Toxoplasma gondii dense granules are morphologically similar to dense matrix granules in specialized secretory cells, yet are secreted in a constitutive, calcium-independent fashion. We previously demonstrated that secretion of dense granule proteins in permeabilized parasites was augmented by the non-hydrolyzable GTP analogue guanosine 5′-O-(thio)triphosphate (GTPγS) (Chaturvedi, S., Qi, H., Coleman, D. L., Hanson, P., Rodriguez, A., and Joiner, K. A. (1998) J. Biol. Chem. 274, 2424–2431). As now demonstrated by pharmacological and electron microscopic approaches, GTPγS enhanced release of dense granule proteins in the permeabilized cell system. To investigate the role of ADP-ribosylation factor 1 (ARF1) in this process, a cDNA encoding T. gondii ARF1 (TgARF1) was isolated. Endogenous and transgenic TgARF1 localized to the Golgi of T. gondii, but not to dense granules. An epitope-tagged mutant of TgARF1 predicted to be impaired in GTP hydrolysis (Q71L) partially dispersed the Golgi signal, with localization to scattered vesicles, whereas a mutant impaired in nucleotide binding (T31N) was cytosolic in location. Both mutants caused partial dispersion of a Golgi/trans-Golgi network marker. TgARF1 mutants inhibited delivery of the secretory reporter, Escherichia coli alkaline phosphatase, to dense granules, precluding an in vivo assessment of the role of TgARF1 in release of intact dense granules. To circumvent this limitation, recombinant TgARF1 was purified using two separate approaches, and used in the permeabilized cell assay. TgARF1 protein purified on a Cibacron G3 column and able to bind GTP stimulated dense granule secretion in the permeabilized cell secretion assay. These results are the first to show that ARF1 can augment release of constitutively secreted vesicles at the target membrane.

Remarkable progress has been made recently in understand-

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Andrea Liendo, Timothy T. Stedman, Huán M. Ngö, Sudha Chaturvedi, Heinrich C. Hoppe, and Keith A. Joiner‡
From the Department of Internal Medicine, Section of Infectious Diseases, Yale University School of Medicine, New Haven, Connecticut 06520-8022

Toxoplasma gondii ADP-ribosylation Factor 1 Mediates Enhanced Release of Constitutively Secreted Dense Granule Proteins*†

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† To whom correspondence should be addressed: Dept. of Internal Medicine, Section of Infectious Diseases, Yale University School of Medicine, LCI 808, 333 Cedar St., New Haven, CT 06520-8022. Tel.: 203-785-4140; Fax: 203-785-3864; E-mail: keith.joiner@yale.edu.

† The abbreviations used are: PV, parasitophorous vacuole; PVM, parasitophorous vacuole membrane; ARF, ADP-ribosylation factor; ARS, ATP-regenerating system; BAP, bacterial alkaline phosphatase; BAP-LDLR, fusion between BAP and the low density lipoprotein receptor; BLA, β-lactamase; HFF, human foreskin fibroblasts; IPA, immunofluorescence assay; PFA, paraformaldehyde; SLO, streptolysin O; TgARF, T. gondii ADP-ribosylation factor; GTPγS, guanosine 5′-O-(thio)triphosphate; DG, dense granules; RACE, rapid amplification of cDNA ends; TGN, trans-Golgi network; EST, expressed sequence tag; PCR, polymerase chain reaction; kb, kilobase pairs; ORF, open reading frame; HA, hemagglutinin; bp, base pair(s); IPTG, isopropyl-1-thio-

β-D-galactopyranoside; DTT, dithiothreitol; PAGF, polyacrylamide gel electrophoresis; LDL, low density lipoprotein; FITC, fluorescein isothiocyanate; AF-1 and -3, adaptor protein complex 1 and 3; ER, endoplasmic reticulum; PLD, phospholipase D; PIP2, phosphatidylinositol 4,5-bisphosphate; NSF, N-ethylmaleimide-sensitive fusion protein; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor; PA, phosphatidic acid.

