Studies with Recombinant *Saccharomyces cerevisiae* CaaX Prenyl Protease Rce1p†

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ABSTRACT: Eukaryotic proteins with carboxyl-terminal CaaX motifs undergo three post-translational processing reactions—protein prenylation, endoproteolysis, and carboxymethylation. Two genes in yeast encoding CaaX endoproteases, *AFC1* and *RCE1*, have been identified. Rce1p is solely responsible for proteolysis of yeast Ras proteins. When proteolysis is blocked, plasma membrane localization of Ras2p is impaired. The mislocalization of undermodified Ras in the cell suggests that Rce1p is an attractive target for cancer therapeutics. Homologous expression of plasmid-encoded *Saccharomyces cerevisiae RCE1* under the control of the *GAL1* promoter gave a 370-fold increase in endoprotease activity over an uninduced control. Yeast Rce1p was detected by Western blotting with a yRce1p antibody or with an anti-myc antibody to Rce1p bearing a C-terminal myc-epitope. Membrane preparations were examined for their sensitivity to a variety of protease inhibitors, metal ion chelators, and heavy metals. The enzyme was sensitive to cysteine protease inhibitors, Zn^{2+}, and Ni^{2+}. The substrate selectivity of yRce1p was determined for a variety of prenylated CaaX peptides including farnesylated and geranylgeranylated forms of human Ha-Ras, Ki-Ras, N-Ras, and yeast Ras2p, **a**-mating factor, and Rho2p. Six site-directed mutants of conserved polar and ionic amino acids in yRce1p were prepared. Four of the mutants, H194A, E156A, C251A, and H248A, were inactive. Results from the protease inhibition studies and the site-directed mutagenesis suggest that Rce1p is a cysteine protease.

The biological activity of a variety of eukaryotic proteins depends on the post-translational modification with a prenyl group (*1*−*3*). One family of modified proteins contains a C-terminal CaaX motif, in which C is cysteine, a is normally a small aliphatic amino acid, and X determines whether a C_{15} farnesyl or a C_{20} geranylgeranyl group is added to the cysteine. Proteins in the Rab family with C-terminal −CC or −CXC sequences are modified at both cysteine residues by geranylgeranyl groups. For many prenylated CaaX proteins, two additional processing steps occur. The −aaX tripeptide is removed by an endoproteolytic CaaX prenyl protease, and the new C-terminal isoprenylated cysteine is carboxylmethylated by a methyltransferase.

Prenylated CaaX proteins include Ras and Rho subfamilies of the Ras superfamily of small GTPases, fungal pheromones, nuclear lamins, a variety of enzymes, a hepatitis delta coat protein, and GTP-binding proteins (*1*−*3*). Ras proteins, in particular, have attracted a great deal of attention. Farnesylation is required for Ras proteins to localize to the intracellular surface of the plasma membrane where they participate in the signal transduction network for cell division (*4*−*6*). Unmodified Ras is cytosolic, and farnesylation is necessary to target the protein to membranes.

Oncogenic forms of ras have been implicated in approximately 30% of human cancers, including more than 90% of pancreatic cancers and 50% of lung cancers. Inhibition of farnesylation of Ras proteins reverses their oncogenic phenotypes. A major effort is currently underway to discover PFTase[1] inhibitors that are effective against Ras-related cancers (*7*), and PFTase inhibitors have impeded tumorgenesis in animal models with no detectable side effects (*8*). Although PFTase inhibitors block farnesylation, some Ras proteins are also substrates for PGGTase I, and the geranylgeranylated oncogenic forms are active. In addition, it has not been established that the inhibition of Ras farnesylation is required for the drugs' antitumor effects. Recent studies indicate that PFTase inhibitors alter the ratio of farnesylated and geranylgeranylated RhoB proteins (*9*) and that an increase in the level of geranylgeranylated RhoB is associated with a loss of growth-transforming activity.

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[1] Abbreviations: Ac-(far)CCMK, acetyl-*S*-farnesylcysteine chloromethyl ketone; Ac-(far)CDK, acetyl-*S*-farnesylcysteine diazomethyl ketone; Biopep, 1-*N*-biotinyl-(13-*N*-succinimidyl-(*S*-farnesyl-L-cysteinyl)-L-valinyl-L-isoleucinyl-L-alanine))-4,7,10-trioxatridecanediamine; Dan, dansyl; DEPC, diethyl pyrocarbonate; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; E64, *N*-[*N*-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]agmatine; far, farnesyl; FMOC, *N*-(9-fluorenylmethoxycarbonyl); gg, geranylgeranyl; MSA, mersalyl acid; ORF, open reading frame; PFTase, protein farnesyltransferase; PGGTase-I, protein geranylgeranyltransferase type I; MMTS, methyl methanethiosulfonate; PHMB, *p*-hydroxymercuribenzoic acid; PHMS, *p*-hydroxymercuriphenylsulfonic acid; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; RPI, *N*-boc-*S*-farnesyl-L-cysteinyl-ψ-(CH_2-NH)-valyl-L-isoleucyl-L-methonine; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; TLCK, N_α-tosyl-L-lysine chloromethyl ketone; TPCK, N_α-tosyl-L-phenylalanine chloromethyl ketone; TPEN, *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine; wt, wild type; YNB, yeast nitrogen base.

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These results suggest a Ras-independent mechanism for antitransforming activity of PFTase inhibitors (*10*).

In contrast to the widespread study of protein prenyltransferases and their inhibitors, less attention has been devoted to CaaX endoproteases. CaaX prenyl protease activity has been studied in yeast (*11*, *12*), rat (*13*), and bovine (*14*) membrane homogenates. The rat and bovine enzymes have been partially purified (*15*−*17*) to give membrane preparations that cleave prenylated C(farnesyl)a_1a_2 tri- and C(farnesyl)-a_1a_2X tetrapeptides.

Two genes encoding proteins with CaaX prenyl protease activity have been identified in yeast; **a**-factor converting enzyme (*AFC1*) and Ras and **a**-factor converting enzyme (*RCE1*) (*18*). *AFC1*-deficient yeast strains were able to process **a**-factor, although at a slightly reduced rate compared to the wild type. In *RCE1*-deficient strains (*RCE1*Δ), CaaX processing was reduced as well. In addition, in *RCE1*Δ mutants, Ras2p was mislocalized in the cell, and heat-shock sensitivity caused by activated Ras2p was suppressed. Afc1 protein (Afc1p) and Rce1 protein (Rce1p) are polytopic integral membrane proteins that are localized to the endoplasmic reticulum (*19*). Afc1p is a putative zinc-dependent metalloprotease containing a consensus HEXXH motif and is sensitive to metal chelators (*18*). Rce1p has no readily identifiable sequence homology with Afc1p, does not contain a consensus metal binding motif, and is not inactivated by metal chelators. Unlike Rce1p, Afc1p has two proteolytic activities that are important for complete processing of yeast **a**-mating factor (*20*). The enzyme removes the −aaX residue following farnesylation and seven amino acids from the N-terminus of the **a**-factor pro peptide (*21*).

