Sorting Signals Required for Trafficking of the Cysteine-Rich Acidic Repetitive Transmembrane Protein in *Trypanosoma brucei*

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The African trypanosome *Trypanosoma brucei* is a unicellular eukaryotic parasite, causing sleeping sickness in humans and related diseases in livestock. *T. brucei* has an intricate life cycle, alternating between the bloodstream form, which is free living in the bloodstream of a mammalian host, and the procyclic (or insect) form, which lives in the transmitting vector, the tsetse fly. By undergoing antigenic variation of the variant surface glycoprotein (VSG) coat, which covers the entire cell surface of the bloodstream form, the parasite survives the host immune attack (10, 16). During differentiation into the procyclic form in the midgut of the tsetse fly, the parasite sheds its VSG coat and replaces it with the procyclin (or the procyclic acidic repetitive protein) coat (42).

*T. brucei*, an extracellular organism, is dependent on host-derived nutrients for its growth and development. Underneath a large part of the plasma membrane is the subpellicular microtubule sheath. This distinct surface structure network prohibits the active pinocytosis of plasma membrane as a means for nutrient uptake. The only surface domain where the microtubule network is absent is the flagellar pocket (FP); the FP is an invagination of the plasma membrane, where the flagellum extends from the cell (4, 49, 50, 53). In trypanosomatids, endocytosis and exocytosis are restricted to the FP. Receptors for the uptake of macromolecules are confined to the FP (39, 53). All vesicular trafficking in this elongated and highly polarized parasite takes place between the nucleus and the FP (11, 19, 31, 39, 40, 53). Membrane-bound proteins, once synthesized, travel from the endoplasmic reticulum (ER) to the Golgi/trans-Golgi network (TGN) and then to the FP membrane. From the pocket, surface coat proteins and some other invariant surface proteins rapidly spread over the entire cell surface, while receptors for the uptake of macromolecules are retained in the FP. We have been investigating mechanisms involved in the sorting of membrane proteins to and from the FP in trypanosomes, a phenomenon that is poorly understood. In the past, most studies on protein trafficking in trypanosomes have focused on the bloodstream form, due to its unique high rate of endocytosis (2, 14, 17, 33, 34, 51, 52). Endocytosis at the FP of the bloodstream form is mediated by large clathrin-coated vesicles (2, 18–20, 32). These vesicles are undetectable in the procyclic form, where endocytosis occurs in a much less efficient manner than in the bloodstream form (26).

Little is known about the molecular details involved in protein sorting during endocytosis and along the secretory pathway in trypanosomes, though trafficking machinery similar to that of higher eukaryotes may operate (33, 34, 40). Based on studies in mammalian systems, a general principle underlying directional transfer is that the sorting information (sorting signals or determinants), usually distinct amino acid sequences or structural domains, resides within the sorted protein. The interaction of sorting determinants with specific components constituting the sorting machinery in the cytoplasm guides the protein to the correct destination. When the final destination is reached, another set of signals is required in order to maintain the protein’s localization (retention) or to allow retrieval from the membrane upon stimuli (such as the interaction with a ligand during endo-
cytosis). Our understanding of the sorting of membrane proteins in trypanosomes is rudimentary. Both the sorting determinants and the corresponding sorting machinery in trypanosomes remain to be identified.

Thus far, only two FP-associated receptor proteins of *T. brucei* have been well characterized: (i) the bloodstream-form transferrin receptor complex, which is a glycosylphosphatidylinositol-anchored protein (3, 29, 44, 46), and (ii) the type I transmembrane protein CRAM (cysteine-rich repetitive acidic transmembrane) of the procyclic form (27, 30, 56). We have been using CRAM as a model to study protein trafficking via the FP of procyclic-form trypanosomes. CRAM is abundantly expressed in the procyclic form and expressed at a relatively low level in the bloodstream form (27). CRAM consists of a putative N-terminal signal peptide followed by the extracellular domain, consisting of a 12-amino-acid-cysteine-rich repeat, a hydrophobic transmembrane domain, and a hydrophobic cytoplasmic extension of 41 amino acids (the C terminus). Cell surface expression of CRAM is exclusively restricted to the FP of trypanosomes. Biochemical analyses demonstrated that CRAM could function as a receptor in trypanosomes and was hypothesized to be a lipoprotein receptor or an essential factor to facilitate the uptake of lipoproteins in the procyclic form (30). Based on our previous deletion mutagenesis, it appeared that the cytoplasmic domain of CRAM plays important roles in the efficient transport of CRAM from the ER to the FP and in the process of receptor-mediated endocytosis (30, 56). To better define the intrinsic sorting signals dictating the trafficking fate of CRAM, we performed a mutagenesis series to delineate amino acids in the CRAM cytoplasmic domain that are involved in various sorting processes in the procyclic form. Since evidence indicated that the clathrin-mediated sorting pathway controls the trafficking fate of CRAM (22, B.-F. Chuang et al., submitted for publication), we determined the possibility of an interaction of CRAM-derived sorting signals with machinery mediated by clathrin and adaptor complexes. In short, we report here the first characterized sorting signal involved in protein trafficking via the FP of trypanosomes and hypothesize that the interaction of *T. brucei* μ1 adaptin (Tbμ1) with the sorting signal may govern the trafficking fate of CRAM.

