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Receptor for Retrograde Transport in the Apicomplexan Parasite *Toxoplasma gondii*

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Toxoplasma gondii **and its apicomplexan relatives (such as** ***Plasmodium falciparum*****, which causes malaria) are obligate intracellular parasites that rely on sequential protein release from specialized secretory organelles for invasion and multiplication within host cells. Because of the importance of these unusual membrane trafficking pathways for drug development and comparative cell biology, characterizing them is essential. In particular, it is unclear what role retrieval mechanisms play in parasite membrane trafficking or where they operate. Previously, we showed that** ***T. gondii*****'s beta-COP (TgBCOP; a subunit of coatomer protein complex I, COPI) and retrieval reporters localize exclusively to the zone between the parasite endoplasmic reticulum (ER) and Golgi apparatus. This suggested the existence of an HDEL receptor in** ***T. gondii*****. We have now identified, cloned, and sequenced this receptor, TgERD2. TgERD2 localizes in a Golgi or ER pattern suggestive of the HDEL retrieval reporter (K. M. Hager, B. Striepen, L. G. Tilney, and D. S. Roos, J. Cell Sci. 112:2631-2638, 1999). A functional assay reveals that TgERD2 is able to complement the** ***Saccharomyces cerevisiae*** **ERD2 null mutant. Retrieval studies reveal that stable expression of a fluorescent exogenous retrieval ligand results in a dispersal of βCOP signal throughout the cytoplasm and, surprisingly, results in βCOP staining of the vacuolar space of the parasite. In contrast, stable expression of TgERD2GFP does not appear to disturb βCOP staining. In addition to TgERD2,** ***Toxoplasma*** **contains two more divergent ERD2 relatives. Phylogenetic analysis reveals that these proteins belong to a previously unrecognized ERD2 subfamily common to plants and alveolate organisms and as such could represent mediators of parasite-specific retrieval functions. No evidence of class 2 ERD2 proteins was found in metazoan organisms or fungi.**

Toxoplasma gondii is an opportunistic pathogen. It belongs to the phylum Apicomplexa, which contains some of the most medically virulent parasitic protozoa (e.g., the causative agent of malaria, *Plasmodium falciparum*) (36, 47). Although small (2 by 8 μm), *T. gondii* possesses the full repertoire of readily identifiable eukaryotic organelles, such as the endoplasmic reticulum (ER) and Golgi apparatus. The nucleus is located centrally, effectively dividing the parasite into apical and basal domains. The ER is concentrated primarily in the basal end, and a single prominent Golgi stack is located immediately apical to the nucleus. In addition to the more traditional eukaryotic secretory machinery mentioned above, *T. gondii* must target proteins to three additional secretory organelles and a residual chloroplast-like organelle called the apicoplast. The anterior cytoplasm of the parasite contains two specialized secretory organelles, micronemes and rhoptries. This complex of specialized substructures gives the phylum Apicomplexa its name (15). The rhoptries and micronemes are located apically, while the dense granules are distributed throughout the parasite. *T. gondii* utilizes proteins from these unique secretory organelles to invade and set up a replication permissive vacuole within the host cell (10, 32, 41).

The central secretory system of *Toxoplasma* as described above is governed by a highly polarized flow of proteins (31). Parasite proteins are synthesized in the ER where they are trafficked to the apical end of the nuclear envelope, *cis*-Golgi stacks, Golgi apparatus, *trans*-Golgi network, and onto the specialized organelles (31). At each checkpoint, decisions are made to either transport or retain specific molecules. For example, dense granules are secreted constitutively, while micronemal and rhoptry proteins are retained until secretion is triggered (11, 20, 25, 31, 56, 66, 68). ER localization, retention, and export motifs have been studied in *Toxoplasma* and are well conserved generally (20, 25). Addition of the *T. gondii* retrieval motif HDEL to a secretory protein causes ER retention (20), providing evidence of a functional retrieval pathway with an HDEL receptor.

In *T. gondii*, the transit area between the ER and Golgi apparatus is condensed. The parasite utilizes the apical end of its nuclear envelope as its transitional ER (tER) (20), possibly because of simple size constraints. In other systems, the tER is a separate entity and functions as a salvage compartment. In yeast and mammalian cells, escaped ER proteins are recovered in coatomer protein complex I (COPI)-coated vesicles from this compartment and the *cis* stacks of the Golgi apparatus (12, 19, 33, 52, 69). Because the majority of parasite βCOP (a subunit of COPI) is restricted to the area between the parasite nuclear envelope and Golgi apparatus (20), we predict localization of retrieval receptors at these sites.

Proteins concentrated in or near the parasite Golgi apparatus, such as the ones discussed above, are of particular interest for several reasons. The Golgi complex plays an important role

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in daughter cell division within the parasite. It is the first organelle to divide, and it is thought to provide both a scaffold for daughter cell growth and the basis for the flattened stacks which form the inner membrane complex (a parasite-specific organelle) (28, 45, 63). As the daughter cells differentiate into invasive or motile stages that will infect new host cells, the parasite Golgi apparatus performs a fundamental role in rhoptry biogenesis (40). The rhoptry is a specialized apical organelle pivotal in parasite invasion. Additionally, the parasite Golgi apparatus is a single stack readily detected by electron microscopy and has recently proven to be an excellent model system for studying Golgi apparatus division and inheritance (54). Therefore, it is important to identify and characterize retrieval mechanisms for misdirected parasite proteins, especially those mechanisms that may intersect with and affect the parasite Golgi apparatus and its stability.

MATERIALS AND METHODS

Cell culture and transfection. Primary human foreskin fibroblasts cells were grown in modified Eagle's medium (Gibco, Grand Island, N.Y.) containing 10% fetal bovine serum (Hazleton Biologics, Lenexa, Kans.) and maintained as previously described (59). For microscopy, 10^5 cells were inoculated directly into 35-mm-diameter wells containing 25-mm-diameter number 1 glass coverslips. Plasmid DNA was electroporated into tachyzoites, cultures were infected, and stable transformants were selected as described previously (59).

Cloning of TgERD2. A *T. gondii* sequence exhibiting strong sequence similarity to ERD2 (ER retention-deficient mutant 2) from other species was identified as expressed sequence tag (EST) number 1163878, which was excised, sequenced, and used to screen a *T. gondii* cDNA library (AIDS repository, National Institutes of Health [NIH]). The EST library and a description of its creation are maintained at http://www.cbil.upenn.edu/ParaDBs (1, 2, 74, 75). Plaques were screened and clones were excised according to the manufacturer's protocol (ZAP-cDNA synthesis kit; Stratagene, La Jolla, Calif.). One full-length clone containing a ~2.6-kb cDNA was identified and fully sequenced. It revealed an open reading frame (ORF) encoding a 226-amino-acid protein (see Fig. 1A).

