Posttranslational Regulation of a Leishmania HEXXH Metalloprotease (gp63)

THE EFFECTS OF SITE-SPECIFIC MUTAGENESIS OF CATALYTIC, ZINC BINDING, N-GLYCOSYLATION, AND GLYCOSYL PHOSPHATIDYLINOSITOL ADDITION SITES ON N-TERMINAL END CLEAVAGE, INTRACELLULAR STABILITY, AND EXTRACELLULAR EXIT*

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Leishmanolysin (EC 3.4.24.36) (gp63) is a HEXXH metalloprotease, encoded by multicopied genes in Leishmania and implicated in the infectivity of these parasitic protozoa. We examined posttranslational regulation of gp63 expression by site-specific mutagenesis of the predicted catalytic/zinc-binding sites in the H²⁶XXH motif, the potential sites of N-glycosylation and glycosyl phosphatidylinositol addition. Mutant and wild-type genes were cloned into a Leishmania-specific vector for transfecting a deficient variant, which produced gp63 20-fold less than wild-type cells. The selective conditions chosen fully restored this deficiency in transfec-
tants with the wild-type gene. Under these conditions, all transfec-
tants were found comparable in both the plasmid copy number per cell and elevation of gp63 transcripts. Mutant and wild-type products in the transfec-
tants were then compared quantitatively and qualitatively by specific immunologic and protease assays. The results indicate the following. 1) Glu-265 in the HEXXH motif is indispensable for the catalytic activity of gp63. The propeptide of the inactive mutant products was cleaved, suggestive of a non-intramolecular event. 2) Substitution of either His residue in HEXXH leads to apparent intracellular degradation of the mutant prod-
ucts, pointing to a role for zinc binding in vivo stability of gp63. 3) The three potential sites of N-glycosyla-
tion at Asn-300, Asn-407, and Asn-534 are all utilized and contribute to intracellular stability of gp63. 4) Substitution of Asn-577 causes release of all mutant products, indicative of its specificity as a glycosyl phosphatidylinositol addition site for membrane anchoring of gp63. It is suggested that expression of gp63 as a functional pro-
tease is regulated by these posttranslational modification pathways.

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1 The abbreviations used are: GPI, glycosyl phosphatidylinositol; PLC, phospholipase C; CRD, cross-reacting determinant; PAGE, poly-
acylamide gel electrophoresis; RER, rough endoplasmic reticulum.

**N-TERMINAL END CLEAVAGE, INTRACELLULAR STABILITY, AND EXTRACELLULAR EXIT**

-GLYCOSYLATION, AND GLYCOSYL PHOSPHATIDYLINOSITOL ADDITION SITES ON
RNA Blot Analysis of gp63 Transcripts—Total RNA was isolated from 1–5 × 10^6 cells in a lysis solution (4 mM guanidinium thiocyanate, 0.5% Sarkosyl, 25 mM sodium citrate, 200 mM sodium acetate, and 100 mM 2-mercaptoethanol) (37). After extractions with acidic phenol/chloroform, RNA was precipitated at 4°C with isopropanol, dissolved in diethyl pyrocarbonate-treated water, and precipitated three times with 4 M LiCl. Denatured samples were dot-blotted in serial dilutions on nylon filters (Cuno) for hybridization with Leishmania gp63 (13) or β-tubulin genes. Northern blot analysis was carried out by standard procedures (37). Probes were labeled by random priming (Amersham Corp.) with [α-32P]dCTP and purified by using the Elutip system (Schleicher & Schuell). Autoradiography was done at −70°C with intensifying screens.

