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MicroReview

Are rhoptries in Apicomplexan parasites secretory granules or secretory lysosomal granules?

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Summary

The club-shaped rhoptries in Apicomplexan parasites are one of the most unusual secretory organelles among the eukaryotes, containing unusual lipid and protein cargo that is specialized for intracellular parasitism. Rhoptries have traditionally been viewed strictly as regulated secretory granules. We discuss in this article recent data on the cargo, function and biogenesis of rhoptries in two parasitic model systems, *Toxoplasma* and *Plasmodium*. Current findings suggest that rhoptries receive products from both biosynthetic and endocytic pathways and, therefore, they are most analogous to secretory lysosomal granules found in mammalian cells.

Introduction

Regulated secretion is essential for proper functions of specialized secretory cells in multicellular organisms. For example, exocrine, endocrine and neuroendrocine cells store and secrete bioactive molecules such as hormones and peptides in secretory granules that are synthesized along the secretory pathway (reviewed by Glombik and Gerdes, 2000; Burgoyne and Morgan, 2003). In contrast, many cells use specialized lysosomes as secretory organelles; hence, both endocytic and secretory routes contribute to the biosynthesis of these lysosomal granules (reviewed by Denzer *et al*., 2000; Blott and Griffith, 2002; Luzio *et al*., 2003). Unlike conventional lysosomes, which are traditionally viewed as dead-end organelles that degrade intracellular proteins and endocytosed macromolecules, the primary function of secretory lysosomes is to process and store effector macromolecules to be exocytosed for extracellular functions.

Protozoans use regulated secretion for a wide range of functions ranging from capture of prey, defence against predators and active parasitism of multicellular organisms (reviewed by Hausmann, 1978). As significant pathogens of humans and livestock, intracellular parasites of the Apicomplexan phylum deploy three different types of secretory organelles (micronemes, rhoptries and dense granules) for active invasion, formation of the parasitophorous vacuole and replication in the host cell (reviewed by Ngô *et al*., 2000; Joiner and Roos, 2002). Whereas dense granules appear to be constitutively secreted, microneme and rhoptry secretion appears to require physiological triggers. Ca^{2+}-mediated microneme secretion mediates saltatory gliding motility and active penetration of host cells, whereas rhoptry secretion coincides with formation of the delimiting membrane (parasitophorous vacuole membrane or PVM) that separates the parasite from the host cytoplasm and thus prevents the parasites from entering the host endocytic pathways, where they may be exposed to degradation.

In this review, we focus on the rhoptry compartment, with two specific aims. The first goal is to provide a synthesis of the structure, function and biogenesis of rhoptries. The second goal is to evaluate the multiple lines of evidence leading to the logical hypothesis that rhoptries have characteristics of secretory lysosomes, i.e. that secretory and endocytic pathways converge for the biogenesis of secretory rhoptries in Apicomplexan parasites.

Secretory granules versus secretory lysosomes

Since the pioneering study of regulated protein secretion in pancreatic exocrine cells (Palade, 1975), secretory granules have been studied extensively, but only recently have secretory lysosomes been delineated from this group. The widespread distribution and functions of secretory granules and lysosomes are best documented

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in mammalian cells (Burgoyne and Morgan, 2003). Secretory granules and secretory lysosomes are fundamentally distinct in their structure and function (Fig. 1), primarily because of the different sorting mechanisms that direct specific molecules to each secretory organelle.

Morphology

As visualized by electron microscopy, secretory granules typically have a dense core that is a highly condensed matrix of secretory materials in an osmotically inert form. Secretory lysosomes have more diverse types of structures; some have a multilamellar or multivesicular appearance (for example, MHC class II compartments in dendritic cells), some exhibit dense cores (for example, platelet-dense granules), and yet others have a unique and distinctive morphology (for example, melanosomes in neural crest in vertebrates).

Cell distribution

Secretory lysosomes are most well documented in professional immune cells that are specialized for phagocytosis and processing and presenting antigens (Blott and Griffith, 2002). They are, however, more widely used in other eukaryotic and mammalian cell lineages. Melanocytes secrete melanin from lysosome-like melanosomes to keratinocytes to produce hair, eye and skin pigmentation. Secretion of active acid hydrolases from sperm cells suggests that the acrosome may be a lysosomal-like granule. Secretion from a lysosomal-like organelle has also been documented in *Dictyostelium*. In a number of protozoan parasites, lysosomal proteases are