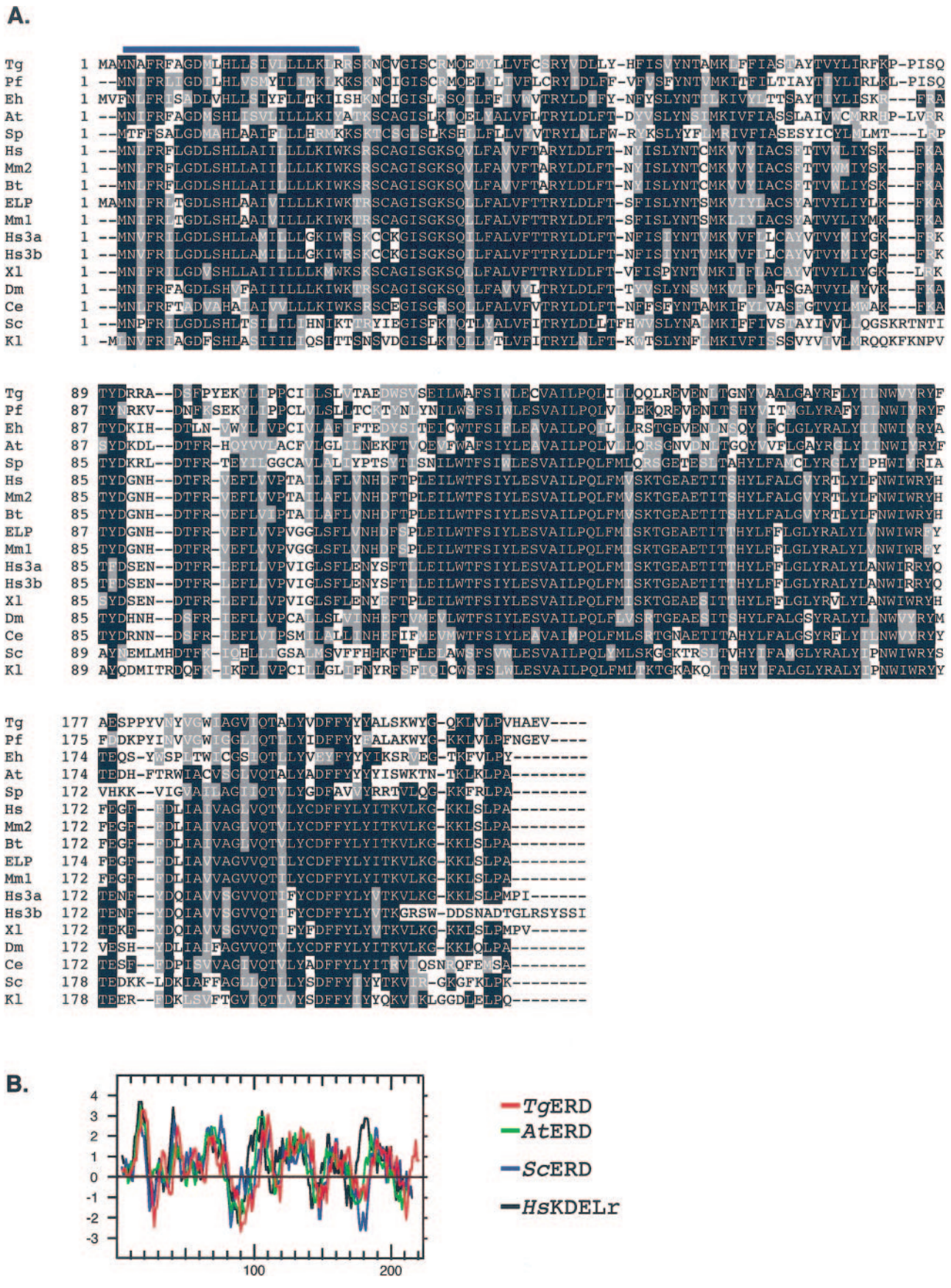
DNA sequence analysis and phylogeny. All plasmid preparations were isolated and assessed for purity according to the manufacturer's protocol (QIAGEN Hi-Speed Plasmid Maxi kit). Probing the GenBank protein sequence database with TgERD2 via PSI-BLAST identified proteins related to TgERD2. All proteins from fully sequenced organisms that were found with expect values (E) of <0.01 and that aligned along the length of ERD2 were included in the phylogenetic analysis; some additional proteins were included to better define points of gene duplication. When sets of proteins with >95% identity were identified in the same organism, only one was included. Similar searches of the GenBank protein, nucleic acid, and EST databases were conducted with class 2 ERD2 proteins as probes as indicated in the text. Preliminary genomic and/or cDNA sequence data were accessed via http://ToxoDB.org and/or http://www.tigr.org/tdb/t_gondii/. Genomic data were provided by The Institute for Genomic Research and the Sanger Center (Wellcome Trust). EST sequences were generated by Washington University. Sequences in Fig. 1 and 5 were aligned using Clustal W1.8 (24) at http://www.ebi.ac.uk/clustalw/, using default parameters. Alignments were prepared for presentation using the Boxshade 3.21 server (http://www.ch.embnet.org/software/BOX_form.html). Phylogenetic analysis was performed on the conserved core of this alignment by the neighbor-joining algorithm as implemented by Clustal W (default parameters). Confidence estimates were performed by bootstrap analysis utilizing 1,000 replicates (see Fig. 5). Pairwise identities were calculated from the core alignment by Clustal W (default parameters). Hydropathy plots were generated with DNA Strider 1.3f14 using Kyte-Doolittle analysis (35).

Yeast strains and complementation of the *erd2* mutant. The *Saccharomyces cerevisiae* parental strain ΔLE26A is a derivative of JCB102 (*MATα ade2 ade3 his3-Δ200 leu2-3,122 ura3-52 TRP1 erd2-ΔNco*) (21). Strain ΔLE26A lacks the chromosomal copy of *ERD2* but maintains viability by the presence of a plasmid bearing the triosephosphate isomerase promoter (TPI) promoter-driven *Kluyveromyces lactis ERD2* gene on a CEN6 plasmid bearing *URA3* and *ADE3*. Complementation was performed using the plasmid-shuffling assay. The parental plasmid containing *URA3* in strain ΔLE26A can be selected against by utilizing the pyrimidine analogue 5-fluoroorotic acid (5-FOA) (65). Once the *KlERD2* plasmid is lost, the yeast will not live unless the test ERD can actively complement the defect. Accordingly, ΔLE26A (the parental strain) was transformed either with TgERD2 or with the positive-control plasmid bearing the TPI promoter-driven *ScERD2* gene by a modified lithium acetate method (29). Two independent colonies from the transformants were replated on prewarmed selective plates (leucine dropout) and incubated at 30°C to enrich for *LEU*-containing plasmids. After two rounds of selection, the surviving colonies were then streaked on plates containing 5-FOA to select against the parental plasmid. The surviving colonies were then streaked on plates lacking uracil or leucine to confirm the loss of the *URA3* plasmid, and their growth was compared with that of the parental strain. H. R. B. Pelham and Michael Lewis (Cambridge, England) kindly provided all yeast strains, protocols, and yeast plasmids used in the assay (21, 62). "Yeast" refers to *S. cerevisiae* throughout the paper.