Western Blot and Immunoprecipitation Analysis—SDS-PAGE-resolved proteins were transferred to nitrocellulose (Schleicher & Schuell) for immunoblot analysis (10, 12) using rabbit antiserum raised against denatured and deglycosylated gp63 (5) that was purified from L. amazonensis (8). For loading control, we used rabbit antiserum raised against p36, a NADPH-oxidoreductase constitutively expressed in Leishmania spp. (38). The second antibody used was either alkaline phosphatase- or horseradish peroxidase-conjugated goat anti-rabbit Fc or IgG (Sigma). Immunoblots were developed in the latter case with ECL reagents for chemiluminescence (Amersham), followed by exposure to X-ray films.

To assess the release of gp63, promastigotes were labeled for 1 h at a density of 10^6 cells/ml in Hanks’ balanced salt solution containing 50–100 μCi of [35S]Met/Cys mixture (DuPont NEN) (specific activity of 100 Ci/mmol). Cells were washed twice in Medium 199 and resuspended in this medium containing 0.5% bovine serum albumin (3 × 10^6 cells/ml). At different intervals after incubation for up to 48 h, spent media were collected and cleared by centrifugation at 50,000 × g. Labeled gp63 were immunoprecipitated from these supernatants for 1 h at 4°C with 1/200 dilution of the anti-gp63 antiserum. Immune complexes were collected by Protein A-Sepharose (Sigma), followed by washing and autoradiography of these samples as performed previously (10, 12).

GPI-PLC Digestion—gp63 was immunoprecipitated from the culture supernatants of transfecteds with mutant N571L using anti-gp63 polyclonal antibodies cross-linked to Protein A-Sepharose with dimethylpyrrolidinomethide. The immunoprecipitates were washed with GPI-PLC digestion buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, and 1% Nonidet P-40, 40% [35S]Met/Cys mixture (DuPont NEN) (specific activity of 100 Ci/mmol). Cells were washed twice in Medium 199 and resuspended in this medium containing 0.5% bovine serum albumin (3 × 10^6 cells/ml). At different intervals after incubation for up to 48 h, spent media were collected and cleared by centrifugation at 50,000 × g. Labeled gp63 were immunoprecipitated from these supernatants for 1 h at 4°C with 1/200 dilution of the anti-gp63 antiserum. Immune complexes were collected by Protein A-Sepharose (Sigma), followed by washing and autoradiography of these samples as performed previously (10, 12).

Laser Densitometry—Banding intensity of gp63 in five autoradiograms from the culture supernatants of transfecants and wild-type gene were normalized against gp63 bands in the untransfected control. Laser densitometry was performed using an LKB laser densitometer (Bromma, Sweden). Values for the glycosylation mutants were normalized against the endogenous gp63, whereas those of the wild-type gene were normalized against gp63 bands in the untransfected control (Table I, Expression level). From a set of five representative data in the gel assays for gelatinolytic activity, the faster migrating overexpressed band (Fig. 3, panel C, open arrows) was scanned versus the endogenous slower migrating band (solid arrows) from the photographic negatives (Table I, Gelatinolytic activity).
RESULTS

Transcription of Mutant and Wild-type Genes in the Transfectants Raises Their gp63 mRNA to a Comparable Level—As a prerequisite to study the effects of mutagenesis on gp63 at the protein level, we first verified that the copy number of the plasmids and transcription of gp63 genes were comparable in all transfectants. DNA dot-blot analysis with a vector-specific probe revealed that all transfectants had an equivalent number of plasmids, i.e., 200–400 copies/cell, as seen previously (12). Structural integrity of the plasmids was also confirmed in all transfectants by restriction mapping (data not shown). In addition, all transfectants with wild-type and the mutant gp63 genes were found to contain an equivalent amount of gp63 mRNA (Fig. 2, A and B, lanes 1 and 4–12) that was elevated ~8-fold over untransfected cells (Fig. 2A, lane 2) or transfectants with vector alone (Fig. 2A, lane 3). Overexpression of gp63 mRNAs to this level in the transfectants is expected from the copy number of plasmids over that of the endogenous gp63 genes estimated per cell (17, 20). Reprobing the blots with the β-tubulin gene provides reference for the comparable quantity of all RNA samples loaded (Fig. 2, panel B). Northern blot analysis further confirms that gp63 transcripts are overexpressed in transfectants with wild-type and representative mutant genes (Fig. 2C, lanes 2–8) when compared to transfectants with the vector alone (lane 1). Significantly, the overexpressed gp63 mRNAs are of comparable patterns, suggestive of their qualitative identity. The ~5.4- and ~8-kilobase pair species are intense and clearly the transcripts from the mutant or wild-type genes electroporated (Fig. 2C, lanes 2–8) because of their absence in the control (lane 1). The remaining faint band of 3 kilobase pairs is present in all samples including the control (Fig. 2C), indicative of its origin from the endogenous gp63 genes. Clearly, site-specific mutations of the gp63 genes introduced affect neither replication of the pX vector nor their transcription, although the interrelationships of the two overexpressed mRNAs observed await further investigation.

