Tandem Duplication of \textit{rab} Genes Followed by Sequence Divergence and Acquisition of Distinct Functions in \textit{Trypanosoma brucei}\textsuperscript{*}

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The Ras superfamily of small G proteins governs unidirectional cellular processes by virtue of GTP hydrolysis and concomitant conformational changes, which are in turn regulated by a number of accessory factors. Members of the Rab subfamily are important for correct targeting and fusion of intra-organellar vesicles loaded with trafficking proteins and lipids. During evolution from a prototype gene, novel functions may be acquired by duplicated daughter genes; for Rab proteins, this can be tested by location, which is specifically related to the function of each Rab. We have found an example of two \textit{rab} genes in \textit{Trypanosoma brucei} (\textit{trab} genes) that clearly arose by tandem duplication, being highly related to each other and remaining juxtaposed in the genome, whose products have dramatically different subcellular locations, indicative of discrete functions. These two \textit{trab} genes, isolated on a single genomic clone, are separated by a short intervening sequence and are in a head-to-tail orientation. The nucleotide sequences of the open reading frames and intervening sequence were determined and show that the genes are paralogues, probably arising from an ancient tandem duplication. Both genes are most homologous to \textit{ypt1} and \textit{sec4} in the \textit{Saccharomyces cerevisiae} genome, while phylogenetic reconstruction indicates that although they have clearly diverged, the proteins are more closely related to each other than to other Rab protein sequences available in the data base. Immunofluorescence microscopy, using antibodies raised against the recombinant Trab proteins, clearly demonstrates that the native Trab proteins have completely distinct subcellular locations in the trypanosome. Trab1p is present in a widespread reticular location similar to BiP, suggesting an endoplasmic reticulum location, while Trab7p is observed in a discrete structure adjacent to the kinetoplast. Most interestingly, the Trab7p-positive compartment also appears to divide at the same time, or just prior to, the kinetoplast, \textit{i.e.} early in mitosis, suggestive of association with structures in the flagellar pocket region. An estimate of the divergence time indicates that the \textit{trab1/trab7} duplication occurred \textit{~}100 million years ago, and therefore, the persistence of this pair suggests an essential role in the survival of \textit{T. brucei}.

\textit{Trypanosoma brucei} spp., the causative agent of nagana in ungulates and sleeping sickness in humans, is a parasitic protozoan of economic and public health importance. Studies of this organism have revealed many unusual biological phenomena later observed in other systems, perhaps most remarkably \textit{trans}-splicing (1) and glycosylphosphatidylinositol protein anchors (2). The paradigm glycosylphosphatidylinositol-anchored protein, the variant surface glycoprotein (VSG),\textsuperscript{3} accounts for \textit{~}10\% of total protein biosynthesis in the mammalian bloodstream form (BSF) of the parasite and provides an essential immunological barrier. The entire VSG coat, comprising \textit{~}10\textsuperscript{7} identical molecules, is replaced during antigenic switching in the BSF by a process of continued biosynthesis of new VSG and internalization/shedding of the old coat protein. Much VSG recycles: internalization is concomitant with surface delivery of newly synthesized VSG in the flagellar pocket (FP). In the insect procyclic form, VSG is replaced by the abundant glycosylphosphatidylinositol-anchored procyclic acidic repetitive protein (PARP) (3).

Rates of surface protein internalization are comparable with that of a mammalian cell, but in a trypanosome, only the FP, a small area of the cell surface, is available for exo- and endocytotic activity due to a subplasmalemmal microtubular network over the rest of the cell body (4). The FP has four specialized adjacent microtubules running parallel down one side of the structure, coincident with a specialized area, the cytosome. This may be the site of exo- and endocytosis since it is associated with vesicles carrying VSG arising from the Golgi complex and with vesicular tubules and flattened cisternae structurally similar to the \textit{trans}-Golgi network. Coated vesicles budding from the FP are observed only in BSF parasites (5). The FP is of interest for several reasons; first, for membrane trafficking of glycosylphosphatidylinositol-anchored glycoproteins; second, for sorting mechanisms, as endo- and exocytosis are presumably simultaneously active within a small membrane area (6, 7); and finally, as a potential site for therapeutic attack.

