expansion of B cells would not be dependent on their clonal specificity, resulting in a situation that may contribute to autoimmunity (*23*). Aside from blocking endocytosis, the only other B-cell activity specific to FcγRII-B1 was capping. This also may be an important feature of B-cell function since capping of sIg leads not only to the co-capping of FcγRII, but also includes several other molecules that may be involved in the activation process; these include MHC class II molecules, CD19, C3d receptor, and cytoplasmic H-*ras* (*13*, *27*–*29*). Thus, the increased propensity of FcγRII-B1 to cap may ensure that the receptor is distributed together with the molecules whose activities it must regulate after sIg-FcγRII cross-linking.

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31. FcγRII-B1 and -B2 encoding cDNAs were isolated (*5*) and obtained from M. Hogarth (Parkville, Victoria, Australia) and J. Ravetch (New York). B2-CT31, B2-CT18 and CT1 encoding cDNA's were derived as described (*9*). The designation refers to the FcγRII isoform (B1 or B2) followed by the remaining length of the cytoplasmic domain (Fig. 1B). B1CT-53 was obtained by introducing a stop codon, by polymerase chain reaction, in position 54 of the B1 cytoplasmic domain. All plasmids were sequenced again after mutagenesis. cDNA's were introduced in pKC3, an SV40 early promoter-based expression vector, and co-transfected by electroporation with pSV1NEO in IIA1.6 cells, as described [K. R. Thomas and M. R. Capecchi, *Cell* **51**, 503 (1987)]. Cells resistant to G418 (1 mg/ml) were cloned in soft agar, and several individual cell lines, expressing similar numbers of wild-type or mutant FcγRII were selected. Receptor-positive cells were enriched, in some cases, by "panning" on bacteriological plates coated with IgG [B. Seed and A. Aruffo, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 3365 (1987)]. Expression was quantified by determining the binding of ^{125}I-labeled Fab fragments of the FcγRII-specific monoclonal antibody 2.4G2 [J. C. Unkeless, *J. Exp. Med.* **150**, 580 (1979)]. All cells (including the A20 parent cell line) expresses between 1×10^5 to 4×10^5 receptors per cell.
32. Culturing of the 24.4 T cell hybridomas and antigen presentation assays were performed as described [M-Z. Lai *et al.*, *J. Immunol.* **139**, 3973 (1987)]. The IC's were formed prior to use by incubating different concentrations of purified λ repressor with (at 15 μg/ml) each of two different monoclonal antibodies to λ repressor (51F and 22D) at 37°C for 15 min. Release of IL-2 was determined by monitoring the growth of the IL-2-dependent cell line CTL.L2 as described (*33*). Bacteriophage λ repressor cI protein fragment 1 to 102 was purified [R. M. Breyer and R. T. Sauer, *J. Biol. Chem.* **264**, 13348 (1989)]; it was provided by R. M. Breyer (Institut Cochin de Genetique Moleculaire, Paris). The monoclonal antibodies to λ repressor were purified from culture supernatants by chromatography (MAB-trap G-protein columns; Pharmacia, Bois d'Arcy, France). The hybridoma T cell 24.4, was isolated from BALB/c mice injected with the 12-24 peptide of λ-repressor as described (*33*).
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Stage-Specific Adhesion of *Leishmania* Promastigotes to the Sandfly Midgut

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Although leishmaniasis is transmitted to humans almost exclusively by the bite of infected phlebotomine sandflies, little is known about the molecules controlling the survival and development of *Leishmania* parasites in their insect vectors. Adhesion of *Leishmania* promastigotes to the midgut epithelial cells of the sandfly was found to be an inherent property of noninfective-stage promastigotes, which was lost during their transformation to metacyclic forms, thus permitting the selective release of infective-stage parasites for subsequent transmission by bite. Midgut attachment and release was found to be controlled by specific developmental modifications in terminally exposed saccharides on lipophosphoglycan, the major surface molecule on *Leishmania* promastigotes.

