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Mammalian microsomal and soluble Ras-processing peptidase activities are distinct

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Abstract Microsomal and soluble peptidases from bovine liver and pig brain hydrolyze the farnesylated, Ras-based CAAX peptide [^{3}H]Ac-fCVIM-*OH*. However, they differ in their sensitivity to substrate-based inhibitors, sulfhydryl and chelating agents, pH and ionic strength optima, and stability. The microsomal activity was exquisitely sensitive to the substrate-based inhibitor Boc-fC[CH_2]VIM-*OH*, moderately sensitive to the sulfhydryl agent *p*CMB, but insensitive to NEM and the metal-chelating agent *o*-phenanthroline. The soluble activity was insensitive to Boc-fC[CH_2]VIM-*OH*, but very sensitive to *p*CMB, NEM and *o*-phenanthroline, suggesting it to be the previously reported (Biochem. Biophys. Res. Commun. 198, 787–794 (1994)) zinc metallopeptidase. The microsomal activity is most likely to be a cysteine peptidase involved in the post-translational processing of Ras proteins.

Key words: Ras protein; Ras-processing peptidase; Ras-based inhibitor

1. Introduction

Ras proteins function as molecular switches in the transduction of extracellular signals from tyrosine kinases, associated with the membrane, to serine/threonine kinases in the cytoplasm [1–3]. The Ras-dependent signal transduction pathways control cell proliferation and differentiation depending on the cell context [4–8]. Ras proteins cycle between an active, GTP-bound form and an inactive, GDP-bound form and are oncogenic when constitutively activated; in its oncogenic, mutated form, Ras is found in 50% of colorectal and 95% of pancreatic human cancers [9]. Critical to Ras function is its processing at the C-terminal Cys-aliphatic-aliphatic-any amino acid sequence (CAAX box). Processing involves farnesylation at the Cys thiol group, removal of the AAX tripeptide by a specific peptidase, and methylation of the exposed Cys carboxyl group [10,11]. Farnesylation is the key reaction for the membrane association of Ras and its ability to transform cells [12,13]. Current research has focused on this reaction, and potent, selective inhibitors of farnesyltransferase are under development [14].

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Abbreviations: AcO, acetate; Ac, acetyl; Boc, *t*-butyloxycarbonyl; DMSO, dimethylsulfoxide; DTT, dithiothreitol; f, farnesyl; [^{3}H]Ac-fCVIM-*OH*, N^{α}-[^{3}H]acetyl-Cys(*S*-farnesyl)-Val-Ile-Met-OH; HPLC, high-pressure liquid chromatography; *I*, ionic strength; MES, 2-[*N*-morpholino]ethanesulfonic acid; NEM, *N*-methylmaleimide; *p*CMB, *p*-chloromercuribenzoate; PMSF, phenylmethylsulfonyl fluoride; TEA, triethanolamine

Recently, two enzymes that cleave AAX from farnesyl-CAAX-containing peptides have been reported. The first was from bovine liver microsomes and cleaved the tetrapeptide [^{3}H]Ac-fCVIM-*OH* [15,16], while the second was from pig brain and cleaved the heptapeptide propionyl-GSP-fC-[^{3}H]VLM-*OH* [17]. The pig brain enzyme was a thiol-dependent zinc metallopeptidase, based on its inhibitor profile, and could be solubilized by freezing and thawing the microsomes.

It was of interest to determine whether the soluble metallopeptidase could also hydrolyze [^{3}H]Ac-fCVIM and be inhibited by Boc-fC[CH_2]VIM-*OH*, a potent substrate-based inhibitor of the microsomal enzyme [18]. We present evidence suggesting that the soluble metallopeptidase has a different inhibitor profile, pH and ionic strength optima than the microsomal peptidase activity, and the latter is most likely a cysteine, Ras-processing peptidase.