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adopter protein complex.
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To dense granules (8), rather than to more conventional constitutive secretory vesicles, containing surface proteins of the parasite such as SAG1, making the dense granule pathway highly unique. Because constitutively secreted dense granule proteins are packaged in a distinctive organelle, T. gondii provides a more tractable system for studying control of vesicle docking and fusion at the plasma membrane than is the case in many other cells.

For this reason, we previously developed a permeabilized cell secretion assay, using the pore forming protein streptolysin O (9), to explore the machinery mediating the unusual exocytic process for dense granules. Release was not only constitutive, but also calcium-independent (9, 10), an important distinction when compared to release of dense matrix granules in mammalian cells. Addition to permeabilized parasites of hamster NSF and bovine α-SNAP increased the secretion of the stably transfected dense granule secretory reporter β-lactamase (BLA). In contrast, bovine Rab GDP dissociation inhibitor served, were first characterized by their ability to activate the dense granule release. ARF proteins, which are broadly conserved with Superscript II Reverse Transcriptase (Life Technologies, Gaithersburg, MD) for use as the PCR template. A 3′-RACE PCR strategy was employed in which the 5′-end (sense strand) oligonucleotide primers TARFUT (5′-AAA CAC GCC TCC TTC TCT TTC TCC TAG CAG C-3′) and TARF1 (5′-GGG CAT CAT GGT TTT GAG CAG CTA C-3′) were used in sequential PCR reactions with the 3′-antisense (antisense) oligo-dT17, polyadenylation site anchor primer. In the first round, TARFUT and oligo-dT17 primers were used for cDNA amplification with Taq DNA polymerase (Roche Molecular Biochemicals, Branchburg, NJ). The products were purified on QIAquick PCR columns (Qiagen, Valencia, CA) and used as template in a second round PCR reaction with primers TARF1 and TARF2 (5′-GGC ATC TTC GCT GTC GCT GTG-3′). The single PCR product was agarose gel-purified and labeled by random oligonucleotide priming with [32P]dCTP and the Rediprime II kit (Amersham Pharmacia Biotech, Piscataway, NJ). Colony hybridization with this probe identified several clones which contained ~0.8-kbp inserts, and sequencing analysis confirmed the presence of a full ARF1 homologue open reading frame (ORF) and 3′-untranslated region. For the parasite, an HA epitope tag with a BglII cloning site was engineered for targeting of the 3′-antisense end of the ARF1 ORF in primer ARFR3 (5′-ACT CGG ATG GGA GAG CGT CAG C-3′) and SGTG2 (5′-GGA GTG GGA GCC AAG CAA G-3′). The TgARF1 RACE clone ORF was PCR-amplified with TARF1, encoding an NcoI site, and ARFR2, and subcloned into the NcoI-BglII cloning site of pcDNA3.1+ utilizing the T. gondii NTAPase3 promoter cassette for expression.2 The TgARF1 cDNA sequence has been deposited in GenBank® (accession number AF227524).

Mutagenesis of TgARF1

Site-directed mutagenesis of TgARF1-HA was performed using a two-independent PCR amplification approach followed by triple ligation. For TgARFQ71L, the 237-bp PCR product from primers TARF1 and ARFR3 (5′-GGC ATC TTC GCT GTC GCT GTG-3′) was digested with NcoI and BglII, and subcloned into the vector pNPTRab11, utilizing the T. gondii NTAPase3 promoter cassette for expression. TgARF1 coding sequence with a C-terminal epitope tag (HA) was subcloned into pET-24d for overexpression in BL21(DE3) E. coli cells, which do not express β-lactamase (BLA). This precludes contamination of purified TgARF1-HA with BLA, which is essential for subsequent assays monitoring BLA release from stably transfected T. gondii. Even highly purified preparations of human ARF1 and ARF1 mutants (obtained from D. Shields, New York, NY) generated in standard vectors have sufficient residual BLA contamination to invalidate secretion assays (12, 13).

TgARF1 was inserted into the pET vector between the restriction sites NcoI and BglII. A plasmid encoding the untagged TgARFPT31N mutation was obtained from Kristen Hager and David Roos (University of Pennsylvania) and used for amplification with TARF1 and ARFR2, digested with NcoI and BglII, and subcloned into pcDNA3.1+ utilizing the NcoI-BglII cloning site of pcDNA3.1+. The two products were subcloned in vector pNPTRab11 following removal of the Rab11 open reading frame with digestion by NcoI and BglII. A plasmid encoding the untagged TgARFQ71L mutation was obtained from Kristen Hager and David Roos (University of Pennsylvania) and used for amplification with TARF1 and ARFR2, digested with NcoI and BglII, and subcloned into pHXNTRab11 as above. 

