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**Recommended Citation**

Mokry, D. Z., Manandhar, S. P., Chicola, K. A., Santangelo, G. M., Schmidt, W. K. (2009). Heterologous Expression Studies of Saccharomyces cerevisiae Reveal Two Distinct Trypanosomatid CaaX Protease Activities and Identify Their Potential Targets. *Eukaryotic Cell, 8*(12), 1891-1900.  
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Heterologous Expression Studies of Saccharomyces cerevisiae Reveal Two Distinct Trypanosomatid CaaX Protease Activities and Identify Their Potential Targets

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Received 13 June 2009/Accepted 16 September 2009

The CaaX tetrapeptide motif typically directs three sequential posttranslational modifications, namely, isoprenylation, prenylation, and carboxyl methylation. In all eukaryotic systems evaluated to date, two CaaX proteases (Rce1 and Ste24/Afc1) have been identified. Although the Trypanosoma brucei genome also encodes two putative CaaX proteases, the lack of detectable T. brucei Ste24 activity in trypanosome cell extracts has suggested that CaaX proteolytic activity within this organism is solely attributed to T. brucei Rce1 (J. R. Gillespie et al., Mol. Biochem. Parasitol. 153:115–124, 2007). In this study, we demonstrate that both T. brucei Rce1 and T. brucei Ste24 are enzymatically active when heterologously expressed in yeast. Using a-factor and GTPase reporters, we demonstrate that T. brucei Rce1 and T. brucei Ste24 possess partially overlapping specificities much like, but not identical to, their fungal and human counterparts. Of interest, a CaaX motif found on a trypanosomal Hsp40 protein was not cleaved by either T. brucei CaaX protease when examined in the context of the yeast a-factor reporter but was cleaved by both in the context of the Hsp40 protein itself when evaluated using an in vitro radiolabeling assay. We further demonstrate that T. brucei Rce1 is sensitive to small molecules previously identified as inhibitors of the yeast and human CaaX proteases and that a subset of these compounds disrupt T. brucei Rce1-dependent localization of our GTPase reporter in yeast. Together, our results suggest the conserved presence of two CaaX proteases in trypanosomatids, identify an Hsp40 protein as a substrate of both T. brucei CaaX proteases, support the potential use of small molecule CaaX protease inhibitors as tools for cell biological studies on the trafficking of CaaX proteins, and provide evidence that protein context influences T. brucei CaaX protease specificity.

Certain isoprenylated proteins are synthesized as precursors having a highly degenerate C-terminal tetrapeptide CaaX motif (C, cysteine; a, aliphatic amino acid; X, one of several amino acids). This motif typically directs three posttranslational modifications that include covalent attachment of an isoprenoid lipid to the cysteine residue, followed by endoproteolytic removal of the terminal three residues (i.e., aaX), and, lastly, carboxyl methyl esterification of the farnesylated cysteine (49, 50). Relevant examples of proteins subject to the above modifications, also referred to as CaaX proteins, include the Ras subunits, prelamin A, members of the Hsp40 family of chaperones, and fungal mating pheromones.

Isoprenylation of CaaX proteins is performed by either the farnesyltransferase (FTase) or the geranylgeranyl transferase I (GGTase I). The particular isoprenoid attached, C15 farnesyl or C20 geranylgeranyl, respectively, depends in part on the sequence of the CaaX motif (8, 26, 31). Proteolysis of isoprenylated intermediates is carried out by the otherwise unrelated Rce1p (Ras converting enzyme 1) and Ste24p (sterile mutant 24) enzymes, collectively referred to as CaaX proteases, which are integral membrane proteins residing within the endoplasmic reticulum (3, 40, 45). Studies to elucidate the specificities of the CaaX proteases have often involved reporters designed from biological substrates (e.g., Ras GTPases) (2, 3, 16, 21, 22, 24, 34). Although these studies suggest that isoprenylated CaaX tetrapeptides alone are sufficient for recognition as a substrate, insufficient evidence exists to assert whether this sequence contains all of the necessary information for substrate specificity. Reporters are typically cleaved by either Rce1p or Ste24p. The Saccharomyces cerevisiae a-factor mating pheromone is a rather unusual biological reporter since it is cleaved by both yeast CaaX proteases. Orthologs of the CaaX proteases from humans, worms, and plants can also cleave a-factor when heterologously expressed in yeast, thereby making a-factor a convenient reporter for comparative analyses of CaaX protease activities (3, 5, 6, 36). Where evaluated using the a-factor reporter, Rce1p and Ste24p display partially overlapping target specificity, and this is an expected property of CaaX proteases in all eukaryotic systems (5, 6, 36, 47). Unlike the isoprenylation and proteolysis steps, carboxyl methyl esterification exclusively relies on a single enzyme, the isoprenylcysteine carboxyl methyltransferase (ICMT) (23, 50). A farnesylated cysteine appears to be the sole recognition determinant of the endoplasmic reticulum-localized ICMT (10, 23, 38). Disruption of the posttranslational modifications associated with CaaX proteins is often perceived as an anticancer strategy.

