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Transmembrane Domain Modulates Sorting of Membrane Proteins in *Toxoplasma gondii**

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Overlapping mechanisms that function simultaneously in the intracellular sorting of mammalian membrane proteins often confound delineation of individual sorting pathways. By analyzing sorting in the evolutionarily simpler organism *Toxoplasma gondii*, we demonstrate a role for transmembrane domain (TMD) length in modulating the signal-dependent segregation of membrane proteins to distinct intracellular organelles. The dense granule localization of the single pass transmembrane protein GRA4 could be completely rerouted to the Golgi and cell surface simply by replacement of its TMD with that from either vesicular stomatitis virus G or the low density lipoprotein (LDL) receptor. Mutational and biochemical analyses suggested that this effect was not caused by any specific sequence motif or strength of membrane association of the GRA4 TMD. Instead, a property imparted by the vesicular stomatitis virus G or LDL receptor TMDs, both of which are longer than the GRA4 TMD, appeared to be a decisive factor. Indeed, shortening the LDL receptor TMD to a length similar to that of GRA4 resulted in dense granule localization, whereas lengthening the GRA4 TMD resulted in rerouting to the Golgi. From these data, we conclude that although the TMD may not necessarily be a sole determinant in membrane protein sorting, its properties can markedly modulate the utilization of more conventional signal-mediated sorting pathways.

A hallmark of eukaryotic cells is their extensive intracellular membrane systems that permit the organized compartmentalization of myriad proteins and their associated biochemical activities. The most well understood paradigm of protein sorting involves the encoding within proteins of specific sequence motifs, termed sorting signals, that are recognized by cellular machinery to mediate their selective segregation and compartmentalization (see Refs. 1 and 2). Many proteins, particularly of complex systems such as mammalian cells, contain multiple overlapping and/or degenerate sorting signals. Understanding how these signals are organized into a hierarchy, are interpreted, and are regulated to mediate high fidelity sorting into various intracellular compartments represents a major challenge of cell biology.

The role of the transmembrane domain (TMD)[1] in sorting membrane proteins remains poorly defined. Although for selected proteins specific TMD residues or motifs direct sorting to or retention within discrete compartments (3–9), there are as yet no general rules from these examples. Statistical analyses correlating features of the TMD to cellular localization have suggested that retention of proteins in the endoplasmic reticulum (ER) or Golgi may be mediated in many cases by TMD length (10–13). In one prevailing model, an increasing gradient of membrane thickness through the secretory pathway from the ER to the plasma membrane would allow proteins with shorter TMDs to be retained in earlier organelles (14). However, because many of the proteins under study are also in larger complexes with other luminal and/or membrane proteins, each of which may contain sorting information, it has been difficult in most cases to clearly demonstrate a physiological role for the TMD in either sorting or retention.

Therefore we explored this problem using a simpler model system, the Apicomplexan parasite *Toxoplasma gondii*. This organism likely possesses fewer redundancies in sorting mechanisms than other eukaryotes (15). The *T. gondii* secretory pathway is minimalized, containing a polarized transitional ER, a single Golgi stack, and limited *N*-linked glycosylation (16). Like all members of the Apicomplexan family, including the *Plasmodium* species, the parasite also contains three other distinct specialized secretory organelles termed rhoptries, micronemes, and dense granules (17). Each of these organelles contains distinct sets of proteins, the segregation and sorting of which are incompletely understood.

All 10 dense granule proteins (18–28) identified in *T. gondii* contain an N-terminal signal sequence for targeting to the ER, and six of these proteins (GRA3 through GRA8) are predicted to contain a single TMD. Curiously, GRA proteins with putative transmembrane domains behave aberrantly in subcellular fractionation assays (29–31), partitioning as partly soluble and partly membrane-associated both within the parasite and following secretion into the vacuolar space. Regardless of whether GRA3–GRA8 are sorted as soluble or as membrane proteins, no common feature or sequences have been identified that may serve to target GRA proteins to dense granules.

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[1] The abbreviations used are: TMD, transmembrane domain; ER, endoplasmic reticulum; BAP, bacterial alkaline phosphatase; LDL, low density lipoprotein; VSVG, vesicular stomatitis virus G; IF, immunofluorescence; LDLR, low density lipoprotein receptor; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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Work from our laboratory (32) and others (33, 34) has demonstrated that soluble proteins, including the heterologous reporters *Escherichia coli* β-lactamase and bacterial alkaline phosphatase (BAP), are targeted to dense granules via the bulk flow pathway and are subsequently secreted into the vacuolar space surrounding the intracellular parasites. Chimeras containing BAP fused to the transmembrane domain and cytosolic tail of either the endogenous dense granule protein GRA4 (to give BAP·GRA4) or vesicular stomatitis virus G protein (to give BAP·VSVG) were targeted to dense granules and Golgi, respectively. Although trafficking beyond the dense granule or Golgi to the cell surface or parasitophorous vacuole was found to be influenced by residues in the cytoplasmic tail (32), the basis for the initial segregation of proteins between the Golgi and dense granules has remained unclear. We have now explored this issue in further detail using GRA4 as a model substrate. Our results have revealed a previously unappreciated modulatory role for the TMD in mediating segregation between the Golgi and dense granule sorting pathways.