Detailed exploration of intracellular trafficking requires suitable markers, but the wide evolutionary distance between trypanosomes and most other eukaryotes is unfortunately reflected in a lack of immunological cross-reactivity between homologues. Marker proteins that are available include trypansom BiP, an endoplasmic reticulum marker (8, 9), and CRAM, a cysteine-rich acidic transmembrane protein found in the FP and associated endocytic vesicles (10). Also available is an antibody to the CB1 epitope of a developmentally regulated 44-kDa protein of unknown function in the endocytic pathway, which is hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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\textsuperscript{1} The abbreviations used are: VSG, variant surface glycoprotein; BSF, bloodstream form; FP, flagellar pocket; PARP, procyclic acidic repetitive protein; PCR, polymerase chain reaction; EST, expressed sequence tag; ORF, open reading frame; kb, kilobase(s); IVS, intervening sequence (between ORFs); TLCK, N\textsubscript{0}-p-tosyl-l-lysine chloromethyl ketone; PAGE, polyacrylamide gel electrophoresis; rTrab, recombinant Trab.
Evolution of rab genes in T. brucei

found in the FP, endosomes, and lysosomes (7).

The Rab family consists of GTPases with ~30% identity to Ras and with most homology confined to four GTP-binding motifs (11). Rab proteins are essential for protein trafficking in all eukaryotic organisms. Family members have low intrinsic GTPase activity, requiring activating proteins (GTPase-activating proteins) to increase turnover and act as effectors (12). GTP- and GDP-bound Ras have distinct conformations (13), and it can be inferred that Rab proteins will similarly undergo a conformational change upon GTP hydrolysis since their structure is highly homologous. Each Rab protein binds a sub-set of organelles; the C terminus is responsible for intracellular localization, while the N terminus recognizes the target organelle and is required for vesicle fusion (14). The paucity of markers suitable for trafficking studies led us to initiate the cloning of rab genes from T. brucei (15) since each Rab protein has a discrete subcellular location within the secretory pathway (16, 17). The study of T. brucei Rab proteins (Trab proteins) will yield new sequences important for targeting proteins to trypanosome organelles, provide information on the compartmentalization of trypanosomes, and generate subcellular markers.

A degenerate PCR cloning approach has yielded eight expressed sequence tags (EST) of small GTP-binding proteins from T. brucei, including Ran/Tc4 (18) and seven Trab homologues (15), while separate studies have identified three additional trab genes (19, 20). We isolated two trab genes on a single genomic clone (15), and here we report the genomic organization of these genes (previously rtb1 and rtb7) and the subcellular location of the encoded proteins.

MATERIALS AND METHODS

Nucleic Acids and Recombinant DNA Methods—Molecular biology-grade water was from an Elgastat Option 4 system (Elga) sterile-filtered through a 0.2-μm filter (Sartorius Corp.). Restriction and DNA-modifying enzymes were from Bioline, Promega, MBI Fermentas, New England Biolabs Inc., and Stratagene. Nitrocellulose was from Schleicher & Schuell (obtained from Anderman & Co., Ltd., London). Radioligostopes were from DuPont NEN and Amersham International. A P1 T. brucei library filter was a gift from Dr. S. Melville (Department of Pathology, Cambridge University, Cambridge, UK). Vector pGEXtk and glutathione-Sepharose 4B were from Pharmacia Biotech Inc., and the λZAPIII kit was from Stratagene. Molecular biology methods and antibody preparations followed standard procedures (21, 22). Plasmids were grown in Escherichia coli XI-L-Blue (Stratagene) following transformation by electroporation with a BTX 600 ECM electroporator. PCR products and gel-embedded DNA were purified using PCR cleanup kits (Promega), and plasmid and λ DNA were purified using QIAGEN kits following the manufacturers' instructions. Small-scale plasmid preparations were performed using the Promega Wizard system. Rabbit antibodies to T. brucei BiP were from J. Bangs (University of Wisconsin, Madison, WI). Secondary antibodies were from Sigma.