The preliminary part of the *Leishmania* life cycle, in which ingested amastigotes from an infected host transform into rapidly dividing extracellular promastigotes, can take place in the blood meal of many different hematophagous arthropods. Within an unsuitable host, however, the parasites are later destroyed or passed out with the feces (*1*). For most leishmanial species, as well as for most other trypanosomatids, the establishment of a true infection within an appropriate vector requires the attachment of parasites to the midgut epithelium (*2*). This attachment is thought to retain parasites in the gut during passage of the digested blood meal and may also be involved in stage differentiation (*3*). The next phase in the development of transmissible infections within the sandfly is the detachment and anterior movement of promastigotes to the cuticle-lined foregut, where some attach by

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forming hemidesmosomes and others remain free for subsequent transmission by bite (2). The growth and movement of promastigotes in the midgut are accompanied by their differentiation from a noninfective stage to a stage that is highly infective to a susceptible vertebrate host (4). The terms "procyclic" and "metacyclic" have been used to refer to these respective invertebrate developmental stages of promastigotes (5). We investigated the relation between stage differentiation and midgut adhesion as well as the role played by lipophosphoglycan (LPG), the major surface glycoconjugate of *Leishmania* promastigotes, in controlling the interaction of promastigote developmental stages with midgut epithelial cells.

A quantitative assay for the attachment of living promastigotes to whole midguts was developed. *Leishmania major* promastigotes were surface-labeled with ^{125}I and incubated with *Phlebotomus papatasi* midguts that had been cut open along the length of the abdominal segment to allow parasite penetration into the lumen (6). Procyclic promastigotes were obtained from logarithmic-phase cultures, and metacyclic forms were purified from stationary cultures as described (7). During the incubation period there was extensive interaction between each developmental stage and the external and luminal surfaces of the midgut epithelium. After washing, however, an average of 8600 (SD = 1200, n = 8) procyclic promastigotes remained bound per midgut, whereas the binding of metacyclic promastigotes was less than 200 (SD = 80, n = 7).

We considered that LPG might control interactions between parasites and epithelial cells because it is the dominant surface molecule on *Leishmania* promastigotes (8). It is densely organized as a glycocalyx that covers the entire cell surface, including the flagellum, and effectively masks exposure of other surface molecules (9). Furthermore, parasite-free LPG has been found deposited on the midgut epithelial cells of *P. papatasi* during infection with *L. major* (10). Structurally, LPG consists of a polymer of repeat units of PO_4^-–6–Gal(β1–4)Manα1 (where Gal is galactose, Man is mannose) linked by a phosphosaccharide core to a novel *lyso*-1-*O*-alkyl phosphatidylinositol lipid anchor (11). For *L. major*, the repeat units are completely substituted in the 3 position of the Gal residue with a variety of saccharide side chains (12). During metacyclogenesis, both the number and saccharide composition of the repeat units are developmentally modified. Specifically, LPG from metacyclic organisms has two to three times the number of repeat units as procyclic LPG, and the majority of the terminal Gal-containing side chains expressed by procyclic LPG are down-regulated during metacyclogenesis in favor of repeat units expressing a terminal α-arabinopyranose (12, 13).

We investigated the role of LPG in mediating procyclic binding by incubating ^{125}I-labeled procyclics with dissected midguts in the presence of purified phosphoglycan (PG), which was prepared from both procyclic and metacyclic LPG by treatment with phosphatidylinositol-specific phospholipase C to remove the lipid. Procyclic PG inhibited parasite attachment in a dose-dependent manner, with complete inhibition with a PG dose as low as 20 μg/ml (Fig. 1A). No inhibition was observed with metacyclic PG in concentrations up to 100 μg/ml. We then explored whether the different inhibitory properties of procyclic and metacyclic PGs could be localized to the different phosphorylated oligosaccharide repeat units. Purified procyclic and metacyclic LPGs were depolymerized with mild acid under conditions that hydrolyze phosphodiester linkages. This treatment released a series of four major and several minor phosphorylated oligosaccharide fragments, which were resolved and purified by ion-exchange high-performance liquid chromatography (HPLC). The four major fragments were used in equimolar concentrations (12 nM) to inhibit procyclic midgut binding (Fig. 1B). The major phosphorylated trisaccharide fragment, which is common to both stages and which contains the terminal side chain Gal(β1–3) linked to the galactosyl residue of the PO_4^-–6–Gal(β1–4)Man backbone unit, was most inhibitory, being comparable to the inhibition achieved with the intact procyclic PG (>90%). Effective inhibition (>70%) was also seen with the phosphorylated tetrasaccharide, which constitutes the major tet-

