Volume 318, number 3, 297–300 FEBS 12182 March 1993

v-Ha-Ras insertion/deletion mutants with reduced protease-inhibitory activity have no transforming activity

Toshie Sawada, Shigeru Sakiyama and Takaki Hiwasa

Division of Biochemistry, Chiba Cancer Center Research Institute, 666-2 Nitona-cho, Chuo-ku, Chiba 260, Japan

Received 4 January 1993

We have purified 26 insertion/deletion mutants of v-Ha-*ras* oncogene products produced by *Escherichia coli* and investigated their protease-inhibitory activity toward papain and cathepsins B and L. K_i values for papain were relatively similar among the mutants, however, those for cathepsins B and L varied up to 10-fold. Among them, four mutants, 1-48 LIR 54-189, 1-110 LIS 112-189, 1-130 PDQ 146-189 and 1-155 LIR 166-189, showed significant reduction in the inhibitory activity toward cathepsin L and these four mutants have lost transforming activity toward NIH3T3 mouse fibroblasts. However, some other mutants also showed no transforming activity in spite of possession of the potent protease-inhibitory activity, suggesting that the protease-inhibitory activity of Ras might be necessary but not sufficient for its biological activity.

v-Ha-*ras*; Oncogene product; Protease inhibitor; Cathepsin; GTP binding

1. INTRODUCTION

ras oncogenes are frequently involved in carcinogenesis of human cancers and the product (Ras) has been well characterized. Guanine nucleotide-binding nature, interaction with GTPase activating protein (GAP) and lipid modification of Ras have been extensively studied [1–3]. However, no causative function of carcinogenesis has been identified. We have previously reported protease-inhibitory activity of Ras which may be a unique feature among oncogene products [4–6]. Ras specifically inhibits cysteine proteinases such as cathepsins B and L [4,6]. It has previously been hypothesized that Ras causes effects as a protease inhibitor [7,8]. In order to examine this hypothesis, the protease-inhibitory activity of various Ras mutants was investigated. The results showed that mutated Ras with reduced protease-inhibitory activity has no transforming activity.

2. MATERIALS AND METHODS

2.1. *Preparation of Ras proteins*

Mutated *ras* plasmids which have the v-Ha-*ras* gene, with both insertions and deletions, have been provided by Dr. Berthe M. Willumsen of University Institute of Microbiology (Copenhagen, Denmark) [9,10]. The mutants were named as described previously [10]. For example, 1-21 PDQ 37-189 has insertion of Pro-Asp-Gln instead of deletion of the amino acid sequence between positions 22 and 36. Ras was purified according to the method of Lacal and Aaronson [11]. *E. coli* bearing *ras* plasmids was cultured at 30°C until OD_{600} reached at 0.4 and then incubated at 42°C for 6 h. Ras protein was extracted from *E. coli* with 8 M urea, 50 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$ and 1 mM DTT, purified through DEAE–Sephacel and Sephadex G-75 columns in 7 M urea, 50 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$ and 1 mM DTT, concentrated by ultrafiltration using PM-10 (Amicon), and then dialyzed extensively against 20 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$ and 1 mM DTT [12].

2.2. *Assay for protease-inhibitory activity*

Papain and bovine spleen cathepsin B were purchased from Sigma Chemical Co. (St. Louis, MO). Porcine kidney cathepsin L and mouse kidney cathepsin B were purified as described previously [12,13]. Protease-inhibitory activity of Ras was measured as follows. Cysteine proteinases such as papain and cathepsins B and L were activated by preincubation at 37°C for 10 min in 20 mM MES (pH 5.5), 2 mM DTT and 1 mM EDTA. These proteinases were then mixed with Ras in 20 mM MES (pH 5.5), 2 mM DTT, 1 mM EDTA and 0.1% Brij-35 and 10–20 mM substrate, and incubated at 37°C for 10 min [14]. Substrates used were Z-Phe-Arg-MCA for papain and cathepsin L, and Z-Arg-Arg-MCA for cathepsin B. The reaction was stopped by addition of an equal volume of 100 mM sodium monochloroacetate, 30 mM sodium acetate and 70 mM acetic acid (pH 4.3) [15]. K_i values were determined according to the method of Dixon and Webb [16].

