Summary

We have described several quantitative and qualitative assays that have been utilized to learn the basic properties of RACE and amphibian and mammalian counterparts. Owing to powerful genetic tractability, high specific activity, and an apparently well-conserved substrate specificity, yeast is an attractive organism in which to study RACE. Efforts are currently in progress to characterize the functional role of the endoproteolytic processing step of many essential proteins.

Acknowledgments

We are indebted to D. S. King for peptide synthesis, mass spectrometry, and unending technical support. We thank L. Wood and A. Moser (University of California, San Francisco) for rat tissues and R. Kim and S.-H. Kim for *Xenopus* oocyte samples and many helpful discussions. We also thank S. Rosenberg and C. C. Yang (Protos, Emeryville, CA) for dansylated peptides and members of the laboratory of J. R. for insightful discussions during the course of the experiments. The work was supported by the California Tobacco-Related Disease Research Program (2FT0030 to M. N. A. and IRT26 to J. R.) and by a National Institute of Environmental Health Sciences Mutagenesis Center grant (P30 ESO 1896).

[21] Yeast STE14 Methyltransferase, Expressed as TrpE–STE14 Fusion Protein in *Escherichia coli*, for *in Vitro* Carboxylmethylation of Prenylated Polypeptides

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Introduction

Prenylated proteins comprise a recently discovered class of posttranslationally modified proteins in eukaryotes, many members of which are initially synthesized in precursor form containing a C-terminal CaaX motif (where C is cysteine, a is usually aliphatic, and X can be one of several amino acids). The CaaX motif acts as a signal for a series of three ordered modifications including prenylation of the cysteine residue via a thioether linkage by either a farnesyl or geranylgeranyl moiety (depending on the identity of X), proteolytic removal of the three C-terminal aaX residues,

and methylesterification of the newly exposed α-carboxyl group.[1-5] Certain other proteins that terminate in CC, CCXX, or CXC are geranylgeranylated, and at least a subset of those proteins are also methylated.[2] C-terminal prenylation and carboxylmethylation reactions appear to occur in all eukaryotic organisms ranging from yeast to mammals, and a diverse group of polypeptides have been shown to be prenylated or prenylated and carboxylmethylated. These proteins include ras, rho, rab, rac, and related GTP-binding proteins, the γ subunit of several heterotrimeric G proteins, nuclear lamins, and fungal mating pheromones such as *Saccharomyces cerevisiae* **a**-factor.[1,2]

Our studies are focused on methylation.[5] In contrast to prenylation, which is known to be critical for the membrane localization of many post-translationally modified proteins, the function of methylation of prenylated proteins is less well understood and may vary from one protein to another. In the case of mammalian and yeast ras proteins, for instance, studies carried out both *in vitro* and *in vivo* suggest that methylation contributes to, but is not essential for, ras membrane association.[6,7] In contrast, for the yeast pheromone **a**-factor, the methyl moiety is required for **a**-factor transport, activity, and intracellular stability.[6,8,9] Methylation may therefore influence specific protein–protein interactions, perhaps mediating recognition of **a**-factor by its transporter, its receptor, and nonspecific cellular proteases.[5,8] Methylation has also been suggested to play an important role in regulating signaling processes, based on the observation that GTPγS can stimulate methylation of soluble species of certain GTP-binding proteins[10,11] (see discussion of G25K below, for instance; for a review, see Ref. 5). Additionally, it has been shown in neutrophils that stimulation with a chemoattractant resulted in a transient increase of carboxylmethylation of several ras-related proteins[12] and that a methylation inhibitor could block certain inducible responses associated with stimulation, possibly suggesting

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a regulatory role for carboxylmethylation in cells of the immune system.[12,13] Because carboxylmethylation can be a reversible reaction, a potential regulatory role for cellular methylesterases has been suggested, but remains unproved. Further studies comparing the functional and physical properties of unmethylated versus methylated versions of the same polypeptide will be important in clarifying the contribution of methylation to protein function and localization.

C-Terminal prenylcysteine methyltransferase activity has been detected *in vitro* in the membrane fraction of cellular extracts from both *S. cerevisiae* and mammalian cells.[14,15] The activity is membrane-associated, *S*-adenosylmethionine-dependent, and can utilize either prenylated polypeptides or the simple modified amino acid *N*-acetyl-*S-trans,trans*-farnesyl-L-cysteine (AFC) as a substrate.[6,13,15,16] Both the yeast and mammalian methyltransferases appear to recognize farnesylated and geranylgeranylated substrates with equal efficiency.[6,13,16,17] The mammalian enzyme cofractionates with an endoplasmic reticulum membrane marker in a rat liver extract.[18] (See [18] in this volume for more details on the mammalian enzyme.)

