The Ras converting enzyme (RCE)\(^3\) is required for the maturation of Ras and certain other lipid-modified proteins, specifically those having a C-terminal CAAX tetrapeptide motif (C, cysteine; A, aliphatic; and X, one of several amino acids) (1, 2). Proteins bearing a CAAX motif (CAAX proteins) typically undergo three ordered post-translational modifications: thiocther attachment of an isoprenoid lipid (farnesyl or geranylgeranyl) to the cysteine, proteolytic removal of the AAAX tripeptide, and carboxyl methyl esterification of the proteolytically exposed isoprenylated cysteine (2). RCE promotes the proteolytic step in this modification pathway. Because of the important role that certain CAAX proteins have in cellular transformation (e.g. Ras and RhoB), agents that inhibit the maturation and activity of CAAX proteins are seen as having chemotherapeutic potential (3, 4). This hypothesis is supported by the recent development of farnesyltransferase inhibitors that prevent isoprenoid attachment to CAAX proteins, which apparently moderates tumor growth (5, 6). Inhibition of the proteolytic step in CAAX protein modification may have similar anti-cancer potential (4, 7).

The founding RCE-encoding gene was identified in Saccharomyces cerevisiae (1). Yeast RCE1 encodes an endoplasmic reticulum membrane-localized protein (Rce1p) that is predicted by hydrophaty analysis to contain multiple membrane spans (8). A number of Rce1p orthologs have since been identified in other eukaryotic organisms (9–12). The pair-wise identity between RCE orthologs ranges from 14 to 27% identity (12). Despite the relatively low degree of primary sequence conservation, all Rce1p orthologs examined to date can substitute for yeast Rce1p in the maturation of the yeast a-factor mating precursor (11, 12). These observations suggest that the RCE family may have conserved substrate specificity.

In the absence of their respective RCE encoding gene, yeast are incapable of producing fully modified Ras and a-factor, and mice are incapable of producing mature forms of certain CAAX proteins, including Ras and lamin B1 (1, 7, 13). Overexpression of RCE results in increased CAAX protease enzymatic activity (10, 14). Furthermore, biochemically enriched RCE has enzymatic activity (14, 15).\(^3\) Through in vitro studies, RCE-dependent activity has been determined to be sensitive to a number of compounds, including general protease inhibitors, organomercurials, and substrate mimetics (11, 14). In particular, RCE orthologs are reported to be inhibited by a non-hydrolyzable substrate mimic, TPCK, organomercurials, and certain divalent metal ions (i.e., zinc and copper) (11, 12, 14, 16). In sum, the evidence strongly supports the protease classification of RCE. However, RCE has not been shown to possess proteolytic activity in a purified system. Thus, the protease classification and mechanism of RCE remains formally unresolved.

RCE has atypical features for a protease. RCE is an endoplasmic reticulum-localized membrane protein that is predicted to possess multiple membrane spans (8). Other established proteases with multiple membrane spans include the STE24 CAAX protease that has a partially overlapping role with RCE, the presenilins that are involved in A\(_{β}\) production and Notch signaling, S2P that is involved in production of the sterol-response element-binding protein, SPP that is involved in clearance of signal sequences, and rhomboid that is involved in the production of Drosophila epidermal growth factor-\(α\) (17, 18). RCE is also atypical in that it lacks a readily identifiable protease motif. Certain amino acids have been identified that are reportedly critical for RCE activity. These include cysteine, glutamate, and histidine residues (14). Combined mutational and inhibitor data have led to the proposal that RCE is a cysteine protease (14). By contrast, a bioinformatic study of eukaryotic and putative prokaryotic RCE orthologs has identified a set of invariably conserved residues that are typically found in metalloenzymes (i.e., histidine and glutamate residues) (19). Whether any of the residues purportedly essential for RCE activity are properly oriented to compose an active site has not yet been determined (i.e., the active site

\(^8\) This work was supported in part through a Georgia Cancer Coalition Distinguished Cancer Clinician/Scientist Scholar award (to W. K. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\(^1\) To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, University of Georgia, 120 Green St., Athens, GA 30602. Tel.: 706-583-8241; Fax: 706-542-1728; E-mail: wschmidt@bmb.uga.edu.

\(^2\) The abbreviations used are: RCE, Ras converting enzyme; TPCK, N-tosylphenylalanylchloromethyl ketone; HA, hemagglutinin.

\(^3\) L. J. Plummer, E. R. Hildebrandt, S. B. Porter, V. A. Rogers, J. McCracken, and W. K. Schmidt, unpublished observation.
should be disposed toward the cytosol so that it can interact with cytosolic protein substrates).

In this study, we have used genetic, mutational, and biochemical analyses to gain insight into the functional properties of yeast Rce1p and its orthologs. We demonstrate that the substrate specificity of RCE enzymes is evolutionarily conserved, which has implications for the identification of novel substrates of this enzyme. We also show that the enzymatic activity of yeast Rce1p requires a glutamate and a pair of histidine residues that are invariably conserved among RCE orthologs but not a conserved cysteine that has been proposed to be a catalytic residue.

**MATERIALS AND METHODS**

**Strains and Media**—The yeast strains used in this study are listed in Table 1. Plasmid-bearing versions of these strains were generated by transformation with the indicated plasmids according to published methods (20). Strains were routinely grown at 30 °C on synthetic complete dropout (SC–) media, as previously described (21).

For studies of CaAAX protease specificity, a strain was created that lacked theα-factor encoding genes (i.e. MFA1 and MFA2) and the CaAAX protease genes (i.e. RCE1 and STE24). This strain (WS164; MATα trp1 leu2 ura3 his3 can1 mfa1-Δ mfa2-Δ rce1::TRP1 ste24::Kan) was derived from SM3689 (MATα trp1 leu2 ura3 his3 can1 mfa1-Δ mfa2-Δ rce1::TRP1), which has previously been described (22). The knockout of the STE24 gene in SM3689 was facilitated by the use of a DNA fragment that was derived from pWS405 (CEN URA3 ste24::Kan), which was created by replacing the STE24 open reading frame in pSM1093 (CEN URA3 STE24) with a Kan cassette (23, 24). Disruption of STE24 was confirmed in 418-resistant clones by PCR of the STE24 locus and by validating the absence of CaAAX protease activity, as measured by a lack of phenome production when the strain was transformed with a plasmid-encoded copy of MFA1.

