Synergistic Induction of Anchorage-independent Growth of NIH3T3 Mouse Fibroblasts by Cysteine Proteinase Inhibitors and a Tumor Promoter*

(Received for publication, December 11, 1995, and in revised form, January 24, 1996)

Takaki Hiwasť, Toshiie Sawada, and Shigeru Sakiyama
From the Division of Biochemistry, Chiba Cancer Center Research Institute, 666-2, Nitona-cho, Chu-o-ku, Chiba 260, Japan

We have previously reported that Ras protein is a potent cysteine proteinase inhibitor. In order to examine whether the cysteine proteinase-inhibitory activity of Ras is involved in carcinogenesis, the effects of the following probes were investigated. Cystatin α is a cysteine proteinase-specific inhibitor and has some amino acid sequence homology with Ras. Ras has a CAAX motif (C, cysteine; A, aliphatic amino acid; X, any amino acid) at the carboxyl terminus, which is indispensable for the biological activity. Thus, cystatin α carrying a CAAX motif (cystatin α-CVLS) was examined. V-Ha-Ras deletion mutant, RasΔ42-49, has undetectable GTP binding activity, yet it retains a similar protease inhibitory activity to that of wild-type v-Ras. These genes were inserted into a eukaryotic inducible expression vector and transfected into NIH3T3 cells. The expression was effectively induced by treatment with a glucocorticoid hormone, dexamethasone. The expression of cystatin α-CVLS or RasΔ42-49 alone induced neither transformation nor morphological changes. However, when their expression was induced in the presence of a tumor-promoting phorbol ester, a remarkable increase in the anchorage-independent growth was observed in cystatin α-CVLS- and RasΔ42-49-transfected clones. These results suggest that cysteine proteinase inhibitors and a tumor promoter synergistically transformed NIH3T3 cells. It is thus possible that the cysteine proteinase-inhibitory activity of Ras might play a key role in the early stage of carcinogenesis.

A number of studies have been made on the action mechanism of ras oncogene product (Ras), and multiple proteins have been proposed as the effectors of Ras protein. These are ras-GAP, protein kinase C, c-Raf-1, ralGDS, and phosphatidylinositol 3-kinase (1–5). Although Ras associates with these proteins in a GTP-dependent manner, the direct effect of Ras remains to be proved. It has been suggested that Ras does not directly activate the kinase activity of Raf but translocates it from cytosol to membrane (6). We have previously reported that Ras is a potent protease inhibitor (7–9). The inhibitory activity of Ras is specific for cysteine proteinases such as cathepsins B and L. Consistently, the amino acid sequence of Ras is partly homologous to those of the cysteine proteinase inhibitor family designated “cystatin superfamily” (10, 11). If the cysteine proteinase-inhibitory activity of Ras is involved in the transformation, catstins might have a similar transforming activity. The present results suggest that inhibition of cysteine proteinases is sufficient for the conversion from a normal cell to an initiated one.

EXPERIMENTAL PROCEDURES

Cell Culture—NIH3T3 mouse fibroblasts and the transfected clones were cultured in Dulbecco’s modified medium supplemented with 5% calf serum.

GTP Binding Assay—Ras proteins (1 μg) were electrophoresed through SDS-polyacrylamide gel and electroblotted onto a nitrocellulose membrane filter. The filter was then incubated with [γ-32P]GTP (5 μCi/ml) in buffer A (50 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 1 mM EGTA, and 0.3% Tween 20) for 30 min at room temperature according to the method of Lapetina and Reep (11). The filter was washed extensively with buffer A and exposed on x-ray film.

Raf Binding Assay—A cDNA fragment (1627 base pairs) of human c-rat-1 (12) obtained by digestion with PvuI and BglII was inserted into a Smal site of a prokaryotic expression vector, pGEX-2T (Pharmacia, Uppsala). Glutathione S-transferase (GST) and GST–c-Raf fusion protein were produced by the transformed Escherichia coli treated with isopropyl-β-D-thiogalactopyranoside for 3 h and were purified by affinity chromatography using glutathione-Sepharose 4B (Pharmacia).

