Characterization of Polymer Release from the Flagellar Pocket of *Leishmania mexicana* Promastigotes

York-Dieter Stierhof, Thomas Ilg, David G. Russell,‡ Heinz Hohenberg,* and Peter Overath

Max-Planck-Institut für Biologie, D-72076 Tübingen, Federal Republic of Germany; *Heinrich-Pette-Institut für Experimentelle Virolgie und Immunologie an der Universität Hamburg, D-20251 Hamburg, Federal Republic of Germany; and ‡Department of Molecular Microbiology, Washington School of Medicine, St. Louis, Missouri

Abstract. Trypanosomatids contain a unique compartment, the flagellar pocket, formed by an invagination of the plasma membrane at the base of the flagellum, which is considered to be the sole cellular site for endocytosis and exocytosis of macromolecules. The culture supernatant of *Leishmania mexicana* promastigotes, the insect stage of this protozoan parasite, contains two types of polymers: a filamentous acid phosphatase (sAP) composed of a 100-kD phosphoglycoprotein with non-covalently associated proteo high molecular weight phosphoglycan (proteo-HMWPG) and fibrous material termed network consisting of complex phosphoglycans. Secretion of both polymers is investigated using mAbs and a combination of light and electron microscopic techniques. Long filaments of sAP are detectable in the lumen of the flagellar pocket. Both sAP filaments and network material emerge from the os- tum of the flagellar pocket. While sAP filaments detach from the cells, the fibrous network frequently remains associated with the anterior end of the parasites and can be found in the center of cell aggregates. The related species *L. major* forms similar networks. Since polymeric structures cannot be detected in intracellular compartments, it is proposed that monomeric or, possibly, oligomeric subunits synthesized in the cells are secreted into the flagellar pocket. Polymer formation from subunits is suggested to occur in the lumen of the pocket before release into the culture medium or, naturally, into the gut of infected sandflies.

---

**Leishmania** are protozoan parasites alternating between the extracellular, flagellated promastigote stage living in the gut lumen of the insect vector, the sand fly, and the intracellular, nonflagellated amastigote stage parasitizing phagolysosomes of mammalian macrophages (Alexander and Russell, 1992; Walters, 1993). Promastigotes grown in axenic culture secrete large amounts of phosphoglycan-containing antigens into the culture medium, which have been collectively termed "excreted factor" (Schnur et al., 1972; Turco and Descoteaux, 1992). One component of "excreted factor" is lipophosphoglycan (LPG), which is also abundantly expressed on the surface of promastigotes (Handman et al., 1984; King et al., 1987; Tolson et al., 1989). The structure of the cell-bound glycoconjugate has been elucidated for several *Leishmania* species (Turco et al., 1989; McConville et al., 1990; Thomas et al., 1992; Ilg et al., 1992). LPG is composed of oligosaccharide caps terminating the non-reducing end of phosphosaccharide repeats; the repeats are connected to lysoalkylphosphatidylinositol via a phosphosaccharide core. The structure of released LPG from *Leishmania mexicana* is very similar to its cellular counterpart (Ilg et al., 1992). A second secreted component, a phosphohydrolase with an acid pH optimum (secreted acid phosphatase; sAP), has been shown to share structural features and immunological epitopes with LPG (Bates et al., 1990; Jaffe et al., 1990; Ilg et al., 1991a). In the case of *L. mexicana*, sAP is a polymeric proteophosphoglycan complex composed of a 100-kD phosphoglycoprotein and a non-covalently associated proteo-high-molecular weight phosphoglycan (proteo-HMWPG; Menz et al., 1991; Ilg et al., 1991a,b). Detailed epitope mapping with anti-sAP mAbs indicate the presence of repeat and cap oligosaccharides shared with LPG as well as sAP-specific peptide and carbohydrate epitopes (Ilg et al., 1993b).

In trypanosomatids, endocytosis and exocytosis is considered to occur exclusively at an invagination of the plasma membrane at the base of the flagellum designated flagellar pocket (Balber, 1990; Webster and Russell, 1993). In the well-studied bloodstream stage of *Trypanosoma brucei*, the lumen of the pocket comprises only 0.2–1.4% of the cellular volume (Fairlamb and Bowman, 1980; Coppens et al., 1987; Webster and Fish, 1989). The membrane area involved in exo- and endocytosis comprises about 0.4% of the plas-
malemia and recycles at a very high rate (Coppens et al., 1987; Seyfang et al., 1990). After insertion into the pocket membrane via exocytosis, membrane components such as the variant surface protein must be able to diffuse laterally towards the surface of the cell body and the flagellum. This implies that the hemidesmosomal junctions found in Trypanosoma (maculae adherens; Vickerman, 1969; Webster and Russell, 1993) connecting the flagellum and the opposing region of the cell body at the entrance of the pocket, do not prohibit the passage of membrane-associated molecules. On the other hand, molecules as large as ferritin enter the pocket and can then be taken up by endocytosis (Langreth and Balber, 1975).

