Solubilization, Partial Purification, and Affinity Labeling of the Membrane-Bound Isoprenylated Protein Endoprotease†

Yulong Chen, Yu-ting Ma, and Robert R. Rando*

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

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ABSTRACT: A previously described [Ma, Y.-T., & Rando, R. R. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6275−6279] membrane-associated isoprenylated protein endoprotease is important in the processing of isoprenylated proteins terminating with CAAX. The enzyme is of substantial interest because specific inhibitors of it block the processing and functioning of *ras in vivo*. The enzyme appears to be an integral membrane protein, as it can only be removed from microsomal membranes with detergent. The enzyme is effectively solubilized by the detergent CHAPSO and can be partially purified (∼10-fold) by anion ion exchange and size exclusion chromatography. Attempts to further purify the enzyme by other column means, including affinity chromatography, were unsuccessful. The partially purified enzyme is very sensitive to thiol reagents but insensitive to other kinds of protease inhibitors, suggesting that the enzyme is a thiol protease. Potent and specific chloroketone containing affinity labeling agents have been developed. These novel inactivators owe their potency to an *S*-farnesylcysteine moiety which is recognized by the enzyme. Specific inhibitors of this type should allow for the identification and cloning of this protease, which is important for signal transduction.

Isoprenylated proteins are posttranslationally modified at the C-terminal CAAX box, or less frequently at either a CXC or a CC sequence, by either a geranylgeranyl or a farnesyl moiety (Manne et al., 1990; Reiss et al., 1990; Schaber et al., 1990). In the case of CAAX containing proteins, endoproteolysis occurs to remove the AAX, followed by the reversible AdoMet-dependent methylation of the newly formed isoprenylated cysteine C-terminus (Scheme 1). These modifications allow isoprenylated/methylated proteins to bind to membranes efficiently (Hancock et al., 1991). Recently, the enzymatic activity responsible for proteolysis in bovine liver and canine microsomal membranes (Ma & Rando, 1992), in yeast membranes (Ashby et al., 1992), and in rat liver microsomes (Jang et al., 1993) has been identified. The membrane-associated endoproteolytic activity readily processes tetrapeptide substrates and is stereospecific or stereoselective with respect to the amino acid residues in the tetrapeptide (Ma et al., 1992). Potent reversible competitive tetrapeptide-based inhibitors for this endoprotease have been synthesized (Ma et al., 1993). Interestingly, standard commercially available protease inhibitors designed to inhibit four classes of proteases (serine, cysteine, aspartyl proteases, and metalloproteases) have been tested and shown not to inhibit the endoprotease in bovine liver microsomal membranes (Ma et al., 1993). These results were thus uninformative in defining the mechanistic class of the isoprenylated protein endoprotease.

The *ras* oncogene product is a CAAX containing small G protein which undergoes *S*-farnesylation, proteolysis, and carboxymethylation (Hancock et al., 1991). Farnesylation is apparently essential for activity, since *ras* mutants unable to undergo farnesylation are inert (Hancock et al., 1991). Moreover, inhibitors of the farnesylation step block the activity of *ras* (Gibbs, 1991; Hancock, 1993). Is endoproteolysis essential for the activity of isoprenylated proteins such as *ras*? Membrane binding studies of fully processed and partially processed *ras* suggest that proteolysis may be important in *ras* function (Hancock et al., 1991). More importantly, potent and specific inhibitors of *ras* endoprotease, such as the reduced tetrapeptide inhibitor RPI[1] (Ma et al., 1993), block *ras* processing and the *ras* dependent stimulation of DNA synthesis in rat 1 cells transfected with the K*ras* val 12 gene under the control of a MMTVLTR promoter (A. Neri et al., personal communication). These kinds of studies strongly suggest that further characterization of the endoprotease will be of substantial interest biologically and pharmacologically. The solubilization, purification, and identification of the endoprotease are important specific goals in this characterization.

Studies are reported here which begin to characterize the endoprotease biochemically. The membrane isoprenylated endoprotease activity from bovine microsomal membranes (Ma & Rando, 1992) is successfully solubilized in the detergent CHAPSO and partially purified. The partially purified enzyme is characterized by using both reversible and irreversible inhibitors as well as enzyme kinetic analysis and chromatographic properties. Sensitivity to inhibitors suggests that the enzyme is a cysteine protease. During the characterization of the solubilized enzyme, we discovered that some of the classic protease affinity labeling agents—halomethyl ketones—selectively and irreversibly inhibited the endoprotease (Chen, 1995). For example, N_α-tosyl-L-phenylalanine chloromethyl ketone (TPCK), a classic irreversible inhibitor for serine proteases (e.g., α-chymotrypsin) (Schoellmann & Shaw, 1963; Powers, 1977; Prorok et al., 1994) and cysteine proteases (e.g., papain) (Bender & Brubacker, 1966; Whitaker & Perez-Villaseñor, 1968; Drenth et al., 1976; Shaw, 1990), irreversibly inhibits the endoprotease with a second-order rate constant $K_{inh}/K_I = 77 \pm 6\ M^{-1}$

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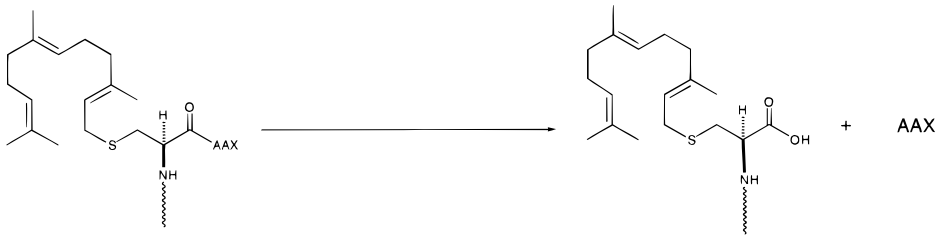
Scheme 1. Endoproteolysis of Isoprenylated Proteins



min^{-1}, while N_α-tosyl-L-lysine chloromethyl ketone (TLCK), an inhibitor for trypsin and trypsin-like enzymes, does not inhibit the enzyme activity under the same conditions. Furthermore, a new chloroketone containing a farnesyl moiety (BFCCMK) was prepared and shown to be a much more potent inactivator of the endoprotease than TPCK is. These haloketone derivatives are the first specific irreversible inhibitors known for the endoprotease. Analogs of this type should be extremely useful both for the molecular identification of the endoprotease and for studies designed to uncover the functional role(s) of the endoprotease.

EXPERIMENTAL PROCEDURES

Materials

Chromatography on gel filtration and ion exchange columns was performed on a LCC-500 FPLC system (LKB-Pharmacia Biotechnology, Inc.). All solutions used in FPLC were filtered using Millipore GS 0.22 μm filters. SDS−polyacrylamide gels were run using a Hoefer SE-250 slab gel electrophoresis unit. Radioactivity was determined on Berthold HPLC radioactivity monitor LB 506 C-1. Fresh bovine liver was obtained from a local slaughter house and stored at −80 °C. CHAPSO was purchased from CALBIOCHEM. TPCK and chymostatin were from Fluka. TLCK and E-60 were from Boeringer & Mannheim. Dithiothreitol (DTT), iodoacetamide (IAA), *N*,*N*′-phenanthroline (1,10-ϕ), and phenylmethanesulfonyl fluoride (PMSF) were from Sigma. H_2N-Ala-Ala-Phe-CMK (H_2NAAFCMK), Z-Ala-Pro-Phe-CMK (ZAPFCMK), Z-Gly-Gly-Phe-CMK (ZGGFCMK), Z-Leu-Tyr-CMK (ZLYCMK), and β-(2-naphthyl)-L-Ala-CMK (2-NAl-Ala-CMK) were from BACHEM Bioscience Inc. *N*-[^{3}H]Acetyl-*S*-farnesyl-L-cysteinyl-L-valyl-L-isoleucyl-L-methionine (Ma et al., 1992) (2.2 Ci/mmol) ([^{3}H]AFCVIM) and *N*-Boc-*S*-farnesyl-L-cysteinyl-ψ(CH_2-NH)-valyl-L-isoleucyl-L-methionine (RPI) (Ma et al., 1993) were gifts from Hoffmann-La Roche. All reagents used in SDS−polyacrylamide gel electrophoresis were obtained from either Bio-Rad or Sigma. All other chemicals were of the highest quality available. Buffer A: 250 mM sucrose, 50 mM TEA, 50 mM KOAc, 6 mM $Mg(OAc)_2$, 1 mM EDTA, 1 mM DTT, and 0.5 mM PMSF (pH 7.5). Buffer B: 20 mM Tris-HCl (pH 7.0) containing 0.1 mM PMSF. Buffer C: 20 mM Tris-HCl (pH 7.0), 1 mM EDTA, and 1 mM 1,10-ϕ. Buffer D: 20 mM Tris-HCl (pH7.0), 1 mM EDTA, and 0.1% CHAPSO. Buffer E: 20 mM Tris-HCl (pH7.0), 1 mM EDTA, 1 mM 1,10-ϕ, and 0.1% CHAPSO.