Plasmid construction. (i) Yeast expression vectors. A HindIII-BamHI fragment containing the TgERD2 ORF was inserted into the *LEU2*-containing 2μm yeast expression plasmid GS315. GS315 contains the TPI promoter in pRS315 (CEN *LEU2*) (37) and a 1-kb insert which can be cut out as a HindIII-BamHI fragment, resulting in a convenient multiple cloning site. The TgERD2 ORF was amplified by PCR using primers ERD/HindIII and ERD/BamHI which introduced HindIII and BamHI sites at the 5′ and 3′ ends, respectively. Primer ERD/HindIII is 5′-ccct*gaagctt*AAGATGGCGATGAATGCCTTTCG, and primer ERD/BamHI is 5′-cgc*ggatcc*CTACACTTCTGCGTGGACGGGCA-3′ (italic letters indicate nucleotides in restriction sites introduced for cloning, capital letters indicate coding sequence, and lowercase letters indicate nonspecific nucleotides added to aid PCR and digestion of the produced fragments).

(ii) Fluorescent constructs. Chimeric *dhfr*-P30-GFP-HDEL/*sag*-CAT plasmids were constructed as previously described (62). All resulting plasmids described below are based on pBluescript KS(+) (Stratagene) and contain either a dihydrofolate reductase (55) or alpha tubulin promoter (49). In all instances, the coding sequence of the gene of interest (GOI) is separated from the promoter by a BglII site and from the fluorescent protein by an in-frame AvrII site. A 3′ untranslated region obtained from the *T. gondii* dihydrofolate reductase-thymidylate synthase gene (55) follows the GOI. The ERD2 ORF was amplified using the primer set ERDBglII and ERDAvrII that introduced BglII and AvrII sites at the 5′ and 3′ ends of ERD2, respectively, while simultaneously removing the stop codon of TgERD2. The resulting BglII-AvrII fragment was subcloned into the BglII and AvrII sites in P30-GFP to replace the P30 gene (67). Plasmid ptubGRASP-RFP/sagCAT was engineered by digesting GRASP (Golgi reassembly stacking protein)-yellow fluorescent protein (YFP) with BglII and AvrII. The resultant BglII-AvrII GRASP fragment was subcloned into the TubP30-RFP plasmid to replace the P30 gene. Graham Warren kindly provided the GRASP-YFP plasmid (54). P30-GFP-HDEL was generated as described previously (20). Primer ERDBglII is 5′-catg*agatct*AAGATGGCGATGAATGCCTTTCG-3′, and primer ERDAvrII is 5′-aactag*cctagg*CACTTCTGCGTGGACGGGCAAC AC-3′ (italic letters indicate nucleotides in restriction sites introduced for cloning, capital letters indicate coding sequence, and lowercase letters indicate nonspecific nucleotides added to aid PCR and digestion of the produced fragments). Boris Striepen generously provided the expression plasmids containing either green fluorescent protein (GFP) or red fluorescent protein (RFP) (67).

Microscopy. For immunofluorescence, infected cells grown on glass coverslips were rinsed in phosphate-buffered saline (PBS), fixed in PBS containing 3% paraformaldehyde (pH 7.4) for 5 min, and permeabilized in 0.25% Triton X-100 (at room temperature) for 5 min or in methanol (at −20°C) for 2 min. After several washes in PBS, samples were blocked in 5.0% bovine serum albumin (BSA) for 30 to 60 min, inverted over 150 μl of primary antibody solution (diluted 1:500 in PBS containing 5% BSA), and maintained in a humidified chamber at room temperature for 60 to 90 min. After additional washes in PBS, coverslips were incubated with either fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody (Sigma) or rhodamine-conjugated goat anti-rabbit secondary antibody (Sigma), as in Fig. 4. Control samples of uninfected cells or primary or secondary antibody alone were also examined. Coverslips were mounted on glass slides using a minimal volume of Fluoromount G. Cells were viewed using a Leica DM IRE2 with a z-drive, built in a motorized fluorescent wheel with appropriate barrier and excitation filters (Chroma Technology, Battleboro, Vt.) and an HCX PL APO 63× oil objective with a numerical aperture of 1.3. Images were captured by an Orca extended-range, interline chip, charge-coupled device camera at 1 × 1 binning, 1,300 by 1,030 pixels (Hamamatsu, Bridgewater, N.J.). A Uniblitz shutter and Uniblitz electronic controller (Vincent Associates, Rochester, N.Y.) controlled exposure time. The images were illuminated with a mercury lamp, captured, and processed by OpenLab 3.2 software (Improvision, Coventry, United Kingdom). All pictures were taken with the same exposure time and binning to ensure a balanced comparison of staining.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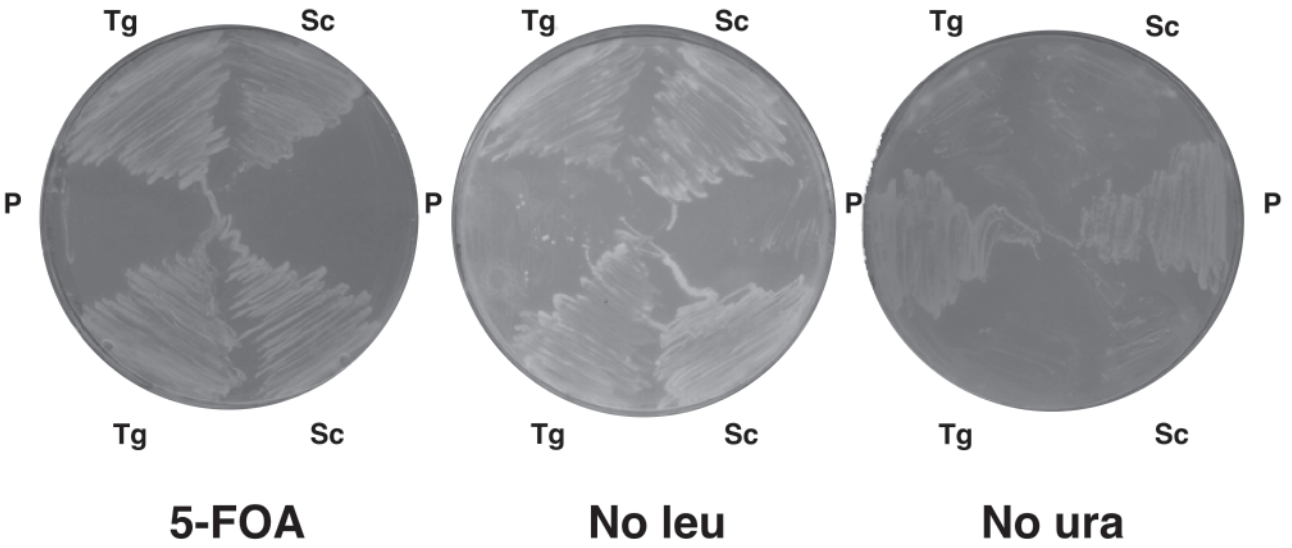


FIG. 2. Complementation of the yeast *erd2* deletion mutant with the *Toxoplasma* ERD2 homologue. The parental yeast strain ΔLE26A (P) and the strain transformed with either the *S. cerevisiae* ERD2 gene (Sc) or the *Toxoplasma* (Tg) ERD2 were streaked on the Leu dropout plate to select for leucine-containing plasmids. The parental plasmid was selected on plates containing the pyrimidine analogue 5-FOA. The surviving colonies, colonies that grew on plates containing 5-FOA, were tested for growth on minimal plates lacking leucine (No leu) or uracil (No ura) and compared with growth of the parental strain (P). Loss of the parental plasmid was scored as an inability to grow on uracil dropout plates.

