A microsomal endoprotease that specifically cleaves isoprenylated peptides

(isoprenylation/synthetic peptides)

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ABSTRACT A microsomal enzymatic activity is described that can specifically cleave the tetrapeptide *N*-acetyl-*S*-farnesyl-L-Cys-L-Val-L-Ile-L-Ser between the isoprenylated cysteine residue and the valine residue. K_m and V_{max} values are measured as 5.8 μ M and 251 pmol/min per mg of protein, respectively. Proteolytic cleavage of the substrate is stereospecific because the substitution of a farnesylated D-cysteine residue for the L-amino acid leads to the abolition of substrate activity. A free carboxyl-terminal group is also required for substrate activity because methyl esterification renders the substrate inert. The tripeptide *N*-acetyl-*S*-farnesyl-L-Cys-L-Val-L-Ile and the dipeptide *N*-acetyl-*S*-farnesyl-L-Cys-L-Val are also hydrolyzed by the protease. Again, stereospecificity is observed at the isoprenylated residue. Hydrolysis of the farnesylated tetrapeptide is not inhibited by a 5-fold excess of the nonfarnesylated tetrapeptide, suggesting that isoprenylation is important for substrate activity. This activity is probably the same as the proteolytic activity proposed to cleave isoprenylated proteins terminating in a Cys-Ali-Ali-Xaa motif, where Ali refers to aliphatic amino acid. These proteins include the ras family of G proteins and the heterotrimeric G proteins. Proteolytic maturation of these essential isoprenylated signal-transducing elements is a key step in their activation.

Hydrophobic posttranslational modification reactions are key elements in many cellular regulatory processes (1). One class of these modifications involves protein isoprenylation, as shown in the biochemical pathways of Scheme I (2–8).

Scheme I

In this set of modifications, a protein with a carboxyl-terminal CAAX (2, 4, 8, 9) or, less frequently, CXC sequence [where C is cysteine, A is aliphatic amino acid (Ali), and X is an undefined amino acid] (10–12) is first isoprenylated at the cysteine residue(s) with either all-*trans*-farnesyl (C_{15}) or all-*trans*-geranylgeranyl (C_{20}) pyrophosphate (13–15). Soluble isoprenyl transferases that carry out these modifications

have already been purified and characterized (16, 17). For modifications at a CAAX motif, proteolysis follows, to generate the isoprenylated cysteine residue as the new carboxyl terminus (18). This set of modifications is completed by the reversible carboxymethylation of the isoprenylated cysteine residue (19–26). Of the reactions in the isoprenylation pathway, only the proteolysis step remains unidentified.

Proteolytic processing could occur by several different routes. The simplest route involves cleavage between the modified cysteine residue and the adjacent aliphatic amino acid to liberate the Ali-Ali-Xaa tripeptide. Alternatively, proteolysis could begin anywhere within the Ali-Ali-Xaa moiety and stop at the modified cysteine residue. Multiple proteases could be involved in this latter scenario. In this communication we identify a microsomal proteolytic activity that produces a single cut between the modified cysteine residue and the Ali-Ali-Xaa tripeptide, using a synthetic tetrapeptide substrate *N*-acetyl-*S*-all-*trans*-farnesyl-L-cysteine (AFC)-Val-Ile-Ser to probe the reaction. This proteolytic activity is probably the same one essential for the maturation and activation of signal-transducing G proteins, including the oncogenic forms of ras. Therefore, this protease is expected to play an important role in cellular regulatory mechanisms.

MATERIALS AND METHODS

Materials. Amino acids and dipeptides were purchased from Bachem. 2-Mercaptoethanol, *o*-phthalaldehyde, all-*trans*-farnesyl bromide, all-*trans*-farnesol, 4,4-dimethylaminopyridine, 1-hydroxybenzotriazole hydrate, 1-(3-dimethylamino)propyl-3-ethylcarbodiimide hydrochloride, *N*-methylmorpholine, and 1-hydroxybenzotriazole hydrate were purchased from Aldrich. HPLC solvents were from J. T. Baker. All chemicals and solvents were of the highest purity available. L-[3H]serine and [3H]acetic anhydride were from New England Nuclear. Dog pancreatic microsomes were purchased from Promega. Fresh bovine calf liver was from a local slaughter house.