EXPERIMENTAL PROCEDURES

All BAP fusion constructs were cloned into the vector pNTP/Sec, which contains an expression cassette for secreted proteins (5′ and 3′ untranslated regions of the NTPase gene, and the NTPase signal peptide) as described earlier (32). The following fragments were generated by the polymerase chain reaction using appropriate templates: residues 23–449 of *E. coli* BAP, a domain of GRA4 containing 8 luminal amino acids and the complete transmembrane (19 amino acids) and C-terminal domain (52 amino acids), and the TMD (23 amino aids) of the LDL receptor. The appropriate combination of these fragments was coligated into the AvrII/BglII cloning site of pNTP/Sec. The leucine substitutions for the BAP·GRA4 transmembrane domain mutants in Fig. 2 were prepared using mutagenic primers and PCR. 5′-poly(L)ext was prepared by introducing complementary, phosphorylated oligonucleotides encoding the residues ALLLLLL after residue 290 (into a NotI site of the GRA4 transmembrane domain) of the 5′-poly(L) construct. Each of the coding regions was subcloned into plasmid SP64 (Promega, Madison, WI) for *in vitro* translation studies. A construct encoding wild type VSVG in the SP64 vector has been described (35). Sequences of all PCR-amplified fragments were verified by dideoxy sequencing at the WM Keck Sequencing Center, Yale University School of Medicine.

The RH strain of *T. gondii* was maintained in Vero cells, and transient transfections by electroporation were as described previously (36). For immunofluorescence (IF), monolayers of human foreskin fibroblasts grown on glass coverslips in 24-well plates were infected with transfected parasites and were processed for IF 20–24 h post-transfection as described (32). BAP chimeras were detected with purified rabbit anti-BAP polyclonal antibody (5 Prime → 3 Prime, Inc., Boulder, CO) as described previously (32). For dense granule colocalization samples were incubated with anti-BAP antibodies and the monoclonal antibody to GRA3 followed by a mixture of fluorescein-conjugated goat anti-rabbit IgG antiserum and rhodamine-conjugated goat anti-mouse antiserum, respectively (Calbiochem-Novachem Corp.). Coverslips were washed in phosphate-buffered saline and mounted in Moviol for examination by epifluorescence microscopy. Images were captured with a cooled charge-coupled device camera (Photometrics, Tucson, AZ) and were processed with Image-Pro Plus software (Media Cybernetics, Silver Spring, MD).

The preparation and use of rabbit reticulocyte lysate and canine pancreatic rough microsomes for *in vitro* translocation assays were as described (37). Translation reactions were at 32 °C for 60 min. Proteolysis reactions used 0.5 mg/ml proteinase K for 60 min on ice. Immunoprecipitations were as described (32) and utilized a rabbit polyclonal antibody to BAP or mouse polyclonal antibodies raised against residues that were either N-terminal or C-terminal to the GRA4 TMD. For alkaline extractions, microsomes from translation reactions were isolated by sedimentation (50,000 rpm for 10 min in a TL100.1 rotor, Beckman Instruments) and were resuspended in 20 times the original translation volume in 0.1 M NaCO3, pH 11.5. After 20 min on ice, the membrane fraction was isolated by centrifugation (100,000 rpm for 20 min in a TL100.1 rotor, Beckman Instruments). Proteins in the supernatant were collected by precipitation with trichloroacetic acid. SDS-PAGE was on 12% Tris-Tricine gels.

In vitro translated material was solubilized under native conditions in 100 mM NaCl, 50 mM Hepes pH 7.4, 1 mM $MgCl_2$, and 1% Triton X-100. This mixture was layered onto a 5–25% glycerol gradient, spun for 14 h at 55,000 rpm in a TLS-55 rotor, fractionated into 11 fractions, and analyzed by SDS-PAGE. Marker proteins migrated as follows: cytochrome C (14 kDa) in fractions 2 and 3, bovine serum albumin (67 kDa) in fractions 6 and 7, native globin (tetramer of 14 kDa) in fractions 6 and 7, GRP94 (dimer of 94 kDa) in fraction 9, and catalase (11 S) in fraction 11. Under these conditions, GRA4 migrated in fraction 5, and BAP·GRA4, BAP·VSVG·GRA4, and BAP·VSVG migrated in fraction 6 and 7.