In *S. cerevisiae,* we and others have shown that the C-terminal prenylcysteine methyltransferase is encoded by the *STE14* gene.[6,14,19] *STE14* was identified in a screen for sterile mutants that failed to mate owing to a defect in **a**-factor production.[20] In a *ste14* mutant methyltransferase activity is absent, as measured by an *in vitro* peptide assay, and *in vivo* physiological substrates such as **a**-factor, RAS1, and RAS2 remain unmethylated.[6,14] The DNA sequence of the yeast *STE14* gene indicates that the STE14 methyltransferase is 239 residues long and contains multiple predicted transmembrane domains (TMDs), indicative of an integral membrane protein.[8,21]

We have expressed *STE14* in *Escherichia coli* as a *trpE–STE14* gene fusion.[6] Extracts of *E. coli* induced for expression of the gene fusion exhibit a methyltransferase activity, designated here as TrpE–STE14, whose properties appear to be the same as those of the native STE14 methyltransferase produced in yeast. The recombinant system has proved useful as a ready source of large amounts of the enzyme. In this chapter, we describe the

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preparation and assay of *E. coli* extracts containing C-terminal prenylcysteine methyltransferase activity. We also describe two uses for the recombinant enzyme, including (1) methylation of a soluble species of the mammalian substrate G25K and (2) use of TrpE–STE14 methyltransferase in a coupled assay to identify proteolytic activities capable of removing the aaX residues from a CaaX-containing substrate. Because of the convenience of preparation of the recombinant TrpE–STE14 enzyme, and the absence of other CaaX modifying activities in *E. coli,* the procedures described here are expected to be of general utility for those who require a source of active methyltransferase to modify prenylated substrates for structural and functional studies.

Expression of *Saccharomyces cerevisiae* STE14 Methyltransferase as Fusion Protein in *Escherichia coli*

trpE–STE14 Expression Vector

For production of the yeast STE14 methyltransferase in *E. coli* under the control of a high-level promoter, we generated a *trpE–STE14* gene fusion using the pATH expression vector system.[22] A 1.5-kb *Cla*I fragment from pSM191[8] bearing *STE14* was ligated into pATH3 that had been digested with *Cla*I and treated with phosphatase. The resulting plasmid, pSM946 (Fig. 1), has the capacity to encode a hybrid protein designated TrpE–STE14(1–239) that contains the N-terminal 322 residues of *E. coli trpE* anthranilate synthase at its N terminus, a 38 residue-long "linker region," and 239 residues comprising the full-length *S. cerevisiae* STE14 methyltransferase at its C terminus. The linker region contains 17 amino acids from the pATH3 polylinker and 21 amino acids encoded by the region upstream of the *STE14* ATG initiation codon. Induction of the gene fusion leads to the production of STE14 methyltransferase activity in *E. coli* as described in detail below.

In addition to pSM946, which contains the full-length *STE14* gene (encoding residues 1–239), we have also used the pATH vectors to construct a series of three other *trpE–STE14* plasmids (pSM947, pSM948, and pSM949) in which only a portion of the *STE14* coding sequence is present (corresponding to residues 119–239, 140–239, and 209–239, respectively), as described in the legend to Fig. 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of induced *E. coli* whole-cell extracts from the series of four fusion-bearing strains followed by Coomassie blue staining indicated that the greater the length of *STE14* contained

[22] T. J. Koerner, J. E. Hill, A. M. Myers, and A. Tzagoloff, this series, Vol. 194, p. 477.