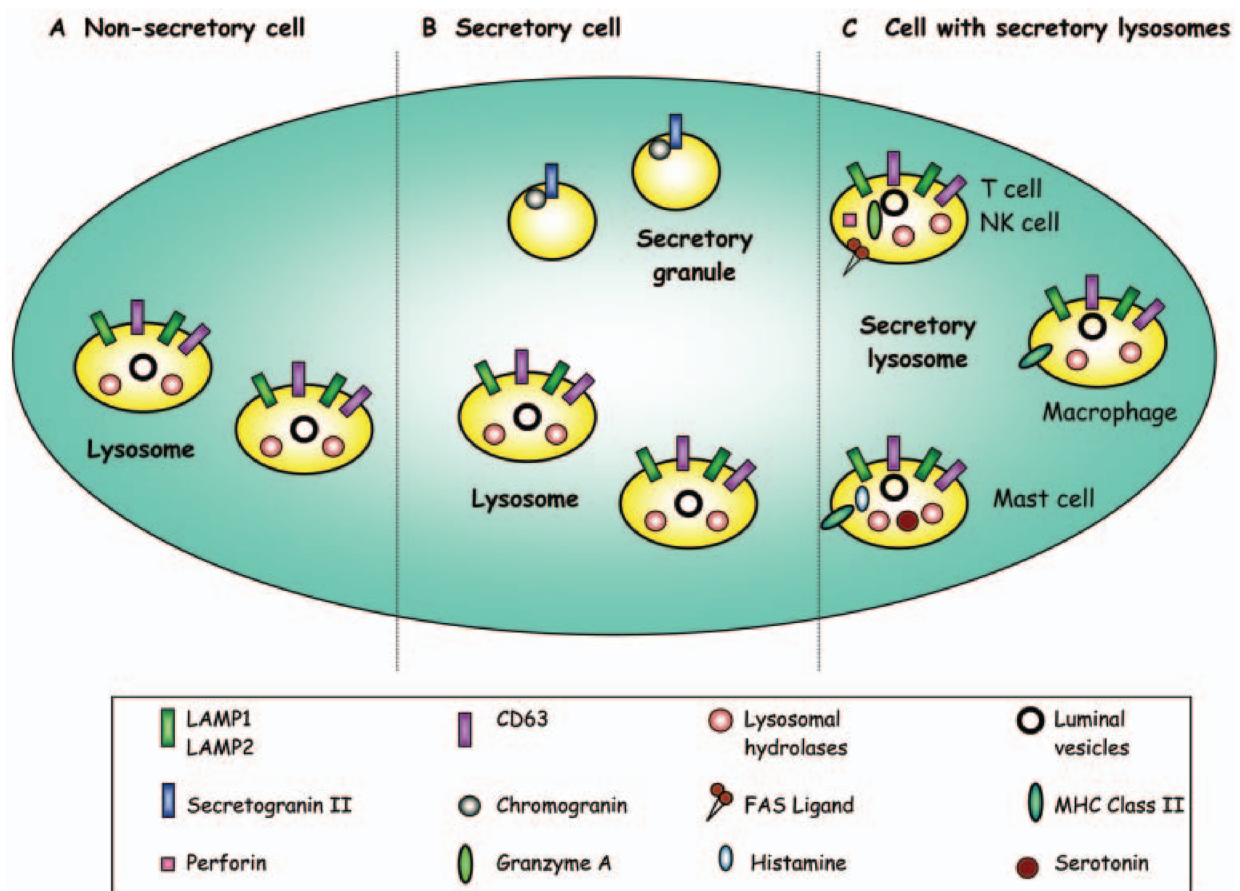

Fig. 1. Contents in cells with conventional lysosomes, secretory granules and secretory lysosomes.
A. All cell types contain a lysosomal compartment, which contains lysosomal hydrolases, transmembrane proteins and internal vesicles. Transmembrane proteins often contain tyrosine and dileucine sorting signals in the cytoplasmic tail. Internal vesicles are invaginations of the limiting membrane carrying selected proteins and lipids.
B. Secretory cells also contain set of secretory proteins specific for each cell type, such as secretogranin II and chromogranins.
C. Cells that have secretory lysosomes contain specific cell type secretory proteins in the modified lysosomal compartment, such as histamine and serotonin in mast cells, MHCII (major histocompatibility complex, class II) in macrophages and Fas ligand, soluble granzyme A and perforin in T and NK cells (modified from Blott and Griffiths, 2002).

secreted to mediate host cell invasion, nutrient acquisition and disruption of the host immune system (Rosenthal, 1999).

Biogenesis

Secretory granules are products of the biosynthetic pathway alone, in which selected proteins are sorted into the regulated secretory pathway at two sorting stations – the *trans*-Golgi network (TGN) and the immature secretory granules (ISG) (Glombik and Gerdes, 2000). Based on their aggregative and membrane-binding properties, the soluble cargo of secretory granules is sorted from constitutively secreted proteins by forming luminal aggregates at low pH and high calcium. These aggregates may bind to membranes and produce budding from the TGN without an identifiable cytoplasmic coat, for delivery into ISG. (It remains controversial whether sorting of soluble cargo is dependent on the sorting receptor, carboxypeptidase E.) Constitutively secreted proteins and other cargo are sorted away from the mature granules at ISG by incorporation into clathrin-coated vesicles (CCV) containing the AP-1 adaptor complex. Maturation of secretory granules involves the proteolytic processing of proteins and peptides by non-lysosomal enzymes in an acidified luminal environment, which is generated by a vacuolar H^+-ATPase (v-ATPase).

In contrast, the secretory lysosome is a product of both endocytic and biosynthetic pathways, serving a dual function of storing both endocytosed and newly synthesized secretory proteins. Lysosomal cargo is transported within CCV, by budding directly from the TGN to endosomes. The sorting event is mediated by protein cytoplasmic tails containing tyrosine- and dileucine-based motifs (reviewed by Boehm and Bonifacino, 2001). These motifs bind directly to cytoplasmic adaptors (AP1-4, GGA, stonins) that sort selected cargo to different stations along the endocytic pathway: endosomes, late endosome or multivesicular bodies (MVB) and then to lysosomes. Of importance, the MVB is a late endosomal compartment that contains enclosed vesicles that have budded internally from the organelle-limiting membrane. Hence, upon fusion with the plasma membrane, small vesicles termed exosomes are secreted into the extracellular environment (Denzer *et al*., 2000).

Protein processing machinery

Another difference between secretory granules and lysosomes is the proteolytic processing machinery. Proteolytic modification of chromogranins and secretogranins in secretory granules is catalysed by subtilisin/kexin-like proprotein convertases PC1 and PC2 (Laslop *et al*., 1998), whereas lysosomes modify specific proteins and degrade bulk materials by a wider range of proteases, including cysteine (cathepsin B, L), aspartic (cathepsin D) and serine proteases (Storrie, 1988).

Synopsis

Many characteristics distinguish mature secretory lysosomes from secretory granules. First, secretory lysosomes contain lysosomal hydrolases that are secreted as extracellular effectors. Secondly, formation of mature secretory lysosomes is dependent on adaptor complexes and sorting of transmembrane cargo containing cytoplasmic-tail tyrosine- and dileucine-targeting signals. Thirdly, the biogenesis of secretory lysosomes is linked to the endocytic pathway, and to distinctive endocytic compartments including the MVB, which contains internal vesicles that can be secreted as exosomes. These are not features of secretory granules. These distinctions form the basis for many of the arguments that follow.

Rhoptries are unusual secretory organelles in their structure

Rhoptries are strikingly unusual in their structure (reviewed by Sam-Yellowe, 1996). Unlike most secretory granules and lysosomal granules in mammalian cells, which are formed into a semi-spheroidal shape, the mature rhoptries are club-shaped with a bulbous base and an extended duct that tapers to a distal tip (Fig. 2A). Although Apicomplexan parasites are relatively small cells, *Toxoplasma* contains 8–12 elongated club-shaped rhoptries, while the pair of rhoptries in *Plasmodium* are more pear-shaped. Occupying 10–30% of total cell volume, rhoptries are second only to the nucleus in terms of cellular volume, emphasizing their essential functions in these small unicellular organisms.

Reconstruction of the development of rhoptries in the budding merozoite of *Plasmodium falciparum* (Bannister *et al*., 2000) indicates that rhoptries first form as a spheroidal structure from fusion of coated vesicles from the Golgi. Structures termed immature rhoptries (or prerhoptries) separate into two distinct zones, including a homogeneous granular basal region and a more heterogeneous apical region with a honeycomb-like appearance, from which an apical duct is elongated. Additional vesicles are fused to the rhoptry duct as both subcompartments progressively become more homogeneously electron dense. In the mature rhoptries, subsets of rhoptry proteins are concentrated either in the base (RAP1-2) or in the apical duct (Rhop 1–3, p225) (Preiser *et al*., 2000).

