Toxoplasma gondii Rab6 Mediates a Retrograde Pathway for Sorting of Constitutively Secreted Proteins to the Golgi Complex*

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Toxoplasma gondii relies on protein secretion from specialized organelles for invasion of host cells and establishment of a parasitophorous vacuole. We identify T. gondii Rab6 as a regulator of protein transport between post-Golgi dense granule organelles and the Golgi. Toxoplasma Rab6 was localized to cisternal rims of the late Golgi and trans-Golgi network, associated transport vesicles, and microdomains of dense granule and endosomal membranes. Overexpression of wild-type Rab6 or GTP-activated Rab6(Q70L) rerouted soluble dense granule secretory proteins to the Golgi and endoplasmic reticulum and augmented the effect of brefeldin A on Golgi resorption to the endoplasmic reticulum. Parasites expressing a nucleotide-free (Rab6(N124I)) or a GDP-bound (Rab6(T25N)) mutant accumulated dense granule proteins in the Golgi and associated transport vesicles and displayed reduced secretion of GRA4 and a delay in glycosylation of GRA2. Activated Rab6 on Golgi membranes colocalized with centrins during mitosis, and parasite clones expressing Rab6 mutants displayed a partial shift in cytokinesis from endodyogeny (formation of two daughter cells) to endopolygeny (multiple daughter cells). We propose that Toxoplasma Rab6 regulates retrograde transport from post-Golgi secretory granules to the parasite Golgi.

The Golgi complex coordinates secretory protein maturation and sorting and is a central intermediary of bidirectional transport between exocytic and endocytic pathways. In most protozoa, the early secretory pathway is well conserved in that of other eukaryotes, performing the critical function of biosynthetic transport to organelles, the cell surface, and the extracellular environment. Members of Apicomplexa, a diverse phylum of obligate intracellular parasites, are distinct from other eukaryotes in harboring three unique polarized secretory organelles, termed micronemes, rhoptries, and dense granules. As exemplified in Toxoplasma gondii, sequential secretion from these organelles is essential for host cell invasion and the concomitant formation of an intracellular parasitophorous vacuole (PV) enveloping the parasite (1). Micronemes and rhoptries are apically tethered and secreted during attachment and penetration of a host cell. Dense granules in Toxoplasma secrete aggregates of soluble and transmembrane proteins constitutively at a basal level and are stimulated for enhanced release following parasite invasion, suggesting a regulated, but Ca2⁺-independent, component (2).

Immunohistochemical analysis indicates that the sorting of dense granule proteins from those targeted to micronemes and rhoptries occurs at the late Golgi cisternae or trans-Golgi network (TGN). Although rhoptries originate from a precursor organelle formed by the Golgi during parasite division, the process regulating biogenesis of Toxoplasma dense granules is completely unknown. Two hypotheses are proposed to account for the biogenesis of regulated secretory vesicles in specialized mammalian cells (reviewed in Ref. 3). In the selective aggregation model, secretory proteins aggregate in the TGN and are sorted for entry into secretory granules by receptors in the TGN membrane. Proteins that fail to aggregate may exit the TGN independently in constitutive secretory vesicles. In the sorting-by-retention model, regulated proteins are retained and sorted from constitutive secretory cargo after formation of immature secretory granules. In this model, immature secretory granules budding from the TGN contain the bulk of biosynthetic cargo and undergo maturation by selective budding of constitutive secretory vesicles containing non-aggregate proteins. Toxoplasma dense granule protein aggregates may dissociate and insert post-translationally into target membranes only after secretion into the PV (4–6). Understanding the biogenesis of Toxoplasma dense granules and the mechanisms involved in regulating dense granule protein transport will provide fundamental insight into the minimal requirements for sorting between regulated and constitutive secretory pathways in eukaryotes.

Molecular evidence and genomic sequencing efforts indicate that the molecular machinery regulating vesicular transport in Apicomplexa is partially conserved in other eukaryotes, particularly early in the secretory pathway. Morphologically, these protozoa possess an endoplasmic reticulum (ER) that is contiguous with the nuclear envelope and a Golgi apparatus that ranges from single dispersed cisternae in malarial parasites (genus Plasmodium) to a stacked apically oriented Golgi in Toxoplasma. Components of the vesicle budding, transport, and fusion machinery have been cloned in Toxoplasma, including N-ethylmaleimide-sensitive fusion protein, multiple Rab

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proteins (7), ARF1 (8), and subunits of the coatomer (9) and adapter complexes (10). Evolutionarily conserved sorting motifs function in the transport of proteins to rhotopins and microtubules (10, 11). In contrast, targeting motifs have not been found in dense granule proteins, and heterologous soluble proteins expressed with an N-terminal signal sequence are constitutively secreted into the PV through dense granules (12).

To further define regulation of dense granule secretion, we sought to identify molecular effectors of dense granule protein transport. In conjunction with effector proteins, monomeric GTPases of the Rab family localize to distinct intracellular compartments and confer one level of specificity on vesicle transport and fusion by mediating tethering or docking of opposing membranes prior to pairing of SNAREs. The small GTPase Rab6 appears to function in intra-Golgi transport (13, 14) and COPI/RARF1-independent retrograde transport from the Golgi to the ER in mammalian cells (15, 16). The yeast homolog of Rab6, Ypt6p, was initially reported to function in the Golgi to the ER in mammalian cells (15, 16). The yeast homolog of Rab6, Ypt6p, was initially reported to function in the Golgi to the ER in mammalian cells (15, 16). The yeast homolog of Rab6, Ypt6p, was initially reported to function in the Golgi to the ER in mammalian cells (15, 16).