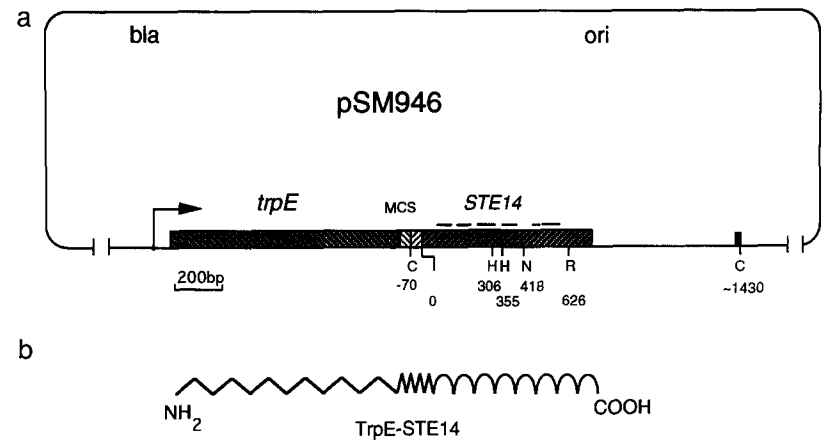

FIG. 1. The *trpE–STE14* expression vector. (a) Plasmid pSM946 encodes a *trpE–STE14* gene fusion and was constructed by cloning a 1.5-kb *Cla*I fragment bearing *STE14* into pATH3 (3.8 kb), as described in the text. Between the *trpE* and *STE14* coding sequences (darkly hatched rectangles) is an in-frame linker region comprising two segments (small lightly hatched rectangles): the pATH3 multiple cloning site (MCS) region (left) and the *STE14* upstream noncoding region between positions −70 and 0 (right). Direction of transcription of the *trpE–STE14* fusion is indicated by an arrow. Only the region lying between the two gap marks (| |), which contains the gene fusion and insert DNA upstream of *STE14*, is drawn to scale as indicated. The relative positions of *bla* and *ori* in the pATH3 vector are indicated. Within the 1500-bp (−70 to approximately +1430) *Cla*I fragment bearing *STE14*, a subset of restriction sites is shown, and the nucleotide position relative to first nucleotide of the *STE14* coding sequence is indicated. Restriction enzyme abbreviations are as follows: C, *Cla*I; H, *Hin*dIII; N, *Nsi*I; and R, *Eco*RI. In plasmids pSM947, pSM948, and pSM949 (referred to in the text), which encode *trpE–STE14* gene fusions bearing only the C-terminal portion of *STE14*, the junctions between *trpE* and *STE14* are at the *Hin*dIII$_{355}$, *Nsi*I$_{418}$, and *Eco*RI$_{626}$ sites, respectively. The solid rectangle at approximately position 1430 indicates a polylinker (containing several unmarked restriction enzyme sites) derived from the progenitor *STE14* plasmid. Short black bars above the *STE14* coding sequence indicate the approximate position of stretches of hydrophobic residues predicted by hydropathy analysis to be transmembrane spanning domains as noted by Sapperstein *et al.*[8] and Ashby *et al.*[21] (b) The hybrid TrpE–STE14 protein predicted to be encoded by pSM946. Components contributed by TrpE, the linker region, and STE14 are indicated by zigzag, jagged, and wavy lines, respectively.

in the fusions, the lower the amount of the hybrid protein or its breakdown product(s) present in the extract. Thus, for the shortest fusion, *trpE–STE14*(209–239), the hybrid protein represents the major polypeptide species present in the extract; for *trpE–STE14*(140–239) the levels are modest, and for *trpE–STE14*(119–239), containing about half of the *STE14* coding sequence, the hybrid protein represents only a minor band (data not shown). With the full-length fusion, *trpE–STE14*(1–239), neither the hybrid protein nor breakdown products could be detected in whole-cell

extracts by Coomassie blue staining, despite the fact that ample enzymatic activity is present, as shown below. One explanation for the precipitous drop-off in the levels of hybrid protein between the shortest and longest *trpE–STE14* fusions is that increasing numbers of TMDs are present in the fusion series (none in the shortest fusion, compared to one, two, or three TMDs in the intermediate length fusions and five to six TMDs in the full-length fusion). The presence of multiple transmembrane domains may cause dramatic destabilization or reduced synthesis of the hybrid protein. Indeed, heterologous proteins containing multiple membrane spans frequently fail to be efficiently recovered in high levels in *E. coli,* possibly because of misfolding or lack of proper membrane insertion followed by degradation.

Of several *E. coli* strains tested after transformation with the *trpE–STE14* constructs, the yield of fusion protein relative to the total cell protein was highest in the strain background BSG24, also designated FZ-392 (obtained from Abbott Laboratories, North Chicago, IL, and derived from strain LE392[23]). A transformant bearing the full-length fusion, designated BSG24[pSM946], has been used for all large-scale preparation of active TrpE–STE14 methyltransferase.

Media for Induction of trpE–STE14

The media used here for induction of the *trpE–STE14* gene fusion are essentially similar to media previously described.[22]

Solutions for Media

10 mg/ml Tryptophan (Trp), 100 mg/ml carbenicillin (carb), and 1 mg/ml thiamin (B1) are prepared in water and filter-sterilized through a 0.45-mm cellulose nitrate membrane. Trp is stored at 4° in the dark and is added to media to a final concentration of 20 μg/ml. Carb is stored at −20°. Note that ampicillin can be used in place of carb

1 mg/ml Indoleacrylic acid (IAA) is prepared in 95% (v/v) ethanol and stored at 4°

1 *M* $MgSO_4$, 0.1 *M* $CaCl_2$, and 50% (w/v) glucose are prepared and sterilized by autoclaving

Media. The media described below are essentially similar to those previously described for induction of pATH fusions.[22]

Modified M9 + Trp medium (per liter): To 60 g Na_2HPO_4, 30 g KH_2PO_4, 5 g NaCl, 10 g NH_4Cl, 50 g casamino acids [vitamin assay

[23] N. E. Murray, W. J. Brammer, and K. Murray, *Mol. Gen. Genet.* **150,** 53 (1977).

quality (Difco, Detroit, MI, No. 0288)], add 981.5 ml water and autoclave. After the medium has cooled, add 1 ml of 1 *M* $MgSO_4$, 1 ml of 0.1 *M* $CaCl_2$, 4 ml of 50% (w/v) glucose, 10 ml of 1 mg/ml thiamin (B1), 0.5 ml of 100 mg/ml carb, and 2 ml of 10 mg/ml Trp. It should be noted that it is important to use vitamin quality casamino acids for efficient induction of the fusion because this mixture of amino acids lacks Trp

Modified M9 − Trp medium: Prepare modified M9–casamino acids medium as described above, except omit Trp

Induction of trpE–STE14

It is important to ensure that excess Trp is alway present during routine propagation of the *TrpE–STE14* fusion-bearing strain in order to repress synthesis of the fusion until the time of induction. The reason for this precaution is that expression of the full-length *trpE–STE14* gene causes *E. coli* cells to elongate and grow poorly, thus providing a selection for low-level producers.

