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

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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 ostium 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)[1], 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., 1991*a*). 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., 1991*a*,*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., 1993*b*).

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-

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1. *Abbreviations used in this paper*: BSE, back-scattered electrons; DAPI, 4,6-diamidino-2-phenylindole; HMWPG, high molecular weight phosphoglycan; LPG, lipophosphoglycan; sAP, secreted acid phosphatase; SE, secondary electrons; SEM, scanning electron microscopy.

malemma 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 the 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., 1991*b*) 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 Immunoprecipitation

Leishmania mexicana mexicana (strain MNYC/BZ/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 as described by Menz et al. (1991).

The mAbs used in this study and their origin are listed in Table I. mAb LT8.2 (IgG2a) reacts with a linear peptide epitope of the 100-kD glycoprotein of sAP and shows a weak reaction with a subfraction of proteo-HMWPG (Ilg et al., 1993*b*). mAb L3.13 (IgG1) is directed against a carbohydrate epitope present on both the 100-kD glycoprotein and proteo-HMWPG; this mAb does not cross-react with LPG (Ilg et al., 1993*b*). mAbs AP3 (IgM), CA7AE (IgM), and LT17 (IgG1) react with both sAP and LPG. mAb AP3 is directed against the oligosaccharide caps terminating the phosphosaccharide repeats of LPG; this epitope is also present on the 100-kD glycoprotein and the proteo-HMWGP of sAP (Ilg et al., 1993*b*). The anti-phosphodisaccharide mAb CA7AE (Tolson et al., 1989) and the anti-phosphotrisaccharide mAb LT17 react with proteo-HMWGP but not the 100-kD glycoprotein (Ilg et al., 1993*b*). Finally, mAb WIC79.3 (IgG1) is an antibody directed against phosphodisaccharide repeats modified by galactosyl residues (Kelleher et al., 1992). The epitope of this antibody can likewise be detected on proteo-HMWPG but not on the 100-kD glycoprotein of sAP. The reactivity of mAbs LT8.2 through WIC79.3 is based on immunoblotting experiments. However, all these antibodies also react with the native sAP filaments as shown by immunoprecipitation experiments with the purified enzyme complex (Ilg et al., 1993*b*). Finally, the control mAb L3.8 (IgG1) recognizes a peptide epitope of the *L. mexicana* surface protease gp63 (Medina-Acosta et al., 1989; Ilg et al., 1993*a*).

Fab fragment preparation from a polyvalent rabbit anti-sAP serum raised as described by Bahr et al. (1993) was performed according to Porter (1959). This antiserum contains antibodies against both carbohydrate and protein epitopes of the complex (Ilg et al., 1993*b*).

Immunoprecipitations of ^{32}P-labeled components released by promastigotes into the culture medium was performed using antibodies coupled to Sepharose (mAb AP3) or bound to protein G–Sepharose (mAbs LT8.2, L3.13, WIC79.3, and L3.8; cf. Ilg et al., 1993*b*).

Negative Staining Electron Microscopy

Purified sAP (Ilg et al., 1991*b*) or promastigote culture supernatant was adsorbed to glow-discharged, pioloform-, and carbon-coated grids and stained with 1% aqueous uranyl acetate. For decoration with antibodies or Fab fragments, the grid surface with bound sAP molecules was first blocked with 0.1% bovine serum albumin in PBS (137 mM NaCl, 3 mM KCl, 1 mM KH_2PO_4, 6 mM Na_2PO_4, pH 7.2) for 10 min and then exposed to mAbs (~10 μg/ml) or Fab fragments (~200 μg/ml) for 45 min at room temperature (followed in one case, Fig. 1 *d*, by incubation with protein A-6 nm gold). After several washings, the grids were subjected to negative staining. For inspection, a Philips 201 electron microscope (objective aperture 30 μm, accelerating voltage 60 kV) was used.