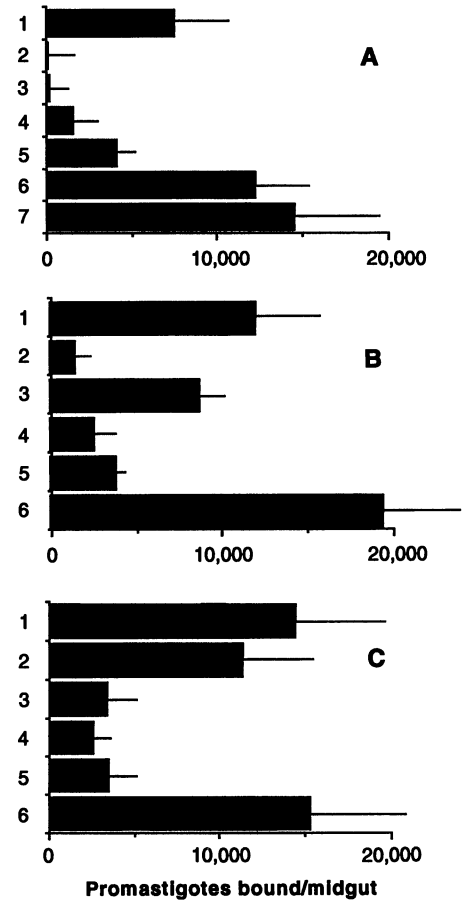

Fig. 1. Inhibition of *L. major* promastigote binding to *P. papatasi* midguts by LPG-derived oligosaccharides. Inhibition of procyclic binding was carried out with LPG purified from *L. major* procyclic and metacyclic promastigotes as described (9). To remove the lipid from the LPG, the glycoconjugate was treated with 0.1 U of PI-PLC from *Bacillus thuringiensis* in 200 μl of water for 18 hours at 37°C. The resulting PG was separated from the cleaved lipid by hydrophobic chromatography. The PGs were further chromatographed on a Sephadex G150 column, and fractions eluting with [^3H]Gal-labeled procyclic and metacyclic PG standards were pooled, dialyzed against water, and lyophilized. To prepare the phosphorylated repeat units, procyclic and metacyclic LPG (2 mg) were depolymerized with 40 mM of trifluoroacetic acid (100°C, 8 min) and then dried in a speedvac evaporator to remove acid. The hydrolysate was suspended in 0.1 M ammonium acetate buffer, pH 7.0, containing 5% 1-propanol, and then applied to a column (4 ml) of octyl-sepharose (Sigma Chemical Co., St. Louis, Missouri) equilibrated in the same buffer. The unbound fraction, containing the phosphorylated oligosaccharide repeat units and neutral cap structures, was lyophilized to remove buffer salts and then fractionated by HPLC on a Dionex model BioLC equipped with a pulsed amperometric detector as reported (12). The phosphorylated oligosaccharide fragments were separated on a CarboPac column (4 × 250 mm), which was initially equilibrated in 63% of buffer A (0.15 M NaOH) and 37% of buffer B (0.15 M NaOH, 0.5 M sodium acetate) and then eluted with a linear gradient over 30 min to 50% of buffer B. Glycan-containing fractions were deionized by chromatography on a column (1 ml) of Ag50 × 12 (H+) and this was followed by drying and repeated flash-evaporation with toluene. Disaccharides differing in anomeric configuration or linkage were obtained from Sigma Chemical Co. (**A**) Opened midguts were incubated with ^{125}I-labeled procyclic promastigotes in the presence of 10 μl of procyclic PG (Pro PG) or metacyclic PG (Met PG). 1, Control; 2, Pro PG (100 μg/ml); 3, Pro PG (20 μg/ml); 4, Pro PG (4 μg/ml); 5, Pro PG (0.8 μg/ml); 6, Met PG (100 μg/ml); 7, Met PG (10 μg/ml). (**B**) Opened midguts were incubated with ^{125}I-labeled procyclic promastigotes in the presence of 12 nm of each depolymerized phosphorylated oligosaccharide. 1, Control; 2, Pro PG (10 μg/ml); 3, PO_4^-–6–Gal(β1–4)Man; 4, Gal(β1–3) linked to PO_4^-–6–Gal(β1–4)Man; 5, Gal(β1–3)Gal(β1–3) linked to PO_4^-–6–Gal(β1–4)Man; 6, Ara(α1–2)Gal(β1–3) linked to PO_4^-–6–Gal(β1–4)Man. (**C**) Opened midguts were incubated with ^{125}I-labeled procyclic promastigotes in the presence of 40 nm of each commercial disaccharide. 1, Control; 2, galactose; 3, Gal(β1–6)Gal; 4, Gal(β1–3)Gal-*O*-Me; 5, Gal(α1–3)Gal-*O*-Me; 6, Man(α1–3)Man-*O*-Me. Me, methyl. Data represent the mean binding ±1 SD of seven to ten midguts per group.

