Published in final edited form as:
J Biol Chem. 2006 February 24; 281(8): 4596–4605. doi:10.1074/jbc.M506284200.

Mutational Analysis of the Ras Converting Enzyme Reveals a Requirement for Glutamate and Histidine Residues

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Abstract

The Ras converting enzyme (RCE) promotes a proteolytic activity that is required for the maturation of Ras, the yeast a-factor mating pheromone, and certain other proteins whose precursors bear a C-terminal C*AAX* tetrapeptide motif. Despite the physiological importance of RCE, the enzymatic mechanism of this protease remains undefined. In this study, we have evaluated the substrate specificity of RCE orthologs from yeast (Rce1p), worm, plant, and human and have determined the importance of conserved residues toward enzymatic activity. Our findings indicate that RCE orthologs have conserved substrate specificity, cleaving CVIA, CTLM, and certain other C*AAX* motifs, but not the CASQ motif, when these motifs are placed in the context of the yeast a-factor precursor. Our mutational studies of residues conserved between the orthologs indicate that an alanine substitution at His^{194} completely inactivates yeast Rce1p enzymatic activity, whereas a substitution at Glu^{156} or His^{248} results in marginal activity. We have also determined that residues Glu^{157}, Tyr^{160}, Phe^{190}, and Asn^{252} impact the substrate selectivity of Rce1p. Computational methods predict that residues influencing Rce1p function are all near or within hydrophobic segments. Combined, our data indicate that yeast Rce1p function requires residues that are invariably conserved among an extended family of prokaryotic and eukaryotic enzymes and that these residues are likely to lie within or immediately adjacent to the transmembrane segments of this membrane-localized enzyme.

The Ras converting enzyme (RCE)[2] is required for the maturation of Ras and certain other lipid-modified proteins, specifically those having a C-terminal tetrapeptide C*AAX* motif (C, cysteine; *A*, aliphatic; and *X*, one of several amino acids) (1,2). Proteins bearing a C*AAX* motif (C*AAX* proteins) typically undergo three ordered post-translational modifications: thioether attachment of an isoprenoid lipid (farnesyl or geranylgeranyl) to the cysteine, proteolytic removal of the *AAX* 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 C*AAX* proteins have in cellular transformation (*e.g.* Ras and RhoB), agents that inhibit the maturation and activity of C*AAX* 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 C*AAX* proteins, which apparently moderates tumor growth (5,6). Inhibition of the proteolytic step in C*AAX* protein modification may have similar anti-cancer potential (4,7).

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[2]The abbreviations used are: RCE, Ras converting enzyme; TPCK, *N*-tosylphenylalanylchloromethyl ketone; HA, hemagglutinin.

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 hydropathy 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 pheromone (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 C*AAX* proteins, including Ras and lamin B1 (1,7,13). Overexpression of RCE results in increased C*AAX* 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 reportedly inhibited by a non-hydrolyzable substrate mimetic, 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 C*AAX* protease that has a partially overlapping role with RCE, the presenilins that are involved in Aβ 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 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.

[3]L. J. Plummer, E. R. Hildebrandt, S. B. Porter, V. A. Rogers, J. McCracken, and W. K. Schmidt, unpublished observation.

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 C*AAX* protease specificity, a strain was created that lacked the **a**-factor encoding genes (*i.e. MFA1* and *MFA2*) and the C*AAX* protease genes (*i.e. RCE1* and *STE24*). This strain (yWS164; *MAT***a** *trp1 leu2 ura3 his4 can1 mfa1-Δ1 mfa2-Δ1 rce1::TRP1 ste24::Kan*^{R}) was derived from SM3689 (*MAT***a** *trp1 leu2 ura3 his4 can1 mfa1-Δ1 mfa2-Δ1 rce1::TRP1*), which has previously been described (22). The knock-out of the *STE24* gene in SM3689 was facilitated by the use of a DNA fragment that was derived from pWS405 (*CEN URA3 ste24::Kan*^{R}), which was created by replacing the *STE24* open reading frame in pSM1093 (*CEN URA3 STE24*) with a *Kan*^{R} cassette (23,24). Disruption of *STE24* was confirmed in G418-resistant clones by PCR of the *STE24* locus and by validating the absence of C*AAX* protease activity, as measured by a lack of pheromone 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 multicopy **a**-factor encoding plasmids have been described previously (12). The C*AAX* protease encoding plasmids are based on pSM1107 (*CEN URA3 HA::STE24*), pSM1314 (*CEN URA3 RCE1::HA*), pJK90 (*2μ URA3* P_{TPI}*-OST4-HA-SUC2-HIS4c*), and pWS479 (*2μ URA3* P_{PGK}*-RCE1::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* P_{PGK}*-His::HA::HsRce1Δ22*) was created by replacing the *STE24* sequence in pSM1282 (*2μ URA3* P_{PGK}*-His::HA::STE24*) with that of *HsRce1Δ22* 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 (*MAT***a** *trp1 leu2 ura3 his4 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 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 (*2μ URA3* P_{PGK}*-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-His4C* reporter were created by recombinational 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 C*AAX* protease orthologs, mating tests were performed either by spotting cultures of washed *MAT***a** cells onto a lawn of *MAT*α cells or by carrying out the serial dilution mating assay. For both approaches, a yeast strain lacking the **a**-factor and C*AAX* protease genes (yWS164) was co-transformed with plasmids encoding an Rce1p or Ste24p ortholog and the indicated **a**-factor C*AAX* 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 H_2O, and spotted (μl spots) onto a lawn of *MAT*α (SM1068) cells that had previously been spread on a minimal agar plate. The *MAT*α cells were prepared as a suspension of cells in YPD (yeast extract, peptone, and dextrose), and 300 μl of this suspension was spread on the minimal plate. The small amount of YPD allows the auxotrophic *MAT***a** and *MAT*α 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, *MAT***a** cultures were grown for 24 h in selective media along with a *MAT*α culture that was grown in YPD. The cultures were all normalized to 1 A_{600} by dilution with appropriate growth media. The *MAT*α cell suspension was loaded into the wells of a 96-well plate (90 μl/well), which was followed by the addition of each *MAT***a** cell suspension to individual wells in the first row of the multiwell 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 multiwell plate. The above steps were repeated for the remaining rows so as to create a 10-fold serial dilution of each *MAT***a** 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 *MAT***a** cells were present for each mutant at each dilution; these plates allow the selective growth of *MAT***a** haploid cells and any diploid cells that may have formed during the processing of the samples.