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**Fig. 1. Molecular analysis of T. gondii rab6.** A, Southern analysis of 10 µg of gDNA derived from *T. gondii* and host Vero cells. Lane M, molecular size standards in kilobase pairs (kbp); lane 1, *T. gondii* HindIII digest; lane 2, Vero HindIII digest; lane 3, *T. gondii* NcoI digest; lane 4, Vero NcoI digest; lane 5, *T. gondii* HindIII and NcoI digest; lane 6, Vero HindIII and NcoI digest. B, left panel, ethidium bromide-stained gel of total *T. gondii* (T) and Vero (V) cellular RNAs. Lane M, RNA ladder. Right panel, Northern blot analysis results indicating a mature *T. gondii* Rab6 mRNA of ~1.3 kb. C, phylogenetic analysis of Rab6 protein homologs. Parsimony analysis using the PAUP program was performed on the alignment in D corresponding to amino acids 6–185 of *T. gondii* Rab6 (180/209 amino acids), excluding the divergent amino and carboxyl termini. A single most parsimonious tree (shown) was found. Bootstrap analysis was then run using 1000 replicates of the alignment, and a consensus tree encompassing 20 trees was produced. Values at tree nodes represent bootstrap confidence values supporting nodal placement. A single most parsimonious tree (shown) was found. Bootstrap analysis was then run using 1000 replicates of the alignment, and a consensus tree was produced. Values at tree nodes represent bootstrap confidence values supporting nodal placement.

Vero cells were subjected to 12% SDS-PAGE and prepared for immunoblotting or GTP binding by electrophoretic transfer to nitrocellulose membranes. Immunoblot analysis was performed with the ECL detection system (Amersham Biosciences) using, as primary antibody, rabbit serum (Zymed Laboratories, Inc.) immunized with a synthetic peptide encompassing *T. gondii* Rab6 (192–206) and displaying strong enzyme-linked immunosorbent assay activity. Membranes were exposed on X-Omat AR autoradiography film (Eastman Kodak Co.). Scanned images were semiquantitatively analyzed with Scion Image software.

**GTP Overlay Assay of Recombinant T. gondii Rab6**—Nitrocellulose transfer blots containing crude recombinant *T. gondii* HA-Rab6, HA-Rab6(Q76L), and HA-Rab6(N124I) proteins were analyzed for GTP-binding activity as previously described (26), except that phosphate buffer was substituted for Tris-HCl in the binding buffer (50 mM Na2HPO4/NaH2PO4, pH 7.0, 5 mM MgCl2, 1 mM EGTA, and 0.3% Tween 20). Blots were preincubated in binding buffer supplemented with 1 mM ATP for 30 min. [γ-32P]GTP was added to a concentration of 1 µCi/ml and incubated for 2 h, and the blots were washed with several changes of binding buffer for 2 h. Gels were dried and exposed at ~70 °C on X-Omat AR film.

**Immunofluorescence Microscope Analysis**—Human foreskin fibroblast cell monolayers grown on coverslips were infected with freshly transfected (106 cells/coverslip) or untransfected (105 cells/coverslip) *T. gondii* cells for 16 h at 37°C. Coverslips were prepared for immunofluorescence assay as previously described (12). Primary antibodies included mouse anti-HA-11 (Covance), rabbit anti-BAP (5 Prime → 3 Prime, Inc.), anti-IMC1 monoclonal antibody (mAb) 45.5 (gift of Gary Ward, University of Vermont, Burlington, VT), and anti-GRA1 mAb T4-1F5 (27). Secondary antibodies included fluorescein isothiocyanate-conjugated sheep anti-mouse IgG (Calbiochem), rhodamine-conjugated goat anti-rabbit IgG (Calbiochem), and Alexa-fluor conjugated equivalents (Molecular Probes, Inc.). Epi-fluorescence and phase-contrast microscope images were captured on a Photometrics SenSys CCD camera and processed using Image Pro Plus software (Media Cybernetics).

**Electron Microscopy**—For immunohistochemistry, infected Vero cell monolayers were fixed with 8% paraformaldehyde in 0.25% HEPES, pH 7.4. Sections (95 nm) were obtained from the Yale Center for Cell Imaging. Immunolabeling was performed with mouse anti-HA mAb (1:100; Babco), followed by rabbit anti-mouse antibody (1:50; Cappel or ICN) and protein A-gold (1:70; J. Slot, University of Utrecht, Holland). The sections were contrasted with neutral uranyl acetate (2%), infiltrated with methyl cellulose (1.8%) and uranyl acetate (0.5%), and examined with a Philips 410 transmission electron microscope. For transmission electron microscopy, infected Vero cell monolayers were fixed in 8% paraformaldehyde and 100 mM cacodylate buffer for 2 h. Blocks were prepared for Epon embedding by the Yale Center for Cell Imaging. Ultrathin 60–80-nm sections were collected on grids, con-
trusted with lead citrate and uranyl acetate, and examined as described above.

**Metabolic Labeling and Quantitative Immunoprecipitation**—Parasite cultures were washed three times with cysteine/methionine-free minimal essential medium (Invitrogen) and metabolically labeled in 1 ml of minimal essential medium containing 5% fetal bovine serum and 100 μCi of [35S]Met/Cys (Promix, Amersham). For analysis of BAP and GRAD2 secretion, parasites were labeled for 30 min. For pulse-chase analysis of GRA4, parasites were labeled for 10 min and chased after three washes for 0, 15, and 30 min. Parasites were freed from host cells by syringe passage in 1 ml of phosphate-buffered saline containing proteinase inhibitors (2) and centrifuged at 1400 × g for 10 min at 4 °C. Cell pellets were lysed in radioimmune precipitation assay buffer (24) containing Complete protease inhibitor mixture (Roche Molecular Biochemicals). Supernatants following the 1400 × g spin (containing T. gondii soluble secreted proteins of the vacuolar space) were used for immunoprecipitation after addition of Nonidet P-40 to 1% as previously described (25) with antibody to BAP, GRA4 (mAb T8-4B9), or GRA2 (mAb T4-1F5) as described above. Gels were dried and exposed to Biomax film (Kodak). Images were scanned and quantitated using Scion Image software.

