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Regulation of ProMMP-1 and ProMMP-3 Activation by Tissue Factor Pathway Inhibitor-2/Matrix-Associated Serine Protease Inhibitor

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Tissue factor pathway inhibitor-2 (TFPI-2)/matrix-associated serine protease inhibitor (MSPI), a 32- to 33-kDa Kunitz-type serine protease inhibitor, inhibits plasmin and trypsin. Because plasmin and trypsin are involved in the activation of promatrix metalloproteases proMMP-1 and proMMP-3, we investigated the role of TFPI-2/MSPI in the activation of these proenzymes. Both plasmin and trypsin activated proMMP-1 by converting the 53-kDa proenzyme to the partially active 43-kDa polypeptide; this activity was inhibited by TFPI-2/MSPI. Similarly, TFPI-2/MSPI inhibited the conversion of 66-kDa proMMP-3 to the activated 45- and 30-kDa polypeptides by plasmin and trypsin. Because plasmin is involved in the physiological activation of proMMP-3, we tested whether TFPI-2/MSPI inhibits the activation of proMMP-3 by HT-1080 fibrosarcoma cells and urokinase-charged HeLa cells. We found that the inhibitor inhibited proMMP-3 activation by HT-1080 cells and urokinase-charged HeLa cells. Collectively, our results suggest that TFPI-2/MSPI indirectly regulates MMP-1- and MMP-3-catalyzed matrix proteolysis by regulating the activation of proMMP-1 and proMMP-3.

Matrix metalloprotease (MMP)-1 (interstitial collagenase) and MMP-3 (stromelysin-1/transin-1) are members of the MMP family of proteases that function in the turnover of extracellular matrix (ECM). MMP-1 degrades fibrillar collagens including types I, II, III, VII, and X, whereas MMP-3 degrades a wide spectrum of substrates including proteoglycans, laminin, fibronectin, and collagen types IV and IX (1, 2). Both enzymes are secreted as inactive proenzymes that require activation. Thus, activation of these proenzymes is a critical step that leads to ECM degradation.

The mechanisms by which secreted proMMP-1 and proMMP-3 are activated *in vivo* are not entirely clear. *In vitro,* trypsin-like proteases activate proMMP-1 and proMMP-3 by a unique stepwise mechanism (3, 4). ProMMP-1 is first converted to a 46-kDa short-lived intermediate, which then is converted to a 43-kDa partially activated MMP-1 by autolytic cleavage. MMP-3 then converts the 43-kDa MMP-1 to a 41-kDa MMP-1 that is fully active. In the case of proMMP-3, initial attack by serine proteases produces a 53-kDa intermediate that is then rapidly converted to 45- and 30-kDa active MMP-3 species (4).

In vivo, plasmin was proposed to be a potential activator of proMMP-1 and proMMP-3 (5–8). In cell model systems, degradation of collagen by synovial and gingival fibroblasts, chondrocytes, capillary endothelial cells, bone cells, and VX2 tumor cells required the presence of plasminogen (Pg) (8). Pg also was required to be present for the activation of proMMP-3 by fibroblasts (9). These results led to the hypothesis that the physiological activation of proMMP-1 and proMMP-3 likely involves a Pg-dependent activation mechanism, i.e., plasmin on the cell surface (9).

Tissue factor pathway inhibitor-2 (TFPI-2)/matrix-associated serine protease inhibitor (MSPI), a 32- to 33-kDa Kunitz-type serine protease inhibitor, is synthesized and secreted by a variety of cells including epithelial, endothelial, and mesenchymal cells (10–15). TFPI-2/MSPI inhibits a variety serine proteases in-

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Abbreviations used: CHO cells, Chinese hamster ovary cells; ECM, extracellular matrix; MMPs, matrix metalloproteases; MSPI, matrix-associated serine protease inhibitor; Pg, plasminogen; t12FB, SV-40 transformed human skin fibroblasts; TFPI-2, recombinant tissue factor pathway inhibitor-2; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; uPA, urokinase-type plasminogen activator.