TgARF1 coding sequence with a C-terminal epitope tag (HA) was subcloned into pET-24d for overexpression in BL21(DE3) E. coli cells, which do not express β-lactamase (BLA). This precludes contamination of purified TgARF1-HA with BLA, which is essential for subsequent assays monitoring BLA release from stably transfected T. gondii. Even highly purified preparations of human ARF1 and ARF1 mutants (obtained from D. Shields, New York, NY) generated in standard vectors have sufficient residual BLA contamination to invalidate secretion assays (12, 13).

Oversexpression and Purification of Recombinant TgARF1-HA Protein

The TgARF1 coding sequence with a C-terminal epitope tag (HA) was subcloned into pET-24d for overexpression in BL21(DE3) E. coli cells, which do not express β-lactamase (BLA). This precludes contamination of purified TgARF1-HA with BLA, which is essential for subsequent assays monitoring BLA release from stably transfected T. gondii. Even highly purified preparations of human ARF1 and ARF1 mutants (obtained from D. Shields, New York, NY) generated in standard vectors have sufficient residual BLA contamination to invalidate secretion assays (12, 13).

TgARF1 was inserted into the pET vector between the restriction sites NcoI (at the initiating Met of ARF) and BamHI. Expression of the protein was induced with isopropylthio-β-galactoside (IPTG, 1 mM) for 4 h at 37 °C and bacterial cells were collected by centrifugation. TgARF1-HA was solubilized from the bacterial pellet using a French press at 8,000 p.s.i. in buffer A (50 mM Tris, 2 mM EDTA, 1 mM DTT and 1 mM phenylmethylsulfonyl fluoride). The lysate was clarified by centrifugation at 18,000 × g. Two different protocols were used to purify recombinant TgARF1. First, the protocol described for human and bovine ARF1 (25) was used, consisting of a DEAE-Sepharose column as a first step. The second purification step consisted of a gel filtration column, AcA 54 Ultrigel, equilibrated in buffer B (10 mM potassium phosphate, pH 7.4, 1 mM EDTA, 100 mM NaCl, 1 mM DTT), the column

2 T. Stedman and K. A. Joiner, unpublished.

1 A. Liendo and K. A. Joiner, unpublished observations.
was developed in the same buffer at a flow rate of 19 ml/h. 2.5-ml fractions were collected. Pooled fractions containing ARF1-HA from the Ultrogel column were concentrated using a Centricon-3 concentrator (Amicon, Beverly, MA). Samples were kept at −80 °C. Second, a purification protocol for soluble TgARF1-HA was developed using as a first step a dye column, Cibacron G3, equilibrated in buffer A. Subsequent wash with 1 M NaCl was performed, and the elution of the active protein was performed with 1 M NaCl. Fractions containing TgARF1-HA were pooled and concentrated using Centricon-3 concentrators (Amicon). The second step was a gel filtration column, AcA 54 Ultrogel, as described above. Purified recombinant TgARF1-HA was frozen immediately at −80 °C.

**SDS-PAGE and Immunoblotting**

The expression of recombinant protein TgARF1-HA was monitored using SDS-PAGE, and immunoblots were performed as previously described (8). Immunoblots were developed using an anti-HA monoclonal antibody (1:1000) or a goat anti-human ARF1 (1:500), followed by goat anti-mouse or rabbit anti-goat IgG-horseradish peroxidase conjugation (1:2000) and analysis using the ECL detection system (Amersham Pharmacia Biotech, UK).

