We have previously demonstrated that *Toxoplasma gondii* has a tyrosine-based sorting system, which mediates protein targeting to the lysosome-like rhoptry secretory organelle. We now show that rhoptry protein targeting is also dependent on a dileucine motif and occurs from a post-Golgi endocytic organelle to mature rhoptries in an adaptin-dependent fashion. The *T. gondii* AP-1 adaptin complex is implicated in this transport because the μ1 chain of *T. gondii* AP-1 (α) was localized to multivesicular endosomes and the limiting and luminal membranes of the rhoptries; (b) bound to endocytic tyrosine motifs in rhoptry proteins, but not in proteins from dense granule secretory organelles; (c) when mutated in predicted tyrosine-binding motifs, led to accumulation of the rhoptry protein ROP2 in a post-Golgi multivesicular compartment; and (d) when depleted via antisense RNA, resulted in accumulation of multivesicular endosomes and immature rhoptries. These are the first results to implicate AP-1 in transport from a post-Golgi compartment to a mature secretory organelle and substantially expand the role for AP-1 in anterograde protein transport.

Obligate intracellular protozoa of the phylum Apicomplexa are highly sophisticated secretory cells. Although the host cell type and the nature of the intracellular vacuole differ when *Plasmodium*, *Toxoplasma*, *Cryptosporidium*, *Eimeria*, *Theilera*, and *Babesia* are compared, these organisms share common secretory organelles: micronemes, rhoptries, and dense granules. Differential secretion from these organelles directs invasion of host cells, formation of an intracellular parasitophorous vacuole, and subsequent modification of the vacuole for replication (reviewed in Ref. 1).

Most unique morphologically and biochemically are the rhoptries. These are flask-shaped secretory organelles that are assembled and positioned at the apical pole of the invasive forms of Apicomplexa (reviewed in Refs. 2–4). At the onset of host cell invasion, prepackaged proteins and membranous materials are recognized by receptors from rhoptries through an apical opening. Rhoptry constituents are likely involved in a plethora of essential functions. These include directing host cell attachment and invasion (3), expansion and maintenance of the parasitophorous vacuole membrane surrounding the intracellular parasite (5), and attachment of host mitochondria and the endoplasmic reticulum to the parasitophorous vacuole membrane (6). Furthermore, rhoptry proteins are targets of the normal immune response; antibodies to rhoptry proteins block invasion; and immunization with selected rhoptry proteins provides partial protection against parasite challenge (2). Hence, the unique rhoptry organelle can serve as a potential target for blocking parasite invasion and growth.

The biogenesis and phylogenetic relationship of rhoptries to secretory organelles in other cells are not well understood. Rhoptries and pre-rhoptries are described as the only acidified organelles in the parasite (7) and are packaged with specialized hydrolyses (8) and cholesterol and membranes for secretion (9, 10), characteristics of a secretory lysosome or lysosome-like organelle (11, 12). We have recently demonstrated that an endocytic tyrosine-based motif (YXXΦ) in the cytoplasmic tail of the rhoptry protein ROP2, when mutated, inhibits delivery of the protein to rhoptries (13). The mutant ROP2 protein accumulates in a compartment of unknown origin, but distinct from the Golgi. Taken together, these results suggest that rhoptries may be analogous to secretory lysosomes.