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cluding trypsin, plasmin, plasma kallikrein, chymotrypsin, cathepsin G, and tissue factor–factor VIIa complex but not α-thrombin, urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (10–13, 16); predominantly associates with ECM so that the inhibitor functions at the cell:ECM interface (11–15); and inhibits matrix degradation and invasion by highly invasive tumor cells (17). In this study, we report that TFPI-2/MSPI inhibited Pg-dependent activation of proMMP-1 and proMMP-3 by tumor cells, suggesting that this inhibitor plays an indirect role in the regulation of MMP-1- and MMP-3-catalyzed ECM proteolysis.

MATERIALS AND METHODS

Materials for this study were obtained as follows. Lysine-plasminogen (Pg) and recombinant urokinase-type plasminogen activator (uPA) were gifts from Dr. Bruce Credo (Abbott Research Laboratories, Abbott Park, IL). Human recombinant TFPI-2/MSPI was a gift from Drs. Walter Kisiel (Department of Pathology, University of New Mexico, Albuquerque, NM) and Donald C. Foster (Zymogenetics Inc., Seattle, WA). Serum free conditioned media from Chinese Hamster ovary (CHO) cells containing ProMMP-3 and polyclonal anti-MMP-3 antibody were gifts from Dr. Lynn Matrisian (Department of Cell Biology, Vanderbilt University, Nashville, TN). Polyclonal anti-MMP-1 antibody was a gift from Dr. William G. Stetler-Stevenson, (Department of Pathology, National Cancer Institute, Bethesda, MD). SV-40 transformed human skin fibroblast (t12FB) cell line was gift from Dr. Charles L. Goolsby (Department of Pathology, Northwestern University, Chicago, IL). HT-1080 fibrosarcoma cells and HeLa cells were purchased from the American Type Culture Collection (Manassas, VA). The ECL Western blotting reagent kit was purchased from Amersham Life Sciences (Buckinghamshire, England).

Cell culture. HT-1080 fibrosarcoma cells, HeLa cells and t12FB were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 50 μg/ml penicillin, and 50 μg/ml streptomycin.

Activation of ProMMP-1 and proMMP-3 by plasmin and trypsin. Serum-free conditioned media (CM) from phorbol 12-myristate-13 acetate-treated t12FB or CHO cells carrying a full length proMMP-3 cDNA that is tagged with protein G-binding domain of IgG (18) were used as sources for proMMP-1 and proMMP-3, respectively. To activate proMMP-1 and proMMP-3 with plasmin, the enzyme plasmin was generated from Pg by activation with uPA. A 100-μl of the reaction mixture consisted of the following: 50 IU uPA, 0.3 μM Pg, 25 μl of a 10-fold concentrated CHO CM or a 50-fold concentrated t12FB CM and incubation buffer (15 mM Tris–HCl, pH 7.4, 0.15 M NaCl, 1 mM $CaCl_2$). The reaction mixtures were incubated for 2 h at room temperature and the reactions were terminated by adding 25 μl of 5× SDS–PAGE sample buffer. To activate proMMP-1 and proMMP-3 with trypsin, 25 μl of CHO CM or t12FB CM were first diluted to 100 μl with incubation buffer and then incubated with 0.5 μg trypsin at 2 h at room temperature. The reaction was terminated by adding SDS–PAGE sample buffer as described above. The proenzyme and activated enzyme species were identified by Western blotting. To determine whether proMMP-1 and proMMP-3 activation by plasmin or trypsin is inhibited by TFPI-2/MSPI, the activation assays were performed in the presence of 25 to 200 nM TFPI-2/MSPI.