The few ultrastructural studies undertaken suggest that the organization of the flagellar pocket of Leishmania promastigotes is similar to that of Trypanosoma (Vickerman and Preston, 1976). Pimenta and De Souza (1987) described hemidesmosome-like flagellum–cell body attachment sites and analyzed the distribution of intramembranous particles in the flagellar pocket membrane of L. amazonensis. In addition, gp63 and different carbohydrate epitopes were localized in the flagellar pocket membrane and lumen of different Leishmania species (Lang et al., 1991; Stierhof et al., 1991; Bahr et al., 1993). Furthermore, acid phosphatase activity derived from a membrane bound (Menz et al., 1991) and/or soluble enzyme has been localized by cytochemistry in the flagellar pocket reservoir of L. donovani, L. amazonensis, and L. mexicana promastigotes (Gottlieb and Dwyer, 1981; Pimenta and De Souza, 1986; Hassan and Coombs, 1987). The soluble, non-polymeric acid phosphatase synthesized by L. donovani (Gottlieb and Dwyer, 1982; Ilg et al., 1991b) has been postulated to be secreted into the lumen of the flagellar pocket and to be released by a process which may require the beating of the flagellum (Bates et al., 1989).

In this study, we directly demonstrate the release of filamentous sAP and of a novel polymeric phosphoglycan-containing antigen from the flagellar pocket into the culture medium of L. mexicana. We propose that both polymers are assembled in the flagellar pocket from mono- or oligomeric subunits supplied by exocytosis.

**Materials and Methods**

**Parasites, mAbs, Antisera, Fab Fragments and Immuno precipitation**

Leishmania mexicana mexicana (strain MNKY/BC2/62/M379) or L. major promastigotes (strain MRHO/IR/76/vaccine strain) were transformed in 2–3 mo intervals from mouse (BALB/c) lesion-derived amastigotes and grown in semi-defined medium 79 to late logarithmic/early stationary phase. The soluble, non-polymeric acid phosphatase synthesized by L. donovani (Gottlieb and Dwyer, 1982; Ilg et al., 1991b) has been postulated to be secreted into the lumen of the flagellar pocket and to be released by a process which may require the beating of the flagellum (Bates et al., 1989).

In this study, we directly demonstrate the release of filamentous sAP and of a novel polymeric phosphoglycan-containing antigen from the flagellar pocket into the culture medium of L. mexicana. We propose that both polymers are assembled in the flagellar pocket from mono- or oligomeric subunits supplied by exocytosis.

The Journal of Cell Biology, Volume 125, 1994
pure copper block cooled with liquid helium. Material was then processed in a Balzer's freeze etch machine and rotary replicated with 2-nm platinum and "backed" with 4-6 nm of carbon. The replicas were freed from promastigote material with hydrofluoric acid and bleach, prior to mounting and examination.

Results

**sAP Filaments in the Culture Supernatant of *L. mexicana* Promastigotes**

Promastigotes secrete sAP during the logarithmic growth phase. In densely grown cultures, the activity in the supernatant is 7 to 10 times higher than the cell bound activity, part of which (40%) is associated with a different, membrane-bound enzyme (Menz et al., 1991). Purified sAP filaments are characterized by a pearl chain-like arrangement of globular particles (subunit diameter 8 nm) presumed to be formed by the linear aggregation of the 100-kD glycoprotein and a surrounding "glycocalyx" supposedly constituting the non-covalently linked proteo-HMWPG (Ilg et al., 1991b). Fig. 1 a depicts such a filament adsorbed directly from the supernatant of cultured promastigotes onto a microscopic grid. While negative staining clearly revealed the central chain, the "glycocalyx" could only be discerned as a faint shadow. The anti-carbohydrate mAb L3.13 strongly labeled the "glycocalyx" resulting in a sausage-like shape (Fig. 1 b). A similar heavy decoration was obtained with mAbs recognizing oligosaccharide caps or phosphosaccharide repeats, structures which are shared by sAP and LPG (e.g., mAbs AP3, CA7AE, and LT7; cf. Table I). The labeling pattern of Fab fragments prepared from a polyclonal rabbit anti-sAP serum containing antibodies against both carbohydrate and protein epitopes defined an upper estimate for the total filament diameter of 40–60 nm (Fig. 1 c). The anti-polypeptide antibody LT8.2 (Table I) produced a different pattern. Binding to adsorbed sAP filaments could be convincingly demonstrated only by subsequent gold labeling (Fig. 1 d). Under these conditions access of the mAb to its binding sites appeared to be sterically hindered because labeling in solution gave rise to heavier decoration (Fig. 1 e). The resulting filament diameter was only 20–40 nm, suggesting that, in contrast to the epitopes of the other mAbs, the binding sites for mAb LT8.2 are located closer to the center of the filaments.