Another implication of these results is that heterotetrameric adaptin complexes (reviewed in Refs. 9, 14, and 15) are potentially involved in protein sorting to rhoptries. In other eukaryotes, tyrosine-based (YXXΦ) or dileucine-based (LL) motifs in the cytoplasmic tail of transmembrane receptors are recognized by adaptor complexes (16–18). The six AP (adaptor protein) complexes (AP-1A/AP-1B, AP-2, AP-3A/AP-3B, and AP-4) are distributed to different organelles in the cell, defining their specific function and cargo selection (15). AP-2 mediates endocytosis from the plasma membrane, whereas AP-1A/AP-1B, AP-3A/AP-3B, and AP-4 sort proteins at the trans-Golgi network (TGN) (13) and/or endosomes. Although AP-1 was originally suggested to function only in anterograde transport...
from the TGN to endosomes, recent evidence suggests that the complex mediates retrograde transport of mannose 6-phosphate receptors from endosomes to the TGN (19, 20). In neuroendocrine, endocrine, and mammary epithelial cells, AP-1 is localized to immature secretory granules, where it functions in the removal of membranes and nonspecific cargo, presumably via a retrograde pathway, to facilitate maturation to mature secretory granules (21, 22). AP-3 mediates direct transport of selected cargo from the TGN to lysosome-like secretory organelles and may also mediate transport from endosomes to lysosome-like organelles (reviewed in Ref. 12, 15, and 23). In contrast, there is no evidence supporting a role for AP-1 in anterograde transport from a post-Golgi compartment to another organelle and especially to a lysosome-like organelle.

On both a functional and genomic basis, it is increasingly clear that the T. gondii secretory pathway can be considered a stripped-down version of the more complicated machinery present in higher eukaryotes (24, 25).2 Coupled with the presence of multiple distinct and easily recognized secretory organelles, the parasite permits insight into processes not readily discerned in other systems. We now show that rhoptry proteins are transported to mature rhoptries from a post-Golgi endocytic compartment in an AP-1-dependent fashion. Other adaptins and adaptors, including AP-3, GGA proteins, stonins, and β-arrestins (reviewed in Ref. 26), cannot participate because they are absent from the T. gondii genome. This result...

2 Available at www.toxodb.org.
substantially expands the role for AP-1 in anterograde transport and more specifically in transport to and biogenesis of lysosome-related organelles.

**EXPERIMENTAL PROCEDURES**

**Cloning of the T. gondii μ1 Chain (Tgμ1) Gene—**A cDNA clone (zz38e01) with strong homology to the mouse μ1A chain was located in the T. gondii expressed sequence tag data base (27). The clone was found to contain the 117 C-terminal codons of a T. gondii μ1 homolog, which we designated Tgμ1. The sequence of the clone was extended by PCR from parasite cDNA using degenerate primers to the conserved sequence YELLDE (amino acids 113–118 of the mouse μ1A sequence), and full-length Tgμ1 was finally obtained by 5’-rapid amplification of cDNA ends from T. gondii cDNA. The Tgμ1 cDNA sequence has been deposited in the GenBank/EBI Data Bank under accession number AY117037. T. gondii and human foreskin fibroblast DNAs were isolated as previously described (13).

The GenBank/EBI accession numbers of adaptin sequences used for the sequence analysis in Fig. 2 are as follows: *Arabidopsis thaliana*
Adaptin-dependent Secretory Organelle Biogenesis

**Fig. 4. Localization of TgA1 by cryo-immuno-electron microscopy.** Shown are electron microscopic images of TgA1-HA parasites demonstrating the gold localization of TgA1 (arrows) predominantly to the trans-most cisterna of the Golgi (G) and vesicles and tubules extending from or juxtaposed to the TGN (GVT) (panels A and B) and to membranes of microtubule (M) and endosomes (E) and also to the membrane and lumen of aberrant rhoptries (R) (panel C). Endosomes are defined as vesicles 200-300 nm in diameter that are clear (panel C, lower inset) or that contain membrane whorls or dense deposits and that are known to contain the early endosomal marker T. gondii Rab5 (44) or VP54 (see Footnote 5). Similar localization was observed for endogenous TgA1 in RH, as detected by affinity-purified anti-TgA1 monoclonal antibody, and for TgA1(D176A)-HA in the stable transgenic parasites (data not shown). The organelar density of gold labeling (gold particles/μm²) for endogenous TgA1 in RH and TgA1(D176A)-HA was determined from a random sample of 20-30 cell profiles (panel D). Density was calculated by estimating the number of gold particles divided by the estimated volume of organelles using a double-square test system. The density was expressed as a percentage of the sum of all organelles and cytoplasm estimates. Only well defined Golgi stacks were categorized as Golgi cisternae, whereas vesicles and tubules adjacent to the TGN were categorized as Golgi-associated vesicles and tubules. N, nucleus. Scale bars = 200 nm.

