Volume 332, number 1,2, 105–110 FEBS 13053 October 1993

Endoproteolysis of non-CAAX-containing isoprenylated peptides

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Received 12 August 1993

A microsomal endoprotease specifically cleaves isoprenylated peptides of the CAAX motif, such as *N*-acetyl-*S*-all-*trans*-farnesyl-L-cysteine (AFC-VIM), at the isoprenylated cysteine residue. It is shown here that endoproteolysis will also occur with peptides which are not of the CAAX type. Peptide substrates modeled after the Delta virus large antigen carboxyl-terminus (CRPQ) are endoproteolytically hydrolyzed by liver microsomes. AFC-RPQ is hydrolyzed with a K_M = 12.4 μM and a V_{max} = 0.27 nmol/min/mg, and AGGC-RPQ is hydrolyzed with a K_M = 7.9 μM and a V_{max} = 0.042 nmol/min/mg. Moreover, a series of potent inhibitors of the endoproteolysis of AFC-AAX-containing peptides are ineffective at inhibiting the hydrolysis of AFC-RPQ and AGGC-RPQ, suggesting the existence of isoforms of the endoprotease.

Endoproteolysis; Isoprenylated peptide; Inhibitor

1. INTRODUCTION

Protein isoprenylation represents a biochemical pathway essential in the activation of a diverse group of proteins. Included in this group are the heterotrimeric G proteins [1,2], the small G proteins (including *ras*) [3,4], and the nuclear lamins [5,6]. It has also recently been reported that the Delta virus large antigen is isoprenylated [7]. Isoprenylation occurs at cysteine residues close to the carboxyl-terminus of the protein to be modified. Proteins with a carboxyl-terminal CAAX (where C = cysteine, A = aliphatic amino acid, and X is an undefined amino acid) [4,8–10] or, less frequently, a CXC sequence [11–13] or a CXXX sequence [7], are first isoprenylated at the cysteine residue(s) with either all-*trans*-farnesyl (C15) or all-*trans*-geranylgeranyl (C20) pyrophosphate [14–16]. In the case of modifications at a CAAX motif, proteolysis follows, to generate an isoprenylated cysteine residue as the new carboxyl-terminus [4]. This set of modifications is completed by the carboxymethylation of the isoprenylated cysteine residue [17–24]. That this latter modification may be physiologically reversible is suggested by the discovery of a methylesterase specific for isoprenylated cysteine methyl ester derivatives [25].

Proteolysis occurs in mammals primarily by endoproteolytic cleavage between the modified cysteine residue and the adjacent aliphatic amino acid, to liberate the intact AAX tripeptide, as shown in Scheme 1 [26,27]. A liver microsomal endoproteolytic activity has been identified using synthetic radiolabeled tetrapeptide substrates such as L-AFC-Val-Ile-Ser [26]. The isoprenyl group is essential for substrate activity, and either stereospecificity or stereoselectivity is observed at AAX [28]. Potent inhibitors for the endoprotease have also been designed [29]. The analog containing BFC-statine-Val-Ile-Met (2) inhibits the endoprotease with a K_I = 64 nM. The analogous pseudopeptide $\psi(CH_2\text{-}NH)$ (1) analog is almost as potent. The endoprotease substrates studied thus far fall into the CAAX motif type. A question that emerges is whether AFC-based tetrapeptides containing hydrophilic amino acids will be endoproteolyzed, and whether the same endoprotease is capable of hydrolyzing the various substrates. The carboxyl-terminal sequence (Cys-Arg-Pro-Gln) of the Delta virus large antigen provides a pertinent example. It is demonstrated here that the tetrapeptides, AFC-RPQ (3) and AGGC-RPQ (4), are specifically processed by endoproteolytic cleavage to produce AFC and the tripeptide RPQ. Thus, the endoproteolysis of non-CAAX sequences proceeds as it does with CAAX sequences. Moreover, potent inhibitors of the endoproteolytic processing of isoprenylated CAAX-containing peptides do not interfere with the processing of AFC-RPQ and AGGC-RPQ, suggesting the presence of isoforms of the endoprotease.