**sAP Filaments Are Released from the Flagellar Pocket**

Fixed parasites were labeled with mAb LT8.2 and FITC-
Table 1. Specificity of Monoclonal Antibodies for L. mexicana sAP

| mAb     | Putative epitope                | 100-kD glycoprotein | Proteo-HMWPG |
|---------|---------------------------------|---------------------|--------------|
| LT8.2*  | Peptide epitope                 | +                   | +/−          |
| L3.13*  | Carbohydrate epitope            | +                   | +            |
| AP3*    | (Man1,2)3,4-Man-PO4-caps        | +                   | +            |
| CA7AE** | -PO4-Galβ1,4Man-1-repeats       | −                   | +            |
| LT17*   | -PO4-[Glcβ1,3]-Galβ1,4Man-1-repeats | −                   | +            |
| WIC79.3*| -PO4-[Galβ1,3]-β1,4 Man 1-repeats | −                   | +            |
| L3.8†   | Peptide epitope of L. mexicana gp63 surface protease | −               | −            |

* Ilg et al., 1993b
† Tolson et al., 1989
‡ Kelleher et al., 1992
§ Medina-Acosta et al., 1989
¶ Ilg et al., 1993a.

mAb L3.8 was used as a control in all studies.

The double exposure revealed antigen in the flagellar pocket (green fluorescence) next to the comma-shaped kinetoplast DNA (blue fluorescence). The reaction of this peptide-specific mAb directly demonstrated the presence of sAP in the pocket. In digitonin permeabilized cells, antibody labeling was essentially restricted to the pocket suggesting that the antigen concentration in intracellular structures was low. Furthermore, sAP in the pocket could be efficiently decorated in live cells demonstrating that macromolecules can enter the pocket from the outside. Another mAb previously reported to give a strong reaction in the flagellar pocket is mAb L3.13 (Stierhof et al., 1991; Ilg et al., 1993b). This mAb not only reacts with sAP but with a second secretory product (see below).

Remarkably, cells were detected in the process of secretion. Curved filaments, typically showing strong labeling with mAb L3.13 (Fig. 2 b, green fluorescence) and a weaker reaction with mAb LT8.2 (Fig. 2 c, red fluorescence) were found close to the opening of the flagellar pocket. These filaments were sometimes in direct continuity with the content labeled secondary antibodies, and DAPI for DNA (Fig. 2 a).

Figure 2. Secretion of sAP filaments (a–e) and networks (f and g) from the flagellar pocket. (a) Flagellar pocket labeling using mAb LTS.2 and FITC-labeled secondary antibodies. (b) Labeling of a sAP secreting cell by mAb L3.13 and FITC-conjugated secondary antibodies. (c and d) Cell secreting sAP filaments labeled with mAb LTS.2 and Cy3-conjugated secondary antibodies. (e) sAP-secreting cell labeled with mAb LT8.2 and Cy3-conjugated secondary antibodies (red fluorescence); subsequent treatment with mAb L3.13 and FITC-labeled
Figure 3. sAP secreting cell observed by immunofluorescence microscopy (a) or immuno SEM (b–e). The same cell (a–d) labeled with mAb LT8.2 and secondary reagents (see Materials and Methods) is shown by immunofluorescence or SEM in the BSE mode. The photograph in d is a high magnification detail with patches and strands of 13-nm gold particles (cf. arrowheads). In e, gold-labeled sAP filaments protrude from the opening of the flagellar pocket (cf. arrow); the immunofluorescence image of this cell shows the corresponding fluorescent strand emerging from the flagellar pocket. fl, Flagellum. Bars: (a and b) 5 μm; and (c–e) 0.5 μm.

of the flagellar pocket (Fig. 2 d). Double labeling resulted in a yellow to orange fluorescence caused by the superposition of the two fluorophores' emission (Fig. 2 e). In order to analyze the secretion process at a higher resolution, immunofluorescence microscopy was combined with scanning electron microscopy (SEM, Figs. 3 and 4). Cells were immobilized on microgrid coverslips and treated with either mAb LT8.2 or L3.13 and FITC-labeled goat anti-mouse IgG followed by rabbit anti-goat IgG and protein A-13-nm gold. Filament-secreting cells were localized by immunofluorescence microscopy before processing for SEM. The procedure permitted the examination of the same cell by both techniques. The sAP filaments labeled with mAb LT8.2 were located around the origin of the flagellum (Fig. 3, a and b).