μ1 (AF009631), μ2 (AC007354.2), and μ4 (AL035356.1); Saccharomyces cerevisiae μ1 (NC_001134.1), and μ3 (NC_001135.1); Caenorhabditis elegans μ1 (P35602) and μ2 (B49837); Dro sophila melanogaster μ1 (AJ006219.1) and μ2 (AF005962.1); ray Discopyge omnata μ2 (150530); mouse μ1 (NM007456.1), μ1B (AF067146.1), and μ2 (I49327); rat μ2 (I49327), μ3A (L07074.1), and μ3B (L07073.1); and human μ1B (NM005498.1), μ2 (D34475.1), μ3A (AF092992.1), and μ4 (AP155158.1).

**Mutuation of the ROP2 Cytoplasmic Tail Dileucine Motif**—The ROP2 cytoplasmic tail contains a dileucine motif at residues 529 and 530, which is known to mediate interaction with the adaptin complex. D176A and W415A mutations were introduced in the cytoplasmic tail of ROP2 using the hemagglutinin (HA)-tagged ROP2 protein transport. An L529A/L530A mutation was introduced into the cytoplasmic tail of ROP2 using the hemagglutinin (HA)-tagged ROP2 construct previously described (13). This construct was transiently transfected into T. gondii and localized as previously reported (13).

**Production of Polyclonal Antibodies**—The TgA1 coding region was subcloned into a pZT28a vector (Amersham Biosciences) to produce a His-TgA1 recombinant protein. His-TgA1 was purified on a column and sent to Cocalico Biologicals, Inc. for production of a rabbit polyclonal antiserum. The antiserum (anti-TgA1) was affinity-purified by adsorption and desorption to isolated recombinant proteins on nitrocellulose membrane as described previously (28). The affinity-purified antiserum recognized a polypeptide migrating at ~47 kDa upon immunoblotting.

**Parasite Growth and Transformation**—The growth conditions and transient and stable transfection of the RH strain of T. gondii grown on monolayers of Vero cells and human foreskin fibroblasts were as described previously (13).

**Antisense RNA-targeted Depletion**—The complete coding region of TgA1 was amplified by PCR and cloned in the antisense orientation into the AurII and EcoRI sites of the ribozyme-histone cassette pNTPZR (29). Transfection, selection, and cloning of antisense clones were done as previously described (29).

**Mutagenesis of TgA1**—An HA epitope tag was introduced into TgA1 between codons 231 and 232 by PCR, yielding TgA1-HA. (Addition of a c-Myc epitope tag at the N terminus of TgA1 resulted in a protein that was diffusely cytosolic and was presumed not to be incorporated into an adaptin complex.) D176A and W415A mutations were introduced in TgA1-HA by PCR, yielding TgA1(D176A)-HA and TgA1(W415A)-HA. These mutations have previously been shown to alter binding of rat μ2 to tyrosine-containing motifs without affecting incorporation of the μ2 chain into the AP-2 complex (30). Both TgA1-HA and TgA1(D176A)-HA were ligated into pNTP/Sec for transient transfection and into pDHFR-TS/C3M2M3 for stable transfection as described previously (13). Sequences of all PCR-amplified fragments were verified by dideoxy sequencing at the W. M. Keck Sequencing Center of the Yale University School of Medicine.

**SDS-PAGE, Immunoblotting, and Immunoprecipitation**—Immunoblotting was performed as previously described (31) using anti-TgA1 antibody (1:100), anti-HA monoclonal antibody (1:200), or anti-ROP2/3 monoclonal antibody T3-4A7 (1:250). Antibody binding was detected by incubation with protein A-horseradish peroxidase (1:5000) and the ECL detection system (Amersham Biosciences). Pulse-chase and immunoprecipitation followed previously described protocols (32).