Binding between Ras proteins and GST-c-Raf was investigated as described previously (13). Purified GST or GST-c-Raf protein (1 μg) was first incubated at 4 °C for 30 min with glutathione-Sepharose in binding buffer (100 mM KCl, 6.33 mM MgCl2, 20 mM Tris-HCl (pH 7.4), 1.0 mg of bovine serum albumin/ml, 25 μM ZnCl2), and the unbound proteins were removed by washing with binding buffer. Guanine nucleotides prebound to Ras were exchanged by incubation of Ras at 30 °C for 15 min in loading buffer (50 mM HEPES (pH 7.4), 5 mM EDTA, 1 mM dithiothreitol, 75 μM of bovine serum albumin/ml, 1 mM GDP or GMP-PNP. GST or GST-c-Raf immobilized on glutathione-Sepharose was then incubated with Ras (1 μg) at 4 °C for 30 min in binding buffer and washed 4 times with wash buffer (100 mM KCl, 6.33 mM MgCl2, 20 mM Tris-HCl (pH 7.4) and 0.25% Triton X-100). The bound materials were analyzed by Western blot using an anti-Ras monoclonal antibody, NCC-RAS-001 (14).

Construction of Plasmids—Rat cystatin α cDNA was provided by Drs. Nobuhiko Katunuma (Tokushima Bunri University) and Yoshima Ike (Mitsui Pharmaceutical Co. Ltd.) (15). A gene for cystatin α bearing a CAAX motif (Tyr-Cys-Val-Leu-Ser) at the carboxyl terminus was also constructed as described previously (16). This cystatin α variant was designated “cystatin α-CVLS” in the following part. V-Ha-Ras gene (v-Ras(WT)) was provided by Dr. Berthe M. Williamsen (University Institute of Microbiology, Copenhagen). Two deletion mutants were constructed as described (17). RasΔ43–45 and RasΔ42–49 have deletions in amino acids 43–45 and 42–49, respectively. Cystatin α, cystatin α-CVLS, v-Ras(WT), RasΔ43–45, and RasΔ42–49 DNAs were inserted into a Smal site of an inducible eukaryotic expression vector, pMSG (Pharmacia). Using this vector, the inserted genes are effectively induced by dexamethasone (Dex).

Transfection—Cystatin α or cystatin α-CVLS DNA (10 μg) was co-transfected with neo (1 μg) into NIH3T3 cells (5 × 103 cells) using Lipofectin (Life Technologies, Inc.). Transfected clones were selected in the presence of G418 (400 μg/ml). NV, NY, NS, NR, NII, and NIII

* This work was supported by a grant-in-aid for scientific research from the Ministry of Education, Science and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed. Tel.: 81-43-254-5431 (ext. 5502); Fax: 81-43-265-4459; E-mail: thiwasa@biolab.kazusa.or.jp.

‡ To whom correspondence should be addressed. Tel.: 81-43-254-5431.
Fig. 1. GTP binding activity of v-Ras(WT), RasΔ43–45, and RasΔ42–49. GTP binding activity was investigated by the filter overlay method. v-Ras(WT) (lane 1), RasΔ43–45 (lane 2), and RasΔ42–49 (lane 3) were electrophoresed and blotted on a nitrocellulose filter, which was then incubated with \[^{[32P]}GTP\] and washed extensively. The autoradiogram is shown. An arrow indicates the position of v-Ras(WT).

Results and Discussion

The cysteine proteinase-inhibitory activity of cystatin \(\alpha\) was more potent than that of Ras (7, 19). However, cystatin \(\alpha\) possesses neither guanine nucleotide binding activity nor a CAAX motif. A CAAX motif at the carboxyl-terminal end of Ras is necessary not only for post-translational farnesylation but also for the biological activity (20). Thus the biological activity of cystatin \(\alpha\) carrying the CAAX motif (cystatin \(\alpha\)-CVLS) was also investigated.

Other probes used were two deletion mutants of v-Ha-Ras, RasΔ43–45 and RasΔ42–49. The most conserved amino acid sequence among the cystatin superfamily is Glu-Val-Val (10), which is also found in Ras at amino acid positions between 43 and 45 (7). These Ras proteins were bacterially expressed and purified. In spite of the deletion of the conserved amino acid sequence, RasΔ43–45 and RasΔ42–49 showed similar protease-inhibitory activities as that of v-Ras(WT) (17). For example, the \(K_v\) values of v-Ras(WT), RasΔ42–49, and RasΔ43–45 were 48, 102, and 40 nm, respectively, toward bovine cathepsin B and 14, 11, and 8 nm, respectively, toward papain. These results are consistent with the previous report that point mutants in the Glu-Val-Val region did not significantly affected the protease-inhibitory activity of cystatin A (21). Thus, the Glu-Val-Val sequence might be important but not essential for the interaction with cysteine proteinases.

