Characterization of a Novel GDP-mannose:Serine-protein Mannose-1-phosphotransferase from *Leishmania mexicana* 

(Received for publication, September 2, 1998, and in revised form, December 3, 1998)

Jonathan M. Moss‡, Gavin E. Reid§, Kylie A. Mullin‡, Jody L. Zawadzki‡, Richard J. Simpson§, and Malcolm J. McConville¶

From the ¶Department of Biochemistry and Molecular Biology, University of Melbourne, Parkville, Victoria 3052, Australia and the §Joint Protein Structure Laboratory, Ludwig Institute for Cancer Research and the Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria 3050, Australia

Protozoan parasites of the genus *Leishmania* secrete a number of glycoproteins and mucin-like proteoglycans that appear to be important parasite virulence factors. We have previously proposed that the polypeptide backbones of these molecules are extensively modified with a complex array of phosphoryglcan chains that are linked to Ser/Thr-rich domains via a common Man₁-PO₄-Ser linkage (Ilg, T., Overath, P., Ferguson, M. A. J., Rutherford, T., Campbell, D. G., and McConville, M. J. (1994) *J. Biol. Chem.* 269, 24073–24081). In this study, we show that *Leishmania mexicana* promastigotes contain a peptide-specific mannose-1-phosphotransferase (pep-MPT) activity that adds Man₁-P to serine residues in a range of defined peptides. The presence and location of the Man₁-PO₄-Ser linkage in these peptides were determined by electrospray ionization mass spectrometry and chemical and enzymatic treatments. The pep-MPT activity was solubilized in non-ionic detergents, was determined by electrospray ionization mass spectrometry.

Parasitic protozoa of the genus *Leishmania* cause a spectrum of human and animal diseases that are transmitted by a sand fly vector. During their development in the digestive tract of the sand fly, these parasites differentiate from non-infective procyclic promastigotes to infective metacyclic promastigotes that target mammalian macrophages when introduced into the host during the insect’s blood meal. Following their internalization into the macrophage phagolysosome, metacyclic promastigotes differentiate into a replicative amastigote stage and eventually rupture the host cell and perpetuate disease by infecting other host cells. A number of cell-surface and secreted virulence factors are thought to be crucial for the survival of these different developmental stages in their respective host environments. These include an abundant glycosylphosphatidylinositol-anchored lipophosphoglycan (LPG)¹ (1–3) and a number of secreted glycoproteins and proteophosphoglycans (PPGs) (reviewed in Refs. 4 and 5). Strikingly, both the cell-surface LPGs and the secreted molecules are elaborated with structurally related phosphoglycan chains that are thought to be the major functional determinants of these molecules.

The secreted glycoproteins and proteoglycans of *Leishmania* have been shown to form distinct macromolecular complexes in the flagellar pocket and extracellular milieu (4). The most intensively characterized of these molecules are the secreted acid phosphatases (sAPs), which aggregate into large pearl-like filamentous polymers (6, 7). Early studies indicated that sAPs were heavily glycosylated and phosphorylated enzymes that contained glycan epitopes characteristic of LPGs (6, 8, 9). We have recently shown that *Leishmania mexicana* sAP is extensively modified with Man₁-PO₄ residues that are linked to serine residues in the polypeptide backbone (10). This unusual type of linkage, in which a monosaccharide is linked to protein via a phosphodiester bridge, has been termed phosphoglycosylation (11) and may be widespread in several lower eukaryotes (12).

In *L. mexicana*, the Man₁-PO₄ residues can be further elaborated with a1–2-linked mannose oligosaccharides or short chains of phosphorylated di- or trisaccharides (10), which are also found in the long phosphoglycan chains (as capping structures or internal repeat units, respectively) of *L. mexicana* LPG (see Fig. 1). Interestingly, the nature of these modifications may be influenced by the size of the Ser/Thr-rich repeat domains in the polypeptide backbone. For example, sAP-1 contains a relatively short Ser/Thr-rich domain and is modified primarily with mannose oligomers, whereas sAP-2, which contains a longer stretch of Ser/Thr-rich repeat sequences, is extensively modified with short phosphoglycan chains (7, 10).

¹ The abbreviations used are: LPG, lipophosphoglycan; PPG, proteophosphoglycan; aPPG, amastigote proteophosphoglycan; sAP, secreted acid phosphatase; MPT, mannose-1-phosphotransferase; pep-MPT, peptide-specific mannose-1-phosphotransferase; CHAPS, 3-[3-chloroamidomethyl]propanesulfonic acid; RP-HPLC, reversed-phase high performance liquid chromatography; HPTLC, high performance thin-layer chromatography; ESI-MS, electrospray ionization mass spectrometry.

* This work was supported by the Australian National Health and Medical Research Council and the Wellcome Trust. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Wellcome Trust Senior Research Fellow and to whom correspondence should be addressed. Tel.: 61-3-9344-5681; Fax: 61-3-9347-7730; E-mail: m.mcconville@biochemistry.unimelb.edu.au.
thought to be attached to serine residues via a Man oligosaccharide cap at the nonreducing terminus (2, 3). In contrast, a Ser/Thr-rich domain in the secreted sAP glycoproteins is modified with a single phosphoglycan chain comprising a long domain of phosphodi- and phosphotri-saccharide repeat units and a mannose oligosaccharide cap at the nonreducing terminus (14, 15).

