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Postisoprenylation Protein Processing: CXXX (CaaX) Endoproteases and Isoprenylcysteine Carboxyl Methyltransferase

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I. Introduction

Multiple eukaryotic proteins that terminate with a "CXXX" sequence undergo a series of posttranslational processing reactions. The CXXX proteins terminate with the amino acids –C–X–X–X, where the "C" is a cysteine, the next two "X" residues are frequently aliphatic amino acids, and the third "X" can be one of several amino acids. Because the middle two X residues are often aliphatic, these proteins have also been referred to as "CaaX" proteins. The first posttranslational modification is the attachment of a farnesyl or geranylgeranyl isoprenoid lipid to the thiol group of the cysteine residue (the "C" of the CXXX sequence) by specific cytosolic protein isoprenyltransferases. Second, the last three amino acids of the protein (i.e., the –XXX) are removed by an isoprenylprotein-specific endoprotease; this step is dependent on the isoprenylation step and is thought to take place on the cytoplasmic surface of the endoplasmic reticulum

(ER). Third, the carboxyl group of the newly exposed isoprenylcysteine is methylated by an ER-associated isoprenylcysteine carboxyl methyltransferase. These three protein-processing steps have been studied most intensively for the yeast mating pheromone **a**-factor and the Ras proteins, a group of small GTP-binding proteins that mediate signal transduction and affect cell growth. However, these modifications occur in a large number of CXXX proteins with diverse biological functions, including the nuclear lamins, the γ subunits of heterotrimeric guanine nucleotide-binding proteins (G proteins), some phosphodiesterases, and other small GTP-binding proteins such as the Rac and Rho proteins.

The Rab family of proteins also undergoes C-terminal isoprenylation. These proteins are important for vesicular trafficking within cells and can be divided into subgroups that contain a Cys–Cys (CC) or a Cys–Xaa–Cys (CXC) motif at the C terminus. Both the CC and CXC subgroups are geranylgeranylated at both cysteines, but neither undergoes endoproteolytic processing. The CXC proteins, but not the CC proteins, are methylated at the C-terminal isoprenylcysteine.

The enzymes responsible for the isoprenylation of the CXXX proteins, protein farnesyltransferase and protein geranylgeranyltransferase I, have been thoroughly characterized in yeast and in higher organisms. These enzymes have attracted attention because the isoprenylation of the Ras proteins is essential, both for their targeting to the plasma membrane and for the ability of mutationally activated Ras proteins to produce a transformed phenotype in cultured cells (*1, 2*). Interest in the protein isoprenyltransferases has been further fueled by reports that protein farnesyltransferase inhibitors retard the growth of cancers (*3–9*) and might hold promise in the treatment of parasitic diseases (*10*). Reviews (*11, 12*) and chapters in this volume have summarized the progress in understanding the protein isoprenyltransferases.

This chapter summarizes progress in understanding the "postisoprenylation" processing of CXXX proteins—the endoproteolytic processing step and the carboxyl methylation step. For much of the 1990s, getting a handle on these steps was slow, at least when compared with the rapid progress in understanding the protein isoprenyltransferases, and there was uncertainty about their physiologic importance. More recently, however, there have been exciting advances in understanding these steps, making it an attractive time to review this area. In a landmark study, Boyartchuk. Ashby, and Rine (*13*) identified two genes from *Saccharomyces cerevisiae, RCE1* and *AFC1,* that are involved in the proteolytic removal of the "–XXX" from two farnesylated CXXX proteins (Ras2p and the precursor to the yeast mating pheromone **a**-factor). This breakthrough made it possible to mine the expressed sequence tag (EST) databases and clone the mammalian

TABLE I

PROPERTIES OF CXXX ENDOPROTEASE ACTIVITIES

Source	Substrate and kinetic data	Conditions affecting activity	Comments	Ref.
Yeast membranes	*N*-Acetyl-KSKTK-(*S*-farnesyl-C)-VIM; specific activity: 158.3 pmol/min/mg protein	Sensitive to sulfhydryl reagents, *N*-ethylmaleimide, $ZnCl_2$; not sensitive to serine and aspartyl proteinase inhibitors or to *o*-phenanthroline	Sensitivity to sulfhydryl reagents and lack of sensitivity to *o*-phenanthroline are similar to the properties of yeast Rcelp (*13, 33*)	Hrycyna and Clarke (*22*)
Yeast membranes; rat liver microsomes	Dansyl-WDPA-(*S*-farnesyl-C)-V[^{3}H]IA; specific activity: 974 ± 147 pmol/min/mg protein	Sensitive to zinc ions; not sensitive to PMSF, *o*-phenanthroline, unfarnesylated **a**-factor peptide, and a large panel of protease inhibitors	Lack of sensitivity to *o*-phenanthroline is similar to what Ashby and co-workers ultimately documented for yeast Rcelp (*13, 33*). Specificity for farnesylated peptides similar to human and yeast Rcel (*13, 15, 33*)	Ashby *et al.* (*24*)
Dog pancreatic microsomes; bovine liver microscomes	*N*-[^{3}H]Acetyl-(*S*-farnesyl-C)-VIS; K_m = 5.76 ± 0.71 μM; V_{max} = 251 ± 8.5 pmol/min/mg protein. *N*-[^{3}H]Acetyl-(*S*-farnesyl-C)-VI and *N*-[^{3}H]acetyl-(*S*-farnesyl-C)-V were also tested	Presence of D-farnesylcysteine in the peptide abolishes substrate activity, as does carboxyl methyl esterification of the peptide. Activity not inhibited by fivefold excess nonfarnesylated peptide	Specificity for farnesylated peptides similar to human and yeast Rcel (*13, 15, 33*)	Ma and Rando (*26*)
Calf liver microsomes	^{3}H-Labeled isoprenylated tripeptides in which the cysteine was modified with a 15-carbon farnesyl group, a 10-carbon geranyl group, or a 20-carbon geranylgeranyl group. Stereospecificity examined with isoprenylated peptides con-	Activity not inhibited by a wide variety of compounds known to inhibit serine proteases, cysteine proteases, metalloproteases, and aspartyl proteases. Substrates with 10-, 15-, and 20-carbon lipids were cleaved, but substrates with D-amino	Lack of sensitivity to *o*-phenanthroline is similar to yeast Rcelp (*13, 33*)	Ma *et al.* (*27, 28*)

Rat liver microsomes. Activity located mainly in ER, as judged by cell fractionation and enzymatic markers	ECB-(*S*-farnesyl-C)-VI[^{3}H]S (ECB, extended chain biotin); specific activity, 2.7 microunits/mg microsomes for VI[^{3}H]S; K_m = 1.1 μM; V_{max} = 7 pmol/min/mg protein. ECB-NPFRQRRFFC (GG)AI[^{3}H]L; specific activity, 0.13 microunits/mg microsomes for AI[^{3}H]L production, K_m = 2.5 μm, V_{max} = 0.4 pmol/min/mg protein	Sensitive to 4-(hydroxymercuri)-benzoate, leupeptin (partial), $ZnCl_2$; not sensitive to unfarnesylated peptide or to a wide panel of protease inhibitors including *o*-phenanthroline, PMSF, EDTA, aprotinin, pepstatin, DFP, and DTT	Sensitivity to sulfhydryl reagents and lack of sensitivity to *o*-phenanthroline are similar to yeast Rcelp (*13, 33*)	Jang *et al.* (*30*)
Bovine liver microsomal membranes. Activity solubilized with CHAPSO, eluted at high molecular weight end of gel-filtration column	*N*-[^{3}H]Acetyl-(*S*-farnesyl-C)-VIM; K_m = 0.65 ± 0.08 μM; V_{max} = 1.96 ± 0.07 nmol/min/mg protein	Major peak of activity not inhibited by *o*-phenanthroline or wide spectrum of protease inhibitors. Partially purified enzyme is sensitive to thiol regents, RPI (reduced peptide inhibitor), PCMB, N_α-tosyl-L-phenylalanine (TPCK), *N-tert*-butyloxycarbonyl-(*S*-farnesyl-L-cysteine)-chloromethyl ketone (BFCCMK), *N*-benzyloxycarbonyl-glycylglycyl-(*S*-farnesyl-L-cysteine) chloromethyl ketone (ZGGFCCMK)	Lack of sensitivity to *o*-phenanthroline is similar to one of the key properties of yeast Rcelp (*13, 33*)	Chen *et al.* (*32*)
Bovine brain microsomal membranes; optimal pH 9.0; solubilized with sodium deoxycholate; appeared to be a 480-kDa protein or complex	Dansyl-KSKTK-(*S*-farnesyl-C)-VIM; K_m = 1.0 μM; V_{max} = 14 pmol/min/mg protein	Insensitive to many protease inhibitors, including leupeptin, chymostatin, E-64, DFP, EDTA; insensitive to *o*-phenanthroline, $ZnCl_2$	Insensitivity to *o*-phenanthroline is similar to one of key properties of yeast Rcelp (*13, 33*)	Nishii *et al.* (*34*)
Pig brain membranes; appeared to be a 70-kDa protein, loosely membrane associated	Propionyl-GSP-(*S*-farnesyl-C)-[^{3}H]-VLM; K_m = 32.5 mM; V_{max} = 60.8 nmol/min/mg protein	Sensitive to *o*-phenanthroline, *p*-CMB, DTT, chymostatin, *N*-ethylmaleimide, $ZnCl_2$, $CuCl_2$, leupeptin (partial); insensitive to PMSF, leupeptin, E-64, pepstatin	Sensitivity to *o*-phenanthroline, is similar to one of key properties of yeast Afc1p (*13, 33*)	Akopyan *et al.* (*31*)
Activity purified fivefold from rat liver microsomes after solubilization in CHAPS	Tested 64 tripeptides [*N*-acetyl-(*S*-farnesyl-C)-X_1X_2] as competitive inhibitors of hydrolysis of *N*-acetyl-(*S*-farnesyl-C)-VI[^{3}H]S. Also tested radiolabeled peptides	Nonisoprenylated peptides do not inhibit enzymatic activity; pH optimum of ~6–6.5. Most potent inhibition is observed with farnesylated tripeptides that have most hydrophobic X_1X_2 dipeptide unit	Peptide hydrolysis results could reflect more than one enzymatic activity	Jang and Gelb (*29*)

orthologs for *RCE1* and *AFC1* (*14–17*). Similarly, the EST databases have been mined to identify the human isoprenylcysteine carboxyl methyltransferase (the ortholog of the *S. cerevisiae* gene *STE14*) (*18*). Strategies for defining the physiologic importance of the endoproteolysis and carboxyl methylation steps in higher organisms have also taken shape. Kim and co-workers (*14*) produced *Rce1* knockout mice and established that the *Rce1* gene is solely responsible for the endoproteolytic processing of the Ras proteins. In this chapter, we provide an overview of earlier work describing the endoproteolysis and carboxyl methylation enzymatic activities, as well as newer work on the identification of the gene products responsible for the endoproteolysis and carboxyl methylation steps in yeast and in mammals.

II. Characterization of Isoprenylprotein Endoprotease Activities

A. Early Characterizations of Isoprenylprotein Endoprotease Activities in Yeast and Mammals

During the 1980s, it became clear that certain yeast mating pheromones with a CXXX motif, including **a**-factor from *S. cerevisiae,* underwent modification with an isoprenoid lipid, endoproteolytic trimming of the C-terminal three amino acids, and carboxyl methylation. In 1988, Clarke and co-workers (*19*) reported that H-Ras, a mammalian CXXX protein, contained a C-terminal methyl ester and hypothesized that the processing of the mammalian Ras proteins and other CXXX proteins involved lipidation, endoproteolysis of the C-terminal three amino acids, and carboxyl methylation of the isoprenylcysteine. The existence of a specific proteolytic processing step received support in 1989, when Gutierrez *et al.* (*20*) provided evidence that the processing of mammalian Ras proteins involves the proteolytic release of the C-terminal three amino acids. At about the same time, Fujiyama *et al.* (*21*) provided direct evidence of the endoproteolytic release of the C-terminal three amino acids from yeast Ras2p.

Once it was clear that the processing of multiple CXXX proteins involved endoproteolytic processing, several groups sought to purify and characterize the enzymatic activity. Each group found that the relevant endoprotease activity was present in the membrane fractions. Table I summarizes the key findings from several of these studies.

Hrycyna and Clarke (*22*) identified three enzymatic activities from *S. cerevisiae* that were capable of removing the C-terminal three amino acids from the synthetic peptide *N*-acetyl-KSKTK-(*S*-farnesyl-C)-VIM. Two enzymatic activities were in the soluble fraction, and one was membrane associated. One soluble activity was due to carboxypeptidase Y, a vacuolar

enzyme (*22*), and the other was due to a vacuolar/Golgi endoproteinase related to rat metalloendopeptidase 24.15, an enzyme with specificity for cleavage after hydrophobic residues (*23*). Those soluble enzymatic activities were judged not to be relevant to the processing of isoprenylated CXXX proteins because neither was localized to the cytosol, where the isoprenylated substrates would be expected to be present, and because they did not require the presence of the isoprenyl group for proteolytic activity. On the other hand, the membrane-associated activity appeared to be a good candidate for the CXXX endoprotease. That activity was markedly inhibited by 1 m*M* *N*-ethylmaleimide and by 0.5 m*M* *p*-hydroxymercuribenzoate, suggesting that the membrane-associated enzyme might be a sulfhydryl-containing protease. The activity was unaffected by inhibitors of serine proteases such as phenylmethylsulfonyl fluoride (PMSF), dichloroisocoumarin, leupeptin, or the aspartyl protease inhibitor pepstatin. The enzyme activity was inhibited by 80% by 0.5 *M* zinc chloride, but was reduced by only 20% by 2.0 m*M* *o*-phenanthroline.

Ashby *et al.* (*24, 25*) also identified multiple enzymatic activities in yeast that were capable of removing the C-terminal three residues (–VIA) intact from the **a**-factor octapeptide dansyl-WDPA-(*S*-farnesyl-C)-VIA, demonstrating that the enzyme was an *endo*protease rather than a carboxypeptidase. The membrane-bound activity was unaffected by PMSF, *o*-phenanthroline, or nonfarnesylated **a**-factor peptide, but was inhibited by a high concentration of zinc ions. They also demonstrated that rat liver membranes contained an endoproteolytic activity that released the intact tripeptide–VIA from the farnesylated octapeptide.

Ma and Rando (*26*) reported an endoprotease activity from liver and pancreatic microsomes that cleaved the –XXX tripeptide from the synthetic tetrapetide substrate *N*-acetyl-(*S*-farnesyl-C)-VIS. The protease also cleaved the tripeptide *N*-acetyl-(*S*-farnesyl-C)-VI as well as the dipeptide *N*-acetyl-(*S*-farnesyl-C)-V (albeit with lower efficiency), but not the *N*-acetyl-(*S*-farnesyl-C)-amide or otherwise identical peptides containing cysteine in the D-configuration. These initial studies established that a dipeptide is required for enzymatic activity and that the cleavage at the scissile bond is stereoselective. Also, they demonstrated that endoproteolysis does not occur when the peptide contained a C-terminal methyl ester. The activity was not inhibited by a wide variety of compounds known to inhibit serine proteases, cysteine proteases, metalloproteases, and aspartyl proteases (*27*).

In a separate study, Ma *et al.* (*28*) tested the calf liver endoprotease activity against isoprenylated tripeptides (CVI) in which the cysteine was modified with a 15-carbon farnesyl group, a 10-carbon geranyl group, or a 20-carbon geranylgeranyl group. All three were cleaved, demonstrating broad substrate specificity with respect to isoprenoid side chains. In contrast,

nonisoprenylated derivatives (the tripeptide CVI or the *tert*-butylthiol derivative of CVI) were not measurably processed. In a separate study, Jang and Gelb (*29*) found that replacement of the farnesyl group with a straight-chain hydrocarbon (*n*-pentadecyl) only modestly affected the endoprotease activity.

Further experiments by Ma *et al.* (*28*) were designed to examine the stereospecificity of the endoprotease for each of the amino acids within the CXXX sequence. The L-D-L and L-L-D analogs of the *N*-acetyl-(*S*-farnesyl-C)-VI tripeptide series were poor substrates for the endoprotease, as were tripeptides containing more than one D amino acid (D-D-D, D-D-L, and L-D-D). The stereospecificity of the X_3 position of CXXX sequence was also explored; only the L-L-L-L derivative was a good substrate for the protease, although the L-L-L-D derivative was processed minimally.

In 1993, Jang *et al.* (*30*) reported the presence of an isoprenyl-specific endoprotease from rat liver microsomes. The activity released a C-terminal tripeptide from the synthetic isoprenylated peptides ECB (extended chain biotin group)-NPFRQRRFF-(*S*-geranylgeranyl-C)-AI[^{3}H]L and ECB-(*S*-farsnesyl-C)-VI[^{3}H]S. A variety of experiments suggested that ^{3}H-labeled dipeptides were produced from tripeptides by secondary proteolysis. Non-isoprenylated peptides at concentrations 10- to 100-fold higher than those of the isoprenylated substrates did not affect the release of the tripeptide. Percoll density fractionation of rat liver membranes indicated that the endoprotease was localized mainly in the ER (in fractions containing the ER marker glucose-6-phosphatase). Enzymatic activity was inhibited by less than 20% by a large panel of inhibitors of serine, cysteine, and aspartic acid proteases and zinc metalloproteases.

B. Solubilization and Partial Purification of Enzymatic Activities

Several groups attempted to purify the isoprenylprotein endoprotease activity, but none was successful in purifying a protein to homogeneity. Akopyan *et al.* (*31*) purified an activity approximately 100-fold from the microsomal fraction of pig brain membranes by chromatography on DEAE Trysacryl M and Sephacryl S-200. The activity, which appeared to have a mass of about 70 kDa, cleaved [^{3}H]VLM from propionyl-GSP-(*S*-farnesyl-C)-[^{3}H]VLM. The activity was strongly inhibited by *p*-chloromercuribenzoate (PCMB), *N*-ethylmaleimide, chymostatin, zinc chloride, and *o*-phenanthroline and thus appeared to be distinct from the previously studied activities.

Chen *et al.* (*32*) partially purified a CXXX endoprotease activity about 10-fold from bovine liver microsomal membranes by solubilization in

CHAPSO 3[(3-cholamidopropyl)dimethylammonio]-2-hydroxylpropane sulfonic acid] and chromatography on Resource Q and Superose 12. They identified two activity peaks after fractionating the solubilized protein on a gel-filtration column. A major peak (with a molecular mass greater than 600 kDa) was not inhibited by *o*-phenanthroline, while a minor peak of activity (with a molecular mass of ~60 kDa) was inhibited by *o*-phenanthroline. The major peak shared most of the properties of the endoprotease in crude microsomal preparations but, unlike the activity described by Akopyan *et al.* (*31*), was not inhibited by chymostatin, dithiothreitol (DTT), or *o*-phenanthroline. The *o*-phenanthroline-sensitive minor peak was not extensively characterized. However, now that Boyartchuk and co-workers have demonstrated the existence of two different yeast CXXX endoproteases (Rce1p and Afc1p) with different sensitivities to *o*-phenanthroline (*13, 33*), it seems possible that the activities within the major and minor peaks produced by Chen *et al.* (*32*) represented complexes containing the *RCE1* and *AFC1* gene products, respectively.

Nishii *et al.* (*34*) purified an endoprotease activity 104-fold from bovine brain microsomes with Sepharose CL-6B gel filtration and DE-52 chromatography. Sodium deoxycholate and sodium cholate solubilized the activity, whereas other detergents (Lubrol PX, Triton X-100, Nikkol, *n*-heptyl-β-thioglucoside, and *n*-dodecyltrimethylammonium bromide) were less effective. The molecular mass of the protein was judged to be 480 kDa by gel filtration on a Sepharose CL-6B column. The partially purified activity cleaved the farnesylated peptide dansyl-KSKTK-(*S*-farnesyl-C)-VIM with a K_m of 1.0 μM and a V_{max} of 14 pmol/min/mg protein. The optimal pH of this activity was 9.0. Neither EDTA nor inhibitors of serine proteases, cysteine proteases, aspartate proteases, and aminopeptidases blocked enzymatic activity. However, the activity was inhibited by *o*-phenanthroline and zinc chloride. Interestingly, *m*- and *p*-phenanthroline (nonchelating isomers of *o*-phenanthroline) were also effective inhibitors, suggesting that the inhibition by *o*-phenanthroline might have nothing to do with its chelating action.