**MATERIALS AND METHODS**

**Trypanosome strains and cell lines.** The procyclic form of *T. brucei* stock 427-60, originally obtained from R. Brun, was maintained in SDM-79 medium at 25°C (13). All CRAM mutagenesis was carried out in the previously described procyclic CRAM-B2 cell line, which contains only one allele of CRAM, as the other allele was deleted (56). The procyclic form 29-13 cell line, which expresses the procyclic CRAM-B2 cell line, which contains only one allele of 427-60, originally obtained from R. Brun, was maintained in SDM-79 medium at 25°C (13). CRAM consists of a putative N-terminal signal peptide followed by the extracellular domain, consisting of a 12-amino-acid cysteine-rich repeat, a hydrophobic transmembrane domain, and a hydrophobic cytoplasmic extension of 41 amino acids (the C terminus). Cell surface expression of CRAM is exclusively restricted to the FP of trypanosomes. Biochemical analyses demonstrated that CRAM could function as a receptor in trypanosomes and was hypothesized to be a lipoprotein receptor or an essential factor to facilitate the uptake of lipoproteins in the procyclic form (30). Based on our previous deletion mutagenesis, it appeared that the cytoplasmic domain of CRAM plays important roles in the efficient transport of CRAM from the ER to the FP and in the process of receptor-mediated endocytosis (30, 56). To better define the intrinsic sorting signals dictating the trafficking fate of CRAM, we performed a mutagenesis series to delineate amino acids in the CRAM cytoplasmic domain that are involved in various sorting processes in the procyclic form. Since evidence indicated that the clathrin-mediated sorting pathway controls the trafficking fate of CRAM (22, B.-F. Chuang et al., submitted for publication), we determined the possibility of an interaction of CRAM-derived sorting signals with machinery mediated by clathrin and adaptor complexes. In short, we report here the first characterized sorting signal involved in protein trafficking via the FP of trypanosomes and hypothesize that the interaction of *T. brucei* μ1 adaptin (Tbμ1) with the sorting signal may govern the trafficking fate of CRAM.

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**Antibodies.** The rabbit-derived anti-Bip antibody was a gift from J. D. Bangs (5). Anti-CRAM antibody and anti-Th29 were as previously described (27, 28). The mouse-derived monoclonal anti-α-tubulin was purchased from Sigma Co. The BB2 monoclonal antibody for detecting the Ty1 tag was a gift from K. Gull’s laboratory (6).
DNA transformation of trypanosomes and confirmation of correct transformants. Ten micrograms of linearized plasmid DNA was electroporated into 5 x 10^7 procyclic trypanosomes using a BTX electroporator, following previously described procedures (43). Phleomycin (2 µg/ml) and/or hygromycin B (40 µg/ml) was added 36 to 48 h after electroporation to select stable transformants. Ten micrograms of linearized plasmid DNA was electroporated into 5 x 10^6 cells/ml. After washes, the plates were incubated with primary antibodies (100 µl/well in PBS) with 0.05% Tween 20 (PBST), for 1 h at room temperature. Following washes, slides were mounted with mounting medium (EMS). The reaction was stopped by the addition of 50 µl/well of 2 M H2SO4, and the optical density was measured at 450 nm with an ELISA photometer (MR5000; Dynatech).

Immunofluorescence microscopy. Slides containing fixed trypanosomes were prepared as previously described (56). After rehydration in glycerine (0.14 M in PBS), slides were blocked with 3% bovine serum albumin (BSA) in PBS for 1 h and then reacted with primary antibody in PBS with 3% BSA for 1 h, followed by washes with PBS. Subsequently, slides were reacted with various fluorophore-conjugated goat-derived anti-rabbit or anti-rat or anti-mouse immunoglobulin Gs (Igs) for 1 h. After washes, slides were mounted with mounting medium (Vectashield H-1200; Vector Laboratories Inc.) containing 4',6-diamino-2-phenylindole (DAPI). Cells were viewed and photographed using a Nikon or a Leica fluorescence microscope. The images were directly captured by a charge-coupled device (CCD) camera and analyzed by the MetaMorph program from Universal Imaging Co.

**A.**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Sequence of the CRAM C-terminus</th>
<th>CRAM Localization</th>
<th>Endocytosis Efficiency</th>
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**B.**

**FIG. 1.** Sequences of the CRAM C-terminal extension in CRAM mutant cell lines. (A) Amino acid sequences. (Top) Site-directed mutagenesis cell lines. B2-W indicates the amino acid sequence of the wild-type CRAM in the CRAM-B2 cell line, and 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, and 16 indicate the mutated CRAM in each corresponding cell line. Changed amino acids are in bold and italics. (Bottom) Ala3-scanning mutagenesis cell lines. 1/2, 3/4, 5/6, 7/8, 9/10, 11/12 and 13/14 indicate the sequence of mutated CRAM in each of corresponding cell lines. The three inserted Ala residues in each cell line are indicated by "aaa." Changes that lead to altered distribution are underlined. The insertions in cell lines 5/6 and 11/12, which resulted in the reduction of endocytosis efficiency, are in bold. On the right are summarized data for the subcellular localization of CRAM and the efficiency of CRAM-mediated endocytosis in each of the indicated cell lines. N, normal compared to wild-type cells; UD, undetermined; L, low efficiency compared to CRAM-B2. (B) Sorting signals in the cytoplasmic domain of CRAM. The solid line indicates the amino acids spanning the transport signal, and the amino acids in bold and italics are essential for the function of the designated transport signal. The dashed line indicates the amino acids spanning the putative endocytosis signal.
Purification and labeling of IgG. IgG molecules from immunized rabbit serum were purified using the Affi-Gel protein A maps II kit from Bio-Rad. The purified IgGs were concentrated using Centricron YM-30 from Millipore. Labeling of IgG with 125I was performed using the IODO-GEN iodination reagent from Pierce. Nonimmune rabbit IgG was purchased from Sigma Co.

Uptake and degradation. For each assay, 1 or 0.5 ml cells (10^7 cells/ml) was used. For the uptake assay, 125I-labeled ligand (IgG) was incubated with trypanosomes in SDM-79 serum-free medium with 3% BSA in the absence or presence of a 20-fold excess of unlabeled ligands at 28°C for 2 to 4 h. Following incubation, trypanosomes were washed three times with SDM-79 medium -0.2% BSA, transferred to a new set of tubes, and spun down. The amount of radioactivity associated with cell pellets was measured in a gamma counter and referred to as the amount of uptake. For the degradation assay, following the incubation of trypanosomes with 125I-ligand at 28°C, trypanosomes were spun down, and the supernatants were collected for determination of the amount of trichloroacetic acid (TCA; 10% wt/vol)-soluble 125I-labeled products. Free 125I was removed from the TCA-soluble fraction by precipitation with 5% (wt/vol) silver nitrate. The amount of radioactivity in the TCA-soluble, noniodide fraction was referred to as the amount of degradation. Total and nonspecific counts were referred to as the measurements from reactions performed in the absence and presence, respectively, of 20-fold excess of unlabeled ligands. Specific counts were obtained by subtraction of nonspecific counts from total counts.