**Plasmids**—The plasmids used in this study are listed in Table 2. The multicopyα-factor encoding plasmids have been described previously (12).

The CaAAX protease encoding plasmids are based on pSM1107 (CEN URA3 HA::STE24), pSM1314 (CEN URA3 RCE1::HA), pK90 (2µ URA3 Pα夫妻-OST4-HA::SUC2-HIS4c), and pWS479 (2µ URA3 Pσσγ-CREC1::HA) (8, 23, 25). pWS360 (CEN URA3 CeRce1::HA) and pWS400 (CEN URA3 AtRCE1::HA) were created by replacing the yeast RCE1 coding region in pSM1314 with the indicated sequence as previously described (11, 12). Similarly, pWS364 (CEN URA3 CeStE24::HA) and pWS413 (CEN URA3 AtStE24::HA) were created by replacing the yeast STE24 coding region in pSM1107 (11, 12). Multicopy versions of these plasmids were created by moving appropriate DNA fragments into pRS426 (2µ URA3) (26), pWS335 (2µ URA3 Pσσγ-His::HA::Rce1::Δ22) was created by replacing the STE24 sequence in pSM1282 (2µ URA3 Pσσγ-::HA::STE24) with that of Rce1::Δ22 by PCR-directed plasmid-based recombinational cloning (10, 17, 27).

Site-directed mutations of yeast RCE1 were created by PCR-directed plasmid-based recombinational cloning using pSM1314 as the vector recipient (27). In brief, mutagenic oligonucleotides designed to contain the desired mutation were used to generate PCR products that were co-transformed into SM3614 (MATα trp1 leu2 ura3 his3 can1 ste24::LEU2 rce1::TRP1) along with pSM1314 that had been linearized with a restriction enzyme near the intended site of mutation. The plasmids that formed in vivo by recombination were isolated from several independent yeast transformants, screened for the presence of a silent
In Vitro CAAX Proteolysis Assays—An established assay was used to monitor in vitro production of a-factor from an a-factor-derived synthetic farnesylated pentadecapeptide (YIKGKFVDPAC[farnesyl]VIA) (17). In brief, the assay involves the mixing of the farnesylated substrate with membranes derived from yeast expressing Rce1p. The components were assembled in a 96-well PCR plate. The substrate component consisted of 5 μM peptide in a volume of 10 μl of 2X assay buffer (200 mM Hepes, pH 7.5, 200 mM NaCl). The membrane component consisted of membranes that were diluted to one of two final concentrations (0.0033 or 0.1 mg/ml) in a final volume of 10 μl immediately before use or were used at stock concentration (1 mg/ml). Membranes were stored and diluted with Lysis buffer (50 mM Tris, pH 7.5, 200 mM sorbitol, 1 mM EDTA, plus protease inhibitors). The membranes were isolated according to published methods (30). Assays were initiated by mixing equal volumes of the substrate and membrane components. After the appropriate period of incubation at 30°C (5–90 min, depending on the experiment), the samples were heated to 95°C for 1 min to kill enzymatic activity, cooled, and supplemented with S-adenosylmethionine (20 μM final) and yeast membranes containing Ste14p (0.1 mg/ml final) to initiate methylation of cleaved products. The Ste14p membranes were derived from a CAAX protease-deficient strain as previously described (17). After 60 min of incubation at 30°C, the samples were supplemented with copper sulfate (1 mM final) to stop the methylation reaction. The a-factor concentration in each sample (26-μl final volume) was determined by evaluating the biological activity associated with each sample via a spot halo test. In this biological response assay, yeast supersensitive to mating pheromone (RC757) undergo growth arrest at levels of pheromone above 7 nM (31). Thus, 2-fold serial dilutions allow for a determination of pheromone amounts in a sample.

An established fluorescence-based assay was used to monitor Rce1p-dependent cleavage of a quenched fluorogenic peptide substrate (32). In brief, the assay involves the mixing of the substrate (ABZ-KSKTKC[farnesyl]Q_{14}IM) with membranes derived from yeast expressing Rce1p. Q_{14} is lysine ε-dinitrophenyl. The components were assembled in a 96-well plate suitable for use in a microtitrator plate fluorometer. The substrate component consisted of 40 μM substrate in a volume of a 50-μl assay buffer (100 mM Hepes, pH 7.5, 5 mM MgCl_{2}); kinetic assays utilized 0.5–20 μM substrate (final concentration). The membrane component used as the source of activity, the same source as described for the a-factor assay, was diluted 1:1 with assay buffer to yield a final volume of 50 μl immediately before use. Assays were initiated by mixing equal volumes of the substrate and membrane components. The fluorescence in these samples was typically measured every 30 s over a 60-min time course at 30°C using a SpectraMax Gemini EM fluorometer with a 250–850 nm cutoff filter (Molecular Devices). The initial linear slopes were used in calculating percent activities and kinetic parameters relative to the wild-type enzyme. Concentrations of product were determined by comparison of the fluorescent signals generated in the sample to a standard curve derived from samples containing the fluorophore alone.

The activity of each mutant, as derived from the assays above, was normalized against its relative expression level. For this purpose, wild-type and mutant Rce1p membrane preparations were subjected to SDS-PAGE and immunoblotting with the anti-HA antibody, and the chemiluminescence intensity of each mutant was quantified by immunoblot using a VersaDoc Imaging System (Bio-Rad). A relative expression factor was calculated for each mutant that was based on the average of values obtained from the quantification of two separate anti-HA immunoblots. The expression level of mutants ranged between 50 and 105% relative to overexpressed wild-type Rce1p.