Hydropathy was assessed by the method of Kyte and Doolittle (38) using MacVector software. Window size was 7 residues. Hydrophilicity was plotted (with hydrophobic residues attaining negative values). The TMD was defined in the present study as the contiguous stretch of residues with a hydrophilic value of less than −0.6. Note that the definition of what segment of a hydrophobic domain is considered the TMD has varied in previous studies. Therefore, the exact number of residues noted in Table I may be slightly different from that cited in previous studies. However, the use of other cutoff values (ranging from 0 to −1.0) for assessing TMD length resulted in similar differences in length between the various constructs and did not affect the close correlation between TMD length and localization.

RESULTS AND DISCUSSION

We tested whether determinants in the TMD of GRA4 were involved in the localization to dense granules. We analyzed three chimeric constructs containing BAP fused to the TMD and cytoplasmic tail derived from either GRA4, VSVG, or low density lipoprotein receptor (LDLR) (Fig. 1*A*). Each chimera was transfected into parasites, which were then used to infect cultured fibroblasts. The subcellular distribution of the chimeras within the intracellular parasites, termed tachyzoites, was visualized by immunofluorescence microscopy (39) (Fig. 1*B*). BAP·GRA4 was localized to dense granules as evidenced by colocalization with the endogenous dense granule protein GRA3 (32). By contrast, expression of either BAP·VSVG or BAP·LDLR·GRA4 in *T. gondii* resulted primarily in an anterior perinuclear staining pattern typical for Golgi localization and distinct from the dense granule pattern revealed by GRA3 staining. That the perinuclear structures are Golgi is supported by the observation that they are disrupted by treatment with the fungal metabolite Brefeldin A (data not shown) as well as by immunoelectron microscopy (32). Overexpression of these two constructs resulted in their delivery to the parasite cell surface, whereas BAP·GRA4 was ultimately delivered to the parasitophorous vacuole and the membrane network located therein (data not shown). Thus, BAP·GRA4 and BAP·LDLR·GRA4, which differ only in their TMD, are distinctly segregated within *T. gondii*.

Constructs were compared biochemically by analyzing translocation and topology at the ER using an *in vitro* translation and translocation system. Both endogenous GRA4 and BAP·GRA4 were efficiently translocated across the ER membrane (Fig. 1*C*) as evidenced by the signal cleavage of GRA4 (note slightly faster mobility of the translation product in the presence, but not absence, of microsomal membranes) and the efficient glycosylation of BAP·GRA4 (resulting in decreased mobility). A small percentage of GRA4 was also glycosylated, which was consistent with the presence of a poorly utilized single potential site for *N*-linked glycosylation. The glycosylation of these proteins was confirmed by an increase in mobility upon treatment with endoglycosidase H or the absence of more slowly migrating forms if translations were performed in the presence of a competitive inhibitor of glycosylation (data not shown). Upon treatment with exogenous protease the translation products were reduced in size by ~4 kDa, which is consistent with the removal of a cytoplasmically oriented tail. This was confirmed by demonstrating that the proteolytically protected domain was immunoprecipitated with N- but not C-terminally directed antibodies (Fig. 1*C*, *lanes 4–7*). Inclusion of 0.5% of the non-denaturing detergent Triton X-100 in the pro-

