es and Medical Microbiology (Saunders, Philadelphia, PA, 1986), p. 783.
14. H. Chmel, M. Bendinelli, H. Friedman, *Pulmonary Infections and Immunity* (Plenum, New York, 1994), p. 286.
15. S. B. Wolbach, *Johns Hopkins Hosp. Bull.* **30**, 104 (1919).
16. E. R. LeCount, *J. Am. Med. Assoc.* **72**, 650 (1919).
17. S. Kumar, K. Tamura, M. Nei, *Molecular Evolutionary Genetics Analysis*, version 1.01 (Pennsylvania State University, University Park, PA, 1993).
18. D. L. Swofford, *PAUP: Phylogenetic Analysis Using Parsimony*, version 3.1.1 (Illinois Natural History Survey, Champaign, IL, 1991).
19. S. F. Altschul, W. Gish, W. Miller, E. W. Meyers, D. J. Lipman, *J. Mol. Biol.* **215**, 403 (1990).
20. C. Röhm, T. Horimoto, Y. Kawaoka, J. Süss, R. G. Webster, *Virology* **209**, 664 (1995).
21. T. Horimoto and Y. Kawaoka, *J. Virol.* **68**, 3120 (1994).
22. Y. Kanegae, S. Sugita, K. F. Shortridge, Y. Yoshioka, K. Nerome, *Arch. Virol.* **134**,17 (1994).
23. O. T. Gorman *et al.*, *J. Virol.* **65**, 3704 (1991).
24. RNA lysates from the paraffin-embedded tissues were produced as previously described [A. E. Krafft *et al.*, *J. Wildlife Dis.* **31**, 410 (1995)]. Reverse transcription was carried out at 37°C for 45 min in 20 μl with the use of 300 U of Moloney murine leukemia virus–RT, 1× RT buffer (Gibco-BRL, Grand Island, NY), 5 μM random hexamers, 200 nM deoxynucleotide triphosphates (dNTPs), and 10 mM dithiothreitol. RT reaction (2 μl) was added to a 20-μl PCR reaction containing 50 mM KCl, 10 mM tris, 2.5 mM $MgCl_2$, 1 μM each primer, 100 nM dNTPs, 1 U of Amplitaq Gold (Perkin-Elmer, Foster City, CA), and 2 μCi of [a-^{32}P]dATP (3000 Ci/mmol). Primers (with IUPAC mixed base codes) used in the PCR reactions were as follows (D = A, G, or T; H = A, C, or T; K = G or T; M = A or C; N = A, C, G, or T; R = A or G; S = C or G; W = A or T; Y = C or T); numbers refer to the complete sequence of each gene in the mRNA orientation: HA137 (117 to 208, 92 bp): forward, 5′-TCWACHGACACTGTTGAYACA-3′; reverse, 5′-CAKAGTTTYCCRTTRTGDCTGTC-3′. HA33 (8 to 208, 200 bp): forward, 5′-GCAGGGGAAAATAAAAACAACC-3′; reverse, same as HA137. HA389 (368 to 536, 169 bp): forward, 5′-CATYGACTATGARGARCTGAG-3′; reverse, 5′-CAGCCATAGYAARTTTCTGT-3′. HA980 (960 to 1093, 133 bp): foward, 5′-CAGAATRTACAYCCAGTCAC-3′; reverse, 5′-ATATTTCCRGCAATGGCTCC-3′. NA53 (34 to 113, 80 bp): forward, 5′-CARAARATAATAACMATTGG-3′; reverse, 5′-GWKAYNAKMWTSSCWATYTGCA-3′. NP540 (520 to 623; 103 bp): forward, 5′-ATGGAYCCCAGRATGTGYTC-3′; reverse, 5′-ARYTCCAWNAYCATTGTYCC-3′. NP918 (898 to 1005, 108 bp): forward, 5′-GTRGCCAGYGGRYAYGACTT-3′; reverse, 5′-TYTCRTTYGGTCTRATDAG-3′. M149 (129 to 142, 113 bp): forward, 5′-GAGGYTCTCATGGARTGGCT-3′; reverse, 5′-TACGCTGCAGTCCTCGCTCA-3′. M811 (794 to 906, 113 bp): forward, 5′-TCATTGGGATCTTGCAC-3′; reverse, 5′-WGGYACTCCTTCCGTAG-3′. Cycling conditions were as follows: 9 min at 94°C; 40 cycles of 94°C for 30 s, 50°C (except HA and NA, which were at 45°C) for 30 s, and 72°C for 30 s; then 5 min at 72°C. One-sixth of the reaction was separated on a 7% denaturing polyacrylamide gel, then dried and visualized by autoradiography. Bands were excised, electroeluted, and ethanol-precipitated. One-fourth of the eluted product was added to a 50-μl PCR reaction (50 mM KCl, 10 mM tris, 2.5 mM $MgCl_2$, 200 nM dNTPs, 1 mM each primer, and 1 U Amplitaq Gold) and cycled as above. The reaction product (2 μl) was cloned into the PCR 2.1 vector (Invitrogen, San Diego, CA) according to the manufacturer's instructions. Direct PCR using M13 primers was done on white colonies, and the products were sequenced by cycle sequencing as described [A. H. Reid, R. E. Cunningham, G. Frizzera, T. J. O'Leary, *Am. J. Pathol.* **142**, 395 (1993)].
