Heparin binds to *Leishmania donovani* promastigotes and inhibits protein phosphorylation

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We show that promastigotes of *Leishmania donovani*, the causative agent of visceral leishmaniasis (kala-azar), possess heparin receptors on their surface. From a linear Scatchard plot of the binding data obtained using [³H]heparin and viable promastigotes, one derives a binding constant of 4.7×10^{-7} M and an estimate of 860 000 receptors per parasite. The [³H]heparin bound to parasites could not be displaced by hyaluronic acid or by three other glycosaminoglycans (dermatan sulphate, chondroitin 4-sulphate and chondroitin 6-sulphate). It was demonstrated that exponential phase promastigotes growing in medium 199 supplemented with fetal bovine serum incorporate ³⁵SO₄ into a cell-associated macromolecule that has the properties of heparin proteoglycan. Heparin inhibits the activity of the cell-surface histone-protein kinase; incubation of viable promastigotes with [γ -³²P]ATP and MgCl₂ (10 mM) in the absence and presence of heparin (0.01–0.5 mg/ml) for 10 min, followed by analysis by SDS/polyacrylamide-gel electrophoresis and autoradiography, revealed that the phosphorylation of 12 or 13 parasite proteins was inhibited by the glycosaminoglycan. These data suggest that heparin may play a role in the host–parasite relationship.

INTRODUCTION

Leishmania donovani is a unicellular eukaryotic parasite and is the causative organism of visceral leishmaniasis, also known as kala-azar. Extracellularly, as it occurs naturally in the gut of its sandfly vector (*Phlebotomus*) or when cultured in liquid media in the laboratory, the organism exists as the motile, flagellated promastigote (Killick-Kendrick *et al.*, 1977). Once inside phagolysosomes of phagocytes of the mammalian host where it proliferates, promastigotes transform into non-motile, non-flagellated amastigotes (Chang, 1979).

In the last 5 years there has been a marked increase in interest in the enzymology of this parasite and the biochemistry of its interaction with host cells (Glew *et al.*, 1988). We demonstrated recently that *L. donovani* promastigotes grown *in vitro* contain large amounts of nucleotide-independent protein kinase activity (Das *et al.*, 1986). Using histones as acceptor to estimate protein kinase activity and monitor the isolation steps, we solubilized and purified the enzyme 1940-fold. In the presence of Mn²⁺ (1 mM), the purified leishmanial kinase can also catalyse the transfer of the γ -phosphate of ATP into tyrosine residues of tubulin, the principal structural protein of microtubules (Mukhopadhyay *et al.*, 1988). Furthermore, we have also shown that approx. two-thirds of the activity of this particular kinase appears to be localized to the outer surface of the parasite (Das *et al.*, 1986).

Studies of the function and significance of the surface protein kinase of the *Leishmania* would be aided by the availability of substances that could alter the activity or specificity of the kinase. Heparin has the potential to be such an effector; it has been shown to be an activator of

glycogen synthase kinase 3 (Ahmad *et al.*, 1986) and an inhibitor of casein kinase II (Hathaway *et al.*, 1980), a cyclic nucleotide-independent protein kinase from rabbit reticulocytes, and a nuclear protein kinase (NII) from hepatomas (Rose *et al.*, 1981).

In the present report we show that an extensively purified preparation of the histone–tubulin protein kinase of *L. donovani* is inhibited by heparin and that exposure of a suspension of autophosphorylating promastigotes to heparin markedly alters their [³²P]phosphoprotein pattern. In addition, we document the presence of high-affinity heparin receptors on the promastigote surface. These observations demonstrate that heparin may provide a useful tool for elucidating the nature and function of proteins and enzymes that play some role in the cell biology of this particular host–parasite interaction.

MATERIALS AND METHODS

Materials

[γ -³²P]ATP (5–10 Ci/mmol) and [G-³H]heparin (0.39 mCi/mg) were purchased from New England Nuclear, Boston, MA, U.S.A. Mixed calf thymus histones, heparins (low molecular mass and unfractionated) and other glycosaminoglycans were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. DEAE-cellulose, Sepharose CL-4B, QAE-Sephadex, Sephadex G-150 and Sephadex G-50 were purchased from Pharmacia Chemicals, Piscataway, NJ, U.S.A. Hydroxyapatite and ampholytes (pH 3–10) were obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A. Lipophosphoglycan (LPG), prepared as described elsewhere (McNeely &

Abbreviations used: TCA, trichloroacetic acid; PAGE, polyacrylamide-gel electrophoresis; BSA, bovine serum albumin; HBS, 25 mM-Hepes buffer in 0.9% NaCl solution; LPG, lipophosphoglycan.