**Immunofluorescence and Cryoimmuno-electron Microscopy Localization of TgA1**—Immunofluorescence localization of endogenous TgA1 using affinity-purified anti-TgA1 antibody (1:20) followed a published protocol (13, 31). TgA1-HA and TgA1(D176A)-HA were localized using anti-HA monoclonal antibody (1:200).

For cryoimmuno-electron microscopy localization, monolayers of Vero cells or human foreskin fibroblasts were infected with wild-type RH or stably transfected parasite clones for 24 h, fixed in 8% paraformaldehyde in 0.25 M HEPES (pH 7.4) for 48 h, scraped off in phosphate-buffered saline, and pelleted in 10% fish skin gelatin. The gelatin-embedded pellets were infiltrated overnight with 2.3M sucrose and 0.5% uranyl acetate in 2% methyl cellulose. Sections were embedded in 0.25M HEPES (pH 7.4) for 48 h, scraped off in phosphate-buffered saline, and pelleted in 10% fish skin gelatin. Ultrathin cryosections were prepared with a Leica Ultracut microtome with cryoattachment and transferred to Formvar-carbon-coated specimen grids. Sections were incubated in phosphate-buffered saline containing affinity-purified rabbit anti-TgA1 antibody (1:20) or mouse anti-HA monoclonal antibody (1:100). Following phosphate-buffered saline washes, the sections were incubated with 1% fish skin gelatin containing 5-nm protein A-gold conjugate (1:70, from the laboratory of J. Slot) for 1 h. Sections were then washed in phosphate-buffered saline, post-fixed in 1% glutaraldehyde, and contrasted with 0.5% uranyl acetate in 2% methyl cellulose. Sections were observed, and images were recorded with a Philips 410
transmission electron microscope. Specificity of localization was determined by stereological analysis of 35–50 random sections of whole cells. The total number of gold particles and organelle surface areas were estimated from positive prints of transmission electron microscope negatives using a double-square test system (33).

Transmission Electron Microscopy Quantitative Analysis of Organelles—Monolayers of Vero cells or human foreskin fibroblasts were infected with RH or stably transfected parasite clones for 24 h, fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.4) for 30 min, washed, and post-fixed in 1% osmium tetroxide in the same buffer. The total number of gold particles and organelle surface areas were calculated from prints of transmission electron microscope negatives using a double-square overlay test system (33).

Yeast Two-hybrid Assay—Yeast two-hybrid assays were performed using the pGBT9 and pACT2 vectors (Clontech) as previously described (13, 16). Cytoplasmic tails at the C termini of putative transmembrane proteins GRA4 (dense granule protein-4; 52 amino acids), GRA7 (41 amino acids), ROP2 (75 amino acids), and ROP4 (85 amino acids), all containing YXXΦ motifs, were PCR-amplified and cloned in-frame behind the Gal4-binding domain in the pGBT9 vector. Tg1, Tg1(D176A), and Tg1(W415A) were cloned in-frame with the Gal4 activation domain in the pACT2 vector.

RESULTS

Mutation of Dileucine Residues in Rop2 Abolishes Targeting to Mature Rhoptries—Members of the Rop2 gene family contain dileucine residues upstream of an endocytic YXXΦ motif previously shown to be involved in targeting to mature rhoptries. Because dileucine residues can also mediate endocytic targeting by binding to adaptin β chains (see Refs. 34 and 35; reviewed in Ref. 11), these residues were mutated to di-alanine, and the ROP2 mutant was expressed in T. gondii. As illustrated in Fig. 1 (A), the L529A/L530A mutant localized to a compartment distinct from, but adjacent to, mature rhoptries, similar to the localization of ROP2 with a deletion of the YXXΦ motif (ROP2Δ) (13). This compartment was distinct from the dense granules, micronemes, and Golgi/TGN of the parasite (Fig. 1B), as was the ROP2Δ mutant (13). Importantly, both the ROP2Δ mutant and the L529A/L530A mutant substantially co-localized with a multivesicular endosomal compartment marked by the endosomal marker VPS4 (vacuolar protein sorting-4). Altogether, these data provide further evidence that rhoptry targeting occurs along the endocytic pathway and is mediated by adaptins. They also implicate adaptin machinery in mediating targeting from a post-Golgi compartment to a mature secretory organelle. We undertook experiments to further support and understand this observation.

