Molecular Cloning and Characterization of a Novel Repeat-containing *Leishmania major* Gene, *ppg1*, That Encodes a Membrane-associated Form of Proteophosphoglycan with a Putative Glycosylphosphatidylinositol Anchor

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*Leishmania* parasites secrete a variety of proteins that are modified by phosphoglycan chains structurally similar to those of the cell surface glycolipid lipophosphoglycan. These proteins are collectively called proteophosphoglycans. We report here the cloning and sequencing of a novel *Leishmania major* proteophosphoglycan gene, *ppg1*. It encodes a large polypeptide of approximately 2300 amino acids. The N-terminal domain of approximately 70 kDa exhibits 11 imperfect amino acid repeats that show some homology to promastigote surface glycoproteins of the psa2/gp46 complex. The large central domain apparently consists exclusively of approximately 100 repetitive peptides of the sequence APSASSSSA(P/S)SSSSS(±S). Gene fusion experiments demonstrate that these peptide repeats are the targets of phosphoglycosylation in *Leishmania* and that they form extended filamentous structures reminiscent of mammalian mucins. The C-terminal domain contains a functional glycosylphosphatidylinositol anchor addition signal sequence, which confers cell surface localization to a normally secreted *Leishmania* acid phosphatase, when fused to its C terminus. Antibody binding studies show that the *ppg1* gene product is phosphoglycosylated by phosphoglycan repeats and cap oligosaccharides. In contrast to previously characterized proteophosphoglycans, the *ppg1* gene product is predominantly membrane-associated and it is expressed on the promastigote cell surface. Therefore this membrane-bound proteophosphoglycan may be important for direct host-parasite interactions.

*Leishmania* are kinetoplastid protozoan parasites with a digenetic life cycle and are responsible for several important human diseases, ranging from mild skin ulcers to fatal visceral disease. Several forms of extracellular flagellated *Leishmania* promastigotes colonize the digestive tract of vector sandflies. After transmission to the mammalian host by the bite of the insect, the parasites transform to the obligatory intracellular, nonflagellated amastigotes, which reside in the phagolysosomes of macrophages (1). Both *Leishmania* life stages synthesize complex and unique glycoconjugates, which are either displayed on their surface (2) or secreted (3). These glycoconjugates, which are thought to have crucial functions for parasite survival, development, and virulence in the sandfly vector and the mammalian macrophage, include a family of phosphoglycan-modified molecules, which comprises lipid-linked, protein-linked, and unlinked forms (4–6). Lipophosphoglycan (LPG)1 is the best studied representative of this family: it consists of 1-alkyl-2-lyso-phosphatidylinositol that is linked via a diphosphoheptasaccharide core to a linear polymer of up to 40 phosphodiester-linked disaccharides that are modified by species-, strain-, and stage-specific side chains. The polymer chain terminates with variable neutral oligosaccharides (2). LPG is expressed in high copy number on the surface of promastigotes (7–10), but its synthesis is strongly down-regulated in amastigotes (11–13). The lipid anchor and in particular the phosphoglycan chain of LPG exhibit a variety of biological activities, which may be important for *Leishmania* survival in the vector sandfly and in the mammalian host. These activities include receptor functions, protection against complement and hydrolases, and the modulation of macrophage killing mechanisms (14–22).

More recently, it has been found that the biologically active phosphoglycan structures of LPG are also present on several highly glycosylated *Leishmania* proteins, collectively termed proteophosphoglycans (PPGs, Refs. 5 and 6). This group of molecules includes secreted acid phosphatase synthesized by promastigotes of all *Leishmania* species except *Leishmania major* (23, 24), non-filamentous proteophosphoglycan of *Leishmania mexicana* amastigotes (25), and a filamentous proteophosphoglycan (fPPG) secreted by promastigotes of all species investigated so far (5, 26). Capped phosphoglycan chains are linked to the polypeptide backbone of these proteins via phosphodiester linkages to serine, an unusual type of protein modification called phosphoglycosylation (27–29). Among the PPGs, fPPG exhibits some unique features; the contour length of filaments observed by electron microscopy in purified preparations may exceed 6 μm. The fPPG protein backbone is largely formed by Ser, Ala, and Pro (together >87 mol%), and

1 The abbreviations used are: LPG, lipophosphoglycan; PPG, proteophosphoglycan; fPPG, filamentous PPG; mPPG, membrane-bound PPG; GPI, glycosylphosphatidylinositol; ELISA, enzyme-linked immunosorbent assay; DIG, digoxigenin; PBS, phosphate-buffered saline; TBS, Tris-HCl-buffered saline; TBS/T, TBS containing Tween 20; TBS/MT, TBS containing Tween 20 and skim milk powder; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; contig, group of overlapping clones; mAb, monoclonal antibody; PVDF, polyvinylidene difluoride; bp, base pairs; kb, kilobase pairs; ORF, open reading frame; RT, reverse transcription; SN, supernatant; P, pellet.
more than 40 mol% of its amino acids are phosphoglycosylated Ser residues. This extensive glycosylation leads to high protease resistance of fPPG and may also be responsible for its filamentous ultrastructure that is reminiscent of mammalian mucins (28). In a recent study, it has been shown that fPPG, initially observed in promastigote cultures (26), is also abundantly present in *Leishmania*-infected sandflies, where it forms a gel of three-dimensional filamentous meshworks that immobilizes the parasites and occludes the lumen of the insect digestive tract (30). This fPPG/parasite plug may be important for efficient transmission of metacyclic promastigotes to the mammalian host during the bite of the vector insect (see Refs. 30 and 31 and references therein).