25. The sequences used in this analysis, obtained from GenBank, EMBL, and DDBJ, were from the following strains: A/Fiji/2/88 (H1N1) [Fiji88], A/Singapore/6/86 (H1N1) [Sing86], A/Czechoslovakia/2/88 (H1N1) [Czech88], A/Yamagata/120/86 (H1N1) [Yamag86], A/Yamagata/32/89 (H1N1) [Yamag89], A/France/15/88 (H1N1) [France88], A/Victoria/43/88 (H1N1) [Vict88], A/Sichuan/4/88 (H1N1) [Sichuan88], A/Massachusetts/1/90 (H1N1) [Mass90], A/Leningrad/109/91 (H1N1) [Len91], A/Stockholm/26/90 (H1N1) [Stock90], A/Leningrad/54/1 (H1N1) [Len54], A/Mongolia/231/85 (H1N1) [Mong85], A/Fort Monmouth/1/47/MA (H1N1) [FtMon47], A/Alma Ata/1417/84 (H1N1) [Sw/AlmaAta84], A/swine/Iowa/1976/31 (H1N1) [Sw/Iowa31], A/swine/Iowa/15/30 (H1N1) [Sw/Iowa30], A/swine/29/37 (H1N1) [Sw/29/37], A/swine/St. Hyacinthe/148/90 (H1N1) [Sw/StHy90], A/swine/Ehime/1/80 (H1N1) [Sw/Ehm80], A/swine/Italy v147/81 (H1N1) [Sw/Italy81], A/swine/St. Hyacinthe/106/91 (H1N1) [Sw/StHy91], A/sw/Illinois/63 (H1N1) [Sw/Ill63], A/NJ/11/76 (X53A) (H1N1) [NJ/1976], A/New Jersey/11/76 (H1N1) [NJ/76], A/Puerto Rico/8/34 (H1N1) [PR34], A/Puerto Rico/8/34 (Cambridge) (H1N1) [PR34(Cam)], A/WSN/33(H1N1) [WSN33], A/swine/Cambridge/39 (H1N1) [Sw/Cam39], A/swine/NI/38 (H1N1) [Sw/NI38], A/turkey/Germany/3/91 (H1N1) [Ty/Germ91], A/DK/HK/196/77 (H1N1) [Dk/HK77], A/DK/WI/259/80 (H1N1) [Dk/Wisc80b], A/duck/Alberta/35/76 (H1N1) [Dk/Alb76], A/DK/WI/1938/80 (H1N1) [Dk/Wisc80a], A/sw/Obihiro/5/92 (H1N1) [Sw/Obih92], A/Hokkaido/2/92 (H1N1) [Hokk92], A/Fort Warren/1/50 (H1N1) [FtWar50], A/Loyang/4/57 (H1N1) [Loyang57], A/FPV/Rostock/34 (H7N1) [FPV/Rost34], A/parrot/Ulster/73 (H7N1) [Parr/Ulls73], A/duck/Ontario/77 (H2N1) [Dk/Ont77], A/tern/Australia/G70C/75 (H11N9) [Tern/Aus75], A/Whale/Maine/1/84 (H13N9) [Whale/Maine84], A/sw/Hong Kong/3/76 (H3N2) [Sw/HK76], A/turkey/Oregon/71 (H7N3) [Ty/Oreg71], A/duck/Alberta/77/77 (H2N3) [Dk/Alb77], A/turkey/Ontario/6118/68 (H8N4) [Ty/Ont68], A/Mallard/Alberta/283/77 (H8N4) [Mall/Alb77], A/duck/England/56 (H11N6) [Dk/Eng56], A/duck/Alberta/28/76 (H4N6) [Dk/Alb76a], A/duck/Alberta/60/76 (H12N5) [Dk/Alb76b], A/shearwater/Australia/72 (H6N5) [Gull/Shear72], A/Ken/1/81 (H3N8) [Eq/Ken81], A/Equine/Alaska/1/91 (H3N8) [Eq/Alask91], A/Cor/16/74 (H7N7) [Eq/Cor74], A/chick/n/Germany/49 (H10N7) [Ck/Germ49], A/California/10/78 (H1N1) [Calif78], A/Udorn/307/72 (H3N2) [Udorn72], A/New Jersey/4/76 (H1N1) [NJ76a], A/Hong Kong/5/83 (H3N2) [HK83], A/Texas/1/77(H3N2) [Texas77], A/Brazil/11/78 (H1N1) [Brazil78], A/USSR/90/77 (H1N1) [USSR77], A/Ann Arbor/6/60 (H2N2) [AnnArbor60], A/Hickox/40 (H1N1) [Hickox40], A/Wilson-Smith/33 (H1N1) [WS33], A/swine/Ohio/23/35 (H1N1) [Sw/Ohio35], A/swine/Iowa/46 (H1N1) [Sw/Iowa46], A/swine/Wisconsin/1/57 (H1N1) [Sw/Wisc57], A/New Jersey/8/76 (H1N1) [NJ76b], A/swine/Italy/2/79 (H1N1) [Sw/Italy79], A/swine/Iowa/17672/88 (H1N1) [Sw/Iowa88], A/swine/Tennessee/24/77 (H1N1) [Sw/Tenn77], A/equine/Prague/1/56 (H7N7) [Eq/Prague56], A/duck/Pennsylvania/1/69 (H6N1) [Dk/Penn69], A/Mallard/NY/6750/78 (H2N2) [Mall/NY78], A/duck/Memphis/928/74 (H3N8) [Dk/Mem74], A/Manitoba/1/53 (H10N7) [Dk/Man53], A/turkey/Ontario/7732/66 (H5N9) [Ty/Ont66], A/equine/Miami/1/63 (H3N8) [Eq/Miami63], A/duck/Bavaria/2/77 (H1N1) [Dk/Bav77], A/duck/Hong Kong/7/75 (H3N2) [Dk/HK75], and A/Anas acuta/Primorje/695/76 (H2N3) [Dk/Prim76].
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Modulation of Ras and a-Factor Function by Carboxyl-Terminal Proteolysis