Antibodies. *Toxoplasma* βCOP-like protein subunit (TgβCOP) is a member of the parasite COPI complex. A new anti-*T. gondii* β-COP antibody was produced by using a peptide corresponding to the C-terminal sequence of *T. gondii* EST number 623080 exhibiting strong sequence similarity to βCOP, (K)LGDRIT VVQRGLTK(20). Mimotopes (a subsidiary of MitoKor, San Diego, Calif.) synthesized, purified, and linked the peptide to keyhole limpet hemocyanin using a glutaraldehyde procedure. The C-terminal peptide was used to immunize previously *T. gondii* seronegative New Zealand White rabbits (20). Polyclonal antisera were produced and affinity column purified by Cocalico Biological, Inc., Reamstown, Pa. Peptide inhibition assays reveal that staining is specific to the peptide the antibody was raised against (data not shown).

RESULTS

Identification of *TgERD2* encoding a putative HDEL receptor. The XDEL motif (X can be S, H, K, or D) is found at the C termini of soluble ER proteins and serves to retrieve escaped ER proteins from a later compartment, often the Golgi apparatus (26, 53). ER proteins in other compartments are sequestered by a transmembrane receptor, encoded by the *ERD2* gene, that self-oligomerizes and binds the XDEL motif (38, 44, 62). A *T. gondii* EST (clone number 1163878) homologous to ERD2 was identified by probing a *T. gondii* sequence database maintained as described in Materials and Methods (1, 2). Using this EST, we screened a λZAP cDNA library and identified a full-length clone. Sequence analysis revealed an ORF encoding a 26.1-kDa protein with 226 amino acids. The protein encoded by this ORF aligned along its length with characterized ERD proteins (Fig. 1A) and was notably similar to the *P. falciparum* PfERD2 protein (16). Signal P analysis indicating a protein in the secretory pathway (6, 7) revealed a 28-amino-acid signal peptide (Fig. 1A). Lastly, seven transmembrane domains (Fig. 1B) characteristic of the ERD receptor predicted by Kyte-Doolittle analysis (35) were also shown to be present.

***Toxoplasma* ERD2 functionally complements the yeast *erd2* mutant.** To determine whether TgERD2 was functional, we performed a complementation test utilizing the plasmid-shuffling assay. The plasmid-shuffling assay works on the principle that cell viability cannot be maintained in the absence of ERD2 in yeast (62). The parental yeast strain ΔLE26A was transformed either with *TgERD2* or with the positive-control *ScERD2* as described in Materials and Methods (29). Trans-

FIG. 1. Isolation of cDNA clone *TgERD2* and sequence analysis of the protein encoded by the DNA. (A) ERD2 sequence alignment. Missing residues (dashes), identical residues (black background), and similar residues (grey background) are indicated. Signal P analysis (http://www.cbs.dtu.dk/services/SignalP/) reveals an N-terminal signal sequence of 28 amino acids indicative of a protein in the secretory pathway (noted by the blue line above the sequence) (6, 7). Multiple sequence alignment was performed at http://www.ebi.ac.uk/clustalw/. Abbreviations: Tg, *Toxoplasma gondii* (AY095348); Pf, *Plasmodium falciparum* (S39609); Eh, *Entamoeba histolytica* (CAC34303); At, *Arabidopsis thalia* (plant) (A49677); Sp, *Saccharomyces pombe* (NP_596529); Hs, *Homo sapiens* (S13293); Mm2, *Mus musculus* (CAC34584); Bt, *Bos taurus* (cow) (A44394); ELP, human ERD2-like protein ELP (A42286); Mm1, *Mus musculus* ELP-like (CAC34585); Hs3a, human KDEL receptor 3 (NP_006846); Hs3b, human KDEL receptor 3 isoform b (NP_057839); Xl, *Xenopus laevis* (O42580); Dm, *Drosophila melanogaster* (O76767); Ce, *Caenorhabditis elegans* (P48583); Sc, *Saccharomyces cerevisiae* (NP_009513); Kl, *Kluyveromyces lactis* (A35618). (B) Comparison of ERD2 hydropathy plots. Hydropathy plots of proteins from four species (TgERD, AtERD, ScERD, and HsKDEL receptor [HsKDELr]) are shown as colored lines. All plots were calculated using the Kyte-Doolittle algorithm (35). The hydropathy plots have similar patterns and show seven potential membrane-spanning domains. The *y* axis represents the hydrophobicity scores (unitless); the *x* axis is the number of amino acids.

