Processing and Trafficking of Leishmania mexicana GP63
ANALYSIS USING GPI8 MUTANTS DEFICIENT IN GLYCOSYLPHOSPHATIDYLINOSITOL PROTEIN ANCHORING*

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GPI8 is a clan CD, family C13 cysteine protease and the catalytic core of the GPI-protein transamidase complex. In Leishmania mexicana, GPI8 is nonessential, and Δgpi8 mutants lack the GPI-anchored metalloprotease GP63, which is the major surface protein of promastigotes. We have identified the active site histidine and cysteine residues of leishmanial GPI8 and generated Δgpi8 lines expressing modified GPI8 proteins. This has allowed us to study the processing and trafficking of GP63 in wild type and Δgpi8 mutants. We show using pulse-chase labeling that in Δgpi8 non-GPI-anchored GP63 was glycosylated and secreted without further processing from the cell with a t1/2 of 120 min. This secretion was prevented by growth of cells in the presence of tunicamycin, indicating that glycosylation is necessary for secretion of non-GPI-anchored proteins. In contrast, in wild type cells the majority of GP63 was rapidly glycosylated, GPI-anchored, and trafficked to the surface with defined processing intermediate forms. Tunicamycin inhibited glycosylation but did not prevent GPI anchor addition or trafficking. These results show that GPI-anchored and unanchored GP63 are trafficked via different pathways. In addition, the balance between GPI anchor addition and secretion of GP63 in Leishmania can vary depending on the activity of the GPI-protein transamidase, which has implications for the host-parasite interaction.

Leishmania is a protozoan parasite that is the causative agent of a variety of human diseases collectively known as the leishmaniasis. The parasite lives within the gut of the sandfly vector as motile proliferative procyclic promastigotes and in the mouth parts as motile nonproliferative metacyclic promastigotes. Within a mammalian host, the parasite lives as a nonmotile amastigote form that proliferates within macrophages. The surface of the promastigote is thought to contribute to survival within the sandfly and also invasion of and initial survival within a mammal (1). The surface is covered with a protective coat known as the glycoalkalx, which is predominantly made up of glycosylphosphatidylinositol (GPI)-anchored1 proteins, lipophosphoglycan, and glycoinositolphospholipids (2, 3). A characteristic of Leishmania and other related trypanosomatids is the great abundance of GPI-anchored molecules on the cell surface, which is in contrast to higher eukaryotes in which the majority of surface proteins are attached via a transmembrane domain.

The major surface protein of Leishmania promastigotes is the 63-kDa GPI-anchored protein known as GP63. It is present at about 5 × 105 molecules/parasite (4). GP63 is present on the amastigote at a greatly reduced level (5, 6). GP63 is a zinc metalloprotease active in a neutral to alkaline pH range (pH 7–10) and is site-specific in its proteolytic activity (7, 8). It is synthesized as an inactive precursor, which is activated via a cysteine switch mechanism (9). The nascent protein has an endoplasmic reticulum (ER)-signaling sequence at the N terminus and adjacent to the regulatory pro-region. A GPI anchor addition site and a hydrophobic tail are present at the C terminus. These three domains are cleaved during the trafficking and processing of the protein.

The addition of a complete GPI anchor to a precursor protein occurs in the ER lumen by the simultaneous cleavage of the protein at the GPI anchor attachment site (near the C terminus) and addition of the GPI anchor in a transamidation reaction (10). The GPI anchor is attached to the protein by amide linkage between the terminal ethanolamine phosphate group of the anchor and the nascent C-terminal carboxyl group of the protein (11). The cleavage and anchor addition occur in one reaction catalyzed by a GPI-protein transamidase complex (12, 13). The complex has been well characterized in mammalian cells and yeast and contains at least four components; GAA1, GPI8, PIG-S (GPI16), and PIG-T (GPI17) (14–19). Studies using photo-cross-linking methods suggest that the complex may indeed contain more proteins (20). Recent studies demonstrated that GPI8 in mammalian cells associates directly with the protein to be anchored (20, 21). GPI8 is thought to be the catalytic subunit of the GPI-protein transamidase complex and belongs to family C13 of the clan CD cysteine proteases (22), which are characterized as having a cysteine nucleophile with

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a catalytic dyad in the order histidine, cysteine (22).