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† Supplemental material for this article may be found at http://ec.asm.org.
‡ Published ahead of print on 9 October 2009.
because of the prominent role of CaaX proteins in cellular transformation (i.e., the Ras GTPases) (49). To date, the most advanced drug discovery efforts have focused on farnesyltransferase inhibitors (FTIs) (9, 53). Inhibitors of the CaaX proteases and ICMT are also being developed (1, 11, 28, 37, 39, 48). Disrupting CaaX protein modifications has therapeutic application to other diseases as well. The relief of prelamin A toxicity by FTIs is a well-documented example (51). Accumulation of the farnesylated but unproteolysed precursor of lamin A results in a progeroid phenotype in individuals lacking Zmp-CaaX proteases and ICMT are also being developed (1, 11, 28, 37, 39, 48). Advanced drug discovery efforts have focused on farnesyltransferase inhibitors (FTIs) (9, 53). 

Materials and Methods

Yeast strains and media. The yeast strains used in the present study are listed in Table 1. To grow in vitro, T. brucei Rce1 and T. brucei Ste24 are active when heterologously expressed in S. cerevisiae and have partially overlapping substrate specificities. The assays rely on various reporters, specifically the green fluorescent protein. All but the GTPase reporter can be effectively cleaved by both T. brucei CaaX proteases. We also demonstrate that the trypanosomal CaaX proteases can be targeted for inhibition by small molecules both in vitro and when heterologously expressed in yeast, suggesting that the trypanosomal CaaX proteases may be attractive drug targets for pharmacological inhibition.

Plasmids. The plasmids used in the present study are listed in Table 2. Yeast expression plasmids encoding T. brucei Rce1 (pWS766) and T. brucei Ste24 (pWS767) were created by PCR-directed recombination-mediated plasmid construction (33). In brief, the open reading frames (ORFs) of T. brucei Rce1 and T. brucei Ste24 (accession nos. XP_843748 and XP_827211, respectively) were used to replace the ORFs of yeast RCE1 and STE24 encoded in pWS479 and pWS802, respectively (36, 45). The trypanosomal ORFs were amplified by PCR from T. brucei genomic DNA (TREU 927). The appropriate PCR product was cotransformed into yeast with pWS79 and pWS154 that had been linearized with SpHl and BglII/NotI, respectively, within the CaaX protease ORF. To facilitate recombination between PCR products and linearized plasmids, the PCR products were engineered to contain 39-bp extensions homologous to yeast genomic DNA. All but the GTPase reporter can be effectively cleaved by both T. brucei CaaX proteases. We also demonstrate that the trypanosomal CaaX proteases can be targeted for inhibition by small molecules both in vitro and when heterologously expressed in yeast, suggesting that the trypanosomal CaaX proteases may be attractive drug targets for pharmacological inhibition.

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brucei Rec1 and T. brucei Ste24. To create the T. brucei Rec1 mutants, pWS766 was linearized with either AsclII or BgIII, as appropriate. Creation of T. brucei Ste24 mutants relied on SacI digestion of pWS767. To facilitate identification of plasmid candidates for sequencing, the oligonucleotides used for PCR were engineered to contain the intended mutation and a silent restriction site near the mutational site.

Yeast expression plasmids encoding a-factor CaaX variants were also created by recombination-based plasmid construction. In most instances, PstI and MluI gapped pWS654 was used as the recipient vector for PCR products amplified from pWS438 (6). The PCR products contained regions of S' and 3' homology for recombination, the desired mutation, and a silent restriction site that was used to facilitate identification of the appropriate recombiant clone for confirmatory DNA sequencing. The a-factor variants containing CAMQ and CTVM motifs were constructed in two steps. First, intermediate plasmids were created by recombination of MluI-gapped pSM1605 with PCR products amplified from pSM1605 using mutagenic oligonucleotides. The resultant plasmids were then modified by standard methods to replace the existing URA3 marker with LEU2.

Constructs encoding glutathione S-transferase (GST)-TbJ1 (pWS782) and GST-TbJ1(C401S) (pWS783) behind the constitutive CUP1 promoter were created by replacement of YDJ1 with Tbj1 using recombination within a GST-YDJ1 expression construct (pWS338) recovered from a GST fusion library (29). To facilitate recombination, pWS338 was linearized with NarI and Bso36I. The construct encoding GST-TbJ1 behind the inducible GAL promoter (pWS990) was created similarly but used a GST-YDJ1 expression vector (pWS253) obtained from a different GST fusion library (19). pWS523 was linearized with NarI and PstI. The constructs encoding other T. brucei Hsp40 proteins (Tbj2-4) behind the constitutive phosphoglycerate kinase (PGK) promoter were also created by recombination, all linearized with Xmal as the recipient vector. PCR products for the above constructs were derived from TREU 927 genomic DNA.

Candidate clones identified by restriction digest were sequenced to confirm the presence of the appropriate Hsp40 gene.

Mating assay. The serial dilution yeast mating assay was performed as previously described (36). In brief, MATa yeast cells expressing the indicated CaaX protease were cultured for 36 h at 30°C in selective media. The cultures were normally grown to an OD600 of 0.6 to 5.5 and used to induce expression of GFP-Ras2p. Where applicable, cells were incubated with a slurry of glutathione-Sepharose 4B resin (10 min, 25°C, 1 mg of total protein/ml. The resulting lysate was cleared (16,000 g for 10 min) and incubated with pretreatment buffer, and the resulting lysate was resuspended in cold spheroplasting buffer (50 mM potassium phosphate [pH 7.5], 1.4 M sorbitol, 10 mM tin, chymostatin, and pepstatin/ml; 500 units of spheroplasting buffer/l of each mixture into a new well containing 90 μl of MATa cells until 5 dilutions were prepared. All mixtures were subsequently spotted (5-μl volumes) onto an SD plate, and the plates were incubated for 72 to 96 h before the results were recorded by using a flatbed scanner. The cell suspensions were also spotted onto SC–lysine solid media, which is selective for MATa and diploid growth, to confirm that MATa cell dilutions were appropriately prepared.