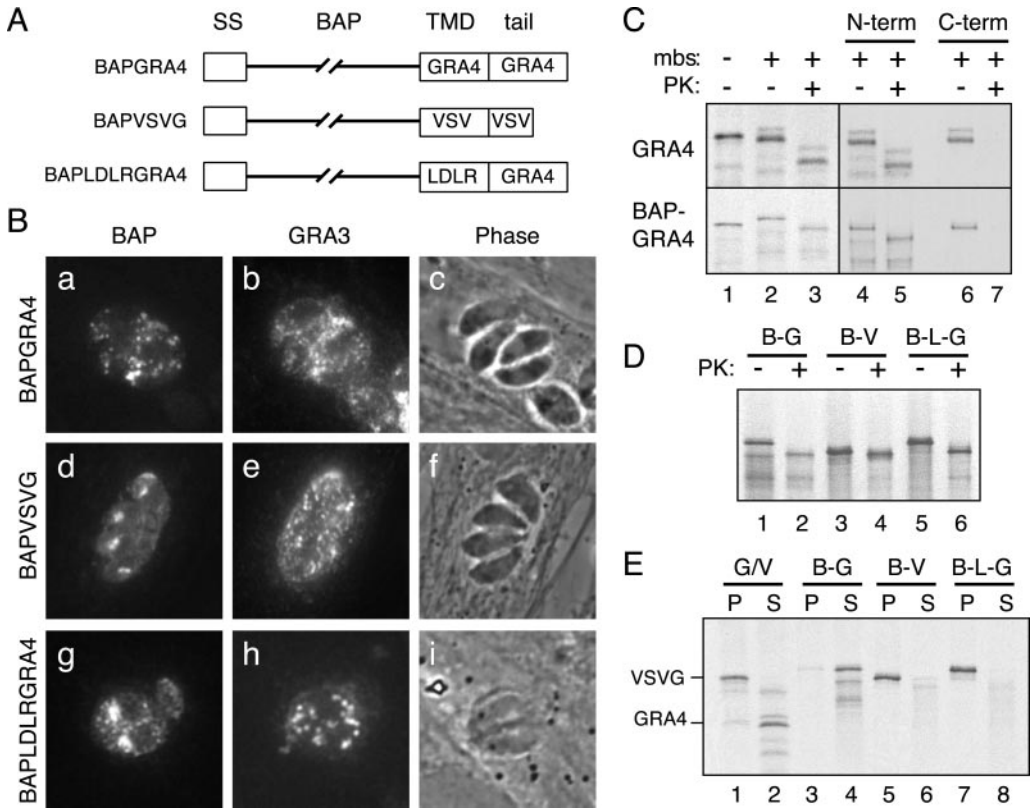

FIG. 1. *A*, diagram of constructs. The signal sequence (*open box*) is from NTPase (23) followed by BAP (*line*), and the TMD and cytoplasmic tails (*boxes* with their sources indicated). *B*, indirect IF of intracellular tachyzoite parasites expressing BAP·GRA4 (*a–c*), BAP·VSVG (*d–f*), and BAP·LDLR·GRA4 (*g–i*). The phase images (*c*, *f*, and *i*) are shown with the fluorescent signals from staining for the expressed protein (BAP, *a*, *d*, and *g*) and the endogenous dense granule protein GRA3 (*b*, *e*, and *h*). *C*, protease protection assay of *in vitro* translated GRA4 (*top panel*) and BAP·GRA4 (*bottom panel*). Translations were in the presence (*lanes 2–7*) or absence (*lane 1*) of microsomal membranes (*mbs*) as indicated. Following translation, samples were either analyzed directly or first digested with proteinase K (*PK*) as indicated. Aliquots of some samples were subjected to immunoprecipitation with an antibody directed against the N terminus (*N-term*, *lanes 4* and *5*) or C terminus of the protein (*C-term*, *lanes 6* and *7*). *D*, BAP·GRA4 (*B-G*), BAP·VSVG (*B-V*) and BAP·LDLR·GRA4 (*B-L-G*) were translated in the presence of microsomal membranes and analyzed for topology by digestion with proteinase K as indicated. Note that the tail of VSVG is shorter than the tail of GRA4, accounting for the smaller shift in migration upon digestion with protease. *E*, the alkaline extractability at pH 11.5 of various proteins from membranes following *in vitro* translocation was assessed (see "Experimental Procedures"). Non-extracted material in the membrane pellet (*P*) is analyzed together with extracted material in the supernatant (*S*). *Lanes 1* and *2* were from translation reactions containing a mixture of both wild type GRA4 and wild type VSVG (*G/V*; their respective positions are indicated at the *left*). The other translation reactions contained BAP·GRA4 (*B-G*, *lanes 3* and *4*), BAP·VSVG (*B-V*, *lanes 5* and *6*) and BAP·LDLR·GRA4 (*B-L-G*, *lanes 7* and *8*).

tease digestion reactions resulted as expected in complete digestion of both GRA4 and BAP·GRA4 (data not shown). A similar analysis of BAP·VSVG and BAP·LDLR·GRA4 revealed that they were in the identical topology (Fig. 1*D*). Importantly, all detergent-extracted chimeras migrated as monomers by sucrose density gradient analysis (data not shown; see "Experimental Procedures" for details). From these data, we conclude that GRA4, BAP·GRA4, BAP·VSVG, and BAP·LDLR·GRA4 all achieve a type I transmembrane topology at the ER with the N-terminal domain in the lumen and a cytosolically disposed C-terminal tail.