As this type of protein glycosylation is unique to Leishmania, the enzymes involved in initiating and assembling these glycans may be potential targets for new anti-leishmanial drugs. At present, several enzyme activities have been detected in cell-free assays and detergent extracts that are involved in the synthesis of the phosphoglycan chains of LPG, but that may also be involved in the synthesis of the shorter phosphoglycan chains of sAP and PPGs. These include two elongating enzymes, a putative α-mannose-1-phosphotransferase (MPT) and a β1→4galactosyltransferase, that transfer Man1-PO4 and Gal from GDP-Man and UDP-Gal, respectively, to form the repeating Galβ1→4Man1-PO4 backbone of both the LPG and PPG glycans (Fig. 1) (19, 20). In addition, a β1→3-galactosyltransferase activity that adds the L. major-specific side chains to these repeat units has also been characterized (21). Interestingly, a separate MPT activity is thought to be involved in adding the first Man1-PO4 residue to the LPG anchor precursor and thus to be required to initiate LPG phosphoglycan biosynthesis (22). As one of the enzymes involved in synthesizing the LPG anchor is localized to the Golgi apparatus (23), it is likely that both the initiating MPT and phosphoglycan chain-elongating and -branching enzymes are also localized in either a Golgi or post-Golgi compartment. This is consistent with the finding that a GDP-Man transporter required by these MPTs is also localized to the Golgi apparatus (24) and that phosphoglycan chain elongation is inhibited by the Golgi-perturbing ionophore, monensin (25).

In this study, we have identified a peptide-specific MPT (pep-MPT) activity from L. mexicana promastigotes that is most likely involved in initiating protein phosphoglycosylation. Mass spectrometry of the glycopeptides containing the Man1-PO4 modification provides the first direct characterization of this novel linkage. We show that pep-MPT is most active against peptides containing the Ser/Thr-rich sequences of endogenous polypeptide acceptors and that it is not inhibited by glycan acceptors of the LPG phosphoglycan-initiating and -elongating MPTs. Furthermore, we provide evidence that this enzyme occurs in a distinct subcompartment of the Golgi apparatus.
apparatus from enzymes involved in phosphoglycan chain elongation.

**EXPERIMENTAL PROCEDURES**

**Materials—**Alkaline phosphatase, GDP-Man, UDP-Gal, and stachyose were from Sigma; jack bean α-mannosidase was from Boehringer Mannheim. The synthetic oligosaccharide acceptor L2 (Galβ1-4Manα1-PO4-CH2OH, CH2=CH2) was generously provided by Professor Michael Ferguson (University of Dundee). GDP-[3H]Man was prepared using previously described methods (26, 27). Briefly, [3H]-GDP-Man-PO4-CH2OH was synthesized enzymatically from [2-3H]Man (NEN Life Science Products) using yeast hexose kinase and then converted to the sugar nucleotide using a mixture of yeast proteins supplemented with snake venom pyrophosphatase and glucose-1,6-diphosphate. The yeast proteins, corresponding to a 50–70% ammonium sulfate cut, were prepared from protease A-deficient yeast strain SC295. GDP-[3H]Man was purified on a column (1 ml) of concanavalin A-Sepharose (Amersham Pharmacia Biotech) that was washed with 15 mM ammonium acetate, 1 mM MgCl2, and 1 mM CaCl2 and then eluted with the same buffer containing 50 mM α-methylmannoside. Fractions containing radioactivity were desalted on a column of Sephadex G-10 eluted in water. Polyclonal antibodies against L. major Rab1 protein (28) and Trypanosoma brucei EPI protein (29) were generously provided by Dr. Emanuela Hanemann (Water and Eliza Hall Institute of Medical Research, Melbourne, Australia) and Dr. James Bangs (Department of Biochemistry, University of Wisconsin, Madison, WI), respectively.

**Synthesis of Peptide Substrates—**Peptides were synthesized using Fmoc (N-9-fluorenylmethoxycarbonyl) chemistry on a Applied Biosystems Model 431A peptide synthesizer. Peptides were cleaved from the resin using 95% trifluoroacetic acid (3 h, 25 °C), recovered by extraction with diethyl ether, and purified on a C18 reversed-phase column (4.6, inner diameter, × 100 mm; Brownlee) eluted with acetonitrile (0–60%) in 0.1 M ammonium acetate over 20 min at a flow rate of 1 ml/min. Reductive methylation of sapp-1 was carried out in 0.2 M borate buffer, pH 9.0 (140 μl), containing 16 mM formaldehyde and 0.1 mM NaBH4 (5 mM) at 0 °C for 10 min (30). The mixture was acidified with 1 M acetic acid to remove excess NaB3H4, and the radiolabeled peptides were recovered after application to a Sep-Pak C18 cartridge (Waters) following elution with 40% acetonitrile.

**Preparation of Parasitic Membranes—**Promastigotes of L. mexicana (strain MNYC/BR/62/M379) were cultivated at 27 °C in RPMI 1640 medium supplemented with 10% fetal bovine serum and harvested at mid-log growth unless otherwise stated. Amastigotes were isolated from infected BALB/c mice by Bates et al. (31) procedure. Cell envelopes were harvested by centrifugation (750 × g, 10 min, 27 °C); washed once with phosphate-buffered saline; resuspended in 50 mM HEPES-NaOH, pH 7.4, containing 50 mM KCl, 10 mM MgCl2, 10 mM MnCl2, 5 mM Na2EGTA, 2 mM dithiothreitol, 1 mM ATP, 0.1 mM phenylmethylsulfonyl fluoride, 2 μM leupeptin, and 0.2 mM Nα,Nα-diisopropylfluorophosphoryl fluoride (buffer A). Enzyme Assays—Fractionation and purification of the enzymes were performed using Finnigan MAT BIOMASSTM software. For tandem mass spectrometry, quadrupole Q1 was operated with a resolution of 2–2.5 Da and Q3 with a resolution of 1–1.5 Da (35). The heated capillary was set at 150 °C. Mass spectra were determined by scintillation counting. Man-PO4-modified and lipo-oligosaccharide L2 acceptors were resolved on analytical thin-layer electrophoresis of 4 μl of the reaction mixture.