4 M. Yang, I. Coppens, S. Wormsley, H. C. Hoppe, and K. A. Joiner, manuscript in preparation.
Cloning of Tgμ1 of the AP-1 Adaptin Complex—Based on a cDNA sequence (clone z38e01) from the T. gondii data base of expressed sequence tags, a full-length medium chain (μ) of a clathrin adaptin was amplified from parasite cDNA. Sequence analysis of the cDNA clones predicted an open reading frame encoding a polypeptide of 430 amino acids with a predicted molecular mass of 47.6 kDa. Alignment and dendrogram analysis of all four known classes of μ chains indicated that this T. gondii homolog separates into the μ1 cluster and shares the highest similarities (75–79%) and identities (58–62%) with the adaptin μ1 chain (Fig. 2) (data not shown). The sequence also contains the signature residues (173VFLD176, Lys 203, Val 399, Leu402, and 414PWVR417) that are known to mediate binding to T. gondii rhoptry (ber/cell). The volume of organelle/volume of cell) into organelle density (organelle number/cell). The arrow indicates either an endosome or an acidocalcisome. Scale bars = 200 nm.

Endogenous Tgμ1 and Tgμ1-HA Localize to the TGN—In mammalian cells, AP-1 is an essential regulator of membrane trafficking by mediating budding of clathrin-coated vesicles from the TGN, immature secretory granules, and endosomes. To define the specific roles of Tgμ1 in the multiple secretory and endocytic pathways in T. gondii, we first examined the localization of endogenous Tgμ1 using affinity-purified anti-Tgμ1 polyclonal antibody. As shown by immunofluorescence microscopy, endogenous Tgμ1 localized in RH parasites predominantly to a region anterior to the nucleus (Fig. 3), consistent with the Golgi/TGN of the parasite, although additional punctate and cytosolic staining was observed. Brefeldin A, which disrupts the T. gondii Golgi cisternae, but not the Golgi scaffold (25, 37), only partially dispersed the Golgi/TGN staining of Tgμ1, suggesting that the protein is partially localized to brefeldin A-resistant compartment(s) in addition to the Golgi.

We then stably expressed wild-type Tgμ1 tagged with the HA epitope (Tgμ1-HA). Putative Golgi/TGN localization and partial redistribution by brefeldin A (Fig. 3) were observed for the transgenic Tgμ1-HA reporter. This result suggests that the transgenic epitope-tagged Tgμ1 is incorporated into an AP-1 complex, which is appropriately localized in the parasite. Tgμ1-HA partially co-localized with the transgenic Golgi/TGN marker bacterial alkaline phosphatase-low density lipoprotein receptor (13) as well as with endogenous T. gondii proteins (dynamin and ADP-ribosylation factor-1) (38) with a predominant Golgi localization (Fig. 3B). Partial co-localization with the rhoptry compartment was also detected (Fig. 3B), although this was more accurately discerned by immunoelectron microscopy (see below).
We analyzed the localization of Tgµ1 by qualitative (Fig. 4, A–C) and quantitative (Fig. 4D) immunolabeling of cryosections. Consistent with the immunofluorescence localization, endogenous Tgµ1 localized to the Golgi/TGN, to juxtaposed coated Golgi-associated vesicles and tubules and endosomes, and to both the rhoptry membrane and membranous lumen. No significant labeling was observed for dense granules, mitochondria, or the nucleus, although microneme labeling was also detected. Altogether, the results suggest that Tgµ1 is localized to multiple compartments in the secretory and endocytic pathways, including mature secretory organelles.