GFP-Ras2p localization assay. An inducible GFP-Ras2p reporter (pWS750) was used to determine the ability of yeast, human, and trypanosomal Rec1 (pWS745) and Ste24 (pWS746, respectively) to promote proper localization of GFP-Ras2p in yeast and to evaluate the effect of chemical agents on Ras2p localization (28). Where yeast and trypanosomal Ste24 were evaluated, pSM1282 (pWS479, pWS335, and pWS766, respectively) to promote proper localization of Ste24 mutants relied on SacI digestion of pWS767. To facilitate identification of the desired mutation, and a silent restriction site near the mutational site.

To quantify the degree of GFP-Ras2p plasma membrane localization compared to 471 micrograms of bovine serum albumin/ml, and compound stocks were prepared at 100 mM in DMIB. Initial velocities were determined at various inhibitor concentrations ranging from 0.46 to 471 μM (11 points minimum). Compounds 1 to 9 were provided by the NCI Developmental Therapeutics Program and are as previously identified (28).

Isolation of yeast membranes. Yeast membranes were isolated by using two slightly modified protocols to lyse cells (37). In general, membranes used for determination of the IC50 values and in vitro-coupled proteolysis methylation assays were isolated directly from cells by bead beating. In brief, mid-log cells were incubated in pretreatment buffer (100 mM Tris [pH 9.4], 10 mM NaCl, 10 mM dithiothreitol; 10 μg/ml) for 10 min, resuspended in lysis buffer (50 mM Tris [pH 7.5], 0.2 M sorbitol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 μg of aprotinin/ml, 3 μg each of leupeptin and pepstatin/ml, 0.4% (w/v) SDS, 0.003% sodium dodecyl sulfate (0.003%) during the incubation period to induce expression of GFP-Ras2p. Where applicable, cells were incubated with a slurry of glutathione-Sepharose 4B resin (10 min, 25°C; three 20-s bursts; 3-min intervals on ice). For all other in vitro assays, membranes were isolated from spheroplasts. In brief, cells were pretreated as described above, and spheroplasts were generated by treatment with Zymolyase (4 μg/ml; Cape Cod, Inc.) for 30 min at 30°C in spheroplasting buffer (50 mM potassium phosphate [pH 7.5], 1.4 M sorbitol, 10 mM NaCl, 25 μg/ml) followed by 10 min on ice. Spheroplasts were harvested (3,000 x g, 10 min, 4°C), washed in spheroplasting buffer, resuspended in cold lysis buffer, and lysed by vortexing in the presence of silicon beads (four 4-min vortex bursts at 4°C, 2-min intervals on ice). Independent of the method used for cell lysis, crude lysates were clarified twice (500 x g, 10 min, 4°C), and membranes were recovered (16,000 x g, 15 min, 4°C) from the clarified lysate. The membranes were resuspended in lysis buffer, resolubilized by centrifugation, resuspended in lysis buffer to the original sample volume, adjusted to 1 mg of total protein/ml by dilution with lysis buffer, and frozen as aliquots at −80°C.

In vitro Tbj1 proteolysis assay. Tbj1 (accession no. XP_951689) was heteroloously expressed as a GST fusion protein (pWS900) in SM3614 (recΔ ste24A) and used for coupled proteolysis-methylation radiolabeling assays. In brief, cells from a 4-liter culture of yeast grown to an OD600 of ~10 in SGA−ura were harvested (5,000 x g for 10 min) and incubated with pretreatment buffer, and the cells were lysed directly as described above except that the lysis buffer was modified by replacing 20 μg of leupeptin, pepstatin, chymostatin, and cho'statin with 20 μg of aprotinin, and trypsin inhibitor/ml. The resulting lysate was clarified (16,000 x g, 15 min) and incubated with a slurry of glutathione-Sepharose 4B resin (10 min, 25°C, 0.675 ml of resin/liter of starting culture). The unbound fraction was decanted after centrifugation of the mixture in a standard 15-ml conical tube.
RESULTS

Both of the trypanosomal CaaX proteases are functional enzymes. The yeast CaaX proteases promote yeast mating, ostensibly through their ability to proteolysate the isoprenylated yeast α-factor precursor (3). CaaX proteases from distinct species can do the same when heterologously expressed in yeast (4–6, 36). Since a previous study had concluded that T. brucei Ste24 may be inactive, we first challenged T. brucei Rec1 and T. brucei Ste24 to cleave the CVIA CaaX motif present on yeast α-factor (20). This analysis revealed that the trypanosomal CaaX proteases could both promote yeast mating, as judged by quantitative serial dilution mating tests (Fig. 1A). This result suggests that the lack of activity previously reported for T. brucei Ste24 may be due to an assay specific or other issue, and not reflective of the intrinsic properties of this enzyme. By our analysis, the trypanosomal CaaX proteases performed as well if not better than their respective yeast counterparts in promoting yeast mating. In part, this observation can be explained by the fact that the yeast and T. brucei CaaX proteases were expressed using different plasmid systems. The yeast CaaX proteases were encoded on low-copy plasmids behind their respective promoters while the T. brucei CaaX proteases were encoded on high-copy plasmids behind the strong constitutive phosphoglycerate kinase promoter. Despite this issue, our result nonetheless indicates an activity for T. brucei Ste24 and suggests that both T. brucei Rec1 and T. brucei Ste24, like their counterparts in other organisms, have the common ability to recognize the α-factor precursor as a substrate (3–6, 36).