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Turco, 1987; Orlandi & Turco, 1987), was generously provided by Dr. S. Turco, University of Kentucky, Lexington, KY, U.S.A.

Organism and growth conditions

A cloned strain of *L. donovani* (MHOM-SU-62-1S) promastigotes was cultivated and harvested as described by Gottlieb & Dwyer (1981), except for the addition of streptomycin (50 µg/ml) and penicillin (50 µg/ml) to the growth medium. An LPG-deficient mutant (LPG⁻) of *L. donovani* and its parent (LPG⁺) were generously provided by Dr. S. Turco, University of Kentucky, and cultured as described above.

Purification of protein kinase

In the initial stages, the enzyme was purified from *L. donovani* promastigotes exactly as described elsewhere (Das *et al.*, 1986). All procedures, unless otherwise specified, were carried out at 4 °C in 10 mM-sodium acetate buffer, pH 6.5, containing 50 µM-leupeptin and 50 µM-aptoprotein to inhibit proteases, and 10 mM-β-mercaptoethanol to stabilize the kinase. After the hydroxyapatite column step (Das *et al.*, 1986), the protein kinase was purified further by histone affinity column chromatography and isoelectric focusing. The histone affinity column was prepared and utilized as described by Saha *et al.* (1988). Isoelectric focusing was performed with the aid of an LKB 8101 electrofocusing column according to the procedure of Vesterberg & Stevenson (1966). Electrofocusing was carried out for 19 h at 3 W in a 100 ml sucrose density gradient [0–50%, (w/v)] containing 2% (v/v) ampholytes (pH 3–10). The kinase focused sharply as a single peak at pH 5.0 (results not shown). The final yield of protein kinase activity relative to the crude extract was 18%, and the specific activity of the kinase was 2.13×10^6 units/mg of protein. The overall purification achieved was 1940-fold.

Protein kinase assay

Protein kinase activity was routinely determined using mixed histones as the phosphate acceptor in a medium which contained the following components in a final volume of 0.1 ml: 10 mM-MgCl₂, 50 mM-NaF (to inhibit phosphatases), 0.2 mM-histone (30 mg/ml), 50 mM-Tris/HCl buffer (pH 7.2), 1 mM-[γ-³²P]ATP, 50 mM-NaCl and 5–20 µl of enzyme solution (0.1–1.0 µg of protein). The reaction was initiated by the addition of ATP, incubated for 10 min at 37 °C and terminated by the addition of ice-cold 10% (w/v) trichloroacetic acid (TCA) followed by 10 µl of bovine serum albumin (BSA) solution (20 mg/ml). The pellet obtained by centrifugation (5000 g, 10 min) was dissolved in water and reprecipitated three more times with 10% TCA. Finally, the precipitate was dissolved in water and an aliquot was taken for measurement of radioactivity (Corbin & Reimann, 1974). One unit of protein kinase activity corresponds to the incorporation of 1 pmol of ³²P/min.

Protein determination

Protein concentration was estimated by the method of Bradford (1976) using BSA as standard.

SDS/polyacrylamide-gel electrophoresis

³²P-Labelled proteins were resolved by SDS/polyacrylamide-gel electrophoresis as described by Laemmli (1970) using 5–20% gels. Proteins were stained with

Coomassie Brilliant Blue (R-250) and press-dried on to Whatman 3 MM paper. Radiolabelled proteins were revealed by autoradiography with the aid of a Dupont Lightning-plus Screen. Some autoradiograms were scanned with the aid of a Hoefer GS300 densitometer and GS350 data analyser.

³²P-labelling of promastigotes using [γ-³²P]ATP

Promastigotes on the seventh day of culture were harvested and washed twice with 25 mM-Hepes buffer, pH 7.2, in 0.9% NaCl (HBS), supplemented with 5 mM-glucose. Washed cells (1×10^7) were incubated at 27 °C in 0.1 ml of a medium consisting of 25 mM-Tris/Hepes buffer (pH 7.2) supplemented with 0.9% NaCl, 5 mM-glucose, 50 mM-NaF, 10 mM-MgCl₂ and [γ-³²P]ATP (20 µCi). The reaction was terminated after 10 min by the addition of the SDS sample buffer [containing 1.5% (w/v) SDS, 0.5 M-Tris-HCl (pH 6.8), 1.5% (v/v) β-mercaptoethanol and 5% (v/v) glycerol] and boiling for 2 min. The incorporation of ³²P into total unfractionated promastigote proteins was determined after TCA precipitation by liquid scintillation counting as described above, and was linear between 0 and 20 min. An aliquot of a duplicate sample was subjected to SDS/PAGE and autoradiography as described above.