**HPLC and HPTLC Analyses—**Underivatized and Man-PO4-modified peptides were separated on a C18 reversed-phase HPLC (RP-HPLC) column (4.6, inner diameter, × 100 mm; Brownlee) eluted with 0–35% acetonitrile in 0.1 mM ammonium acetate at 1 ml/min over 30 min. Peptides were detected by UV absorbance at 280 nm (for sapp 1, 2, 4–6) or by MS spectroscopy. Radioactivity was determined by scintillation counting. Man-PO4-modified peptide and lipo-oligosaccharide L2 acceptors were resolved on aluminum-backed Silica Gel 60 HPTLC sheets (Merck) developed in chloroform, methanol, and 0.25% KCl (10:10:3, v/v) over 10 cm. HPTLC sheets were sprayed with ENHANCE (NEN Life Science Products) and exposed to Biomax MR film (Eastman Kodak Co.) at 80 °C.

**Thin-layer Electrophoresis—**Man-PO4-modified labeled peptides were resolved from underivatized [3H]labeled peptides by thin-layer electrophoresis on plastic-backed cellulose thin-layer sheets (20 × 20 cm; Macherey Nagel). Aliquots of the reaction mixture (containing 100,000 cpm) were spotted onto the cellulose sheets, which were then saturated with pyridine/acetic acid/water (1:10:89, v/v) and subjected to electrophoresis (500 V, 30 mA, 75 min) under a layer of petroleum spirit and pyridine/acetic acid/water (1:10:89, v/v) in each electrophoresis chamber. Radioactivity on the dried cellulose sheets was detected using an automatic TLC scanner (Berthold) and/or by fluorography as described above.

**Enzyme and Chemical Analyses of Phosphoglycosylated Peptides—**Phosphorylated peptides were digested with jack bean α-mannosidase (30 μl, 50 units/ml) in 0.1 M sodium acetate buffer, pH 5.0 (18 h, 37 °C), with or without subsequent digestion in alkaline phosphatase (2000 units/ml) in ammonium bicarbonate, pH 8.5 (7 h, 37 °C). For recovery of [3H]labeled glycans, the enzyme digests were desalted on a C18 Sep-Pak (Waters) cartridge pre-equilibrated in 0.1 M ammonium bicarbonate (50 mM Na2HPO4, pH 7.5, 2000 units/ml) or in ammonium bicarbonate or water (1:10:89, v/v) in each electrophoresis chamber. Radioactivity on the dried cellulose sheets was detected using an automatic TLC scanner (Berthold) and/or by fluorography as described above. Incorporation of radioactivity into phosphoglycosylated peptides was quantitated by scraping the cellulose containing the bands using the fluorograph as template, extracting the cellulose with 30% acetonitrile (50 μl), and counting the radioactivity by liquid scintillation counting of the extracts.

**Electrospray Ionization Mass Spectrometry—**Unmodified and phosphorylated peptides were analyzed with a triple quadrupole mass spectrometer (Finnigan MAT Model TSQ-700) equipped with a Finnigan MAT electrospray ionization (ESI) source and rapid capillary RP-HPLC. The column used in this study (0.2, inner diameter, × 150 mm; Vydac C18) was fabricated and operated as described elsewhere (34). The column was developed with a linear 30-min gradient at 1.6 μl/min from 0 to 100% solvent B, where solvent A was 0.1% trifluoroacetic acid and solvent B was 60% acetonitrile containing 0.1% trifluoroacetic acid (35). The ESI needle was operated at −4.5 kV. The sheath liquid was methanol/acetone/0.1% acetic acid (40:40:20, v/v) at a flow rate of 400 μl/min over Agilent Capillary HTs. H-Labeled peptides were analyzed by thin-layer electrophoresis as described above, whereas [3H]labeled glycans were analyzed by HPTLC using 1-propanol/acetone/water (9:6:5, v/v) as the solvent system (33).
the ion selected for collision-induced dissociation by 0.04. The daughter ion offset voltage was set at twice the collision cell offset value. The parent ion offset voltage was set at one-third the value of the daughter ion offset voltage.

Characterization of Endogenous Polypeptide and LPG Acceptors—Sonicated microsomal membranes were incubated with GDP-[3H]Man in the presence of exogenous peptide acceptors and UDP-Gal as described above. Membranes were recovered by centrifugation (14,000 × g, 5 min), washed with water to remove residual peptide, and recentrifuged, and the supernatants were combined for recovery of the peptide on a Sep-Pak C18 cartridge and further analyzed by RP-HPLC as shown in Fig. 2. Incubation of the membranes with sapp-1 (containing two Ser-rich motifs) generated at least five 3H-labeled peaks that eluted earlier than the unmodified peptide on RP-HPLC. These peaks were not detected when membranes were incubated in the absence of either the exogenous peptide or GDP-Man (data not shown).

Treatment of the total peptide mixture or the individual HPLC-purified peaks with either jack bean α-mannosidase (data not shown) or mild trifluoroacetic acid (to selectively cleave hexose 1-phosphate linkages) generated a single labeled component in each case that comigrated with mannose on HPTLC (Fig. 2C). These data suggested that sapp-1 was being variably modified with one or more Man1-P0 residues. This was confirmed by positive ion ESI-MS of the HPLC-purified phosphomannosylated peaks 1 and 2 in Fig. 2. Multiply charged ions were obtained that were in agreement with the calculated mass for sapp-1 modified with one or two hexose phosphate residues, respectively (Table II). As the ionization of these phosphomannosylated peptides was relatively poor, a smaller peptide (sapp-2) containing a single Ser/Thr-rich repeat unit and a C-terminal lysine residue was used as substrate. As with sapp-1, several [3H]Man-labeled peptides were generated when this peptide was incubated with sonicated membranes (Fig. 3A). Positive ion ESI-MS of the two major peaks contained both [M + H]" and [M + 2H]" ions corresponding to sapp-2 with either one (Fig. 3B) or two (Fig. 3C) hexose-P0 residues (Table II). Fragment ions corresponding to the loss of one or two hexose residues were also evident in these mass spectra, indicating that the Man-P0 linkage is relatively labile under the ionization conditions employed. Taken together, these data indicate that peptides containing the same Ser/Thr-rich domains as endogenous polypeptides are modified with one or more single Man1-P0 residues, providing the first evidence for the presence of a pep-MPT activity.