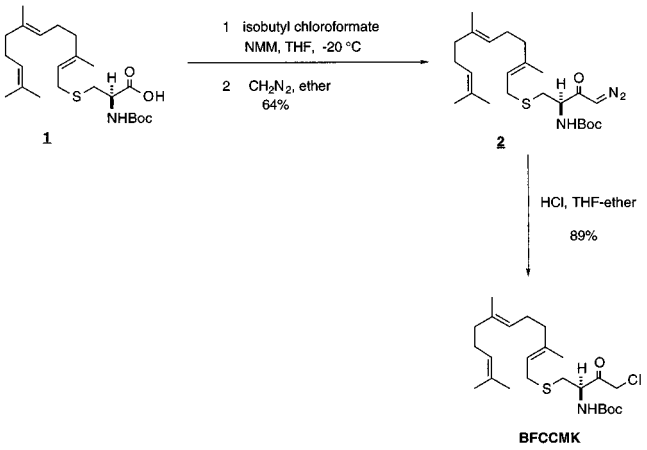
Methods

Syntheses of Halomethylketone Endoprotease Inhibitors. S-all-trans-Farnesyl-L-cysteine, *N*-Boc-*S-all-trans*-farnesyl-L-cysteine and *S-all-trans*-farnesyl-L-cysteine methyl ester were prepared as reported (Ma et al., 1993, 1994). N_ϵ-Biotinyl-N_α-tosyl-L-lysine chloromethyl ketone (BTLCK) was prepared from (+)-biotin 4-nitrophenyl ester and N_α-tosyl-L-lysine chloromethyl ketone by the conventional solution peptide coupling method (Bodanszky & Bodanszky, 1994). The procedures for the preparations and the properties of the *S*-farnesylated inhibitors are given below. The overall synthetic schemes for BFCCMK and GGFCCMK are also shown below.

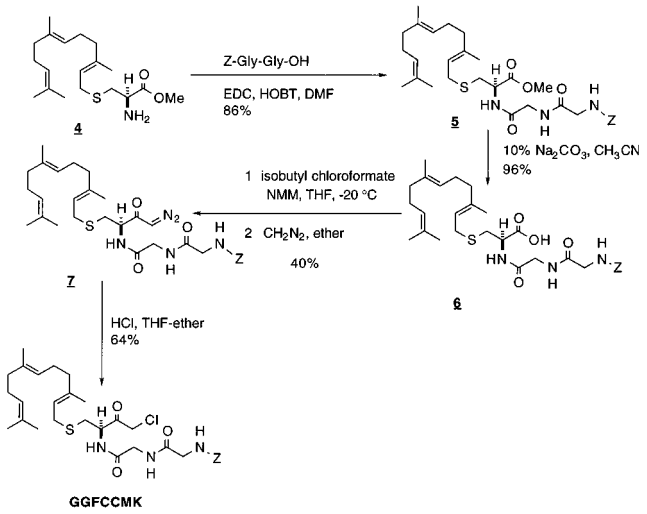
*N-Boc-S-all-trans-Farnesyl-L-cysteine Diazomethyl Ketone (**2**)* was prepared from *N*-Boc-*S-all-trans*-farnesyl-L-cysteine (**1**) (3.47 g, 8.15 mmol), NMM (0.9 mL, 824 mg, 8.15 mmol), and isobutyl chloroformate (1.06 mL, 1.113g, 8.15 mmol) in 30 mL of dry THF at −20 °C with stirring for 30 min, followed by the addition of ethereal diazomethane by the method of Green and Shaw (1981). The crude product was purified on a silica gel column (hexane/ethyl acetate, 90:10, v/v) to give a yellowish oil (2.35 g, 64%). $R_f = 0.58$ (hexane/ethyl acetate, 2:1, v/v).

^{1}H NMR ($CDCl_3$, 500 MHz): 5.59 (1H, brs, $COC\underline{H}N_2$), 5.34 (1H, brs, NH), 5.22 (1H, t, $J = 7$ Hz), 5.08 (2H, s), 4.29 (1H, brs, α-H), 3.18 (2H, m), 2.80 (2H, m), 1.96−2.06 (8H, m), 1.64 (6H, s), 1.58 (6H, s), 1.44 (9H, s). HRCIMS (NH_3 gas): 467.3056 ($M + NH_4^+$, calcd. for $C_{24}H_{39}N_3O_3S$,

[1] Abbreviations: AdoMet, *S*-(5′-adenosyl)-L-methionine chloride; AFC, *N*-acetyl-*S*-farnesyl-L-cysteine; AFCVIM, *N*-acetyl-*S*-farnesyl-L-cysteinyl-L-valyl-L-isoleucyl-L-methionine; APMSF, 4-(amidinophenyl)methanesulfonyl fluoride; BFCCMK, *N-tert*-Boc-*S*-farnesyl-L-cysteine chloromethyl ketone; BFCMK, *N*-biotinyl-L-phenylalanine chloromethyl ketone; Boc, *tert*-butyloxylcarbonyl; BNPP, bis(*p*-nitrophenyl)phosphate; BTLCK, N_ϵ-biotinyl-N_α-tosyl-L-lysine chloromethyl ketone; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CMC, critical micellization concentration; CMK, chloromethyl ketone; CTAB, cetyltrimethylammonium bromide; DFP, diisopropyl fluorophosphate; DMF, dimethyl formamide; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; E-64, *N*-[*N*-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]agmatine; EDTA, ethylenediaminetetraacetic acid; EDC, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide; 1,10-ϕ, *N*,*N*′-phenanthroline; H_2NAAFCMK, L-alanyl-L-alanyl-L-phenylalanine chloromethyl ketone; HOBT, 1-hydroxybenzotriazole hydrate; IAA, iodoacetamide; ICE, interleukin-1β converting enzyme; 2-NAL-ALA-CMK, β-(2-naphthyl)-L-alanine chloromethyl ketone; NMM, *N*-methylmorpholine; PCMB, *p*-chloromercuribenzoate; PMSF, phenylmethanesulfonyl fluoride; RPI, *N*-Boc-*S*-farnesyl-L-cysteinyl-ψ-(CH_2-NH)-valyl-L-isoleucyl-L-methionine; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TEA, triethanolamine; THF, tetrahydrofuran; TLCK, N_α-tosyl-L-lysine chloromethyl ketone; TPCK, N_α-tosyl-L-phenylalanine chloromethyl ketone; Tris-HCl, 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride; Z, benzyloxylcarbonyl; ZAPFCMK, *N*-benzyloxylcarbonyl-L-alanyl-L-prolylphenylalanine chloromethyl ketone; ZGGFCCMK, *N*-benzyloxylcarbonylglycylglycyl-*S*-farnesyl-L-cysteine chloromethyl ketone; ZGGFCMK, *N*-benzyloxylcarbonylglycylglycyl-L-phenylalanine chloromethyl ketone; Z-gly-gly-OH, *N*-benzyloxylcarbonylglycylglycine; ZLYCMK, *N*-benzyloxylcarbonyl-L-leucyl-L-tyrosyl chloromethyl ketone; A, alanine; Ali, aliphatic amino acid; aa, amino acid; C, cysteine; CAAX, Cys-Ali-Ali-Xaa; F, phenylalanine; G, glycine; L, leucine; P, proline; X, an undefined amino acid; Y, tyrosine.



467.3054, 18%), 450 (M + H^+, 7%), 439 (M + NH_4^+ − N_2, 35%), 229 (100%).



N-Boc-S-all-trans-Farnesyl-L-*cysteine Chloromethyl Ketone (BFCCMK)* (**3**) was prepared from the diazomethyl ketone **2** (1.00 g, 2.22 mmol) in 20 mL of dry THF and ethereal hydrogen chloride (4.44 mL, 4.44 mmol) at room temperature by the method of Kettner and Shaw (1981). The crude product was purified on a silica gel column (hexane/ethyl acetate, 90:10, v/v) to give a yellowish oil (902 mg, 89%). R_f = 0.65 (hexane/ethyl acetate, 2:1, v/v). ^{1}H NMR ($CDCl_3$, 500 MHz): 5.30 (1H, brs, NH), 5.20 (1H, t, J = 7 Hz), 5.08 (2H, t, J = 5.5 Hz), 4.59 (1H, dd, J = 5, 7.5 Hz, α-H), 4.34 (2H, s, COC$\underline{H}_2$Cl), 3.17 (2H, m), 2.90 (1H, dd, J = 5.5, 14 Hz), 2.82 (1$\underline{H}$, dd, J = 6.5, 14 Hz), 2.12−1.94 (8H, m), 1.67 (6H, s), 1.59 (6H, s), 1.44 (9H, s). HRCIMS (NH_3 gas): 475.2961 (M + NH_4^+, calcd. for $C_{24}H_{44}N_2O_3$SCl, 475.2759, 25%), 457.2417 (M^+, calcd. for $C_{24}H_{40}NO_3$SCl, 457.2415, 8%), 439 (M + NH_4^+ − Cl, 65%), 422 (M^+ − Cl, 60%), 205 (100%).