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 (YIIKGVFWDPAC[farnesyl]VIA) (17). In brief, the assay involves the mixing of the farnesylated substrate with membranes derived from yeast overexpressing 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 2× 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 C*AAX* 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_LIM) with membranes derived from yeast expressing Rce1p; Q_L is lysine ϵ-dinitrophenyl. The components were assembled in a 96-well plate suitable for use in a microtiter 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 chemi-luminescence 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.

Computational Methods

The sequence of *S. cerevisiae* Rce1p was analyzed by five different hydrophobic segment and membrane topology prediction methods: PHDhtm (33), HMMTOP 2.1 (34), THHMM 2.0 (35), TMPred (36), and TopPred 1.0 (37,38).

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 C*AAX* motif. In this study, we have evaluated the ability of the C*AAX* proteases from budding yeast (*S. cerevisiae*), plant (*Arabidopsis thaliana*), worm (*Caenorhabditis elegans*), and humans to cleave three distinct C*AAX* motifs that have been proposed to be nonspecific (CVIA), RCE-specific (CTLM), or

STE24-specific (CASQ) (Fig. 1) (39). Our analysis, using **a**-factor C*AAX* 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. 1*A*). Overexpressed RCE orthologs display the same substrate selectivity, although overexpressed yeast Rce1p appears to have marginal activity toward CASQ (Fig. 1*C*). 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. 1*B*). 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. 1*D*). Overall, the selectivity of STE24 orthologs is consistent with CVIA and CASQ being preferred motifs.

These analyses were extended to seven additional C*AAX* 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 C*AAX* 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. 4*A*).

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^{156}, His^{194}, and His^{248}). Four additional residues (Glu^{157}, Tyr^{160}, Phe^{190}, and Asn^{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.[3] Glu^{156}, His^{194}, and His^{248} have been reported to be required for Rce1p activity (14,39), whereas unpublished observations have been made regarding the importance of Glu^{157} and Asn^{252}.[4] 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. 4*C*). 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. 4*B*). 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. 4*C*). 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 C*AAX* 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 *MAT*α 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. 5*A* 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. 5*B* 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. 5*C* 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 C*AAX* 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 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 C*AAX* 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 C*AAX* variants (CVIA, CTLM, or CASQ) in a strain lacking both C*AAX* proteases and the two **a**-factor genes (12). The mutants co-expressed with wild-type **a**-factor C*AAX* motif (CVIA) had a mating profile similar to that previously observed (compare Figs. 4*A* and 6*A*). 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. 6*B*). 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.[3]

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. 7*A* and Table 5). For this study, an HA/Suc2/His4c reporter was fused 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. 7*B*). 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. 7*B*). Longer truncations removed category II–IV residues (*e.g.* His^{248} and His^{194}), and not surprisingly, these truncations were inactive as judged by mating tests (Fig. 7*B*).[3] 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.[3]

DISCUSSION

In this study, we have addressed two main issues regarding the enzymology of the C*AAX* 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, CTLM, CTVM, CSVM, and CTSM C*AAX* proteins are likely to be RCE substrates, whereas CASQ C*AAX* proteins are likely to be STE24 substrates.