GST pull-down assay. GST fusion proteins were isolated from Escherichia coli BL21 cells harboring pGEX-derived plasmids using glutathione-agarose beads from Amersham Biosciences following the manufacturer’s instructions. Protein concentrations were estimated with the Coomassie Plus protein assay reagent from Pierce Co. The 35S-labeled proteins were prepared by using the in vitro transcription/translation system from Promega Co. After centrifugation at 12,000 rpm for 5 min, 5 μl of each of the translation products was incubated with 10 μg of a GST fusion protein in 500 μl of binding buffer (0.05% Triton X-100, 50 mM HEPS [pH 7.3], 10% glycerol, 100 mM KCl, 2 mM MgCl2, 0.1 mM CaCl2, 50 μM dithiothreitol, and 0.1% BSA) at room temperature for 2 h. Then, glutathione-Sepharose beads (15 μl) were added to each reaction mixture, and the mixtures were incubated for 30 min. Beads were washed three times with binding buffer without BSA and eluted with Laemmli sample buffer. The released proteins were separated on sodium dodecyl sulfate (SDS)–polyacrylamide gels, and the proteins were detected by autoradiography.

Yeast two-hybrid assay. The yeast strain CG1945 (Clontech) was used for the yeast two-hybrid assay. Transformation was done as described in the instructions for the MATCHMAKER two-hybrid kit (Clontech). Yeast cells were cotransformed with GAL4bd- and GAL4ad-derived plasmids. Transformants were first selected in the absence of leucine (–Leu) and tryptophan (–Trp). Individual colonies were then streaked on either –Leu/–Trp/+His (histidine-containing) plates or –Leu/+Trp/–His plates containing 20 mM 3-amino-1,2,4-triazole (3-AT). For growth assays, transformants were streaked on plates lacking Leu, Trp, and His with 20 mM 3-AT. For liquid β-galactosidase assays, cultures of transformants were prepared according to the MATCHMAKER instructions. β-Galactosidase activities were measured using a luminescent β-galactosidase detection kit II (Clontech) and TD 20/20 luminometer (Turner Biosystems). Yeast cell density was measured by the optical density at 600 nm. Background signals from samples containing no β-galactosidase were subtracted from the signal for each sample. The number of relative luminescent units (RLU) was used to represent β-galactosidase activity.

RESULTS

Construction of procyclic trypanosome cell lines expressing various mutated CRAM proteins. The cytoplasmic domain of CRAM spans 41 amino acids at the CRAM C terminus (Fig. 1). Our previous analyses demonstrated that the last 19 amino acids of the CRAM C terminus are essential for efficient transport of CRAM from the ER to the FP, indicating that it may contain putative transport signals (56). Truncation of more than 19 (up to 40) amino acid residues from the CRAM C terminus led to the escape of CRAM to the cell surface and the surface of the flagellum, suggesting that this region might be important in the retention of the protein at the FP (56). Further kinetic analysis of endocytosis demonstrated that the cytoplasmic extension of CRAM is also essential for CRAM-mediated endocytosis, suggesting the presence of putative endocytosis or internalization signals (30). To delineate functional domains involved in different sorting processes, we performed a mutagenesis series at the CRAM C terminus of the endogenous CRAM gene in the CRAM-B2 cell line. Cell line CRAM-B2 contained only a single allele of CRAM; its second CRAM allele was deleted and replaced by the phleomycin resistance gene, as previously described (56). By PCR-based mutagenesis, mutations were introduced into the DNA fragment encoding the CRAM C terminus. Each of the DNA fragments encoding mutated CRAM C termini was linked to a hygromycin resistance gene (Hph) driven by a procyclin promoter and was used as a targeting sequence, allowing the integration of mutations into the endogenous CRAM C terminus of T. brucei via homologous recombination, as previously described (Fig. 1; see Materials and Methods for details).

Two types of mutant cell lines were established. Seven mutant cell lines (1/2, 3/4, 5/6, 7/8, 9/10, 11/12, and 13/14) were created by Ala, Gly scanning mutagenesis, which inserts three consecutive Ala residues into the CRAM C terminus at 5-amino-acid intervals (Fig. 1; for details, see Materials and Methods). Fifteen additional mutant cell lines were created by site-directed mutagenesis with nucleotide mutations that led to a single amino acid change, based on the rationale of replacing bulky or charged amino acids with the small and noncharged amino acid Ala or Gly. These point mutation cell lines are referred to as cell lines 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, and 16 (Fig. 1; for details, see Materials and Methods). In each clonal cell line, the correct integration and mutation event was confirmed by Southern genomic blot analysis and nucleotide sequence analysis of PCR-amplified genomic DNA (data not shown). Cell line CRAM-0, which was previously established and which carries the gene encoding a wild-type CRAM C terminus, was used as a control cell line. All cell lines analyzed exhibited normal growth efficiency compared with the wild-type procyclic forms.

Expression of mutated CRAM in transformed trypanosome cell lines. The level of mutated CRAM expression in each transformed cell line was compared to that in wild-type trypanosomes and the parental CRAM-B2 cell line by Northern blot analysis (data not shown) and Western blot analysis (Fig. 2A). As predicted, in CRAM mutants, due to the extended 3’ untranslated region resulting from the integration of the plasmid DNA, each mutated CRAM mRNA is ~0.8 kb longer than the wild-type CRAM mRNA, as previously described (56), and their expression levels are, as anticipated, similar to or equivalent to ~85% of that in the CRAM-B2 cell line (data not shown).