Methods. Bovine liver microsomes were obtained by the published procedure (27). Kinetic measurements were done by incubating protein at 0.2 mg/ml with peptide substrates in 200 mM Hepes buffer/100 mM NaCl/5 mM $MgCl_2$, pH 7.4, all in a final volume of 50 μ l. The peptide substrates were added in dimethyl sulfoxide (DMSO) to a final concentration of 4% (vol/vol) at 37°C. The enzymatic reaction was quenched at the appropriate time by adding 0.5 ml of chloroform/methanol, 1:1 (vol/vol). Phase separation was achieved by adding 0.5 ml of 1 M citric acid. The chloroform layer was separated and evaporated under nitrogen, and the residue was dissolved in 1-hexane/2-propanol/trifluoro-

Abbreviations: DMSO, dimethyl sulfoxide; AFC, *N*-acetyl-*S*-all-*trans*-farnesyl-L-cysteine; D-AFC, *N*-acetyl-*S*-all-*trans*-farnesyl-D-cysteine; Ali, aliphatic amino acid; CAAX, Cys-Ali-Ali-Xaa.

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acetic acid/cold AFC. Formation of the product ^3H -labeled AFC was followed by HPLC analysis at 210 nm (23). The samples were injected onto a normal-phase HPLC column (Dynamax 60, Rainin, Woburn, MA) connected to a model LB 506-C online radioactivity monitor (Berthold, Nashua, NH). The column was eluted with 1-hexane/2-propanol/trifluoroacetic acid, 85:15:0.1 (vol/vol/vol) at 1.5 ml/min.

When following the processing of AFC-Val-Ile- ^3H Ser, hydrophobic derivitization of the peptide and amino acid products was required to separate them quantitatively. Amino-terminal derivitization with *o*-phthalaldehyde/2-mercaptoethanol was used here (28). A solution of 27 mg of *o*-phthalaldehyde in 0.5 ml of absolute ethanol and 5 ml of 0.1 M sodium tetraborate was prepared. 2-Mercaptoethanol (50 μl) was added, and 200 μl of the final solution was mixed with 50 μl of the amino or peptide solution. The reaction was allowed to proceed for 2 min and was then injected onto the HPLC column, and separation of the components was effected by gradient elution and two buffered solutions. Buffer A contained 800 ml of 0.05 M sodium phosphate, pH 5.5, and 200 ml of methanol, and buffer B contained 200 ml of the phosphate buffer and 800 ml of methanol. A Rainin C₁₈ column (10.5 $\times$ 1 cm) was used, and detection was at 254 nm. The exact HPLC conditions used are given in the appropriate figure legend.

Syntheses. AFC was prepared by the published procedure (23).

Syntheses of Radioactive Peptides. *Synthesis of N-[^3H]acetyl-S-all-trans-farnesyl-L-Cys-L-Val-L-Ile and N-[^3H]acetyl-S-all-trans-farnesyl-D-Cys-L-Val-L-Ile.* A mixture of S-all-trans-farnesyl-L-Cys-L-Val-L-Ile methyl ester (20 mg, 36 μmol), [^3H]acetic anhydride (0.02 mmol, ≈ 10 mCi, 500 mCi/mmol; 1 Ci = 37 GBq), triethylamine (3.3 μl , 0.024 mmol), and a catalytic amount of 4,4-dimethylaminopyridine in 20 ml of methylene chloride was stirred at room temperature overnight. The mixture was filtered, and the solvent was evaporated. The residue was separated by HPLC (Rainin silica, 250 $\times$ 4.6 mm, 1.5 ml/min, hexane/isopropanol, 85:15; UV detection: 210 nm). Retention time was 4.27 min, and yield was 3.57 mCi, 14.28 μmol , 71%.

