# Proteophosphoglycans of *Leishmania mexicana*

## Molecular cloning and characterization of the *Leishmania mexicana ppg2* gene encoding the proteophosphoglycans aPPG and pPPG2 that are secreted by amastigotes and promastigotes

Ulrich GÖPFERT, Nathan GOEHRING, Christian KLEIN and Thomas ILG[1]

Max-Planck-Institut für Biologie, Abteilung Membranbiochemie, Corrensstrasse 38, D-72076 Tübingen, Germany

Intracellular amastigotes of the pathogenic protozoon *Leishmania mexicana* secrete an extensively phosphoglycosylated proteophosphoglycan (aPPG) into the phagolysosome of mammalian host macrophages, that appears to fulfil important functions for the parasites. Promastigotes (the sandfly vector forms) of the same species secrete a proteophosphoglycan with identical protein backbone but exhibiting stage-specific phosphoglycosylation patterns [Klein, Göpfert, Goehring, Stierhof and Ilg (1999) Biochem. J. **344**, 775–786]. In this study we report the cloning of the novel repeat-containing proteophosphoglycan gene *ppg2* by antibody screening of a *Leishmania mexicana* amastigote cDNA expression library. *ppg2* is equally expressed in promastigotes and amastigotes at the mRNA level. Targeted gene replacement of both alleles of the single copy gene *ppg2* results in the loss of pPPG2 expression in promastigotes. Antisera against *Escherichia coli-expressed ppg2* recognize the deglycosylated forms of aPPG as well as pPPG2. These results confirm that *ppg2* encodes the protein backbones of aPPG and pPPG2. An unusual finding is that *ppg2* exhibits two stable allelic forms, *ppg2a* and *ppg2b*. Their main difference lies in the number of central 72 bp DNA repeats (7 versus 8). *ppg2a* and *ppg2b* encode polypeptide chains of 574 and 598 amino acids, respectively, that show no homology to known proteins. The novel 24 amino acid Ser-rich peptide repeats encoded by the 72 bp DNA repeats are targets for Ser phosphoglycosylation in *Leishmania mexicana*.

Key words: gene replacement, O-glycosylation, peptide repeats.

## INTRODUCTION

Developmentally regulated glycoconjugates of the digenetic parasitic protozoa *Leishmania* are considered to be major determinants of virulence in the sandfly vector and the mammalian host [1,2]. Among these glycoconjugates, a family of phosphoglycan-modified molecules has been a major focus of attention. This family consists of the structurally well-studied lipophosphoglycan (LPG), phosphoglycan (PG) and several protein-bound PGs collectively termed proteophosphoglycans (PPGs). The PPGs include secreted acid phosphatase (SAP), filamentous proteophosphoglycan (fPPG) and membrane-bound proteophosphoglycan (mPPG), all of which are primarily synthesized in the sandfly vector stages (promastigotes) of the parasites, as well as a non-filamentous proteophosphoglycan (aPPG) that is a major secretory product of the mammalian stage (amastigote) (reviewed in [3–5]).

It has been proposed that the assembly of PGs in the parasites may provide targets for the rational development of new *Leishmania*-specific drugs [1]. Considerable efforts have been made to elucidate the biosynthetic pathway of the lipid-linked PGs in LPG. Some studies have used biochemical approaches [6–13] while others have depended on screening for LPG-negative mutants [14] and expression cloning of genes restoring LPG biosynthesis [15–17].

Much less is known about the biosynthesis of the PPGs, since a prerequisite for such studies is sequence information about their protein backbones. The genes encoding SAP from *L. mexicana* (*lmsap1* and *lmsap2* [18]) and *L. donovani* (*Ld-sAcP-1* and *Ld-sAcP-2* [19]) have been cloned and shown to be closely related. More recently, the gene of *L. major* mPPG (*ppg1* [20]) has also been identified. *ppg1* is a distinct member of a multigene family unrelated to the SAP genes that also most likely includes the gene(s) encoding fPPG ([20], and unpublished results). A hallmark of the deduced products of these five PPG genes is the presence of Ser-rich repeats varying in length from 32 amino acids in the case of SAP1 to more than 1500 amino acids in the case of mPPG (reviewed in [4,5]). For *L. mexicana* SAP1, SAP2 and *L. major* mPPG, it has been shown that these peptide repeats serve as substrates for the novel and *Leishmania*-specific attachment of phosphoglycans to Ser residues via phosphodiesters, a modification called phosphoglycosylation [20–23]. In a recent study using *L. mexicana* SAP repeat peptides as substrates, the first step of phosphoglycosylation in *L. mexicana* has been identified and shown to involve a transfer of Man$\alpha$1-P to Ser residues [24a].

In this study we describe the identification and cloning of the novel PPG gene *ppg2* from *L. mexicana*. Part of the *ppg2* sequence predicts Ser-rich 24 amino acid repeats that direct phosphoglycosylation when fused to another protein, but are

Abbreviations used: LPG, lipophosphoglycan; PG, phosphoglycan; PPG, proteophosphoglycan; aPPG, amastigote proteophosphoglycan; pPPG2 promastigote proteophosphoglycan 2; fPPG, filamentous PPG; mPPG, membrane-bound PPG; SAP, secreted acid phosphatase; PCR, polymerase chain reaction; DIG, digoxigenin; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; TBS, Tris/HCl buffered saline; TBS/T, TBS containing 0.1 % Tween-20; TBS/MT, TBS containing 0.1 % Tween-20 and 5 % skim milk powder; pNPP, *para*-nitrophenylphosphate; SDS, sodium dodecyl sulphate; SDS/PAGE, discontinuous polyacryl gel electrophoresis in the presence of SDS; EDTA, ethylendiamine tetraacetate.

[1] To whom correspondence should be addressed (e-mail thomas.ilg@tuebingen.mpg.de).

The DNA sequences identified in this study have been submitted to the EMBL database under the accession numbers AJ245667, AJ245668 and AJ245669.

unrelated to the phosphoglycosylated peptides of SAP and mPPG. Immunoblots, Northern blots and targeted gene replacement experiments suggest that *ppg2* encodes the protein backbones of aPPG and pPPG2 [24b] secreted by *L. mexicana* amastigotes and promastigotes, respectively.

## MATERIALS AND METHODS

### Parasites

Promastigotes of *L. mexicana* MNYC/BZ/62/M379, *L. donovani* LV9, *L. amazonensis* LV78 and *L. major* LRC137 were grown in semi-defined medium 79 (SDM) supplemented with 4% inactivated fetal calf serum as described [25]. *L. mexicana* amastigote-infected tissue was obtained from dorsal lesions of CBA mice infected 3–6 months' previously with $5 \times 10^6$–$10^7$ stationary phase promastigotes in the shaven rump at the base of the tail.

### DNA and RNA techniques

Agarose gel electrophoresis, isolation of λ-phage DNA, purification of genomic *Leishmania* DNA, Southern and Northern blotting, λ-phage and bacterial colony lifts, restriction enzyme digests, DNA ligations and transformation of *E. coli* were performed according to standard methods [26]. *L. mexicana* promastigote RNA was prepared by the acidic phenol extraction method [27]. Plasmid DNA was isolated by alkaline lysis followed by anion-exchange chromatography (Qiagen, Hilden, Germany). DNA fragments isolated from agarose gels were purified by adsorption to glass beads (Gene-Clean, Bio101, Vista, CA, U.S.A.). Digoxigenin (DIG)-labelling of DNA fragments was performed either by random priming (cDNA1.1-DIG) or by the polymerase chain reaction (PCR) as described by the manufacturer (Roche, Mannheim, Germany). The following primer pairs were used for the generation of DIG-labelled probes by PCR: P1 and P2 (see below) for 5′-UTR-DIG, P3 and P4 (see below) for 3′-UTR-DIG, CCTTCCCATCGGTTGCTTTA and ATTGTCTTCCGCCTTCCAGT for 5′-region DIG, GCGGAAGACAATCCGCCTAT and TATCAGCTACCCCTTCTCCA for repeats DIG, TGGAGGGAGGGCGTCTGTGA and TCTGAGGCGAACAAAGAGAA for 3′-region DIG, the *hyg* gene-derived GGTCGCGGAGGCCATGGATG and a T7 promoter primer binding to flanking vector sequence of pBSK$^+$ (AATACGACTCACTATAGGG) for the hyg-DIG probe, the neo gene-derived TGCCGGGGCAGGATCTCCTG and T7 promoter primer for the neo-DIG probe. DIG-labelled RNA was synthesized by *in vitro* transcription of plasmid inserts using a RNA DIG labelling kit (Roche). DIG-labelled DNA or RNA on blots was detected using anti-DIG-$F_{ab}$ fragments coupled to alkaline phosphatase (Roche) and CDP-Star™ (Tropix, Bedford, MA, U.S.A.) as chemiluminescent substrate according to the manufacturer's instructions. PCR was performed using the Expand™ High fidelity PCR system (Roche).