secondary antibodies (green fluorescence). (f) Network-secreting cell labeled with both antibodies as in e. (g) Cell aggregate labeled with both antibodies as in e. Simultaneous labeling of the DNA with DAPI results in a blue fluorescence of the comma-shaped kinetoplast (k) located close to the flagellar pocket (fp) (a–d). Arrowheads point to the flagellar pocket. The photographs shown in a, b, and c were taken under additional weak phase contrast illumination in order to visualize the cell body. n, nucleus. Bar, 10 μm.
Figure 4. sAP secreting cell labeled with mAb L3.13 and secondary reagents (see Materials and Methods) observed by immunofluorescence (a) or SEM in the BSE mode (b and c). c is a detail of b showing the 13-nm gold particles bound to the filaments. Bars: (a and b) 5 μm; and (c) 0.5 nm.

Higher magnification (Fig. 3, c and d) allowed the visualization of the gold particles bound to the surface of the filaments, which emerged from the ostium of the flagellar pocket (Fig. 3 e). As mentioned above, mAb L3.13–labeling resulted in a stronger and more uniform filament decoration when analyzed by immunofluorescence microscopy (Fig. 4 a) or by SEM (Fig. 4, b and c).

Fibrous Networks, a Second Type of Secretory Product
L. mexicana promastigotes showed a high tendency to form rosettes and higher aggregates that were particularly prominent in densely grown cultures (Fig. 5 a) and that have also been described in the insect vector (Jadin and Creemers, 1967; Walters et al., 1987). Typically, most flagella were directed towards the center of the rosettes, which contained a network of fibers (cf. detail in Fig. 5 b). These fibers were released from the flagellar pocket (Fig. 5 c) forming interwoven meshes, which typically remained associated with the anterior end of the cells (Fig. 5 d; see also Fig. 2 f).

The following ultrastructural properties of fibers allowed a differentiation from sAP filaments: straight, cable-like appearance, variable diameter, and the occurrence of branches (Fig. 5, b, c, and f). Furthermore, the two polymers differed in their antigenic properties. In the same double labeling experiment described for the sAP (Fig. 2 e), the network associated with either single promastigotes (Fig. 2 f) or the center of rosettes (Fig. 2 g) showed a green fluorescence demonstrating that this material did not react with mAb LT8.2 and did therefore not consist of sAP filaments. In addition, the network in the center of the aggregates could not be labeled by several other anti–sAP mAbs (2/E1, 2/9a, and 11/5b) directed against linear and conformational peptide epitopes, which readily recognized sAP filaments (not shown). These experiments could be confirmed on the electron microscopic level. In negatively stained preparations or
Figure 5. Characterization of networks. (a) SEM image (SE mode) of an unlabeled flattened rosette of *L. mexicana* promastigotes. (b) Detail of fibrous mesh in the center of the rosette shown in (boxed area). (c) Network-secreting cell labeled with mAb L3.13 and protein A-13-nm gold and viewed in the SE mode. The arrowheads point to some unlabeled regions of the otherwise heavily gold-decorated fibers emerging from the opening of the flagellar pocket (arrow). The corresponding immunofluorescence image of this cell shows a labeling pattern typical for networks (e.g., Fig. 2 f). (d) Network attached to a promastigote cell labeled with mAb L3.13 and FITC-conjugated secondary antibodies. (e and f) Negatively stained fibres of networks adsorbed to a grid and either treated with mAb L3.13 and protein A-13-nm gold (e) or left unlabeled (f). p, promastigote cell. Bars: (a and d) 5 μm; and (b, c, e, and f) 0.5 μm.
on ultra-thin resin sections networks were labeled by mAb L3.13 (Fig. 5 e) but not by mAb LT8.2 (not shown).

*L. major* promastigotes do not secrete acid phosphatase (Lovelace and Gottlieb, 1986). The flagellar pocket of these cells could be labeled by mAb L3.13 but not by mAb LT8.2. This species likewise forms aggregates in culture and in the insect vector (Lawyer et al., 1990). Network material in rosettes similar to that observed for *L. mexicana* could be demonstrated by light and electron microscopy after labeling with mAb L3.13 and with antibodies directed against cap oligosaccharides or phosphodi-accharide and galactosylated phosphotrisaccharide repeats (Table I). However, the abundance of network material in cell aggregates of *L. major* was less than in *L. mexicana*. With the exception of *L. donovani*, network material could be demonstrated in cell aggregates of several other species (*L. amazonensis*, *L. braziliensis*, *L. tropica*, and *L. aethiopica*; results not shown).