Although the *Toxoplasma* rhoptry is more subcompartmentalized in appearance, in that the bulbous base is consistently mottled and the duct is electron dense, a distinctive polarization of protein distribution is not as well

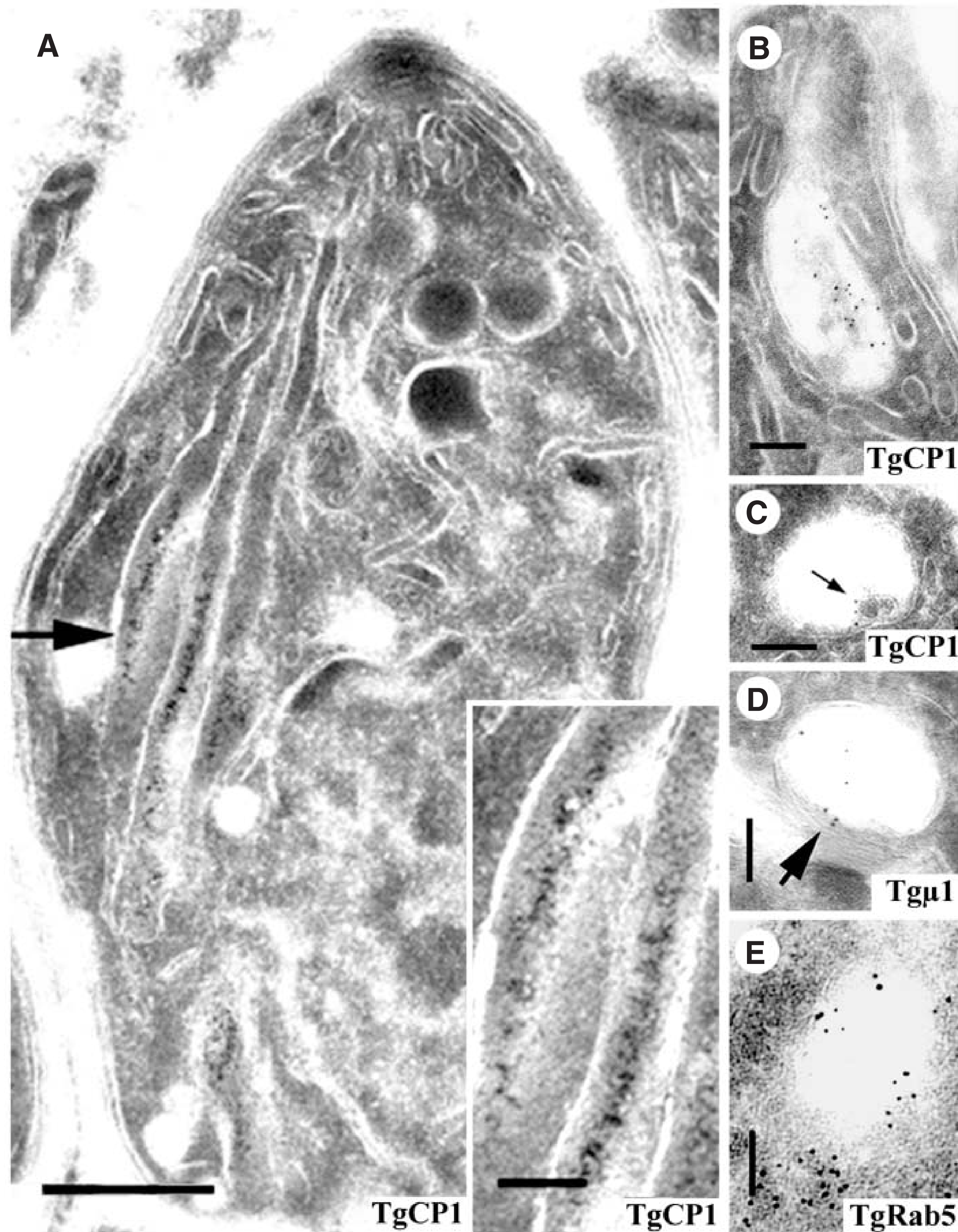

Fig. 2. Protein localization to rhoptries and multivesicular body-like endosomes by cryoimmunoelectron microscopy. A *T. gondii* cysteine protease (TgCP1), identified as a lysosomal cathepsin B, is localized to the core region of the base of mature rhoptries (A and inset) and is found as residual cargo associated with vesicles in the core region of a discharged rhoptry (B). Multivesicular bodies (MVB) are lucent vacuoles of 400–900 nm that typically contain internal vesicles. MVBs are labelled with toxopain-1 (C) and endosomal markers Tgμ1 (D) and TgRab5 (E). Electron micrographs are modified from Que *et al*. (2002) (A and C), Ngô *et al*. (2003) (D) and Robibaro *et al*. (2002) (E). Scale bars: A = 500 nm, A (inset) = 250 nm, B–E = 200 nm.

defined as in *Plasmodium*. Earlier ultrastructural localization indicates that native ROP1 is localized to the rhoptry base (Saffer *et al*., 1992). When expression of the soluble rhoptry antigen ROP1 is abolished (Soldati *et al*., 1995), the rhoptry base is no longer mottled, suggesting that there is a domain-specific function of ROP1 in the two subcompartments. It is also unclear in *Toxoplasma* whether all 8–12 rhoptries have formed from a centralized immature rhoptry, or whether each mature rhoptry originates from a separate immature rhoptry. Serial reconstruction of parasites undergoing endodyogeny supports the latter model as each forming daughter contains at least six visible immature rhoptries (Pelletier *et al*., 2002). Whether there is a heterogeneity of rhoptries in *Toxoplasma* with differential cargo and functions has not been addressed. The discharge of only a subset of the 8–12 rhoptries during host cell invasion suggests, however, at least a differential level of exocytotic competency.

The remarkable structure and morphogenesis of rhoptries are hence unlike any known secretory lysosome or granule of higher mammalian systems. The mechanism of how elongated spindle secretory organelles are formed and maintained is probably more universal in protozoans (Hausmann, 1978), such as trichocysts in ciliates (Plattner and Kissmehl, 2003).

Rhoptry functions are explicit to intracellular parasitism

Few other secretory organelles have been designed with as wide a range of functions as the rhoptries in the Apicomplexan parasites. Secreted macromolecules from the parasite rhoptries are involved in the selection and adhesion to host cells, in forming an intracellular environment for parasite survival and in 'hijacking' host organelles (see discussion below for references).

In *Plasmodium*, rhoptries appear to be secreted simultaneously with micronemes, as micronemes are docked and fused to the rhoptry ducts; hence, compound exocytosis of both secretory organelles contributes to all stages of parasitic invasion. In *Toxoplasma*, micronemes are triggered first to mediate host cell attachment and penetration, whereas rhoptry contents are secreted secondarily for contributing to PVM biogenesis and host parasite interactions, although it is still unclear whether micronemes undergo heterotypic fusion with the rhoptry duct during exocytosis in *Toxoplasma*.

Although rhoptry morphogenesis appears to be principally conserved between *Toxoplasma* and *Plasmodium*, each parasite loads a different set of protein cargoes into their secretory rhoptries (summarized by Sam-Yellowe, 1996). None of the malaria rhoptry proteins (summarized by Preiser *et al*., 2000) have homologues in *Toxoplasma*, and vice versa.

A striking example in the divergence of cargoes between the two related parasites is the subtilases, in which two isozymes (SUB1, SUB2) are found in *Plasmodium* dense granules (Blackman *et al*., 1998; Hackett *et al*., 1999). In *Toxoplasma*, SUB1 and SUB2 are sorted and secreted from the micronemes and rhoptries respectively (Miller *et al*., 2001; 2003).