### Construction of a λZAPII *L. mexicana* amastigote cDNA library

Lesion tissue from *L. mexicana*-infected CBA mice (6.3 g) was homogenized by repeated passage through a steel net and resuspended in 25 ml SDM containing 10 μg/ml bovine pancreas ribonuclease A (RNase A) to digest host RNA. Large tissue fragments were removed from the supernatant by low-speed centrifugation (100 ***g***, 5 min). The pellet was resuspended in 5 ml SDM containing 10 μg/ml RNase A, homogenized and centrifuged as described above and the pellet discarded. The combined supernatants were centrifuged (2000 ***g***, 15 min), the amastigote-containing pellet was resuspended in 10 ml SDM containing 10 μg/ml RNase A and homogenized by the use of a potter homogenizer (5 strokes, pistill size B) to destroy residual host cells. After centrifugation (2000 ***g***, 15 min) and one washing step with 15 ml SDM without RNase A (2000 ***g***, 15 min), the amastigote-containing pellet (1 g) was inspected microscopically for mouse lymphocyte contamination, which was $< 0.1\%$. Purification of total amastigote RNA (340 μg) was performed essentially as described by Chomczynski and Sacchi [27] and poly(A) RNA (12.4 μg) was subsequently isolated by oligo(dT) cellulose chromatography [26]. cDNA was synthesized from amastigote poly(A) RNA, ligated to *Eco*RI adaptors and the 5′ ends phosphorylated by T4-polynucleotide kinase according to the manufacturer's instructions (cDNA synthesis kit, Stratagene). Excess adaptors were removed by chromatography on Sephacryl S300 spun columns (Amersham–Pharmacia, Freiburg, Germany). 50 ng cDNA was ligated to *Eco*RI-cut and dephosphorylated λZAPII phage DNA (Stratagene), *in vitro* packaged into λ phage (Gigapack Gold II, Stratagene) and plated using *E. coli* XL1 Blue McrA$^-$, McrB$^-$ as bacterial host strain. The resulting unamplified λZAPII *L. mexicana* amastigote cDNA library contained $3.8 \times 10^6$ independent recombinant phages.

### Antibody screening of a λZAPII *L. mexicana* amastigote cDNA library and cloning of genomic copies of the *ppg2* gene

Nitrocellulose filter lifts (Schleicher und Schuell, Dassel, Germany) of isopropylthio-β-D-galactoside-induced phage plaques [26] derived from a λZAPII *L. mexicana* amastigote cDNA library were used for antibody screening for the aPPG gene. The filters were washed with Tris-buffered saline (TBS, 50 mM Tris/HCl, pH 7.5, 150 mM NaCl), 0.05% Tween-20 for 30 min followed by blocking of non-specific binding sites by incubation with phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8 mM $Na_2HPO_4$, 1.4 mM $KH_2PO_4$, pH 7.4), 10% skim milk powder, 0.05% Tween-20 (1 h, 25 °C). Anti-aPPG mouse serum M1.1 [24b] was added to the filters at a 1:1000 dilution in PBS, 10% skim milk powder, 0.05% Tween-20 (2 h, 25 °C). After 3 washing steps in PBS, 0.05% Tween-20, positive clones were visualized by incubation with alkaline phosphatase-coupled goat anti-rabbit IgG antibodies (Dianova, Hamburg, Germany) diluted 1:5000 in PBS, 10% skim milk powder, 0.05% Tween-20 followed by 5 washings with PBS, 0.05% Tween-20 and colour development using 0.5 mM 5-bromo-4-chloro-3-indolylphosphate and 0.5 mM nitroblue tetrazolium dissolved in 50 mM $NaHCO_3$/NaOH, pH 9.6, 2 mM $MgCl_2$. Positive phages were subjected to two more rounds of antibody screening to obtain pure clones. The generation of filamentous phages and pBSK-phagemids from λZAPII phage clones containing *ppg2*-cDNA was performed by *in vivo* excision using a helper phage as described by the manufacturer (λZAPII cloning kit, Stratagene). For the construction of dedicated genomic plasmid libraries *L. mexicana* DNA was digested with *Eco*RI and separated on 0.7% agarose gels. Regions on the gels that corresponded to positive bands on Southern blots using DIG-labelled *ppg2* cDNA as a probe, were cut out, the DNA fragments were purified and cloned into *Eco*RI-cut pBSK$^+$. Filter lifts (cationized nylon, Roche) from transformed *E. coli* XL1-Blue colonies were hybridized with DIG-labelled aPPG cDNA and several clones (pBSK$^+$*ppg2a*/*Eco*RI or pBSK$^+$*ppg2b*/*Eco*RI) containing the complete *ppg2* gene were isolated. Sequence analysis of both DNA strands was performed by the dideoxy chain termination method [28] using the Autoread™ sequencing kit (Amersham–Pharmacia, Freiburg, Germany), the Thermosequenase sequencing kit (Amersham–Pharmacia) or a Dye