Analysis of membrane integration by alkaline extraction revealed surprising differences between the constructs (Fig. 1*E*). Both GRA4 and BAP·GRA4 were unexpectedly and nearly quantitatively extracted upon treatment with 0.1 M $NaCO_3$, pH 11.5. By contrast, neither wild type VSVG (a known integral membrane protein control) nor the chimeras BAP·VSVG or BAP·LDLR·GRA4 were extracted with carbonate (Fig. 1*E*). Thus, carbonate extraction, in contrast to topology, is a feature that distinguishes between these constructs in a manner that correlates with their subcellular localization. Furthermore, the extractability of GRA4 may provide an explanation for the previous enigmatic observations that GRA proteins containing transmembrane domains, when liberated from parasites or infected cells by mechanical disruption, fractionate by differential centrifugation as partially soluble and partially membrane-associated molecules (29–31). In the case of GRA5 these earlier results had been interpreted to suggest that the molecule inserts post-translationally into membranes of the parasitophorous vacuole following secretion as a soluble protein (31). In view of the current results, it is possible instead or in addition that transmembrane-anchored GRA proteins (GRA3–GRA8) are synthesized as membrane proteins that behave anomalously in traditional subcellular fractionation assays. Most importantly for the current study, however, is the fact that the TMD of GRA4, in addition to its unusual behavior upon carbonate extraction, appears to be directly involved in its sorting to dense granules.

Two principal ways in which the TMD may control localization of a membrane protein may be envisioned. Specific sequence elements within the TMD could be recognized by machinery that mediates sorting. Alternatively, biophysical features that distinguish the GRA4 TMD from those of VSVG and LDLR could be exploited to mediate sorting. To distinguish between these alternatives, especially as they relate to differences in alkaline extraction (Fig. 1*E*), we generated a series of mutations within the GRA4 TMD of the BAP·GRA4 reporter (Fig. 2*A*). These mutations were designed to disrupt putative sorting sequence motifs as well as alter the biophysical characteristics of the TMD. *In vitro* analysis revealed that each of

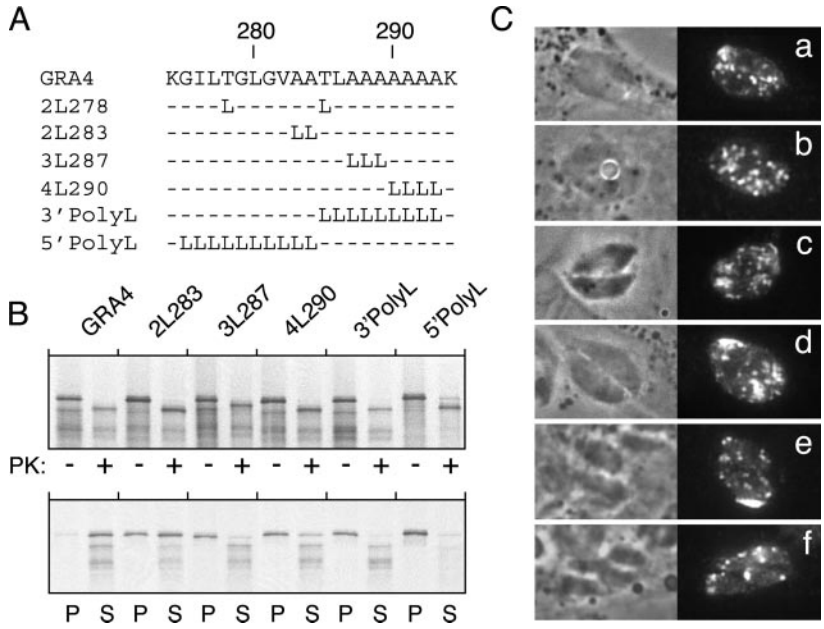

FIG. 2. *A*, sequences of the wild type GRA4 TMD indicating various leucine mutations introduced into this domain. *B*, BAP·GRA4 constructs containing each of the mutations described in *A* (as indicated above the gel) were analyzed for topology by protease protection (*top panel*) or membrane integration by alkaline extraction (*bottom panel*) as in Fig. 1, *D* and *E*, respectively. *C*, indirect IF for BAP of tachyzoites expressing BAP·GRA4 containing the following mutations: *a*, 2L278; *b*, 2L283; *c*, 3L287; *d*, 4L290; *e*, 3′-poly(L); and *f*, 5′-poly(L). Both the phase contrast (*left*) and fluorescent image (*right*) are shown. Colocalization with an endogenous dense granule marker (GRA3) was confirmed in each case (data not shown). *PK*, proteinase K.