**Mutation of Residues in Tgµ1 Responsible for Tyrosine Binding Does Not Alter Tgµ1 Localization**—We next sought to disrupt Tgµ1 function to assess effects on transport through the secretory pathway and on organelle biogenesis. We have previously shown that Tgµ1 binds to the ROP2 cytoplasmic tail in a fashion dependent upon the YEQL motif in the ROP2 cytoplasmic tail (13). We therefore introduced mutations in Tgµ1 at residues previously shown in mammalian adaptins to bind to the YXXΦ motif, specifically D176A and W415A (18, 30), and stably expressed these mutants in *T. gondii*. The Tgµ1(D176A)-HA mutant localized to the Golgi/TGN region and to punctate brefeldin A-resistant clusters (Fig. 3), suggesting that the protein was incorporated into an adaptin complex. In contrast, the W415A mutant was diffusely distributed in the cytosol (data not shown), suggesting that the protein failed to associate with other chains of the AP-1 complex. Quantification of immunostained cryosections indicated that Tgµ1(D176A)-HA was still associated predominantly with the Golgi/TGN, Golgi-associated vesicles and tubules, rhoptries, and micronemes (Fig. 4D). Overall, the relative association of Tgµ1(D176A)-HA with organelles was nearly identical to that of endogenous Tgµ1, further supporting a broad organelar distribution for AP-1 in *T. gondii* irrespective of interaction with cargo.

**Binding of Tgµ1(D176A)-HA to Tyr-based Motifs Is Inhibited Compared with That of Tgµ1-HA**—We sought to confirm that introduction of the D176A and W415A mutations altered binding of Tgµ1 to tyrosine-based motifs. Binding of Tgµ1 to the cytoplasmic tails containing putative YXXΦ motifs of dense granule (GRA4, YAEL; and GRA7, YRHF) and rhoptry (ROP2, YEQL; and ROP4, YREM) proteins was tested by yeast two-hybrid analysis. Tgµ1 bound to the cytoplasmic tail of ROP4 to a greater extent than to that of ROP2. In contrast, Tgµ1 did not bind to YXXΦ motifs within the putative cytoplasmic tails of the dense granule proteins GRA4 and GRA7 (Fig. 5A). The avidity of Tgµ1 binding to the ROP2 tail was increased when a triplicated YEQL motif was added to the truncated tail (ROP2.3Y) (Fig. 5, A and B), as previously reported (13). Importantly, both D176A and W415A mutations impaired binding to ROP2.3Y and ROP4 (Fig. 5B), confirming the functional roles of the conserved residues within Tgµ1 and the utility of the mutants as dominant-negative inhibitors of endogenous µ1 function. Because the W415A mutant was not incorporated into an AP-1 complex, the remainder of the experiments were conducted with the D176A construct.

**Dominant-negative Tgµ1(D176A) Alters Sorting of Rhoptry Proteins**—We evaluated the effects of stably overexpressing the wild-type and D176A constructs on protein sorting and organelle biogenesis in *T. gondii*. The most striking phenotype as shown by immunofluorescence microscopy was an alteration in the pattern of staining with antibody to ROP2 (Fig. 6). In the majority of parasites overexpressing Tgµ1-HA, ROP2 labeling was no longer restricted to the defined apical cluster of mature rhoptries observed in wild-type parasites (upper panels), but was more fragmented into vesicles and tubules (middle panels). In parasites overexpressing dominant-interfering Tgµ1(D176A)-HA, the labeling of ROP2 decreased drastically in intensity and was detectable as thin tubules and vesicles.
extending nearly the length of the cell. In contrast, there were no detectable differences in the targeting of glycosylphosphatidylinositol-anchored proteins targeted to the cell surface (SAG1 (surface antigen glycoprotein-1)) or of dense granule proteins (GRA3 and GRA4) to mature dense granules (data not shown). Of note, there were no differences in the kinetics or extent of processing of precursor ROP2 to mature ROP2 when native and Tg/H92621(D176A)-HA-expressing parasites were compared (data not shown). This indicates that ROP2 transport to a post-Golgi processing compartment is not substantially blocked by the dominant-negative Tg/H92621(D176A) construct.

