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c-Ha-*ras* gene products are potent inhibitors of cathepsins B and L

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c-Ha-*ras* proteins produced by *Escherichia coli* inhibited the activities of cathepsins B and L which had been partially purified from rat kidney. Furthermore, amino acid sequence homology between c-Ha-*ras* proteins and thiol proteinase inhibitors has been found.

c-Ha-*ras* gene; Thiol proteinase; Cathepsin B; Cathepsin L; Cystatin

1. INTRODUCTION

Both c-Ha-*ras* proteins and cAMP-dependent protein kinase have been suggested to be involved in initiation of two-stage carcinogenesis [1–4]. Recently, we have found that the proteolytic degradation of cAMP-binding proteins is effectively inhibited by c-Ha-*ras* proteins and that the degradation of cAMP-binding proteins can possibly be carried out by thiol proteinases because it is inhibited by leupeptin and antipain (in preparation). In the present paper, we report that the activities of cathepsins B and L were effectively inhibited by c-Ha-*ras* gene products. Amino acid sequence homology between c-Ha-*ras* proteins and thiol proteinase inhibitors also supports the above results.

2. MATERIALS AND METHODS

2.1. *Purification of* ras *gene products*

Normal human and mutated Ha-*ras* proteins (p21s) were produced in *Escherichia coli* with plasmids bearing chemically synthesized *ras* genes. p21(Gly-12) and p21(Val-12) are truncated p21s (Met-1 to Leu-171) and have glycine and valine, respectively, at position 12. Purification of p21s was carried out according to the method of Gibbs et al. [5,6] and described in [7].

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2.2. *Assay for cathepsin activity*

Assay for cathepsin activity was carried out according to the method of Barrett and Kirschke [8]. Benzyloxycarbonyl-arginyl-arginine 4-methyl-7-coumarylamide (Z-Arg-Arg-NMec) and benzyloxycarbonyl-phenylalanyl-arginine 4-methyl-7-coumarylamide (Z-Phe-Arg-NMec) were obtained from Peptide Institute Inc. (Osaka, Japan) and used as substrates for cathepsins B and L, respectively.

2.3. *Partial purification of cathepsins*

Cathepsins L and B were partially purified from rat kidney according to the method of Barrett and Kirschke [8–10]. A 20–65%-saturation-$(NH_4)_2SO_4$ fraction of the homogenate was applied to a column of CM-Sephadex C-50 pre-equilibrated in buffer A (20 mM sodium phosphate buffer, pH

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5.8, 1 mM EDTA). After washing with buffer A, the column was eluted with 0.2 M KCl in buffer A followed by 0.6 M KCl in buffer A. The 0.6 M KCl fraction contains cathepsin L [8–10], and was used as a cathepsin L fraction. The purity of cathepsin L was approx. 2%. The 0.2 M KCl fraction which contains cathepsins B and H was then applied to a column of DEAE-cellulose (DE52, Whatman) pre-equilibrated in buffer B (20 mM sodium phosphate buffer, pH 6.0, 1 mM EDTA). The unadsorbed fraction contains cathepsin H [10]. After washing with buffer B, cathepsin B was eluted at approx. 0.05 M NaCl. The active fractions were pooled and used as a cathepsin B fraction. The purity of cathepsin B was higher than 40%.

3. RESULTS AND DISCUSSION

We used truncated p21s instead of full-length proteins because the carboxy-terminal region of p21 varies among Ha-*ras*, Ki-*ras* and N-*ras* gene products and seems to have no essential role except anchoring p21 to the plasma membrane [11–13].