The relative amounts of the CRAM protein produced in the cell lines were compared by Western blot analysis (Fig. 2A). The CRAM protein in all mutant cell lines exhibited an electrophoretic mobility of ~200 kDa, similar to that of the wild-type CRAM in wild-type procyclic forms and in the CRAM-B2 cell line, when detected by anti-CRAM antibodies (anti-P1-55 [27]) (Fig. 2A; the CRAM protein is glycosylated). In addition, most cell lines expressed levels of CRAM similar to that of CRAM-B2, with the exception of cell line 4, which obviously had a higher level of CRAM. As a control, these blots were downloaded from http://ec.asm.org/ on October 11, 2017 by guest.
reacted with the antibody recognizing α-tubulin to demonstrate that a similar amount of protein was loaded in each lane (Fig. 2A). To better quantitate the relative amount of CRAM protein in each cell line, we performed ELISA using the endoplasmic network-associated protein Tb-29 as an internal control (Fig. 2B) (28). The result indicated that most cell lines had similar levels of CRAM, equivalent to ~80 to 95% of that in the CRAM-B2 cell line (Fig. 2). The CRAM expression level in cell line 4 is ~2.2-fold higher than that in the CRAM-B2 cell line. Currently, we do not know the mechanisms involved in the up-regulation of CRAM in cell line 4. We speculated that the mutation in cell line 4 might have increased the stability or half-life of the corresponding protein. Due to contamination, cell line 5 was not included in the analysis of the protein expression level.

Subcellular localization of mutated CRAM in the procyclic form of T. brucei. The effect of each mutation in the trafficking fate of CRAM was examined by subcellular localization of CRAM in each mutant cell line, using indirect immunofluorescence analysis (IFA). We first examined the overall distribution of CRAM in each cell line, using paraformaldehyde-fixed and permeabilized cells. The IFA data below are not quantitative, and the relative amount of CRAM in each cell line was measured by ELISA, as described above. For each type of CRAM mutant, at least three individually transformed cell lines were examined.
Among all mutant cell lines, only four, cell lines 10 (containing D-5A), 13 (containing V-12G), 14 (containing V-10G), and 9/10 (containing the Ala 3 insertion between amino acids 13 and 12), had an altered distribution of CRAM (Fig. 1 and 3). In these four cell lines, the CRAM protein is no longer concentrated at the FP but is spread throughout the cell, excluding the nucleus and kinetoplast. In all the rest of the CRAM mutant cell lines, as in the wild-type trypanosome, CRAM is located exclusively at the FP, indicating that mutations in these cell lines did not affect the trafficking fate of CRAM. Representative images from each group of phenotypes are presented in Fig. 3. These images represent the superimposition of the staining with anti-CRAM antibody (green) and the DNA-specific dye DAPI (blue). The large and small blue dots locate the positions of nucleus and kinetoplast, respectively. In Fig. 3A, panel a shows wild-type CRAM in the CRAM-B2 cell line; the green staining shows CRAM concentrated at the area of the FP, which is closely adjacent to the kinetoplast. Panels b and c are images from cell line 3 (containing E-29A) and cell line 3/4 (containing an Ala 3 insertion between the −28 and −27 position), respectively, and in these mutant cells CRAM remained at the FP. Even though cell line 4 expresses a relatively high level of CRAM, CRAM was mainly confined to the FP (data not shown).
Panels d, e, and f show images from cell lines 10, 13, and 9/10, respectively. Obviously, in these cell lines, CRAM spreads all over the cell.

To better understand the distribution of mistargeted CRAM in cell lines 10, 13, 14, and 9/10, we colocalized CRAM with the ER-specific marker protein Bip (anti-Bip antibody was a gift from J. D. Bangs) (5). It was obvious that the majority of CRAM in cell lines 10, 13, 14, and 9/10 was colocalized with Bip at the ER. One set of representative images from cell line 10 is shown in Fig. 3B. Panel a demonstrates the general distribution of CRAM (green) in cell line 10, and panel b shows the costaining pattern with anti-Bip and DAPI; panel c shows the superimposition of all images. The retention of mutated CRAM in the ER of cell lines 10, 13, 14, and 9/10 suggests that mutations in these cell lines may have affected the function of sorting determinants responsible for the trafficking fate of CRAM. Thus, these results indicate that the amino acid sequence spanning −5 to −14 of the CRAM C terminus is essential for the efficient transport of CRAM from the ER to the FP, and we refer to this region as a transport signal. Importantly, the amino acids at −12 (V), −10 (V), and −5 (D) are essential for the function of the identified transport signal.

To determine whether some of the mutations may have resulted in the release of CRAM protein to other parts of cell surface than the FP, we performed live trypanosome staining with anti-CRAM antibody at 4°C and surface staining of fixed and nonpermeabilized cells as well as fluorescence-activated cell sorting analysis. We confirmed that CRAM did not spread onto the outside of the cell surface in all mutant cell lines generated (data not shown). Thus, these mutant cell lines did not allow us to identify putative signals in the CRAM C terminus that might be involved in the retention of CRAM at the FP.

Comparison of endocytosis efficiency of mutated CRAM proteins with that of the wild-type CRAM. We previously used an anti-CRAM IgG, which was raised against the extracellular repeat domain, as a ligand to characterize CRAM-mediated endocytosis and subsequently found that the cytoplasmic extension of CRAM is essential for the processes of internalization and transport of the CRAM–anti-CRAM IgG complex to the endocytic compartment (30). To further identify the potential sorting signal involved in CRAM-mediated endocytosis, we compared the efficiency of uptake and degradation of 125I-anti-CRAM IgG in all CRAM mutant cell lines that exhibited normal localization of CRAM at the FP with that in the CRAM-B2 cell line (Fig. 4). Cell lines 10, 13, 14, and 9/10 no longer contain CRAM at the FP, limiting a fair comparison of endocytosis. However, when the amounts of degraded 125I-anti-CRAM IgG released from all cell lines were compared, we found that cell lines 5/6 (containing an Ala3-scanning mutant cell lines).
CRAM on cell growth. 