The various efforts to characterize and purify the isoprenylprotein endoprotease activity are confusing because there are abundant and significant discrepancies in the key properties of the enzymatic activity (e.g., sensitivities to inhibitors, molecular weight). Almost all these differences remain unexplained. Some of the differences could relate to differences in animal species or the tissue that was examined. Also, it is possible that the differences relate to the existence of more than one CXXX endoprotease (*13, 33*). In any case, now that two endoproteases have been cloned and can be overexpressed in cell culture systems, it should be possible to characterize their biochemical properties in more detail. Also, it is now possible to

characterize CXXX endopeptidase activities from mammalian cells that lack either the *AFC1* or *RCE1* gene products (*14*).

C. Development of Endoprotease Inhibitor Compounds

The laboratory of R. Rando pioneered the development of specific CXXX endoprotease inhibitors (*27, 28, 32, 35*). Soon after it became clear that the endoprotease activity (or activities) trims CXXX proteins after the farnesylcysteine residue, Ma and co-workers (*28*) tested the ability of various farnesylcysteine derivatives to block the endoprotease activity. In the initial series of experiments, they demonstrated that an aldehyde analog, *N-tert*-butyloxycarbonyl-*S*-farnesylcysteine aldehyde, was an effective competitive inhibitor of the endoprotease. In an attempt to identify more potent inhibitors, they prepared farnesylcysteine derivatives that contained several amino acid residues. The potency of these compounds increased as the number of amino acids in the analog increased from two to four. *N-tert*-Butyloxycarbonyl-(*S*-farnesyl-C)-ψ(CH_2-NH)-VIM (also designated RPI, for reduced peptide inhibitor) inhibited the calf liver endoprotease activity with a K_i of 86 nM. As described further below, RPI inhibits the recombinant human RCE1 enzyme (*15*).

In a subsequent study, Chen *et al.* (*32*) identified structurally distinct compounds that irreversibly inhibited the endoprotease activity. In characterizing a bovine liver endoprotease, they found that N_α-tosyl-L-phenylalanine chloromethyl ketone (TPCK) was an irreversible inhibitor; this irreversible inhibition could be blocked with the reversible inhibitor RPI. To develop more potent irreversible inhibitors, they synthesized and tested farnesylcysteine analogs of the chloromethyl ketone inhibitors: BFCCMK [*N-tert*-butyloxycarbonyl-(*S*-farnesyl-C)-chloromethyl ketone] and ZGGFCCMK [*N*-benzyloxycarbonylglycylglycyl-(*S*-farnesyl-C)-chloromethyl ketone]. The former compound exhibited a 15-fold increase of the second-order rate constant (K_{inh}/K_i = 1164 M^{-1} min^{-1}) compared with that of N_α-tosyl-L-phenylalanine chloromethyl ketone (K_{inh}/K_i = 77 M^{-1} min^{-1}), and the K_i of its inhibitor–enzyme complex was 30 μM compared with 1.1 mM for N_α-tosyl-L-phenylalanine chloromethyl ketone.

More recently, Chen (*36*) has reported the development of new chloromethyl ketone inhibitors. One of these, UM96001 (*N-tert*-butyloxycarbonyl-2 amino-DL-hexadecanoyl-chloromethyl ketone), was based on the structure of BFCCMK, but the farnesyl group was replaced with a dodecyl group, and the thioether unit (which is chemically labile) was replaced with a methylene unit. The latter modification provided more chemical stability as well as increased cell permeability. As discussed in more detail below, UM96001 was reported to block the growth of Ras-transformed rodent

and human cell lines (*36*). It is important to point out, however, that much more work is required to document the specificity of these compounds for the CXXX endoproteases.

D. Influence of –XXX Sequence on Endoproteolysis

Jang and Gelb (*29*) tested the ability of a panel of 64 different tripeptides [*N*-acetyl-(*S*-farnesyl-C)-X_1X_2] to compete for the CXXX endoprotease-mediated hydrolysis of *N*-acetyl-(*S*-farnesyl-C)-VI[^{3}H]S. Their studies indicated that the endoprotease activity prefers large hydrophobic residues in the X_1 and X_2 positions. When X_1 was a large hydrophobic residue, potent inhibition was observed when X_2 was hydrophobic; less inhibition was seen when X_2 was more hydrophilic (e.g., glutamine or serine); and little if any inhibition was observed when X_2 was anionic (e.g., aspartate). Inhibition was also modest when both X_1 and X_2 were small hydrophobic residues, or when both were hydrophilic, uncharged residues. Tripeptides with aspartate in the X_2 position were weak inhibitors, regardless of what residue was in the X_1 position; tripeptides with lysine and arginine were tolerated in the X_1 position, as long as X_2 was a large hydrophobic residue. They also analyzed hydrolysis of tripeptides directly with radiolabeled tripeptides and tetrapeptides, and reached similar conclusions, except that the radiolabeled peptides containing arginine were poor substrates.

Kato and co-workers (*2*) analyzed posttranslational modifications with K-Ras4B constructs with a number of different amino acid substitutions in the CXXX sequence. Changing the wild-type –VIM sequence to –VYM principally affected the postisoprenylation processing steps. When the –VYM mutant was expressed in NIH 3T3 cells, a significant percentage of the protein appeared to be farnesylated but not further processed (i.e., no endoproteolysis or carboxyl methylation). It is difficult to understand why the –VYM mutant exhibited defective postisoprenylation processing, given that both X_1 and X_2 were hydrophobic amino acids.

Heilmeyer and co-workers (*37*) have demonstrated that the α and β subunits of phosphorylase kinase, which terminate in CAMQ and CLVS, respectively, are farnesylated but not further processed in muscle tissue. The absence of endoproteolytic processing in these proteins is mysterious, especially because they contain hydrophobic amino acids in the X_1 and X_2 positions. Jang and Gelb (*29*) analyzed the hydrolysis of a radiolabeled CLVS peptide in an *in vitro* system and found that it was a good substrate for the rat microsomal enzyme activity.

Ma and Rando (*38*) also explored the endoproteolysis of isoprenylated peptide *N*-acetyl-(*S*-farnesyl-C)-RPQ and *N*-acetyl-(*S*-geranylgeranyl-C)-RPQ modeled after an isoprenylated delta virus CXXX protein. This pep-

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S. cerevisiae     MFDLKTILDHPNIPWKLIISGFSIAQFSFESYLTYRQYQKLSETKLPPVLEDEIDDETFHKSRNYSR  67
S. pombe          MSPGLCFLKEISVIQATPKPTTRSFANCCKMGILQHLMHILDIPGFPWKIVIAGFSIGKYAWDLYLRRRQVPYLLREKPPAILAEHVDEKKYQKALSYAR  100
E. nidulans       0
E. tenella        0
D. discoideum     0
N. crassa         LARFLDRPLFPWKKLIIGFSLANYFIEGFLGYRQYQVLKKTKVPKVLEHEVSQEVPDKSQAYGR  64
A. thalina        0
G. max            0
B. malayi         0
D. melanogaster   0
C. elegans        MDASCLFKALLATNWALFLWDQYITFRQYKAHKNAVKRPNEVKELIGEEDYKKARDYKI  59
M. musculus       0
H. sapiens        MGMWASLDALWEMPAEKRIFGAVLLFSWTVYLWETFLAQRQRRIYKTTTHVPPELGQIMDSETFEKSRLYQL  72
H. pylori         MLDIWIDMIICIFYLLFFTTPYIVGDILQLKFIRQKLCEKPVLLPQKDYEEAGNYAI  57
B. subtilis       MRKWIAAAGLAYVLYGLFFYWYFFLSGDSAIPEAVKGTQADPASFMKPSELAVAEQYSN  59

S. cerevisiae     AKAKFSIFGDVYNLAQKLVFIKYDLFPKIWHMAVS---LLNAVLPVRFHMVSTVAQSLCFLGLLSSLSTLVDLPLSYYSHFVLEEKFGFNKLTVQLWITD  164
S. pombe          DKSWFSTIVSTFTLAVDLLIIKYDGLSYLWNITKFPWMDKLAASSSRFSLSTSITHSCVFMFGLTLFSRLIQIPFNLYSTFVIEEKYGFNKSTLKIFVID  200
E. nidulans       EEKFGFNKQTVKLWVSD  17
E. tenella        AAASTFNKKTAILFIKD  17
D. discoideum     0
N. crassa         AKAKFEFFSGIYGQIQNILFYQ DVLEKLWSFAGN---LLVRFAPARF  109
A. thalina        MAIPFMETVVGFMIVMYIFETYLDLRQLTALKLPTLPKTLITD----------------LPFSLYSTFVIESRHGFNKQTIWMFIRD  71
G. max            0
B. malayi         0
D. melanogaster   0
C. elegans        DNHLFGFFHSWFNQLLLTAQLIGCYYPFLWYATASYPLHVAVFLSINSIIETIID----------------LPWDLYSTFIIEDAHGFNKQTIGFYFVD  142
M. musculus       0
H. sapiens        DKSTFSFWSGLYSETEGTLILLFGGIPYLWRLSGR------FCGYAGFGPEYEITQSLVFLLLATLFSALTGLPWSLYNTFVIEEKHGFNQQTLGFFMKD  166
H. pylori         RKMQLSIISQILDGVIFAGWVFFGLTHLEDLTHYLNLPETLGYLVFALLFLAIQS--------------VLALPISYYTTMHLDKEFGFSKVSLSLFFKD  143
B. subtilis       VKNFLFFIGVPLDWFLFFVLIVSGVSKKIKKWIEAAVPFRFLQTVGFVFVLSLIT--------------TLVTLPLDWIGYQVSLDYNISTQTTASWAKD  145

S. cerevisiae     MIKSLTLAYAIGGPILYLFLKIFDKFPTDFLWYIMVFLFVVQILAMTIIPVFIMPMFNKFTPLEDGELKKSIESLADRVGFPLDKIFVIDGSKRSSHSNA  264
S. pombe          LLKELSLGGLLMSVVVGVFVKILTKFGDNFIMYAWGAYIVFGLILQTIAPSLIMPLFYKFTPLENGSLRTQIEELAASINFPLKKLYVIDASRRSTHSNA  300
E. nidulans       MLKGQMLGIVLGAPIISAVLKIVQKIGTSCFYYLWLFGVFVQVFAITIYPIAILPLFNKLSPLEPGAIKTGVENLAKKLNFPLQELHVIDGSKRSAHSNA  117
E. tenella        KLLGLGLTAAIGAPILCAVNWLIKWGGENFHFWLWGFSVLTTFGMIVIYPNFIAPLFNKFKVLGDRELRSKIDALAEQLNFPLCEVYEMDGSKRSSHSNA  117
D. discoideum     DTIFVVDNSKRDGHMNA  17
N. crassa         109
A. thalina        MIKGTFLSVILGPIVAAIIFIVQKGGPYLAIYLWAFMFILSLVMMTIYPVLIAPLFNKFTPLPDGDLREKIEKLASSLKFPLKKLFVVDGSTRSSHSNA  171
G. max            MSFTFGLSIVMMTLYPVLIAPLFNKFTPLPDGQLREKIEKLASSLNYPLKKLFVVDGSTRSSHSNA  66
B. malayi         TVYPEFIAPLFDKYVPLPESELKQKIEKLARSLNFPLKKILVVYGSKRSAHSNA  64
D. melanogaster   FIWLWIFTGVISLVLLTLYPIFIAPLFDKYTPLEKGALRQSIEDLAASLKFPLTKLFVV  59
C. elegans        KIKKMLVGFALTMPIVYGIEWIIVNGGPYFFVYIWLFVSVVVLLMTIYPTFIAPLFDKYFPLPDGDLKTKIEQLAASLSYPLTELYVVNGSKRSAHSNA  242
M. musculus       0
H. sapiens        AIKKFVVTQCILLPVSSLLLYIIKIGGDYFFIYAWLFTLVVSLVLVTIYADYIAPLFDKFTPLPEGKLKEEIEVMAKSIDFPLTKVYVVEGSKRSSHSNA  266
H. pylori         FFKGLLLTLGVGLLLIYTLIMIIEHVEH--WEISSFFVVFVFMILANLFYPKIAQLFNQFTPLNNRDLESQIESMMDKVGFKSQGIFVMDASKRDGRLNA  241
B. subtilis       QVISFWISFPIFTLCVLVFYWLIKRHEKKWWLYAWLLTVPFSLFLFFIQPVIIDPLYNDFYPLKNKELESKILELADEANIPADHVYEVNMSEKTNALNA  245

S. cerevisiae     YFTCLPFTSKRIVLFDTLVNSNS------------------------------TDEITAVLAHEIGHWQKNHIVNMVIFSQLHTFLIFSLFTSI  328
S. pombe          FFYCLPWNKG-IVLFDTLVKNHT------------------------------EPELIAILGHELGHWYMSHNLINTIIDYGMSLFHLFLFAAF  363
E. nidulans       121
E. tenella        YFYGFWRWKR-IVIFDTLLHLPH------------------------------DQILAIL  146
D. discoideum     YFYGLFGPKR-IVLYDTLVN LD------------------------------KEELLAVMGHEFGHYKMSHTLKQMLLVQVHLVTLLYAFSLL  80
N. crassa         109
A. thalina        YMYGFFKNKR-IVLYDTLIQQCK-----------------NED------------------EIVAVIAHELGHWKLNHTTYSFIAV-------------  221
G. max            YMYGFFKNKR-IVLYDTLIQQCK-----------------DDE------------------EIVAVIAHELGHWKLNHTVYTFVAMQILTLLQFGGYTLV  130
B. malayi         YLYGFWNNKR-IVLYDTLFGEEMRAKLKETACFPTENEEKSYDKG--DEEIKERKLGMQDDEVLAVLGHEFGHWALWHAVIQLFFAEINLLLLLAIFAKF  161
D. melanogaster   59
C. elegans        YMYGFWKNKR-IVLYDTLLSGAEKEKVHELYVAAGEKIEETEN-------DKKR--GMNNDEVVAVLGHELGHWALWHTLINLVITEVNLFFSFAVFGYF  332
M. musculus       13
H. sapiens        YFYGFFKNKRIVLFDTLLEEYSVLNKDIQEDSGMEPRNEEEGNSEEIKAKVKNKKQGCKNEEVLAVLGHELGHWKLGHTVKNIIISQMNSFLCFFLFAVL  366
H. pylori         YFGGLGKNKR-VVLFDTLISKVG------------------------------TEGLLAILGHELGHFKNKDLLKNLGIMGGLLALVFALIAHL  304
B. subtilis       YVTGIGANKR-IVLWDTTENKLD------------------------------DSEILFIMGHEMGHYVMKHVYIGLAGYLLVSLAGFYVIDKL  308

S. cerevisiae     YRNTSFYNTFGFFLEKSTGSFVDPVITKEFPIIIGFMLFNDLLTPLECAMQFVMSLISRTHEYQADAYAKKLGYK-QNLCRALIDLQIK----------N  417
S. pombe          IRNNSLYTSFNFITEK-------------PVIVGLLLFSDALGPLSSILTFASNKVSRLCEYQADAFAKQLGYA-KDLGDGLIRIHDD----------N  438
E. nidulans       121
E. tenella        146
D. discoideum     INDDQLYQQFGFVSSKD-------------SVLVGLTLFMFLYSPIDRIFSLLINIFSRKYEFQADDFAVELGFL-N--SNHLFKLHFK----------E  154
N. crassa         109
A. thalina        ----------------------------------QHTVIPLQHLVSFGLNLVSRAFEFQADAFAVKLGYA-KDLRPALVKLQVREDNNRTQTE  281
G. max            RNSADLYRSFGFDTQP-------------VLI-GLIIFQHTVIPLQQLVSFGLNLVSRSFEFQADGFAKKLGYA-SGLRGGLVKLQE----------EN  204
B. malayi         YQSTSLFHGIWVSMI  176
D. melanogaster   59
C. elegans        YKWEALYQGFGYHDTP-------------PVIGMMLIFQFVLALYNQLASICMVIHSRSAEFGADEFAANLGHG-ENLIGALTKLGV----------DN  407
M. musculus       IGRRELFAAFGFYDSQP-------------TLIGLLIIFQFMF-----------------------------------T  XG----------S  50
H. sapiens        IGRKELFAAFGFYDSQP-------------TLIGLLIIFQFIFSFYNEVLSFQLTVLSRRFEFQADAFAKKLGKA-KDLYSALIKLNKD----------N  442
H. pylori         P--PLVFEGFNVSQTP--------------ASLITILLLFLPVFSFYAMPLIGFFS---RKNEYNADKFGASLSSK-ETLAKALVSIVNE----------N  375
B. subtilis       YKRTVRLTRSMFHLEGR-------------HDLAALPLLLLLFSVLSFAVTPFSNAVSRYQENKADQYGIELTENREAAVKTFQDLAVT----------G  385

S. cerevisiae     LSTMNVDPLYSSYHYSHPTLAERLTALDYVSEKKKN  453
S. pombe          LSPLEFDSLYTSYYHSHPILVDRLNAIDYTTLKKNN  474
E. nidulans       121
E. tenella        146
D. discoideum     LGCLVYDPLYSAYHHSHPTLVERSNNIDKKVALYKLKNK  193
N. crassa         109
A. thalina        LSAMNTDPLYSAYHYSHPPLVERLRAIDGEDKKTD  316
G. max            LSAMN  209
B. malayi         176
D. melanogaster   59
C. elegans        LSMPINDSLYSWCTHTHPPVVERVAAVRAFQANNN  442
M. musculus       VFLPN------------SPEPQI  61
H. sapiens        LGFPVSDWLFSMWHYSHPPLLERLQALKTMKQH  475
H. pylori         KAFPYSHPFYVFLHFTHPPLLERLKALDYEIE  407
B. subtilis       LSQVDPPVLVKIFRGSHPSIMERIQHAEKEENAPEHQDADK  426
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tide was referred to as a "non-CaaX" peptide because it has a nonaliphatic amino acid, arginine, in the X_1 position. They found that liver microsomes released an intact –RPQ peptide from the farnesylated substrate, and that none of the standard group-specific protease inhibitors (e.g., antipain, aprotinin, bestatin, chymostatin, EDTA, leupeptin, pepstatin, *o*-phenanthroline) affected the reaction. This result was similar to the properties of the endoprotease that processed peptides with conventional CXXX sequences (*26, 28*). However, the farnesylcysteine-based peptide inhibitors (e.g., RPI), which are potent inhibitors of the endoproteolysis of typical CXXX peptides, such as *N*-acetyl-(*S*-farnesyl-C)-VIM, did not block the hydrolysis of *N*-acetyl-(*S*-farnesyl-C)-RPQ or *N*-acetyl-(*S*-geranylgeranyl-C)-RPQ. This result raised the possibility of an additional endoprotease activity that participates in the hydrolysis of isoprenylated CXXX peptides with hydrophilic amino acids.

III. Identification of Two Yeast Genes, *AFC1* (*STE24*) and *RCE1*, Involved in Endoproteolytic Processing of Isoprenylated CXXX Proteins

The fact that the "CXXX endoprotease" was refractory to biochemical purification prompted interest in a genetic screen for the responsible gene. Yeast defective in the farnesylation or methyl esterification of **a**-factor are sterile, and it seemed likely that a defect in the middle step of **a**-factor processing, the endoproteolysis step, would also produce sterility. Because methods for identifying sterile yeast mutants were well established, it might reasonably have been predicted that the identification of "**a**-factor endopro-

FIG. 1. Amino acid sequence alignment of yeast *AFC1* (*STE24*) and orthologs in other species. GenBank accession numbers for the full-length sequences: human, AAC68866; *S. cerevisiae,* P47154; *H. pylori,* AAD06444; *C. elegans,* CAB03839; *S. pombe,* Q10071; *A. thaliana* AAB61028. GenBank EST sequences: *D. melanogaster,* AA567990; *B. malayi,* AA585633; *E. tenella,* A1757461; *E. nidulans,* AA965341; mouse, AA498259; *G. max,* AI759796; *D. discoideum,* AUO37739; *N. crassa,* AI330202; *B. subtilis,* NC000964. The HEXXH sequence (residues 297–301 in the *S. cerevisiae* sequence) is conserved in all species. Protein sequences were aligned with Macvector 6.5, using a CLUSTALW alignment. Pairwise alignment was performed with a BLOSUM30 matrix, and multiple alignment was performed with a BLOSUM series matrix. The resulting alignment output was saved as a Word-98 document, and additional identities were shaded. The document was then imported into Illustrator.

tease" would have been a trivial task. Such optimism would, of course, have rested on the existence of a single CXXX endoprotease gene.