TABLE I
Localization and biochemical behavior of GRA4 constructs

Construct	TMD length[a]	Localization	Topology	Integrated[b]
				%
GRA4	17	DG[c]	Type I TM	<10
BAPGRA4	17	DG	Type I TM	<10
2L297	17	DG	ND[d]	ND
2L303	17	DG	Type I TM	40
3L307	17	DG	Type I TM	>90
BAPLDLRΔGRA4	17	DG	Type I TM	80
5′-poly (L)	19	DG	Type I TM	>90
4L310	20	DG	Type I TM	80
3-poly(L)	20	DG	Type I TM	>90
BAPLDLRGRA4	23	Golgi	Type I TM	>90
BAPVSVG	24	Golgi	Type I TM	>90
5′-poly(L)ext	26	Golgi	ND	ND

[a] TMD length was defined as the number of contiguous amino acids scoring less than −0.6 on the Kyte-Doolittle scale (38).
[b] Percent integration (rounded to the nearest 10%) was assessed by extraction at pH 11.5 as in Figs. 1*E* and 2*B*.
[c] DG, dense granule.
[d] ND, not determined.

the mutations was made as a type I transmembrane protein (Fig. 2*B*, *top panel*). By contrast, replacement of various residues within the TMD with leucines resulted in an alteration of the alkaline extractability of the protein (Fig. 2*B*, *bottom panel*). The 2L283 mutant was ~50% extracted, whereas the other mutants were largely resistant to extraction. In particular, the 5′-poly(L) and 3′-poly(L) constructs in which entire halves of the TMD were replaced with leucines were as resistant to extraction as the VSVG or LDLR TMDs (compare Fig. 2*B* with Fig. 1*E*).

All of the constructs localized to dense granules (Fig. 2*C*). These data argue strongly against a sequence motif within the TMD mediating targeting to dense granules, as none of the changes that together encompassed every residue within the TMD altered localization. More surprising however was the finding that altering the extractability of BAP·GRA4, which was a principal feature distinguishing it from BAP·VSVG and BAP·LDLR·GRA4, also did not affect localization. For example, despite the fact that the 3′-poly(L) and BAP·LDLR·GRA4 constructs are identical in their luminal domains, cytosolic tails, topology, and resistance to alkaline extraction, they are nonetheless efficiently segregated to different compartments within the parasite.

Sequence comparisons between the LDLR and VSVG TMDs did not reveal known sorting motifs or sequence similarities. Thus, it seemed unlikely that positive sorting signals in these TMDs segregated them from the construct containing the GRA4 TMD. Therefore we searched for other systematic differences between the various constructs that might correlate with their differential sorting. Notably, all of the TMDs from constructs shown in Figs. 1 and 2 that localize to dense granules were 20 or fewer residues in length, whereas the LDLR and VSVG TMDs were 23 and 24 residues in length, respectively (Table I). This distinction is readily illustrated by Kyte-Doolittle hydropathy plots of the TMD regions of BAP·GRA4, 3′-poly(L), BAP·LDLR·GRA4, and BAP·VSVG (Fig. 3*A*), where residues comprising the hydrophobic core of the TMD are indicated with *black bar*s. These observations raised the possibility that TMD length may be another feature that influences sorting between the Golgi *versus* dense granules.

We tested this hypothesis in two ways. We reasoned that we should be able to redirect a Golgi-localized protein (such as BAP·LDLR·GRA4) to dense granules simply by shortening the TMD. Conversely, lengthening the TMD of a dense granule-localized protein (such as the 5′-poly(L) construct) should result in localization to the Golgi. The LDLR TMD of

BAP·LDLR·GRA4 construct was shortened by 6 residues to generate BAP·LDLR·Δ6GRA4. Hydropathy analysis showed this shortened TMD to now be of similar length, hydrophobicity, and character to the native GRA4 TMD (Fig. 3*B*). Importantly, the deletion construct was still made as a type I transmembrane protein that was largely resistant to alkaline extraction (Table I). Notably, however, this construct localized to dense granules in contrast to the clear Golgi localization for BAP·LDLR·GRA4 (Fig. 4, *a–f*). To perform the complementary experiment, the 5′-poly(L) TMD was lengthened by the insertion after residue 290 of 7 hydrophobic amino acids (ALLLLLL, to generate 5′-poly(L)ext; hydropathy plot shown in Fig. 3*B*). This extension of the TMD resulted in an apical, perinuclear localization upon expression in parasites (Fig. 4, *g–i*). This localization pattern is consistent with the Golgi and is clearly distinguished from dense granules.