N-Benzyloxycarbonylglycylglycyl-S-all-trans-farnesyl-L-*cysteine Methyl Ester (**5**)* was prepared from *S-all-trans*-farnesyl-L-cysteine methyl ester (**4**) (1.69 g, 4.75 mmol) and Z-Gly-Gly-OH (1.26 g, 4.75 mmol) by the conventional solution peptide coupling method (Bodanszky & Bodanszky, 1994). The crude product was purified on a silica gel column (hexane/acetone, 70:30, 50:50, v/v) to give an oil (2.386 g, 86%). R_f = 0.52 (hexane/acetone 1:1). ^{1}H NMR ($CDCl_3$, 500 MHz): 7.34 (5H, m), 6.84 (1H, m, NH), 5.53 (1H, brs, NH), 5.18 (1H, t, J = 7.5 Hz), 5.12 (2H, s), 5.08 (2H, t, J = 6.5 Hz), 4.75 (1H, dd, J = 6.5, 12.5 Hz), 4.02 (2H, m), 3.91 (2H, brs), 3.74 (3H, s), 3.18 (1H, dd, J = 9, 13.5 Hz), 3.10 (1H, dd, J = 7.5, 13.5 Hz), 2.94 (1H, dd, J = 5, 14.5 Hz), 2.83 (1H, dd, J = 6, 14.5 Hz), 2.12−1.95 (8H, m), 1.67 (3H, s), 1.65 (3H, s), 1.59 (6H, s).

N-Benzyloxycarbonylglycylglycyl-S-all-trans-farnesyl-L-*cysteine (**6**)* was prepared by saponification of the methyl ester **5** (1.64 g, 2.79 mmol) with 40 mL of 10% sodium carbonate/acetonitrile (1/1, v/v) with stirring at room temperature for 40 h. HCl (5%) was added to the mixture until pH 3 and then extracted with ethyl acetate (3 × 60 mL). The combined organic layer was washed with 5% HCl, with saturated sodium bicarbonate, and with brine and then dried and evaporated to give a white solid (1.50 g, 94%). R_f = 0.05 (hexane/acetone, 1:2, v/v). ^{1}H NMR (DMSO-d_6, 500 MHz): 8.21 (1H, d, J = 8 Hz, NH), 8.06 (1H, t, J = 5.5 Hz), 7.48 (1H, t, J = 6 Hz), 7.74 (3H, s), 7.29 (1H, brs), 5.14 (1H, t, J = 7.5 Hz), 5.05 (2H, m), 5.01 (2H, s), 4.39 (1H, dd, J = 6.5, 12.5 Hz), 3.75 (2H, brs), 3.64 (2H, d, J = 5.5 Hz), 3.17 (1H, dd, J = 8.5, 13.5 Hz), 3.10 (1H, dd, J = 7.5, 13.5 Hz), 2.80 (1H, dd, J = 4.5, 13.5 Hz), 2.63 (1H, dd, J = 8, 13.5 Hz), 2.40−1.88 (8H, m), 1.61 (3H, s), 1.60 (3H, s), 1.53 (6H, s).

N-Benzyloxycarbonylglycylglycyl-S-all-trans-farnesyl-L-*cysteine Diazomethyl Ketone (**7**)* was prepared from **6** (2.23 g, 3.90 mmol) by the same method as previously described for **2**. The crude material was purified on a silica gel column (hexane/acetone 70:30, 60:40, 50:50, v/v) to give a yellowish gel (922 mg, 40%). R_f = 0.70 (hexane/acetone 1:1, v/v). ^{1}H NMR ($CDCl_3$, 500 MHz): 7.35 (5H, s), 7.03 (1H, d, J = 6.5 Hz, NH), 6.85 (1H, brs, NH), 5.65 (1H, brs, COC$\underline{H}N_2$), 5.54 (1H, brs, NH), 5.20 (1H, t, J = 8 Hz), 5.12 (2$\underline{H}$, s), 5.08 (2H, t, J = 4.5 Hz), 4.58 (1H, brs, α-H), 3.99 (2H, m), 3.88 (2H, d, J = 4 Hz), 3.20 (1H, dd, J = 9, 13.5 Hz), 3.13 (1H, dd, J =7, 13.5 Hz), 2.91 (1H, dd, J = 4.5, 13.5 Hz), 2.78 (1H, dd, J = 7, 13.5 Hz), 2.12−1.95 (8H, m), 1.67 (3H, s), 1.66 (3H, s), 1.59 (6H, s). HRFABMS: 620.2883 (M + Na^+, calcd. for $C_{31}H_{43}N_5O_5$SNa, 620.2880, 95%).

N-Benzyloxycarbonylglycylglycyl-S-all-trans-farnesyl-L-*cysteine Chloromethyl Ketone (ZGGFCCMK)* was synthesized from **7** (900 mg, 1.51 mmol) by the same method described for BFCCMK. The crude product was purified on a silica gel column (hexane/acetone, 70:30, 60:40, 50:50, v/v) to give a yellowish solid (585 mg, 64%). R_f = 0.75 (hexane/acetone, 1:1, v/v). ^{1}H NMR ($CDCl_3$, 500 MHz): 7.35 (5H, s), 7.10 (1H, d, J = 5.5 Hz, NH), 6.84 (1H, t, J = 5.5 Hz, NH), 5.54 (1H, t, J = 3.5 Hz, NH), 5.19 (1H, t, J = 8 Hz), 5.12 (2H, s), 5.08 (2H, t, J = 6 Hz), 4.83 (1H, dd, J = 7, 13.5 Hz, α-H), 4.34 (2H, s, COC$\underline{H}_2$Cl), 4.00 (2H, brs), 3.87 (2H, d, J = 4.5 Hz), 3.19 (1H, dd, J = 8, 13 Hz), 3.12 (1H, dd, J = 7, 13 Hz), 2.95 (1H, dd, J = 5.5, 14 Hz), 2.79 (1H, dd, J = 7, 14 Hz), 2.12−1.95 (8H, m), 1.67 (3H, s), 1.66 (3H, s), 1.59 (6H, s). HRFABMS: 628.2588 (M + Na^+, calcd. for $C_{31}H_{44}N_3O_5$SClNa, 628.2588, 94%).

Preparation and Solubilization of the Membrane-Bound Endoprotease. Bovine liver microsomal membranes were prepared according to the method of Walter and Blobel (1983). A typical adapted procedure was briefly as follows: The frozen bovine liver (100 g) was thawed and minced into small pieces which were placed in the chamber of a Waring blender (model 5010s). Three volumes of cold buffer A were added to the liver, and the liver was blended for 1 min three times. The supernatant was transferred to eight centrifugation tubes (each 45 mL) and centrifuged for 10 min at 1000*g* (Beckman JA-20 rotor). Floating fatty

materials were removed, and the rest of the supernatant was further centrifuged at 10000*g* for 10 min. The supernatant (7 mL in each tube) was collected and mixed with 1 mL of 2.0 M sucrose in each tube. The mixture was centrifuged at 113000*g* for 2.5 h (Beckman Ti 50 rotor). The supernatant of 113000*g* was discarded, and the pellets were rinsed with buffer B (2 × 2 mL for each tube). The pellets were then suspended in buffer C (70 mL) and homogenized three strokes with a Talboys electrical homogenizer which consists of a Tline Laboratory Stirrer, Model 101, a shaft with a Teflon Pestle, and a glass tube (Talboys Engineering Corp., Emerson, NJ). The homogenized microsomal membranes were centrifuged at 113000*g* for 1 h (Beckman Ti 50 rotor). The pellets were suspended in 20 mL of buffer C and homogenized three strokes with the electric homogenizer. The homogenized bovine liver microsomal membranes were stored at −80 °C. The membrane preparation was incubated in 20 mM Tris-HCl (pH 7.0), 1 mM EDTA, 0.1 mM PMSF, 1 mM DTT, and 1% CHAPSO at 4 °C for 1 h. The mixture was centrifuged at 4 °C for 2.5 h at 113000*g* (Beckman Ti 50 rotor) or for 45 min at 304000*g* (Beckman TLA-100.3 rotor). The clear supernatant was carefully collected; ca 40% of membrane proteins were solublized (e.g., for a typical run, 16 mg of solubilized proteins were obtained from 44 mg of membranes).

Partial Purification of the Isoprenylated Protein Endoprotease. All purification steps were carried out at 4 °C. *(A) Ion Exchange Chromatography.* The solubilized protein preparation (16 mg) was chromatographed on a Resource Q column (6 mL) (Pharmacia) using an FPLC system (Pharmacia LKB Biotechnology). The chromatography was performed as described in the legend of Figure 3A. Fractions between 0.4 and 0.5 M NaCl contained the majority of the isoprenylated protein endoprotease activity. These fractions (63−69) were pooled; the pool was concentrated by an Amicon CentriPrep-10 concentrator; the final concentrate was stored at −80 °C. *(B) Superose 12 Column.* The concentrate (2.2 mg) from the Resource Q column was applied to a Superose 12 column (124 mL) equilibrated with 20 mM Tris-HCl (pH 7.0) containing 1 mM EDTA, 100 mM NaCl, and 0.1% CHAPSO. The endoprotease activity was eluted as described in the legend of Figure 3B.

Protease Activity Assay. The solubilized endoprotease activity was assayed, adapting from the published method for assay of the activity in membrane preparations (Ma & Rando, 1992). An aliquot (1.0 μL) of the solubilized protein preparation was diluted in 47 μL of buffer D (see Materials) at 4 °C. Radioactive tetrapeptide substrate [^{3}H]AFCVIM (*N*-[^{3}H]acetyl-*S*-farnesyl-L-cysteinyl-L-valyl-L-isoleucyl-L-methionine of specific activity 2.2 Ci/mmol) in DMSO (2 μL) was added at time zero (final concentration was 2 μM for most assays). After the reaction mix was vortexed well, it was incubated at 37 °C for 30 min. The reaction was quenched with 0.5 mL of chloroform/methanol (1/1, v/v) and 0.5 mL of 1 M citric acid. After mixing thoroughly for 2 min, the organic layer was separated and evaporated by nitrogen steam. The residue containing starting substrate and enzymatic product (*N*-[^{3}H]acetyl-*S*-farnesylcysteine, [^{3}H]-AFC) was analyzed on a normal-phase HPLC column (Dynamax 60A, Rainin) with hexane/isopropyl alcohol/TFA (92/8/0.0001, by volume) and a Berthold LB 506-C HPLC radioactivity monitor.

