without any LdSar1 protein was used as a control. Presence of GFP-Ldgp63 in the budded vesicles was determined by Western blot using anti-GFP antibody. All results are representative of three independent preparations. Results of control (*) and LdSar1:WT or LdSar1:T34N were analyzed by paired *t* test and levels of significance are indicated by *P* value.

FIGURE 7. Determination of the role of LdSar1 in the trafficking and secretion of Ldgp63 in *Leishmania*. A. *Leishmania* promastigotes overexpressing LdSar1:WT-GFP or LdSar1:T34N-GFP were transfected with RFP-Ldgp63 as described in “Experimental Procedures”. Cells expressing only RFP-Ldgp63 were used as a control. Cells were examined by confocal microscopy. Green represents LdSar1-GFP and LdSar1:T34N-GFP. Red is RFP-Ldgp63. All results are representative of three independent experiments. B. To determine the role of COPII proteins in the secretion of endogenous gp63 by *Leishmania*, the levels of gp63 associated with cells and secreted in the spent media by *Leishmania* promastigotes overexpressing LdSar1:WT-GFP or LdSar1:T34N-GFP was determined by western blot analysis using anti-Ldgp63 antibody as described in “Experimental Procedures”. Results

are representative of three independent experiments. Results of control (*) and indicated overexpressed cells were analyzed by paired *t* test and levels of significance are indicated by *P* value. C. To determine the requirement of different motifs of Ldgp63 to exit from ER, *Leishmania* promastigotes overexpressing GFP-Ldgp63^{L594A/L595A}, GFP-Ldgp63^{V597S} or GFP-Ldgp63^{E548A/E549A} were analysed by confocal microscopy. All results are representative of three independent experiments.

FIGURE 8. LdSar1-mediated secretion of Ldgp63 is necessary for the survival of *Leishmania* in macrophages. A. THP1 differentiated macrophages were infected with LdSar1:WT or LdSar1:T34N overexpressed transgenic parasites as described in “Experimental Procedures”. Parasite load in the infected macrophages was microscopically estimated at indicated time. Results are expressed as numbers of parasites present in 100 macrophages \pm S.D. from three independent experiments Results of control (*) and LdSar1:WT or LdSar1:T34N overexpressed cells were analyzed by paired *t* test and levels of significance are indicated by *P* value. B. Confocal images of the representative experiment showing the parasite load in macrophages. *Leishmania* and macrophage nucleus were stained with propidium iodide (Red).

Fig.1B.