PCR—PCR was performed in 50-μl reactions with 25 pmol of each primer in ammonium buffer (Bioline), 2.5 mM MgCl2, and 1 unit of Taq polymerase in a Model 480 thermal cycler (Perkin-Elmer) as follows: one cycle at 98°C for 5 min; one cycle at 95°C for 5 min; and 35 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 3.5 min. Templates were 1 μl of a 1:100 dilution of R111 λ phage lysate or 1 μl of a λZAPIII plaque equilibrated in 0.4 ml of SM phage dilution medium (21) or 10 ng of plasmid; phages were boiled for 10 min prior to PCR. The primers for determining orientation of trab1 and trab7 were RAT1FUS, RAT7PCR, RAT7AS, RAT1AS, and trab1 3′, GAGCAAGGAAATTCATCAACACCA, and trab7 3′, GGACAGAAGAATTCAATCAGACCC. For expression of recombinant Trab proteins, each ORF was amplified from pBS.R7X1 (see below), digested with EcoRI/BamHI for 16 h at room temperature, and ligated into EcoRI/BamHI-cleaved pGEX2tk.

Southern Blotting—0.5-μg aliquots of R111 λ DNA were cut with the AvoII, BglII, ClaI, HindIII, HincII, PstII, SacII, SstII, XbaI, or XhoI restriction enzyme, and duplicate filters were prepared by high salt transfer Southern blotting (21). Filters were probed at high stringency (0.05× SSC and 0.1% SDS at 75°C) with trab1 or trab7 ESTs cut from pBlueScript with EcoRI/BamHI, gel-purified, and labeled using a random hexamer priming kit (Boehringer Mannheim). To map R111, 0.5-μg aliquots were cut with one or two restriction enzymes (AvoII, ClaI, SacII, XbaI, and XhoI), and hybridization and washes were performed using the λZAPIII probe (15) labeled with [γ-32P]ATP using polynucleotide kinase at low and high stringency (6× SSC and 0.1% SDS at room temperature, and 1× SSC and 0.1% SDS at 65°C, respectively). Single and double restriction digests of pBS.R7X1 were blotted and probed with trab1 and trab7 ESTs. To estimate the total number of trab genes in the T. brucei genome, bacteriophage library filters (SM5 No. 12) of T. brucei strain TREU 927/4 genomic DNA in grid format, with ~3× haploid genome coverage, was probed with the WD primer at low stringency. Autoradiography was with X-Omat 5 film (Eastman Kodak Co.) at ~85°C with an intensifying screen; exposure times were 16 h to 7 days. Construction of pBS.R7X1—A 6-kb XbaI fragment from R111 (15) was subcloned into pBlueScript using a λZAPIII kit; recombinant λ plasmids (from the whole R111 XbaI digest) were screened on duplicate filters with trab1 and trab7 EST probes. Secondary screening was by PCR (15), and one clone was rescued to produce plasmid pBS.R7X1.

DNA Sequencing—Sanger sequencing of pBS.R7X1 through the ORFs and intervening region (IVS) was performed using a deaza-GTP kit (Pharmacia) and primer walking in both directions. Dye terminator cycle sequencing of uncanstal regions and constructs was performed using a Taq thermal cycle sequencing kit and a Model 377 DNA sequencer (Perkin-Elmer) with polyethylene glycol-polymerized DNA (6.5% polyethylene glycol 6000 and 0.4 mM NaCl on ice 20 min; centrifuged at 13,000 × g for 30 min at 4°C; washed with 70% EtOH). Antibodies—Expression plasmids pGEX2tk.Trab1 and pGEX2tk.Trab7 in E. coli were grown in L-broth and induced with 1 mM isopropyl-1-thio-galactopyranoside. Fusion proteins were affinity-purified on glutathione-Sepharose 4B and thrombin-cleaved to produce recombinant Trab1p and Trab7p, as described (23), with the following modifications. Protease inhibitors (peptatin, leupeptin, antipain, and chymostatin, all at 2 μg/ml protease inhibitor mixture) and TLCK (dry) were added to the initial lysate. Cleared lysate was equilibrated with glutathione-Sepharose for 16 h at 4°C. Thrombin treatment was at room temperature for 2 h, with more thrombin added after 1 h. Protein was quantitated by SDS-PAGE and Comassie Blue staining. Typical yields were 0.4–4 mg of rTrab protein/liter of E. coli, with a purity of 95%. Rabbits were immunized three times with a total of 3 mg of rTrabBlp or 0.9 mg of rTrab7p using the MPL + TDM + CWS Adjuvant System (Sigma). Animals were exsanguinated, and serum was stored at -4°C in Trasylol. For affinity purification, 0.2 g of Trab7p (Department of Pharmacology, Cambridge University, Cambridge, UK) was activated and resuspended with an equal volume of coupling buffer (100 mM NaHCO3 and 500 mM NaCl, pH 8.5) containing 0.5 mg of rTrab protein in phosphate-buffered saline and coupled overnight at 4°C. Efficiency was ~45%. Purified antibodies were prepared from 50% ammonium sulfate-precipitated serum and stored at -85°C.

