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Secretory Pathway of Trypanosomatid Parasites

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INTRODUCTION

The Trypanosomatidae comprise a large group of parasitic protozoa, some of which cause important diseases in humans. These include *Trypanosoma brucei* (the causative agent of African sleeping sickness and nagana in cattle), *Trypanosoma cruzi* (agent of Chagas' disease in Central and South America), and *Leishmania* spp. (agent of visceral and [muco]cutaneous leishmaniasis throughout the tropics and subtropics). All of these parasites are transmitted by insect vectors and invade a range of different tissues or cell types in their mammalian hosts. Parasite survival within these environments requires the regulated surface transport of highly abundant coat glycoproteins and glycolipids as well as a large number of other, less abundant plasma membrane transporters, surface enzymes, and receptors that are delivered to the cell surface via a specialized invagination in the plasma membrane, termed the flagellar pocket. In this article we describe the ultrastructural organization of the trypanosomatid secretory pathway and review recent information on protein sorting signals that direct transport to the cell surface and the endocytic and lysosomal compartments, as well as the organization and function of glycosylation enzymes in the secretory pathway. As these parasites represent a highly divergent eukaryote lineage, these studies provide new insights into the extent to which the basic

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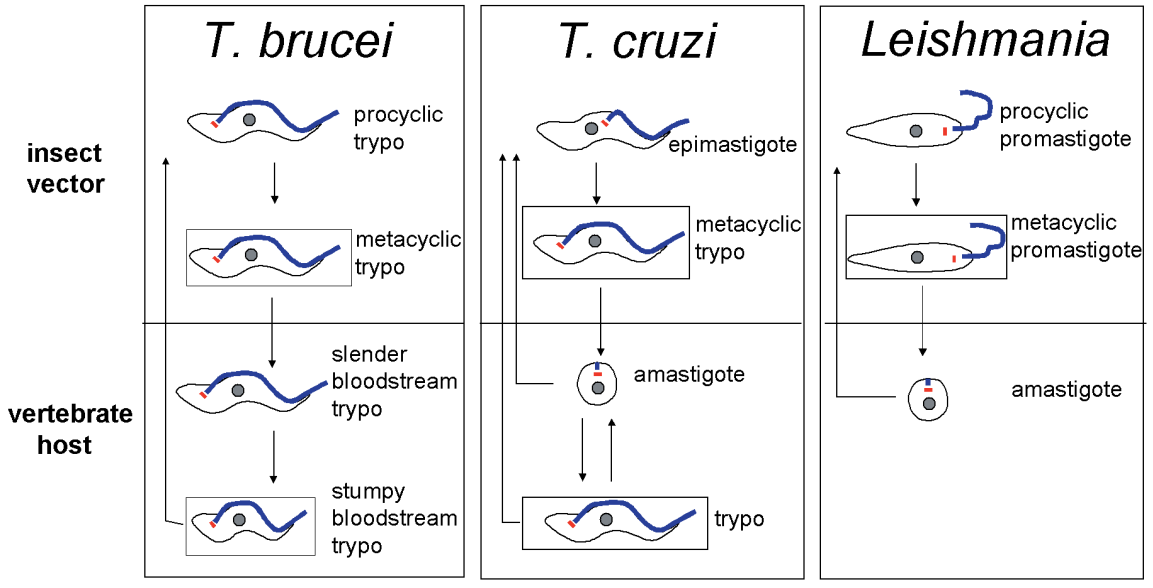

FIG. 1. Life cycles of trypanosomatid parasites. The insect vectors for *T. brucei*, *T. cruzi*, and *Leishmania* are the tsetse fly (*Glossina*), the reduviid bug (*Triatoma*), and sand flies (*Phlebotomus* and *Lutzomyia*), respectively. The mammalian stages of *T. brucei* exist primarily in the bloodstream. In contrast, most of the mammalian developmental stages of *T. cruzi* and *Leishmania* spp. reside within the cytoplasm of a wide range of host cells or the phagolysosome of host macrophages, respectively. Proliferative and nonproliferative (boxed) stages and the locations of the flagellum (blue) and kinetoplast (red) relative to the nucleus (grey) are indicated. *T. cruzi* and *Leishmania* amastigotes may undergo periods of proliferative and nonproliferative growth. It should be noted that most studies on the secretory functions of these parasites have been carried out on proliferative stages. Trypo, trypomastigote.

molecular machinery underlying secretory and endocytic processes has been conserved throughout eukaryotic evolution. Unusual features of the trypanosomatid secretory pathway, such as the polarized delivery of secretory material to the flagellar pocket and requirement for protein sorting to distinct lytic and storage vacuoles, as well as the presence of several unusual or unique glycosylation pathways are emphasized. A detailed understanding of these processes, some of which may represent adaptations of these organisms to parasitic lifestyles, may lead to the development of new antiparasitic strategies. The reader is also referred to other excellent reviews that have focused on particular aspects of protein trafficking in trypanosomatids (19, 61, 175, 250) and other protists (29).

SURFACE COATS OF TRYPANOSOMATIDS

Trypanosomatid parasites go through a number of distinct developmental stages during their digenetic life cycles in the insect vector and mammalian hosts (Fig. 1). The characteristic shapes of these developmental stages are maintained by an array of subpellicular microtubules that underlie the plasma membrane. A single flagellum emerges from a deep invagination in the plasma membrane, termed the flagellar pocket, that can be located either anterior (pro- and epimastigote stages) or posterior (trypomastigote stages) to the nucleus (Fig. 1). In some developmental stages (amastigote stages), the flagellum does not emerge from the flagellar pocket, while in others it may be tightly attached to the plasma membrane along the anterior-posterior axis of the cell. The flagellar pocket is a semisecluded compartment that is accessible to a range of proteins, including very large macromolecular complexes, but not to cellular components of the mammalian host immune system (250). The flagellar pocket membrane is not subtended by the subpellicular microtubule array and is thought to be the major site of exocytosis of secretory cargo (glycoproteins, proteoglycans, and glycolipids) that forms protective surface coats (Fig. 2). These surface coats are compositionally diverse but have a common feature in that they are all dominated by glycosylphosphatidylinositol (GPI)-anchored glycoproteins and/or free GPI glycolipids. The compositions of the surface coats of *T. brucei*, *T. cruzi*, and *Leishmania* spp. are briefly described below.

T. brucei

Early electron microscopy studies revealed that the plasma membrane of infective *T. brucei* bloodstream forms (BF) was covered in an electron-dense coat (357). This coat was subsequently shown to comprise a single ~58-kDa glycoprotein (approximately 10^7 copies/cell) termed the variant surface glycoprotein (VSG) (Fig. 2). The VSG coat protects the parasites from the alternative pathway of complement-mediated lysis, shields other cell surface proteins from the host immune system, and, by the process of antigenic variation, allows these parasites to persist for long periods in the host bloodstream (259). However, small proteins (e.g., trypsin) and essential nutrients can still permeate the VSG coat and reach the plasma membrane (42). Each VSG is attached to the plasma membrane by a GPI anchor that facilitates the dense packing of VSG dimers into coat arrays with minimal perturbation of the plasma membrane. The utilization of a GPI anchor may also allow VSGs to occupy spaces, such as the lipid core of polytopic transporters, from which integral membrane proteins may be excluded. The GPI anchors and N-linked glycans of

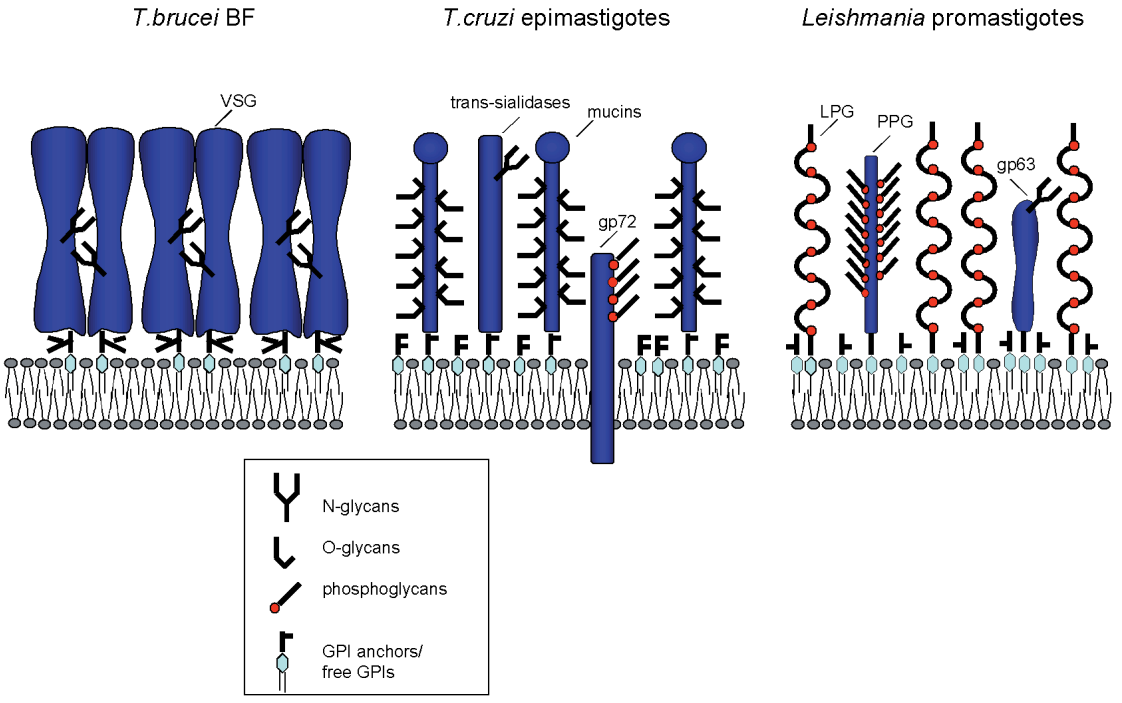

FIG. 2. Surface coats of trypanosomatids. The surface coats of trypanosomatid parasites are dominated by GPI proteins and/or non-protein-linked GPI glycolipids. The nature of the common and species-specific modifications (N-linked glycans, O-linked glycans, and phosphoglycans) that occur in the secretory pathway of these parasites is highlighted. In *Leishmania*, some free GPIs are also phosphoglycosylated to form LPG.

VSG are variably modified with glycan side chains that further contribute to the function of the VSG as a macromolecular diffusion barrier (100, 210, 380). While VSG is evenly distributed over the plasma membrane (cell body, flagellum, and flagellar pocket), other GPI proteins on the surface of *T. brucei* BF can have a punctate distribution or be entirely restricted to the flagellar pocket (183, 238). The surface coat of *T. brucei* BF also contains a number of type 1 integral membrane proteins (the so-called invariant surface glycoproteins) (378) that likewise may be distributed over the entire cell body or sequestered within the flagellar pocket (Table 1). Finally, the plasma membrane contains a large number of polytopic transporters that underlie this surface coat and are inaccessible to surface antibodies in live trypanosomes (Table 1) (38).

In contrast to *T. brucei* BF, the major insect (procyclic) stages of *T. brucei* are covered by a structurally distinct family of GPI proteins, termed the procyclins (287). The procyclins are encoded by multigene families and contain an extended rod-like polypeptide domain made up of repetitive amino acid sequences (EP procyclin contains 21 to 27 Glu-Pro repeats, while GPEET contains 5 or 6 Gly-Pro-Glu-Glu-Thr repeats) that terminate in a globular N-glycosylated N-terminal domain (287). The polypeptide backbone of the procyclins may be modified with phosphate groups (53, 209), while the GPI anchor is elaborated with large, branched poly-*N*-acetyllactosamine (poly-NAL) glycans (102). The poly-NAL chains on the GPI anchors are further capped with sialic acid by a cell surface *trans*-sialidase, conferring additional negative charges to the procyclin coat (53, 95, 209, 269). The procyclin coat is thought to protect this stage from hydrolases in the tsetse fly midgut and to be required for maturation to the metacyclic stage (354). It is therefore surprising that *T. brucei* procyclics lacking this surface coat, after deletion of a gene involved in GPI biosynthesis, survive in culture and can establish an infection (albeit slowly) in the tsetse fly (230). This is in contrast to the situation in BF, where deletion of GPI biosynthesis is lethal (230). However, it remains possible that the procyclins are still required for *T. brucei* infection of tsetse flies in the wild (where levels of infection are very low) and/or subsequent transmission to the mammalian host (99). The procyclic surface coat also contains a number of type 1 membrane proteins and flagellar pocket receptors (although fewer than in the BF stages), and it is likely that the procyclics contain a similar repertoire of plasma membrane transporters (Table 1).

T. cruzi

The surface coats of the major developmental stages of *T. cruzi* are dominated by two heterogeneous families of GPI-anchored glycoproteins, the mucin-like glycoproteins and the *trans*-sialidase family of glycoproteins (49, 67, 303) (Fig. 2; Table 1). Both classes of glycoproteins are encoded by large multigene families. The mucins contain polypeptide backbones with Thr-rich domains that are extensively modified with short O-linked glycans (4, 9, 67, 109, 304) (Fig. 2). A primary function of some members of the *trans*-sialidase glycoproteins (60 to 250 kDa) is to transfer sialic acid from host glycoproteins to terminal galactose residues in the mucin O-linked glycans. Other members of this family lack *trans*-sialidase activity but may be important ligands for host cell receptors (49, 67). The sialylation of the mucin coat is thought protect the extracellular stages of *T. cruzi* from complement lysis and opsonization

TABLE 1. Secretory pathway modifications of surface and secreted trypanosomatid antigens (only major proteins and glycoconjugates or proteins that have defined post-translational modifications are listed)

Molecule(s)	Stage[a]	Modification[b]	Distribution or function	Reference
T. brucei molecules				
GPI proteins				
Variant surface glycoprotein	BF	GPI, N-glycans	Major coat glycoprotein	259
Alanine-rich protein (BAPP)	BF	GPI, N-glycan	Punctate surface domains	238
Gp63	BF	GPI?	Surface metalloproteinase	231
Transferrin receptor (ESAG-6, -7)	BF	GPI, N-glycan, NAL	Flagellar pocket	338
ESAG-2	BF	GPI	Surface protein	259
ESAG-3	BF	GPI	Surface protein	259
TgsGP	BF	GPI, N-glycan, NAL	Flagellar pocket	33
Procyclin	PF	GPI, N-glycan, P, NAL	Major coat antigen	209, 287
Greag-1	PF	GPI		57, 90, 238
trans-Sialidase	PF	GPI	Surface enzyme	95
Membrane proteins				
Invariant surface antigens (Isg65, -70, -75)	BF	N-glycan, NAL	Surface glycoproteins	259
Isg100	BF	N-glycan	Flagellar pocket, intracellular	239
115-kDa ectophosphatase	BF	N-glycan	Surface glycoprotein	17
66-kDa acid phosphatase	BF	N-glycan	Surface and/or flagellar pocket	302
Adenylate cyclase (ESAG4)	BF		Flagellum	251
Fla1	BF, PF	N-glycan, PG	FAZ, flagellar pocket	183, 241
LDL-binding protein	BF, PF		Flagellar pocket	27
CRAM (HDL-binding protein?)	BF		Flagellar pocket	375
P67/Cb1-gp	All	N-glycan, NAL	Flagellar pocket, lysosomes	167
Glycolipids				
Free GPIs	All		Major cellular glycolipids	279
T. cruzi molecules				
GPI proteins				
Mucins (35/50 antigen, Ssp3)	E, T, M	GPI, O-glycan	Major coat glycoproteins	4, 109, 261, 304
trans-Sialidase family (gp85, 1G7, Ssp-4, TCNA)	All	GPI, N-glycan	Coat glycoproteins	67
Amastin	A	GPI?, O-glycan?	Cell surface and intracellular vesicles	347
160-kDa protein (gp160, CRP-160)	T	GPI	Involved in complement resistance	240
86- to 93-kDa protein (T-DAF)			Involved in complement resistance	345
Membrane protein				
Gp72	All	N-glycan, PG	Flagellar attachment zone	64, 139
Secreted protein				
Tc-Tox	T	None	Acid active, pore-forming hemolysin	13
Glycolipids				
Free GPIs (LPPG)	E, T		Major cellular glycolipids	71
Leishmania molecules				
GPI proteins				
Leishmanolysin/gp63/PSP	Pro	GPI, N-glycan	Major surface glycoprotein	76, 306
PSA-2/GP40	Pro, A	GPI, N-glycan	Surface glycoprotein	135, 285
PPG	Pro	GPI, N-glycan, PG	Surface proteoglycan	156
Membrane proteins				
3′ and 5′ nucleotidase	Pro	N-glycan	Surface protein	69
Receptor adenylate cyclase	Pro		Flagellum	300
Amastin	A		Polytopic surface protein	373
Secreted glycoproteins				
Acid phosphatase	Pro	N-glycan, PG	Major secreted glycoprotein	157
PPG	Pro, A	N-glycan, PG	Major secreted glycoprotein	153
Chitinase	Pro		Secreted protein	319
Glycolipids				
LPG	Pro		Major surface macromolecule	205
Free GPIs (GIPLs)	Pro, A		Major cellular glycolipids	202

Continued on following page

TABLE 1—*Continued*

Molecule(s)	Stage[a]	Modification[b]	Distribution or function	Reference
General transporters common to most or all trypanosomatids				
Glucose transporter			Polytopic membrane protein; distinct isoforms on flagellum and cell body	26, 174
Inositol, H^+ transporter			Polytopic membrane protein	83
Proline transporter			Polytopic membrane protein	379
Nucleoside transporters			Polytopic membrane protein; some isoforms on flagellum	299, 355
Biopterin transporter			Polytopic membrane protein	170, 259
ABC transporters			Polytopic membrane proteins	38, 174
Na^+/K^+-ATPase			Polytopic membrane protein	96
Ca^{2+}-ATPase			Polytopic membrane protein	30, 190
Mg^{2+}-ATPase				225
V-H^+-ATPase			Multiprotein complex	31
H^+-pyrophosphatase			Polytopic membrane protein	288

[a] BF, bloodstream trypomastigotes; PF, procylic trypomastigotes; Pro, promastigote; A, amastigote; E, epimastigote; T, trypomastigote.
[b] N, N glycosylated; O, O glycosylated; PG, phosphoglycosylated; P, phosphorylated; NAL, poly-*N*-acetyllactosamine glycans.

with anticarbohydrate antibodies in human serum and to play a role in *T. cruzi* invasion of a wide range of animal cells (49, 67, 261, 304). *T. cruzi* trypomastigotes invade animal cells by stimulating the fusion of secretory lysosomes with the plasma membrane of the target cell. After being internalized into these lysosomes, the trypomastigotes escape into the cytosol by secreting an acid-activated hemolysin (324). The surface *trans*-sialidases of the parasite facilitate this process by removing sialic acid from lysosomal membrane glycoproteins, thereby increasing the sensitivity of these membranes to the hemolysin. As the sialic acids are transferred to the trypomastigote coat, this process may also protect the parasite from its own hemolysin (133). In addition to the mucins and the *trans*-sialidase glycoproteins, *T. cruzi* trypomastigotes express a number of other GPI proteins that are involved in inactivating opsonic complement components (Table 1). Finally, all developmental stages of *T. cruzi* synthesize a highly abundant class of free GPIs (also referred to as glycoinositol-phospholipids [GIPLs] or lipopeptidophosphoglycan [LPPG]) that are likely to form a densely packed glycocalyx beneath the mucin coat (70, 71) (Fig. 2).

