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Leishmania and *Trypanosoma* surface glycoproteins have a common glycophospholipid membrane anchor

(variant surface glycoprotein/p63 antigen/phosphatidylinositol/phospholipase C/crossreacting determinant)

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ABSTRACT **The variant surface glycoprotein (VSG) of the African trypanosomes is the major membrane protein of the plasma membrane of the bloodstream stage of the parasite. It is anchored in the plasma membrane by a glycolipid covalently bound to the C-terminal amino acid of the protein. The VSG is released through the action of a phosphatidylinositol-specific phospholipase C that removes dimyristoylglycerol and exposes the carbohydrate antigenic determinant common to all VSGs. Promastigotes of *Leishmania* have a predominant surface glycoprotein, termed p63, that is anchored in the plasma membrane in a similar way. A water-soluble form of p63 can be generated through the action of phosphatidylinositol-specific phospholipase C from trypanosomes or from *Bacillus cereus*. Either treatment exposes on the *Leishmania* p63 an antigenic determinant recognized by antibody prepared against the trypanosomal crossreacting determinant. These findings indicate that p63 and VSG have a common membrane anchor and are structurally related.**

The plasma membranes of the protozoan parasites of the genus *Leishmania*, and of the African trypanosomes, contain a major glycoprotein termed p63 in *Leishmania* promastigotes and VSG (variant surface glycoprotein) in bloodstream trypomastigotes of trypanosomes. Whereas sequence diversity among VSGs is responsible for the antigenic variation of African trypanosomes, p63s are apparently conserved among *Leishmania* species (1–4). These glycoproteins contain a phospholipid and both can be purified either in amphiphilic or in hydrophilic forms (5–7). The amphiphilic form of VSG contains an unusual glycoinositol phospholipid that anchors the VSG in the membrane (8). The hydrolysis of this lipid moiety by an endogenous lipase generates the hydrophilic form of VSG during lysis of the cell (5, 6, 8). Taking into account the similarities between p63s and VSGs, we have investigated whether both surface glycoproteins share this singular mode of membrane attachment. We now report that the trypanosomal lipase is capable of removing the lipid anchor of *Leishmania* p63, generating hydrophilic forms of p63 and VSG with similar kinetics. Moreover, after the removal of this membrane anchor by the lipase, p63 displays the complex carbohydrate known as the crossreacting determinant (CRD), which is present in the hydrophilic form of all VSGs (5, 9). Our findings show that p63 and VSG have a common membrane anchor and are structurally related.

MATERIALS AND METHODS

Materials. Promastigotes of *Leishmania major* LEM-513 and *Leishmania donovani* LEM-75 were maintained in modified Schaefer's medium (10), supplemented with heat-inactivated fetal bovine serum (2.5%). The modifications are described in ref. 11. *Trypanosoma brucei* variants ILTat 1.25 (12) and MITat 1.6 (also known as 121; ref. 13) were maintained as frozen stocks and were harvested from the blood of infected mice or rats by chromatography on DEAE-cellulose (DE-52, Whatman). The membrane form of the VSG (mfVSG) was prepared as described in ref. 9, and the amphiphilic form of p63 (ap63) as described in ref. 7. Biosynthetic labeling of promastigotes of *L. major* LEM-513 with [^{3}H]myristic acid and purification of radiolabeled ap63 by phase-separation in Triton X-114 (TX-114) and chromatography on DEAE-cellulose and Mono Q (Pharmacia) were performed as described (7). Phospholipase from *T. brucei* ILTat 1.25 was obtained by solubilization of VSG-depleted membranes in *n*-octyl glucoside, followed by covalent chromatography on Affi-Gel 501 (Bio-Rad), or by TX-114 extraction from VSG-depleted membranes, followed by phase-separation at 37°C (J.W. and C.B., unpublished method). Purified MITat 1.6 mfVSG was radioiodinated on an affinity column of antibody as described (14), and ap63 was radioiodinated on living *L. major* promastigotes and then purified to homogeneity (3, 7). Phospholipase C, type III, from *Bacillus cereus* was obtained from Sigma. Antibody to the CRD of *T. brucei* was prepared and affinity-purified as described (5, 9). *Staphylococcus aureus* protein A conjugated to horseradish peroxidase was from Miles–Yeda (Rehovot, Israel). Endoglycosidase F was a gift from J. Kaufman (Basel Institute of Immunology).