As it turned out, two yeast genes are involved in the removal of the C-terminal three amino acids from **a**-factor, and a genetic approach to the identification of responsible genes was vexatious. However, Boyartchuk and co-workers (*13*) overcame the obstacles and identified two genes, *RCE1* and *AFC1*, that are required for the endoproteolytic processing of **a**-factor as well as at least one other CXXX protein, Ras2p. Afclp (for **a**-factor convertase) is a zinc protease that participates in the endoproteolytic processing of **a**-factor, while Rce1p (for Ras and **a**-factor-converting enzyme) participates in the processing of both Ras2p and **a**-factor. As outlined below (see Section IV), *AFC1* has also been identified as *STE24*, a gene involved in the N-terminal processing of **a**-factor (*39*). In this chapter, to avoid confusion, we have frequently designated the gene *AFC1* (*STE24*) [or *STE24* (*AFC1*)].

The key to discovering *RCE1* and *AFC1* rested on the identification of an **a**-factor substrate that was recognized by only one of the two endoproteases. Heilmeyer and co-workers (*37*) had shown that the α subunit of rabbit muscle glycogen phosphorylase kinase (a CXXX protein that terminates in –AMQ) is farnesylated but not further processed. Boyartchuk and colleagues (*13, 33*) produced farnesylated peptides that terminate in –AMQ and found that they were not endoproteolytically processed by *o*-phenanthroline-treated yeast microsomes. However, when the –AMQ sequence was incorporated into an **a**-factor (*MFA1*) construct and transformed into yeast, biologically active **a**-factor was produced and secreted! That observation suggested the possibility of more than one CXXX endoprotease in yeast and provided the basis for a sensitized genetic selection for CXXX endoprotease mutants. Yeast expressing –AMQ **a**-factor were mutagenized, and an autocrine arrest selection strategy was used to isolate sterile mutants (*13*). This approach resulted in the identification of a sterile yeast mutant (*afc1*) that was defective in processing the –AMQ form of **a**-factor. Of note, the *afc1* mutation had minimal effects on pheromone production in yeast that expressed wild-type **a**-factor, suggesting the existence of a second enzymatic activity capable of processing **a**-factor.

The *AFC1* gene was cloned by complementation (*13*). The gene specifies a 453-amino acid protein (Fig. 1) with multiple predicted transmembrane domains indicative of a polytopic integral membrane protein (Fig. 2A). At amino acids 297–301, Afclp exhibits a perfect match with the HEXXH (H, His; E, Glu) motif of a group of zinc-dependent metalloproteases and shares other sequence similarities with neutral zinc metalloproteases (*13*). Mutating either of the conserved histidines in the HEXXH domain blocks

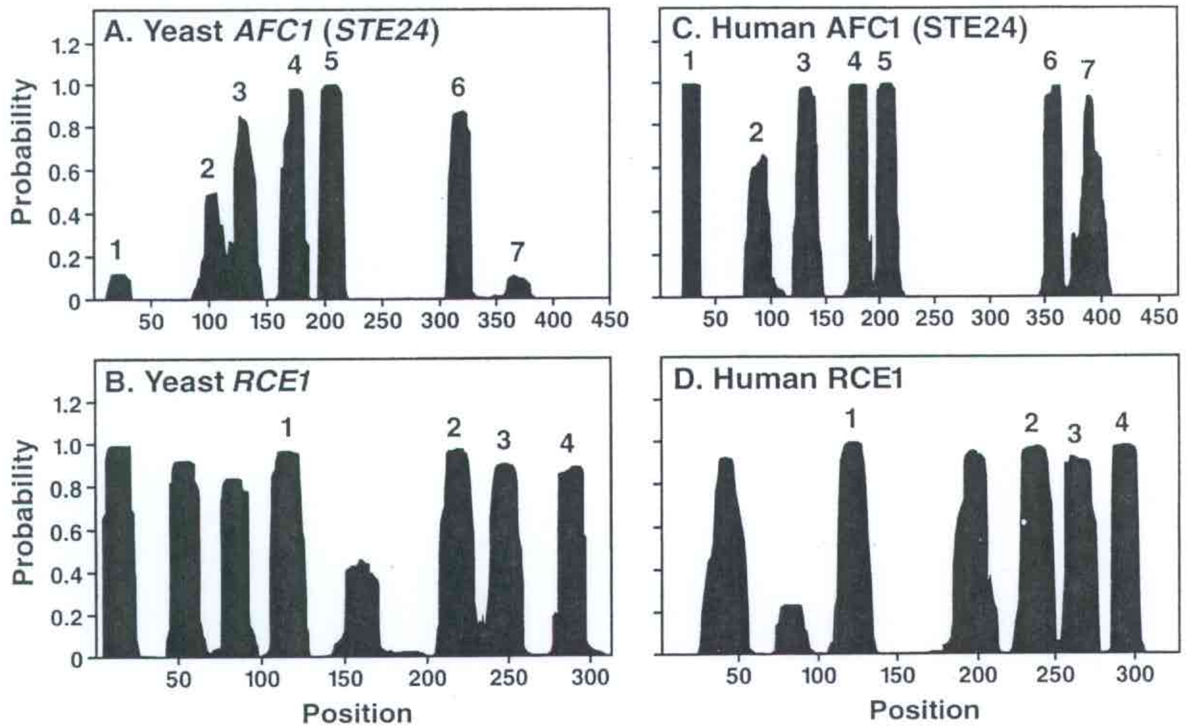

FIG. 2. Predicted transmembrane domains in the endoproteases. (A) Yeast Afc1p (Ste24p); (B) yeast Rce1p; (C) human *AFC1* (*STE24*); (D) human RCE1. Predicted transmembrane domains were determined with the TMHMM transmembrane domain analysis program (http://genome.cbs.dtu.dk/services/TMHMM-1.0/). To aid in the alignment, potential transmembrane domains with yeast/human sequence identities are indicated by numbers 1–7 for Afc1p and 1–4 for Rce1p.

the ability of *AFC1* to complement the mating defect of *AFC1*-deficient yeast (*afc1*Δ) expressing the –AMQ form of **a**-factor (*13*).

Yeast null mutants for *AFC1* produced reduced amounts of wild-type **a**-factor but were not sterile, indicating the existence of another gene capable of processing **a**-factor. Membranes from *afc1*Δ yeast exhibited a slight but reproducible decrease in their capacity to remove the last three amino acids from a farnesylated **a**-factor synthetic peptide. This slight decrease in endoprotease activity in the *afc1*Δ yeast was similar to the modest decrease observed by treating wild-type yeast membranes with *o*-phenanthroline. The residual CXXX endoprotease activity in the *afc1*Δ yeast membranes was insensitive to *o*-phenanthroline, suggesting that the remaining endoprotease activity was not zinc dependent. The properties of yeast *AFC1* (*STE24*) are summarized in Table II.

There were no *AFC1* (*STE24*) homologs in the yeast genome, indicating that the residual **a**-factor processing in *afc1*Δ yeast must have been due to a structurally distinct gene. To isolate the other CXXX endoprotease, *afc1*Δ yeast were mutagenized and screened for mutations that blocked the residual production of **a**-factor. In addition, yeast 2μ libraries were screened for plasmids that at high copy would partially restore **a**-factor production

TABLE II

PROPERTIES OF YEAST *AFC1* (*STE24*)-ENCODED PROTEINS

Cleaves C-terminal three amino acids (–VIA) from precursor to yeast mating pheromone **a**-factor. No other substrates yet reported
Cannot cleave –XXX from mutant mating factor terminating in sequence –TLM (C-terminal three amino acids from *STE18*-encoded γ subunit of heterotrimeric G protein), but can cleave C-terminal –XXX from mutant mating factor terminating in sequence –AMQ (C-terminal three amino acids from α subunit of rabbit muscle glycogen phosphorylase kinase)
Cleaves N-terminal seven amino acids from precursor to yeast mating pheromone **a**-factor
Enzymatic activity is zinc dependent and is blocked by *o*-phenanthroline
Located within ER by cell fractionation studies
Null mutant reduced mating efficiency in α cells. Overexpression of Axl1p suppresses mating defect
453-amino acid protein; 36% identity with human AFC1 amino acid sequence
Multiple predicted transmembrane domains
An HEXXH domain, which is shared with many other zinc proteases, is critical for enzymatic activity
Contains C-terminal dilysine motif, but that sequence is not required for ER localization

in *afc1*Δ yeast expressing –AMQ **a**-factor. Both approaches led to the identification of a second endoprotease gene, *RCE1*. *RCE1* encodes a 329-amino acid protein (Fig. 3) that is predicted to have multiple transmembrane domains indicative of an integral membrane protein (Fig. 2B). Unlike Afc1p, an analysis of the Rce1p protein sequence did not reveal sequences characteristic of any of the defined classes of proteases. However, there appeared to be remote similarities with the type IIb signal peptidase, which cleaves signal sequences from proteins containing nearby lipid modifications (*13*). Key properties of yeast *RCE1* are listed in Table III.

Yeast lacking the *RCE1* gene (*rce1*Δ) are viable, with a modest decrease in **a**-factor production, and there was little effect on mating efficiency. In these respects, the phenotypes of *rce1*Δ and *afc1*Δ were similar. The membranes from *rce1*Δ yeast manifested moderately decreased CXXX endoprotease activity in *in vitro* assays involving farnesylated **a**-factor peptides—more so than with the *afc1*Δ yeast. Of note, the residual CXXX endoprotease activity in *rce1*Δ yeast was sensitive to *o*-phenanthroline, consistent with Afc1p being a zinc protease. Yeast lacking both *RCE1* and *AFC1* (*afc1*Δ*rce1*Δ) are completely defective in the production of mature **a**-factor and are sterile, indicating that both *RCE1* and *AFC1* (*STE24*) process **a**-factor.

In contrast to the redundant functions of *RCE1* and *AFC1* in **a**-factor processing, genetic evidence indicates that *RCE1*, but not *AFC1*, is critical for the processing of Ras proteins, and that *RCE1* deficiency attenuates

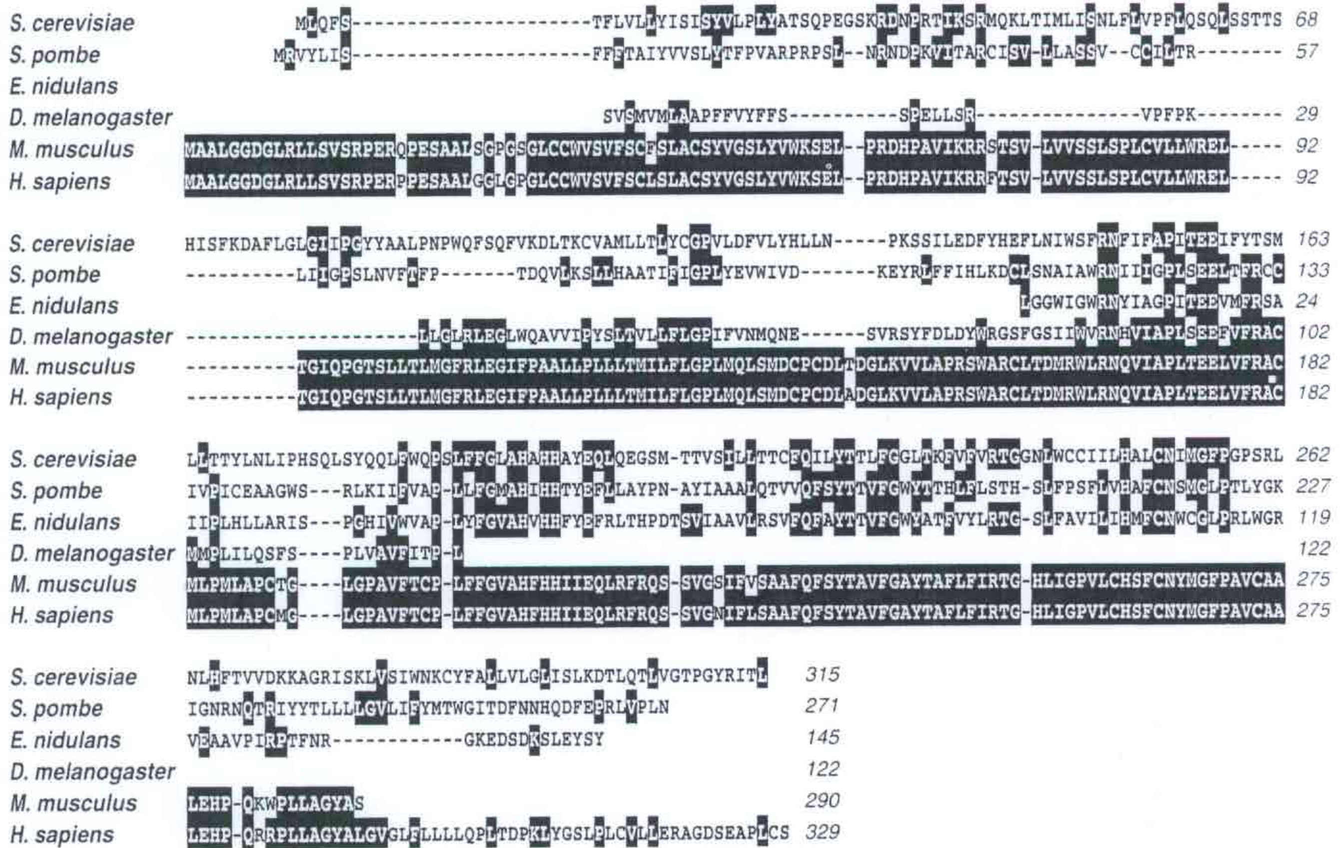

FIG. 3. Amino acid sequence alignment of yeast RCE1 and orthologs in other species. GenBank accession numbers: *E. nidulans*, AI210465; *D. melanogaster*, AI514743; human, NP005124; *S. cerevisiae*, Q03530; *S. pombe*, CAA22596; mouse, unpublished data (E. Kim and S. Young). Protein sequences were aligned as described in the legend to Fig. 1.

the activity of the Ras proteins (*13*). In yeast, Ras2p mutations that impair GTP hydrolysis (e.g., $RAS2^{Val19}$) induce sensitivity to heat shock and starvation (*13*). In *rce1*Δ yeast, the heat shock sensitivity elicited by a mutationally activated Ras2p is suppressed by a factor of 100, compared with wild-type yeast. The *afc1*Δ mutant had little if any effect on heat shock sensitivity, while the *afc1*Δ*rce1*Δ mutant was indistinguishable from that of the *rce1*Δ mutant. These experiments indicated that the endoproteolytic processing of Ras2p by Rce1p attenuated Ras function. However, the *rce1*Δ mutant almost certainly did not completely block all Ras functions, given that *rce1*Δ yeast are viable (*13*) and yeast lacking the Ras proteins are not (*40*).

To assess further the functional importance of *RCE1* in Ras function, Boyartchuk *et al.* (*13*) studied the effects of *RCE1* deficiency in yeast with a temperature-sensitive *RAS2* allele, *ras2-23*. Yeast harboring a *ras2-23* allele as their only Ras allele grow well at 30°C but exhibit a reduced growth rate at 34°C. The deletion of *RCE1* completely blocked the growth of the *ras2-23* mutant at 34°C (*13*). The deletion of *AFC1* had no effect on the growth of the *ras2-23* mutant.

TABLE III

PROPERTIES OF YEAST RCE1

Properties of yeast RCE1
Cleaves C-terminal three amino acids (–VIA) from precursor to yeast mating pheromone **a**-factor. Required for endoproteolytic processing of Ras2p. No other substrates reported
Insensitive to inhibition by *o*-phenanthroline
Extremely low activity against nonfarnesylated proteins
Cannot cleave –XXX from a mutant mating factor terminating in sequence –AMQ (C-terminal three amino acids from α subunit of rabbit muscle glycogen phosphorylase kinase), but can cleave C-terminal –XXX from mutant mating factor terminating in sequence –TLM (C-terminal three amino acids from *STE18*-encoded γ subunit of heterotrimeric G protein)
Unlike Afc1p (Ste24p), Rce1p does not cleave N terminus of **a**-factor
Appears to be located almost exclusively in ER
315-amino acid protein; 27.6% identical with the human RCE1 sequence
Multiple predicted transmembrane domains
Null mutant does not affect mating of α cells but causes slight decrease in **a**-factor production
Null mutant results in mislocalization of GFP–Ras2p fusion protein, with less reaching the plasma membrane. Null mutant largely blocks the phenotypes elicited by mutationally activated Ras2p

The effect of endoproteolysis on Ras2p localization within yeast was tested with a green fluorescent protein (GFP)–*RAS2* fusion construct in which GFP was fused to the N terminus of Ras2p, leaving the CXXX motif of Ras2p intact and available for processing. In wild-type yeast, fluorescence microscopy revealed that the fusion construct was targeted to the periphery of the cells (i.e., the plasma membrane). In keeping with that result, cell fractionation revealed that the wild-type fusion protein sedimented with the P100 membrane fraction. In contrast, the fusion protein in the *rce1*Δ cells was widely dispersed within the cell, with the majority of the fluorescence appearing inside the cell (i.e., either in the cytosol or associated with an internal membrane compartment). Thus, the endoproteolytic processing of the Ras proteins by Rce1p appears to contribute importantly to their proper localization within the cell.

RCE1 has few sequence similarities with *AFC1* or other proteases. Thus, it is reasonable to question whether Rce1p is truly a protease or simply a cofactor for another enzyme. Although this issue has not been settled definitively, the evidence supports the idea that it is a protease. The levels of endoprotease activity in yeast correlated with the amount of *RCE1* expression (*13*). Moreover, as described below, overexpression of the human ortholog of *RCE1* in insect cells produced an enormous increase in endoprotease activity (*15*).

The studies by Boyartchuk and co-workers (*13, 41*) revealed that both

AFC1 and *RCE1* are active in processing **a**-factor, while only *RCE1* appears to be involved in Ras metabolism. Their *in vitro* endoproteolysis assay, which assessed the cleavage of a farnesylated **a**-factor peptide, indicated that ~35% of the total CXXX endoprotease activity in yeast membranes is due to *AFC1,* with *RCE1* accounting for the remainder. However, any conclusion regarding the extent to which Rce1p and Afc1p participate in "total endoproteolysis activity" should be viewed with caution, simply because the CXXX endoprotease activities of Afc1p and Rce1p are almost certainly dependent on the sequence of the CXXX peptide or protein used in the assay (*41*).

There may be as many as 86 CXXX proteins in the yeast genome, as judged by search of the yeast protein database (http://genome-www2.stanford.edu/cgi-bin/SGD/PATMATCH/nph-patmatch?class = pept). A critical issue in the enzymology of Rce1p and Afc1p is to define their unique and overlapping protein substrates. It is unclear whether the majority of the yeast CXXX proteins are processed by a combination of both Rce1p and Afc1p, or whether many proteins are processed solely by Afc1p or solely by Rce1p. However, the tools are clearly in hand to address these issues.

IV. Role for *AFC1* (*STE24*) in Proteolytic Processing of N Terminus of a-Factor in *Saccharomyces cerevisiae*

The *S. cerevisiae* mating pheromone **a**-factor, a 12-amino acid farnesylated peptide, is the product of functionally redundant genes, *MFA1* and *MFA2*. These two genes encode precursor proteins of 36 and 38 amino acids, and the production of mature **a**-factor depends on a series of processing steps: isoprenylation, endoproteolytic removal of the C-terminal three amino acids (–VIA), carboxyl methylation of the isoprenylcysteine, and the proteolytic removal of the N-terminal 21 amino acids (following –KKDN). It has become clear that the removal of the N-terminal 21 residues involves two sequential steps: the initial removal of the first 7 amino acids (following –STAT), followed by the removal of 14 additional amino acids (following –KKDN) (*42–44*). The first of these amino-terminal processing steps has been shown to be carried out by *STE24* (*39*), while the second is carried out by *AXL1*- and *STE23*-encoded proteins. Remarkably, *STE24* was recognized to be identical to *AFC1*; thus, in addition to its role in cleaving the –XXX from the **a**-factor precursor, Ste24p (Afc1p) plays a distinct role in the N-terminal processing of **a**-factor. Figure 4 illustrates the five steps in the biogenesis of mature **a**-factor, showing the two distinct roles of Ste24p (Afc1p).