Quantitative and Qualitative Assays of gp63 Differentiate Electroporated Gene Products from Endogenous gp63 in the Transfectants—To assess the effects of site-specific mutations on gp63, mutant and wild-type products of the transfectants were compared quantitatively and qualitatively (Fig. 3; Table I). For adequate comparison, all samples were loaded using the same cell number (Fig. 3A). Western blot analysis of a constitutively expressed Leishmania NADPH-oxidoreductase; panel B, immunoblot with rabbit polyclonal sera raised against affinity-purified gp63, showing relative level of gp63 expression by wild-type and mutant genes in the transfectants; panel C, gelatin-containing gel for proteolytic activities of the same samples. Lane 1, parental gp63-deficient variant; lanes 2I and 2II, two independent transfectants with the wild-type gene; lanes 3–15, transfectants with the gp63 site-specific mutants loaded in the same order as those in Fig. 1 and Table I.

The relative amounts of gp63 proteins were assessed by Western blot analysis (Fig. 3B). To optimize the capacity of this assay for quantitative evaluation, polyclonal antiserum raised against denatured and deglycosylated gp63 was used (S). Laser densitometry of the gp63 bands developed by ECL reagents on x-ray films provided further quantitative evaluation (Table I, Expression level). In addition, wild-type and mutant products in the transfectants were quantitatively and qualitatively as-
The wild-type or Y254D products (Table I and Fig. 3 (Table I, Caseinolytic activity). Assay has an added advantage in that it separates the products caseinolysis of glutaraldehyde-fixed cells in solution. The gel after electrophoresis of cell lysates in non-denaturing gels and sequence, e.g. neutral endopeptidase (39), angiotensin converting enzyme (40), and E. coli protease III (41).

Although the products of E265D are enzymatically inactive, they appear to have been appropriately processed posttranslationally, judging from their identity in electrophoretic mobility to the wild-type gp63 (Fig. 3B, lane 5 versus lane 2). This suggests that the cleavage of propeptide from the nascent gp63 does not require an “intramolecular autocleavage,” as found for the proenzyme activation in the matrixin family (25). The proposal that gp63 may undergo a similar process of autocleavage (24) via the “cysteine switch” mechanism (23) remains possible, assuming that the propeptide is cleaved by an “intermolecular” processing event. In this instance, it may involve the action of either a different protease or the endogenous wild-type enzyme on the mutant. Additional work is needed to elucidate this important step in the regulation of gp63 maturation into a functional protease.