The unique specificities of RCE and STE24 enzymes suggest that these enzymes have distinct cellular functions. Indeed, mouse knock-out experiments reveal that RCE is required for proper embryonic and heart development, whereas STE24 is required for proper bone and musculature development (7,40–42). The identification of RCE-specific substrates, in particular, is likely a prerequisite for the future development of RCE inhibitors as cancer therapeutics. Although several RCE-specific substrates such as Ras2p have been identified, STE24-specific substrates have remained elusive. Only the yeast **a**-factor precursor (CVIA) and mammalian prelamin A (CSIM) have been confirmed as substrates for STE24 enzymes, with the former also being a substrate of RCE (40,41,43). Despite the fact that prelamin A has a C*AAX* motif similar to those that are Rce1p-specific, both its C*AAX* cleavage and a second cleavage associated with lamin A biosynthesis require STE24.

Our observations regarding the conserved substrate specificities of the C*AAX* proteases suggest that bioinformatic approaches may eventually be useful for cataloging the substrate targets of RCE and STE24. However, our studies indicate that some of the specificity predictions made for C*AAX* proteases and farnesyltransferases are not valid (39,44). For instance, we observed that CTSM was Rce1p-specific and that CTSQ was a poor C*AAX* motif despite predictions that both should be recognized by Rce1p and Ste24p. In the case of CTSQ, we cannot distinguish between farnesylation and/or proteolytic defects. Furthermore, our mating assay does not allow us to directly compare *in vivo* farnesylation levels of the different C*AAX* motifs, so we cannot rule out the possibility that our results are affected by differences in farnesylation. Clearly, the precise determinants of C*AAX* protease specificity require further study.

The second major finding of this study is the identification of seven residues that are important for the enzymatic activity of Rce1p (Fig. 4 and Table 3). Using site-directed mutational analysis, we determined that Glu^{156}, His^{194}, and His^{248} (category III and IV residues) are most critical for Rce1p activity, whereas Glu^{157}, Tyr^{160}, Phe^{190}, and Asn^{252} (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.* Glu^{156}, His^{194}, and His^{248}) of RCE, and certain category II residues that are required for optimal activity (*i.e.* Glu^{157}, Tyr^{160}, and Phe^{190}), are also highly conserved in a group of prokaryotic and eukaryotic 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 Glu^{157} 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 Glu^{157} mutant is unstable and undergoes inactivating structural changes upon extraction from yeast. Third, it is possible that Glu^{157} 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 metalloproteases, 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 (Cys^{251}) 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 Cys^{251} 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-tosylamido-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 farnesylaldehyde analogs are covalent modifiers of RCE, and if so, to which residue (s) they bind, will help resolve their mechanism of inhibition and ultimately clarify whether Rce1p utilizes 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 His^{194} 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.

This work was supported in part through a Georgia Cancer Coalition Distinguished Cancer Clinician/Scientist Scholar award (to W. K. S.).

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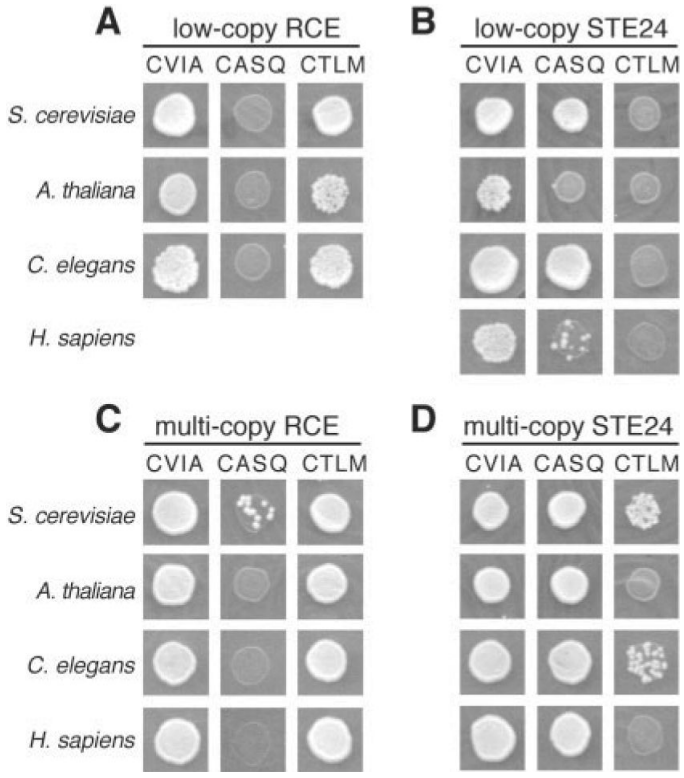

FIGURE 1. The partial overlapping substrate specificity of C*AAX* proteases is conserved