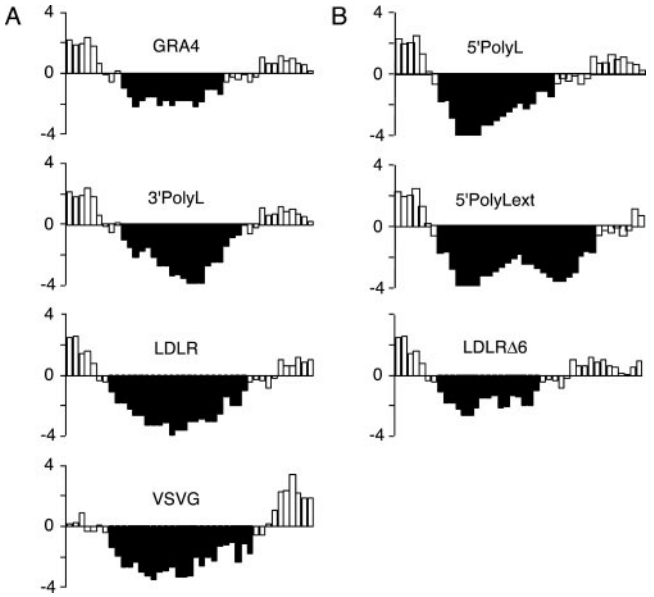

FIG. 3. **Hydropathy analysis of the indicated TMD regions in the context of their respective BAP fusion constructs (see "Experimental Procedures").** Hydrophilicity is plotted, and residues in the TMD are indicated by the *black sections*.

The data presented in this study allow us to conclude that transmembrane domain length can modulate sorting between the dense granule and Golgi pathways. The strongest argument for this conclusion is the observation that matched constructs invariant in their luminal and tail domains were nonetheless differentially localized on the basis of their TMDs. Overall hydrophobicity of the various TMDs tested did not appear to be a major determinant for sorting, because neither hydropathy analyses (Fig. 3), topology (Figs. 1*D* and 2*B*), nor alkaline extractability (Figs. 1*E* and 2*B*) correlated to localization behavior (Table I). Furthermore, no sequence homology or structural features within the TMD correlated to localization, arguing against specific recognition of sequences within the TMD or TMD-mediated protein-protein interactions involved in sorting. By contrast, the only feature that could be altered to influence localization involved the length of the TMD. Although the precise aspects of TMD length that most affect localization remain to be elucidated, the collective data argue that sorting mediated by either luminal or cytoplasmic tail motifs of a membrane protein can be modulated by the TMD.

This modulating effect could be mediated by changes in the thickness of the membrane bilayer in different compartments of the secretory pathway. In this model, a gradient of cholesterol (and perhaps other lipids) in the membranes from the ER to the cell surface results in a progressive increase in membrane thickness. Thus, shorter TMDs may not be as stable in a thick bilayer, resulting in their preferential retention in earlier compartments of the secretory pathway. As described above, resident Golgi enzymes in mammalian cells appear to have statistically shorter TMDs than plasma membrane proteins (12), suggesting that perhaps they are prevented from progressing further in the secretory pathway. Unfortunately, this