Trypanosomes—Culture-adapted BSF T. brucei strain 427, a gift from S. Croft (London School of Hygiene and Tropical Medicine), was grown in HMI-18 (24) at 37°C in a 5% CO2 incubator. Procyclic form T. brucei strain 427 was grown in SDM79 (25); media and supplements were from Sigma. For boiling SDS lysates, freshly harvested trypanosomes were added to SDS-PAGE sample buffer (26) at 95°C, heated for 5 min, and reduced with dithiothreitol. For hypotonic lysates, trypanosomes were resuspended in 10 mM Tris, pH 7.5, protease inhibitor mixture, phenylmethylsulfonyl fluoride, and TLCK, followed by two cycles of freeze/thaw and centrifugation at 13,000 × g for 15 min at 4°C.

Western Blotting—Western blotting was performed on proteins electrophoresed on 17% SDS-polyacrylamide minigels and wet-blotted to 100 V for 3 h in transfer buffer (Hoefler Pharmacia Biotech Inc.) onto 0.45-μm nitrocellulose. Filters were blocked in 3% or 5% bovine serum albumin, phosphate-buffered saline, and 0.1% Tween 20 at 4°C for 16 h. All remaining manipulations were at room temperature. Blots were probed with primary antibodies (in a 2–4 ml block) for 1 h, washed four times with phosphate-buffered saline/Tween 20, and then incubated with secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG diluted 1:2000 in blocking buffer for 2 h) and visualized with enhanced chemiluminescence (ECL) (Amersham Corp.) exposed to x-ray film. Purified antibodies at concentrations of 0.43 mg/ml (anti-Trab1p) and 0.17 mg/ml (anti-Trab7p) were typically used diluted at 1:1000 and 1:200, respectively.

Immunofluorescence Analysis—Immunofluorescence analysis was described (8) with the following modification. Cells were fixed for 80 min with 1.5% formaldehyde and 0.32% glutaraldehyde (v/v). Purified
RESULTS

Organization of trab1 and trab7 Genes—Limited restriction endonuclease mapping of R111, a genomic clone containing both trab1 and trab7 (15), showed the insert was at least 26 kb long. The genomic organization of the trab genes was further explored by PCR using forward (F) and reverse (R) primers to each EST. We tested all four possible orientations: trab1 and trab7 oriented head-to-tail with trab1 first (1F/7R), head-to-head with trab7 first (7F/1R), head-to-head reversed (1F/7F), or tail-to-tail (1R/7R). A 2.4-kb product was obtained using only the second primer combination, 7F/1R (Fig. 1A, second lane), indicating that trab7 preceded trab1 in a head-to-tail orientation, with an IVS of ~1.8 kb between ORFs. Identity of the PCR product was confirmed by digestion with XhoI and HincII (Fig. 1B), previ-
were present in the \textit{trab1} and \textit{trab7} genes. Although the structure of the \textit{trab7} genes is \textit{double-underlined}, with acceptor dinucleotides indicated by asterisks (*, mini-exon splice site; **, putative polyadenylation site). Three noncoding regions are homologous to one another (boxed) and were located using COMPARE. Regions with homology to the repeat units of the mini-exon donor gene (double-underlined; GenBank™ Data Bank accession number X00833) were located by BLAST search of an unidentified ORF found in the IVS against the nucleotide data base. Homology to this region in the \textit{trab1} 3'-untranslated region was located using BESTFIT (70\% nucleotide homology).