Structure/Function Studies of Rce1p

Restriction site that was typically introduced along with the mutation, and candidates carrying the restriction site were sequenced to confirm the presence of the mutation. SacI DNA fragments derived from the pSM1314-based plasmids (containing RCE1 coding and 3’-untranslated regions) were subcloned into pWS479 to create overexpression vectors. pWS479 was created by subcloning the SacI fragment of pSM1314 containing part of the open reading frame of RCE1:HA and its 3’-untranslated regions into the same sites of pWS123 (2 μg URA3 P_{pce}-RCE1::His). pWS123 was created by subcloning a PCR fragment containing RCE1 into the BamHI and NotI sites of pSM703 such that the oligonucleotides also introduced a polyhistidine tag at the 3’-end of the open reading frame. pSM703 has been described before (28).

Fusions of yeast RCE1 to the OST4-His14C reporter were created by recombinant cloning. In brief, PCR products encoding full-length or truncated RCE1 with ends homologous to pJK90 were co-transformed with pJK90 that had been linearized with SmaI (25). Recombinant plasmids were recovered and analyzed by restriction enzyme digestion to identify the desired clones.

Yeast Mating Assays—The ability of Rce1p mutants to promote a-factor production was assessed by genetic tests in which the growth of diploids is indicative of mating and a functional Rce1p enzyme (11, 29). For the substrate specificity study of CAAX pro tease orthologs, mating tests were performed either by spotting cultures of washed MATa cells onto a lawn of MATa cells or by carrying out the serial dilution mating assay. For both approaches, a yeast strain lacking the a-factor and CAAX protease genes (yWS164) was co-transformed with plasmids encoding an Rce1p or Ste24p ortholog and the indicated a-factor CAAX variant (11), and the co-transformed strain was cultured in selective media. For the washed cells approach, cells were harvested by centrifugation, washed once with sterile H2O, and spotted (1 μl spots) onto a lawn of MATa (SM1068) cells that had previously been spread on a minimal agar plate. The MATa cells were prepared as a suspension of cells in YPD (yeast extract, peptone, and dextrose), 300 μl of this suspension was spread on the minimal plate. The small amount of YPD allows the auxotrophic MATa and MATa haploid yeast to survive for a short amount of time during which mating events can occur but does not allow the growth of visible haploid colonies. For the serial dilution approach, the serial dilution mating test was performed as described below. For both approaches, the formation of diploid colonies was scored after 2–3 days of growth at 30°C.

For the genetic analysis of Rce1p mutants (site-directed and truncation), a serial dilution mating test was used. In brief, MATa cultures were grown for 24 h in selective media along with a MATa culture that was grown in YPD. The cultures were all normalized to 1 A_{600} by dilution with appropriate growth media. The MATa cell suspension was loaded into the wells of a 96-well plate (90 μl/well), which was followed by the addition of each MATa cell suspension to individual wells in the first row of the multowell plate (10 μl/well). The cell populations were mixed using a multichannel pipetter, and a volume (10 μl) of each first row suspension was transferred to the corresponding second row well of the multowell plate. The above steps were repeated for the remaining rows so as to create a 10-fold serial dilution of each MATa culture. The cell suspensions from all the wells were spotted onto a minimal agar plate, and the formation of diploid colonies was scored after 2–3 days of growth at 30°C. The serial dilutions were also spotted onto SC-K (lysine) plates to confirm that equivalent amounts of MATa cells were present for each mutant at each dilution; these plates allow the selective growth of MATa haploid cells and any diploid cells that may have formed during the processing of the samples.
RESULTS

RCE Orthologs Have Conserved Substrate Specificity—Yeast Rce1p and Ste24p have partially overlapping substrate specificity (39). Both enzymes cleave the a-factor precursor, which contains the CVIA CAAX motif. In this study, we have evaluated the ability of the CAAX proteases from budding yeast (S. cerevisiae), plant (Arabidopsis thaliana), worm (Caenorhabditis elegans), and humans to cleave three distinct CAAX motifs that have been proposed to be non-specific (CVIA), RCE-specific (CTLM), or STE24-specific (CASQ) (Fig. 1) (39). Our analysis, using a-factor CAAX variants and a genetic assay, reveals that RCE orthologs have highly conserved substrate specificity. Yeast, plant, and worm RCEs readily cleave the CVIA motif but fail to cleave the CASQ motif when these enzymes are expressed from low copy plasmids (Fig. 1A). Overexpressed RCE orthologs display the same substrate selectivity, although overexpressed yeast Rce1p appears to have marginal activity toward CASQ (Fig. 1C). Overall, the data is consistent with CVIA and CTLM being the preferred motifs for RCE orthologs.

STE24 orthologs also appear to have conserved substrate specificity. All readily cleave the CVIA motif, and none cleave the CTLM motif when these enzymes are expressed from low copy plasmids (Fig. 1B). Yeast and worm STE24 readily cleave the CASQ motif, whereas human and plant STE24 have reduced or no activity, respectively, against CASQ. Overexpression of these enzymes reveals that the plant and human enzymes are indeed capable of cleaving the CASQ motif and that the yeast and worm enzymes have slight activity toward CTLM (Fig. 1D). Overall, the selectivity of STE24 orthologs is consistent with CVIA and CASQ being preferred motifs.

These analyses were extended to seven additional CAAX motifs using the yeast and human forms of RCE and STE24 (Fig. 2). For these motifs, substrate specificity appeared conserved between the yeast and human forms of each enzyme. There were four different types of CAAX motifs. One type consisted of motifs that were cleaved by both RCE and STE24 (CVIA, CAMQ, CALQ, and CALM). Within this group, the human enzymes sometimes promoted mating more efficiently, but this is likely due to overexpression of these enzymes. A second type was recognized exclusively by RCE (CTLM, CTVM, CSVM, and CTSM). A third was recognized only by STE24 (CASQ). The final type was poorly recognized by either protease regardless of species (CTSQ). Inefficient utilization of the CTSQ motif may be due to lack of farnesylation of this motif and/or poor cleavage by either protease.

Rce1p Activity in Vivo Requires Glutamate and Histidine Residues—A primary sequence alignment of yeast (budding and fission), human, fly, worm, and plant RCE orthologs reveals a limited number of invariably and highly conserved residues (Fig. 3). As part of this study, the importance of these conserved residues to the function of yeast Rce1p was evaluated. We determined that the bulk of conserved residues examined (15 out of 22) had no effect on Rce1p function as judged by a genetic test that is based on yeast a-factor mating pheromone production (Fig. 4A).