Gands.

compartment, resulting in a smaller amount of degraded li-

gands being delivered to the lysosomal experimental variations, because a reduced uptake generally leads to reduction of uptake in cell line 15 is most likely due to exper-
dents presented here, the uptake ability of cell line 15 ap-
peared to be reduced to ~75%, though the amount of degra-
dation was not significantly reduced. We think that the 
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The uptake ability of cell line 15 appeared to be reduced to ~75%, though the amount of degradation was not significantly reduced. We think that the reduction of uptake in cell line 15 is most likely due to experimental variations, because a reduced uptake generally leads to a smaller amount of ligands being delivered to the lysosomal compartment, resulting in a smaller amount of degraded ligands.

Impact of overexpression of the cytoplasmic domain of CRAM on cell growth. We hypothesize that the interaction of sorting signals in the CRAM C terminus with the transport machinery may govern the trafficking fate of CRAM. Thus, expression of an excess amount of the CRAM-derived sorting signal may compete for the sorting machinery and, as a result, may affect the fate of CRAM. To test this hypothesis, we made the pLew10C plasmid to overexpress the CRAM C terminus in procyclic forms. pLew10C, which is a pLew82-derived plasmid (a gift from G. Cross), contains the inducible T7 promoter/TetO system driving the expression of 10 tandem copies of the CRAM C terminus, which replaced the luciferase gene in pLew82. At the N and C termini of the 10 copies of the CRAM C-terminal peptide in pLew10C are the Ty1 epitope tags, encoding 10 amino acids: Glu Val His Thr Asn Gln Asp Pro Leu Asp (6). The Ty1 tags provide the initiation codon ATG and the termination codon TAA, for proper expression of the peptide, as well as allowing monitoring of the expression level of the ectopic CRAM C-terminal peptide using the specific monoclonal antibody BB2 (a gift from K. Gull)(6). Linearized pLew10C was introduced into the procyclic 29-13 cell line, which expresses the T7 RNA polymerase and tetracycline repressors (55), and stable transformants were established. The resulting cell line was referred to as CRAM10C (uncloned population).

We first examined cell growth and the expression level of the Ty1-tagged 10× CRAM C-terminal protein (referred to as TY10C) after various periods of induction by tetracycline (Fig. 5A). A high level of expression of TY10C, which is anticipated to be ~47 kDa, began to be observed 2 days after induction, though even under noninducing conditions, a significant amount of BB2-reactive material was expressed (Fig. 5B). Surprisingly, 2 days after overexpression of TY10C, cell growth efficiency was reduced (Fig. 5A). We estimate that upon induction of the expression of TY10C, the generation time of the population).

To address whether the fate of CRAM is affected upon overexpression of TY10C, we compared the expression level and the subcellular localization of CRAM before and after induction of TY10C. Interestingly, after 4 days of induction of TY10C expression, the steady-state level of the CRAM protein was significantly reduced (Fig. 5B), while the expression levels of tubulin and the endoplasmic membrane network structural protein Tb-29 were unaffected (Fig. 5B). When the distribution of CRAM was examined by IFA, we found that only ~44% of

FIG. 5. Impact of overexpression of the CRAM C terminus on cell growth and on CRAM expression in the procyclic-form trypanosome. (A) Growth curves. The growth curves of CRAM10C (closed symbols) and lew82 (open symbols) cell lines were determined in the absence (triangles) or presence (squares) of 1 µg/ml of tetracycline (for induction of TY10C). Cells were continuously maintained at log phase. If needed, medium was added to expand the culture. The total number of cells in each culture was calculated at different time points. (B) Western blot analysis. Total protein lysates derived from procyclic trypanosomes (~2 × 10^7 trypanosomes each) before (~) and 2, 3, 5, and 7 days (d) after induction of the TY10C expression were size separated in 6% polyacrylamide gels and electrophoretically transferred to nitrocellulose filters. The blots were sequentially probed with the BB2 monoclonal antibody against the Ty1 tag of TY10C (TY) (6), anti-CRAM antibody (CRAM), anti-Tb-29 procyclin (Tb-29) and anti-α-tubulin (Tubulin). Following the first antibody reaction, filters were treated with horseradish peroxidase-labeled goat anti-rabbit or anti-mouse IgG. The signals were detected using an enhanced chemiluminescence detection system. “C” represents the total cell lysate derived from the procyclic 29-13 cell line. (C) Localization of CRAM in TY10C overexpressers. Trypanosome slides were first incubated with rabbit-derived anti-CRAM and the mouse monoclonal BB2 antibody, followed by reaction with FITC-conjugated goat anti-rabbit IgG and rhodamine-conjugated goat anti-mouse IgG. Green, CRAM; red, TY10C; blue, DAPI staining. Arrowhead, cell with a relatively high level of TY10C.
the cells in the TY10C overexpresser population could be positively stained by anti-CRAM antibody, and their CRAM signal remained concentrated at the FP, while in the noninduced cells, >85% of cells exhibited an intense CRAM signal at the FP. In addition, we did not find CRAM localization to other organelles (i.e., lysosomal-endosomal compartments) or CRAM escaping to the outside of the cell surface (data not shown). When the TY10C overexpresser cells were stained with the BB2 antibody, it appeared that TY10C was distributed all over the cell, and its expression level varied among individual cells. Interestingly, most cells with a relatively low level of TY10C exhibited clear and discrete CRAM staining at the FP, while most cells with a relatively high level of TY10C had an undetectable level of CRAM. A representative image is shown in Fig. 5C (the cell with a high level of TY10C is indicated by an arrowhead). Thus, although this experiment was not able to provide a clear conclusion for the effect of TY10C overexpression on the final localization of CRAM to the FP, overexpression of TY10C may have significantly affected the biosynthesis and/or the turnover rate of the CRAM protein in procyclic forms. We do not know the causes of the reduced growth efficiency of TY10C overexpression cells, though it should not be a direct consequence of the reduction of CRAM, since CRAM-null mutants can grow efficiently (57). In addition, the nonhomogenous phenotype in the population after induction of TY10C overexpression may explain the less dramatic reduction of growth efficiency (57). We hypothesized that the interaction between sorting signals of CRAM and transport vehicles may dictate the fate of CRAM in trypanosomes. To validate this hypothesis, we determined whether Tb\(\mu\)1 interacts with the cytoplasmic domain of CRAM and, as a control, with a mammalian-derived tyrosine-based signal (37, 38).