Gel Electrophoresis. NaDodSO$_4$/polyacrylamide gel electrophoresis was done according to the method of Laemmli (15). Gels were fixed and stained with 0.1% Coomassie blue or were prepared for ^{3}H fluorography by immersion in Amplify (Amersham). For immunoblotting, samples were fractionated by gel electrophoresis and were electrophoretically transferred to nitrocellulose paper, which then was stained with Ponceau S and photographed. After destaining, the nitrocellulose was saturated with 1% gelatin in TBS (10 mM Tris·HCl/150 mM NaCl, pH 7.4) and was then incubated overnight with anti-CRD (2 μg/ml) prior to detection with *S. aureus* protein A-horseradish peroxidase.

Phospholipase Digestions. Removal of [^{3}H]myristate from ap63 was monitored by incubation of 2 μg of the labeled ap63 in 160 μl of TBS with 0.5 mM dithiothreitol and 0.05% Triton X-100 (TX-100) for 1 hr at 30°C in the presence (0.45 μl/ml) or absence of affinity-purified lipase. After digestion, samples were heated and reduced prior to analysis by electrophoresis in a 7.5–15% polyacrylamide gradient gel. To monitor conversion of the amphiphilic forms of p63 and VSG

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Abbreviations: TX-114, Triton X-114; TX-100, Triton X-100; VSG, variant surface glycoprotein; mfVSG and sVSG, membrane-form and soluble VSG, respectively; CRD, crossreacting determinant of VSG; ap63 and hp63, amphiphilic and hydrophilic forms, respectively, of the p63 antigen of *Leishmania*.

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to hydrophilic forms, the two substrates were incubated in the presence or absence of TX-114-extracted lipase in a final volume of 50 μl of TBS containing 0.4% TX-114 and 0.04% sodium deoxycholate, for 30 min at 30°C. At the end of the reaction, 100 μl of 3% TX-114 in TBS was added. Samples were left for 3 min on ice, and then phase-separation was induced by incubation for 2 min at 37°C. After centrifugation at 15,000 × *g* for 30 sec at room temperature, the phases were collected in separate tubes and both adjusted to contain the same amounts of TBS and TX-114. These samples were then prepared for $NaDodSO_4$/polyacrylamide gel electrophoresis, without prior heating or reduction. To monitor the time course of conversion of the amphiphilic form to the hydrophilic form, 0.5-μg samples of ^{125}I-labeled ap63 (1200 cpm/μg) or mfVSG (15,500 cpm/μg) were incubated in TBS/0.05% Triton X-100 (final volume 20 μl) containing various concentrations of Affi-Gel-purified lipase from *T. brucei* ILTat 1.25, for 30 min at 30°C. After the incubation, phase-separation was carried out as described above. The radioactivity recovered in each phase was determined, and the fraction of the iodinated protein measured in the aqueous phase was normalized to the amount obtained at the plateau with 50 μl of lipase per ml (100% = maximal solubilization). The number of counts found in the aqueous phase at 50 μl of lipase per ml for these two preparations of p63 and VSG was, respectively, 80% and 40% of the input. These differences are attributed to the iodination procedure, since the percent of input radioactivity released varies for different mfVSG and ap63 preparations (data not shown). To monitor exposure of the CRD, purified ap63 and mfVSG were incubated in a final volume of 20 μl of TBS/0.06% TX-100, either in the absence or in the presence of phospholipase C (6 units/ml) from *B. cereus* or TX-114-extracted lipase (14 μl/ml) from *T. brucei* MITat 1.6, for 30 min at 30°C. At the end of the incubation, the samples were heated and reduced prior to $NaDodSO_4$/polyacrylamide gel electrophoresis and immunoblotting.

Endoglycosidase F Digestion of p63. Purified *L. major* ap63 (2 μg) was incubated in 10 mM Tris·HCl/10 mM EDTA/0.03% TX-100, pH 8.8, for 16 hr at 37°C, in the presence or absence of *Flavobacterium meningosepticum* endoglycosidase F. Affi-Gel-purified *T. brucei* phospholipase was then added (final concentration 0.5 μl/ml) to half of each sample and the incubations continued for 2 hr at 30°C. Samples were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis after heating and reduction. Proteins were transferred to nitrocellulose, stained, and, after destaining, subjected to immunoblotting with anti-CRD.