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Prenylated proteins contain a covalently linked cholesterol intermediate near their carboxyl-termini. Maturation of most prenylated proteins involves proteolytic removal of the last three amino acids. Two genes in *Saccharomyces cerevisiae*, *RCE1* and *AFC1*, were identified that appear to be responsible for this processing. The Afc1 protein is a zinc protease that participates in the processing of yeast **a**-factor mating pheromone. The Rce1 protein contributes to the processing of both Ras protein and **a**-factor. Deletion of both *AFC1* and *RCE1* resulted in the loss of proteolytic processing of prenylated proteins. Disruption of *RCE1* led to defects in Ras localization and signaling and suppressed the activated phenotype associated with the allele *RAS2^{val19}*.

Eukaryotic cells differ from prokaryotes in their use of specialized compartments and membranes to localize metabolic pathways, structural components, and regulatory processes. Some peripheral membrane proteins depend for proper membrane localization on co- or posttranslational covalent attachment of a lipid moiety to the protein. The most recently discovered such modification involves the covalent attachment of cholesterol biosynthetic intermediates to proteins and is referred to generically as protein prenylation (*1*).

Prenylation is catalyzed by one of three different prenyl-transferase enzymes, which attach either the 15-carbon farnesyl or the 20-carbon geranylgeranyl moieties to a cysteine near the COOH-terminus of the protein. Which enzyme prenylates which substrate is determined by the amino acid sequence at the COOH-terminus of the substrate. A pair of COOH-terminal cysteine residues, together with other determinants, direct geranylgeranylation by a type II pro-

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tein geranylgeranyl transferase (*2*). In contrast, a CAAX motif, in which C is cysteine, A is any aliphatic amino acid, and X is any amino acid, is able to direct either geranylgeranylation or farnesylation by one of two different type I protein prenyl-transferases. If the X position is occupied by leucine or phenylalanine, the protein is geranylgeranylated (*3*). Other amino acids at this position cause farnesylation.

Prenylated proteins include the Ras oncogenes, fungal pheromones, the γ subunits of trimeric guanosine triphosphate (GTP)–binding proteins (G proteins), and a wide range of small GTP-binding proteins that participate in vesicular trafficking (*4*). Prenylation is critical to the activity of many of these proteins. Farnesylation of the Ras protein of yeast and humans is necessary for its membrane localization and transforming activity (*5*). Inhibitors of protein farnesyl transferases have been developed as potential treatments for Ras-based tumors (*6*). Farnesylation of the mating pheromone **a**-factor of the yeast *Saccharomyces cerevisiae* is the first step in a series of posttranslational modifications that convert the 36–amino acid precursor to the 12–amino acid farnesylated mature **a**-factor. Farnesylation appears to be essential for all subsequent modifications of **a**-factor, including proteolytic trimming by NH_2- and COOH-terminal proteases and export by the Ste6 protein (*1*, *7*).

In prenylated proteins with a CAAX motif, prenylation is almost invariably followed by two additional posttranslational modifications: The three COOH-terminal residues are proteolytically removed, and the carboxyl group of the resulting prenylcysteine is carboxyl-methyl esterified (*8*). The key unresolved issue in protein prenylation concerns the identity and role of the protease or proteases responsible for the COOH-terminal trimming. An endoproteolytic activity that cleaves the terminal tripeptide from prenylated substrates, which we refer to as RACE (Ras and **a**-factor converting enzyme), has been identified in membrane fractions of yeast, *Xenopus*, and mammalian cells. The processing of peptides by RACE is dependent on the prenylation of the substrate (*9*). Previously, the inability to identify the gene (or genes) encoding RACE made it impossible to establish unequivocally the relation between CAAX proteolysis and the function of prenylated proteins. We have now identified a pair of proteins required for proteolysis of prenylated proteins in *S. cerevisiae* and have established the role that each plays in the in vivo and the in vitro processing of Ras and **a**-factor precursors.

The production of and response to mating pheromones is critical for mating of haploid yeast cells. Yeast mutants defective in either farnesylation of the **a**-factor precursor or prenylcysteine methylesterification cannot produce active pheromone and are sterile (*10*). Because these steps precede and follow COOH-terminal proteolysis of **a**-factor, respectively, a mutation in the COOH-terminal protease should also cause sterility. To isolate such sterile mutants, we adapted an autocrine arrest selection and conditionally expressed the **a**-factor receptor in cells of the **a**-mating type (*11*). Cells that both synthesize and respond to their own **a**-factor arrest in the G_1 phase of the cell cycle and fail to divide. However, mutants defective in either the synthesis of or response to **a**-factor survive. To further sensitize the selection, we removed the two structural genes that encode **a**-factor in yeast and used a mutant form of the **a**-factor gene as the source of the pheromone (*12*). The three COOH-terminal amino acid codons, specifying isoleucine, valine, and alanine (CVIA) of **a**-factor, were replaced with codons for alanine, methionine, and glutamine (CAMQ), respectively (*13*). This CAMQ sequence is present at the COOH-terminus of the α subunit of rabbit muscle glycogen phosphorylase kinase, which is farnesylated but not proteolyzed (*14*). Thus, the CAMQ extension on **a**-factor could either block the proteolytic processing of **a**-factor or reduce its efficiency and thus provide a sensitized screen for mutants defective in CAAX proteolysis. A yeast strain lacking wild-type **a**-factor genes and expressing this mutant **a**-factor was able to mate and produced nearly wild-type amounts of the pheromone (Fig. 1A). The autocrine arrest selection performed with this strain identified a mutation in a previously unknown gene *afc1-2* (**a**-factor converting enzyme) that is required for the processing of **a**-factor. The *afc1-2* mutation reduced mating efficiency and production of **a**-factor in strains with the CAMQ form of the **a**-factor gene. An *afc1-2* mutant with the wild-type **a**-factor gene had only a modest decrease in **a**-factor production (Fig. 1B). The residual **a**-factor produced in the *afc1* mutant indicated either that *afc1-2* was a leaky mutation or that a second protease capable of processing **a**-factor existed.