We first determined whether the cytoplasmic domain of CRAM can interact with \(\mu\)-adaptins by in vitro binding assay and then confirmed an interaction by yeast two-hybrid analysis. For the in vitro binding assay, in vitro-synthesized \(^{35}\)S-labeled \(\mu\)-adaptins were incubated with various GST-linked sorting signals, and bound materials were pulled down by glutathione beads and analyzed by gel electrophoresis (Fig. 6). The interaction of mouse \(\mu\)2 with the GST-tyrosine-based signal was used as a positive control. It appeared that Tb\(\mu\)1, like mouse \(\mu\)2, interacted with a mammalian tyrosine-based signal—GST-(SDYQRL)\(_3\) protein (Fig. 6, middle and right panels) and did not interact with the mutated tyrosine-based signal—GST-(SDAQRL)\(_3\) protein, in which the tyrosine (Y) was replaced by an alanine (A) (Fig. 6, middle and right). However, the \(^{35}\)S-labeled luciferase, as a negative control, did not interact with either GST-(SDYQRL)\(_3\) or GST-(SDAQRL)\(_3\), demonstrating specificity (Fig. 6, left). Using the same assay, we found that Tb\(\mu\)1 also interacted with the GST-CRAM cytoplasmic tail in addition to GST-(SDYQRL)\(_3\) (Fig. 6, right), while mouse \(\mu\)2 was unable to efficiently interact with the GST-CRAM cytoplasmic tail under the same conditions (Fig. 6, middle). In addition, we reproducibly found that the quality and the quantity of the in vitro-synthesized Tb\(\mu\)1 were not as good as those of mouse \(\mu\)2, as results for the loading control showing that several truncated or incomplete Tb\(\mu\)1 proteins existed in the synthesized Tb\(\mu\)1. The poorly synthesized Tb\(\mu\)1 may account for the relatively weak pull-down results.

![Figure 6](http://ec.asm.org/)

**FIG. 6. In vitro binding of Tb\(\mu\)1 with the tyrosine-based sorting signal and the cytoplasmic domain of CRAM.** \(^{35}\)S-labeled luciferase (\(^{35}\)S-Luc), mouse \(\mu\)2 (\(^{35}\)S-M\(\mu\)2), or Tb\(\mu\)1 (\(^{35}\)S-Tb\(\mu\)1) was incubated with GST-SDYQRL, GST-SDAQRL, and GST-CRAM. One-tenth of the amount of in vitro-translated proteins that were used for each reaction was loaded in the first lane of each panel to demonstrate the quantity and quality of each translated protein. In vitro pull-down materials were analyzed on 10% SDS–polyacrylamide gels and visualized by autoradiography. —, blank lane.
To further confirm the results observed with the in vitro binding assay, we tested whether Tbμ1 can interact with SDYQRL and the peptide spanning the last 31 amino acids of the CRAM cytoplasmic tail (CRAM31) in vivo by a yeast two-hybrid assay. The sorting signals were fused to GAL4bd; the μ-adapts were linked to GAL4ad. The occurrence of interactions was revealed by the ability of the yeast transformed with both GAL4bd- and GAL4ad-derived constructs to grow on His^− plates and to express β-galactosidase activity.

FIG. 7. Yeast two-hybrid interaction of Tbμ1 with the tyrosine-based sorting signal and the cytoplasmic domain of CRAM. (A) Yeast cells were cotransformed with plasmids encoding GAL4bd fused to the sequences indicated above the panels and plasmids encoding GAL4ad only (ACT2), GAL4ad fused to Tbμ1 (Tbμ1), or GAL4ad fused to mouse μ2 (indicated by Mμ2). Transformed yeasts were streaked on +His or −His plates. Growth on −His plates represents the interaction of cotransformed plasmids. “CRAM” indicates the GAL4bd construct containing the C-terminal 31 amino acids of CRAM, also referred to as CRAM31. (B) Quantitation. Transformants were cultured in −Leu−Trp−His+20 mM 3-AT medium, and β-galactosidase activity was measured accordingly. Each value is the mean of three determinations, and each set of experiments was repeated three times. β-Galactosidase activity is shown as RLU on a logarithmic scale. In the graph on the right, the background β-galactosidase activity from the interaction of GAL4ad-ACT2 with each GAL4bd-derived plasmid was subtracted prior to comparison. “CRAM” represents CRAM31. (C) Comparison of the interaction of Tbμ1 with the wild-type CRAM C terminus and different versions of the mutated CRAM C terminus by yeast two-hybrid assay. Yeast cells were cotransformed with a plasmid encoding GAL4ad fused to Tbμ1 (Tbμ1) and plasmids encoding GAL4bd only (AS2) or GAL4bd fused to different versions of the CRAM C-terminus sequences as indicated (see Materials and Methods for a detailed description of each construct). Tbμ1 represents the growth of yeast transformed with the GAL4ad-Tbμ1 construct only. Transformed yeasts were streaked on histidine-containing (+Leu+/Trp+/His+/20 mM 3-AT) or histidine-deficient (−Leu−Trp−His+/20 mM 3-AT) plates. Three individually transformed colonies are shown for each combination. Growth on −Leu−Trp−His+/20 mM 3-AT plates represents the interaction of cotransformed plasmids.
(SDYQRL)$_3$ and SDAQRL as positive and negative controls, respectively. Like mouse $\mu_2$, Tb$_1$ significantly interacted with SDYQRL/(SDYQRL)$_3$ but not SDAQRL (Fig. 7A). Based on the quantitation of $\beta$-galactosidase activity, it appeared that the interaction of Tb$_1$ with (SDYQRL)$_3$ is comparable to that of mouse $\mu_2$ with SDYQRL or (SDYQRL)$_3$, while interaction of Tb$_1$ with the SDYQRL is ~100-fold lower than that of mouse $\mu_2$ with SDYQRL or (SDYQRL)$_3$ (Fig. 7B). The two-hybrid assay also demonstrated that Tb$_1$ significantly interacted with the CRAM cytoplasmic tail, and this interaction is comparable to that of Tb$_1$ with (SDYQRL)$_3$, as shown by the $\beta$-galactosidase activity (Fig. 7B). Thus, we demonstrated that Tb$_1$ can interact with the cytoplasmic domain of CRAM both in vitro and in vivo in yeast.