RESULTS

Both *Leishmania* p63 and *T. brucei* VSG contain a covalently attached phospholipid that can be hydrolyzed by phospholipase C of *B. cereus* (8, 10). In order to test whether the lipid moiety attached to p63 is susceptible to the trypanosomal phospholipase, purified p63 biosynthetically labeled with [^3H]myristic acid (7, 10) was incubated in the presence or absence of this enzyme and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. Similar amounts of protein were detected by Coomassie blue staining in both samples of p63 (Fig. 1*A*). However, the fluorographic image (Fig. 1*B*) of the same gel reveals that the tritiated label present on undigested p63 (lane 1) was removed from the protein by incubation with the lipase (lane 2). This experiment shows that the radiolabeled phospholipid found in *Leishmania* p63 is a substrate for *T. brucei* phospholipase.

It was also possible to evaluate the lipase digestion by using phase-separation in the presence of the nonionic detergent TX-114 (11). When an aqueous solution of TX-114 is warmed at 37°C, two phases separate; hydrophilic proteins are found in the aqueous phase, and molecules with amphiphilic properties are recovered in the detergent phase. This method has been used to separate two forms of both p63 and VSG (7, 9). Thus, ap63 and mfVSG, following incubation in the presence or absence of trypanosomal lipase, were phase-separated, and the contents of each phase were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. In the absence of lipase, ap63 and mfVSG were essentially recovered as expected in the detergent-enriched phases (Fig. 2 *Insets*, lanes 2), whereas after digestion with the lipase, both proteins were recovered in the aqueous phase (lanes 3). This result confirms the change in the amphipathic nature of both glycoproteins and also illustrates the typical decreased electrophoretic migration of unheated, unreduced hydrophilic p63 (hp63) and of soluble VSG (sVSG) (5, 7). By using ^{125}I-labeled ap63 and mfVSG, the relative rate of appearance of the respective hydrophilic products was quantitatively assessed as shown by the curves in Fig. 2. From the slopes of the curves, it appears that the specific activity of the lipase is approximately 3 times greater on mfVSG than on ap63. Given the intrinsic complexity of the glycophospholipid (8, 16), the possibility that radiolabeling might introduce some level of substrate damage, and the possibility that protein conformation in the two substrates might differ considerably, we believe that such small differences in reaction rate do not support a difference in substrate specificity.

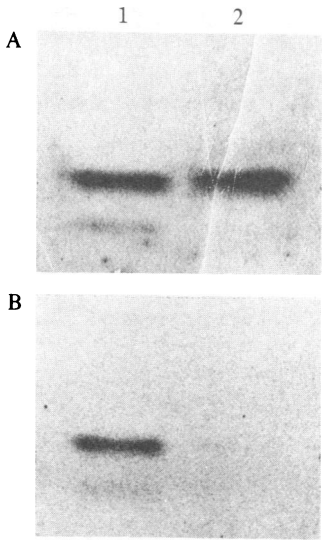

FIG. 1. Trypanosomal phospholipase removes the lipid biosynthetically incorporated into p63. Promastigotes of *L. major* LEM-513 were biosynthetically labeled with [^3H]myristic acid, and ap63 was purified by phase-separation in TX-114, followed by chromatography on DEAE-cellulose and Mono Q. Phospholipase from *T. brucei* ILTat 1.25 was obtained by solubilizing VSG-depleted membranes in *n*-octyl glucoside, followed by affinity purification on Affi-Gel 501. Two 2-μg aliquots of labeled p63 were incubated in the presence or absence of the lipase as described in *Materials and Methods*; after digestion, the two samples were heated and reduced and analyzed by $NaDodSO_4$/PAGE. Lanes 1 and 2 show, respectively, undigested p63 and lipase-digested p63 stained with Coomassie brilliant blue (*A*) or revealed by ^3H fluorography (*B*).

A second approach to demonstrate the similarity of both anchoring moieties was to examine the binding of both forms of p63 and VSG to an antibody recognizing the so-called crossreacting determinant (CRD) found on the VSG. The CRD is contained within a carbohydrate attached to the α-carboxyl group of the C-terminal amino acid through an amide linkage to ethanolamine (16–18). Since the CRD can be easily detected on sVSG but is a cryptic determinant in mfVSG, detection with anti-CRD on immunoblots has been used to probe VSG conversion (5, 19). Therefore, we decided to test on immunoblots the binding of anti-CRD to ap63 of *L. major* and *L. donovani* and to mfVSG of *T. brucei* before and