Although the substrate selectivities of Afc1p and Rce1p overlap to some extent, for example, both enzymes process the CaaX motif of yeast **a**-mating factor, in vivo studies indicate that yeast Rce1p is responsible for processing yeast Ras proteins (*18*). Interestingly, null mutations of *RCE1* did not seem to affect cell viability. Recently, studies with *RCE1*-deficient mice indicated that blocking endoproteolytic processing and carboxymethylation caused a mislocalization of Ras proteins to the cytosol (*22*). The results from the yeast and mouse studies suggest that selective inhibitors of Rce1p are attractive targets for the potential development of cancer therapeutics because inhibition of CaaX prenyl proteases might show activity against oncogenic forms of Ki4B-Ras that are geranylgeranylated in a compensatory manner upon inhibition of PFTase (*23*). Recently, the gene for a human CaaX protease (*hRCE1*) was identified (*24*), and the yeast and human genes were produced in S*f9* insect cells by infection with a recombinant baculovirus. We now describe construction of a yeast strain for expression of recombinant yRce1p that gave a 10-fold higher level of specific activity compared to previous systems as well as studies of recombinant yRce1p and related site-directed mutants.

MATERIALS AND METHODS

Enzymes for molecular biology were purchased from New England Biolabs or Gibco-BRL. KlenTaq polymerase was from ClonTech. Oligonucleotide primers were synthesized by Bob Schackmann, Huntsman Cancer Institute, DNA/Peptide Core Facility. DNA sequencing was performed by the Sequencing Services Laboratory (University of Utah). Avidin resin was purchased from Pierce. All chemicals were purchased from Aldrich or Sigma unless otherwise noted. Standard molecular biology procedures and *E. coli* transformations were carried out as described by Sambrook et al. (*25*). Large-scale plasmid preparations (>100 μg) were performed with the purification kit from Qiagen. PCR products and DNA fragments were purified on agarose gels with a Geneclean II kit (Bio 101). The yeast shuttle plasmid pYE-R was obtained from J. Rine (*18*). The yeast expression plasmid p426GAL1 (*26*) was obtained from ATCC. The plasmid pMev1 and yeast strain ABY53 (*18*) were obtained from Acacia Biosciences. The plasmid pJEL167 and yeast strain BCY123 (*27*) were obtained from Dr. Janet Lindsley (University of Utah). The yRce1p antibody was a gift from Dr. Susan Michaelis (The Johns Hopkins University School of Medicine). *Saccharomyces cerevisiae* yeast transformations were performed using the lithium acetate method (*28*). *Pichia* yeast transformations were done using the electroporation method as described in the *Pichia* expression kit (Invitrogen).

SDS−PAGE was performed in 12% polyacrylamide gels by the method of Laemmli (*29*), followed by staining with Coomassie Brilliant Blue. Protein concentrations were determined by the modified Lowry assay using the Bio-Rad DC Protein Assay kit (*30*). Liquid scintillation spectrometry was performed using CytoScint scintillation cocktail (ICN Laboratories). Dansyl peptides were prepared by standard solid-phase synthesis methods using FMOC chemistry and were prenylated using the procedure of Pompliano et al. (*31*). Concentrations of prenylated dansyl peptides were determined as described by Cassidy et al. (*32*).

DNA Amplification and Cloning

PCR Amplification of yRCE1 and Cloning into pPICZαA. Restriction sites were introduced at both ends of the *yRCE1* gene by PCR. Plasmid pYE-R containing *yRCE1* served as the template for PCR. The sense and antisense primers were (XHORCESEC) 5′-GAAGGGGTATCTCTCGAGAAAA-GAGAGGCTGAAGCT*ATG*CTACAATTCTCAACATTTC-TAGTGC-3′ and (RCENOTMYC) 5′-TATACAAGTACGT-GCGGCCGCAAGGGTTATTCTATACCAGG -3′. It was necessary to reconstruct the end of the α-factor signal sequence between the *Xho*I site (underlined) and the ATG start codon (italicized) in sense primer XHORCESEC. The antisense primer RCENOTMYC contained a *Not*I site (underlined) downstream of the end of the open reading frame to facilitate cloning. The stop codon was deleted to fuse the myc epitope in the vector to *yRCE1*. The PCR product was purified by agarose gel electrophoresis and digested with *Xho*I and *Not*I. The 1 kb DNA fragment for *yRCE1* was cloned into the pPICZαA vector (Invitrogen), and the resulting construct, pPyRcemyc, was sequenced to verify the fidelity of the PCR.

Construction of Plasmids pMev-αyRcemyc and p426Gal-αyRcemyc. Plasmid pPyRcemyc was used as the template to introduce appropriate restriction sites at the ends of *yRCE1* by PCR. DNA primers were (NHEALPHA) 5′-GGCATA-CAGCGCTAGCATGAGATTTCCTTCAATTTTTACTGC-3′ and (ECO1RCE) 5′-CGTCCACTTGGAATTC*TCA*GACG-GCGCTATTCAGATCC-3′. The primer NHEALPHA (upstream) included an *Nhe*I site (underlined) before the start

of the ORF. The downstream primer ECOR1RCE contained an *Eco*RI site (underlined) downstream of the stop codon (italicized) to facilitate cloning. The PCR product was digested with *Nhe*I and *Eco*RI, and the DNA was ligated into pMev1, which had been digested with *Eco*RI and *Xba*I as a *Nhe*I−*Eco*RI cassette to prepare pMev-αyRcemyc. The PCR product was also ligated into the *Spe*I and *Eco*RI restriction sites of p426GAL1 to place *yRCE1* under the control of the GAL1 promoter. The structure of the constructs was verified by sequencing.

Construction of Plasmid pJEL-αyRcemyc. PCR was used to introduce appropriate restriction sites flanking the *yRCE1* ORF for cloning into the pJEL vector. Plasmid pPyRcemyc was the template for PCR to introduce *Bam*HI and either *Xho*I or *Sal*I restriction sites flanking *yRCE1*. DNA primers were (GARCEBAM) 5′-GATATTGCAGGATCCGTAACCATGTCAAGATTTCCTTCAATTTTTACTGC-3′, (GYRCEBAM) 5′-CCAATTGCAGGATCCGTAACCATGTCACTACAATTCTCAACATTTCTAGTGC-3′, (GXHOMYC) 5′-CGTCCACTTGCTCGAG*TCA*GACGGCGCTATTCAGATCC-3′, and (GSALMYC) 5′-CGTCCACTTGGTCGAC*TCA*GACGGCGCTATTCAGATCC-3′. Primers GARCEBAM and GYRCEBAM (upstream) included a *Bam*HI site (underlined) before the start of the ORF. The downstream primers GXHOMYC and GSALMYC contained an *Xho*I site or a *Sal*I site (underlined) downstream of the stop codon (italicized). The PCR products were initially subcloned into pTAdv (ClonTech) to give pTAdv-yRcemyc and pTAdv-αyRcemyc, and the fidelity of the reactions was confirmed by sequencing. The yRcemyc DNA fragment from pTAdv-yRcemyc was excised as a *Bam*HI−*Xho*I fragment and ligated into pJEL167 to give expression construct pJEL-yRcemyc. Similarly, αyRcemyc was excised from pTAdv-αyRcemyc as a *Bam*HI−*Sal*I cassette and cloned into pJEL167 to give pJEL-αyRcemyc.