In 1995, Adames *et al.* (*45*) used a genetic screen for reduced mating

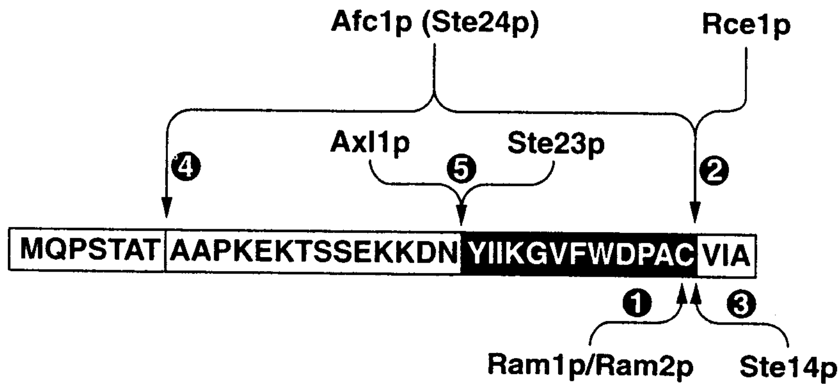

Fig. 4. A schematic illustrating the two distinct roles of Afclp (Ste24p) in **a**-factor metabolism. First, the **a**-factor precursor is farnesylated by Ramlp/Ram2p. Second, the –VIA tripeptide is cleaved by both Afclp (Ste24p) and Rcelp. Third, the isoprenylcysteine is carboxyl methylated. Fourth, the N-terminal seven amino acids are cleaved by Afclp. Fifth, an additional 14 amino acids are cleaved by Axllp and Ste23p. In the absence of Afclp (Ste24p) and Rcelp, P0* accumulates. P0* is farnesylated but not further processed (e.g., no CXXX endoproteolysis and no carboxyl methylation of the isoprenylcysteine). P1, which is fully modified at the C terminus (i.e., having undergone isoprenylation, –XXX endoproteolysis, and carboxyl methylation), accumulates in the absence of Afclp (Ste24p). P2, which lacks the N-terminal seven amino acids, accumulates in the absence of Axllp and Ste23p. [Reproduced, with permission, from Ashby, M. N. (1998). *Curr. Opin. Lipidol.* **9,** 99–102.]

efficiency in *S. cerevisiae* to identify *AXL1* (or *STE22*). Axllp has significant sequence identity to the insulin-degrading enzymes and belongs to a family of metalloproteases with a preference for small peptide substrates. In *axl1*Δ yeast, pulse–chase/metabolic labeling studies of **a**-factor formation revealed the early appearance of an **a**-factor intermediate, P1, that was fully modified at the C terminus (i.e., an **a**-factor intermediate that had undergone isoprenylation, –XXX endoproteolysis, and carboxyl methylation). P1 was converted to another **a**-factor intermediate, P2, which lacks the N-terminal seven amino acids. P2 accumulated in *axl1*Δ yeast, with only small amounts undergoing further processing to mature **a**-factor (*42, 44*). In wild-type yeast, P1 is converted to P2, but the P2 is then processed to mature **a**-factor (by the removal of 14 additional amino acids). These experiments, together with those from the laboratory of S. Michaelis (*42, 44*), revealed two important points about **a**-factor processing. First, the three sequential modifications of the C terminus of **a**-factor occur early and are completed before the proteolytic processing of the N terminus. Second, the Axllp-mediated cleavage of the **a**-factor precursor represents the second of two N-terminal proteolytic processing steps. The removal of the initial seven amino acids occurs before the Axllp proteolysis step.

The characterization and kinetic analysis of **a**-factor intermediates have been more completely described in a series of experiments by Chen *et al.* (*44*). A search of the *S. cerevisiae* sequence database revealed a gene

homologous to *AXL1*, designated *STE23*, which also participates in the second N-terminal proteolysis step (*45*). Yeast mutants lacking *AXL1* (*axl1*Δ) retain a markedly reduced mating efficiency while mutants lacking both *AXL1* and *STE23* (*axl1*Δ*ste23*Δ) are sterile.

The identification of *AXL1* and *STE23* raised a key question: What protease cleaves the amino-terminal seven amino acids from the **a**-factor precursor? Multiple genetic screens for sterile yeast mutants in several laboratories failed to identify the protease. However, in 1997, 5 months before the report of the CXXX endoproteases by Boyartchuk *et al.* (*13*), Fujimura-Kamada *et al.* (*39*) solved the riddle. Using a sensitized genetic screen for sterile yeast, they identified a new gene, *STE24*, that was essential for the removal of the N-terminal seven amino acids from the **a**-factor precursor. *STE24* had multiple predicted membrane-spanning domains and shared important sequence similarities with a variety of different zinc-dependent proteases from diverse organisms ranging from bacteria to humans.

Yeast lacking *STE24* (*ste24*Δ) exhibited reduced mating efficiency but were not sterile. Pulse–chase experiments of **a**-factor maturation in *ste24*Δ yeast revealed a striking defect in the conversion of P1 (the intermediate that is fully modified at the C terminus) to P2 (the intermediate lacking the N-terminal seven amino acids). Why aren't the *ste24*Δ yeast sterile? The likely explanation is that some Axl1p/Ste23p-mediated cleavage of **a**-factor occurs, albeit at reduced efficiency, in the absence of Ste24p (Afc1p). Fujimura-Kamada *et al.* (*39*) demonstrated that overexpression of *AXL1* suppressed the mating defect in *ste24*Δ yeast, suggesting that the Ste24p-mediated cleavage of the first seven amino acids greatly facilitates, but is not absolutely required for, the subsequent proteolytic cleavage between amino acids 21 and 22.

With the publication of the CXXX endoprotease paper by Boyartchuk and co-workers (*13*), it was evident that the sequence of *AFC1* was identical to that of *STE24*. Thus, using a screen for reduced mating efficiency, the Michaelis group had identified *STE24* as a gene essential for the N-terminal processing of **a**-factor (39). Within months, Boyartchuk, Ashby, and Rine (13) used a screen for sterile mutants to identify *AFC1*, and concluded that it was one of two genes responsible for the C-terminal processing of **a**-factor. Thus, two groups applying similar genetic screens identified the same gene and reached different conclusions regarding its function. In a pair of follow-up papers (*14, 16*), this seeming discrepancy has been resolved. Both groups went on to show that Afc1p (Ste24p) is involved in two distinct steps of **a**-factor processing: the –XXX endoproteolytic cleavage, and the cleavage of the N-terminal seven amino acids.

Tam *et al.* (*16*) explored the dual roles of Ste24p in **a**-factor maturation by examining the processing of various *MFA1* truncations in yeast. Because conventional *MFA1* truncation mutants yield low levels of **a**-factor expression, they assembled ubiquitin–*MFA1* fusion constructs, which result in higher levels of expression. The basic strategy was to rely on the endogenous ubiquitin proteases within yeast to cleave the N-terminal ubiquitin sequences from the fusion proteins, releasing truncated **a**-factor proteins. When a ubiquitin fusion construct containing full-length *MFA1* protein (Ubi–P1) was transformed into *ste24*Δ*mfa1*Δ *mfa2*Δ yeast, P1 accumulated, and only minute amounts of mature **a**-factor were produced. However, if the same yeast were transformed with ubiquitin fused to *MFA1* lacking the first seven amino acids (Ubi–P2), high levels of mature **a**-factor were produced and secreted. Thus, the removal of the first seven amino acids of **a**-factor obviated the requirement for Ste24p-mediated processing of the N terminus. As expected, *rce1*Δ*ste24*Δ*mfa1*Δ*mfa2*Δ yeast transformed with Ubi–P2 secreted no mature **a**-factor, because both CXXX endoproteases were absent. These experiments established that Ste24p has two distinct roles in **a**-factor processing and that the two roles could be uncoupled and characterized.

Tam and co-workers (*16*) went on to study **a**-factor biogenesis in wild-type, *ste24*Δ, *rce1*Δ, and *ste24*Δ*rce1*Δ yeast with pulse–chase experiments. In wild-type yeast, P1 (the intermediate with only C-terminal modifications) appeared quickly and was rapidly converted to P2 and then to mature **a**-factor. As predicted, *ste24*Δ yeast accumulated P1, and there was minimal conversion of P1 to mature **a**-factor. In *ste24*Δ*rce1*Δ yeast, a novel **a**-factor intermediate, designated P0*, accumulated. P0* is farnesylated but not further processed (e.g., no CXXX endoproteolysis and no carboxyl methylation of the isoprenylcysteine). Interestingly, P0* appeared transiently in *rce1*Δ yeast but was rapidly converted to P2 and mature **a**-factor, without the appearance of the P1 intermediate. Thus, in the absence of Rce1p, Ste24p appears to cleave the –XXX and the seven N-terminal amino acids in rapid succession, seemingly without releasing the intermediate (P1). The *rce1*Δ yeast secreted approximately half-normal amounts of mature **a**-factor.

Boyartchuk and Rine (*41*) likewise used ubiquitin–*MFA1* fusion constructs to dissect the dual roles of Afc1p (Ste24p) in **a**-factor maturation. In addition, they analyzed ubiquitin–*MFA1* fusion constructs in which the C-terminal –XXX sequence was mutated, so that the C-terminal cleavage reaction was carried out only by Afc1p or only by Rce1p, but not both proteins. Both Rce1p and Afc1p carry out the C-terminal processing of fusion proteins terminating in the wild-type *MFA1* sequence (–VIA). However, changing the C-terminal sequence to –AMQ (the C-terminal three

amino acids from the α subunit of rabbit muscle glycogen phosphorylase kinase) eliminated C-terminal processing by Rce1p, while changing the sequence to –TLM (the C-terminal three amino acids from the *STE18*-encoded γ subunit of a heterotrimeric G protein) eliminated C-terminal processing by Afc1p.

By comparing mating efficiencies of *mfa1*Δ*mfa2*Δ, *mfa1*Δ*mfa2*Δ*afc1*, and *mfa1*Δ*mfa2*Δ*afc1*Δ*rce1*Δ yeast that had been transformed with various ubiquitin–*MFA1* fusion constructs, they demonstrated that Afc1p plays a role in the N-terminal processing of **a**-factor, in addition to its role in CXXX endoproteolysis. For example, a full-length –TLM **a**-factor construct produced less mature **a**-factor in *afc1*Δ yeast than in wild-type yeast. This result is likely explained by defective N-terminal processing in the *afc1*Δ cells. It cannot be explained by differences in C-terminal processing, since the –TLM construct is processed exclusively by Rce1p. Boyartchuk and Rine (*41*) observed less **a**-factor production with a ubiquitin fusion construct lacking the seven N-terminal amino acids than with a construct containing the entire **a**-factor protein, leading them to conclude that the N-terminal Afc1p-mediated cleavage step increases the efficiency of mature **a**-factor production.

The finding that the *AFC1* (*STE24*) gene product participates in a second proteolytic cleavage reaction in **a**-factor raises the possibility that this gene product could play dual processing roles for other yeast CXXX proteins, or maybe even that it has proteolytic processing roles for non-CXXX proteins. Thus far, however, no other substrates for Afc1p (Ste24p) have been reported.

V. Characterization of Mammalian Orthologs for *AFC1* (*STE24*) and *RCE1*

A. Characterization of Mammalian RCE1

When yeast *RCE1* was identified and reported, related sequences already existed in the human and mouse EST databases. Mammalian orthologs were cloned by Kim and Young, by Otto and Casey, and by Ashby. Ultimately, Kim, Young, and co-workers collaborated with Otto and Casey to define the roles of the mammalian orthologs in the endoproteolytic processing of mammalian CXXX proteins (*14, 15*). Kim, Young, and co-workers assessed the role of the mouse *Rce1* gene in the processing of Ras proteins by knocking out *Rce1* in mice (*14*), while Otto, Casey, and co-workers focused on the cloning, expression, and characterization of human

TABLE IV

PROPERTIES OF MAMMALIAN RCE1

RCE1 expressed in Sf9 cells processes farnesylated K-Ras, farnesylated H-Ras, farnesylated N-Ras, the farnesylated heterotrimeric $G\gamma_1$ subunit, geranylgeranylated K-Ras, and geranylgeranyl-Rap1B. There is no processing of these substrates by membranes from mouse fibroblasts lacking *Rce1* gene expression
250-fold greater specificity of the enzyme for isoprenylated peptides than for nonisoprenylated peptides
RPI, a reduced farnesyl-peptide analog, is an effective inhibitor of human RCE1, with an IC_{50} of approximately 5 n*M*
Located exclusively in membrane fractions of mammalian cells
RCE1 is expressed in all mouse and human tissues, including early-stage embryos, with highest levels in the heart and skeletal muscle
329-amino acid protein; 27.6% identity with *S. cerevisiae* Rce1p amino acid sequence; 42% identity and 68% similarity between residues 166 and 270
Protein has multiple predicted transmembrane domains
Achieving high levels of human Rce1 activity in Sf9 cells required deletion of N-terminal 22 amino acids of protein
Mice lacking *Rce1* expression (*Rce1*$^{-/-}$) die during embryonic development, beginning on day 15. No gross defects in organogenesis
Endoproteolytic processing of Ras proteins is absent in *Rce1*$^{-/-}$ fibroblasts and embryos
Rce1 deficiency in fibroblasts produces a gross mislocalization of a GFP–K-Ras fusion protein within the cell
Membranes from *Rce1*$^{-/-}$ fibroblasts cannot carry out C-terminal processing of farnesylated K-Ras, farnesylated H-Ras, farnesylated N-Ras, farnesylated heterotrimeric $G\gamma_1$ subunit, geranylgeranylated K-Ras, or geranylgeranyl-Rap1B

RCE1 in cultured cells (*15*). Key properties of human RCE1 and its mouse ortholog (Rce1) are listed in Table IV.

1. *Expression and Characterization of Human RCE1*

Using the yeast *RCE1* sequence, Otto and co-workers identified an apparent ortholog in a human EST database and then cloned the corresponding cDNA (*15*). Figure 3 shows the alignment of the human RCE1 protein, along with the RCE1 proteins from several other species. Like yeast Rce1p, the human protein is predicted to contain multiple transmembrane domains (Fig. 2D). Northern blots revealed that RCE1 is expressed ubiquitously, with the highest levels of expression in placenta, heart, and skeletal muscle. Although the predominant transcript length was 1.3 kb, larger transcripts were also observed, probably reflecting alternatively or aberrantly spliced transcripts. Heterogeneity in transcript length was even more obvious in mouse tissues (*15*). The significance of the larger transcripts, if any, is unknown.

High levels of yeast and human RCE1 proteins were expressed in Sf9

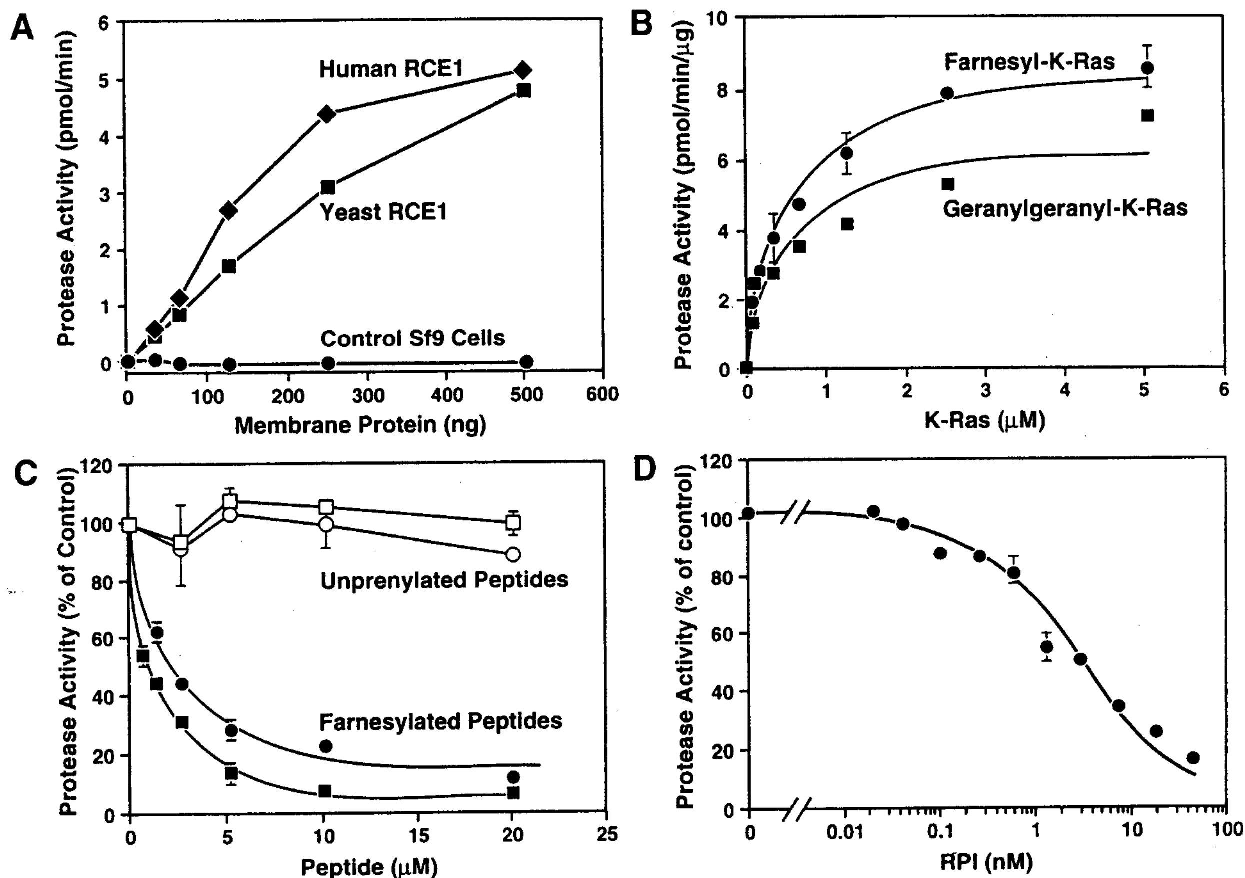

FIG. 5. Characterization of human RCE1 activity. (A) Yeast and human RCE1 gene products possess isoprenyl protein protease activity. Protease assays were conducted with increasing amounts of membranes prepared from noninfected Sf9 cells (●), or from Sf9 cells infected with recombinant baculoviruses containing the cDNA for either yeast (■) or human RCE1 (◆). Purified farnesyl-K-Ras (2 μM) was used as substrate. Isoprenyl protein protease activity was assessed with a coupled proteolysis/methylation assay (*15*). (B) Kinetics of proteolysis for farnesyl-K-Ras and geranylgeranyl-K-Ras. RCE1 membrane protein (15 ng) was incubated with increasing levels of farnesyl-K-Ras (●) and geranylgeranyl-K-Ras (■). (C) Competition of RCE1 activity by isoprenylated peptides. Isoprenylated peptides [farnesyl-CVIM (●) and farnesyl-GSPCVLM (■)] or nonisoprenylated peptides [CVIM (○) and GSPCVLM (□)] were included in the standard protease reaction mixture with 0.5 μM purified farnesyl-K-Ras. Activity is expressed as the percentage of that observed for an untreated control. (D) Inhibition of RCE1 activity by the farnesyl-peptide analog RPI. RPI was diluted in 10% DMSO, and added to the standard protease reaction mixture containing 0.5 μM purified farnesyl K-Ras. Activity is expressed as the percentage of that observed for an untreated control. [Reproduced, with permission, from Otto, J. C., Kim, E., Young, S. G., and Casey, P. J. (1999). *J. Biol. Chem.* **274**, 8379–8382.]

(*Spodoptera frugiperda* ovary) insect cells with recombinant baculoviruses (Fig. 5A). Of note, high-level expression of the human RCE1 protein could not be achieved without first deleting the first 22 amino acids of the protein. To assess the activity of yeast and human RCE1 proteins, extracts from the Sf9 cells were mixed with recombinant isoprenylated CXXX proteins. In this assay, the recombinant RCE1 proteins cleave the –XXX from the

isoprenylated proteins, rendering the proteins susceptible to carboxyl methylation with recombinant Ste14p and *S*-adenosyl[*methyl*-^{3}H]methionine. Using this coupled endoproteolysis/methylation assay, Otto and co-workers demonstrated that human RCE1 processes farnesylated K-Ras, farnesylated H-Ras, farnesylated N-Ras, the farnesylated heterotrimeric G protein $G\gamma_1$ subunit, geranylgeranylated K-Ras, and geranylgeranyl-Rap1B. Both farnesylated and geranylgeranylated K-Ras exhibited a K_m value of approximately 0.5 μM and similar k_{cat} values (Fig. 5B). Isoprenylated CXXX peptides, but not nonisoprenylated peptides, were able to compete for the processing of isoprenylated K-Ras (Fig. 5C). In addition, a previously identified inhibitor of endoproteolytic processing, RPI (a reduced farnesylpeptide analog), was an extremely effective inhibitor of RCE1, with an IC_{50} of approximately 5 nM (Fig. 5D).