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Abbreviations: DMSO, dimethylsulfoxide; AFC, *N*-acetyl-*S*-all-*trans*-farnesyl-L-cysteine; AGGC, *N*-acetyl-*S*-all-*trans*-geranylgeranyl-L-cysteine; BFC, *N*-*t*-Boc-all-*trans*-farnesyl-L-cysteine; DMF, dimethylformamide; DMAP, 4,4-(dimethylamino)pyridine; PNB, *p*-nitrobenzyl; Arg, arginine; Pro, proline; Gln, glutamine.

2. EXPERIMENTAL

2.1. *Materials*

Fresh bovine calf liver was obtained from a local slaughterhouse.

N-Acetyl-L-proline and *N*-acetyl-L-glutamine were purchased from the Sigma Chemicals Co. Geranylgeraniol was purchased from TCI Inc. All-*trans*-farnesyl bromide, piperidine, and trifluoroacetic acid were acquired from Aldrich Chemical. [^{3}H]Acetic anhydride (spec. act. = 500 mCi/mmol) was purchased from New England Nuclear. *N*-Acetyl-L-arginine-L-proline-L-glutamine was synthesized by Dr. Chuck Dahl at the Harvard Medical School peptide synthesis facility. Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy was recorded on a Varian VRX 500S Spectrometer operating at a proton frequency of 499.843 MHz. Dimethylsulfoxide (DMSO-d_6) was used as the ^{1}H NMR solvent. The residual proton absorption of the deuterated solvent was used as the internal standard. HPLC solvents were from J.T. Baker Inc. All chemicals and solvents purchased were of the highest purity commercially available.

2.2. *Protease assays*

Substrates were dissolved in DMSO and incubated with bovine liver microsomal membranes (0.2 mg of protein/ml) in 100 mM potassium phosphate buffer (pH 7.0) at 37°C for 60 min. The final DMSO concentration in the assays was 4% (v/v). The reaction was quenched with 500 μl of $CHCl_3$/MeOH (1:1, v/v), *N*-[^{3}H]AFC was extracted after thoroughly mixing for 1 min. Phase separation was achieved by adding 500 μl of 1 M citric acid to the mixture. The chloroform layer was removed, dried, and evaporated under nitrogen. The residue was dissolved in hexane/isopropanol/TFA (85:15:0.1) and non-radioactive AFC standard was added for UV detection (210 nm). Samples were injected on a normal-phase HPLC column (Dynamax 60A; Rainin) and elution was performed with the same solvent at a flow rate of 1.5 ml/min. Radioactivity was determined using an on-line Berthold (Nashua, NH) LB 506-C HPLC radioactivity monitor.

2.3. *Syntheses*

Complete synthetic procedures used for the synthesis of *N*-[^{3}H]-acetyl-*S*-all-*trans*-farnesyl-L-cysteine-L-arginine-L-proline-L-glutamine and *N*-[^{3}H]acetyl-*S*-all-*trans*-geranylgeranyl-L-cysteine-L-arginine-L-proline-L-glutamine will be published separately. The ^{1}H NMR data and procedures for the last step in the synthesis of [^{3}H]AFC-Arg-Pro-Gln and [^{3}H]AGGC-Arg-Pro-Gln are described below.