Light and Electron Microscopy

Cells were fixed onto polylysine-coated microgrid coverslips with a coordinate system (CelLocate, Eppendorf, Netheler; and Hinz, Hamburg, Germany) by combining a drop of promastigote cell suspension with an equal drop of fixative (4% formaldehyde, 0.1% glutaraldehyde [GA] in PBS) on the glass surface. After 1 h at room temperature and several washings with PBS, non-specific binding sites were blocked with 0.5% bovine serum albumin, 0.2% gelatine in PBS for 20 min. Thereafter, the coverslips were treated with mAbs in blocking buffer and FITC- or Cy3 (cyanine dye)-labeled goat anti-mouse IgG/IgM (Dianova, Hamburg, Germany) in blocking buffer. After several washings with PBS, specimens were postfixed with 0.1% GA, stained for DNA using 4,6-diamidino-2-phenylindole (DAPI), and examined by light microscopy. For double-labeling experiments at the light microscopic level, cells were first labeled with mAb LT8.2 and Cy3-conjugated goat anti-mouse antibodies (Dianova), fixed with 0.1% GA and then incubated with mAb L3.13 and FITC-conjugated goat anti-mouse IgG/IgM. For double-labeling experiments at the light microscopic and scanning electron microscopic (SEM) level, cells were first treated with mAbs LT8.2 or L3.13 and FITC-conjugated secondary goat anti-mouse antibodies followed by incubation with rabbit serum against goat IgG, and protein A- 13-nm gold complexes (Slot and Geuze, 1985). On-section labeling of Lowicryl-embedded parasites was performed as described before (Stierhof et al., 1991).

For immunofluorescence microscopy, cells were embedded in Mowiol 4.88 (Hoechst, Frankfurt/Main, Germany; Rodriguez and Deinhardt, 1960) or 50% glycerol and viewed in a Zeiss Axioplan equipped with epifluorescence illumination and a Plan-Neofluar 100×/1.30 oil Ph3 objective. For SEM, cells were postfixed by sequential treatment with 2.5% GA and 1% osmium tetroxide in PBS, dehydrated with ethanol and subjected to critical point drying. Thereafter, cells were either covered with a carbon or platinum/carbon layer, for imaging backscattered electrons (BSE; Figs. 3, *b–e* and 4, *b* and *c*) or sputtered with gold/palladium for imaging secondary electrons (SE; Fig. 5, *a–c*). A Hitachi S-800 field emission SEM equipped with an annular YAG single crystal BSE detector (Autrata et al., 1992) operating at an accelerating voltage of 20 kV (30-μm objective aperture) was used. Control labelings omitting the specific antibodies mAbs LT8.2 and L3.13 resulted in completely unlabeled specimens. Incubation with mAb L3.8 specific for the cell surface protease gp63 (Medina-Acosta et al., 1989) did not result in any filament or network labeling.

Preparation of *L. mexicana* promastigotes for freeze-etch electron microscopy was performed as described previously (Heuser et al., 1979). In brief, cells were fixed using 2% GA in 30 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid, 100 mM NaCl, 0.5 mM $CaCl_2$, pH 7.0, washed in three to four changes of double distilled water, and pelleted in a microcentrifuge. The promastigotes were quick-frozen by impact against an ultra-

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., 1991*b*). 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 LT17; 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-