Leishmania spp.

The major surface macromolecule on the surface of the promastigote (insect) stage of *Leishmania* spp. is the hyperglycosylated GPI glycolipid, termed lipophosphoglycan (LPG) (205, 212). These molecules contain a highly conserved GPI anchor and a long phosphoglycan backbone that can be elaborated with species- and stage-specific glycan side chains (202, 205, 212). In all *Leishmania* spp. that are pathogenic in humans, LPG is expressed at very high levels ($\sim 5 \times 10^6$ molecules/cell) and forms a distinct glycocalyx that can be readily detected by electron microscopy (265). The LPG coat may protect the promastigote stage from lysis by the alternative complement cascade and extracellular hydrolases (277, 296) and may be a ligand, either directly or indirectly, for insect midgut and mammalian macrophage receptors (212, 297). Polymorphisms in LPG structure appear to contribute to the vector tropism of different *Leishmania* species (297). LPG is essential for the infectivity of *Leishmania major* promastigotes in both the insect and mammalian hosts (297, 335) but is dispensable for infectivity of *Leishmania mexicana* promastigotes in mice (152, 335, 351). The latter result may reflect functional redundancy with other surface molecules or differences in the stringency of the mouse model system for these different *Leishmania* species (152, 335, 351). The surface coat of *Leishmania* promastigotes also contains a number of GPI proteins and proteophosphoglycans (PPGs) (Table 1). Most of these proteins appear to be masked on live promastigotes by the LPG coat (166), and their precise function remains unclear. *Leishmania donovani* mutants lacking six of the seven gene copies for the major GPI protein, gp63 (promastigote surface protease, or leishmanolysin), are more sensitive to complement lysis (165). However, *L. mexicana* mutants lacking all GPI proteins can still establish an infection in animals (146, 165). Like *T. cruzi*, *Leishmania* promastigotes also synthesize very high levels of free GPIs that are 10-fold more abundant ($\sim 5 \times 10^7$ copies/cell) than LPG. While most of these glycolipids are found in the exoplasmic leaflet of the plasma membrane (201, 377), a significant pool may be located in the inner leaflet of the plasma membrane (280). These glycolipids are thus likely to be essential components of the surface coat, but their precise function remains unclear (Fig. 2). Attempts at obtaining GPI-negative *Leishmania* promastigotes were initially unsuccessful, suggesting that these glycolipids may be essential for viability (160). However, an *L. mexicana* mutant that is unable to synthesize all GPI-anchored macromolecules (protein and LPG) or mature free GPIs and that is viable in rich media has recently been generated (114). Remarkably, this mutant is weakly infective in animals, although this result is less surprising given that the mammalian (amastigote) stage of these parasites down-regulates the expression of most of these molecules and can acquire glycolipids from the host cell (200) (see below).

Leishmania spp. amastigotes reside within the phagolysosome compartment of mammalian macrophages and are unique amongst the trypanosomatids in lacking a prominent surface glycocalyx of GPI proteins or other GPI-anchored macromolecules (16, 200, 218, 265, 295, 370). Indeed, very few

proteins can be labeled on the surfaces of live amastigotes by biotinylation and iodination protocols (370). Instead, this stage is coated with a surface layer of free GPIs and host-derived glycosphingolipids, most likely acquired from the inner leaflet of the macrophage phagolysosome membrane (200, 308, 343, 370). These glycolipids may protect essential plasma membrane transporters from proteolysis in the host cell lysosome, while the lack of surface-exposed proteins could constitute a form of immune evasion. Specifically, this surface architecture may reduce the opportunity for parasite peptides to be transported to the surface of infected macrophages in association with major histocompatibility complex class II proteins for presentation to the host T cells (249, 371).

Leishmania promastigotes and amastigotes also differ from other trypanosomatids in secreting copious amounts of soluble PPGs (153). The secreted (and surface-bound) PPGs characteristically contain very long polypeptide backbones which are modified with phosphoglycans similar to those added to LPG (157). Individually or after self-association, the PPGs form very large macromolecular filamentous structures that may facilitate promastigote aggregation and the transmission of large parasite clusters within the sand fly bite (340). The secreted PPGs may also deplete the level of lytic complement components in the mammalian lesion via nonproductive activation of the complement pathway (262) and modulate processes in the macrophage, such as the number and size of parasitophorous vacuoles and cytokine production (153, 263).

ORGANIZATION OF THE TRYPANOSOMATID SECRETORY PATHWAY

Early Secretory Pathway

Most proteins destined for the cell surface or lysosomes of trypanosomatids are initially assembled in the endoplasmic reticulum (ER). The ER is also the site of synthesis of most of the membrane phospho- and glycolipids. A number of markers for the trypanosomatid ER have been identified, including the major protein chaperones BiP and calreticulin, which assist newly synthesized proteins to fold in the ER lumen (21, 25, 164, 172), enzymes involved in the assembly of dolichol-linked oligosaccharides and GPI protein anchor precursors (54, 226, 271), and an ER-type Ca^{2+}-ATPase that maintains high Ca^{2+} levels in the ER lumen (111, 189). In rapidly dividing trypanosomatids, the ER can account for 60% of the internal membranes (65). At the ultrastructural level the ER comprises the nuclear envelope and a connected system of cisternal or tubular membranes that are often closely associated with the plasma membrane (25, 159, 232, 265, 333, 365). These studies, as well as subcellular fractionation approaches, have revealed the presence of a number of functionally distinct ER subdomains. The first of these domains is the nuclear envelope that surrounds the large centrally located nucleus and remains intact throughout the cell cycle. Highly purified nuclei and nuclear envelopes have been isolated from *T. brucei* BF and procyclics and shown to contain numerous nuclear pore complexes that span the inner and outer membranes and are morphologically indistinguishable from those in the nuclear envelopes of other eukaryotes (290). One of the functions of the inner membrane is to maintain the organization of the nuclear lamina which contains the NUP-1 antigen, a large (350-kDa) coiled-coil protein that may be the trypanosomatid homologue of mammalian lamins (290). The outer membrane of the *T. brucei* nuclear envelope is studded with numerous ribosomes, suggesting that it is part of the rough ER (290). However, in *Leishmania* promastigotes, the nuclear envelope also contains a number of markers that are associated with a smooth ER fraction, suggesting that the nuclear envelope may comprise more than one domain (226). The cortical ER is continuous with the nuclear envelope but may also comprise a number of domains. These include the classical rough and smooth domains, which are often difficult to distinguish in electron micrographs because of the high density of free polysomes in the cytosol. However, two distinct ER domains can be resolved when *L. mexicana* promastigote microsomes are fractionated on sucrose density gradients. Both populations of microsomes contain the ER chaperone BiP (which is thought to be a marker of the entire ER), while the light fraction is highly enriched in enzymes involved in GPI and phospholipid biosynthesis (226). Light and electron microscopy studies indicated that these light and dense ER microsomal fractions form a mosaic throughout the cortical ER and nuclear envelope (226). Interestingly, some of these ER markers are transported to an unusual tubular compartment, termed the multivesicular tubule (MVT) in late-log- and stationary-phase promastigotes (226). Although initially thought to be part of the early secretory pathway, recent studies have shown that the MVT is a mature lysosome (120, 226, 365) (Fig. 3). In *T. brucei* and *T. cruzi* trypomastigotes, a distinctive subdomain of the smooth ER is consistently found attached to four specialized microtubules in the subpellicular array (357). These microtubules can be distinguished from other subpellicular microtubules based on their polarity and tubulin composition and directly underlie the flagellar attachment zone (FAZ) (127). Intimate connections between ER subdomains and the subpellicular microtubules also occur in other trypanosomatids that lack an adherent flagellum and FAZ (264). To date, no markers have been localized to these ER subdomains and their function is unknown.

Rapidly dividing trypanosomatids also contain a specialized transitional ER (tER) that lies directly opposite the single Golgi apparatus proximal to the flagellar pocket (Fig. 3). The tER comprises a cisternal extension of the cortical ER and is likely to be the major site at which proteins and lipids are exported to the Golgi (85, 103, 108, 226, 264, 365). A recent study on high-pressure-frozen *L. mexicana* promastigotes has provided remarkable new insights into the organization of the tER and its connection with the Golgi apparatus (365). The ribosome-free membrane of the tER facing the *cis*-Golgi contains many omega-shaped budding profiles, while the narrow space (100 nm) between the tER and the *cis*-Golgi is full of transport vesicles and an electron-dense matrix. There is no evidence that cytoskeletal elements are required for vesicular transport, although a single (or pair of) cytoplasmic microtubule(s) may be involved in maintaining the association of the tER with the Golgi (226, 365). This intimate association between the tER and Golgi may be essential to sustain the very high levels of protein and lipid transport to the cell surface. It is not known whether transport vesicles can also bud from other regions of the ER. However, in the absence of a con-

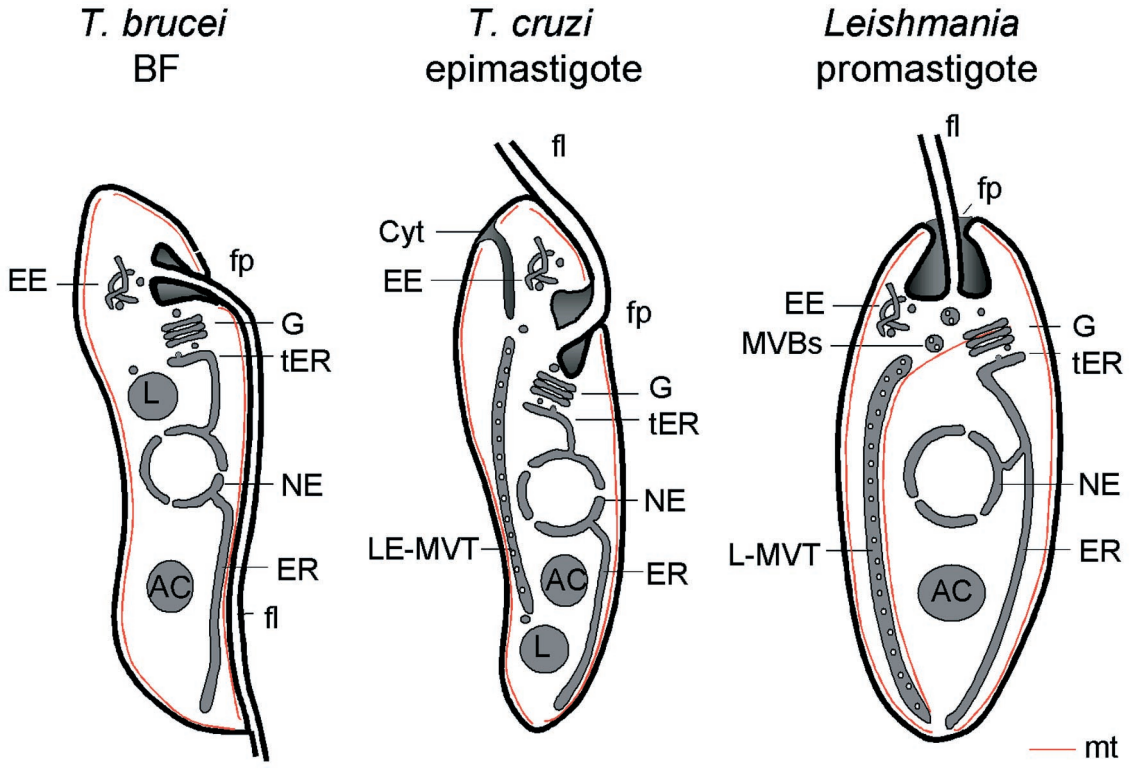

FIG. 3. Schematic representations of the secretory and endocytic organelles of *T. brucei* BF, *T. cruzi* epimastigotes, and *Leishmania* promastigotes. Most of the organelles involved in secretion and the early endocytic pathway are organized around the flagellar pocket. Significant stage- and species-specific differences exist in the organization and morphology of late-endosome and lysosomal organelles that are highlighted in these schemes. In all cases, the ER comprises the nuclear envelope (NE) and a cortical reticulum (ER) that is connected to the specialized tER proximal to the Golgi apparatus (G) and the flagellar pocket (fp). Early endosomes (EE; depicted as a complex tubule-vesicle network) are also invariably located near the flagellar pocket. In *T. brucei* BF, the nature of late endosomes has not been clearly defined and mature lysosomes have a predominantly perinuclear location. In *Leishmania* promastigotes, a population of MVBs, which may correspond to late endosomes, forms near the Golgi apparatus and fuses with the lysosome-MVT (L-MVT), which extends along the anterior-posterior axis of the cell. In *T. cruzi* epimastigotes, a morphologically related MVT functions as an intermediate, late-endosome (LE-MVT) compartment that transports markers from the cytostome (Cyt; a second invagination in the plasma membrane that is specialized for endocytosis) to the mature lysosomes at the aflagellate end of the cell. Acidocalcisomes (AC) constitute a second class of acidified vacuoles which contain resident proteins that are initially synthesized in the ER. fl, flagellar; mt, microtubules. See the text for references.

spicuous transcellular microtubule- or actin-based cytoskeleton in these parasites, these vesicles would have to reach the Golgi apparatus by the much less efficient process of diffusion (36).

Like its counterparts in other eukaryotes, the single Golgi apparatus of trypanosomatids consists of a stack of 3 to 10 cisternae and a polymorphic *trans*-Golgi network (TGN) (93, 108, 226, 365). Transport through the Golgi apparatus is thought to occur via two possible and not mutually exclusive mechanisms: vesicular transport between cisternae or the maturation and progressive movement of cisternae towards the *trans* face of the Golgi (cisternal maturation) (121). Cisternal maturation may be important in trypanosomatids, based on ultrastructural studies that show the apparent formation of new cisternae in the space between the tER and the *cis* face of the Golgi (365). Moreover, cisternal maturation may be required for the transport of very large macromolecular complexes, such as the filamentous PPGs of *Leishmania* promastigotes and amastigotes (341). The transport vesicles that are associated with the margins of the trypanosomatid Golgi may be involved in either the retrograde transport of resident proteins from older to younger cisternae or the anterograde transport of cargo proteins and lipids. Interestingly, and in contrast to the situation in animal cells, the Golgi apparatus of trypanosomatids and several other protists (32) does not break down during the cell cycle but undergoes medial fission during mitosis, along with other organelles (i.e., basal body, flagellum, kinetoplast, and mitochondria) (105, 365).