2.3.1. Synthesis of *N*-[^{3}H]acetyl-*S*-all-*trans*-farnesyl-L-cysteine-L-arginine-L-proline-L-glutamine

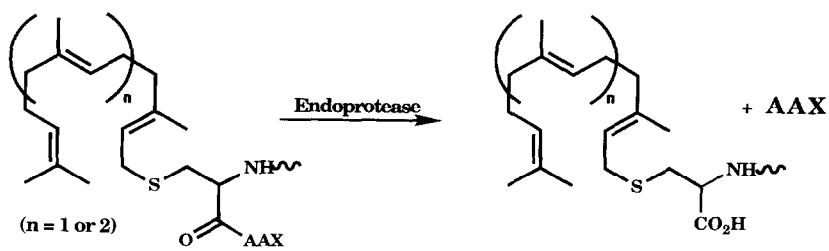
A mixture of *S*-all-*trans*-farnesyl-L-cysteine-ω-nitro-L-arginine-L-proline-L-glutamine-*p*-nitrobenzyl ester (15 mg), [^{3}H]acetic anhydride (5 mCi, 500 mCi/mmol), and a catalytic amount of DMAP in methylene chloride (8 ml) was stirred at room temperature for 28 h. The solvent was evaporated and the residue was purified by silica gel chromatography (hexane/acetone 70:30, 50:50; then eluted with acetone and methanol successively). The acetylated peptide was re-dissolved in 90% acetic acid (5 ml), and zinc dust (200 mg) was added. The mixture was stirred at room temperature for 22 h after which the zinc dust was removed by filtration. The aqueous solution was lyophilized, and the residue was purified by reverse-phase HPLC (column, C-18, 10.5 × 1 cm; flow rate, 2.5 ml/min; UV detection, 210 nm. Solvent A: 10 mM TFA in 20–100% water. Solvent B: 10 mM TFA in acetonitrile. Gradient program: 20% in 30 min, 100% B in 5 min, 100–20% B in 5 min, 20% B in 5 min). The retention time for the product was 27.85 min. Yield, 0.78 mCi, 31%. ^{1}H NMR (500 MHz, DMSO-d_6) of the authentic non-radioactive peptide: 8.20–8.11 (3 H, m), 8.08 (1 H, d, J = 8 Hz), 7.22 (1 H, s), 6.78 (1 H, s), 5.13 (1 H, t, J = 7.5 Hz), 5.05 (2 H, brs), 4.49 (1 H, t, J = 6 Hz), 4.40 (1 H, dd, J = 8.5, 14.5 Hz), 4.33 (1 H, t, J = 5 Hz), 4.09 (1 H, dd, J = 8, 14 Hz), 3.63 (1 H, m), 3.50 (1 H, m), 3.23 (1 H, m), 3.12 (2 H, d, J = 7 Hz), 3.07 (1 H, m), 2.70 (1 H, dd, J = 5.5, 13.5 Hz), 2.52 (1 H, dd, J = 4, 13.5 Hz), 2.20–2.10 (3 H, m), 2.04–1.86 (13 H, m), 1.82 (3 H, s), 1.78–1.64 (4 H, m), 1.61 (3 H, s), 1.59 (3 H, s), 1.53 (6 H, s).

2.3.2. Synthesis of *N*-[^{3}H]acetyl-*S*-all-*trans*-geranylgeranyl-L-cysteine-L-arginine-L-proline-L-glutamine

A mixture of *S*-all-*trans*-geranylgeranyl-L-cysteine-ω-nitro-L-arginine-L-proline-L-glutamine-*p*-nitrobenzyl ester (28 mg), [^{3}H]acetic anhydride (5 mCi, 500 mCi/mmol), and a catalytic amount of DMAP in methylene chloride (8 ml) was stirred at room temperature for 40 h. Solvent was evaporated and the residue was purified by silica gel chromatography (hexane/acetone 70:30, 50:50; then eluted with acetone and methanol successively). The acetylated peptide was re-dissolved in 90% acetic acid (5 ml) and zinc dust (200 mg) was added. The mixture was stirred at room temperature for 24 h after which the zinc dust was removed. The aqueous solution was lyophilized and the residue was purified by reverse-phase HPLC (column, C-18, 10.5 × 1 cm; flow rate, 2.5 ml/min; UV detection, 210 nm. Solvent A: 10 mM TFA in water. Solvent B: 10 mM TFA in acetonitrile. Gradient program: 20–100% B in 30 min, 100% B in 5 min, 100–20% B in 5 min, 20% B in 5 min). The retention time for the product was 21.69 min. Yield, 0.51, 20%. ^{1}H NMR (500 MHz, DMSO-d_6) of the authentic non-radioactive peptide: 8.63 (1 H, brs), 8.20–8.12 (3 H, m), 8.08 (1 H, d, J = 8 Hz), 7.21 (1 H, s), 6.78 (1 H, s), 5.13 (1 H, t, J = 7.5 Hz), 5.05 (3 H, t, J = 6.5 Hz), 4.50 (1 H, brs), 4.40 (1 H, dd, J = 7.5, 12.5 Hz), 4.33 (1 H, dd, J = 4, 8.5 Hz), 4.10 (1 H, dd, J = 8, 14 Hz), 3.64 (1 H, m), 3.49 (1 H, m), 3.23 (1 H, m), 3.13 (2 H, d, J = 8 Hz), 3.04 (1 H, m), 2.70 (1 H, dd, J = 5.5, 13.5 Hz), 2.52 (1 H, dd, J = 4, 13.5 Hz), 2.22–2.10 (3 H, m), 2.04–1.88 (17 H, m), 1.82 (3 H, s), 1.78–1.64 (4 H, m), 1.61 (3 H, s), 1.59 (3 H, s), 1.53 (9 H, s).