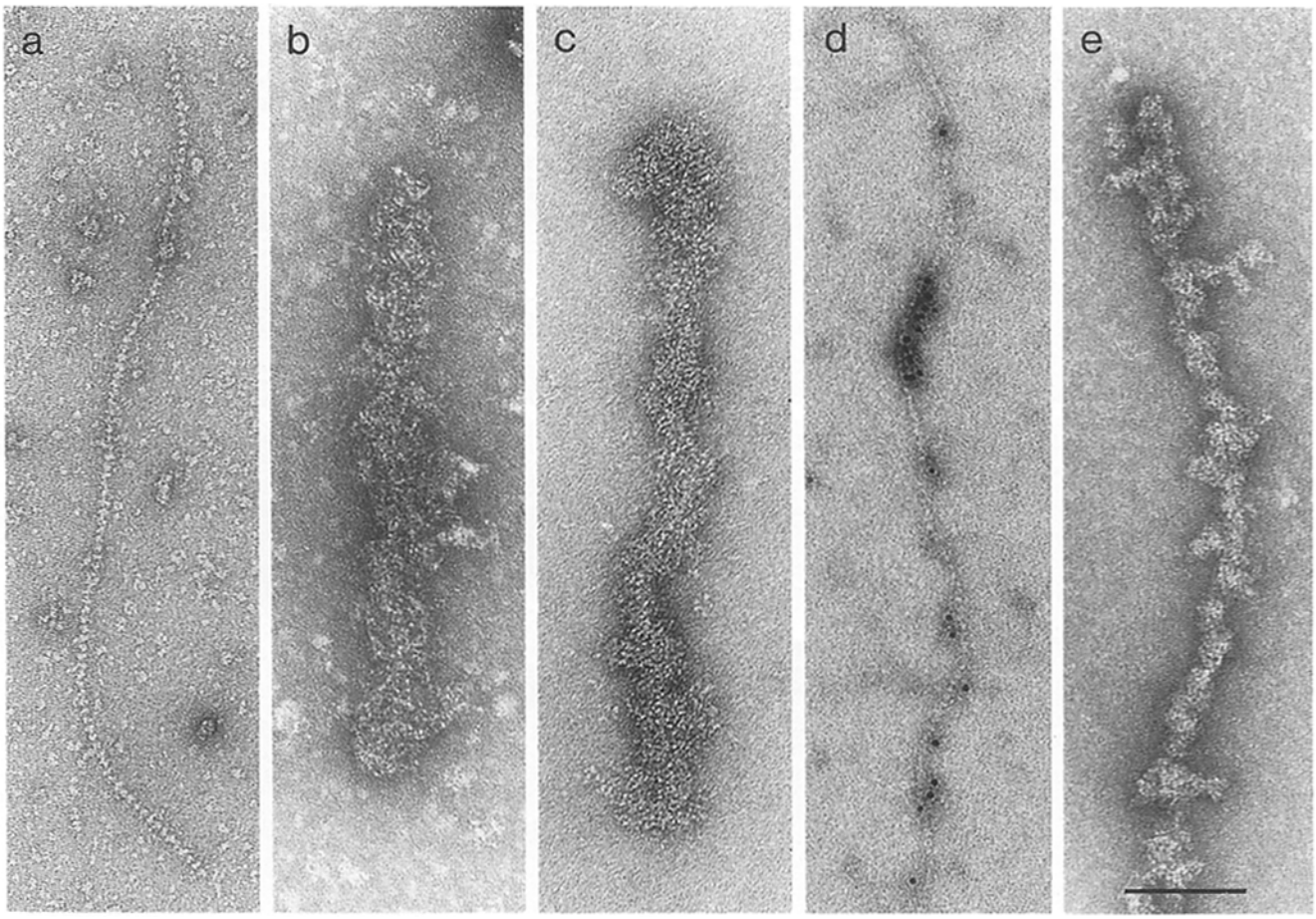

Figure 1. Filaments of sAP revealed by negative staining electron microscopy. sAP was adsorbed to grids from serum-free *L. mexicana* culture supernatant and stained directly (*a*) or after labeling with mAb L3.13 (*b*). Fab fragments isolated from rabbit anti-sAP IgG (*c*), or mAb LT8.2 (*d*) were incubated with purified sAP absorbed to grids. mAb LT8.2 binding was confirmed by subsequent incubation with protein A–6-nm gold. In e, purified sAP was labeled with mAb LT8.2 in solution before adsorption to the grid. Bar, 100 nm.

Table I. 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*	(Manα1,2)$_{1-2}$-Man-PO$_4$-caps	+	+
CA7AE*‡	-PO$_4$-6Galβ1,4Manα1-repeats	−	+
LT17*	-PO$_4$-6[Glcβ1,3-]Galβ-1,4Manα1-repeats	−	+
WIC79.3*§	-PO$_4$-6[Galβ1,3-]$_{1-3}$Galβ1,4 Manα1-repeats	−	+
L3.8‖¶	Peptide epitope of *L. mexicana* gp63 surface protease	−	−

* Ilg et al., 1993*b*
‡ Tolson et al., 1989
§ Kelleher et al., 1992
‖ Medina-Acosta et al., 1989
¶ Ilg et al., 1993*a*.
mAb L3.8 was used as a control in all studies.

labeled secondary antibodies, and DAPI for DNA (Fig. 2 *a*). 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., 1993*b*). 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