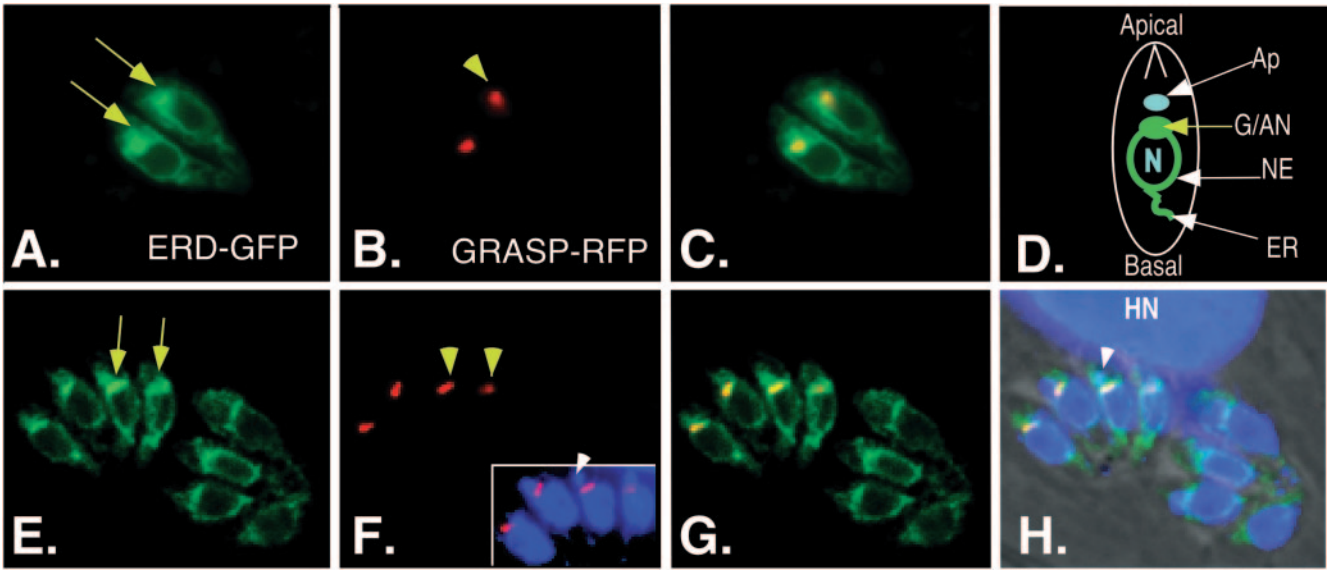
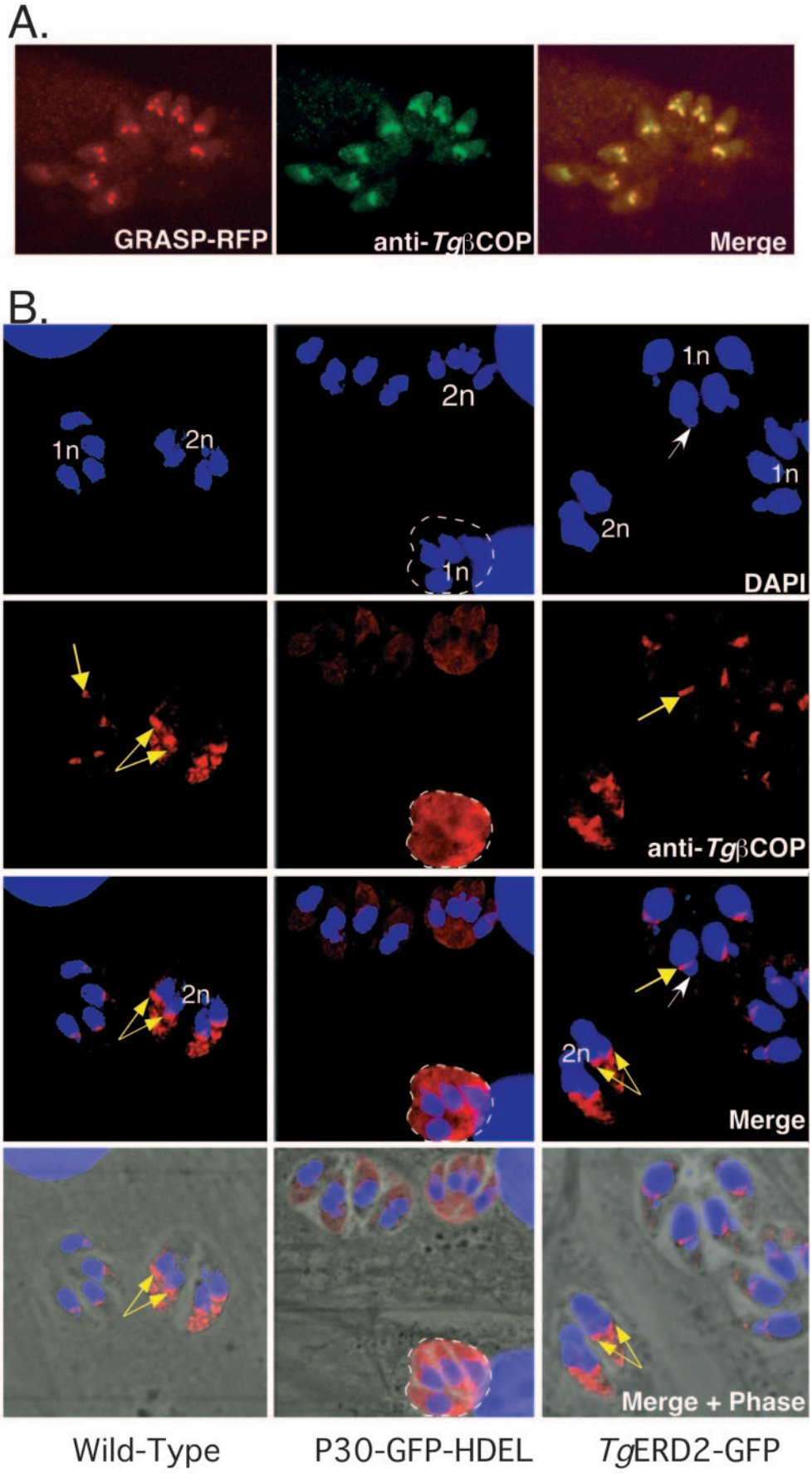


FIG. 3. TgERD2-GFP localizes to the Golgi apparatus. Parasites orient with their apical ends pointed outward (48). Parasites were cotransfected with TgERD2-GFP and GRASP-RFP constructs (A to C), or the GRASP-RFP construct was transfected into transgenic parasites stably expressing TgERD2-GFP (E to H). (A) Localization of TgERD2-GFP (green). Live image analysis reveals that TgERD2-GFP localizes primarily to the apical end of the parasite nucleus (yellow arrows) with some staining in the nuclear envelope and ER (the model for organelle orientation is shown in panel D). (B) Localization of GRASP-RFP (red). The construct is expressed as a tight red dot in a restricted area, which is marked by a yellow arrowhead. (C) Merging the GRASP-RFP and TgERD2-GFP images confirm that the green-tagged receptor and the red-tagged Golgi apparatus matrix marker overlap significantly, as evidenced by the intense yellow staining (see also panel G) (46). (D) Diagram of *T. gondii* and its intracellular organelles. The apicoplast (Ap) and nucleus (N) are two compartments containing DNA that stain with the dye DAPI (blue). The parasite Golgi apparatus (G) is invariably interposed between the apical end of the nuclear envelope (AN) and the nucleus. As in other organisms, the ER of the parasite is contiguous with the nuclear envelope (NE); however, the bulk of it is concentrated in the basal end of the parasite. (E to H) GRASP-RFP construct was transfected into transgenic parasites stably expressing TgERD2-GFP. (E) TgERD2-GFP localization is similar to the transiently expressing parasites with staining at the apical end of the nuclear envelope of the parasite (yellow arrows). Some staining is also seen in the nuclear envelope. (F) Yellow arrowheads point to the intense GRASP-RFP staining, which can be seen in the inset sandwiched between the DAPI (blue) staining compartments of the nucleus and apicoplast (white arrowhead). (G) Merging the GRASP-RFP and TgERD2-GFP images confirm that the green-tagged receptor and the red-tagged Golgi apparatus matrix marker overlap significantly, as evidenced by the intense yellow staining (also see panel C) (46). (H) Merging all fluorescence staining images with an overlay of the phase-contrast image. HN, host nucleus.

fected colonies were tested for growth on plates containing the pyrimidine analogue 5-FOA (65). Yeast cells expressing *URA3* are unable to metabolize 5-FOA. Therefore, the loss of *URA3* is required for growth of yeast on plates containing 5-FOA. Once the *KlERD2* plasmid is lost, the yeast will not live (grow on the plate containing 5-FOA) unless the test ERD can actively complement the defect (Fig. 2). Both the positive control (*S. cerevisiae*) and the test construct (*T. gondii*) were able to grow on plates containing 5-FOA (Fig. 2). In contrast, the parental strain was unable to grow on the plates containing 5-FOA. The surviving colonies were subsequently tested to confirm the loss of the *URA3* plasmid by checking for the ability of the strains to grow on plates lacking uracil or leucine. The strains that were transformed with the *LEU2* plasmid (*T. gondii* and *S. cerevisiae*) were able to grow on plates lacking leucine (Fig. 2), but only the strain containing the *URA3*-bearing plasmid (parental strain) was able to grow on the plate lacking uracil (64). Thus, unlike the human homologue, TgERD2 can complement the yeast *erd2* null mutant (Fig. 2).