The GPI-protein transamidase complex of trypanosomatids has not been characterized fully, although a cell-free assay for GPI anchoring in trypanosomes has been used to establish a reaction mechanism for GPI anchor addition (23). The Leishmania mexicana GPI8, unlike the mammalian and yeast homologues, has no transmembrane domain and appears to be a soluble homologue of the yeast and mammalian enzymes (24). A L. mexicana mutant lacking GPI8 (Δgpi8) was deficient of GPI-anchored proteins, including GP63, yet remained viable in culture and was capable of infecting macrophages and also a mammalian host (24). The production of this L. mexicana Δgpi8 line provided an opportunity to study the effect that GPI anchor deficiency has on the processing and trafficking of a GPI protein. Secretory transport in trypanosomatids is thought to follow the general pathway found in higher eukaryotes (25, 26), although endocytosis and exocytosis to the cell surface occurs via the flagellar pocket (27; reviewed in Ref. 28). In this study, we have defined the active site residues of L. mexicana GPI8 and demonstrated that GPI8 interacts with other proteins to carry out the transamidation reaction. The trafficking of GP63 in wild type and Δgpi8 cell lines was compared, and the role that the GPI anchor plays in the forward transport of GPI-anchored proteins was examined.

EXPERIMENTAL PROCEDURES
Parasites—L. mexicana wild type (MNYC/BZ/M379) and Δgpi8 promastigotes were maintained in culture at 25 °C in modified Eagle’s medium containing 10% (v/v) heat-inactivated fetal calf serum. Neomycin (G418; Invitrogen) was added at 25 µg ml\(^{-1}\) typically and up to 500 µg ml\(^{-1}\) as required. Where necessary tunicamycin (Sigma) was added at 5 µg ml\(^{-1}\). Transfection of promastigotes with an episome was as described previously (24).

Site-directed Mutagenesis—Site-directed mutagenesis was carried out on plasmid pCL289 (24) using the QuikChange site-directed mutagenesis kit (Stratagene). Primer sequences for each of the four sets of mutations were as follows (mutated nucleotides are in bold): H63A, OLC331, 5′-GCCGCAATGGGCAATGCAGG-3′, and OL331, 5′-GCCGCAATGGGCAATGCAGG-3′; C94G, OLC336, 5′-GACGATTCCTTGGCACTTGGCCTGGG-3′, and OL336, 5′-GACGATTCCTTGGCACTTGGCCTGGG-3′; H174A, OLC340, 5′-CTAACGTCGCGG-3′, and OL340, 5′-CTAACGTCGCGG-3′; C216G, OLC345, 5′-GGCCGAAATGCGCCATTGGCGG-3′, and OL345, 5′-GGCCGAAATGCGCCATTGGCGG-3′. Primers were allowed to overhang by 5′, and the oligonucleotides were annealed. The samples were mixed for 12 h at 4 °C and the supernatant was overlaid onto a sucrose cushion (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, and protease inhibitors) and precleared. The medium samples had an equivalent volume of 2× IDB buffer added and were then made up to 1 ml with 1× IDB, 100 µl of protein G-Sepharose (resuspended to 0.5 mg ml\(^{-1}\) in IDB), and 10 µl of L.38 antibody were added to the samples and incubated for 12 h at 4 °C, and the beads subsequently washed three times with GP63 wash buffer (0.1% Triton X-100, 0.02% SDS, 150 mM Tris-HCl, pH 7.5) and then in TEN. 40 µl of 2× SDS-PAGE loading buffer was added to the samples prior to further analysis.