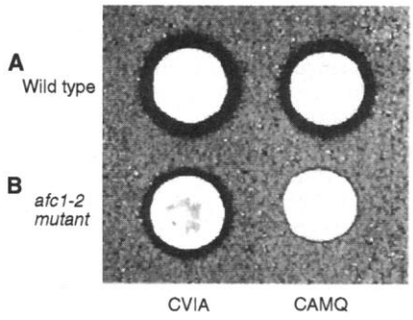

Fig. 1. Amount of biologically active **a**-factor in wild-type and *afc1-2* mutant strains depends on the amino acid sequence of the COOH-terminus of the **a**-factor gene. The wild-type CVIA sequence at the COOH-terminus of the *MFA1* gene was changed to CAMQ (*13*). Both forms of the gene were introduced in the strain deleted for the *MFA1* and *MFA2* **a**-factor genes. Levels of **a**-factor production were assayed by spotting samples of the cell suspension on a lawn of **a**-factor supersensitive *MATα sst2* cells (*30*). Released **a**-factor formed halos of growth-arrested *MATα* cells of sizes proportional to the amounts of **a**-factor processing. (**A**) Wild-type strain (JRY5312); (**B**) the *afc1-2* mutant (JRY5313).

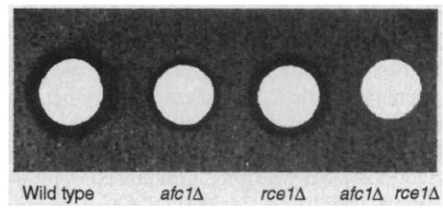

Fig. 2. Production of active **a**-factor depends on both Afc1p and Rce1p. Halo assays were performed on cell suspensions from isogenic strains differing as indicated. The MAT**a** strains used in the test were as follows: wild type (JRY5314), *afc1Δ::HIS3* (JRY5315), *rce1Δ::TRP1* (JRY5316), and *afc1Δ::HIS3 rce1Δ::TRP1* (JRY5317) (*12*).

The sequence of the *AFC1* gene, cloned by complementation from a recombinant library, revealed that the 453–amino acid Afc1 protein was likely to be a polytopic integral membrane protein. The COOH-terminal sequence of the protein (KKKN; K is Lys and N is Asn) matched the consensus KKXX sequence that mediates retrieval of endoplasmic reticulum proteins from the Golgi (*15*). Most importantly, Afc1p had a perfect match at position 297 to 301 to the HEXXH motif (H is His and E is Glu) of Zn-dependent metalloproteases and matched a larger consensus sequence characteristic of neutral Zn metalloproteases. To test the significance of this motif, we made two mutants by site-directed mutagenesis, each of which had one of the conserved histidines changed to alanine. Both mutations completely blocked the ability of the gene to complement the **a**-factor–processing defect of *afc1-2* mutants, establishing that this protease motif was essential for Afc1p function. A null allele of *AFC1* (*afc1Δ*) was made by replacing a part of the open reading frame by the selectable marker *HIS3* (*16*). Disruption of the *AFC1* gene showed that it was not required for viability, caused no mating defect in α cells, and affected **a**-factor production in **a** cells (Fig. 2).

The presence of a sequence motif suggestive of a Zn-dependent protease in Afc1p was surprising in that previous stud-

ies of the RACE activity of membrane fractions established that the activity was only slightly affected by the Zn chelator 1,10-phenanthroline. To determine whether *AFC1* encoded the enzyme responsible for the RACE activity, we compared membrane fractions from wild-type and *afc1*Δ mutants for their ability to cleave the COOH-terminal three amino acids from a prenylated CAAX peptide by two different in vitro assays (*17*). In both assays, the *afc1*Δ mutation resulted in a slight but reproducible decrease in RACE activity (Fig. 3). The decrease in activity caused by the *afc1*Δ mutation was similar in magnitude to the decrease in activity of wild-type extracts caused by chelation of Zn. Moreover, removal of Zn caused no further decrease in activity in the *afc1*Δ extracts. A simple explanation for these observations is that RACE activity is a composite of two different CAAX proteases: a Zn-dependent activity encoded by *AFC1* and a second protease encoded by a different gene. The active **a**-factor produced by an *afc1* mutant would presumably result from the second protease.

The *AFC1* sequence has no homologs in the yeast genome, indicating that residual **a**-factor processing was performed by a structurally distinct protease. To search for additional CAAX prenyl proteases, we used an *afc1* mutant strain to screen for mutations that would block the remaining **a**-factor production. In addition, multicopy libraries were used to screen for plasmids that, at high copy, could partially restore **a**-factor production in *afc1* mutants with the CAMQ form of **a**-factor. Both routes led to the identification and cloning of the same gene, *RCE1* for Ras and **a**-factor converting enzyme. The sequence of *RCE1* revealed no sequence homologies adequate to imply a function for the protein. As with *AFC1*, *RCE1* appeared to be a polytopic integral membrane protein, with a slight similarity to type IIb signal peptidases, which cleave signal sequences from proteins containing nearby lipid modifications. As a test of whether *RCE1* encodes or regulates a CAAX prenyl protease, a null allele of *RCE1* (*rce1*Δ) was constructed by replacing the entire open reading frame with the selectable marker *TRP1*. Disruption of the *RCE1* gene revealed that, like *AFC1*, *RCE1* was not essential for viability, had no effect on the mating ability of α cells, and caused only a modest decrease in **a**-factor production. The double mutant *rce1*Δ *afc1*Δ grew as well as wild type. Thus, CAAX proteolysis is not essential for viability. However, the double mutant was completely defective in the production of mature **a**-factor (Fig. 2). Therefore the functions of *RCE1* and *AFC1* are at least partially overlapping, and both proteins contribute to the production of mature **a**-factor.

As with *afc1* mutants, the *rce1* mutants showed reduced CAAX prenyl protease activity in two different in vitro assays (*17*) and was more defective than the *afc1* mutant. Moreover, the activity that remained in an *rce1*Δ mutant was completely blocked by removal of Zn, as expected for the Zn-dependent protease encoded by *AFC1* (Fig. 3). The double mutant had low levels of CAAX prenyl protease activity indistinguishable from background levels. Thus, *AFC1* and *RCE1* appeared to encode a pair of CAAX prenyl proteases that lack any sequence similarity but have overlapping substrate specificity.

