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STRUCTURAL ANALYSIS OF A MATURE *hst-1* PROTEIN WITH TRANSFORMING GROWTH FACTOR ACTIVITY

Kiyoshi Miyagawa[1], Sadao Kimura[2], Teruhiko Yoshida[1], Hiromi Sakamoto[1], Fumimaro Takaku[3], Takashi Sugimura[1], and Masaaki Terada[1]

[1]Genetics Division, National Cancer Center Research Institute, 1-1, Tsukiji 5-chome, Chuo-ku, Tokyo 104, Japan

[2]Institute of Basic Medical Science, University of Tsukuba, Tsukuba, Ibaraki 305, Japan

[3]Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, 3-1, Hongo 7-chome, Bunkyo-ku, Tokyo 113, Japan

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SUMMARY: A recombinant *hst-1* protein produced in silkworm cells by a recombinant baculovirus, previously shown to be a potent mitogen for NIH3T3 cells and human endothelial cells, also stimulated anchorage-independent growth of NRK-49F cells. Amino acid sequence analysis revealed that the amino-terminal sequence with 58 amino acids was cleaved off in silkworm cells. These results indicated that the mature *hst-1* protein consisting of 148 amino acids had transforming growth factor activity.

An *hst-1* transforming gene, originally identified in DNAs from gastric cancers by its ability to cause transformation of NIH3T3 cells upon transfection (1), has now been identified from a wide variety of tumors including Kaposi's sarcoma and normal tissues as

ABBREVIATIONS: bFGF, basic fibroblast growth factor; aFGF, acidic fibroblast growth factor; kbp, kilobase-pair; HPLC, high performance liquid chromatography; kDa, kilodalton; SDS-PAGE, sodium dodecyl-sulfate gel electrophoresis; DMEM, Dulbecco's modified Eagle medium; TGF, transforming growth factor; PDGF, platelet-derived growth factor; EGF; epidermal growth factor.

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a transforming gene (2-7). The *hst-1* protein deduced from the nucleotide sequence has a significant homology with basic fibroblast growth factor (bFGF), acidic fibroblast growth factor (aFGF), mouse *int-2* protein, FGF5, the *hst-2*/FGF6 protein and keratinocyte growth factor (8-11). The *hst-1* gene is mapped to human chromosome 11q13, the same chromosomal localization for the *int-2* gene, and these two genes are co-amplified in some human cancers (12, 13), indicating that the *hst-1* and *int-2* genes are very closely located on the human chromosome. Cosmid mapping showed that these two genes are actually only 35 kbp apart with the same transcriptional orientation (14).

We have previously reported the production of the *hst-1* protein in silkworm cells using a recombinant baculovirus (15). The recombinant *hst-1* protein was purified to homogeneity with a combination of heparin affinity chromatography and reversed-phase high performance liquid chromatography (HPLC). The purified *hst-1* protein was a potent mitogen for NIH3T3 cells and human umbilical vein endothelial cells, and also induced malignant transformation of NIH3T3 cells including morphological changes and anchorage-independent growth in soft agar (15). The molecular weight of the purified protein was about 17.5 kilodalton (kDa) as judged by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), smaller than the 22.0 kDa predicted by the nucleotide sequence (16). This result suggested that the *hst-1* protein was processed in silkworm cells. We report here the primary structure of the processed *hst-1* protein with anchorage-independent growth stimulation activity on NRK-49F cells.

MATERIALS AND METHODS

Materials: The recombinant *hst-1* protein produced in silkworm cells by a recombinant baculovirus was purified to homogeneity by heparin affinity chromatography and reversed-phase HPLC as described (15).
Colony formation assay: An underlayer was established with 2 ml of

Dulbecco's modified Eagle medium (DMEM) containing 10% calf serum and 0.5% agar in a 35-mm dish. Five thousand NRK-49F cells were seeded onto the overlayer in 2 ml of DMEM containing 10% calf serum and 0.3% agar in the presence of various concentrations of the *hst-1* protein. After incubation for 8 days, the number of colonies was counted.

Amino-terminal sequence analysis: The purified recombinant *hst-1* protein was successfully recovered in the presence of 1% SDS after HPLC purification followed by lyophilization. Subsequently about 150 pmol of the *hst-1* protein was subjected to automated Edman degradation.