A *MAT***a** strain (yWS164) lacking the genes encoding **a**-factor (*MFA1* and *MFA2*) and the C*AAX* proteases (*RCE1* and *STE24*) was co-transformed with plasmids encoding the indicated *MFA1* C*AAX* variant and an RCE (*A* and *C*) or STE24 ortholog (*B* and *D*). The co-transformed strains were evaluated for the ability to mate with a *MAT*α strain (SM1068). The mating test was performed by spotting a volume of a *MAT***a** culture onto a lawn of *MAT*α cells that had been spread on minimal solid media. Only the prototrophic diploids that arise from mating events can survive on the selective media, and diploid formation requires C*AAX* protease-dependent production of **a**-factor by the *MAT***a** cells. Thus, growth indicates that the ortholog recognizes the respective **a**-factor C*AAX* variant as a substrate. The orthologs were expressed from either low copy (*CEN*, *A* and *B*) or multicopy (2μ, *C* and *D*) plasmids. The RCE and STE24 orthologs were encoded behind the native yeast *RCE1* or *STE24* promoters, respectively, with the exception of human Rce1, which was encoded behind the phosphoglycerate kinase promoter (*i.e.* 2μ P_{PGK}).

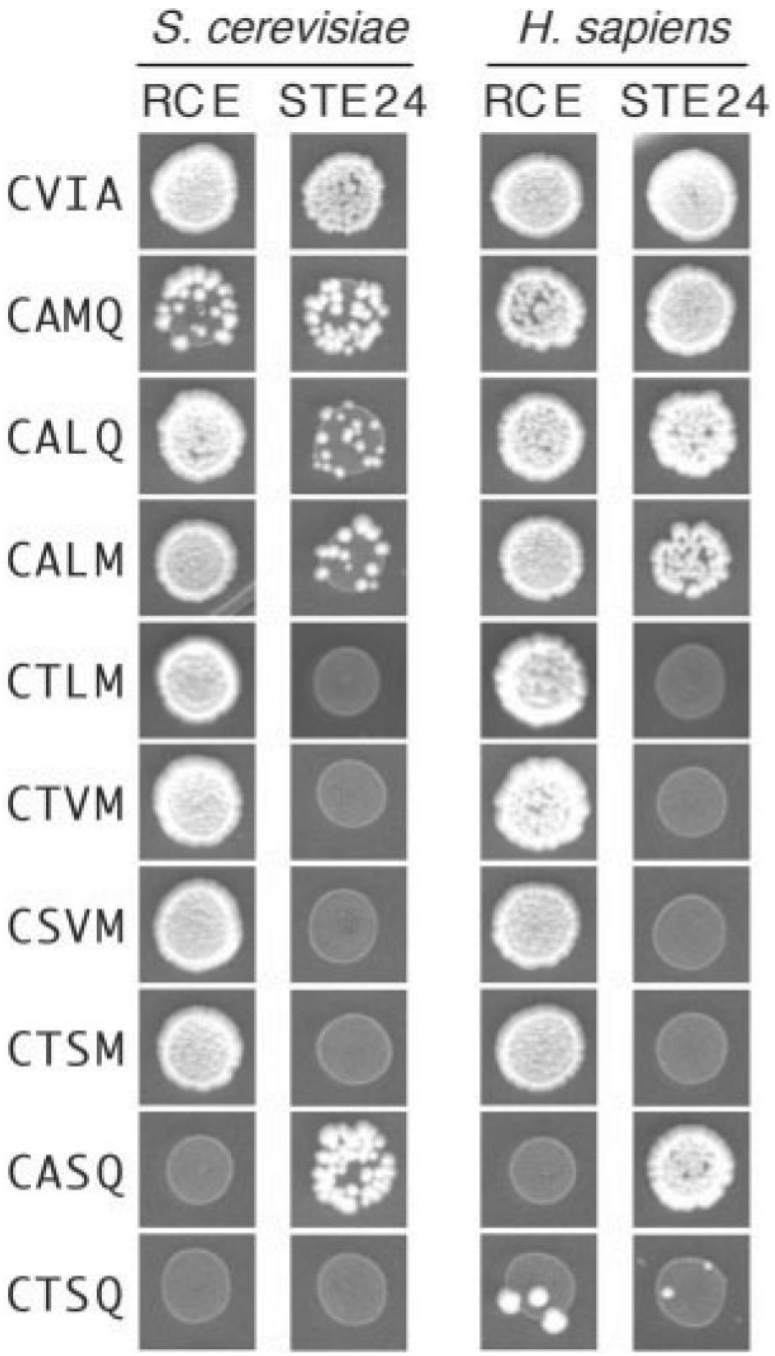

FIGURE 2. Further evidence for the conservation of C*AAX* protease specificity across species

Additional *MFA1* C*AAX* variants were evaluated as in Fig. 1. Yeast (*S. cerevisiae*) *RCE1* and *STE24* were expressed from low copy plasmids, whereas human (*H. sapiens*) *RCE* and *STE24* were expressed from multicopy plasmids. The co-transformed strains were evaluated for the ability to mate with a *MAT*α strain (SM1068) using the serial dilution mating test. Only the first dilution spot is shown.