In this paper we describe the identification of a *L. major* gene family whose members share common repetitive sequences encoding exclusively Ser, Ala, and Pro in ratios very similar to those found in fPPG. One of the genes (ppg1) was cloned and sequenced, and its expression and functional elements were analyzed. Our results suggest that ppg1 encodes a novel membrane-bound and possibly glycosylphosphatidylinositol (GPI)-anchored PPG that is present on the surface of *L. major* promastigotes.

**EXPERIMENTAL PROCEDURES**

**Parasites—**Promastigotes of *L. major* LRC-L137, clone 121, were grown in *vitro* in semisolid medium 79 (32) supplemented with 5% heat-inactivated fetal bovine serum. Cells were harvested at densities of 5 × 10⁹ to 10⁷/ml.

**DNA and RNA Techniques—**Agarose gel electrophoresis, isolation of λ-phage DNA, purification of genomic *Leishmania* DNA and total RNA, Southern and Northern blotting, λ-phage and bacterial colony lifts, antibody screening of a λgt11 expression gene library, restriction enzyme digests, DNA ligations, and transformation of *Escherichia coli* were performed according to standard methods (33, 34). Plasmid DNA and DNA fragments from agarose gels were isolated using commercial kits according to the manufacturers’ instructions (Qiagen, Hilden, Germany; Gene-Clean, Bio101, Vista, CA). Digoxigenin (DIG) labeling of DNA or RNA fragments was performed by random priming and by the polynucleotide chain reaction (PCR) or by in vitro transcription, respectively (Roche Molecular Biochemicals, Mannheim, Germany). DIG-labeled RNA or DNA probe was detected using anti-DIG-Fab monoclonal antibody or DIG-Fab goat antirabbit antibody (DIG labeled at the 3′-end with biotin) applied to the membrane (Roche) and CDP-StarTM (Tropix, Bedford, MA) as chemiluminescent substrate according to the manufacturer’s instructions. PCR was performed using the ExpandTM high fidelity PCR system (Roche). Reverse transcriptase PCR (RT-PCR) was performed from total *L. major* promastigote RNA using the Masterscript™ RT-PCR kit (Epitrend Technologies, Madison, WI).

**Cloning of ppg1—**A λgt11 expression library constructed from sheared genomic *L. major* DNA (36) was propagated in *E. coli* Y1090 and plated on agar dishes. Nitrocellulose filter lifts of λgt11 phage plaques were used for antibody screening by the PPG genes using a rabbit antiserum against HF-dephosphorylated secreted L. major TPPG (28) that had been preadsorbed four times with *E. coli* lysate (33). *L. major* DNA inserts were excised from DNA of positive λgt11 phages and cloned into NcoI/NdeI-cut pGEM5z+ (37). Isolated inserts were ligated and then used for Southern blot analysis or hybridization on bacterial filter lifts (see below). For the construction of dedicated genomic plasmid libraries *L. major* DNA was digested with *XhoI* or *SphI* and separated on 0.7% agarose gels. DNA fragments that corresponded to positive bands on Southern blots using the DIG-labeled λ-phage inserts as probes, were cloned into *XhoI*-cut pBSK*′* or *SphI*-cut pGEM5z+, respectively. Filter lifts from transformed *E. coli* XL1-Blue colonies were hybridized with DIG-labeled λ-phage inserts and the clones pBSK*′* ppg1/XhoI and pGEM5z*′* ppg1/SphI containing overlapping inserts were isolated (compare Fig. 3A). To obtain the entire ppg1 gene on one plasmid the XbaI/XhoI fragment of pGEM5z*′* ppg1/SphI (Fig. 3A) was ligated into XbaI/XhoI-cut pBSK*′*. This construct was cut with *XhoI* and ligated with the XhoI fragment from pBSK*′* ppg1/XhoI (Fig. 3A), yielding pBSK*′* ppg1/ORF. The correct orientation of the insert was confirmed by restriction digests and sequencing. Sequence analysis of the clones was performed by the dyeoxy chain termination method (38). Both DNA strands were sequenced in nonrepetitive regions. For sequencing of the large repetitive region, the central 4.75-kb Sau3AI fragment was isolated from pBSK*′* ppg1/XhoI and ligated into BamHI-cut pBSK*′*. The resulting construct (pBSK*′* ppg1/Sau3AI) was sequenced from both ends. Internal regions of the repeats were characterized by constructing exonuclease III/mung bean nuclease deleted clones (New England Biolabs, Schwalbach, Germany) of pBSK*′* ppg1/Sau3AI followed by sequencing. RT-PCR of the ppg1 3′-flanking region of this mutant oligonucleotide primed by a 5′-GTCTACAGGCTCTTGCTACCTG-3′ complementary to the *L. major* minienzyme consensus sequence (35) and the primer oligonucleotide TCCGTCCTTCCCTCATCTATCT-TC with an initial reverse transcription step of 60 °C for 20 min, followed by 1 min of denaturation at 95 °C, 1 min of annealing at 55 °C, and 1 min of extension at 72 °C for 35 cycles. Determination of the ppg1 splice site involved an initial RT-PCR reaction using an oligonucleotide complementary to the *L. major* minienzyme consensus sequence (35) and the primer oligonucleotide TCCGTCCTTCCCTCATCTATCT-TC containing within ppg1 coding region. Conditions were as described above except that the extension was done for 25 cycles and was followed by a second round of nested PCR using the same minienzyme primer and an oligonucleotide situated immediately downstream of the ppg1 start codon (GC-GTCTAGCATGATCCAAACGTTTGGT). The product was cloned into pBSK*′* and its primary structure determined by DNA sequencing.