Collision-induced dissociation of the monophosphomannosylated sapp-1 and sapp-2 peptides provided negligible or incomplete sequence information on the site(s) of Man-P0 substitution, reflecting the relatively poor ionization of these peptides. However, complete sequence information was obtained for monophosphomannosylated sapp-3, a peptide with a single serine-rich motif and two C-terminal lysine residues (Tables I and II). The tandem mass nontectrometry of the unmodified sapp-3 [M + 2H]" ion (m/z 995.4) resulted in the loss of mannose and fragmentation along the peptide backbone to produce a series of mass spectra, indicating that none of the C-terminal residues in the peptide, including Ser15 and Ser16, were phosphorylated. In contrast,
Mannose-1-phosphotransferase Activity from L. mexicana

Fig. 2. RP-HPLC analysis of phosphomannosylated peptides synthesized in vitro. Following incubation of promastigote microsomal membranes with sapp-1 and GDP-[3H]Man, unmodified and phosphomannosylated peptides were purified on a Sep-Pak C18 cartridge and resolved by RP-HPLC employing a gradient of acetonitrile (0–35%). Peptides were detected by UV absorbance and resolved by RP-HPLC employing a gradient of acetonitrile (0–35%) phomannosylated peptides were purified on a Sep-Pak C18 (HPTLC analysis, and acid-labile (phosphodiester-linked) glycans were analyzed by (Glc, Gal, and Man) and disaccharides (two series of y9–11 ions, differing in mass by 80 Da (corresponding increase in ion intensity of the y9–12 ions for the unmodified peptide and the corresponding increase in ion intensity of the y9–12 ions for the phosphorylated peptide (Fig. 4C) suggest that all four Ser residues in the central domain of this peptide were partially phosphorylated. Whereas the b series of fragment ions was less complete, the identification of the expected b2–3 series for the unmodified peptide indicates that neither Ser1 nor Ser2 is phosphorylated. These data indicate that sapp-3 is heterogeneously modified with Man1-PO4 on Ser7–10 and also suggest that none of the Thr residues are modified.

Properties of the pep-MPT Activity—pep-MPT activity in detergent-solubilized microsomes was not affected by inclusion of amphomycin (0.6 mg/ml), a potent inhibitor of dolichol-phosphate mannose synthesis, indicating that the mannose phosphate residue was being directly transferred from GDP-Man (data not shown). Although pep-MPT activity was solubilized from microsomal membranes (>80%) with a range of non-ionic detergents (see “Experimental Procedures”), Triton X-100 was the most effective at retaining enzyme activity (Fig. 5A) (data not shown). However, enzyme activity was considerably more labile after detergent solubilization, as maximal activity occurred at 16 °C, rather than at 32 °C, when non-solubilized membranes were assayed (data not shown). The detergent-solubilized pep-MPT activity displayed a pH optimum at 7.4 (Fig. 5B) and had an apparent K_m for GDP-Man of 180 μM (Fig. 5C). All activity was abolished when 5 mM EGTA was included in the reaction mixture in the absence of added divalent cations, but was restored to maximal levels with the addition of 10 mM MnCl_2 (i.e. 5 mM free Mn_2^{2+}) (Fig. 5D). MgCl_2 also stimulated activity, but was not as effective as MnCl_2 and did not significantly further stimulate the effect of adding MnCl_2 alone (Fig. 5D). Addition of 5 mM free Ca_2^{2+} had no effect on the pep-MPT activity in the absence (Fig. 5D) or presence (data not shown) of MnCl_2 or MgCl_2. Interestingly, the pep-MPT activity varied markedly in a growth- and stage-dependent manner (Fig. 5A). Rapidly growing (procyclic) promastigotes contained 5- or 10-fold higher activity than either late stationary (metacyclic) promastigotes or freshly isolated lesion amastigotes, which varied markedly in a growth- and stage-dependent manner (Fig. 5A). Two different series of y9–11 ions, differing in mass by 80 Da (corresponding to both unmodified and phosphorylated forms of Ser8–10), and one series of y12–16 ions corresponding to the phosphorylated peptide were observed in the mass spectrum (Fig. 4, A and B). Additional fragment ions corresponding to loss of H_3PO_4 from the phosphorylated peptides, diagnostic ions for serine- and threonine-phosphorylated peptides (37), were also present (y_n’ in Fig. 4, A and B). The progressive decrease in ion intensity of the y9–12 ions for the unmodified peptide and the corresponding increase in ion intensity of the y9–12 ions for the phosphorylated peptide (Fig. 4C) suggest that all four Ser residues in the central domain of this peptide were partially phosphorylated. Whereas the b series of fragment ions was less complete, the identification of the expected b2–3 series for the unmodified peptide indicates that neither Ser1 nor Ser2 is phosphorylated. These data indicate that sapp-3 is heterogeneously modified with Man1-PO4 on Ser7–10 and also suggest that none of the Thr residues are modified.