N-[^3H]acetyl-S-all-trans-farnesyl-L-Cys-L-Val-L-Ile methyl ester (1.19 mCi, 4.76 μmol , 250 mCi/mmol) was dissolved in 12 ml of methanol. KOH/MeOH (5%, 12 ml) was added at 0°C, and the mixture was stirred at 0°C for 1 hr and then at room temperature for 18 hr. Basic hydrolysis under these conditions served to racemize the chiral center at the cysteine residue. Acetic acid was added to pH 7. The methanol was evaporated, and the residue was extracted with ethyl acetate twice. The combined organic phase was washed with 10% HCl, then with water, then dried over anhydrous sodium sulfate, filtered, and evaporated. Total activity was 1.04 mCi (87%). Purification was carried out by HPLC on Rainin silica 250 $\times$ 4.6 mm column at a flow rate of 1.5 ml/min, using hexane/isopropanol/trifluoroacetic acid, 92:8:0.01, as eluant. UV detection was at 210 nm. Retention time of the D-L-L isomer was 8.1 min, and for the L-L-L isomer it was 10.2 min. ^1H -NMR (DMSO- d_6 , 500 MHz) of the authentic nonradioactive peptide data was as follows: AFC-L-Val-L-Ile: δ 8.13 (1 H, d, J = 8.5 Hz), 7.91 (1 H, d, J = 8 Hz), 7.79 (1 H, d, J = 9 Hz), 5.14 (1 H, t, J = 8 Hz), 5.04 (2 H, br s), 4.46 (1 H, dd, J = 6, 10 Hz), 4.25 (1 H, dd, J = 6, 9 Hz), 4.11 (1 H, dd, J = 6, 8 Hz), 3.12 (2 H, d, J = 7.5 Hz), 2.72 (1 H, dd, J = 6, 14 Hz), 2.52 (1 H, dd, J = 7.5, 14 Hz), 2.06–1.88 (9 H, m), 1.83 (3 H, s), 1.74 (1 H, m), 1.61 (3 H, s), 1.59 (3 H, s), 1.53 (6 H, s), 1.38 (1 H, m), 1.17 (1 H, m), 0.78–0.86 (9 H, m). N-acetyl-S-all-trans-farnesyl-D-Cys-L-Val-L-Ile: δ 8.09 (1 H, d, J = 8 Hz), 8.07 (1 H, d, J = 10 Hz), 7.95 (1 H, d, J = 8 Hz), 5.15 (1 H, t, J = 8 Hz), 5.04 (2 H, t, J = 6 Hz), 4.57 (1 H, dd, J = 6.5, 8 Hz), 4.28 (1 H, dd, J = 7, 9 Hz), 4.09 (1 H, dd, J = 6, 8 Hz), 3.16 (2 H, dd, J =

4, 8 Hz), 2.65 (1 H, dd, J = 6.5, 13.5 Hz), 2.51 (1 H, dd, J = 7.5, 13.5 Hz), 2.04–1.88 (9 H, m), 1.81 (3 H, s), 1.75 (1 H, m), 1.61 (6 H, s), 1.53 (6 H, s), 1.40 (1 H, m), 1.19 (1 H, m), 0.78–0.88 (9 H, m).