[³H]Heparin binding to promastigotes

In the standard binding assay *L. donovani* promastigotes (2×10^7 cells), suspended in 0.1 ml of HBS (pH 7.2) supplemented with 5 mM glucose, were incubated at 27 °C for 30 min in the presence of [³H]heparin (330 000 c.p.m.; 0.39 mCi/mg) at the concentrations of heparin specified. The reaction was terminated by the addition of 10 vol. of ice-cold HBS and rapid centrifugation at 20 000 g for 2 min in a microcentrifuge. The pellet was washed three times with ice-cold HBS, and after resuspending the cells in 0.12 ml of distilled water, the radioactivity in a 50 µl aliquot was measured by liquid scintillation counting. Blanks were obtained by adding an excess (0.1 mg) of non-labelled heparin to the promastigote suspension immediately before addition of [³H]heparin. In a typical experiment, in which about 26 000 c.p.m. of [³H]heparin was bound to 2×10^7 promastigotes, the amount of total radioactivity in the blank was always less than 700 c.p.m. In the competition experiment (Table 2) in which hyaluronic acid and various glycosaminoglycans were tested for their ability to compete with [³H]heparin for binding sites on promastigotes, 0.1 mg (1 mg/ml) of each of the glycosaminoglycans specified was added to the standard [³H]heparin incubation medium immediately prior to the addition of the labelled heparin.

Biosynthesis of a ³⁵SO₄-labelled heparin-like product by promastigotes

L. donovani promastigotes (approx. 10^9 cells) were harvested after 7 days of culture, washed with HBS and resuspended in 10 ml of fresh medium 199 supplemented with 2 mCi of Na₂³⁵SO₄. After these cells had been incubated for 24 h at 27 °C, they were collected, washed with HBS supplemented with 1 mM-Na₂SO₄, and extracted with 4 M-guanidine/HCl in 20 mM-Tris/HCl, pH 7.0. After removal of insoluble material by centrifugation (12 000 g, 15 min), the radiolabelled macromolecules in the supernatant were separated from free ³⁵SO₄ by chromatography on a Sephadex G-50 column equili-

brated with 4 M-guanidine/HCl in 20 mM-Tris/HCl (pH 7.0). The void volume fractions were combined, dialysed against distilled water and lyophilized. The lyophilized material was dissolved in 6 M-urea containing 0.15 M-NaCl and 0.05 M-Tris/HCl (pH 6.8) and applied to a DEAE-cellulose column (1.6 cm × 2.5 cm) equilibrated with the same buffer. Glycoproteins were eluted by washing the column with 0.15 M-NaCl in the same buffer. The column was then washed with 1.15 M-NaCl in the same buffer to elute $^{35}\text{SO}_4$ -labelled macromolecules, including proteoglycans. The 1.15 M-NaCl eluates were pooled, dialysed against water and lyophilized. The first peak was not analysed further. The second peak from the DEAE-cellulose column, the presumed proteoglycan fraction, was treated with 1 M-sodium borohydride in 0.05 M-NaOH (Carlson, 1968) to release O-linked polysaccharides by β -elimination. After neutralization with glacial acetic acid, the sample was dialysed against distilled water and lyophilized. Finally, it was applied to a Sepharose CL-4B column (1 cm × 45 cm), which was equilibrated with 4 M-guanidine/HCl in 20 mM-Tris/HCl, pH 7.0. The single radioactive peak near the void volume was collected, dialysed against distilled water, lyophilized, digested with nitrous acid (Höök *et al.*, 1975; Shively & Conrad, 1976) and rechromatographed on the same column.

RESULTS

Purification of protein kinase

The purification scheme for *L. donovani* histone protein kinase is summarized in Table 1. Using the six steps indicated in Table 1, the overall purification achieved was 1940-fold and the yield of activity, based on the histone kinase assay, was 18%.

Phosphorylation of endogenous proteins by live *L. donovani* promastigotes

Parasite proteins can be phosphorylated by briefly (10 min) incubating freshly isolated intact promastigotes with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. When stationary phase promastigotes on the seventh day of culture were washed, resuspended in HBS containing 5 mM-glucose and incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and 10 mM- MgCl_2 , subsequent analysis by SDS/PAGE revealed phosphorylation of more than a dozen proteins (Fig. 1); the smallest radiolabelled band

was 14 kDa and the largest labelled protein that entered the gel was 170 kDa in size.