The activities of partially purified cathepsins B and L were inhibited by p21s. Lineweaver-Burk plots showed that p21s inhibited cathepsins B and L non-competitively (fig.1). K_i values of p21s for cathepsins B and L were 64 and 22 nM, respectively. No significant difference of the inhibitory activity between p21(Gly-12) and p21(Val-12) was observed (fig.1). The inhibitory activity for cathepsin H was very weak (K_i > 300 nM). p21s did not

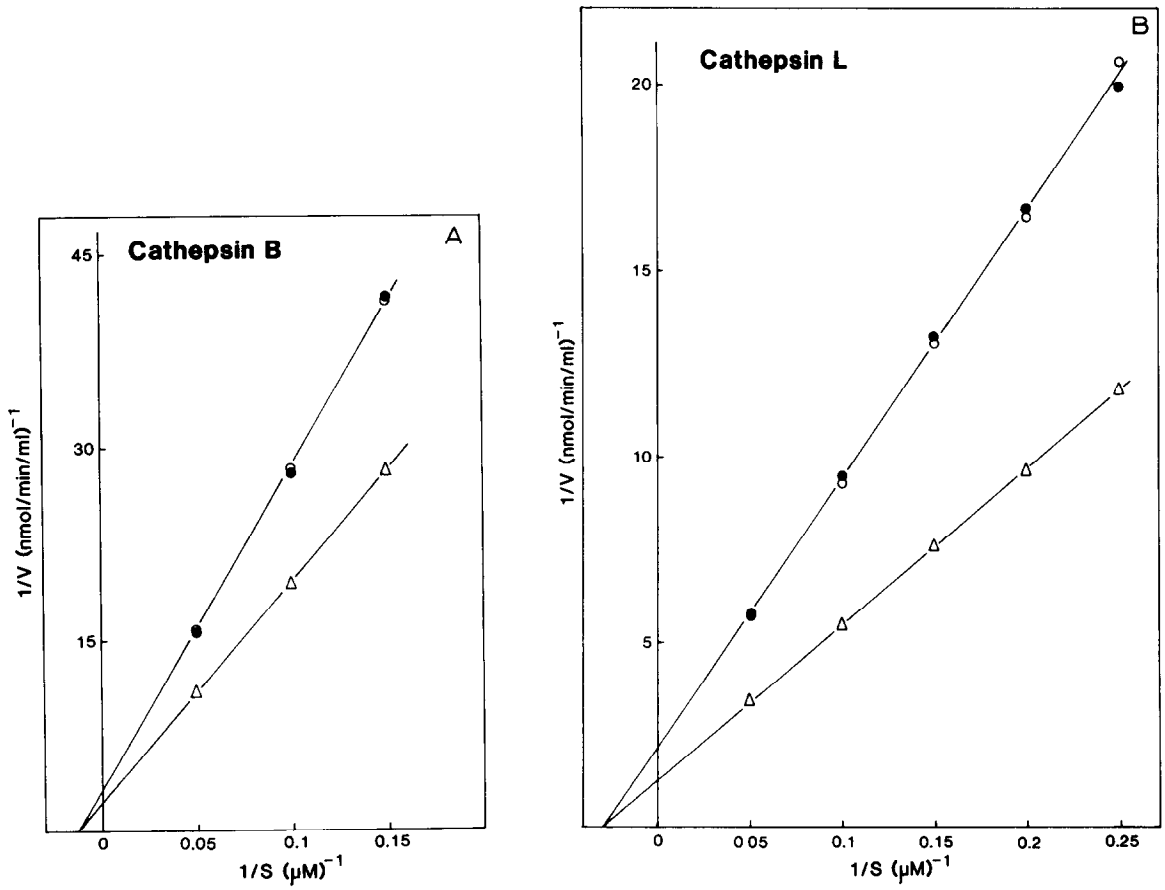

Fig.1. Kinetic analysis of the reaction of cathepsins B and L with p21s. (A) Lineweaver-Burk plots of Z-Arg-Arg-NMec concentration against cathepsin B activity in the presence (○,●) or absence (△) of p21. (○) p21(Gly-12), 0.6 μg/ml; (●) p21(Val-12), 0.6 μg/ml. (B) Lineweaver-Burk plots of Z-Phe-Arg-NMec concentration against cathepsin L activity in the presence (○,●) or absence (△) of p21. (○) p21(Gly-12), 0.3 μg/ml; (●) p21(Val-12), 0.3 μg/ml.