Disruption of Tg/H92621 Function Alters Endosome and Rhoptry Structure—The above alterations in staining for ROP2 were defined more precisely and quantitatively by electron microscopy. In comparison with RH, rhoptries in Tg/H92621(D176A)-HA parasites were thinner (Fig. 7B), with an overall 2.1-fold decrease in size (volumetric density of 0.013 for rhoptries in RH versus 0.006 for those in Tg/H92621(D176A)-HA) (Fig. 7A). As shown by immunoelectron microscopy, the localization of ROP2 was substantially altered in the Tg/H92621(D176A)-HA-expressing parasites (Fig. 8). Thin elongated rhoptries extending nearly the length of the cell were stained. Large tubular and multivesicular structures consistent with endosomes were most prominently labeled, in comparison with native parasites. ROP2 accumulated in electron-dense structures resembling the sites to which ROP2 with mutations in the YXXΦ motif localized (Fig. 8, ROP2Δ panel). On the other hand, minimal accumulation of ROP2 label was observed in the T. gondii Golgi, largely excluding the notion that AP-1 mediates direct transport from the Golgi to rhoptries. Overall, the results indicate that the AP-1 adaptin mediates transport of ROP2 from a post-Golgi endosomal compartment to the mature rhoptry organelle.

Targeted Depletion of Tg/H92621 Alters Golgi, Endosome, and Rhoptry Structures as Well as Parasite Segregation—Finally, we investigated the consequences of lowering the expression level of Tg/H92621 using the recently described antisense mRNA strategy (29). Immediately after isolation of Tg/H92621 antisense stable clones, up to 90% depletion of Tg/H92621 was detected by immunoblotting (Fig. 9A). In contrast, no alteration in expression of the dense granule protein nucleoside-triphosphate hydrolase was observed, confirming the specificity of the antisense effect.

Dramatic effects on parasite morphology were observed. As shown by differential interference contrast microscopy (Fig. 9B), >50% of the vacuoles contained large irregular cells suggestive of defective parasite division. This morphologic defect was shown by
electron microscopy instead to reflect parasites that had completed division, but in which the plasma membranes of neighboring cells were still closely associated; hence, parasites were aggregated in the vacuole (data not shown). Staining for ROP2 was aberrant, clumped, and irregularly distributed.

Parasites depleted of Tgα1 had swollen Golgi cisternae and an accumulation of lucent endocytic vesicles containing internal vesicles and membranes and also of immature rhoptries (Fig. 10A, IR), containing the honeycombed appearance characteristic of rhoptry luminal contents. There was a 7–8-fold increase in volumetric density of both endosome-like structures as well as immature rhoptries (Fig. 10B) in the μIAS6 clone. There was no significant alteration in the density of dense granules and micronemes. These results substantially extend the evidence that Tgα1 mediates rhoptry biogenesis from a post-Golgi compartment with characteristics of multivesicular endosomes and immature rhoptries while also supporting an additional role for Tgα1 in transport from the Golgi.

**DISCUSSION**

Delivery of ROP2 and ROP4 from the TGN to rhoptries can occur by three possible routes. The first pathway is direct transport from the TGN to mature rhoptries. This pathway is unlikely because ROP2 mutated to remove either tyrosine-based or dileucine motifs is not retained in the TGN, but rather in a post-Golgi organelle (Ref. 13 and this study). The second possibility is that rhoptry proteins are delivered first to the plasma membrane and then internalized to the endosomal/lysosomal and rhoptry pathway. We do not favor this explanation because neither native nor mutant ROP2 is observed at the plasma membrane at steady state as shown by immunofluorescence (13) and immunoelectron (this study) microscopy localization. The third possibility is that rhoptry proteins are delivered from the TGN to endosomal compartment and subsequently to the mature rhoptry. The data presented in this work strongly support this notion and directly implicate AP-1 in the process. The data contained herein (in particular, see Fig. 10) also support the expected involvement of AP-1 in transport from the Golgi. Our model for the role of *T. gondii* AP-1 in protein transport is illustrated in Fig. 11.