Guanine nucleotide binding activities of these two deletion mutants were also compared with that of v-Ras(WT). Fig. 1 shows the results of GTP binding examined by the filter overlay assay using \[^{[32P]}GTP\] (11). v-Ras(WT) showed a potent GTP binding activity while RasΔ42–49 and RasΔ43–45 showed no detectable GTP binding activity. The GTP binding activity was also examined by rapid filtration according to the method of Gibbs et al. (22), and the results showed both RasΔ42–49 and RasΔ43–45 had less than 1% guanine nucleotide binding activity of that of v-Ras(WT) (17).

We have also investigated the in vitro association of Ras and c-Raf-1. c-Ha-Ras bound to GST-c-Raf only in the presence of GMP-PNP, a non-hydrolyzable GTP analogue (Fig. 2). No significant binding was observed toward GST. Under the same conditions, v-Ras(WT) bound to GST-c-Raf irrespective of the GDP- or GMP-PNP-bound form. Guanine nucleotides sometimes do not affect the association between Ras and c-Raf under certain experimental conditions as reported previously (23, 24). RasΔ42–49 showed similar results as those of v-Ras(WT), suggesting that the region between amino acid positions 42 and 49 is not necessarily required for Rap binding. However, RasΔ43–49 failed to bind to GST-c-Raf probably because the deletion caused some additional alteration in the three-dimensional conformation of Ras.

DNAs for cystatin \(\alpha\), cystatin \(\alpha\)-CVLS, v-Ras(WT), RasΔ42–49, and RasΔ43–45 were inserted into a downstream region of mouse mammary tumor virus-long terminal repeat of a eukaryotic expression vector, pMSG, and transfected into NIH3T3 cells. Western blot analysis demonstrated that the transfected genes were effectively induced after treatment with Dex (9, 16, 17). Induction of cystatin \(\alpha\), cystatin \(\alpha\)-CVLS, RasΔ42–49, and RasΔ43–45 did not cause any significant morphological changes whereas induction of v-Ras(WT) resulted in drastic morphological changes as well as the appearance of dense transformed foci (not shown).

Because anchorage-independent growth is one of the specific phenotypes observed in malignant cells (18), growth of transfected clones in soft agar medium was investigated. Vector-transfected clones, NV1, NV2, and NV3, as well as the parent NIH3T3 cells did not grow in soft agar medium while v-ras(WT)-transfected clones, NR24 and NR37, showed high colony-forming efficiencies in the presence of Dex (Fig. 3). These clones grew in soft agar medium even in the absence of Dex probably due to the leaked expression of Ras in the absence of Dex (9, 16). On the other hand, induction of cystatin \(\alpha\), cystatin \(\alpha\)-CVLS, RasΔ42–49, and RasΔ43–45 by Dex did not result in any discernible change in the colony-forming ability. This implies that induction of cystatin \(\alpha\), cystatin \(\alpha\)-CVLS,
Fig. 4. **Phase morphology of transformed colonies.** Colonies formed by NY-21 (A), NS-13 (B), NII-3 (C), and NR-37 (D) in soft agar in the presence of both Dex and TPA are shown. Original magnification was ×60. The bar in C represents 0.2 mm.

RasΔ42-49, or RasΔ43-45 alone is insufficient to induce anchorage-independent transformation.

These proteins might play a role at a certain stage in multi-stage carcinogenesis. Two-stage carcinogenesis, which consists of initiation and promotion, was first proposed by Berenblum (25). Initiated cells produced by irradiation or treatment with chemical carcinogens are easily transformed in the presence of tumor promoter phorbol ester, TPA, and the transformation can be evaluated by focus-forming assay (26, 27). Some of the chemical carcinogens that induce initiation have been shown to induce specific mutations in protooncogenes, and thus the involvement of Ras in the early stage of carcinogenesis was suggested (28–30). Consequently, cystatin α might have a role at the stage of initiation and cooperate with a tumor promoter to induce full transformation. Thus, the anchorage-independent growth in the presence of TPA was also investigated (Fig. 3). The culture of cystatin α-CVLS- and RasΔ42-49-transfected clones in the presence of both Dex and TPA resulted in a remarkable increase in colony-forming efficiency, which was significantly higher than that in the absence of either TPA or Dex (p < 0.01). The presence of TPA alone was not sufficient to induce anchorage-independent transformation in these transfectants. Vector-transfected control clones and NIH3T3 cells showed no significant increase in colony numbers. Induction of cystatin α showed the intermediate results and the colony-forming efficiencies of NY clones in the presence of Dex and TPA were between 10 and 30% (0.01 < p < 0.1) versus that in the absence of TPA. This suggests that the expression of cystatin α partly induces the changes corresponding to initiation.