A perplexing aspect of the Sf9 expression system is that it required the truncation of the N-terminal 22 amino acids of the human protein. This finding has not been explained. One possibility is that the presence of the N-terminal 22 amino acids simply reduces the absolute level of RCE1 protein expression in Sf9 cells. Alternatively, it is possible that the N terminus of RCE1 might normally bind to a "protein partner." When that protein partner is absent (as might be the case in Sf9 cells), the amino-terminal domain might cause RCE1 to misfold, diminishing its activity. In any case, the cloning of human RCE1 and its expression in cultured cells represent important advances, as they lay the foundation for purifying the enzyme, performing structure–function studies, defining the catalytic domain of the protein, and identifying additional CXXX protein substrates.

Multiple RCE1 orthologs now exist in the databases. Comparison of the amino acid sequences encoded by *S. cerevisiae RCE1* and its human and mouse orthologs reveals the greatest sequence similarity in the C-terminal half of the protein (Fig. 3). When the predicted transmembrane helical regions of the human and yeast forms are compared (Fig. 2B and D) with the aligned sequences (Fig. 3), only four of the predicted transmembrane domains appear to match with aligned sequences—three near the C terminus and one in the central region of the molecule (designated 1, 2, 3, and 4 in Fig. 2, B and D).

2. *Assessing Physiologic Role of Rce1 Gene Product in Mice*

Kim, Young, and co-workers (*14*) sought to assess the physiologic role of *Rce1* in higher organisms by producing and characterizing *Rce1* knockout mice. The yeast *RCE1* sequences were used to identify potential orthologs within the mouse EST databases, and those were used to clone most of the mouse cDNA. The mouse and human proteins are highly conserved throughout their lengths, with 95% of the amino acid residues being identi-

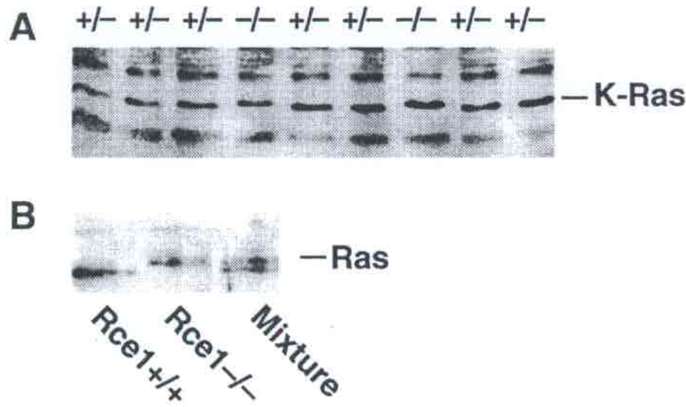

FIG. 6. Abnormal electrophoretic mobility of Ras proteins in lysates from $Rce1^{-/-}$ embryos and $Rce1^{-/-}$ primary embryonic fibroblasts. (A) Western blot of lysates of $Rce1^{+/+}$, $Rce1^{+/-}$, and $Rce1^{-/-}$ embryos. (B) A Western blot of an equal mixture of $Rce1^{+/+}$ and $Rce1^{-/-}$ embryo lysates with antibody Ab-4, demonstrating the expected doublet band. [Reproduced, with permission, from Kim, E., Ambroziak, P., Otto, J. C., Taylor, B., Ashby, M., Shannon, K., Casey, P. J., and Young, S. G. (1999). *J. Biol. Chem.* **274,** 8383–8390.]

cal. Southern blots of mouse genomic DNA with one of the EST probes suggested the presence of a single copy of *Rce1* in the genome. Strain 129/Sv mouse genomic clones were identified and used to construct a sequence-replacement gene-targeting vector. *Rce1*-deficient mice were generated by standard gene-targeting techniques (*14*).

Heterozygous knockout mice ($Rce1^{+/-}$) were healthy and fertile. However, virtually all of the homozygous *Rce1* knockout embryos ($Rce1^{-/-}$) died, beginning on about embryonic day 15 (E15). $Rce1^{-/-}$ mice were born only rarely, and they invariably died within 1 week. The reason for the embryonic lethality remains mysterious. Inspection of the $Rce1^{-/-}$, $Rce1^{+/+}$, and $Rce1^{+/-}$ embryos after E15.5 revealed no consistent differences in morphology, organogenesis, stage of development, color, or size. The knockout mice did not appear to die from a defect in hematopoiesis. The $Rce1^{-/-}$ embryos were pink, and histologic analysis revealed abundant numbers of mature erythrocytes in their blood vessels. Moreover, lethally irradiated mice were successfully rescued with hematopoietic stem cells from the livers of $Rce1^{-/-}$ embryos (*14*).

Fibroblasts were cultured from the $Rce1^{-/-}$ embryos. Despite an absence of Ras endoproteolytic processing, the growth rates of $Rce1^{-/-}$ and $Rce1^{+/+}$ fibroblasts did not appear to be grossly different, at least when they were grown in medium containing a high concentration of fetal bovine serum.

To determine if mouse *Rce1* participates in the processing of Ras proteins, the electrophoretic mobilities of the Ras proteins from $Rce1^{-/-}$, $Rce1^{+/+}$, and $Rce1^{+/-}$ embryo lysates were analyzed on SDS–polyacrylamide gels (Fig. 6A). The electrophoretic mobility of the Ras proteins from $Rce1^{-/-}$

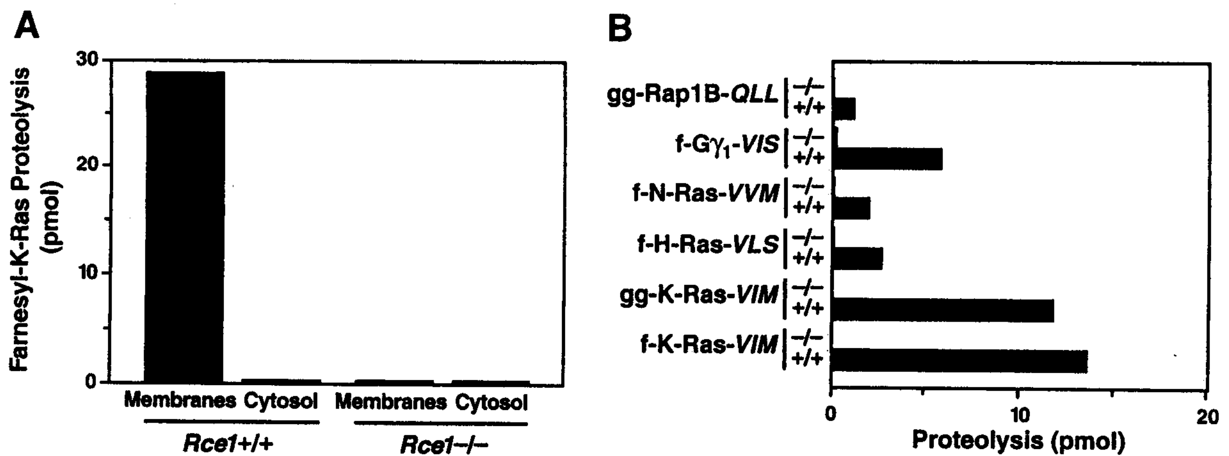

FIG. 7. Direct assays of the ability of $Rce1^{-/-}$ fibroblasts to endoproteolytically process isoprenylated CXXX proteins. (A) An assay of the ability of cytosolic and membrane fractions from $Rce1^{-/-}$ fibroblasts to endoproteolytically process farnesyl-K-Ras. Membrane and cytosolic fractions (80 μg) from $Rce1^{+/+}$ and $Rce1^{-/-}$ fibroblasts were incubated with farnesyl-K-Ras (2 μM). The processed farnesyl-K-Ras was then methylated with 5.0 μM [^{3}H]AdoMet (1.5 Ci/mmol) and 20 μg of membranes from Sf9 insect cells expressing high concentrations of the yeast isoprenyl protein carboxyl methyltransferase encoded by *STE14*. [^{3}H]Methyl-isoprenylated proteins were collected on filters, and methylation was quantified by scintillation counting. Proteolysis is described as the number of picomoles of [^{3}H]methyl groups transferred to the isoprenylated protein. (B) Endoprotease activity in membranes from $Rce1^{+/+}$ and $Rce1^{-/-}$ fibroblasts against a panel of isoprenylated proteins. The isoprenyl group attached to proteins is indicated by an "f-" for farnesyl or a "gg-" for geranylgeranyl. The –XXX sequence for each protein is indicated in italics. The concentrations of farnesyl-K-Ras and geranylgeranyl-K-Ras were 1.0 μM. The approximate concentration of geranylgeranyl-Rap1B was 0.2 μM, and the approximate concentrations of farnesyl-H-Ras, farnesyl-N-Ras, and farnesyl-Gγ_1 were 1 μM. [Reproduced, with permission, from Kim, E., Ambroziak, P., Otto, J. C., Taylor, B., Ashby, M., Shannon, K., Casey, P. J., and Young, S. G. (1999). *J. Biol. Chem.* **274**, 8383–8390.]

fibroblasts and embryos was distinctly abnormal. In fact, Ras proteins with normal electrophoretic mobilities were undetectable. When equal amounts of lysates from $Rce1^{-/-}$ and $Rce1^{+/+}$ embryos were mixed and analyzed on an SDS–polyacrylamide gel, a doublet Ras band was observed (Fig. 6B).

The abnormal electrophoretic mobility of the Ras proteins strongly suggests that the *Rce1* product is responsible for Ras endoproteolytic processing. Because endoproteolysis is required for subsequent carboxyl methylation of the isoprenylcysteine, it would be assumed that this would not occur with Ras proteins from $Rce1^{-/-}$ fibroblasts. To test this prediction, $Rce1^{+/+}$ and $Rce1^{-/-}$ fibroblasts were metabolically labeled with L-[*methyl*-^{3}H]methionine, and the Ras proteins were then immunoprecipitated from the cells. As expected, the Ras proteins from the $Rce1^{+/+}$ cells contained an ^{3}H-labeled cysteine methyl ester, whereas Ras proteins from the $Rce1^{-/-}$ cells did not (*14*). Membranes from $Rce1^{-/-}$ fibroblasts also lacked the capacity to process farnesylated K-Ras in a coupled proteolysis/carboxyl

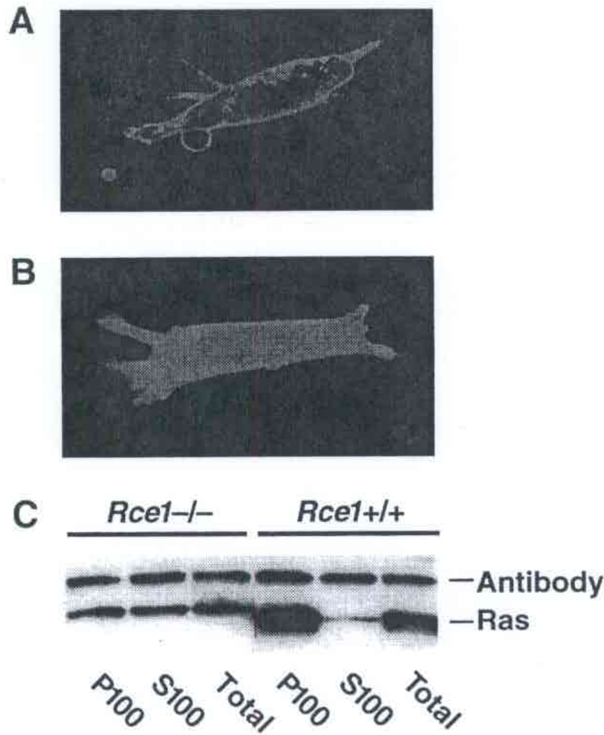

FIG. 8. Mislocalization of Ras proteins in *Rce1*$^{-/-}$ fibroblasts. (A) Fluorescence image of a GFP–K-Ras fusion protein in an *Rce1*$^{+/+}$ fibroblast, showing localization of the fusion protein at the plasma membrane. (B) Fluorescence image of a GFP–K-Ras fusion protein in an *Rce1*$^{-/-}$ fibroblast, showing localization of the fusion protein to the cytosol or internal membranes. (C) Mislocalization of Ras proteins as judged by cell fractionation. Cells were fractionated into cytosolic (S100) and membrane (P100) fractions by ultracentrifugation. The Ras proteins were immunoprecipitated from the S100 and P100 fractions, as well as from the total cellular lysates, and analyzed on a Western blot of an SDS–polyacrylamide gel. [Reproduced, with permission, from Kim, E., Ambroziak, P., Otto, J. C., Taylor, B., Ashby, M., Shannon, K., Casey, P. J., and Young, S. G. (1999). *J. Biol. Chem.* **274,** 8383–8390.]

methylation assay (*14, 15*) (Fig. 7A). The membranes from *Rce1*$^{-/-}$ fibroblasts lacked significant levels of processing activity for the farnesylated heterotrimeric $G\gamma_1$ subunit, farnesylated H-Ras, farnesylated N-Ras, geranylgeranylated K-Ras, and geranylgeranyl-Rap1B (Fig. 7B).

The Western blot studies indicated that Ras endoproteolytic processing was abolished in the *Rce1* knockout mice. To determine whether the loss of *Rce1* affected the intracellular localization of Ras, both immunofluorescence microscopy and cell fractionation studies were performed. An enhanced GFP–K-Ras fusion protein was transformed into *Rce1*$^{-/-}$ and *Rce1*$^{+/+}$ embryonic fibroblasts. In *Rce1*$^{+/+}$ cells, the fluorescence was localized to the plasma membrane (Fig. 8A). In contrast, most of the fluorescence in the *Rce1*$^{-/-}$ cells was cytosolic or associated with internal membranes (Fig. 8B). A substantial difference in the subcellular localization of Ras was also evident by immunoblot analysis of P100 and S100 fractions from *Rce1*$^{-/-}$ and *Rce1*$^{+/+}$ fibroblasts. Virtually all the Ras proteins in *Rce1*$^{+/+}$

cells were located in the P100 fraction (Fig. 8C), whereas a large fraction of the Ras proteins in *Rce1*$^{-/-}$ cells was in the S100 fraction (Fig. 8C).

The most reasonable interpretation of these results is that the mislocalization of the Ras proteins in the setting of *Rce1* deficiency results from a failure to clip the C-terminal three amino acids from the proteins. The extent to which the mislocalization is due to the absence of isoprenylcysteine carboxyl methylation remains to be established. In the future, that issue could be clarified by examining the intracellular distribution of Ras proteins in mice lacking the isoprenylcysteine carboxyl methyltransferase. It is also possible that *Rce1* deficiency impairs Ras palmitoylation. A significant part of the membrane association of the Ras proteins depends on palmitoylation (*46–48*), and it has been suggested that retardation in the endoproteolysis and methylation of Ras might affect the extent of Ras palmitoylation (*49*). It would not be particularly surprising if the absence of endoproteolysis and carboxyl methylation in *Rce1*$^{-/-}$ cells affected the extent of Ras palmitoylation.

3. *Potential Relevance of Rce1 as Therapeutic Target for Treatment of Ras-Induced Cancers*

The striking mislocalization of Ras2p in *rce1Δ* yeast, combined with the reduction in heat-shock sensitivity elicited by a mutationally activated Ras2p, led Boyartchuk *et al.* (*13*) to suggest that RCE1 might be an attractive target for the treatment of human cancers associated with activated forms of Ras. Could such a strategy be successful? On the one hand, some of the findings with the *Rce1*-deficient mice might lead one to be dubious about the potential of RCE1 inhibition to retard cell growth. For example, the *Rce1*$^{-/-}$ fibroblasts grew normally; *Rce1*$^{-/-}$ hematopoietic stem cells engrafted, and embryonic development appeared normal until late in gestation. On the other hand, other findings were more encouraging. The GFP–K-Ras fusion construct was profoundly mislocalized in mammalian cells—probably even more so than with the GFP–Ras2p fusion in *rce1Δ* yeast, making it plausible that Ras function would be abnormal in those cells. In yeast, the effects of the *RCE1* knockout mutation were most impressive in the cells that expressed a mutationally activated Ras protein, so it is possible that the same will be true in humans. To date, no studies of Ras signaling in *Rce1*$^{-/-}$ fibroblasts have been reported, nor have there been any reports on the susceptibility of *Rce1*$^{-/-}$ fibroblasts to transformation with activated forms of Ras. Even when these experiments are completed, there is no guarantee that the results obtained in fibroblasts will be generalizable to the epithelial cell types most frequently involved in human cancers.

Potentially relevant to the suitability of RCE1 as a pharmacological target

are the 1992 studies by Kato and co-workers (*2*), who examined the behavior of a Ras mutant that did not undergo efficient proteolysis/carboxyl methylation. They produced a panel of K-Ras4B constructs with a variety of amino acid substitutions in the CXXX sequence. Certain substitutions, such as changing the wild-type –VIM sequence to –VDM, abolished farnesylation, prevented membrane binding of the Ras, and eliminated Ras transforming activity in NIH 3T3 cells. A –VYM mutant principally affected the postisoprenylation processing steps. When that mutant was expressed in NIH 3T3 cells, some nonfarnesylated protein was produced, but a significant percentage of the protein was farnesylated but not further processed (i.e., no endoproteolysis or carboxyl methylation). No fully processed Ras was observed. Of the –VYM Ras protein that had undergone farnesylation, 50% was located in the membrane fractions (vs. >90% with wild-type Ras). Moreover, the –VYM construct displayed transforming capacity, although at a reduced efficiency compared with the wild-type construct. These results led the authors to conclude that isoprenylation, but not the subsequent processing events, were crucial for Ras transforming capacity. While these experiments appeared to be convincing, several caveats should be borne in mind. First, the expression vector that they used undoubtedly produced high levels of Ras expression, and it is possible that the transformation that they observed with the –VYM mutant represented a nonphysiologic consequence of flooding the system with high levels of Ras expression. The effects of blocking endoproteolysis might have been more significant at a physiologic level of Ras expression. Second, as noted earlier, it is not clear whether results with fibroblasts can be generalized to all cell types.

There has been one report suggesting that irreversible Ras endoprotease inhibitors block the growth of Ras-transformed rodent cells (*36*). At 5 μM concentrations, both BFCCMK [*N-tert*-butyloxycarbonyl-(*S*-farnesyl-C)-chloromethyl ketone] and UM96001 (*N-tert*-butyloxycarbonyl-2-amino-DL-hexadecanoylchloromethyl ketone) blocked the growth of three different Ras-transformed cells (two transformed with Kirsten murine sarcoma virus, and one human endometrial cancer cell line with an activating mutation in K-Ras) but did not affect the growth of nontransformed NIH 3T3 cells. Low concentrations of these compounds also blocked the anchorage-independent growth of transformed cells. While these results were encouraging, it is important to note that the report contained no evidence of the specificity of these compounds and no biochemical evidence that the drugs truly interfered with Ras endoproteolysis.

B. Characterization of Mammalian AFC1 (STE24)

Two groups have reported the cloning and initial characterization of the human ortholog of yeast *AFC1* (*STE24*) (*16, 17*). The human *AFC1* (*STE24*)

TABLE V

PROPERTIES OF HUMAN *AFC1* (*STE24*)

No protein substrates have been identified. Its role, if any, in processing isoprenylated CXXX proteins has not been established
The human enzyme is almost certainly capable of carrying out both N-terminal and C-terminal processing of precursor to yeast mating pheromone **a**-factor
Expressed in all mouse and human tissues examined, with the highest levels reported in kidney, prostate, testis, and ovary
Immunofluorescence microscopy with hemagglutinin-tagged AFC1 (STE24) indicated predominant localization in ER
An ~3-kb transcript encodes a 475-amino acid protein; 62% similarity and 36% identity with *S. cerevisiae AFC1* (*STE24*) amino acid sequence
Multiple predicted transmembrane domains
Contains an HEXXH domain, which is shared by the yeast enzyme and many other zinc proteases. Contains a degenerate dilysine ER retention sequence

cDNA encodes a 475-amino acid protein with 36% identity and 62% similarity to yeast Afc1p (Ste24p). Like its yeast counterpart, the putative human ortholog contains the zinc metalloprotease motif HEXXH, as well as multiple predicted transmembrane domains (Fig. 2C). Figure 1 shows the predicted sequence of the human AFC1 (STE24) protein in comparison with other related sequences in the databases. High levels of human AFC1 (STE24) transcripts were detected in all tissues, including heart, brain, kidney, spleen, intestine, testis, ovary, and prostate (*17*). Immunofluorescence studies in HEK-293 cells transiently transformed with a hemagglutinin-tagged AFC1 (STE24) construct revealed an ER-like staining pattern similar to that of calnexin (an ER protein), indicating that the human AFC1 (STE24) protein is localized to the ER and Golgi (*17*). These results are consistent with a prior report by Schmidt *et al.* (*50*) demonstrating an ER localization of Ste24p (Afc1p) in yeast. Key properties of mammalian AFC1 (STE24) are listed in Table V.