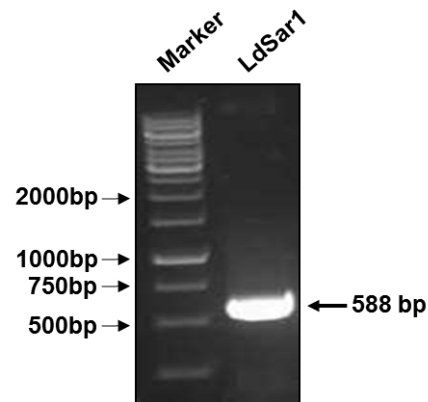


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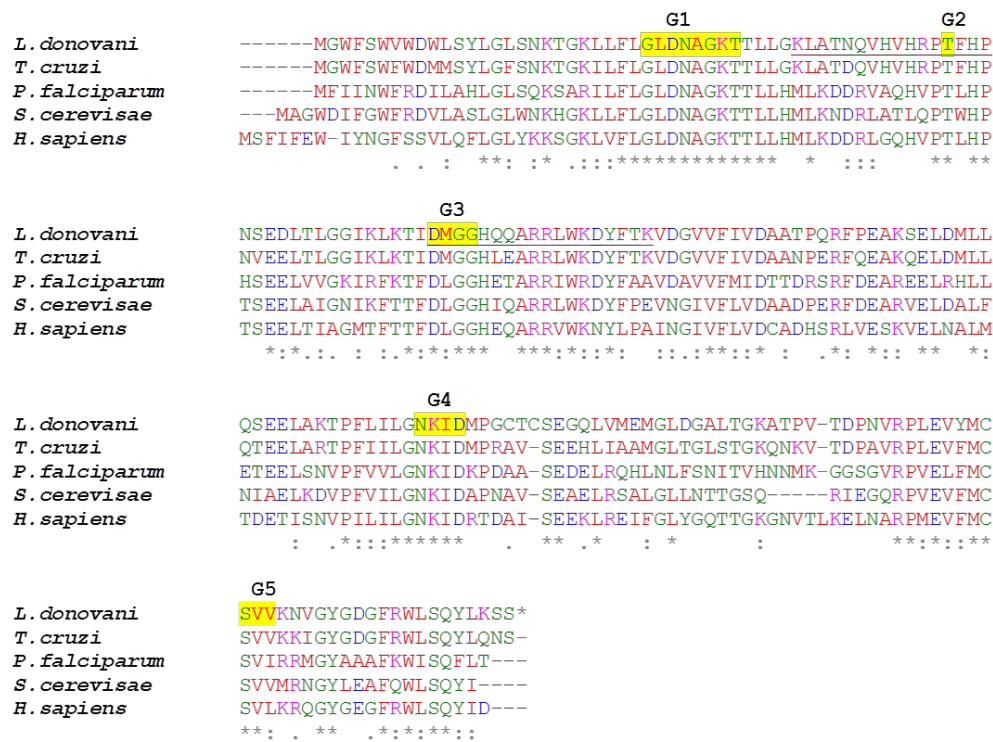


Fig.1C.

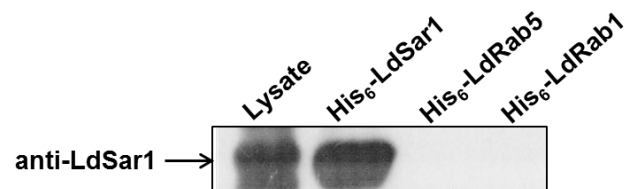


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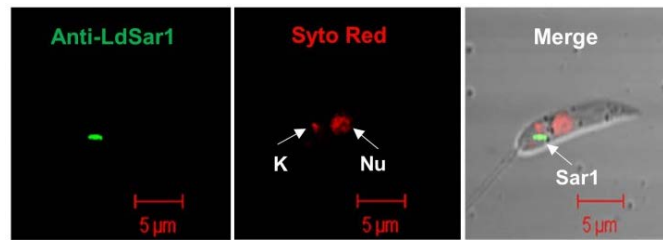


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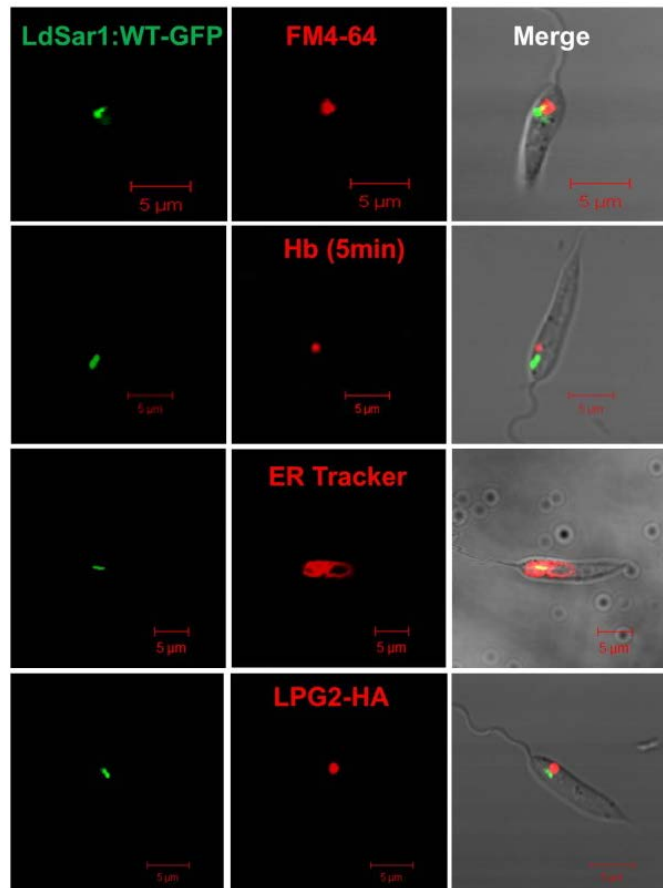


Fig.2A.