Our preliminary genetic analysis of Rce1p site-directed mutants revealed that three residues were required for the activity of Rce1p when
it was expressed from its natural promoter (Glu\textsuperscript{156}, His\textsuperscript{194}, and His\textsuperscript{248}). Four additional residues (Glu\textsuperscript{157}, Tyr\textsuperscript{160}, Phe\textsuperscript{190}, and Asn\textsuperscript{252}) were required for optimal activity. The E157A, Y160A, F190A, and N252A mutants were phenotypically 10 to 20\% less active at promoting mating than wild-type Rce1p, whereas the N252A mutant was 50–100\% less active. These differences cannot be attributed to differential expression levels, because the mutants were expressed similarly to wild-type Rce1p as judged by quantitative immunoblot analysis.\textsuperscript{1,3} Glu\textsuperscript{156}, His\textsuperscript{194}, and His\textsuperscript{248} have been reported to be required for Rce1p activity,\textsuperscript{14, 39} whereas unpublished observations have been made regarding the

it was expressed from its natural promoter (Glu\textsuperscript{156}, His\textsuperscript{194}, and His\textsuperscript{248}). Four additional residues (Glu\textsuperscript{157}, Tyr\textsuperscript{160}, Phe\textsuperscript{190}, and Asn\textsuperscript{252}) were required for optimal activity. The E157A, Y160A, and F190A mutants were phenotypically 10 to 20\% less active at promoting mating than wild-type Rce1p, whereas the N252A mutant was 50–100\% less active. These differences cannot be attributed to differential expression levels, because the mutants were expressed similarly to wild-type Rce1p as judged by quantitative immunoblot analysis.\textsuperscript{1,3} Glu\textsuperscript{156}, His\textsuperscript{194}, and His\textsuperscript{248} have been reported to be required for Rce1p activity,\textsuperscript{14, 39} whereas unpublished observations have been made regarding the
The importance of Glu(157) and Asn(252). The importance of Tyr(160) and Phe(190) for Rce1p function has not been previously noted. Importantly, our genetic analysis did not reveal significant requirements for His(197) or Cys(251), as has previously been reported (14, 39). A quantitative assessment of our mating results is presented in Table 3.

To probe the activity of non-mating mutants in more detail, we overexpressed these mutants using a strong promoter (i.e. PGK) and evaluated their activity by our genetic test (Fig. 4C). Overexpression, which raised the intracellular levels of WT and mutant Rce1p enzymes ~100-fold,3 partially rescued the ability of two of the three most severely compromised mutants to promote mating. Yeast expressing Rce1p H248A had a significant but still compromised ability to mate, whereas E156A had very poor mating competence. Despite overexpression, Rce1p H194A remained incapable of promoting mating. Our genetic analysis thus revealed four categories of mutants. Category I mutants were essentially wild-type in function (e.g. H197A and C251A), category II mutants had diminished but significant activity (i.e. E157A, Y160A, F190A, and N252A), category III mutants had activity only when overexpressed (i.e. E156A and H248A), and the sole category IV mutant (i.e. H194A) was invariably inactive.

To investigate the requirements for a negative charge at Glu(156), we evaluated two additional mutations, E156D and E156Q (Fig. 4B). E156D had marginal activity in the mating assay, and E156Q had no activity. Overexpression of these mutants significantly boosted the activity of E156D, whereas E156Q remained inactive (Fig. 4C). Thus, it appears that Rce1p has a specific requirement for a negative charge at the Glu(156) position.

In Vitro Analyses of Wild-type and Mutant Yeast Rce1p—Several in vitro assays have been described for the measurement of Rce1p activity (1, 8, 10, 11, 14, 17, 32). In this study, we used two distinct assays to monitor the activity of category II–IV Rce1p mutants and a subset of category I mutants to obtain additional quantitative measurements of enzymatic activity and to assess whether observed defects in function for some mutants were substrate specific. First, we utilized a sensitive a-factor production assay to verify the a-factor production defects observed for certain mutants (17). For this assay, a synthetic peptide representing a-factor with a CAAX motif is used as the substrate. The substrate is converted to mature, biologically active pheromone in an Rce1p-dependent manner. Detection of the pheromone in the sample takes advantage of the observation that MATA yeast undergo growth arrest when exposed to a-factor (31). Because the minimal concentration of a-factor that elicits a response in this assay is known, serial dilutions of a sample yield an estimate of the a-factor concentration in a sample. Using the a-factor assay, wild-type Rce1p had an activity of 2.1 nmol of a-factor produced per minute per milligram of Rce1p membrane. The in vitro activities of the mutants generally mirrored that observed in our genetic tests. Category I mutants had significant activity ranging from 30 to 113% relative to wild-type Rce1p (Fig. 5A and Table 3). Three category II mutants (Y160A, F190A, and N252A) had significantly reduced activity, which was observable only when the reaction conditions were altered to contain 30× more enzyme (Fig. 5B and Table 3). One category II mutant (E157A) and the category III and IV mutants (E156A, H194A, and H248A) had no measurable in vitro activity despite using 300× higher input enzyme and an 18× longer incubation time to maximize the sensitivity of the assay (Fig. 5C and Table 3). Except for E157A, the results obtained with the in vitro a-factor production assay paralleled those using genetic methods. This study also revealed that our genetic tests, although highly sensitive, overestimated the relative activity of category I mutants having modest defects in activity (e.g. mutants with ~30% activity appeared wild-type).