Tam *et al.* (*16*) initially cloned the human *STE24* cDNA ortholog as a part of their study to document the dual roles of Ste24p in the processing of **a**-factor. Interestingly, the human STE24 cDNA partially corrected the mating defect in *ste24Δrce1Δ* yeast and fully complemented the modest mating defect of single *ste24Δ* mutants. Those observations suggest strongly that the human protein can mediate both the C-terminal and N-terminal **a**-factor processing reactions.

While the human AFC1 (STE24) protein clearly has the capacity to cleave yeast **a**-factor, no **a**-factor orthologs have been identified in mammals. Thus, the natural substrate(s) for human AFC1 (STE24) is unknown. One possibility is that the natural substrate actually is an as-yet-unidentified **a**-

factor ortholog. Another possibility is that human AFC1 (STE24) participates in the C-terminal processing of a variety of CXXX proteins, both alone and in combination with RCE1. A potential candidate for a substrate is prelamin A, an isoprenylated CXXX protein that undergoes an N-terminal processing step after the removal of the –XXX sequence (*51*). Finally, it is conceivable that human AFC1 (STE24) is not even involved in the processing of mammalian CXXX proteins, but instead has a different role. Along these lines, it is noteworthy that human AFC1 (STE24) shares important sequence similarities (e.g., the HEXXH domain and multiple transmembrane-binding domains) with a gene from *Helicobacter pylori,* a bacterial organism in which protein isoprenylation and CXXX protein modifications are assumed to be nonexistent. It is conceivable that the role of AFC1 in CXXX protein endoproteolysis is an evolutionary adaptation unique to yeast, and that it plays a different and completely unrelated role in mammalian cell biology. For example, for all we know, AFC1 could be responsible for the γ-secretase activity that results in the production of β-amyloid peptide; that endoproteolytic activity is thought to be located in the ER. Knocking out the mouse *Afc1* gene ultimately might help to identify the protein substrates and the physiologic importance of this protease in higher organisms.

As illustrated in the sequence alignments in Fig. 1, *AFC1* (*STE24*) orthologs from a variety of organisms can be identified in the databases. Each of the apparent orthologs contains the HEXXH sequence involved in the binding of zinc, and each appears to be an integral membrane protein. As judged by the TMHMM transmembrane domain analysis program, both the yeast and the human enzymes appear to share seven transmembrane helices, although a common high probability is found only for putative helices 3, 4, 5, and 6 (Fig. 2A and C).

VI. Modification of C-Terminal Isoprenylcysteine Residues by Methyl Esterification Reactions in CXXX Proteins

The intellectual origin for the study of isoprenylated proteins, as well as the realization that S-lipidated C-terminal cysteine residues in polypeptides could be methyl esterified, can arguably be traced to the structural determination of the peptidyl mating factors of two relatively obscure jelly fungi. The A-10 mating factor of *Tremella mesenterica* was found to contain an α-methyl ester of a C-terminal cysteine residue, which was also modified by an oxidized C_{15} *S*-farnesyl group (*52, 53*). A similarly methylated C-terminal residue was found on the *A*(Ia) mating factor of *Tremella brasiliensis* (*54*). Although evidence of the

isoprenylation of mammalian proteins was available at the time (*55*), the connection between these two findings was clouded initially by the suggestion that mammalian proteins were modified by a distinct type of chemistry in which larger isoprenyl groups (C_{45} to C_{95}) were O-linked to hydroxyl-containing amino acids (*56*).

A milestone in the recognition of the similarity between the modifications of the fungal mating factors and mammalian proteins, and the eventual identification of methyl esterified C_{15} farnesylated and C_{20} gernanylgeranylated cysteines at the C termini of eukaryotic proteins, was the characterization of a mutant gene in *S. cerevisiae* (alternatively designated *STE16, DPR1,* or *RAM1*). That mutant gene, which was later shown to encode the β subunit of protein farnesyltransferase, was shown to be involved in the posttranslational modification of both the yeast mating pheromone **a**-factor and the Ras proteins (*57*). What common structural feature of the mating pheromone and the Ras proteins might allow them to be recognized by the same protein modification system? Powers *et al.* (*57*) suggested that it was within the C terminus of the proteins, where each contained a cysteine, followed by two aliphatic residues and then a C-terminal amino acid residue. This suggestion raised the possibility that a similar type of chemistry occurs with the *Tremella* mating factors and the yeast proteins. However, at that point, the lack of a precise structure for the *S. cerevisiae* **a**-factor and the C terminus of the Ras proteins precluded a direct comparison. In fact, it was initially speculated that the *Ram1p/Dpr1p*-catalyzed modification was a fatty acylation (*57*). In addition, evidence was presented that the mature yeast **a**-factor included a fatty acid-modified cysteine with no trimming of the terminal three amino acids (*58*) and that mammalian Ras proteins were similarly modified (*59*). What was clear was that the processing of the yeast Ras proteins was complex and that more than one reaction was likely involved (*60*).

Independently, the chemical nature of methyl esterified proteins in prokaryotic and eukaryotic cells was beginning to be established. It had been shown that bacterial chemoreceptors were modified on the side chain of L-glutamate residues by type I protein carboxyl methyltransferases, and that a variety of spontaneously damaged mammalian proteins was modified on the side chain of D-aspartyl and L-isoaspartyl residues by type II protein carboxyl methyltransferases [for a review, see Clarke (*61*)]. However, in two methyl esterified proteins, the site of methylation did not appear to fit with the products of either type of enzyme. These were the α subunit of the retinal cGMP phosphodiesterase (*62*) and nuclear lamin B (*63*). With the realization that the genes encoding both these proteins contained a C-terminal CXXX sequence, it was possible to hypothesize that the yeast mating pheromone **a**-factor, the Ras proteins, cGMP phosphodiesterase,

and nuclear lamin B might all share a similar posttranslational modification pathway, and all might have a C-terminal structure similar to that in the *Tremella* mating factors (*19*). If this were the case, a key prediction would be that the **a**-mating factor and the Ras proteins would be methyl esterified. This hypothesis was first tested with the mammalian Ha-Ras protein, and the prediction was upheld. Figure 9, reproduced from an article by Clarke and co-workers (*19*), demonstrates the incorporation of methyl groups into the Ha-Ras protein. These results made it possible to formulate a proposal that the previously recognized CXXX motif (*57*) would direct separate lipidation, proteolytic, and methylation reactions (*19*).

The demonstration that the C-terminal residue of yeast **a**-factor was in fact a farnesylcysteine α-methyl ester (*64*) and the determination that the conserved cysteines in mammalian CXXX proteins were isoprenylated rather than fatty acylated (*65–67*) provided support for the proposed three-part posttranslational modification pathway for CXXX proteins, with the initial lipidation reaction being the addition of a short isoprenyl chain to the conserved cysteine residue. It was difficult, however, to demonstrate directly the presence of the methyl ester in many of the CXXX proteins (*68*). Although evidence of a methyl ester consistent with a C-terminal cysteine residue was presented for both mammalian Ha-Ras (*19*) and yeast Ras2p (*69*), the first direct demonstration of the methyl ester was accomplished for a group of 23- to 29-kDa proteins in retinal rods. With these proteins, a combination of proteolysis and oxidation was used to obtain free cysteic acid methyl ester (*70*). This approach was then used to show that the rod cGMP phosphodiesterase, the γ subunit of large G proteins, and the small G protein G25K also contained α-methyl esters of C-terminal cysteines (*71–73*). Subsequently, high-performance liquid chromatography (HPLC) methods were developed for the direct identification of farnesylcysteine methyl ester and geranylgeranyl methyl ester after complete proteolysis of proteins (*74, 75*).

The discovery of the C-terminal methyl esterified cysteines set off a search for the enzymes that would catalyze these reactions. A synthetic peptide related to the C terminus of the *Drosophila* Ras protein (LARYKC) was used to show that membrane fractions of rat liver, kidney, brain, and spleen could catalyze the methyl esterification of the S-geranylated, S-farnesylated, and S-geranylgeranylated forms of the peptide, but not the unmodified form (*76*). This result showed that the isoprenylation step occurred before the methylation step. Subsequent studies showed that the crucial feature for the recognition of the protein substrate by the methyltransferase was the isoprenylated cysteine residue itself and that a variety of peptides, modified cysteine residues, and nonamino acid analogs could serve as substrates (*77–80*). The availability of these synthetic substrates

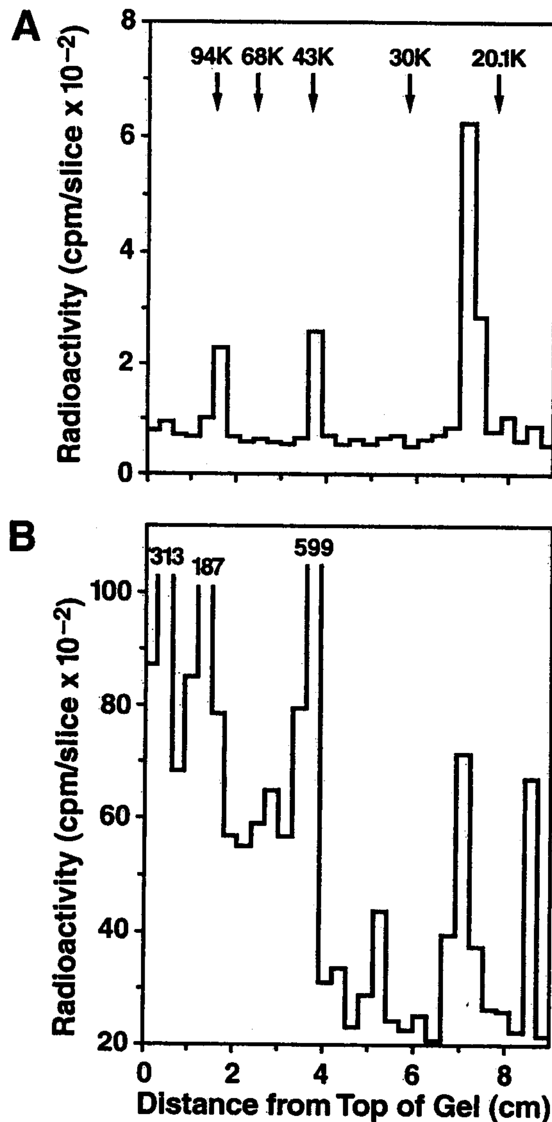

FIG. 9. Immunoprecipitation of Ha-Ras proteins from transformed fibroblasts labeled with [*methyl*-^{3}H]methionine. Immunoprecipitates were denatured in SDS and the polypeptides were size fractionated on a 10% polyacrylamide–SDS gel. Dried gel slices were assayed for base-labile volatile radioactivity (A) and for total radioactivity (B). The amount of methyl groups incorporated into the Ras polypeptide was calculated from the radioactivity migrating from 6.9 to 7.5 cm from the top of the gel. Assuming a background of 40 cpm and 83% efficiency in methanol transfer from the gel slice to the scintillation fluid, the Ras polypeptide band contains 1000 cpm as methyl esters. The total radioactivity (which includes both methyl esters and methionine residues in the protein) in these same fractions is 6987 cpm if a background of 2000 cpm is assumed. These results suggest that approximately one radiolabeled methyl group is present for every six labeled methionine residues. The positions of the molecular weight standards (in $M_r \times 10^{-3}$) are indicated by arrows. [Reproduced, with permission, from Clarke, S., Vogel, J. P., Deschenes, R. J., and Stock, J. (1988). *Proc. Natl. Acad. Sci. U.S.A.* **85,** 4643–4647.]

then allowed for the characterization of both the yeast and mammalian methyltransferases, which are discussed in the following sections.

VII. Isoprenylation and Carboxyl Methylation in Proteins Containing Cys–Xaa–Cys and Cys–Cys Motifs at Their C Termini

Not all proteins that are isoprenylated at the C terminus have the canonical precursor CXXX sequence. Some members of the Rab small G protein family involved in vesicular transport have a C-terminal Cys–Xaa–Cys sequence that is geranylgeranylated at both cysteine residues and methyl esterified. Examples of these proteins include the YPT5, Rab3a, and Rab4 proteins (*81–84*). Some differences have been reported in the affinity of inhibitors for the CXXX and CXC methyl-accepting substrates, and it has been suggested that the methyltransferase responsible for methylating the CXC proteins is distinct from the *STE14* isoprenylcysteine methyltransferase described below, which is responsible for the methylation of CXXX proteins (*85*). On the other hand, Beranger *et al.* (*86*) have presented evidence suggesting that only a single isoprenylcysteine methyltransferase exists, at least in yeast. They expressed mammalian Rab6, a CXC protein, into wild-type yeast and *ste14*Δ mutant yeast, and then grew the yeast in the presence of [^{35}S]methionine and *S*-adenosyl-L-[*methyl*-^{3}H]methionine. As expected, both the wild-type and *ste14*Δ yeast incorporated [^{35}S]methionine into Rab6. The wild-type yeast incorporated ^{3}H-labeled methyl esters into Rab6, as judged by the release of ^{3}H-labeled methanol from the Rab6 gel slices. In contrast, no ^{3}H-labeled methanol was released from Rab6 gel slices from *ste14*Δ mutant cells. Their results strongly suggest that the isoprenylcysteine methyltransferase for the CXC proteins is identical to the one for the CXXX proteins, at least in yeast. Further work is needed in this area. It will be important to confirm these results and also determine whether the yeast CXC proteins such as the *YPT5* gene product are methyl esterified in *ste14*Δ yeast.

Other Rab proteins, including YPT1, YPT3, and Rab2, have C-terminal Cys–Cys sequences that are also modified by geranylgeranylation at both of the cysteine residues. Interestingly, these proteins do not appear to be methylated by Stel4p or any other methyltransferase, and they appear to have a free α-carboxyl group on the C-terminal cysteine (*82, 83, 87*). Smeland and co-workers (*88*) demonstrated that changing the CXC sequence on Rab3a to a CC sequence abolished carboxyl methylation, while replacing the CC sequence of Rab1a with a CXC motif resulted in a carboxyl methylated protein. When the CC terminus of Rab1a was changed to Ser–Cys, the protein was methylated. Thus, it appears that two adjacent

geranylgeranylcysteines in the CC proteins can prevent the methyltransferase from catalyzing methyl ester formation at the C-terminal residue (*88*).

VIII. Characterization of Yeast Protein Modification Catalyzed by *STE14*-Encoded Methyltransferase

A. Identification of *Saccharomyces cerevisiae STE14* Gene as Structural Gene for Methyltransferase

Methyltransferase activity was initially detected in a crude membrane fraction of wild-type *S. cerevisiae* at levels similar to those observed previously in rat microsomes, using an assay containing *S*-adenosyl-L-methionine and the methyl-accepting peptide *S*-farnesyl-LARYKC (*89*). As with the mammalian enzyme (*76*), little or no activity was detected in cytosolic fractions, and the nonfarnesylated peptide was not found to be a methyl acceptor. With the knowledge that the methyl ester on **a**-factor was important for its activity (*64*), it was possible to ask whether any of the previously described yeast **a**-sterile mutants might lack the methyltansferase. Normal methyltransferase activity was present in *STE6*- and *STE16*-deficient yeast, but no activity was found in the *STE14*-deficient mutants (*89*). Because the sequence of the peptide substrate in these experiments was derived from a Ras protein, this experiment suggested that one methyltransferase catalyzed both **a**-factor peptide and Ras methylation reactions. Furthermore, transformation of wild-type and mutant cells with a plasmid containing the *STE14* gene resulted in the overproduction of active methyltransferase (*89*).

Subsequent studies confirmed that the *S. cerevisiae STE14* gene was the structural gene for the isoprenylcysteine methyltransferase [protein-*S*-isoprenylcysteine *O*-methyltransferase (EC 2.1.1.100)] (*90*). Normally, bacteria such as *Escherichia coli* lack the isoprenylcysteine methyltransferase. However, when the yeast *STE14* gene was expressed as a fusion protein in *E. coli,* the membrane fraction of the bacteria catalyzed the methyl esterification of a synthetic peptide (*90*).

Key properties of yeast STE14 are listed in Table VI.

B. Methylation of a-Mating Factor, *RAS1* and *RAS2* Gene Products, and Other Yeast Polypeptides by *STE14*-Encoded Methyltransferase

By overexpressing **a**-factor in yeast, it was possible to show that neither the intracellular nor the extracellular forms of **a**-mating factor are methylated in *ste14Δ* yeast (*90*). Similarly, when the *RAS1* and *RAS2* genes were

TABLE VI

PROPERTIES OF YEAST *STE14*

Major, and likely only, methyltransferase that methylates the carboxyl group of isoprenylcysteine of CXXX proteins
Likely responsible for methylating the C-terminal isoprenylcysteine of proteins that terminate in Cys–Xaa–Cys
Methylates *N*-acetyl farnesylcysteine and short farnesylated peptides efficiently
Methylates both farnesylated and geranylgeranylated substrates
Null mutants do not methylate **a**-factor and are sterile
Null mutants lack methylation of Ras1p and Ras2p. Nevertheless, the heat shock sensitivity and glycogen accumulation phenotypes are identical in *ras2^{val19}* mutants and *ras2^{val19}/ste14Δ*) double mutants
Localized in the ER membranes by immunofluorescence and subcellular fractionation
239-amino acid protein, apparent molecular mass of 24 kDa by SDS–polyacrylamide gels
Protein has multiple predicted transmembrane domains, but no *S*-adenosylmethionine-binding motif

overexpressed in *ste14*Δ yeast, no methyl esters could be detected in either protein (*90*). To examine the possible methylation of other polypeptides, Hrycyna *et al.* (*91*) incubated wild-type and *ste14*Δ yeast with *S*-adenosyl-L-[*methyl*-^{3}H]methionine. Yeast incubated with *S*-adenosyl-L-[*methyl*-^{3}H]methionine take up the label and use it for endogenous transmethylation reactions. Hrycyna *et al.* (*91*) size fractionated the resulting ^{3}H-methylated polypeptides (representing proteins that have been methylated by Ste14p as well as by other protein methyltransferases) on SDS–polyacrylamide gels, and then analyzed the [^{3}H]methyl ester content of the various proteins. In wild-type yeast, methyl esterified polypeptides were detected at 49, 38, 35, 33, 31, and 26 kDa (*91*). In *ste14*Δ yeast, methylated proteins were not observed in the 38-, 33-, 31-, and 26-kDa regions, suggesting that polypeptides migrating at these positions are substrates for the *STE14*-encoded isoprenylcysteine methyltransferase. Under the conditions of this experiment, **a**-mating factor would not be detected and the *RAS1* and *RAS2* gene products would be expected to make up at least a portion of the radioactivity at 38 kDa (*91*). Thus, it is clear that the *STE14* methyltransferase can modify a variety of yeast proteins in addition to the Ras proteins. As described above, there are perhaps more than 80 yeast CXXX proteins, including the products of the *RHO1, RHO2, RHO3, RHO4, BUD1/RSR1, STE18,* and *CDC42* genes (*90*.) All the *STE14*-dependent methylated polypeptides were localized in the membrane fraction, and their methylation was inhibited by the protein synthesis inhibitor cyclohexamide (*91*).