The greatest number of radiolabelled bands and the highest amount of incorporation of radioactivity from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ were obtained with promastigotes incubated in the presence of Mg^{2+} ions; in contrast, barely detectable phosphorylation was obtained when Ca^{2+} replaced Mg^{2+} (results not shown). Considerable incorporation of ^{32}P into promastigote proteins was also obtained in the presence of Mn^{2+} . Interestingly, although the $[\text{P}]\text{phosphoprotein}$ profiles of cells incubated separately with Mg^{2+} and Mn^{2+} revealed that many of the same protein bands had been labelled (e.g. the 50 and 66 kDa bands), there were some noteworthy differences. For example, in the presence of Mg^{2+} the 40 and 45 kDa promastigote proteins were labelled extensively, whereas relatively little label was incorporated into these particular proteins when Mn^{2+} served as the divalent cation activator. All subsequent phosphorylation experiments were performed using Mg^{2+} as the divalent cation.

The effect of heparin on histone protein kinase activity

The effects of heparin, hyaluronic acid and several glycosaminoglycans on the activity of the purified leishmanial protein kinase were compared using the standard protein kinase assay in which histone served as the phosphate acceptor. Unfractionated heparin (0.5 mg/ml) inhibited the leishmanial protein kinase by 93%. The other five members of the glycosaminoglycan family, namely heparan sulphate (49.7% inhibition), dermatan sulphate (38.4%), chondroitin 4-sulphate (69.7%), chondroitin 6-sulphate (60.5%) and hyaluronic acid (35.2%), were less inhibitory than heparin. When the inhibition by heparin was analysed over a range of concentrations (10–50 μg per assay), it was found that 50% inhibition required approx. 25 μg of heparin per assay (0.25 mg/ml) (Fig. 2). This experiment was repeated using a low molecular mass (4000–6000 kDa) preparation of heparin in place of unfractionated heparin; as shown in Fig. 2, the low molecular mass heparin was just as effective an inhibitor of the leishmanial protein kinase as the unfractionated heparin preparation.

The inhibition of leishmanial protein kinase by heparin exhibited the features of a competitive inhibitor when the ATP concentration was held constant and the histone concentration was varied (results not shown); the K_m

Table 1. Summary of the purification of *L. donovani* protein kinase

Purification step	$10^{-3} \times$ Total activity (units*)	Total protein (mg)	$10^{-3} \times$ Specific activity (units/mg)	Purification (fold)	Recovery (%)
Crude extract	359	350	1.1	1.0	100
High-speed supernatant	329	150	2.2	2.0	91
QAE-Sephadex	323	18.8	17.2	15.6	90
Sephadex G-150	305	9.46	32.2	29.3	85
Hydroxyapatite	193	3.70	52.3	47.7	54
Histone affinity column	186	0.75	248	225	51
Isoelectric focusing	63.9	0.03	2130	1940	18

* One unit of kinase activity equals 1 pmol of ^{32}P incorporated into histone/min.

Fig. 1. Phosphorylation of parasite proteins by incubation of viable promastigotes with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$

L. donovani promastigotes (1×10^7 cells) from a 7-day culture were harvested, washed twice with HBS supplemented with 5 mM-glucose and then resuspended in 0.5 ml of the same medium to which was added MgCl_2 (10 mM), NaF (50 mM) and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (20 μCi). After 10 min at 27 °C, the reaction was terminated by rapid freezing. An aliquot (40 μl) of the radiolabelled cells was dissolved in SDS and subjected to SDS/PAGE and autoradiography to identify SDS/PAGE radiolabelled proteins. The X-ray film was developed (a) and scanned with a densitometer. Lane 1, promastigotes incubated in the presence of 1 mM-EDTA and 10 mM- MgCl_2 ; lane 2, promastigotes incubated in the absence of MgCl_2 and in the presence of 1 mM-EDTA. (b) Densitometer scans of the X-ray film; the continuous line is the trace of lane 1 (plus MgCl_2) and the dotted line is the trace of lane 2 (minus MgCl_2). The peaks have been specified according to molecular mass, as determined by the migration of protein standards whose molecular masses are indicated at the far left side of the Figure.

Fig. 2. Effect of heparin on protein kinase activity

Protein kinase activity was determined in the presence of various concentrations of low (continuous line) and high (dashed line) molecular mass heparin using histone as the phosphate acceptor as described in the Materials and methods section.

was increased but the V_{max} was unchanged, and the K_i for heparin was estimated to be 0.24 mg/ml. In contrast, when the histone concentration was fixed and the ATP concentration was varied, the Lineweaver-Burk plots of the resultant heparin inhibition data were linear and exhibited the characteristics of non-competitive inhibition; the K_i value for heparin was estimated to be 0.38 mg/ml (results not shown).