For production of an *E. coli* extract expressing *trpE–STE14,* strain BSG24[pSM946], stored in 15% (v/v) glycerol at −80°, is patched onto Luria broth (LB) plates containing 50 μg/ml carb and 20 μg/ml Trp (made by spreading 50 μl of the Trp stock solution onto the surface of a standard LB plate containing 50 μg/ml carb). The source plate is incubated overnight at 30° and can be stored for several weeks at 4°. To initiate production of the extract, a generous inoculum of cells is made from the plate into 100 ml of modified M9 + Trp medium. The culture is grown with aeration into late log phase (~3–4 hr) to an OD_{600} of 0.7 to 1.2, harvested at 10,000 *g* for 10 min at 4°, washed once with 50 ml of modified M9 − Trp medium, and resuspended in 1 liter of that medium. After incubation for 1.5 hr at 30° with vigorous shaking, 5 ml of the IAA stock solution is added, and incubation of the culture with shaking is allowed to proceed for an additional 6 hr.

Preparation of Escherichia coli Protein Extracts Exhibiting trpE–STE14-Dependent Methyltransferase Activity

After induction as described above, cells are harvested at 10,000 *g* for 10 min at 4°, and the cell pellet is weighed and resuspended at a concentration of 0.5 g cells/ml in *E. coli* lysis buffer [5 m*M* sodium phosphate, pH 7.0, 5 m*M* EDTA, 10% (v/v) glycerol, 25 μ*M* phenylmethylsulfonyl fluoride (PMSF), and 15 m*M* 2-mercaptoethanol, with the last two components being added just prior to use.] The cells are broken by one pass through a French press at 16,000 psi, and unbroken cells and large debris are removed by a low-speed centrifugation at 4°. The supernatant (total cell

extract) is then subjected to a high-speed spin at 100,000 *g* for 60 min at 4°. The pellet from this high-speed spin is composed of a crude membrane fraction together with insoluble proteins and contains the methyltransferase activity derived from *trpE–STE14;* no methyltransferase activity can be detected in the supernatant.[14,15] The pellet is resuspended in cold *E. coli* lysis buffer to a concentration of approximately 15–20 mg/ml and stored at −80°. Repeated freeze–thaw cycles do not appear to affect enzyme activity substantially. It should be pointed out that we have not determined whether methyltransferase activity resides in *E. coli* membranes or in the insoluble fraction, nor have we established whether it is the full-length TrpE–STE14 hybrid protein or a breakdown product that confers enzymatic activity.

We assay methyltransferase activity in the *E. coli* pellet fraction using the synthetic substrate AFC[6,13] under conditions described in Fig. 2. Other peptides containing an *S*-farnesylcysteine residue at the C terminus can also be conveniently substituted for AFC in the assay.[6,15] Although essentially no background methyltransferase activity is observed in the pellet fraction from the parental strain BSG24, which lacks the expression plasmid, a high level of methyltransferase activity is conferred by the pellet fraction from BSG24[pSM946], which encodes the full-length *trpE–STE14* fusion (Fig. 2 and Ref. 6). It has been determined in other studies that *E. coli* lacks the other two necessary enzymes in the protein prenylation pathway, namely, the prenyltransferase(s)[6,24,25] and C-terminal protease(s).[26,27] Therefore, expression of TrpE–STE14 in *E. coli* has provided an efficient means of producing large quantities of active C-terminal carboxylmethyltransferase that can be used *in vitro* to methylate substrates in the absence of other prenylation-associated processing activities.

In Vitro Methylation of Proteins Using TrpE–STE14 Methyltransferase

In addition to its ability to methylate AFC and prenylated peptides, as described in the preceding section, the *E. coli* pellet fraction containing TrpE–STE14 can also efficiently methylate prenylated protein substrates *in vitro.* We have used the pellet fraction, prepared essentially as described above, to synthesize [^{3}H]methyl-labeled derivatives of low molecular weight

[24] B. He, P. Chen, S. Y. Chen, K. L. Vancura, S. Michaelis, and S. Powers, *Proc. Natl. Acad. Sci. U.S.A.* **88,** 11373 (1993).
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[26] C. A. Hrycyna and S. Clarke, *J. Biol. Chem.* **267,** 10457 (1992).
[27] M. N. Ashby, D. S. King, and J. Rine, *Proc. Natl. Acad. Sci. U.S.A.* **89,** 4613 (1992).