**RESULTS**

**Identification and Phylogeny of the T. gondii Rab6 Homolog**—Because Rab6 functions in Golgi transport in yeast and mammalian cells, we sought to identify this protein in Toxoplasma as a potential regulatory target for secretory protein trafficking. Combinatorial RACE/PCR amplification was used to clone a T. gondii rab6 cDNA. In genomic digests, a rab6 cDNA probe strongly hybridized to a single T. gondii restriction fragment in each case (Fig. 1A), indicating that rab6 is a single-copy gene in T. gondii. Northern blot analysis indicated a major RNA transcript size of ~1.3 kb, along with a larger, possibly unprocessed transcript (Fig. 1B). In neither case did the rab6 probe hybridize to nucleic acid controls derived from host cell cultures, confirming that Rab6 is of parasite origin.

The Toxoplasma rab6 cDNA encodes a 209-amino acid protein (Fig. 1D) with a predicted molecular mass of 23.7 kDa. Of the available sequences, the Toxoplasma Rab6 protein is most homologous to Rab6 of the Apicomplexa malarial parasites Plasmodium falciparum and Plasmodium berghei (76–77% similarity and ~72% identity using BESTFIT analysis). The phylogenetic relationship of T. gondii Rab6 to other Rab6 homologs was examined by alignment (Fig. 1D) and parsimony analysis using the PAUP program (Fig. 1C). The Rab family domains are well conserved, including the nucleotide-binding pocket domains and switch regions, which confer nucleotide-dependent conformational changes and interaction with Rab effectors (Fig. 1D). Distinct from Saccharomyces and Plasmodium, Toxoplasma Rab6 encodes a conservative Ser85 residue within a motif essential for interaction with a cytoskeleton-associated Rab6 effector, Rabbisn6-6 (29). The hypervariable C-terminal region and extreme N terminus of Rab6, important in conferring functional specificity and intracellular distribution between Rab proteins, are divergent among Rab6 homologs. A single most parsimonious phylogenetic tree, well supported by bootstrap analysis, was generated with representatives of animal, plant, and fungal Rab6 homologs (Fig. 1C).

Recombinant T. gondii HA-Rab6 and HA-Rab6(Q70L), but Not HA-Rab6(N124I), Are Competent GTP-binding Proteins—To initiate a functional analysis of T. gondii Rab6, we developed expression plasmids encoding Toxoplasma Rab6 and Rab6 GTP-binding domain mutants predicted to be altered in their GTP-binding affinity. An N-terminal 9-HA epitope tag was fused to the Toxoplasma rab6 ORF or to point mutants Rab6(Q70L) and Rab6(N124I). Rab6(Q70L) is predicted to be deficient in GTPase activity, whereas Rab6(N124I) is predicted to lack affinity for guanine nucleotide, based upon homology to known Ras-related protein mutations (30, 31). Soluble bacterial lysates were probed by immunoblotting with antiserum to a synthetic peptide (T. gondii Rab6-(192–206) (Fig. 2B, lanes 2–4) or with antibody to the HA epitope (data not shown); in

**Fig. 2. Western and GTP blot assays of recombinant Toxoplasma Rab6 and Rab6 mutant proteins.** A. protein gel of bacterially expressed Rab6 proteins induced by isopropyl-β-D-thiogalactosidase. Lane M, molecular mass standards; lane 1, pET15b expression control; lane 2, HA-Rab6; lane 3, HA-Rab6(Q70L); lane 4, HA-Rab6(N124I). Induced protein at ~30 kDa is visible in Rab6 expression lysates. B, Western blot analysis of gel in A probed with rabbit anti-T. gondii Rab6(192–206). The 30-kDa band is present in Rab6 lysates (lanes 2–4), but not in control pET15b lysate (lane 1). C, [α-32P]GTP blot overlay of identical gel run in parallel to that in A.

**Fig. 3. Toxoplasma HA-Rab6 and HA-Rab6(Q70L) localization in parasites and Golgi localization in CHO cells.** For orientation, the apical end of a single tachyzoite in each transfected parasite vacuole is labeled with an asterisk. Transiently expressed HA-Rab6 (A) and HA-Rab6(Q70L) (C) were localized to the single T. gondii Golgi stack (arrows), cytoplasm, and dense granules (arrows with asterisks). The corresponding phase-contrast images for A and C are shown in B and D. Transiently expressed T. gondii Rab6 partially colocalized with giantin (E and F) and GRASP65 (H–J) in CHO cells. T. gondii HA-Rab6 transiently expressed in CHO cells was localized to a juxtanuclear compartment (E and H). Also shown is the cisternal labeling of the Golgi with giantin (F) and with GRASP65 (I). Merged images show colocalization of Toxoplasma Rab6 (red) with giantin (green) (G) or with GRASP65 (green) (J) along Golgi cisternae.
each case, proteins with an apparent molecular mass of 30 kDa were recognized. GTP-binding activity was assessed for HA-Rab6, HA-Rab6(Q70L), and HA-Rab6(N124I). Both HA-Rab6 (Fig. 2C, lane 2) and HA-Rab6(Q70L) (lane 3) bound [γ-32P]GTP nearly equally, but HA-Rab6(N124I) (lane 4) did not bind detectable amounts of GTP, confirming the anticipated GTP-binding characteristics of the mutants.