Synthesis of N-[^3H]acetyl-S-all-trans-farnesyl-L-Cys-L-Val-L-Ile-L-Ser. A solution of N-[^3H]acetyl-S-all-trans-farnesyl-L-Cys-L-Val-L-Ile (1.12 μmol , 250 mCi/mmol), L-serine hydrochloride methyl ester (20 mg, 0.13 mmol), and 1-hydroxybenzotriazole hydrate (17.4 mg, 0.13 mmol) in 3 ml of dimethylformamide was cooled to 0°C. 1-(3-Dimethylamino)propyl-3-ethylcarbodiimide hydrochloride (17.2 mg, 0.09 mmol) and N-methylmorpholine (14.1 μl , 13 mg, 0.13 mmol) were added at 0°C. The mixture was stirred at 0°C under argon for 2 hr and at room temperature for 24 hr. Water (20 ml) was added, and the mixture was extracted with ethyl acetate (4 $\times$ 20 ml). The combined organic layer was washed with 10% HCl, saturated sodium bicarbonate, and water; dried over anhydrous magnesium sulfate; filtered; and evaporated. The residue was dissolved in 3 ml of acetonitrile, and 3 ml of 10% aqueous sodium carbonate was added. The mixture was stirred at room temperature for 24 hr. Ten percent HCl was added to adjust the pH to 2. The mixture was then extracted with ethyl acetate (20 ml three times), and the combined organic layer was washed with water, dried over anhydrous magnesium sulfate, filtered, and evaporated. Purification was done by HPLC on a Rainin silica 4.6 $\times$ 250-mm column at a flow rate of 1.5 ml/min, with hexane/isopropanol/trifluoroacetic acid, 85:15:0.1 as eluant. UV detection was at 210 nm. Recovery of radioactivity was 28.2 μCi , 1.13×10^{-4} mmol. Retention time was 6.49 min. Data from ^1H -NMR (500 MHz, DMSO- d_6) of the authentic non-radioactive peptide are as follows: δ 8.14 (1 H, d, J = 8 Hz), 7.97 (1 H, d, J = 7 Hz), 7.86 (1 H, d, J = 9 Hz), 7.76 (1 H, d, J = 8.5 Hz), 5.16 (1 H, t, J = 8.5 Hz), 5.05 (2 H, m), 4.48 (1 H, br dd, J = 6.5, 14 Hz), 4.25 (1 H, t, J = 8 Hz), 4.20 (1 H, br t, J = 8 Hz), 4.10 (1 H, br d, J = 7 Hz), 3.66 (1 H, dd, J = 6, 11 Hz), 3.56 (1 H, dd, J = 4, 11 Hz), 3.08 (2 H, m), 2.73 (1 H, dd, J = 5.5, 13.5 Hz), 2.52 (1 H, dd, J = 4, 11 Hz), 1.90–2.10 (8 H, m), 1.84 (3 H, s), 1.73 (2 H, m), 1.62 (3 H, s), 1.60 (3 H, s), 1.54 (6 H, s), 1.41 (1 H, m), 1.30 (1 H, m), 0.80 (12 H, m). N-[^3H]acetyl-S-all-trans-farnesyl-D-Cys-L-Val-L-Ile-L-Ser was prepared by the same method as described above, starting with N-[^3H]acetyl-S-all-trans-farnesyl-D-Cys-L-Val-L-Ile.

RESULTS

Enzymatic Hydrolysis of a Synthetic Tetrapeptide. In the experiments reported here, a tetrapeptide having the sequence AFC-Val-Ile-Ser (all L amino acids) **1** was chosen as a synthetic substrate for the protease. This sequence is taken from the carboxyl terminus of the γ subunit of the retinal heterotrimeric G protein transducin (29–32). The γ subunit of transducin has already been shown to be farnesylated and methylated (5, 20). Incubation of the above mentioned tetrapeptide (labeled with an [^3H]acetyl group) with a calf liver microsomal enzyme preparation led to the time-dependent formation of AFC (Fig. 1). When the concentration of sub-

strate **1** was varied, saturation was observed, and the K_m and V_{max} were measured to be $5.76 \pm 0.72 \mu\text{M}$ and 251.17 ± 8.51

FIG. 1. Time-dependent hydrolysis of *N*-[³H]acetyl-S-farnesyl-L-Cys-L-Val-L-Ile-L-Ser. *N*-[³H]acetyl-S-farnesyl-L-Cys-L-Val-L-Ile-L-Ser (10 μ M) was incubated with bovine liver microsomal membranes, and its processing to [³H]AFC was followed. At indicated times, 50- μ l samples were withdrawn from the incubation mixture, and the reaction was terminated with 500 μ l of chloroform/methanol, 1:1 (vol/vol). [³H]AFC was extracted after thoroughly mixing the mixture for 1 min and extracting with chloroform. Phase separation was achieved by adding 500 μ l of 1 M citric acid. The chloroform layer was evaporated under nitrogen, resuspended in hexane/isopropanol/trifluoroacetic acid, 85:15:0:1, and nonradioactive AFC standard was added for detection at 210 nm. The sample was injected onto a silica HPLC column (Dynamax 60A, Rainin, Woburn, MA) and eluted with the same solvent. Radioactivity was counted with an online Berthold (Nashua, NH) LB 506-C HPLC radioactivity monitor.