**Expression Constructs—**For expression of *ppg1* encoding the *ppg1* ORF that includes part of the ampicillin gene was ligated with the XbaI/SphI-cut *Leishmania* expression vector pX (39) yielding pXppg1/ORF. For the generation of fusion constructs of the repetitive ppg1 domains to the 3′-region of a *L. mexicana* membrane-bound acid phosphatase (lmmnb, Ref. 40) whose transmembrane anchor sequence had been removed (modified lmmnb = modMAP, Ref. 41), the expression constructs were made using modMAP (41, 45). The correct orientation was confirmed by restriction digests and sequencing. For expression constructs, *L. major* promastigotes were transformed by electroporation as described previously (42) and grown in semisemidefinite medium 79 plus 5% heat-inactivated fetal bovine serum that was supplemented with 20–100 μg/ml G418 to select for plasmid-harboring parasites.

**Analytical Procedures—**Discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE, Ref. 45) was performed on 4% stacking gels over 12% separating gels. For electrophoresis of protein from ppg1/polyacrylamide gels (24) polyvinylidene difluoride (PVDF) membranes (Millipore, Eschborn, Germany) were used. For immunodetection of antigens on PVDF membranes, nonspecific binding sites were blocked for 1 h at 25 °C in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5% skimmed milk powder, 0.1% Tween 20 (TBS/MT). The monoclonal mouse antibody (mAb) LT6 (hybridoma supernatant), which recognizes the PO₄⁻1-phosphoglycan repeats, the biotinylated purified mAb L7.25, which recognizes the phosphoglycan cap structures (Man1–2)₃–Man9–1PO₄⁻ (24), the mAb WIC79.3 (ascites fluid), which recognizes PO₄⁻–[Galβ1–3]–6-Galβ1–4Manol–1-phosphoglycan repeats (44), and affinity-purified rabbit anti-C-region antibodies (1 mg/ml) were used as primary antibodies at dilutions of 1:20, 1:1000, 1:2000 and 1:1200, respectively in TBS/MT. After 3 washes with in 50 mM Tris-HCl, pH 7.5, 0.1% Tween 20 (TBS/MT), goat anti-mouse IgG or goat anti-rabbit IgG antibodies coupled to horseshadise peroxidase (Di-anova) or Extravidin Peroxidase (Sigma) were used at a 1:10000 dilution in TBS/MT. After 5 washes with TBS/MT, the bound enzyme conjugates were detected by chemiluminescence using the ECL detection system (Amersham-Pharma). Two-site ELISA of culture supernatants from *L. major* promastigotes transfected with pXmodMAP constructs were performed using mAb AP4-coated (50 μg/ml G418 to select for plasmid-harboring parasites). Membrane-bound Proteophosphoglycan of *L. major* 31411

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lowed by incubation with the biotinylated mAbs L7.25, WIC79.3, and WIC108.3 (1–2 μg/ml in TBS/MT, 100 μl/well, 1 h, 25 °C). After three washing steps with TBS/T, the ELISA plates were incubated with Extravidin-alkaline phosphatase (Sigma), diluted 1:5000 in TBS/T, 5% fish gelatin (Serva, Heidelberg, Germany), (100 μl/well, 1 h, 25 °C) followed by four washes with TBS/T, one wash with 1 M diethanolamine-HCl, pH 9.8, 1 mM MgCl₂, and incubation with 5 mM pNPP in the same buffer. Bound enzyme conjugate was measured by determination of the OD₄₀₅.

Production of Rabbit Antisera against Recombinant PPG1 Protein—A 1026-bp SalI/HindIII DNA fragment of the ppg1 5' region (N-domain) and a 905 bp SalI/HpaI fragment of the ppg1 3' region (C-domain) (Fig. 3A) were ligated in frame into SalI/HindIII-cut and SphI/Smal-cut pQE31, respectively. Hexa-His-tagged recombinant proteins expressed in E. coli M15 were purified using Ni-NTA-agarose columns according to the manufacturer’s instructions (Qiagen). Rabbits were immunized with 200 μg of purified recombinant protein by standard protocols (28). For immunoblotting studies, anti-N-domain antibodies as well as anti-C-domain antibodies were affinity-purified on recombinant protein that had been electrotransferred to PVDF membranes after SDS-PAGE as described previously (40).