Guanine nucleotide-binding activity of Ras was quantitated using [^{3}H]GTP according to the method described previously [10,17].

3. RESULTS AND DISCUSSION

The protease-inhibitory activity of Ras is highly specific, i.e. it inhibits the proteolytic acitivity of papain and cathepsins B and L but does not inhibit that of cathepsins H, C and D, chymotrypsin or thrombin ([4–6,14], data not shown). Fig. 1 shows typical inhibition

Correspondence address. T. Hiwasa, Division of Biochemistry, Chiba Cancer Center Research Institute, 666-2, Nitona-cho, Chuo-ku, Chiba 260, Japan. Fax: (81) (43) 264 5431.

Abbreviations: DTT, dithiothreitol; *E. coli, Escherichia coli;* GAP, GTPase activating protein; MES, 2-(*N*-morpholino)ethanesulfonic acid; Ras, *ras* oncogene product; Z-Arg-Arg-MCA, 7-(benzyloxycarbonyl-arginyl-arginyl-amino)-4-methylcoumarin; Z-Phe-Arg-MCA, 7-(benzyloxycarbonyl-phenylalanyl-arginyl-amino)-4-methylcoumarin.

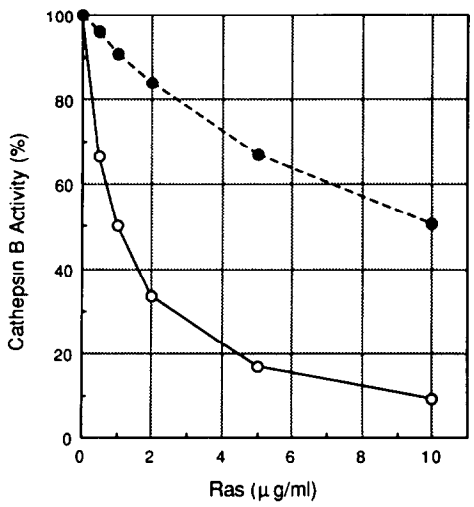

Fig. 1. Inhibition profiles of Ras toward bovine cathepsin B. Cathepsin B activity in the presence of varying concentrations of Ras (o) or a mutant, 1-48 LIR 54-189 (●), was measured and is expressed as a percentage of that in the absence of Ras.

profiles of wild-type v-Ha-Ras and a mutant, 1-48 LIR 54-189, toward cathepsin B. Ras inhibited cathepsin B activity dose-dependently and the inhibition was non-competitive.

The K_i values for papain and cathepsins B and L are shown in Fig. 2. The K_is for papain were not largely altered among mutants, suggesting that there is no indispensable region for the inhibition of papain. The docking model between papain and cystatin reported by Turk and Bode [18] showed there are multiple contact points between them. The three-dimensional structure of Ras resembles that of cystatin [19–21]. Taken together, loss of one contact point might be insufficient to significantly affect the inhibitory acitivity of Ras toward papain unless large conformational changes were induced. These similar K_i values also imply that most Ras proteins were correctly re-folded after denaturation.

K_i values for bovine cathepsin B varied 10-fold among mutants (Fig. 2B). Especially, 1-48 LIR 54-189 showed very weak inhibitory activity toward cathepsin B (also shown in Fig. 1). The reduced inhibitory activity of this mutant was reproducibly observed among several lots of the preparation. This mutant can bind guanine nucleotides and also interact with GAP, however, it has no transforming activity toward NIH3T3 cells [10]. These suggest that the deleted region (amino acid positions between 49 and 53) interacts not only with cathepsin B but also with the target. Fujita-Yoshigaki et al. have recently reported that the possible target, but not GAP, interacts with c-Ha-Ras at amino acid positions 45 and 48 [22], which is very close to the deleted region. K_i values for mouse cathepsin B were similar to those for bovine cathepsin B (data not shown).

There were also large differences in the inhibitory activity against cathepsin L among Ras mutants (Fig. 2C). Again, 1-48 LIR 54-189 showed a very high K_i value toward cathepsin L. Furthermore, three other mutants, 1-110 LIS 112-189, 1-130 PDQ 146-189 and 1-155 LIR 166-189, had very weak inhibitory activity. All of these mutants had no transforming activity [10] (Table I). Thus, at least four domains are required for Ras to inhibit cathepsin L effectively, i.e. amino acid positions between 49 and 53, 109 and 111, 139 and 142, and 162 and 165.