Triton X-114 Fractionation—Triton X-114 fractionation was performed following the method of Bordier (29). The cells were lysed in 200 µl of Triton X-114 buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% precondensed Triton X-114) on ice for 15 min and precleared by centrifugation at 10,000 × g for 10 min at 4 °C, and the supernatant was overlaid onto a sucrose cushion (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 6% w/v sucrose, 0.06% precondensed Triton X-114) in a fresh Eppendorf tube. The samples were incubated at 30 °C for 3 min and centrifuged at 300 × g for 3 min at room temperature, and the upper aqueous layer was removed to a fresh tube. 0.5% precondensed Triton X-114 was added to this sample and incubated at 4 °C for 10 min, and then this upper layer was overlaid back on the original sucrose cushion. The sample was incubated at 30 °C for 3 min and centrifuged for 3 min at 300 × g at room temperature. The whole aqueous phase was removed to a fresh tube, leaving a small Triton X-114 pellet containing the membrane fraction. The aqueous phase was treated with 2% precondensed Triton X-114, incubated at 4 °C for 10 min, transferred to 30 °C for 3 min, and centrifuged at 300 × g at room temperature for 3 min. The aqueous phase containing the soluble cell fraction was then transferred to a fresh tube, and the soluble and membrane fractions were subjected to further analysis.

Processing of GP63 on Concanavalin A—The cells were grown to stationary phase, the cells were harvested, and the medium was filtered and retained. The cells were lysed in 1× ConA binding buffer (10 mM Tris-HCl, pH 7.4, 0.5% Triton X-114, 80 µl of 2× ConA-Sepharose, 1, 000 µl of 2× ConA binding buffer) and protease inhibitors (200 µg ml\(^{-1}\) Pefabloc SC, 5 µg ml\(^{-1}\) pepstatin A, 40 µg ml\(^{-1}\) leupeptin) were added. To the medium 1 volume of 2× ConA binding buffer, 500 µl of ConA-Sepharose, and protease inhibitors were added. The samples were mixed for 12 h at 4 °C and washed four times in ConA binding buffer, and glycosylated proteins were eluted with an appropriate volume of elution buffer (1 mM methyl α-D-mannopyranoside in ConA binding buffer).

PL-PLC Treatment—The cells were pulse-chase labeled, and two samples were collected for each time point, washed three times in ice-cold phosphate-buffered saline, and snap frozen. These samples were lysed in 200 µl of Triton X-114 buffer but with the absence of ConA-Sepharose and the addition of 0.05% Triton X-100, incubated at room temperature for 10 min, and centrifuged to preclear. The samples were then transferred to a fresh tube, 4 µl of PI-PLC (Sigma) was added to one sample from each time point, and all of the samples were incubated at 37 °C for 1 h. 0.5% precondensed Triton X-114 was added to each sample, and the samples were incubated at 4 °C subsequent to Triton X-114 fractionation as described previously.

RESULTS
Identification of Active Site Residues of L. mexicana GPI8—We demonstrated previously that L. mexicana GPI8 is a member of the C13 family of cysteine endopeptidases and a component of the GPI-protein transamidase of the parasite (24). We identified two cysteine (Cys94 and Cys 216) and two histidine (His63 and His174) residues of the L. mexicana GPI8 deletion mutant (Δgpi8) to give lines
Leishmanial GPI8 Is Essential for Transamidase Activity—In yeast and mammalian cells GPi8 has been shown to form a complex with other proteins to form an active GPI-protein transamidase (20, 30). In an attempt to define the role of GPI8 in L. mexicana, the gene encoding nonfunctional GPI8C216G was expressed from an episome in wild type cells (to give cell line WT[pXgpi8C216G]). This cell line was compared with wild type promastigotes expressing episomal GPI8 (cell line WT[pXgpi8]). Equal expression of GPI8 was verified in the two cell lines by metabolic labeling with Expre35S35S and subsequent immunoprecipitation with an α-GPI8 antibody (Fig. 2A). The expression of GPI-anchored GPI8 was examined by Western blot analysis (Fig. 2B). WT cells expressing the functional episomal copy of GPI8 (Fig. 2B, lanes 3–5) were found to express GPI8 at levels similar to the wild type parasites (lane 1). An increase in the concentration of G418, and hence GPI8 levels, did not alter the levels of GPI8 (compare lanes 3–5). However, cells expressing the mutated form of GPI8 showed decreased levels of GPI8 in the cells (compare lanes 1 and 6), and the amount of GPI8 increased as GPI8C216G expression increased (compare lanes 6–8). Overexpression of GPI8 did not appear to affect GPI8 surface expression as examined by immunofluorescence, whereas expression of GPI8C216G in wild type cells drastically reduced the amount of GPI8 on the cell surface (data not shown). At the highest concentration of antibiotic (500 μg ml⁻¹), surface expression of GPI8 was almost undetectable. Thus expression of GPI8C216G in wild type promastigotes produced a pronounced dominant-negative effect, which provides compelling evidence that GPI8 is required for transamidation activity and is likely to be part of a GPI-protein transamidase complex in Leishmania.