3. RESULTS

Previously we had found that tetrapeptides which conform to the AFCAAX or AGGCAAX motifs are endoproteolytically processed by bovine liver microsomes after the modified cysteine residue to generate either AFC or AGGC [26,29]. Because the isoprenylated Delta virus large antigen possesses a carboxyl-terminal sequence, CRPQ, we decided to determine if the liver enzyme(s) was able to cleave the appropriately modified sequence in the same way as tetrapeptides ending with CAAX. To these ends, the radioactive peptides shown in Scheme 2 were synthesized and studied as substrates for the liver microsomal enzyme(s). As shown in Fig. 1A and B, both molecules proved to be enzymatically processed with the indicated K_M and V_{max}



Scheme 1. Endoproteolysis in the isoprenylation pathway.

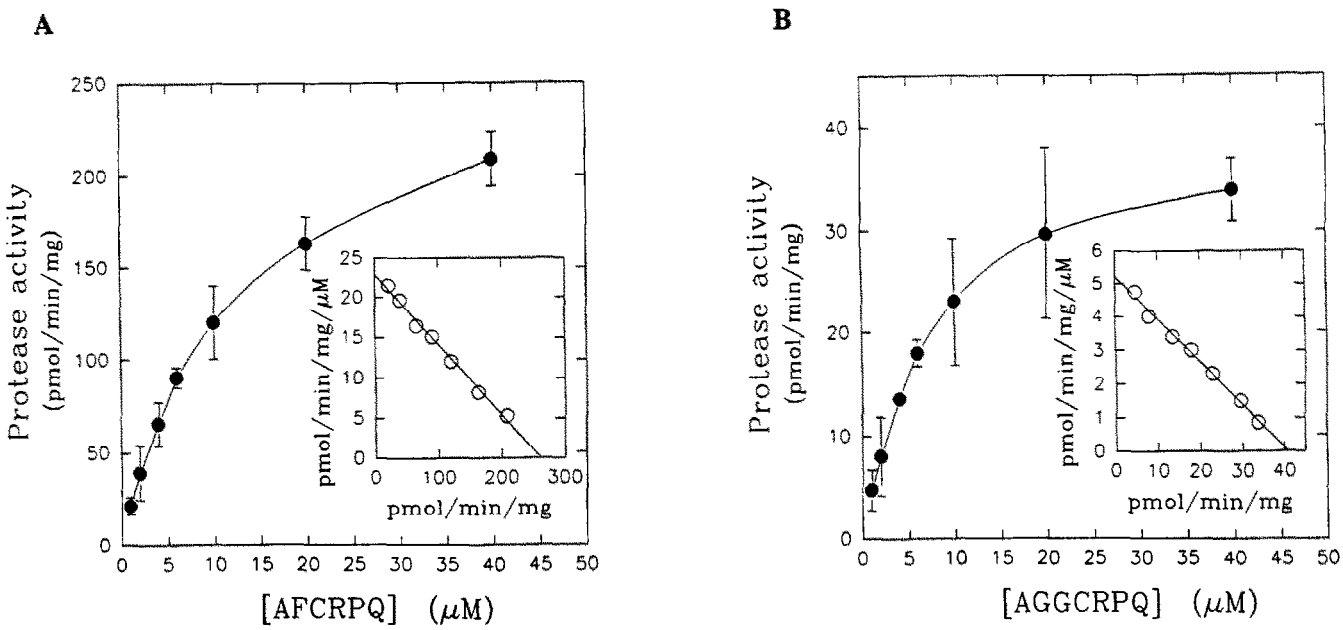


Fig. 1. (A) Protease activity with *N*-[^{3}H]acetyl-*S*-farnesyl-L-Cys-L-Arg-L-Pro-L-Gln as substrate. Michaelis–Menten and Eadie–Hofstee plots (inset) of the formation of *N*-[^{3}H]AFC as a function of *N*-[^{3}H]acetyl-*S*-farnesyl-L-Cys-L-Arg-L-Pro-L-Gln concentration. Symbols represent mean values of four determinations, and error bars represent the standard deviation from the mean. Error bars not shown are within symbols. (B) Protease activity with *N*-[^{3}H]acetyl-*S*-geranylgeranyl-L-Cys-L-Arg-L-Pro-L-Gln as substrate. Michaelis–Menten and Eadie–Hofstee plots (inset) of the formation of *N*-[^{3}H]AGGC as a function of *N*-[^{3}H]acetyl-*S*-geranylgeranyl-L-Cys-L-Arg-L-Pro-L-Gln concentration. Symbols represent mean values of four determinations, and error bars represent the standard deviation from the mean. Error bars not shown are within symbols.

values (Scheme 2). The enzymatic assays employed measure the formation of either [^{3}H]AFC or [^{3}H]AGGC by HPLC and so cannot be used to determine if the proteolytic cleavage is mediated in an endo or exo fashion. To determine the mechanism of the cleavage reaction, the products of the proteolysis reaction had to be modified in such a way that they could be separated and detected using HPLC techniques. Acetylation was the modification chosen for this