Only a few resident protein markers have been identified for the trypanosomatid Golgi (131, 184, 220). The fluorescent lipid BODIPY-ceramide has been used to label the Golgi in live *T. brucei* BF [105, 602], while this dye is specifically sequestered within the tubular lysosome of *Leishmania* promastigotes (159), highlighting possible differences in the lipid composition of secretory pathway membranes in different trypanosomatids. The different cisternae of the trypanosomatid Golgi appear to be biochemically and functionally differentiated, based on the polarized distribution of some Golgi marker proteins (56), the separation of different Golgi fractions in subcellular fractionation studies (125, 224), and differences in the cytochemical staining of some cisternae (94). Moreover, *T. brucei* BF glycoproteins receive complex N-linked glycan modifications that

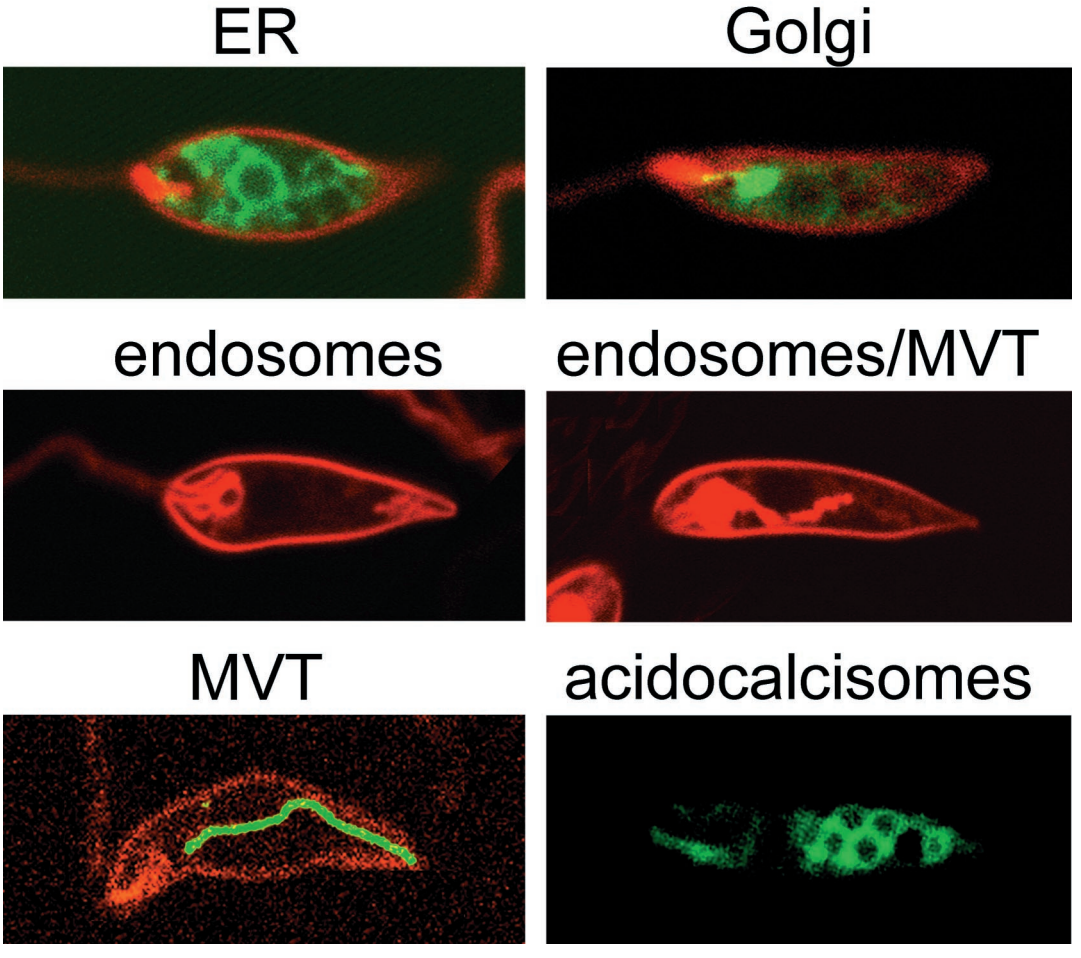

FIG. 4. Secretory and endocytic organelles of *L. mexicana* promastigotes. Individual organelles were visualized in live *L. mexicana* promastigotes that expressed or were labeled with fluorescent markers, as follows. The ER is defined by a GFP chimera containing an N-terminal signal sequence and a C-terminal ER retention signal. The single Golgi apparatus is defined by a GFP chimera containing a C-terminal GRIP (TGN-binding) domain. The early endosomes are labeled with the vital dye FM 4-64 (20 min at 10°C). Endosomes and lysosomes (lysosome-MVT) are labeled with FM 4-64 (2 h at 27°C). The lysosome-MVT is labeled with a GFP chimera containing the ER glycosyltransferase dolichol-phosphate-mannose synthase, which accumulates in the lysosome-MVT in late-log- and stationary-phase promastigotes. The acidocalcisomes are labeled with the acidotrophic dye Lysotracker. Live promastigotes expressing the GFP chimeras were surface labeled with TRITC (tetramethyl rhodamine isocyanate)-concanavalin A to highlight the cell body, the flagellum, and the flagellar pocket at the anterior end of the cell (226).

are assembled in different Golgi cisternae in animal cells (210, 252). Although the latter processing steps do not occur to the same extent in *T. cruzi* or *Leishmania* spp. (see below), many of the proteins in the latter species are modified with complex O-linked glycans or phosphoglycans by enzymes that also appear to be compartmentalized in the Golgi (131, 224). Some of these Golgi-specific processing steps can be inhibited with the ionophore monensin (20, 28), although this drug does not affect the surface transport of GPI proteins such as VSG (20, 85). Interestingly, brefeldin A, a fungal metabolite that induces the collapse of the Golgi into the ER in many animal cells and some other protozoa, has little or no effect on the structure of the Golgi or secretory transport in trypanosomatids (108) (K. A. Mullin and M. J. McConville, unpublished data). In *T. cruzi* epimastigotes, brefeldin A treatment actually results in an increase in the number of Golgi cisternae, a phenotype that is markedly more pronounced in *T. cruzi* mutants that are resistant to cysteine protease inhibitors (93). In this case, brefeldin A may affect post-Golgi sorting steps, including the recycling of lysosomal cargo receptors, leading to the accumulation of proteins in the early secretory pathway and the amplification of Golgi compartments.

The ER localization of several trypanosomatid proteins is likely to depend on the efficient retrieval of these proteins from the Golgi. For example, the *T. brucei* ER chaperone BiP contains a C-terminal tetrapeptide (MDDL [single amino acid code]) that is both necessary and sufficient for ER retention (21, 25). This motif also acts as an ER localization signal when appended to a secreted form of the green fluorescent protein (GFP) in *L. mexicana* promastigotes (159) (Fig. 4). By analogy with the HDEL/KDEL ER localization signal in *Saccharomyces cerevisiae* and animal cells, these proteins are probably recognized by a transmembrane receptor in the Golgi apparatus and recycled back to the ER in COP1 retrograde transport vesicles (316, 346). Similarly, some trypanosomatid ER membrane proteins (e.g., the ER-type Ca^{2+}-ATPase [111]) contain

a C-terminal KKXX motif that may direct these proteins into Golgi retrograde transport vesicles for delivery back to the ER. These observations support the notion that trypanosomatids have an efficient ER-to-Golgi retrograde transport pathway.

The Late Secretory Pathway—from TGN to the Flagellar Pocket

In higher eukaryotes, the TGN is a major sorting station, where proteins destined for the cell surface are separated from those progressing to vacuolar and lysosomal compartments (366). Several lines of evidence suggest that the *trans*-most cisterna of the trypanosomatid Golgi is functionally equivalent to the TGN. First, the *trans*-most cisterna of the trypanosomatid Golgi is usually more highly dilated than earlier cisternae and is associated with a range of vesicular, cisternal, and multivesicular structures, suggestive of a sorting role (365). Second, cargo proteins such as VSG accumulate in this compartment when *T. brucei* BF are incubated at 20°C rather than 37°C (85). Third, the *trans*-cisterna can be selectively labeled with fluid-phase endocytic markers (176, 361, 365), suggesting that it is intimately connected to endocytic organelles by anterograde and retrograde transport vesicles (366). Finally, a number of protein markers, including a putative β1-adaptin (221) and reporter proteins containing a TGN targeting signal, termed the golgin-97, RanBP2α, Imh1p, and p230/golgin-245 (GRIP) domain, specifically localize to the *trans*-cisternae of *T. brucei* and *L. mexicana* Golgi (M. J. McConville, S. C. Ilgoutz, B. Foth, R. D. Teasdale, and P. A. Gleeson, submitted for publication), respectively, suggesting that it is a functionally distinct domain of the Golgi apparatus (Fig. 4).

The nature of the major transport intermediates that direct proteins from the Golgi to the flagellar pocket has yet to be precisely defined. In *T. brucei* BF, VSG is transported to the flagellar pocket in a complex system of cisternal and tubulovesicular membranes (85, 363). The unequivocal identification of these membranes as exocytic intermediates is complicated by the fact that VSG is also present in morphologically similar endosomal structures that are concentrated in the same region of the cell. However, some of these membranes are not labeled with fluid-phase endocytic markers and are thus likely to be primarily involved in exocytic transport (85, 363). In *L. mexicana* promastigotes, gp63 may be transported from the TGN to the flagellar pocket in large translucent vacuoles (226, 365). However, small transport vesicles are also commonly observed in the region between the Golgi apparatus and the flagellar pocket and may be involved in exocytosis. Thus, it is possible that transport to the flagellar pocket membrane involves a number of different carriers, possibly containing distinct proteins and lipid cargo, as has been found in other eukaryotes (136, 228, 267).

In some trypanosomatids, the lumen of the flagellar pocket may contain a gel-like matrix as well as many membrane profiles (176). In *T. brucei* BF, this matrix is rich in GPI proteins such as VSG and the transferrin receptor (119, 298, 338). The major GPI proteins of *T. brucei* procyclics and *T. cruzi* trypomastigotes are also abundantly present in the flagellar pocket lumen (119). It remains to be determined whether these membrane proteins are released into the lumen as micelles or as monomers. There is evidence that this matrix reduces the rate at which macromolecules diffuse out of the flagellar pocket (176).

Pathways from the TGN to Lysosomes and Vacuoles

Most trypanosomatid developmental stages contain at least two distinct classes of acidified vacuoles, the lysosomes and the acidocalcisomes (Fig. 3). The lysosomes are lytic vacuoles that receive material from the Golgi apparatus via a pleiomorphic population of endosome intermediates. The morphology of the lysosomes varies markedly in different trypanosomatid stages. In *T. brucei* BF and *T. cruzi* trypomastigotes, mature lysosomes comprise a series of prominent spherical vacuoles that characteristically have a perinuclear distribution (85, 176, 184, 332, 334, 374). In *T. cruzi* epimastigotes, the lysosomes (also termed reservosomes) contain electron-dense, lipid-rich cores and accumulate near the aflagellate end of the cell (270, 332) (Fig. 3). In rapidly dividing *Leishmania* promastigotes, the mature lysosomes exist as a highly unusual MVT, termed here the lysosome-MVT, that extends from the flagellar pocket to the posterior end of the cell (120, 226, 365) (Fig. 3 and 4). The *Leishmania* lysosome-MVT was first observed in live *L. mexicana* promastigotes expressing a GFP chimera of the ER glycosyltransferase, dolichol-phosphate-mannose synthase (DPMS), suggesting that it may have been part of the early secretory pathway (159). However, subsequent studies clearly demonstrate that it is a mature lysosome (120, 226, 365). In high-pressure-frozen and conventionally fixed *L. mexicana* promastigotes, the lysosome-MVT contains many small luminal vesicles and is clearly distinct from the ER and Golgi apparatus (120, 226, 365). This structure is highly reminiscent of late endosomes and lysosomes of other eukaryotes (126, 180). Moreover, the lysosome-MVT is the terminal compartment for a number of endocytic markers (FM 4-64, biotinylated surface proteins, and horseradish peroxidase) and is clearly distinct from the ER and Golgi apparatus. It also contains resident lysosomal proteases and is the site of degradation of several reporter proteins. Finally, glycoconjugates that are assembled in the Golgi apparatus can accumulate in the lysosome-MVT lumen, confirming that it is a post-Golgi compartment. Morphologically similar MVT structures occur in the related trypanosomatid *Crithidia fasciculata* (44) and in *T. cruzi* epimastigotes (270). However, the *T. cruzi* MVT is thought to correspond to a prelysosomal compartment and is termed here the late-endosome-MVT (Fig. 3). Interestingly, the *L. mexicana* lysosome-MVT matures into multiple electron-dense vacuoles as rapidly dividing promastigotes reach stationary growth (226, 265). In the amastigote stage, the lysosomes form large electron-dense vacuoles (termed megasomes) that occupy a significant proportion of the cytoplasm (63, 352). The morphology of late-endosome and mature-lysosome compartments can thus vary enormously within developmental stages of the same species. These developmental changes undoubtedly reflect the changing requirements for lysosomal degradation in regulating protein turnover and nutrient acquisition during the parasite life cycle.

Trypanosomatid proteins may be transported from the Golgi apparatus to the lysosomes via the flagellar pocket (the indirect route) or by a direct intracellular route (Fig. 5). While the ultrastructure of the endocytic membranes has been investigated in many trypanosomatids (see below), very little is known

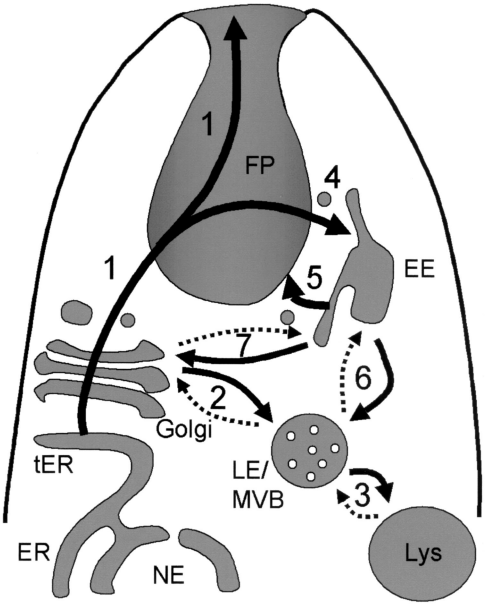

FIG. 5. Proposed protein transport pathways in the secretory and endocytic pathways of trypanosomatids. Proteins are synthesized in the rough ER, comprising domains of the nuclear envelope (NE) and the cortical ER (ER), and then exported to the Golgi apparatus via a specialized tER. Transport from the Golgi to the flagellar pocket membrane may occur in a pleiomorphic population of vesicles and cisternal vacuoles (route 1). After delivery to the flagellar pocket, plasma membrane proteins can be directed to the cell body and/or the flagellum. Resident lysosomal proteins and ER membrane proteins destined for degradation can be transported to lysosomes via a direct internal route from the Golgi apparatus (1, 2, and 3) or after delivery to the flagellar pocket and internalization in early endosomes (1, 4, 6, and 3). Both routes may involve the internalization of membrane proteins into the lumen of MVBs as microinvaginating vesicles (2 and 6). Lysosomal resident proteins and the degradation products of lysosomes may be exocytosed via various retrograde pathways (3, 6, and 5; dotted lines) or by direct fusion with the flagellar pocket membrane. Some surface proteins are recycled through the endosomes and returned to the flagellar pocket membrane (4 and 5). Evidence for these pathways is drawn from studies on *T. brucei*, *T. cruzi*, *Leishmania*, and *C. fasciculata* (see the text for details and references). Solid lines indicate pathways that are supported by biochemical or ultrastructural studies. Dotted lines are speculative but are based on pathways that have been defined in other eukaryotes. See the text for references.

about the vesicular intermediates that deliver proteins to the lysosome from either the endosomes (indirect route) or the TGN (direct route). Putative vesicular intermediates have been identified in *T. brucei* (176, 184), *Trypanosoma vivax* (50), and *Trypanosoma congolense* BF (356). Recent studies with *L. mexicana* promastigotes have also identified a population of multivesicular bodies (MVBs) that form opposite the TGN and fuse with the lysosome-MVT (226). The involvement of MVBs in this step would be analogous to the recently delineated MVB pathway in yeast and animal cells (126, 180, 244, 283). MVBs are thought to form in the endocytic pathway by a process that involves the invagination of microvesicles from the outer membrane of endosomes that pinch off to form discrete structures in the MVB lumen. When MVBs fuse with the lysosome, these internal vesicles are delivered into the lysosome lumen and eventually degraded by soluble hydrolases (proteases, lipases, and glycosidases). Proteins and lipids destined for degradation are incorporated into the internal vesicles of the MVBs, whereas proteins that function at the lysosome surface remain in the MVB limiting membrane and can be recycled back to the early endosomes or the TGN (180). Several recent observations suggest that the MVBs in *Leishmania* and other trypanosomatids play a similar role. In particular, ER proteins such as DPMS (an integral membrane protein that is oriented on the cytoplasmic leaflet of the ER) are sorted into the lumen of the MVB and lysosome-MVT of stationary-phase *L. mexicana* promastigotes (226). Some peripheral leishmanial membrane proteins that associate with phosphoinositide lipids on the cytoplasmic leaflet of the endosomes are also delivered into the lumen of the lysosome (J. Callaghan and M. J. McConville, unpublished results). However, not all proteins are packaged into these lumen vesicles. For example, the *T. brucei* type 1 membrane proteins tGLP-1 and p67 are distributed between the Golgi apparatus and the limiting membrane of the lysosome in *T. brucei* BF (184). This distribution suggests that some proteins may undergo multiple cycles of transport between the Golgi apparatus to the limiting membrane of the lysosome (Fig. 5). Retrograde transport of proteins out of the lysosome is supported by the observation that proteolytically processed forms of p67 can be detected at the cell surface of *T. brucei* BF (41).

Retrograde transport from the lysosome is likely to be important in recycling trypanosomatid flagellar pocket receptors. For example, the transferrin and lipoprotein receptors of *T. brucei* BF appear to be transported to either a prelysosome or mature lysosome compartment, where their ligands are released and degraded (186, 339). However, the receptors themselves are not degraded but are apparently recycled back to the flagellar pocket for further rounds of transferrin and lipoprotein uptake and internalization (339). This contrasts with the situation in animal cells, where the transferrin receptor recycles between the plasma membrane and a population of early endosomes. In this case the transferrin remains bound to the human transferrin receptor throughout the cycle (iron is released from the diferric [holo]transferrin-receptor complex in the early endosomes) and the iron-free (apo)transferrin is released only once the complex returns to the cell surface (350). After being delivered to the lysosome of *T. brucei* BF, the transferrin degradation products are secreted into the extracellular milieu (339). Similarly, the degradation products of antibodies internalized to the lysosome with other flagellar pocket receptors are also secreted (186). These observations indicate that the contents of the lysosome as well as lysosomal membrane proteins can be delivered back to the plasma membrane (Fig. 5). Delivery to the plasma membrane could occur via a retrograde pathway or by direct fusion of lysosomes with the flagellar pocket membrane (12).