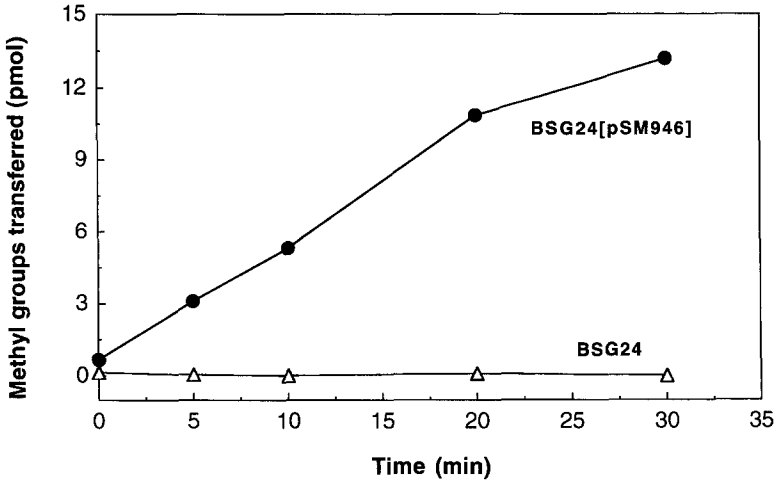

FIG. 2. Expression of the full-length *STE14* gene product as a TrpE fusion protein in *E. coli* generates C-terminal farnesylcysteine methyltransferase activity. Crude membrane fractions (10 μl) from the parent *E. coli* strain, BSG24 (Δ), and from a transformant with a plasmid containing *trpE* fused to the full-length *STE14* coding region, BSG24[pSM946] (●), were assayed with 20 μl of 50 μM [^{14}C]AdoMet (ICN, 50 mCi/mmol) and 20 μl of 100 mM Tris-HCl (pH 7.5) in a total volume of 50 μl with or without the methyl-accepting substrate AFC (1 nmol) that had been dried down in a 1.5-ml microcentrifuge tube. The samples were incubated at 37° for the indicated times and subjected to the vapor diffusion assay, essentially as previously described [I. M. Ota and S. Clarke, *J. Biol. Chem.* **264,** 12879 (1989); H. Xie, H. K. Yamane, R. C. Stephenson, O. C. Ong, B. K.-K. Fung, and S. Clarke, *Methods: A Companion to Methods in Enzymology*(*San Diego*) **1,** 276 (1990)]. To carry out the assay, the reactions were stopped by the addition of 60 μl of 1 *M* NaOH, 1% (w/v) sodium dodecyl sulfate and vortexed immediately to initiate base hydrolysis. An aliquot (100 μl) was spotted onto a 2 × 8 cm piece of folded filter paper (Bio-Rad, Richmond, CA, No. 165-0962) and placed in the neck of a 20-ml scintillation vial containing 7–10 ml scintillation fluid. Volatile [^{14}C]methanol, released by cleavage of the methyl esters by base, diffuses into the scintillation fluid, whereas other nonvolatile material remains on the filter paper. After 2 hr at room temperature, the filter paper was removed and radioactivity was determined in a liquid scintillation counter. The small amount of methyl esters (<0.1 pmol) formed in the absence of AFC was subtracted to give the values presented. Similar results were obtained when the peptide *S*-farnesyl-LARYKC (L-Leu-L-Ala-L-Arg-L-Tyr-L-Lys-[*S-trans,trans*-farnesyl-L-Cys]) was used as a substrate. The protein concentrations of the BSG24 and the BSG24[pSM946] pellet fractions were 15.5 and 17.8 mg/ml, respectively. (Reproduced from ref. 6, with permission from Oxford University Press.)

GTP-binding proteins for use in experiments designed to compare the properties of methylated and unmethylated versions of these proteins.

Here we describe the use of TrpE–STE14 for methylation of a particular GTP-binding protein, G25K (also known as CDC42Hs), a member of the

rho-subfamily of ras-related GTP-binding proteins.[28] Although the function of G25K in mammalian cells has not yet been determined, the counterpart in yeast, CDC42, is known to be required for normal bud orientation during cell growth.[29] G25K is present in brain in both soluble and membrane-bound forms. The soluble form of G25K can be purified as a heterodimer with the rho GDP dissociation inhibitor (GDI) protein[10,11] and contains an unmethylated C-terminal geranylgeranylcysteine residue.[10] A soluble pool of several other ras-related GTP-binding proteins, such as the rac and rho proteins, also appears to be associated with a GDI protein,[12,30] and these proteins may also prove to be unmethylated. It is clearly of interest to carry out *in vitro* carboxylmethylation of these GTP-binding proteins in order to compare the activity of the methylated and unmethylated species and to examine their association with GDI and membranes.

Assay for Methylation of G25K by TrpE–STE14

To determine whether G25K is a substrate for methylation by TrpE–STE14, we have carried out methylation assays using the unmethylated soluble species of G25K purified from brain.[10] The reactions are performed in microcentrifuge tubes in a final volume of 0.1 ml, containing 40 m*M* Tris-HCl, pH 7.5, 2.5 μM *S*-adenosyl-L-[*methyl*-^{3}H]methionine ([^{3}H]AdoMet, 12 Ci/mmol), 0.1 m*M* GTPγS, and 25 μg of the BSG24[pSM946] *E. coli* pellet fraction with or without 25 ng G25K (1 pmol). Reactions are incubated for increasing lengths of time at 37° up to 30 min, as shown in Fig. 3, and are stopped by the addition of 11 μl of 50% trichloroacetic acid (TCA). The samples are placed on ice for 10 min and then centrifuged at 18,000 *g* for 10 min to pellet the precipitate. The supernatant is removed, and the pellet is washed with 0.2 ml of ice-cold 5% TCA. The presence of [^{3}H]methyl esters in the pellet is determined by a vapor diffusion assay, essentially as described in detail elsewhere.[31] Briefly, [^{3}H]methyl esters are hydrolyzed to methanol by the addition of 50 μl of 1 *M* NaOH. The microcentrifuge tube is then immediately placed into a scintillation vial containing 5 ml scintillation fluid, and the vial is capped. Volatile radioactivity is allowed to equilibrate with the scintillation fluid overnight at room temperature, and radioactivity is quantitated in a scintillation counter.