Mutation of Either His Residue in HEXXH of gp63 Leads to No Detectable Products in Vivo: Possible Role of Zinc Binding for Intracellular Stability of gp63—Interestingly, substitutions of either His-264 or His-268, the two zinc-binding ligands in the HEXXH motif, resulted in no detectable overexpression of gp63 and hence little enzyme activity above background in the transfecteds (Table I and Fig. 3B, C, and lanes 3, 4, 6, and 7). There was very little extracellular exit of the mutant proteins into the culture medium of these transfecteds (data not shown), as was the case of the wild-type transfecteds (Fig. 4A, pX-gp63). Two mutants were prepared for each His residue by conserved substitution with two different amino acids, i.e. H264Y, H264F, H268Y, and H268N (Fig. 1), and all gave the same results. It is thus unlikely that the negative results are due to mutations of other residues introduced inadvertently into the gene sequence during mutagenesis. The results obtained are unexpected in the sense that several other metalloproteases remained intact, albeit with loss of proteolytic activity when the His residues in their HEXXH motif are similarly mutated (39, 42). Perhaps, gp63 requires metal to maintain its appropriate conformation for intracellular stability and becomes susceptible to degradation in the absence of its metal-binding ligands. The fact that substituting either of the two His residues in HEXXH produces the same effect indeed suggests a need of their coordinated action, as in the case of zinc binding. If gp63 does require zinc for stability, it would be strictly an in vivo requirement. It has been shown previously that dialysis of purified gp63 against

Table I  

| Lane no. | Plasmid | Expression level | Caseinolytic activity (ng azocasein digested/h/5 x 10⁷ cells = S.D.) | Gelatinolytic activity |
|---------|---------|------------------|---------------------------------------------------------------|---------------------|
| 1       | None    | 10               | 2 ± 1                                                          | 15                  |
| 2, 1 & II | gp63/I | 183              | 46 ± 1                                                         | 9                   |
| 3       | H264Y   | 10               | 1 ± 0                                                          | 8                   |
| 4       | H264F   | 12               | 2 ± 0                                                          | 7                   |
| 5       | E265D   | 166              | 7 ± 2                                                          | 2                   |
| 6       | H268Y   | 12               | 5 ± 0                                                          | 1                   |
| 7       | H268N   | 10               | 5 ± 2                                                          | 1                   |
| 8       | Y254D   | 12               | 45 ± 1                                                         | 2                   |
| 9       | N577L   | 10               | 6 ± 1                                                          | 9                   |
| 10      | N300Q   | 77               | 41 ± 3                                                         | 11                  |
| 11      | N407Q   | 74               | 37 ± 1                                                         | 8                   |
| 12      | N534Q   | 73               | 41 ± 1                                                         | 8                   |
| 13      | N300Q/N407Q | 13          | 34 ± 2                                                         | 7                   |
| 14      | N407Q/N534Q | 16        | 54 ± 2                                                         | 11                  |
| 15      | N300Q/N407Q/N534Q | 21     | 40 ± 3                                                         | 5                   |

a See Figs. 1 and 3 for mutated residues and corresponding lanes 1–15, respectively.

b Relative quantity of gp63 assessed by densitometry as described under “Experimental Procedures.”

c Cell surface proteolytic activity of glutaraldehyde-fixed cells assayed under conditions as described under “Experimental Procedures.”
removal of its metal (5) or after chemical modification of its His residues via zinc binding in the catalytic activity of gp63 is not formally proven here due to the absence of mutant products. A role for the 2 His ligation proteins and oncoproteins (43, 44). A role for the 2 His metallocproteins, none of these contains HE.

O-phenanthroline to remove the metal results in a loss of its catalytic activity, but not its molecular integrity, since an addition of zinc ions fully restores activity to the dialyzed apoenzyme (5). Although zinc has been reported to stabilize some metalloproteins, none of these contains HEXXX, e.g. viral replication proteins and oncoproteins (43, 44). A role for the 2 His residues via zinc binding in the catalytic activity of gp63 is not formally proven here due to the absence of mutant products. However, this function is strongly suggested by the findings that purified gp63 loses >95% of its catalytic activity after removal of its metal (5) or after chemical modification of its His residues with 0.5 mM diethylpyrocarbonate (data not shown). These results suggest that gp63 may be regulated by its zinc binding via the His residues in HEXXX not only for its proteolytic activity but also for its molecular integrity in vivo.