For the above in vivo and in vitro assessments of Rce1p activity, we used a-factor-based assays. To exclude the possibility that we were observing substrate-specific effects, we monitored the ability of Rce1p mutants to cleave a quenched fluorogenic K-Ras-derived CAAX peptide substrate (32). The cleavage of this substrate can be monitored in real-time, thus allowing for kinetic analyses. Using this assay, we determined the activity of wild-type Rce1p to be 3.4 nmol per minute per milligram

---

**TABLE 3**

| Category | Enzyme | Mating assay | Halo assay | Fluorescence assay |
|----------|--------|--------------|------------|--------------------|
| I        | WT     | 100          | 100        |                    |
|          | H196A  | 100          | 72         | 34                 |
|          | H197A  | 100          | 30         | 53                 |
|          | C251A  | 100          | 113        | 107                |
| II       | E157A  | 10           | <10<sup>-3</sup> | <0.5 |
|          | Y160A  | 10           | 2.6        | <0.5               |
|          | F190A  | 10           | 3.1        | <0.5               |
|          | N252A  | 1            | 0.9        | <0.5               |
| III      | E156A  | <0.1         | <10<sup>-3</sup> | <0.5 |
|          | H248A  | <0.1         | <10<sup>-3</sup> | <0.5 |
| IV       | H194A  | <0.1         | <10<sup>-3</sup> | <0.5 |

---

<sup>4</sup>Percent activities reported are derived from the data presented in Fig. 4A (mating assay), Fig. 5 (halo assay), or from the fluorescence-based assay. Values shown for in vitro assays have been normalized for mutant Rce1p expression levels relative to wild-type Rce1p.

---

**FIGURE 5.** Activity of Rce1p mutants as determined by an in vitro assay for a-factor production. Membrane preparations containing the indicated Rce1p mutant were used to synthesize a-factor in vitro from a farnesylated pentadecapeptide precursor. The reactions were spotted onto a lawn of MATA (RC757) cells. The presence of a-factor is visualized as a zone of growth inhibition. The assay conditions were adjusted for input amounts of protein or incubation time to keep the output within the linear range of the assay. Assays with mutants contained either 0.033 μg (A), 1 μg (B), or 10 μg (C) of membrane sample and were carried out for 5 min (A and B) or 90 min (C). In every experiment, a sample containing wild-type Rce1p (WT) was evaluated using the conditions described for panel A samples; this sample served as a normalization control. A sample lacking Rce1p (vector; pRS316) that served as a negative control was also evaluated. The membranes used in this study were all from cells with 2μ P<sub>rep</sub>-based RCE1 expression vectors. Samples were spotted as 2-fold serial dilutions (top to bottom), with the top spot representing undiluted sample.


**Structure/Function Studies of Rce1p**

### Table 4

| Enzyme | \( K_m \) |
|--------|----------|
| WT     | 1.72 ± 0.67 |
| H196A  | 3.26 ± 0.43 |
| H197A  | 1.44 ± 0.21 |
| C251A  | 2.34 ± 0.58 |

**FIGURE 6.** Effect of site-directed mutations on the substrate specificity of Rce1p. The Rce1p mutants listed in Table 3 were co-expressed with one of three a-factor CAAX variants in YWS164 and evaluated for the ability to promote mating as described in Fig. 4; the negative control strain contained pBS316 (vector) instead of an Rce1p encoding plasmid. Yeast were co-transformed with either wild-type MFA1, which has the CVIA motif (A), a CTLM variant (B), or a CASQ variant (not shown). Differences in the ability to promote mating were observed for category II mutants. The MFA1 plasmids used in this study were high copy (\( \mu_s \) + / – 0.67), the Rce1p mutants were encoded behind the native RCE1 promoter on a low copy vector.

**FIGURE 7.** The C terminus of yeast Rce1p is not essential for activity. A, schematic of truncations evaluated in this study and summary of ability to promote yeast mating. The truncations are shown to scale; the HA-Suc2-His4c reporter is not drawn to scale. B, yeast lacking both of the CAAX proteases (SM3614; rce1 Δ) were transformed with plasmids encoding Rce1p (WT) or the indicated Rce1p fusion and were evaluated for the ability to mate as described in Fig. 4. The plasmids used in this study were either an empty vector (A), a low copy vector encoding Rce1p and its natural promotor (WT), or multicopy vectors encoding Rce1p fusions behind the constitutive TPI promoter. Full-length Rce1p is 315 amino acids.

of membrane. We also evaluated the activity of certain category I mutants (H196A, H197A, and C251A) previously reported to have defects in enzymatic activity, category II mutants (E157A, Y160A, F190A, and N252A), and category III–IV mutants (E156A, H194A, and H248A) (Table 3). Category I mutants had substantial activity (34% or greater relative to wild-type), including Rce1p C251A that had previously been reported to be an inactive mutant (14). These mutants were scrutinized more carefully by detailed in vitro kinetic analysis, which revealed that Category I mutants had \( K_m \) values similar to that of wild-type Rce1p (Table 4). \( K_m \) values for category II–IV mutants could not be determined, because they had no measurable activity in this assay, despite some having readily measurable activity in the a-factor based assays (E157A, Y160A, F190A, and N252A). The inability to measure the activities of category II mutants could be due to the lower sensitivity of the Ras-based assay, which we have determined to have a threshold detection level of 0.5%. Attempts to enhance the activity of category II–IV mutants by increasing the incubation time did not yield measurable activity. Overall, the results obtained with the Ras-based assay complemented our findings using a-factor based assays. In addition, our findings establish that pheromone production-based in vivo and in vitro tests can be used as highly sensitive measures of Rce1p proteolytic activity.

Category II Mutations Alter the Substrate Specificity of Rce1p—We were prompted to investigate whether category II mutants impacted the substrate specificity of Rce1p, because we observed that these mutants were active against the a-factor substrate but appeared less active against the Ras-based substrate (Table 3). To address this issue, we took advantage of the a-factor CAAX motif variants used initially to establish that Rce1p orthologs had conserved substrate specificity (Fig. 1). Category II–IV mutants and a subset of category I mutants were co-expressed with one of the three a-factor CAAX variants (CVIA, CTLM, or CASQ) in a strain lacking both CAAX proteases and the two a-factor genes (12). The mutants co-expressed with wild-type a-factor CAAX motif (CVIA) had a mating profile similar to that previously observed (compare Figs. 4A and 6A). In these strains, the modest defect in mating for the category II mutants is rescued by the overexpression of a-factor. The CTLM motif was utilized as well as CVIA in the presence of wild-type Rce1p, suggesting that both motifs are efficiently farnesylated and proteolyzed. Generally, most of the Rce1p mutants evaluated were capable of cleaving the CTLM motif (Fig. 6B). However, there were notable exceptions. All four of the category II mutants (i.e., E157A, Y160A, F190A, and N252A) had a reduced ability to cleave CTLM a-factor by comparison to CVIA a-factor, with Rce1p N252A being mating incompetent. None of the mutants were capable of promoting mating when co-expressed with a-factor having the STE24-specific CASQ motif.