A divergence time of 100 million years ago was calculated from the nucleotide ORF sequences of \textit{trab1} and \textit{trab7} using the method of Li (28). This suggests that these genes are paralogues arising from an ancient duplication event. The homology of the amino acid sequence (64\% similar) is greater than that of the nucleotide sequence (49\%), so although synonymous nucleotide substitutions were saturated (28), considerable constraints on the divergence of the protein sequences are evident, indicating that the two gene products are important for \textit{T. brucei} survival. The ORF sequences of \textit{trab7} and \textit{trab1} were used to search the data base, and both are clearly Rab homologues at the nucleotide and amino acid levels. However, \textit{Trab1p} and \textit{Trab7p} could not be unambiguously assigned as particular Rab homologues, which suggests a trypanosome-specific function for \textit{Trab1p} and \textit{Trab7p}. Because of species redundancy in the GenBank™ Data Bank, we also determined which genes in another unicellular eukaryote, \textit{S. cerevisiae}, were most similar to \textit{trab1} and \textit{trab7}. \textit{trab1} was most homolo-
gous to sec4, while trab7 was most similar to ypt1, which are themselves closely related. We analyzed the relationship of trab1p and trab7p to one another and to other homologous Rab sequences by phylogenetic reconstruction. trab1p and trab7p always cosegregate as a monophyletic group whether assessed against their closest Rab homologues (Fig. 3A and data not shown) or compared with kinetoplastid Rab sequences (Fig. 3B), including the putative Rab5 homologues trab5Ap (Tbrab5b (19)) and trab5Bp.

**Protein Sequences of trab1p and trab7p—**Hypothetical translations of the trab1p and trab7p ORFs indicate that the protein sequences are 43% identical and 64% similar. Both sequences contain all of the major homology boxes typical of a Rab protein, including most of the residues involved in GTP/GDP binding and GTPase-activating protein binding (Fig. 4A). trab1p also has a typical C-terminal amino acid motif for double geranylgeranylation, CCGG.

Interestingly, trab7p contains several atypical features. Most significantly, the GTPase motif characterized by the WD box, normally WDTAQGE for Rab proteins (PM3 in Fig. 4A), is altered in trab7p to WDTSGQD. The Glu → Asp change is not uncommon, but the Ala → Ser change (corresponding to a G → T nucleotide transversion in the first position of the codon) is highly unusual and possibly functionally significant; human RhoE also has an Ala → Ser substitution at this position and binds GTP but not GDP. Additionally, both trab7p and RhoE have Ser at codon 12 (Ras numbering); combined with the Ala → Ser substitution, this results in lack of GTPase activity in RhoE (35). Also, constitutively active viral Ki-Ras has an Ala → Thr mutation in the WD box (36). Overall, these data suggest that trab7p may possess abnormal GTPase activity.

trab7p has a 12-amino acid insertion when compared with Ha-Ras, Ki-Ras, Ran, and at least 10 other Rab proteins (Fig. 4A and data not shown). Alignments place this insertion as an extension of loop 8 (nomenclature of Wittinghofer and Pai (13)). Residues important in the adjoining secondary structures are conserved in both trab1p and trab7p (Fig. 4B). The 12-residue loop of trab7p was hypothetically large enough, by three-dimensional modeling, to interact with the adjacent nucleotide-binding site (data not shown).

**Membrane Association of trab Proteins—**The unusual C-terminal sequence of trab7p suggested that this protein may not be isoprenylated efficiently, and therefore, we wished to test if the protein was membrane-associated. trab1p, by contrast, with its canonical C-terminal isoprenylation motif, is predicted to be isoprenylated and therefore membrane-associated. Procyclic cells were hypotonically lysed and centrifuged, and the soluble and particulate material was resolved by SDS-PAGE. trab1p and trab7p were located by Western blotting, and proteins were quantitated in identical samples (see “Materials and Methods”). Trypanosome proteins fractionated equally into soluble and particulate material, indicating efficient cell lysis, while both trab1p and trab7p appeared solely in particulate fractions (Fig. 5B). These data indicate that trab7p is membrane-associated. In addition, our data are consistent with previous observations that some Rab proteins are exclusively membrane-bound and have no cytoplasmic pool (37).

**Subcellular Locations of trab1p and trab7p—**To begin to ascribe a function to trab1p and trab7p, we next chose to investigate the subcellular locations of the two proteins. We used affinity-purified antibodies to immunolocalize the native proteins by immunofluorescence analysis. Anti-trab1p staining revealed a reticular pattern in both procyclic and BSF cells (Fig. 6, A and C) very similar to the appearance of cells stained for BiP, an endoplasmic reticulum luminal marker (Fig. 6, A and C). 10 μg of rTrab1p premixed with primary antibody for 10 min was sufficient to abolish the signal in BSF cells, demonstrating specificity (data not shown). Therefore, an endoplasmic reticulum location is tentatively assigned to trab1p.