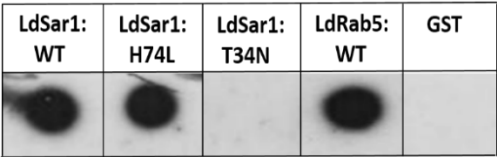


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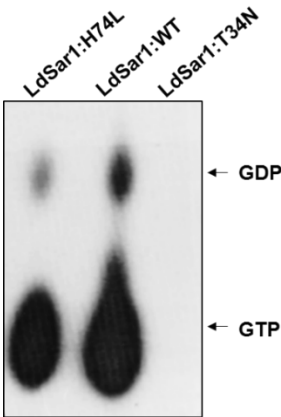


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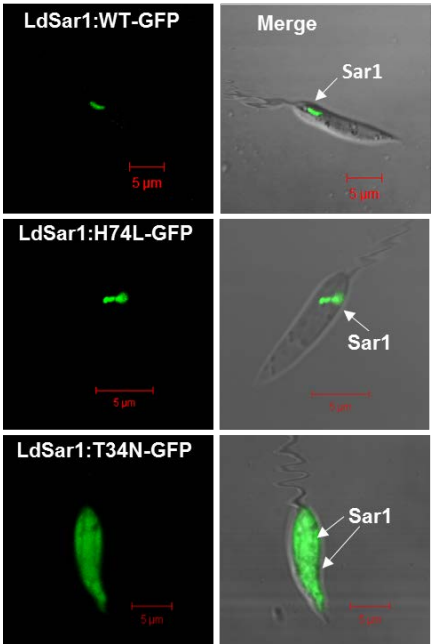


Fig.3A.