Table I summarizes the protease-inhibitory activity of Ras mutants together with their transforming activity toward NIH3T3 cells and guanine nucleotide-binding activity. Transformation-deficient mutants have lost either guanine nucleotide-binding or potent protease-inhibitory activity. Three mutants, 1-21 PDQ 37-189, 1-27 SDQ 30-189 and 1-34 LIR 38-189, have no transform-

Table I

Protease-inhibitory activity, transforming activity and guanine nucleotide-binding activity of v-Ha-Ras mutated proteins

Ras mutants	Protease-inhibitory activity[a]	Transforming activity[b]	GTP-binding activity[c]
v-Ha-Ras (1-189)	+++	++	++
1-21 PDQ 37-189	+++	−	++
1-27 SDQ 30-189	+++	−	++
1-34 LIR 38-189	+++	−	++
1-40 PD 43-189	+++	−	++
1-48 LIR 54-189	+	−	+
1-63 SDQ 73-189	+++	++	++
1-68 ADQ 77-189	++	++	+
1-71 TDQ 83-189	++	−	−
1-89 SDQ 98-189	++	−	++
1-96 LIR 104-189	+++	++	++
1-101 PDQ 109-189	++	++	+
1-106 ADQ 109-189	+++	++	++
1-106 ADQ 112-189	++	+	−
1-110 LIR 112-189	+	−	+
1-110 LIS 120-189	+++	−	−
1-119 PDQ 126-189	+++	+	−
1-119 PDQ 130-189	++	−	−
1-123 LIR 130-189	++	++	+
1-123 LIR 132-189	++	++	++
1-130 PDQ 139-189	++	++	++
1-130 PDQ 146-189	+	−	−
1-142 ADQ 152-189	+++	−	−
1-148 LIR 154-189	+++	−	−
1-153 ADQ 162-189	+++	−	+
1-155 LIR 166-189	+	−	−
1-165 LIR 180-189	+++	++	+

[a] Protease-inhibitory activity toward cathepsin L is shown. +++, K_i < 100nM; ++, 100 nM < K_i < 200 nM; +, K_i > 200 nM.

[b] Relative transforming activities toward NIH3T3 cells [9,10] are shown. Number of transformed foci is more than 10% (++), 0.1–10% (+), and less than 0.1% (−) of that induced by the wild-type.

[c] Guanine nucleotide-binding activity of purified mutated Ras proteins. The binding activities were measured by using [^{3}H]GTP and almost comparable to those reported previously [10]. GTP-binding activity is more than 10% (++), 0.2–10% (+), and less than 0.2% (−) of that of wild-type Ras.