**Toxoplasma Rab6 Localizes to the Golgi in Both Parasite and CHO Cells**—In transiently or stably transfected *Toxoplasma* tachyzoites, both HA-Rab6 (Fig. 3A) and HA-Rab6(Q70L) (Fig. 3C) proteins localized, as determined by indirect immunofluorescence assay, predominantly to a discrete irregular structure apical to the parasite nucleus. HA-Rab6 signal was detected in lower amounts throughout the parasite cytoplasm and in one or more dense granules (Fig. 3, A and C). We reasoned that Rab6 motifs necessary for Golgi localization would be functionally conserved among eukaryotes, given the general ability to functionally complement subsets of Rab proteins between yeast and mammals. To compare the distribution of *Toxoplasma* Rab6 within a higher organism, *T. gondii* Rab6 was transiently expressed in CHO-K1 cells and localized with endogenous markers, including the Golgi-associated tethering proteins giantin and GRASP65, the ER protein calnexin, and mannose 6-phosphate receptor protein, a marker principally of late endosomes. In addition to labeling a cytosolic pool, *T. gondii* HA-Rab6 localized in a juxtanuclear pattern typical of the Golgi (Fig. 3, E and H) and colocalized in this region with giantin (Fig. 3, E–G). Likewise, HA-Rab6 signal overlapped GRASP65 on the Golgi (Fig. 3, H–J). In contrast, HA-Rab6 exhibited minimal colocalization with the mannose 6-phosphate receptor and calnexin (data not shown).

To more precisely define Rab6 localization in *Toxoplasma*, HA-Rab6(Q70L) parasite clones were subjected to immuno-electron microscopy (Fig. 4, A–E). Activated Rab6 was most heavily concentrated on late *Toxoplasma* Golgi cisternae (Fig. 4, A and F). Specifically, Rab6 was distributed on the apical (trans) cytosolic face of the late Golgi cisternae and the TGN, on the cisternal rims of the trans- and medial-Golgi cisternae, and on peripheral vesicles closely associated with the cisternal rims. Labeling of the cis-Golgi, the intermediate compartment between the Golgi and the nuclear envelope, and the transitional ER of the nuclear envelope was minimal. Rab6 also strongly labeled microdomains on membranes of a subset (~42%, n = 38) of dense granule sections (Fig. 4, B, C, and E, arrows). Rab6 was sporadically associated with microdomains on membranes of large electron lucent vesicles, putatively of endosomal function, but was not associated with membranes of mature rhoptries, micronemes, apicoplasts, or mitochondria (Fig. 4, A and E). This distribution implicates activated Rab6 in a transport process to the late Golgi in *Toxoplasma*, possibly involving dense granules.

**Localization of Toxoplasma Rab6** Rab6 in both the parasite (Fig. 5) and transiently transfected CHO cells (data not shown) was also sensitive to the fungal metabolite brefeldin A (BFA), an inhibitor of the ARF1 GTPase exchange protein. Following addition of 5 μg/ml BFA, transiently expressed HA-Rab6 (Fig. 5C) collapsed to the perinuclear envelope, along with a reticular and cytosolic labeling pattern throughout the parasite (Fig. 5, F and D). Activated HA-Rab6(Q70L) was likewise dispersed by BFA treatment to a prominent perinuclear and reticular pattern, suggesting a complete collapse of the parasite Golgi into the ER, similar to tubulovesicular retrograde Golgi-derived elements in mammalian cells. In total, these results indicate that *Toxoplasma* Rab6 contains evolutionarily conserved Golgi-targeting signals, despite significant C-terminal divergence of Rab6 proteins.

**Fig. 4. Immunolocalization of Rab6 in T. gondii HA-Rab6(Q70L) clones.** Immunogold labeling was enriched at the lateral ends of the trans-most Golgi (Go) cisternae (A), on microdomains of dense granule (DG) membranes (B, C, and E), on lucent vacuole membranes (E), and associated with cytoplasmic centrosomes (Ce) (D). No labeling was observed in rhoptries (Rh), micronemes (Mi), apicoplasts (Ap), and the nucleus (Nu). Control cryosections of wild-type parasites with equivalent antibody dilutions were completely free of immunogold label (data not shown). Also shown is the frequency distribution of HA-Rab6(Q70L) antigen (Ag) on cryosections (F). 10 random cryosections encompassing 34 parasites were quantitated for total gold distribution. DG, dense granules; cytopl + sv, labeling of the cytoplasm and small vesicle (these were co-quantitated because vesicle membranes were frequently difficult to differentiate); ER + tER, labeling of the ER and perinuclear envelope; ERGIC, intermediate compartment between the Golgi and nuclear envelope; endosome, electron lucent vacuoles; mic, micronemes; rhop, rhoptries; mito, mitochondrion; apicoplast, apicoplast; IMCPM, inner membrane complex and plasma membrane.