The Last 39 Residues of Rce1p Are Dispensable for Rce1p Enzymatic Activity—The C-terminal regions are not highly conserved among members of the RCE family. To examine the importance of this region to the enzymatic activity of yeast Rce1p, we evaluated the effect of C-terminal truncations on enzyme activity. The truncations were generally initiated within predicted loops as judged by hydropathy analysis of yeast Rce1p (Fig. 7A and Table 5). For this study, an HA-Suc2-His4c reporter was fusied to the end of each truncation that was evaluated (25). Fusion of the reporter to full-length Rce1p did not block the function of Rce1p (Fig. 7B). Analysis of the truncation mutants revealed that only Rce1p 1–276 retained activity. This truncation had slightly reduced activity relative to wild-type Rce1p with and without the reporter as judged by serial dilution mating tests (Fig. 7B). Overall, these results indicate that the C-terminal 39 amino acids...
of Rce1p are dispensable for activity even though this region likely contains a transmembrane segment. A truncation strategy was also applied to the N-terminal region of Rce1p, but all N-terminal truncations were invariably inactivating, suggesting that these deletions may affect protein stability or topology.

**DISCUSSION**

In this study, we have addressed two main issues regarding the enzymology of the CAAAX proteases. First, we have determined that the partially overlapping substrate specificity described for the yeast enzymes Rce1p and Ste24p is conserved in plant, worm, and human orthologs (Fig. 1) (39). Our study suggests that, in systems other than yeast, Rce1p and Ste24p is conserved in plant, worm, and human orthologs (category II residues) are required for optimal activity and may play a role in modulating the substrate specificity of yeast Rce1p. In the absence of a structure for any RCE, we hypothesize that category III and IV residues are part of the yeast Rce1p active site. However, we cannot exclude the possibility that mutations at these sites are altering some other aspect of Rce1p physiology, such as proper localization, interaction with other proteins, or orientation in the endoplasmic reticulum membrane. Interestingly, the category III and IV glutamate and histidine residues (i.e. Glu156, His194, and His248) of RCE, and certain category II residues that are required for optimal activity (i.e. Glu157, Tyr160, and Phe190), are also highly conserved in a group of prokaryotic and euukaryotic enzymes that have been postulated to be RCE orthologs (19). It therefore seems likely that these conserved residues are either active site residues or are important for maintaining the structure of the active site.

Although our genetic and in vitro assays generally yielded similar data, a notable exception to this rule was Rce1p E157A. This category II mutant had considerable in vivo function yet was non-functional in both in vitro assays. At this time, we cannot explain this observation, although several scenarios are plausible. One is that Glu157 plays a critical role in substrate binding. This residue may stabilize interactions with portions of the native substrates that are missing in our in vitro substrates. A second possibility is that the Glu157 mutant is unstable and undergoes inactivating structural changes upon extraction from yeast. Third, it is possible that Glu157 is required for maintaining an interaction between Rce1p and a co-factor that is otherwise soluble. In the mutant, the co-factor would be lost during the membrane isolation procedure used to recover Rce1p for in vitro assays. The importance of this residue toward Rce1p function remains an intriguing issue.

Our results for category III mutants (E156A and H248A) are also intriguing. These mutants were found to be active in vivo when overexpressed but had no discernable activity in either of our in vitro assays. We do not believe that the observed activity in vivo indicates that the mutant enzymes have robust activity. Rather, the activity observed merely highlights the sensitivity of our genetic assay. This sensitivity is exaggerated under overexpression conditions where Rce1p levels are ~100-fold higher. This increased activity is clearly evident for E157D, which has ~100-fold more activity when overexpressed (Fig. 4, compare panels B and C). By taking into account overexpression, we estimate that E156A is 100,000-fold less active than wild-type, and H248A is at least 1000-fold less active. Thus, category III mutants are indeed highly compromised for activity.

Given our findings, we propose that the RCE catalytic site is comprised of a glutamate and two histidine residues. Although a histidine residue is typically part of the catalytic triad of serine and cysteine proteases, a requirement for two histidine residues and at least one glutamate is a hallmark of metalloproteases. For metalloproteases, a metal
ion and a glutamate residue typically orient a water molecule that ultimately performs peptide bond hydrolysis. Because the glutamate residue is not directly performing catalysis, it is reasonable that a negatively charged aspartate could substitute for the glutamate residue such that some activity is retained. This has been observed for certain metallopeptases, and we find this to be the case for Rce1p (45–47). Consistent with the hypothesis that RCE is a metalloenzyme, we have independently confirmed that 1,10-phenanthroline and excess zinc inhibit RCE activity (14). However, we have not been able to inactivate yeast Rce1p with extensive EDTA treatment or reactivate 1,10-Phenanthroline-treated RCE with metal ions.3 Ultimate proof of RCE being a metalloenzyme will require metal analysis of the purified active enzyme or demonstrable reactivation of the purified apo form with a particular metal. The fact that RCE has multiple membrane spans makes either of these potential avenues a daunting challenge due to the inherent difficulties associated with the purification of membrane proteins in active form.