The transformed colonies formed by NY, NS, and NII clones were similar to those formed by NR clones. However, the sizes of the former were smaller than those of the latter (Fig. 4), i.e., the diameters of most colonies of NY, NS, and NII clones were between 0.05 and 0.2 mm while those of NR clones were larger than 0.2 mm. The colony-forming efficiency of NR clones could be underestimated because some colonies were overlapped. The results obtained in the present study are summarized in Table I. Although cystatin α-CVLS alone cannot induce full transformation, it induced anchorage-independent transformation in the presence of a tumor promoter. RasΔ42-49 also induced anchorage-independent growth at a high efficiency in the presence of TPA in spite of the drastic decrease in the GTP binding activity. This mutant retains the cysteine proteinase-inhibitory activity, which is similar to that of v-Ras(WT). Consequently, suppression of cysteine proteinases may be sufficient for the conversion of a normal cell into an initiated one. The specific role of cysteine protease inhibitors at the stage of initiation is consistent with the possible involvement of ras oncogenes in the stage of initiation (28–30).

Cystatin α-CVLS also induced initiation without any GTP binding activity. The expression of RasΔ43-45 resulted in a slight increase in the colony formation even in the presence of TPA. Since this mutant cannot bind to c-Raf-1, association with Raf protein might be necessary for the transformation. Cystatin α-CVLS induced similar transformation without binding to c-Raf-1, possibly because the protease-inhibitory activity of cystatin α is higher than that of v-Ras(WT) (7, 19).

Even if the suppression of cysteine proteinases induces initiation, the present results cannot identify the target protease of Ras and cystatin α. Higher colony-forming efficiencies of NS clones as compared with those of NY clones suggest that the target protease is localized at a membrane fraction. In order to identify the substrates of the target protease, we have investigated the intracellular contents of Ras-binding proteins in the transfected cells before or after treatment with Dex. They were c-Raf-1, A-Raf, MEK, MEK2, protein kinase C, rasGAP, and phosphatidylinositol 3-kinase p85. However, none of them increased reproducibly after induction of Ras or cystatin α (data not shown). It is thus unlikely that Ras and cystatin α protect these proteins from degradation. Further study is necessary to determine the target protease and its substrates, which are involved in the induction of initiation.

**Acknowledgments**—We are grateful to Drs. Berthe M. Willumsen (University Institute of Microbiology, Copenhagen), Nobuhiko Katunuma (Tokushima Bunri University), and Yoshimasa Ike (Mitsui Pharmaceutical Inc.) for providing plasmids. Human c-ras-1 gene was obtained from the Japanese Cancer Research Resources Bank.

**REFERENCES**

1. Trahey, M., and McCormick, F. A. (1987) Science 238, 542–545
2. Diaz-Meco, M. T., Lozano, J., Munoz, M. M., Bier, E., Frutos, E., Sanz, L., and Moscat, J. (1994) J. Biol. Chem. 269, 31706–31710
3. Warne, P. H., Vidana, P. R., and Downward, J. (1993) Nature 364, 352–355
4. Rodrigue-Vidana, P., Warne, P. H., Dhand, R., Vanhaesebroek, B., Goud, I., Fry, M. J., Waterfield, M. D., and Downward, J. (1994) Nature 370, 527–532
5. Kikuchi, A., Demo, S. D., Ye, Z.-H., Chen, Y.-W., and Williams, L. T. (1994) Mol. Cell. Biol. 14, 7483–7491
6. Leeser, S. J., Paterson, H. F., and Marshall, C. J. (1994) Nature 369, 411–414
7. Hiwasa, T., Yokoyama, S., Sa, H.-M., Noguchi, S., and Sakiyama, S. (1987) FEBs Lett. 211, 23–26
8. Hiwasa, T., Sakiyama, S., Noguchi, S., Sa, H.-M., Miyazawa, T., and Yokoyama, S. (1987) Biochem. Biophys. Res. Commun. 146, 731–738
9. Hiwasa, T. (1995) Proteases Involved in Cancer, pp. 153–160, Monduzzi Editore, Bologna, Italy
10. Rawlings, N. D., and Barrett, A. J. (1990) J. Mol. Evol. 30, 60–71
11. Lapetina, E. G., and Reep, B. R. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 2263–2265
12. Bonner, T. I., Kerby, S. B., Sutrathe, P., Gunnell, M. A., Mark, G., and Rapp, U. R. (1984) Mol. Cell. Biol. 5, 1400–1407
13. Pumiglia, K., Chow, Y.-H., Fabian, J., Morrison, D., Dedeker, S., and Ove, R. (1995) Mol. Cell. Biol. 15, 398–406
14. Hiwasa, T., Sawada, T., Tanaka, K., Chiba, T., Tanaka, T., Kominami, E., Katunuma, N., and Sakiyama, S. (1991) Biochem. Biophys. Acta 1079, 579–585
15. Katunuma, N., Yamato, M., Kominami, E., and Ike, Y. (1988) FEBS Lett. 238, 116–118
16. Hiwasa, T., Ma, J., Ike, Y., Katunuma, N., and Sakiyama, S. (1995) Cell. Biochem. Funct. 13, 293–296