This differential sorting of secreted effectors may reflect the differences between *Toxoplasma* and *Plasmodium* in host cell range. Most of the malarial rhoptry proteins are implicated in erythrocyte recognition and invasion. These include the low-molecular-weight complex of RAP 1–3, the high-molecular-weight complex of Rhop 1–3, rhoptry multigene families of Pf60 and Py235 (Preiser *et al*., 2000) and RAMA (Topolska *et al*., 2003). For example, the secreted oligomeric complex of Rhop-H and SERA (120 kDa serine-rich protein) binds to erythrocyte membranes, vesicles and liposomes. Vesicles containing phosphatidylethanolamine can inhibit the association of rhoptry protein complexes with mouse erythrocytes (Sam-Yellowe, 1996). Monoclonal antibodies to some malarial rhoptry proteins block merozoite invasion of red blood cells *in vitro* and inhibit parasite replication *in vivo*.

In *Toxoplasma*, nine major rhoptries proteins (ROP1–9) have been identified. The primary sequences of ROP1, ROP2, ROP3, ROP4, ROP8 and ROP9 indicate that only ROP2, ROP3, ROP4 and ROP8 are highly similar and contain predicted transmembrane motifs. Two rhoptry hydrolases (cathepsin B, subtilase) have been identified recently and are discussed in a later section. Soluble ROP1 is found in secreted exosomes (Hakansson *et al*., 2001) and is a component of the parasite protein fraction that enhances invasion *in vitro* (Saffer *et al*., 1992). As ROP1 knock-out parasites show no defects in invasion kinetics, or virulence in mice, ROP1 function remains elusive. Although ROP9 carries an integrin-like RGD motif (Reichman *et al*., 2002), its potential role in mediating host cell interaction is undetermined.

Peculiar to *Toxoplasma* invasion of nucleated eukaryotic cells is the recruitment and adherence of host mitochondria and endoplasmic reticulum (ER) to adhere to the parasite vacuole. Transmembrane ROP2 is secreted from the rhoptries and inserted into the PVM with its N-terminus facing the host cytosol (Beckers *et al*., 1994). A mitochondrial targeting signal in the N-terminus of ROP2 has been proposed to mediate the tight association of host mitochondria and ER (Sinai and Joiner, 2001). Depletion of endogenous ROP2 by antisense RNA expression confers a significant (90%) decrease in host mitochondria association to the PVM (Nakaar *et al*., 2002).

A common feature between *Toxoplasma* and *Plasmodium* invasion is the formation of the PVM to enclose the parasite in the host cell. Secretion of proteins, lipids and multivesicular structures contribute to the formation of the

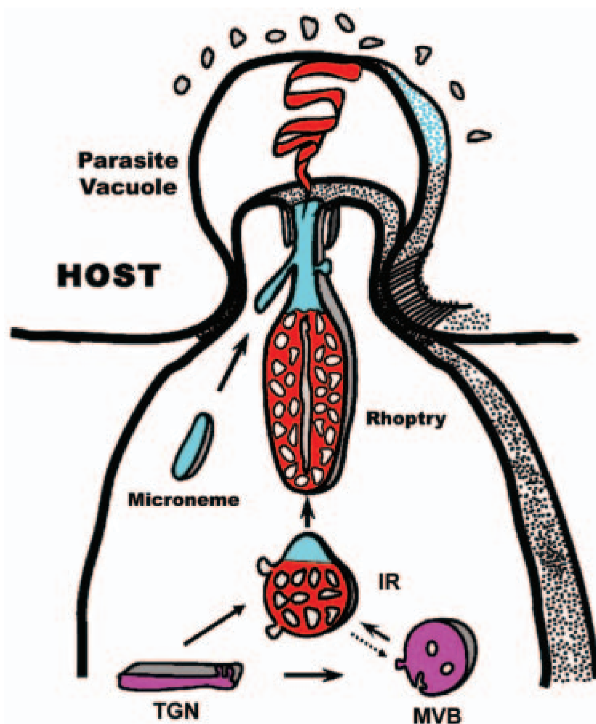

Fig. 3. Biogenesis and exocytosis of rhoptry contents. This model proposes that rhoptries are formed by contributions from both secretory and endocytic pathways. Secretory cargo from the TGN is transported to the mature rhoptry via the immature rhoptry. Some cargo is delivered directly from the TGN to the immature rhoptry (possibly subtilase), while other cargo transits the endosomal pathway through an MVB en route to the immature rhoptry. The MVB intersects the rhoptry biogenesis pathway by both delivering cargo to and probably receiving cargo from the immature rhoptry. Selected cargo is then subcompartmentalized to form the tapering apical duct (blue), a region that is potentially fusogenic with secreting micronemes, most notably in *Plasmodium*. When activated by an unknown trigger, rhoptries secrete internal vesicles (exosomes), which then fuse with the parasitophorous vacuole membrane (Hakansson *et al.*, 2001).

nascent PVM, which is a hybrid between parasite and host materials (Fig. 3). Sibley and coworkers (Hakansson *et al.*, 2001) demonstrated that exosomes and membranous sheaths secreted from *Toxoplasma* rhoptries into the host cytoplasm will subsequently undergo heterotypic fusion with the nascent parasitophorous vacuole, contributing parasite lipids and proteins to the PVM (Fig. 3).

Rhoptry proteins are processed in an acidified lumen

All rhoptry proteins identified to date are synthesized as larger precursors (prepro-protein) with a typical signal sequence that is cleaved presumably after entry into the endoplasmic reticulum. A second processing step entails the proteolytic cleavage of an N-terminal pro domain to generate a mature polypeptide. In *Toxoplasma*, matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry has identified E83/A84 as the cleavage site in soluble pro-ROP1 (Bradley and Boothroyd, 1999). The rhoptry subtilase (TgSUB2) has the same cleavage site as ROP1, as demonstrated by a E686A mutation resulting in a reduction in TgSUB2 processing (Miller *et al.*, 2003). A similar EA dipeptide is detected in the N-terminal regions of transmembrane ROP2 and ROP8, although the mature N-terminus of the protein has not been determined. Disruption of the secretory pathway by brefeldin A treatment, or temperature block, inhibits the normal processing of rhoptry proteins that occurs within 20 min of protein synthesis (Howard and Schmidt, 1995; Soldati, 1998). Hence, pro-domain trimming occurs late in the rhoptry pathway, presumably at the immature/maturing rhoptry.

Typically, proper protein processing and packaging in secretory granules, or hydrolysis of macromolecules in lysosomes, requires an acidified luminal environment that is generated by proton pumping by a vacuolar H^+-ATPase (v-ATPase). Studies using the electron microscopic probe DAMP (Shaw *et al.*, 1998) indicate that the only acidic organelles in *Toxoplasma* are immature (pH 3.5–5.5) and mature (pH 5.0–7.0) rhoptries or multivesicular bodies (pH 5.3–6.5). No DAMP labelling is observed in the rhoptry duct or at the base of 25% of mature rhoptries. Although the electron-dense appearance in the lumen of these two subdomains suggests that the cargoes are more condensed, non-partitioning of DAMP may reflect the novel rhoptry content, which is presumed to be a tightly packaged gel of proteins and lipids. Cytochemical studies also suggest that rhoptries contain an acidic phosphatase and Ca^{2+}-, Mg^{2+}-ATPase activities, as well as a high concentration of calcium (Bouchot *et al.*, 2001).