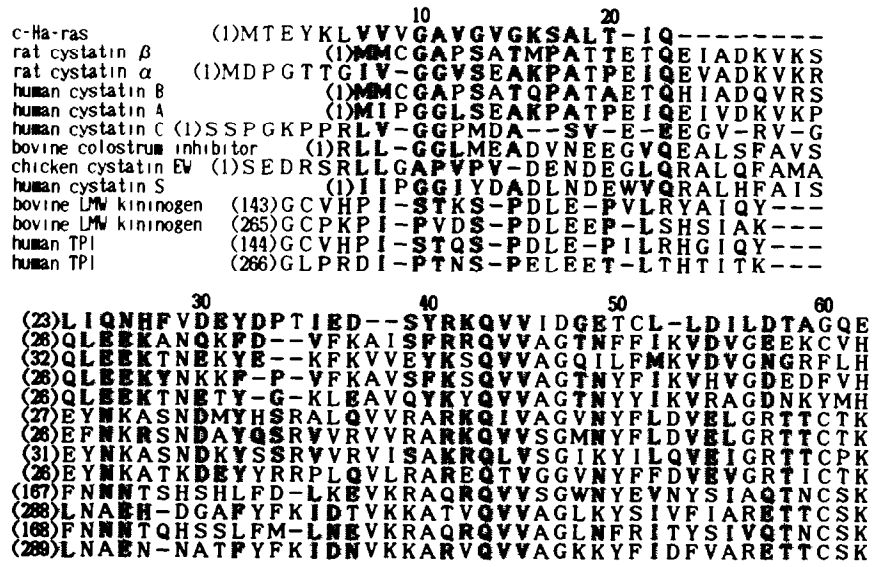

Fig.2. Comparison of amino acid sequences of c-Ha-*ras* (p21(Val-12)) with cystatins, including rat cystatin β [14], rat cystatin α [15], human cystatin B [16], human cystatin A [16], human cystatin C [17], bovine colostrum inhibitor [18], chicken cystatin EW [19,20], human cystatin S [21], bovine low-M_r kininogen [22], and human TPI [23]. The following Dayhoff conservative categories [31] were used: C (Cys); A (Ala), G (Gly), P (Pro), S (Ser), and T (Thr); R (Arg), H (His), and K (Lys); N (Asn), D (Asp), Q (Gln), and E (Glu); I (Ile), L (Leu), M (Met), and V (Val); F (Phe), W (Trp), and Y (Tyr). Identical or analogous residues are shaded. Numbers in parentheses represent the amino acid position of the beginning of each protein. Gaps, shown by dashes, were introduced for optimal alignment.

significantly inhibit the activity of trypsin or *Staphylococcus* V8 protease (not shown).

Cystatin is a well-known thiol proteinase inhibitor. Many kinds of cystatins have been reported [14–23]. The amino acid sequence between positions 24 and 59 of c-Ha-*ras* is highly homologous to those of cystatins (fig.2). This region is 61% homologous (19% identical and 42% conservative substitutions) with that of rat cystatin β. The region between positions 41 and 45, -$^{Arg}_{Lys}$-$^{Lys}_{Arg}$-Gln-Val-Val-, is highly conserved among the cystatin superfamily and thought to be a functional domain. Although the region between positions 25 and 46 of p21 had no homology with guanine nucleotide-binding proteins such as transducin, elongation factor-2, elongation factor-Tu, etc. [24], this region is highly conserved among the *ras* gene family [11,12]. Moreover, it has been suggested that this region is indispensable for the transforming activity of p21 [25,26]. Therefore, it is plausible that thiol proteinase-inhibitor activity of p21 may be common among the *ras* gene family and play an important role in its cellular functions. The region between positions 7 and 22 of c-Ha-*ras* also has some homology with cystatins (fig.2).

In general, K_i values of cystatin for cathepsins L and H are much lower than that for cathepsin B [27,28], while K_i of p21 for cathepsin B (64 nM) is slightly higher than that of p21 for cathepsin L (22 nM) or that of cystatin for cathepsin B (15 nM [29], 16 nM [30]).

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