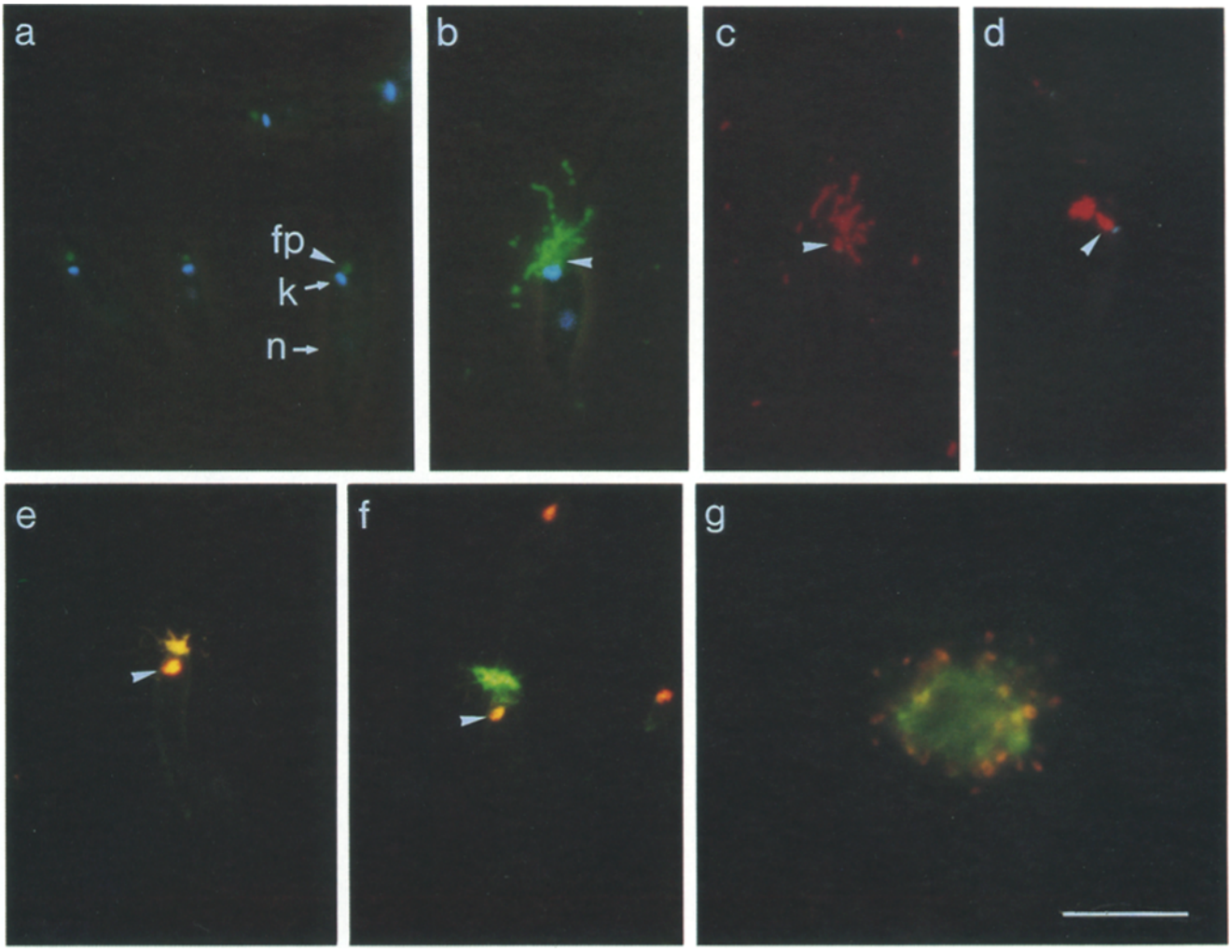

Figure 2. Secretion of sAP filaments (*a–e*) and networks (*f* and *g*) from the flagellar pocket. (*a*) Flagellar pocket labeling using mAb LT8.2 and FITC-labeled secondary antibodies. (*b*) Labeling of a sAP secreting cell by mAb L3.13 and FITC-conjugated secondary antibodies. (*c* and *d*) Cell secreting sAP filaments labeled with mAb LT8.2 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