The acidocalcisomes constitute the second class of acidified vacuoles in trypanosomatids (reviewed in reference 79). Similar vacuoles (also known as volutin granules or polyphosphate bodies) are present in apicomplexan parasites and some green algae (292). Unlike the lysosomes, the acidocalcisomes lack proteinase activities and appear to be storage organelles containing the major cellular reserves for phosphorus (mainly as polyphosphates), calcium, sodium, and a number of other cations (Zn^{2+} and Mg^{2+}) (79, 80). They may also play a role in protecting these unicellular eukaryotes from osmotic stress

(293). The acidocalcisomes appear to be empty by conventional electron microscopy but have a high electron density when cells are prepared without fixation and dehydration (79). They can be detected by fluorescence microscopy in living parasites that have been stained with acidotrophic or cationic dyes (acridine orange, Lysotracker, or DAPI [4′,6′-diamidino-2-phenylindole]) (226, 293). The intracellular distribution of acidocalcisomes varies in different developmental stages, alternatively clustering near the flagellum (*T. cruzi* trypomastigotes), the cell periphery (*T. cruzi* amastigotes), or the aflagellate end of the parasite (*L. mexicana* promastigotes) (79, 226). They can also be closely associated with other intracellular organelles, such as the ER and mitochondria, consistent with their being the dynamic storage reservoirs of Ca^{2+} and phosphate (217). These vacuoles are not labeled with endocytic markers in rapidly dividing trypanosomatid stages, suggesting that the lysosomes or earlier endocytic compartments do not fuse with the acidocalcisomes (217, 226, 313). However, in drug-treated parasites, endocytic markers can be internalized into acidocalcisomes (353), possibly as a result of nonselective autophagic degradation of intracellular organelles under these conditions. The limiting membrane of the trypanosomatid acidocalcisomes contains a V-H^+ pyrophosphatase, a Na^+/H^+ exchanger, a Ca^{2+}-H^+ exchanger, a Ca^{2+}-ATPase, and a V-H^+-ATPase that maintain the acidic pH and concentrate various cations in the lumen (79, 311, 312). Resident proteins such as the V-H^+ pyrophosphatase and V_0 components of the V-H^+-ATPase contain N-terminal signal sequences (143) and can also have dual localizations in the plasma membrane, suggesting that they are delivered to the acidocalcisomes via the Golgi apparatus or, alternatively, that the acidocalcisomes can fuse with the plasma membrane (293). Very little is known about the biogenesis of acidocalcisomes. A novel kinesin-like protein (TbKIFC1) that may associate with intermediates in acidocalcisome biogenesis and direct these structures along the subpellicular microtubules has recently been identified in *T. brucei* (87). Down-regulation of kinesin expression by double-stranded-RNA interference methods partially inhibited acidocalcisome function (87), although the effect of kinesin down-regulation on other post-Golgi compartments was not examined. These observations suggest that proteins are sorted into at least three distinct classes of transport vesicles in the Golgi apparatus that are targeted to the flagellar pocket, the endosome-lysosome system, and the acidocalcisomes.

Intersection of Secretory and Endocytic Pathways

Parasite survival within insect and vertebrate hosts is dependent on the uptake of extracellular nutrients and the removal of opsonic host proteins from the cell surface. Endocytosis in trypanosomatids is restricted to either the flagellar pocket membrane or, where present, the cytostome. The early endosomes commonly consist of a system of pleiomorphic tubules and cisternal structures that are invariably localized around the flagellar pocket and in close proximity to the Golgi apparatus (176, 361, 362, 365) (Fig. 3). However, the rate of endocytosis varies enormously in different trypanosomatids. In *T. brucei* BF, the entire flagellar pocket volume may be endocytosed six to eight times per h, equivalent to the internalization of the flagellar pocket membrane every 2 min (65). Endocytosis is mediated primarily by a population of clathrin-coated vesicles that bud from the flagellar pocket and deliver a range of fluid-phase markers (i.e., ferritin, horseradish peroxidase, fluorescein isothiocyanate-bovine serum albumin, gold-labeled bovine serum albumin, and lucifer yellow) and membrane markers (VSG and transferrin-, low-density lipoprotein-, and high-density lipoprotein-receptor complexes) to a system of cisternal elements and collecting tubules (176, 221). This tubule-vesicle complex contains distinct populations of endosomes, as defined by the distribution of several Rab protein homologues (TbRab4, TbRab5A, TbRab5B, and TbRab11) (104), which are likely to be active in sorting membrane and soluble components for transport to the lysosomes or back to the flagellar pocket. Compartments defined by TbRab4, -5A, and -5B are associated with endosomes that are involved in the internalization steps and possibly transport to late-endosome and lysosome compartments (104). In contrast, TbRab11 delineates a distinct population of endosome membranes that are closely associated with these compartments but which appear to be involved in recycling GPI proteins, such as the VSG and transferrin receptor, to the cell surface (163). Unexpectedly, some of these Rab proteins can be detected at the aflagellate end of *T. brucei* BF, raising the possibility that they may be involved in long-distance transport (104, 221). In *T. brucei* procyclics and most other trypanosomatids, the rate of endocytosis is much lower, although uptake of plasma membrane markers can still be readily detected (94, 120, 176, 178, 226, 362), and the morphology of the endocytic organelles is considerably less complex (106, 163, 221, 362, 365). The lower rate of endocytosis may reflect the absence of clathrin-coated endocytic vesicles in these developmental stages (176, 221). However, endocytosis is significantly increased in *T. brucei* procyclics overexpressing constitutively active forms of TbRab5A, suggesting that these vesicles can sustain a high level of endocytosis (251a).

Plasma membrane proteins and flagellar pocket receptors that are internalized into the tubular-vesicular endosomes may be recycled back to the flagellar pocket or transported to the late-endosome and lysosome compartments (43, 85, 132, 323, 361) (Fig. 5). Transport from the tubular-vesicular (early) endosomes to the lysosome is specifically inhibited when *T. brucei* BF and *Leishmania* promastigotes are incubated at 10 to 12°C (43, 132, 226), suggesting that this step is more temperature sensitive than the recycling step. By analogy with the situation in other eukaryotes (180), transport from the endosomes to the lysosomes may involve the late-endosome–MVB pathway described above. These organelles would thus represent the point at which the endocytic and secretory pathways converge (Fig. 5). Interestingly, the GPI proteins of trypanosomatids can also be internalized into the lysosome, along with other flagellar pocket receptors. Overath and colleagues estimated that approximately 25% of *L. mexicana* gp63 accumulates in the lysosome-MVT of the promastigote stage (365). A significant steady-state pool of GPI-anchored PPG is also present in these organelles (E. Handman, A. Piani, J. Curtis, T. Ilg, M. J. McConville, and B. Foth, submitted for publication). In another interesting series of studies, anti-VSG antibodies opsonized to the cell surface of *T. brucei* BF were found to be rapidly endocytosed into the endocytic pathway and delivered to perinuclear lysosomes (318, 364). Removal of a single layer

of bound antibody was complete within 30 min under physiological conditions. As both bi- and monovalent antibodies and antibody fragments are internalized, it is likely that VSG is constitutively endocytosed at a very high rate and bound antibodies are subsequently degraded in the lysosome. However, it remains unclear whether all the endocytosed VSG reaches late-endosome and lysosome compartments or whether only opsonized VSG is diverted to these compartments. VSG is subsequently recycled back to the flagellar pocket in Rab11-positive endosomes (163). The internalization of VSG into late-endocytic compartments is likely to play a key role in preventing the accumulation of opsonic antibody on the surface coat of *T. brucei* BF (18, 242, 318, 364) and may provide a rationale for why the rate of endocytosis in this developmental stage is massively up-regulated compared to that in other trypanosomatids.

Endocytosis via the Cytostome

Some trypanosomatids contain a second specialized invagination in their plasma membranes, termed a cytostome, that constitutes an alternative site of endocytosis. Cytostomes are absent from *T. brucei* and *Leishmania* but are prominent in *T. cruzi* epimastigotes and amastigotes (Fig. 3). The cytostome of *T. cruzi* epimastigotes is located near the flagellar pocket and extends into the cytoplasm as a narrow tubule (the cytopharynx) with an acidified lumen (270, 334). Lectin binding and freeze fracture studies demonstrate that the plasma membrane around the cytostome and in the region between the cytostome and the flagellar pocket is distinct from the rest of the plasma membrane and that these structures overlie a set of specialized microtubules (332). Uncoated vesicles bud from the end of the cytopharynx and fuse with an MVT that extends along the anterior-posterior axis of the cell (270) (Fig. 3). Although this organelle is morphologically similar to the lysosome-MVT of *L. mexicana* promastigotes, it appears to be an intermediate compartment in the delivery of endocytic markers to a population of electron-dense lysosomes (reservosomes) at the posterior end of the cell (270) (Fig. 3). The structure of the cytostome and endocytic organelles has also been studied in *C. fasciculata*. In this case, the cytostome is located inside the flagellar pocket, and endocytic vesicles that bud from this structure deliver their contents to a series of MVBs that arise near the Golgi and subsequently fuse with an MVT reminiscent of that found in *L. mexicana* (44). Collectively, these studies demonstrate that the cytostome, rather than the flagellar pocket, is the major site of endocytosis in these trypanosomatids and that endocytosed material is delivered to terminal lysosomes via MVBs or MVT structures (44, 247, 270, 332, 334). It remains to be determined whether the cytostome is also involved in exocytosis and the role of the endosomes in the cytostome-lysosome pathway.

Components of the Vesicle Transport Machinery

In other eukaryotes, the fusion of transport vesicles with their target membrane is regulated by proteins of the soluble NSF attachment proteins (SNAP) receptor (SNARE) family (236). A v-SNARE on the vesicle interacts with a t-SNARE on the target organelle, and their interaction is required for membrane fusion. The fusion reaction and subsequent recycling of the SNAREs is regulated by soluble proteins, such as *N*-ethylmaleimide-sensitive factor (NSF) and SNAP, as well as a number of other proteins, including Rab GTPases and Sec1-like proteins (336). Analyses of the trypanosomatid genomes have identified 11 putative homologues of different Rab proteins, seven of which have been implicated in different mammalian membrane trafficking steps (Table 2). In contrast, only two SNARE proteins could be reliably identified based on sequence homology (unpublished observation). This result is likely to reflect the degree of conservation of these proteins across species. Comparison of functional homologues of these proteins between yeast and mammals shows that the Rab GTPases have a higher level of conservation than the SNARE proteins. The identification of additional SNAREs will require biochemical studies or the use of more sensitive sequence homology searches.

A homologue of NSF and another member of the AAA (ATPase associated with different cellular activities) family of proteins, p97/cdc48p, have been identified in *T. brucei* (289) (Table 2). These proteins are thought to be responsible for unraveling highly stable SNARE complexes and thus play a key role in priming or recycling SNAREs for the next round of vesicle fusion. While NSF acts in most vesicle transport steps, p97 is thought to be involved in regulating the homotypic fusion of ER and Golgi membranes and the biogenesis of these organelles. The *T. brucei* p97 gene complements the yeast cdc48 gene, encodes a protein that forms hexameric complexes, and contains two functional ATPase domains (173, 289). This protein is essential for parasite growth, and the regulated expression of dominant negative forms (in which one of the ATPase domains is mutated) results in gross changes in cell shape (173). However, it does not appear to play a direct role in exocytosis of soluble or GPI proteins, and its potential function in regulating organellar division remains to be determined.

In addition to the membrane targeting and fusion machinery, a number of coat protein complexes drive the initial formation of a membrane vesicle and also play a key role in selecting protein cargo and cargo receptors (336). Vesicle formation requires the recruitment of the coat proteins from the cytosol either as individual proteins or as preformed complexes. Following vesicle formation, the coat proteins are released, allowing the SNAREs and other tethering proteins to interact with their counter receptors. Well-characterized coat proteins include clathrin and the adaptor 1 and 2 complexes (Golgi-to-plasma membrane and plasma membrane–to–early-endosome transport, respectively), COPI (Golgi-to-ER and intra-Golgi transport), and COPII (ER-to-Golgi transport) (336). Additional coat proteins are thought to be involved in regulating the transport of TGN tubular vesicles to the plasma membrane and retrograde transport from the endosomes to the TGN (180, 314). Open reading frames that encode putative homologues of many of these coat proteins are present in the *T. brucei* and *L. major* genome databases (Table 2). In *T. brucei* BF, endocytosis is largely mediated by coated vesicles that contain clathrin heavy chain (221, 320). Potential adaptor proteins and an α-dynamin homologue that may facilitate this process are present in the databases (Table 2). Expression of the *T. brucei* clathrin heavy chain is markedly up-regulated in

TABLE 2. Putative homologues of membrane trafficking proteins identified in the genomes of *T. brucei* and *L. major*

Transport pathway	Class	Protein	EBI database homolog (reference) in[a]:	
			T. brucei	*L. major*
ER to Golgi	COPII	Sec13p	ND	AQ902337
		Sec23p	AZ212341, AQ941243, AQ946342, AQ942118, AZ215819	AQ902560
		Sec24p	AQ639904, AQ659295, AQ660713, AQ657751	ND
	Rab GTPase	Rab1	AQ656291	L12031 (56)
		Rab2	AQ661511	ND
	SNAREs	Syntaxin 5	AQ643295	
Golgi to ER, intra-Golgi	COPI	α-COP	AL441840	AQ852115
		β-COP	AJ271083[b]	ND
		β′-COP	AJ250726[c]	ND
		γ-COP	AQ641446	ND
		ζ-COP	AJ271084[b]	AC021894[d]
	Rab GTPase	Rab6	AC008031	AC084317[e]
Post-Golgi	Clathrin	Clathrin heavy chain	AQ948970, AQ948967, AQ652557 AQ652561, AQ941349, AQ644077	ND
		Dynamin-2	AQ646871, AQ950923	ND
	AP1 adaptors	γ-Adaptin	AQ649197, AQ649199	ND
		β-Adaptin	AF152173 (221)	AQ843984, AQ844080
		μ1 chain	AQ949131	
		σ1 chain	AQ655666, AZ213671	ND
	AP3 adaptors	δ-Adaptin	AQ654960, AQ652661, AQ947042	ND
		μ3 chain	AQ945902, AQ641127	ND
		σ3 chain	AQ637868	ND
Endosome to Golgi	Retromer	Vps5p	AA736241	AC013257[e]
		Vps26p	AQ950582	ND
		Vps29p	AQ952117	ND
		Vps35p	AQ653574, AQ940473	AL446005
Endocytosis, endosomal transport	AP2 adaptors	α-Adaptin	ND	AQ849482, AQ849788
	AP4 adaptors	δ-Adaptin	AQ654960, AQ652661, AQ947042	
	Rab GTPase	Rab4	U24677 (89)	AL499614
		Rab5	U24678 (89)	AL359774
		Rab7	AF146042	AL139262 (161)
		Rab11	AF152531 (163, 177)	ND
	Sec1-like	Munc18	AQ639772, AQ639299	
		Vps33p	B07420	
		Vps45p	AQ948874	
	SNAREs	Syntaxin 16	AQ643792, AQ651850	AQ848167
	NSF		AL359782	AL354272

[a] *T. brucei* and *L. major* sequence databases, located at the European Bioinformatics Institute (EBI; http://www.ebi.ac.uk/blast2/parasites.html), were searched for sequences that showed significant homology to proteins involved in membrane trafficking. Four additional Rab GTPases were identified: Rab18 (*T. brucei*, AF131291 [163, 177]), Rab21 (*T. brucei*, AL359774), Rab23 (*L. major*, AQ848776), and Rab28 (*T. brucei*, AA960734). ND, not detected.

[b] A. G. Maier, S. Schulreich, M. Bremser, and C. Clayton, unpublished GenBank sequence.

[c] H. D. Webb, A. F. Gaud, and M. Carrington, unpublished GenBank sequence.

[d] P. J. Myler, E. Sisk, J. Cawthra, F. Handley, C. Vogt, L. Robertson, P. McDonagh, and K. Stuart, unpublished GenBank sequence.

[e] P. J. Myler, E. Sisk, J. Ruiz, P. Cosenza, A. Cruz, K. Stuart, and E. A. Worthey, unpublished GenBank sequence.

the BF stage compared to the procyclic stage, consistent with the finding that the endocytic vesicles with a conspicuous cytoplasmic coat are absent in the latter stage (176, 221, 361). The nature of the cytoplasmic coat on these endocytic vesicles remains to be determined. Clathrin-coated vesicles may also mediate transport between the Golgi apparatus and the endosomes in *T. brucei* BF, as suggested by the partial distribution of the clathrin heavy chain to the Golgi region and the identification of a putative β1-adaptin homologue (221). Unlike the clathrin heavy chain, β1-adaptin was expressed at similar levels in both *T. brucei* BF and procyclics, indicating that this pathway is similarly active in both stages (221). Protein coats have also been observed on post-Golgi vesicles in *L. mexicana* promastigotes (365). Finally, putative homologues of COPII and COPI coat complexes have been identified in the trypanosome and leishmania genomes (Table 2), and a high-molecular-weight COPI complex has been partially purified from *T. brucei* procyclics (195). Interestingly, secretory transport in *T. brucei*, *T. cruzi*, and *Leishmania* is not inhibited by brefeldin A, a potent inhibitor of COPI coat formation in many animal and protozoan cells (93, 108) (Mullin and McConville, unpublished), suggesting that one or more proteins involved in ER-

Golgi transport in trypanosomatids differ from their mammalian counterparts. Overall, trypanosomatids have numerous proteins encoded in their genomes related to proteins that function in the various membrane trafficking stages in both yeast and mammalian cells. Based on the range of proteins identified, trypanosomatids would be predicted to have the same membrane transport pathways, utilizing similar protein machinery, to those already identified in other eukaryotes.