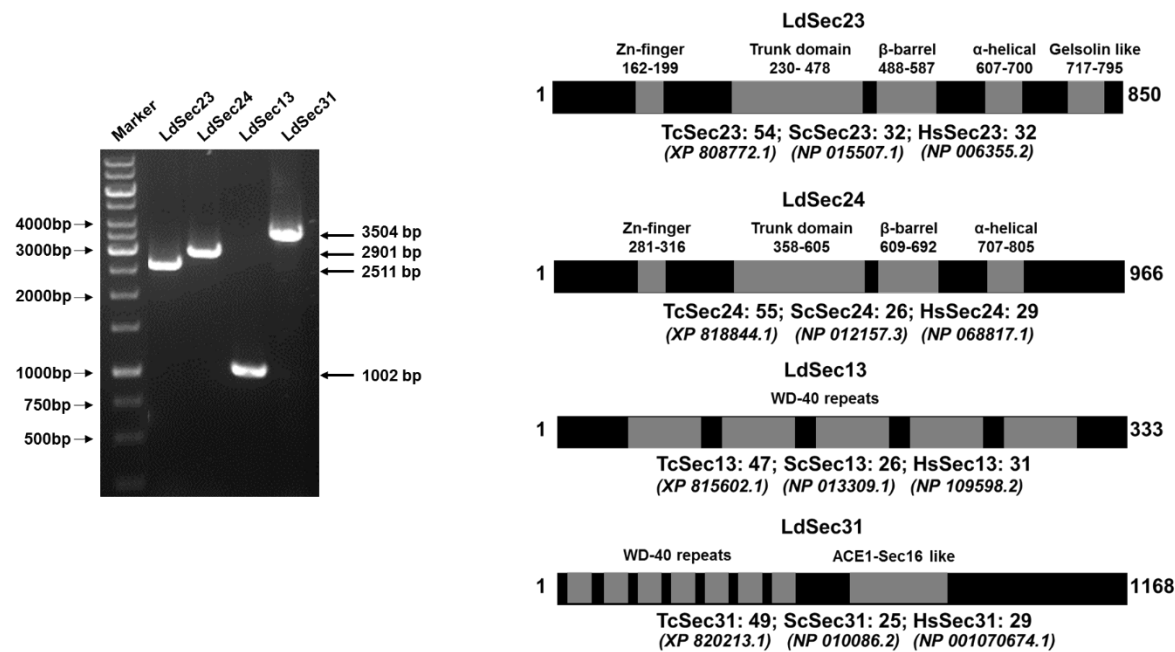


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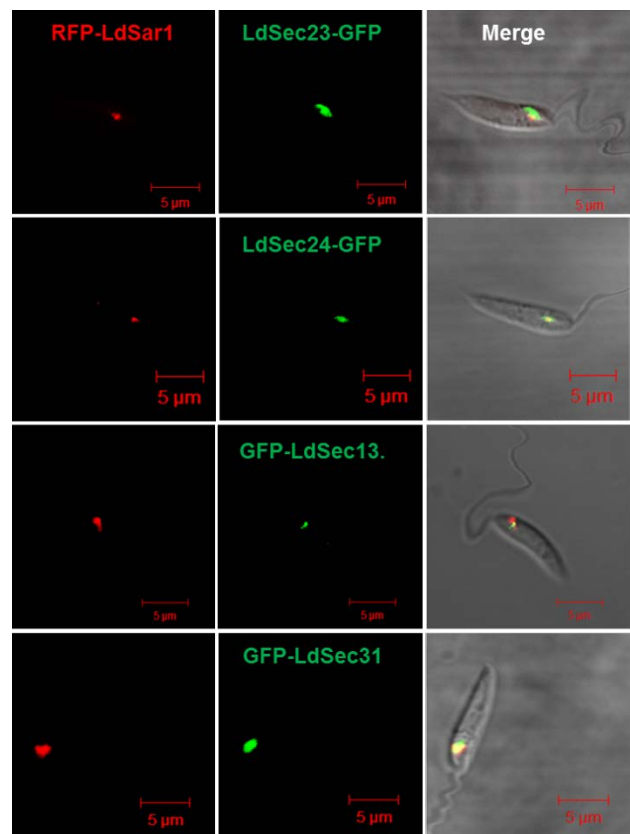


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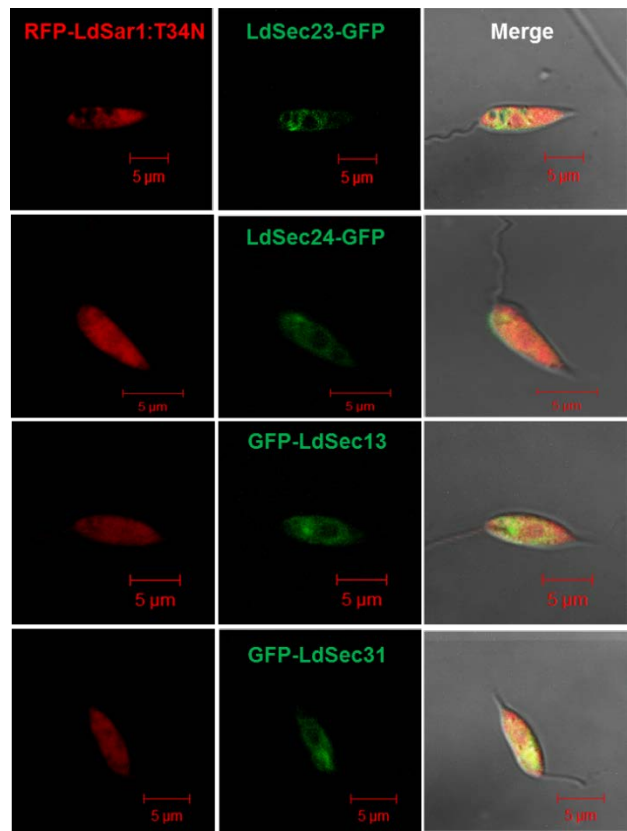