The effect of heparin on phosphorylation of parasite proteins using $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ by live promastigotes

Having shown above (Fig. 1) that live promastigotes are capable of incorporating ^{32}P from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into their own surface proteins, presumably through the action of the parasite's ectokinase (Das *et al.*, 1986), we were interested in determining what effect heparin might have on the ^{32}P phosphorylation pattern. To this end, we incubated live stationary-phase promastigotes in the presence of Mg^{2+} and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, with and without heparin, for 10 min and then analysed for ^{32}P phosphoproteins by SDS/PAGE and autoradiography. As shown in Fig. 3, the presence of 0.5 or 1.0 mg of heparin/ml in the phosphorylation medium markedly decreased the incorporation of ^{32}P from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into essentially all protein bands. In fact, as little as 0.01 mg of heparin/ml significantly reduced the extent of phosphorylation of parasite proteins (results not shown). Of the total of six

Fig. 3 Effect of heparin on promastigote phosphorylation

Two different concentrations (0.5 mg/ml and 1.0 mg/ml) of high molecular mass heparin (6000–20 000 kDa) were tested for their effect on phosphorylation of intact *L. donovani* promastigotes in the presence of [γ - 32 P]ATP in HBS supplemented with 5 mM-glucose. SDS/PAGE and autoradiography were carried out as described in the Materials and methods section. Lane 3, promastigotes alone; lanes 1 and 2 indicate the phosphorylation patterns in the presence of 1.0 mg and 0.5 mg of heparin/ml, respectively.

glycosaminoglycans tested (1 mg/ml), none except heparin had any consistent and reproducible inhibitory effect on the 32 P phosphorylation pattern exhibited by control promastigotes (results not shown). Occasionally, hyaluronic acid caused a slight reduction in the labelling of all of the phosphoprotein bands. These observations indicate once again that the heparin effects observed in the present study are relatively specific and are not a general property of polyanionic polysaccharides. Low molecular mass heparin was only slightly less effective than unfractionated heparin in modifying the 32 P phosphorylation pattern generated by incubating promastigotes with [γ - 32 P]ATP (results not shown). The fact that all of the proteoglycans used in this study inhibited the activity of the purified leishmanial protein kinase while only heparin caused marked inhibition of the incorporation of 32 P from [γ - 32 P]ATP into promastigote proteins suggests that the phosphorylation of most of the parasite proteins was attributable to protein kinases other than the one we purified.

The cell surface of *L. donovani* contains LPG, a negatively charged glycoconjugate. Superficially, this glycoconjugate resembles a glycosaminoglycan in that it is constructed of an anionic disaccharide repeat unit of $\text{PO}_4 \rightarrow 6\text{Gal}(\beta 1 \rightarrow 4)\text{Man}\alpha 1$ (Turco, 1988). Stimulated by the observation that LPG inhibits the activity of protein

kinase C from rat brain (McNeely & Turco, 1987), we performed an experiment to determine whether LPG, like heparin, was capable of altering the phosphorylation pattern of promastigotes incubated with [γ - 32 P]ATP. As shown in Fig. 4, *L. donovani* LPG did indeed markedly alter the 32 P phosphorylation pattern obtained when Sudan strain promastigotes were incubated with [γ - 32 P]ATP. The incorporation of 32 P into most of the parasite phosphoproteins was inhibited by LPG (e.g. 50, 92, 116, 150 and 170 kDa bands). However, the labelling of some proteins with 32 P (e.g. the 45 and 66 kDa bands) was unaffected by LPG. Interestingly, there was one band, namely the 60 kDa protein, which took up considerably more label in the presence of 0.5 mg of LPG/ml (see Fig. 4, lane 1) than it did in the absence of exogenous LPG (lane 3).

Demonstration of the binding of [3 H]heparin to promastigotes

If the effects of heparin on protein phosphorylation described above are the result of the polysaccharide's binding to the parasite surface, it should be possible to demonstrate such binding using radiolabelled [3 H]heparin. In preliminary experiments, we found that when promastigotes were incubated with [3 H]heparin for 15–30 min in Hepes buffer, they would take up radioactivity and retain the isotope even through repeated washings with iso-osmotic buffer. A binding study was therefore performed in which increasing concentrations (2–40 μ g/ml) of radiolabelled heparin were incubated with 2.0×10^7 *L. donovani* promastigotes. As shown in Fig. 5, the promastigotes appear to have relatively high affinity for [3 H]heparin, and heparin binding is saturable; maximum binding was obtained with approx. 3–4 μ g of heparin per assay (30–40 μ g/ml) and half-maximum binding was observed when the heparin concentration of the medium was 0.6 μ g per assay (6 μ g/ml). A Scatchard

Fig. 4. Effects of LPG on promastigote autophosphorylation

Pure *L. donovani* LPG was tested for its effect on the phosphorylation of intact *L. donovani* promastigotes by [γ - 32 P]ATP using the procedure described in the Materials and methods section. SDS/PAGE and autoradiography were carried out as described in the legend to Fig. 1. Lane 3, promastigotes alone; lanes 1 and 2 indicate the presence of 0.5 mg and 0.2 mg of LPG/ml, respectively.