rasaccharide species of procyclics and is formed by side chain substitution of the backbone sequence with Gal(β1–3)-Gal(β1–3). In contrast, the phosphorylated disaccharide PO_4–6–Gal(β1–4)Man, which constitutes the backbone sequence of all the repeat units, was a poor inhibitor (<20%). Finally, the predominant tetrasaccharide obtained from metacyclic LPG, which contains the side chain Ara(α1–2)Gal(β1–3) (where Ara is arabinose), actually enhanced procyclic binding.

These results suggest that the binding of procyclic promastigotes to midgut epithelial cells is mediated by LPG that contains terminally exposed Gal residues. To determine the contribution of reducing sugars to the recognition of midgut epithelial cells, we studied procyclic binding in the presence of equimolar concentrations (40 nM) of Gal or commercially obtained disaccharides that differed in linkage or anomeric configuration, or both. Although Gal itself was a poor inhibitor (20%), effective inhibition was achieved with each of three disaccharides that contained a Gal residue at the nonreducing end (Fig. 1C). The linkage and anomeric configuration of the sugars did not appear to be important. No inhibition was seen with a disaccharide that contained a terminal Man.

We studied the binding of procyclic and metacyclic PGs to *P. papatasi* midguts by incubating purified PGs with dissected, opened midguts and then by immunofluorescent staining with monoclonal antibodies specific for the two developmental forms of PG (Fig. 2). Midguts incubated with procyclic PG were intensely stained throughout the abdominal region and portions of the thoracic midgut. The region just posterior to the stomodeal valve was not stained. Midguts incubated with metacyclic PG were poorly stained in both the posterior and the anterior regions. Ultrastructural studies were done by means of electron microscope (EM)–gold immunostaining techniques to label PG-incubated midguts before sectioning (Fig. 3). Particles bound to procyclic PG–incubated midguts were distributed along the entire surface of the microvilli. Few particles were bound to metacyclic PG–incubated or control midguts (*14*).