Rhoptries contain cysteine and serine proteases

Specific inhibitors of cysteine (cathepsin inhibitor III) and serine (TPCK and subtilisin inhibitor III) proteases cause swelling of the ER and nuclear envelope, collapse of the Golgi and specific disruption of rhoptry biogenesis in *Toxoplasma* (Shaw *et al.*, 2002). Subtilisin inhibitor III also generates multivesicular bodies (see below) that accumulate luminal vesicles.

The subtilisin-type serine protease (TgSUB2) is localized to the *Toxoplasma* rhoptries (Miller *et al.*, 2003). As TgSUB2 undergoes autocatalytic cleavage and is co-precipitated with soluble ROP1, the subtilase is proposed to be involved in rhoptry biogenesis as a maturase. *Toxoplasma* rhoptries contain at least one cysteine protease, a cathepsin B that is termed toxopain-1 (Que *et al.*, 2002). Toxopain-1 is one candidate for processing of rhoptry proteins, based on the evidence that this protease is localized to acidic compartments in which active protein modification occurs, i.e. multivesiculated endosomes (Fig. 2B), immature rhoptries and the core region of rhop-

try base (Fig. 2A and inset). Cysteine protease inhibitors disrupt rhoptry biogenesis and partially retard the processing kinetics of transmembrane ROP2.

Up to 40% of *Toxoplasma* toxopain-1 is secreted from extracellular parasites, and EM localization indicates that it is localized in the parasitophorous vacuolar space. Inhibitors of cysteine and serine proteases cause an accumulation of abnormal membranous bodies in the parasite vacuole. Although cysteine and serine protease inhibitors block tachyzoite invasion and replication in the host cells (Conseil *et al.*, 1999; Que *et al.*, 2002), it is not yet clear whether secreted proteases are involved in the proteolytic degradation of vacuolar materials.

Subtilase and cathepsin B have not been identified in the *Plasmodium* rhoptries, but an unusual 76 kDa GPI-anchored serine protease (rhoptry associated membrane antigen or RAMA) that is associated with cholesterol and sphingolipid-rich rafts has been localized to the luminal face of the rhoptry membrane (Topolska *et al.*, 2003). RAMA is proposed to be cleaved by phospholipase C and, after activation, to be inserted into the erythrocyte membrane and the PVM (Braun-Breton *et al.*, 1988; Topolska *et al.*, 2003).

Overall, the bulk of the evidence presented here and summarized elsewhere (reviewed by Ngô *et al.*, 2000; Joiner and Roos, 2002) indicates that rhoptries receive material from both biosynthetic and endocytic pathways (Fig. 3). This feature provides additional support for the argument that rhoptries are analogous to secretory lysosomes.

Rhoptries contain an unusual lipid content

The plasma membrane of the host cell contributes the bulk (>80%) of the lipids and selected proteins that are required for the formation of the PVM upon *Toxoplasma gondii* entry into the cell (Suss-Toby *et al.*, 1996). Thereafter, membranous sheaths and exosomes secreted from the rhoptries may supply critical parasite proteins and lipids for the nascent formation of the hybrid PVM (Hakansson *et al.*, 2001). A recent study presented by Haldar and coworkers (Hiller *et al.*, 2003) suggests that a *Plasmodium* stomatin (Pfstomatin) secreted from the rhoptries and an erythrocyte stomatin-like protein-2 (SLP) are strong candidates for the assembly of non-caveolar detergent-resistant membrane (DRM) rafts during the formation of the *Plasmodium* PVM. Although stomatin has yet to be identified in *Toxoplasma*, this model is consistent with the report that *Toxoplasma* invasion of host cells is independent of caveolin (Coppens and Joiner, 2003).

Toxoplasma gondii rhoptries are atypical in having a high cholesterol–phospholipid ratio of 1.3–1.5:1 (Foussard *et al.*, 1991; Coppens and Joiner, 2003). The cholesterol–phospholipid ratio of rhoptries is too high for lipid bilayer stability, indicating that some of the cholesterol may be organized in a crystalline mosaic. Electron-lucent components in immature rhoptries and the base of mature rhoptries may represent lipid and protein aggregates, as lipids are most probably extracted by non-polar solvents during preparation for electron microscopy. Luminal proteins are tightly associated with lipids as their extraction requires ionic detergent (Etzion *et al.*, 1991). Furthermore, *Plasmodium rhopH*-3 is more susceptible to trypsin only after phospholipase treatment. As the rhoptries mature, lipid and protein interactions may increase and therefore appear as a more homogeneously electron-dense lumen. The possible tighter packaging of proteins and lipids may also explain the unusual neutral pH of the condensed duct region, or that the complexes are inaccessible to the acidity probe DAMP (Shaw *et al.*, 1998).

Based on the high cholesterol content, rhoptry cholesterol has been speculated to be essential to the formation of the nascent parasite vacuole membrane. When rhoptry cholesterol is depleted, however, parasite invasion is not impaired. In contrast, depletion of cholesterol from the host cell plasma membrane blocks parasite organelle exocytosis and parasitic invasion (Coppens and Joiner, 2003). Rather than serving as an extracellular effector, cholesterol may therefore play a more critical role in rhoptry biogenesis. As exosomes secreted from MVB are composed mainly of DRM domains that are enriched in sphingolipids and cholesterol, assembly of DRM rafts has been proposed to drive the internalization of the limiting membrane of late endosomes to form the internal vesicles and sheaths of MVB (Wubbolts *et al.*, 2003). In *Toxoplasma*-infected host cells, incubation with progesterone disrupts the mobilization of cholesterol from the lysosome/late endosome in both host and parasites. Progesterone induces an accumulation of internal membranes inside *Toxoplasma* MVB, a reduction in cholesterol delivered to the rhoptries and inhibition of parasite growth (Coppens and Joiner, 2003).

A long-standing puzzle of how transmembrane proteins such as ROP2 can be localized in the lumen of rhoptry rather than the rhoptry-limiting membrane can be explained by a model in which MVBs contribute to rhoptry biogenesis. The predicted primary amino acid sequence of ROP2 encodes a type I integral membrane protein. ROP2 would be synthesized co-translationally as a transmembrane protein, and would be transported to the rhoptry-limiting membrane through vesicular intermediates. In these intermediates, the N-terminus of the ROP2 would be luminal, and the C-terminus would be oriented towards the cytosol. Inward budding of the rhoptry-limiting membrane to form MVBs would generate exosomes with the ROP2 N- terminus out of and the C-terminus in the exosome lumen. After invasion, fusion of released exosomes with the nascent PVM, in the process described by Sibley

and coworkers (Hakansson *et al.*, 2001), would insert ROP2 into the PVM in the experimentally observed orientation –N-terminus in the host cell cytosol (Fig. 3). Inward budding associated with MVB formation thus provides an attractive explanation for both the location and the topology of ROP2 and ROP2 family members.