**Transientsly Overexpressed Toxoplasma Rab6 and Rab6 Mutants Block Transport of Dense Granule Proteins**—To examine the role of *Toxoplasma* Rab6 in transport and secretion of dense granule proteins, HA-Rab6, HA-Rab6(Q70L), and HA-Rab6

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**Rab6 Function in Toxoplasma 5437**

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**Transiently Overexpressed Toxoplasma Rab6 and Rab6 Mutants Block Transport of Dense Granule Proteins**—To examine the role of Toxoplasma Rab6 in transport and secretion of dense granule proteins, HA-Rab6, HA-Rab6(Q70L), and HA-Rab6
(N124I), or GDP-restricted HA-Rab6(T25N) were coexpressed along with the secretory reporter protein BAP. This protein, fused to the nucleoside-triphosphate hydrolase-3 signal sequence, behaves as a soluble secreted dense granule protein in Toxoplasma tachyzoites (12). Secretory BAP localized to dense granules and the PV upon secretion (Fig. 5, A and D). In the presence of transiently overexpressed HA-Rab6 (Fig. 5B) or BFA (Fig. 5, D and G), BAP secretion into the PV and distribution in dense granules were partially inhibited, with signal accumulating in the Golgi and the ER-associated perinuclear envelope. Rab6 overexpression augmented the BFA-induced retrograde appearance of BAP in the parasite ER within the first 15 min of treatment (Fig. 5J).

The role of activated and inhibitory Rab6 mutants in secretory transport was assessed by transient overexpression in transfected parasites. GTP-activated mutant HA-Rab6(Q70L) (Fig. 6A) induced a similar but more pronounced accumulation of BAP in the Golgi and the ER-associated perinuclear envelope (Fig. 6B) in comparison with overexpressed wild-type Rab6. HA-Rab6(N124I) was cytosolic in distribution (Fig. 6D), whereas HA-Rab6(T25N) was partially Golgi-localized (Fig. 6G). Each of these mutants induced dispersal of BAP within the parasite in a small vesicular and reticular pattern independent of dense granules (Fig. 6, E and H), with BAP accumulating at or near the late Golgi in the case of HA-Rab6(T25N) (Fig. 6H), although secreted BAP was still detectable in parasite vacuoles (Fig. 7A). Transient overexpression of HA-Rab6(N124I) was toxic because parasite vacuoles often contained rounded degenerative bodies of atypical parasite morphology (data not shown).

Parasites expressing Rab6 mutants were further analyzed for transport of endogenous dense granule proteins GRA1–4. In the case of HA-Rab6(T25N), GRA1 accumulated in the same Golgi-proximal anterior compartment (Fig. 7B, arrows), colocalizing with BAP (Fig. 7C). Although BAP was dispersed from dense granules, these remained weakly positive for GRA1; both secreted proteins were still apparent in the PV (Fig. 7, B and C, near the asterisks). Identical results were obtained when parasites were labeled with endogenous GRA2–4, suggesting an attenuated effect on transport of dense granule protein aggregates in comparison with soluble secretory BAP. In contrast, transport of glycosylphosphatidylinositol-anchored proteins to the cell surface and microneme or rhoptry proteins from the ER and Golgi was uninhibited in the presence of the Rab6 mutants (data not shown). Altogether, these results indicate that alteration of Rab6 function by overexpression leads to partial disruption in the transport of constitutively secreted proteins between dense granules and the Golgi.

Dense Granule Secretion Is Differentially Inhibited in Transient and Stable Rab6 Mutants—To determine whether bulk secretion into the PV was altered by Rab6 levels, secretion of BAP was quantitatively assessed in intracellular parasites by metabolic pulse labeling. BAP secreted from parasites transiently overexpressing HA-Rab6 or HA-Rab6(Q70L) was ~50–60% of that secreted from parasites expressing BAP alone, whereas expression of HA-Rab6(N124I) reduced BAP secretion by ~10% (Fig. 8A), despite the loss in labeling of dense granules. This result suggests that constitutive secretion may not require transport through dense granules, although this is the default route in wild-type parasites (2, 12). We then examined

Fig. 5. Overexpressed Rab6 augments the brefeldin A-induced retrograde transport of dense granule proteins. A, in untreated parasites, BAP was secreted through dense granules and accumulated in the PV. In B–I, treatment with BFA induced intracellular retention, ultimately accumulating BAP in the perinuclear ER. In parasites transiently overexpressing HA-Rab6 (C, arrows), BAP secretion through dense granules was partially inhibited, accumulating in the Golgi and perinuclear ER (B, arrows and asterisks). Both BAP (E and H) and transiently expressed HA-Rab6 (F and I) were partially collapsed to the perinuclear ER following treatment with 5 μg/ml BFA for 15 or 60 min. In J, shown is the quantitation of the appearance of BAP in the parasite ER following BFA (5 or 20 μg/ml) addition. Perinuclear and retrograde labeling was more pronounced and rapidly induced in cells overexpressing HA-Rab6.
the effect of low level expression of Rab6 mutants in tachyzoites by isolating stable parasite clones coexpressing HA-Rab6(Q70L) or Rab6(N124I) along with BAP. These clones retained endogenous Rab6 along with a single integrated copy of the mutant proteins expressed from the low level tachyzoite Sag1 promoter (data not shown). In contrast to parasites transiently overexpressing HA-Rab6(Q70L), BAP secretion was essentially unaltered in HA-Rab6(Q70L) clones, whereas HA-Rab6(N124I) parasite clones exhibited a similar attenuation upon secretion of BAP compared with those transiently expressing the mutant (n = four independent experiments) (Fig. 8B).