purpose. *N*-Ac-Gln, *N*-Ac-Pro, *N*-Ac-Arg, and *N*-Ac- Arg-Pro-Gln could be readily separated by HPLC, as shown in Fig. 2A. The proteolysis products of the farnesylated peptide, [^{3}H]AFCRPQ, were reacted with [^{3}H]acetic anhydride. When the resultant radioactive peptides were separated by HPLC, the results shown in Fig. 2B were obtained. Clearly the tetrapeptide was endoproteolyzed to generate [^{3}H]*N*-Ac-RPQ as the major product (Fig. 2). Therefore, the proteolysis of the hydrophilic peptide proceeded in an endo manner, which liberates labeled AFC (or AGGC) and RPQ.

It was of interest to determine if protease inhibitors, some of which inhibit the processing of isoprenylated CAAX-containing peptides, would interfere with the proteolysis of the hydrophilic isoprenylated peptides described here. First, none of the standard group-specific protease inhibitors tested, which included antipain, APMSF, aprotinen, bestatin, chymostatin, E-64, EDTA-Na_2, leupeptin, pepstatin, phosphoramidon, and 1,10-phenanthroline, affected the proteolysis of AFC-RPQ. This result was also observed with the proteases which process the isoprenylated CAAX-containing peptides [29]. However, the latter proteolytic activity was potently inhibited by a series of pseudo (ψ) peptide and statine derivatives, some of which are shown below (Table I). None of these inhibitors had any effect on the processing of AFC-RPQ when tested under conditions that potently inhibited the processing of AFC-VIM. This result is not consistent with the idea that a single enzyme is involved in the hydrolysis of all isoprenylated proteins, and shows that different forms of the protease are involved in the hydrolysis of hydrophobic and hydrophilic peptides.

4. DISCUSSION

The experiments described here demonstrate that AFC-RPQ and AGGC-RPQ are cleaved endoproteolytically by a liver microsomal activity. The amino acid sequence from which this peptide is derived is based on the isoprenylated Delta virus large antigen [7]. It is not clear whether this protein is farnesylated or geranylgeranylated [7]. Nevertheless, we report here that both the farnesylated and geranylgeranylated peptides are endoproteolyzed.