Disruption of the RCE1 Gene in BCY123. A DNA fragment containing an *rce1::TRP1* disruption was obtained by PCR from ABY53 (*MATa, rce1*Δ*::TRP1, afc1*Δ*::HIS3*). The PCR primers were (RCE1KO) 5′-AGATGCCACCTTCTCTCTAC-3′ and (RCE1TREV) 5′-AGCTGCCTATGTTGTAGGTT-3′. The PCR product was purified using the Wizard PCR purification kit (Promega) and stored at −20 °C. Strain BCY123 (*MATa, pep4::HIS3, prb1::LEU2, bar1::HIS6, can1, ade2, trp1, ura3, his3, leu2-3,112 lys2::PGAL1/10-GAL4, prc1*) was transformed with the PCR product by the lithium acetate method, spread on SC-tryptophan plates, and incubated at 30 °C for 2 days. Individual colonies were restreaked on SC-tryptophan plates. Genomic DNA was isolated from 10 colonies using the Easy DNA kit (Invitrogen), and the *rce1::TRP1* disruption was confirmed by PCR using primers (TRP1REV) 5′-CACGCCAACCAAGTATTTCG-3′ and (MR274P) 5′-TGTTATGGCCGCAGTGGAAATAG-3′. The strain was named JDY101.

Growth of Yeast Strains with Plasmids Containing yRCE1

pMev-αyRcemyc. Single colonies from freshly streaked plates were used to inoculate 3 mL cultures of SC-Ura (Cas) (0.67% YNB, 2% (w/v) Casamino acids, 2% (w/v) glucose), supplemented with adenine (20 mg/L) and tryptophan (20 mg/L) and lacking uracil, which were grown at 30 °C overnight. A portion of these cultures was used to inoculate flasks containing 300 mL of SC-Ura (Cas), which were grown at 30 °C until the $o.d._{600} = 1.0$. Cells were centrifuged at 5000*g*, washed with cold sterile water, and stored at −80 °C.

p426Gal and pJEL Strains. Cultures were grown essentially as described by Worland and Wang (*33*). Single colonies from freshly streaked plates were used to inoculate 10 mL cultures of synthetic minimal media lacking uracil and supplemented with 2% (w/v) glucose to repress PGAL1. Cultures were grown to late log phase. A 3 mL portion was used to inoculate 300 mL of synthetic minimal media lacking uracil and supplemented with 3% (w/v) glycerol and 2% (w/v) lactic acid. The cultures were grown at 30 °C for 20−24 h ($o.d._{600} = 0.5-0.7$). Galactose was added to a final concentration of 2%, and the cells were grown for an additional 6 h. Cells were pelleted by centrifugation at 5000*g* for 15 min, washed with cold sterile water, and stored at −80 °C.

Preparation of Membrane Fractions. Alkaline carbonate-leached membranes (AC-P139) were prepared according to the procedure of Ashby and Rine (*12*), with slight modifications. Briefly, 4 mL of frozen cell paste was thawed on ice and resuspended in 8 mL of SSTϕ buffer (0.3 M sorbitol, 0.1 M NaCl, 10 mM Tris−HCl, pH 7.4, 5 mM EDTA, pH 8, 1 mM PMSF, 0.5 mM 1,10-phenanthroline). Acid-washed glass beads (212−300 μm) were added (8 mL), and the suspension was chilled on ice for 5 min prior to vortexing at maximum setting for 1 min followed by chilling on ice for 1 min. The vortex-chilling cycle was repeated 10 times. The homogenate was clarified by centrifugation at 3500*g* for 15 min at 4 °C. The centrifugation was repeated for the supernatant. The final 3500*g* supernatant was transferred to ultracentrifuge tubes, mixed with 1/10 volume of 2 M sodium carbonate, pH 11.5, and spun at 139000*g* (40 000 rpm, TY65 rotor) for 1 h at 2 °C. The P139 pellet was gently washed twice with cold SSTϕ buffer (8 mL) and resuspended in 8 mL of cold SSTϕ buffer with a Dounce homogenizer. The sample was spun again at 139000*g* for 1 h at 2 °C. The pellet was washed as before and was resuspended in 2 mL of cold SSTϕ buffer. The AC-P139 membrane preparation was divided into portions, quick frozen in dry ice/acetone, and stored at −80 °C.

Protease Assay. Biotin/Avidin-Binding Assay. Assays (50 μL) contained AC-P139 membranes (1−5 μg of protein) and 1-*N*-biotinyl-(13-*N*-succinimidyl-(*S*-farnesyl-L-cysteinyl)-L-valinyl-L-isoleucinyl-[^{14}C]-L-alanine))-4,7,10-trioxatridecanediamine (Biopep) in 100 mM Tris, pH 7.4, 0.5 mM 1,10-phenanthroline, and 1 mM PMSF. Inhibitors were added in either buffer or DMF (2.5 μL). Reactions were initiated by the addition of Biopep in 2.5 μL of DMF. After incubation for 15−30 min at 37 °C, the enzyme was inactivated by heating at 80 °C for 5 min. The incubation mixtures were briefly cooled on ice, avidin resin (150 μL, Pierce) was added, and the material was allowed to stand at room temperature for 15 min with frequent mixing. Assay buffer (150 μL) was added, the tubes were centrifuged for 1 min, and radioactivity in 200 μL of supernatant was measured.

Western Blot Analysis of yRce1p. Proteins from AC-P139 membrane preparations were resolved by SDS−PAGE, transferred to PVDF membranes, and probed with a yRce1p antibody. Membranes were suspended on 12% SDS−PAGE gels according to standard protocols. One gel was stained

with Coomassie Brilliant Blue, and the other gel was electroblotted to a PVDF membrane using the Mini Trans-Blot Electrophoretic Transfer Cell System from BioRad. The membrane was probed following the protocol for the Western Breeze chemiluminescence detection system (Novex) using either yRce1p antibody (1:2000 dilution) and anti-rabbit IgG-AP conjugate or anti-myc antibody (Invitrogen) as the primary antibody (1:5000 dilution) and antimouse IgG-AP conjugate as the secondary antibody.