These data implied that Rce1p was a CAAX protease; however, Rce1p could also be a regulator of the second CAAX protease. To determine whether *RCE1* was actually the structural gene for the second CAAX protease, we expressed a tagged *RCE1* gene from different strength promoters and tested whether different expression levels result in different levels of activity (*13*). Each promoter produced a different level of CAAX protease activity in an *afc1*Δ *rce1*Δ double mutant, and the amount of activity was linearly proportional with respect to the amount of protein, suggesting that Rce1p itself was the CAAX protease (*18*). Nevertheless, from these data one cannot exclude the formal possibility that Rce1p is a limiting noncatalytic subunit of a CAAX protease.

Yeast contain two Ras genes that are highly homologous to and functionally interchangeable with the Ras oncogenes of humans. The Ras genes of yeast are required for viability (*19*). Similarly, the role of Ras farnesylation is equally important in humans and yeast. We explored what role, if any, removal of the COOH-terminal amino acids from farnesylated Ras played in the function of the Ras protein by testing the phenotypes of Ras mutants in *afc1* and *rce1* mutant strains.

Like oncogenic alleles of human Ras, mutant alleles of yeast *RAS2* that block GTP hydrolysis cause extreme sensitivity to heat shock and to starvation (*20*). The heat-shock sensitivity of cells containing the *RAS2*^{val19} activated allele and null alleles of *RCE1* and *AFC1* was used to determine whether removal of the COOH-terminal tripeptide from farnesylated Ras2p affected the activity of Ras. The *rce1*Δ mutation suppressed the heat-shock sensitivity caused by activated Ras2p by a factor of about 100 (Fig. 4). Thus, a loss in CAAX proteolysis caused by *rce1*Δ reduced but did not eliminate Ras function. In contrast, the *afc1*Δ mutation caused only a modest effect on heat-shock sensitivity, and the *afc1*Δ *rce1*Δ double mutant was indistinguishable from the *rce1* single mutant.

In an independent test of the role of CAAX proteolysis, we evaluated whether loss of CAAX proteolysis would affect *RAS2* mutations that reduce Ras function in cells. The *ras2-23* allele is a partial loss-of-function allele that causes temperature-sensitive growth (*21*). The mutant grows well at 30°C, less well at 34°C, and cannot grow at all at 37°C. Deletion of *RCE1* rendered *ras2-23* mutants unable to grow at

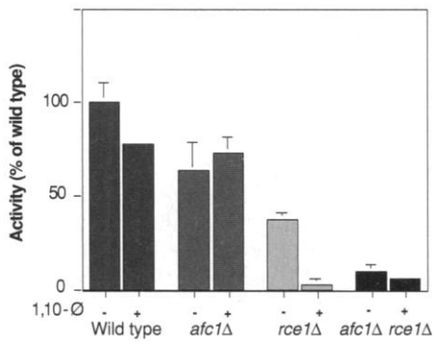

Fig. 3. Direct CAAX proteolysis assays. P100 membrane fractions were prepared from late logarithmic phase cultures of wild-type (JRY5314), *afc1*Δ::*HIS3* (JRY5315), *rce1*Δ::*TRP1* (JRY5316), and *afc1*Δ::*HIS3* *rce1*Δ::*TRP1* (JRY5317). The proteolytic activity was determined by measurement of the release of tritiated tripeptide from the farnesylated substrate (*17*). Zinc chelation experiments were performed in the presence of 4 mM 1,10-phenanthroline (1,10-Ø). The proteolytic activity was calculated relative to that of the wild-type strain in the absence of 1,10-Ø.

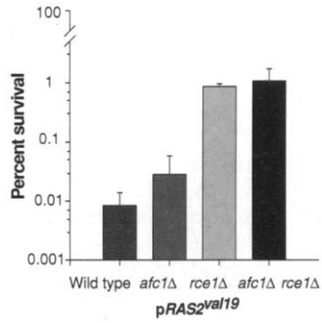

Fig. 4. Heat-shock sensitivity assay of Ras2p function. Yeast cells (*12*) containing the activated form of *RAS2* (*RAS2*^{val19}, pJR1040) were grown for 45 hours at 30°C in YM medium plus 2% glucose and 2% casamino acids (Difco). The cultures were heat-shocked for 90 s at 55°C in a PCR thermocycler (PTC100, MJ Research, Watertown, Massachusetts), and plated on selective medium for viability. Values are presented as the percent of viable cells relative to the culture before heat shock and are the average of duplicate cultures from two independent transformants. The error bars indicate the standard deviation. Strains carrying a plasmid with the wild-type *RAS2* gene (pJR1039) showed no decrease in viability in an analogous experiment.

34°C (Fig. 5). As in the previous assay, the *afc1* mutation had no detectable effect. Thus, CAAX proteolysis, mediated by Rce1p, plays a modulatory role on Ras2p, decreasing the function of Ras but not eliminating it.

Ras proteins are localized to the inner surface of the plasma membrane where they interface between signals from outside cells and the various response mechanisms inside cells. Agents or mutations that block prenylation of Ras block its membrane association and thereby block the function of Ras (*22*). Prenylation of other GTP-binding proteins also appears to be essential for their proper membrane localization and consequently their function (*23*). Therefore, we examined whether mutations in genes encoding CAAX prenyl proteases affected the subcellular localization of their substrates.

The green fluorescent protein (GFP) is a versatile protein module that can be fused to other proteins, enabling their localization by fluorescence microscopy (*23*). We used a fully bifunctional *GFP-RAS2* fusion gene to monitor the localization of Ras2p in wild-type and mutant cells. In this fusion protein, the GFP sequences are fused to the NH_2-terminus of the Ras protein, leaving the CAAX motif intact and available for posttranslational processing (*13*). In wild-type cells, the GFP-Ras2p was localized to the periphery of cells reflecting its association with the plasma membrane (Fig. 6), and cellular fractionation experiments confirmed that the fusion protein sediments with the P100 membrane fraction (*18*). There was still some GFP-Ras2p at the plasma membrane of the *rce1*Δ mutant (Fig. 6). However, a significant portion of the fusion protein was evident at internal locations, with the fluorescence outlining what appeared to be internal membranes. Thus, proteolytic processing of the CAAX motif of Ras2p contributed to the proper subcellular localization of Ras.