**Parasites**

*T. gondii* tachyzoites were maintained by serial passage in monolayers of either African Green Monkey (Vero) cells or human foreskin fibroblasts (HFF) grown in modified Eagle’s minimal medium or α-minimal essential medium, respectively, supplemented with 7.5% fetal bovine serum. The RH strain and a stable transgenic clone of the RH strain, expressing the soluble foreign secretion reporter *E. coli β-lactama* (BLA) were described previously (8). The stable transgenic clone of the RH strain, expressing bacterial alkaline phosphatase fused with the LDL receptor (BAP-LDLR) is as previously described (26). For experiments with extracellular parasites, infected cells were scraped, and parasites were isolated by two passages through a 27-gauge needle.

**Parasite Permeabilization with SLO and Cytosol Depletion**

Permeabilization of extracellular parasites with SLO was performed using a protocol described earlier (9). Assessment of permeabilization was done by staining SLO permeabilized parasites with 4 μg/ml propidium iodide for 5 min at room temperature. The percentage of positive nuclear staining was quantitated by fluorescence microscopy.

**Immunofluorescence Assay (IFA)**

*Use of HA Epitope Tag to Localize the Transgenes in Transiently Transfected Parasites*—The detailed technique is described in Karsten et al. (8). Briefly, confluent HFF cell monolayers (12-mm coverslips) were infected with transiently transfected parasites. Transient transfection was performed by electroporation. After 16–24 h, cells were fixed and permeabilized with 3% paraformaldehyde in phosphate-buffered saline and 0.1% Triton X-100 and incubated with anti-HA monoclonal antibody (Babco, Richmond, CA) (1:200), followed by FITC or Texas Red-labeled secondary antibodies to detect HA-tagged proteins on DG protein release, we next asked whether there were any qualitative or quantitative differences in the morphology of DG exocytosis, in the presence or absence of GTPγS, using the permeabilized cell system (9). We took advantage of
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Table I: Wortmannin does not alter release of T. gondii dense granule proteins

| Treatment | GRA3 release | BLA release |
|-----------|--------------|-------------|
| Control   | 0.29 ± 0.05  | 0.52 ± 0.12 |
| Wortmannin| 0.26 ± 0.08  | 0.56 ± 0.09 |

Fig. 1: GTPγS augments the formation of coated vesicles and the exocytosis of dense granules. Extracellular parasites were permeabilized with SLO, incubated with GTPγS, ARS, and tannic acid, then prepared for transmission electron microscopy. Although the cytoplasm appears depleted (A, B) in comparison to control non-permeabilized cells (not shown), membranes of the nucleus (N), perinuclear endoplasmic reticulum, and rhoptries (R) are undamaged. Treatment with GTPγS promotes a significant increase in the budding of coated vesicles (small arrow) from the ER and plasma membrane (PM) as illustrated in C (a higher magnification of the bar region in A). Dense granules (DG) arrested by tannic acid in their docking and fusion to the cell surface are readily observed (arrowhead in A, B). CC, cortical cisternae. Magnifications: A, × 6,500; B, × 4,000; C, × 20,000.

Fig. 2: Steps in dense granule exocytosis. Dense granules in extracellular parasites treated as described in the legend to Fig. 1 were classified in three stages. Preformed dense granules (A) were not associated with the cortical cisternae and had an intact delimiting membrane (arrowhead). Docking dense granules (B and C) were in contact with the cortical cisternae, often via membranous threads (unlabeled arrow in B), with no or minimal discontinuity in the cortical cisternae. An electron dense band was commonly observed at the junction of the docked DG and the cortical cisternae (arrow in C). Fusing dense granules (D, E) were associated with a discontinuity in the cortical cisternae. Contents from the dense granule lumen are secreted into the extracellular space, occasionally with evagination of the plasma membrane (E), and loss of clear definition of the delimiting membrane surrounding the dense granule. The secreted contents often have a membranous appearance (F). Magnification, × 30,000. These events were assessed quantitatively, and the results are provided in the text.