Non-GPI-anchored GPI8 Is Secreted—To determine whether GPI8 was being degraded or secreted in the Δgpi8 mutant, WT promastigotes, Δgpi8, and lines containing mutated GPI8 were labeled for 6 h with Expre35S35S and GPI8 immunoprecipitated from their respective culture supernatants. With WT parasites (Fig. 3A, lane 1), a small amount of three isoforms of GPI8 were detected (of 63, 64, and 65 kDa, which are therefore designated 63s, 64s, and 65s (for secreted)), with the 63s isoform being the most abundant. In contrast, a large amount of the 65s isoform was detected in the medium in which Δgpi8 cells had been grown (lane 2). This 65s form secreted from Δgpi8 cells was subsequently found to be an isoform of GPI8

FIG. 2. Expression of GPI8C216G in wild type promastigotes produces a dominant-negative effect. A, GPI expression in wild type cells (WT) expressing episomal copies of GPI8 was confirmed. The cells grown in 125 μg ml⁻¹ G418 were analyzed by Expre35S35S labeling and immunoprecipitation with anti-GPI8 polyclonal antibody. B, Western blot analysis of GPI8 expression in L. mexicana lysates. The cells were grown in increasing concentrations of G418 to select for increased plasmid copy number and a higher level of expression of GPI8 or GPI8C216G. The Western blot was performed with an anti-GPI8 monoclonal antibody.
that lacks a GPI anchor. Re-expression of GP8 in the Δgpi8 null mutant resulted in only small amounts of GP63 being secreted, with isoform sizes comparable with those secreted from WT cells (lane 3). Large quantities of the 65s isoform were detected also in the Δgpi8 cell lines expressing GP8C216G (lane 7) and GP8H174A (lane 6), as well as GP8C94G (lane 5). However, the Δgpi8 cell line expressing GP8H63A mimicked the situation in wild type cells with little GP63 secreted. Thus a high level of secretion was associated with cells having a non-functional GPI-protein transamidase.

The presence of GP63 was also analyzed in the cell lysates (Fig. 3B) by immunoprecipitation with the same anti-GP63 antibody. Three isoforms of GP63 were detected in WT cells (63, 64, and 65, kDa, which are therefore designated 63c, 64c, and 65c (for cell-associated)), with the 63c isoform being most abundant. In the Δgpi8 cell line, only the 65c isoform was detected (lane 2). The two smaller isoforms of GP63 (63c and 64c) were present in Δgpi8[pXGP8] lysates (lane 3) at approximately equal levels. Δgpi8 cell lines expressing GP8H174A (lane 6) or GP8C216G (lane 7) were similar in cell-associated profiles to Δgpi8 with the 65c isoform predominating, whereas the Δgpi8 cell line expressing GP8H63A (lane 4) was the same as wild type with abundant 63c isoform. The Δgpi8 cell line expressing GP8C94G (lane 5) gave an intermediate pattern, with all three isoforms of GP63 present. These data confirm that GP8C216G and GP8H174A are nonfunctional enzymes, whereas GP8H63A is fully functional and GP8C94G is partially functional.