Role of the Microtubule Cytoskeleton

Microtubules are the major cytoskeletal elements of trypanosomatids (reviewed in reference 127). There is increasing evidence that the subpellicular microtubules that underlie the plasma membrane, as well as a limited number of specialized transcellular microtubules, may play a role in regulating the polarized distribution and biogenesis of secretory and endocytic pathway organelles. They may do this in two ways: by sterically restricting exo- and endocytic functions to certain plasma membrane domains and/or by acting as cytoplasmic scaffolds for specific organelles or transport intermediates.

The subpellicular array comprises more than 100 microtubules that are aligned along the anterior-posterior axis of the cell and are highly cross-linked both to each other and to the overlying plasma membrane. Microtubule attachment to the plasma membrane may be mediated by acylated microtubule-binding proteins (142, 305) or polytopic integral membrane proteins (331). It is generally assumed that the subpellicular microtubules limit the accessibility of vesicular traffic to all domains of the plasma membrane except the flagellar pocket (and cytostome, where it exists) and thus impose a degree of polarization on the distribution of secretory and endocytic organelles. However, large secretory granules can reach the plasma membranes of other protozoan parasites that contain a highly organized cortical (membrane or microtubule) skeleton (235), and some domains of the ER can clearly penetrate between the subpellicular microtubules in trypanosomatids (264). These indirect lines of evidence raise the possibilities that the subpellicular microtubules may play a more active role in directing vesicular traffic to the flagellar pocket and/or that specific protein or lipid determinants on the flagellar pocket membrane act as targeting signals. In support of an active role for the subpellicular microtubules, a number of microtubule-binding proteins have recently been identified that are directed to different ends of these microtubule arrays and may function as motor proteins. For example, a GFP chimera containing the microtubule-binding protein trypanin (previously called T-lymphocyte-triggering factor) accumulates at the posterior face of the flagellar pocket of *T. brucei* BF. Remarkably, a GFP fusion containing the mammalian homologue of trypanin accumulates at the extreme posterior end of the trypomastigotes, where the subpellicular microtubules terminate (144, 145). The *T. brucei* and mammalian proteins may bind to different motor proteins and/or different microtubules within the subpellicular array. In particular, the localization of the *T. brucei* GFP-trypanin chimera suggests that it might bind to the microtubule quartet, a specialized set of four microtubules that underlie the FAZ. These microtubules arise near the flagellum basal body before being intercalated into the subpellicular array, have the polarity opposite to that of other subpellicular microtubules, contain γ-tubulin, and are more resistant to high-salt extraction than the subpellicular microtubules (286, 294, 372). The possible involvement of the FAZ microtubules in organelle positioning is supported by the tight association of ER subdomains with these microtubules along part of their length (357). Finally, a kinesin-like motor protein that binds to microtubules in vitro and membrane-bound organelles that may be required for the biogenesis of mature acidocalcisomes in vivo has been identified in *T. brucei* (87). In the presence of NH_4Cl, these structures move to the minus end of subpellicular microtubules, suggesting that such movement is dependent on intracellular pH gradients.

While the studies described above suggest that the subpellicular microtubules may play a role in directing membrane transport, most organelles in the trypanosomatid secretory and endocytic pathways are not associated with the subpellicular array. However, there is evidence that in *C. fasciculata* and *Leishmania* promastigotes, the subcellular positions of some of these organelles, including the Golgi apparatus and the lysosome-MVT, may be maintained by one or two cytoplasmic microtubules that extend along the anterior-posterior axis of the parasite (44, 220, 226, 365). Whether these cytoplasmic microtubules are related to or are the same as the microtubule quartet of *T. brucei* and *T. cruzi* trypomastigotes is not known. Compounds that disrupt intracellular pH gradients cause the rapid collapse of the lysosome-MVT, possibly as a result of the dissociation of these labile membranes from these microtubules (159, 226). These microtubules may also have a role in segregating the Golgi apparatus and populations of endosomes into daughter cells during mitosis, as this processes is tightly coordinated with the division of the basal body (105, 163).

PROTEIN TRANSPORT IN THE SECRETORY PATHWAY

Surface Transport of GPI-Anchored Glycoproteins

The major surface proteins of trypanosomatids are initially synthesized with a cleavable N-terminal signal sequence and a C-terminal GPI attachment signal that is rapidly replaced (within 1 min) with a GPI anchor in the ER lumen (10, 20, 22, 23, 68, 97, 182, 198, 226). After addition of the GPI anchor, these proteins are transported to the cell surface with comparatively rapid kinetics. For example, VSG is transported to the surface of *T. brucei* BF with a half time ($t_{1/2}$) of approximately 15 min (20, 85), while gp63 is transported to the surface of *L. mexicana* promastigotes with a $t_{1/2}$ of 40 min (280). If anchor addition is prevented, by either deletion of the GPI transamidase (146) or depletion of GPI anchor precursors 116, 214, 230; M. Ellis, J. D. Hilley, D. K. Sharma, S. Lillico, G. H. Coombs, and J. C. Mottram, Mol. Parasitol. Meet. XI, abstr. 135, 2000), the newly synthesized proteins are either slowly secreted or degraded. The rapid surface transport kinetics of VSG and gp63 raises the possibility that these proteins may be selectively incorporated into ER transport vesicles and exported at a rate that is higher than bulk flow. Efficient export from the ER is supported by immunoelectron microscopy studies showing that steady-state levels of VSG and gp63 in the ER are very low, despite the fact that these proteins are synthesized at very high rates in metabolic labeling experiments (97, 365). Second, VSG is transported to the cell surface of *T.*

brucei BF with faster kinetics than that of the integral membrane protein, p67 ($t_{1/2}$, 15 versus ~60 min) (41). Third, Bangs and colleagues have shown that GPI-anchored VSG is transported to the plasma membrane with faster kinetics than a soluble form of VSG (lacking a GPI signal sequence) when these proteins are ectopically expressed in *T. brucei* procyclic stages ($t_{1/2}$, ~1 versus 5 h) (24, 207). Moreover, the soluble forms of VSG accumulate in the ER, suggesting that the addition of a GPI anchor is required for efficient export via the tER. Finally, gp63 is transported to the surface of *L. mexicana* promastigotes with faster kinetics (twofold) than the more abundant free GPIs, which may be transported by bulk flow (280).

Recent studies with yeast suggest that the major GPI protein of *Saccharomyces cerevisiae*, Gas1p, is selectively incorporated into a subpopulation of COPII-coated ER transport vesicles and that this transport step is dependent on COPI coat components and ongoing ceramide synthesis (82, 148, 227, 344). Two models, which are not mutually exclusive, have been proposed to account for the selective recruitment of GPI proteins into these ER vesicles (228). The first of these models posits that Gas1p is recognized by the p24 family of cargo receptors (169, 227). Homologues of these proteins have been identified in the *T. brucei* genome (194; R. D. Teasdale and M. J. McConville, unpublished data) and may selectively recognize partially conserved features in the VSG polypeptide or the GPI moiety itself. It is worth noting that some conformational epitopes in VSG are lost upon removal of the GPI anchor (52), possibly accounting for the slower transport of nonanchored forms of VSG that may no longer be recognized by an ER receptor. The presence of putative cargo receptors in the tER may also explain why gp63 is transported to the cell surface with faster kinetics than the free GPIs of *L. mexicana* (Ralton et al., submitted) and why many foreign proteins get trapped in the ER when ectopically expressed in *T. brucei* BF (U. Bohme, E. Wirtz, M. Duszenko, and G. A. M. Cross, Mol. Parasitol. Meet. X, abstr. 201, 1999). In the second model, the efficient export of Gas1p from the ER of yeast reflects the association of this protein with sphingolipid-rich membranes or rafts in the ER that are subsequently incorporated into COPII vesicles (15). In support of this model, anterograde transport of yeast Gas1p, but not integral membrane proteins, requires ongoing sphingolipid biosynthesis (148, 344). However, more recent studies have shown that the major raft-forming sphingolipid of yeast is made in the Golgi apparatus rather than the ER (181) and that sphingolipids are also required for fusion of ER vesicles with the Golgi (344). Finally, inhibitors of sphingolipid biosynthesis do not affect the rate of surface transport of gp63 in *L. mexicana* promastigotes (280). It is therefore likely that the forward transport of GPI proteins in *Leishmania* does not involve a lipid-based sorting mechanism and that the requirements for surface transport of GPI proteins in yeast and trypanosomatids are different.

Several recent studies suggest that the GPI anchors could also act as a cell surface sorting signal in the late secretory pathway of trypanosomatids. For example, when expressed at high levels, GPI proteins are efficiently transported to the cell surface while many integral membrane reporter proteins accumulate in the lysosome. Examples of integral membrane proteins that accumulate in the lysosome-MVT of *Leishmania* spp. when expressed at high levels include the membrane-bound acid phosphatase (normally found in endosomes) (365), a chimera of GFP containing the transmembrane domain of plasma membrane 3′-nucleotidase (120), and several GFP chimeras containing ER glycosyltransferases (226). Replacement of the transmembrane domains of the acid phosphatase and GFP with a GPI signal sequence results in the efficient expression of this protein at the cell surface. These studies raise the possibility that the lysosome is the default pathway for many integral membrane proteins, as is the case in yeast (62), and that addition of a GPI anchor redirects proteins into exocytic transport vesicles in the Golgi apparatus. Alternatively, GPI proteins may be sorted from integral membrane proteins in the flagellar pocket (41). In the latter case, addition of a GPI anchor may reduce the rate at which the protein is endocytosed or increase the efficiency with which it is recycled to the flagellar pocket. Interestingly, *L. mexicana* promastigotes express a number of different isoforms of gp63 that all contain a GPI attachment signal and are targeted predominantly to the cell surface. In contrast, the amastigote stage expresses a single isoform of gp63 that lacks a GPI attachment signal (but contains a C-terminal hydrophobic domain) and is localized to the lysosome (16). Thus, the stage-specific expression of protein isoforms with or without a GPI attachment signal may lead to the redistribution of the same protein to the plasma membrane or the lysosome.

The sorting of heterologous GPI proteins to the plasma membrane is likely to involve a lipid-based sorting mechanism. In this respect, a number of recent studies have shown that trypanosomatid GPI proteins become associated with detergent-resistant membranes (DRMs) during transit to the cell surface (73, 238, 280). DRMs have been found in all eukaryotes that have been investigated and are thought to correspond to dynamic micro- or macrodomains in intracellular and plasma membranes that are present largely in a liquid-ordered, rather than a liquid-disordered (fluid), state (46, 47). In common with the DRMs of other eukaryotes, the *Leishmania* promastigote DRMs are highly enriched in sphingolipids (inositolphosphoceramide), sterols (primarily ergosterol), and GPI proteins and free GPIs. They have a light buoyant density in sucrose density gradients and are depleted of integral membrane proteins (73, 280). Interestingly, newly synthesized gp63 is incorporated into DRMs upon arrival at the cell surface of *L. mexicana* promastigotes but at a much earlier point in *L. major* promastigotes (73, 280), suggesting that the lipid composition of secretory pathway membranes varies between species. More strikingly, DRMs can be detected in *T. brucei* only when these parasites are extracted in dilute (0.5 rather than 1%) Triton X-100 (73, 237). Thus, if DRMs do act as sorting platforms in the late secretory pathway of trypanosomatids, they are unlikely to have a specific lipid composition.

Surface Transport of Integral Membrane Proteins

The plasma membranes of trypanosomatids also contain a number of functionally diverse type 1 and polytopic membrane proteins (Table 1). These proteins may contain sorting signals that prevent them from being transported to the lysosome. In the case of *L. donovani* 3′-nucleotidase, this putative sorting signal may reside in the luminal domain as the full-length protein is efficiently transported to the plasma membrane even

when overexpressed (68), while GFP chimeras containing the transmembrane and cytoplasmic domains of 3′-nucleotidase are primarily transported to the lysosome-MVT (120).

Other studies have provided evidence that efficient export of some integral membrane proteins from the ER is signal mediated. Deletion of 19 amino acids from the cytoplasmic tail of the *T. brucei* high-density lipoprotein receptor (cysteine-rich repetitive acidic transmembrane protein [CRAM]) results in the accumulation of the mutated protein in the ER (375). However, further studies are needed to discount the possibility that ER retention is not due to a defect in the folding of this protein.

Sorting in the Flagellar Pocket

The flagellar pocket appears to constitute an important sorting compartment in its own right. While the major GPI proteins and surface transporters are swept out of the flagellar pocket to the cell body and/or the flagellum, a number of cell surface receptors and resident lysosomal proteins are retained within the flagellar pocket and enter the endocytic recycling and/or lysosomal transport pathways. Recent studies suggest that sorting signals may be present in both the cytoplasmic tails and luminal domains of flagellar pocket proteins. In the case of the high-density lipoprotein receptor, CRAM, sequences in the cytosolic tail of this protein (near the transmembrane domain) are both necessary and sufficient for the steady-state localization of CRAM and a reporter protein to the flagellar pocket of *T. brucei* procyclics (375). This domain is also required for the endocytosis and transport of CRAM to the lysosome (186). Thus, the flagellar pocket localization of CRAM in *T. brucei* procyclics could reflect the continuous internalization of this receptor into the endosome-lysosome system and recycling back to the flagellar pocket.

In contrast, the *T. brucei* BF transferrin receptor is a heterodimer of ESAG-6 (a GPI protein) and ESAG-7 (a highly related protein lacking a GPI signal) and thus lacks a cytoplasmic polypeptide domain (183, 298, 337). The retention and internalization signals in the receptor must therefore reside in either the luminal or GPI domains of these proteins. Nolan and colleagues recently proposed that endocytosis of a number of flagellar pocket receptors and lysosomal proteins in *T. brucei* BF is regulated by a lectin-like receptor that binds to luminally oriented N-linked glycans on these proteins (237). This hypothesis is based on the finding that several of these receptors (including the transferrin receptor, ISG100, p67/CB1-gp, and ESAG-2) are strongly recognized by the tomato lectin that binds branched poly-NAL glycans and that the uptake of transferrin is inhibited by GlcNAc-containing oligosaccharides (237). Furthermore, when the dimeric ESAG-6 and -7 receptor complex is expressed in *T. brucei* procyclics, these proteins are modified with high-mannose N-glycans and are distributed over the entire cell body (183). However, direct evidence for a poly-NAL receptor has yet to be obtained, and it remains to be determined how such a mechanism could work given that the flagellar pocket will also contain very high levels of VSG with similar poly-NAL chains. If a lectin receptor does exist, it is likely to be restricted to *T. brucei* BF, as this is the only trypanosomatid to synthesize poly-NAL N-glycans (see below).

Some surface proteins, such as the glucose transporters of *Leishmania enriettii*, are sorted to either the cell body plasma membrane or the flagellum (174). Glucose transporter isoform 2 is directed to the cell body by a short N-terminal cytoplasmic tail that mediates the attachment of this protein to the subpellicular microtubules that underlie the cell body (331). On the other hand, glucose transporter isoform 1 has a longer (139-amino-acid) cytoplasmic domain that contains a flagellar-membrane-targeting motif (266, 330, 331). From alanine-scanning mutagenesis, this targeting motif was restricted to five contiguous amino acids. Furthermore, it was proposed that flagellum localization may be mediated by continuous recycling of the isoform 2 transporter between the flagellum and the flagellar pocket. It will be of interest to determine whether these proteins associate with cytoplasmic raft complexes that have been shown to move up and down the flagellum via interactions with flagellar microtubules and motor proteins in other protists (260). A number of other proteins have been localized to the flagellum of other trypanosomatids, including the receptor adenylate cyclase (ESAG-4) from *T. brucei* and the cytosolically oriented Ca^{2+}-binding proteins of *T. brucei* and *T. cruzi* (122). These proteins lack the cytoplasmic peptide motifs of the leishmanial transporters and appear to be targeted to the flagellum by additional mechanisms.

Sorting in the Endosomes

Only a few protein markers for the trypanosomatid endosomes have been identified. These include a number of Rab proteins (Table 2) (106), the membrane-bound acid phosphatase of *L. mexicana* promastigotes (367), and the *Leishmania tarentolae* ABC transporter, glycoprotein-like protein A (PGPA) (179). Overexpression of the *L. mexicana* membrane-bound acid phosphatase in wild-type cells results in the transport of this protein to the cell surface and the lysosome-MVT (365). The endosomal localization of this protein may thus depend on saturable endocytic and recycling steps. As a truncation mutant of the acid phosphatase comprising only the luminal domain is secreted, this signal(s) must reside in the cytoplasmic or transmembrane domains of this protein (367).