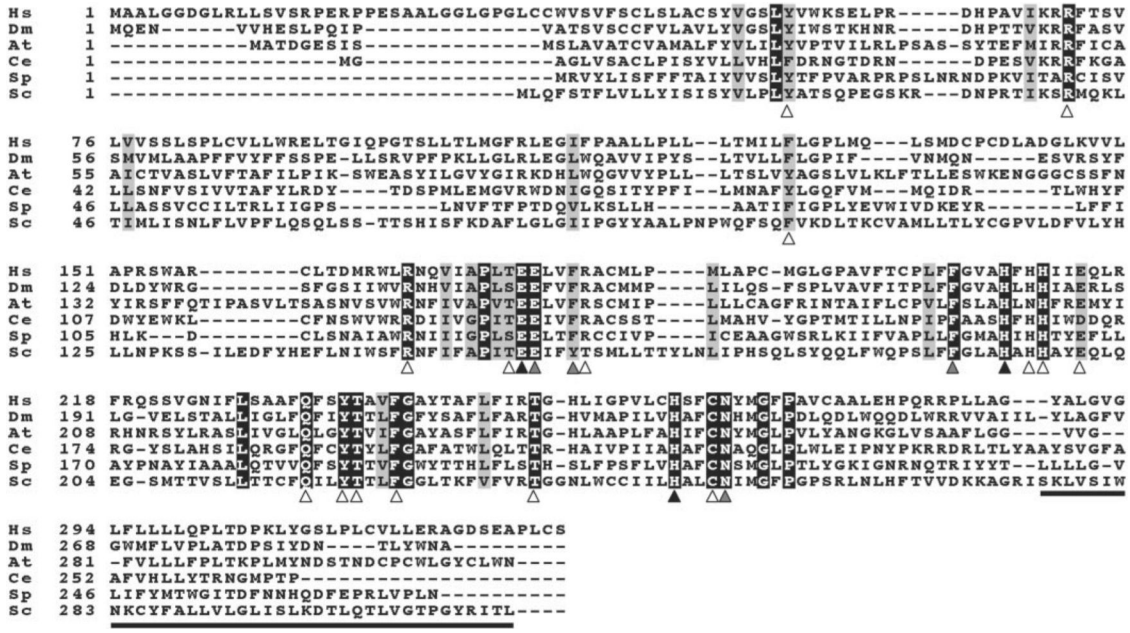

FIGURE 3. ClustalW alignment of Rce1p orthologs

The 21 invariable residues are highlighted in *black*. The 16 highly conserved residues are highlighted in *gray*. Residues targeted for site-directed mutation are marked with a *triangle*. Mutations that inactivated, reduced, or had no effect on activity as judged by pheromone production tests are marked with *black*, *gray*, or *unfilled triangles*, respectively (see Figs. 4 and 5). The underscored C-terminal region can be deleted without inactivating yeast Rce1p (see Fig. 7).

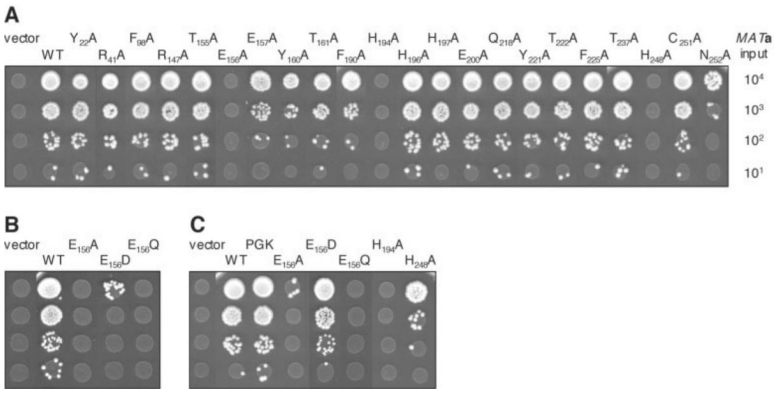

FIGURE 4. Effect of site-directed mutations on Rce1p activity

The indicated invariable and highly conserved residues of Rce1p were targeted for site-directed mutation to alanine, or other amino acids as indicated. Low copy (*A* and *B*) or multicopy (*C*) plasmids encoding the indicated Rce1p mutants or a vector only control (pRS316) were transformed into yeast lacking C*AAX* proteases (SM3614; *rce1*Δ *ste24*Δ), and the ability of the mutants to promote **a**-factor production was determined as judged by serial dilution mating tests. The approximate number of *MAT***a** cells mixed with a constant number of *MAT*α cells is indicated to the *right* of *panel A* and is the same for *panels B* and *C*. Growth of prototrophic diploids is indicative of mating, which is directly dependent upon the activity of Rce1p in this assay. No differences in mating ability relative to wild-type Rce1p were observed for the majority of mutants. *A*, Rce1p E156A, H194A, and H248A mutants were incapable of promoting mating when encoded on a low copy plasmid behind the native yeast *RCE1* promoter, whereas E157A, Y160A, F190A, and N252A mutants were observed to have reduced mating ability. *B*, Rce1p E156D has reduced mating ability while the E156Q mutant was unable to promote mating. *C*, marginal mating activity was observed for Rce1p E156A when it was encoded on a multicopy plasmid behind the phosphoglycerate kinase promoter (*PGK*), whereas significantly increased activity was observed for Rce1p E156D and H248A.