Fig. 5. Binding of heparin to promastigotes

Promastigotes (2×10^7) were incubated in HBS supplemented with glucose and increasing amounts of [^3H]heparin. The amount of radioactivity bound to the cells was determined as described in the Materials and methods section. A Scatchard plot constructed from the binding data is shown in the inset. From the slope of the line and the intercept on the x-axis, a binding constant of 4.7×10^{-7} M (based on a mean molecular mass of 15000) and a stoichiometry of 864000 receptors per parasite were estimated.

plot of the data yielded a straight line (inset of Fig. 5), from which we estimated the stoichiometry of binding (8.64×10^5 molecules of heparin per parasite). The binding constant (K_d) was estimated to be 4.7×10^{-7} M.

The fact that the [^3H]heparin associated with the washed promastigote pellet could be displaced essentially instantaneously and completely by an excess of unlabelled heparin indicated that labelled heparin was probably bound to the surface of the organism and that it had not been internalized. The binding of [^3H]heparin to *L. donovani* promastigotes was completely reversible, for if at the end of the 30 min period during which the parasites had been incubated with [^3H]heparin we added a 100-fold excess of non-labelled heparin, 96% of the bound radioactivity could be discharged from the cells (Table 2). When this particular experiment was repeated using the other five (non-labelled) glycosaminoglycans (Table 2), we found that none was capable of releasing the bound [^3H]heparin from the cells. In fact, with chondroitin 4-sulphate, chondroitin 6-sulphate and dermatan sulphate, inexplicably, the addition of any one of these glycosaminoglycans actually increased the amount of [^3H]heparin bound to the promastigotes. The only polyanionic polysaccharide tested which was capable of competing with tritiated unfractionated heparin for binding sites on the promastigotes was a commercial low molecular mass (4–6 kDa) preparation of heparin that had been generated by treating the latter with nitrous acid; the addition of 100 μg (1.0 mg/ml) of low molecular mass heparin to the standard binding assay discharged about 80% of the [^3H]heparin from the parasites.

We also found that the LPG-deficient mutant (LPG⁻)

of *L. donovani* bound five times more [^3H]heparin than its wild-type parent (results not shown), indicating that the heparin receptors on the parasite surface are not LPG molecules. This observation also raises the possibility that the LPG molecules on the wild-type organism may somehow mask the heparin receptors on the surface of the parasite. Furthermore, the [^3H]heparin that is bound to the surface of either the mutant or wild-type cells

Table 2. Displacement of [^3H]heparin from promastigotes by various glycosaminoglycans

Promastigotes (2×10^7) were incubated with 5 μg of [^3H]heparin in 0.1 mM-HBS supplemented with 5 mM-glucose as described in the Materials and methods section. At 30 s after the addition of the [^3H]heparin, 0.1 mg of each of the substances indicated above were added to separate incubations. The blank was 675 c.p.m. and was subtracted from the results obtained with each sample.

Addition	[^3H]Heparin bound (c.p.m./ 2×10^7 promastigotes)
None	26000
Unfractionated heparin	400
Low molecular mass heparin	6750
Dermatan sulphate	36800
Chondroitin 4-sulphate	40600
Chondroitin 6-sulphate	44300
Hyaluronic acid	26100

Fig. 6. Sephadex CL-4B chromatography of proteoglycan

Pooled fractions from the DEAE-cellulose column were dialysed, lyophilized and chromatographed on a Sephadex CL-4B molecular sieve column (1 cm $\times$ 45 cm) after β -elimination (described in the Materials and methods section). The column was eluted with 4 M-guanidine/HCl in 20 mM-Tris/HCl buffer, pH 7.0. Fractions (1 ml) were collected and 0.1 ml aliquots of each were counted to determine $^{35}\text{SO}_4$ (a). After the fractions were pooled as indicated (shaded bar), dialysed, lyophilized and subjected to HNO_2 treatment, an aliquot was then run on the same column. Fractions (1 ml) were collected and radioactivity in 0.2 ml aliquots was counted (b). V_0 and V_T refer respectively to the void and retention volumes of the column.

could not be displaced by an excess of unlabelled LPG, suggesting that LPG is not bound to the parasite through the heparin receptors.