In contrast, cells stained with anti-trab7p antibody revealed one or two discrete and highly compact structures in each trypanosome cell. The trab7p-positive compartment was located close to the kinetoplast in both procyclic and BSF trypanosomes (Fig. 6, E–J). Prior incubation of the antibodies
with 10 μg of rTrab7p abolished the signal completely (data not shown). Most significantly, in cells containing two kinetoplast DNA networks (as revealed by Hoechst stain), two Trab7p-positive structures were observed positioned at either end of the kinetoplast (Fig. 6, G–J). The location of Trab7p is similar to that described for the basal bodies of trypanosomes (38), but clearly, further analysis is required to refine this assignment. Taken together, the immunolocalization data demonstrate radically different subcellular locations for Trab1p and Trab7p. Therefore, a tandem duplication resulted in acquisition of functional differences in these highly related Rab homologues.

DISCUSSION

We have identified a pair of small GTPase genes juxtaposed in a head-to-tail orientation in the genome of T. brucei. We were unable to find evidence for a reverse transcriptase-mediated duplication mechanism, e.g. mini-exon or poly(A) tail vestiges. While there are two genes present in the locus (trab1 and trab7), five other trab genes and the gene for Ran/TC4 are dispersed and exhibit no linkage with each other (15, 18), suggesting that the trab1/trab7 pair is unusual. In addition, all our data suggest that trab1/trab7 are not part of a more extensive trab gene array. Phylogenetic reconstruction indicates that Trab1p and Trab7p are more similar to each other than to any other Rab sequence, evidence that they are paralogues. Taken together, these data strongly argue that these genes are the result of an ancient tandem duplication event, allowing polycistronic expression is a common strategy in T. brucei and may facilitate coordination of gene expression. Indeed, trab1 and trab7 are transcribed at low levels and in equal amounts (15).

By probing of a genomic library of known complexity, the total number of Trab-related genes was estimated to be ~20. Since 11 trab clones and ESTs have been reported to date, it is likely that a representative sampling of trab sequences, covering most of the trypanosome intracellular trafficking pathways, is already in hand. This number is similar to that obtained for S. cerevisiae, also a unicellular eukaryote, and is in agreement with our earlier estimate of total trab numbers (15). Therefore, these data suggest that, as a first approximation, T. brucei possesses a secretory pathway of equivalent complexity to yeast.

We propose that the continued linkage of trab1 and trab7 is due to functional selection. Conservation of protein sequence contrasted with saturated synonymous nucleotide replacement strongly suggests a functional selection on the gene products. While both Rab sequences are homologous to a selection of Rab homologues, conservation of protein sequence is also consistent with a species-specific function. The immunolocalization data for Trab7p, indicating association with the kinetoplast and FP, are clearly consistent with this proposal. In addition, we were unable to detect Trab1p or Trab7p in COS-7 cells, either by Western blotting or by immunofluorescence analysis, or in L. major, by Western blotting. While the former result was not unexpected, the lack of immunological cross-reactivity between Trab proteins and Leishmania Rab proteins is also consistent with a species-specific function.

Trab1p has canonical Rab sequences, including a C-terminal isoprenylation signal, and shows diffuse reticular staining consistent with a Rap1p/Rab1p function. The homology to sec4 (and the closely related ypt1) detected by searching the S. cerevisiae genome may reflect this. In contrast, Trab7p has several un-
usual features. An important alteration in Trab7p is an Ala → Ser substitution in the WD box, potentially resulting in a GTP-bound, constitutively active protein like RhoE (35) or viral Ki-Ras, which has an Ala → Thr mutation (36). A second change in Trab7p is a 12-amino acid loop adjacent to the nucleotide-binding pocket. We believe it is likely that Trab7p binds only GTP and does not hydrolyze it, but clearly, this needs to be shown empirically. The subcellular location of Trab7p is also highly unusual, consisting of a single spot in nondividing cells located close to the kinetoplast. This structure divides at a similar time to the kinetoplast, clearly preceding nuclear division, suggesting association of the Trab7p compartment with basal body/FP structures. Like the Trab7p compartment, the basal body and FP are located close to the kinetoplast and divide with it, prior to nuclear division (38).