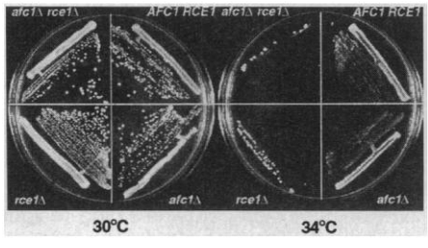

Fig. 5. Effect of the deletions of *RCE1* and *AFC1* genes on the temperature sensitivity of cells with the *ras2-23* allele (*21*). All strains are *ras1*Δ and have the *ras2-23* temperature-sensitive allele (JRY5318) combined with the following deletion alleles: *afc1*Δ::*HIS3* (JRY5319), *rce1*Δ::*TRP1* (JRY5320), and *afc1*Δ::*HIS3* *rce1*Δ::*TRP1* (JRY5321). Strains were grown for 3 days on rich yeast medium plates at 30°C and 34°C.

Our results provided most of the missing pieces in understanding the trio of posttranslational processing steps shared by prenylated proteins. Afc1p is an integral membrane prenyl protein protease with a metalloprotease sequence motif essential for its activity. Afc1p was responsible for some, but not all, processing of **a**-factor precursor in vivo, and in vitro it contributed about 35% of the prenyl-protein protease activity detectable with the peptide substrates used here. Rce1p also appears to be an integral membrane prenyl protein protease and to be responsible for processing the remaining **a**-factor precursor on the basis of the complete absence of **a**-factor processing in the double mutant. In vitro, Rce1p accounted for about 60% of the detectable activity. Rce1p and Afc1p are adequate to account for all **a**-factor proteolysis in vivo and all detectable prenyl protein protease activity in vitro. Thus, there may be only two prenyl protein proteases in yeast. However, the absence of any sequence similarity between these two proteins and the possibility of substrate specificity tempers this conclusion. There appears to be some difference in the substrate specificity of Afc1p and Rce1p. Both proteins contribute to the processing of **a**-factor precursor. However, Ras2p processing required Rce1p but did not require Afc1p.

The farnesyl transferases from all organisms examined are soluble cytoplasmic enzymes (*25*). In contrast, the carboxyl-methyltransferase Ste14p is membrane-associated and is likely to be an integral membrane protein (*26*). The sequences of Afc1p and Rce1p reported here indicate that they both are integral membrane proteins, consistent with the fractionation data. Thus, it appears that prenylation of precursor proteins leads to their association with a membrane where they are subsequently trimmed of their AAX extensions and methylated.

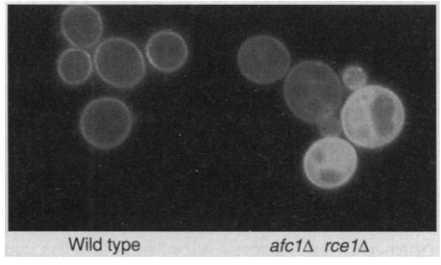

Fig. 6. In vivo fluorescence of GFP-Ras2p fusions. Wild-type (JRY5314) and *afc1*Δ::*HIS3*; *rce1*Δ::*TRP1* double mutant (JRY5317) strains carried a plasmid containing *RAS2* coding sequence fused in frame to *GFP* under the control of the constitutive promoter (*13*). Cells were grown overnight in selective medium, and GFP-Ras2p localization was examined directly with a Zeiss Axioskop microscope at a magnification of ×100. The fields were captured by a charge-coupled device camera with NorthernExposure software (Phase3 Imaging Systems).

Previously, it was assumed that prenylation led to membrane association of the protein at the plasma membrane where the last two processing steps occur. However, our data suggest a different hypothesis with interesting consequences. In the *rce1*Δ mutant cells, a portion of the GFP-Ras2p protein was associated with internal cellular membranes, indicating either that this fraction of the GFP-Ras2p was mislocalized, or that Ras2p associates first with internal membranes and then is transported to the plasma membrane. At present the second alternative seems the more likely one on the basis of the experiments indicating that the methyl-transferase of mammalian cells is in the endoplasmic reticulum–microsome fraction and that the microsome fraction contains a high-affinity binding site for prenylated peptides (*27*).

If prenylated Ras2p initially associates with internal membranes, then there must be some mechanism for moving it to the plasma membrane. To date there is no evidence that, once processed, Ras2p leaves the membrane fraction, although a short-lived, soluble processed form cannot be formally excluded. Thus, we are led to consider a model in which Ras2p may be transported from internal membranes to the plasma membrane by membrane transport vesicles. If this model is correct, then unlike secreted proteins, which are packaged in the lumen of transport vesicles, Ras2p would have to be associated with the external, cytoplasmic face of transport vesicles.

At face value, this model would seem to conflict with published reports that secretion of **a**-factor in yeast is independent of the secretory pathway (*28*). In these experiments **a**-factor secretion was not blocked upon a shift to the restrictive temperature in temperature-sensitive mutants that had mutations of the secretory pathway. The **a**-factor precursor undergoes the same COOH-terminal processing steps as Ras2p, and one would expect that both proteins reach the plasma membrane the same way, with **a**-factor subsequently released by the Ste6p transporter protein. However, because the Ste6p transporter itself requires the secretory pathway to reach the plasma membrane, there may be technical issues with the published work that merit reevaluation.

Because mutations in prenyl-transferases suppress the activated or transformed phenotype caused by mutant Ras proteins, a substantial effort has gone into developing inhibitors of prenyl-transferases as potential anticancer compounds (*6*). Some of these have proven effective in mice, but their value in humans has not been established (*29*).