Determination of K_m and V_{max}. K_m and V_{max} values for [^{3}H]AFCVIM in the presence of 425 ng of the partially purified protein preparation in 50 μL of reaction solution were obtained by fitting the saturation experimental data points to the Michaelis−Menten equation (Lineweaver−Burke plots gave essentially the same K_m and V_{max} values) (Figure 5).

Polyacrylamide Gel Electrophoresis. SDS−PAGE was performed using a Hoefer minigel SE-250 or standard gel apparatus and discontinuous 10% analytical gels, prepared according to the method of Laemmli (1970). Protein bands were detected by either Coomassie Blue or silver stain.

Protein Concentration Determination. Protein concentrations were determined by the modified Lowry Assay (Lowry et al., 1951) using the Bio-Rad *DC* Protein Assay kit.

Inhibition of the Isoprenylated Protein Endoprotease by Halomethyl Ketones. A typical procedure of inhibition is given here to illustrate the details of this procedure. A CHAPSO-solubilized protein preparation (2.5 mg/mL) or a partially purified protein preparation (0.17 mg/mL) was incubated with chloromethyl ketone inhibitors at 37 °C for 30 min. Aliquots were removed at timed intervals and diluted with assay buffer E for the solubilized preparation or with assay buffer D for the partially purified protein preparation in 20-fold. The final protein and substrate concentrations were 0.12 mg/mL (or 8.5 μg of partially purified protein preparation per milliliter) and 2.0 μM, respectively, except as otherwise noted; the residual enzyme activity was assayed as described above. TPCK and BFC-CMK were characterized by kinetic measurements. The bimolecular rate constant K_{inh}/K_I and pseudo-first-order kinetic rate constant K_{inh} were obtained using the Kitz−Wilson method (Kitz & Wilson, 1962). According to Kitz−Wilson method, the irreversible modification of an enzyme by an active-site directed inhibitor is given by

$$I + E \overset{K_I}{\rightleftharpoons} E{\cdot}I \xrightarrow{K_{inh}} E{-}I' \qquad (1)$$

where $K_I = [E]{\cdot}[I]/[E{\cdot}I]$ and $-d([E] + [E{\cdot}I])/dt = K_{inh}[E{\cdot}I]$. [I] and [E] are inhibitor and enzyme concentrations at a certain time, respectively. Under conditions where $[I] \gg [E_0]$, which is the enzyme concentration at time zero, [I] is constant. Under conditions where the enzyme−inhibitor solution is extensively diluted before the assay, a simplified eq 2 can be obtained as follows:

$$\ln [E]/[E_0] = -K_{app}t \qquad (2)$$

Where $[E]/[E_0]$ is remaining enzyme activity (%) (REA%) after the enzyme is preincubated for time "*t*" under the conditions and where $K_{app} = K_{inh}/(1 + K_I/[I])$. Equation 2 shows that the remaining enzyme activity (%) vs time in the irreversible inhibition reaction is governed by first order kinetics.

From eq 2, we have

$$1/K_{app} = 1/K_{inh} + K_I/K_{inh}[I] \qquad (3)$$

If [I] is close to K_I, a plot in accordance with eq 3 should be a straight line that intercepts the positive *y*-axis. The interception is equal to $1/K_{inh}$; the slope of the line is equal to K_I/K_{inh}.

Inhibition of the Enzyme by Non-Halomethyl Ketone Inhibitors. An aliquot (1−5 μL) of the enzyme preparation was diluted with buffer D to 47 μL at 4 °C. An inhibitor solution (1 μL) in DMSO was added, and the reaction solution was mixed well at time zero. After the reaction

Table 1: Enzyme Activity from the Washed Membranes and Supernatant[a]

incubation buffer	total activity: pellets (pmol min^{-1})	total activity: supernatants (pmol·min^{-1})	total activity: supernatants normalized to detergent extract	total activity: combination of pellets and supernatants
1% CHAPSO	200 ± 14	527 ± 17	100 ± 3	663 ± 27
20 mM Tris-HCl	370 ± 8	12 ± 26	2 ± 5	135 ± 35
0.5 M NaCl	1087 ± 70	22 ± 12	4 ± 2	927 ± 43
0.5 M NaBr/0.05% Tween-20	893 ± 2	3 ± 17 (33±7)	0.6 ± 3 (6±1)	747 ± 20
4 M urea	610 ± 28	10 ± 9 (20 ± 13)	2 ± 4 (4 ± 2)	620 ± 37
membranes	703 ± 143			

[a] The membranes were frozen at −80 °C before they were used. The microsomal membranes were thawed at 4 °C, homogenized manually, and incubated with 20 mM Tris-HCl buffer (pH 7.0) containing 0.1 M PMSF, 1 mM EDTA, and 1 mM DTT, with 0.5 M NaCl in the same Tris buffer, with 0.5 M NaBr in the Tris buffer containing 0.05% Tween-20, and with 4 M urea in the Tris buffer, respectively. The incubated mixtures were centrifuged at 304000*g* for 45 min. Data were presented in mean ± SD from two determinations, and data in parentheses were activity of the supernatants from NaBr and urea-treated mixtures which were dialyzed against buffer D, respectively. Enzyme activity was assayed as described in Methods. The other independent run from a different batch of bovine liver and membrane preparation gave similar results.

mix was incubated at 37 °C for 15 min, 2 μL of [^{3}H]-AFCVIM was added (the final concentration = 2.0 μM), and this mixture was vortexed well. The reaction mix was then incubated at 37 °C for 30 min. The residual enzyme activity was assayed as described above.

RESULTS

Solubilization of the Endoprotease Activity. Initial experiments were aimed at determining whether the protease behaved as an integral membrane protein or as a protein weakly associated with the membrane. Homogenized bovine microsomal membranes stored at −80 °C were thawed at 4 °C and mixed with 20 mM Tris-HCl buffer at pH 7.0. The mixture was homogenized manually and incubated at 4 °C for 45 min. The incubated membranes at 4 °C were centrifuged at 304000*g* for 45 min. Both pellets and supernatant were assayed for the endoprotease activity. As expected, it was found that the main enzymatic activity remained in the membrane pellets (Ma & Rando, 1992); no appreciable activity was released to supernatant (Table 1). In addition, both the chaotropic agent sodium bromide (0.5 M) in the above buffer containing 0.05% Tween-20 and the strong denaturing agent urea (4 M) in the above buffer were used separately to extract the membranes. After the supernatants from 0.5 M NaBr and 4 M urea treatments were dialyzed against buffer D, no appreciable proteolytic activity was found in either of the supernatants (Table 1). These findings are consistent with the notion that the endoprotease from bovine microsomal membranes is an integral membrane protein.

Initial studies on a variety of detergents showed that the endoprotease activity was extensively solubilized from bovine microsomal membranes by octyl glucoside, Triton X-100, CHAPS, CHAPSO, sodium cholate, and by CTAB (A. Chaudhuri and R. R. Rando, unpublished experiments). We chose to investigate CHAPSO further, as it effectively released 77% of the endoprotease activity from membranes at its optimum conditions (*vide infra*). Moreover, CHAPSO has a high CMC value (8 mM at 0−0.05 M Na^+) (Hjelmeland et al., 1983) and does not interfere with the monitoring of columns at 280 nm. Figure 1A shows that the ratio of detergent to protein (w/w) is critical for efficient solubilization; the optimum ratio for 8.1 mg of protein/mL was 1.2/1 (16 mM CHAPSO). The solubilized endoprotease is stable in a broad range of detergent concentrations. The endoprotease activity was maximal at 0.1% CHAPSO in 20 mM Tris-HCl buffer at pH 7.0 with 1 mM EDTA (Figure 1B). At this concentration, the enzyme activity remained in supernatant after it was centrifuged for 113000*g* for 1 h.

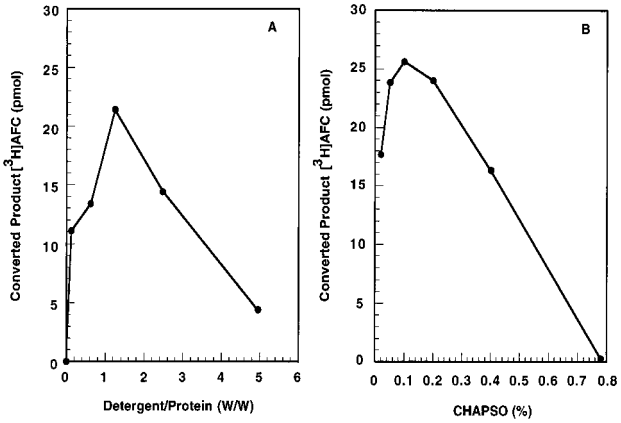


FIGURE 1: (A) CHAPSO solubilization of the isoprenylated protein endoprotease activity. Bovine liver microsomal membranes were suspended at 4 °C to 8.1 mg of protein/mL in 20 mM Tris-HCl (pH 7.0), 1 mM EDTA, 0.1 mM PMSF, and 1 mM DTT. In 3.0 mL centrifuge tubes, a 400-μL sample in each tube was mixed well with 100 μL of CHAPSO solutions to give the final concentrations shown in panel A. After the mixtures were incubated at 4 °C for 1 h, they were centrifuged at 304000*g* for 45 min. Aliquots (1 μL) of supernatants were incubated with 2 μM of [^{3}H]AFCVIM for 30 min and assayed for the converted product [^{3}H]AFC as described in Methods. (B) Effect of CHAPSO concentration on the endoprotease activity. CHAPSO-solubilized membrane proteins (3.2 μg) were incubated with 2 μM tetrapeptide substrate, [^{3}H]AFCVIM. The assay of enzyme-converted product, [^{3}H]AFC, was described in Methods. The CHAPSO concentration was the final concentration in the reaction solution.