To define the region of the CRAM cytoplasmic domain that is responsible for the interaction with Tb$_1$, we compared the interactions of Tb$_1$ with CRAM31, CRAM19 (the peptide spanning the last 19 amino acids of the CRAM C terminus, covering the transport signal and the putative internalization signal), CRAM19-10 (CRAM19 with D-5A), CRAM19-13 (CRAM19 with V-12G), CRAM19-14 (CRAM19 with V-10G), and CRAM19-9/10 (CRAM19 with an Ala$_3$ insertion between the −13 and −12 amino acids) by yeast two-hybrid assays (Fig. 7C). For each set of two-hybrid experiments, the results for three independently selected colonies are shown (Fig. 7C). It appeared that like CRAM31, CRAM19 efficiently interacted with Tb$_1$, while CRAM19-10, CRAM19-13, CRAM19-14, and CRAM19-9/10 lost their ability to interact with Tb$_1$. These results demonstrate the interaction of the CRAM-derived sorting signal with Tb$_1$ and mutations at −5, −10, and −12, which inhibited trafficking of CRAM from the ER to the FP in trypanosomes, also abolished the ability of the CRAM-derived sorting signal to interact with Tb$_1$ in yeast. We hypothesize that the interaction of the Tb$_1$ with the CRAM sorting signal may dictate the trafficking fate of CRAM.

**DISCUSSION**

In trypanosomatids, endocytosis and exocytosis occur exclusively at the FP, where receptors for uptake of macromolecules are located. Accumulated studies suggest that trafficking machinery similar to that of higher eukaryotes might operate in trypanosomes (19, 31, 33, 34, 40). Trafficking between organelles, either in the secretory pathway or during endocytosis, is mediated by membrane-bound vesicular structures. In higher eukaryotes, the trafficking fate of individual membrane proteins is dictated by their intrinsic signals (8, 9, 24, 25, 41, 47). Little is known about the sorting processes in trypanosomes, and surface receptors that contain sorting signals resembling those in higher eukaryotes, such as the tyrosine-based signal (NPXY or YXXΦ) and the dileucine-based signal [DE]XXL[LI] DXXLL, have not yet been identified (8, 9, 25, 41, 47). Nevertheless, sequences resembling the potential dileucine-based signal can be identified in the lysosomal membrane protein p67 of *T. brucei*, though its biological function remains to be determined (1, 23). Motifs similar to the tyrosine- and dileucine-based signals can be found in the C terminus of the endosome/lysosome-associated membrane-bound acid phosphatase (MBAP) of *Leishmania mexicana*, though apparently the C-terminal IIV motif of MBAP, but not the tyrosine residue, is required for endosomal targeting (54).

Thus far, CRAM is the only transmembrane receptor-like protein at the FP in *T. brucei* that has been characterized. Our previous studies suggested that sequences of the cytoplasmic domain or C terminus of CRAM govern the trafficking fate of CRAM in the endocytosis and along the secretory pathway (56). With this finding, we continued to delineate the domains and amino acids in the CRAM C terminus that are essential for targeting CRAM to the FP and/or for CRAM-mediated endocytosis and, as a result, defined the sorting determinants involved in trafficking of CRAM in trypanosomes. Apparently, the sequence spanning amino acid −5 to amino acid −14 of the CRAM C terminus is responsible for transport of CRAM from the ER to the FP, and therefore, we refer to this region as a transport signal. Within the transport signal, mutations of amino acids at −12 (V), −10 (V), and −5 (D) prohibited the export of CRAM from the ER, leading to the accumulation of mutant CRAM mainly in the ER. It is possible that mutant CRAM proteins may be able to exit the ER but fail to move forward at the Golgi complex, resulting in retrograde transfer to the ER. However, in the latter event, we would anticipate a gradient distribution of CRAM from the Golgi complex to the ER or perhaps other organelles. Thus, the phenotype of accumulation of mainly mutant CRAM proteins in the ER favors the hypothesis that the transport signal mediates exit from the ER. In addition, the amino acid sequence from −5 to −23 of the CRAM C terminus is required for efficient CRAM-mediated endocytosis, and we refer to this region as a putative endocytosis signal. Obviously the transport signal overlaps with the putative endocytosis signal. We speculated that the same signal may govern both inbound and outbound trafficking of CRAM in trypanosomes. Another unusual property is that the sorting signals in the CRAM C terminus do not obviously resemble the sorting signals identified in mammalian systems. However, when the amino acid sequence of the CRAM C terminus was compared to those in the data bank, interestingly, a 5-of-11-amino-acid homology was found in the −15 to −4 amino acid sequence of the CRAM C terminus and the tyrosine-based sorting signal domain responsible for internalization in the human low-density lipoprotein (LDL) receptor (residues 801 to 812) and the LDL receptor-related protein (residues 4482 to 4493) (15) (Fig. 8). The CRAM-derived sorting signal does not have the Y residue; at the corresponding position is a Thr (T). Amino acids surrounding the Y or T share a high degree of homology. The significance of this homology is unclear. However, when the Y in the human LDL receptor protein (residue 807) was replaced by a T, this protein still retained its capability for internalization, though with a reduced efficiency (15).