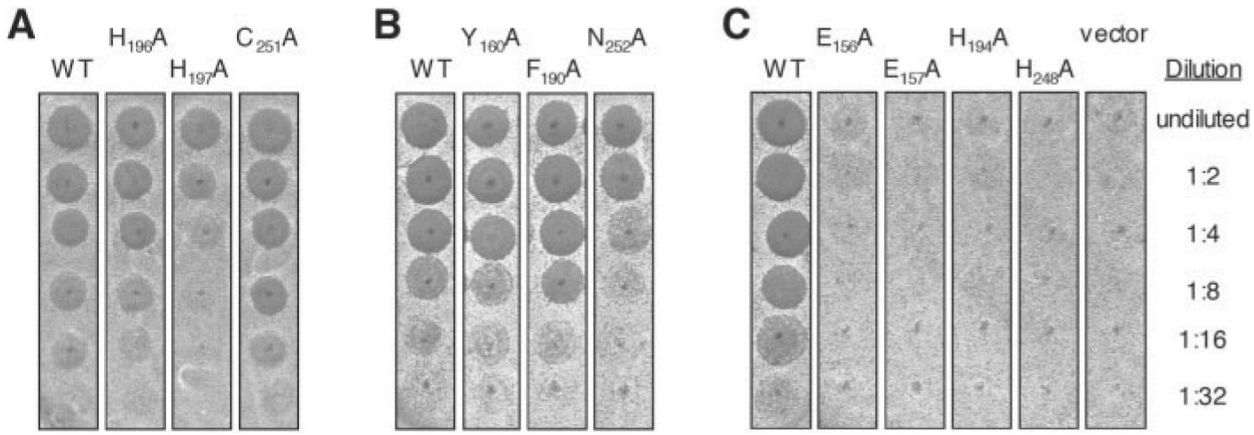

FIGURE 5. Activity of Rce1p mutants as determined by an *in vitro* assay for a-factor production

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 *MAT*α (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 *2*μ P_{PGK}-based *RCE1* expression vectors. Samples were spotted as 2-fold serial dilutions (*top* to *bottom*), with the *top spot* representing undiluted sample.

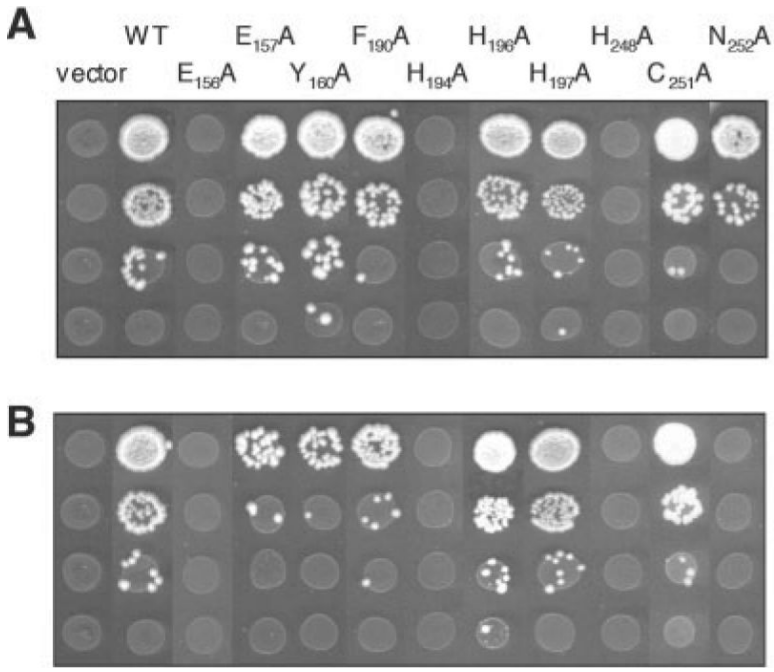

FIGURE 6. Effect of site-directed mutations on the substrate specificity of Rce1p

The Rce1p mutants listed in Table 3 were co-expressed with one of three **a**-factor C*AAX* variants in yWS164 and evaluated for the ability to promote mating as described in Fig. 4; the negative control strain contained pRS316 (*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 (2μ-based), and the Rce1p mutants were encoded behind the native *RCE1* promoter on a low copy vector.