Cloning and Sequence Analysis of ARF1—The Toxoplasma data base of expressed sequence tags (EST) revealed a cDNA sequence (W66111) with partial homology to human ARF1. Using a PCR RACE approach, we amplified from parasite cDNA the full-length T. gondii ARF1 homologue. Sequence analysis of cDNA clones revealed an open reading frame encoding a polypeptide of 183 amino acids, with a predicted molecular mass of 21 kDa and an isoelectric point of 6.8. The protein sequence contains prototypical GTP and Mg\textsuperscript{2+} binding sites and switch regions (Fig. 3) characteristic of RAS-related proteins, and an additional C-terminal signature sequence for the ADP-ribosylation factor family. BLASTN and BLASTP analyses of the sequence data bases through NCBI revealed strongest homology to ARF1 proteins of plants, animals, yeast, and protozoa, and we named the T. gondii homologue TgARF1 accordingly. The highly conserved switch regions of the ARF1 subfamily serve in part as effector binding sites for phospholipase D. Although the T. gondii sequence varies little in these conserved domains from those in the well characterized mammalian and yeast sequences, a Ser-83 residue adjacent to the switch 2 domain is at a site conferring differential stimulation of phospholipase D1 between yeast and mammalian ARF1 (30).

TgARF1-HA Localizes to the Golgi/TGN by Immunofluorescence and Immuno-electron Microscopy—We examined the localization of TgARF1 in T. gondii. A C-terminal HA epitope tag was added, and the TgARF1-HA construct was transiently expressed in T. gondii. Staining with an anti-HA antibody revealed that TgARF1-HA localized predominantly to a region anterior to the nucleus, consistent with the Golgi/TGN of the parasite (Fig. 4A). No staining was apparent on dense granules.
ules. This localization pattern was confirmed with rabbit anti-serum to TgARF1, raised against the recombinant protein expressed in E. coli (Fig. 4D). Brefeldin A, which disrupts the T. gondii Golgi (31), induced a redistribution of TgARF1 to the nuclear envelope and ER (not shown). These results are all concordant with the known localization of ARF1 to the Golgi complex in mammalian cells.

This result was confirmed by two separate approaches. First, thin section cryoimmunoelectron microscopy was done using the rabbit anti-TgARF1 antiserum. As shown in Fig. 5, TgARF1 localized to the Golgi stacks. No TgARF1 staining of dense granules was observed. Second, a stable line of T. gondii expressing bacterial alkaline phosphatase fused with the LDL receptor (BAP-LDLR) was transiently transfected with TgARF1-HA. As previously assessed by immunoelectron microscopy, BAP-LDLR localizes to the Golgi/TGN of the parasite (26). Transiently transfected TgARF1-HA co-localized with BAP-LDLR in the Golgi region (Fig. 6A), confirming localization of TgARF1-HA to the Golgi/TGN of the parasite.

**Expression and Localization of TgARF1 Mutants**—We generated two mutants of TgARF1, which are impaired in the GTP binding or hydrolysis cycle. By analogy to the Ras-Q61L mutation, which inhibits GTP hydrolysis but not binding, a Q71L mutation was introduced in T. gondii TgARF1-HA (25) and transiently expressed in the organism. The Q71L mutant localized to the Golgi as well as to dispersed punctate structures (Fig. 4B) in comparison to the discrete Golgi/TGN structure observed with wild type TgARF1-HA. Thus, the Q71L mutant appears to behave as in mammalian cells, where this mutant has distinct effects on the structure and/or function of ER, Golgi apparatus, and endocytic pathway (32). We also generated a T31N mutant of T. gondii TgARF1, a mutant predicted to be defective in GTP binding (19). Following transient transfection of the epitope-tagged protein, the T31N mutant was diffusely distributed throughout the parasite cytosol (Fig. 4C), likely due the inability to bind GTP. Together, these results demonstrate that the localization of TgARF1 within the parasite is coupled to the GTP cycle, as has been described for ARF1 in other systems (reviewed in Ref. 33).

**Effect of TgARF1 Mutants on Organelle Structure**—The effect of overexpression of TgARF1Q71L-HA on the localization of BAP-LDLR was assessed. TgARF1Q71L-HA was transiently transfected into the BAP-LDLR stable line. In contrast to wild-type TgARF1-HA, this mutant altered the localization of BAP-LDLR, consistent with partial disruption of the Golgi/TGN (Fig. 6B), as shown above. Mechanistically, this alteration in BAP-LDLR localization may be due to both an enhancement of retrograde transport and to augmented anterograde flow from the TGN. A similar partial dispersion of the BAP-LDLR signal was seen with the a T31N mutant (not shown).