Fig.4A

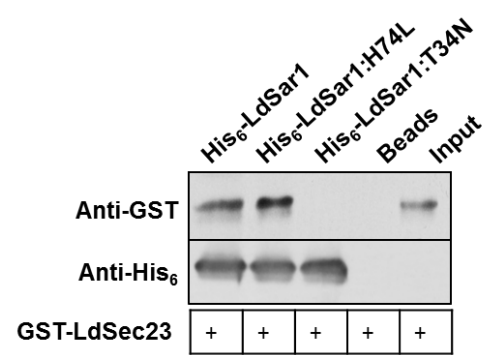


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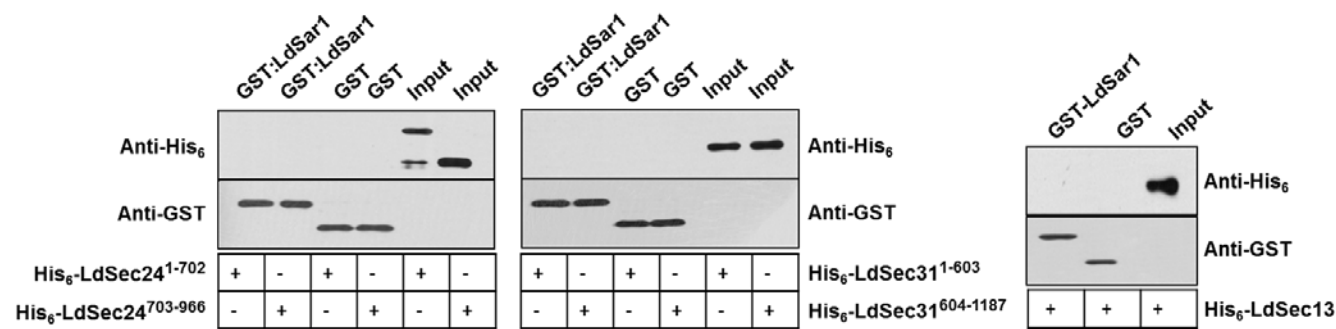


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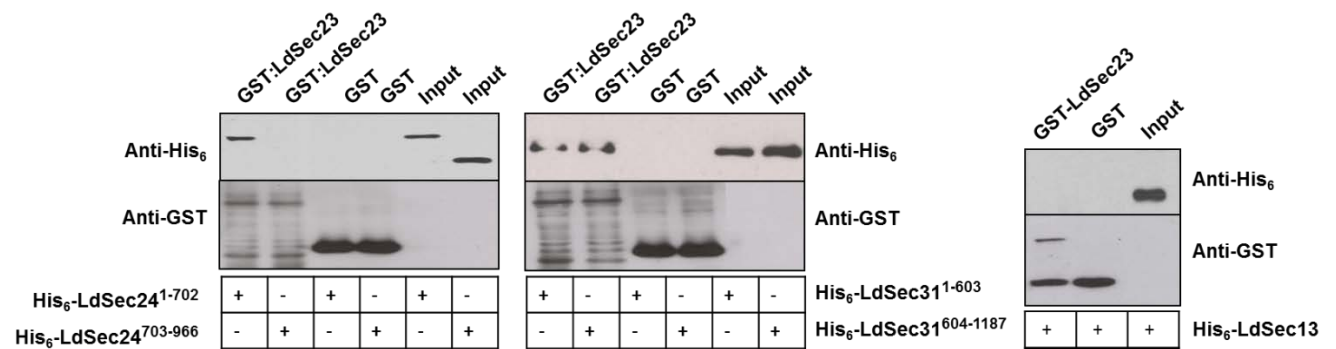


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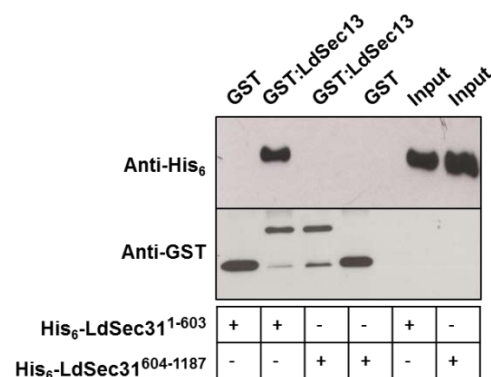


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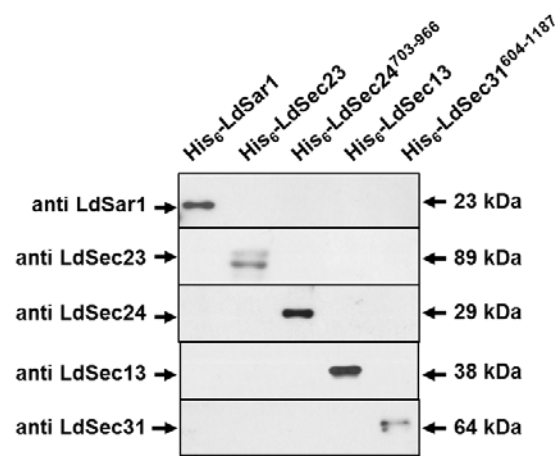