Soluble GP63 Is Secreted Rapidly from Δgpi8—Clearly, lack of an active GPI affects the processing and trafficking of the GPI-anchored protein GP63 when compared with WT cells. To examine these variations in more detail, wild type promastigotes and Δgpi8 and Δgpi8[pXGP8C216G] cells were labeled with Expre35S35S for 12 min and then chased in cold medium for a period up to 300 min. GP63 was immunoprecipitated from the culture medium (Fig. 4A) or cells (Fig. 4B) and analyzed by SDS-PAGE. Only a very low level of 35S-labeled GP63 was secreted from wild type parasites, whereas high levels of secreted GP63 could be detected over a 180-min chase period for both Δgpi8 and Δgpi8[pXGP8C216G]. After 300 min, the level of GP63 secretion from WT cells was estimated to be only 13% of that secreted from Δgpi8 cells, with the t1/2 for Δgpi8 GP63 secretion estimated to be 120 min (Fig. 4C). This clearly demonstrates the higher rate of secretion of newly synthesized GP63 in cells with a nonfunctional GPI8. Two forms of GP63 were secreted from wild type cells, a 65s isoform and a 63s isoform (Fig. 4A, WT, time 180).

The processing of GP63 within the cells was also examined by partitioning the cells into soluble and membrane-associated fractions by either extraction with NaCO3 (data not shown) or Triton X-114 (Fig. 4B). Both methods of fractionation produced similar results. In wild type cells, GP63 partitioned exclusively into the membrane-associated fraction at all time points. At the start of the chase, the major isoform of GP63 was 65c. After 20 min, the majority of 65c had been chased into 64c, and by 40 min 64c was in the process of being chased into 63c. By 180 min all detectable label was in the 63c isoform. In contrast to wild type cells, only the 65c isoform was detected in the Δgpi8 and Δgpi8[pXGP8C216G] lines (Fig. 4B). Moreover, most of 65c remained in the soluble phase in Δgpi8 and Δgpi8[pXGP8C216G] cells.

Nascent GP63 Is Rapidly GPI-anchored—The timing of GPI anchor addition during the processing of GP63 was examined by determining which forms of GP63 have a GPI anchor. WT promastigotes were labeled with Expre35S35S for 12 min and then chased in cold medium for a period up to 180 min. The samples were taken at 0, 20, and 180 min, and the lysates were treated with or without PI-PLC followed by Triton X-114 fractionation and GP63 immunoprecipitation (Fig. 5). At the start of the chase, the major 65c band was present in the membrane-associated fraction (lane 2), but after PI-PLC treatment it was found in the soluble fraction (lane 3), consistent with the GPI anchor having been removed. The samples from time 40 min and time 180 min showed the same pattern for the maturing GP63 following PI-PLC treatment. All forms of GP63 identified were found only in the soluble fraction following treatment. This demonstrates that GP63 GPI addition occurred very rapidly subsequent to translation and translocation to the ER.
Processing of GP63 in Leishmania

![Fig. 5. PI-PLC treatment demonstrates early anchor addition to GPI-anchored GP63.](image)

![Fig. 6. Glycosylation affects the processing and secretion of GP63.](image)

should also be noted that a fourth minor isoform of GP63 could occasionally be detected (lanes 6 and 7).

Glycosylation of GP63 Occurs Prior to Addition of the GPI Anchor—Glycosylation of GP63 was examined by testing whether the protein bound to concanavalin A, which interacts with N-linked glycans (Fig. 6A). GP63 did not bind to Sepharose beads nonspecifically (data not shown). A much higher level of glycosylated GP63 bound to ConA from WT promastigotes (lane 1) than Δgpi8 cells (lane 2). In contrast glycosylated GP63 was present at a higher level in the medium of Δgpi8 cells (lane 3) compared with that from WT cells (lane 4), and the size of GP63 precipitated was larger in Δgpi8. This demonstrates that GP63 is N-glycosylated in both wild type and Δgpi8 cells and that N-linked glycosylation can occur in the absence of GPI-protein transamidase activity.

