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Degradation of epidermal growth factor receptors by cathepsin L-like protease: inhibition of the degradation by c-Ha-*ras* gene products

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Extract of NIH3T3 mouse fibroblasts contains a protease which can cleave epidermal growth factor receptor (EGF receptor). This protease was tentatively named cathepsin X and purified to near homogeneity. The characteristics of cathepsin X were similar to those of cathepsin L and the proteolytic activity of cathepsin X was inhibited by c-Ha-*ras* gene products.

c-Ha-*ras*; Cathepsin L; EGF receptor

1. INTRODUCTION

During the course of investigation on cAMP-dependent protein kinases [1,2], we have found that c-Ha-*ras* gene products (p21s) produced by *Escherichia coli* have cysteine proteinase-inhibitor activity [3,4]. Although p21s have been found to suppress the proteolytic degradation of a cAMP-binding protein [3], it is possible that p21s can inhibit the degradation of other proteins which are more closely related to cell growth. First of all, the degradation of EGF receptor was investigated by purifying a protease which can cleave EGF receptor followed by examining whether the proteolytic activity of the protease can be inhibited by p21s.

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Abbreviations: p21, a c-Ha-*ras* gene product; EGF receptor, epidermal growth factor receptor; Z-Phe-Arg-NMec, benzyloxycarbonyl-phenylalanyl-arginine 4-methyl-7-coumarylamide; Z-Arg-Arg-NMec, benzyloxycarbonyl-arginyl-arginine 4-methyl-7-coumarylamide; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; EGTA, [ethylenebis(oxyethylenenitrilo)]-tetraacetic acid; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; E-64, *N*-[*N*-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]agmatine; TLCK, *p*-tosyl-L-lysine chloromethylketone; TPCK, *p*-tosyl-L-phenylalanine chloromethylketone

2. MATERIALS AND METHODS

2.1. *Preparation of p21s and cathepsin L*

p21s were produced in *Escherichia coli* with plasmids bearing human c-Ha-*ras* genes and purified as described previously [3–5]. p21(G,171) and p21(V,171) are truncated p21s of ^{1}Met to ^{171}Leu and have glycine and valine, respectively, at position 12. Cathepsin L was purified from rat kidney according to [6].

2.2. *Immunoprecipitation of EGF-receptor*

A431 cells grown on a 100-mm dish were labeled with [^{35}S]methionine and [^{35}S]cysteine for 16 h at a concentration of 0.2 mCi/ml. Cell extract was prepared with buffer A (0.5% Nonidet P-40, 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF)) as described [7] and incubated at 37°C for 30 min with an appropriate amount of the protease fraction. EGF receptors were immunoprecipitated with anti-EGF receptor monoclonal antibody (Transformation Research Inc., Framingham, MA) as described previously [8]. The solubilized materials were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [9].

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2.3. *Purification of cathepsin X*

Cathepsin X was purified from NIH3T3 mouse fibroblasts as follows. Approximately 1×10^9 cells were harvested and washed 4 times with phosphate-buffered saline. The cell pellet was mixed with 15 ml of buffer A, incubated at 0°C for 10 min, and centrifuged at $10000 \times g$ for 10 min. The supernatant was dialyzed against buffer B (20 mM sodium acetate, pH 5.0, 50 mM NaCl, 1 mM EDTA and 5 mM β-mercaptoethanol) and applied to a column (2.5 cm × 30 cm) of CM-Sephadex C-50 pre-equilibrated in the same buffer. After washing with buffer B, the column was eluted with a linear gradient (0.05 to 0.5 M) of NaCl in buffer B. Cathepsin X was eluted at 0.1 to 0.3 M NaCl concentration. The active fractions were concentrated with ultrafiltration (Amicon, PM10), dialyzed against buffer C (20 mM sodium phosphate buffer, pH 6.0, 1 mM EDTA and 1.4 mM β-mercaptoethanol), and applied to a column (1.5 cm × 15 cm) of DEAE-cellulose (DE52, Whatman) pre-equilibrated in buffer C. After washing with buffer C, the column was eluted with a linear gradient (0–0.4 M) of NaCl in buffer C (2 × 100 ml). Cathepsin X was eluted at approx. 0.05 M NaCl. The active fractions were directly applied to a column (1.2 cm × 8 cm) of hydroxyapatite (Nakarai Chemicals Co. Ltd, Osaka, Japan). After washing with buffer C, the column was eluted with a linear gradient (20–500 mM) of sodium phosphate buffer (pH 6.0) in the presence of 1 mM EDTA and 1.4 mM β-mercaptoethanol. Cathepsin X was eluted at a phosphate concentration of approx. 350 mM. The active fractions were combined, concentrated with ultrafiltration, dialyzed against buffer C, and further purified with AcA44 (LKB) gel filtration. The activity of cathepsin X to cleave the 150-kDa EGF-receptor was investigated by treatment of A431 cell extract with cathepsin X followed by immunoprecipitation of EGF receptor as described above.