TgERD2-GFP localizes most intensely at the parasite Golgi apparatus. Our hypothesis suggests that the apical end of the nuclear envelope serves as the tER of the parasite. Therefore, we anticipated that tER markers, such as the HDEL receptor, TgERD2, should localize intensely in this region and the parasite Golgi apparatus. At the light microscopy level, it is difficult to distinguish between closely positioned structures such

FIG. 4. COPI localization in wild-type (untransfected) and transgenic parasite cell lines. (A) GRASP-RFP (red) and TgβCOP (green) staining overlap in wild-type parasites. This is indicated by the intense yellow staining of the overlay in the rightmost panel. (B) Wild-type, P30-GFP-HDEL, or TgERD2-GFP transgenic parasites were stained with both a nuclear dye, DAPI (blue), to visualize nuclear DNA and apicoplast DNA (small white arrow) and also anti-TgβCOP (red) dye. DAPI staining shows the stage of the cell cycle that the parasites are in (indicated by the DNA content [either 1*n* or 2*n*]). HN, host cell nucleus. Stains reveal parasitophorous vacuoles with parasites in various stages of division. Anti-TgβCOP (red, yellow arrows) follows COPI localization. For direct comparison between the parasite cell lines, compare similar nuclear content vacuoles, i.e., 1*n* and 1*n*. Double yellow arrows indicate a dividing Golgi body in 2*n* parasites. While wild-type parasites in panels A and B show a restricted area of COPI (TgβCOP) staining, transgenic parasites stably expressing P30-GFP-HDEL display a diffuse βCOP distribution. The staining pattern has become completely cytosolic and in ~30% of the vacuoles, TgβCOP is detected within the parasitophorous vacuole (white dotted line). TgβCOP staining appears relatively unchanged in parasites stably expressing TgERD2-GFP compared to wild-type parasites.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TgERD2-GFP