**Filaments in the Lumen of the Flagellar Pocket**

Electron microscopy of fixed and subsequently quick-frozen, freeze-fractured and deep-etched *L. mexicana* promastigotes gave an impressive view of sAP-like filaments located in the lumen of the flagellar pocket (Fig. 6). The strands appeared to be composed of subunits of similar size and periodicity as those of sAP filaments (Fig. 1 a). In addition, on-section labeling experiments using mAb LT8.2 showed an occasional chain-like arrangement of protein A-gold particles located on strands in the lumen of the flagellar pocket (Fig. 7 a). The flagellar pocket of *L. major* was devoid of filaments (Fig. 7 b); this observation correlated with the absence of sAP synthesis in this species. Pockets of both species lacked fibers corresponding to network material; possibly, these structures were removed during sample preparation for electron microscopy.

**Metabolic Labeling of Secretory Products**

The novel secretory product designated network has not yet been isolated and, therefore, evidence for its chemical composition is indirect. Immunofluorescence experiments showed that networks react with mAbs specific for oligosaccharide caps and phosphosaccharide repeats. A secreted component possibly corresponding to fiber subunits could be detected by metabolic labeling experiments using [32P]phosphate (Fig. 8). As shown in this autoradiograph of a polyacrylamide gel, the culture supernatant of *L. major* contained a high molecular weight component (lane 5), which could be immunoprecipitated by mAbs L3.13, AP3 and WIC79.3 (lanes 6–8) but not by the control mAb L3.8 (lane 9). In the case of *L. mexicana*, the labeling pattern in the high molecular weight region was more complicated due to the presence of sAP with its two components, the 100-kD phosphoglycoprotein and proteo-HMWPG (Fig. 8, lane 1). However, there was a third component remaining close to the start of the gel, which could be immunoprecipitated by mAbs L3.13 (Fig. 8, lane 3) and AP3 (lane 4) or LT17 (not shown, cf. Table I) but not by LT8.2 (lane 2). Therefore, this high molecular weight component has similar antigenic properties as network material: it carries the epitopes for mAb L3.13 and antibodies...
Figure 7. (a) On-section labeling of *L. mexicana* promastigote embedded in Lowicryl K4M using mAb LTS.2 and protein A-13-nm gold. Gold particles are located on strands. (b) Deep-etched freeze-fracture image of the flagellar pocket of *L. major* promastigote. Flagellum (fl), kinetoplast (k); the arrow points to the ostium of the pocket. Bar, 0.5 μm.

directed against capped phosphosaccharide repeats but lacks the peptide epitope characteristic for the 100-kD component of sAP.

**Discussion**

The structural and immunological properties of the two polymeric secretory products described in this study can be summarized as follows: the sAP filaments formed by *L. mexicana* but not by *L. major* are composed of a central chain of globular particles with a surrounding "glycocalyx." They frequently assume a curved shape and show little tendency for self-aggregation. sAP filaments can be evenly and intensely decorated with mAbs directed against linear and conformational polypeptide epitopes of the 100-kD phosphoglycoprotein, an unknown carbohydrate epitope (mAb L3.13) as well as with mAbs recognizing oligosaccharide caps and phosphosaccharide repeats. In contrast, networks formed by both *L. mexicana* and *L. major* are composed of straight, cable-like fibers without detectable subunit structure. The fibers form interwoven meshes in the center of promastigote aggregates and their decoration by mAbs is not uniform along their length. They react with mAb L3.13 and mAbs directed against oligosaccharide caps and phosphosaccharide repeats but not with antibodies reactive with linear or conformational epitopes of the enzymatically active component of the sAP filaments. This leads to the conclusion that sAP filaments and networks are separate polymeric entities.

Using metabolic labeling experiments, we have detected a high molecular weight-phosphoglycan in the culture supernatant of both *L. mexicana* and, more clearly, *L. major*, which we consider to be a candidate for the component forming networks. By analogy to the 100-kD phosphoglycoprotein and the proteo-HMWPG component of sAP, we assume that the phosphoglycans of network material are linked to a protein backbone. Chemical analysis of both components of *L. mexicana* sAP shows an amino acid composition rich in serine and serine-phosphate and the presence of oligosaccharide caps and phosphosaccharide repeats (Ilg et al., 1994). Furthermore, the sequence of the gene for the 100-kD phosphatase predicts a defined repetitive region very rich in serine residues (Ilg et al., 1994). Analysis of proteo-HMWPG isolated from *L. mexicana* amastigotes, which like *L. major* promastigotes do not form sAP, revealed an amino acid composition rich in serine and serine-phosphate as well as the presence of capped phosphosaccharide repeats (Ilg et al., 1994). Our present hypothesis is that the network material, both components of sAP and the amastigote proteo-HMWPG belong to a new class of proteins covalently modified by phosphoglycans.