Immunoprecipitation of PPG—50–100 μl aliquots of protein A-Sepharose beads (Amersham Pharmacia Biotech) were incubated in 1 ml of TBS/T with 2 μg at 25 °C with 200 μl of rabbit anti-SalI/HindIII serum, rabbit anti-C-domain serum, or rabbit preimmune serum. The beads were washed five times with TBS/T, 2% bovine serum albumin (BSA). L. major promastigotes (2 × 10⁸) were solubilized in 1 ml 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, containing protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 20 μl leupeptin, 10 μl o-phenanthroline) and 10 units/ml benzonuclease (Merck, Darmstadt, Germany). Insoluble material was removed by centrifugation, and the supernatants were added to the antibody-loaded protein A-Sepharose beads. After incubation for 16 h at 4 °C, the beads were washed five times with TBS/T, resuspended in 100 μl of 1% triton X-100, 6.8%, 2% SDS, 50 mM dithiothreitol and heated to 100 °C for 10 min. Samples (30 μl) were loaded onto SDS-polyacrylamide gels, electrotransferred onto PVDF membranes, and subjected to immunodetection.

Isolation of Gene Fragments from L. major Genomic DNA Encoding for Ser-, Ala-, and Pro-rich Peptide Repeats of PPG—Multiple attempts to generate peptide sequence information from L. major fPPG were unsuccessful (28), which precluded a gene cloning strategy using degenerate oligonucleotides. Therefore, a 1026-nt expression library constructed from sheared genomic L. major DNA was screened with a rabbit antisera directed against the 40% HF-deglycosylated protein backbone of fPPG (anti-HFP serum; Ref. 28). Three positive L. major clones were identified, subcloned into plasmid vectors, and mapped by restriction enzyme digests and partially sequenced. These clones appeared to consist exclusively of repetitive DNA sequence potentially encoding Ser-, Ala-, and Pro-rich peptide repeats of PPG.

RESULTS

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positive on Southern blots with the $\lambda gt11$ repeat probe (Fig. 2A).
To this end, two dedicated plasmid libraries of size-fractionated $L. major$ genomic DNA containing a 5.9-kb $XhoI$ fragment (Fig. 2A, lane 1) and a 20-kb $SphI$ fragment (data not shown), respectively, were constructed and screened with the $\lambda gt11$ repeat probe. Overlapping $XhoI$- and $SphI$ clones apparently containing a complete open reading frame were identified and a detailed restriction map was constructed (Fig. 3, A and B). One peculiar feature of the gene-containing Eco47 III/$XhoI$ $L. major$ DNA fragment (Fig. 3B) was the presence of a central Sau3AI/NcoI fragment (>4.6 kb), which was the smallest mapped fragment to react with the repeat DNA probe on Southern blots (data not shown; compare Figs. 2A and 3A, and see below). The nonrepetitive DNA in the 5'-region from this central fragment starting from the Eco47 III site and in the 3'-region from the central fragment ending at a $XhoI$ site was sequenced completely on both strands (Figs. 3B and 4). An open reading frame was identified upstream and just before of the central repeats that encompassed 1926 bp. The splice addition site of its mRNA was determined after sequencing a fragment derived from the region near the putative start codon (Fig. 4). The open reading frame upstream of the central repeats encoded a potential signal sequence for import into the membrane anchor addition (Figs. 4 and 5, A and C).

Characterization of the Central Repetitive $ppg1$ Domain—

The central Sau3AI/NcoI DNA fragment of $ppg1$ (Fig. 3B) was most unusual in being devoid of any palindromic hexanucleotide restriction sites (data not shown) and most palindromic tetranucleotide restriction sites, except for those of the enzymes AluI, HinPPII (Fig. 6A), and BstUI (data not shown). By sequencing both ends of the DNA fragment and by sequencing of exonuclease III deletion clones, more than 90% (>4.2 kb) of the very GC-rich (>73%) primary structure of the central repeat domain could be determined, but the perfection of the repeat sequence precluded the accurate identification of overlaps between individual sequencing runs. The data suggest, however, that the central repeat domain consists exclusively of an estimated 100 closely related 48- and 45-bp repeats, like those identified in the $\lambda gt11$ expression clones of the initial screen (Figs. 1 and 4). Digestion of the central repeat region (Fig. 3B) by the restriction enzyme AluI that cuts once per repeat leads to the exclusive appearance of fragments whose size is consistent with this assumption (Fig. 5B). The few variations that occur in the repeats mainly involve the third position of some codons and do not lead to amino acid changes, except for an occasional transition at a first codon position (C to T) leading to a change of Pro to Ser (Fig. 1).