Carboxyl-terminal isoprenylation is essential for Rab function and occurs in T. brucei (44), and in all Rab homologues reported in T. brucei (44), (KWRBC), canonical isoprenylation sequences are found, i.e. SCAC (Trab4Bp), GCAC (Trab5Ap), GCCG (Trab5Bp), GGCG (Trab1p), and DSCC (Ypt1p from kinetoplastid L. major). Despite its unusual signal sequence, native Trab7p is membrane-associated, which suggests that it is a substrate for the trypanosome isoprene transferases. It remains to be determined which isoprene is present on Trab7p.

The divergence of Trab7p from the canonical Rab sequence and the association with a kinetoplast-specific organelle are suggestive of a novel role for Trab7p in the trypanosome cell. We propose that trab7 arose by duplication of a progenitor trab gene, with Trab1p now fulfilling a more basic endoplasmic reticulum-associated function and Trab7p co-opted into a specialized role. Trab1p and Trab7p are 43% identical and 64% similar, reminiscent of Arf1 and Arf6, which are 66% homologous and also have different locations (45). Because of the absence of clear homologues for Trab1p and Trab7p, the precise functions of these proteins must be determined directly, which

![Image](314x318 to 557x729)

**FIG. 5.** Antibody detection of Trab proteins and their membrane localization. A, affinity-purified antibodies raised against rTrab1p and rTrab7p (lanes 1 and 7, respectively) detected specific antigens in T. brucei lysates from both procyclic and BSF trypanosomes in Western blotting. No cross-reactivity was observed between antibodies to Trab1p and Trab7p. Primary antibody was omitted from the negative controls (lanes 0). Migration positions of molecular mass standards (in kDa) are indicated to the left. B, Trab1p and Trab7p were detected by Western blotting in procyclic trypanosome lysates following separation into particulate (P) and soluble (S) fractions (inset). Antigens reactive with anti-Trab1p and anti-Trab7p antibodies were detected only in the insoluble particulate fraction. The percentage of each antigen detected in particulate and soluble fractions (main panel) was quantitated by scanning the developed Western blot using NIH Image. Trypanosome lysis was efficient, as judged by the release of proteins to the soluble fraction, quantitated by scanning a Coomassie Blue-stained gel (main panel, □). Rabbit antisera raised against rTrab1p (■) or rTrab7p (□) were affinity-purified and used in Western blots against whole trypanosome lysates or fractionated proteins from 10^7 cells (see “Methods and Materials”). Detection was by NiCl_2/diaminobenzidine.

![Image](314x318 to 557x729)

**FIG. 6.** Subcellular locations of Trab1p and Trab7p in T. brucei. Native Trab proteins were detected in whole trypanosome cells by indirect immunofluorescence using affinity-purified antibodies specific for each Trab protein. Procyclic (A, B, E–H, K, and L) or BSF (C, D, I, and J) cells were stained with anti-Trab1p antibody (A and C), anti-T. brucei Bip antibody (B and D), anti-Trab7p antibody (E, G, I, and J), or Hoescht stain (F, H, J, and L) of the same fields as E, G, I, J, respectively. Anti-Trab1p staining in procyclic cells gives reticular staining comparable to that given by anti-T. brucei Bip antibody (B). Identical staining patterns are seen in BSF cells (C and D). Note that the exposure of D is lower than that of B; staining intensities were actually equivalent. Anti-Trab7p antibodies detect a discrete compartment close to the kinetoplast in procyclic cells, and two Trab7p entities are seen in cells with two kinetoplasts (E–H). Trab7p entities are also found in BSF cells (I and J). Primary antibody was omitted from the negative controls, shown at the same exposure as stained cells (K).
is being actively pursued. Efforts to further localize the individual proteins and to gain more insight into their function by overexpression and direct study of the GTPase activities of these potentially important trypanosome proteins are currently under way. In summary, we report the presence of a tandem pair of rab genes in the protozoan parasite *T. brucei*. Immunolocalization and sequence data suggest a species-specific role for at least one (Trab7p). The trab1/trab7 pair has remained together for a considerable period of time, indicative of functional importance.

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