Mutation of the GPI Addition Site Resulting in Extracellular Exit of gp63, Demonstrating Its Site Specificity as a Membrane Anchor—Mutation of Asn-577, the predicted GPI addition site of gp63 (27), also produces unexpected and dramatic results. The release of the mutant products appears complete, since the cell-associated gp63 is essentially of the background level in the wild-type gene (Fig. 4A, Pur-gp63, lanes N577L + GPI-PLC), but not in the released mutant products (Fig. 4B, α-CRD, lane N577L + GPI-PLC). It thus appears that the absence of the GPI moiety accounts for the extracellular exit of the mutant products. The released mutant gp63 is gelatinolytic (data not shown), indicating that the GPI moiety is not involved in the catalytic activity of gp63. Previously, transfection of Leishmania with the gene encoding GPI-PLC of T. brucei was found to deprive gp63 of a GPI anchor, also resulting in the release of this molecule (28).

The GPI addition site of gp63 differs in several aspects from that of other GPI-anchored membrane molecules. The abundant release of N577L mutant products indicates that the GPI anchor is the dominant, if not the only, mechanism for membrane anchoring of the gp63 in question and that the GPI addition site is quite specific. In contrast, other GPI-anchored molecules after similar mutations often become sequestered and degraded intracellularly (29, 30), except in rare cases where an alternative GPI addition site may be used (45). The results obtained from the N577L mutant raise the possibility that the addition of GPI to gp63 may regulate this molecule to act as a protease on the surface and/or after extracellular exit. This mode of regulation as a general phenomenon for GPI-anchored molecules deserves further study.

All Three Potential Sites of N-Glycosylation Predicted from the Gene Sequence Are Glycosylated and Contribute to the Intracellular Stability of gp63—Conserved substitutions of the three potential N-glycosylation sites of gp63 at Asn-300, Asn-407, and Asn-534 with Gln provided evidence of glycosylation at all sites. All these mutant products increase in electrophoretic mobility when compared with the endogenous fully glycosylated gp63 (Fig. 3, B and C, lanes 10–15, open arrow versus solid arrow). Moreover, the level of this increase in mobility depends on mutations at single sites (Fig. 3, B and C, lanes 10–12) or simultaneously at two different sites (lanes 13 and 14) or at all three sites (lane 15). The electrophoretic mobility of the mutant products thus increases proportionally with the number of N-glycosylation sites ablated. The completely deglycosylated mutant protein is ~54 kDa, consistent in size with enzymatically or chemically deglycosylated gp63 (8, 34). The deglycosylated mutant products are distinguishable from one another and from fully glycosylated gp63, due to their different mobility in native gels with gelatin substrates (Fig. 3C, lanes 10–15) as well as in denaturing SDS-PAGE for Western blot analysis (Fig. 3B, lanes 10–15). This property of the mutant products facilitates their quantitative and qualitative comparison with endogenous gp63 and with the wild-type gene or other mutant products.

Clearly, the deglycosylated mutant products (Fig. 3, B and C, lanes 10–15) are less abundant than those from the wild-type

**Fig. 4.** Extracellular exit of gp63 and characterization of the released gp63 from the transfectant with N577L, the GPI addition site mutant. Panel A, autoradiography of immunoprecipitated gp63 from culture supernatants of radiolabeled transfectants with either the wild-type gene (left panel) or the GPI mutant (N577L) (right panel). Spent medium was collected for immunoprecipitation with anti-gp63 (upper) and anti-CRD (lower) antisera. Panel B, immunoblot analysis of gp63 immunoprecipitated from supernatants of N577L transfectants (right two lanes) and from purified wild-type (Pur-) gp63 (left two lanes) with anti-gp63 (upper) and anti-CRD (lower) antisera. + and −, with or without GPI-PLC digestion.