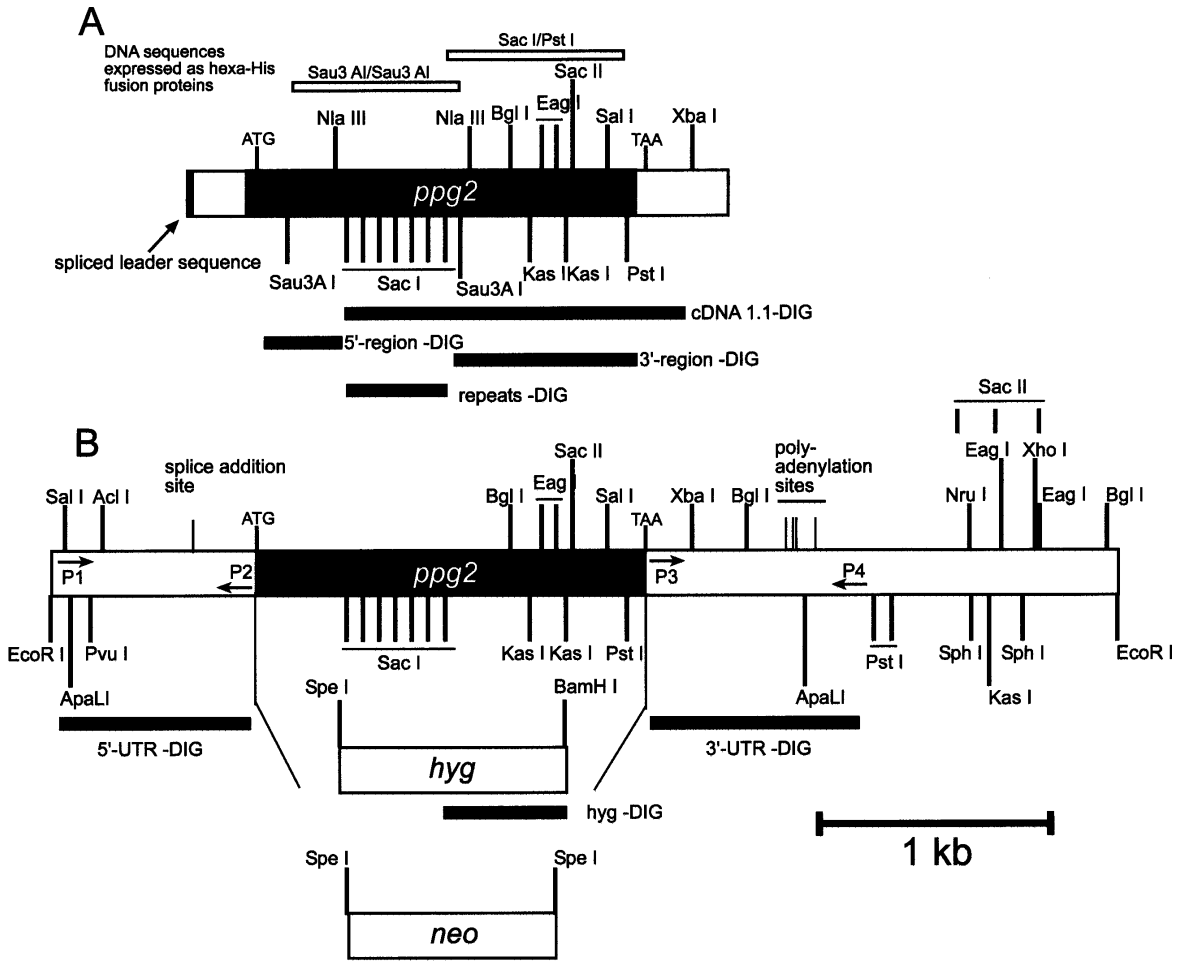



**Figure 1 Schematic diagram of a *ppg2* cDNA clone and of the ppg2 genomic locus**

Map of cDNA 6.1 (**A**) and the genomic *ppg2* locus (**B**) based on restriction enzyme sites relevant for Figures 4 and 5 and for the generation of *ppg2* null mutants. For the enzymes *Nla*III and *Sau*3AI only the sites closest to the repeat region are indicated. DIG-labelled DNA probes used for hybridizations are shown by the thin black bars. DNA fragments expressed in *E. coli* as hex-His fusion proteins are shown as thin white bars. The positions of the start and the stop codons (**A, B**), the spliced leader sequence (**A**), the splice addition site (**B**) and the polyadenylation sites (**B**) are indicated. Primers used for the PCR amplification of 5′-untranslated and 3′-untranslated DNA fragments of the *ppg2* locus as well as the antibiotics resistance genes (*neo* and *hyg*) that were used for the generation of *ppg2* gene replacement constructs are highlighted by arrows and white bars, respectively.

Terminator sequencing kit (Applied Biosystems) and either an ALF DNA-sequencing system (Amersham–Pharmacia) or an ABI 373 DNA-sequencing system (Applied Biosystems).

### Production of rabbit antisera against recombinant PPG2 protein

A 809 bp *Sac*I/*Pst*I DNA fragment and a 924 bp *Sau*3AI fragment of *ppg2a* were ligated in frame into the *Sac*I/*Pst*I-cut or *Bam*HI-cut pQE31 expression plasmids (Qiagen), respectively. Induction of transformed *E. coli M15* cells with 2 mM isopropylthio-$\beta$-galactoside led to high level expression of hexa-His-tagged recombinant protein, which was purified using Ni–NTA–agarose columns according to the manufacturer's instructions (Qiagen). Rabbits were immunized with 200 $\mu$g of purified recombinant protein dissolved in 8 M urea, 50 mM $NaH_2PO_4$, pH 4.8, emulsified with 1 ml 50% (v/v) complete Freund's adjuvant in phosphate-buffered saline (PBS), pH 7.4, for primary immunizations and 50% incomplete Freund's adjuvant (v/v) in PBS for all subsequent booster immunizations. Serum was obtained 10–14 days after each booster immunization yielding antisera against *E. coli*-expressed polypeptide of the *Sac*I/*Pst*I fragment (anti-PPG2 C-domain serum) and antisera against *E. coli*-expressed polypeptide of the *Sau*3AI fragment (anti-PPG2 N-domain/repeats serum).

### Double targeted gene replacement of the *appg* gene

A neomycin phosphotransferase gene (*neo*)-containing DNA fragment was prepared by digesting pX-neo [29] with *Spe*I. A hygromycin B phosphotransferase gene (hyg)-containing DNA fragment was obtained by *Spe*I/*Bam*HI double digestion of pX63-Hyg [30]. The 5′-untranslated DNA region (5′-UTR) just upstream of the start codon of the *ppg2* gene was amplified by PCR using the primers P1 (ATAAGAAT**GCGGCCGC**AGC-AGACGCTAAATGA) and P2 (AAT**ACTAGT**CTTCTCGC-CGTGTTTCTTGT) to create flanking *Not*I and *Spe*I restriction sites in the resulting 500 bp fragment. For the 3′-untranslated DNA region (3′-UTR) just downstream of the *ppg2* stop codon, PCR amplification was performed using the primers P3

(AAT**ACTAGT**AA**GGATCC**ATGAAACACAGCACAAAC-GA and P4 (AAT**GAATTC**GGCGCGCCCTGTTTTGCGG-GACTC to create flanking *Spe*I, *Bam*HI and *Eco*RI restriction sites in the resulting 776 bp fragment. The *Not*I/*Spe*I-cut PCR fragment of the 5′-UTR was ligated into *Not*I/*Spe*I-cut pBSK$^+$. The resulting plasmid was digested with *Spe*I and *Eco*RI and ligated to the *Spe*I/*Eco*RI-cut PCR fragment of the 3′-UTR. This plasmid was then digested with either *Spe*I alone or double-digested with *Spe*I and *Bam*HI and ligated with the *neo*-containing *Spe*I fragment or the *hyg*-containing *Spe*I/*Bam*HI fragment, respectively. This resulted in the gene replacement constructs pBSK*ppg2-neo* and pBSK*ppg2-hyg*. Correct orientation of the *neo* gene was confirmed by restriction digests and sequencing. To generate linear DNA for gene replacement, pBSK*ppg2-neo* was cut with *Apa*LI and pBSK*ppg2-hyg* was cut with *Acl*I and *Bgl*I (see Figure 1). The purified DNA fragments (4–10 $\mu$g) were transfected by electroporation into *L. mexicana* promastigotes that were washed with cold electroporation buffer (21 mM Hepes, 137 mM NaCl, 5 mM KCl, 0.7 mM $Na_2HPO_4$, 6 mM glucose, pH 7.5) and resuspended to $10^8$ cells/ml. Electroporation was performed in 2 mm BTX disposable cuvettes at 450 V, 500 mF and R1 resistance setting (13 $\Omega$) using a BTX Electro Cell Manipulator (ECM600) [18] that generates pulse times of 2.3–2.8 ms. After 10 min on ice the cells were resuspended in 5 ml SDM/5% iFCS and incubated for 24–48 h at 27 °C. Selection was then initiated by the addition of 10 $\mu$g/ml G418 or 20 $\mu$g/ml hygromycin B (Roche), or both. Cloning was performed by dilution of 2.5 ml of the culture to 120 ml SDM/5% iFCS containing the respective selection agent and distribution onto 96 well plates (100 $\mu$l/well).