Figure 8. 32P-labeled secretory products in the culture supernatant of *L. mexicana* and *L. major* promastigotes. Promastigotes were labeled with [32P]P, as described by Ilg et al. (1993b). After removal of the cells by centrifugation, the supernatant was either concentrated by ultrafiltration or aliquots were subjected to immunoprecipitation by mAbs. The samples were then subjected to 7.5–20% SDS-PAGE and autoradiography. Lanes 1–4 and 5–9 refer to secreted products from *L. mexicana* or *L. major*, respectively. (Lanes 1 and 5) Culture supernatant; (lanes 2–4) immunoprecipitates using mAbs LT8.2, L.3.13, and AP3, respectively; (lane 6–9) immunoprecipitates using mAbs L.3.13, AP3, WIC79.3 and L3.8 (control, see Table 1), respectively. Standard proteins in kD are indicated.
Cell aggregates and fibrous material have been described in association with parasites in their natural habitat, the sand fly (for review see Walters, 1993). Thus, Walters et al. (1987) noted that in the cardia/stomodeal valve region of Lutzomyia midguts large aggregates of L. mexicana promastigotes are embedded in a carbohydrate-rich gel which, ultrastructurally, appeared as a matrix of electron dense strands. Furthermore, gel-like material similar to the networks described here were observed in the esophagus and in the pharynx, a region in the foregut of Phlebotomus papatasii, infected with L. major (Lang et al., 1991; Killick-Kendrick et al., 1988) or in association of heavily parasitized midguts (Lawyer et al., 1990). Lang and co-workers demonstrated that the fibrous material could be heavily decorated in situ with mAb WIC79.3 (cf. Table I), an antibody recognizing LPG and network material. While these authors interpreted this result to indicate LPG deposition on the fibrous material, we would like to suggest that it represents secreted network material, which carries structures cross-reactive with oligosaccharide caps and phosphosaccharide repeats of LPG.

In principle, assembly of sAP filaments from mono- or oligomeric subunits could occur in three compartments: in the culture medium, in intracellular vesicles committed to exocytosis or in the flagellar pocket. These possibilities will be considered one by one.

Polymer Formation in the Medium. sAP filaments as well as networks can be demonstrated in direct association with the anterior end of promastigotes and sometimes just emerging from the flagellar pocket. Furthermore, essentially all of the enzyme activity in the medium is in a polymeric state (Ilg et al., 1991b) although the concentration in the supernatant of densely grown cultures is only 0.5 mg/l (5 × 10^{-9} M for a 100-kD protein). These observations are consistent with the notion that polymerization takes place before rather than after release from the flagellar pocket.

Intracellular Formation of Polymers. Intracellular assembly would imply that filaments up to several μm in length should be present in vesicular structures between the Golgi stacks and the flagellar pocket. So far, we have failed to demonstrate any prominently labeled intracellular structures by either light or electron microscopy using mAbs LTB2 or L3.13. Dense packing in vesicles would also require that sAP filaments assume a highly curved shape. The smallest radius of curvature observed so far in negatively stained or frozen hydrated preparations is about 50 nm (Ilg et al., 1991b).

Assembly in the Flagellar Pocket. We favor a scenario, where monomeric or possibly oligomeric subunits are constantly released into the lumen of the pocket by exocytosis; there, they polymerize to filaments, which then leave the cell via the pocket's ostium. In support of this view, we have demonstrated sAP filaments in the lumen of the pocket. Promastigotes contain roughly 1,500 molecules of the 100-kD protein, which could form 30 filaments with an average of 50 subunits. As judged by immunofluorescence microscopy, the protein is largely located in the lumen of the pocket. Accordingly, the concentration in the pocket is expected to be about 5 × 10^{-6} M (0.5 mg/ml; assumed pocket volume 0.4 μm^3), which is 1,000-fold higher than in the spent medium of a stationary phase culture. Polymer formation in the pocket is expected to be a thermodynamically and kinetically favored reaction. Polymerization of fibrous material forming networks could likewise occur in the flagellar pocket. Whether polymer release from the flagellar pocket is continuous or if the pocket evacuates intermittently is presently not clear. In the case of L. donovani promastigotes the beating of the flagellum may be required for the release of the nonpolymeric acid phosphatase from the flagellar reservoir (Bates et al., 1989).