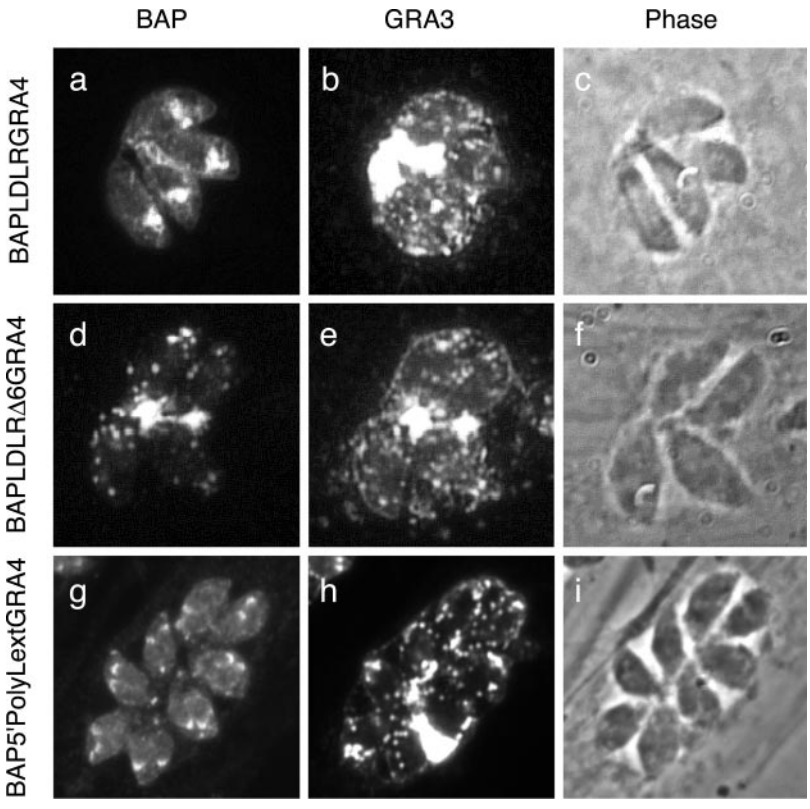

FIG. 4. **Indirect IF of tachyzoites expressing BAP·LDLR·GRA4 (*a*, *b*, and *c*), BAP·LDLR·Δ6GRA4 (*d*, *e*, and *f*), and BAP5′·poly(L)ext·GRA4 (*g*, *h*, and *i*).** Parasites were double stained for the transfected construct (BAP) and an endogenous marker for dense granules (GRA3). Note that BAP·LDLR·Δ6GRA4 shows extensive colocalization of BAP and GRA3 in numerous dense granules and the intravacuolar network, sites of accumulation of dense granule but not Golgi proteins after secretion. By contrast, the other two constructs show distinct localization patterns for the BAP and GRA3 proteins that are characteristic for Golgi and dense granules, respectively.

model has been confounded by observations that many of the Golgi enzymes interact with each other, suggesting the alternate possibility that "kin recognition" results in complexes too large to enter transport carrier exiting the Golgi. Similar confounding variables have prevented a clear understanding of how resident ER membrane proteins such as components of the oligosaccharyl transferase complex are retained. The present study argues that endogenous dense granule proteins of *T. gondii* are sorted partly on the basis of their TMD lengths (all of which are less than 20 amino acids by the criteria applied in this study).

It is not yet clear where proteins destined for dense granules are segregated from other secreted molecules, such as glycosylphosphatidylinositol-anchored proteins destined for the parasite surface. Sorting into dense core vesicles has classically been a feature of the *trans*-Golgi network or more distal compartments, in particular the immature secretory granule (Refs. 40 and 41). However, two observations suggest that these may not be the sites of dense granule protein sorting in *T. gondii*. First, the parasite does not contain compartments with morphological characteristics of immature secretory granules. Second, no processing of dense granule proteins occurs, which is unlike the situation in many secretory cells. In fact, TMD length-mediated sorting of dense granule proteins may occur at the ER because the ER membrane (by analogy to mammalian cells) is likely to be cholesterol-poor and relatively thin. Thus, proteins that are marginally stable in the ER membrane may not be able to progress to the Golgi where the membrane may be thicker. Indeed, the GRA4 TMD, although sufficiently stable to quantitatively attain a membrane spanning orientation at the ER, is nonetheless entirely extractable with a high pH (Fig. 1).

Our results suggest indirectly that the GRA4 cytoplasmic tail is sufficient to mediate sorting to dense granules if juxtaposed to the GRA4 transmembrane domain. In contrast, the GRA4 cytoplasmic tail is not sufficient to mediate dense granule sorting for constructs with long heterologous transmembrane domains (for example BAP·LDLR·GRA4). The sorting motif within the GRA4 cytoplasmic tail may be the tyrosine-containing Y*XX*Ø (tyrosine-*X*-*X*-bulky hydrophobic) sequence. We have demonstrated previously that selected Y*XX*Ø motifs are recognized by the μ1 chain of the *T. gondii* AP-1 adaptor complex (42, 43) and that mutation of these motifs alters targeting of the rhoptry proteins ROP2 and ROP4 (42). Although secretion of GRA4 is impaired by mutation of the Y*XX*Ø motif (32), the GRA4 cytoplasmic tail is not recognized by the *T. gondii* μ1 chain (43) suggesting that another adaptor may be involved. Clearly, mutation of the GRA4 cytoplasmic tail within the context of the various constructs used herein is a logical future direction of study. Such experiments are likely to shed light on the molecular mechanism in which TMD-mediated sorting of GRA4 (demonstrated in this study) is influenced by coexisting conventional sorting signals proposed in previous studies (32, 43).

Finally, one interesting although speculative implication of these findings is the possibility that the sorting pathway utilized by particular proteins could be altered in response to changes in the environment or metabolic state of the cell. For example, TMD-mediated sorting, if it depends on lipid bilayer properties, could be either dominant or recessive to other sorting signals depending on cellular parameters such as cholesterol abundance. In this manner, the subcellular distribution and hence potential function of selected proteins could change in response to cellular need. In the context of the Apicomplexan parasites, such marked changes in metabolism may accompany different stages of a complicated life cycle that includes multiple diverse environments and hosts. Further studies will be required to determine whether such potential types of regulated sorting are exploited by organisms in a physiologically productive manner.

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