Rhoptries are associated with the endocytic pathway

Members of the highly conserved Rab/Ypt family of GTPases are key regulators of membrane fusion and provide useful markers for identifying compartments of both endocytic and secretory pathways. *Toxoplasma* and *Plasmodium* contain endocytic rabs that are prototypic markers for early (rab5), recycling (rab11) and late (rab7) endosomes (reviewed by Robibaro *et al.*, 2001). In *Toxoplasma*, the early endosomal rab5 (TgRab5) is localized to tubulovesicular structure adjacent to the Golgi, the Golgi itself and large lucent vesicles containing intraluminal membrane-bound vesicles and tubules (Fig. 1E) (Robibaro *et al.*, 2002).

The large lucent vesicles are most likely MVB). Surface-labelled cationized ferritin is internalized in a small population (10–20%) of parasites and found in these structures (H. M. Ngô and K. A. Joiner, unpublished data). Lucent vesicles are not frequently observed with internal vesicles and membranes, unless the endocytic pathways are disrupted by chemical or molecular manipulations. Parasites treated with the subtilisin inhibitor III or progesterone accumulate MVB-like structures (Que *et al.*, 2002; Coppens and Joiner, 2003). Expression of dominant-positive Rab5Q103L, but not wild-type or dominant-negative Rab5, also augments the appearance of MVBs (Fig. 2E). Dominant-positive mutants of rab5 augment the uptake of cholesterol quantitatively by 1.8-fold, and cholesterol accumulation is enhanced in the parasite rhoptries, lipid bodies and the inner membrane complex. These organelles also contain the AP-1 adaptor complex (Fig. 2D; Ngô *et al.*, 2003), which mediates anterograde and retrograde vesicular trafficking from early endosomes. Dominant-negative expression of the medium chain of AP-1 (Tgμ1D176A) elevates the number of MVBs. All these features link the endocytic pathway in *T. gondii* to MVB.

Vacuolar protein sorting mutant 4 (VPS4) is a class E ATPase involved in MVB formation in yeast and mammalian cells. VPS4 homologues are found in both *T. gondii* (TgVPS4) and *P. falciparum* (PfVPS4). When mutated to block ATP hydrolysis, PfVPS4 expressed in *T. gondii* or mammalian cells results in the formation of enlarged multivesicular structures enriched in cholesterol, completely analogous to aberrant MVB seen in other systems (M. Yang *et al.*, 2004). These structures partially co-localize with the endosomal marker TgRab5. Most importantly, TgROP2 mutated in the cytoplasmic tail and thereby blocked in delivery to mature rhoptries (M. Yang *et al.*, submitted) also accumulates in these aberrant PfVPS4 compartments. Bioinformatic analysis indicates that *Plasmodium* and *Toxoplasma* contain all components of the endosome-associated complex III (VPS 20/Snf7, VPS2/24, DID2 and VPS60) that interact with VPS4 in yeast to catalyse the release of the entire machinery from the membrane. These results illustrate that MVB are part of the endocytic pathway, and that MVB intersect the rhoptry biogenesis pathway (Fig. 3). These are features expected of secretory lysosomes, but not of secretory granules.

Rhoptry formation is mediated by adaptor-based coated vesicles

In Apicomplexan parasites, delivery of cargo from the single discoidal Golgi to the prerhoptry MVB and rhoptry duct appears to be mediated by coated vesicles, some of which resemble CCV (Bannister *et al.*, 2000). Recent insights into the sorting signals of rhoptry proteins support the role of adaptor-based vesicles in rhoptry biogenesis in a similar mechanism to that found in lysosomal targeting.

Members of the ROP2 gene family (ROP2, ROP4, ROP8) are type I transmembrane proteins, which contain in their cytoplasmic tails both tyrosine-based and dileucine sorting signals. Deletion of either signal allows ROP2 or ROP4 to exit the TGN but significantly diminishes their delivery to mature rhoptries, resulting in an accumulation in MVB (Hoppe *et al.*, 2000). As mentioned above, the structure stains positively for both TgRab5 and VSP4, confirming its nature as an endocytic compartment analogous to MVB (M. Yang *et al.*, submitted).

The *Toxoplasma* μ chain (Tgμ1) of adaptor complex 1 (TgAP-1) has been localized to the TGN and associated coated vesicles and tubulovesicular structures, MVB, immature and mature rhoptries (Ngô *et al.*, 2003). Disruption of the tyrosine-binding pocket of Tgμ1 by alanine substitution (D176A, W425A) diminishes Tgμ1-specific binding to cytoplasmic tails of rhoptry transmembrane proteins (ROP2, ROP4) as determined by yeast two-hybrid interaction assay. Dominant-negative expression of Tgμ1D176A disrupts rhoptry biogenesis. Depletion of Tgμ1 by antisense RNA expression results in swelling of the Golgi and endosomes and disrupts both delivery of ROP2 and other family members as well as formation of rhoptries. Hence, TgAP-1 is responsible for delivering specific cargo to mature rhoptries, rather than in sorting cargo away from developing rhoptries. Again, these are all features characteristic of secretory lysosomes, rather than secretory granules.

Although specific signals mediating targeting of soluble proteins to rhoptries are not definitely identified, deletion

mapping indicates that they are embedded in two domains of ROP1, the propeptide (amino acids 24–85) and a central peptide (amino acids 198–345) (Bradley and Boothroyd, 2001; Striepen *et al.*, 2001). The prodomains of most lysosomal proteins are glycosylated in the Golgi complex and targeted to lysosomes by MPRs, which recognize the modified asparagine-linked oligosaccharides. As minimal N-glycosylation occurs in Apicomplexan parasites, ROP1 is not post-translationally modified, and the genome does not encode the MPR sorting machinery, sorting of soluble proteins must use a non-MPR mechanism.

One possible MPR-independent sorting process depends on the membrane-binding motif (nine residues) at the N-terminus of prodomains in mammalian procathepsin L (McIntyre and Erickson, 1993) and trypanosome cruzipain (Huete-Perez *et al.*, 1999). It remains to be determined whether *Toxoplasma* ROP1 has membrane-binding potential as tentatively reported (He *et al.*, 2001) and, if so, whether the sorting motifs of rhoptry-soluble proteins will bind to the luminal domain of the ROP2 transmembrane protein family. In *Plasmodium*, soluble RAP2 and RAP3 form an oligomeric complex with RAP1, which contains at least one predicted transmembrane motif. A gene deletion that generates a truncated RAP1, containing the N-terminal 344 amino acids of the 782-amino-acid wild-type RAP1, abolishes heterocomplex formation between RAP1 and 2. Consequently, RAP2 is retained in the lumen of ER and possibly Golgi, whereas RAP1 is still faithfully targeted to the rhoptries (Baldi *et al.*, 2000). Tyrosine sorting signals (YAKL, YFAF) are predicted at the C-tail of RAP1 that can potentially interact with adaptor complex and adaptor-like proteins.

As the *T. gondii* ROP1 prodomain targets a VSG fusion to both the rhoptry base and duct whereas mature ROP1 is restricted to the basal subcompartment (Saffer *et al.*, 1992; Bradley and Boothroyd, 2001), the motif that mediates the basal retention of ROP1 is encoded in the mature protein. It remains to be determined whether the octapeptide repeat regions in ROP1 and ROP9 that are enriched in proline may mediate this sorting function.