Carboxy-terminal sequence analysis: About 300 pmol of the purified *hst-1* protein was digested by 0.5 μg of Lysyl-endopeptidase in 50 μl of 0.1 M ammonium bicarbonate (pH 8.0) at 37°C for 4 h, and digested fragments were purified by a reversed-phase column (Chemcosorb 3ODS-H, 4.6 X 75 mm) with a linear gradient of 0 to 50% acetonitrile in the presence of 0.1% trifluoroacetic acid. A few digested fragments with high recovery were subjected to automated Edman degradation.

RESULTS

Transforming growth factor activity: As shown in Fig. 1, the recombinant *hst-1* protein induced anchorage-independent growth of NRK-49F cells in soft agar. The *hst-1* protein stimulated anchorage-independent growth of NRK-49F cells in a concentration-dependent manner (Fig. 2). The half-maximal stimulation was observed at the concentration of 0.8 ng/ml.

Amino acid sequence analysis: The amino-terminal sequence of the biologically active *hst-1* protein was Pro-Val-Ala-Ala-Gln-Pro-Lys which

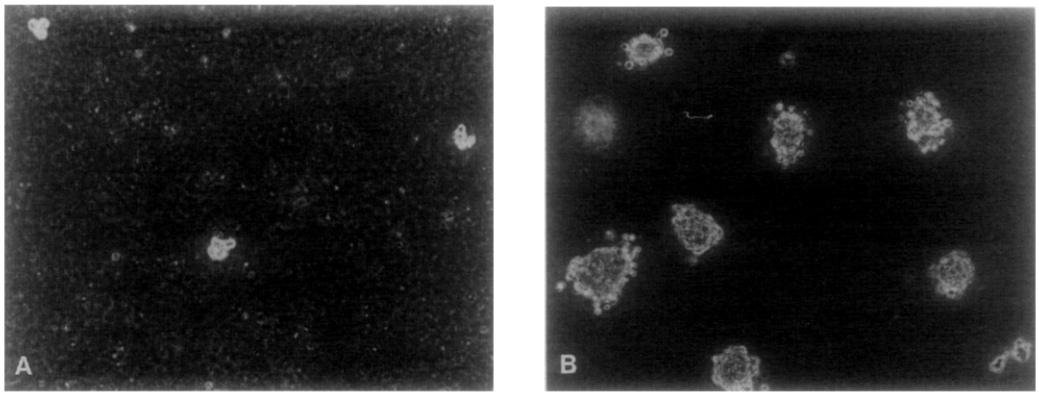

Fig. 1. Colony formation of NRK-49F cells stimulated by the *hst-1* protein in soft agar. Five thousand NRK-49F cells were incubated in the absence (A) or in the presence (B) of 10 ng/ml of the *hst-1* protein for 8 days in soft agar, and were photographed.

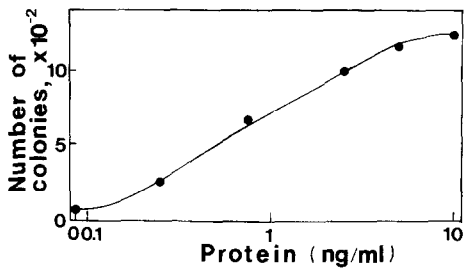

Fig. 2. Effect of the *hst-1* protein on anchorage-independent growth of NRK-49F cells. Colony formation of NRK-49F cells in soft agar at various concentrations of the *hst-1* protein was performed as described in the text. After incubation for 8 days, the number of the colonies per dish (>5,000 μm^2) was counted. Values are the means of triplicate determinations.

corresponds to the amino acid positions 59-65 of the *hst-1* protein deduced from the nucleotide sequence (16). The carboxy-terminus was also determined after digestion with Lysyl-endopeptidase. The sequence Val-Thr-His-Phe-Leu-Pro-Arg-Leu which corresponds to the amino acid positions 199-206 of the deduced *hst-1* protein was recognized. This result indicated that the carboxy-terminus of the *hst-1* was not processed. Therefore, the processed form of the *hst-1* protein consists of 148 amino acids which corresponds to the amino acid positions 59-206 with a calculated molecular weight of 16.2 kDa (Fig. 3). This molecular weight is a little smaller than 17.5 kDa as judged by SDS-PAGE (15). However, no other bands whose amounts were sufficient for Edman degradation were detected, indicating that the 17.5 kDa band actually corresponded to the *hst-1* protein with the calculated molecular weight of 16.2 kDa.