Genomic Structure and Transcription of $ppg1$ and Related Genes—Southern blots of $L. major$ genomic DNA digested with a selection of restriction enzymes were hybridized with a DIG-labeled probe of the $ppg1$ sequence downstream (3') of the central repeats (NcoI/NcoI-DIG, compare Fig. 3B). These blots yielded a band pattern consistent with the presence of a single gene copy (Figs. 2C and 3, A and B). However, using a DIG-labeled probe of the domain upstream (5') from the central repeats, only about 50% of the observed fragments could be identified as being derived from $ppg1$ (Fig. 2B, marked with asterisks). In this Southern blot, the very large fragments (>23 kb), except for those derived from $XhoI$ and from NcoI digests, are most likely derived from related, but different genes, as are some smaller fragments in AatII, PstI, and $XhoI$ digests (Figs. 2B and 3A). With a DIG-labeled repeat probe, only a minority of the fragments could be identified as derived from $ppg1$ (Fig. 2A), while most fragments, in particular those of very large size (>23 kb, except for NcoI digests), as well as those of smaller size (1–3 kb) must also be derived from genes related but different from $ppg1$ (Figs. 2A and Fig. 3, A and B). Most remarkably, on Southern blots of $L. major$ DNA digested with palindromic tetranucleotide-cutting restriction enzymes probed with DIG-labeled $ppg1$ repeat DNA, the very large bands (>23 kb) were still present, except for those enzymes cutting frequently in the repeats, which only gave rise to very small fragments (Fig. 6C). These results suggest that in $L. major$ a multigene family is present whose members contain $ppg1$-type repeat sequences. These repeats may, in some of the genes, be longer than 23 kb. However, $ppg1$ is a distinct member of this multigene family in possessing a unique 3'-region (Fig. 2C).
To assess the expression of the ppg genes on the RNA level, a DIG-labeled RNA probe of the ppg1 repeats was used on a Northern blot of RNA from L. major promastigotes and mouse lesion-derived amastigotes (Fig. 7, A and B). This probe does not distinguish between different members of the ppg gene family. A broad smear showing maximal intensity in the size region larger than the 9.5-kb marker was observed in both samples, indicating the very large size of the ppg gene transcripts, which is in agreement with the Southern blot data (Fig. 7B; see also Fig. 2). A distinct transcript of the size predicted for the ppg1 gene (6.85 kb) was not observed, which may be due to the presence of a long 3'-untranslated region. To investigate specifically the ppg1 gene expression, RT-PCR was performed on L. major promastigote and amastigote total RNA using oligonucleotide primers derived from the specific 3'-region of the ppg1 gene. Fragments of the predicted size and sequence were amplified in both RNA preparations indicating that ppg1 gene transcripts are present in both L. major promastigotes and amastigotes (Fig. 7C).

Functional Analysis of the Putative GPI Membrane Anchor Addition Sequence—To determine whether the putative GPI anchor addition signal is functional, a DNA fragment encompassing this region was ligated in frame to the 3'-region of a modified L. mexicana membrane-bound acid phosphatase gene, which lacks the sequence encoding the...
transmembrane helix (modMAP; Refs. 40 and 41). The modMAP gene and the fusion construct were cloned into the Leishmania expression vector pX yielding pX-modMAP and pX-modMAP-GPI, respectively (Fig. 8A). L. major promastigotes transfected with a pX control vector contained, like untransfected cells, only a small amount of acid phosphatase and did not secrete any enzyme activity into the medium (Fig. 8B). Parasites transfected with pX-modMAP produced large amounts of this enzyme activity (>600 milliunits/ml culture, 5 × 10⁷ cells/ml) and secreted most of it (>97%) into the culture medium (Fig. 8B). L. major promastigotes transfected with pXmodMAP-GPI also synthesized considerable amounts of enzyme (>250 milliunits/ml culture, 5 × 10⁷ cells/ml), which was, however, largely associated with the cells (<80%) (Fig. 8B). In immunofluorescence experiments using a mAb directed against the modMAP tag (41, 45), these pXmodMAP-GPI transfected parasites showed a strong surface labeling (Fig. 9A), while wild type or pXmodMAP-transfected promastigotes did not show any signal on their cell surface (data not shown). All of the cell-associated modMAP-GPI protein was found in the membrane-containing pellet after ultracentrifugation of disrupted promastigotes (data not shown) and 90% of the activity partitioned into the detergent-rich phase in a Triton X-114 phase separation experiment (Fig. 8C). After incubation with GPI-specific phospholipase C from Trypanosoma brucei, however, the majority (>90%) of the acid phosphatase activity partitioned into the upper detergent-poor phase, which was a strong indication for the presence of a GPI anchor on modMAP-GPI (Fig. 8C).