The data in Fig. 3 demonstrate that TrpE–STE14 can indeed methylate G25K, as evidenced by the stimulation of incorporation of radioactivity

[28] S. Munemitsu, M. A. Innis, R. Clark, F. McCormick, A. Ullrich, and P. Polakis, *Mol. Cell. Biol.* **10,** 5977 (1990).
[29] D. I. Johnson and J. R. Pringle, *J. Cell Biol.* **111,** 143 (1990).
[30] R. Regazzi, A. Kikuchi, Y. Takai, and C. B. Wollheim, *J. Biol. Chem.* **267,** 17512 (1992).
[31] D. Chelsky, N. I. Gutterson, and D. E. Koshland, Jr., *Anal. Biochem.* **141,** 143 (1984).

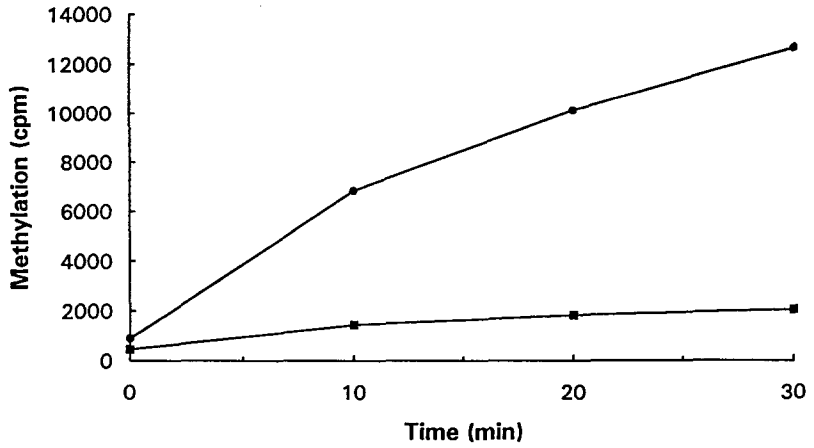

FIG. 3. Methylation of G25K by TrpE–STE14. Methylation assays using the high-speed pellet fraction from *E. coli* BSG24[pSM496] induced for *trpE–STE14* were performed as described in the text, either without (■) or with (●) 25 ng G25K. The reactions were incubated at 37° for the indicated times, then stopped with 5% TCA. The extent of methylation was determined by quantitation of the base-labile volatile radioactivity in the TCA precipitate using a diffusion assay as described by Chelsky *et al.*[31]

into methyl esters upon addition of G25K to the BSG24[pSM946] pellet fraction. When proteins in the mixture are separated by SDS–PAGE, the radioactivity is incorporated into a single band which corresponds to G25K (data not shown). The binding of GTPγS to G25K has been shown previously to lower the K_m for G25K as a substrate for methylation by the brain methyltransferase,[10] resulting in an increased level of methylation of G25K. The methylation of G25K by the TrpE–STE14 methyltransferase is also stimulated by GTPγS. In the absence of GTPγS, G25K methylation is decreased by about 60% under the conditions of the assay.

Preparative Methylation of G25K

The TrpE–STE14 methyltransferase produced in *E. coli* is also useful for large-scale preparation of [^{3}H]methyl-labeled G25K that can be used to compare the properties of the methylated species with the unmethylated counterpart. In this case, the methylation reaction is performed in a volume of 0.6 ml containing 0.1 *M* sodium phosphate, pH 6.8, 2 m*M* EDTA, 0.1 m*M* GTPγS, 2 μ*M* [^{3}H]AdoMet (80 Ci/mmol), 1 mg/ml bovine serum albumin (BSA), 0.5 mg of the BSG24[pSM946] *E. coli* pellet, and 0.4–1 μg G25K. The reaction mixture is incubated at 37° for 90 min, followed by centrifugation at 4° at 18,000 *g* for 10 min. The supernatant is then

applied to a PD-10 gel-filtration column (Pharmacia, Piscataway, NJ) equilibrated with column buffer (50 m*M* sodium phosphate, pH 6.8, 100 m*M* NaCl, 5% glycerol, 1 mg/ml BSA). After the sample is loaded, the column is eluted using the column buffer, and 0.5 ml fractions are collected. Figure 4 indicates that methylated G25K (fractions 4 and 5) can be well separated from the large peak of radioactive AdoMet eluting in the later fractions. The fractions containing methylated G25K are pooled and stored at −70°.

This procedure results in the preparative synthesis of methyl-labeled G25K under nondenaturing conditions and in the absence of detergents. The methylation reaction and PD-10 chromatography can also be performed in the absence of BSA, although the yield of methylated G25K is reduced under those conditions. We have used this methyl-labeled material to examine the binding of methylated G25K to membranes and as a substrate in assays designed to identify specific C-terminal prenylcysteine methylesterases.