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MSGPGTAAVALLPAVLLALLAPWAGRGGAAAPTAPNGTLEAELERRWESL   50
VALSLARLPVAAQPKEAAVQSCACDYLLGIKRLRRLYCNVGIGFHLQALP  100
DGRIGGAHADTRDSLLELSPVERGVVSIFGVASRFFVAMSSKGKLYGSPF  150
FTDECTFKEILLPNNYNAYESYKYPGMFIALSKNGKTKKGNRVSPTMKVT  200
HFLPRL                                               206
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Fig. 3. Primary structure of the *hst-1* protein produced by a recombinant baculovirus. The deduced amino acid sequence is shown by a single-letter notation for amino acids. Primary structure of the recombinant *hst-1* protein determined by amino-terminal and carboxy-terminal sequence analyses is boxed. The signal peptide and the potential N-glycosylation site are indicated by underline and asterisks, respectively. Two cysteine residues conserved in FGF-related proteins are indicated by arrows.

DISCUSSION

Transforming growth factor-α (TGF-α) has an ability to induce anchorage-independent growth of NRK-49F cells (17), and transforming growth factor-β (TGF-β) also has the same ability in the presence of epidermal growth factor (EGF) or TGF-α (18). Platelet-derived growth factor (PDGF) is known to induce anchorage-independent growth of NRK-49F cells in the presence of extracts of human platelet, but PDGF alone does not elicit this effect (19). Bovine brain-derived growth factor, whose chemical and physical properties are similar to those of aFGF, also induces the same effect in the presence of EGF (20). As shown in Fig.1 and 2, the *hst-1* protein, like TGF-α, induced colony formation of NRK-49F cells in the absence of EGF, and EGF did not stimulate the activity of the *hst-1* protein (data not shown). The present study indicates that the *hst-1* protein alone, as in the case of TGF-α, has the ability to transform NRK-49F cells.

We have previously shown the structural characteristics of the *hst-1* protein predicted by the nucleotide sequence(8). Unlike bFGF and aFGF, the *hst-1* protein has a signal peptide and a potential N-glycosylation site. However, two cysteine residues of the *hst-1* protein are conserved in other homologous proteins. Furthermore, clusters of basic amino acids or groups of basic and aromatic amino acids (positions 81 to 85 and 170 to 173) which are considered to be important for heparin-binding and the inverse of minimal fibronectin cell recognition site (position 100 to 103) are found in the *hst-1* protein. As shown in Fig. 3, present structural analysis revealed that the signal peptide and the potential N-glycosylation site are cleaved off, but that the two cysteine residues and two possible functional domains described above are conserved in the processed form of the *hst-1* protein. The present study indicates that the processed *hst-1* protein with the calculated molecular weight of 16.2 kDa is biologically active. Previously, Delli Bovi *et al.* reported that the *hst-1* protein produced in COS cells was processed (7). The signal peptide was also cleaved off in COS cell, but, in contrast to the *hst-1* protein produced in silkworm cells, the potential N-glycosylation site was not cleaved off. The

processed *hst-1* protein secreted from COS cells consisted of 175 or 176 amino acids. The potential N-glycosylation site located at the amino acid positions 36 to 38 was conserved in the secreted *hst-1* protein, and this protein was glycosylated. The conditioned medium from COS cells producing the *hst-1* protein was shown to have growth promoting activity on NIH3T3 cells and bovine capillary endothelial cells. This report indicated that this form of the *hst-1* protein also has growth factor activity. However, it is unknown whether or not the *hst-1* protein produced in COS cells has a potential equal to that of the 16.2 kDa *hst-1* protein produced in silkworm cells.

Our results show that a long amino-terminal extension unique to the *hst-1* protein is cleaved off in silkworm cells. In the mature form, the 16.2 kDa *hst-1* protein shares about 40% homology with bFGF, aFGF and the selected regions of other homologous proteins. It is now necessary to analyze the biological difference of these homologous proteins in normal cell function, tumor development and embryogenesis.