2.4. *Immunoblot analysis*

Immunoblot analysis was carried out according to [10]. Cathepsin X and rat cathepsin L were subjected to SDS-PAGE (11% acrylamide), transferred to a nitrocellulose filter, and probed with rabbit anti-rat cathepsin L antibody [6]. The filter was incubated with goat anti-rabbit IgG antibody coupled with horseradish peroxidase and then incubated with the 4-chloro-1-naphthol substrate.

2.5. *Assay for the proteolytic activity of cathepsin X*

The activity of cathepsin X to cleave the synthetic substrate, benzyloxycarbonyl-phenylalanyl-arginine 4-methyl-7-coumarylamide (Z-Phe-Arg-NMec) was measured in the absence or presence of p21(G,171) or p21(V,171) as described [3,4,11]. The activities of each protease were adjusted to 0.2 mU (one unit of enzyme activity is defined as the amount of enzyme necessary to release 1 μmol of 7-amino-4-methylcoumarin per min at 37°C) and the incubation was carried out at 37°C for 10 min.

3. RESULTS

Human epidermoid carcinoma A431 cells overexpress EGF receptor [12,13]. A typical 170-kDa/150-kDa doublet of EGF receptors was observed after immunoprecipitation of A431 cell extracts with anti-EGF receptor antibody (fig.1, lane 1). NIH3T3 mouse fibroblasts contain high activity to cleave the 150-kDa EGF receptor at acidic conditions. This EGF receptor-cleaving proteinase was tentatively named 'cathepsin X', which binds CM-Sephadex and is eluted with 0.1 to 0.2 M NaCl (not shown). The elution profile of cathepsin X from CM-Sephadex was similar to that of rat cathepsin L [6]. Cathepsin X was further purified by the methods of DEAE-cellulose and hydroxyapatite column chromatography and AcA44 gel filtration.

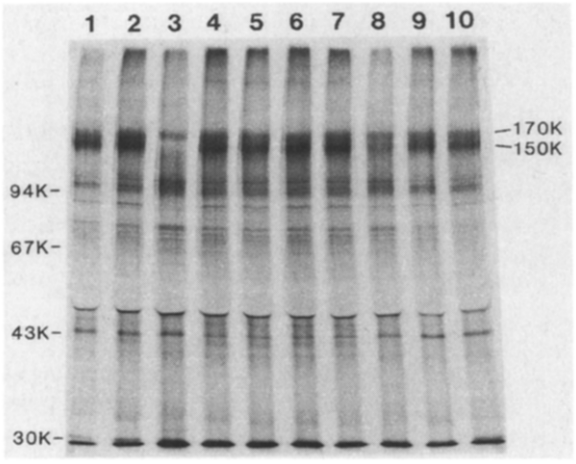

Fig.1. Cleavage of EGF-receptor by cathepsin X. A431 cell extracts were incubated at 30°C for 30 min (lanes 2–10) in the presence of 1 mU cathepsin X (lanes 3–10) and various inhibitors (lanes 4–10). The inhibitors and their concentrations were as follows: lane 4, E-64, 10 μM; lane 5, antipain, 10 μM; lane 6, leupeptin, 10 μM; lane 7, TLCK, 10 μM; lane 8, TPCK, 10 μM; lane 9, p21(G,171), 50 μg/ml; lane 10, p21(V,171), 50 μg/ml. The extract without incubation is shown in lane 1. Other conditions of incubation and immunoprecipitation were carried out. The positions of molecular mass markers and two types of EGF receptors are shown on the left and right, respectively.