Preparation of Site-Directed Mutants of yRce1p. The ORF contained in pMev-αyRcemyc was mutagenized with the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) following the kit directions. The following oligonucleotide pairs were used for the mutagenesis: H194A, (DW33) TTTTTTGGACTTGCTGCTGCACACCATGCTTAT, (DW34) ATAAGCATGGTGTGCAGCAGCAAGTCCAAAAAA, H196A, (DW35) GGACTTGCGCACGCAGCACATGCTTATGAGCAA, (DW36) TTGCTCATAAGCATGTGCTGCGTGCGCAAGTCC; H197A, (DW37) CTTGCGCACGCACACGCAGCTTATGAGCAATTA, (DW38) TAATTGCTCATAAGCTGCGTGTGCGTGCGCAAG; H248A, (DW44) TGCTGCATAATCCTGGCAGCCCTTTGCAATATC, (DW45) GATATTGCAAAGGGCTGCCAGGATTATGCAGCA; E156A, (DW48) TTTGCACCAATAACTGCAGAAATATTTTACACG, (DW49) CGTGTAAAATATTTCTGCAGTTATTGGTGCAAA; C251A, (DW60) ATCCTGCATGCGCTAGCCAATATCATGGGGTTT, (DW61) AAACCCCATGATATTGGCTAGCGCATGCAGGAT. All constructs were sequenced to verify that the correct mutations had been incorporated.

RESULTS

Disruption of RCE1 in Yeast Strain BCY123. BCY123 is a protease-deficient yeast strain with the *GAL4* gene linked to a *GAL1* promoter for synthesis of recombinant proteins (*27*). BCY123 has functional chromosomal copies of *AFC1* and *RCE1*. The activity of wt Afc1p in membrane preparations was effectively suppressed with 1,10-phenanthroline (*18*), and the chromosomal copy of *RCE1* was disrupted by transformation with the *rce1*Δ*::TRP1* construct used to disable *RCE1* in ABY53 (*18*). The presence of the disruption was confirmed in colonies grown on minimal plates lacking tryptophan. Primers were designed so that the sense primer was located upstream of the disruption site, and the antisense primer was located within the *TRP1* gene. PCR of genomic DNA from ABY53 gave a band at 1.8 kb (Figure 1). Lane 3 contained genomic DNA from ABY2, a strain with wild-type *RCE1*. As expected, no PCR product was seen. Lanes 4−7 contained DNA from four different colonies of JDY101, transformants of BCY123 with the *rce1*Δ::*TRP1* knockout. Membranes prepared from JDY101 did not have detectable levels of CaaX endoprotease activity in the presence of 1,-10-phenanthroline.

Production of yRce1p in S. cerevisiae. Three promoter systems were examined for expression of *yRCE1* in yeast: *MEV1* (pACA1) (*18*), *GAL1* (p426GAL1) (*26*), and a second *GAL1* promoter (pJEL167, a derivative of YEpTOP2PGAL1) (*27*). All of the constructs contained a myc-epitope fused to the C-terminus of the protein for detection of the expressed protein by Western blotting. In view of reports (*34*−*36*) that expression of some membrane receptors was facilitated by inclusion of the α-factor leader sequence (*34*−*36*), some of our constructs also had the α-factor signal sequence fused to the N-terminus of yRce1p.

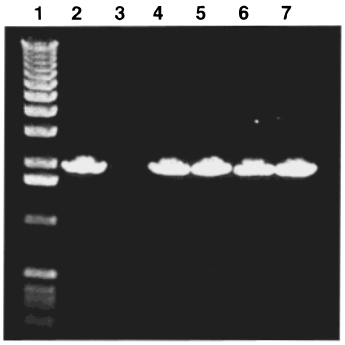

FIGURE 1: Confirmation of the *rce1::TRP1* disruption in BCY123. PCR primers and reaction conditions are found in the Materials and Methods. Lane 1: kb ladder. Lane 2: genomic DNA from ABY53. Lane 3: genomic DNA from ABY2. Lanes 4−7: Genomic DNA isolated from separate colonies of BCY123 *rce1::TRP1* (JDY101).

Table 1 shows the specific activities for recombinant Rce1p isolated from different yeast strains. The JEL system under the *GAL1* promoter without the α-factor signal sequence (pJel-yRcemyc) gave the highest level of overproduction with a specific activity 300−375-fold higher than those of similar preparations from two yeast strains, JRY0947 and JRY1551, containing wt copies of *RCE1*. The JEL construct with the α-factor signal sequence was 2.5 times less active. No activity was detected in an uninduced control or in a control containing p426GAL1. Constructs based on the p426GAL1 and *MEV1* promoters gave membranes whose yRce1p activity was 9−15-fold lower than that of the JEL system. Initial velocities for yRce1p were measured with membranes produced from JDY101/pJel-αyRcemyc. The data were fit to the Michaelis−Menten equation to give a $K_M = 1.3 \pm 0.3$ μM and $V_{Max} = 46 \pm 3$ nmol min^{-1} mg^{-1}.

Immunodetection of yRce1p. yRce1p was detected in yeast membranes by immunoblotting with a polyclonal yRce1p antibody (*19*). As shown in Figure 2, membranes from all strains except uninduced JDY101/pJel-αyRcemyc (lane 4) gave detectable signals. A single band at 29 kDa was seen in lane 1 for yRce1p, while prominent bands at 29 and 39 kDa were seen in lanes 2 and 3 for αyRce1p and yRce1p. Proteins in lanes 1−3 were derived from plasmid-encoded αyRcemyc under the *MEV1*, *GAL1*, and JEL/*GAL1* promoters, respectively. These results suggest that the α-factor leader sequence was completely (lane 1) or partially (lanes 2 and 3) removed. The cleavage may have occurred at a Kex2 endoprotease cleavage site that had been inserted between the end of the α-factor signal sequence and the start of the *yRCE1* gene (*37*). The sizes of the bands corresponding to αyRce1p (39 kDa) and yRce1p (29 kDa) are smaller than the predicted sizes of 45.9 and 38.6 kDa, respectively. However, a similar result was recently reported for strains expressing HA-tagged yRce1p at low levels in which the aberrant mobility of Rce1p was attributed to the multispanning nature of the protein (*19*). A similar result was observed by probing with the anti-myc antibody (data not shown).

Characterization of yRce1p Endoprotease Activity. The ability of several classical protease inhibitors to reduce yRce1p activity was examined for the carbonate-leached

Table 1: yRce1p CaaX Protease Activity of Carbonate-Leached Membranes[a]

strain	specific activity (nmol min^{-1} mg^{-1})	strain	specific activity (nmol min^{-1} mg^{-1})
JRY0947	0.4	JDY101/pJel-αyRcemyc	46
JRY1551	0.3	JDY101/pJel-αyRcemyc (uninduced)	<0.02
ABY53/pMev-αyRcemyc	12	JDY101/pJel-yRcemyc	112
JDY101/p426Gal-αyRcemyc	7	JDY101/p426Gal1	<0.03

[a] Assays were run with AC-P139 membranes (0.15−18 μg of protein) as described in the Materials and Methods.