The pep-MPT Activity Is Distinct from the LPG Phosphoglycan-initiating and -elongating MPTs—Although pep-MPT utilizes exogenously added peptides, it was possible that this

2 J. E. Ralton and M. J. McConville, unpublished data.
activity was due to two previously characterized leishmanial MPT activities that are thought to be required for initiation and elongation of the LPG chain biosynthesis (20, 22). These oligosaccharide MPT activities add Man-PO₄ to terminal galactose residues on either the LPG anchor precursor or phosphodisaccharide repeat units on the growing LPG phosphoglycan chain, respectively (20, 22). A second assay was developed to assess whether synthetic oligosaccharide acceptors for these MPTs competed with the peptide substrates for pep-MPT activity. In this assay, N-terminally ³H-labeled sapp-1 peptide (at a concentration of 2 × Kᵣₐᵣₑ) and unlabeled GDP-Man were added to microsomal membranes in the absence or presence of unlabeled oligosaccharide or peptide substrates. In the absence of competitive substrate, ~30% of the ³H-labeled sapp-1 peptide was modified with Man-PO₄ residues over a 30-min incubation period, as shown by the appearance of additional labeled bands on thin-layer electrophoresis that migrated more rapidly toward the cathode (Fig. 6A). The additional bands corresponded to the sapp-1 peptide with one, two, or three Man₈PO₄ residues, as sequential treatment of the peptide mixture with jack bean α-mannosidase and alkaline phosphatase collapsed these bands back to a single labeled species that comigrated with unmodified sapp-1 (Fig. 6B). As expected, the addition of 1 mM unlabeled sapp-1 resulted in a 55% decrease in

---

**Fig. 3.** Positive ion ESI-MS mass spectra of mono- and diphosphomannosylated sapp-2 peptides. A, RP-HPLC profile (Årug) of unmodified and phosphomannosylated sapp-2 peptides; B and C, positive ion ESI-MS of monophosphomannosylated and diphosphomannosylated peaks, respectively. Several minor ions in the spectrum shown in B (m/z 1798.9, 1637.2, and 900.2) were derived from a contaminating peptide with the same sequence as sapp-2 minus one of the glycine residues.
the extent to which the reductively methylated $^{3}$H-labeled sapp-1 peptide was modified (Fig. 6C). In contrast, the oligosaccharide acceptors for the LPG-specific MPTs, L2 (Galβ1–4Manα1-PO4-(CH2)8CH2) and stachyose (Galα1–6Galα1–6Glcα1–2Fru), did not inhibit the pep-MPT activity when present at a 6–30-fold excess over the concentration of the sapp-1 peptide (Fig. 6C). The concentration of L2 used in these experiments was higher than that used previously ($600 \text{ mM}$) to achieve close to maximal saturation of the putative elongating MPT (20). Moreover, in separate experiments, unlabeled sapp-4 was found not to inhibit the phosphomannosylation of L2. As shown in Fig. 7, incubation of detergent-solubilized microsomal membranes with L2 (1 mM) and GDP-[$^{3}$H]Man generated a labeled product with a slower HPTLC mobility compared with unmodified L2 (compare lanes 1 and 3). This product has previously been shown to correspond to L2 modified with a single terminal Man-1-PO4 residue (20). Significantly, the modification of L2 by the putative elongating MPT was not affected by the inclusion of sapp-4 (up to 0.5 mM) in the assay (Fig. 7, compare lanes 1 and 2). As the apparent $K_{m}$ of pep-MPT for sapp-4 is 0.05 mM (Table III), these data support the notion that the elongating MPT and pep-MPT are separate enzyme activities. Similar experiments were not performed with substrates of the initiating MPT (stachyose or LPG anchor) because high concentrations of GDP-Man (1 mM) (22) are needed to detect this activity, precluding the use of GDP-[$^{3}$H]Man.

**Substrate Specificity of pep-MPT**—The minimal requirements for pep-MPT activity were investigated using a number of peptide substrates. As shown in Table III, peptides with two Ser/Thr-rich repeats (i.e. sapp-1) were slightly better substrates than peptides with one Ser/Thr-rich repeat (i.e. sapp-4) ($K_{m}$ 520 and 50$^{\text{mM}}$, respectively). Replacement of the four Ser residues in sapp-4 with Thr (sapp-5) virtually abrogated all substrate potential, confirming the specificity of MPT for Ser residues (Table III). Interestingly, replacement of the two acidic amino acids in the sequences flanking the serines (sapp-6) resulted in a 17-fold increase in the $K_{m}$ of the pep-MPT activity, suggesting that these residues may contribute to MPT substrate recognition (Table III). In the competition assay, unlabeled sapp-4 (1 mM) was slightly less effective than unlabeled sapp-1 (50\% versus 55\%) at reducing the rate of phosphoglycosylation of $^{3}$H-labeled sapp-1 (Fig. 6C). However, both sapp-5 and sapp-6 and a peptide containing 11 serine residues (Ser11 peptide) inhibited this reaction by 10\%. These data suggest that replacement of Ser residues with Thr or removal of the acidic flanking residues in the repeat sequences abrogates recognition of the sAP-based peptides by pep-MPT.

**Subcellular Distribution of pep-MPT**—Initial experiments showed that pep-MPT activity was sedimented in a 100,000 $\times g$ microsomal pellet and that activity was detected only when intact microsomal membranes were disrupted by sonication or detergent lysis, indicating that pep-MPT is a membrane-associated enzyme and that the catalytic domain has a luminal orientation. To investigate the subcellular location of this enzyme and thus the site at which protein phosphoglycosylation is initiated, L. mexicana microsomal membranes were prepared by nitrogen cavitation and fractionated by equilibrium velocity sucrose gradient centrifugation. Previously character-
Mannose-1-phosphotransferase Activity from *L. mexicana*

**Fig. 5.** Properties of *L. mexicana* pep-MPT. **A**, levels of pep-MPT activity in promastigotes from early logarithmic (L) and late stationary (S) phase cultures and from lesion-derived amastigotes (A). Specific activity was measured in sonicated membranes with or without solubilization in 1% Triton X-100 (TX-100). Total pep-MPT activity in the detergent extracts was ~80% of that in the sonicated membranes in the absence of detergent. **B–D**, the effect of pH, GDP-Man concentration, and various cations, respectively, on pep-MPT activity. The standard assay buffer was used in all experiments, except for the cation dependence experiment, where buffer A minus MgCl₂ and MnCl₂ was used and cations were supplemented as indicated.