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REFERENCES

1. Sakamoto, H., Mori, M., Taira, M., Yoshida, T., Matsukawa, S., Shimizu, K., Sekiguchi, M., Terada, M., and Sugimura, T. (1986) Proc. Natl. Acad. Sci. USA 83, 3997-4001.
2. Yoshida, T., Sakamoto, H., Miyagawa, K., Little, P. F. R., Terada, M., and Sugimura, T. (1987) Biochem. Biophys. Res. Commun. 142, 1019-1024.
3. Koda, T., Sasaki, A., Matsushima, S., and Kakinuma, M. (1987) Jpn. J. Cancer Res. 78, 325-328.
4. Nakagama, H., Ohnishi, S., Imawari, M., Hirai, H., Takaku, F., Sakamoto, H., Terada, M., Nagao, M., and Sugimura, T. (1987) Jpn. J. Cancer Res. 78, 651-654.
5. Yuasa, Y. and Sudo, K. (1987) Jpn. J. Cancer Res. 78, 1036-1040.
6. Sakamoto, H., Yoshida, T., Nakakuki, M., Odagiri, H., Miyagawa, K., Sugimura, T., and Terada, M. (1988) Biochem. Biophys. Res. Commun. 151, 965-972.
7. Delli-Bovi, P., Curatola, A. M., Newman, K. M., Sato, Y., Moscatelli, D., Hewick, R. M. , Rifikin, D. B., and Basilico, C. (1988) Mol. Cell. Biol. 8, 2933-2941.
8. Yoshida, T., Miyagawa, K., Odagiri, H., Sakamoto, H., Little, P. F. R., Terada, M., and Sugimura, T. (1987) Proc. Natl. Acad. Sci. USA 84, 7305-7309.

9. Zhan, X., Bates, B., Hu, X., and Goldfarb, M. (1988) Mol. Cell. Biol. 8, 3487-3495.
10. Marics, I., Adelaide, J., Raybaud, F., Mattei, M.-G., Coulier, F., Planche, J., De Lapeyriere, O., and Birnbaum, D. (1989) Oncogene 4, 335-340.
11. Finch, P. W., Rubin, J. S., Miki, T., Ron, D., and Aaronson, S. A. (1989) Science 245, 752-755.
12. Tsutsumi, M., Sakamoto, H., Yoshida, T., Kakizoe, T., Koiso, K., Sugimura, T., and Terada, M. (1988) Jpn. J. Cancer Res. 79, 428-432.
13. Yoshida, M. C., Wada, M., Satoh, H., Yoshida, T., Sakamoto, H., Miyagawa, K., Yokota, J., Koda, T., Kakinuma, M., Sugimura, T., and Terada, M. (1988) Proc. Natl. Acad. Sci. USA 85, 4861-4864.
14. Wada, A., Sakamoto, H., Katoh, O., Yoshida, T., Yokota, J., Little, P. F. R., Sugimura, T., and Terada, M. (1988) Biochem. Biophys. Res. Commun. 157, 828-835.
15. Miyagawa, K., Sakamoto, H., Yoshida, T., Yamashita, Y., Mitsui, Y., Furusawa, M., Maeda, S., Takaku, F., Sugimura, T., and Terada, M. (1988) Oncogene 3, 383-389.
16. Taira, M., Yoshida, T., Miyagawa, K., Sakamoto, H., Terada, M., and Sugimura, T. (1987) Proc. Natl. Acad. Sci. USA 84, 2980-2984.
17. Anzano, M. A., Roberts, A. B., Smith, J. M., Sporn, M. B., and De Larco, J. E. (1983) Proc. Natl. Acad. Sci. USA 80, 6264-6268.
18. Sporn, M. B., Roberts, A. B., Wakefield, L. M., and Assoian, R. K. (1986) Science 233, 532-534.
19. Assoian, R. K., Grotendorst, G. R., Miller, D. M., and Sporn, M. B. (1984) Nature 309, 804-806.
20. Huang, S. S., Kuo, M.-D., and Huang, J. S. (1986) Biochem. Biophys. Res. Commun. 139, 619-625.