The two cell lines were grown in medium containing 5 μg ml⁻¹ tunicamycin to inhibit N-linked glycan formation. Pulse-chase labeling was used to examine the processing of GP63 under these conditions (Fig. 6, B and C). GP63 was processed differently in WT cells grown in the presence or absence of tunicamycin. A smaller form (of ~63 kDa, which is therefore designated 63ct (for cellular material with tunicamycin)) was present at time 0, and this was chased into a 60-kDa form (60ct) after 180 min. Only a single minor intermediate form of GP63 was identified in cells grown in the presence of tunicamycin, compared with the one major (64c) and one minor band identified in normally grown WT cells. The Δgpi8 cells grown in the presence of tunicamycin also expressed a smaller GP63 (63ct). However, this was not chased to a 60-kDa form. The size difference between the proteins isolated at time 0 from cells grown in the presence and absence of tunicamycin correlates with the lack of N-glycosylation. The lower number of detectable intermediate forms present in WT cells grown in the presence of tunicamycin indicates that one of the isoforms detected during GP63 maturation may be a result of N-glycan processing. GP63 could not be detected in the medium of either Δgpi8 or wild type cells when grown in the presence of tunicamycin (Fig. 6C), showing that N-linked glycosylation is important for the secretion of GP63 from both WT and Δgpi8.

DISCUSSION

We have identified His^{174} and Cys^{216} as the L. mexicana GPI8 catalytic dyad characteristic of the C13 family of clan CD cysteine proteases. Mutation of either residue leads to the loss of GPI-anchored GP63 on the surface of the parasite, demonstrating that these residues are essential for activity of GPI8 and therefore for GPI anchoring in *Leishmania*. These findings complement previous results demonstrating that a sulfhydryl group acts as the active site residue for the GPI-protein transamidase of *Trypanosoma brucei* (23) and that recombinant *L. mexicana* GPI8 can be inactivated by thiol alkylating agents in a cell-free system (10). Moreover, His^{174} and Cys^{216} of *L. mexicana* GPI8 align with those identified recently as the active site histidine and cysteine residues in yeast and human GPI8 (30, 31). Removal of GPI-protein transamidase activity from trypanosome membranes by a high pH wash and reconstitution of that activity with recombinant GPI8 suggested that in trypanosomatids GPI8 is part of a complex and that this complex may be dynamic (10). The findings reported here that expression of the active site mutant GPI8C216G in wild type parasites produced a pronounced dominant-negative effect, with GPI-protein transamidase activity being severely down-regulated, provide further evidence that *L. mexicana* GPI8 is part of a larger protein complex. In yeast and higher eukaryotes, GPI8 is thought to form a stable but dynamic complex with at least three other components, GAA1, GPI16/PIG-S, and GPI17/PIG-T (16, 19, 31). No homologue of other transamidase complex members has yet been cloned from protozoa, although analysis of sequence data has identified a possible GAA1 homologue in *Leishmania major* (GenBank TM accession number CAB86709 (32)) and a similar protein in *T. brucei* (GenBank TM accession number AC087702).

We reported previously that GP63 could not be detected in the culture medium of Δgpi8 cells (24). In the current study we used pulse-chase labeling and immunoprecipitation assays that are considerably more sensitive than the previously employed methods, and this enabled us to show that GP63 was indeed secreted (Fig. 4). Thus *Leishmania* differ from higher eukaryotes in which non-GPI-anchored proteins accumulate in the ER (33, 34) before being exported to the cytosol for degrad-

![Fig. 5. PI-PLC treatment demonstrates early anchor addition to GPI-anchored GP63.](image)

![Fig. 6. Glycosylation affects the processing and secretion of GP63.](image)
port (38). In addition, replacement of the hydrophobic tail of the variable surface glycoprotein with a transmembrane domain resulted in ER accumulation followed by lysosomal degradation (39). Our results clearly demonstrate that retention in the ER of the unanchored form of GP63 does not occur in Leishmania. These findings are in agreement with previous studies utilizing expressed forms of GP63 that had been mutated to prevent GPI anchor addition (40).