The purity of the AcA44-purified cathepsin X was higher than 95% and the molecular mass of the major band was 31 kDa (fig.2a) which is similar to that of the single-chain form of cathepsin L [6]. Anti-rat cathepsin L antibody reacted with the 31-kDa cathepsin X as well as cathepsin L, which showed the 25-kDa heavy-chain form (fig.2b).

The 150-kDa EGF receptor was selectively cleaved by cathepsin X and the cleavage was inhibited by p21s as well as *N*-[*N*-(L-3-*trans*-car-

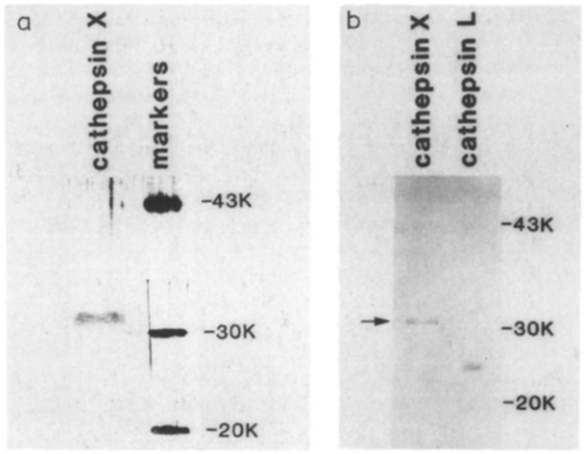

Fig.2. SDS-PAGE and immunoblot analysis of purified cathepsin X. (a) SDS-PAGE of cathepsin X. Cathepsin X purified from NIH3T3 cells was resolved by SDS-PAGE (10% acrylamide), and stained with silver (Silver Staining Reagent, Daiichi Chemicals, Japan). (b) Immunoblot analysis of purified cathepsin X. Cathepsin X and rat cathepsin L were resolved by SDS-PAGE (11% polyacrylamide), transferred to a nitrocellulose filter, and analyzed by immunoblotting for reactivity with anti-rat cathepsin L antiserum.

boxyoxiran-2-carbonyl)-L-leucyl]agmatine (E-64) [14], antipain, leupeptin and *p*-tosyl-L-lysine chloromethylketone (TLCK) (fig.1). *p*-tosyl-L-phenylalanine chloromethylketone (TPCK) inhibited it partially.

Z-Phe-Arg-NMec which is a common substrate for cathepsins B and L [11] was also cleaved by cathepsin X. The cleavage of Z-Phe-Arg-NMec by cathepsin X was inhibited by p21s dose-dependently (fig.3). The inhibition profile of cathepsin X by p21s was comparable to that of cathepsin L. Cathepsin X failed to cleave Z-Arg-Arg-NMec (not shown). These results suggest that cathepsin X is quite similar to cathepsin L.

4. DISCUSSION

Many characteristics of cathepsin X were similar to those of rat cathepsin L; e.g., elution profiles from CM-Sephadex and DEAE-cellulose, a molecular mass of 31 kDa, reaction with anti-rat cathepsin L, cleavage of Z-Phe-Arg-NMec, and inhibition by various protease inhibitors including p21s. Therefore, cathepsin X may be cathepsin L itself or other cathepsin L-related protease such as cathepsin S [15].

Cathepsin X is the major protease which can cleave EGF-receptor and p21s can inhibit cathepsin X-induced degradation of EGF receptor. Taken together, it is possible that p21s can protect EGF receptor against the specific protease. Although the exact situation of the contact among EGF receptor, p21 and cathepsin X has not been elucidated, the contact may take place in vivo, for

