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Inhibition of cathepsin L-induced degradation of epidermal growth factor receptors by c-Ha-ras gene products

Takaki Hiwasa*, Shigeru Sakiyama*, Shigeyuki Yokoyama**, Jong-Myung Ha**, Junko Fujita**, Shigeru Noguchi***, Yoshiaki Bando°, Eiki Kominami°, and Nobuhiko Katunuma°

*Division of Biochemistry, Chiba Cancer Center Research Institute, 666-2, Nitona-cho, Chiba 280, Japan

**Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan

***Biology Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104, Japan

°Department of Enzyme Chemistry, Institute for Enzyme Research, University of Tokushima, Tokushima, Tokushima 770, Japan

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Summary The inhibitory activities of c-Ha-ras gene products (p21s) toward several cysteine proteinases have been investigated. The activity of cathepsin L was inhibited by p21s most effectively while those of cathepsin B and papain were slightly inhibited by p21s. p21s did not show any inhibitory activity toward cathepsin H. In order to connect the protease-inhibitor activity of p21s with cell growth, the degradation of epidermal growth factor receptors (EGF-receptors) was investigated. EGF-receptors were preferentially cleaved by cathepsin L but not by cathepsin B or H. The cleavage of EGF-receptors by cathepsin L was inhibited by p21s dose-dependently. These results raise the possibility that p21s can suppress the degradation of growth-related proteins such as EGF-receptors and thereby affect cell growth.

Although products of ras gene family have guanine nucleotide-binding activity (1,2) and GTPase activity (3-5), these activities are not always correlated to their transforming activities (6-8).

Abbreviations: p21, a c-Ha-ras gene product; EGF-receptor, epidermal growth factor receptor; kDa, kilodalton; Z-Phe-Arg-NMec, benzyloxycarbonyl-phenylalanyl-arginine 4-methyl-7-coumarylamide; Arg-NMec, arginine 4-methyl-7-coumarylamide; PBS, phosphate-buffered saline; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Ca^{2+}-protease, calcium-dependent cysteine protease.

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This suggests that some additional activities may be possessed by the ras proteins. During the course of investigation on cAMP-dependent protein kinases, We have found the proteinase-inhibitor activity in p21s (9-12). p21s suppressed the proteolytic degradation of a cAMP-binding protein (11) and inhibited the activities of partially purified cathepsins B and L (12). Furthermore, p21s have amino acid-sequence homology with the conserved regions of cystatins which are also cysteine proteinase inhibitors (12). In the present study, we report that EGF-receptors are preferentially degraded by cathepsin L and that the degradation can be suppressed by p21s.

Materials and Methods

Materials p21s were produced in Escherichia coli with plasmids bearing human c-Ha-ras genes and purified by utilizing DEAE-Sephacel and Sephadex G-75 according to the method of Gibbs et al. (6) as described previously (13). Cathepsins B and H were purified from rat liver according to the methods of Towatari et al. (14) and Kirschke et al. (15), respectively. Cathepsin L was purified from rat kidney according to the method of Bando et al. (16). Papain was purchased from Sigma Chemical Co. Ltd. (St. Louis, MO).

Assay for cysteine proteinase activity The activities of cathepsin B, L and H were measured in the absence or presence of p21(G, 171) or p21(V, 171) according to the method of Barrett and Kirschke (11,12,17). The activity of papain was assayed under the same condition as for cathepsin B. The substrates used were Z-Phe-Arg-NMec for cathepsins B and L and papain and Arg-NMec for cathepsin H. 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 µmole of 7-amino-4-methylcoumarin per minute at 37°C) and the incubation was carried out at 37°C for 10 min.

Immunoprecipitation of EGF-receptors Human epidermoid carcinoma 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. After washing cells with phosphate-buffered saline (PBS), cells were mixed with 0.25 ml of buffer A (0.5 % Nonidet P-40, 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM DTT and 1 mM PMSF), incubated at 0°C for 10 min, and centrifuged at 10,000 x g for 10 min. Eight µl of the supernatant were incubated in 100 µl of reaction mixture containing 40 mM sodium phosphate buffer, pH 6.0, 2 mM DTT, 2 mM EGTA, 1 mU of each cathepsin and an appropriate amount of p21. Incubation was carried out at 30°C for 30 min. Ten µl of anti-EGF-receptor monoclonal antibody (Transformation Research Inc., Framingham, MA) and 400 µl of RIPA buffer (30 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1 % sodium deoxycholate, 1 % Triton X-100, 0.1 % SDS and 1 mM PMSF) containing 10 µM each of antipain and leupeptin were added to the mixture and incubated for 90 min at room temperature.