Targeting of Lysosomal Proteins

Most newly synthesized lysosomal hydrolases and integral membrane proteins are delivered to the trypanosomatid lysosomes via the secretory pathway. This is supported by the following findings. First, most resident lysosomal proteins characterized to date contain a canonical N-terminal signal sequence that directs nascent polypeptides into the lumen of the ER (150, 167). Second, these proteins are modified with N-glycans and terminal carbohydrate modifications that occur only in the ER and Golgi lumens, respectively (41, 55, 255). Third, unprocessed lysosomal proteases accumulate in the Golgi or flagellar pocket (45, 93). Finally, inhibition of these processing steps with protease inhibitors results in dramatic changes in Golgi structure (93). Transport of proteins to the lysosomes of trypanosomatids may occur via two routes, the direct intracellular pathway from the Golgi apparatus to the lysosomes, most likely via an endosome intermediate (see above), and the indirect surface pathway, where proteins are first transported to the flagellar pocket before being trans-

ported to the lysosome via the endocytic pathway (Fig. 5). The extent to which some proteins are transported along one or the both of these pathways is developmentally regulated (168).

Cysteine proteases. The cysteine proteases are a major class of soluble lysosomal hydrolases in all trypanosomatids. These proteinases are initially synthesized as prepropolypeptides with an N-terminal signal peptide, a 100- to 122-amino-acid proregion, a central catalytic domain, and, in some cases, an unusual 100-amino-acid C-terminal extension (55, 150). The N-terminal propeptide is thought to maintain these enzymes in an inactive state and to facilitate folding in the ER. The propeptide is also required for lysosomal targeting of cruzain (the major cysteine protease of *T. cruzi*) and is capable of directing reporter proteins such as GFP to lysosomes in both *T. cruzi* and *Leishmania* (150). These studies suggest that the prodomain is recognized by an unidentified receptor in the early secretory pathway and proteolytically removed during transit to or upon arrival in the lysosome. If cleavage of the cruzain propeptide is inhibited, by either expression of forms with a mutated cleavage site or incubation of *T. cruzi* epimastigotes in cysteine protease inhibitors (150), the propeptide accumulates in the Golgi apparatus rather than being secreted. Prolonged treatment of *T. cruzi* epimastigotes with cysteine protease inhibitors induces dramatic changes in structure of the Golgi apparatus as well as the ER (84, 93, 315), possibly accounting for the toxicity of these compounds to many trypanosomatids (92, 208, 315). These data suggest that the major cysteine proteases of *T. cruzi* epimastigotes are transported to the lysosomes via the direct intracellular route. In contrast, unprocessed cysteine proteases accumulate in the flagellar pocket of a protease-deficient *L. mexicana* mutant (45). It was concluded that these proteases normally transit via the flagellar pocket, although it remains possible that the flagellar pocket localization is due to the saturation of an intracellular cargo receptor. Interestingly, the prodomains of trypanosomatid cysteine proteases contain a nine-residue domain that shares close homology with a related domain in mammalian procathepsin L. This domain is apparently required for lysosomal traffic via a mannose-6-phosphate-independent pathway. As there is no evidence that trypanosomatids contain a mannose-6-phosphate pathway (45, 84), these parasites may constitute an interesting model system for investigating lysosomal targeting mechanisms that may have been conserved throughout eukaryotic evolution (150). Finally, there is evidence that some fully processed cysteine proteases are secreted (84, 246). The secretion of these proteases may represent the saturation of cargo receptors in the secretory pathway or the exocytosis of lysosomal contents (12).

Lysosomal membrane proteins. The paradigm for lysosomal membrane protein transport in trypanosomatids is the *T. brucei* type 1 membrane glycoprotein, p67, which has a domain structure similar to that of the lysosomal acidic membrane proteins of animal cells (167). In *T. brucei* BF, newly synthesized p67 is initially detected as a 100-kDa ER glycoform that is modified with 14 N-linked oligosaccharides. The N-glycans of p67 are extended and elaborated with a novel galactose-containing glycan (the CB1 epitope) in the Golgi apparatus to form the 150- to 180-kDa "Golgi" glycoform. This form is subsequently processed into smaller fragment by proteases in the endosome and lysosome compartments. By following these processing steps in conjunction with surface biotinylation, Brickman and Balber (41) and Kelley and colleagues (168) have shown that a significant fraction of p67 in *T. brucei* BF is transported from the Golgi apparatus to the flagellar pocket before being internalized via the endocytic pathway and delivered to lysosomes. Furthermore, processed forms of p67 can be detected at the plasma membrane, supporting the notion that some lysosomal proteins are transported back to the flagellar pocket (41). In contrast, *T. brucei* procyclics synthesize only an ER glycoform that is transported directly from the TGN to the lysosomes (168). p67 contains a number of potential targeting signals (dileucine and acidic amino acids) in its C-terminal cytoplasmic domain (167), and truncation mutants lacking this domain are transported to the cell surface of *T. brucei* procyclics (D. L. Alexander and J. D. Bangs, Mol. Parasitol. Meet., abstr. 59, 2000). The C-terminal domain is thus likely to play a role in sorting p67 in the Golgi apparatus into the lysosomal pathway.

Nonclassical Secretion

Most cell surface and secreted proteins are delivered to the plasma membrane via the secretory pathway. However, there is increasing evidence that alternative pathways exist in eukaryotic cells for secreting proteins which lack an N-terminal signal sequence but which contain alternative targeting signals. These so-called nonclassical secretory pathways include the regulated exocytosis of lysosomal compartments (i.e., interleukin 1β, basic fibroblast growth factor) (11, 12), the formation of exvaginating plasma membrane vesicles (i.e., galactin-1) (211), and the extrusion of polypeptides across internal or plasma membranes by polytopic ABC transporters (i.e., yeast **a**-factor). Recent studies on a family of hydrophilic acylated surface proteins (HASPs) from *Leishmania* spp. suggest that one or more of these pathways may also exist in trypanosomatids. The HASPs (previously called GBP and GA/CP) were initially detected in screens for genes that are selectively expressed on infective metacyclic promastigote and amastigote stages of *Leishmania* spp. The sequences of the HASP genes predict a family of hydrophilic proteins, 10 to 44 kDa in size, that lack an N-terminal signal sequence. In early studies, these proteins were found to be associated with either the entire cell body or the flagellar pocket (281), to be accessible to surface probes (i.e., biotinylation and surface antibodies), and to be hydrophobic proteins based on their tendency to partition into Triton X-114 detergent phases (326). Recent biochemical studies show that these proteins are N-terminally myristoylated and palmitoylated and that both modifications are required for surface expression (1, 74). An 18-amino-acid N-terminal peptide can direct the reporter protein GFP to the cell surface, while mutation of the single cysteine residue in this domain (the putative site of palmitoylation) resulted in the accumulation of the GFP in a compartment that was identified as the Golgi apparatus (74). A tentative model for secretion of the HASPs has been proposed in which these proteins are cotranslationally myristoylated before being transported to the cytoplasmic leaflet of the Golgi apparatus, where they are palmitoylated. The diacylated protein may then be translocated across the membrane of the Golgi apparatus or a post-Golgi compartment and secreted via the flagellar pocket. This process appears to be relatively inefficient (only 10 to 20% of

HASPB can be detected at the cell surface), and nothing is known about the translocation mechanism. Remarkably, HASPB is also transported to the surface of CHO cells, suggesting that this pathway of nonclassical secretion has been broadly conserved during eukaryotic evolution. The extent to which other trypanosomatid proteins are secreted by this pathway is unknown. *T. brucei* GPI-phospholipase C (PLC) is generally considered a cytoplasmic protein, but recent studies suggest that it may access GPI-anchored proteins that are located in the lumen of the secretory pathway or on the cell surface (119). Like HSAPB, the *T. brucei* GPI-PLC lacks an N-terminal signal sequence and is multiply acylated (14, 256). Whether this enzyme is similarly translocated across intracellular or plasma membranes remains to be examined.

MODIFICATION OF PROTEIN AND LIPIDS IN THE SECRETORY PATHWAY

N-Glycosylation

Assembly and processing of N-glycans in the ER. Most proteins in the secretory pathway of trypanosomatids are modified with N-linked glycans (252). As in other eukaryotes, these oligosaccharides are assembled on a dolichol lipid in the ER and are then transferred en bloc to newly synthesized proteins in the lumen of the ER. Work carried out by Parodi, Previato, and colleagues has established that the steps in dolichol-linked oligosaccharide biosynthesis are the same as in other eukaryotes (reviewed in reference 252). This pathway is initiated with the transfer of GlcNAc-1-PO_4 from UDP-GlcNAc to dolichol phosphate to form GlcNAc-P-P-dolichol. The gene for the *L. mexicana* GlcNAc-1-phosphotransferase has been cloned (60) and localized to the bulk ER (K. A. Mullin, S. C. Ilgoutz, and M. J. McConville, unpublished observations). This enzyme is essential for growth of *Leishmania* (60) and has been used as a selectable marker in gene expression studies with the drug tunicamycin (187). The endogenous dolichols of *T. brucei* and *Leishmania* are shorter than those found in animal cells (containing 11 or 12 rather than ~20 five-carbon isoprene units) (188), but there is no evidence that the trypanosomatid GlcNAc-1-phosphotransferase selectively utilizes shorter dolichols in vitro. Subsequent steps in dolichol-linked oligosaccharide biosynthesis involve the addition of a second GlcNAc residue followed by six to nine mannose residues. In contrast to the situation in all other eukaryotes, none of the trypanosomatid dolichol-linked oligosaccharides are capped with glucose residues, as these parasites do not synthesize the sugar donor (dolichol-phosphate-glucose) for these reactions. Thus, most pathogenic trypanosomatids transfer $Man_9GlcNAc_2$ onto newly synthesized proteins (Fig. 6). *Leishmania* spp. also lack some of the dolichol-P-Man-dependent mannosyltransferases and transfer smaller $Man_{6-7}GlcNAc_2$ oligosaccharides to protein. Not surprisingly, the trypanosomatid oligosaccharidyltransferase complex shows little preference for glucosylated $Man_9GlcNAc_2$ oligosaccharides over the truncated versions in vitro (252). This is in contrast to the situation in yeast and mammalian cells, where the oligosaccharidyltransferase preferentially transfers glucosylated $Man_9GlcNAc_2$ structures to nascent proteins.

Once dolichol-linked oligosaccharides are transferred to protein in the lumen of the ER, they are transiently modified with one or two glucose residues by a soluble UDP-Glc-dependent glucosyltransferase. These terminal glucose residues are subsequently removed by the concerted action of glucosidase I and II. This cycle of ER glucosylation and deglycosylation of N-linked glycans was first identified in trypanosomatids (facilitated by the fact that the parasites transfer nonglucosylated oligosaccharides to protein) but is now known to occur in many other eukaryotes (253, 254, 349). The glucosyltransferase is thought to recognize unfolded or misfolded proteins in the ER lumen. Glucosylated proteins subsequently interact with the lectin chaperones calnexin and calreticulin, which facilitate their folding and/or oligomerization. Trypanosomatids contain a calreticulin homologue, and the intracellular transport of *T. cruzi* lysosomal proteins is strongly impeded in the presence of glucosidase inhibitors (168, 171). Direct binding between *T. cruzi* calreticulin and a major lysosomal protease as also been demonstrated (172). On the other hand, inhibitors of N-glycosylation have little effect on the processing of *Leishmania* gp63 (110). Moreover, most of the mature N-glycans on gp63 and the secreted PPGs of *L. mexicana* retain one or more terminal glucose residues (157, 248), suggesting that the removal of these residues is not essential for glycoprotein export from the ER.

Golgi modifications of N-linked glycans. Further processing of N-linked glycans in the ER and Golgi apparatus is highly variable in different trypanosomatids (Fig. 6). Some trypanosomatid parasites that infect only insects or nonmammalian hosts contain N-linked glycans with highly unusual (i.e., ribose, rhamnose, or galactofuranose) terminal glycan modifications (215). In contrast, the N-glycans of secretory and lysosomal proteins in *Leishmania*, *T. cruzi,* and *T. brucei* procyclic stages undergo minimal modifications in the Golgi apparatus (Fig. 6). These high-mannose glycans may be trimmed by a soluble α1,2-mannosidase in the ER to generate mature glycans with the structures (+/-Glc)$Man_{4-8}GlcNAc_2$ (252). Although most N-linked glycans in these parasites are of the high-mannose type, many trypanosomatids retain the enzymatic machinery for making more complex N-glycan structures. For example, some lysosomal and surface proteins of *T. cruzi* (e.g., cruzain and Tc85) are modified with galactose-containing N-linked glycans (66, 255) (Fig. 6). Similarly, wild-type *T. brucei* procyclic stages bind galactose-binding lectins and concanavalin A-resistant mutants that cap the high-mannose glycans of the procyclins with the single N-acetyllactosamine unit, Galβ1-4GlcNAc, have been isolated (5, 151). In contrast, the major surface and lysosomal glycoproteins of *T. brucei* BF are elaborated with complex N-linked glycans, primarily biantennary glycans with branched and linear poly-NAL sequences, similar to those of higher eukaryotes (210, 376). Some of these glycans may be capped with the immunogenic Galα1-3Gal epitope, which is recognized by antibodies in human serum. These processing steps occur within several resolvable compartments in the Golgi apparatus (124). Lectin and antibody-binding studies suggest that the addition of these complex glycans is protein dependent, as some flagellar pocket receptors appear to contain distinct glycans (237). Not all the VSGs in *T. brucei* BF contain complex glycans. The mammalian cytokine tumor necrosis factor has been shown to bind to VSGs that retain a $Man_{6-9}GlcNAc_2$ N-linked glycan (193). Binding is restricted to the flagellar pocket, where these glycans appear to be more

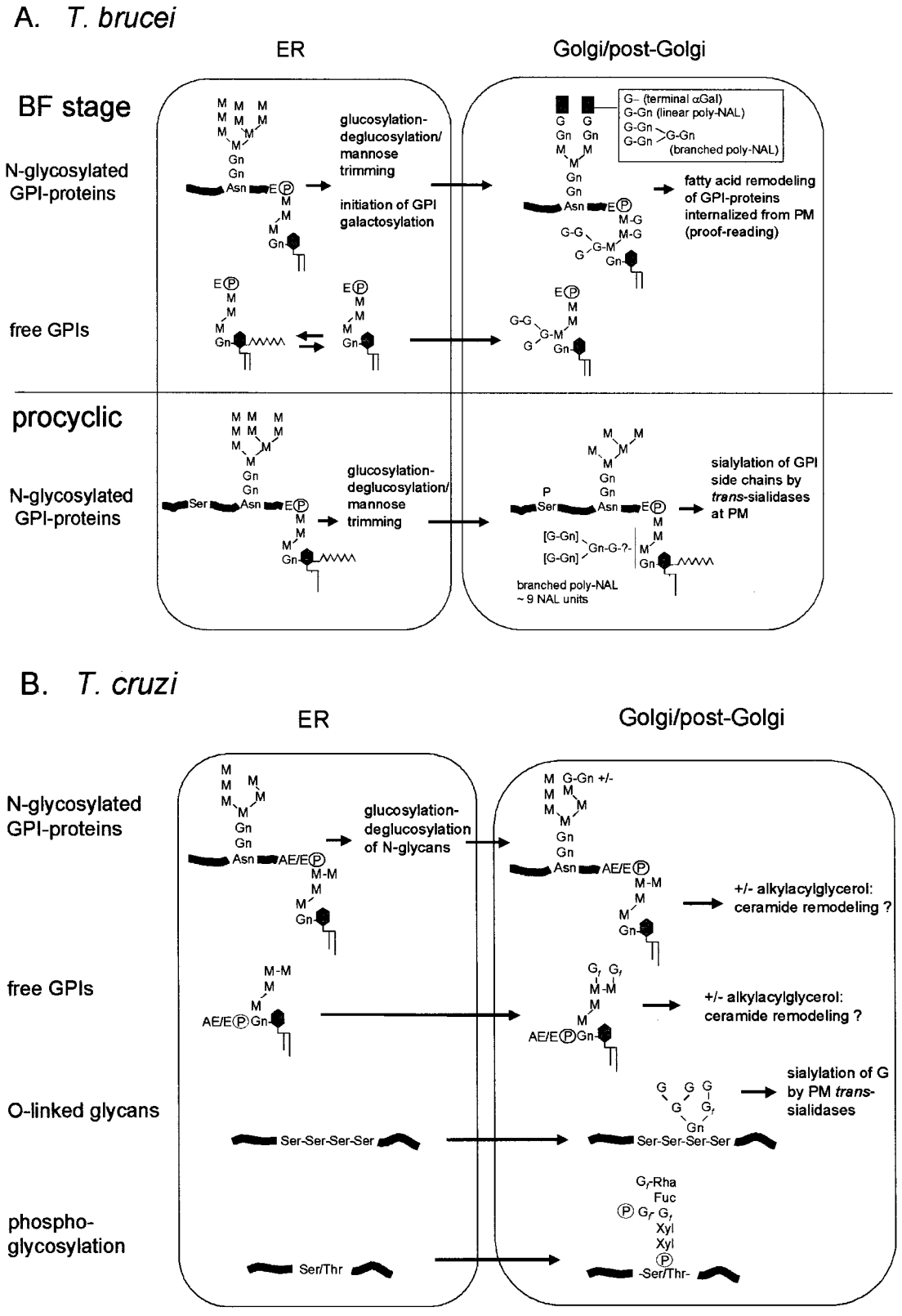

FIG. 6. Secretory pathway modifications of proteins and GPIs in trypanosomatids. Species- and stage-specific differences in the assembly and processing of N-linked glycans, protein-linked and free GPIs, and O-linked glycans and phosphoglycosylation are highlighted. Examples of only the major glycosylation reactions are shown. The presence and location of alkylacylglycerol-ceramide remodeling reactions in *T. cruzi* are speculative, as is the localization of O glycosylation and phosphoglycosylation reactions in this parasite. Ara, arabinopyranose; AEP, aminoethylphosphonate; EP, ethanolamine phosphate; G, galactose (pyranose); G_f, galactofuranose; Glc, glucose; Gn, GlcN/GlcNAc; M, Man; Rha, rhamnose; Xyl, xylose. Note that N-glycans can also be added to the mucins and PPGs of *Leishmania* and *T. cruzi*.