These properties of CHAPSO make it ideal, among the detergents tested, for the solubilization of the endoprotease. Three conventional criteria were used to demonstrate that the enzyme was successfully solubilized by CHAPSO: (i) The endoprotease activity was maintained in the supernatant after centrifugation both at 304000*g* for 45 min and at 113000*g* for 2.5 h; (ii) the CHAPSO-solubilized enzyme activity passed through a Millex GV 0.22 μm filter (Millipore) with ∼100% recovery; (iii) after chromatography of the supernatant on a Sephadex 200 HR 10/30 column, the endoprotease activity was detected between the void volume and the column volume (Figure 2). It should be noted that a second small amount of proteolytic activity was also found with an apparent molecular mass of approximately 60 kDa, which amounted to approximately 10% of the major peak, but which chromatographed at a smaller molecular size than

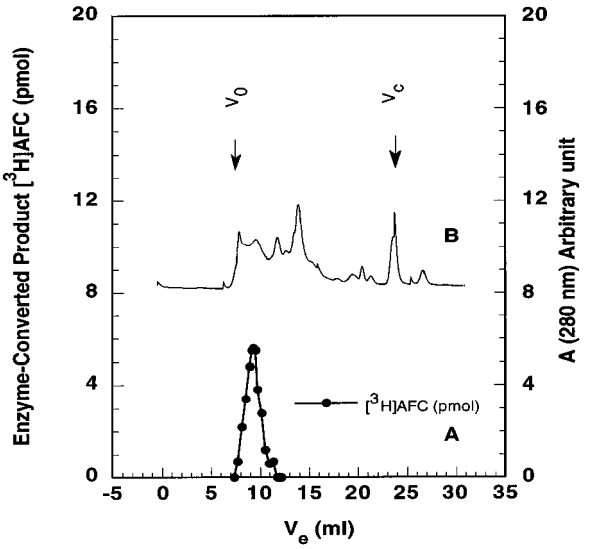


FIGURE 2: Gel filtration chromatography of the solubilized isoprenylated endoprotease on Sephadex 200. The solubilized microsomal membrane protein preparation (664 μg, 200 μL) was loaded to a calibrated Sephadex 200 HR 10/30 column equilibrated with a buffer of 20 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, and 0.5% CHAPSO at pH 7.0. Fractions of 0.2 mL each were collected and 25-μL aliquots of certain fractions were assayed. (A) The enzyme-converted product [^{3}H]AFC was presented by closed circles (●); data were an average of two independent runs. (B) The void volume (V_0) of the column was at 8.32 mL and the column volume (V_c) was 24.0 mL. The major enzyme activity was eluted at V_e = 9.35. The molecular weights of the major activity was determined using the calibration curve from the molecular weight standards. The major activity (641 kDa) was eluted between markers of thyroglobulin (Thyro; 669 kDa) and ferrin (Fer; 440 kDa).

the major peak. This smaller peak was not investigated further, for several reasons. Quantitatively, it is not significant. The activity is potently inhibited by 1,10-ϕ, a metal chelator without effect on the primary peak of activity and without effect on the previously described mammalian (Ma & Rando, 1992) and yeast endoproteolytic activity (Ashby et al., 1992). It also is not known if the smaller peak of activity is comprised of an endoproteolytic activity or is simply made up of nonspecific proteases. Finally, the major peak of activity is profoundly inhibited by the reduced peptide inhibitor RPI (Ma et al., 1993), which also blocks *ras* processing *in vivo* (A. Neri et al., personal communication). All further studies described here will be relevant to the major solubilized peak of activity.

Partial Purification of the Isoprenylated Protein Endoprotease. (A) *Chromatography of the Endoprotease by Ion Exchange Chromatography.* Solubilized microsomal membrane protein (16 mg) was chromatographed on a Resource Q anion exchange column (6 mL). Figure 3A shows the elution profile of the isoprenylated protein endoprotease activity from the column. The activity of fractions was assayed in the presence of buffer E. The activity appeared as a single sharp peak that was eluted at approximately 0.4 M sodium chloride. The peak fractions from the Resource Q column were pooled and concentrated for gel filtration chromatography (*vide infra*).

(B) Chromatography of the Endoprotease on a Gel Filtration Column. The pool from the Resource Q column was chromatographed on a Superose 12 column (124 mL). Figure 3B shows the elution profile of the endoprotease activity from the gel filtration column. The activity was eluted at the high molecular weight end, and no low molecular weight activity was observed (*vide supra*). The peak fractions were pooled and saved at −80 °C for later use.

Table 2 summarizes the results of a typical purification procedure that started with 44 mg of bovine liver microsomal membrane proteins. After detergent extraction, Resource Q chromatography, and Superose 12 gel filtration chromatography, the endoprotease was partially purified to 9-fold activity with a yield of approximately 10%. The final specific activity was 1.8 nmol of [^{3}H]AFC per min per

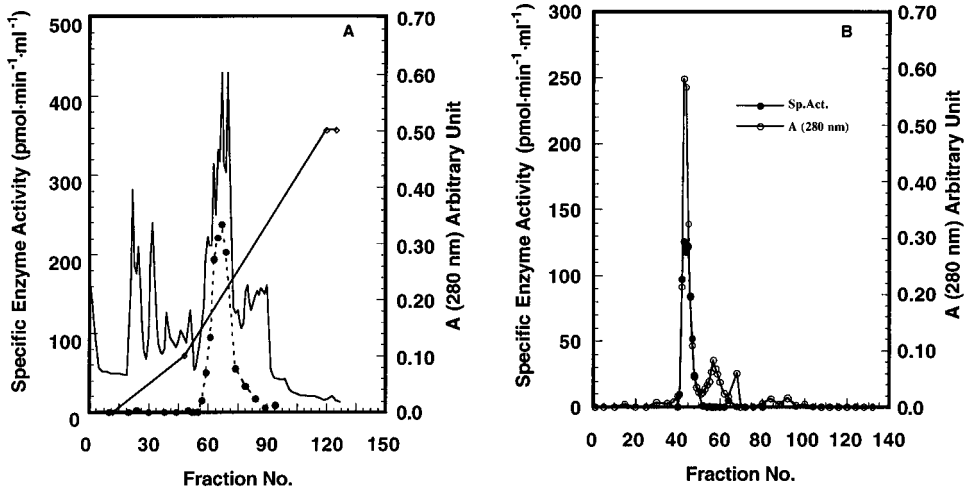


FIGURE 3: (A) Chromatography of the solubilized endoprotease on a Resource Q column. The solubilized microsomal endoprotease preparation (16 mg of protein) was applied to a Resource Q column (6 mL) equilibrated with buffer D at 4 °C. The column was washed with 12 mL of buffer D (fractions 1−12), followed by a 36-mL linear gradient from 0 to 0.20 M NaCl (fractions 13−48). The enzyme was eluted with a 75-mL linear gradient of 0.2−1.0 M NaCl in buffer D at a flow rate of 1 mL/min (fractions 49−123). Finally the column was washed with 5 mL of 1 M NaCl in buffer D. Fractions of 1 mL were collected. A 10-μL aliquot of every interval fraction (●) was assayed for endoprotease activity as described in Methods. The protein content of each interval fraction (−) was estimated from the absorbance at 280 nm. The NaCl gradient is shown by the open diamond shape legend (◇) with 0.0−0.2 and 0.2−1.0 M. (B) Chromatography of the isoprenylated protein endoprotease activity on Superose 12. Active fractions from the Resource Q column (fractions 63−69) were pooled, and the pool was concentrated by an Amicon Centriprep-10 concentrator to 1.0 mL. The concentrated sample (2.2 mg of protein) was applied to a 124-mL Superose 12 column equilibrated with buffer D at 4 °C. The column was run at 0.5 mL/min. A 20-μL aliquot of each interval fraction was assayed for the isoprenylated protein endoprotease activity (●), and the protein content of each interval fraction (○) was estimated by the absorbance at 280 nm.

Table 2: Partial Purification of an Isoprenylated Protein Endoprotease from Bovine Liver

purification step	protein (mg)	specific activity (units/mg)[a]	total activity (units)	purification (fold)	recovery (%)
microsomal membranes	44.1 ± 3.5	202 ± 7	8908	1	100
detergent extract	16.2 ± 1.2	430 ± 13	6966	2.1	77
Resource Q	2.2 ± 0.1	1000 ± 24	2200	5.0	25
Superose 12	0.51 ± 0.06	1824 ± 118	930	9.0	10

[a] The endoprotease activity was assayed as described in Methods. Specific activity (units/mg) is expressed as picomoles of *N*-[^{3}H]acetyl-*S*-farnesyl-L-cysteine per min per milligram of protein; total activity (units) is picomoles of *N*-[^{3}H]acetyl-*S*-farnesyl-L-cysteine per min. Data were presented as mean ± SD (n = 3−6).