To confirm these results for endogenous dense granule proteins, we examined the secretion of GRA4 by quantitative immunoprecipitation in parasite clones expressing HA-Rab6(Q70L) or HA-Rab6(T25N). Once again, the activated Rab6 mutant had little effect on dense granule secretion compared with untransfected parasites, whereas stable expression of the dominant inhibitory mutant was attenuated upon secretion of GRA4 (Fig. 8C). To address the possibility that protein modification in the parasite Golgi could be altered in the presence of the Rab6 mutants due to retrograde imbalance of glycosylation enzymes, the O-linked glycosylation of GRA2 was examined by metabolic pulse-chase. Within minutes of synthesis, GRA2 is modified by O-linked glycosylation from an ~26-kDa precursor to an ~28-kDa mature protein (27, 32). At 15 min of chase, a kinetic lag was observed in the mobility of GRA2 in parasites stably expressing HA-Rab6(T25N), whereas GRA2 mobility in parasites expressing HA-Rab6(Q70L) was unaltered compared with wild-type parasites (Fig. 8D). By 30 min of chase, nearly 100% of GRA2 was modified in all parasite clones (Fig. 8D). In summary, elevated overexpression of wild-type and GTP-activated Rab6 blocks constitutive protein secretion, whereas GDP-bound and nucleotide-free Rab6 mutants appear to attenuate dense granule protein transport and glycosylation. Consequently, Rab6 appears to regulate a retrograde pathway involving transport of dense granule proteins to the Golgi.

**Rab6 Marks Golgi Fission, and Rab6 Mutants Alter Cytokinesis in Toxoplasma Tachyzoites**—Upon both plaque assay and uracil incorporation analysis, growth or invasiveness of activated and inhibitory Rab6 clones was reduced in comparison with wild-type parasites (Fig. 9). Although Rab6 mutants did not alter transport of rhoptry proteins to the post-Golgi organelle, HA-Rab6(Q70L) and HA-Rab6(N124I) parasites were enriched in tubulovesicular precursor rhoptry organelles (data not shown). Because rhoptry biogenesis is itself developmentally regulated, we examined the parasite clones for developmental alterations. Daughter Toxoplasma merozoites form in pairs within the maternal parasite by a process of internal budding termed endodyogeny. This process is initiated through a pair of cytoplasmic centrosomes, which form the intranuclear spindle poles and behave as a microtubule-organizing center (33). During mitosis, the Golgi stacks partition in two near the centrosomes. This was easily visualized in parasites expressing Golgi-localized HA-Rab6(Q70L). The medial fission of the Golgi stacks occurred in close juxtaposition with the duplicated centrosomes and appeared to be complete prior to nuclear division (Fig. 10, K–N). During cytokinesis, activated Rab6 and centrin were perfectly colocalized (Fig. 10M, arrow with asterisk), suggesting a tethering of the late Golgi with the centrosomes.

Concurrently with Golgi partitioning, a scaffold for daughter cell assembly is formed from subpellicular microtubules and an associated network of flattened membranes, the IMC. During cytokinesis, the IMC migrates posteriorly toward and around each nuclear pole, partitioning the replicated nucleus into the two forming daughters. Strikingly, some HA-Rab6(Q70L) and HA-Rab6(N124I) parasite vacuoles exhibited unusual patterns of division, forming multiple daughters (Fig. 10A), indicative of replication by endodyogeny rather than endodyogeny. The IMC of each daughter enveloped a complete set of organelles, including Golgi, apicoplasts, and pre-rhoptries, indicating that appropriate organellar replication and segregation were occurring concurrent with nuclear division. The frequency of endodyogeny was quantitated using antibody to IMC1, an antigen of the IMC

**Fig. 6.** Transient overexpression of Rab6 mutants in *T. gondii* inhibits trafficking of BAP to dense granules and secretion into the PV. In parasites overexpressing GTP-activated HA-Rab6(Q70L) (A), BAP accumulated in the Golgi, perinuclear envelope, and ER (B, arrow). In parasites transiently overexpressing HA-Rab6(N124I), which is predominately cytosolic (D), BAP accumulated in a dispersed vesicular pattern throughout the parasite (E). In parasites transiently overexpressing HA-Rab6(T25N) (G), BAP was dispersed in vesicles and accumulated at or juxtaposed to the Golgi (H, arrow). Corresponding phase-contrast images are shown in C, F, and I.

**Fig. 7.** Rab6(T25N) alters transport of endogenous dense granule proteins and BAP. A, BAP in parasites coexpressing Rab6(T25N) was diffuse and accumulated near the Golgi (punctate label centralized in each tachyzoite). B, GRA1 partially accumulated near the Golgi with reduced dense granule labeling. C, merged images show colocalization (yellow) of BAP (red channel) and GRA1 (green channel) in the region of the Golgi and after secretion into the PV (near the asterisk). D, shown is a phase-contrast image. Identical distributions were evident for dense granule proteins GRA2–4 in parasites transfected with Rab6(T25N) or Rab6(N124I).
in both adult and forming daughter parasites, thus serving as a hallmark of daughter cell formation by microscopy (34). In nearly all untransfected tachyzoites, parasites budded by endodyogeny, with two forming daughters distinguishable (Fig. 10B), although on rare occasions (2% of all vacuoles containing dividing parasites) (Fig. 10F), multiple daughter parasites could be found in a single parent. However, in HA-Rab6(Q70L) (data not shown) and HA-Rab6(N124I) (Fig. 10E–J, arrows) clones, vacuoles harboring parasites replicating by endopolygeny were 5 and 10 times more common, respectively (Fig. 10F). These results suggest that altered Rab6 function enhances uncoupling of cytokinesis and karyokinesis in developing parasites.