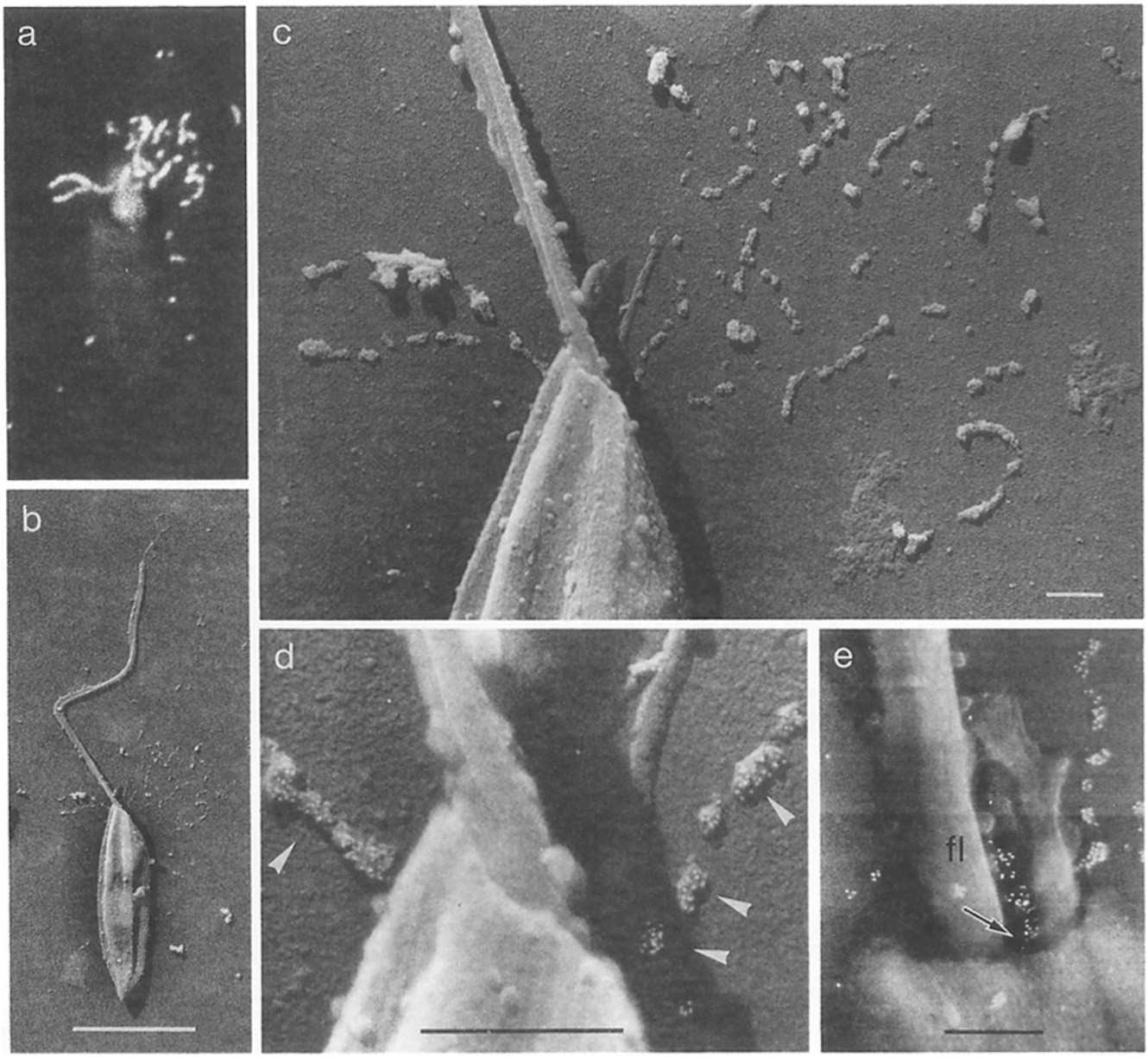

Figure 3. sAP secreting cell observed by immunofluorescence microscopy (*a*) or immuno SEM (*b–e*). The same cell (*a–d*) labeled with mAb LT8.2 and secondary reagents (see Materials and Methods) is shown by immunofluorescence or SEM in the BSE mode. The photograph in d is a high magnification detail 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.