The Rce1p family of prenyl protein pro-

teases offer a potentially better target for therapeutic intervention than the prenyl-transferases. Like the prenyl-transferases, blocking of Rce1p function reduces but does not eliminate Ras function. Moreover, null mutations in either *RCE1* or *AFC1* or in both genes cause no obvious growth or viability defects, whereas mutations in prenyl-transferases cause cells to be either growth defective or dead, depending on the mutation. Thus, if these results in yeast translate to human cells, inhibitors of CAAX proteases may be more valuable therapeutic agents than inhibitors of prenyl-transferases.

REFERENCES AND NOTES

1. W. R. Schafer and J. Rine, *Annu. Rev. Genet.* **26**, 209 (1992); S. Clarke, *Annu. Rev. Biochem.* **61**, 355 (1992).
2. F. R. Khosravi *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 6264 (1991); B. T. Kinsella and W. A. Maltese, *J. Biol. Chem.* **267**, 3940 (1992); M. C. Seabra, J. L. Goldstein, T. C. Sudhof, M. S. Brown, *ibid.*, p. 14497.
3. A. A. Finegold *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 4448 (1991); B. T. Kinsella, R. A. Erdman, W. A. Maltese, *ibid.*, p. 8934.
4. P. J. Casey, *Curr. Opin. Cell. Biol.* **6**, 219 (1994); J. E. Rothman and F. T. Wieland, *Science* **272**, 227 (1996).
5. J. F. Hancock, K. Cadwallader, C. J. Marshall, *EMBO J.* **10**, 641 (1991); K. Kato *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6403 (1992); A. D. Cox, M. M. Hisaka, J. E. Buss, C. J. Der, *Mol. Cell. Biol.* **12**, 2606 (1992).
6. F. Tamanoi, *Trends Biochem. Sci.* **18**, 349 (1993); N. E. Kohl *et al.*, *J. Cell Biochem. Suppl.* **22**, 145 (1995).
7. W. R. Schafer *et al.*, *Science* **249**, 1133 (1990).
8. L. Gutierrez, A. I. Magee, C. J. Marshall, J. F. Hancock, *EMBO J.* **8**, 1093 (1989); R. S. Marr, L. C. Blair, J. Thorner, *J. Biol. Chem.* **265**, 20057 (1990); A. Fujiyama and F. Tamanoi, *ibid.*, p. 3362; S. D. Perez, B. A. Gilbert, E. W. Tan, R. R. Rando, *Biochem. J.* **284**, 835 (1992); R. C. Stephenson and S. Clarke, *J. Biol. Chem.* **267**, 13314 (1992).
9. M. N. Ashby, D. S. King, J. Rine, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 4613 (1992); Y. T. Ma and R. R. Rando, *ibid.*, p. 6275; C. A. Hrycyna and S. Clarke, *J. Biol. Chem.* **267**, 10457 (1992); G. F. Jang, K. Yokoyama, M. H. Gelb, *Biochemistry* **32**, 9500 (1993).
10. A. Fujiyama, K. Matsumoto, F. Tamanoi, *EMBO J.* **6**, 223 (1987); C. A. Hrycyna, S. K. Sapperstein, S. Clarke, S. Michaelis, *ibid.* **10**, 1699 (1991).
11. A. Bender and G. F. Sprague, *Cell* **47**, 929 (1986).
12. A parent strain for the autocrine selection JRY5312 (*MAT***a** *HML***a** *HMR***a** *sst2*Δ *ade2-1 lys2 leu2-3, 112 ura3-1 STE3*::*GAL1-STE3*::*HIS3 mfa1*Δ::hisG *mfa2*Δ::hisG) was derived from W303-1a (JRY2334). For the halo and protease assays and heat shock and localization experiments, we used the following W303-1a derivatives: JRY5314 (*MAT***a** *his3 leu2-3,112 trp1 ura3-1*), JRY5315 (JRY5314 *afc1*Δ::*HIS3*), JRY5316 (JRY5314 *rce1*Δ::*TRP1*), JRY5317 (JRY5314 *afc1*Δ::*HIS3 rce1*Δ::*TRP1*). The temperature sensitivity experiments were performed in W303 derivatives: JRY5318 (*MAT***a**, *ade2-1 leu2-3,112 trp1, ura3-1 ras1*Δ::*HIS3 ras2-23*^{ts}), JRY5319 (JRY5318 *afc1*Δ::*HIS3*), JRY5320 (JRY5318 *rce1*Δ::*TRP1*), and JRY5321 (JRY5318 *afc1*Δ::*HIS3 rce1*Δ::*TRP1*). All strains were generated by standard genetic and molecular methods (*16*).
13. The plasmid carrying the wild-type (CVIA) (pJR1555) form of **a**-factor was created by insertion of the Xba I–Sal I fragment of the *MFA1* gene into pRS416 [R. S. Sikorsky and P. Hieter, *Genetics* **122**, 19 (1989)] *URA3 CEN* plasmid. The CAMQ variant (pJR1556) of *MFA1* was created by site-directed mutagenesis with the synthetic oligonucleotide 5′-GGGACCCAG-CATGTGCTATGCAATAGTTTCTGCG-3′. Wild-type *RAS2* (pJR1039) and dominant *RAS2*^{val19} (pJR1040) genes were contained in the 1.9-kb Cla I–Hind III fragment inserted into pRS316 vector. The *GFP-RAS2* fusion plasmid was created by subcloning the Bam HI–Sal I fragment containing the open reading frame of *RAS2* fused in frame to *GFP* under control of the glyceraldehyde-3-phosphate dehydrogenase (*GPD*) promoter {pJW192 [J. L. Whistler, thesis, University of California at Berkeley (1996)]}, into Bam HI– and Sal I–cut YEplac195 vector [R. D. Gietz and A. Sugino, *Gene* **74**, 527 (1988)]. The Rce1p-GFP fusions were constructed by engineering Xba I restriction sites flanking the *RCE1* open reading frame by polymerase chain reaction (PCR) amplification with synthetic oligonucleotides (5′-ACGTA-AAAATCTAGAAAGGGTTAT-3′ and 5′-AACAG-CAATGTCTAGATTCTCAAC-3′). The PCR product was subcloned into an Xba I site of the constructs containing ~1-kb promoter fragments in front of the GFP open reading fame [*GAL1*- pACA51, *ERG12*-pACA5, and *MEV1*-pACA1 (Acacia Biosciences)].