**TABLE I**

| Protein                | GTP binding activity | Protease inhibitory activity | CAAX motif | c-Raf binding activity | Induction of initiation | Full transforming activity |
|------------------------|----------------------|------------------------------|------------|------------------------|------------------------|---------------------------|
| Cystatin α             | −                    | +                            | −          | +                      | +                      | −                         |
| Cystatin α-CVLS        | +                    | +                            | +          | +                      | +                      | +                         |
| v-Ras(WT)              | +                    | +                            | +          | +                      | +                      | +                         |
| RasΔ42-49              | +                    | +                            | +          | +                      | +                      | +                         |
| RasΔ43-45              | +                    | +                            | +          | +                      | +                      | +                         |

* The results of GTP binding are shown in Fig. 1 and were reported in Ref. 17.
* Protease inhibitory activities were reported in Refs. 7, 17, and 19.
* c-Raf-1 binding activities are shown in Fig. 2.
* Full transforming activity is the colony-forming efficiency in the absence of both TPA and Dex as shown in Fig. 3.
Induction of Initiation by Protease Inhibitors

17. Sawada, T., Sakiyama, S., and Hiwasa, T. (1995) Proteases Involved in Cancer, pp. 177–181, Monduzzi Editore, Bologna, Italy
18. Hiwasa, T., Tanigawara, C., and Sakiyama, S. (1987) Cancer Res. 47, 953–959
19. Takeda, A., Kobayashi, S., and Samejima, T. (1983) J. Biochem. (Tokyo) 94, 811–820
20. Hancock, J. F., Magee, A. L., Childs, J. E., and Marshall, C. J. (1989) Cell 57, 1167–1177
21. Nikawa, T., Towatari, T., Ike, Y., and Katunuma, N. (1989) FEBS Lett. 255, 309–314
22. Gibbs, J. B., Schaber, M. D., Allard, W. J., Sigal, I. S., and Scolnick, E. M. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 5026–5030
23. Moodie, S. A., Willumsen, B. M., Weber, M. J., and Wolfman, A. (1993) Science 260, 1658–1661
24. Brtva, T. R., Drugan, J. K., Ghosh, S., Terrell, R. S., Campbell-Burk, S., Bell, R. M., and Der, C. J. (1995) J. Biol. Chem. 270, 9809–9812
25. Berenblum, I. (1941) Cancer Res. 1, 44–48
26. Herschman, H. R., and Brankow, D. W. (1986) Science 234, 1385–1388
27. Mordan, L. J., Martiner, J. E., and Bertram, J. S. (1983) Cancer Res. 43, 4062–4067
28. Bizub, D., Wood, A. W., and Skaikha, A. M. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 6048–6052
29. Balmain, A., Ramsay, M., Bowden, G. T., and Smith, J. (1984) Nature 307, 658–660
30. Barbacid, M. (1987) Annu. Rev. Biochem. 56, 779–827
31. Santos, E., Nebreda, A. R., Bryan, T., and Kempner, E. S. (1988) J. Biol. Chem. 263, 9853–9858
Synergistic Induction of Anchorage-independent Growth of NIH3T3 Mouse Fibroblasts by Cysteine Proteinase Inhibitors and a Tumor Promoter
Takaki Hiwasa, Toshie Sawada and Shigeru Sakiyama

J. Biol. Chem. 1996, 271:9181-9184.
doi: 10.1074/jbc.271.16.9181

Access the most updated version of this article at http://www.jbc.org/content/271/16/9181

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 29 references, 15 of which can be accessed free at http://www.jbc.org/content/271/16/9181.full.html#ref-list-1