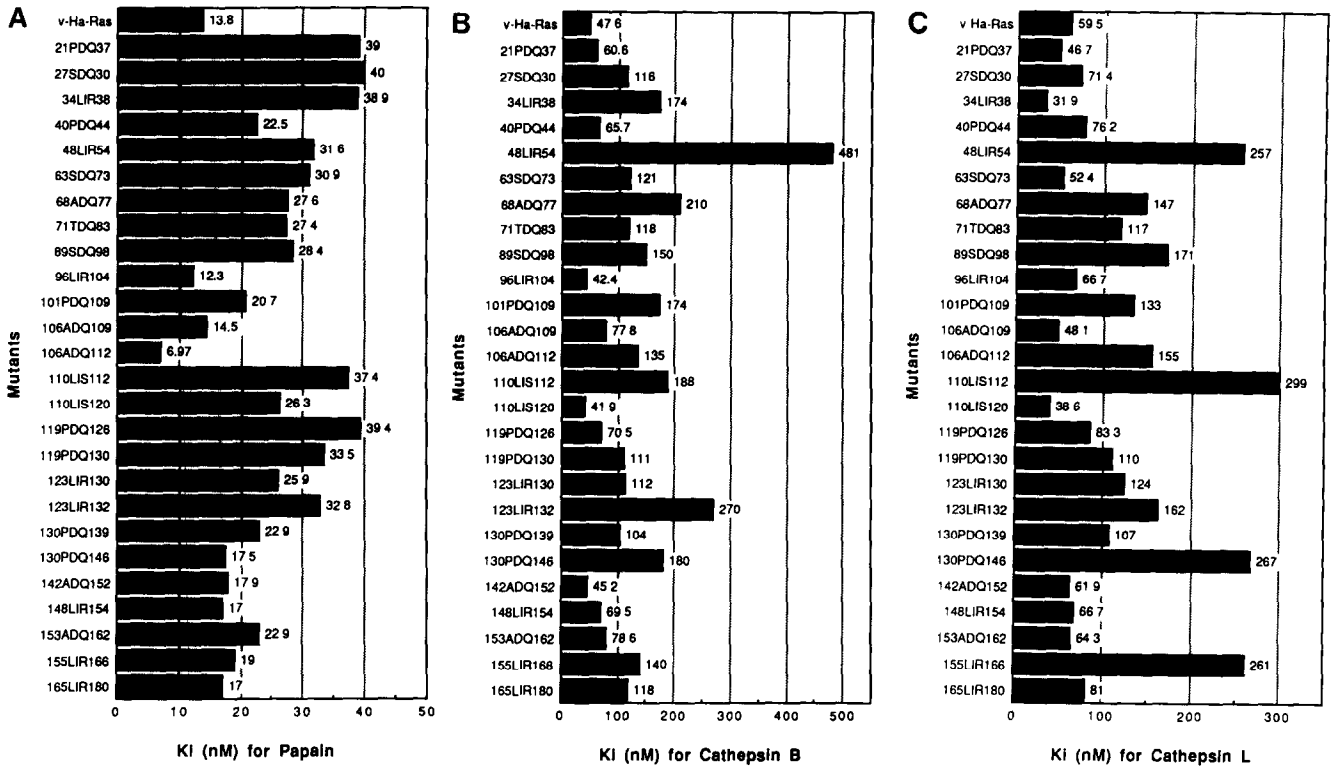

Fig. 2. K_i values of v-Ha-Ras and it's insertion/deletion mutants for papain (A), bovine cathepsin B (B) and porcine cathepsin L (C). K_i values were determined as described in section 2. Mutant names were abbreviated. For example, 1-21 PDQ 37-189 was described as 21PDQ37. Numbers in the figures reprersent K_i values (nM).

ing activity possibly because of the deletion in the interaction domain with GAP [23,24]. We cannot explain why three mutants, 1-40 PD 43-189, 1-89 SDQ 98-189 and 1-153 ADQ 162-189, have completely lost the transforming activity. Thus, protease-inhibitory activity of Ras alone may be necessary, but not sufficient, for its biological activity.

The present manuscript shows that reduced inhibitory activity of Ras toward cathepsin L is related to the loss of the biological activity, and that the target of both Ras and cathepsins may associate with Ras at a similar domain, namely amino acid positions between 49 and 53. These data alone might be insufficient to define cathepsin L and/or cathepsin B as the target of Ras. It remains to be determined how protease-inhibitory activity of Ras is regulated by guanine nucleotides and/or GAP, and how activating point mutations in Ras could affect the protease-inhibitory activity. However, our recent work has shown that K_i of c-Ha-Ras toward cathepsin B is 3-fold higher than that of v-Ha-Ras, although it's K_is for papain and cathepsin L are similar to those of v-Ha-Ras (manuscript in preparation). Thus, it is not too far from the truth to say that the target of Ras is cathepsins B and/or L, and even if the target is not cathepsins, the target may be structurally related to cathepsins because they bind the same region of Ras.

Acknowledgements: We thank Dr. Berthe M. Willumsen (University Institute of Microbiology, Copenhagen, Denmark) for providing us with v-Ha-*ras* mutant plasmids. This work was supported by Grants-in-Aid from the Ministry of Health and Welfare for the Comprehensive 10-Year Strategy for Cancer Control, Japan.