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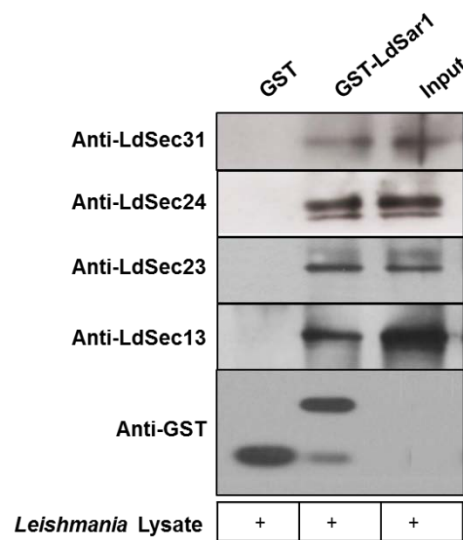


Fig.5A.



Fig5B.

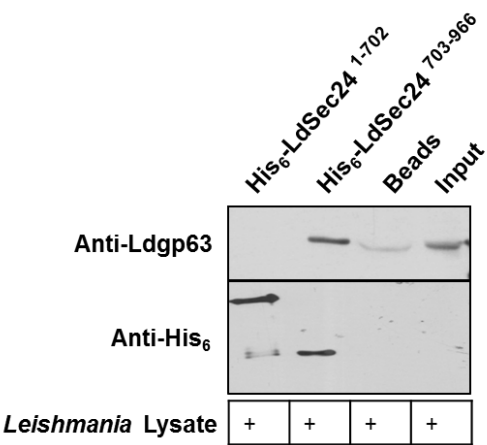


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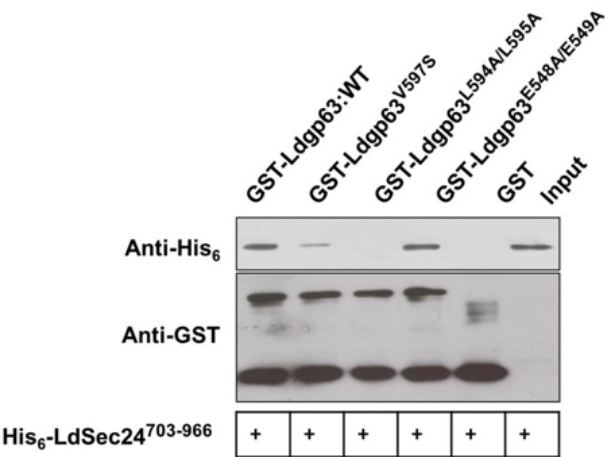


Fig.6A.

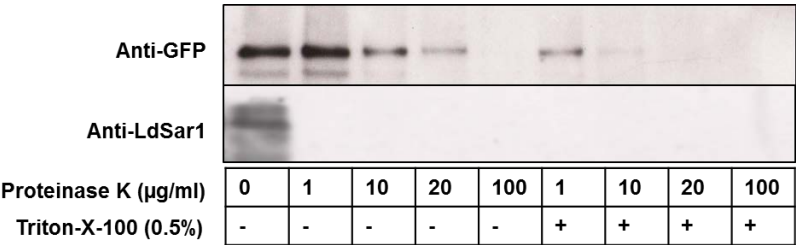


Fig.6B.

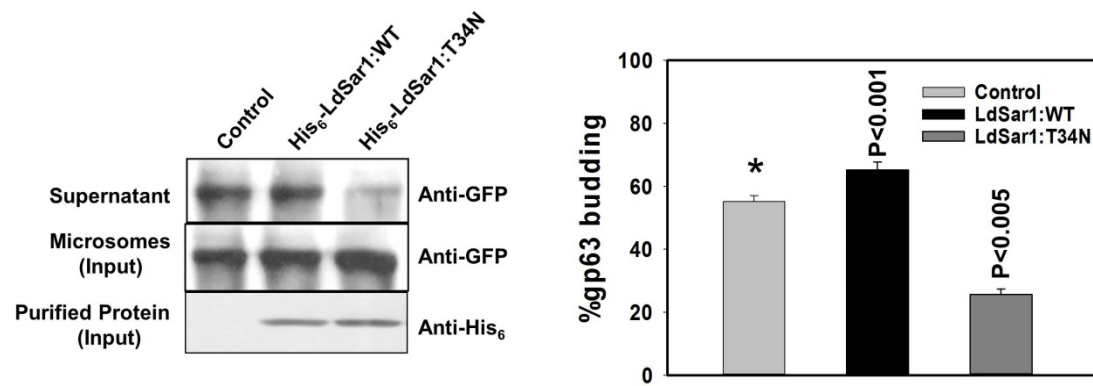


Fig.7A.