**DISCUSSION**

The highly polarized secretory pathway of *T. gondii*, complete with a single stack of apically oriented Golgi cisternae, provides a simple model for protein transport in eukaryotes. We have identified the *T. gondii* homolog of the highly conserved GTPase Rab6 as the first marker of the parasite Golgi and as a potential mediator of retrograde transport from dense granule secretory organelles. Whereas Rab6 paralogs function in retrograde transport from post-Golgi-, intra-Golgi-, and Golgi-to-ER transport pathways in mammalian cells, and Ypt6p functions in recycling of Golgi proteins from the endosomal pre-vacuole in yeast, *Toxoplasma* Rab6 appears to regulate in part post-Golgi transport of constitutively secreted proteins to the parasite Golgi. Our results further suggest that Rab6 function may be required for normal cytokinesis or that parasite cell division may be sensitive to perturbation of the Rab6-mediated retrograde pathway.

We propose that the role of Rab6 in *Toxoplasma* dense granule protein secretion in *T. gondii* transiently overexpressing Rab6 GTP-binding domain mutants. A, inhibition of BAP secretion in parasites transiently overexpressing HA-Rab6 or HA-Rab6 mutants. Shown are the results from quantitative immunoprecipitation of intracellular and secreted BAP protein in parasites transiently expressing BAP alone or coexpressing BAP and HA-Rab6 or HA-Rab6 mutants and expressed as fractions secreted over 60 min. B, BAP secretion in *Toxoplasma* HA-Rab6(Q70L) and HA-Rab6(N124I) stable clones. Shown are the results from quantitative immunoprecipitation of intracellular and secreted BAP protein in parasites stably expressing BAP alone or coexpressing BAP and HA-Rab6(Q70L) or HA-Rab6(N124I) and expressed as fractions secreted over 60 min. The results shown are representative of four independent experiments with two clonal lines for each Rab6 mutant. C, GRA4 secretion in *Toxoplasma* HA-Rab6(Q70L) and HA-Rab6(T25N) stable clones. Upper panel, 40-kDa GRA4 immunoprecipitates from intracellular and PV fractions as follows. Lanes 1 and 2, hypoxanthine-xanthine-guanine phosphoribosyltransferase (HXGPRT)-knockout parental strain intracellular and secreted fractions; lanes 3 and 4, Rab6(Q70L) clone intracellular and secreted forms; lanes 5 and 6, Rab6(T25N) clone intracellular and secreted fractions. Lower panel, quantitation of GRA4 immunoprecipitates expressed as fractions secreted over 60 min. D, glycosylation of GRA2 in *Toxoplasma* wild-type (WT), HA-Rab6(Q70L), and HA-Rab6(T25N) stable clones. Shown are the results from quantitative immunoprecipitation of intracellular GRA2 expressed as fractions of a 28-kDa mature protein (double asterisks) to a total of a 26-kDa precursor (single asterisk) + a mature form following a 12-min metabolic pulse with or without a chase of 15 and 30 min. Gel results for Rab6(Q70L) (lanes 1–3; 0-, 15-, and 30-min chases, respectively) and Rab6(T25N) (lanes 4–6; 0-, 15-, and 30-min chases, respectively) are shown at the top. P, pellet; S, supernatant.

**Fig. 8.** Rab6 inhibits dense granule protein secretion in *T. gondii* transiently overexpressing Rab6 GTP-binding domain mutants. A, inhibition of BAP secretion in parasites transiently overexpressing HA-Rab6 or HA-Rab6 mutants. Shown are the results from quantitative immunoprecipitation of intracellular and secreted BAP protein in parasites transiently expressing BAP alone or coexpressing BAP and HA-Rab6 or HA-Rab6 mutants and expressed as fractions secreted over 60 min. B, BAP secretion in *Toxoplasma* HA-Rab6(Q70L) and HA-Rab6(N124I) stable clones. Shown are the results from quantitative immunoprecipitation of intracellular and secreted BAP protein in parasites stably expressing BAP alone or coexpressing BAP and HA-Rab6(Q70L) or HA-Rab6(N124I) and expressed as fractions secreted over 60 min. The results shown are representative of four independent experiments with two clonal lines for each Rab6 mutant. C, GRA4 secretion in *Toxoplasma* HA-Rab6(Q70L) and HA-Rab6(T25N) stable clones. Upper panel, 40-kDa GRA4 immunoprecipitates from intracellular and PV fractions as follows. Lanes 1 and 2, hypoxanthine-xanthine-guanine phosphoribosyltransferase (HXGPRT)-knockout parental strain intracellular and secreted fractions; lanes 3 and 4, Rab6(Q70L) clone intracellular and secreted forms; lanes 5 and 6, Rab6(T25N) clone intracellular and secreted fractions. Lower panel, quantitation of GRA4 immunoprecipitates expressed as fractions secreted over 60 min. D, glycosylation of GRA2 in *Toxoplasma* wild-type (WT), HA-Rab6(Q70L), and HA-Rab6(T25N) stable clones. Shown are the results from quantitative immunoprecipitation of intracellular GRA2 expressed as fractions of a 28-kDa mature protein (double asterisks) to a total of a 26-kDa precursor (single asterisk) + a mature form following a 12-min metabolic pulse with or without a chase of 15 and 30 min. Gel results for Rab6(Q70L) (lanes 1–3; 0-, 15-, and 30-min chases, respectively) and Rab6(T25N) (lanes 4–6; 0-, 15-, and 30-min chases, respectively) are shown at the top. P, pellet; S, supernatant.