Biosynthesis of a $^{35}\text{SO}_4$ -labelled heparin-like substance by *L. donovani* promastigotes

We investigated the possibility that *L. donovani* promastigotes synthesize heparin. Stationary phase (7th day) promastigotes that had been incubated overnight in medium 199 (in the presence of fetal bovine serum) supplemented with $^{35}\text{SO}_4$ were harvested, washed and extracted with 4 M-guanidine/HCl containing 20 mM-Tris/HCl, pH 7.0. The guanidine/HCl-solubilized material was chromatographed in 4 M-guanidine/HCl on a Sephadex G-50 column in order to separate free $^{35}\text{SO}_4$ and low molecular mass radiolabelled materials from the high molecular mass proteoglycan and glycoprotein components. The radiolabelled material in the void

volume of the Sephadex G-50 column was chromatographed on a DEAE-cellulose column (results not shown); the 0.15 M-NaCl-eluted radioactive peak probably corresponds to glycoproteins. The second, smaller radioactive peak which eluted at the higher salt concentration (1.15 M-NaCl) corresponds to the proteoglycan fraction (Hassell *et al.*, 1980).

The proteoglycan-containing fraction from the DEAE-cellulose column was treated with 1 M-sodium borohydride in 0.05 M-NaOH to release O-linked polysaccharides by β -elimination. The radioactive alkali/borohydride-treated product eluted as a single peak near the void volume of the Sepharose CL-4B column, indicating that the material is of high molecular mass (Fig. 6a). This is the position where glycosaminoglycans would be expected to elute (Iozzo, 1989). The elution position of the voided nitrous acid-treated material shifted to the position of the retention volume (Fig. 6b) of the column, indicating that the material is nitrous acid-sensitive, which is a property shared with heparin and heparan sulphate (Iozzo, 1989), but not with chondroitins 4-sulphate or 6-sulphate, or dermatan sulphate.

DISCUSSION

The principal result of the present study is the finding that heparin binds tightly to the surface of *L. donovani* promastigotes. The binding of radiolabelled heparin to the parasite seems to be relatively specific, since hyaluronic acid and several other glycosaminoglycans including dermatan and chondroitin sulphates do not compete with or displace [^3H]heparin (Table 2). From the Scatchard plot of the binding data, it appears that a single stationary phase (7-day) promastigote can accommodate just under one million molecules of heparin.

The initial stimuli for determining if heparin would bind to *L. donovani* were the reports which demonstrated that this glycosaminoglycan modifies the activity of several protein kinases (Hathaway *et al.*, 1980; Rose *et al.*, 1981; Ahmad *et al.*, 1986). The investigation was also prompted by our objective to identify tools and reagents which might help to elucidate the function and pathophysiological significance of parasite kinases and protein phosphorylation to the host-parasite interaction.

Although heparin inhibits the activity of the purified leishmanial kinase (Fig. 2) and does clearly alter the ^{32}P protein phosphorylation pattern obtained when intact promastigotes are incubated with [γ - ^{32}P]ATP (Fig. 3), one cannot conclude that the altered phosphorylation pattern is due exclusively or directly to inhibition of the parasite's cell surface protein kinase by heparin. In fact, the observations that binding of heparin to promastigotes reaches saturation at 30–40 μg of heparin/ml (Fig. 5), whereas inhibition of histone protein kinase activity requires more than a 10-fold higher concentration of the proteoglycan (Fig. 2), indicate that the heparin receptors detected using [^3H]heparin are probably not identical to the protein kinase, and that the altered [^{32}P]phosphoprotein profiles caused by heparin cannot be accounted for simply by direct inhibition of the protein kinase by heparin. Thus it is more likely that heparin binds not only to the ectokinase but also to protein substrates that are normally phosphorylated by the protein kinase *in vivo* when heparin is absent; if heparin were to form a complex with a potential phosphoprotein substrate it could block the phosphorylation of that particular pro-

tein, especially if heparin binding hinders access of the kinase or substrates (e.g. ATP) to the hydroxy amino acid residue which would otherwise be phosphorylated. Regardless of whether one of these two likely mechanisms is actually operating, it is clear that one can use heparin to control the degree, and to some extent the pattern, of parasite protein phosphorylation.