14. L. J. Heilmeyer *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 9554 (1992).
15. E. C. Gaynor, H. S. Te, T. R. Graham, M. Aebi, S. D. Emr, *J. Cell Biol.* **127**, 653 (1994).
16. A null allele of *afc1* was constructed by replacing the 218–base pair (bp) Bgl II fragment of *AFC1* (GenBank accession Z49617) with a 1764-bp Bam HI fragment containing the yeast *HIS3* gene. A deletion of the entire *RCE1* gene (GenBank accession Z49260) was created by PCR amplification of the *TRP1* coding sequence with synthetic oligonucleotides (5′-CCTTTGATGATTTTATTACCTTTATTT-TAAGTTACTAAAATATCGAGATTGTACTGAGAG-TGCACC-3′ and 5′-AAACAGTTGTCATGGAGC-CTTCCTGTAATTGCTCATAAGCATGGTGTGCGG-TATTTCACACCGC-3′). The PCR product was used directly in one-step gene replacement to create *rce1*Δ::*TRP1* [R. Rothstein, *Methods Enzymol.* **194**, 281 (1991)].
17. All assays were performed as described [M. N. Ashby and J. Rine, *Methods Enzymol.* **250**, 235 (1995)]. In direct CAAX proteolysis assays, 2 μg of membrane extract was incubated in 50 μl of 80 mM tris-HCl (pH 7.4), 1 mM phenylmethylsulfonyl fluoride (PMSF) reaction buffer with 0.5 nmol of a synthetic peptide KWD-PA(S-*trans*-*trans*-farnesyl)CV[4,5-^{3}H]IA (47 mCi/mmol) (K, Lys; W, Trp; D, Asp; P, Pro; A, Ala) and 20 μg of bovine serum albumin (BSA) for 1 hour at 37°C. After heat inactivation the RACE activity was measured by scintillation counting of cleaved tripeptide eluted from C_{18} cartridges (Analtech). In coupled proteolysis-methylation assays, 50 μg of protein was incubated with 1 nmol of Dansyl-WDPA(S-*trans*-*trans*-farnesyl)CVIA substrate and 1 nmol of [^{14}C]SAM (M, Met) in 100 mM tris-HCl (pH 7.4), 1 mM PMSF reaction buffer for 1 hour at 37°C. After addition of 50 μl of 1 M NaOH–1% SDS, the amount of newly formed hydrolyzed methyl esters was measured by scintillation counting.
18. V. L. Boyartchuck, M. N. Ashby, J. Rine, data not shown.
19. T. Kataoka *et al.*, *Cell* **40**, 19 (1985).
20. D. Broek *et al.*, *ibid.* **41**, 763 (1985).
21. H. Mitsuzawa, I. Uno, T. Oshima, T. Ishikawa, *Genetics* **123**, 739 (1989).
22. J. E. Buss, P. A. Solski, J. P. Schaeffer, M. J. MacDonald, C. J. Der, *Science* **243**, 1600 (1989); W. R. Schafer *et al.*, *ibid.* **245**, 379 (1989).
23. J. Inglese, W. J. Koch, M. G. Caron, R. J. Lefkowitz, *Nature* **359**, 147 (1992); C. A. Parish and R. R. Rando, *Biochemistry* **35**, 8473 (1996).
24. C. W. Cody, D. C. Prasher, W. M. Westler, F. G. Prendergast, W. W. Ward, *Biochemistry* **32**, 1212 (1993).
25. B. He *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 11373 (1991); L. E. Goodman *et al.*, *ibid.* **87**, 9665 (1990).
26. M. N. Ashby, P. R. Errada, V. L. Boyartchuk, J. Rine, *Yeast* **9**, 907 (1993); S. Sapperstein, C. Berkower, S. Michaelis, *Mol. Cell. Biol.* **14**, 1438 (1994).
27. R. C. Stephenson and S. Clarke, *J. Biol. Chem.* **267**, 13314 (1992); J. A. Thissen and P. J. Casey, *ibid.* **268**, 13780 (1993).
28. K. Kuchler and J. Thorner, *Curr. Opin. Cell Biol.* **2**, 617 (1990).
29. N. E. Kohl *et al.*, *Nature Med.* **1**, 792 (1995).
30. G. F. Sprague Jr., *Methods Enzymol.* **194**, 77 (1991).

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Crystal Structure of Protein Farnesyltransferase at 2.25 Angstrom Resolution

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Protein farnesyltransferase (FTase) catalyzes the carboxyl-terminal lipidation of Ras and several other cellular signal transduction proteins. The essential nature of this modification for proper function of these proteins has led to the emergence of FTase as a target for the development of new anticancer therapy. Inhibition of this enzyme suppresses the transformed phenotype in cultured cells and causes tumor regression in animal models. The crystal structure of heterodimeric mammalian FTase was determined at 2.25 angstrom resolution. The structure shows a combination of two unusual domains: a crescent-shaped seven-helical hairpin domain and an α-α barrel domain. The active site is formed by two clefts that intersect at a bound zinc ion. One cleft contains a nine-residue peptide that may mimic the binding of the Ras substrate; the other cleft is lined with highly conserved aromatic residues appropriate for binding the farnesyl isoprenoid with required specificity.

Posttranslational modification by a 15-carbon farnesyl isoprenoid is essential for the activity of a number of proteins that are central in the functioning of eukaryotic cells. These include Ras guanosine triphosphatases (GTPases), nuclear lamins, and several proteins involved in visual signal transduction (*1*). Addition of the farnesyl