Pulse–chase experiments showed no turnover of methyl esters in the 38-kDa polypeptide and only slow turnover in the 33/31-kDa species. These

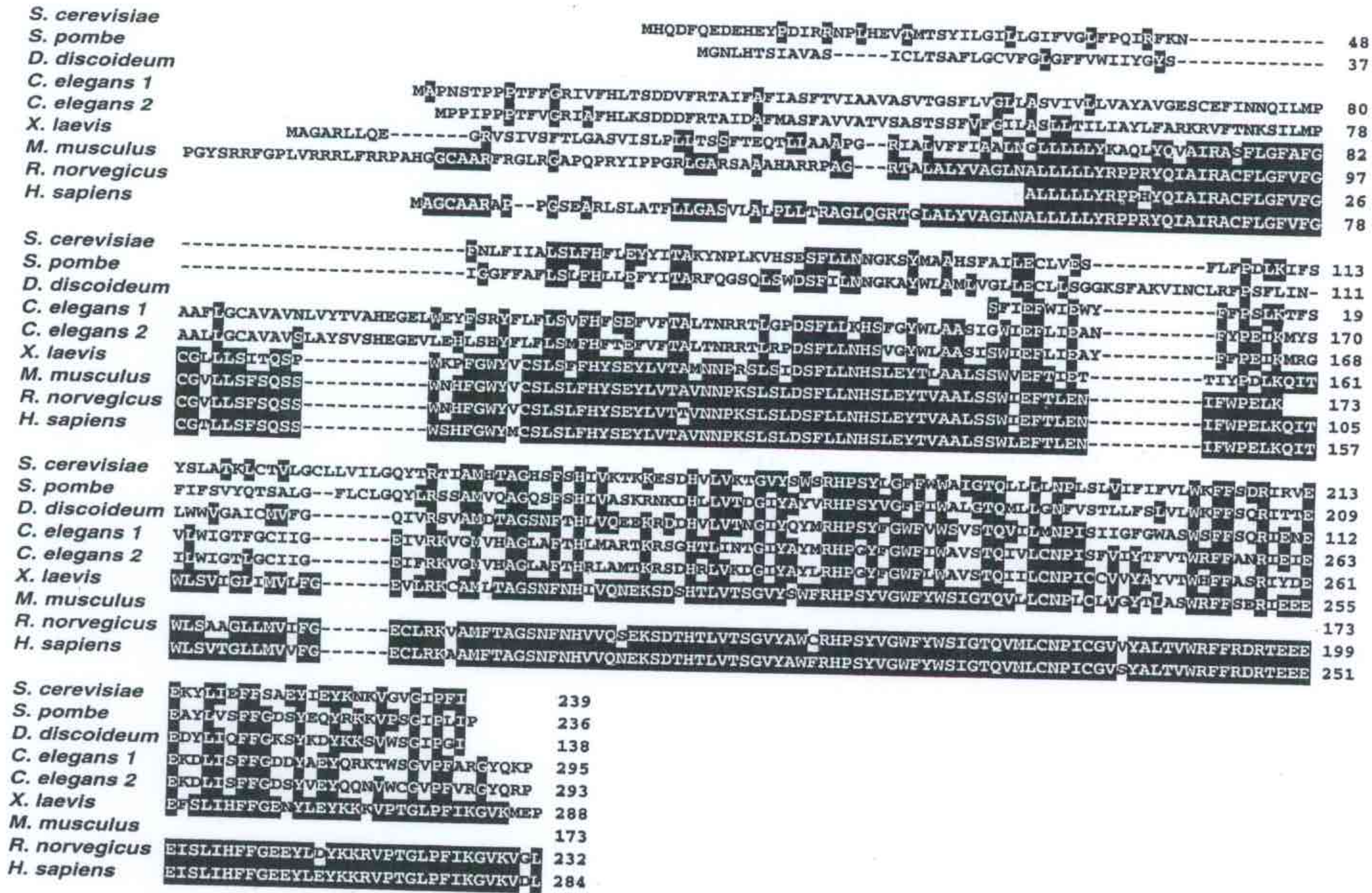

FIG. 10. Amino acid sequence alignment of yeast Ste14p and orthologs in other eukaryotes. GenBank accession numbers: *C. elegans* 1, AAB42280; *C. elegans* 2, AAB37832; *S. pombe*, BAA18999; *X. laevis*, BAA19000; *S. cerevisiae*, AAA16840; human, AF064084. GenBank EST accession numbers: mouse, AA022288; rat, AAD42926; *D. discoideum*, C89921. Protein sequences were aligned as described in the legend to Fig. 1.

results suggest that the isoprenylcysteine methylation reaction largely occurs immediately after protein translation and that the methylation is not readily reversible. These studies also indicate that a single isoprenylcysteine methyltransferase is present in *S. cerevisiae* and can methylate **a**-factor and other protein products.

C. CHARACTERIZATION OF *STE14* GENE PRODUCT

The DNA sequence of the yeast gene encoding the isoprenylcysteine methyltransferase was initially presented at a meeting (*92*) and was then published in full form (*43, 93*). Yeast Ste14p contains 239 residues and is rich in hydrophobic amino acids. Its amino acid sequence is shown in Fig. 10, along with orthologs in other species. The yeast enzyme, like its human counterpart, is predicted to have multiple transmembrane domains (Fig.

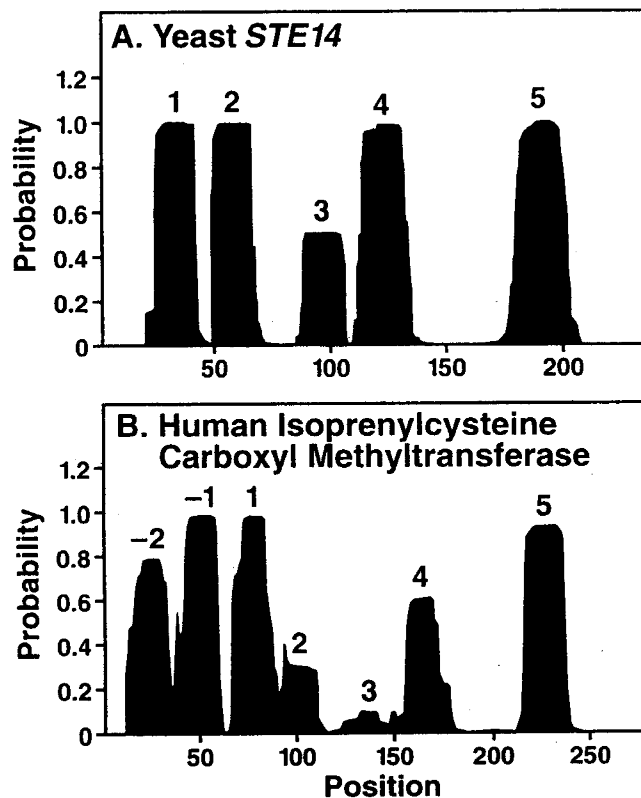

Fig. 11. Predicted transmembrane domains for yeast Ste14p (A) and its human ortholog, human isoprenylcysteine carboxyl methyltransferase (B). Predicted transmembrane domains were determined with the TMHMM transmembrane domain analysis program (http://genome.cbs.dtu.dk/services/TMHMM-1.0/). To aid in the alignment, potential transmembrane domains with yeast/human sequence identities are indicated by numbers 1–5. The regions labeled −1 and −2 in the human sequence are not found in the yeast sequence.

11). By immunofluorescence microscopy and cellular fractionation studies, the enzyme is localized to the ER (*94*). A similar localization was found for the mammalian enzyme (see below) (*18*, *100*).

D. Physiological Role of Isoprenylcysteine Methyltransferase in Yeast

Several general possibilities have been suggested for the function of the methylation reaction. First, the conversion of a carboxylate anion to a methyl ester residue would be expected to make the C terminus more hydrophobic, rendering it more likely to partition into the hydrophobic membrane (for reviews, see Refs. *23* and *95*). Second, once the three C-terminal amino acids have been removed, methylation of the isoprenylcysteine might protect the precursor polypeptide from proteolytic digestion (*22*). Finally, methyl esterification could serve as a recognition signal for specific receptor proteins in the plasma membrane or other intracellular membranes (*68*). Most of the isoprenylated proteins are involved in signal

transduction, and it seems possible that methylation could modulate specific protein–protein interactions and thereby affect upstream or downstream signaling partners.

The availability of the *ste14Δ* mutants in *S. cerevisiae* provided an opportunity to address the function of methylation in this organism. Methylation of **a**-factor appears to be important for several reasons. First, the wild-type **a**-factor is at least 200-fold more active than the nonmethylated **a**-factor in *ste14Δ* yeast (*90*). That result was not particularly surprising, given that *ste14Δ* mutants were originally isolated because of a sterile phenotype (*96*). In addition, the lack of **a**-factor methylation results in enhanced proteolytic degradation inside cells, and an essentially complete block in the export of the protein from cells (*43*).

Aside from the dramatic loss of mating ability, *ste14Δ* yeast are viable and seem to be phenotypically normal (*43, 90*). Considering the importance of Ras1p and Ras2p in cell growth and survival, this result is rather surprising. To assess whether *ste14Δ* yeast exhibit a partial loss of Ras activity, *ste14Δ* yeast were constructed in a $ras2^{Val19}$ strain. The latter mutation results in the expression of a constitutively active Ras protein, which causes heat shock sensitivity and the loss of ability to accumulate glycogen. Mutations in the genes for protein farnesyltransferase have been found to block the expression of the phenotypes elicited by the $ras2^{Val19}$ mutant because farnesylation of the Ras proteins is essential for activity (*57, 97*). However, this was not the case with the $ste14\Delta/ras2^{Val19}$ double mutants, indicating that the loss of methylation did not significantly affect the activity of the mutationally activated Ras proteins (*90*). These results suggest that the yeast Ras proteins retain functional activity in the absence of methyl esterification.

Even though the *ste14Δ* mutation had no major effect on the activity of the activated yeast Ras protein, a significant effect was found on the processing, stability, and membrane attachment, of Ras2p (Fig. 12) (*90*). In wild-type cells, the processing involves the conversion of a nonfarnesylated 41-kDa cytosolic precursor (p41) to a 40-kDa farnesylated species (p40) that can become palmitoylated and associate with the membrane (*60, 98*). In *ste14Δ* cells, the conversion of p41 to p40 appeared to be inhibited. Only a small amount of the p40 polypeptide is made in the cytosol, and even less becomes associated with the membrane (Fig. 12) (*90*). In addition, the total amount of protein was decreased and the fraction of protein in the membrane fraction was greatly reduced.

The inhibition of the p41-to-p40 conversion was especially puzzling because this conversion is thought to involve the farnesylation of an unmodified precursor protein, a reaction that should be unaffected in *ste14Δ* cells. There are several possible solutions to this paradox. One possibility is that

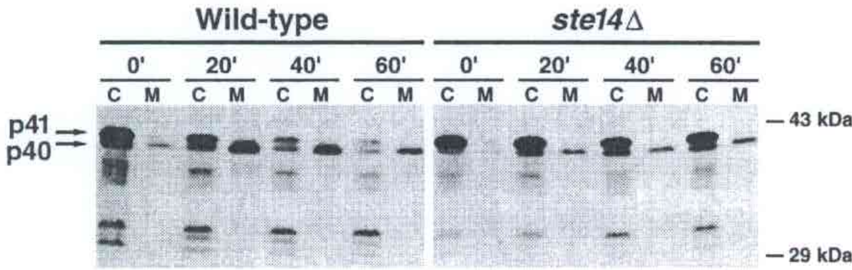

Fig. 12. Kinetics of Ras2p membrane localization in wild-type and *ste14Δ* yeast. Wild-type and *ste14Δ* yeast containing the plasmid pADH-*RAS2* were labeled with [^{35}S]methionine for 2 min at 30°C, and chased for the indicated times. Total cell extracts from the pulse–chase experiment were prepared and immunoprecipitated with the anti-Ras monoclonal antibody Y13-259 and immunoprecipitates were analyzed by SDS–PAGE on a 10% polyacrylamide gel. [Reproduced, with permission, from Hrycyna, C. A., Sapperstein, S. K., Clarke, S., and Michaelis, S. (1991). *EMBO J.* **10,** 1699–1709.]

the isoprenyltransferases or the *RCE1* protease or both are in a membrane-bound complex with the *STE14* methyltransferase, so that the loss of the methyltransferase might adversely affect the activity of the farnesyltransferase or the endoprotease. Thus, the accumulation of p41 protein might represent the unmodified precursor or the farnesylated but nonproteolyzed protein. In mammalian cells, the loss of RCE1 activity results in the production of Ras proteins with distinctly reduced electrophoretic mobility (*14*). A second possibility is that isoprenylation and protease reactions occur normally in the *ste14Δ* mutant but that the absence of methylation of the farnesylcysteine renders the protein more susceptible to proteolytic cleavage by some element of the intracellular protein degradation pathway (*22, 23*). Thus, the p41 in the *ste14Δ* mutant could represent a C-terminal truncated protein in which the farnesylcysteine (and perhaps additional residues) have been exoproteolytically cleaved. This form may migrate in the same position as the unmodified precursor p41 in the wild-type cells. This hypothesis would account for the apparent persistence of the p41 precursor species in the *ste14Δ* mutant yeast (Fig. 12) (*22*). With state-of-the-art mass spectroscopy, it should be possible to isolate the p41 protein from *ste14Δ* cells and determine its precise structure, distinguishing between these possibilities. Other possibilities could also be proposed to explain these data (*90*).

Finally, the absence of overt phenotypes in *ste14Δ* cells, such as retarded cell growth, indicates that the other protein substrates for the isoprenylcysteine methyltransferase can function in the absence of the C-terminal methyl ester or are not essential for viability. Nevertheless, it would be of interest to determine whether the absence of carboxyl methylation affects the intracellular stability of other CXXX proteins or their ability to become associated with membranes.

IX. Methyl Esterification of Isoprenylated Mammalian Proteins

A. Specificity of Isoprenylcysteine Methyltransferase

A key element in the recognition of the protein substrate is the isoprenyl group. For the rat liver microsomal enzyme, the K_m for the unmodified LARYKC peptide was greater than 45 m*M*. In contrast, the K_m values for the *S*-geranyl derivative (C_{10}), the *S*-farnesyl derivative (C_{15}), and the *S*-geranylgeranyl (C_{20}) derivative were 389, 2.2, and 10.9 μM, respectively. When the peptide was modified at the sulfhydryl group with C_8, C_{10}, C_{13}, and C_{15} *n*-alkyl derivatives, some activity was observed but the estimated K_m values (based on a V_{max} comparable to that of the farnesyl peptide) were 480–1760 μM, reflecting relatively weak substrate recognition compared with the isoprenylated derivatives (*76*).

N-Acetylfarnesylcysteine is itself a good substrate for the methyltransferase (*77, 78, 80, 99*). The minimal recognition element is simply an isoprenylated thiopropionate moiety, and no amino acid or peptide sequences are needed at all (*80, 99*). K_m values for *N*-acetylfarnesylcysteine have been measured at 20 μM (*78*) and 22 μM (*99*), while values of 14 μM (*80*) and 20 μM (*77*) have been reported for *S*-farnesylthiopropionic acid. Thus, the affinity of the methyltransferase for these substrates is only about an order of magnitude less than that of a farnesylated peptide substrate (*76*) and suggests that most of the recognition by the methyltransferase is due to the carboxyl group being in the correct spatial relationship to the isoprene group. The K_m value for *N*-acetylgeranylgeranylcysteine has been reported as 7 μM (*77*), a value similar to that of the farnesyl analog. Kinetic evidence has been presented suggesting that the mammalian methyltransferase can recognize both farnesyl and geranylgeranyl substrates (*79*).

B. Intracellular Localization of Enzyme

From the initial report of the isoprenylcysteine methyltransferase activity, it was clear that the enzyme was membrane associated; the highest specific activities were in the microsomal fraction, containing largely ER membranes but also contains lesser amounts of plasma membrane and other internal membranes (*76*). Subsequent fractionation of rat liver microsomal membranes by Percoll density gradient centrifugation revealed that the methyltransferase largely comigrated with ER markers and not with markers for the plasma membrane or the Golgi apparatus (*100*). Studies have confirmed its localization to the ER (*18*). One report suggested that human neutrophils possess a plasma membrane-localized enzyme that is activated by anionic phospholipids (*101*), but this activity has not been characterized

further. There appears to be only a single *STE14* gene in mammals, as judged by analyses of the EST databases. If a distinct plasma membrane-associated enzyme truly exists, it may be encoded by a structurally distinct gene. Further work is required to establish the existence of a second type of isoprenylcysteine methyltransferase activity in mammalian cells.

Attempts to solubilize the *STE14*-related enzyme in active form have not been successful (*100*). This enzyme appears to form an integral part of the membrane and its activity is lost when the membranes are treated with a variety of mild detergents that have been used successfully to solubilize other membrane proteins in active form (*100*).

C. Effect of GTP on Methylation Reactions

In a number of *in vitro* systems, methylation reactions attributable to the *STE14*-related methyltransferase have been enhanced by the presence of a nonhydrolyzable GTP analog, GTPγS (*102–105*). GTPγS does not appear to stimulate the methyltransferase directly (*100*) but serves to activate a class of small G proteins, such as G25K, for membrane attachment, which then allows for more efficient methylation by the membrane-bound Ste14p (*106*).

D. Methyltransferase Inhibitors as Functional Probes *in Vivo*

There has been an intensive effort to utilize specific inhibitors of the isoprenylcysteine methyltransferase to understand the functional roles of this enzyme. For example, 5′-methylthioadenosine has been reported to inhibit the carboxyl methylation and assembly of nuclear lamin B (a CXXX protein) (*107*), as well as a class of small G proteins (*108*). The origin of this effect may be complex, however, because the isoprenylcysteine methyltransferase does not appear to be inhibited by this compound in *in vitro* assays (*100*).

Other studies have utilized compounds that result in the accumulation of *S*-adenosylhomocysteine and other derivatives that inhibit both the isoprenylcysteine methyltransferase (*76*) and other types of *S*-adenosyl-L-methionine-dependent methyltransferases. From the time when it was established that receptor function in bacterial chemotaxis requires the methylation of a group of L-glutamate residues [for a review, see (*61*)], attempts have been made to show that mammalian cell chemotaxis also requires methylation. Early work showed that chemoattractants could transiently increase the level of methyl esterification of rabbit neutrophils (*109, 110*). Treatment of a mouse macrophage cell line with 3-deaza-adenosine, a precursor of the methyltransferase inhibitor 3-deaza-adenosylhomocys-

teine, inhibited their chemotaxis (*111*), although this effect was later attributed largely to an effect on protein synthesis (*112*). The discovery that methyl-esterified G proteins mediate mammalian chemotaxis sparked a renewed interest in this topic. It was found, for example, that the treatment of human HL-60 cells for 24 or 48 hr with peroxidate-oxidized adenosine, a compound that results in the intracellular accumulation of *S*-adenosylhomocysteine, inhibits the fMet-Leu-Phe-dependent release of superoxide ion (*113*). However, while suggestive of possible functional roles of the isoprenylcysteine methyltransferase reaction, these studies must be interpreted with caution because of the large number of other methylation reactions that could also be inhibited.

Another approach to more specifically inhibit the mammalian isoprenylcysteine methyltransferase in intact cells involves the use of methyl-accepting small molecules such as *N*-acetylfarnesylcysteine (*77–80*) and *N*-acetylgeranylgeranylcysteine (*79, 114*) as cell-permeable competitive inhibitors. These compounds would be expected to inhibit the isoprenylcysteine methyltransferase without inhibiting other types of methyltransferases. However, these compounds may affect other cellular processes. For example, the fact that they contain isoprenyl groups could lead them to bind to other cellular proteins that normally interact with isoprenylated proteins.

The potential benefits and risks of this approach are illustrated in studies on the effect of *N*-acetylfarnesylcysteine on the chemotactic response of mammalian cells. It was initially reported that macrophage chemotaxis was inhibited about 80% in the presence of *N*-acetylfarnesylcysteine (*78*). This study suggested that the methylation of an isoprenylated residue may be a crucial component of the signaling system. Similar results were found in human HL-60 cells (*115*) and with the chemoattractant-mediated activation of the G_i protein in neutrophils (*116*). In another study, however, it was shown that *N*-acetylfarnesylcysteine and its analogs could either inhibit the chemotactic peptide-stimulated release of superoxide ion in human neutrophils or stimulate peptide-independent release (*117*). Because similar results were obtained with analogs that inhibit the isoprenylcysteine methyltransferase (e.g., *N*-butyrylfarnesylcysteine) and those that do not (e.g., *N*-pivaloylfarnesylcysteine), it did not appear that these effects are mediated entirely or in large part by their effect on the methyltransferase (*117*). These results were supported by the finding that general methyltransferase inhibitors did not affect superoxide ion release (*117*), although this result does conflict with that reported in a similar experiment by another group (*113*). Finally, Scheer and Gierschik (*118*) found that stimulation of GTPγS binding and GTP hydrolysis by fMet-Leu-Phe in well-washed membranes of HL-60 granulocytes (which were presumably free of the methyl donor *S*-adenosylmethionine) were still affected by *N*-acetylfarnesylcysteine, sug-

gesting again that *N*-acetylfarnesylcysteine affected a cellular mechanism unrelated to the isoprenylcysteine methyltransferase. Subsequent work by these authors suggested that *N*-acetylfarnesylcysteine exerted its activity by interfering with the interaction of activated receptors with G proteins, probably with their $\beta\gamma$ subunits (*119*).