The mixture was further incubated with 50 µl of protein A-Sepharose CL-4B (Pharmacia) for 60 min at room temperature, and then washed four times with PBS containing 0.1 % bovine serum albumin, 0.2 % NaN_3, 0.5 % Nonidet P-40 and 0.1 % SDS. The pellet of centrifugation at 1,000 x g for 3 min was mixed with SDS mixture (0.125 M Tris-HCl, pH 8.2, 1 % SDS, 3 % β-mercaptoethanol and 15 % glycerol) and boiled for 5 min. The solubilized materials were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (18).

Measurement of radioactivity of the 170-kDa EGF-receptor After the autoradiography, the gel portion corresponding to the 170-kDa EGF-receptor was cut out ane treated with 1 ml of Protosol (New England Nuclear) at 55°C for 7 h. The radioactivity was then measured as described previously (19).

Results and Discussion

Normal human and activated p21s were produced by Escherichia coli and purified as described previously (13). We used truncated p21s because full-length p21s are highly insoluble in aqueous solution and form aggregates easily. p21(G, 171) and p21(V, 171) are truncated p21s (^{1}Met to ^{171}Leu) and have glycine and valine, respectively, at position 12. p21(G,171) is a protooncogene product and p21(V,171) is a oncogene type. The effects of these p21s toward cysteine proteinases were investigated and shown in Fig. 1. The activity of cathepsin L was inhibited by p21s most effectively, while those of cathepsin B and papain were weakly inhibited. None of inhibition by p21s was observed toward cathepsin H. This specific inhibition of cathepsin L is a quite unique property which has not been observed in other cysteine-proteinase inhibitors (20,21). The inhibitory activity of p21(V, 171) was slightly higher than that of p21(G, 171). The half maximal inhibition dose (ID_{50}) of p21s toward cathepsin L was approximately 5 µg/ml. The effect of guanine nucleotides was also investigated and the results showed guanine nucleotides had no direct effect toward the protease-inhibitor activity of truncated p21s (data not shown).

For the next step of interpretation of the effects of p21s, it is necessary to know possible substrates of cathepsin L. It is possible that p21s may affect the degradation of some growth-