2. Materials and methods

2.1. Materials

Bovine liver and pig brains were obtained from Pel-Freeze (Rogers, AR). DTT, HEPES, NEM, PMSF, *p*CMB, and farnesol were obtained from Sigma (St. Louis, MO). [^{3}H]Ac-fCVIM-*OH* (spec. act. 2.2 μCi/nmol) was synthesized by Dr. Satish Choudry of the Radioisotope Synthesis group and Boc-fC[CH_2]VIM-*OH* [18] by Dr. Steve Ferguson of the Oncology department. Hydroxyapatite (CHTII), methyl HIC, Affigel 501 (an organomercurial agarose gel), and DC protein assay kit were from BioRad (Richmond, CA). Dihydroxyboryl-*m*-phenyl-matrix gel (PBA-60) for boronate affinity column, and dye matrix affinity column kit (Blue A, Red A, Orange A, Green A, Blue A) were from Amicon (Beverly, MA). HiTrap HIC test kit (phenyl Sepharose and octyl Sepharose) were from Pharmacia (Piscataway, NJ). Dynamax 60A, 8 μm silica gel column (4.3×250 mm) and guard column were from Rainin Instruments (Woburn, MA). Waters 600E HPLC and WISP 710B were from Millipore Corp. (Bedford, MA). FLO-ONE for Windows Radio-HPLC workstation software package, IBM PC, Beta Flo-one detector and Ultima M scintillation cocktail were from Packard Instrument Co. (Meriden, CT). All solvents (analytical/HPLC grade) were from Fisher Scientific (Pittsburgh, PA).

2.2. Preparation of bovine liver and pig brain microsomes

Bovine liver microsomes were prepared by the method of Ma et al. [16] and frozen in aliquots at −70°C. Pig brain microsomes were prepared according to Akopyan et al. [17] with the following modifications. Pig brain (30 g) was minced in 3 vols. of 50 mM TEA, pH 7.5, containing 250 mM sucrose, 50 mM potassium acetate, 6 mM magnesium acetate, 0.02% sodium azide, 0.1 mM DTT, 0.5 mM PMSF, 10 μM leupeptin, and 0.05 units/ml aprotinin. The resuspension buffer was 25 mM TEA, pH 7.2, 0.1 mM DTT, 0.5 mM $ZnCl_2$, 0.02% sodium azide, 0.5 mM PMSF, 10 μM leupeptin, and 0.05 units/ml aprotinin. The ultracentrifugation step did not include a sucrose cushion.

2.3. Solubilization of microsomal peptidase activity by freeze-thawing

Soluble peptidase activity was prepared from microsomes by the method of Akopyan et al. [17]. The frozen microsomes were thawed by swirling in a 37°C bath, cooled by stirring in an ice bath, set on ice

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for 1 h, and centrifuged at 100 000 × *g* for 4 h (4°C). The resulting supernatant was used immediately in peptidase activity assays.

2.4. Peptidase activity assay

Incubation mixtures contained in a total volume of 50 μl: 25 pmol of [^{3}H]Ac-fCVIM-*OH* in 2 μl of DMSO, 5 μg of peptidase preparation, test compound (10-fold concentrated solution in 100% DMSO), and assay buffer (200 mM HEPES pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$). Reactions were initiated with the addition of [^{3}H]Ac-fCVIM-*OH* and, after 60 min at 37°C, terminated with the addition of 0.5 ml of chloroform/methanol (1:1, by vol.) followed by 0.5 ml of 1 M citric acid for phase separation. The lower, organic layer was collected, dried in a SpeedVac (Savant Instruments, Farmingdale, NY) at room temperature or 43°C, and analyzed by normal-phase HPLC [16,19,20]. Substrate and product peaks were monitored with an on-line Flo-one Beta radiochromatography detector using automatic data analysis. All assays were performed at least in duplicate.

2.5. pH and ionic strength profiles

For the pH profile, 156 mM MES-156 mM HEPES-300 mM diethanolamine was used as a single buffer (I=0.30) to separate the effects of ionic strength from pH [21]. For the ionic strength profile, the buffer used was 26 mM MES-26 mM HEPES-50 mM diethanolamine, pH 7.2 (I = 0.05), and the ionic strength was varied with KCl. Effects of buffer ions, phosphate, acetate and Tris were examined using 26 mM MES-26 mM HEPES, 50 mM diethanolamine, pH 7.2, and the appropriate buffer ion at 100 mM (pH 7.2), with ionic strength being adjusted to 0.3 with KCl.

3. Results and discussion

Hancock et al. [22] reported that microsomal preparations from canine pancreas removed the terminal three amino acids from Ras in vitro. More recently, Ma and co-workers reported that microsomal preparations from bovine liver removed the intact terminal tripeptide from synthetic, farnesylated tetrapeptide substrates mimicking the C-terminus of Ras [15,16] and achieved a modest, partial purification of the microsomal enzyme [23]. We also used radiolabeled, farnesylated peptides to assay proteolytic activity and infer potential Ras-processing peptidase.