Identification of Proteolytic Activities Involved in Maturation of Prenylated Polypeptide Precursors

The STE14 methyltransferase expressed in *E. coli* has also proved to be an invaluable tool in the identification and characterization of proteolytic activities responsible for cleavage of the three C-terminal amino acids from

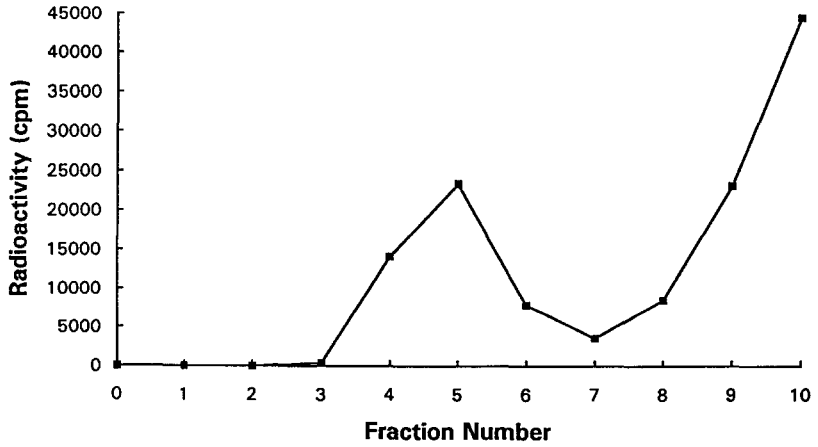

FIG. 4. Separation of methylated G25K from [^{3}H]AdoMet by gel-filtration chromatography. After methylation using the high-speed pellet fraction from BSG24[pSM946], methylated G25K was separated from [^{3}H]AdoMet by chromatography over a PD-10 gel-filtration column, as described in the text. The sample was applied to the column, then eluted with column buffer. Fractions (0.5 ml) were collected, and the radioactivity in each fraction was determined (10-μl aliquots). Fractions 4 and 5, containing the methylated G25K, were pooled.

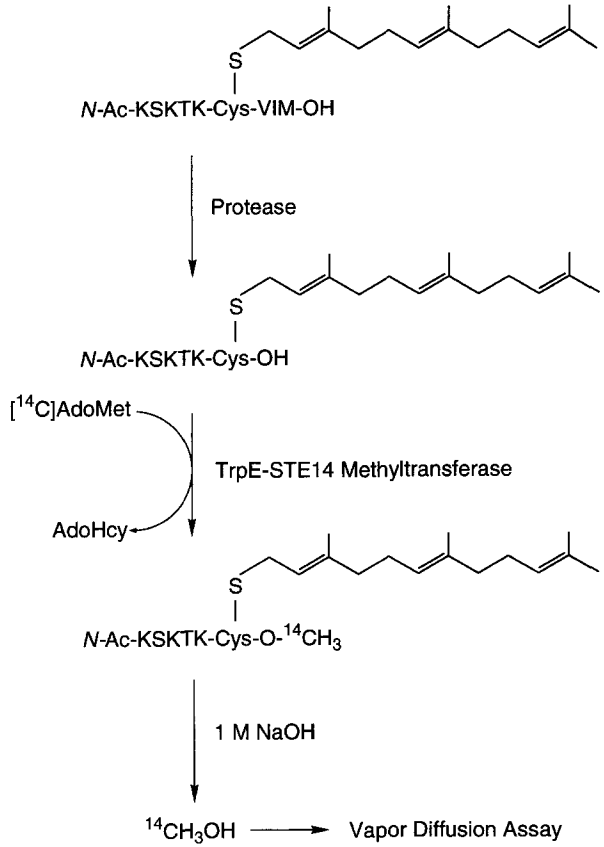

FIG. 5. Scheme for employing the TrpE–STE14 methyltransferase in a coupled assay to detect C-terminal protease activity. Cleavage of the peptide bond between farnesylcysteine and valine is necessary for the synthetic peptide *N*-acetyl-KSKTK[*S*-farnesyl-Cys]VIM to serve as a substrate for the TrpE–STE14 methyltransferase.[26,32] In the presence of an excess of both [^{14}C]AdoMet and a preparation of the high-speed pellet fraction from *E. coli* BSG24[pSM946] expressing the *trpE–STE14* fusion, [^{14}C]methyl esters are formed that can be base-hydrolyzed to yield [^{14}C]methanol, which is quantified by the vapor diffusion assay. Using this indirect assay, protease activity can be detected in membrane and soluble extracts from *S. cerevisiae* as described in the text and shown in Table I. (Although not indicated here, the acetyl group of the peptide substrate contains a ^{14}C label that facilitates purification.) (Reproduced from ref. 32, with permission from the publisher.)

prenylated CaaX-containing peptides in yeast.[26,32] As shown in Fig. 5, an *in vitro* coupled assay was developed to follow proteolytic activity present in both the soluble and membrane fractions of *S. cerevisiae.* The assay is

[32] C. A. Hrycyna and S. Clarke, *Biochemistry* **32,** 11293 (1993).