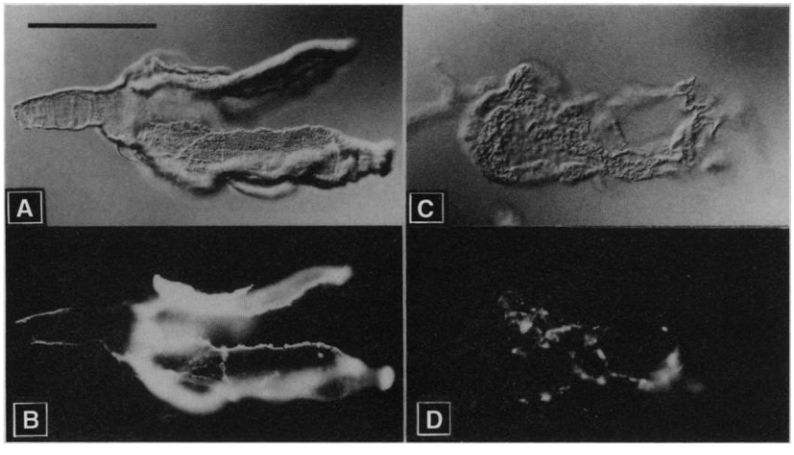

Fig. 2. Immunofluorescent staining of purified *L. major* phosphoglycan bound to *P. papatasi* midguts. Opened, dissected midguts were fixed with 4% formaldehyde and 0.1% glutaraldehyde in PBS at 4°C for 20 min. After several washes in PBS they were incubated for 45 min with purified procyclic PG (10 μg/ml) or metacyclic PG (10 μg/ml) in PBS, washed, reacted with a 1:100 dilution of ascites containing monoclonal antibodies WIC79.3 or 3F12 (specific for *L. major* procyclic and metacyclic PGs, respectively), and then incubated with fluorescein antimouse immunoglobulin G (IgG). (**A** and **B**) Bright field and ultraviolet (UV) exposures of procyclic PG–incubated midgut. (**C** and **D**) Bright field and UV exposures of metacyclic PG–incubated midgut. Bar: 0.5 mm.

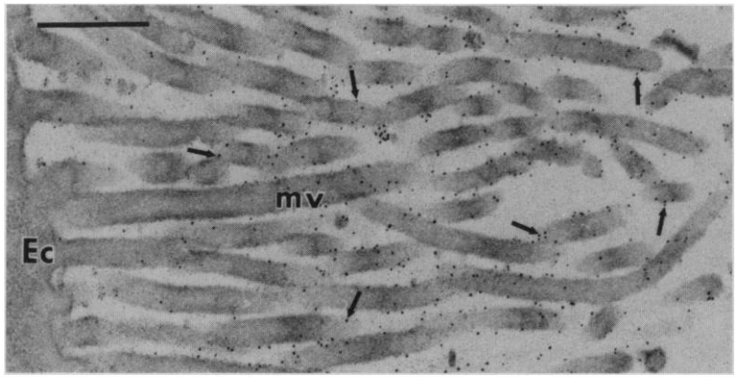

Fig. 3. Immunogold labeling of phosphoglycan bound to epithelial cell microvilli (indicated by arrows). Opened, dissected midguts were fixed for immunocytochemistry as described in Fig. 2, incubated with procyclic PG, reacted with WIC79.3, and then incubated with 10 nm of antimouse IgG colloidal gold for 45 min. The samples were embedded in epon for sectioning and microscopy as described (*20*). Ec, epithelial cell; mv, microvilli; bar, 0.5 μm.

These data demonstrate that procyclic promastigotes display an inherent capacity to bind to midgut epithelial cells that is lost during their transformation to metacyclic forms and that this property is controlled by developmental modifications in surface-exposed oligosaccharides. Factors extrinsic to the parasite, such as modification of the gut epithelia during the course of infection, or competitive inhibition by nutrient sugars, or even released LPG, no longer seem necessary to explain midgut binding and release, although such additional influences cannot be discounted.

Our studies show that the phosphoglycan derived from procyclic LPG binds directly to epithelial cell microvilli and completely inhibits parasite attachment to the midgut. The only other molecule to have been implicated in these interactions is a flagellar-membrane protein (*15*). These conclusions were based on the ability of a monoclonal antibody to inhibit the binding of flagella preparations to frozen sections of sandfly midguts. The protein itself was not used, and the inhibitory effect of the antibody did not exceed 60%, which suggests that other molecules were also involved in the process or that the inhibition observed was due to steric interference of the actual ligand or both.