Because microsomal Ras protease activity from bovine liver was not amenable to purification by conventional methods, we became interested in the freeze-thaw technique of Akyopan et al. [17] that released a soluble peptidase activity from pig brain microsomes resembling endopeptidase EC 3.4.24.15, a thiol-dependent zinc metallopeptidase. We found that this soluble activity could be further purified on organomercurial affinity column (Hitz and Georgopapadakou, unpublished results). However, its inhibitor profile was different from that of the microsomal activity (Table 1). For example, the microsomal activity was exquisitely sensitive to the farnesylated peptide gap inhibitor Boc-fC[CH_2]VIM-*OH*, relatively resistant to the unfarnesylated CAAX peptides Ac-CVIS-*OH* and Ac-CVIM-*OH*, moderately sensitive to the sulfhydryl agent *p*CMB, and resistant to the metal chelator *o*-phenanthroline. The soluble activity was relatively resistant to Boc-fC[CH_2]VIM-*OH* [18], very sensitive to *p*CMB, and sensitive to *o*-phenanthroline (but not to *m*-phenanthroline ($IC_{50} > 1$ mM), a non-chelating isomer). The soluble activity thus resembled a cytosolic a-factor peptidase from *Saccharomyces cerevisiae* reported previously, although the yeast enzyme was insensitive to sulfhydryl agents [24]. With both soluble and microsomal activities, the main affinity determinant appeared to be the farnesyl group, since farnesylated tetrapeptides were better inhibitors than the corresponding nonfarnesylated tetrapeptides. The specificity of AcfCVIS-*OH* over AcCVIS-*OH* was >250-fold for the microsomal activity but only ~5-fold for the soluble activity suggesting that the former may be more relevant to the removal of the C-terminal tripeptide from farnesylated Ras.

Since Akopyan [17] used pig brain microsomes, the inhibitor specificity of the soluble and microsomal peptidase activities from that source were also compared (Table 1). As with bovine liver, the soluble activity was resistant to inhibition by Boc-fC[CH_2]VIM-*OH* while the microsomal activity was exquisitely sensitive. Accordingly, the specificity of farnesylated over non-farnesylated tetrapeptides (AcfCVIS-*OH* vs. Ac-CVIS-*OH*) was ~2-fold with the soluble but ≥21-fold for the microsomal peptidase activity. As previously reported [17], the soluble activity was sensitive to *o*-phenanthroline. When the preincubation time with *o*-phenanthroline was increased to 60 min at room temperature, the IC_{50} decreased from 300 μM to < 100 μM. In other systems, inhibition by EDTA has also been reported to be time dependent [25], although in the present study this chelator did not inhibit soluble peptidase activity up to 1 mM. It is noted that the metalloprotease carboxypeptidase B, though sensitive to *o*-phenanthroline, is also resistant to EDTA [26]. As with bovine liver, the soluble peptidase activity from pig brain was more sensitive to the sulfhydryl agents *p*CMB and NEM than was the microsomal activity.

The two peptidase activities from pig brain were next char-

Table 1
Inhibitor profiles of soluble and microsomal bovine liver and pig brain peptidase activities using [^{3}H]Ac-fCVIM-OH as substrate

Compound	IC_{50} (μM)					
	Bovine liver peptidase			Pig brain peptidase		
	Soluble	Microsomal		Soluble	Microsomal	
Boc-fC[CH_2]-VIM-*OH*	>100[a]	0.003	(0.086)	variable[a]	NA[b]	0.0047
AcfCVIS-*OH*	10[c]	4[c]	(6[d])	140	(30 000[e])	10
AcfCVIM-*OH*	NA	0.5[f]	(3[d])	NA	NA	NA
AcCVIS-*OH*	55	>1000	NA	103	NA	≥210
AcCVIM-*OH*	112	500	NA	79	(<300[g])	192
Farnesol	NA	30	NA	>100	NA	>100
o-Phenanthroline	470	>1000	(>600)	300	(100)	>1000
*p*CMB	2	10	(500[h])	6	(<10)	38
NEM	NA	500	NA	26	(<100)	>1000

Values represent averages from at least two separate experiments, each involving duplicate assays. Literature values for microsomal bovine liver peptidase [18,19] and soluble pig brain peptidase [17] are shown in parentheses.
[a]Biphasic inhibition curve; [b]NA, data not available; [c]K_i; [d]K_m; [e]K_m of propionyl-GSPfCVLM-*OH*; [f]K_i using Ac-[^{3}H]fCVIS-*OH* as substrate [19]; [g]inhibition of a soluble yeast a-factor protease by unfarnesylated a-factor [22]; [h]IC_{50} for *p*-hydroxymercuribenzoate.