Mutational analysis of the trypanosomal CaaX proteases.

To confirm that the mating observed in our genetic test was indeed dependent on the proteolytic activities of the T. brucei CaaX proteases and not some unrecognized activity, we next evaluated the effect of mutations predicted to inactivate the enzymes. Despite having a formally unresolved mechanism, a glutamate and two histidine residues are known to be essential for yeast Rec1p activity (12, 36). These residues are conserved in Rec1p orthologs, including T. brucei Rec1, but their functional importance in any ortholog outside of yeast had not been previously addressed (35). Mutation of T. brucei Rec1 at these conserved sites resulted in the loss of activity as measured by the yeast mating assay (Fig. 1B), further supporting a role for histidine and glutamate residues in the Rec1p mechanism. In the instance of yeast Ste24p, histidine and glutamate residues are also required (18). These amino acids are part of a putative HExxH zinc-coordination motif that is common among zinc-dependent metalloproteases. Expectedly, T. brucei Ste24 required this motif for activity (Fig. 1C). For both T. brucei CaaX proteases, the mutations had no observable effect on protein expression relative to the native proteins, as judged by immunoblots (D. Z. Mokry and W. K. Schmidt, unpublished data). Our observations as a whole thus support that both T. brucei Rec1 and T. brucei Ste24 have proteolytic activity.

Specificity of the trypanosomal CaaX proteases.

To gain additional insight into the enzymatic properties of the trypanosomal CaaX proteases, we applied our genetic approach to assess the target specificity of the enzymes by measuring their ability to recognize disparate CaaX motifs (Fig. 1D). Variants of the α-factor precursor were created that contained CaaX motifs previously documented to be Rec1-specific (e.g., CTLM and CTVM), Ste24-specific (e.g., CASQ), and nonspecific (e.g., CVIA and CAMQ) in the context of CaaX proteases from other species, namely, yeast and human (36, 47). Consistent with these prior observations, T. brucei Rec1 was observed to be specific for the CTLM and CTVM motifs. T. brucei Ste24 was specific for CASQ, and both were active against the native CVIA motif (see Fig. 1A). The CAMQ motif was recognized...
Rce1 recognized both motifs, the CTML and CVIM motifs found on the Ras-related protein. Trypanosomes do not appear to have a true Ras ortholog, associated with predicted trypanosomal CaaX proteins. Beev was evaluated as targets for T. brucei Rce1, primarily by T. brucei Rce1 activity (17). Although T. brucei Rce1 recognized both motifs, T. brucei Ste24 also recognized CVIM, indicating that the T. brucei CaaX proteases have overlapping specificity for this motif. Interestingly, CVIM is found on mammalian Ras and is considered to be an Rce1p-specific motif in mammalian systems. We also evaluated CaaX motifs found on putative trypanosomal Hsp40 proteins. This family of proteins was targeted because the CASQ motif, which is present on the farnesylated Hsp40 chaperone Ydj1p, is preferentially cleaved by various Ste24 orthologs. In our genetic system, none of these motifs (CTQQ, CVHQ, and CTAQ) were cleaved by T. brucei Rce1, and only CVHQ was cleaved by T. brucei Ste24. This pattern was also observed in the context of yeast and human CaaX proteases, with the exception of the CTAQ motif, which was readily cleaved by Rce1 from these organisms. The reason for the lack of recognition of the CTAQ motif (found on two trypanosomal homologs of yeast Ydj1p) and CTAQ motifs by the T. brucei CaaX proteases is unknown but may relate to the protein context in which they were evaluated (see below and Discussion). When considering our results as a whole and in the context of the α-factor reporter, the trypanosomal CaaX proteases appear to have substantial but not complete conservation of substrate specificity with respect to that observed with CaaX proteases from other systems.

Trypanosomal Rce1 can cleave a yeast GTPase. Both yeast and human Rce1p preferentially, if not exclusively, recognize GTPases of the Ras superfamily as substrates. Considering that the T. brucei CaaX proteases displayed target specificity profiles by our genetic test that were largely similar to the profiles displayed by the yeast and human CaaX proteases, we next evaluated whether T. brucei Rce1 had conserved the ability to recognize Ras GTPases as substrates. Attempts to heterologously express trypanosomal GTPases GFP-RLP and GFP-RHP in yeast did not yield a discernible membrane association for either GTPase, suggesting a potential defect with isoprenylation, so we addressed the ability of T. brucei Rce1 to interact with the yeast Ras2p GTPase. Ras2p normally decorates the cytosolic face of the yeast plasma membrane (Fig. 2A). This localization pattern is highly dependent on the status of Ras2p posttranslational processing (3, 28). In the absence of CaaX protease activity (rce1Δ ste24Δ), subcellular punctate structures are observed with a GFP-Ras2p reporter (Fig. 2B). As determined through qualitative and quantitative methods, plasmid-based expression of yeast Rce1p, but not Ste24p, can restore proper GFP-Ras2p localization in this genetic background (Fig. 2C and D, respectively, and Table 3). We took advantage of this observation to investigate whether either of the trypanosomal CaaX proteases could properly modify a GTPase. When the trypanosomal CaaX proteases were evaluated in the context of GFP-Ras2p, proper localization of the reporter was observed with T. brucei Rce1 but not T. brucei Ste24 (Fig. 2E and F, respectively, and Table 3).