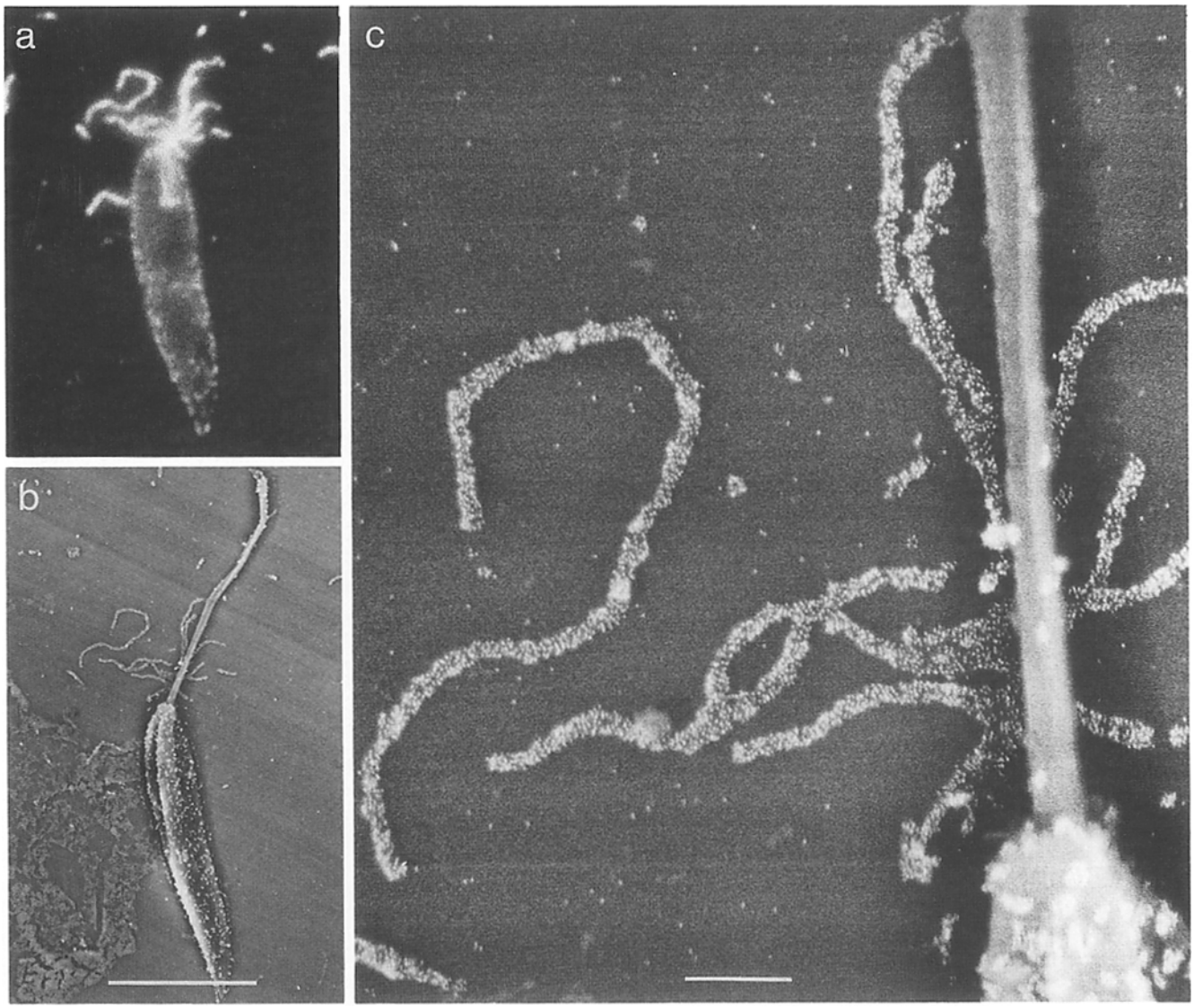

Figure 4. sAP secreting cell labeled with mAb L3.13 and secondary reagents (see Materials and Methods) observed by immunofluorescence (*a*) or SEM in the BSE mode (*b* and *c*). *c* is a detail of *b* showing the 13-nm gold particles bound to the filaments. Bars: (*a* and *b*) 5 μm; and (*c*) 0.5 nm.