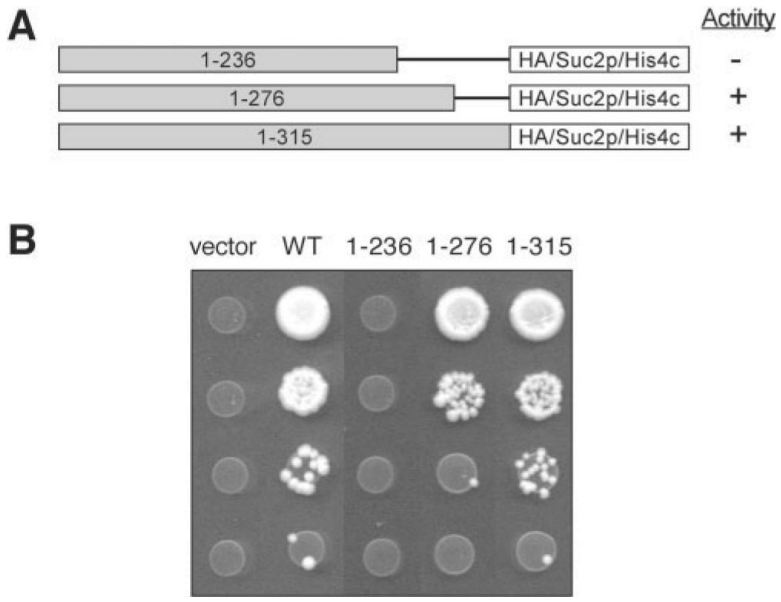

FIGURE 7. The C terminus of yeast Rce1p is not essential for activity

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 C*AAX* proteases (SM3614; *rce1*Δ *ste24*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 (*vector*), a low copy vector encoding Rce1p and its natural promoter (*WT*), or multicopy vectors encoding Rce1p fusions behind the constitutive *TPI* promoter. Full-length Rce1p is 315 amino acids.

TABLE 1

Strains used in this study

Strain	Relevant genotype[a]	Reference
RC757	*MAT*α *sst2–1 rme1 his6 met1 can1 cyh2*	(49)
SM1068	*MAT*α *lys1*	(22)
SM3614	*MAT*a *trp1 leu2 ura3 his4 can1 ste24*Δ::*LEU2 rce1*Δ::*TRP1*	(22)
STY50	*MAT*α *leu2 ura3 his4 trp1 suc2*::*LEU2*	(25)
yWS164	*MAT*a *trp1 leu2 ura3 his4 can1 mfa1-*Δ*1 mfa2-*Δ*1 rce1*:: *TRP1 ste24*::*Kan*^{R}	(12)

[a]The *MAT*a strains used in this study are isogenic to SM1058 (*MAT*a *trp1 leu2 ura3 his4 can1*) (21).

TABLE 2

Plasmids used in this study

Plasmid	Genotype	Reference
pJK90	*2μ URA3* P_{TPI}*-OST4-HA-SUC2-HIS4c*	(25)
pRS316	*CEN URA3*	(26)
pRS426	*2μ URA3*	(26)
pSM703	*CEN URA3* P_{PGK}	(28)
pSM1107	*CEN URA3 HA* :: *STE24*	(23)
pSM1282	*2μ URA3* P_{PGK}*-His::HA::STE24*	(17)
pSM1314	*CEN URA3 RCE1* :: *HA*	(8)
pWS123	*2μ URA3* P_{PGK}*-RCE1::His*	This study
pWS164	*2μ URA3 HA::STE24*	This study
pWS182	*2μ URA3 HA::HsZmpSte24*	This study
pWS183	*CEN URA3 HA* :: *HsZmpSte24*	This study
pWS335	*2μ URA3* P_{PGK}*-His::HA::HsRce1Δ22*	This study
pWS360	*CEN URA3 CeRCE1* :: *HA*	(11)
pWS364	*CEN URA3 CeSTE24* :: *HA*	(11)
pWS398	*CEN URA3 RCE1::HA* (C251A)	This study
pWS400	*CEN URA3 AtRCE1* :: *HA*	(12)
pWS404	*2μ URA3 RCE1::HA*	(8)
pWS405	*CEN URA3 ste24* :: Kan^R	(12)
pWS413	*CEN URA3 AtSTE24* :: *HA*	(12)
pWS438	*2μ LEU2 MFA1*	(12)
pWS440	*2μ LEU2 MFA1-CASQ*	(12)
pWS441	*2μ LEU2 MFA1-CTLM*	(12)
pWS444	*2μ URA3 CeRCE1::HA*	This study
pWS445	*2μ URA3 CeSTE24::HA*	This study
pWS448	*2μ URA3 AtRCE1::HA*	(12)
pWS450	*2μ URA3 AtSTE24::HA*	(12)
pWS454	*CEN URA3 RCE1::HA* (E156A)	This study
pWS455	*CEN URA3 RCE1::HA* (H197A)	This study
pWS456	*CEN URA3 RCE1::HA* (H196A)	This study
pWS457	*CEN URA3 RCE1::HA* (H194A)	This study
pWS458	*CEN URA3 RCE1::HA* (H248A)	This study
pWS459	*CEN URA3 RCE1::HA* (N252A)	This study
pWS479	*2μ URA3* P_{PGK}*-RCE1::HA*	This study
pWS480	*2μ URA3* P_{PGK}*-RCE1::HA* (C251A)	This study
pWS481	*2μ URA3* P_{PGK}*-RCE1::HA* (N252A)	This study
pWS487	*2μ URA3* P_{PGK}*-RCE1::HA* (H197A)	This study
pWS488	*2μ URA3* P_{PGK}*-RCE1::HA* (H196A)	This study
pWS492	*CEN URA3 RCE1::HA* (Y22A)	This study
pWS493	*CEN URA3 RCE1::HA* (R147A)	This study