The situation in Leishmania appears relatively complex, as evidenced by the observation that GP63 was processed and trafficked differently in wild type cells and the mutants containing modified GPI8 or entirely lacking the protein (Fig. 4). The level of GP63 secretion correlated with GPI-protein transamidase activity. Cells with a functional copy of GPI8 (WT, Δgpi8[pXGPI8] or Δgpi8[GPI8H174A]) secreted a small quantity of GP63 (65s and 63s) into the medium, whereas cells without a functional GPI8 (Δgpi8, Δgpi8[GPI8H174A], or Δgpi8[GPI8C94G]) secreted a high level of the 65-kDa (65a) form only. Analysis of the Δgpi8[GPI8C94G] cells revealed an intermediate phenotype with GP63 being processed and transported to the cell surface (data not shown) as well as secreted into the medium (Fig. 3). This suggests that this mutated GPI-protein transamidase is dysfunctional, being active but less so than the wild type protein. This could reflect differing abilities to form the functional complexes, perhaps because the Cys94 residue is necessary for optimal folding of the GPI8 and/or is directly involved in its interaction with other proteins of the complex. Mutation of His54 in yeast GPI8 and Cys82 in human GPI8 also led to a partial loss of GPI-protein transamidase function (30, 31), suggesting that protein-protein interactions are affected directly or indirectly by these residues. Our results with Leishmania suggest that the relative amounts of GP63 (or other GPI-anchored proteins such as the proteophosphoglycan (41)) directed to the surface of the parasite or secreted could be controlled by regulation of GPI8 activity. This may have an influence on the ability of the parasite to survive in the variety of environmental conditions that it encounters.

Characterization of the processing of GP63 in wild type promastigotes by pulse-chase labeling revealed that the first detectable cell-associated product was a 65-kDa form (Fig. 4B, 65c) that was GPI-anchored and glycosylated. This finding shows that addition of the GPI anchor to GP63 must occur very rapidly following translation and translocation into the ER and subsequent to processing of the N-terminal signal sequence. We have shown by immunofluorescence microscopy that GPI8 co-localizes with QM, a 60 S ribosomal protein, which suggests the GPI-protein transamidase has a close association with the rough ER (42). In eukaryotes the translocon contains the machinery for N-linked glycosylation, so it is likely that this event occurs during translocation into the ER. Our results support the proposal that the GPI-protein transamidase is closely associated with the translocon (20) and that GPI anchor processing occurs very soon post-translation (21).

The GPI-anchored 65c form was processed further to a 64c protein within 20 min and a mature 63c form within 40 min (Fig. 5). By 180 min, all of the label had been chased into the 63c form. These events can be accounted for by secondary glycosylation events (which are inhibited by tunicamycin; Fig. 6) and the cleavage of the pro-region to activate the enzyme. The processing events in the Δgpi8 line differed from those occurring in wild type parasites in that the cell soluble associated 65c protein present at time 0 did not undergo any processing events throughout the chase, as assessed by size variation. Thus although the GP63 in the Δgpi8 cell line was glycosylated during translocation (Fig. 6), no modifications could be detected thereafter.

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\text{Cell surface expression of GP63} \quad (45, 46) \quad \text{may influence the survival of the parasite in its host.}
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These results show that GPI-anchored GP63 and unan-
chored GP63 are trafficked via different pathways: a classical pathway whereby GP63 is GPI-anchored, processed, and trafficked to the cell surface and a direct secretion pathway whereby non-GPI-anchored GP63 is rapidly exported from the cell without further modification (Fig. 7). The secretion pathway is directed by N-glycans, because the loss of glycosylation results in loss of secretion. In contrast, trafficking of the GPI-anchored GP63 to the cell surface appears to be regulated exclusively by the presence of a GPI anchor, because the loss of glycosylation does not affect forward trafficking. This study does not address whether GPI anchors act as a signal for the forward transport of proteins or whether they play an active role in protein transport. GPI-anchored proteins become insoluble to detergent extraction during trafficking through the secretory pathway, forming detergent-resistant membranes. Lipid microdomains are thought to occur in protozoa (47–49), and detergent-resistant membranes of Leishmania are enriched in components characteristic of eukaryotic lipid rafts: inositol phosphorylceramide, sterols such as ergosterol, and GPI-anchored molecules (both GP63 and LPG). Lipid rafts may be relevant to the forward trafficking and processing of GPI proteins in Leishmania, and studies are ongoing to investigate these in wild type and GPI-deficient parasites.

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