### Generation of gene fusion constructs of modMAP with *ppg2a* repeats

The DNA repeat region of the *ppg2a* gene was amplified by PCR with the primers TCT**AGATCT**CGGAAGACAATCCGCC-TATC and TCC**GGATCC**CTTCTCCAGGTTGCTTCT to create flanking *Bgl*II and *Bam*HI restriction sites. The 633 bp PCR fragment was digested with *Bgl*II and *Bam*HI and ligated in frame into *Bg*lII-cut pXmodMAP [20,31]. The correct orientation of the resulting construct (pXmodMAP-*ppg2a*-repeats) was checked by restriction digests and by sequencing. pXmodMAP and pXmodMAP-*ppg2a*-repeats were transfected by electroporation into *L. mexicana* promastigotes. For selection of transfectants 10–50 $\mu$g/ml G418 was added to the growth medium. Two-site ELISA of culture supernatants from *L. mexicana* promastigotes transfected with pXmodMAP constructs were performed using mAb AP4-coated (50 $\mu$l/well, 20 $\mu$g/ml, 50 mM $NaHCO_3$, pH 9.6, 1 h 25 °C) flexible polyvinylchloride ELISA plates (Becton–Dickinson, Heidelberg, Germany). mAb AP4 recognizes a conformational peptide epitope of *L. mexicana* modMAP [25]. The plates were washed twice with 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20 (TBS/T, 200 $\mu$l/well) and non-specific binding sites were blocked with TBS/T containing 5% skim milk powder (TBS/MT, 200 $\mu$l/well, 1 h, 25 °C). After washing with TBS/T *L. mexicana* culture supernatants (undiluted and diluted, 1:2, 1:5, 1:10 and 1:20 in SDM/5% iFCS) were added (100 $\mu$l/well, 1 h, 25 °C). Bound acid phosphatase activity was then determined after 3 washing steps with TBS/T (200 $\mu$l/well) by incubation with 5 mM *p*-nitrophenylphosphate (pNPP) in 100 mM Na acetate, pH 5.0 (100 $\mu$l/well, 1 h, 37 °C). 80 $\mu$l aliquots were then transferred to 20 $\mu$l 1 M NaOH and the optical density at 405 nm ($O.D._{405}$) was measured. For subsequent ELISA detection, the wells were washed twice with TBS/T followed by incubation with the biotinylated mAbs L7.25, AP3 and WIC108.3 (1–2 $\mu$g/ml in TBS/MT, 100 $\mu$l/well, 1 h, 25 °C). After washing steps with TBS/T, the ELISA plates were incubated with Extravidin-alkaline phosphatase (Sigma), diluted 1:5000 in TBS/T containing 5% fish gelatine (Serva, Heidelberg, Germany, 100 $\mu$l/well, 1 h, 25 °C) followed by 4 washes with TBS/T, 1 wash with 1 M diethanolamine/HCl, pH 9.8, 1 mM $MgCl_2$ and incubation with 5 mM pNPP in the same buffer. Bound enzyme conjugate was measured by determination of the $O.D._{405}$

### Miscellaneous techniques

Discontinuous SDS/polyacrylamide electrophoresis (SDS/PAGE), immunoblotting, 40% HF dephosphorylation, electron microscopy and purification of pPPG2 was performed as described in ref. [24b].

## RESULTS

### Identification and isolation of *ppg2* cDNAs from a λZAP II *L. mexicana* amastigote cDNA expression library

Direct N-terminal amino acid sequencing of *L. mexicana* aPPG before and after mild acid deglycosylation as well as amino acid sequencing after proteolytic digests of aPPG did not generate peptide sequence information useful for the design of degenerate oligonucleotide primers. Therefore a λZAP II *L. mexicana* cDNA expression library was constructed from poly(A) RNA of mouse lesion-derived amastigotes. This expression library was screened with a mouse antiserum (M1.1) that recognizes 40% HF-deglycosylated and dephosphorylated aPPG [24b]. Six positive λZAP II phage clones (cDNAs 1.1, 3.1, 6.1, 7.1, 16.1, 17.1) were identified, the DNA inserts subcloned into pBSK$^-$, mapped by restriction enzyme digests and sequenced. One of the cDNAs (6.1, Figure 1A) was sequenced completely from both strands (Figure 2). This cDNA is 2394 bp in length and contains 16 bp of the common spliced leader sequence present on all *Leishmania* mRNAs [32] at its 5′ end, which allowed the determination of the first site of pre-mRNA processing (Figures 1 and 2A). An open reading frame of 1722 bp begins at position 299: a short non-repetitive 5′ region (360 bp) leads to a central region (504 bp) consisting of seven perfect 72 bp repeats, which then is followed by a longer non-repetitive 3′ region (858 bp). The N-terminus contains a potential signal sequence (Figures 2 and 3A), the central repeat region encodes Ser-rich 24 amino acid peptide repeats (Figures 2 and 3A) that are clearly distinct from the Ser-rich repeats of *L. mexicana* SAP, fPPG and mPPG (Figure 3B) and the non-repetitive C-terminal domain contains both a very hydrophobic sequence of 17 amino acids (Figure 3A) and a single potential *N*-glycosylation site (Figure 2). Partial sequencing of the other five cDNA clones indicated that they contained, in part, truncated open reading frames identical to that of cDNA 6.1. In contrast to cDNA 6.1, however, they possessed poly(A) tails at their 3′-termini which allowed the determination of the second site of pre-mRNA processing (Figure 1). The DNA sequence and the deduced peptide sequence of cDNA 6.1 did not show significant homologies to any known sequences in the database. In particular, no sequence homology was detected to the recently described *ppg1* gene [20]. Therefore, this novel proteophosphoglycan gene was named *ppg2*.

### Genomic structure, expression and species-specificity of the *ppg2* gene

Southern blot analysis of genomic *L. mexicana* DNA using a large portion of the *ppg2* open reading frame and some 3′-

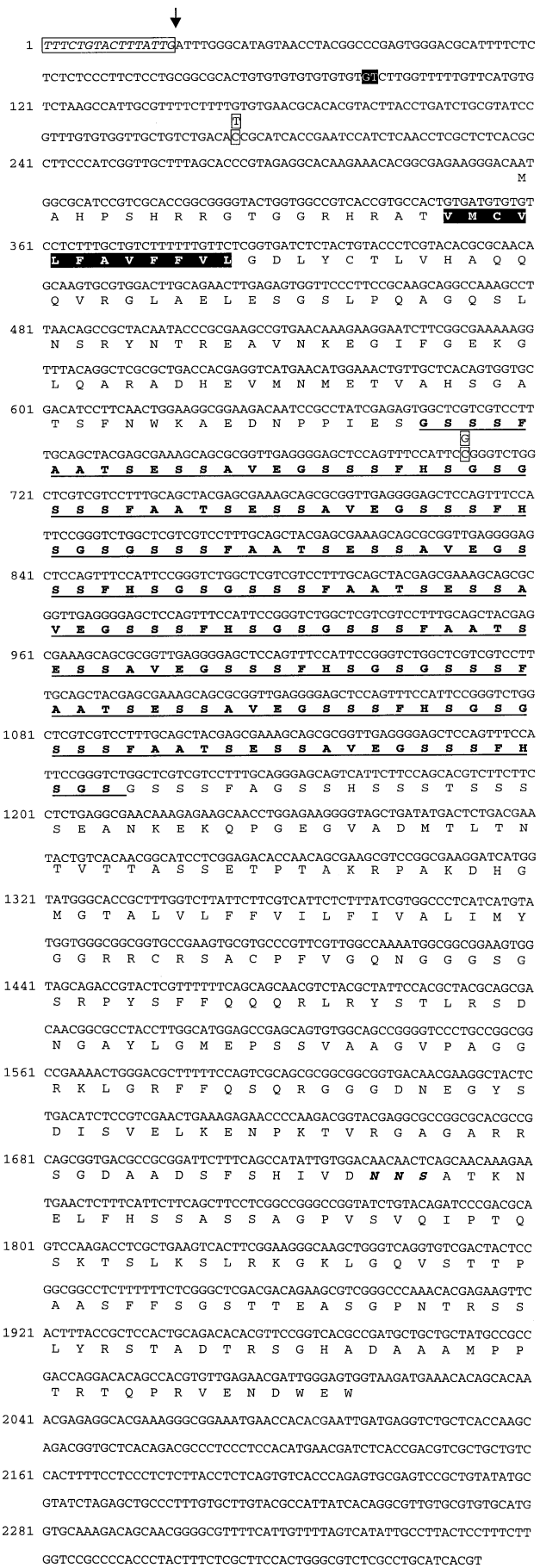
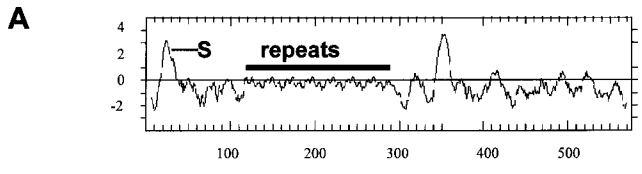


**Figure 2 Nucleotide sequence of the cDNA 6.1 (*ppg2a*) and predicted amino acid sequence**

The spliced leader sequence is highlighted by boxed letters in italics and the position of the spliced leader addition site is marked by an arrow. The h-region [34] of the putative signal sequence for protein import into the endoplasmic reticulum is in white bold letters on black background. The central repetitive domain is in bold letters and underlined while the single putative *N*-glycosylation site is in bold letters and italics. Differences between the two alleles *ppg2a* and *ppg2b* in the 5′-untranslated region are indicated by frames. In *ppg2b*, the highlighted GT is not present while the highlighted C is exchanged for T. In addition *ppg2b* possesses in its open reading frame eight 72 bp repeats versus seven in the case of *ppg2a*.