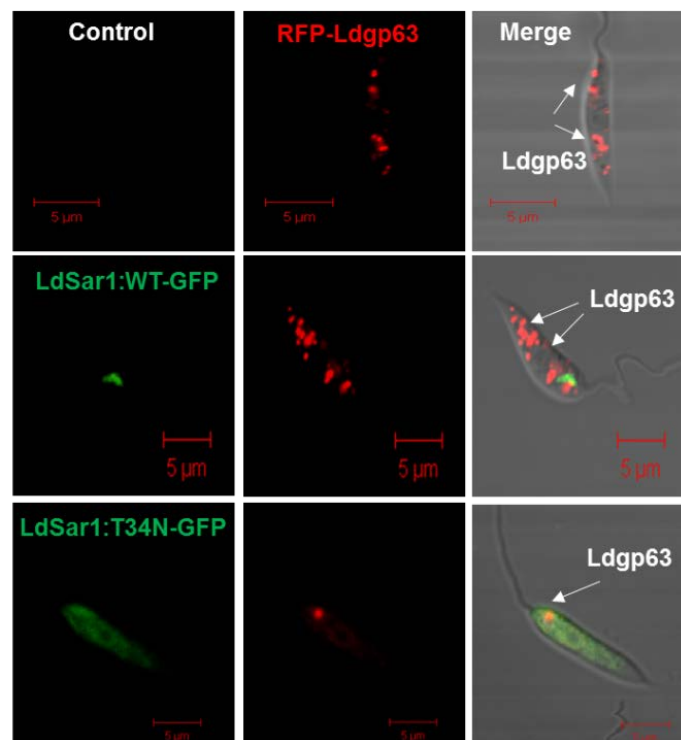


Fig.7B.

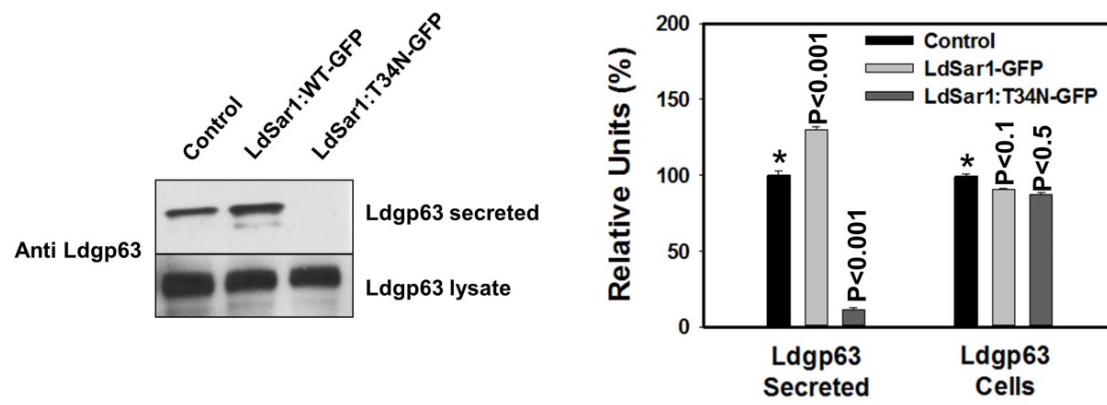


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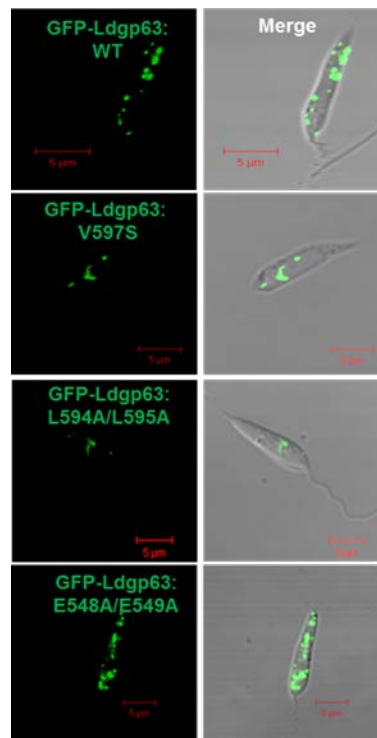


Fig.8A.