Trypanosomal Hsp40 protein Tbj1 can substitute for yeast Ydj1p and is cleaved by both Tb CaaX proteases. Primarily through use of α-factor as a reporter molecule, the CaaX proteases have been demonstrated to possess partially overlapping substrate specificity (47). However, the only two established substrates for the Ste24p family of CaaX proteases are the precursors of the yeast α-factor mating pheromone and mammalian lamin A molecules. Since trypanosomes are not known to express orthologs of either of these molecules and, given the apparent specific ability of T. brucei Ste24 to cleave

![Image](http://ec.asm.org/Downloaded from)

**FIG. 2.** T. brucei Rce1 promotes proper Ras2p localization. Yeast strains that were wild type (EG123) (A) or deficient for endogenous yeast CaaX proteolytic activity (yWS164) (B to F) were each cotransformed with a plasmid encoding a GFP-Ras2p reporter (pWS750) and either an empty vector (A and B) or a plasmid encoding yeast Rce1p (C), yeast Ste24p (D), T. brucei Rce1 (E), or T. brucei Ste24 (F). For each condition, a small cluster of cells are shown as imaged after 6 h of induction of the reporter in SGal−ura media. The CaaX proteases were encoded in pSM1107, pSM1314, pWS766, and pWS767; pRS316 was used as the empty vector.

**TABLE 3. Quantification of GFP-Ras2p plasma membrane localization**

| Backgrounda | CaaX protease | % PM localization ± SEMb |
|-------------|---------------|--------------------------|
| Wild type   | Vector        | 93.2 ± 1.5               |
| rce1Δ ste24Δ | Vector        | 37.6 ± 4.2               |
| rce1Δ ste24Δ | S. cerevisiae Rce1 | 80.7 ± 1.2             |
| rce1Δ ste24Δ | T. brucei Rce1 | 83.0 ± 1.4               |
| rce1Δ ste24Δ | S. cerevisiae Ste24 | 42.3 ± 5.8         |
| rce1Δ ste24Δ | T. brucei Ste24 | 38.6 ± 2.0               |

a Strains used were EG123 (wild type) or isogenic yWS164 (rce1Δ ste24Δ) that were cotransformed with plasmids encoding GFP-Ras2p (pWS750) and, as indicated, an empty vector (pRS316) or plasmid encoding a CaaX protease (pSM1107, pSM1314, pWS766, and pWS767).

b Values for plasma membrane (PM) localization were calculated as s/n/π, where s is the sample standard deviation and n is number of observations.
CaaX motifs associated with Hsp40 proteins from different organisms (i.e., CASQ of yeast Ydj1p and CVHQ of Tbj4), we postulated that isoprenylated Hsp40 proteins are substrates of Ste24p. In obvious conflict with this prediction is our observation that yeast are temperature sensitive when yeast Ydj1p is absent or fails to be isoprenylated (7). A query of the T. brucei genome database yielded four putative Ydj1 orthologs, respectively, as determined by CLUSTAL W2 analysis. Heterologous expression of Tbj1 and Tbj2 rescued growth of a ydj1Δ strain at elevated temperatures to an extent indistinguishable from the wild type. Tbj3 rescued growth less robustly and, for all practical purposes, Tbj4 failed to rescue growth (Fig. 3A). Tbj1 and Tbj2 both possess the CTQQ CaaX motif, whereas Tbj3 and Tbj4 possess the motifs CVHQ and CTAQ, respectively. The inability of Tbj4 to complement growth was surprising, since it contains more sequence homology to yeast Ydj1p than both Tbj2 and Tbj3. Sequence analysis of this clone and others isolated independently during construction of the Tbj4 expression plasmid consistently revealed an 18-nucleotide deletion near the amino terminus. While this may be the cause for the lack of complementation observed, it is also possible that the sequence for this protein is incorrectly annotated within the T. brucei genome database. The sequence we obtained for this clone has been submitted to the National Center for Biotechnology Information (accession no. FJ_611958).

To specifically determine the impact of protein farnesylation on Tbj1 function, we mutated its CaaX motif cysteine to a serine and evaluated the effect. Tbj1(C401S) could not rescue the temperature sensitivity of the ydj1Δ yeast strain (Fig. 3B), which is the same effect observed for yeast Ydj1p when similarly mutated (7). We interpret this observation to indicate that the function of Tbj1 in trypanosomes has an absolute requirement for the cysteine residue within the CaaX motif, thereby suggesting that Tbj1 is indeed isoprenylated.