**DISCUSSION**

We have characterized a novel pep-MPT activity from *L. mexicana* promastigotes that is likely to be responsible for initiating the phosphoglycosylation of a major class of secreted glycoproteins and mucin-like molecules. The phosphoglycans may account for 20–90% of these secreted molecules and are structurally variable, ranging from single Man-PO₄ residues to exceedingly complex and highly branched phosphoglycans, depending on the nature of the polypeptide backbone and parasite developmental stage (10, 14, 15). However, all these glycans are thought to be linked to the polypeptide backbone by a common Man₁→PO₄-Ser linker sequence (10, 14, 15). This linkage was indicated by the detection of O-phosphoserine in sAPs and PPGs and the resistance of these residues to alkaline phosphatase unless the masking glycans were removed with α-mannosidase or mild acid hydrolysis (10, 14, 15). However, it has been difficult to confirm the nature of this linkage by mass spectrometry or NMR, as the heavily glycosylated Ser/Thr-rich domains are essentially resistant to proteolysis, preventing the generation of small well-defined glycopeptides that are amenable to such analyses. In this context, the synthesis of Man₁→PO₄-modified peptides by *L. mexicana* membranes and the characterization of these peptides by electrospray collision-induced dissociation mass spectrometry and chemical enzymatic treatments have provided unequivocal confirmation of this novel linkage. Although the Man₁→PO₄-Ser linkage is apparently unique to leishmanial glycoconjugates, it is related to phosphodiester-linked glycans from other lower eukaryotic glycoconjugates. For example, the cysteine proteases of Dictyostelium discoideum are modified with GlcNAc₁→PO₄ residues in the Triton X-100 pellet corresponds to tightly bound polypeptide acceptors. Unlike the endogenous LPG acceptors, the Triton X-100-insoluble acceptors were modified primarily with Man-PO₄ and only to a small extent with the Galβ₁→4Man disaccharide (Fig. 9, lane 2). Thus, this second pool of acceptors may have limited access to the enzymes involved in the synthesis of the disaccharide repeat units.

**M. J. McConville, unpublished data.**
linked to Ser, whereas secreted glycoproteins from *Trypanosoma cruzi* may be modified with structurally complex oligosaccharides containing a putative Xyl-1-PO\(_4\)-Thr (Ser) linker sequence (11, 12). The abundance of this type of modification in several lower eukaryotes suggests that the glycan-PO\(_4\)-Ser motif may be more common than previously suspected.

We have provided evidence that *L. mexicana* pep-MPT transfers Man\(_a\)1-PO\(_4\) from GDP-Man to Ser-rich peptide sequences that are found in *L. mexicana* sAP. The transfer of Man\(_a\)1-PO\(_4\) was supported by the finding that product formation was not inhibited by amphomycin, suggesting that dolichol-phosphate mannose is not utilized as a sugar donor by this enzyme. In addition, there was no evidence for the modification of the peptide in the absence of GDP-Man indicating that this linkage is not being assembled by the sequential action of a serine kinase followed by a novel mannosyltransferase. Based on ESI-MS product analysis and the use of various peptide acceptors, the following conclusions were made concerning the substrate specificity of pep-MPT. First, this enzyme only adds Man\(_a\)1-PO\(_4\) to Ser residues; a peptide containing Thr instead of Ser was neither a substrate nor an acceptor. Second, the enzyme shows a preference for peptide sequences containing multiple Ser residues. Third, the enzyme is specific for Ser residues and does not transfer Man\(_a\)1-PO\(_4\) to Thr residues.

**FIG. 6.** *Substrate specificity of pep-MPT.* The substrate specificity of pep-MPT was tested using a competition assay. Triton X-100-solubilized microsomal membranes were incubated with \(^3\)H-labeled sapp-1 and GDP-Man in the absence or presence of the indicated competitive substrate (1 mM), and the labeled products were analyzed by thin-layer electrophoresis. A, when products were analyzed after a 30-min incubation (30') in the absence of competitive substrate, several additional labeled bands (not present in the zero (0') time point) were generated that migrated toward the cathode. B, sequential digestion of the products in the 30-min time point with jack bean \(\alpha\)-mannosidase (JBAM) and alkaline phosphatase (AP) collapsed these bands back to unmodified sapp-1, indicating that these bands were differentially modified with Man\(_a\)1-PO\(_4\). C, inhibition of \(^3\)H-labeled sapp-1 phosphomannosylation by glycan acceptors of the LPG-specific MPTs (L2 and stachyose) and various unlabeled peptide substrates (sapp-1, sapp-4, sapp-5, sapp-6, and Ser11) is shown using standard incubation conditions. The structures of L2 and stachyose are Gal\(\beta\)1-4Man\(_a\)1-PO\(_4\)-(CH\(_2\))\(_8\)CH\(_2\) and Gal\(_\alpha\)1-6Gal\(_\alpha\)1-6Glc\(_\beta\)1-2Fru, respectively. The mean values of three experiments are shown. O indicates the sample loading origin on the thin-layer electrophoresis sheets.