REFERENCES

[1] Scolnick, E.M., Papageorge, A.G. and Shih, T.Y. (1979) Proc. Natl. Acad. Sci. USA 76, 5355–5359.
[2] Trahey, M. and McCormick, F. (1987) Science 238, 542–545.
[3] Hancock, J.F., Magee, A.I., Childs, J.E. and Marshall, C.J. (1989) Cell 57, 1167–1177.
[4] Hiwasa, T., Yokoyama, S., Ha, J.-M., Noguchi, S. and Sakiyama, S. (1987) FEBS Lett. 211, 23–26.
[5] Hiwasa, T., Sakiyama, S., Noguchi, S., Ha, J.-M., Miyazawa, T. and Yokoyama, S. (1987) Biochem. Biophys. Res. Commun. 146, 731–738.
[6] Hiwasa, T., Sakiyama, S., Yokoyama, S., Ha, J.-M., Fujita, J., Noguchi, S., Bando, Y., Kominami, E. and Katunuma, N. (1988) Biochem. Biophys. Res. Commun. 151, 78–85.
[7] Hiwasa, T. (1988) Biol. Chem. Hoppe-Seyler 369 (Suppl.), 239–241.
[8] Hiwasa, T., Sakiyama, S., Kominami, E., Katunuma, N. and Tanaka, T. (1989) in: Intracellular Proteolysis: Mechanism and Regulations (N. Katunuma and E. Kominami, eds.) pp. 328–337, Japan Scientific Societies Press, Tokyo.
[9] Willumsen, B.M., Papageorge, A.G., Hubbert, N., Bekesi, E., Kung, H.-F. and Lowy, D.R. (1985) EMBO J. 4, 2893–2896.
[10] Willumsen, B.M., Papageorge, A.G., Kung, H.-F., Bekesi, E., Robins, T., Johnsen, M., Vass, W.C. and Lowy, D.R. (1986) Mol. Cell. Biol. 6, 2646–2654.

[11] Lacal, J.C. and Aaronson, S.A. (1986) Mol. Cell. Biol. 6, 4214–4220.
[12] Hiwasa, T., Sawada, T. and Sakiyama, S. (1990) Carcinogenesis 11, 75–80.
[13] Hiwasa, T., Sawada, T., Sakiyama, S., Kominami, E. and Katunuma, N. (1989) Biol. Chem. Hoppe-Seyler 370, 1215–1220.
[14] Hiwasa, T. (1992) in: Recent Advances in the Chemistry and Molecular Biology of Cancer Research (Q. Dai, M.-A. Armour and Q. Zheng, eds.) Springer-Verlag and Science Press, Heidelberg (in press).
[15] Barrett, A.J. and Kirschke, H. (1981) Methods Enzymol. 80, 535–561.
[16] Dixon, M. and Webb, E.C. (1979) Enzymes, 3rd edn., pp. 332–380, Longman Group, London.
[17] Gibbs, J.B., Schaber, M.D., Allard, W.J., Sigal, I.S. and Scolnick, E.M. (1988) Proc. Natl. Acad. Sci. USA 85, 5026–5030.
[18] Turk, V. and Bode, W. (1991) FEBS Lett. 285, 213–219.
[19] Milburn, M.V., Tong, L., DeVos, A.M., Brünger, A., Yamaizumi, Z., Nishimura, S. and Kim, S.-H. (1990) Science 247, 939–945.
[20] Krengel, U., Schlichting, I., Scherer, A., Schumann, R., Frech, M., John, J., Kabsch, W., Pai, E.F. and Wittinghofer, A. (1990) Cell 62, 539–548.
[21] Hiwasa, T., Sawada, T., Tanaka, K., Chiba, T., Tanaka, T., Kominami, E., Katunuma, N. and Sakiyama, S. (1991) Biomed. Biochim. Acta 50, 579–585.
[22] Fujita-Yoshigaki, J., Shirouzu, M., Koide, H., Nishimura, S. and Yokoyama, S. (1991) FEBS Lett. 294, 187–190.
[23] Schaber, M.D., Garsky, V.M., Boylan, D., Hill, W.S., Scolnick, E.M., Marshall, M.S., Sigal, I.S. and Gibbs, J.B. (1989) Protein: Struct. Funct. Genet. 6, 306–315.
[24] Adari, H., Lowy, D.R., Willumsen, B.M., Der, C.J. and McCormick, F. (1988) Science 240, 518–521.