In our hypothesis, the flagellar pocket would serve as a secluded yet extracellular vessel for the assembly of macromolecules promoting this structure to a unique secretory organelle of Leishmania and, possibly, other trypanosomatids. In multicellular organisms, secretion of monomeric or oligomeric macromolecules can be followed by their polymerization in the extracellular space. Relevant examples are the formation of collagen fibrils and other components of the extracellular matrix. Therefore, the flagellar pocket appears to fulfill a function in a single cell that in higher organisms presupposes a highly differentiated, multicellular state.

We thank Dorothée Harbecke and Jürgen Berger for excellent technical assistance, Manuela Fuchs for correcting the manuscript and the Deutsche Forschungsgemeinschaft for support. D. G. Russell is extremely grateful to John Heuser and Robyn Roth for their extensive assistance in preparing material for examination of the flagellar pocket by freeze-etch electron microscopy.

D. G. Russell is supported by the National Institutes of Health grant AI-26889.

Received for publication 17 November 1993, and in revised form 25 January 1994.

References

Austra, R., R. Hermann, and M. Müller. 1992. An efficient single crystal detector in SEM. Scanning. 14:127-135.

Alexander, J., and D. G. Russell. 1992. The interaction of Leishmania species with macrophages. Adv. Parasitol. 31:175-254.

Bahr, V., Y.-D. Stierhof, T. Ilg, M. Demar, M. Quinten, and P. Overath. 1993. Expression of lipophosphoglycan, high-molecular-weight phosphoglycan and glycoprotein 63 in promastigotes and amastigotes of Leishmania mexicana. Mol. Biochem. Parasitol. 58:107-122.

Balzer, A. E. 1990. The pellicle and the membrane of the flagellum, flagellar adhesion zone, and flagellar pocket: functionally discrete surface domains of the blood-stream form of African trypanosomes. Crit. Rev. Immunol. 10:177-201.

Bates, P. A., I. Hermes, and D. M. Dwyer. 1989. Leishmania donovani: immunocytological localization and secretory mechanism of soluble acid phosphatase. Exp. Parasitol. 68:335-346.

Bates, P. A., I. Hermes, and D. M. Dwyer. 1990. Golgi-mediated posttranslational processing of secretory acid phosphatase by Leishmania donovani promastigotes. Mol. Biochem. Parasitol. 39:247-256.

Coppen, I., F. R. Oppendoes, P. J. Courtney, and P. Baudhain. 1987. Receptor-mediated endocytosis in the blood-stream form of Trypanosoma brucei. J. Protozool. 34:465-473.

Fairlamb, A. H., and I. B. R. Bowman. 1980. Trypanosoma brucei: Maintenance of concentrated suspensions of bloodstream trypanomastigotes in vitro using continuous dialysis for measurement of endocytosis. Exp. Parasitol. 49:366-380.

Gottlieb, M., and D. M. Dwyer. 1981. Leishmania donovani: surface membrane acid phosphatase activity of promastigotes. Exp. Parasitol. 52:117-128.

Gottlieb, M., and D. M. Dwyer. 1982. Identification and partial characterization of extracellular acid phosphatase of Leishmania donovani promastigotes. Mol. Cell Biol. 2:76-81.

Handman, E. C. L., Greenblatt, and J. W. Godin. 1984. An amphipathic sulphated glycolconjugate of Leishmania: characterization with monoclonal antibodies. EMBO (Eur. Mol. Biol. Organ.) J. 3:2301-2306.

Hassan, H. F., and G. H. Coombs. 1987. Phosphonooesterases of Leishmania mexicana mexicana and other flagellates. Mol. Biochem. Parasitol. 23:285-296.

Heuser, J. E., T. S. Reese, M. J. Dennis, L. Y. Yan, W. N. Jan, and L. Evans. 1979. Synaptic vesicle exocytosis captured by quick-freezing and correlated with quantalet transmitter release. J. Cell Biol. 81:275-300.

Ilg, T., R. Eiges, P. Overath, M. J. McConville, J. Thomas Oates, J. Thomas, S. W. Homans, and M. A. J. Ferguson. 1992. Structure of Leishmania mexicana lipophosphoglycan. J. Biol. Chem. 267:6834-6840.