**FIG. 7.** sapp-4 does not inhibit phosphomannosylation of L2. Microsomal membranes were solubilized in 1% Triton X-100 and incubated with the synthetic lipo-oligosaccharide acceptor L2 (1 mM) (lane 1) or both L2 (1 mM) and sapp-4 (0.5 mM) (lane 2) or sapp-4 (0.5 mM) (lane 3), together with GDP-\(^3\)HMan (0.5 mM). After incubation (30 min, 16 °C), the reaction was stopped, and the labeled lipo-oligosaccharide and peptide acceptors were recovered on a Sep-Pak C\(_18\) cartridge (20) and analyzed by HPTLC in chloroform, methanol, and 0.25% KCl (10: 10:3, v/v).

| Peptide | \(K_m\) | \(V_{max}\) |
|---------|---------|---------|
| sapp-1  | 20      | 6.77    |
| sapp-4  | 50      | 6.06    |
| sapp-5  | ND\(^c\) | ND      |
| sapp-6  | 850     | 6.60    |

\(^a\) \(K_m\) and \(V_{max}\) values calculated from the negative x axis and the slope of the plot of \(s/v\) against \(s\), respectively.  
\(^b\) \(V_{max}\) expressed as picomoles of Man-P transferred per \(\mu\)g of protein.  
\(^c\) ND, not determined due to lack of detectable activity.
A pep-MPT. cis the endoplasmic reticulum marker BiP, the promastigotes were lysed by nitrogen cavitation, and microsomes in a 3000 × 3 fractionation profile of protein; B, sedimentation profile of the endoplasmic reticulum marker BiP, the cis-Golgi marker Rab1, and pep-MPT.

endogenous sAPs and PPGs (10, 14). Third, amino acids flanking the contiguous Ser sequences may be important for pep-MPT recognition and influence the pattern of substitution. For example, sAP peptides lacking the flanking Glu or Asp residues (i.e. sapp-6) were poorly utilized by pep-MPT in direct assays and were poor inhibitors in competition assays. It is possible that these residues may be required to maintain a suitable conformation or the solubility of the peptide, as polyserine peptides become increasingly insoluble above six residues in length.

The pep-MPT characterized here appears to be distinct from the two oligosaccharide-specific MPTs that are involved in adding Man-PO4 to the LPG anchor precursor (initiating MPT) or preformed phosphoglycan chains on lipid or peptide acceptors (elongating MPT) (20, 22). The initiating MPT can be assayed using either dephosphorylated LPG core or the structurally related oligosaccharide, stachyose, as acceptor (22). In contrast, the elongating MPT adds Man1-PO4 to the minimal disaccharide unit Galβ1–4Manα1-PO4 that is present in the synthetic hydrophobic oligosaccharide acceptor L2 (20). Neither stachyose nor L2 inhibited the pep-MPT activity when present at 6–30-fold excess over the peptide acceptors (Fig. 6C). Moreover, unlabeled sapp-4 peptide did not inhibit the phosphomannosylation of L2 when present at concentrations that inhibit the phosphomannosylation of the 3H-labeled sapp-1 peptide (Fig. 7). pep-MPT could also be distinguished from the other MPTs by its solubility and stability in various detergents (20, 22). On the other hand, all three MPTs appear to utilize GDP-Man as the sugar donor and have a similar pH optimum and requirement for divalent cations. Although the substrate specificities of these MPTs appear to be distinct, it is notable that both pep-MPT and elongating MPTs are maximally active against substrates that contain negatively charged groups near the aglycon acceptor (i.e. acidic amino acids or phosphate, respectively) (this study and Ref. 20), suggesting that these activities may have arisen from a common progenitor enzyme.

Marked differences were observed in the levels of pep-MPT activity during promastigote growth, with 5- or 10-fold higher levels of activity being observed in rapidly dividing procyclic promastigotes compared with late stationary phase promastigotes and lesion-derived amastigotes, respectively. There is no evidence that the low pep-MPT activity of the metacyclic promastigotes or the intracellular amastigotes is associated with a reduced level of phosphoglycosylation of secreted polypeptides. Indeed, aPPGs are much more extensively modified with phosphoglycans than promastigote sAPs or promastigote PPGs (15). It is thus possible that the low pep-MPT activity in non-dividing promastigotes and the intracellular amastigotes may reflect a low level of secretory activity in these developmental stages or the presence of other pep-MPT activities that preferentially recognize distinct peptide sequences in aPPGs (14, 15).

Subcellular fractionation studies suggested that pep-MPT was localized in a post-endoplasmic reticulum compartment and that it had an overlapping (but not coincident) distribution with the Golgi marker Rab1. Rab1 has been localized primarily to cis-Golgi cisternae in L. major (28), but may also be localized to endoplasmic reticulum-Golgi transport vesicles, where it is thought to be involved in regulating the organization of vesicle proteins (38). The broader distribution of the Rab1 marker may reflect the partial distribution of this protein in lighter transport vesicles from which pep-MPT is excluded. Localization of pep-MPT to the Golgi apparatus is consistent with the recent demonstration that the leishmanial Golgi apparatus contains a GDP-Man transporter that is required for phosphoglycan biosynthesis (24). Similarly, the initiating and elongating MPTs
involved in LPG biosynthesis are also thought to be localized to the Golgi apparatus based on the finding that one of the enzymes involved in LPG anchor biosynthesis has been localized to the Golgi apparatus by electron microscopy (23). In this respect, it is of interest that Man-PO₄ residues on the endogenous polypeptide pool were quantitatively modified with at least one repeat unit. Although it is possible that the Man-PO₄-modified peptide is a poor substrate for these elongating enzymes, the low rate of the peptide or a pool of endogenous polypeptide acceptors were negligibly or poorly elaborated with phosphoglycan repeat units. In contrast, Man-PO₄ residues on the endogenous LPG acceptors were quantitatively modified with at least one repeat unit. It is likely that the Man-PO₄-modified peptide is a poor substrate for these elongating enzymes, the low rate of elongation of Man-PO₄ on the endogenous polypeptide pool suggests that pep-MPT and possibly other MPTs involved in LPG biosynthesis are also thought to be localized to the Golgi apparatus based on the finding that one of the enzymes involved in LPG anchor biosynthesis has been localized to the Golgi apparatus by electron microscopy (23).