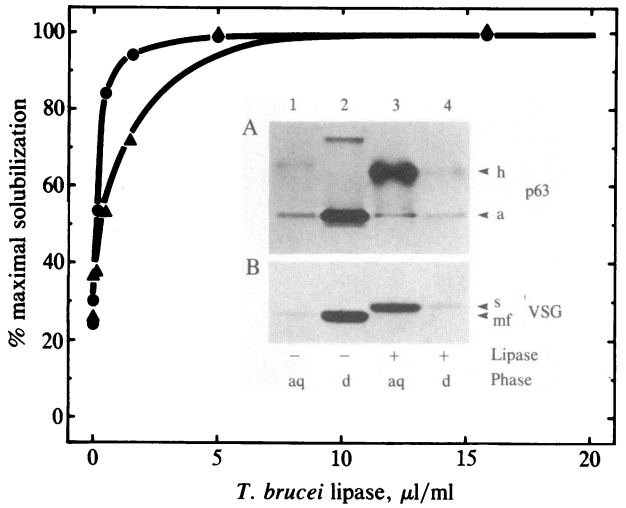

FIG. 2. Trypanosomal lipase generates hydrophilic forms of p63 and VSG with the same kinetics, and both products show typical decreased electrophoretic mobility on $NaDodSO_4$/PAGE. ap63 and mfVSG were purified from *L. major* LEM-513 and *T. brucei* ILTat 1.25 and MITat 1.6. (*Insets*) Both ap63 (*A*) and mfVSG (*B*) were incubated in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of TX-114-extracted lipase and then subjected to phase-separation (aq, aqueous phase; d, detergent phase) prior to $NaDodSO_4$/PAGE without prior heating or reduction of the samples. After electrophoresis, the proteins were stained with Coomassie blue. Arrowheads point to hydrophilic (h) and amphiphilic (a) p63, as well as to water-soluble (s) and membrane-form (mf) VSG. (*Graph*) Curves show the appearance of the hydrophilic forms of the glycoproteins as a function of the concentration of the phospholipase in the assay. This was performed using radioiodinated MITat 1.6 mfVSG (●) or ap63 (▲) as substrate. Following lipase digestion and phase-separation, the radioactivity recovered in each phase was determined.

after digestion with trypanosomal lipase or *B. cereus* phospholipase C. In comparing the amount of protein antigen (Fig. 3*A*) and the binding of anti-CRD (Fig. 3*B*), it becomes clear that the amphiphilic forms of *L. major* p63, *L. donovani* p63, and VSG did not bind the antibody (lanes 1, 4, and 7). However, following digestion by either *B. cereus* (lanes 2, 5,

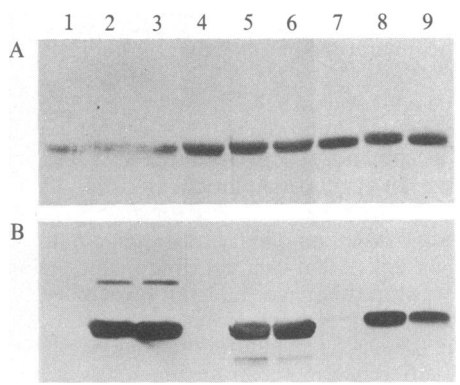

FIG. 3. After removal of the membrane anchor by lipase, p63 exposes CRD found in sVSG. Purified ap63 and mfVSG were treated with phospholipase C from *B. cereus* or *T. brucei*, as described in *Materials and Methods*, prior to analysis by $NaDodSO_4$/PAGE and immunoblotting. Lanes: 1–3, *L. major* p63; 4–6, *L. donovani* p63; 7–9, *T. brucei* ILTat 1.25 VSG. The samples shown in lanes 1, 4, and 7 were treated with no enzyme; those in lanes 2, 5, and 8 were digested with *B. cereus* phospholipase C; and those in lanes 3, 6, and 9 were digested with *T. brucei* lipase. (*A*) Ponceau S staining pattern of the proteins on the nitrocellulose. (*B*) Binding of anti-CRD to this immunoblot, revealed after incubation with peroxidase-conjugated protein A.