RCE has also been proposed to be a cysteine protease, but we posit that this classification needs reconsideration. In support of a cysteine protease classification, yeast Rce1p has been reported to require a cysteine (Cys251) that is invariably conserved between RCE members (14). Furthermore, RCE orthologs have been shown to be sensitive to certain cysteine protease inhibitors (e.g. TPCK), and farnesylaldehyde derivatives that should mimic the transition state of a cysteine protease (11, 12, 14, 16). However, several observations contest a cysteine-based mechanism. First, we have found that Cys251 of yeast Rce1p is not required for enzyme function, despite it being the only conserved cysteine among Rce1p orthologs (Figs. 3 and 4). Moreover, the kinetic properties of Rce1p C251A appear unaltered (Tables 3 and 4). Although the reported inhibitory effects of TPCK and farnesylaldehydes are consistent with a cysteine protease-based mechanism for Rce1p, these data must be carefully considered. Foremost, one must take into account that these compounds readily react with residues besides cysteine. For example, TPCK covalently modifies histidine residues that are part of the catalytic triad of cysteine and serine proteases having a hydrophobic binding pocket (e.g. chymotrypsin). In a similar manner, TPCK may interact with a hydrophobic pocket in RCE. This interaction may position TPCK such that a covalent adduct is formed with a residue that is part of or near the active site. Alternatively, TPCK may bind non-covalently such that it effectively competes with the binding of substrates. Consistent with this hypothesis is the observation that certain hydrophobic protease inhibitors reduce RCE activity (i.e. 1,10-phenanthroline and the organomercurials mersalyl acid, p-hydroxymercuribenzoic acid, and p-hydroxymercuriphenylsulfonic acid), whereas more water-soluble protease inhibitors have little inhibitory effect (e.g. 1-chloro-3-tertosyl-amido-7-amino-2-heptanone (TLCK) and E64) (11, 14). Likewise, farnesylaldehydes can react quite readily and nonspecifically with serine and lysine residues to form hemi-acetals and Schiff bases, respectively. Determining whether TPCK and farnesylaldehydes are consistent with a cysteine protease-based mechanism for Rce1p, these data must be carefully considered. Foremost, one must take into account that these compounds readily react with residues besides cysteine. For example, TPCK covalently modifies histidine residues that are part of the catalytic triad of cysteine and serine proteases having a hydrophobic binding pocket (e.g. chymotrypsin). In a similar manner, TPCK may interact with a hydrophobic pocket in RCE. This interaction may position TPCK such that a covalent adduct is formed with a residue that is part of or near the active site. Alternatively, TPCK may bind non-covalently such that it effectively competes with the binding of substrates. Consistent with this hypothesis is the observation that certain hydrophobic protease inhibitors reduce RCE activity (i.e. 1,10-phenanthroline and the organomercurials mersalyl acid, p-hydroxymercuribenzoic acid, and p-hydroxymercuriphenylsulfonic acid), whereas more water-soluble protease inhibitors have little inhibitory effect (e.g. 1-chloro-3-tertosyl-amido-7-amino-2-heptanone (TLCK) and E64) (11, 14). Likewise, farnesylaldehydes can react quite readily and nonspecifically with serine and lysine residues to form hemi-acetals and Schiff bases, respectively. Determining whether TPCK and farnesylaldehydes are consistent with a cysteine-based mechanism.

A formal possibility also exists that RCE utilizes a novel proteolytic mechanism that requires glutamate and histidine residues. Perhaps the unique microenvironment of the Rce1p active site near or possibly within membranes dictates the need for a novel mechanism. Although a novel mechanism may seem contentious, the recent discovery of a proteolytic mechanism that is distinct from the four established mechanisms (i.e. aspartic, cysteine, serine/threonine, and metallo) reveals that novel proteolytic mechanisms remain to be discovered (48). This new fifth mechanism utilizes a glutamate and glutamine dyad, thus establishing the importance of glutamates as active site residues for proteases outside the realm of the metalloprotease family.

Regardless of the mechanism used by RCE, the essential glutamate and histidine residues must be in close proximity in order for these residues to be part of the enzyme active site. In the absence of structural data for RCE, we suspect that these residues reside in separate interacting transmembrane segments. Although, intramembrane active sites are atypical for proteases, such active sites have been proposed for several proteases, including the presenilins, S2P, SPP, and rhomboid. We propose that RCE has a membrane-embedded active site, because the essential residues of yeast Rce1p are predicted to lie near the edge or within predicted membrane spans by web-based transmembrane span prediction algorithms, although the case for His324 is not as strong (Table 5). Whether the atomic distances between these residues are sufficiently short to allow interactions awaits structural information on this enzyme. Nevertheless, this analysis is consistent with Rce1p having a membrane-embedded active site that is critically positioned to interact with substrates that are membrane-associated by virtue of their isoprenoid modification. This analysis, however, does not address whether inactivating mutations are altering catalytic residues, the structure, or other aspects of Rce1p that are required for its function (e.g. localization).

Although significant progress has been made toward understanding the function of RCE, several outstanding issues remain to be resolved. These include the proteolytic mechanism, topology, structure, and identification of substrates of RCE and, ultimately, the physiological importance of RCE function. These issues will surely be resolved in the near future for this rather confusing enzyme.

Acknowledgments—We are grateful to Dr. Claiborne Glover, Dr. Jim Travis, and the Travis laboratory for technical advice, access to equipment, and critical discussions, Andrea Lapham-Cardenas, Ray Chen, and Daniel Mandel for expert technical assistance, and Wyeth Pharmaceuticals for providing the K-Ras peptide substrate.

REFERENCES
1. Boyartchuk, V. L., Ashby, M. N., and Rine, J. (1997) Science 275, 1796–1800
2. Young, S. G., Ambroziak, P., Kim, E., and Clarke, S. (2001) in The Enzymes (Tamanoi, F., and Siggian, D., eds) pp. 155–213, Academic Press, New York
3. Gibb, J. (1991) Cell 65, 1–4
4. Bergo, M. O., Ambroziak, P., Gregory, C., George, A., Otto, J. C., Kim, E., Nagase, H., Casey, P. J., Balmain, A., and Young, S. G. (2002) Mol. Cell. Biol. 22, 171–181
5. Baum, C., and Kirschmeier, P. (2003) Curr. Oncol. Rep. 5, 99–107
6. Zhu, K., Hamilton, A. D., and Sebit, S. M. (2003) Curr. Opin. Investig. Drugs 4, 1428–1435
7. Kim, E., Ambroziak, P., Otto, J. C., Taylor, B., Ashby, M., Shannon, K., Casey, P. J., and Young, S. G. (1999) J. Biol. Chem. 274, 8383–8390
8. Schmidt, W. K., Tam, A., Fujimura-Kamada, K., and Michaelis, S. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11175–11180
9. Freije, J. M., Bay, P., Pendas, A. M., Cadinanos, J., Crespo, P., and Lopez-Otin, C. (1999) Genomics 58, 270–280
10. Otto, J. C., Kim, E., Young, S. G., and Casey, P. J. (1999) J. Biol. Chem. 274, 8379–8382
11. Cadinanos, J., Schmidt, W. K., Fueyo, A., Varela, I., Lopez-Otin, C., and Freije, J. M. (2003) Biochim. J. 370, 1047–1054
12. Cadinanos, J., Varela, I., Mandel, D., Schmidt, W. K., Díaz-Perales, A., Lopez-Otin, C., and Freije, J. M. (2003) J. Biol. Chem. 278, 42091–42097
13. Maske, C. P., Hollinshead, M. S., Higgin, N. C., Bergo, M. O., Young, S. G., and Vaux, D. J. (2003) J. Cell. Biol. 162, 1223–1232
14. Dolelce, J. M., Steward, L. E., Dolelce, E. K., Wong, D. H., and Poulter, C. D. (2000) Biochemistry 39, 4096–4104
15. Chen, Y., Ma, Y. T., and Rando, R. R. (1996) Biochemistry 35, 3227–3237
16. Ma, Y. T., Gilbert, B., and Rando, R. (1993) Biochemistry 32, 2386–2393
17. Tam, A., Schmidt, W. K., and Michaelis, S. (2001) J. Biol. Chem. 276, 46798–46806
18. Weihofen, A., and Martoglio, B. (2003) Trends Cell Biol. 13, 71–78
19. Pei, J., and Grishin, N. V. (2001) Trends Biochem. Sci. 26, 275–277
20. Eibl, R. (1992) BioTechniques 13, 18–20