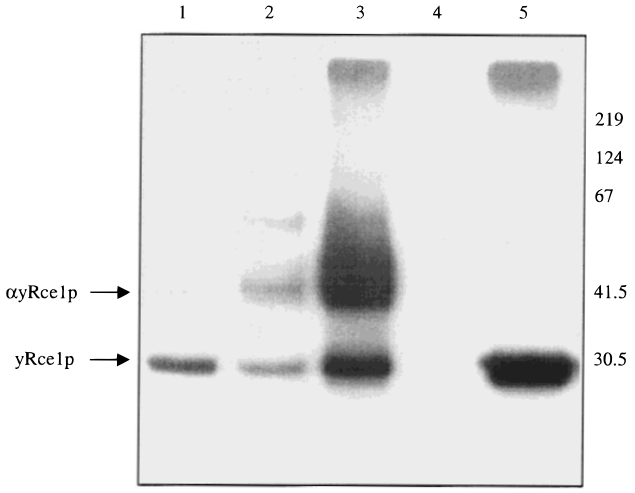

FIGURE 2: Immunodetection of yRce1p by Western blotting using chemiluminescent detection. Western blots were performed as described in the Materials and Methods. Lane 1: ABY53/pMev-αyRcemyc. Lane 2: JDY101/p426Gal-αyRcemyc. Lane 3: JDY101/pJel-αyRcemyc. Lane 4: JDY101/pJel-αyRcemyc, uninduced. Lane 5: JDY101/pJel-yRcemyc.

membrane preparations. RPI, a peptidomimedic inhibitor of mammalian CaaX endoproteases (*38*), gave an $IC_{50} = 103 \pm 9$ nM. Otherwise, the enzyme was insensitive to the protease inhibitors except those typically active against cysteine and serine proteases. Metal chelators had no effect on enzyme activity (Table 2). Although enzyme activity decreased substantially at *o*-phenanthroline concentrations >1.0 mM, a control experiment with *p*-phenanthroline, a nonchelating isomer, gave similar losses of activity. We attribute the decrease of activity to nonspecific effects of high concentrations of phenanthroline rather than to chelation of a required metal ion. Extended incubation of metal chelators with enzyme for up to 12 h did not further reduce enzyme activity (data not shown).

Protease inhibitors bestatin, antipain, chymostatin, pepstatin A, leupeptin, E64, and PMSF had no effect on the activity of yRce1p (Table 3). However, compounds that are typically active against serine and cysteine proteases inhibited the enzyme. These include chloromethyl ketones (TPCK and TLPK), organomercurials (PHMB, PHMS, and MSA), a typical cysteine-modifying reagent (MMTS), and metal ions. Of the chloromethyl ketones, TPCK was considerably more potent than TLCK. In a 30 min preincubation, 35 μM TPCK reduced CaaX protease activity by 50%, whereas 1−2 mM concentrations of TLCK were required to achieve similar levels of inhibition. Rando and co-workers had previously reported that chloromethyl ketones inhibited CaaX protease activity in membranes from bovine liver (*15*). yRce1p activity was reduced to background levels (≤1%) by 22 μM concentrations of the organomecurial reagents, and 50% inhibition was achieved with 1 μM PHMS. Although not as potent as the mecurials, 50 mM MMTS resulted in 40% inhibition. The effect of several heavy metals on yRce1p activity was also determined. Both Zn^{2+} and Cu^{2+} strongly inhibited the enzyme with the $IC_{50} = 11.6 \pm 0.1$ μM for $ZnCl_2$ (Table 4). Ni^{2+}, Fe^{2+}, and Co^{2+} were less potent.

We synthesized *N*-acetyl-*S*-farnesylcysteine chloromethyl ketone (Ac-(far)CCMK) and *N*-acetyl-*S*-farnesylcysteine diazomethyl ketone (Ac-(far)CDK) according to procedures described by Chen et al. (*15*). Both ketones were good inhibitors of yRce1p. Fifty percent inhibition was achieved at 2.0 μM Ac-(far)CCMK after preincubation at 37 °C for 30 min, and a similar level of inhibition under the same conditions was achieved with 15.2 μM Ac-(far)CDK.

Substrate Specificity of yRce1p. A selection of dansylated prenylated peptides was synthesized to evaluate the substrate selectivity of yRce1p. Representative CaaX sequences from yeast included CVIA (**a**-mating factor), CIIS (Ras2p), and CIIL (Rho2p), while mammalian sequences included CVLS (Ha-Ras), CVIM (Ki-Ras), and CVVM (N−Ras). In each case both farnesylated and geranylgeranylated derivatives were synthesized. The prenyl peptides were first screened as inhibitors for proteolysis of Biopep to ensure that they were bound to yRce1p. All of the compounds inhibited yRce1p. As shown in Table 5, IC_{50} values were mostly in the low micromolar range. There were no clear trends with respect to which prenyl group was preferred in each CaaX pair.

The rates for proteolysis of the prenylated peptides by yRce1p were measured by an HPLC assay that permitted us to identify the N-terminal hydrolysis product (*12*). The prenylated peptides were incubated separately in the presence and absence of AC-P139 membranes. The products were extracted into 1-butanol and analyzed by reversed-phase HPLC. For each substrate, proteolysis gave a more polar (decrease in retention time) N-terminal product, whose identity was confirmed by electrospray mass spectroscopy. The reactivities of the prenylated peptides relative to dan-WDPA(far)CVIA are shown in Table 5. All of the farnesylated CaaX peptides were substrates for yRce1p. CVIA was the most reactive CaaX motif. The farnesylated CVLS, CVIM, and CIIS derivatives were 4−8 times less reactive. Dan-G(far)CIIL was the least reactive farnesylated CaaX derivative. However, in this case, we are most likely not working under saturating conditions, because of the limited solubility of the substrate. Thus, the intrinsic reactivity of dan-G(far)CIIL may be somewhat higher than we report. In general, the geranylgeranylated peptides were not good substrates. Although we were only able to detect products for dan-WDPA(gg)CVIA and dan-KTK(gg)CVVM, it is clear that the geranylgeranylated peptides were cleaved in these two cases. In fact, dan-WDPA(gg)CVIA was as

Table 2: Effect of Metal Ion Chelators on Rce1p Activity[a]

metal chelator	concn (mM)	% activity remaining	metal chelator	concn (mM)	% activity remaining
EDTA	25.0	99	*o*-phenanthroline	2.5	70
EGTA	25.0	88		5.0	46
TPEN	0.5	97	*p*-phenanthroline	0.5	93
o-phenanthroline	0.5	90		5.0	70
	1.0	92			

[a] A 4 μL portion of enzyme diluted 20-fold with assay buffer (100 mM Tris, pH 7.4) was added to 43.5 μL of assay buffer containing the appropriate chelator. The assay tubes were preincubated at 37 °C for 20 min. Reactions were initiated by the addition of 2.5 μL of Biopep (25 μM) and incubated at 37 °C for 30 min. The residual enzyme activity was determined using the avidin assay as described in the Materials and Methods.