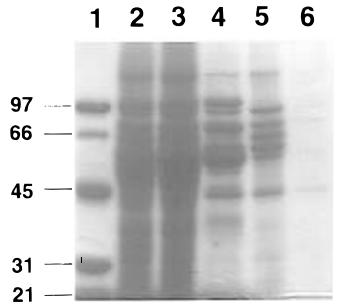


FIGURE 4: Partial purification of the membrane-bound isoprenylated protein endoprotease from bovine liver microsomal membranes. Samples from each of the purification steps were analyzed by SDS−PAGE on a 10% polyacrylamide resolving gel along with Bio-Rad low molecular mass markers (lane 1: 97, 66, 45, 31, 21 kDa) as described in Methods. The protein bands were visualized by staining with Coomassie brilliant blue for 45 min and destaining for 24 h. The samples analyzed were crude microsomal membranes (lane 2), detergent-solubilized preparation (lane 3), Thiopropyl Sepharose 6B pool (lane 4), DEAE Sephacel pool (lane 5), Mono Q pool (lane 6).

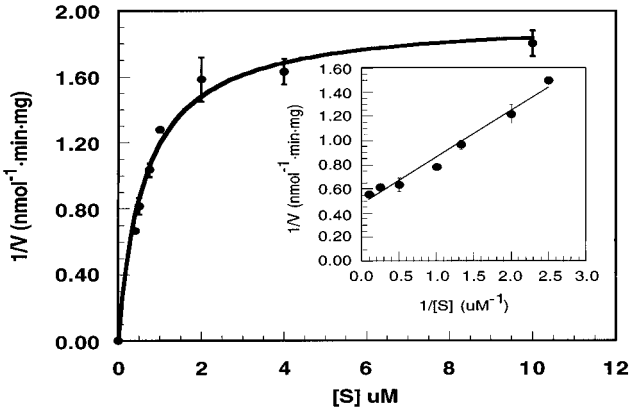


FIGURE 5: Substrate saturation curve for the partially purified bovine microsomal isoprenylated protein endoprotease. Each reaction mixture contained 425 ng of the partially purified protein and varying amounts of ^{3}H-labeled tetrapeptide substrate, [^{3}H]AFCVIM. Assays were carried out in triplicate at 37 °C for 30 min. The [^{3}H]-AFC radioactivity was measured as described in Methods.

milligram of protein (in the presence of 10 μM of [^{3}H]-AFCVIM tetrapeptide substrate).

The low recovery of activity reported above, coupled with relatively modest purification of the endoprotease, suggests that the enzyme becomes relatively unstable as it is purified. Several other column materials were tested for further purification, with very modest results. Columns studied included Thiopropyl Sepharose 4B, Zn^{2+}-charged chelating column, DEAE, and hydrophobic interaction columns. Among the tested hydrophobic columns, a Butyl Agarose column was the best; it produced 2.0-fold purification with 63% recovery of activity. None of these purification efforts produced a more highly purified enzyme than the scheme shown in Table 2. Attempts at enzyme purification by affinity chromatography using reduced peptide inhibitors (Ma et al., 1993) met with no success. Purified enzyme could not be eluted from the column, under a variety of conditions (A. Chaudhuri, unpublished experiments). Another procedure, which led to about the same level of purification as shown in Table 2, utilized Thiopropyl Sepharose 4B, DEAE, and Mono Q columns. This procedure produced a 7-fold purification. Figure 4 shows SDS−PAGE gel analysis of the protein bands from this purification procedure. Only a few protein bands in the gel were visualized by Coomassie Blue after this purification procedure (Figure 4). By comparison of SDS−PAGE analysis from the product of the last step of purification in Table 2 (data no shown) with Figure 4, the two preparations gave different band patterns. Two more column steps from the purification scheme in Figure 4 did not proved a greater purification than the procedures used in Table 2 in terms of specific activity although the product from the purification scheme shown in Figure 4 was less complex with respect to the numbers of protein bands on the SDS−PAGE gel. It seems quite clear that further purification of the enzyme will be problematic.

Kinetic Experiments. The partially purified endoprotease was used to determine the V_{max} and K_m values for the radioactive tetrapeptide substrate, [^{3}H]AFCVIM. Figure 5 shows that the enzyme was saturated by the substrate. The experimental saturation data points fit the Michaelis−Menten equation well, as described in Methods. K_m and V_{max} values for [^{3}H]AFCVIM were measured to be 0.65 ± 0.08 μM and 1.96 ± 0.07 nmol/(min·mg of protein), respectively. The results are to be contrasted with results obtained with whole membranes (Ma et al., 1992).

Characterization of the Solubilized Endoprotease Activities. The solubilized protein materials can be stored at −80 °C for a few months without significant loss of the endoprotease activity. Neither DTT (1−5 mM) nor glycerol (20%) affects the enzyme's activity. A variety of inhibitors was tested on the CHAPSO-solubilized preparations and on the partially purified enzyme from the purification scheme shown in Table 2 (Table 3). The membrane-bound enzyme proved to be relatively insensitive to a wide spectrum of protease inhibitors (Ma et al., 1993). However, after the endoprotease was solubilized, it became more sensitive to some of the inhibitors. A typical example is *p*-chloromercuribenzoate (PCMB), which inhibited the endoprotease 41% at 0.5 mM, when membrane-bound (Ma et al., 1993). The solubilized enzyme is completely inactivated by 100 μM PCMB (Table 3). DTT (5 mM) was able to regenerate 44% of the inhibited enzyme activity from the detergent extract after it was inhibited completely by 0.5 mM PCMB (Table 3), while no regeneration of the inhibited activity was observed after extensive dialysis and dilution. It appeared that the purer the enzyme, the greater the sensitivity to PCMB. The

Table 3: Inhibition of the Isoprenylated Protein Endoprotease with a Variety of Inhibitors[a]

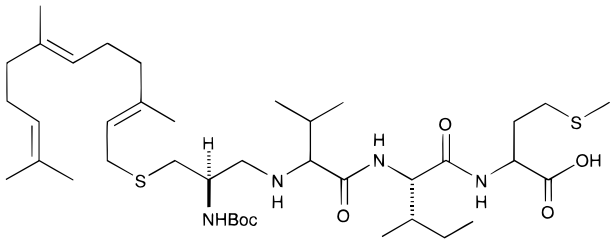
inhibitors	concentration	enzyme activity (%) detergent extract	enzyme activity (%) partially purified
control (no inhibitor)		100 ± 5	100 ± 5
BNPP	1 mM	100 ± 8	
Chymostatin	0.33 mM	104 ± 8	99 ± 1
DTT	5 mM	108 ± 8	106 ± 6
E-64	0.11 mM	113 ± 0	
	0.5 mM		103 ± 4
1,10-ϕ	1 mM	90 ±10	102 ± 14
PCMB	0.5 mM	0 ± 5	
	+5 mM DTT	44 ± 4	
	0.1 mM	0 ± 6	
	5 μM		33 ± 3
PMSF	1 mM	93 ± 3	

[a] All data were presented in mean ± SD from 2−6 determinations.

partially purified enzyme activity was inhibited to 67% by 5 μM PCMB (Table 3). The partially purified enzyme was, however, not inhibited by chymostatin (0.33 mM), 1,10-ϕ (1 mM), or DTT (5 mM) (Table 3). These latter results are interesting because they serve to differentiate clearly between the microsomal enzyme described here and the putative soluble endoprotease from extracted pig brain which is sensitive to these inhibitors (Akopyan et al., 1994).

Inhibition of the Endoprotease Activity by Chloromethyl Ketones. As attempts at fully purifying the enzyme were not successful, an alternate method of potentially identifying the endoprotease was sought. One possible approach is the specific affinity labeling of the endoprotease. During the characterization of the solubilized enzyme, it was found that N_α-tosyl-L-phenylalanine chloromethyl ketone (TPCK), a classic affinity irreversible inhibitor of the serine protease α-chymotrypsin, irreversibly inhibited the endoprotease activity with $K_{inh}/K_I = 77 \pm 6\ M^{-1}\ min^{-1}$ and $K_{inh} = (1.4 \pm 0.6) \times 10^{-3}\ s^{-1}$ (Figure 6A), while N_α-tosyllysine chloromethyl ketone (TLCK), a specific irreversible inhibitor of trypsin and trypsin-like enzymes, did not inhibit the enzyme activity under the same conditions (Figure 6B) (Chen, 1995).

The specificity of the inhibition of the endoprotease by TPCK was further explored. The finding that the inhibition with TPCK obeyed saturation kinetics (Figure 6A) and that the chemically similar TLCK did not inactivate the enzyme already strongly suggests that the inhibition mechanism is specific in nature. The irreversible inhibition of the endoprotease activity by TPCK was blocked by the endoprotease-specific reversible inhibitor RPI. The enzyme activity



RPI, a Potent Competitive Inhibitor of the Endoprotease (Ma et al., 1993)

was prevented from inhibition by 82% in the presence of 500 nM RPI, a potent competitive inhibitor of the endoprotease, shown above. However, if the enzyme was preincubated with TPCK first, and then 500 nM RPI was added, no enzyme activity was regenerated from the inhibited enzyme. We also observed that the enzyme activity was not regenerated after a gel filtration chromatography of the inhibited enzyme solutions, which were preincubated with TPCK at different times, and after a 24-h dialysis of the inhibited enzyme solutions. The data presented above support the view that TPCK is a specific active-site-directed irreversible inhibitor of the isoprenylated protein endoprotease. After we established that TPCK was a specific irreversible inhibitor for this endoprotease, we started exploring the other potent chloromethyl ketone inhibitors.