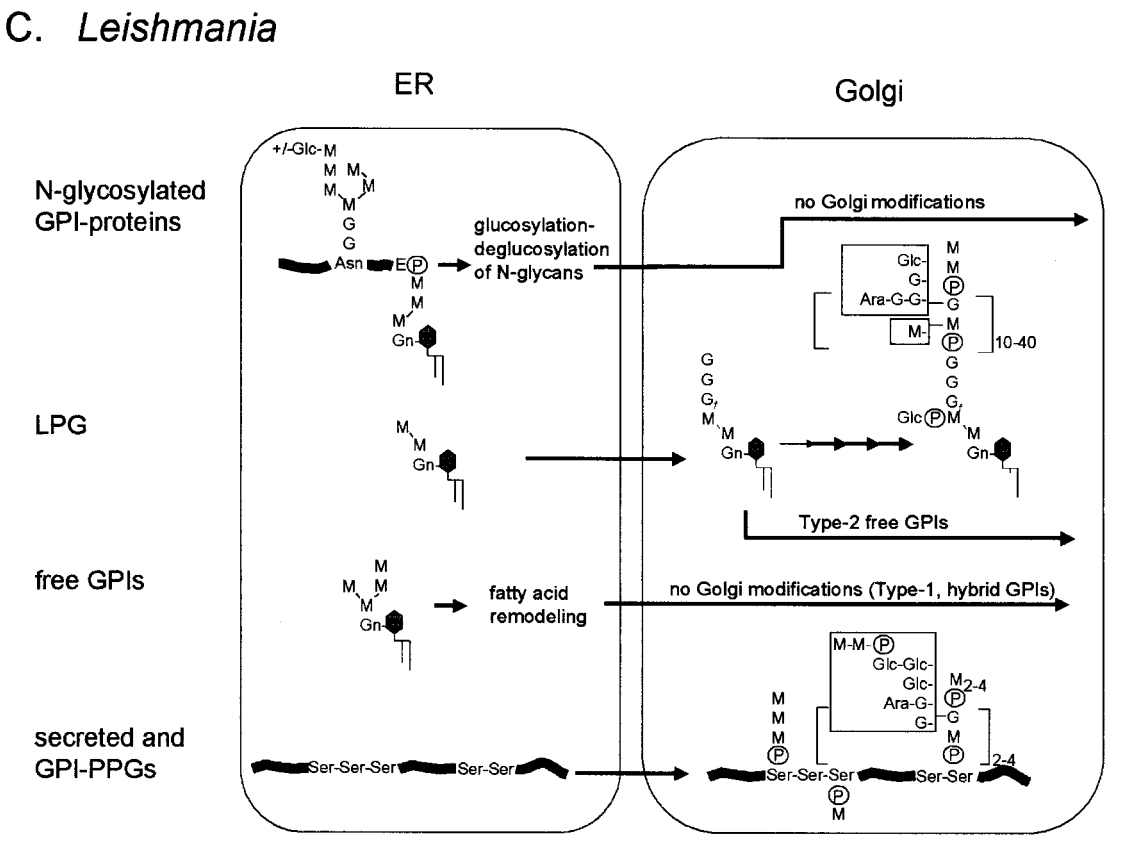

FIG. 6—*Continued.*

exposed, and the bound TNF is subsequently internalized into lysosomes (193).

Biosynthesis of GPI Protein Anchors

Assembly of intermediates in the ER. GPI anchor precursors are assembled in the ER and subsequently transferred to newly synthesized proteins by a luminally oriented GPI transamidase (101, 204). The synthesis of GPI anchor precursors is highly up-regulated in *T. brucei* BF and procyclics, and these parasites have been used as model experimental systems to delineate the essential steps in GPI biosynthesis that have been conserved in all eukaryotes (100). All of the GPIs synthesized in *T. brucei* appear to be potential anchor precursors, although the rate of synthesis of these glycolipids is at least 10-fold higher than is required to sustain normal rates of VSG synthesis (279). The precise fate of these excess intermediates (whether they are metabolized or transported out of the ER) is unclear. GPI biosynthesis is essential for the growth of *T. brucei* BF in culture, possibly because the VSG coat stabilizes the plasma membrane or because of the essential role of other GPI proteins such as the transferrin receptor (230). In contrast, GPI protein anchor biosynthesis is not essential for growth of *T. brucei* procyclics (230) or for *L. mexicana* promastigotes and amastigotes (146). The major steps in the biosynthesis of *T. brucei* BF protein anchor precursors are shown in Fig. 7, while the enzymology of these reactions has been comprehensively reviewed in reference 101. Protein anchor biosynthesis involves (i) the addition of GlcNAc to phosphatidylinositol (PI) and subsequent de-N-acetylation of the GlcNAc to form GlcN-PI, (ii) the transfer of three mannose residues from dolichol-phosphate-mannose to form Man_3GlcN-PI, (iii) the acylation of the inositol ring and transfer of ethanolamine phosphate (EtN-P) to form EtN-P-Man_3GlcN-acyl-PI, and (iv) the sequential remodeling of the *sn*-1 and *sn*-2 fatty acids (primarily $C_{18:0}$) with myristate ($C_{14:0}$). The latter remodeling reactions may be initiated on precursors containing either diacyl-PI or inositol-acylated *lyso*-PI lipid moieties (128, 222). The final products of this pathway are glycolipid A and glycolipid C (an inositol-acylated form of glycolipid A) (128). Only glycolipid A is added to VSG in vitro and in vivo (197), and the precise function of glycolipid C is still unclear. The inositol acylation of glycolipid A to form glycolipid C is a highly dynamic and reversible process. It has been proposed that glycolipid C could constitute a pool of reserve precursors (128–130), or it may be the first intermediate in a catabolic pathway to remove excess intermediates (216).

A similar pathway of GPI biosynthesis occurs in *T. brucei* procyclics, *Leishmania* promastigotes, and *T. cruzi* epimastigotes. The mature GPI anchor precursor in *T. brucei* procyclics contain an inositol-acylated *lyso*-PI lipid moiety (107). As this end product is identical to the first intermediate in the *T. brucei* BF fatty acid remodeling pathway (222), it is likely that the procyclic stage lacks one or more enzymes or cofactors involved in transferring myristol groups to the *sn*-2 position. In *Leishmania* spp., the protein anchor precursors are assembled on a very minor pool of alkylacyl-PI rather than diacyl-PI

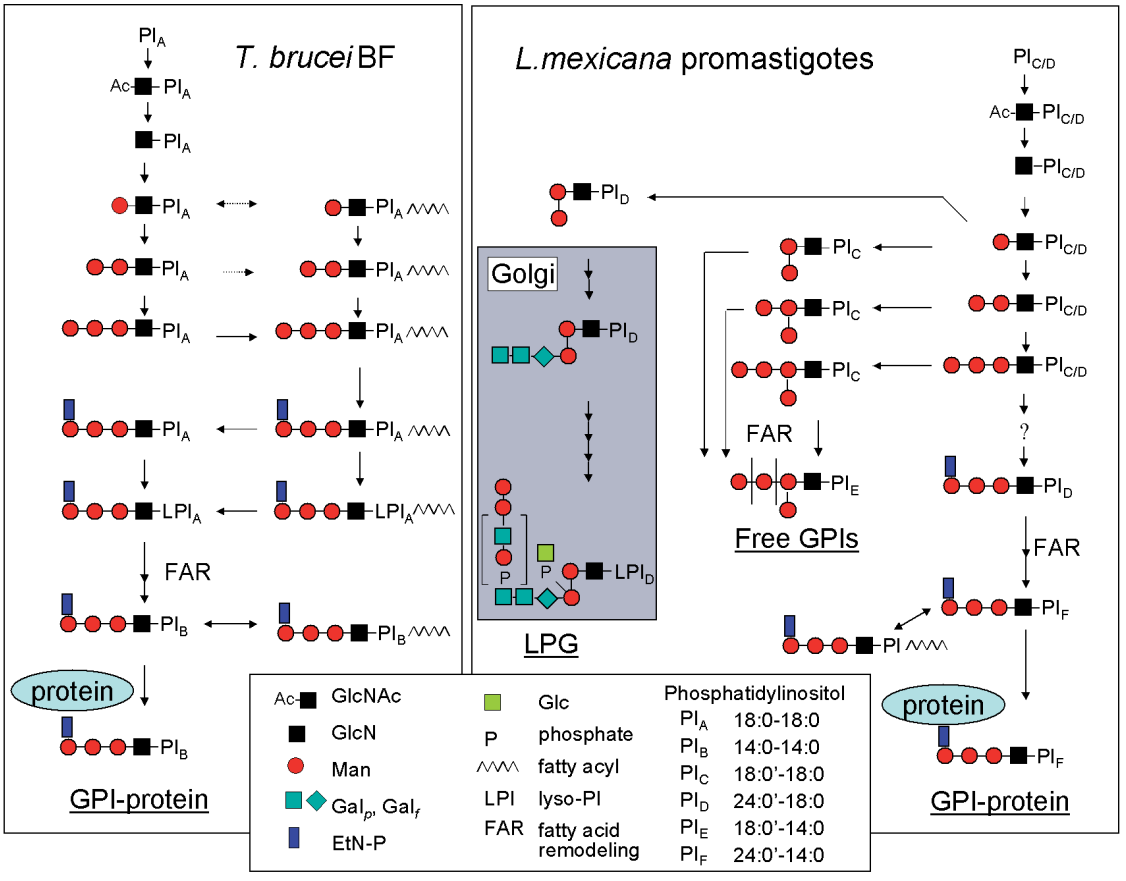

FIG. 7. GPI biosynthetic pathways in *T. brucei* BF and *Leishmania* promastigotes. The *T. brucei* pathway has been reviewed in reference 101 and incorporates data from recent studies (222). The *L. mexicana* pathway is from references 146 and 278. The protein anchor pathway is not as well characterized in *L. mexicana*, and it is unclear whether inositol acylation is required for addition of the terminal ethanolamine phosphate (indicated by "?"). The final lipid composition of these GPIs reflects the initial incorporation of distinct PI molecular species (indicated by PI_A, etc.) and/or fatty acid remodeling reactions. The fatty acid remodeling steps involve the removal of a fatty acid from the *sn*-1 or *sn*-2 position of the glycerol backbone and transfer of a new fatty acid from an acyl-CoA donor. All GPI intermediates in *T. brucei* BF contain a diacylglycerol lipid, and both fatty acids are remodeled. In contrast, GPI intermediates in most *Leishmania* spp. contain alkylacylglycerol lipids (the alkyl group is indicated by a prime) and only the *sn*-2 acyl group is remodeled. All the reactions shown here, with the exception of those involved in the assembly of the LPG anchor and phosphoglycan chains, occur in the ER. This figure was adapted from reference 99.

molecular species. Interestingly, the *sn*-2 fatty acid in the *Leishmania* GPI protein anchor precursors is also exchanged for myristate, although the mature precursors retain a very long ($C_{24:0}$ or $C_{26:0}$) alkyl chain at the *sn*-1 position and are therefore more hydrophobic than the VSG anchor (278). A proportion of the *Leishmania* protein anchor pool is also inositol acylated. The addition of this modification appears to be highly regulated, as it does not occur on the far more abundant and structurally related free GPIs in the same cell (146). The protein anchor precursors are also assembled on alkylacyl-PI in *T. cruzi* (3, 141). GPI proteins in the epimastigote stage retain an alkylacyl-PI, while GPI proteins in the infective metacyclic stages can contain either alkylacyl-PI (on IG7, gp90) or inositolphosphoceramide (3, 6, 140). The presence of these protein-specific anchor lipids could reflect the presence of different GPI precursors. Alternatively, all proteins may receive the same anchor and then be subjected to protein-specific alkylacylglycerol:ceramide remodeling reactions, analogous to those identified in yeast (34, 284) (Fig. 6B). Interestingly, differences in the lipid moiety may define the rate of turnover of surface-expressed proteins. Proteins containing a ceramide moiety are shed from the surface of *T. cruzi* as membrane blebs, while those containing alkylacylglycerol are apparently excluded from this process (123, 304). The GPI anchors of the mucins in *T. cruzi* trypomastigotes contain an alkylacyl-PI with unsaturated fatty acids ($C_{18:1}$ or $C_{18:2}$) (8). These GPIs are among the most potent stimulators of macrophage proinflammatory cytokines and may be important in inducing some of the pathology seen in Chagasic patients (8). Finally, some *T. cruzi* GPI protein anchor precursors may be modified with terminal aminoethyl-phosphonate, which forms a novel bridge between the protein and the mature GPI anchor (3, 141) (Fig. 6B).

GPI anchor precursors are rapidly attached to newly synthesized VSG in the ER lumen (23, 97). In yeast and animal cells, this process is catalyzed by a heterodimeric complex of two integral membrane proteins, GPI8p and Gaa1p, which removes the C-terminal signal sequence of the nascent protein and simultaneously attaches the newly exposed C terminus to the ethanolamine of the GPI precursor. GPI8p shares se-

quence homology with other endopeptidases and is proposed to be the catalytic component. This reaction has recently been reconstituted in vitro by using a trypanosome cell-free system (322) and an in vitro translation system (86). Unexpectedly, the GPI8p activity in trypanosome membranes can be readily extracted in high pH salt buffers, suggesting that it is a soluble protein (321). Consistent with this finding, the *L. mexicana* GPI8 gene is predicted to encode a hydrophilic protein that lacks a distinct transmembrane domain (146). The leishmanial GPI8p also lacks an obvious ER retention signal and may depend on interactions with other components of the GPI-transamidase complex (i.e., Gaa1p, PIG-S, and PIG-T) for ER retention (245).

Compartmentalization and topology of GPI biosynthesis. It is now well established that many of the enzymes involved in phospholipid and sterol biosynthesis are compartmentalized within distinct subdomains of the ER (2, 360). In *L. mexicana* promastigotes, the enzymes involved in GPI biosynthesis appear to be enriched within a light ER subcompartment (159, 226). These membranes are also enriched in enzymes involved in PI and aminophospholipid biosynthesis (226) (Mullin and McConville, unpublished). The compartmentalization of these enzymes may be required to sustain the extraordinarily high levels of GPI biosynthesis in these parasites. However, de novo-synthesized PI is incorporated into free GPIs only after a long lag time in vivo and not at all in vitro, suggesting that the entry of PI into this pathway is highly regulated (Mullin and McConville, unpublished).

Intermediates in GPI biosynthesis are thought to be assembled on both sides of the ER membrane. While the initial steps in GPI biosynthesis (up to the formation of GlcN-PI) occur on the cytoplasmic side of the ER, later steps are catalyzed by luminally oriented enzymes (192, 204, 358). However, early studies by Vidugiriene and Menon indicated that a significant fraction of the newly synthesized mannosylated intermediates in *T. brucei* BF become oriented on the cytoplasmic leaflet of the ER (359). Similarly, Mensa-Wilmot and colleagues have provided evidence that the protein anchor intermediates in *L. major* promastigotes are quantitatively hydrolyzed in vivo if the *T. brucei* GPI-PLC is ectopically expressed in these cells (213, 214). As the GPI-PLC lacks an N-terminal signal sequence and is thought to associate with the cytoplasmic leaflet of intracellular membranes (48), these data supported the notion that some protein anchor intermediates are flipped to the cytoplasmic leaflet of the ER. Surprisingly, the levels of expression of free GPIs and LPG are unchanged in *L. major* and *L. mexicana* promastigotes expressing the GPI-PLC, suggesting that intermediates in these pathways have a different topology from the protein anchor intermediates (160, 214). However, recent studies suggest that this is not the case (Ralton et al., submitted) and that the phenotype induced by the ectopic expression of GPI-PLC may be more complex than previously thought. Indeed there is evidence that this lipase may be secreted into the flagellar pocket in *T. brucei* BF (119). As the GPI-PLC is multiply acylated (14, 256), it may be secreted into the flagellar pocket via the nonclassical pathway outlined above. The selective down-regulation of GPI proteins in *Leishmania* expressing this enzyme could thus be due to hydrolysis of GPI proteins but not free GPIs and LPG within the secretory-endocytic pathway or in the flagellar pocket. Finally, the fact that endogenous and ectopically expressed GPI-PLC does not hydrolyze free GPIs in *T. brucei* BF and *Leishmania* spp., respectively, although it can hydrolyze other PI lipids (243), suggests that this enzyme does not associate with ER (and Golgi) membranes.

Golgi and post-Golgi modifications of protein-linked GPIs. The GPI anchor of different classes of VSG can be variably modified with 0 to 10 galactose residues during transit to the cell surface. While the first galactose residue may be added in the ER, subsequent αGal residues are probably added in the Golgi apparatus (22, 196) (Fig. 6A). The anchors of different VSG subclasses are modified with galactose to different extents (none in class III, zero to five residues in class I, and up to eight residues in class II), suggesting that the galactosyltransferases act as spatial probes, filling in the space between the membrane and the VSG coat (101, 380). In support of this notion, a small proportion of the free GPIs in *T. brucei* BF are modified with galactose residues to the same extent, regardless of VSG subtype (196). In *T. congolense* BF, the VSG anchor is modified with a novel Galβ1-6GlcNAc side chain that is added to the first core mannose residue (118). In addition, the lipid moieties of the VSG anchors can undergo further rounds of fatty acid remodeling in the Golgi or post-Golgi compartment, in which preexisting myristic acid is replaced with another myristic acid (54). These reactions are biochemically distinct from the ER fatty acid remodeling reactions and presumably constitute a proofreading mechanism (54).