Ilg, T., D. Harbecke, and P. Overath. 1993a. The lysosomal gp63-related protein in Leishmania mexicana amastigotes is a soluble metalloproteinase with
an acidic pH optimum, FEBS (Fed. Eur. Biochem. Soc.) Lett. 327:103-107.
Ilg, T., D. Harbecke, M. Wiese, and P. Overath. 1993a. Monoclonal antibodies directed against Leishmania secreted acid phosphatase and lipophosphoglycan: partial characterization of private and public epitopes. Eur. J. Biochem. 217:603-615.
Ilg, T., B. Menz, G. Winter, D. G. Russell, R. Enges, D. Schell, and P. Overath. 1993b. Monoclonal antibodies to Leishmania mexicana promastigote antigens. J. Secreted acid phosphatase and other protein share epitopes with lipophosphoglycan. J. Cell Sci. 99:175-180.
Ilg, T., Y.-D. Stierhof, R. Etges, M. Adrian, D. Harbecke, and P. Overath. 1991b. Secreted acid phosphatase of Leishmania mexicana: A filamentous phosphophosphatidyl polymer. Proc. Natl. Acad. Sci. USA. 88:8774-8778.
Ilg, T., Y.-D. Stierhof, M. Wiese, M. J. McConville, and P. Overath. 1994. Characterization of phosphophosphacan-containing secretory products of Leishmania. Parasitology. 108: Supplement 31.
Jadin, J. M., and J. Creemers. 1967. Etude de l'ultrastructure et de la biologie de Leishmania donovani. Bi. Parasitol. 55:362-372.
Jafso, C. L., L. M. Peres, and L. F. Schnur. 1990. Lipophosphoglycan and lipil, T., Y.-D. Stierhof, M. Wiese, M. J. McConville, and P. Overath. 1994. Characterization of phosphophosphacan-containing secretory products of Leishmania. Parasitology. 108: Supplement 31.
Jadin, J. M., and J. Creemers. 1967. Etude de l'ultrastructure et de la biologic de Leishmania donovani. Bi. Parasitol. 55:362-372.

Porter, R. R. 1959. The hydrolysis of rabbit γ-globulin and antibodies with crystalline papain. Biochem. J. 73:119-126.
Rodriguez, J., and F. Deinhardt. 1960. Preparation of a semipermanent mounting medium for fluorescent antibody studies. Virology. 12:316-317.
Schmutz, L. F., A. Zuckerman, and C. L. Greenblatt. 1972. Leishmanial serotypes as distinguished by the gel diffusion of factors excreted in vitro and in vivo. Isr. J. Med. Sci. 5:932-942.
Seyfang, A., D. Mecke, and M. Dussenbro. 1990. Degradation, recycling, and shedding of Trypanosoma brucei variant surface glycoprotein. J. Protozool. 37:546-552.
Slot, J. W., and H. J. Geuze. 1985. A new method for preparing gold probes for multiple-labeling microscopy. Eur. J. Cell Biol. 38:87-93.
Stierhof, Y.-D., H. Schwarz, B. Menz, D. G. Russell, M. Quinten, and P. Overath. 1991. Monoclonal antibodies to Leishmania mexicana promastigote antigens. II. Cellular localization of antigens in promastigotes and infected macrophages. J. Cell Sci. 99:181-186.
Thomas, J. R. M., J. E. Thomas-Oates, S. W. Homans, M. A. J. Ferguson, P. A. J. Gorin, K. D. Greis, and S. J. Turco. 1992. Refined structure of the lipophosphoglycan of Leishmania donovani. J. Biol. Chem. 267:6829-6833.
Tolson, D. L., S. J. Turco, R. P. Beecroft, and T. W. Pearson. 1989. The immunocytochemical structure and surface arrangement of Leishmania donovani lipophosphoglycan determined using monoclonal antibodies. Mol. Biochem. Parasitol. 35:109-118.
Turco, S. J., and A. Descotesaux. 1992. The lipophosphoglycan of Leishmania parasites. Annu. Rev. Microbiol. 46:65-94.
Turco, S. J., P. A. Orlandi, S. W. Homans, M. A. J. Ferguson, R. A. Dwek, and T. W. Rademacher. 1989. Structure of the phosphosaccharide-core of the Leishmania donovani lipophosphoglycan. J. Biol. Chem. 264:6711-6715.
Vickerman, K. 1969. On the surface coat and flagellar adhesion in Leishmania. Parasitology. 59:87-93.

Vickerman, K. 1969. On the surface coat and flagellar adhesion in Leishmania. Parasitology. 59:87-93.

Walters, L. L., G. B. Modi, R. B. Tesh, and T. Burrage. 1987. Host-parasite interaction of Leishmania mexicana amazonensis. J. Submicrosc. Cytol. 18:127-132.

Weber, P., and W. R. Fish. 1989. Endocytosis by African trypanosomes. II. Occurrence in different life-cycle stages and intracellular sorting. Eur. J. Cell Biol. 49:303-310.
Weber, P., and D. G. Russell. 1993. The flagellar pocket of trypanosomatids. Parasitol. Today. 9:201-206.