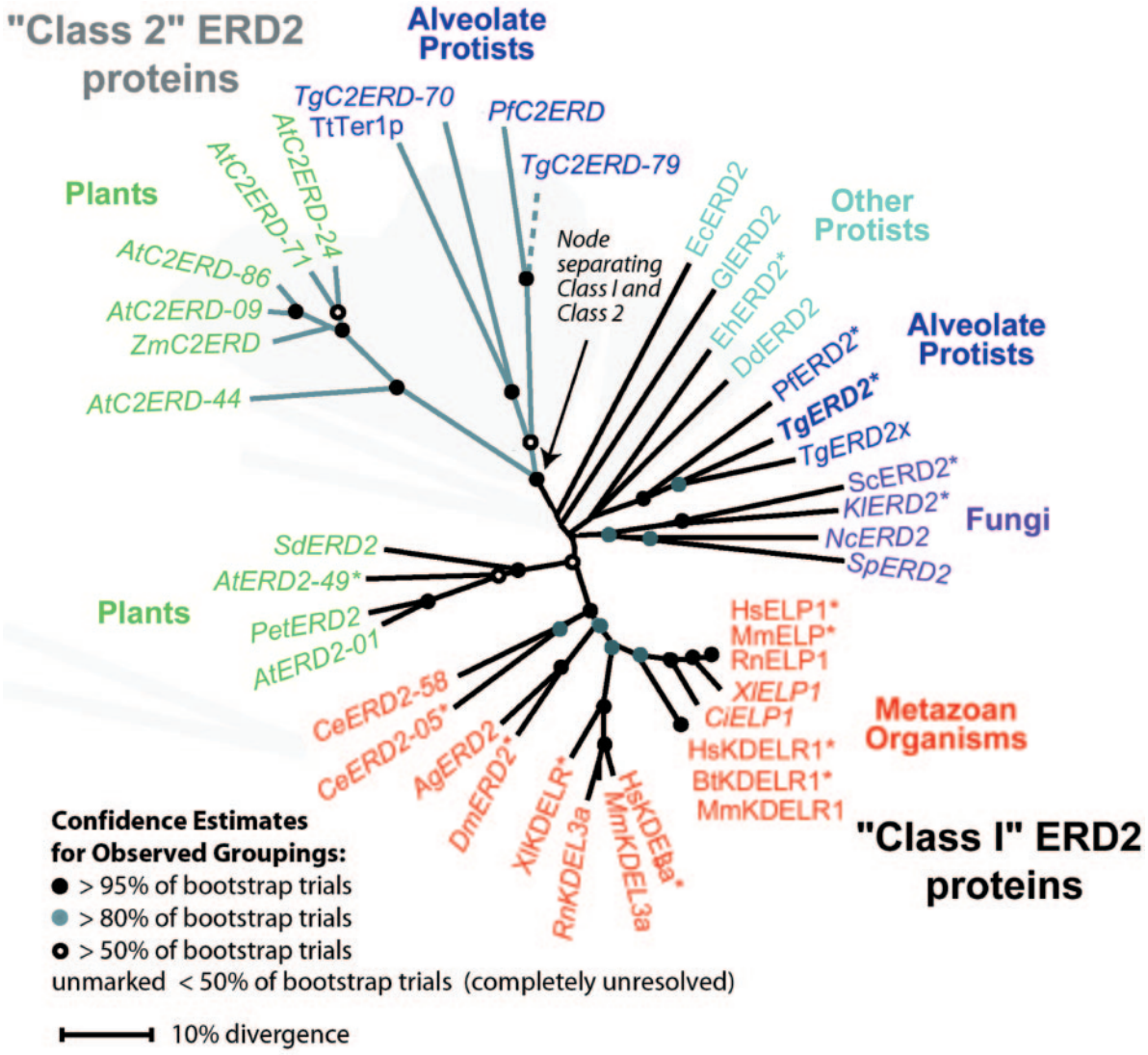

FIG. 5. Phylogenetic analysis reveals two classes of ERD2-related proteins. Phylogenetic analysis of ERD2-related sequences. *Toxoplasma gondii* ERD2 (TgERD2, gi22656361) is shown in bold type. Published gene names are used where they exist. Most uncharacterized sequences (indicated by italic font) have been named either ERD2 or "class 2" ERD2 proteins depending on which group they belong to; when more than one sequence of a particular group exists in a given organism, the last two digits of the NCBI gi number are included in the name after a hyphen. Vertebrate sequences have been given the name of their apparent human orthologue. Sequences with an asterisk were used in the alignment shown in Fig. 1. Only those organisms possessing a plastid-like organelle or those thought to have had a plastid or plastid-like organelle possess "class 2" ERD2 proteins. For metazoan organisms, the *Anopheles gambiae* (mosquito) AgERD2 (gi21290772), *Bos taurus* (cow) BtKDELR1 (gi462021), *Caenorhabditis elegans* CeERD2-05 (gi25295805) and CeERD2-58 (gi17552358), *Ciona intestinales* CiELP1 (gi27525270), *Drosophila melanogaster* DmERD2 (gi17137442), *Homo sapiens* HsELP1 (gi284076), HsKDELR1 (gi5803048), and HsKDEL3a (gi6857802), *Mus musculus* (mouse) MmELP1 (gi13385318), MmKDELR1 (gi19527170), and MmKDEL3a (gi19353820), *Rattus norvegicus* (rat) RnELP1 (gi27662352) and RnKDEL3a (gi27662728), and *Xenopus laevis* XlELP1 (gi28277295) proteins were analyzed. For plants, the *Arabidopsis thaliana* AtERD2-49 (gi18397049), AtERD2-01 (gi21592401), AtC2ERD-71 (gi15222871), AtC2ERD-24 (gi15223724), AtC2ERD-86 (gi21537286), AtC2ERD-09 (gi18420309), and AtC2ERD-44 (gi 28393144), *Scherffelia dubia* (algae) SdERD2 (gi14285310), and petunia PetERD2 (gi6685428) proteins were analyzed. For fungi, *Encephalitozoon cuniculi* EcERD2 (gi19074408), *K. lactis* KlERD2 (gi119544), *Neurospora crassa* NcERD2 (gi18376020), *S. cerevisiae* ScERD2 (gi6319431), and *Schizosaccharomyces pombe SpERD2* (gi19113321) proteins were analyzed. For protozoan organisms, the *Dictyostelium discoideum* DdERD2 (gi2882906), *Entamoeba histolytica* EhERD2 (gi6685402), *Giardia lamblia* GlERD2 (gi29246388), *Plasmodium falciparum* PfC2ERD (gi23619247) and PfERD2(gi23619458), *Toxoplasma gondii* TgERD2 (gi22656361), TgERD2x, TgC2ERD-70, and TgC2ERD-51 (gi27725951), and *Tetrahymena thermophila* Ter1p (gi18913082) proteins were analyzed. Note that this search identified two *Toxoplasma* ESTs (gi27725951, which is 66% identical to Pf23619247 over 107 amino acids and which corresponds to genomic TGG_6447; gi27726970, which is 35% identical to TtC2ERD Ter1 over 185 amino acids and which corresponds to genomic TGG_6573) as well as a maize mRNA (gi21213525 [91% identical to At15223724] and a rice genomic fragment [gi28971740]) as likely members of this novel subfamily.

as the parasite Golgi apparatus and the apical end of the nuclear envelope. Therefore, we created a fluorescent recombinant fusion reporter for the Golgi apparatus. (Fig. 3B and F). GRASP55 is a mammalian Golgi structural or matrix protein and has a highly similar homologue in the *Toxoplasma* EST database (54, 61). Localization of (human) GRASP-YFP to the parasite Golgi apparatus has been confirmed both by fluorescence microscopy and immunoelectron microscopy (54). In both transiently (Fig. 3A to C) and stably transfected parasites (Fig. 3E to H), TgERD2-GFP localized most intensely to the apical end of the nuclear envelope (Fig. 3A and E). Some expression was observed in the rest of the nuclear envelope and ER (use the model in Fig. 3D for orientation of organelles). This apical region corresponds to a region on or near the parasite Golgi apparatus as evidenced by the GRASP-RFP overlap (Fig. 3C and G). ERD2 localization to the Golgi apparatus has been observed in both mammalian and yeast cells (3, 8, 27, 37, 38, 62, 71). Previous to studies utilizing GRASP, the parasite Golgi apparatus was defined as the area sandwiched between two DNA-containing compartments. These two compartments, which are easily identified on the fluorescent level by 4′,6′-diamidino-2-phenylindole (DAPI) staining (Fig. 3F, inset), consist of the large parasite nucleus and the smaller apicoplast compartment (Fig. 3D and F). GRASP-RFP (Fig. 3F, inset) and TgERD2-GFP (Fig. 3H) clearly localized within this zone.

HDEL ligand-induced redistribution of coatomer. Expression of constructs encoding either retrieval ligands or their receptors results in brefeldin A-like effects in mammalian cells (30, 38, 42). The Golgi apparatus is disturbed, and coatomer is redistributed to the cytosol (3, 27). To determine whether such mechanisms existed in *Toxoplasma*, we monitored the localization of parasite coatomer in several different transgenic parasites. *Toxoplasma* βCOP-like protein subunit (TgβCOP) is a member of the parasite COPI complex (20). TgβCOP stains the Golgi region in untransfected ("wild-type") parasites (Fig. 4). Parasites stably expressing exogenous retrieval ligand (P30-GFP-HDEL) result in a redistribution of coatomer (Fig. 4). In these cell lines, TgβCOP staining becomes diffuse and cytosolic (Fig. 4B). We were surprised to see that TgβCOP (red) also stains the vacuolar space outside of these parasites. This phenotype is observed in both mixed and clonal populations expressing P30-GFP-HDEL (data not shown). In contrast, stable expression of exogenous receptor, TgERD2-GFP did not result in coatomer redistribution either in interphase cells (1*n* DAPI staining) or in dividing cells (2*n* DAPI staining) (Fig. 4B).

Phylogenetic analysis of TgERD2. Pairwise sequence comparisons (not shown) were consistent with the hypothetical model that TgERD2 identified above is the *Toxoplasma* ortholog of the characterized yeast and human ERD2 proteins. To test this model and better understand the evolution of ERD2, we performed a phylogenetic analysis of all proteins detectably similar to TgERD2 in all fully sequenced organisms (sequences from some unfinished organisms were included to provide additional definition to the tree). The topology of most parts of the resulting tree (Fig. 5) is consistent with analyses of organism evolution (5), suggesting that most organisms have a single ERD2 gene and that TgERD2 is the *Toxoplasma* orthologue of these genes. Closer examination reveals additional complexity. Duplications of the ERD2 gene have occurred along some lineage(s), particularly in vertebrates (which have three ERD2 genes apparently resulting from two duplications that occurred after the flies and vertebrates diverged) and *Caenorhabditis elegans* (which has two genes resulting from an independent duplication event).

These conclusions are consistent with those of previous analysis. However, we were surprised to see that *Plasmodium* and *Arabidopsis* contain additional ERD2-related proteins that group together, separate from the other ERD2-related proteins (upper left quadrant of the tree in Fig. 5, "Class 2"). This grouping, which is well-supported by bootstrap analysis, suggests that these divergent organisms share a novel subfamily of ERD2-related proteins and that related organisms will also contain members of this subfamily. To test this prediction, we probed the entire GenBank DNA and EST databases with these two sequences. This search and subsequent phylogenetic analysis identified two *Toxoplasma* ESTs as well as a *Tetrahymena* mRNA, a maize mRNA, and a rice genomic fragment (gi28971740) as members of this novel subfamily which we have termed class 2 ERD2 proteins. No evidence of class 2 ERD2 proteins was found in metazoan organisms or fungi. Genes corresponding to both of the *Toxoplasma* class 2 ESTs exist in the unfinished *Toxoplasma* genome database (TGG_6447 and TGG_6573), confirming that these are legitimate *Toxoplasma* sequences. This genomic sequence search also revealed the existence of an additional *Toxoplasma* class I ERD2 gene (TgERD2x, TGG_6633), but because no ESTs corresponding to this gene have been reported, it is not clear whether this sequence is expressed.

DISCUSSION

Transmembrane retrieval receptor in *T. gondii*. This study identifies and characterizes the first *Toxoplasma* transmembrane retrieval receptor for retrograde trafficking in the secretory pathway of *T. gondii*. Because *Toxoplasma* has parasite-specific organelles that play central roles in the process of invasion and pathogenesis, detailed understanding of its secretory pathway is both intriguing from the standpoint of comparative cell biology and essential from the standpoint of developing drugs to fight these organisms. Here we confirm that a functional HDEL receptor exists and that *Toxoplasma* has retrieval pathways. TgERD2 provides an important tool for further dissection of the parasite secretory pathway. Membrane trafficking studies in *T. gondii* have been hampered by the lack of transmembrane markers, forcing researchers to rely on heterologous chimeric reporters (25). Now, with our characterization of TgERD2, there is a definitive marker that allows for in-depth studies on the trafficking of transmembrane bound as well as soluble cargo.