B

| *Leishmania* PPG gene | Ser-rich peptide repeat sequence | length |
|---|---|---|
| *L. mexicana sap1* [1] | [T][T](S/T)(S/T)(S/T)(S/T)SSEG | 7-9 AA |
| *L. mexicana sap2* [1] | [T][T](S/T)(S/T)(S/T)(S/T)SSEG | 7-9 AA |
| | [T](A/T)(S/T)(S/T)(S/T)SSD(A/V) | 8-9 AA |
| *L. donovani SAcP-1/SAcP-2* [2] | (T/I)T(T/A)SSSEG | 8 AA |
| | VTTASSSD | 8 AA |
| *L. major ppg1* and fPPG genes [3] | APSASSSSAPSSSSS[S] | 15-16 AA |
| *L. mexicana ppg1* and fPPG genes [4] | APSSSSSAPSSSS | 13 AA |
| *L. mexicana ppg2* [5] | GSSSFAATSESSAVEGSSSFHSGS | 24 AA |

**Figure 3 Analysis of the *L. mexicana ppg2*-derived peptide sequence**

(**A**) Hydrophobicity plot of the *ppg2* open reading frame according to [41]. The position of the signal sequence for import into the endoplasmic reticulum (S) and the central peptide repeats are indicated. (**B**) Comparison of the *ppg2* repeat sequence with repeat sequences from other PPG genes; [1] taken from [18]; [2] taken from [19]; [3] taken from [20]; [4] U. Göpfert and T. Ilg, unpublished results; [5] this study.

untranslated region as a hybridization probe demonstrated that *ppg2* is present in a single copy per haploid genome (cf. Figure 1B with Figure 4). However, when *ppg2* was isolated from a dedicated plasmid library of genomic *L. mexicana* DNA, it was noted that two types of genes could be distinguished. Sequencing of their open reading frames showed that their main difference was in the number of 72 bp repeats, which was either seven (*ppg2a*, as in the case of cDNA 6.1) or eight (*ppg2b*). Additional minor differences between *ppg2a* and *ppg2b* involved the deletion of a dinucleotide ($G_{102}T_{103}$), a transition ($C_{209} \rightarrow T$) in the 3′-UTR between the splice addition site and the start codon and a silent transversion ($C_{712} \rightarrow G$) inside the open reading frame (Figure 2). To demonstrate that these observations were not a cloning artifact, genomic *L. mexicana* DNA as well as two representative copies of *ppg2a* and *ppg2b* cloned into pBSK were digested with tetranucleotide-cutting enzymes that do not cut within the repeat region and subjected to Southern blot analysis (Figure 5A). (We have noticed that recombinations of *ppg2* and also *ppg1* repeat sequences leading to shorter and, occasionally, to longer repeat regions are frequently seen in $\lambda$ phages and M13 phages, even when the phages were grown in recA$^-$ *E. coli* strains. When cloned into plasmid vectors these repeat sequences appear to be more stable.) The results showed that the same size

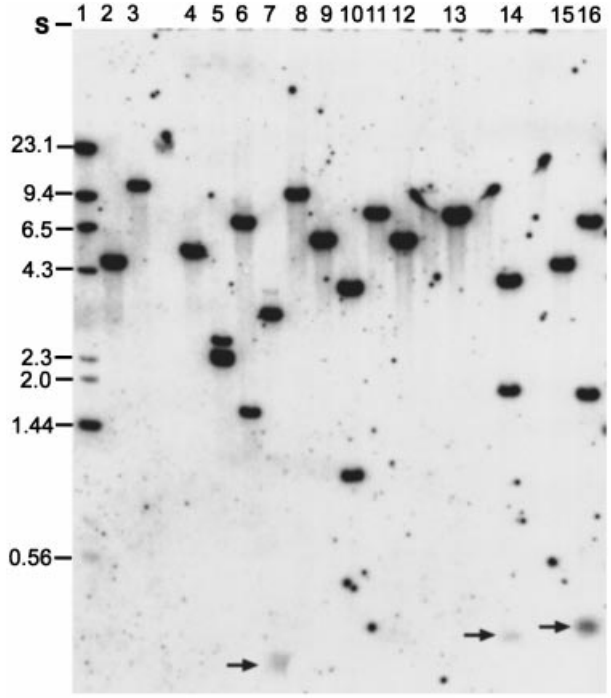



**Figure 4 Detection of the *ppg2* gene in *L. mexicana* genomic DNA by Southern blot**

*L. mexicana* genomic DNA was digested with restriction enzymes, separated by 0.7% agarose gel electrophoresis, blotted onto positively charged nylon membranes and hybridized with the DIG-labelled cDNA 1.1 fragment (Figure 1A). Lane 1, 200 ng DIG-labelled λ*Hin*dIII DNA standard fragments (size indicated in kbp); lanes 2–16, 2 μg genomic *L. mexicana* DNA digested with the following restriction enzymes: 2, *Xho*I; 3, *Xba*I; 4, *Sph*I; 5, *Sal*I; 6, *Sac*II; 7, *Sac*I; 8, *Pvu*II; 9; *Pvu*I; 10, *Pst*I; 11, *Nsi*I; 12, *Nru*I; 13, *Nco*I; 14, *Kas*I; 15, *Eco*RI; 16, *Eag*I. The weak signals of very small DNA fragments (lanes 7, 14, 16) are marked with arrows.

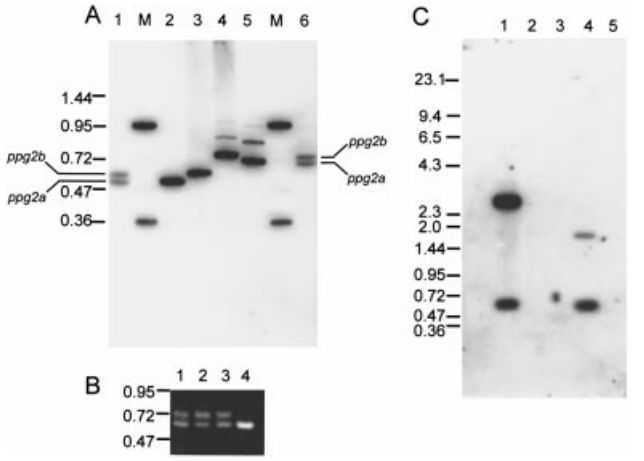



**Figure 5 Stable heterozygosity of the *L. mexicana ppg2* locus and species-specificity of *ppg2***

(**A**) *L. mexicana* genomic DNA (5 μg, lanes 1 and 6), pBSK$^+$-*ppg2a-Eco*RI (20 ng, lanes 2 and 5) and pBSK$^+$-*ppg2b-Eco*RI (20 ng, lanes 3 and 4) were digested with the restriction enzymes *Nla*III (lanes 1–3) or *Sau*3AI, (lanes 4–6), separated on a 1.8% agarose gel, blotted onto cationized nylon and probed with the repeats-DIG probe (Figure 1). The size of DNA standard fragments is indicated in kbp. some size marker fragments hybridize with traces of DIG-labelled pBSK vector sequence. (**B**) PCR using primers flanking the *ppg2* repeat region with *L. mexicana* genomic DNA isolated from a cloned parasite line in 1992 (lane 1), 1996 (lane 2) and 1998 (lane 3). DNA of the closely related species *L. amazonensis* was used as a control (lane 4). (**C**) Southern blot (0.8% agarose gel) of genomic DNA (2 μg) from *L. mexicana* (lane 1), *L. major* (lane 2), *L. donovani* (lane 3), *L. amazonensis* (lane 4) and Crithidia fasciculata (lane 5) digested with *Pst*I and hybridized with a mixture of the *ppg2* probes 5′-region-DIG, repeats-DIG and 3′-region-DIG (Figure 1).