The sequence CRPQ, of course, does not fit in with the well-known CAAX motif, a common signal for isoprenylation [3]. Nevertheless, AFC-RPQ and AGGC-RPQ are endoproteolyzed by the liver microsomal ac-

tivity, which brings up the question as to whether isoforms of the proteases exist, or whether the same

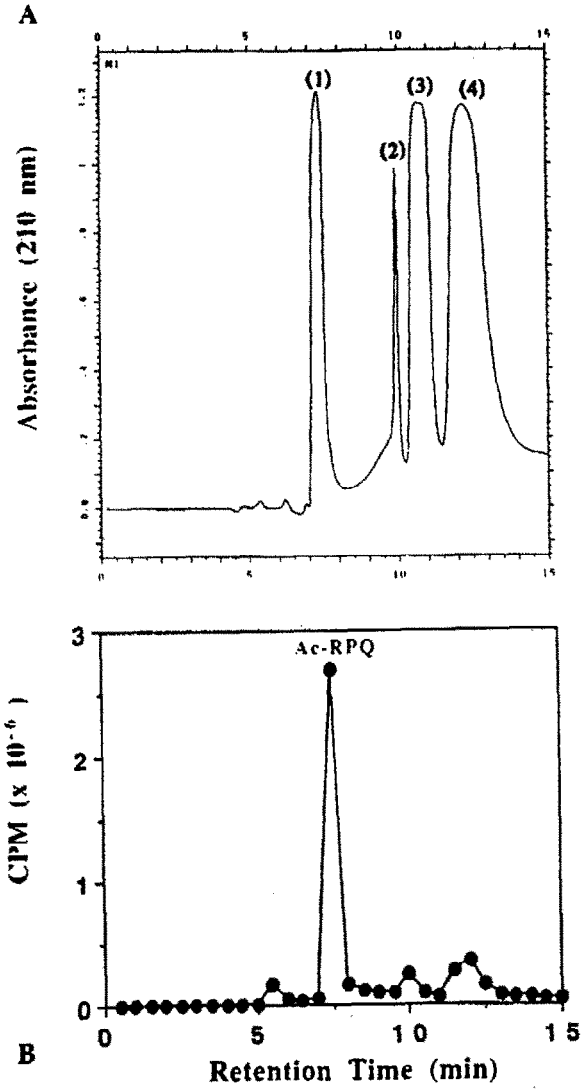
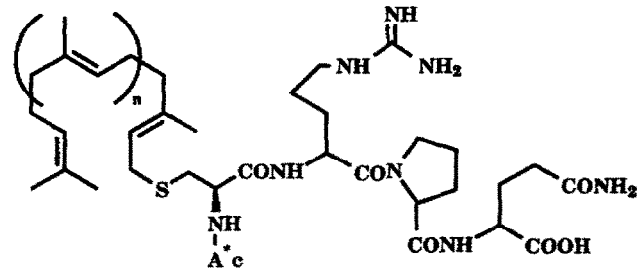


Fig. 2. Formation of L-Arg-L-Pro-L-Gln from AFC-L-Arg-L-Pro-L-Gln. AFC-L-Arg-L-Pro-L-Gln (10 mM) was incubated with bovine liver microsomal membranes at 37°C for 1 h. The reaction was quenched with 200 ml of chloroform/methanol (1:1, v/v) and 200 ml of water. The aqueous layer was filtered and lyophilized. Ten assay mixtures were pooled together. The residue was suspended in 3 ml of methylene chloride, and 2 mCi of [^{3}H]acetic anhydride (500 mCi/mmol) was added. The mixture was stirred at room temperature overnight. Water (1 ml) was added and methylene chloride was evaporated. The aqueous part was lyophilized and the residue was re-dissolved in 1 ml of water/acetonitrile (1:1, v/v). A mixture of 0.1 ml of this solution and a standard solution of Ac-Arg, Ac-Pro, Ac-Gln, and Ac-Arg-Pro-Gln was injected onto a reverse-phase HPLC system (Rainin, C-18, 10.5 × 1 cm, UV detection at 210 nm, and flow rate 2.5 ml/min), starting the linear gradient at the same time. The gradient solvent for reservoir A was water containing 10 mM TFA, and for reservoir B was acetonitrile saturated with 10 mM TFA. Elution was 0–100% B in 35 min, 100% B in 10 min, 100–0% B in 5 min, and 10 min re-equilibration. Each fraction (1.25 ml) was collected and counted in scintillation fluid (12 ml) by using a Beckman LS-1800 radioactivity counter. (A) HPLC trace: peak 1, Ac-RPQ; 2, Ac-Arg; 3, Ac-Gln; 4, Ac-Pro. (B) Radioactivity associated with the different fractions.