In summary, the biogenesis of rhoptries is mediated by adaptor-based vesicular trafficking, most closely related to the sorting mechanism for secretory lysosomes. Future studies will hopefully identify the transmembrane proteins that mediate the sorting of soluble proteins into coated vesicles for forward transport to the rhoptries.

Conclusion: are rhoptries secretory granules or secretory lysosomes?

Three possible scenarios currently exist for the biogenesis of rhoptries. The secretory granule model indicates that rhoptry proteins and lipids aggregate at the TGN via sorting receptors/escorters, or DRM rafts, and are exclusively sorted to the immature rhoptry. An alternative model is that rhoptries are formed exclusively via the endocytic pathway. Finally, the ‘secretory lysosome’ model suggests that both secretory and endocytic pathways contribute to formation of the organelle (Fig. 3). The preponderance of evidence supports the last model. Altogether, we believe that rhoptries represent one of the earliest models of secretory lysosomes.

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References

Baldi, D.L., Andrews, K.T., Waller, R.F., Roos, D.S., Howard, R.F., Crabb, B.S., and Cowman, A.F. (2000) RAP1 controls rhoptry targeting of RAP2 in the malaria parasite *Plasmodium falciparum*. *EMBO J* **19:** 2435–2443.

Bannister, L.H., Hopkins, J.M., Fowler, R.E., Krishna, S., and Mitchell, G.H. (2000) Ultrastructure of rhoptry development in *Plasmodium falciparum* erythrocytic schizonts. *Parasitology* **121:** 273–287.

Beckers, C.J.M., Dubremetz, J.F., Mercereau-Puijalon, O., and Joiner, K.A. (1994) The *Toxoplasma gondii* rhoptry protein ROP2 is inserted into the parasitophorous vacuole membrane, surrounding the intracellular parasite, and is exposed to the host cell cytoplasm. *J Cell Biol* **127:** 947–961.

Blackman, M.J., Fujioka, H., Stafford, W.H., Sajid, M., Clough, B., Fleck, S.L., *et al.* (1998) A subtilisin-like protein in secretory organelles of *Plasmodium falciparum* merozoites. *J Biol Chem* **273:** 23398–23409.

Blott, E.J., and Griffiths, G.M. (2002) Secretory lysosomes. *Nature Rev Mol Cell Biol* **2002** (3)**:** 122–131.

Boehm, M., and Bonifacino, J.S. (2001) Adaptins. The final recount. *Mol Biol Cell* **12:** 2907–2920.

Bouchot, A., Jaillet, J.D., Bonhomme, A., Alessandro, N.P., Laquerriere, P., Kilian, L., *et al.* (2001) Detection and localization of a Ca2+-ATPase activity in *Toxoplasma gondii*. *Cell Struct Funct* **26:** 49–60.

Bradley, P.J., and Boothroyd, J.C. (1999) Identification of the pro-mature processing site of *Toxoplasma* ROP1 by mass spectrometry. *Mol Biochem Parasitol* **100:** 103–109.

Bradley, P.J., and Boothroyd, J.C. (2001) The pro region of *Toxoplasma* ROP1 is a rhoptry-targeting signal. *Int J Parasitol* **31:** 1177–1186.

Braun-Breton, C., Rosenberry, T.L., and da Silva, L.P. (1988) Induction of the proteolytic activity of a membrane protein in *Plasmodium falciparum* by phosphatidyl inositol-specific phospholipase C. *Nature* **332:** 457–459.

Burgoyne, R.D., and Morgan, A. (2003) Secretory granule exocytosis. *Physiol Rev* **83:** 581–632.

Conseil, V., Soete, M., and Dubremetz, J.F. (1999) Serine protease inhibitors block invasion of host cells by *Toxoplasma gondii*. *Antimicrob Agents Chemother* **43:** 1358–1361.

Coppens, I., and Joiner, K.A. (2003) Host but not parasite cholesterol controls *Toxoplasma* entry by modulating organelle discharge. *Mol Biol Cell* **14:** 3804–3820.

Denzer, K., Kleijmeer, M.J., Heijnen, H.F., Stoorvogel, W., and Geuze, H.J. (2000) Exosome: from internal vesicle of the multivesicular body to intercellular signaling device. *J Cell Sci* **113:** 3365–3374.

Etzion, Z., Murray, M., and Perkins, M. (1991) Isolation and characterization of rhoptries of *Plasmodium falciparum*. *Mol Biochem Parasitol* **47:** 51–62.

Foussard, F., Leriche, M.A., and Dubremetz, J.F. (1991) Characterization of the lipid content of *Toxoplasma gondii* rhoptries. *Parasitology* **102:** 367–370.

Glombik, M.M., and Gerdes, H.H. (2000) Signal-mediated sorting of neuropeptides and prohormones: secretory granule biogenesis revisited. *Biochimie* **82:** 315–326.

Hackett, F., Sajid, M., Withers-Martinez, C., Grainger, M., and Blackman, M.J. (1999) PfSUB-2: a second subtilisin-like protein in *Plasmodium falciparum* merozoites. *Mol Biochem Parasitol* **103:** 183–195.

Hakansson, S., Charron, A.J., and Sibley, L.D. (2001) *Toxoplasma* evacuoles: a two-step process of secretion and fusion forms the parasitophorous vacuole. *EMBO J* **20:** 3132–3144.

Hausmann, K. (1978) Extrusive organelles in protists. *Int Rev Cytol* **25:** 197–276.

He, C.Y., Shaw, M.K., Pletcher, C.H., Striepen, B., Tilney, L.G., and Roos, D.S. (2001) A plastid segregation defect in the protozoan parasite *Toxoplasma gondii*. *EMBO J* **20:** 330–339.

Hiller, N.L., Akompong, T., Morrow, J.S., Holder, A.A., and Haldar, K. (2003) Identification of a stomatin orthologue in vacuoles induced in human erythrocytes by malaria parasites: a role for microbial raft-protein in apicomplexan vacuole biogenesis. *J Biol Chem* **278:** 48413–48421.

Hoppe, H.C., Ngo, H.M., Yang, M., and Joiner, K.A. (2000) Targeting to rhoptry organelles of *Toxoplasma gondii* involves evolutionarily conserved mechanisms. *Nature Cell Biol* **2:** 449–456.

Howard, R.F., and Schmidt, C.M. (1995) The secretary pathway of *Plasmodium falciparum* regulates transport of p82/RAP1 to the rhoptries. *Mol Biochem Parasitol* **74:** 43–54.

Huete-Perez, J.A., Engel, J.C., Brinen, L.S., Mottram, J.C., and McKerrow, J.H. (1999) Protease trafficking in two primitive eukaryotes is mediated by a prodomain protein motif. *J Biol Chem* **274:** 16249–16256.

Joiner, K.A., and Roos, D.S. (2002) Secretory traffic in the eukaryotic parasite *Toxoplasma gondii*: less is more. *J Cell Biol* **157:** 557–563.