JOURNAL OF BIOLOGICAL CHEMISTRY VOLUME 281 • NUMBER 8 • FEBRUARY 24, 2006
21. Michaelis, S., and Herskowitz, I. (1988) Mol. Cell. Biol. 8, 1309–1318
22. Tam, A., Nouvet, F., Fujimura-Kamada, K., Slunt, H., Sisodia, S. S., and Michaelis, S. (1998) J. Cell Biol. 142, 635–649
23. Fujimura-Kamada, K., Nouvet, F. J., and Michaelis, S. (1997) J. Cell Biol. 136, 271–285
24. Brachmann, C. B., Davies, A., Cost, G. J., Caputo, E., Li, J., Hieter, P., and Boeke, J. D. (1998) Yeast 14, 115–132
25. Kim, H., Melen, K., and von Heijne, G. (2003) J. Biol. Chem. 278, 10208–10213
26. Sikorski, R. S., and Hieter, P. (1989) Genetics 122, 19–27
27. Oldenburg, K. R., Vo, K. T., Michaelis, S., and Paddan, C. (1997) Nucleic Acids Res. 25, 451–452
28. Zhang, Y., Nijbroek, G., Sullivan, M. L., McCracken, A. A., Watkins, S. C., Michaelis, S., and Brodsky, J. L. (2001) Mol. Biol. Cell 12, 1303–1314
29. Nijbroek, G. L., and Michaelis, S. (1998) Methods Enzymol. 292, 193–212
30. Schmidt, W. K., Tam, A., and Michaelis, S. (2000) J. Biol. Chem. 275, 6227–6233
31. Marcus, S., Caldwell, G. A., Miller, D., Xue, C.-B., Naider, F., and Becker, J. M. (1991) Mol. Cell. Biol. 11, 3603–3612
32. Hollandier, I., Frommer, E., and Mallon, R. (2000) Anal. Biochem. 286, 129–137
33. Rost, B., Fariselli, P., and Casadio, R. (1996) Prot. Sci. 5, 1704–1718
34. Tusnady, G. E., and Simon, I. (1998) J. Mol. Biol. 283, 489–506
35. Krogh, A., Larsson, B., von Heijne, G., and Sonnhammer, E. L. (2001) J. Mol. Biol. 305, 567–580
36. Hofmann, K., and Stoffel, W. (1993) Biol. Chem. Hoppe-Seyler 374, 166
37. von Heijne, G. (1992) J. Mol. Biol. 225, 487–494
38. Claros, M. G., and von Heijne, G. (1994) Comput. Appl. Biosci. 10, 685–686
39. Trueblood, C. E., Boyarzchuk, V. L., Picologlou, E. A., Rozema, D., Poulter, C. D., and Rine, J. (2000) Mol. Cell. Biol. 20, 4381–4392
40. Bergo, M. O., Gavino, B., Ross, J., Schmidt, W. K., Hong, C., Kendall, L. V., Mohr, A., Meta, M., Genant, H., Jiang, Y., Wisner, E. R., Van Bruggen, N., Carano, R. A., Michaelis, S., Grifye, S. M., and Young, S. G. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 13049–13054
41. Pendas, A. M., Zhou, Z., Cadinanos, J., Freije, J. M., Wang, J., Hultenby, K., Astudillo, A., Wernerson, A., Rodriguez, F., Tryggvason, K., and Lopez-Otin, C. (2002) Nat. Genet. 31, 94–99
42. Bergo, M. O., Lieu, H. D., Gavino, B. J., Ambroziak, P., Otto, J. C., Casey, P. J., Walker, Q. M., and Young, S. G. (2004) J. Biol. Chem. 279, 4729–4736
43. Corrigan, D. P., Kunzczak, D., Ruzinol, A. E., Thewke, D. P., Hercyna, C. A., Michaelis, S., and Sinensky, M. S. (2005) Biochem. J. 387, 129–138
44. Reid, T. S., Terry, K. L., Casey, P. J., and Rees, L. S. (2004) J. Mol. Biol. 343, 417–433
45. Le Moust, H., Roques, B. P., Crine, P., and Boileau, G. (1993) FEBS Lett. 324, 196–200
46. Vazeux, G., Wang, J., Corvol, P., and Llorens-Cortes, C. (1996) J. Biol. Chem. 271, 9069–9074
47. Bzymek, K. P., and Holz, R. C. (2004) J. Biol. Chem. 279, 31018–31025
48. Fujinaga, M., Cherney, M. M., Oyama, H., Oda, K., and James, M. N. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 3364–3369
49. Powers, S., Michaelis, S., Broek, D., Santa Anna, S., Field, J., Herskowitz, I., and Wigler, M. (1986) Cell 47, 413–422