**Effects of TgARF1 Mutants on Localization of Dense Granule Proteins**—We assessed the effects of TgARF1 mutants on transport of proteins to dense granules. Native TgARF1, and TgARF1 mutants ARF1T31N and ARF1Q71L, were transiently overexpressed in a parasite clone stably expressing the soluble dense granule secretory reporter BAP (8) or in wild type parasites. As shown in Fig. 7, in parasites transfected with TgARF1T31N, discrete labeling of BAP (Fig. 7B) or GRA3 (Fig. 7K) in dense granules was reduced and replaced by a reticular
endoplasmic reticulum-like staining pattern. Similarly, in parasites transfected with the TgARF1Q71L mutant, labeling of dense granules for BAP (Fig. 7E) or for GRA3 (Fig. 7N) was partially replaced by perinuclear and posterior reticular staining. Less GRA3 was detected at the parasitophorous vacuole membrane, with both the TgARF1T31N (Fig. 7K) and TgARF1Q71L (Fig. 7N) mutants. Staining for GRA3 in the wild type TgARF-1 transfectants was variably altered, with mild effects in most cells (Fig. 7H). These results suggest that one predominant effect of both Q71L and T31N mutants is to block delivery of soluble proteins to dense granules and to induce accumulation of the secretory proteins in the ER. This observation precluded an assessment of ARF1 effects on post-Golgi release of dense granule proteins.

Expression and Purification of Recombinant TgARF1 Protein—We therefore opted to analyze the effects of TgARF1 on dense granule secretion in the permeabilized cell assay, using purified TgARF1-HA protein. TgARF1-HA was expressed in E. coli using the pET system. Induction with 1 mM IPTG of BL21(DE3) cells carrying the TgARF1-HA/pET-24d construct resulted in a time-dependent increase in the accumulation of TgARF1-HA protein, which continued for at least 120 min (not shown). The induced protein migrated at 21 kDa on SDS-PAGE.

Previous work overexpressing human and bovine ARF1 in E. coli has shown that recombinant ARF1 proteins are easily solubilized from the bacterial pellet with TX-100 and lysozyme treatment or using a French press cell. Our results confirmed these facts. Recombinant TgARF1-HA was solubilized from IPTG-induced bacterial cell pellets (Fig. 8, lane 2). Different protocols were attempted to purify recombinant TgARF1-HA. First, the protocol described for human and bovine ARF1 (25) was used. This protocol consists of a DEAE-Sephacel column as a first step, where typically almost all of the bacterial proteins are absorbed, while ARF1 is not retained. In our experiments, recombinant TgARF1-HA was adsorbed to the DEAE matrix along with the bacterial proteins (not shown), necessitating
further purification on an AcA 54 Ultrogel column (not shown). Second, we used an immunoaffinity column, containing monoclonal anti-HA antibodies cross-linked to a Sepharose matrix (Babco, Richmond, CA). Even though the antibody recognized TgARF1-HA in immunoblots, the recombinant TgARF1-HA was not absorbed to the column under the various conditions tested (not shown). Finally, purification of the soluble TgARF1-HA was done using a Cibacron G3 column, equilibrated with buffer A. Cibacron G3 is a triazine dye column, used as an affinity resin for binding nucleotide-dependent enzymes. TgARF1-HA, purified by the standard DEAE protocol using DEAE columns. Although all of these proteins were successfully purified by the standard DEAE method (not shown), the purified proteins lacked GTP binding activity (Fig. 9, lanes 3). Likely explaining the failure of the proteins to bind to dye resin columns. Control samples (containing no protein or bovine serum albumin) did not bind the nucleotide (Fig. 9, lane 6).