Table 3: Inhibition of yRce1p with Various Protease Inhibitors[a]

inhibitor	type	concn (μm)	% activity remaining
bestatin	amino peptidase	100	96
antipain	papain, trypsin, cathepsin A, B	75	87
chymostatin	chymotrypsin	100	95
pepstatin a	aspartyl	50	103
PMSF	serine	1	96
leupeptin	serine and cysteine	10	85
TPCK	serine/cysteine	300	6
TLCK	serine/cysteine	300	68
		1000	54
PHMB	cysteine	22	1
PHMS	cysteine	22	1
MSA	cysteine	22	1
MMTS	cysteine	50	60
E64	cysteine	44	97

[a] A 4 μL portion of enzyme diluted 20-fold with assay buffer (100 mM Tris, pH 7.4) was added to 43.5 μL of assay buffer containing the appropriate inhibitor, added in 1 μL of DMSO (except EDTA, EGTA, phenanthroline, TPEN, organomercurials, and MMTS were aqueous). The assay tubes were preincubated at 37 °C for 20 min. Reactions were initiated by the addition of 2.5 μL Biopep (25 μM) and incubated at 37 °C for 15−30 min. The residual enzyme activity was determined using the avidin assay as described in the Materials and Methods.

Table 4: Inhibition of Rce1p with Various Metals[a]

inhibitor	% activity remaining	inhibitor	% activity remaining	inhibitor	% activity remaining
$ZnCl_2$	1	$Cu(NO_3)_2$	3	$FeSO_4$	72
CuCl	3	$NiSO_4$	52	$CoCl_2$	52

[a] A 4 μL portion of enzyme diluted 20-fold with assay buffer (100 mM Tris, pH 7.4) was added to 43.5 μL of assay buffer containing the appropriate metal ion at a concentration of 87 μM. The assay tubes were preincubated at 37 °C for 20 min. Reactions were initiated by the addition of 2.5 μL of Biopep (25 μM) and incubated at 37 °C for 30 min. The residual enzyme activity was determined using the avidin assay as described in the Materials and Methods.

reactive as all of the farnesyl derivatives except dan-WDPA-(far)CVIA.

Site-Directed Mutagenesis of yRce1p. Alignments of amino acid sequences for Rce1p from *S. cerevisiae* (*18*), human (*24*), mouse (*22*), and *Schizosaccharomyces pombe*, as well as partial sequences from *Caenorhabditis elegans* and *Neurospora crasa* (Figure 3) showed several regions with substantial sequence similarities. The completely conserved histidine, cysteine, and glutamate residues were replaced with alanine by site-directed mutagenesis in plasmid pMev-αyRcemyc. The transformed strains were evaluated in the halo assay (*18*) for their ability to process **a**-mating factor using a tester strain that is supersensitive to **a**-factor. In the presence of mature **a**-factor, the strain arrested and a halo of growth inhibition was observed. In the absence of CaaX proteolysis, no halo formed. Six yRce1p mutants produced no or small halos: H194A, H196A, H197A, H248A, E156A, and C251A. Membranes from yeast strain ABY53/pMev-αyRcemyc (*rce1*Δ::*TRP1*, *afc1*::*HIS3*) were prepared for the six mutant proteins, and their specific activities were measured using the biotin/avidin assay (Table 6). Two of the histidine mutants, H196A and H197A, had substantial levels of proteolytic activity compared to wild-type yRce1p. However, none of the remaining four mutants, H194A, E156A, H248A, and C251A, were active.

DISCUSSION

yRce1p and yAfc1p are membrane-bound endoproteases necessary for post-translational processing of prenylated proteins in yeast with C-terminal CaaX motifs. yAfc1p was first identified because of its role in processing **a**-mating factor. The enzyme catalyzes two separate cleavage steps: removal of the −aaX tripeptide from farnesylated **a**-factor precursor and removal of the seven amino acid N-terminal fragment. yRce1p was identified from its special role in processing farnesylated Ras proteins in yeast. Like yAfc1p, yRce1p removes the −aaX tripeptide from farnesylated CaaX proteins, but does not have the N-terminal activity reported for yAfc1p. CaaX protease activity has also been detected in bovine and rat liver membrane preparations. Recently, Otto et al. reported production of recombinant yRce1p and a human homologue in S*f9* insect cells (*24*).

We investigated several systems for producing yRce1p for biochemical studies. Preliminary attempts in *E. coli* gave inclusion bodies. We next attempted production in the methylotrophic yeast strain *Pichia pastoris*. The *Pichia* system was chosen because the induction system is tightly regulated by methanol, cultures grow to high cell densities (*39*), and a variety of active recombinant membrane-bound proteins have been obtained with this system (*34*−*36*, *40*, *41*). We also incorporated an N-terminal α-factor signal sequence, which is reported to facilitate the expression of active membrane receptors (*36*, *42*). However, several attempts to obtain functional yRce1p in *P. pastoris* were unsuccessful, and Western blot analysis of proteins in the membranes did not give bands corresponding to αyRcemyc. The *S. cerevisiae RCE1* gene contains two AT-rich sequences, ^{371}ATTTATTAAAT^{381} and ^{442}AATTTTATATTT^{453}, that are similar to sequences previously shown to cause premature termination of transcription in *P. pastoris* (*43*). Northern blot analysis of αyRcemyc mRNA had bands corresponding to 438, 560, and 703 bases, but no signal for 1630 bases for full-length αyRcemyc mRNA (data not shown). The bands for the mRNA fragments were not seen in samples isolated from uninduced cell growths. These

Table 5: Inhibition of yRce1p by Prenylated Peptides and Relative Rates of the Prenylated Peptides with yRce1p

peptide	IC_{50} (μM)[a]	relative reactivity[b]	peptide	IC_{50} (μM)[a]	relative reactivity[b]
Dan-WDPA(far)CVIA	6.2	1	DanWDPA(gg)CVIA	1.8	0.12
Dan-SAK(far)CVLS	5.3	0.12	Dan-SAK(gg)CVLS	*c,e*	<0.01
Dan-KTK(far)CVIM	1.5	0.25	Dan-KTK(gg)CVIM	2.3	0.02
Dan-GLP(far)CVVM	3.1	0.13	Dan-GLP(gg)CVVM	9.5	<0.01
Dan-G(far)CIIS	8.1	0.13	Dan-G(gg)CIIS	5.7	<0.01
Dan-G(far)CIIL	*c,d*	0.06	Dan-G(gg)CIIL	*c,f*	<0.01