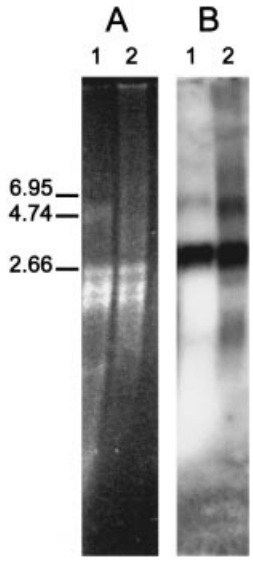



**Figure 6 Analysis of *ppg2* expression in *L. mexicana* promastigotes and amastigotes by Northern blotting**

10 μg of *L. mexicana* promastigote (lane 1) and amastigote total RNA (lane 2) were prestained with ethidium bromide and separated by formaldehyde–1% agarose gel electrophoresis (**A**), then blotted onto a cationized nylon membrane and hybridized with a mixture of the *ppg2* probes 5′-region-DIG, repeats-DIG and 3′-region-DIG (**B**). The size of RNA standards is indicated in kb.

heterogeneity in the *ppg2* repeat region that was observed in isolated plasmid clones was also found in genomic *L. mexicana* DNA. Finally PCR was performed with genomic DNA isolated 7 years ago, 3 years ago and recently from a cloned line of *L. mexicana* continuously passaged in culture and in mice using primers flanking the *ppg2* repeat region. DNA fragments of the expected sizes for the *ppg2a* and the *ppg2b* repeat regions were observed in agarose gel electrophoresis in equal intensity in all samples. Taken together, the results of DNA sequencing, Southern blots and PCR suggest that *L. mexicana* exhibits allelic forms of *ppg2* and that this heterozygosity of the *ppg2* gene locus is a stable phenomenon (Figure 5B). *L. amazonensis* that was used as a control does not show this heterozygosity (Figure 5B).

Northern blot analysis of *L. mexicana* promastigote and amastigote total RNA shows a mRNA of approx. 2.8 kb in both parasite life stages (Figure 6). This size is in agreement with the results of cDNA cloning (Figure 1). A minor band between 5 and 6 kb may represent partially processed pre-mRNA (Figure 6). The relative band intensities on the Northern blot suggests that *ppg2* mRNA expression is very similar in promastigotes and amastigotes (Figure 6).

Southern blots of genomic DNA from several Trypanosomatid species with the *ppg2* open reading frame as a probe indicated that the presence of this gene is restricted to *L. mexicana* and *L. amazonensis* (Figure 5C). No significant signal was observed with DNA from *L. major*, *L. donovani*, *C. fasciculata* (Figure 5C), *L. tropica* and *L. braziliensis* DNA (not shown), even at low stringency of hybridization and washing. This suggested that if *ppg2* is present in these species it has diverged considerably from the *L. mexicana* sequence.

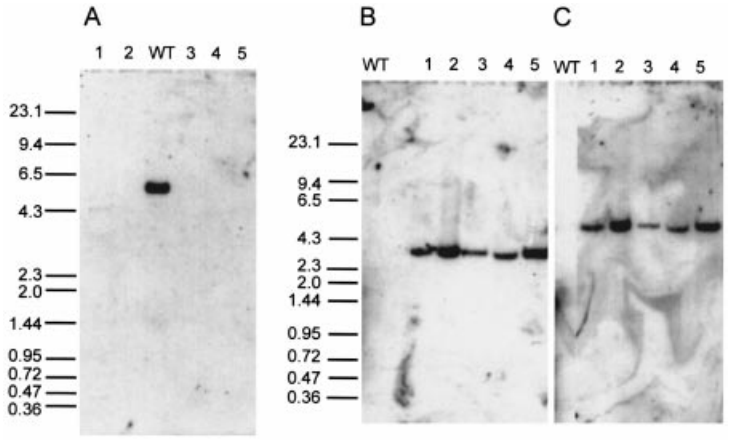



**Figure 7 Southern blot analysis of *L. mexicana* Δ*ppg2* mutants**

Genomic DNA from *L. mexicana* WT (WT) and five Δ*ppg2* mutants was digested with *Eco*RI, separated by 0.7% agarose gel electrophoresis, blotted onto a cationized nylon membrane and hybridized with *ppg2* repeats-DIG probe (**A**), *hyg*-DIG probe (**B**) and *neo*-DIG probe (**C**) (see Figure 1). The size of DNA standards is indicated in kbp.

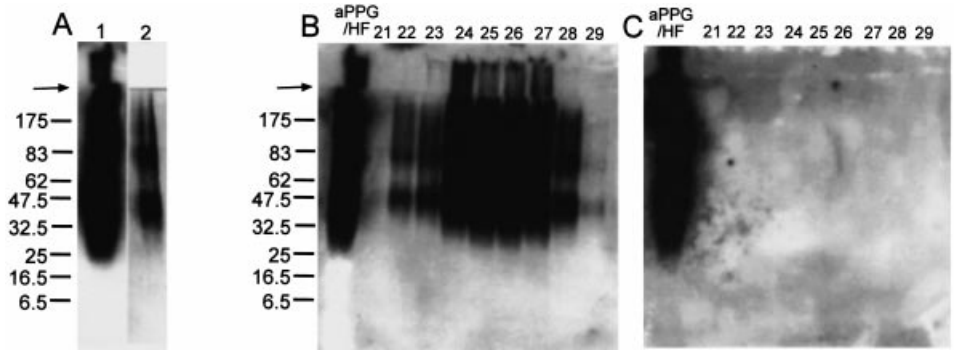



**Figure 8 Immunoblot analysis of 40% HF-treated aPPG and pPPG2 from *L. mexicana* WT and from *L. mexicana* Δ*ppg2***

(**A**) 7.5–20% SDS/PAGE/immunoblots of 40% HF-treated *L. mexicana* aPPG were probed with rabbit anti PPG2-NR serum (lane 1) and rabbit anti PPG2-C serum (lane 2). (**B**) pPPG2 was purified from *L. mexicana* WT (**B**) and Δ*ppg2* promastigotes (**C**). 1/250 vol. of the fractions from the final Superose 6 pPPG2 purification step (see [24b]) were treated with 40% HF, separated by 7.5–20% SDS/PAGE, electroblotted onto PVDF membranes and probed with mouse antiserum M 1.1. 40% HF-treated *L. mexicana* aPPG (aPPG/HF, 10 $\mu$g) served as a control. The arrow indicates the boundary between the stacking gel and the separating gel. The position and the molecular mass of standard proteins is indicated in kDa.

### Generation of antisera against recombinant PPG2 protein and targeted deletion of the *ppg2* gene

Antisera raised in rabbits against *E. coli*-expressed fragments of the PPG2 polypeptide encompassing the N-terminal domain and the central repeats (anti-PPG2-NR serum) and the C-terminal domain (anti-PPG2-C serum) were used for immunoblots of purified *L. mexicana* aPPG. Remarkably both antisera failed to recognize native aPPG (not shown). They did, however, strongly react with 40% HF-deglycosylated and dephosphorylated aPPG (Figure 8A) as well as with the 40% HF-treated promastigote counterpart, pPPG2 [24b].