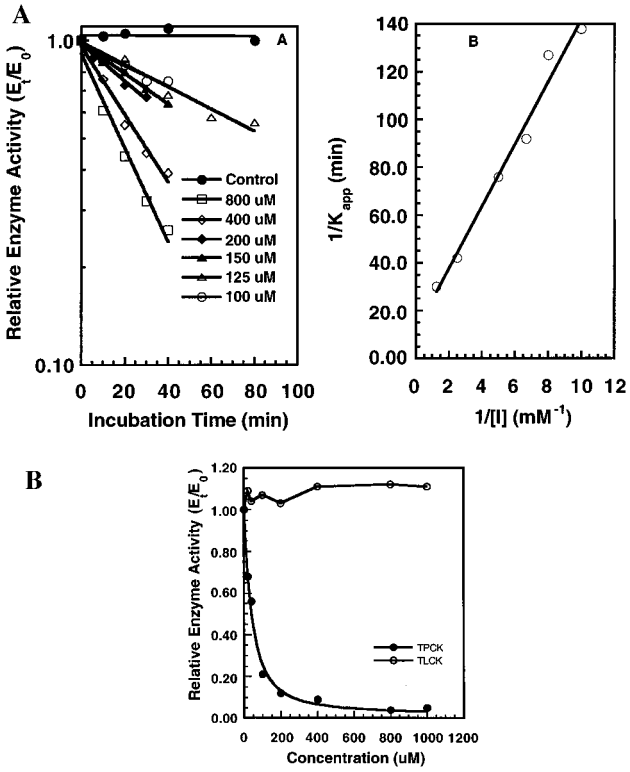
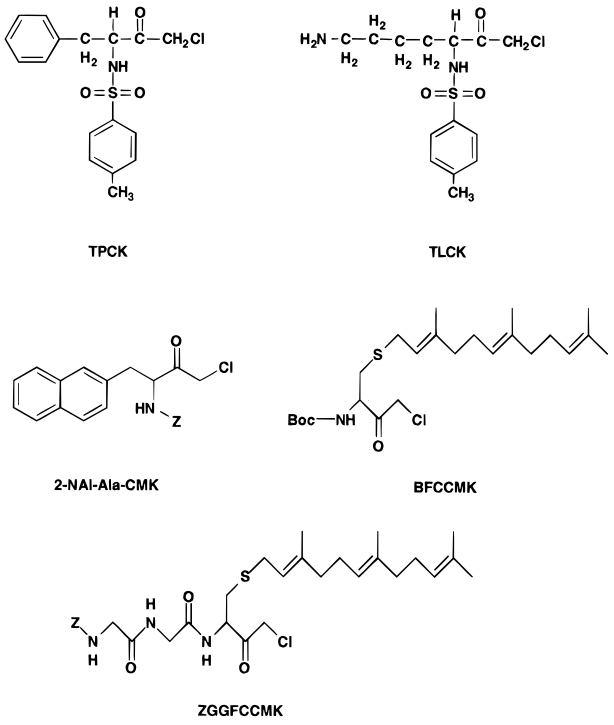


FIGURE 6: (A) Time-dependent inhibition. (B) Kitz−Wilson plot (the upper panel) for the inhibition of the isoprenylated protein endoprotease by TPCK. The inhibition studies were carried out at 37 °C using the solubilized endoprotease preparation. Aliquots of the inhibitor−enzyme solution were taken at interval times and diluted by a factor of 20, and the enzyme activity was assayed as described in Methods. (B) (the lower panel) The concentration-dependent inhibition of the endoprotease by TPCK. The inhibition studies were carried out at 37 °C. The crude CHAPSO-solubilized endoprotease preparations (0.12 mg/mL) were preincubated for 60 min in 50 μL of buffer E in the presence of 284 μM TPCK or TLCK with 6% DMSO and 2% 2-propanol. After the incubation, the reaction solution was diluted by a factor of 20, and the remaining enzyme activity of the inhibitor−enzyme solution was assayed as described in Methods. Data were represented in mean from duplicate runs.

Since the P_1 position of natural substrates of this endoprotease is a farnesylcysteine residue, we designed and tested two farnesylcysteine chloromethyl ketone inhibitors, BFC-CMK and ZGGFCCMK, along with the structurally related anthranoyl derivative (Scheme 2 and Table 4). The results showed that BFCCMK and ZGGFCCMK were good inhibitors. The former has a 15-fold increase of the second-order rate constant ($K_{inh}/K_I = 1164\ M^{-1}\ min^{-1}$) compared with that of TPCK ($K_{inh}/K_I = 77\ M^{-1}\ min^{-1}$), and the K_I of the inhibitor−enzyme complex under the given conditions is $K_I = 30\ \mu M$ (Figure 7). This is to be contrasted with a $K_I =$ 1.1 mM for TPCK. The fact that an analog like BFCCMK is a potent inactivator of the enzyme is important, because this observation strengthens the view that the enzyme inhibited does indeed recognize a farnesyl-L-cysteine moiety.

Scheme 2. Structures of Affinity Inactivators of the Endoprotease



To further establish the specificity of the inactivation process, several commercially available haloketones were studied as putative inactivators of the endoprotease. The structures of these analogs are shown and their activities are shown in Table 5. In these studies, the solubilized protein material was preincubated with a chloromethyl ketone inhibitor at 284 μM (unless otherwise noted) for 30 min; then 2.5-μL aliquots of the incubated solution were taken and diluted 20-fold for assay of the residual enzyme activity. Of the chloromethyl ketone inhibitors tested, 2-Nal-Ala-CMK has a similar inhibitory potency to that of TPCK (Table 4). Save for the dipeptide (ZLYCMK) and tripeptide derivatives (ZGGFCMK and ZAPFCMK) derived from TPCK, the other halomethyl ketones tested are virtually inert. In aggregate, these data demonstrate that the inactivation of the endoprotease by TPCK and BFCCMK is specific in nature.

DISCUSSION

We had previously reported on the identification of a liver microsomal endoprotease activity capable of cleaving after the isoprenylated cysteine moiety of isoprenylated peptides (Ma & Rando, 1992). The enzyme proved not to be substantially inhibited by various group-specific protease inhibitors but was weakly inhibited by thiol reagents (Ma et al., 1993). *N*-Boc-*S*-Farnesyl-L-cysteine aldehyde competitively inhibited the enzyme with a K_I in the low micromolar range, suggesting that the enzyme might be a serine or thiol protease (Ma et al., 1993). The enzyme is potently and competitively inhibited by reduced peptides, such as RPI (Ma et al., 1993). Any further characterization of the enzyme requires its effective solubilization and at least partial purification. In this paper we report on these latter issues and further explore the inhibition of the enzyme. In the past we had developed potent competitive inhibitors of the enzyme (Ma et al., 1993). In the current work, specific irreversible affinity labeling agents have been developed for the enzyme.

Studies on the solubilization of the enzyme show that it is likely to be an integral membrane protein, because only detergent solubilization effectively solubilizes the enzyme. True solubilization of the enzyme could be demonstrated with several detergents, although, for reasons already stated, CHAPSO was studied in the most detail. Effective solubilization was shown by demonstrating that the solubilized enzyme is recalcitrant to centrifugation, passes through a 0.22 μm filter, and can enter a Sephadex column. The solubilized enzyme is quite stable and can be stored at −80 °C for months without substantial activity loss. Neither DTT nor glycerol affects the stability of the enzyme.

Having the enzyme solubilized in a stable form allows for an attempt at purifying the endoprotease. Some success was achieved, and a partially purified enzyme with *ca.* 10-fold greater specific activity than that in the membrane preparation was obtained. Although the level of purification is modest, the protein itself might be much purer than it appears, as it is certain that substantial activity is lost during purification. Attempts at the substantial purification of the enzyme did not meet with success, even though several different classes of columns were used. Generally we found that while an individual column might provide appreciable purification on the crude solubilized material, the column proved to be completely ineffective when used in series with other columns. It is likely that any substantial purification removes stabilizing entities or enzyme subunits, which would lead in either case to inactivation of the protease.

It is important to note that a putative isoprenylated peptide protease from pig brain has been reported which is apparently readily removed from the membranes by freeze-thawing (Akopyan et al., 1994). This uncharacterized enzyme is potently inhibited by 1,10-ϕ (1 mM), chymostatin (0.1 mM), and DTT (1−10 mM) (Akopyan et al., 1994). We do not believe that this enzymatic activity is related to the one reported here, because the endoprotease under discussion here is unaffected by 1,10-ϕ (1 mM), chymostatin (0.33 mM), and DTT (5 mM). In addition, the enzymatic activity described here is not removed from membranes by freeze-thawing. Thus, the microsomal enzymatic activity described here and the pig brain enzyme appear to be unrelated. It is interesting to note though that a small additional peak of peptidase activity capable of cleaving the substrate AFCVIM was found during Sephadex chromatography. Moreover, this peptidase activity is also eluted at low salt (<0.4 M) on a Mono Q column (Y.-L. Chen and R. R. Rando, unpublished experiments). This activity was not investigated further, as it was a minor peak of unknown specificity and proved to be inhibited by 1,10-ϕ—an inhibitor known not to inhibit the previously identified isoprenylated protein endoprotease (Ma et al., 1992; Ashby et al., 1992). It is possible that this minor activity and the "soluble" pig brain activity are the same.