[a] AC-P139 membranes (0.8 μg) were added to a mixture of assay buffer (100 mM Tris, pH 7.4, 0.5 mM 1,10-phenanthroline, 1 mM PMSF) and the appropriate prenyl peptide. Reactions were initiated by addition of BioPep to a final concentration of 2.0 μM, and the reaction mixtures were incubated for 15 min at 37 °C. The residual enzyme activity was determined with the avidin assay as described in the Materials and Methods. [b] Reactions containing 180 μL of assay buffer (100 mM Tris pH 7.4, 0.5 mM 1,10-phenanthroline, 1 mM PMSF), 10 μL of peptide substrate (50 μM), and 10 μL of AC-P139 yRce1p membranes (3−60 μg) were incubated at 37 °C for 45 min. The reaction mixtures were extracted with 250 μL of water-saturated butanol, followed by incubation at room temperature for 15 min. The tubes were centrifuged for 1 min, and the organic layers were transferred to new tubes. The aqueous layers were extracted with an additional 150 μL of butanol as before. The butanol layers were combined and evaporated, and the samples were stored at 4 °C until HPLC analysis. Samples were dissolved in 50−100% CH_3CN/0.1%TFA and analyzed on a Vydac C18 column (4.6 × 250 mm) using a linear gradient from 0.1% TFA/45% CH_3CN/55% H_2O (v/v) to 0.1% TFA/80% CH_3CN/20% H_2O (v/v) over 14 min for the farnesylated peptides and a linear gradient from 0.1% TFA/20% CH_3CN/80% H_2O (v/v) to 0.1% TFA/80% CH_3CN/20% H_2O (v/v) over 20 min for the geranylgeranylated peptides. Detection was at 214 nm. [c] Limited solubility above 50 μM concentrations prevented an accurate determination of IC_{50}. [d] 53% inhibition at 50 μM Dan-G(far)CIIL. [e] 41% inhibition at 50 μM Dan-SAK(gg)CVLS. [f] 67% inhibition at 50 μM Dan-G(gg)CIIL.

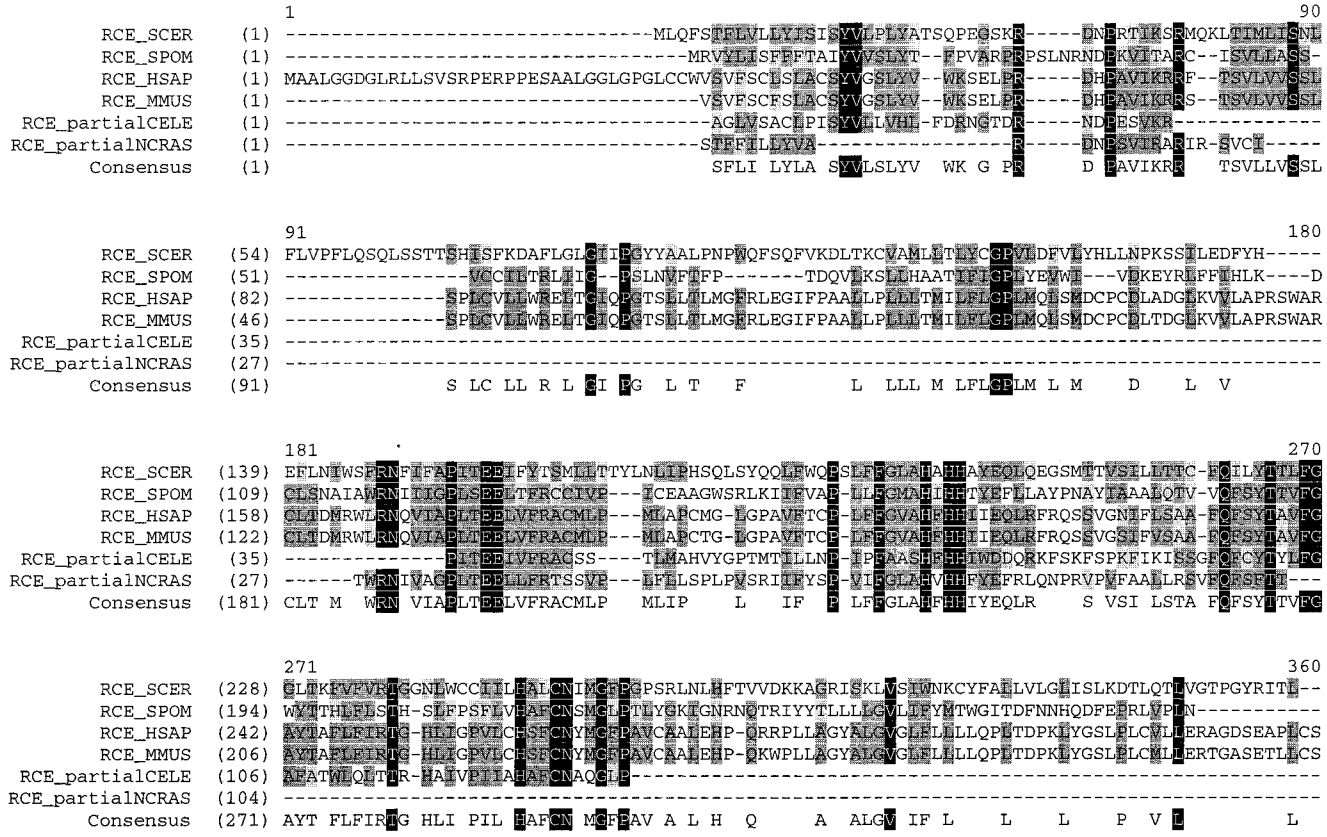

FIGURE 3: Multiple sequence alignment of *S. cerevisiae*, *S. pombe*, *Homo sapiens*, and *Musculus musculus* Rce1 proteins and predicted partial *C. elegans* (CEF48F5) and *N. crasa* (NM7G10-T7) Rce1 proteins. Absolutely conserved residues are negatively imaged, highly conserved residues are shaded, and weakly conserved residues are lightly shaded. The alignment was prepared with the ALIGNX module of Vector NTI Suite.

results suggest that premature transcription termination had occurred in our construct.