pmol/min per mg of protein, respectively (Fig. 2). AFC formation did not occur when the tetrapeptide was *N*-acetyl-S-all-*trans*-farnesyl-D-cysteine (D-AFC)-Val-Ile-Ser 2, nor when the carboxyl group of the serine residue was methylated. These data demonstrate that the proteolysis is stereospecific and that a free terminal-carboxyl group is required in the substrate. A pH-versus-rate profile for the proteolysis reaction showed a broad maximum at $\approx$ pH 7 (Fig. 3).

A similar stereospecific proteolytic enzymatic activity was also found in dog pancreatic microsomes. Under extended incubation conditions, where 54% of the tetrapeptide substrate 1 was cleaved by these microsomes, no proteolysis of D-AFC-Val-Ile-Ser 2 could be detected (<1%). It is noteworthy that dog pancreas microsomes have already been shown to process farnesylated but unproteolyzed ras protein into the mature protein containing the carboxyl-terminal farnesylated cysteine residue (18).

Specific Enzymatic Cleavage Occurs at the Modified Cysteine Residue. The data described above show that tetrapeptide 1 is enzymatically processed by microsomal preparations to generate AFC. All further studies reported here were done with the calf liver microsomal preparation because it could be obtained readily. It was of special interest to determine where the initial cleavage reaction occurred. To accomplish this, tetrapeptide 1 was synthesized with a tritiated serine residue at the carboxyl terminus. This tetrapeptide was subjected to proteolysis by the microsomal enzyme, and the products were treated with *o*-phthalaldehyde and 2-mercaptoethanol to generate the fluorescently tagged peptides (28). These peptides could be separated easily on reverse-phase HPLC. Fig. 4A shows that initial incubation produced virtually only the tripeptide Val-Ile-Ser. Further incubation with the enzyme caused further proteolysis of the tripeptide, to produce the dipeptide (Fig. 4B). These data show that the initial proteolytic cut is between the AFC moiety and the adjacent

FIG. 2. Protease activity with *N*-[³H]acetyl-S-farnesyl-L-Cys-L-Val-L-Ile-L-Ser as substrate. Michaelis-Menten and Eadie-Hofstee plots (Inset) of the formation of [³H]AFC as a function of *N*-[³H]acetyl-S-farnesyl-L-Cys-L-Val-L-Ile-L-Ser (AFCVIS) concentration. Symbols represent mean values of two determinations, and error bars represent the SEM. Error bars not shown are within symbols.

valine residue. Further processing of the Ali-Ali-Xaa tripeptide probably occurs by means of nonspecific proteolysis.

Farnesylated Di- and Tripeptide Substrates of the Protease. The experiments described above show that the protease is stereospecific at the scissile bond and also requires a free carboxyl-terminal group in the tetrapeptide substrate. Substrate specificity was further explored by using the tripeptide AFC-Val-Ile and the dipeptide AFC-Val. The measured K_m and V_{max} values for the tripeptide are $12.6 \pm 0.27 \mu$ M and 65.6 ± 5.7 pmol/min per mg, respectively (Fig. 5). Clearly, the tripeptide is a weaker substrate for the protease than the tetrapeptide. Kinetic constants were not obtained for the dipeptide AFC-Val, but it is clearly a substrate for the protease as well. Stereospecificity is also observed with both the di- and tripeptides because substitution of D-AFC for L-AFC in these molecules led to complete abolition of substrate activity. Finally, AFC amide is not a substrate for

FIG. 3. Dependence of protease activity on pH. The protease activity was determined with 4 μ M *N*-[³H]acetyl-S-farnesyl-L-Cys-L-Val-L-Ile-L-Ser as substrate in the following buffers: sodium acetate (pH 4.5 and 5.5), sodium phosphate (pH 6.0 and 7.0), Hepes (pH 7.4), and Tris (pH 8.0 and 8.7). Incubation time was 30 min. Symbols represent mean values of two determinations, and error bars represent the SEM. Error bars not shown are within symbols.