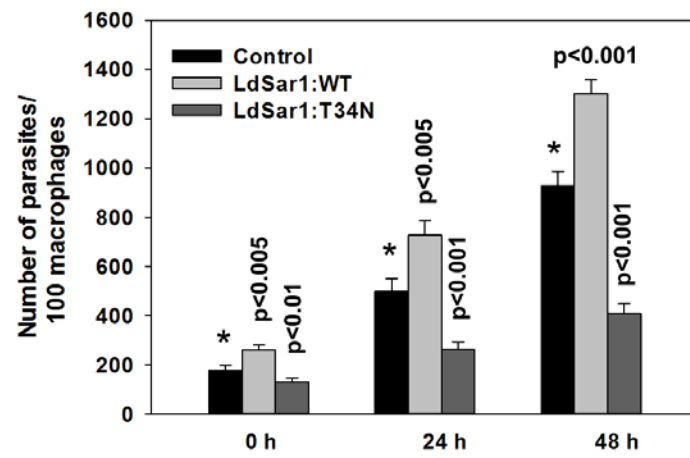
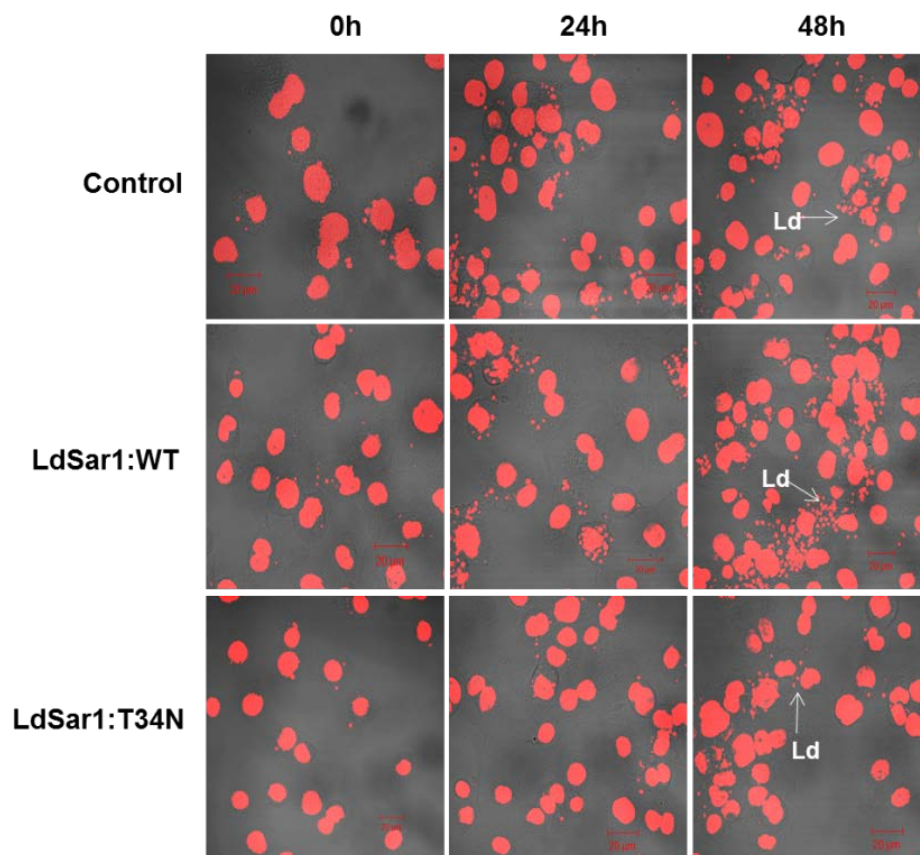


Fig.8B.



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

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