In mammalian cells, clathrin and adaptor complexes (APs) mediate receptor-mediated endocytosis and various sorting pathways (7-9, 24, 25). Sorting of transmembrane proteins, either from the plasma membrane or the Golgi to endosomes-lysosomes or from the Golgi or endosomes-lysosomes to the plasma membrane, is governed by specific sorting signals located at the cytoplasmic extension of the proteins (24, 25, 47). Selective recognition of these sorting signals by the $\mu$-adaptin of a specific AP facilitates the incorporation of receptors into transport vesicles and subsequently delivers receptors to their
intended destinations. At least four different classes of APs have been identified in mammalian cells, referred to as AP-1, AP-2, AP-3, and AP-4 (7–9). T. brucei contains homologues of AP-1, AP-3, and AP-4 but does not have AP-2, which facilitates clathrin-coated vesicle formation at the plasma membrane during endocytosis in mammalian cells (7, 48; Chuang et al., submitted). Our simultaneous studies of sorting machinery in trypanosomes demonstrated that clathrin is required for trafficking of CRAM from and to the FP in procyclic forms of T. brucei (8). In addition, our recent study indicated that Tb1p is involved in trafficking of CRAM between the TGN (or endosomes) and the FP membrane, a function similar to mediating the polarized transport of membrane proteins to the basolateral surface of epithelial cells from the TGN by AP-1 (7, 9, 24, 25; Chuang et al., submitted). In view of these observations, we determined whether the CRAM sorting signal can interact with Tb1p. Both the in vitro binding assay and the in vivo yeast two-hybrid assay demonstrated that the CRAM sorting signal can efficiently interact with Tb1p. Mutations of the CRAM sorting signal, at amino acids that are essential for trafficking CRAM, abolished the interaction of the sorting signal with Tb1p, demonstrating specificity. However, whether the interaction of Tb1p with the CRAM sorting signal is essential for the outbound and/or for the inbound trafficking of CRAM is not clear. Thus, the exact steps along the intracellular trafficking path of CRAM which are governed via the interaction with Tb1p remain to be defined. Apparently Tb1p can also interact with the tyrosine-based signal. We do not know whether the CRAM-derived sorting signal and the tyrosine-based sorting signal compete for the same binding domains in Tb1p. Since, in higher eukaryotes, the dileucine-based signals in clathrin-dependent trafficking interact with APs and do not compete with the tyrosine-based signal for binding to APs (9, 12), the possibility that the CRAM-derived sorting signal and the tyrosine-based signal do not share the same binding domain in Tb1p is not excluded. Further experiments will be pursued to delineate the molecular details of the interaction of CRAM-derived sorting signals with the sorting machinery.

To demonstrate the biological significance of the identified sorting signal, we thought that expression of an excess amount of the CRAM-derived sorting signal might compete for the sorting machinery and thus affect the trafficking fate of CRAM. Surprisingly, overexpression of the CRAM C-terminal peptide drastically reduced the growth efficiency of procyclic forms and the steady-state level of the CRAM protein. Due to the diminished level of CRAM in cells overexpressing the CRAM C-terminal peptide, this approach did not allow us to address the role of the sorting signal in the trafficking of CRAM. Nevertheless, the outcome suggested that overproduction of the CRAM C-terminal peptide may have affected the biosynthesis or the turnover rate of CRAM. We hypothesize that the presence of a vast amount of CRAM C-terminal peptide may compete for machinery needed for maturation of CRAM in the ER and/or for efficient exit from the ER. Under the quality control, newly synthesized nascent CRAM or partially processed CRAM (incomplete posttranslational modifications) may be degraded within the ER prior to exit, leading to a low level expression of CRAM (21, 45). It is also possible that the overexpressed CRAM C-terminal peptide competes for chaperone molecules governing CRAM stability and subsequently reduces the stability of CRAM in different intracellular trafficking pathways (3). These possibilities are purely hypothetical and remain to be investigated. We do not think that the reduction of CRAM expression resulted in the reduced growth efficiency in cells that overexpressed CRAM C-terminal peptide, because CRAM knockout cell lines did not have growth defects (57). It is most likely that the function of other factors essential for cell growth were affected by the overexpression of CRAM C-terminal peptide.

As mentioned above, we found that the point mutation cell line 4, which contained nucleotide changes leading to E-24A, accompanied by a newly generated SstI site, had an elevated expression of CRAM at the protein level. We do not know the exact mechanism leading to the elevation of CRAM in cell line 4. However, the mutation and the high level expression of CRAM did not affect the localization of CRAM to the FP, did not result in a spilling over of CRAM to other parts of the cell surface (pellicular surface), including the surface of the flagellum, and also did not lead to significant accumulation of CRAM in the ER and/or the Golgi compartment in procyclic trypanosomes. This phenomenon is unlike what is known for the distribution of the transferrin receptor in the bloodstream form of T. brucei. The bloodstream-form trypanosome can regulate the localization of the transferrin receptor in a flexible manner (35, 36). Up-regulation of the transferrin receptor results in the escape of the transferrin receptor from the FP onto the pellicular surface and a large increase of the transferrin receptor in the ER and Golgi. These differences could be attributed to the differences of membrane dynamics and/or different mechanisms involved in routing and rooting of different membrane proteins in different stages of T. brucei.

In summary, we systematically dissected sequences involved in the trafficking of CRAM via the FP of trypanosomes and characterized the first sorting signal required for localization of a transmembrane receptor protein, CRAM, to the FP in the procyclic-form trypanosome. As anticipated for trypanosomes,
the sequence of the sorting signal is unique and unlike those in other eukaryotes. Nevertheless, the CRAM-derived sorting signal efficiently interacts with Tpl1, which strengthens the hypothesis of the involvement of clathrin and adaptors in the trafficking of CRAM.

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