While we had earlier designed potent competitive inhibitors of the endoprotease (Ma et al., 1993), such as the reduced tetrapeptide RPI which has a K_I in the 50 nM range (Ma et al., 1993), no potent active-site directed irreversible inhibitors of the enzyme were available. It is shown here that TPCK, a well-known inactivator of chymotrypsin-like and papain-like enzymes (Schoellmann & Shaw, 1963; Whitaker & Perez-Villaseñor, 1968), irreversibly inhibits the protease by an affinity labeling mode. Inhibition by TPCK was blocked by the active-site-directed competitive inhibitor RPI, and gel filtration and extensive dialysis of the inhibited enzyme did

Table 4: Inhibition of the Isoprenylated Protein Endopeptidase by Halomethyl Ketone Inhibitors[a]

inhibitors	concentration (μM)	enzyme activity (%)	K_{inh} (s^{-1})	K_I (μM)	K_{inh}/K_I (M^{-1} min^{-1})
control		100 ± 3			
TPCK	284	43 ± 5	$(1.4 \pm 0.6) \times 10^{-3}$	1100 ± 500	77 ± 6
TLCK	1000	94 ± 15			
2-Nal-Ala-CMK	284	53 ± 1			
BFCCMK	284	27 ± 2	$(5.9 \pm 0.4) \times 10^{-3}$[b]	30 ± 2[b]	1164 ± 60[b]
ZGGFCCMK	284	44 ± 1			

[a] Inhibition procedures and assay conditions were described in Methods. Data were represented in mean ± SD from two independent runs (four determinations) to six independent runs. The enzyme activity in the absence of inhibitor was used as a control and normalized to 100%. [b] The values were obtained from the Kitz−Wilson plot using the partially purified enzyme preparation (Figure 7).

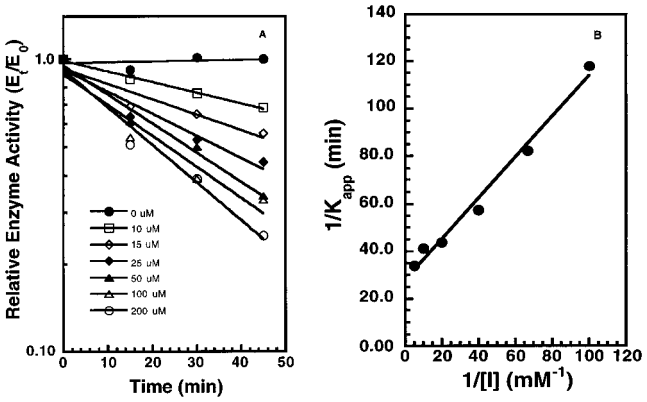
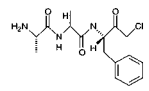
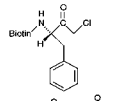
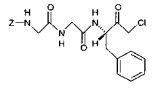
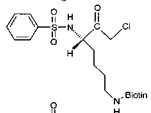
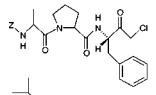
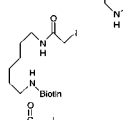
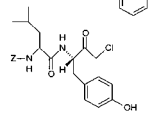
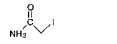


FIGURE 7: (A) Time-dependent inhibition. (B) Kitz−Wilson plot for the inhibition of the partially purified endoprotease by BFCCMK. The inhibition studies were carried out at 37 °C . Aliquots of the inhibitor−enzyme solution were taken at interval times and diluted by a factor of 20, and the enzyme activity was assayed as described in Methods. Data were presented in mean from duplicate runs.

Table 5: Inhibition of Isoprenylated Protein Endoprotease by Halomethyl Ketone Inhibitors[a]

Inhibitors	Structures	Enz.Act. (%)/Conc.	Inhibitors	Structures	Enz.Act. (%)/Conc.
CONTROL		100±3	H_2NAAFCMK		103/284 μM
BFCMK		98±8/284 μM	ZGGFCMK		86.2±0.9/284 μM
BTLCK		101±9/1mM	ZAPFCMK		73.6±0.9/284 μM
Iodoacetyl-LC-Biotin		101±2/400 μM	ZLYCMK		68.6±3.5/284 μM
IAA		98±1/400 μM			

[a] The experimental conditions were the same as described in the footnotes to Table 4.

not regenerate enzyme activity. It is interesting to compare the second-order rate constants from the inhibition of the endoprotease with those from the inhibition of α-chymotrypsin and papain by TPCK. The second-order rate constant ($K_{inh}/K_I = 1.3$ M^{-1} s^{-1}) from the reaction of TPCK with the endoprotease is comparable with those from reactions of chymotrypsin ($K_{inh}/K_I = 7.7$ M^{-1} s^{-1}) (Shaw & Ruscica, 1971) or papain ($K_{inh}/K_I = 1.96$ M^{-1} s^{-1}) (Whitaker & Perez-Villaseñor, 1968) with TPCK. By comparison, the second-order rate constant for the reaction of the model thiol compound cysteine with chloromethyl ketones is approximately 10^4 lower (Whitaker & Perez-Villaseñor, 1968). Taken together with the saturation kinetics observed in the Kitz−Wilson plot (Figure 6A), our results support the idea that the inhibition of the endoprotease by TPCK involves the formation of a Michaelis inhibitor−enzyme complex with a $K_I = 1.1$ mM (Figure 6A) and that the irreversible inhibition reaction occurs at the active-site of the endoprotease.

While a full structure−activity study on chloroketone inactivators was not performed, studies on a few inhibitors suggest a required structural pattern for successful inhibition. Both TPCK and 2-Nal-Ala-CMK inhibit the endoprotease well (Table 4), but TLCK does not (Figure 6B). This result is consistent with the fact that the primary binding pocket of the enzyme is hydrophobic, in order to accommodate the isoprenyl group of its natural substrates. It was of interest to synthesize and study analogs which actually contained a farnesyl moiety as the hydrophobic unit. In particular, BFCCMK proved to be quite a potent inactivator of the endoprotease, with a K_I of approximately 30 μM. The K_I for TPCK was approximately 1 mM, so the addition of the farnesyl moiety was salutary. This result is expected, given that the endoprotease is designed to hydrolyze isoprenylated proteins. Moreover, we had found earlier that *N*-Boc-*S*-*all-trans*-farnesyl-L-cysteine aldehyde proved to be a fairly potent competitive inhibitor of the enzyme (Ma et al., 1993). This inhibitor probably owes a good part of its potency to the aldehyde's ability to generate a tetrahedral intermediate with the active-site serine or cysteine residue (Vinitsky et al., 1992). The chloroketone moiety is similar to the aldehydic moiety with respect to its ability to form a tetrahedral intermediate with the active-site serine/cysteine (Powers, 1977; Prorok et al., 1994; Bender & Brubacher, 1967; Drenth et al., 1976; Shaw, 1990).

Only hydrophobic amino acid containing chloroketones inactivated the enzyme. BFCCMK, a chloroketone containing a farnesylcysteine moiety, proved to be the most potent inactivator of the endoprotease. A large number of chloroketone analogs which were not structurally similar to this analog were inert as endoprotease inactivators. This is important, because it further supports the idea that the mode of inhibition observed here is specific in nature.

The results with the inhibitors described above strongly suggest that the endoprotease is either a thiol protease or serine protease. Inhibition studies using the thiol group specific chemical reagent PCMB to inactivate the enzyme suggest that the enzyme may be a thiol protease. The enzyme is not inhibited by the serine reagent PMSF (1 mM), by chymostatin (0.33 mM), by BNPP (1 mM), or by the other serine protease inhibitors APMSF (20 μM), aprotinin (0.6 μM), leupeptin (1.0 μM), or DFP (1.0 mM) (Ma et al.,

1993), suggesting that the endoprotease is not a serine protease. The endoprotease is also not inhibited by chelating reagents EDTA (10 mM) and 1,10-ϕ (1 mM). However, the enzyme is also insensitive to E-64 (0.5 mM) and IAA (0.4 mM), both of which inhibit some thiol proteases (Hanada et al., 1978; Gurd, 1972). It should be noted though that many cysteine proteases are sensitive to only a subset of thiol alkylating reagents. For example, the cysteine protease apopain (Nicholson et al., 1995) is inhibited by *N*-ethylmaleimide and iodoacetamide (IAA) but not by E-64, TLCK, and TPCK (Nicholson et al., 1995). The cysteine protease ICE is insensitive to E-64 and antipain but is inactivated by PCMB, iodoacetamide, and TPCK (Wilson et al., 1994). Of course, unambiguous evidence on the nature of the mechanistic class to which this endoprotease belongs will require the purification and/or cloning of the enzyme. While the traditional approach of purification of this enzyme may be exceedingly difficult to carry out successfully, indirect approaches using specific affinity labeling of the enzyme by chloroketones should be possible. Experiments of this type are the subject of current studies in this laboratory.

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