To unambiguously assign the *ppg2* gene to aPPG/pPPG2, targeted deletion was performed. Both alleles were sequentially replaced by selective marker genes conferring neomycin (*neo*) or hygromycin B (*hyg*) resistance to the parasites. Successful *ppg2* gene replacement was confirmed by Southern blots on genomic DNA from five independent neomycin- and hygromycin B-resistant clones isolated by limiting dilution using DIG-labelled DNA probes of the *ppg2* open reading frame (Figure 7A), the 5′-UTR and 3′-UTR of *ppg2* (not shown) as well as *hyg* (Figure 7B) and *neo* (Figure 7C).

Purifications of pPPG2 [24b] were performed in parallel from culture supernatant of *L. mexicana* WT and one of the *L. mexicana* Δ*ppg2* mutants. The fractions in the final Superose 6 gel filtration step corresponding to the pPPG2 peak were subjected to 40% HF dephosphorylation and deglycosylation followed by SDS/PAGE/immunoblotting using mouse antiserum M1.1. While in the case of *L. mexicana* WT Superose 6 fractions the expected immunoblot signal pattern for pPPG2 was observable, no signal could be detected in the case of the mutant parasites (Figures 8B and 8C). In addition upon electron microscopical inspection of the Superose 6 fractions, the short filaments typical for pPPG2 were completely absent in the case of *L. mexicana* Δ*ppg2*, while the abundance and morphology of the macromolecules SAP and fPPG appeared to be unchanged (data not shown).

### The *ppg2* repeat region-encoded peptide is a target for phosphoglycosylation

aPPG and pPPG2 are proteins that are heavily phosphoglycosylated via Ser residues of the protein backbone [24b,33]. It appeared likely that the central Ser-rich repeat regions pre-

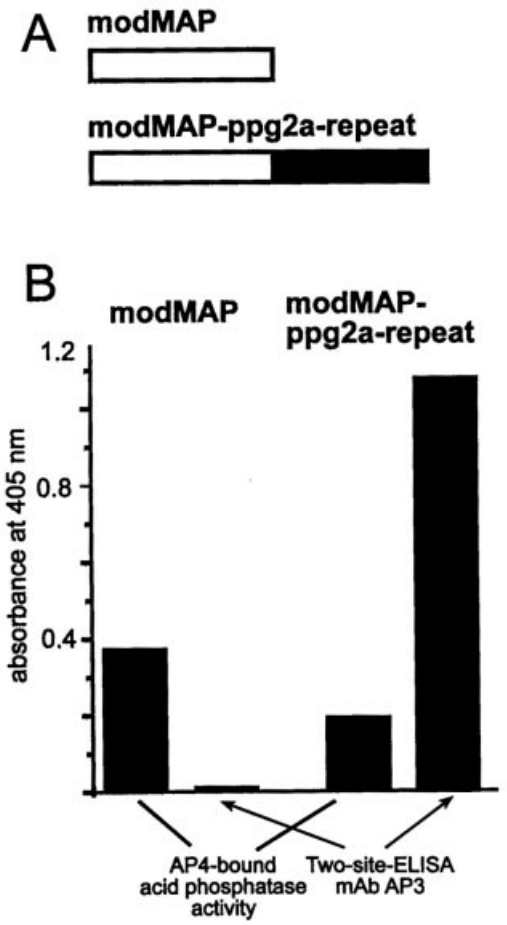



**Figure 9 Functional analysis of the *ppg2* central repeats**

(**A**) Schematic diagram of the gene fusion construct of modMAP with the *L. mexicana ppg2a* repeats. (**B**) ELISA ($O.D._{405}$, bars 2 and 4) of mAb AP4-bound acid phosphatase released by *L. mexicana* promastigotes that had been transfected with pXmodMAP or pXmodMAP-*ppg2*-repeat ($O.D._{405}$, bars 1 and 3). The bars represent the average of triplicate assays.

dicted by the *ppg2a* and *ppg2b* gene sequence were the target for this protein modification. To investigate this possibility, the central repeat region of the *ppg2a* gene was amplified by PCR to generate suitable restriction sites, fused in frame to the gene encoding a secreted version of a membrane-bound acid phosphatase (modMAP) from *L. mexicana* [31] and cloned into the *Leishmania* expression vector pX yielding the construct pXmodMAP-*ppg2a*-repeat (Figure 9A). *L. mexicana* promastigotes were transfected with this construct and pXmodMAP as a control. Both transfected cell lines released acid phosphatase activity that could be immobilized by the mAb AP4, while none was detectable in untransfected parasites. Similar amounts of the mAb AP4-bound secreted enzymes modMAP and modMAP-*ppg2*-repeat were probed with biotinylated anti-mannooligosaccharide cap mAb AP3. While modMAP alone reacted only very weakly with mAb AP3, modMAP-*ppg2a*-repeat showed a very strong ELISA signal (Figure 9B). This result demonstrated that the *ppg2a*-encoded 24 amino acid peptide repeats are the targets for phosphoglycosylation in *L. mexicana* promastigotes.

## DISCUSSION

In this study we have identified and cloned *ppg2*, a novel proteophosphoglycan gene from *L. mexicana* that shows no homology to any sequence in the database. Several results of this and the accompanying study [24b] suggest that *ppg2* encodes the common backbone of the proteophosphoglycans aPPG and pPPG2 that are secreted by *L. mexicana* amastigotes and promastigotes, respectively: (1) *ppg2* was identified using a mouse antiserum raised against aPPG; (2) rabbit antisera raised against different *E. coli*-expressed fragments of the *ppg2*-encoded polypeptide recognize the deglycosylated protein backbones of both aPPG and pPPG2; (3) targeted gene deletion of *ppg2* results in the loss of pPPG2 expression in *L. mexicana* promastigotes. The *ppg2* gene is equally transcribed in promastigotes and amastigotes, which is in agreement with the results on protein expression in [24b]. As expected for a secreted molecule, the *ppg2* gene product contains a putative signal sequence for import into the endoplasmic reticulum at the N-terminus [34] with 4 positive charges and 3 histidines in its n-region, which is typical for *Leishmania* proteins [35]. *ppg2* contains perfect 72 bp repeats encoding an array of 24 amino acid peptide repeats that consist of Ser (45.8 mol%), Gly, Ala (12.5 mol% each), Phe, Glu (8.3 mol% each) Thr, Val, and His (4.2 mol% each). Expression of these repeats fused to the reporter enzyme modMAP [31] in *L. mexicana* promastigotes shows that the 24 amino acid peptide repeats direct phosphoglycosylation. The large C-terminal domain contains a strongly hydrophobic stretch of 17 amino acids followed by several non-repetitive Ser-rich areas that may also be targets for phosphoglycosylation. Whether the hydrophobic peptide forms a transmembrane helix is currently not known. In this case, however, release of pPPG2 and a PPG from the cells would require proteolytic cleavage immediately after the peptide repeats. Alternatively, this hydrophobic peptide sequence may be buried inside the molecule and be present in the secreted product. Amino acid analysis cannot definitely distinguish between these two possibilities and aberrant migration of polypeptides containing Ser-rich repeats on SDS/polyacrylamide gels ([22], and unpublished results) precludes accurate molecular mass determinations of the deglycosylated backbone by SDS/PAGE. Further experiments will be required to solve this question. Surprisingly, the *ppg2* gene exists in two allelic forms, *ppg2a* and *ppg2b*, which differ only in the number of repeats (7 versus 8) and a silent point mutation and encode proteins of 574 and 598 amino acids, respectively. How and why *L. mexicana* maintains these allelic forms of *ppg2* over several years of continuous culture is unknown, given the high frequency of recombination and the demonstrated loss of heterozygosity in *Leishmania* [17,36].