Functional Analysis of the Central ppg1 Repeat Sequence—The central 4.75-kb Sau3AI fragment of ppg1 (Fig. 3B), which consists largely of repetitive sequence, was ligated in frame to modMAP gene and cloned into pX yielding pX-modMAP-repeat (Fig. 8A). L. major promastigotes transfected with this gene fusion construct synthesized acid phosphatase activity (>150 milliunits/ml culture, 5 × 10⁷ cells/ml). Approximately 70% of the enzyme was detected in the culture supernatant (Fig. 8B). Immunofluorescence microscopy of transfected promastigotes using mAb AP4 revealed a strong flagellar pocket labeling (Fig. 9, B and C) indicating secretion of the enzyme via this parasite organelle. It appeared likely that the Ser/Ala/Pro-rich peptide repeats encoded by the ppg1 Sau3AI DNA fragment were target peptide sequences for the extensive phosphoglycosylation
FIG. 6. Analysis of the central repeat domain of ppg1 by agarose gel electrophoresis and Southern blot. A, 1.8% agarose gel electrophoresis of the repeat-containing 4.75-kb Sau3AI ppg1 DNA fragment digested with various restriction enzymes cutting palindromic tetranucleotide sites separated by 0.7% agarose gel electrophoresis. The blot was probed with DIG-labeled DNA fragment digested with the following restriction enzymes: 4, RsaI; 5, NlaIII; 6, Msel; 7, HpaII; 8, HinPII; 9, HaeIII; 10, BfaI; 11, AluI; 12, undigested fragment. B, 2.5% agarose gel electrophoresis of the repeat-containing 4.75-kb Sau3AI ppg1 DNA fragment. 1, undigested; 2, AluI-digested; 3, DNA marker fragments (VIII, Roche Molecular Biochemicals) whose size in bp is indicated. C, Southern blot of L. major genomic DNA incubated with PstI and restriction enzymes cutting palindromic tetranucleotide sites separated by 0.7% agarose gel electrophoresis. The blot was probed with DIG-labeled ScaI/NcoI fragment (Fig. 3B). 1, PstI; 2, AluI; 3, BfaI; 4, HaeIII; 5, HinPII; 6, HpaII; 7, Msel; 8, NlaIII; 9, RsaI; 10, Sau3AI. Fragments derived from the ppg1 gene are marked with asterisks.

observed on fPPG (28). To test this hypothesis, similar amounts of the modMAP and modMAP-repeat proteins from promastigote culture supernatants were captured by the mAb AP4 onto microtiter plates and then analyzed for their binding of anti-phosphoglycan repeat (WIC79.3 and WIC108.3) and cap antibodies (L7.25) in an ELISA. While modMAP showed only marginal binding of L7.25 and no binding of the mAbs WIC79.3 and WIC108.3, all three antibodies strongly bound to modMAP-repeat, which demonstrated its modification by capped phosphoglycan chains (Fig. 8D). The enzymes modMAP and modMAP-repeat were purified by affinity chromatography on a column of mAb AP4 coupled to Sepharose CL-4B and inspected by electron microscopy. While modMAP consisted only of small globular particles of approximately 4 nm in diameter (Fig. 10c), modMAP-repeat also exhibited these globular particles, but they were attached to filamentous structures with a contour length of up to 250 nm (Fig. 10b). The identity of these molecules was confirmed by labeling with mAb AP4, which bound to the globular domain of modMAP-repeat (Fig. 10c).

Membrane Association and Phosphoglycosylation of ppg1—To investigate the expression of ppg1 in L. major promastigotes at the protein level, an antisera was raised against a recombinant peptide from the C-terminal non-repetitive domain of ppg1 that was not shared with other members of the PPG multigene family (anti-C-domain serum, see Figs. 2C and 3B). Using affinity-purified antibodies isolated from this serum, SDS-PAGE/immunoblots of total cell lysates from PBS-washed L. major promastigotes showed a strong signal in the region corresponding to the stacking gel and the top of the separating gel (Fig. 11, lane 1), a position typical for PPGs (25, 28, 29). This cell-bound PPG1 protein remained associated with the membrane pellet after disruption of the cells and ultracentrifugation (Fig. 11A, lanes 2 and 3). Further washing of the membranes with 10 mM EDTA-containing solutions and 10 mM EDTA plus 250 mM NaCl-containing solutions solubilized some of the compound (Fig. 11A, lanes 4 and 6, respectively), but most PPG1 protein remained membrane-bound (Fig. 11A, lanes 5 and 7). Importantly, the anti-C-domain serum did not recognize the secreted fPPG from L. major supernatant (Fig. 11A, lane 8). Therefore, we propose the term mPPG for this novel membrane-bound PPG1 from L. major. Using the anti-phosphoglycan mAb LT6, which does recognize secreted fPPG but not cell surface LPG of L. major procyclic promastigotes (Fig. 11B, lane 8; compare also Refs. 28 and 30), the typical PPG signal pattern is also obtained on SDS-PAGE/immunoblots of L. major lysates (Fig. 11B, lanes 1–7). A significant proportion of this PPG remains associated with the membrane pellet throughout the different washing steps (Fig. 11B, lanes 3, 5, and 7), which indicates that it may be identical to mPPG. In immunofluorescence microscopy of fixed L. major promastigotes, most cells showed a strong signal on their cell surface using anti-C-domain serum (Fig. 9E) compared with preimmune serum (Fig. 9D), indicating that at least part of membrane-bound mPPG is localized on the parasite cell surface. In labelings with the mAb LT6, most cells showed the expected surface fluorescence, which was, however, highly heterogeneous in its intensity (Fig. 9F). Cell lysates of L. major promastigotes that were transfected with a pX expression vector carrying a copy of ppg1 (pXppg1-ORF) showed a much stronger signal in the stacking gel region on SDS-PAGE/immunoblots using anti-C-domain antibodies than wild type cells (Fig. 11, compare A and C, lanes 1), which confirmed that ppg1 encodes
mPPG. Most of the mPPG in the overexpressing *L. major* strain remained also associated with the membrane fraction after repeated washing and ultracentrifugation (Fig. 11C, lanes 3, 5, and 7), but, more pronounced than in wild type cells, a significant proportion could be extracted by EDTA-containing buffer (Fig. 11C, lanes 4 and 6). In addition to a signal in the stacking gel region, some bands reactive with anti-C-domain antibodies were visible that migrated in SDS-PAGE with an apparent molecular mass lower than 30 kDa (Fig. 11C) and most likely represented partial proteolysis of the mPPG C-terminal part.