FIG. 4. Formation of L-Val-Ile-[^3H]Ser from AFC-L-Val-L-Ile-L-[^3H]Ser. AFC-L-Val-L-Ile-L-[^3H]Ser (10 μM , 500 mCi/mmol) was incubated with bovine liver microsomal membranes at 37°C for 2 hr (A). The reaction was terminated with 200 μl of chloroform/methanol, 1:1 (vol/vol) and 200 μl of water. The aqueous layer was lyophilized and resuspended in 50 μl of phosphate buffer, pH 5.5. *o*-Phthalaldehyde/2-mercaptoethanol reagent (200 μl) was added, and the mixture was thoroughly mixed for 2 min. Standard *o*-phthalaldehyde/2-mercaptoethanol derivatives of L-serine, L-Ile-L-Ser, and L-Val-L-Leu-L-Ser (50 μl) were also added and are denoted as S, IS, and VIS, respectively. The resulting mixture (300 μl) was injected into the reverse-phase HPLC system (Rainin, C_{18} , 10.5 $\times$ 1 cm, UV detection at 254 nm, and flow rate 2.5 ml/min), starting the linear gradient at the same time. The gradient mixture for reservoir I was 0.05 M sodium phosphate buffer, pH 5.5/methanol, 4:1 (vol/vol) and for reservoir II was buffer/methanol, 1:4. Elution was 0–10% B in 10 min, 10–100% B in 25 min, 100–0% B in 10 min, and 10-min reequilibration. Each fraction (2.5 ml) was collected and counted in scintillation fluid (7.5 ml) by using a Beckman LS-1800 radioactivity counter. (Upper) HPLC tracings. (Lower) Radioactivity associated with the different fractions. The lyophilized aqueous layer from the reaction was incubated again with bovine liver microsomal membranes at 37°C for 2 hr. The reaction was terminated, and the products were derivitized and analyzed as in A. Results of the experiment are shown in B.

the enzyme, demonstrating that a second amino acid moiety is required, in addition to AFC, in the substrate.

As expected, the proteolysis of [^3H]AFC-Val-Ile-Ser was inhibited by the dipeptide substrate (AFC-Val) and by the tripeptide substrate (AFC-Val-Ile). Incubation of 4 μM substrate along with either 20 μM of the dipeptide or tripeptide led to an $\approx 50\%$ diminution in substrate cleavage. Interestingly, deletion of the farnesyl moiety from the tetrapeptide substrate to yield *N*-acetyl-L-Cys-Val-Ile-Ser did not produce an inhibitor of the enzyme. Incubation of the tetrapeptide substrate with a 5-fold excess of *N*-acetyl-L-Cys-Val-Ile-Ser did not interfere with substrate processing. This result strongly suggests that the isoprenyl moiety is important in enabling the substrate to bind to the enzyme.

DISCUSSION

Four separate enzymatic activities comprise the isoprenylation pathway. The soluble isoprenyl transferases have been purified and characterized (16, 17). The membrane-associated methyltransferase(s) has been assayed in a variety of tissues, and a considerable amount is already known about

its specificity for substrates and inhibitors (33). The existence of a specific methyltransferase has been inferred from studies on the hydrolysis of methylated G proteins and the hydrolysis of synthetic isoprenylated substrates (23). Of the reactions in the isoprenylation pathway, only the proteolytic activity remained unidentified until the current work. This activity is critical in the pathway, and its absence leads to isoprenylated proteins unable to associate efficiently with membranes (18).