Recombinant yRce1p was successfully produced in *S. cerevisiae* with strains where the chromosomal copy of *RCE1* was disrupted. The three different yeast promoters, *MEV1*, *GAL1*, and JEL/*GAL1*, all gave recombinant yRce1p. However, the JEL/*GAL1* system gave 10-fold higher levels of yRce1p activity in isolated membranes than our other constructs or the baculovirus system (*24*). In contrast to previous reports for membrane-associated proteins, the levels of yRce1p were 3-fold higher for constructs without an α-factor leader. Western blot analysis showed that most of the protein obtained from constructs with the α-factor signal sequence did not have the leader sequence, presumably as a result of proteolysis at the Kex2 cleavage site.

yRce1p is tightly bound to membranes. An analysis of predicted secondary structures based on multiple sequence alignments suggests that the protein has seven transmembrane

Table 6: Specific Activity of yRce1p and Site-Directed Mutants[a]

enzyme	specific activity (nmol min^{-1} mg^{-1})	relative specific activity	enzyme	specific activity (nmol min^{-1} mg^{-1})	relative specific activity
wt (ABY53/pMev-αyRcemyc)	11 ± 1.4	1	H197A	0.9 ± 0.2	0.08
E156A	$(1.3 \pm 0.2) \times 10^{-2}$	$<10^{-3}$	H248A	$(1.0 \pm 0.03) \times 10^{-2}$	$<10^{-3}$
H194A	0.018 ± 0.002	0.0016	C251A	$(1.0 \pm 0.2) \times 10^{-2}$	$<10^{-3}$
H196A	5.5 ± 0.9	0.5	vector control (pACA1)	$(1.3 \pm 0.2) \times 10^{-3}$	$<10^{-3}$

[a] Reactions contained 0.1−20 μg of AC-P139 membranes and 25 μM Biopep in 100 mM Tris, pH 7.4, 1 mM PMSF, 0.5 mM phenanthroline, 1 mM EDTA, 100 μM bestatin, 100 μM pepstatin, 100 μM antipain, 100 μM E−64, and 100 μM chymotrypsin. Reactions were run at 37 °C for 15−60 min; enzyme activity was measured as described in the Materials and Methods.

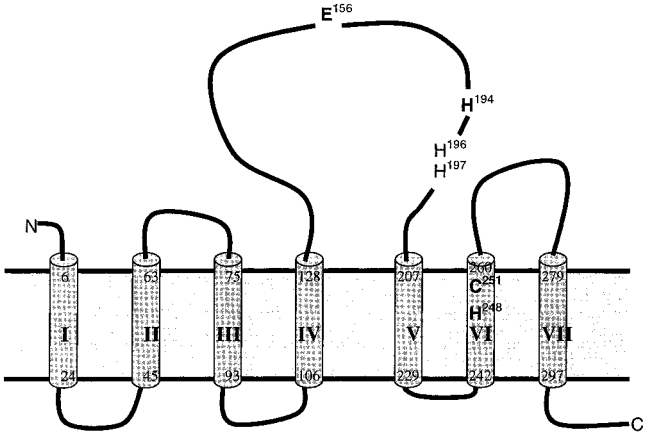

FIGURE 4: Graphical illustration of predicted membrane spanning regions of *S. cerevisiae* Rce1p. Transmembrane regions were predicted with TMHM (*46*) (http://www.cbs.dtu.dk /services/TMHMM-1.0/) and are illustrated as numbered cylinders. Loop regions are indicated by ribbons. Conserved and potentially functional residues examined by site-directed mutagenesis that are localized on the same side of the ER membrane are included: E156; HXHH, 194−197; H248 and C251 (in transmembrane region VI).

helices. As illustrated in Figure 4, helices IV and V are connected by a large loop on the cytoplasmic surface that contains conserved residues ^{156}E, ^{194}H, ^{196}H, and ^{197}H. Two additional conserved residues, ^{251}C and ^{248}H, are located in helix VI. The histidine-rich region in the large loop does not precisely correlate with the metal-binding sites in known Zn^{2+} proteases but is somewhat reminiscent of Zn^{2+} sites in other proteins (*44*). Since Afc1p exhibits the hallmarks of a Zn^{2+} protease, we examined the potential role of metal ion catalysis by yRce1p and found that the enzyme was insensitive to a variety of metal chelators (Table 2) and was inhibited by higher concentrations of Zn^{2+} and Cu^{2+} (Table 4). Although the absence of inhibition by the metal chelators is "negative evidence" for a non-metal-mediated proteolysis, no decrease in activity was seen for yRce1p under conditions similar to those we used previously to remove the tightly bound catalytic site zinc in protein farnesyltransferase (*45*).

Screening with a variety of protease inhibitors revealed that yRce1p is insensitive to bestatin, antipain, chymostatin, pepstatin A, leupeptin, E64, and PMSF. However, chloromethyl ketones TPCK and TLCK inhibited the enzyme. TPCK was more potent than TLCK. Rando and co-workers found TPCK protease activity in a bovine liver preparation and attributed the difference to binding of the phenylalanyl side chain of TPCK in the hydrophobic isoprenoid pocket (*15*). We synthesized two farnesylated cysteine analogs, chloromethyl ketone Ac-(far)CCMK and diazo ketone Ac−(far)CDK. Both compounds inhibited yRce1p, and the chloromethyl ketone inhibitor was almost an order of magnitude more potent than the diazo ketone. Typically, peptidyl diazomethanes are selective for cysteine proteases (*46*). Inhibition of Rce1p by Ac−(far)CDK is consistent with the enzyme being a cysteine protease. The sensitivity of Rce1p toward MMTS and organomercurials supports this suggestion.

Site-directed mutagenesis experiments were also consistent with the suggestion that Rce1p is a cysteine protease. Four of the mutants (H194A, H248A, E156H, and C251A) were inactive in the biotin/avidin assay. Although the membrane preparations used were impure, the chromosomal copies of *RCE1* and *AFC1* were disabled in the yeast hosts used for expression of the mutants. Cysteine proteases typically have a catalytic triad consisting of cysteine, histidine, and aspartate residues. In the gene sequences now available for Rce1ps, only one cysteine and four histidines are completely conserved, along with two glutamates. Alanine mutants for a subset of these, ^{251}C, ^{194}H, ^{196}H, ^{197}H, ^{248}H, and ^{156}E, were marginally active or inactive in the halo assay for **a**-factor processing. Our biochemical assays suggest that four of the residues, ^{251}C, ^{194}H, ^{248}H, and ^{156}E, are required for endoprotease activity. Assuming that yRce1p is indeed a cysteine protease, it is likely that the sulfhydryl moiety in ^{251}C is the active-site nucleophile, ^{194}H or ^{248}H facilitates deprotonation of the thiol moiety, and ^{156}E replaces the aspartate normally found in the cysteine protease catalytic triad. Our data do not allow us to determine which of the two histidines is part of the catalytic triad. Both ^{251}C and ^{248}H are located in helix VI. The predicted location of the residues within the bilayer may be an artifact of the software used to predict the location of membrane-spanning helices in yRce1p. However, it is also possible that the active site of yRce1p is below the surface of the membane to accommodate a substrate whose scissle bond is flanked by a hydrophobic prenyl chain and hydrophobic amino acids.

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