To further test our hypothesis of protein context being important for specificity, we investigated whether T. brucei Rce1 and/or T. brucei Ste24 could cleave the CaaX motif of Tbj1 using a coupled proteolysis-carboxylmethylation assay. In this type of assay, the extent of CaaX proteolytic activity is indirectly monitored by the extent of ICMT-dependent carboxyl methylation, which is monitored by using a radioactive tracer. For the purposes of this experiment, the source of the Tbj1 substrate was a cell extract prepared from CaaX protease-deficient yeast that expressed Tbj1 heterologously. Although the use of a purified form of the Tbj1 precursor would have been preferred, attempts to purify an adequate quantity of Tbj1 from yeast cell extracts were unsuccessful due to the instability of the precursor during purification. Use of the cell extract revealed that both T. brucei CaaX proteases can cleave the CaaX motif of GST-Tbj1 (Table 4). This observation stands in contrast to that observed for the cleavage of the CaaX motif in the context of the yeast a-factor reporter (see Fig. 1D), which is not recognized by either T. brucei CaaX protease. Hence, we propose that contextual information exists outside the CaaX motif that aids in directing CaaX processing.

### Table 4. Coupled proteolysis-carboxyl methylation in vitro assay reveals cleavage of Tbj1 by both T. brucei CaaX proteases

| Protease | cpma | SEMa | Pa |
|----------|------|------|----|
| Vector   | 345.5| 18.8 |    |
| T. brucei Rce1 | 499.2| 31.2 | 0.002|
| T. brucei Ste24 | 454.0| 35.7 | 0.032|

a Values represent mean of six data points derived from two independent experiments (three replicates per experiment).

b The standard error of the mean was calculated from the equation $\sigma/s$, where $\sigma$ is sample standard deviation and $n$ is number of observations.

c $P$ values calculated by analysis of variance (single factor) relative to vector condition.
were pSM1282, pWS479, pWS766, and pWS767. ples were prepared in the SM3614 background. The plasmids used units/min for yeast Rce1 and Ste24, respectively. All membrane sam-
activity, and open bars represent Ste24 activity. The maximal activities where maximal activity was observed. Closed bars represent Rce1 activity for each CaaX protease is reported relative to the condition 

coupled to a lysine (QL) placed at either the 
a1
X
a2
X

b1
X
b2
X

FIG. 4. Trypanosomal CaaX proteases have similar specificities to-
ward a synthetic K-Ras4B-based substrate. Yeast membranes enriched for the indicated trypanosomal (A) or yeast (B) CaaX protease were evaluated for their ability to cleave a farnesylated nonapeptide based on the K-Ras4B C terminus. The peptide contains an amino benzoic acid fluorophore that intensifies in fluorescence after cleavage of the CaaX motif (CVIM), which contains a dinitrophenol quencher that is coupled to a lysine (Q) placed at either the a1, a2, or X position. The activity for each CaaX protease is reported relative to the condition where maximal activity was observed. Closed bars represent Rce1 activity, and open bars represent Ste24 activity. The maximal activities were 15.16 and 11.02 relative fluorescence units/min for trypanosomal Rce1 and Ste24, respectively, and 93.27 and 25.10 relative fluorescence units/min for yeast Rce1 and Ste24, respectively. All membrane sam-
plasms used were pSM1282, pWS479, pWS766, and pWS767.

The trypanosomal CaaX proteases can be pharmacologi-
ly inhibited. Protein isoprenylation is considered a target for antiparasitic drug discovery (13). Postisoprenylation en-
zymes appear to hold similar potential as drug discovery tar-
gets, as evidenced by the observation that RNAi-mediated gene silencing of T. brucei Rce1 or T. brucei ICMT impairs the growth of trypanosomes (20). Hence, we decided to determine the inhibitor profiles of the trypanosomal CaaX proteases us-
ing in vitro and in vivo approaches with small molecules known to inhibit CaaX proteases from other species (11, 12, 28).

For our in vitro approach, we first determined the utility of K-Ras4B-based, internally quenched, fluorogenic peptide sub-
strates previously used to assess the function of the yeast and human orthologs have similar quencher position prefer-
cing in vitro and in vivo approaches with small molecules known to inhibit CaaX proteases from other species (11, 12, 28). Unlike the profiles of yeast Rce1p and Ste24p, the trypanoso-
mal CaaX proteases had largely overlapping profiles, with both proteases preferring the quencher at the X position (Fig. 4).

Using an optimized fluorogenic reporter (CVIQx), we evalu-
ated the relative in vitro sensitivities of the trypanosomal CaaX proteases to TPCK, a dipeptidyl (acyloxy)methyl ketone (FKBK), EDTA, EGTA, and a set of small molecule com-
ounds previously demonstrated to inhibit yeast and human Rce1p (28, 37). This analysis revealed that T. brucei Rce1p was inhibited by all of the compounds, with the exception of EDTA and EGTA (Table 5). The observed inhibitor profile of T. brucei Rce1p was similar if not identical to that of yeast and human Rce1p (28). Unlike its yeast and human counterparts, T. brucei Ste24 was sensitive to TPCK. Otherwise, T. brucei Ste24 behaved as predicted, being relatively insensitive to the effects of EDTA, EGTA, weakly inhibited by compound 1, and strongly inhibited by the other compounds evaluated. To gain a more detailed understanding of the observed inhibitor effects on T. brucei Rce1p, we determined IC50 values for the small molecule compound set (Table 6). The compounds largely had IC50 values of <10 μM, with the exception of compounds 2, 4, and 5, which had IC50 values of >80 μM.