In principle, the proteolysis could proceed by several different routes. A direct cleavage after the isoprenylated cysteine residue to split off the terminal tripeptide is the most straightforward route. Several other mechanisms involving various modes of sequential cleavage reactions could also be imagined. All that is required in these latter mechanisms is that proteolytic cleavage cease at the isoprenylated cysteine residue. In this article, a specific membrane-bound activity is found with characteristics that suggest it represents the protease central to the isoprenylation pathway.

In the experiments described here, it is shown that a simple tetrapeptide 1 modeled after the carboxyl terminus of transducin γ subunit serves as a specific substrate for a microsomal protease from both dog pancreas and bovine liver. Dog

FIG. 5. Protease activity with *N*-[³H]acetyl-*S*-farnesyl-L-Cys-L-Val-L-Ile as substrate. Michaelis-Menten and Eadie-Hofstee plots (Inset) of the formation of [³H]AFC as a function of [³H]AFC-L-Val-L-Ile (AFCVI) concentration. Symbols represent mean values of two determinations, and error bars represent the SEM. Error bars not shown are within symbols.

pancreas microsomes have already been shown to specifically cleave farnesylated, but nonproteolyzed, ras protein to the mature protein (18). The V_{max} and K_m values for tetrapeptide with the calf liver microsomes are 5.8 μ M and 0.251 nmol/min per mg of protein, respectively. The protease specifically cleaves the tetrapeptide between the farnesylated cysteine and the adjacent valine residue to produce AFC and the tripeptide Val-Ile-Ser. Upon extended incubation, further hydrolysis of the tripeptide occurs. This further proteolysis is presumably due to nonspecific microsomal proteases. Because the specific proteolytic cleavage of the tetrapeptide occurs at the AFC moiety, the reaction would be expected to be sensitive to the stereochemistry at the modified cysteine residue. Indeed, the proteolytic cleavage reaction is stereospecific because the tetrapeptide with a D-AFC residue substituting for the L-cysteine form is not processed. A free carboxyl terminus is also required because the methyl ester of the tetrapeptide substrate is not hydrolyzed.

In addition, the tripeptide AFC-Val-Ile and the dipeptide AFC-Val were also hydrolyzed by the protease. The tripeptide was cleaved to generate AFC and the dipeptide Val-Ile. This result is consistent with the nature of the cleavage in the tetrapeptide substrate. The dipeptide and tripeptide substrates also inhibit the protease-mediated cleavage of the tetrapeptide, showing that the same activity is involved in the processing of the different substrates. Interestingly, a 5-fold excess of the nonisoprenylated tetrapeptide *N*-acetyl-L-Cys-Val-Ile-Ser did not inhibit the proteolytic cleavage of [³H]AFC-Val-Ile-Ser. This result strongly suggests that the isoprenyl group is required for substrate activity—an expected result.

The limited structure-activity studies done so far suggest that the isoprenylated cysteine residue is an important determinant of substrate activity. The fact that AFC-Val-Ile-Ser, AFC-Val-Ile, and AFC-Val are substrates for the enzyme, although the latter two peptides are weaker substrates than the tetrapeptide, suggests that the nature of the amino acid residues attached to the modified cysteine residue is not critical. This conclusion is, perhaps, to be expected, because the protease(s) presumably must process many different isoprenylated proteins, with diverse CAAX sequences at their carboxyl termini. That different proteases exist to process the large (>100) and expanding number of distinct isoprenylated proteins is unlikely. Along these lines, it is interesting to note that the CAAX sequence of the tetrapeptide substrate used was derived from retinal transducin,

which would not normally be processed by liver or pancreatic microsomes. However, further structure/activity studies will decide whether quantitative differences exist among different peptides with respect to substrate activity. It will also be interesting to determine whether specificity is observed for an all-*trans*-farnesyl moiety versus an all-*trans*-geranylgeranyl moiety. Finally, the finding that simple isoprenylated peptides serve as substrates for the protease suggests that the design of specific inhibitors of the enzyme may be relatively straightforward.

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