**Fig. 9.** Replication and survival competence of parasite clones stably expressing HA-Rab6(Q70L) and HA-Rab6(N124I). A, uracil incorporation assay measuring DNA synthesis within *Toxoplasma* cells. After infection for 24 h in host cells, intracellular parasites were labeled with [3H]uracil for 1 h. B, plaque assay determining survival rates after 9 days in culture. *Toxoplasma* plaques were scored relative to 100 parasites plated on human foreskin fibroblast (HFF) host cell monolayers (n = three experiments). Relative to BAP clones, HA-Rab6(Q70L) (42%) and HA-Rab6(N124I) (26%) parasites were maturation-impaired. RH, wild type *Toxoplasma*. 
ule protein transport is retrograde from a post-Golgi compartment, based on several observations. Generally, activated forms of Rab proteins concentrate on the target membrane for vesicle transport. Both wild-type and GTP-activated Rab6 localize to the late Golgi cisternae and inhibit constitutive secretion of dense granule proteins only when highly overexpressed, resulting in Golgi and ER retention of the dense granule proteins. In this instance, Rab6 augments the effect of BFA on retrograde transport to the ER, resulting in resorption of the Golgi cisternae and associated cargo. BFA inhibits GTPase exchange proteins mediating ARF1 recruitment that is essential for COPI-regulated vesicle budding (35).

Toxoplasma ARF1 mutants induce a similar block in the early transport of dense granule proteins (8), indicating that both COPI-mediated and Rab6-mediated pathways function in dense granule protein transport.

In contrast to activated Rab6, dominant inhibitors Rab6(N124I) and Rab6(T25N) alter post-Golgi transport of dense granule proteins, accumulating these proteins in the Golgi and altering glycosylation rates, indicative of an imbalance in Golgi modification enzymes. However, the viability of the mutants and the relatively modest effects on constitutive secretion in stable clones indicate that Rab6 function is dispensable for secretion in Toxoplasma, in common with Ypt6p in yeast. Nonetheless, the specific association of Rab6 with dense granule membranes and small vesicles near the trans-face of the Golgi complex, in addition to the entrapment of dense granule proteins in the Golgi induced by dominant-negative mutants, implicates a transport pathway linking the two organelles. This pathway may serve a recycling function from mature dense granules or alternatively play a direct role in dense granule biogenesis or maturation through protein sorting.

A novel Rab6-mediated Golgi-to-ER retrograde transport in mammalian cells was initially proposed based upon the BFA-like phenotypes of activated Rab6 on Golgi enzymes (14). This pathway was further defined by following transport of Shiga and Shiga-like toxins to the ER (15) and the finding that

![Fig. 10. Rab6 mutants are altered in cytokinesis, and activated Rab6 colocalizes with centrin during cytokinesis.](image-url)
fluorescent protein–Rab6 transits along microtubule tracks between the Golgi and ER sites at the mammalian cell periphery (16). It is thought that this retrograde pathway may regulate recycling of proteins lacking ER retrieval motifs, recycling of lipids, and the slow continuous cycling of resident Golgi proteins. A post-Golgi function for mammalian Rab6 in recycling between early or recycling endosomes and the TGN has now distinguished the highly homologous Rab6A ‘isoform from Rab6A, which is implicated in Golgi-to-ER transport (36). Evidence for Rab6 association with post-Golgi secretory vesicles is less clear, although Rab6 has been postulated to play a role in transport of rhodopsin on post-Golgi vesicles in retinal rod photoreceptor cells (37) and to function at an early step in the biosynthesis of small synaptic vesicles of embryonic neurons (38) and was found to be associated with secretory granules in rat atrial myocytes (39).

The role of the yeast homolog of Rab6, Ypt6p, is increasingly well understood. Yeast late Golgi membrane proteins cycle through a pre-vacuolar endocytic compartment and are retrieved to the vacuole. Ypt6p mutants disrupt the cycling of the endoprotease Kex2p and Vps10p, the sorting receptor for the vacuolar hydrolase carboxypeptidase Y, which is consequently partially missorted to the plasma membrane (40). Recent identification of effector and binding proteins acting on Ypt6p has elucidated a mechanistic role for GTP-activated Ypt6p, the trimeric VPS52-VPS53-VPS54 complex, and the SNARE Tlg1p in sorting and docking of transport vesicles to the late Golgi. The Ypt6p GTP exchange factor has been identified as a stable heteromeric complex of Ric1p-Rgp1p peripherally associated with Golgi membranes (20). After GDP/GTP exchange, activated Ypt6 and binds to microtubules both in vitro and in vivo. Rab6 may regulate the association and dissociation of Rabkinesin-6 with the microtubule cytoskeleton and, as a complex, control movement of Golgi membranes and transport vesicles along microtubules (43). However, Rab6-KIFL is also proposed to function in cell division during cytokinesis (44, 45). Rab6-KIFL was shown to localize to the mid-zone of the mitotic spindle and to the cleavage furrow and midbody during late stages of mitosis, and overexpression of the protein induced a defect in cleavage furrow formation, blocking cytokinesis.

A second effector, GAPCenA, has been identified by a yeast two-hybrid screen and serves as a Rab6-specific GTPase-activating protein, with the dual capacity to transiently interact with centrosomes (46). GAPCenA bears homology to spindle checkpoint proteins in yeast, can form complexes with γ-tubulin, and has a role in microtubule nucleation. Intriguingly, assembly of the Toxoplasma conoid, an initial step in parasite cytokinesis, involves γ-tubulin. Although they remain to be identified, Rab6 effectors similar to Rabkinesin-6/Rab6-KIFL and GAPCenA may be operative in Toxoplasma, and alterations in parasite cytokinesis might be due to a dominant-interfering effect of Rab6 mutants on these effectors. In total, it appears that Rab6 or its effectors may play bimodal roles, functioning not only in protein transport, but also in cell division.

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Rab6 Function in Toxoplasma