Three different types of proteophosphoglycans can now be distinguished with respect to their unrelated phosphoglycosylated peptide repeat units: (1) the *sap* type with 7–9 amino acid repeats; (2) the *ppg1* type with 13–16 amino acid repeats and (3) the *ppg2* type with 24 amino acid repeats (see Figure 3). In all three cases, the extensive phosphoglycosylation leads to an extended filamentous structure of the repeats that can be readily visualized by electron microscopy [20,22,37]. This situation is reminiscent of the different mammalian mucins which also exhibit no similarity to each other with respect to the sequence or the number of amino acids in their repeats but form similar rod-like structures in the O-glycosylated regions (reviewed in [38]).

Moss and co-workers [24a] have recently identified the first enzyme of proteophosphoglycan biosynthesis in *L. mexicana* microsomes, a GDP-mannose:serine-protein mannose-1-phosphate transferase that adds Man$\alpha$1-$PO_4$ to serine residues of peptides derived from SAP repeats. Their results suggest that two acidic residues within the SAP peptide repeat may contribute to substrate recognition [24a]. The *ppg2*-encoded 24 amino acid repeats of aPPG and pPPG2 identified in this study also contain

two Glu residues and could therefore possibly be efficient substrates for this enzyme activity. We have, however, recently identified the *L. mexicana* counterparts of the *L. major ppg1* gene [20] and its phosphoglycosylated Ser-rich repeats are completely devoid of charged amino acids (Ilg, T., unpublished experiments). Therefore it appears likely that several GDP-mannose:serine-protein mannose-1-phosphate transferases with distinct peptide substrate specificities exist in *L. mexicana*.

The results of this study and that presented in [24b] demonstrate that the structure and the complexity of PPG2 glycans is not a function of its protein backbone, but rather determined by other factors such as stage-specific expression of glycosyltransferases and transporters or availability of precursors and substrates. The function of the pPPG2 glycoform for the promastigotes is currently unknown; however, it can be speculated that its secretion by promastigotes in the sandfly's digestive tract may contribute to the formation of the parasite-derived gelatinous plug whose scaffold is formed by fPPG [4]. It has been proposed that complement activation via the mannose-binding protein pathway and induction of vacuoles in macrophages by the amastigote-derived aPPG may be important for the establishment and maintenance of *L. mexicana* infections in the mammalian host [39,40]. The generation of *ppg2* gene knockout mutants in this study will allow the investigation of these functional aspects in more detail.

We would like to thank Professor Dr Peter Overath for comments on the manuscript and Monika Demar for expert technical assistance. N.G. is supported by a student grant from the J. William Fulbright Program and a scholarship from Amherst College.

## REFERENCES


1 Turco, S. J. and Descoteaux, A. (1992) Annu. Rev. Microbiol. **46**, 65–94
2 McConville, M. J. and Ferguson, M. A. J. (1993) Biochem. J. **294**, 305–324
3 Mengeling, B. J., Beverley, S. M. and Turco, S. J. (1997) Glycobiology **7**, 873–880
4 Ilg, T., Handman, E., Ng, K., Stierhof, Y.-D. and Bacic, A. (1999) Trends Glycosci. Glycotech. **11**, 1–19
5 Ilg, T., Handman, E. and Stierhof, Y.-D. (1999) Biochem. Soc. Trans. **27**, 48–55
6 Carver, M. A. and Turco, S. J. (1991) J. Biol. Chem. **266**, 10974–10981
7 Carver, M. A. and Turco, S. J. (1992) Arch. Biochem. Biophys. **295**, 309–317
8 Huang, C. and Turco, S. J. (1993) J. Biol. Chem. **268**, 24060–24066
9 Ng, K. F., Handman, E. and Bacic, A. (1994) Glycobiology **4**, 845–853
10 Ng, K. F., Handman, E. and Bacic, A. (1996) Biochem. J. **317**, 247–255
11 Brown, G. M., Millar, A. R., Masterson, C., Brimacombe, J. S., Nikolaev, A. V. and Ferguson, M. A. J. (1996) Eur. J. Biochem. **242**, 410–416
12 Ma, D., Russell, D. G., Beverley, S. M. and Turco, S. J. (1997) J. Biol. Chem. **272**, 3799–3805
13 Mengeling, B. J., Zilberstein, D. and Turco, S. J. (1997) Glycobiology **7**, 847–853
14 King, D. L. and Turco, S. J. (1988) Mol. Biochem. Parasitol. **28**, 285–294
15 Ryan, K. A., Garraway, L. A., Descoteaux, A., Turco, S. J. and Beverley, S. M. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 8609–8613
16 Descoteaux, A., Luo, Y., Turco, S. J. and Beverley, S. M. (1995) Science **269**, 1869–1872
17 Beverley, S. M. and Turco, S. J. (1998) Trends Microbiol. **6**, 35–40
18 Wiese, M., Ilg, T., Lottspeich, F. and Overath, P. (1995) EMBO J. **14**, 1067–1074
19 Shakarian, A. M., Ellis, L. S., Mallinson, D. J., Olafson, R. W. and Dwyer, D. M. (1997) Gene **196**, 127–137
20 Ilg, T., Montgomory, J., Stierhof, Y.-D. and Handman, E. (1999) J. Biol. Chem., in press
21 Ilg, T., Overath, P., Ferguson, M. A. J., Rutherford, T., Campbell, D. G. and McConville, M. J. (1994) J. Biol. Chem. **269**, 24073–24081
22 Ilg, T., Stierhof, Y.-D., Craik, D., Simpson, R., Handman, E. and Bacic, A. (1996) J. Biol. Chem. **271**, 21583–21596
23 Mehta, D. P., Ichikawa, M., Salimath, P. V., Etchison, J. R., Haak, R., Manzi, A. and Freeze, H. H. (1996) J. Biol. Chem. **271**, 10897–10903
24a Moss, J. M., Reid, G., Mullin, K. A., Zawadzki, J. L., Simpson, R. J. and McConville, M. J. (1999) J. Biol. Chem. **274**, 6678–6688
24b Klein, C., Göpfert, U., Goehring, N., Stierhof, Y.-D. and Ilg, T. (1999) Biochem. J. **344**, 775–786
25 Menz, B., Winter, G., Ilg, T., Lottspeich, F. and Overath, P. (1991) Mol. Biochem. Parasitol. **47**, 101–108
26 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
27 Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. **162**, 156–159
28 Sanger, F., Nicklen, S. and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. **12**, 5463–5467
29 LeBowitz, J. H., Coburn, C. M., McMahon-Pratt, D. and Beverley, S. M. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 9736–9740
30 Cruz, A., Coburn, C. M. and Beverley, S. M. (1991) Proc. Natl. Acad. Sci. U.S.A. **89**, 7170–7174
31 Wolfram, M., Fuchs, M., Wiese, M., Stierhof, Y.-D. and Overath, P. (1996) Eur. J. Immunol. **26**, 3153–3162
32 Kapler, G. M., Coburn, C. M. and Beverley, S. M. (1990) Mol. Cell. Biol. **10**, 1084–1094
33 Ilg, T., Craik, D., Currie, G., Multhaup, G. and Bacic, A. (1998) J. Biol. Chem. **273**, 13509–13523
34 Von Heijne, G. (1985) J. Mol Biol. **184**, 99–105
35 Al Qahtani, A., Teilhet, M. and Mensa-Wilmot, K. (1998) Biochem. J. **331**, 521–529
36 Gueirosfilho, F. J. and Beverley, S. M. (1996) Mol. Cell. Biol. **16**, 5655–5663
37 Stierhof, Y.-D., Wiese, M., Ilg, T., Overath, P., Haener, M. and Aebi, Ue (1998) J. Mol. Biol. **282**, 137–148
38 Gendler, S. J. and Spicer, A. P. (1995) Annu. Rev. Physiol. **57**, 607–634
39 Peters, C., Stierhof, Y.-D. and Ilg, T. (1997) Infect. Immun. **65**, 783–786
40 Peters, C., Kawakami, M., Kaul, M., Ilg, T., Overath, P. and Aebischer, T. (1997) Eur. J. Immunol. **27**, 2666–2672
41 Kyte, J. and Doolittle, R. F. (1982) J. Mol. Biol. **157**, 105–132


Received 24 June 1999/20 August 1999; accepted 10 September 1999