The *N*-acetylfarnesylcysteine inhibitor approach has also been used to study the role of isoprenylcysteine methylation in platelets. It was initially shown that *N*-acetylfarnesylcysteine inhibits platelet aggregation in response to collagen, ADP, and arachidonic acid (*120*). However, it was then demonstrated that *N*-benzoylfarnesylcysteine and *N*-pivaloylfarnesylcysteine, neither of which is a substrate or an inhibitor of the enzyme, could inhibit platelet aggregation as effectively as, or more effectively than, *N*-acetylfarnesylcysteine (*121*). This latter result again points to the possibility that these methylation inhibitors may also affect other pathways, and suggests that the isoprenylcysteine methyltransferase may not be involved in a direct signal transduction pathway (*121*).

Inhibitor studies with *N*-acetylfarnesylcysteine and related compounds are potentially attractive because of their specificity for the isoprenylcysteine methyltransferase. Unfortunately, these compounds appear to affect "nonmethyltransferase" pathways in a nonspecific fashion. The fact that the inhibitor compounds contain isoprenyl groups may lead them to bind to cellular proteins that interact with isoprenylated proteins. To the extent that this is the case, it might be difficult to distinguish the effect of methyltransferase inhibition from other nonspecific effects. Overall, the take-home lesson from these studies is that it may not be possible to conclude much about the *in vivo* action of the methyltransferase by treating cells with this class of compounds, except perhaps when the result is negative (i.e., when there is no effect on a function).

E. *In Vitro* Studies of Effect of Methyl Esterification on Protein Function

A number of experimental procedures have been used to compare directly the activity of proteins with and without the C-terminal methyl ester and quantitate the effect of this modification on protein function. However, with the notable exception of studies on the activity of **a**-factor in *S. cerevisiae* (*43, 64, 90, 122*), these investigations have largely shown that methylation either does not affect, or affects minimally, the activities of isoprenylated proteins. These studies are described in detail below.

One approach has been to use isolated proteins and membrane fractions from cells preincubated with general methyltransferase inhibitors. With that approach, it has been possible to show that methylation of the γ subunit

of the $\beta\gamma$ complex can enhance the ability of pertussis toxin to ADP-ribosylate the α subunit by about twofold while not enhancing the membrane attachment of the $\beta\gamma$ subunit itself (*113, 123*).

Another approach has taken advantage of the ability of a pig liver esterase preparation to catalyze the hydrolysis of C-terminal isoprenylcysteine methyl esters in the intact protein (*85*). With this approach, it was possible to produce the nonmethylated form of the transducin $\beta\gamma$ subunits and then to compare the capacities of the nonmethylated and fully methyl esterified proteins to stimulate GTPγS exchange in the presence of the transducin α subunit and photoactivated rhodopsin. In the presence of a detergent, no differences in GTPγS exchange were observed with the methylated and nonmethylated proteins (*85*). However, when membrane preparations were used, the methylated transducin $\beta\gamma$ was about twofold more efficient than the nonmethylated form (*85*). Of note, the methylated transducin $\beta\gamma$ was more than 10-fold more potent in activating phosphatidylinositol-specific phospholipase C and phosphoinositide 3-kinase. Similar experiments showed that methylation of the γ subunit of transducin $\beta\gamma$ can enhance its affinity for phosducin by about twofold (*124*). It has also been possible to separate methylated and nonmethylated forms of transducin $\beta\gamma$ by chromatography and then examine their *in vitro* activites (*125*). Here, it was found that methylation could faciliate functional association of transducin $\beta\gamma$ with the light-activated receptor (*125*). Interestingly, transducin $\beta\gamma$ is generally fully methylated in rod cells, raising the possibility that its activity may not be modulated *in vivo* by interconverting the methylated and nonmethylated forms (*125*).

The *in vitro* activity of the $\beta\gamma$ subunit of the nontransducin large G proteins has been assayed by using the pig liver esterase to produce demethylated geranylgeranylcysteine-containing γ subunits (*95*). Methylation had no effect on the ability of the $\beta_1\gamma_2$ subunit to interact with the α subunit, as shown by a pertussis toxin-catalyzed ADP-ribosylation assay. A small effect was seen on the ability of $\beta\gamma$ to activity phosphoinositol-specific phospholipase C or phosphoinositide 3-kinase (*95*). Once again, no striking effects of methyl esterification on protein function were observed.

Functional studies of the mammalian smg-25A/rab3A protein (a CXC isoprenylated protein) have also been reported (*126*). Although this protein may be modified by a methyltransferase distinct from the *STE14*-encoded enzyme (see earlier discussion of this topic), the C terminus still contains an α-methyl ester of an isoprenylated cysteine. It is possible to produce the unmodified precursor of this polypeptide in bacterial cells and then to isoprenylate it *in vitro* so that it contains the geranylgeranyl group but not the methyl ester. When the properties of the nonmethylated protein were compared with those of the fully methylated cellular-derived species, no

differences were observed in its membrane-binding properties or in its interactions with its GDP/GTP exchange protein (*126*).

What conclusions can we draw from the general lack of a large-scale effect of methylation on the functions of proteins? Two avenues can be explored. First, the effect of methylation, although subtle in *in vitro* studies, might be crucial in fine-tuning the responses of these proteins *in vivo*, especially in signal transduction cascades. Second, the effect of the methylation reaction might simply ensure the stability of the protein and its isoprenylated C terminus. As hypothesized above for the *S. cerevisiae* Ras and as demonstrated for **a**-factor (*43*), the methylation reaction can provide a C-terminal cap that may prevent proteolytic "trimming" of the C terminus. Of note, RhoA, a small G protein, was found to have a decreased half-life when methylation was inhibited (*134*). The availability of a mouse knockout of the isoprenylcysteine methyltransferase will probably make it possible to address these hypotheses more directly.

F. Reversibility of Protein Isoprenylcysteine Methylation

There is little evidence that C-terminal methyl esters on isoprenylated proteins can be physiologically hydrolyzed to the free C-terminal carboxyl group. Pulse–chase experiments have indicated slow if any turnover (*20, 91*) and where measured, it appears that the bulk of the cellular protein is fully modified (*125*). *In vitro,* methyl esters are readily cleaved by pig liver esterase (*127*), and while this observation has been nicely exploited as an experimental technique (see discussion above), it is not clear that the enzyme catalyzes this type of reaction *in vivo.* Yeast carboxypeptidase Y can also cleave the methyl ester linkage (*22*), but this vacuolar enzyme probably does not come in contact with isoprenylated proteins *in vivo.* No nonvacuolar activities were found in yeast that could catalyze the hydrolysis of *N*-acetylfarnesylcysteine methyl ester (*22*). The hydrolysis of *N*-acetylfarnesylcysteine methyl ester and methylated transducin can be catalyzed by unidentified proteins in the membrane fractions of bovine retinal rods (*128, 129*), and at least two activities have been found in rabbit brain that catalyze the hydrolysis of methyl esters from the small G protein G25K (*130*). Interestingly, one of the activities found in the latter report was identified as the lysosomal protease cathepsin B (*130*). As with the yeast vacuolar carboxypeptidase Y, it would not appear that this hydrolytic activity would be physiologically important. However, the second soluble enzyme activity (and a similar activity in the membrane fraction) may be more physiologically relevant (*130*). There are a large number of potential methylated substrates in cells, and a large number of potential methylesterases. Matching potential physiological substrates and enzymes to determine whether

some isoprenylated proteins undergo reversible methylation will be a challenge. If reversible methylation occurs, modulation by the relative activity of methyltransferases and methylesterases could be used to effect distinct biological responses. Further work in this area is warranted.

G. Comparison of Amino Acid Sequences of Mammalian and Yeast Methyltransferases

After Ste14p from *S. cerevisiae* was identified and characterized, a 426-base pair (bp) mouse cDNA with homology to *STE14* appeared in the EST databases. This sequence was used to clone the human isoprenylcysteine carboxyl methyltransferase cDNA (*18*). The 3595-bp cDNA encoded a 284-amino acid protein that is 26% identical to yeast Ste14p (Fig. 10). Transfection of the human isoprenylcysteine carboxyl methyltransferase clone into *ste14*Δ yeast partially restored the mating defect, indicating that that the human enzyme can carry out the carboxyl methylation of **a**-factor. Like Ste14p, the human enzyme is predicted to have multiple transmembrane domains (Fig. 11).

Transient transfection of the human isopreylcysteine carboxyl methyltransferase expression vector into Cos-1 cells strikingly increased the methylation of *N*-acetylfarnesylcysteine and *N*-acetyl-*S*-geranylgeranylcysteine. Transfection of the carboxyl methyltransferase enhanced the methylation of the Rho GTPase in Cos-1 cells, and membranes from transfected Cos-7 cells increased the methylation of partially purified neutrophil Rac2 and RhoA. Key properties of the human isoprenylcysteine carboxyl methyltransferase are listed in Table VII.

In Fig. 10, we provide an updated alignment for the encoded amino acid sequences of the *S. cerevisiae STE14* gene, its human ortholog, as well as encoded sequences for the *Schizosaccharomyces pombe* (*94, 131*), *Xenopus laevis* (*131*), mouse, and rat isoprenylcysteine methyltransferase, and the sequences for putative *Dictyostelium discoidium* and *Caenorhabditis elegans* enzymes [compare with Fig. 7 of Romano *et al.* (*94*)]. It is interesting to note that two closely related genes are present in the nematode *C. elegans*. What is most impressive, however, is the conservation of amino acid sequences in all but a relatively small N-terminal region of each protein. The encoded proteins are rich in hydrophobic amino acids, consistent with the tight membrane association of the yeast and mammalian methyltransferases. Analysis of putative transmembrane helical regions by the TMHMM posterior program revealed seven potential regions for the human enzyme and five for the *S. cerevisiae* enzyme (Fig. 11). Two potential N-terminal helices in the human sequence were not found in the yeast sequence. The other five do align, although common high probabilities are found only for

TABLE VII

PROPERTIES OF HUMAN ISOPRENYLCYSTEINE CARBOXYL METHYLTRANSFERASE

Methylates C-terminal isoprenylcysteine in CXXX proteins
Might be responsible for methylating C-terminal cysteine of Rab proteins that terminate in cys–Xaa–Cys
When expressed in Cos-1 cells, methylates neutrophil Rho GTPases and isoprenylcysteine analogs *N*-acetyl-*S*-farnesylcysteine and *N*-acetyl-*S*-geranylgeranylcysteine
The human enzyme complements biochemical defect in *ste14*Δ yeast, indicating that human enzyme can carboxyl methylate **a**-factor
Located in ER
Human enzyme (284 amino acids) is 26% identical to the yeast protein Ste14p. Molecular mass is 33 kDa, as judged by SDS–polyacrylamide gels
Predicted to contain multiple transmembrane domains; the first 65 amino acids are 36% identical to amino acids 750–821 of the human band 3 anion transporter
Sequences homologous to *S*-adenosylmethionine-binding regions that are found in aspartyl and glutamyl protein carboxyl methyltransferases are not apparent in sequence of enzyme

the first and fifth of these sequences. This program does not clearly predict the location (cytosolic or intraluminal) of the domains between the transmembrane domains. Given a localization of the enzyme in the ER (*18, 94, 100*), it would be expected that the residues important for binding the precursor isoprenylated protein and the cosubstrate *S*-adenosyl-L-methionine would be located on the cytoplasmic rather than the luminal side of the membrane. This is because the precursor proteins are modified by cytosolic protein isoprenyltransferases and because no evidence has been presented that there is an intraluminal pool of *S*-adenosyl-L-methionine in addition to the cytoplasmic pool. The sequence of the isoprenylcysteine methyltransferase does not contain any of the four sequence motifs (regions I, post-I, II, and III) that have been described for a number of soluble methyltransferases (*132*).

We have been intrigued by the high conservation in the encoded amino acid sequences of the genes of the various organisms between putative transmembrane helices 4 and 5 and in the C terminus of the human isoprenylcysteine methyltransferase. The putative transmembrane helix 5 may actually represent a hairpin structure so that both the region between helices 4 and 5 and the C terminus are cytosolic and may form the active site of the enzyme. We are especially intrigued by the sequence corresponding to amino acids 205–221 in the *S. cerevisiae* protein, where eight residues are conserved in all species (FFXXRXXXEEXXLXXFF). The four aromatic phenylalanine residues can represent sites of π–π interactions with the

aromatic purine base of *S*-adenosylmethionine; interactions of the positively charged sulfonium atom of *S*-adenosylmethionine with aromatic amino acid residues (cation–π interactions) are also possible. Further evidence of the role of the C-terminal region in the methyltransferase activity is its disruption when a hemagglutinin-epitope tag is inserted at residue 226 (*94*). Closer to the N terminus, the conserved sequences are largely found in the putative transmembrane regions and may not be associated with the active site of the enzyme.

Because successful three-dimensional structure determination of intrinsic membrane proteins such as the isoprenylcysteine methyltransferase is problematic (at least at the present time), a combination of sequence comparisons, *in vitro* mutagenesis studies, and chemical modification studies may be useful for determining the amino acid residues important in the recognition of isoprenylated substrate proteins and the catalysis of methyl ester formation. For example, chemical modification studies have been used to try to identify active site residues. The mammalian enzyme has been shown to be inactivated by *N*-ethylmaleimide in the absence but not in the presence of *S*-adenosylmethionine or *S*-adenosylhomocysteine (*100*). *N*-Ethylmaleimide preferentially modifies cysteine residues although it can also modify lysine and histidine residues, suggesting that one or more of these residues is present in the binding site for *S*-adenosylmethionine. A similar protection from inactivation by *S*-adenosylhomocysteine by reagents with specificity for arginine and tryptophan residues has been reported (*133*). It would be useful to ask if the yeast enzyme may also be similarly inactivated so that specific residues may be targeted for analysis by site-directed mutagenesis techniques.

We have also been interested in the presence of genes in two bacterial species that appear to be related to the genes for the isoprenylcysteine methyltransferases. As shown in Fig. 13, *STE14*-like sequences are found in the prokaryotes *Pseudomonas denitrificans* and *Mycobacterium tuberculosis*. An analysis of these sequences with the TMHMM transmembrane domain analysis program (http://genome.cbs.dtu.dk/services/TMHMM-1.0/) revealed that *P. denitrificans* contains four transmembrane domains, while the *M. tuberculosis* sequence contains two. Biochemical studies will be needed to determine if these enzymes are indeed isoprenylcysteine methyltransferases or whether they may represent distinct methyltransferases or simply play other enzymatic functions. It is clear that *E. coli* does not contain an isoprenylcysteine methyltransferase (*90*), and it has been assumed that this type of methylation occurs only in eukaryotic cells. If some prokaryotes do have an isoprenylcysteine methyltransferase activity, it would indicate that protein isoprenylation reactions are more widespread in nature than previously thought.

```
S. cerevisiae     MHQDFQEDE------------------HEYPDIRR 17
H. sapiens        MAGCAARAPPGSEARLSLATFLLGASVLALPLLTR 35
P. denitrificans      MSTTLP------------------TILMLVV 13
M. tuberculosis                                        0

S. cerevisiae     N-------------------------PLHEVTMTS 27
H. sapiens        AGLQGRTGLALYVAGLNALLLLLYRPPRYQIAIRA 70
P. denitrificans  G---------------------------LSTLALA 21
M. tuberculosis                                        0

S. cerevisiae     YILGILLGIFVGL-FPQIRFKNFNLFIIALSLFHF 61
H. sapiens        CFLGFVFGCGTLLSFSQSSWSHFGWYMCSLSLFHY 105
P. denitrificans  ILRRVQSGRSPVVLTYGDDAEGFAGRLFRLIVAAL 56
M. tuberculosis                                        0

S. cerevisiae     LEYYITAKYNPLKVHSESFLLNNGKSYMAAHSFAI 96
H. sapiens        SEYLVTAVNNPKSLSLDSFLLNHSLEYTVAALSSW 140
P. denitrificans  VIHLLAVAMLPASVDAA---LG------------- 75
M. tuberculosis    MLQRTNVVQP---------LN------------- 12

S. cerevisiae     LECLVESFLFPDLKIFSYSLATKLCTVLGCLLVIL 131
H. sapiens        LEFTLENIFWPELKQITW------LSVTGLLMVVF 169
P. denitrificans  ---RIPALDTPILHAVGLA-----LMTLGGGLTML 102
M. tuberculosis   ----TLRMVWIQVAGIIP-------ATAGIAATVY 36

S. cerevisiae     GQYTRTIAMHTAGHSFSHIVKTKKESDHVLVKTGV 166
H. sapiens        GECLRKAAMFTAGSNFNHVVQNEKSDTHTLVTSGV 204
P. denitrificans  SQWA-------MRHSWKIGIP-EKQ-DAPLVTSGL 128
M. tuberculosis   AQLA-------MGDSWRIGVD-EQE-NTTLVRTGP 62

S. cerevisiae     YSWSRHPSYLGFFWWAIGTQLLLLNPLSLVIFIFV 201
H. sapiens        YAWFRHPSYVGWFYWSIGTQVMLCNPICGVSYALT 239
P. denitrificans  YAFSRNPIYVGMVTALIGTVMAVPN-VISVALALS 162
M. tuberculosis   FKWVRHPIYTAMMAFGLGLLLVTPNLVALAGFILL 97

S. cerevisiae     LWKFFSDRIRVEEKYLIEFFSAEYIEYKNKVGVGI 236
H. sapiens        VWRFFRDRTEEEEISLIHFFGEEYLEYKKRVPTGL 274
P. denitrificans  AWISISYQIRMEETHLAKVFGDAYGAYCRRVRRWV 197
M. tuberculosis   VATLEVHVRRVEEPYLLRTHSAVYRGYTASVGRFV 132

S. cerevisiae     PFI         239
H. sapiens        PFIKGVKVDL  284
P. denitrificans              197
M. tuberculosis   PGVGLIR     139
```

Fig. 13. Amino acid sequence alignment of yeast Ste14p and similar sequences in two prokaryotes. Protein sequences were aligned with Macvector 6.5, using a CLUSTALW alignment. Pairwise alignment was performed with a BLOSUM30 matrix and multiple alignment was performed with a BLOSUM series matrix. The document was then imported into Illustrator. Identical residues are shaded; similar residues are boxed. An analysis of the *P. denitrificans* and *M. tuberculosis* sequences with the TMHMM transmembrane domain analysis program revealed that the *P. denitrificans* contained four predicted transmembrane domains (amino acids 4–22, 43–65, 84–106, and 143–165), while the *M. tuberculosis* sequence contained two (amino acids 18–40 and 76–98). GenBank accession numbers: *P. denitrificans,* P29940; *M. tuberculosis,* CAB03782; *S. cerevisiae,* AAA16840; human, AF064084.

X. Conclusions

There has been substantial progress in understanding the postisoprenylation processing of CXXX proteins. The yeast genes responsible for the endoproteolytic processing of **a**-factor and the Ras proteins have been reported, and orthologs in other species have been identified. The impor-

tance of *RCE1* for the endoproteolysis of mammalian Ras proteins has been established with the *Rce1* knockout experiment and also with biochemical studies. However, many questions remain. For example, almost nothing has been reported regarding structure–function relationships for RCE1 in any organism. It is not clear why *Rce1* knockout mice die during embryonic development. The list of protein substrates for Rce1 is undoubtedly incomplete. Moreover, the protein substrates for which the endoproteolytic processing step carries significant functional consequences has not been established. Nor is it clear whether AFC1 (STE24) plays a role in the endoproteolytic processing of isoprenylated proteins in higher organisms. If it does, the unique and overlapping roles of RCE1 and AFC1 (STE24) in cleaving CXXX proteins need to be established. In addition, the role of AFC1 (STE24), if any, in the proteolytic processing of non-CXXX proteins needs to be investigated.

Advances have also been made in our understanding of isoprenylcysteine methyltransferase. The identity of the mammalian isoprenylcysteine methyltransferase has been established, and orthologs have been identified in multiple organisms. Many of its protein substrates have been established. Once again, however, there are many unanswered questions. There is little information on the structure of this enzyme (e.g., the location of the binding sites for the isoprenylcysteine or *S*-adenosylmethionine). In addition, its importance in mammals needs to be investigated with a gene-knockout experiment.

Much of the interest in the endoproteolysis and carboxyl methylation steps has stemmed from the fact that they are involved in the processing of the Ras proteins, which have been strongly implicated in the development of human cancers. Inhibitors of these steps might be effective in retarding the growth of cancers. It is quite possible that inhibition of these steps could inhibit Ras function in some cell types, but not others. We also believe that effective endoproteolysis/methylation inhibitors might limit cell growth by interfering with the function of "non-Ras" CXXX proteins. In the future, these possibilities could be addressed decisively by using animal models in which these processing steps have been selectively inhibited in certain tissues.

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