In *T. brucei* procyclics, the GPI anchors of the procyclins are extensively modified with large (5 to 30 hexoses) heterogeneous glycan side chains (102) (Fig. 6A). These glycan side chains consist of poly-NAL structures that are linked to the GPI backbone and are capped with sialic acid by the cell surface *trans*-sialidase (269). The addition of the poly-NAL to procyclic anchors is presumed to occur in the Golgi apparatus and is not protein specific, as similar side chains are added to the anchor of ectopically expressed VSG (257, 380). In contrast, the protein-linked GPIs of *T. cruzi* and *Leishmania* do not appear to receive any further modifications in the Golgi apparatus and contain only the single mannose extension (added in the ER) or no modifications, respectively (Fig. 6B and C). The lack of modifications on these anchors is in contrast to the extensive Golgi-specific modifications that are added to the more abundant free GPIs of these parasites.

Notable differences between the *T. brucei* and mammalian and yeast GPI pathways include (i) the timing of inositol acylation and deacylation, (ii) the presence of additional ethanolamine phosphate residues on the glycan backbone of mammalian and yeast but not trypanosomatid GPIs, and (iii) the myristic acid remodeling of *T. brucei* and *Leishmania* anchors (100). Several potential inhibitors that exploit differences in the substrate specificity of the trypanosome and mammalian enzymes have been developed (317, 325, 327, 329). Inhibitors of the fatty acid remodeling reactions also have potent trypanocidal activity (81). Recently, Morita and colleagues showed that *T. brucei* BF can synthesize a major fraction of myristic acid de novo rather than scavenge fatty acids from the host bloodstream as previously thought and that these de novo-synthesized fatty acids are efficiently incorporated into GPIs (223). The fatty acid synthetase activity is associated with microsomal membranes, utilizes butyryl-coenzyme A (butyryl-CoA) instead of acetyl-CoA as a primer, and is inhibited by the

antibiotic thiolactomycin, a highly specific inhibitor of prokaryote (but not animal) type II fatty acid synthetases. If de novo-synthesized fatty acids rather than fatty acids taken up from the media are the major source of myristic acid for GPI biosynthesis, these enzymes may be interesting drug targets (223, 258).

Biosynthesis of *Leishmania* LPG

The LPGs are the major surface macromolecules of most human-pathogenic *Leishmania* species, although levels of expression are lower in members of the *Leishmania braziliensis* complex (229) and not detectable in the lizard parasite *Leishmania alderi* (273). The *Leishmania* LPGs contain a highly conserved GPI anchor with the structure Galα1-6Galα1-3Gal_fβ1-3[Glcα1-PO_4-6]Manα1-3Manα1-4GlcN-*lyso*-alkyl-PI. The lipid moiety has the same very long ($C_{24:0}$ or $C_{26:0}$) alkyl chains as the protein anchors (205). This anchor structure is subsequently modified with a long phosphoglycan chain comprising 10 to 40 Galβ1-4Manα1-PO_4 repeat units. These repeat units are further elaborated with species- and stage-specific side chains and capped with a mannose-rich oligosaccharide (39, 205) (Fig. 6C and 7). Many of the steps in the assembly of the LPG anchor and phosphoglycan chain have been studied in vivo and in vitro (58, 59, 278), and a number of genes encoding putative transferases and nucleotide transporters have been identified by complementation of LPG mutants (35; D. E. Dobson, L. D. Scholtes, E. V. Kelli, B. J. Mengeling, D. Sullivan, D. L. Sacks, S. J. Turco, and S. M. Beverley, Mol. Parasitol. Meet. XI, abstr. 101, 2000). The LPG anchors are assembled on the same minor pool of alkylacyl-PI as the protein anchor precursors (278) (Fig. 7). However, this pathway diverges from the protein anchor pathway with the addition of the second mannose residue in α1-3 rather than α1-6 linkage. The α1-3-linked mannose is probably added on the cytoplasmic face of the ER by a GDP-Man-dependent mannosyltransferase (160, 280). In contrast, all the other GPI mannosylation reactions are thought to occur in the ER lumen (204). The LPG anchor precursor is subsequently elongated with a galactofuranose residue involving the product of the LPG1 gene (149) (Fig. 7). LPG1p is a type 2 integral membrane protein that is primarily oriented in the lumen of the Golgi apparatus (131). Thus, early LPG intermediates must be flipped from the cytosolic face of the ER to the luminal face of the ER or the Golgi for this step to occur. The remaining steps in LPG anchor synthesis include the addition of two more Gal residues and a Glc-PO_4 side chain and deacylation of the lipid moiety (Fig. 7). However, the ordering and enzymology of these reactions have not been carried out. The phosphoglycan moiety of LPG is subsequently assembled by the multiple rounds of Man-1-PO_4 and Gal (donated from GDP-Man and UDP-Gal, respectively) additions to the mature anchor and growing chain (58, 59). Mutants lacking the Golgi GDP-Man transporter (the product of the LPG2 gene) are unable to synthesis these phosphoglycan chains, supporting the notion that GDP-Man is the donor for all the mannose additions and that phosphoglycan synthesis occurs in the Golgi apparatus (147, 191). At least two mannose-1-phosphate transferases are required for the assembly of the first repeat and subsequent repeat units, respectively (75, 291). The elongating transferase acts only on unmodified repeat units, demonstrating that the backbone repeat units must be formed before the side chains are added (291). Several enzymes involved in the addition of the species- and stage-specific side chains have recently been characterized by using in vitro assays (40, 233, 234), and genes have been identified by complementation of mutants (51). In *L. major*, families of galactosyl- and arabinosyltransferases are required for addition of a unique set of glycan side chains to the LPG phosphoglycan backbone. As *L. major* procyclic promastigotes differentiate into nondividing metacyclic promastigotes, the average length of the LPG chains increases (~2-fold) and the galactose side chains become extensively capped with arabinose-pyranose (Ara_p) residues (206, 301). The arabinose units are transferred from GDP-Ara_p (307), which is synthesized in the cytosol and possibly transported into the Golgi by the LPG2p GDP-Man transporter (147). How the developmentally regulated changes in the average length of the LPG phosphoglycan chains and the degree of Ara capping are regulated during promastigote growth remain intriguing questions.

Biosynthesis of Free GPIs

Free GPIs are the major cellular glycolipids in all trypanosomatids investigated to date. In *T. brucei* BF, the free GPIs are structurally and functionally indistinguishable from the protein anchor precursors and thus appear to be excess products of this pathway. However, in *Leishmania*, *T. cruzi*, and many other trypanosomatids, the free GPIs are structurally distinct from the protein anchors and are clearly metabolic end products (71, 72, 200, 201, 203, 282, 310, 377). The function of the free GPIs remains enigmatic. Recent gene knockout studies suggest that they may be essential for growth of *L. mexicana* promastigotes under some growth conditions (160) but not others (112–114). Down-regulation of free GPIs in *T. cruzi* amastigotes by ectopic expression of a GPI-PLC also inhibited growth (115). The free GPIs of *Leishmania* spp. are classified as type 1, type 2, or hybrid, depending on whether they contain the same glycan backbone structure as the protein anchors, the LPG anchors, or the core structures of both anchors, respectively (201). Most species of *Leishmania* express hybrid-type free GPIs that are assembled on a distinct pool of PI precursors from those incorporated into the protein and LPG anchor precursors (278, 309) (Fig. 7). The steps involved in the assembly of these glycolipids are the same as those involved in protein anchor biosynthesis except that each of the mannosylated intermediates in this pathway may be modified with an α1-3-linked mannose branch (201, 278). Addition of this residue on the cytoplasmic leaflet of the ER may prevent subsequent addition of the α1-6-linked mannose or further extension of this residue, leading to the accumulation of a family of free GPIs with two to four mannose residues (278, 377). The free GPIs of *L. major* and *L. alderi* promastigotes have the same glycan head group as the LPG anchor (199, 273, 276, 328). Recent gene deletion experiments suggest that the regulation of the LPG anchor and free GPI biosynthetic pathways is more complex than previously thought. For example, deletion of the *L. major* LPG1 gene encoding the putative galactofuranose transferase abolishes galactofuranose addition to the LPG anchor precursors but not the free GPIs (335). *L. major* may thus contain two galactofuranosyltransferases that differ-

entially recognize intermediates in the LPG anchor and free GPI biosynthetic pathways. The differential expression of these different isoforms could account for the selective down-regulation of LPG anchor but not free GPI biosynthesis in the amastigote stage (16, 200, 308).

Following the assembly of the glycan head group and remodeling of the *sn*-2 fatty acid (278), the free GPIs are transported to the exoplasmic leaflet of the plasma membrane. In *L. mexicana*, the free GPIs reach the plasma membrane more slowly than the GPI protein gp63 ($t_{1/2}$, 70 min compared to 40 min) but appear to be transported by the same (temperature-sensitive) vesicular pathway (280). The distinct transport kinetics of these molecules could reflect the facilitated transport of gp63 out of the ER (see above) while the free GPIs are transported by bulk flow. Upon reaching the plasma membrane, the hybrid free GPIs of *L. mexicana* are incorporated into detergent-resistant domains in the exoplasmic leaflet of the plasma membrane and may themselves be important constituents in maintaining these domains (280).

Protein O Glycosylation

Trypanosomatids do not appear to modify any of the proteins in the secretory pathway with O-linked GalNAc or mannose residues, the major types of O-linked glycans in vertebrate cells and fungi, respectively. In contrast, several mucin-like glycoproteins on the surfaces of *T. cruzi* epimastigotes, metacyclic trypomastigotes, and amastigotes are extensively modified with O-linked αGlcNAc residues that are added to Thr-rich sequences in these proteins (3, 9, 117, 272, 274, 304) (Fig. 6). This type of modification is unique among the trypanosomatids and is different from addition of O-βGlcNAc to intracellular proteins (134). Most of the O-GlcNAc on the *T. cruzi* mucins is extended with one to five galactose residues that form short linear and branched glycan chains (3, 9, 274, 348) (Fig. 6B). Terminal β-Gal residues in these glycans are the major acceptors for the cell surface *trans*-sialidases that transfer sialic acid (one or two residues per chain) from host glycoconjugates (304). The structures of these O-linked glycans can exhibit some strain-specific polymorphisms. In particular, in the *T. cruzi* Y strain, all the O-linked galactose residues are in the pyranose configuration, while in the G strain some of these residues are in the furanose configuration (9). Moreover, some of the O-glycans on the high-molecular-weight mucins of the mammalian stages are modified with O-glycans that terminate in Galα1-3Galβ1-4GlcNAc (9). These αGal epitopes are highly immunogenic to humans and are recognized by lytic antibodies in the sera of patients with acute and chronic Chagas' disease (9).

The GlcNAc-transferase that initiates O glycosylation in *T. cruzi* is associated with purified Golgi membranes, utilizes UDP-GlcNAc as the sugar donor, and adds GlcNAc residues to Thr residues in a synthetic dodecapeptide containing the consensus sequence of the *T. cruzi* MUC gene (220, 275). The O glycosylation of the *T. cruzi* mucins is clearly important in forming a protective surface glycocalyx (77). Addition of O-linked glycans may confer structural rigidity to the mucin polypeptide, ensuring that the hypervariable N-terminal domain is presented on the surface of the mucin coat, which may direct the immune response away from conserved domains within the coat (268). As mentioned above, the sialylation of the mucin O-glycans is also important for parasite invasion and protecting the extracellular forms from lysis by complement and the parasite's own hemolysin (261).

Protein Phosphoglycosylation

A number of cell surface and secreted trypanosomatid proteins are elaborated with a distinct class of phosphooligosaccharides. Phosphoglycosylation is initiated by transfer of a sugar-phosphate to Ser or Thr residues in the context of serine/threonine-rich sequences (157, 369). Protein phosphoglycosylation probably occurs in all trypanosomatids and is the most abundant form of protein glycosylation in *Leishmania* spp. (153). Protein phosphoglycosylation has not been identified in any metazoan organisms to date, although there is increasing evidence that this type of modification (often involving different sugar-phosphate linkages) may be common in other unicellular eukaryotes, such as *Dictyostelium discoideum* and the protozoan parasite *Entamoeba histolytica* (137, 219).

This unusual type of glycan modification was initially detected on the abundant secreted acid phosphatase (SAP) of *L. donovani* and *L. mexicana*. Early immunochemical and biochemical analyses indicated that these heavily glycosylated proteins were modified with similar phosphoglycans to those found on the LPG of the same parasites (28, 162). Subsequent studies showed that serine residues in the protein backbone of the SAP were modified with Man-1-PO_4, which was extended with short linear chains of α1-2-linked mannose or longer chains of repeating Galβ1-4Manα1-PO_4-phosphodisaccharides (157, 185, 224). The SAPs belong to a heterogeneous family of PPGs, which include the promastigote filamentous PPG, the GPI-anchored cell surface PPG, and the nonfilamentous amastigote PPG (153, 155, 156). The distinct polypeptide backbones of these PPGs contain Ser/Thr-rich or Ser/Pro-rich domains that are extensively modified with phosphoglycan chains (156, 369). The length and composition of the phosphoglycan chains vary enormously in different PPGs (157, 158). While the 100-kDa form of *L. mexicana* SAP is primarily modified with short (one to six residues long) mannose oligosaccharides, the secreted PPGs of the amastigote stage are elaborated with exceedingly complex branched phosphoglycans (154). The assembly of phosphoglycan chains may thus depend on the properties of the protein carrier, the rate at which these proteins are transported through the secretory pathway, as well as the complement of glycosyltransferases expressed in each species (368). While some of the SAPs of Old World *Leishmania* spp. are monomeric, the SAPs of New World species (*L. mexicana*, *Leishmania amazonensis*, and *L. braziliensis*) assemble into highly unusual polymeric chains, containing up to 200 subunits (~2 μm long) (342). These chains are not present in the Golgi but appear to assemble in the flagellar pocket (341). As the filamentous PPGs (encoded by a 23-kb gene and containing 96% carbohydrate) are as long as ~2 μm (153), it is highly likely that the majority of these molecules are retained with the lumen of the Golgi cisternae and transported across the Golgi stack by cisternal maturation. Similarly, transport from the TGN to the plasma membrane may be in large cisternal vacuoles (226, 365).

In *T. cruzi*, the stage-specific glycoprotein gp72 is heavily

phosphoglycosylated (Fig. 6B). This glycoprotein is required for attachment of the flagellum to the cell body of epimastigotes and metacyclic stages (64). Approximately 50% of the mass of the protein comprises complex glycans (containing xylose, rhamnose, fucose, and galactofuranose residues) that are recognized by the antibody WIC 29.26 (138). These glycans are released by mild acid hydrolysis and are probably linked to phosphothreonine and some phosphoserine residues in the polypeptide backbone (138). Recent studies by Haynes et al. and Ferguson and colleagues suggest that these glycans have the partial structure $Gal_f\beta1$-4Rha$\alpha1$-2Fuc$\alpha1$-2/3[PO_4-5/6$Gal_f\beta1$-2/3]$Gal_f\beta1$-4Xyl$\beta1$-4Xyl-PO_4-Thr/Ser (138; M. Ferguson, personal communication). Interestingly, *T. brucei* BF and procyclics express a homologue of GP72 (Fla1) that also associates with the flagellar attachment zone and is modified with both N-glycans and (in the case of BF) acid-labile oligosaccharides (241). To date, there is no information on either the function or the site of synthesis of these novel oligosaccharides.

CONCLUSIONS

Considerable progress has been made in the last few years in defining the ultrastructure of the secretory and endocytic pathways of the trypanosomatids and the nature of protein and lipid transport routes within these pathways. These studies have been greatly facilitated by the identification of organelle-specific markers and cargo proteins, the characterization of the biosynthetic functions of the ER and Golgi apparatus, and, more recently, the identification of genes (via genome searches) that regulate organelle biogenesis and intraorganelle transport. Collectively, these studies demonstrate that the basic features of the trypanosomatid secretory-endocytic pathways are very similar to those found in other eukaryotes, despite the fact that these organisms represent one of the most divergent eukaryotic lineages. In this respect, the trypanosomatids may prove to be interesting experimental systems for investigating aspects of protein and lipid trafficking and glycan and lipid biosynthesis that are highly up-regulated and may have been conserved throughout eukaryotic evolution, such as the transport of GPI proteins. These studies are facilitated by the fact that all the major organelles in the secretory and endocytic pathways can be readily visualized at the level of light and electron microscopy. On the other hand, some aspects of the trypanosomatid secretory and endocytic pathways are clearly unusual. It remains to be determined how the highly polarized secretory and endocytic pathways of these parasites are maintained in the presence of a minimalist system of cytoplasmic microtubules and the nature of sorting signals that direct proteins to the unusual acidocalcisomes. The recent discovery of the exotic lysosome-MVT of *Leishmania* promastigotes and the related late-endosome-MVT of *T. cruzi* epimastigotes highlights the importance of visualizing these structures in live cells and the need to undertake comparative studies of all developmental stages. With the sequencing of the genomes of *T. brucei*, *L. major*, and *T. cruzi* well advanced, the stage is now set to increase our understanding of the molecular basis underlying protein and lipid transport and organelle biogenesis in these important parasites. This information may in turn lead to the development of new antiparasite strategies.

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