Laslop, A., Weiss, C., Savaria, D., Eiter, C., Tooze, S.A., Seidah, N.G., and Winkler, H. (1998) Proteolytic processing of chromogranin B and secretogranin II by prohormone convertases. *J Neurochem* **70:** 374–383.

Luzio, J.P., Poupon, V., Lindsay, M.R., Mullock, B.M., Piper, R.C., and Pryor, P.R. (2003) Membrane dynamics and the biogenesis of lysosomes. *Mol Membr Biol* **20:** 141–154.

McIntyre, G.F., and Erickson, A.H. (1993) The lysosomal proenzyme receptor that binds procathepsin L to microsomal membranes at pH 5 is a 43-kDa integral membrane protein. *Proc Natl Acad Sci USA* **90:** 10588–10592.

Miller, S.A., Binder, E.M., Blackman, M.J., Carruthers, V.B., and Kim, K. (2001) A conserved subtilisin-like protein TgSUB1 in microneme organelles of *Toxoplasma gondii*. *J Biol Chem* **276:** 45341–45348.

Miller, S.A., Thathy, V., Ajioka, J.W., Blackman, M.J., and Kim, K. (2003) TgSUB2 is a *Toxoplasma gondii* rhoptry organelle processing proteinase. *Mol Microbiol* **49:** 883–894.

Nakaar, V., Ngô, H.M., Aronson, E.P., Coppens, I., Stedman, T.T., and Joiner, K.A. (2002) Pleiotropic effect due to targeted depletion of secretory rhoptry protein ROP2 in *Toxoplasma gondii*. *J Cell Sci* **116:** 2311–2320.

Ngô, H., Hoppe, H.C., and Joiner, K.A. (2000) Differential sorting and post-secretory targeting of proteins in parasitic invasion. *Trends Cell Biol* **10:** 67–72.

Ngô, H.M., Yang, M., Pypaert, M., Hoppe, H.C., and Joiner, K.A. (2003) Biogenesis of secretory organelles in an apicomplexan parasite is dependent on the AP-1 adaptin. *J Biol Chem* **278:** 5343–5352.

Palade, G. (1975) Intracellular aspects of the process of protein synthesis. *Science* **189:** 347–358.

Pelletier, L., Stern, C.A., Pypaert, M., Sheff, D., Ngo, H.M., Roper, N., *et al.* (2002) Golgi biogenesis in *Toxoplasma gondii*. *Nature* **418:** 548–552.

Plattner, H., and Kissmehl, R. (2003) Molecular aspects of membrane trafficking in paramecium. *Int Rev Cytol* **232:** 185–216.

Preiser, P., Kaviratne, M., Khan, S., Bannister, L., and Jarra, W. (2000) The apical organelles of malaria merozoites: host cell selection, invasion, host immunity and immune evasion. *Microbes Infect* **2:** 1461–1477.

Que, X., Ngo, H., Lawton, J., Gray, M., Liu, Q., Engel, J., *et al.* (2002) The cathepsin B of *Toxoplasma gondii*, toxopain-1, is critical for parasite invasion and rhoptry protein processing. *J Biol Chem* **277:** 25791–25797.

Reichmann, G., Dlugonska, H., and Fischer, H.G. (2002) Characterization of TgROP9 (p36), a novel rhoptry protein of *Toxoplasma gondii* tachyzoites identified by T cell clone. *Mol Biochem Parasitol* **119:** 43–54.

Robibaro, B., Hoppe, H.C., Yang, M., Coppens, I., Ngo, H.M., Stedman, T.T., *et al.* (2001) Endocytosis in different lifestyles of protozoan parasitism: role in nutrient uptake with special reference to *Toxoplasma gondii*. *Int J Parasitol* **31:** 1343–1353.

Robibaro, B., Stedman, T.T., Coppens, I., Ngo, H.M., Pypaert, M., Bivona, T., *et al.* (2002) *Toxoplasma gondii* Rab5 enhances cholesterol acquisition from host cells. *Cell Microbiol* **4:** 139–152.

Rosenthal, P.J. (1999) Proteases of protozoan parasites. *Adv Parasitol* **43:** 105–159.

Saffer, L.D., Mercereau-Puijalon, O., Dubremetz, J.F., and Schwartzman, J. (1992) Localization of a *Toxoplasma gondii* rhoptry protein by immunoelectron microscopy during and after host cell penetration. *J Protozool* **39:** 526–530.

Sam-Yellowe, T.Y. (1996) Rhoptry organelles of the apicomplexa: their role in host cell invasion and intracellular survival. *Parasitol Today* **12:** 308–316.

Shaw, M.K., Roos, D.S., and Tilney, L.G. (1998) Acidic compartments and rhoptry formation in *Toxoplasma gondii*. *Parasitol* **117:** 435–443.

Shaw, M.K., Roos, D.S., and Tilney, L.G. (2002) Cysteine and serine protease inhibitors block intracellular development and disrupt the secretory pathway of *Toxoplasma gondii*. *Microbes Infect* **4:** 119–132.

Sinai, A.P., and Joiner, K.A. (2001) The *Toxoplasma gondii* protein ROP2 mediates host organelle association with the parasitophorous vacuole membrane. *J Cell Biol* **154:** 95–108.

Soldati, D. (1998) Processing of *Toxoplasma* ROP1 protein in nascent rhoptries. *Mol Biochem Parasitol* **96:** 37–48.

Soldati, D., Kim, K., Kampmeier, J., Dubremetz, J.F., and Boothroyd, J.C. (1995) Complementation of a *Toxoplasma gondii* ROP1 knock-out mutant using phleomycin selection. *Mol Biochem Parasitol* **74:** 87–97.

Storrie, B. (1988) Assembly of lysosomes: perspectives from comparative molecular cell biology. *Int Rev Cytol* **111:** 53–105.

Striepen, B., Soldati, D., Garcia-Reguet, N., Dubremetz, J.F., and Roos, D.S. (2001) Targeting of soluble proteins to the rhoptries and micronemes in *Toxoplasma gondii*. *Mol Biochem Parasitol* **113:** 45–63.

Suss-Toby, E., Zimmerberg, J., and Ward, G.E. (1996) *Toxoplasma* invasion: the parasitophorous vacuole is formed from host cell plasma membrane and pinches off via a fission pore. *Proc Natl Acad Sci USA* **93:** 8413–8418.

Topolska, A.E., Lidgett, A., Truman, D., Fujioka, H., and Coppel, R.L. (2003) Characterisation of a membrane-associated rhoptry protein of *Plasmodium falciparum*. *J Biol Chem* **279:** 4648–4656.

Wubbolts, R., Leckie, R.S., Veenhuizen, P.T., Schwarzmann, G., Mobius, W., Hoernschemeyer, J., *et al.* (2003) Proteomic and biochemical analyses of human B cell-derived exosomes. Potential implications for their function and multivesicular body formation. *J Biol Chem* **278:** 10963–10972.

Yang, M., Coppens, I., Wormsley, S., Baevova, P., Hoppe, H.C., and Joiner, K.A. (2004) The *Plasmodium falciparum* Vps4 homolog mediates multivesicular body formation. *J Cell Sci* (in press).