Previously, we showed that the parasite reporter P30-GFP (67), normally secreted into the parasitophorous vacuole, is retained within the parasite ER and nuclear envelope upon the addition of the parasite retrieval motif HDEL (20). This was the first evidence that a functional HDEL receptor involved in retrograde traffic existed in the *T. gondii* parasite. In this study, we show that the HDEL receptor localizes to the parasite tER and Golgi apparatus (Fig. 3). In other systems, the formation of this receptor-ligand complex drives the recruitment of

coatomer proteins and the budding of vesicles destined for retrograde trafficking (3, 4, 38, 44). We have shown previously that TgβCOP (a major COPI component) localized to the apical face of the nuclear envelope (20) and now show that TgβCOP also localizes to the parasite Golgi apparatus (Fig. 4A). These findings are significant in that they emphasize the importance of the essential relationship between ER exit or retrieval sites and the Golgi apparatus (54, 76).

Receptor-ligand interactions: effects on *Toxoplasma* membrane trafficking. To explore the relationship between retrograde trafficking, coated vesicle formation, and parasite Golgi apparatus stability, we examined the localization of TgβCOP in transfected parasite cell lines expressing the HDEL retrieval ligand. We found that TgβCOP staining became cytosolic in cell lines expressing the P30-GFP-HDEL retrieval ligand (Fig. 4B). This observation is consistent with previous studies showing that upregulation of the retrograde trafficking pathway results in brefeldin A-like effects. Intriguingly, upregulation of retrograde traffic by expression of retrieval ligand (P30-GFP-HDEL) also results in staining of the parasitophorous vacuole, indicating an apparent secretion of TgβCOP out of the parasites (Fig. 4). The significance of this observation is unclear; future studies will address this phenomenon.

Next, we examined the effect of stable exogenous expression of the retrieval receptor TgERD2. Previous studies in mammalian cells have shown that overexpression of human ERD2 results in βCOP dispersal (27). However, we did not observe any discernible change in localization of TgβCOP in parasites expressing TgERD2-GFP (Fig. 4). There are many possible explanations for this observation. One explanation is that the receptor becomes nonfunctional upon tagging. We consider this unlikely given that several studies in other systems have shown that epitope-tagged and fluorescently tagged ERD2 functions and localizes similarly to wild-type ERD2 (27, 44, 60, 70, 71). A second explanation is that the stable cell lines express TgERD2-GFP at low levels (near endogenous). All TgERD2-GFP clonal cell lines (10 lines) obtained displayed similar GFP intensity levels, suggesting that TgERD2-GFP expression is limited. Such limitation might occur by specific mechanisms regulating TgERD2 levels. Alternatively, overexpression could simply be lethal. A third explanation is that βCOP dispersion occurs only in response to excess ligand, not excess receptor. This effect could be specific to organisms such as *Toxoplasma*, but it could also have wider relevance. For example, some mammalian studies have indicated that ligand binding serves as a prerequisite for recruitment of the retrograde trafficking coatomer complex and movement of COPI coated vesicles carrying the retrieval receptor, ERD2 (3, 4, 43, 44).

Complementation of the yeast null mutant. The ability of TgERD2 to complement the yeast *erd2* mutant stands in contrast to human ERD2 (37). The level of amino acid identity between species does not explain this difference in functionality. Despite 51% amino acid identity between the yeast and human proteins, human erd2.1 is not able to complement the loss of ERD2 function in yeast (37). Conversely, TgERD2 possesses only 38.6% identity with ScERD2 and is able to complement the yeast *erd2* mutant. A more likely explanation is that yeast contains only one ERD2 protein and mammals have three (Fig. 5) (37, 39, 53, 70). This observation suggests that the failure of human erd2.1 to complement the yeast ERD2 mutation may result from functional specialization among human ERD2 proteins. In other words, human erd2.1 may fail to replace yeast ERD2 because erd2.1 is specialized to perform a subset of ERD2 functions. Whatever the case may be, our results indicate that *Toxoplasma* ERD2 is capable of accomplishing the full range of the required ERD2 functions in yeast.

Novel ERD2-related proteins in alveolate protists and plants. All of our results, including the ability of TgERD2 to rescue the yeast ERD2 mutation, are consistent with the idea that TgERD2 is the *Toxoplasma* orthologue of the yeast ERD2 protein and that TgERD2 has the full functionality of the yeast ERD2 protein (Fig. 2).

The results of our phylogenetic analysis of ERD2-related proteins are consistent with this conclusion (Fig. 5), but our analysis also reveals that the ERD2 family is more complex than has been previously assumed. Figure 5 shows that the protists *Toxoplasma*, *Plasmodium*, and *Tetrahymena* contain additional ERD2-related proteins that group into a distinct subfamily. These organisms are members of a distantly related group of protists called alveolate protists, which either have plastid-derived organelles or are believed to have possessed them in the past (National Center for Biotechnology Information [NCBI] taxonomy database) (17). Members of the novel class 2 ERD2 subfamily are also found in flowering plants (Fig. 5). No evidence for this group was found in metazoan organisms or fungi, including those that are fully sequenced. This previously unrecognized ERD2 subfamily appears to be limited to organisms that have plastids or plastid-like organelles. Should localization studies of these class 2 ERD2s bear out this hypothesis, we suggest naming these proteins PERLs (PERLs for plastid-associated ERD2-like proteins).

The functional significance of these class II proteins is not yet clear. We have verified that these genomic sequences are expressed as transcripts, using reverse transcription-PCR (X. Ye and K. M. Hager, unpublished data). Given that they have been conserved in organisms as divergent as alveolate protists and plants, it seems likely that they are functionally significant. It is reasonable to postulate that the alveolate class 2 ERD2 proteins are involved in membrane trafficking functions specific to these organisms, such as those associated with the plastid (9, 14, 18, 22, 23, 57, 72). The alveolate plastid generally has four membranes (13, 50, 51, 58) and is believed to have been acquired by endosymbiosis of a plant cell (red or green algae) (17, 34, 50, 51, 73). It has been postulated that the external membrane is derived from the host, the outermost internal membrane is derived from the ancestral plant, and the remaining internal two membranes are derived from the chloroplast of the plant (13, 58, 73). In light of this understanding and the observation that plants also contain these class 2 ERD2 proteins, it is tempting to speculate that class 2 ERD2 proteins are involved in hypothetical transport functions occurring between the cytoplasm of plants (which includes the interior of the apicoplast between the second and third membranes) and the chloroplast (the interior two membranes of the apicoplast). Resolving the function of alveolate and plant class 2 ERD2 family of ERD proteins will be an intriguing problem for further study. Because this novel set of ERD proteins are not found in metazoan organisms and are postulated to func-

tion in pathways important for parasite survival and virulence, they are attractive drug development targets.

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