Scheme 2. $n = 1$ (AFC-RPQ): $K_M = 12.38 \pm 0.54$ μM, $V_{max} = 270.54 \pm 5.1$ pmol/min/mg. $n = 2$ (AGGC-RPQ): $K_M = 7.90 \pm 1.12$ μM, $V_{max} = 41.77 \pm 2.21$ pmol/min/mg.

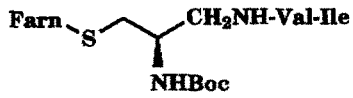
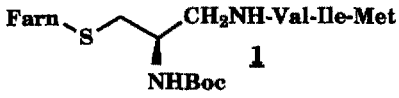
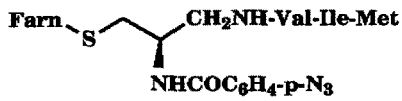
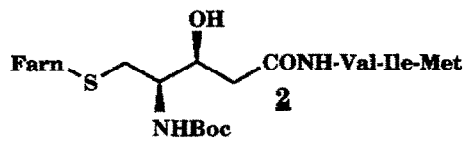
enzyme processes peptides both within the CAAX motifs and without. This issue has been addressed with respect to the isoprenyl transferases. Sequence-specific isoforms certainly exist which use either farnesyl pyrophosphate or geranylgeranyl pyrophosphate as substrates [14–16]. The methyltransferase [30] and methylesterase are membrane-bound enzymes and have not yet been purified. Nevertheless, in the case of the methyltransferase, cross-inhibition studies indicate that the enzyme(s) is at least non-selective with respect to the geranylgeranyl moiety vs. the farnesyl moiety, and that individual isoforms with isoprene-specific activities may not exist [30]. Although the membrane-bound endoprotease is readily solubilized with CHAPSO (Chaudhuri, A. and Rando, R.R., unpublished experiments), it has not been completely purified, so that the question of possible isoforms has not been approached.

Potent tetrapeptide-based inhibitors of the endoprotease with respect to its ability to hydrolyze AFC-VIM have been developed [29]. For example, **1** and **2** (Table 1) have K_I values of 86 nM and 64 nM, respectively [29]. When the inhibitors were studied for their inhibitory potencies with respect to endoproteolysis of AFC-RPQ and, in some cases, AGGC-RPQ, they proved to be inert as inhibitors (Table I). The same concentrations of inhibitors potently inhibit the endoproteolysis of AFC-VIM. It can be concluded from these studies that the same form of the enzyme which hydrolyzes AFC-VIM and is susceptible to inhibition by analogs based on this sequence does not hydrolyze AFC-RPQ-containing peptides. Thus, isoforms of the endoprotease are likely to be present which could have important regulatory significance. The selective inhibition of these putative isoforms could be of substantial interest for uncovering the physiological role(s) of proteolysis in the isoprenylation pathway.

Acknowledgements: The excellent technical assistance of Ms. Mei-Huei Tu is gratefully acknowledged. This work was supported by the National Institutes of Health Public Health Service Grant EY-03624 and by a grant from Hoffmann-LaRoche Inc.

Table I

CAAX-based endoprotease inhibitors do not affect processing of CRPQ-based substrates[a]

Inhibitor	Conc. tested	% inhibition
	50 μM	0
	50 μM	0
	50 μM	0
	50 μM	0
	50 μM	0
	50 μM	0

The inhibitors were preincubated with the calf liver microsomal membrane (0.2 mg of protein/ml) in 100 mM phosphate buffer (pH 7) for 10 min. *N*-[^{3}H]AFC-RPQ (10 μM) was added and the incubation was continued for 1 h. The work-up and enzymatic assays were performed as indicated in section 2.

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