The phosphoglycosylation of mPPG with LPG/fPPG-type glycans was demonstrated by detection of mPPG immunoprecipitates (Fig. 8). Functional analysis of the *ppg1* central repeats and the putative GPI anchor addition signal. A, schematic diagram of gene fusion constructs of *L. mexicana* modMAP and parts of the *L. major* *ppg1* gene. B, acid phosphatase activity associated with cells and culture supernatant of *L. major* promastigotes transfected with pX constructs as indicated. The bars represent the average of duplicate assays. C, Triton X-114 phase separation of membrane-bound acid phosphatase from *L. major* transfected with pXmodMAP-GPI, with and without previous incubation with GPI-specific phospholipase C from *T. brucei*, and of enzyme activity released into the culture supernatant. The bars represent the average of duplicate assays. D, ELISA of mAb AP4-bound acid phosphatase (AP) released from *L. major* promastigotes transfected with pXmodMAP-repeat and pXmodMAP using the anti-phosphoglycan cap and repeat mAbs L7.25, WIC79.3, and WIC108.3. The bars represent the average of quadruplicate assays. The standard error is indicated.

**Fig. 9.** Immunofluorescence microscopy of *L. major* promastigotes and electron microscopy of purified modMAP-repeat. A–F, immunofluorescence. A, *L. major* pXmodMAP-GPI labeled with mAb AP4; B, *L. major* pXmodMAP-repeat labeled with mAb AP4; C, same specimen as in B, but stained for DNA with 4,6-diamidino-2-phenylindole. The arrows in B and C indicate the position of the AP4 fluorescence signal and the position of the kinetoplast, respectively; D, *L. major* wild type labeled with rabbit preimmune serum (diluted 1:100); E, *L. major* wild type labeled with rabbit anti-C domain serum (diluted 1:100); F, *L. major* wild type labeled with mAb LT6. Arrows point to promastigotes with weak surface fluorescence.

**Fig. 10.** Electron microscopy of glycerol-sprayed and rotary metal shadowed modMAP and modMAP-repeat proteins. A, unlabeled modMAP; B, unlabeled modMAP-repeat; the arrow points to the globular modMAP domain; C, mAb AP4-labeled modMAP-repeat; the left arrow indicates the modMAP globular domain with bound mAb AP4. The bar represents 100 nm.
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Fig. 11. Analysis of soluble and membrane-associated components of *L. major* promastigotes by 7.5–20% SDS-PAGE/immunoblot. Soluble (S) and membrane-associated (P) components were obtained as described under “Experimental Procedures,” separated by SDS-PAGE, and electroblotted onto PVDF membranes. Lane 1, promastigote lysate; lane 2, S1; lane 3, P1; lane 4, S2; lane 5, P2; lane 6, S3; lane 7, P3. In lanes 1–7, the equivalent of 10⁸ promastigotes was loaded in each lane. Lane 8, 2 µg of purified *L. major* mPPG. The border between the stacking gel and the separating gel is marked by an arrow. The molecular masses of standard proteins and the expected positions of PPGs are indicated.

Fig. 12. Analysis of *ppg1* immunoprecipitates from *L. major* cell lysates by 7.5–20% SDS-PAGE/immunoblot and schematic diagram of mPPG. Immunoprecipitates from *L. major* cell lysates using rabbit preimmune serum (lanes 1), rabbit anti-N-domain serum (lanes 2), and rabbit anti-C-domain serum (lanes 3) were separated on 7.5–20% SDS-polyacrylamide gels, electroblotted onto PVDF membranes, and probed with mAb L7.25 (A), mAb WIC79.3 (B), and mAb LT6 (C). The border between the stacking gel and the separating gel is marked by an arrow. The molecular masses of standard proteins and the position of mPPG are indicated. D, schematic drawing of plasma membrane-associated mPPG and LPG. The putative length of the O-phosphoglycosylated mPPG repetitive domain and the LPG phosphoglycan chain are indicated.