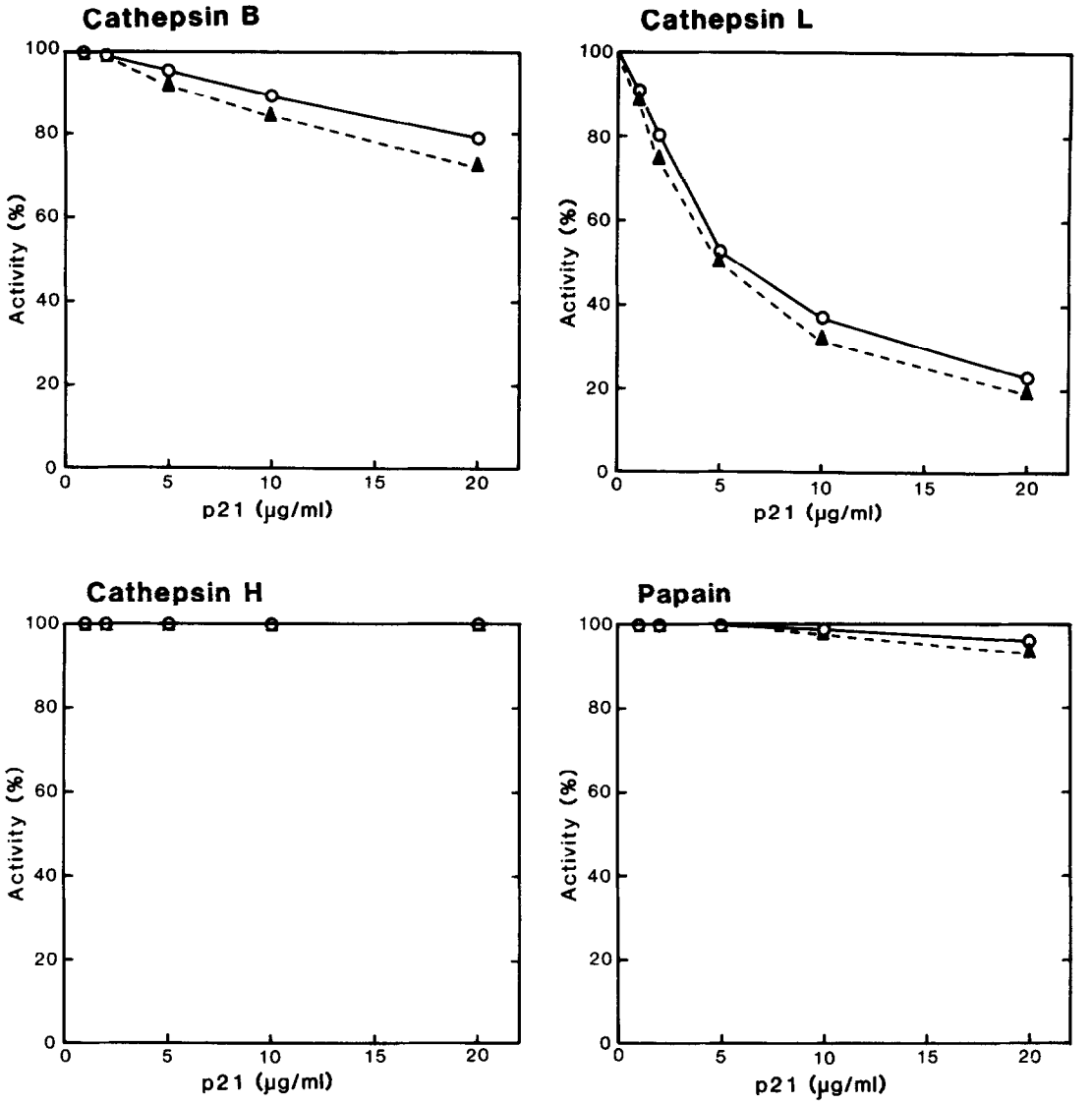


Fig. 1. Inhibition of various cysteine proteinases by p21s. The activities of cathepsins B, L, and H and papain were measured in the presence of varying amounts of p21s. The protease activity in the absence of p21s is expressed as 100 % and each point represents the average value of two assays. (o), p21(G, 171); (▲), p21(V, 171).

relating molecules such as growth factor receptors. Among several possible proteins, EGF-receptor was selected.

Human epidermoid carcinoma A431 cells overexpress EGF-receptors (22,23). A typical 170-kDa/150-kDa doublet of EGF-receptors was observed after immunoprecipitation of A431 cell extracts with anti-

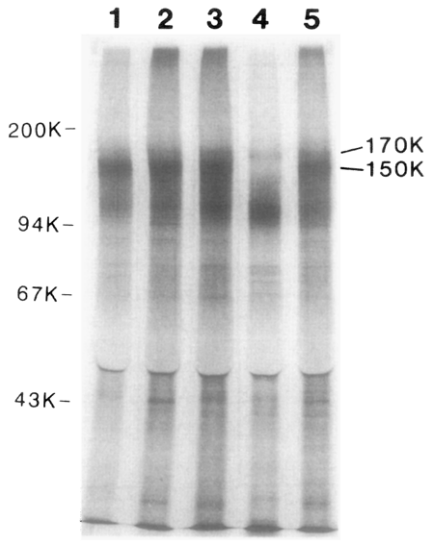
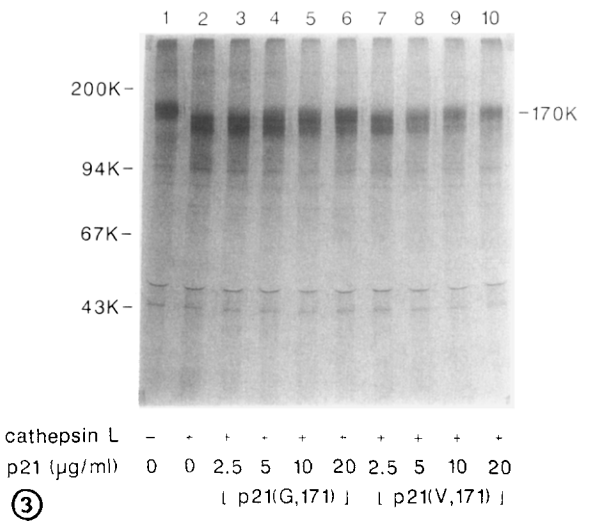


Fig. 2. Effects of cysteine proteinases on EGF-receptor. Effects of three cathepsins on EGF-receptor. A431 cell extracts (20 μg protein) were incubated at 0°C (lane 1) or 30°C for 30 min (lanes 2 - 5) in the presence of 1 mU each of cathepsin B (lane 3), cathepsin L (lane 4), or cathepsin H (lane 5). After the incubation, EGF-receptor was immunoprecipitated with anti-EGF-receptor monoclonal antibody. The immunoprecipitated materials were analyzed by SDS-PAGE (7.5 % acrylamide). The autoradiogram of the gel is shown. Molecular mass markers shown at the left are myosin (200 kDa), phosphorylase b (94 kDa), albumin (67 kDa) and ovalbumin (43 kDa) (Pharmacia Fine Chemicals, Piscataway, NJ). Positions of two types of EGF-receptors (170 kDa and 150 kDa) are also shown at the right.