Recombinant TgARF1 Stimulates BLA Release from Permeabilized Parasites—Finally, we examined the influence of recombinant TgARF1-HA on the release of the secretory reporter BLA in permeabilized T. gondii. Extracellular tachyzoites were permeabilized with 1 unit/ml streptolysin O, cytosol was depleted, and BLA release was measured, as described previously (9) and under “Materials and Methods.” Three separate experiments are illustrated in Fig. 10, A, B, and C. Slightly different experimental variables were tested in the three experiments. ARS alone but not GTPγS alone augmented BLA release slightly, as previously reported (9). Treatment with 100 μg/ml TgARF1-HA (purified on Cibacron G3 and stored at −80 °C) resulted in 1.4-fold (Fig. 10A, lanes 2 and 4), 1.8-fold (B, lanes 2 and 4), or 2.0-fold (C, lanes 3 and 4) enhancement of release of the secretory reporter BLA from permeabilized parasites in the presence (but not the absence) of ARS. Addition of GTPγS to ARS and TgARF1-HA did not substantially augment BLA release (Fig. 10A, lanes 4 and 5). Addition of TgARF1-1 to GTPγS-treated parasites, in the presence of ARS, did not substantially augment BLA release in comparison to GTPγS plus ARS alone (Fig. 10B, lanes 4 and 5). Treatment of non-permeabilized cells with recombinant TgARF1-HA did not result in stimulation of secretion, either in the absence or presence of ARS and GTPγS (Fig. 10A, lanes 6 and 7). TgARF1-HA purified by DEAE chromatography, and lacking GTP binding activity, did not stimulate BLA release from permeabilized parasites (not shown). These results illustrate that TgARF1 augments release of dense granule proteins, and that the magnitude of the effect is similar to that of GTPγS alone or in combination with GTPγS.

DISCUSSION

Our data suggest that T. gondii TgARF1 stimulates post-Golgi secretion from preformed DG. This represents a specialized function for TgARF1 in this apicomplexan parasite. A related function has been described for ARF1 in selected secretory cells, in which ARF1 mediates the release of preformed granules in the presence of GTPγS (21, 22). Nonetheless, a fundamental difference between our system and release observed in mast cells, chromaffin cells, or PC12 cells is that DG release in the parasite is a constitutive process, which is not triggered by calcium. Hence, our results have direct relevance to constitutive secretion. Moreover, we have been able to identify an effect of TgARF1 at the final step of constitutive vesicle release because of the unique features of the T. gondii system.

In mast cells, ARF1-mediated enhancement of granule secretion is thought to occur via activation of phospholipase D. PLD hydrolyses phosphatidylcholine to generate PA and choline. The conversion of phosphatidylcholine to PA alters the lipid bilayer properties, replacing a non-fusogenic phospholipid with a fusogenic one (34), potentially stimulating preformed granules to fuse and release their contents to the extracellular environment. In addition, PA generated by PLD regulates a phosphatidylinositol 5-kinase, resulting in enhanced synthesis of phosphatidylinositol 4,5-bisphosphate (PIP2). Elevated lev-
Fig. 7. Transiently overexpressed ARF1 mutants block transport and secretion of dense granule proteins. Parasites stably expressing the soluble dense granule secretory reporter BAP (A–F) or wild type parasites (G–O) were transiently transfected with TgARF1T31N (A–C, J–L), TgARF1Q71L (D–F, M–O), or wild type TgARF1-HA (G–I). The apical ends of parasite vacuoles expressing the ARF1 proteins as detected with antibody to the HA epitope tag are denoted in each image (asterisks in A, D, G, J, M). TgARF1Q71L is predominately localized to the Golgi and perinuclear envelope (arrows in D, M) whereas TgARF1T31N localization is predominately cytosolic with minor Golgi association (arrows in A, J). Transiently expressed TgARF1-HA is concentrated in the Golgi and cytosol (G). BAP and GRA3 proteins label dense granules in untransfected parasites (small arrows in B, E, H, K). Transport of BAP (B, E) or GRA3 (H, K, N) to dense granules is partially blocked, and the dense granule proteins accumulate in a reticular ER-like pattern throughout the ARF1T31N-transfected parasites (arrows in B, K) and ARF1Q71L parasites (arrows in E, N). Additionally, less secreted GRA3 signal is detected at the parasitophorous vacuolar membrane in parasites expressing ARF1T31N and ARF1Q71L than in untransfected parasites (long arrows in K, N). Transiently overexpressed ARF1HA induces only partial accumulation of GRA3 in the Golgi region (H).

eels of PIP₂ stimulate mast cell exocytosis. In contrast, in both chromaffin and PC12 cells, ARF1 stimulates granule release in a process not dependent on PLD activation. It is of note that ethanol, which diverts PA to PE and blocks PIP₂ generation in mammalian cells, also inhibits dense granule secretion in T. gondii (35). This result suggests that TgARF1 augments DG release in a PLD-dependent fashion. Successful purification of functionally active TgARF1d17-HA capable of binding GTPγS would have allowed a direct test of this hypothesis (19, 23, 24) in the permeabilized cell system, but this was not possible (Fig. 9).