based on the observation that carboxylmethylation of an prenylcysteine residue, of necessity, can only occur after proteolytic cleavage of the three terminal amino acids of a prenylated CaaX-containing substrate. For the assay, we have used the synthetic peptide *N*-acetyl-KSKTK[*S*-farnesyl-Cys]VIM (*N*-acetyl-L-Lys-L-Ser-L-Lys-L-Thr-L-Lys-[*S-trans,trans*-farnesyl-L-Cys]-L-Val-L-Ile-L-Met), derived from the sequence of the COOH terminus of the human Ki-ras-2B protein, where the proteolytic removal of the three terminal amino acids VIM renders it a substrate for the TrpE–STE14 methyltransferase (Fig. 5).[26,32] For the assay to be solely indicative of protease activity, it is necessary to have an excess of methyltransferase in the reaction mixture. Addition of the STE14 methyltransferase produced in *E. coli* provides a means of ensuring that the proteolytic cleavage reaction is, in fact, rate limiting. The use of this novel coupled assay has allowed us to identify several distinct candidate activities for the proteolytic removal of the three C-terminal amino acids from farnesylated polypeptide precursors in *S. cerevisiae,* including both soluble and membrane-associated activities, and to provide evidence that the methyltransferase and protease activities are distinct (Table I).[26]

To carry out the assay for protease activity, 1.5 nmol of the synthetic farnesylated peptide substrate *N*-acetyl-KSKTK[*S*-farnesyl-Cys]VIM[26] is first dried in a 1.5-ml polypropylene microcentrifuge tube and resuspended in 20–27 μl of 100 m*M* Tris-HCl buffer (pH 7.5). Potential sources of protease activity from membrane and soluble extracts of *Saccharomyces cerevisiae* are incubated with 3–5 μl (45–100 μg) of the *E. coli* BSG24[pSM946] pellet fraction containing TrpE–STE14 and 20 μl of 50 μ*M S*-adenosyl-L-[*methyl*-^{14}C]methionine ([^{14}C]AdoMet, ICN, Costa Mesa, CA, 50 mCi/mmol), and the Tris buffer is added to a final volume of 50 μl. After incubation at 37°, the reaction is stopped by the addition of 55 μl of 1 *M* NaOH, 1% (w/v) sodium dodecyl sulfate. The solution is vortexed, 85 μl is immediately spotted onto a piece of folded thick filter paper, and ^{14}C-labeled methyl esters are detected by a vapor diffusion assay using the conditions described in the legend to Fig. 2.

We have used the assay to demonstrate that yeast contains at least three distinct activities capable of C-terminal proteolytic cleavage of the prenylated peptide substrate, two soluble activities and one membrane-associated activity. One of the soluble activities is the vacuolar protease carboxypeptidase Y (Table I and Ref. 26). The other soluble activity has been identified as a metalloendopeptidase encoded by the *YCL57w* gene on chromosome III of *S. cerevisiae.*[32] The membrane-associated activity may, in fact, represent the physiologically relevant species in the posttranslational processing of farnesylated polypeptides *in vivo* as it is the only

TABLE I

PROTEASE ACTIVITIES RESPONSIBLE FOR C-TERMINAL PROCESSING OF FARNESYLATED CaaX-CONTAINING PEPTIDE[f]

Methyltransferase source	Protease source	Peptide Substrate	Protease specific activity[b]	
			No added protease (pmol/min/mg of membrane protein)	Plus added protease source (pmol/min/mg of protease protein)
TrpE–STE14 pellet[a]	—	None	0.78	—
TrpE–STE14 pellet	—	*S*-Farnesyl-Ac-KSKTKCVIM	1.1	—
TrpE–STE14 pellet	Yeast membranes[c]	*S*-Farnesyl-Ac-KSKTKCVIM	—	69.8
TrpE–STE14 pellet	Yeast cytosol[d]	*S*-Farnesyl-Ac-KSKTKCVIM	—	158.3
TrpE–STE14 pellet	Purified CPY[e]	*S*-Farnesyl-Ac-KSKTKCVIM	—	1385.4

[a] Membranes prepared from *Escherichia coli* strain BSG24[pSM946] expressing the full-length *STE14* gene product as a *TrpE* fusion protein.

[b] Each value represents averages from 4 to 5 separate experiments. For values with added protease, the background of *E. coli* membranes plus peptide substrate (1.1 pmol/min/mg membrane protein) was subtracted.

[c] *Saccharomyces cerevisiae* strain SM1188 (*MAT***a** *leu2 ura3 his4 can1 ste14Δ::TRP1*). From C. A. Hrycyna, S. K. Sapperstein, S. Clarke, and S. Michaelis, *EMBO J.* **10,** 1699 (1991).

[d] *Saccharomyces cerevisiae* strain ABYS1 (*MAT***a** *pra1 prb1 prc1 cps1 ade*). From T. Achstetter, O. Emter, C. Ehmann, and D. H. Wolf, *J. Biol. Chem.* **259,** 13334 (1984).

[e] Purified carboxypeptidase Y from bakers' yeast (Sigma, St. Louis, MO).

[f] Reproduced from ref. 26, with permission from the publisher.

proteolytic activity described to date shown to be specific for farnesylated CaaX peptide substrates.[27]

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