EGF-receptor antibody (Fig. 2). Incubation of the cell extracts in the absence or presence of cathepsin B or H at 30°C for 30 min caused no effect on EGF-receptors. However, incubation with cathepsin L resulted in remarkable decrease of the radioactivity of EGF-receptors (Fig. 2, lane 4). Under the same condition, few other proteins derived from A431 cell extracts were digested by cathepsin L (data not shown). It appeared that the 150-kDa EGF-receptor is more sensitive toward cathepsin L than the 170-kDa receptor (Fig. 2).

It has been reported that the 170-kDa EGF-receptor is cleaved to the 150-kDa form by a calcium-dependent cysteine protease (Ca^{2+}-protease) (24-26). It may be necessary to know whether cathepsin L can degrade the 170-kDa EGF-receptor in the absence of Ca^{2+}-protease. Inclusion of EGTA throughout the preparation of cell



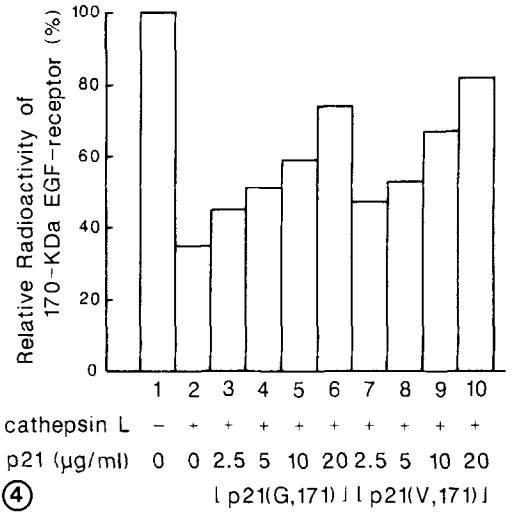


Fig. 3. Effects of p21s on the cleavage of EGF-receptor by cathepsin L. Preparation of A431 cell extract and all of the subsequent experiments were carried out in the presence of 5 mM EGTA. Cell extracts were incubated at 30°C for 30 min in the absence (lane 1) or presence of 1 mU of cathepsin L (lanes 2 - 10) and p21s (lanes 3 - 10). p21s and their concentrations were as follows; lane 3, p21(G, 171), 2.5 μg/ml; lane 4, p21(G, 171), 5 μg/ml; lane 5, p21(G, 171), 10 μg/ml; lane 6, p21(G, 171), 20 μg/ml; lane 7, p21(V, 171), 2.5 μg/ml; lane 8, p21(V, 171), 5 μg/ml; lane 9, p21(V, 171), 10 μg/ml; lane 10, p21(V, 171), 20 μg/ml. Immunoprecipitated EGF-receptors were analyzed by SDS-PAGE and the autoradiogram is shown. Molecular mass markers and the 170-kDa EGF-receptors are shown at the left and right, respectively.

Fig. 4. Relative radioactivity of the 170-kDa EGF-receptor. Radioactivity of the 170-kDa EGF-receptor shown in Fig. 3 was measured. The column numbers correspond to the lane numbers of Fig. 3. The radioactivity of column 1 was 1,113 dpm and expressed as 100 %.

extract prevented the appearance of the 150-kDa EGF-receptor (Fig. 3, lane 1) as reported previously (26). The cell extract was treated with cathepsin L and the results showed that the 170-kDa EGF-receptor can be degraded by cathepsin L alone (Fig. 3, lane 2). The degradation was suppressed by p21(G, 171) and p21(V, 171) dose-dependently (Fig. 3, lanes 3 - 10). The radioactivity of the 170-kDa receptor shown in Fig. 3 was measured and is shown in Fig. 4. The inhibition profile of the degradation of EGF-receptor is almost comparable to that of cathepsin L activity (Fig. 1), suggesting that p21s can inhibit cathepsin L-induced degradation of EGF-receptors.

Recently, it has been reported that p21s have some interactions with insulin receptor (27,28) and protein kinase C (29,30).

Therefore, it is probable that p21s are associated with various kinds of cell surface receptors. The contact between receptors and cathepsin L may take place at the time of receptor-mediated endocytosis. After cell surface receptors bind to their ligands, they are rapidly endocytosed and subsequently degraded in lysosomes by lysosomal proteases (31,32). If the degradation of the internalized receptors is inhibited by p21s which are possibly associated with them, the receptors may be protected against proteases and stimulate cell growth constitutively.

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