We have observed that certain small molecule inhibitors de-
scribed above can induce delocalization of GFP-Ras2p in vivo when applied to yeast cultures expressing yeast Rce1p and that this phenotype is similar to that observed in the absence of

| Compound | Mean % activity ± SEM |
|----------|-----------------------|
| TPCK     | 21.6 ± 5.1            |
| FKBK     | 34.4 ± 6.0            |
| EDTA     | 92.0 ± 11.1           |
| EGTA     | 94.6 ± 5.5            |
| 1        | 30.7 ± 4.8            |
| 2        | 22.1 ± 4.7            |
| 3        | 18.6 ± 2.7            |
| 4        | 23.9 ± 9.5            |
| 5        | 34.2 ± 9.4            |
| 6        | 19.3 ± 4.0            |
| 7        | 18.4 ± 3.8            |
| 8        | 16.1 ± 3.3            |
| 9        | 15.4 ± 3.7            |

TPCK, FKBK, EDTA, and EGTA were used at 200 μM. Compounds 1 to 9 are as previously identified and were used at 100 μM (28).

Values are relative to an appropriate untreated control treated with H2O (EDTA and EGTA) or DMSO (TPCK, FKBK, compounds 1 to 9).
CaaX protease activity (28). Thus, we predicted that chemical treatment of yeast heterologously expressing trypanosomal Rce1 as the only CaaX protease would result in a similar phenotype. Indeed, six compounds induced delocalization (Fig. 5A). Compounds 4 and 9 strongly induced delocalization, with 91%, and 79% of the cell population responding, respectively. Compounds 3, 6, 7, and 8 also induced delocalization, but less dramatically, with 48, 47, 62 and 59% of the cell population responding, respectively. Examples of the delocalized patterns, regardless of being the majority or minority phenotype, are shown in the respective panels of the figure.

Compounds 1, 2, and 5 had no effect on GFP-Ras2p localization at the concentrations evaluated, which may represent a lack of cell permeability by these compounds.

We were intrigued by the observation that compounds 7 and 8 could induce GFP-Ras2p delocalization, even if only in a minority of the population, because of evidence suggesting that human Rce1 was less sensitive to these compounds (28). To further investigate the relative effects of these compounds on trypanosomal and human Rce1, we performed both in vivo and in vitro dose-response studies. Consistent with expectations, Trypanosoma brucei Rce1 was more sensitive to compounds 7 and 8 than human Rce1 when evaluated using our GFP-Ras2p localization assay (Fig. 5B). We observed that a 5.3 μM concentration of compound 7 was sufficient to induce delocalization of GFP-Ras2p in the context of T. brucei Rce1, but this concentration had no apparent effect on human Rce1. In fact, an 11.2 μM concentration of compound was required to delocalize the reporter to the same extent in the context of human Rce1. Similarly, a lower concentration of compound 8 was needed to comparably delocalize GFP-Ras2p in the context of T. brucei Rce1 compared to human Rce1 (10.8 and 25 μM, respectively). Consistent with our in vivo results, in vitro IC50 determinations revealed that compound 7 was nearly 10-fold more selective for T. brucei Rce1 than its human counterpart (Table 6). The opposite, however, was true for compound 8, which was about 2-fold less potent against T. brucei Rce1. The reason for the lack of correlation with this compound is unknown. Together, our observations indicate that GFP-Ras2p is an effective reporter for T. brucei Rce1 activity, that the subcellular distribution of GFP-Ras2p can be used as an indicator for determining the effectiveness of compounds for disrupting T. brucei Rce1 activity in vivo, and that the reporter can be used to demonstrate differential targeting specificity by Rce1 inhibitors (i.e., human versus trypanosomal Rce1).

**DISCUSSION**

The results of this study are consistent with the conclusion that two separate CaaX protease activities are present in *T. brucei* corresponding with genes encoding orthologs of Rce1p and Ste24p found in other eukaryotic systems. This observation can be contrasted with that of a previous investigation into the enzymatic properties of these proteins that revealed a proteolytic activity in association with *T. brucei* Rce1 but not *T. brucei* Ste24 (20). We propose that the lack of activity observed in the earlier study for *T. brucei* Ste24 may be a direct consequence of an inappropriate reporter for the enzyme. By our own evidence, we find that *T. brucei* Ste24 is active against several CaaX motifs when it is evaluated in the context of the yeast a-factor reporter, including the CVIM motif present on the substrate used in the prior trypanosomal study (Fig. 1). From these observations, we hypothesize that contextual information is present within the non-CaaX portion of reporters that helps specify *T. brucei* CaaX protease specificity. The idea of contextual information being required by the CaaX proteases has been previously proposed (47) and is further supported by our observation that CTOQ is suitable as a CaaX motif in the context of Tbj1, but not yeast a-factor (Fig. 1D and Table 4). Moreover, it has been demonstrated that yeast Ste24p cannot cleave the CIIS motif in its natural protein context (i.e., Ras2p), but can when appended to the a-factor precursor (3, 47). Collectively, these observations challenge the predictive utility of the a-factor reporter system, or any single reporter background for that matter, as a means by which to assess CaaX protease specificity.