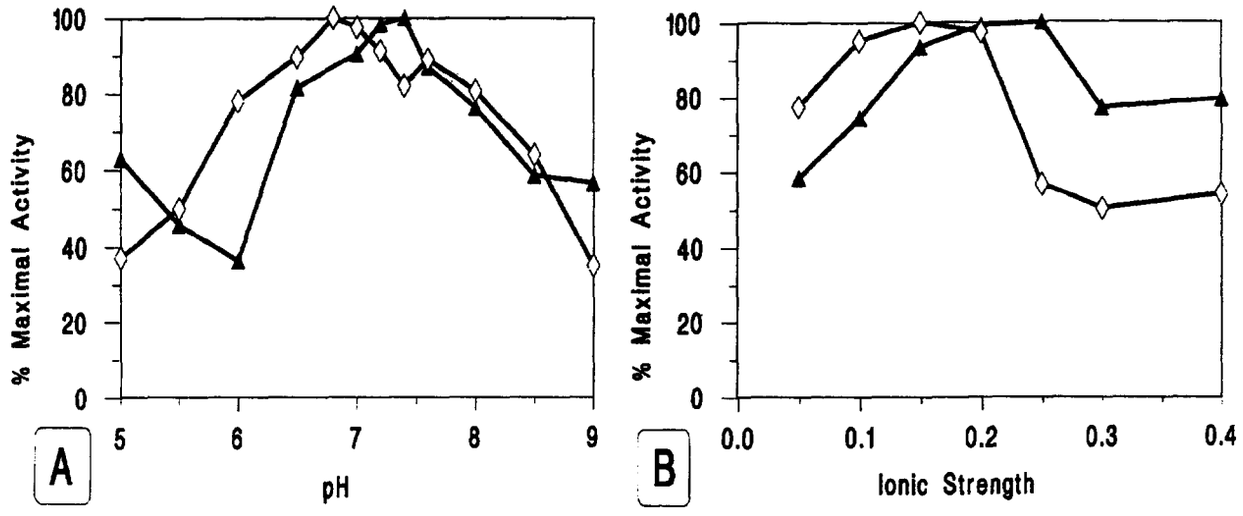

Fig. 1. pH (A) and ionic strength (B) profiles of pig brain microsomal (◇) and soluble (▲) peptidase preparations. In (A), ionic strength was kept constant as described in Section 2. In (B), ionic strength was varied with KCl. Points represent averages values from two separate experiments, each involving duplicate assays.

acterized with respect to pH and ionic strength optima (Fig. 1). For the soluble activity, the pH optimum was 7.2–7.4 (Fig. 1A), similar to that reported for the soluble peptidase from pig brain [17] and rat brain [27]. The microsomal activity reproducibly exhibited two maxima, at pH 6.8 and 7.6, which differ from the single maximum at pH 7.0 reported for the microsomal activity from bovine liver [15]. Interestingly, while the microsomal activity was not inhibited by any of the buffers used, the soluble activity was inhibited by the potentially chelating phosphate and acetate buffers (IC_{50} 100 mM), consistent with it being a metallopeptidase. The ionic strength optima for the two peptidase activities were also reproducible distinct, the soluble activity showing a maximum at higher ionic strength than the microsomal activity (Fig. 1B).

The detergent solubilized [23], microsomal activity was unstable during most chromatography steps (boronate, hydroxyapatite, dye affinity, methyl- phenyl- and octyl-hydrophobic interaction chromatography, and organomercurial affinity columns), resulting in low activity yields (Hitz and Georgopapadakou, unpublished results).

In summary, we have compared a microsomal and soluble peptidase activity from bovine liver and pig brain and found them to be distinct, and the microsomal activity to be most likely the relevant, Ras-processing peptidase. It should be noted that the microsomal activity may represent more than one isozyme [28]. Validation of the Ras-processing activity of the microsomal peptidase must await in vitro and in vivo studies with appropriately radiolabeled, farnesylated Ras.

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