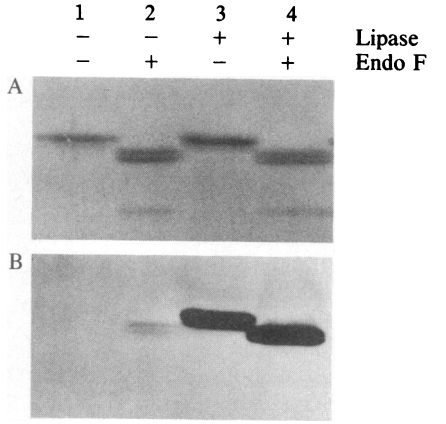

FIG. 4. Endoglycosidase F digestion of p63 and immunoenzymatic detection of CRD. After purified *L. major* ap63 was incubated with (lanes 2 and 4) or without (lanes 1 and 3) *F. meningosepticum* endoglycosidase F, samples were incubated in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of *T. brucei* phospholipase. Samples were analyzed by $NaDodSO_4$/PAGE after heating and reduction, and proteins were transferred to nitrocellulose paper and processed as in Fig. 3. (*A*) Ponceau S protein stain. (*B*) Immunoenzymatic detection of CRD.

and 8) or trypanosomal (lanes 3, 6, and 9) lipase, the CRD becomes exposed and binds the antibody with equally high affinity for all the three glycoproteins. The difference in migration rate between undigested and lipase-digested p63 is not so obvious as it is in Fig. 2 (*Inset A*) because the samples were heated and reduced prior to electrophoresis (7). The higher molecular weight proteins detected by anti-CRD on lanes 2, 3, 5, and 6 of Fig. 3 (and also seen in Fig. 2 *Inset A*, lane 2) have peptide maps (one-dimensional separation after cleavage with *N*-chlorosuccinimide) identical to those of p63 and are believed to correspond to dimers of p63 (data not shown). The experiment shown in Fig. 3 presents strong evidence that the complex carbohydrate found at the C terminus of all VSGs is also present on the major surface proteins of the two *Leishmania* species examined. In addition, the absence of anti-CRD binding to the phospholipid-containing p63 indicates that the lipid is bound to p63 through the CRD, as in the VSGs. At present, we have no further data on the structure of the p63 glycolipid, although in common with endoglycosidase H-treated VSGs, endoglycosidase F-treated hp63 still displays the CRD (Fig. 4), suggesting that this moiety is not an N-linked oligosaccharide (20).

Chemical analyses of VSGs have shown that the substrate of the *T. brucei* lipase contains a phosphatidylinositol moiety (8). Using ap63 as a substrate, we found that the *B. cereus* lipase activity was inhibited by 5 mM Zn^{2+} but not by 1 mM EDTA or *o*-phenanthroline (data not shown). This behavior is characteristic of the phosphatidylinositol-specific phospholipase C of *B. cereus* and suggests that the phospholipid of p63 may also contain inositol (refs. 21 and 22, and see discussion in ref. 8).

DISCUSSION

The role for a lipid anchor on p63 within the life cycle of the parasite *Leishmania* is not immediately obvious. Recent work suggests that p63 may play a role in leishmanial infection of the host cell, insofar as the binding of promastigotes to macrophages is inhibited by pretreatment of the macrophages with purified p63 (23). It may be that the carbohydrate component of the glycolipid anchor plays a role in this interaction. In contrast with the trypanosomes, an endogenous *Leishmania* lipase, which has as its substrate ap63, has not so far been characterized. Since macrophages

are known to contain phosphatidylinositol-specific phospholipase C of high activity (24), it may be that if the membrane anchor of p63 is cleaved at all, it is cleaved by a macrophage enzyme. In the African trypanosomes, VSGs are shed when bloodstream trypanosomes differentiate to a form lacking a surface coat (25). This presumably takes place through activation of the lipase in the bloodstream stage, since lipase synthesis is repressed following transformation (26). Shed sVSG in the bloodstream of the mammalian host of *T. brucei* is probably released from senescent, short, stumpy forms (27). It may be that after release from the membrane, sVSG and perhaps hp63 could play some role in the immunopathology of the diseases, as has been proposed for the *Leishmania* glycolipid, also implicated in macrophage binding (28).

This unusual membrane anchor could also provide a means for covering the surface of the parasite with a very large number of copies of a protein species (that can be rather diverse in amino acid sequence, in the case of VSGs) with minimal perturbation of the lipid bilayer of the plasma membrane. From the biosynthetic point of view, it is conceivable that the hydrophobic extension encoded in VSG mRNA might signal for the addition of a glycolipid and subsequent targeting of the protein to the cell surface. Other proteins, such as Thy-1 antigen (29) and *Torpedo* acetylcholinesterase (30), might be processed by a similar biosynthetic pathway. Furthermore, this type of glycolipid anchor might provide a more general and common mode of efficient release of membrane-bound proteins, by either endogenous or exogenous phospholipase.

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