Our most contextually correct data set for the *T. brucei* CaaX proteases establishes that both can mediate maturation of the *T. brucei* Hsp40-family protein Tbj1 (Table 4). However, it would be inappropriate to extrapolate that Tbj2, Tbj3, and Tbj4 are also processed in this manner because their CaaX motifs have not been assessed in their proper protein context. We also cannot infer that *T. brucei* Rce1 specifically mediates maturation of the *T. brucei* RLP GTPase, despite an obvious preference for cleavage of its CaaX motif (CTML) by *T. brucei* Rce1 in our a-factor assay. Likewise, *T. brucei* Rce1 might
specifically cleave *T. brucei* RHP despite its motif (CVIM) being cleaved by both *T. brucei* CaaX proteases in our yeast assay. It appears that determination of specific CaaX protease processing preferences must be experimentally addressed on an individual basis within their native context using methods similar to those applied in this and other studies (4, 27, 34). This will certainly be a challenging task given that inspection of the *T. brucei* genome using the TriTrypDB server reveals over 200 proteins with a canonical CaaX motif, which can be reduced to 61 by applying filters that eliminate pseudogenes, hypothetical proteins, and those with putative signal sequences that would not be expected to be associated with isoprenylated CaaX proteins (see Table S1 in the supplemental material). It remains to be determined which among these presumptive CaaX proteins undergoes isoprenylation, let alone CaaX proteolysis. Among the *T. brucei* GTPases previously identified, only a few have CaaX motifs (17). These include the Ras-like GTPase *T. brucei* RLP (CTML), the Rho-like GTPase *T. brucei* RHP (CVIM), the Rab-like GTPase *T. brucei* Rab23 (CSVM), and the Rag-like GTPase *T. brucei* Rab28 (CAVM). All other identified trypansomal GTPases, including those of the Arf and Ran families, do not contain CaaX motifs, while additional members of the Rab family possess canonical dicysteine geranylgeranylation motifs. Future studies may ascertain the role of *T. brucei* Rce1 and/or *T. brucei* Ste24 in the maturation of trypansomal GTPases containing a CaaX motif and help elucidate the reported essential role of *T. brucei* Rce1 (20).

Our study cautions the use of a single reporter system to specifically determine which CaaX protease cleaves a particular motif. Nonetheless, such reporters still retain value for assessing whether a particular set of CaaX protease orthologs has conserved or dissimilar specificity. For example, the observation that yeast and human Rce1p better recognize certain motifs (Fig. 1D, CAMQ and CTAQ) than does *T. brucei* Rce1 in the context of the a-factor reporter implies that *T. brucei* Rce1 has an intrinsic specificity difference from its relatives. Similar arguments can be made for *T. brucei* Ste24 by comparing its specificity profile to that of its orthologs, although the observed differences are less dramatic (Fig. 1D, CASQ and CAMQ).

The inhibition of *T. brucei* Rce1 could hold therapeutic potential, since the loss of *T. brucei* Rce1 function appears to be correlated with the loss of parasite viability (20); the impact of Ste24 loss of function has not yet been addressed. Should trypansomal and human Rce1 ultimately have overlapping substrate specificities, substrate-based inactivators of *T. brucei* Rce1 will likely target the human enzyme, thereby leading to unintended and undesirable side effects for patients. We have observed some differences, however, in the activities of trypansomal and human Rce1 that may indicate that specific targeting of the parasitic enzyme may be possible. First, we determined that *T. brucei* Rce1 does not readily cleave CTAQ and CAMQ motifs in the context of the a-factor reporter, and thus does not have the exact specificity profile of its human and yeast counterparts (Fig. 1D). Second, we observed that *T. brucei* Rce1 has a distinct preference, by comparison to the yeast and human enzymes, for the optimal placement of a lysine dinitrophenol quenching group on a synthetic fluorogenic peptide reporter (Fig. 4) (37). Lastly, we identified two small molecule agents that inhibit *T. brucei* Rce1 preferentially over the human enzyme in vivo (Fig. 5). The specificity and inhibitor profiles of *T. brucei* Rce1 seem to reflect enzymatic differences between it and human Rce1, suggesting the exciting prospect that trypanosome-specific agents can be identified and developed. This conclusion is predicated on the observed specificity differences holding true independent of protein context.

In conclusion, our study continues to support the observation that eukaryotic systems generally possess two distinct CaaX proteolytic activities. The purpose for this redundancy is not immediately obvious and is likely tied to the need to accommodate the great variety of substrate CaaX proteins encoded in eukaryotic genomes. We argue, based on our results, that these substrates possess information both within their CaaX motifs and protein backbones that provides specificity for one or both proteolytic systems.

ACKNOWLEDGMENTS

We are grateful to T. Ochsenreiter for providing genomic *T. brucei* DNA, M. P. Terns for access to a Zeiss Axioskop 2 Mot Plus microscope, T. M. Dore and members of the Schmidt laboratory (all of the University of Georgia) for critical discussions and technical assistance, and R. G. Mallon (Wyeth Research) for initial samples of fluorogenic K-Ras4B peptides.

This study was supported by an R01 grant (GM067092) from the National Institutes of Health (W.K.S.) and an associated research supplement (K.A.C.).

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