There are several non-mutually exclusive alternatives to the scenario presented above. It is possible that TgARF1 is involved in a triggered component of dense granule release. In this scenario, the secretion of preformed dense granules measured in the permeabilized cell assay in the absence of GTPγS would correspond to the constitutive component of DG release and would not be dependent on ARF1 activity. The putative burst of DG release following invasion would be an ARF1-dependent process, analogous to the situation already described in selected secretory cells (21, 22). Nonetheless, the relationship of T. gondii dense granules to dense core secretory granules in mammalian cells is not clear, and there are many unusual features of the T. gondii system. The process is calcium-independent, and no physiological trigger has been identified for dense granule release (9, 10). No immature secretory granule precursors (36) are visible in T. gondii. Soluble proteins are routed by the bulk flow pathway quantitatively to dense granules (8). The presence of putative membranous material within the dense granule matrix (Fig. 2) is also unique. Altogether, these features suggest that T. gondii dense granules, despite their morphologic appearance, are evolutionarily distinct from dense core granules in mammalian secretory cells. As another alternative, ARF1 may augment release of a sub-population of dense granules, although no convincing evidence yet exists for dense granule heterogeneity. TgARF1 may also enhance formation of nascent secretory vesicles at the trans-Golgi network (19, 20), in addition to or even instead of augmenting release of intact DG. Finally, different pathways may be triggered by GTPγS and by TgARF1 in the permeabilized cell system. In particular, the effects on GTPγS on release of intact dense granules could be mediated either via a Rab protein or another member of the ARF family, such as ARF6 (34). Although all of these possibilities exist, and several processes may operate concurrently, the morphology, kinetics, and pharmacological inhibition profile suggest at a minimum that TgARF1 is enhancing constitutive release of preformed dense granules.

TgARF1 was not detected on the dense granule membrane, either by immunofluorescence or immunoelectron microscopy. This does not obviate a role for TgARF1 in mediating release of preformed dense granules, especially if a TgARF1-regulated phospholipase D activity localizes to the cortical cisternae or plasma membrane (37). Furthermore, translocation of ARF1 to
secretory granule membranes of rat parotid acinar cells, specifically in the presence of GTPγS, has been previously demonstrated (38), and such a process may also occur in *T. gondii*. Our data suggest that TgARF1 is also involved in maintaining the structure of the *T. gondii* Golgi and TGN. In mammalian cells, ARF1 supports the GTP-dependent association of COPI with Golgi membranes (39–41). Localization of TgARF1 to TgARF1-HA to the Golgi/TGN is consistent with such a function in *vivo* in the parasite. Although it is apparent that transfected TgARF1 affects delivery of secretory reporters to dense granules (Fig. 7), likely by effects on the Golgi, this is not likely to be the case in the permeabilized cell assay.

The functions for ARF proteins in the secretory and endocytic pathways of mammalian cells are now quite broad. Although it is assumed that TgARF1 will have most analogous functions in *T. gondii*, this is yet to be formally established. The parasite has a Golgi-ER retrieval system that is reminiscent of the process in higher eukaryotes (42) and has components of a COP1 coat (31), confirming the expectation from the morphologic consequences of Brefeldin A treatment. TgARF1 expresses the AP-1 adaptor complex, and sorts proteins in an AP-1-dependent fashion, suggesting that TgARF1 will also participate in this step (26). AP-3-dependent sorting is also ARF1-dependent (43, 44), and circumstantial evidence (26) argues in favor of an AP-3-dependent sorting pathway in *Toxoplasma*. We expect that the unusual features of the *T. gondii* secretory and endocytic pathways may allow further insights into ARF1 functions, as these pathways are explored in the parasite.

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