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gene or other mutant genes (lanes 2, 5, and 8). The low level of deglycosylated products is not due to their release, since only negligible amounts of gp63 were found in the medium of these transfectants (data not shown) as the wild-type transfectants (Fig. 4A, pX-gp63). Densitometry of the banding intensity further indicates that the levels of gp63 in single deglycosylation mutants (Table I, lanes 10–12) and double or triple deglycosylation mutants are 2.5- and 13-fold less, respectively, than that of the fully glycosylated gp63 from the wild-type gene (Table I, lanes 21 and 22). The reduction is more profoundly affected by the number of glycosylation sites obliterated than their position in the molecule. It is noteworthy that the endogenous gp63 is also reduced in these transfectants, especially those with double or triple deglycosylation mutants (Fig. 3, B and C, lanes 13–15, solid arrow) (see “Discussion”). The results thus suggest that the extent of N-glycosylation of gp63 affects its cellular level, presumably by stabilizing its structural integrity against proteolytic degradation (46). The role of glycosylation is indeed thought to endow nascent peptides with proper local conformation for correct folding during cotranslation or transport into the RER (47). N-Glycosylation of gp63 does not seem to be required for their expression on the cell surface, since deglycosylated products appear on the intact transfectants, as suggested by their increased caseinolytic activity (Table I, lanes 10–15) and surface reactivity with anti-gp63 antibodies (data not shown).

All N-glycosylation mutant products, including those completely deglycosylated, are proteolytically active under the assay conditions used with either gelatin or azocasein (Table I and Fig. 3C, lanes 10–15) or fibrinogen (data not shown) as the substrate. Interestingly, semi-quantitative analysis of proteolytic activity by laser densitometry of zymograms suggests that deglycosylation may actually increase the specific gelatinolytic activity of gp63 (data not shown), although the total activity of deglycosylated gp63 appears lower than the wild-type protein (Fig. 3C and Table I, Gelatinolysis, lanes 10–15 versus lane 2). Also, the surface caseinolytic activity of the transfectants with the deglycosylation mutants is only slightly lower, if at all, than that of the transfectants with the wild-type gene (Table I, Caseinolysis, lanes 10–15 versus lane 2). These observations suggest that the specific caseinolytic activity of deglycosylated mutant products increases on the cell surface, since the amounts of these products decrease significantly with increasing level of deglycosylation. Our results are thus at variance with previous findings of no changes either in the quantity of gp63 or its specific proteolytic activities with enzymatically and chemically deglycosylated gp63 (34). Perhaps deglycosylation by non-genetic means is less complete, leading to the different observations reported. Glycosylation of other proteases may impart either positive or negative effects on their enzymatic activities. For example, the cleavage of plasminogen by tissue plasminogen activator increases by 2–4-fold in the absence of one of its three N-linked glycans (32), while the activity of neutral endopeptidase decreases after deleting three of its five N-glycans (33). In both cases, the differences observed are thought to result from changes in intrinsic catalytic activity, but not substrate binding. To address this issue in the case of gp63, it is necessary to purify genetically deglycosylated products for comparison with wild-type proteins to determine their kinetics of enzyme activity against specific substrates.

**DISCUSSION**

Here, we examined regulatory functions of posttranslational modifications on the Leishmania HEXXH metalloprotease. Insofar as is known, it is the only family of these proteases so extensively modified postranslationally by both N-glycosylation and GPI addition in addition to the N-terminal end processing. While a number of metalloproteases are known to be heavily glycosylated, few are GPI-anchored, i.e. human carboxypeptidase M and yeast vacuolar dipeptidase, but neither contains HEXXH (see Ref. 1). Additional significance more specific to this group of organisms includes their apparent absence of transcriptional regulation of gene expression (22) and the implication of gp63 in the infectivity of these parasites (6). Thus, postranslational modifications represent potentially important steps in regulating the expression of this and possibly other similar molecules.