Plasmid	Genotype	Reference
pWS494	*CEN URA3 RCE1::HA* (F190A)	This study
pWS495	*CEN URA3 RCE1::HA* (E200A)	This study
pWS497	*CEN URA3 RCE1::HA* (T155A)	This study
pWS498	*CEN URA3 RCE1::HA* (Y221A)	This study
pWS505	*2μ URA3* P_{PTI}*-RCE1(1–708)-HA-SUC2-HIS4c*	This study
pWS506	*2μ URA3* P_{PT}*I-RCE1(1–828)-HA-SUC2-HIS4c*	This study
pWS507	*2μ URA3* P_{PTI}*-RCE1(1–915)-HA-SUC2-HIS4c*	This study
pWS508	*CEN URA3 RCE1::HA* (F98A)	This study
pWS509	*CEN URA3 RCE1::HA* (T237A)	This study
pWS510	*CEN URA3 RCE1::HA* (T222A)	This study
pWS526	*CEN URA3 RCE1::HA* (R41A)	This study
pWS537	*CEN URA3 RCE1::HA* (T161A)	This study
pWS559	*2μ URA3* P_{PGK}*-RCE1::HA* (E156A)	This study
pWS561	*2μ URA3* P_{PGK}*-RCE1::HA* (H194A)	This study
pWS563	*CEN URA3 RCE1::HA* (F225A)	This study
pWS564	*CEN URA3 RCE1::HA* (Q218A)	This study
pWS565	*CEN URA3 RCE1::HA* (Y160A)	This study
pWS579	*2μ URA3* P_{PGK}*-RCE1::HA* (H248A)	This study
pWS580	*2μ URA3* P_{PGK}*-RCE1::HA* (F190A)	This study
pWS581	*2μ URA3* P_{PGK}*-RCE1::HA* (Y160A)	This study
pWS616	*CEN URA3 RCE1::HA* (E157A)	This study
pWS621	*2μ URA3* P_{PGK}*-RCE1::HA* (E157A)	This study
pWS651	*CEN URA3 RCE1::HA* (E156Q)	This study
pWS652	*2μ LEU2 MFA1-CTSQ*	This study
pWS653	*2μ LEU2 MFA1-CTSM*	This study
pWS654	*2μ LEU2 MFA1-CALQ*	This study
pWS655	*2μ LEU2 MFA1-CALM*	This study
pWS662	*CEN URA3 RCE1::HA* (E156D)	This study
pWS666	*2μ URA3* P_{PGK}*-RCE1::HA* (E156Q)	This study
pWS667	*2μ URA3* P_{PGK}*-RCE1::HA* (E156D)	This study

TABLE 3

In vitro activity of Rce1p mutants relative to wild-type activity

Category	Enzyme	Mating assay[a]	Halo assay	Fluorescence assay
I	WT	100	100	100
	H196A	100	72	34
	H197A	100	30	53
	C251A	100	30	107
II	E157A	10	$<10^{-3}$	<0.5
	Y160A	10	2.6	<0.5
	F190A	10	3.1	<0.5
	N252A	1	0.9	<0.5
III	E156A	<0.1	$<10^{-3}$	<0.5
	H248A	<0.1	$<10^{-3}$	<0.5
IV	H194A	<0.1	$<10^{-3}$	<0.5

[a]Percent activities reported are derived from the data presented in Fig. 4*A* (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.

TABLE 4

Kinetic analysis of category I Rce1p mutants using the Ras-based peptide in the fluorescence assay

Enzyme	K_m
	μ M
WT	1.72+/−0.67
H196A	3.26+/−0.43
H197A	1.44+/−0.21
C251A	2.34+/−0.58

TABLE 5

Predicted transmembrane segments of yeast Rce1p

Numbering reflects the primary amino acid sequence of yeast Rce1p —, a transmembrane segment was not predicted for this region.

Span	TM pred	TCDB	Top pred	TMHMM	Predict protein
1	1–22	7–25	1–23	4–23	5–22
2	44–64	45–63	44–64	44–61	44–61
3	70–90	75–93	74–94	76–93	—
4	102–123	105–123	157–177	105–127	104–121
5	149–173	158–176	157–177	—	150–168
6	177–196	—	—	—	177–194
7	212–229	207–227	206–226	208–230	212–229
8	232–256	241–259	240–260	237–259	234–251
9	282–305	279–299	278–298	279–298	281–298