In summary, we have confirmed the nature of the peptide linkage of the unusual phosphoglycans that are added to the major secretory proteins of L. mexicana and identified a novel pep-MPT activity. Similar types of phosphoglycosylation occur in all developmental stages of the parasite, including the amastigotes that perpetuate disease in the mammalian host and that have been shown to be functionally important. Consequently, it is likely that pep-MPT and possibly other MPTs are potential targets for new anti-leishmanial drugs.

Acknowledgments—we thank Professor Michael Ferguson for providing the synthetic L2 acceptor, Drs. Emanuela Handman and James Bangs for antibodies, and Professor Tony Bacic for critical reading of the manuscript.

REFERENCES
1. Turco, S. J., and Descoteaux, A. (1992) Annu. Rev. Biochem. 61, 65–94
2. McConville, M. J., and Ferguson, M. A. J. (1993) Biochem. J. 294, 305–324
3. McConville, M. J., Schnur, L. F., Jaffe, C., and Schneider, P. (1995) Biochem. J. 310, 807–818
4. Ilg, T., Stierhof, Y.-D., Wiese, M., McConville, M. J., and Overath, P. (1994) Parasitology 108, S63–S71, and references therein
5. Mengeling, B. J., Beverley, S. M., and Turco, S. J. (1997) Glycobiology 7, 873–880
6. Ilg, T., Stierhof, Y.-D., Etges, R., Adrian, M., Harbecke, D., and Overath, P. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 8774–8778
7. Weise, M., Ilg, T., Lottspeich, F., and Overath, P. (1995) EMBO J. 14, 1067–1074
8. Jaffe, C. L., Perez, L. M., and Schnur, L. F. (1990) Mol. Biochem. Parasitol. 41, 223–240
9. Ilg, T., Harbecke, D., Wiese, M., and Overath, P. (1993) Eur. J. Biochem. 217, 603–615
10. Ilg, T., Overath, P., Ferguson, M. A. J., Rutherford, T., Campbell, D. G., and McConville, M. J. (1994) J. Biol. Chem. 269, 24073–24081
11. 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
12. Haynes, P. A. (1996) Glycobiology 8, 1–5
13. Ilg, T., Stierhof, Y.-D., McConville, M. J., and Overath, P. (1995) Eur. J. Cell Biol. 66, 205–215
14. Ilg, T., Stierhof, Y.-D., Craik, D., Simpson, R., Handman, E., and Bacic, A. (1996) J. Biol. Chem. 271, 21583–21596
15. Ilg, T., Craik, D., Currie, G., Multsaap, G., and Bacic, A. (1998) J. Biol. Chem. 273, 15509–15525
16. Stierhof, Y.-D., Ilg, T., Russell, D. G., Hohenberg, H., and Overath, P. (1994) J. Cell Biol. 125, 321–331
17. Peters, C., Kawakami, M., Ilg, T., Overath, P., and Aeberhardt, T. (1997) Eur. J. Immunol. 27, 2666–2672
18. Peters, C., Stierhof, Y.-D., and Ilg, T. (1997) Infect. Immun. 65, 783–786
19. Carver, M. A., and Turco, S. J. (1991) J. Biol. Chem. 266, 10974–10981
20. 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
21. Ng, K., Handman, E., and Bacic, A. (1996) Glycobiology 4, 845–853
22. Mengeling, B. J., Zilberstein, D., and Turco, S. J. (1997) Glycobiology 7, 847–853
23. Ha, D. S., Schwarz, J. K., Turco, S. J., and Beverley, S. M. (1996) Mol. Biochem. Parasitol. 77, 57–64
24. Ma, D., Russell, D. G., Beverley, S. M., and Turco, S. J. (1997) J. Biol. Chem. 272, 3799–3805
25. Bates, P. A., Hermes, I., and Dwyer, D. M. (1990) Mol. Biochem. Parasitol. 39, 127–1256
26. Israel, A. Y., Tae, M. S., Krag, S. S., and Robbins, P. W. (1976) Anal. Biochem. 74, 484–487
27. Rush, J. S., Shelling, J. G., Zingg, N. S., et al. (1993) J. Biol. Chem. 268, 31310–31317
28. Cappai, R., Osborn, A. H., Gleson, P. A., and Handman, P. (1993) Mol. Biochem. Parasitol. 62, 73–82
29. Bangs, J. D., Uyetake, L., Brickman, M. J., Balber, A. E., and Boothroyd, J. C. (1993) J. Cell Sci. 105, 1101–1113
30. Tack, B. F., Dean, J., Elat, D., Lorenz, P. E., and Schechter, A. N. (1986) J. Biol. Chem. 255, 8842–8847
31. Bates, P. A., Robertson, C. D., Tetley, L., and Combs, G. H. (1993) Parasitology 105, 193–202
32. Wessel, D., and Flugge, I. U. (1986) Anal. Biochem. 138, 141–143
33. Schneider, P., Balbon, J. E., McConville, M. J., and Ferguson, M. A. J. (1994) Anal. Biochem. 210, 102–112
34. Moritz, R. L., and Simpson, R. J. (1992) J. Chromatogr. 599, 119–130
35. Moritz, R. L., Eddes, J. S., Reid, G. E., and Simpson, R. J. (1996) Electrophoresis 17, 909–917
36. McConville, M. J., Thomas-Oates, J. E., Ferguson, M. A. J., and Homans, S. W. (1999) J. Biol. Chem. 275, 18611–18623
37. Schiller, J. B., and Carr, S. A. (1996) Anal. Chem. 68, 3413–3421
38. Finn, S. N., Nuoffer, C., McCaffery, J. M., Plutner, H., Davidson, H. W., Farquhar, M. G., and Balch, W. E. (1994) J. Cell Biol. 125, 239–252