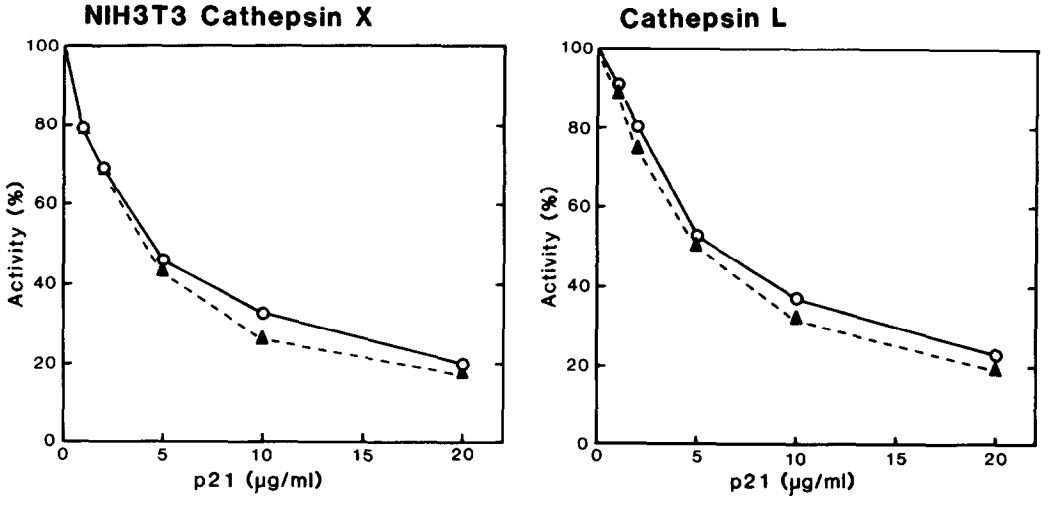

Fig.3. Inhibition of the activity of cathepsin X by p21s. The activities of cathepsins X and L were measured in the presence of varying amounts of p21s. The protease activity in the absence of p21s is expressed as 100%. Each point represents the average value of two assays. (○) p21(G,171); (▲) p21(V,171).

example, at the time of receptor-mediated endocytosis. After EGF receptor binds to EGF, it is rapidly endocytosed and subsequently degraded by lysosomal proteases [16,17]. If the degradation of the internalized EGF receptor is inhibited by p21s in vivo, the stabilized receptor may stimulate cell growth constitutively.

Finally, it is probable that cathepsin L-like protease may be involved in the degradation of other kinds of growth factor receptors and that p21s can generally suppress the down-regulation of many kinds of receptors.

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REFERENCES

[1] Hiwasa, T. and Sakiyama, S. (1986) Biochem. Biophys. Res. Commun. 139, 787–793.
[2] Hiwasa, T., Tanigawara, C. and Sakiyama, S. (1987) Cancer Res. 47, 953–959.
[3] Hiwasa, T., Sakiyama, S., Noguchi, S., Ha, J.-M., Miyazawa, T. and Yokoyama, S. (1987) Biochem. Biophys. Res. Commun. 146, 731–738.
[4] Hiwasa, T., Yokoyama, S., Ha, J.-M., Noguchi, S. and Sakiyama, S. (1987) FEBS Lett. 211, 23–26.
[5] Miura, K., Inoue, Y., Nakamori, H., Iwai, S., Ohtsuka, E., Ikehara, M., Noguchi, S. and Nishimura, S. (1986) Jap. J. Cancer Res. 77, 45–51.
[6] Bando, Y., Kominami, E. and Katunuma, N. (1986) J. Biochem. 100, 35–42.
[7] Hiwasa, T., Fujimura, S. and Sakiyama, S. (1982) Proc. Natl. Acad. Sci. USA 79, 1800–1804.
[8] Sakiyama, S., Nakamura, Y. and Yasuda, S. (1986) Jap. J. Cancer Res. 77, 965–969.
[9] Laemmli, U. (1970) Nature 227, 680–685.
[10] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[11] Barrett, A.J. and Kirschke, H. (1981) Methods Enzymol. 80, 535–561.
[12] Fabricant, R.N., DeLarco, J.E. and Todaro, G.J. (1977) Proc. Natl. Acad. Sci. USA 74, 565–569.
[13] Carpenter, G., King, L.E. and Cohen, S. (1979) J. Biol. Chem. 254, 4884–4891.
[14] Towatari, T., Tanaka, K., Yoshikawa, D. and Katunuma, N. (1978) J. Biochem. 84, 659–671.
[15] Kirschke, H., Ločnikar, P. and Turk, V. (1984) FEBS Lett. 174, 123–127.
[16] Beguinot, L., Lyall, R.M., Willingham, M.C. and Pastan, I. (1984) Proc. Natl. Acad. Sci. USA 81, 2384–2388.
[17] Krupp, M.N., Connolly, D.T. and Lane, M.D. (1982) J. Biol. Chem. 257, 11489–11496.