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

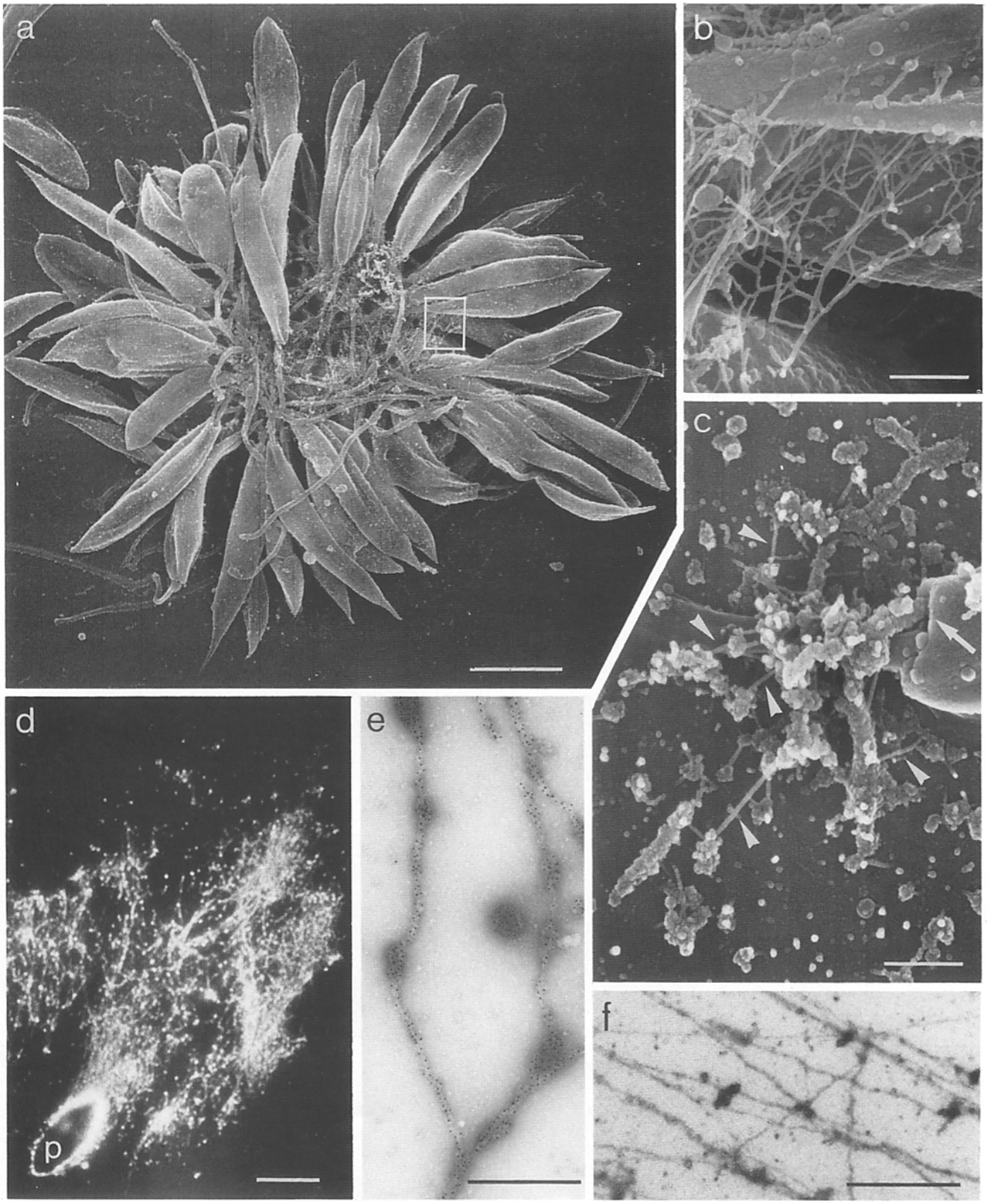

Figure 5. Characterization of networks. (*a*) SEM image (SE mode) of an unlabeled flattened rosette of *L. mexicana* promastigotes. (*b*) Detail of fibrous mesh in the center of the rosette shown in *a* (*boxed area*). (*c*) Network-secreting cell labeled with mAb L3.13 and protein A–13-nm gold and viewed 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 phosphodisaccharide 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 [^{32}P]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