The affinity of promastigotes for heparin observed is within the range of values $[(1.2-8.8) \times 10^{-7} \text{ M}]$ reported by Raja *et al.* (1988) for the binding constants for the interaction of hyaluronic acid with rat liver endothelial cells. Furthermore, the hyaluronic acid receptors on these endothelial cells also recognizes heparin. The heparin receptor on *L. donovani* promastigotes appears to be much more specific than the hyaluronate/heparin receptor of endothelial cells. Leung *et al.* (1989) demonstrated a single class of heparin-binding sites for human monocytes, with about two million binding sites per cell and a dissociation constant of $1.9 \times 10^{-7} \text{ M}$. They suggested that heparin and heparin-like glycosaminoglycans might mediate the interaction of monocytes and macrophages with blood vessels. In addition, Kjellén *et al.* (1977) described heparin receptors on the surface of rat hepatocytes which appear to be similar to those we have found on the surface of *L. donovani* promastigotes.

While heparin may prove to be a useful tool in studies of the pathobiology of leishmaniasis, it is debatable at this point if the heparin effects we have observed in this study are pathophysiologically relevant to *Leishmania* infections in man, since heparin has not yet been identified in blood. However, the local concentration of heparin at certain sites in the body may reach relatively high values. For example, Scully *et al.* (1986) and Stevens *et al.* (1985) have estimated that a stimulated mast cell can release as much as 25 pg of heparin, and that if this heparin is released evenly into a 0.1 mm sphere around the cell, heparin concentrations greater than 10^{-7} M will be achieved. Thus the concentration of heparin in the region surrounding a mast cell may be sufficiently high to permit binding of the glycosaminoglycan to the heparin receptors on the surface of *L. donovani*. Another possibility is that the parasite manufactures its own heparin. The finding that promastigotes in culture incorporate $^{35}\text{SO}_4$ into a cell-associated macromolecule that has the characteristics of heparin (Fig. 6) indicates that the organism may synthesize heparin or a heparin-like substance and secrete it on to its surface, where it could be bound by the heparin receptors which we have herein demonstrated as being present on the parasite. Further studies are needed before the identity of the $^{35}\text{SO}_4$ -labelled macromolecule with heparin can be claimed.

We have no knowledge of how heparin might influence the degradation of *Leishmania* by macrophages. It was shown recently that heparin is a competitive antagonist of inositol-trisphosphate-mediated Ca^{2+} fluxes in smooth muscle cells (Ghosh *et al.*, 1988). It would be worthwhile determining if inositol-trisphosphate affects the binding of heparin to promastigotes and if heparin-coated promastigotes affect Ca^{2+} concentrations inside the parasite or host cells.

The finding (Fig. 4) that purified LPG inhibits the phosphorylation of many parasite proteins when promastigotes are incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ demonstrates that the organism produces a substance which has the potential of being a regulator of the activity of its own cell surface protein kinase. Thus, the earlier suggestion

(Turco, 1988) that LPG may play an important protective role for *L. donovani* by inhibiting critical host cell protein kinase activities should be broadened to include the possibility that LPG regulates the phosphorylation of the parasite's own proteins as well. However, since the histone kinase activities of the LPG^- mutant and its wild-type parent (LPG^+) were nearly identical (results not shown), the effect is probably not the result of direct inhibition of the global catalytic activity of the leishmanial protein kinase, but rather an effect on the enzyme's specificity with respect to particular phosphate acceptors.

There are at least two other explanations for the different ^{32}P phosphorylation patterns in LPG^- and LPG^+ promastigotes. First, the presence of LPG in the LPG^+ cells might be masking certain proteins which would otherwise be phosphorylated. Secondly, the absence of LPG^- could cause a redistribution of proteins on the surface of the parasite, such that some phosphate acceptors are moved out of reach of the kinase.

In pursuing the use of heparin to investigate the biochemistry and cell biology of the interaction between *L. donovani* and phagocytes (Pearson & Steigbigel, 1981) it should be possible to isolate the heparin-binding proteins that are localized to the surface of the promastigote using a heparin affinity column. Bergey & Stinson (1988) recently employed a heparin-Sepharose 6B column to isolate heparin-inhibitable basement membrane binding proteins from an extract of *Streptococcus pyogenes*. Purification of heparin-binding proteins from promastigotes should facilitate investigations into the function and pathophysiological significance of these proteins. To ascertain which domain of heparin is specifically involved in binding to the promastigote, further studies should be carried out with derivatives and subfragments of heparin.

With regard to pathophysiological significance, it is tempting to speculate that the function of the heparin on the surface of *Leishmania* may be to facilitate the attachment of parasites to host phagocytes. It is interesting in this regard that human monocytes contain about two million heparin receptors on their surface (Leung *et al.*, 1989). Perhaps heparin serves as a bridge which links the parasite to its host cell, namely the macrophage.

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