We had previously suggested (24, 39) that rhoptries are lysosome-like organelles, analogous to melanosomes and platelet-dense granules (11, 12). This raised the possibility that ROP2 was sorted by AP-3, especially because of the acidic “AP-3-like” YXXØ binding motif (EYEQL) (23) within the ROP2 cytoplasmic tail. Three lines of evidence argue against a role for AP-3 in rhoptry protein transport. First and by far the most important, no μ3 or β3 chains are present in the *T. gondii* genome (of interest, the same is true in the closely related *Plasmodium falciparum*). Second, as shown by two-hybrid analysis, ROP2 (13) and ROP4 (5) bind substantially less well to human μ3 than to human μ1. Finally, a chimera between the ROP2 ectodomain/transmembrane domain and the cytoplasmic tail of human tyrosinase (targeted to melanosomes in an AP-3-dependent fashion) (40) did not localize to rhoptries when expressed in *T. gondii*. (6) Taken together, these results implicate AP-1 rather than AP-3 in the transport of rhoptry proteins from a post-Golgi endosomal compartment to mature rhoptry organelles. The absence from the *T. gondii* genome of other adaptors that mediate endocytic transport, including GGA proteins, stonins, and β-arrestins (26), lends further credence to the argument that Tgα1 is responsible for protein transport to the *T. gondii* rhoptry.

The crystal structure of a rat μ2 fragment (residue 158–435) bound to tyrosine-containing peptides resolved the critical residues that interact with the aromatic ring (Phe174 and Trp421) and phenolic hydroxyl group (Asp176, Lys203, and Arg423) of the critical tyrosine (18). An independent mutational analysis of dominant-interfering μ2 confirmed in vitro that Asp176 and Trp421 are critical for the internalization of TGN38 and the epidermal growth factor receptor (30), albeit no changes in the cell morphology or protein localization were detected. We adopted this mutational strategy to analyze the role of the adaptin μ1 chain in mediating protein sorting in *T. gondii*. The partial phenotype of the dominant-interfering D176A mutant in *T. gondii* is almost certainly due to the presence of endogenous adaptin μ1 chain and also to the capacity of other residues (Phe174, Lys203, and Arg417 in *T. gondii*) within Tgα1 to interact with the YXXØ motif. Although this partial phenotype might not have been apparent in other eukaryotic cells, the unique structure and polarized distribution of rhoptries allowed us to uncover the sorting defect.

It is now well established that the antisense RNA strategy inherently does not generate complete inhibition or complete loss of function of a gene and therefore is not suitable for the creation of a null mutant (41–43). Targeted depletion of the *T. gondii* adaptin μ1 chain by antisense RNA allowed the stable clone to survive through at least three passages of the host cell prior to our isolation and analyses of single clones. At this

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5 M. Yang, I. Coppens, S. Wormsley, H. C. Hoppe, and K. A. Joiner, unpublished data.
6 K. Paprotka and K. A. Joiner, unpublished data.
stage, the mutants grew at an irregular and slow rate, and depletion of Tgα1 eventually resulted in the substantial abortion of parasite vacuoles in the host cell. On the other hand, when maintained in continuous passage, μl levels returned to those in wild-type parasites, and normal growth and morphology were restored, similar to what we observed in clones depleted of ROP2 by antisense mRNA. Protein recovery that is accompanied by loss of phenotypes further supports the specificity of defects due to Tgα1 depletion.

We have now identified two separate circumstances in which blocking transport of ROP2 to the mature rhoptry alters rhoptry morphology (this work). These results are most consistent with a model in which transmembrane ROP2 (and perhaps other associated proteins) is required for establishing and maintaining structural integrity of the rhoptry. Whether components analogous to ROP2 exist in other lysosome-like organelles and whether their transport is AP-1-dependent remains to be determined.

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