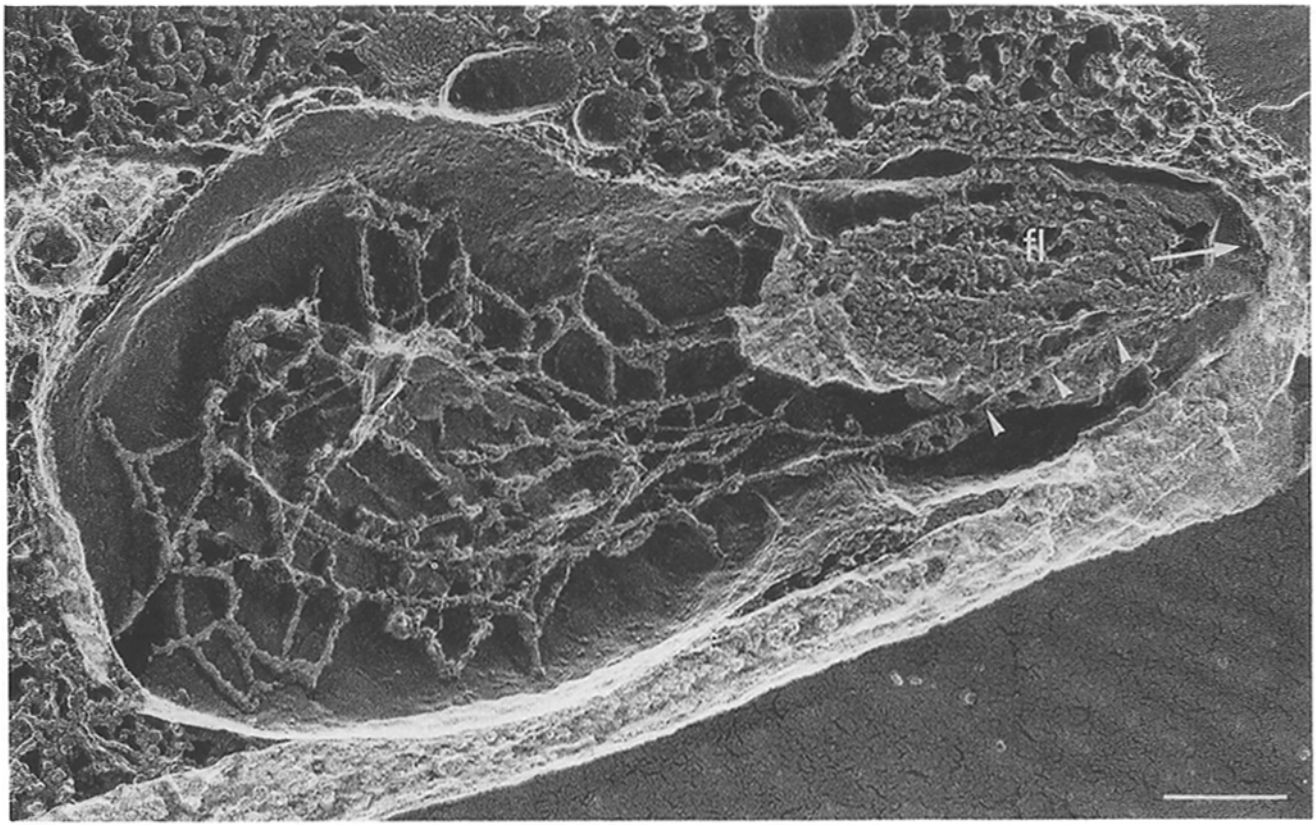

Figure 6. Deep-etched freeze-fracture image of the flagellar pocket of *L. mexicana* promastigote. The arrow points to a strand extending towards the opening of the flagellar pocket. Flagellum (*fl*). Bar, 200 nm.

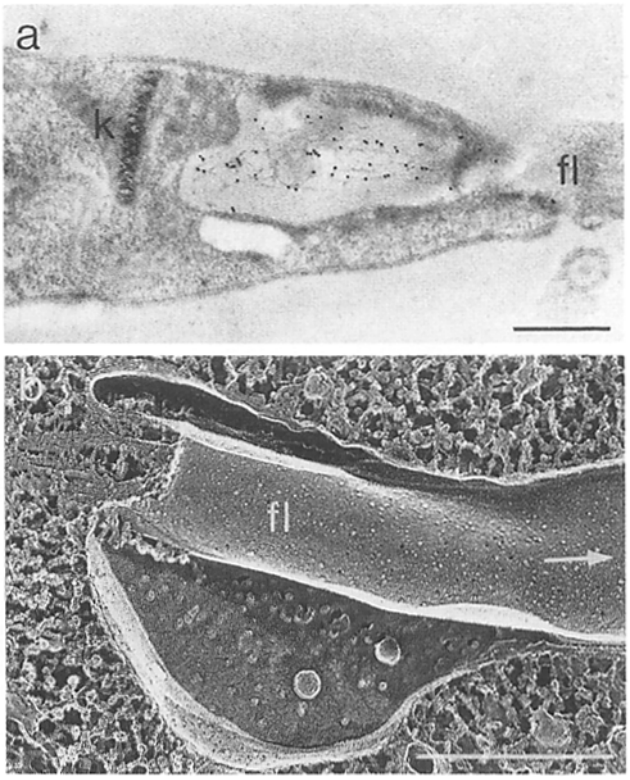

Figure 7. (*a*) On-section labeling of *L. mexicana* promastigote embedded in Lowicryl K4M using mAb LT8.2 and protein A-13-nm gold. Gold particles are located on strands. (*b*) Deep-etched freeze-fracture image of the flagellar pocket of *L. major* promastigote. Flagellum (*fl*), kinetoplast (*k*); 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.

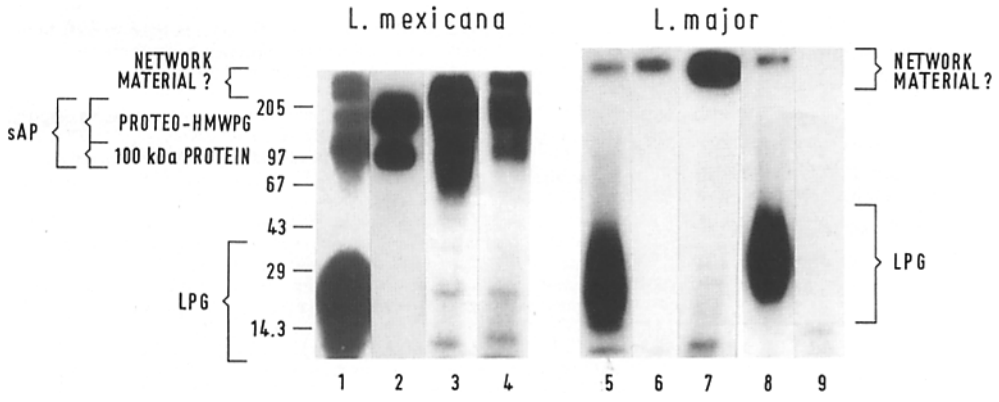

Figure 8. ^{32}P-labeled secretory products in the culture supernatant of *L. mexicana* and *L. major* promastigotes. Promastigotes were labeled with [^{32}P]P_i as described by Ilg et al. (1993*b*). After removal of the cells by centrifugation, the supernatant was either concentrated by ultrafiltration 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 I), 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 papatasi*, 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., 1991*b*) 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 LT8.2 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., 1991*b*).

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 fullfill a function in a single cell that in higher organisms presupposes a highly differentiated, multicellular state.

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