The finding that specific oligosaccharides mediate interactions between *L. major* and *P. papatasi* supports previous suggestions that gut-associated lectins or lectin-like molecules, which have been described for the insect vectors of many trypanosomatids, including *Leishmania*, might play a role as parasite attachment sites (*16*). It would appear that the primary recognition site of the *P. papatasi* lectin is the terminal β-galactopyranose residue and that substitution or capping of this residue with α-arabinopyranose, as occurs during metacyclogenesis, effectively masks this epitope and thus explains the loss of binding by metacyclic forms. It would also appear that linkage of the Gal side chain residues to the backbone structure contributes substantially to the binding energy of the complex because equimolar concentrations of Gal failed to inhibit binding, which is in agreement with previous observations (*15*). Although the major tetrasaccharide repeat units that contain ter-

minal Gal side chains are down-regulated on metacyclic LPG in favor of terminal Ara-containing tetrasaccharides, there are still significant amounts of terminal Gal within the trisaccharide repeats of metacyclic LPG. It is possible that the trisaccharide repeat units contribute little to the binding of either of the developmental forms because they are buried near the core anchor domains or are made inaccessible by adjacent tetrasaccharide repeat units that have more extended side chains. Alternatively, the trisaccharides may contribute to procyclic PG binding but be made inaccessible by elongation or rearrangement of the phosphoglycan during metacyclogenesis, or both.

If the molecular mechanism that controls interactions between *L. major* and *P. papatasi* also operates in other combinations of *Leishmania* and sandfly, then the LPG of other species could undergo similar modifications during metacyclogenesis. Developmental differences in LPG terminal side chain sugars have recently been found for *L. donovani* and *L. amazonensis* (*17*). Because the stage-specific sugars themselves tend to be species specific, their attachment sites within the fly might also vary, and it is these polymorphisms that could account at least in part for the species specificity of vectorial capacity observed in nature.

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Reversal of the Orientation of an Integral Protein of the Mitochondrial Outer Membrane

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The NH_2-terminus of the signal-anchor sequence of an integral, bitopic protein of the outer mitochondrial membrane was extended both in amino acid length (from 11 to 38 amino acids) and net charge (from +4 to +8)—changes that confer on the NH_2-terminus characteristics of a strong matrix-targeting signal. The protein was inserted into the outer membrane but in an inverted orientation (N_{cyto}-C_{in}). These findings suggest that, in common with other membrane systems, the orientation of a protein in the outer mitochondrial membrane can be determined by a signal that causes retention of the NH_2-terminus on the cytosolic side of the membrane.

We have focused on a simple bitopic integral protein of the outer mitochondrial membrane in yeast, OMM70 (also called MAS70) (*1*, *2*). The topogenic information in OMM70 resides within a stretch of 29 amino acids at the NH_2-terminus, which anchors the protein in the outer membrane by a predicted 19–amino acid transmembrane segment (amino acids 11 through 29) in the N_{in}-C_{cyto} orientation, where the NH_2-terminus is in the mitochondrion and the COOH-terminus is in the cytoplasm (*1*, *3*). A hybrid protein, pOMD29, was created by fusing amino acids 1 through 29 of OMM70 in-frame to a cytosolic reporter protein, dihydrofolate reductase (*4*) (Fig. 1). The protein pOMD29 was efficiently imported into the outer membrane of intact mitochondria with the expected transmembrane orientation (N_{in}-C_{cyto}) (*3*, *4*). It did not target to endoplasmic reticulum (ER) microsomes. The transmembrane segment (amino acids 11 through 29) was essential for targeting, whereas the positively charged amphiphilic NH_2-terminus contained little targeting information for import but cooperated with the transmembrane segment to enhance the overall efficiency of targeting and insertion (*4*).

We suggest that the topogenic domains of pOMD29 (OMM70) operate as the functional equivalent of the signal-anchor sequence (*5*) found in type II and type III proteins (*6*) inserted into the ER, in which the signal (targeting) sequence is coincident with, or overlaps, the membrane anchor segment. An important consequence of a signal-anchor function is that the sequence that specifies targeting and initial translocation across the membrane is also the sequence that abrogates this process and results in lateral release of the segment to the surrounding lipid bilayer (*7*). Proteins like OMM70 contain a signal-anchor sequence selective for the outer membrane; this allows for the analysis of determinants (*8–10*) that specify transbilayer orientation of the protein.

Import of pOMD29 into the outer membrane of intact mitochondria from rat heart was dependent on adenosine triphosphate (ATP) (Fig. 2) and protease-sensitive surface components (*3*). After centrifugation at the end of import incubations, input pOMD29 sedimented only in the presence of mitochondria (Fig. 2), and, of the mito-

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