DISCUSSION

Promastigotes of most *Leishmania* species secrete a network-forming filamentous PPG that accumulates in the center of cell aggregates in culture (26). In infected sandflies, PPG is present in the lumen of the digestive tract, where it may play a crucial role for parasite transmission from the insect to the mammalian host (30). In the present study we have attempted to clone the PPG gene by antibody screening of a *L. major* genomic expression library. This approach led to the identification of repetitive DNA fragments that encode exclusively for Ser, Ala, and Pro in ratios in line with the known composition of PPG. However, Southern blot analysis indicated that such repeats are present in several *L. major* genes. One of these genes was cloned and characterized in this study; it encodes a large protein of approximately 2300 amino acids with three distinct domains. The N-terminal polypeptide chain of 642 amino acids contains a putative signal sequence for import into the endoplasmic reticulum with 5 positive charges in its N-region (46), which is typical for signal peptides of *Leishmania* proteins (47). It also exhibits 11 imperfect 24 amino acid repeats located between amino acid 153 and amino acid 600. These peptide repeats show some homology to repeats of the psa2/ppg46 gene family of GPI-anchored promastigote cell surface proteins (48, 49). The N-terminal domain continues into a large central domain of approximately 1500 amino acids, which is encoded by approximately 4.5-kb GC-rich repetitive DNA. The perfection of the DNA repeats precluded the determination of overlaps and the generation of a contig during DNA sequencing. However, the sequence information of more than 90% of the repeats and restriction enzyme digests suggest that the central domain consists exclusively of approximately 100 closely related 45- and 48-bp repeats encoding exclusively for Ser, Ala, and Pro (Figs. 1 and 4). Remarkably, less than half of the codons in these repeats show variations, and the variation is confined to transitions of the third position (G to A; T to C) and do not cause amino acid changes, except for an occasional first position transition (C to T) leading to a change of PRO to Ser (Fig. 1). Southern blots of genomic DNA purified from one *L. major* strain over a period of 4 years show no evidence of rearrangements in the central *ppg1* domain. How and why *L. major* maintains this long stretch of almost perfect repeats is currently unknown.

The presumed role of the derived Ser-, Ala-, and Pro-containing peptide repeats (Fig. 1) as attachment sites for phosphoglycosylation was confirmed by fusing the central repetitive *ppg1* domain to the gene encoding a modified acid phosphatase (*modMAP*) of *L. mexicana* (40, 41) resulting in the chimeric construct *modMAP*-repeat. Expression of *modMAP*-repeat in *L. major* yields a phosphoglycosylated product and antibody binding data suggest that the modification pattern resembles that of PPG (28). In addition, our study shows that the phosphoglycosylated APSASSSSA(P/S)SSSSS(±S) repeats give rise to filamentous structures that are similar to the extended phosphoglycosylated domains of SAP2 (50) and PPG (28) as well as the highly glycosylated domains of mammalian mucins and proteoglycans (51).
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Southern blot analysis suggests that the DNA sequence encoding the short C-terminal PPG1 domain of approximately 16 kDa is unique. Antibodies directed against this domain do not recognize the network-forming secreted fPPG, which suggests that this polypeptide is not encoded by ppp1, but most likely by other as yet unidentified members of the ppg gene family. The PPG1-specific anti-C domain antibodies react on immunoblots with a predominately membrane-associated high molecular weight product of L. major promastigote lysates, and they bind to the surface membrane of fixed promastigotes. These results suggest that the ppp1 gene encodes a novel surface membrane-bound PPG species, for which we propose the name mPPG. There is evidence that the membrane association of mPPG is via a GPI anchor. The C-terminal hydrophobic peptide sequence of mPPG reassembles known GPI anchor addition sequences. Second, fusion of this C-terminal mPPG peptide to a secreted version of a L. mexicana membrane-bound acid phosphatase (41) and its expression in L. major promastigotes lead to GPI-phosphophasel C-sensitive membrane association and cell surface localization of this enzyme. Attempts to directly demonstrate a GPI anchor on mPPG by GPI-phosphophasel C digestion and Triton X-114 phase separation of promastigote membranes led to inconclusive results, possibly due to poor solubility of membrane-bound mPPG in this detergent. Although we have evidence that mPPG can be biosynthetically labeled with [3H]ethanolamine,2 unequal confirmation of the GPI anchor on mPPG will most likely require purification of this molecule for structure analysis.

A variety of studies over the last 15 years have established LPG as the dominant cell surface glycoconjugate of Leishmania promastigotes and many functions have been proposed for this complex glycolipid (2, 52). The identification of a membrane-bound form of PPG, which shares several phosphoglycan repeat and cap structures with LPG, raises the question whether it may also exhibit some of its biological and pharmacological activities. In particular, mPPG may be, due to its unusual structure, an ideal molecule as a complement acceptor and as a potential receptor and complement binding sites, since it may also exhibit some of its biological and pharmacological activities. In particular, mPPG may be, due to its unusual structure, an ideal molecule as a complement acceptor and as a potential receptor and complement binding sites, since it may also exhibit some of its biological and pharmacological activities.

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