Several favorable factors made our approach to study posttranslational modifications of gp63 by site-specific mutagenesis possible. 1) The gp63 genes are highly conserved and functional residues in the putative motifs already identified (13–19) for mutagenesis (see Fig. 1); 2) the use of Leishmania-specific vector pX (36) for the transfections allowed consistent transcription of the electroporated genes (Fig. 2); 3) the gp63-deficient variant (10) used provides a suitable recipient host in the absence of Leishmania gp63 null mutants (20, 48); 4) electroporated gene products are sufficiently abundant for the specific immunologic and protease assays used to achieve adequate quantitative and qualitative comparisons (Fig. 3 and Table I). The results obtained here with the postranslational regulation of gp63 include several observations that have not been reported previously with other glycosylated HEXXH metalloproteases or GPI-anchored molecules. Specifically, gp63 appears not to require an intramolecular autolytic mechanism for cleaving its N-terminal propeptide, requires zinc binding and N-glycosylation to maintain its intracellular integrity, and is released with the loss of its GPI anchor. How these events, presumably all initiated in the RER, are coordinated to regulate gp63 are not known, but several observations pose interesting questions that are worthy of further consideration.

Interestingly, substitution of either His residue in HEXXH leads to no detectable mutant gp63 in the transfectants with all four independently prepared mutants (Table I and Fig. 3B and C, lanes 3, 4 and 6, 7), while it is only reduced in transfectants with all the deglycosylation mutants (lanes 10–15, open arrow). Most unexpected is the observation that the endogenous gp63 is simultaneously reduced, but only in the transfectants with deglycosylation mutants (Fig. 3, B and C, lanes 10–15, solid arrow). This disparity seen between the two groups of mutants may signify functional differences of zinc binding and N-glycosylation in their respective contributions to the intracellular stability of gp63. Mutations of the His residues in HEXXH presumably deprives gp63 of a zinc atom. Such mutations may simply change the conformation of gp63 more drastically than deglycosylation, thereby rendering His mutants more susceptible than the deglycosylation mutants to intracellular degradation. However, this scenario does not accommodate the simultaneous reduction of the endogenous gp63 seen only in the transfectants with deglycosylation mutants. One possible explanation may lie in the fact that gp63 is a homopolymer in its native state (3, 8). If monomeric deglycosylated mutant products and endogenous fully glycosylated gp63 in the transfectants co-polymerize randomly, it is possible to envision the formation of mutant/wild-type heteropolymers, leading to their intermediate susceptibility to intracellular degradation and thus a partial reduction of both wild-type and mutant gp63 seen (Fig. 3, B and C, lanes 10–15). Conformational changes of the His mutants may be so drastic that they become too short-lived or too deformed to co-polymerize with the endogenous gp63, thereby leaving the latter undisturbed (Fig. 3B, lanes 3, 4, 6, and 7, solid arrow).

It appears that both the N and C termini of gp63 are processed differently from other proteases or GPI-anchored pro-
gp63 has been reported to play a role in conditions encountered by regulation of its expression. It is conceivable that the extreme tance of multiple posttranslational modifications of gp63 in the contrast to other GPI-anchored molecules. This transport mechanism of the gp63 mutants deserves further study, especially with respect to the cleavage of their hydrophobic C-terminal end for exit from the vesicles with the plasma membrane, however, the mutant products may be transported in vesicles along the normal route for surface expression. Upon fusion of these vesicles with the plasma membrane, however, the mutant products therein are set free because they lack a GPI anchor. This transport mechanism of the gp63 mutants deserves further study, especially with respect to the cleavage of their C-terminal end and their insusceptibility to degradation, in contrast to other GPI-anchored molecules.

The work presented here underscores the potential importance of multiple posttranslational modifications of gp63 in the regulation of its expression. It is conceivable that the extreme conditions encountered by Leishmania during their life cycle in both insect and mammalian hosts may up- or down-regulate their N-glycosylation, GPI addition, and terminal end processing and, hence, the expression of gp63. Indeed, up-regulation of gp63 has been reported to play a role in Leishmania infection (5, 6, 10, 21).

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