Activation of proMMP-3 by tumor cells. HT-1080 fibrosarcoma cells or HeLa cells with or without uPA charging, were used for activating proMMP-3. HeLa cells were charged with uPA because the receptors for uPA were free to bind exogenous uPA (19). Charging the HeLa cells with uPA was done as follows. HeLa cells were grown to confluence, detached with 15 mM Tris–HCl, 0.15 M NaCl, and 1 mM EDTA, and washed once with RPMI 1640 with 0.1% bovine serum albumin (BSA). The cells (2×10^7) were incubated at room temperature with 5000 IU uPA in 5 ml of RPMI 1640 with 0.1% BSA for 30 min with end-over-end rotation. The unbound uPA was removed by washing the cells three times with Hank's balanced salt solution (HBSS) with 0.1% BSA and the cells used for activating proMMP-3. HeLa cells, uPA-charged HeLa cells or HT-1080 fibrosarcoma cells (2×10^6) were incubated with 0.4 μM Pg and 50 μl of 10-fold concentrated CHO CM for 30 min at room temperature in 400 μl of HBSS, 0.1% BSA solution with end-over-end rotation. At the end of incubation, supernatants were collected, diluted to 1× with 5× SDS–PAGE sample buffer, and a 30 μl aliquot was assayed for activated MMP-3 species by Western blotting. To determine the effect of TFPI-2/MSPI, some reactions were performed in the presence of 50 to 100 nM TFPI-2/MSPI. To assess whether the presence of Pg is essential for the activation of proMMP-3 by HT-1080 cells or uPA-charged HeLa cells, the cells were incubated with proMMP-3 in the absence of Pg.

Western blotting. Proteins were reduced with β-mercaptoethanol, boiled for 3 min, separated by SDS–PAGE with 10 to 12% polyacrylamide gels, and electroblotted onto nitrocellulose membranes as described elsewhere (20). After electroblotting, the membranes were blocked with 4% nonfat dry milk in TBST (10 mM 15 mM Tris–HCl, pH 7.40, 0.15 M NaCl, 0.05% Tween 20) for 2 h at room temperature. Then, the membranes were incubated either for 2 h at room temperature or overnight at 4°C with normal rabbit serum or anti-MMP-1 or anti-MMP-3 antibodies, diluted 1:2000 in TBST containing 1% BSA. After several washes with TBST, the membranes were incubated for 1 h with a peroxidase-conjugated secondary antibody diluted 1:3000 in TBST with 1% BSA. The immunoreactive proteins were identified by using the ECL Western blotting system according to the manufacturer's instructions. The proteins were quantified by scanning the bands with an imaging densitometer (Model GS 670, Bio-rad, Richmond, CA).

RESULTS

TFPI-2/MSPI inhibited the activation of proMMP-1 and proMMP-3 by plasmin and trypsin. To assess the role of TFPI-2/MSPI in the activation of proMMP-1 and proMMP-3 by plasmin and trypsin, we activated the proenzymes in the presence of the inhibitor and identified the activated MMP-1 and MMP-3 species by Western blotting. Incubation of the 53-kDa proMMP-1 with plasmin or trypsin resulted in its conversion to the 43-kDa partially activated MMP-1 (Figs. 1A and 1B). However, the presence of 50 to 200 nM TFPI-2/MSPI prevented the conversion of the 53-kDa proMMP-1 to the 43-kDa MMP-1 by plasmin (Fig. 1A) or trypsin (Fig. 1B). Similarly, TFPI-2/MSPI blocked the production of the 45- and 30-kDa activated MMP-3 polypeptides from the 66-kDa proMMP-3 by plasmin (Fig. 1C) or trypsin (Fig. 1D). Incubation of proMMP-1 or proMMP-3 with uPA alone or Pg alone did not produce the activated polypeptides, suggesting that plasmin is responsible for converting the 53-kDa proMMP-1 to the 43-kDa MMP-1, and the 66-kDa proMMP-3 to the 45- and 30-kDa MMP-3.

Pg and uPA were required for proMMP-3 activation by tumor cells. To assess the importance of Pg and uPA in the activation of proMMP-3, we performed

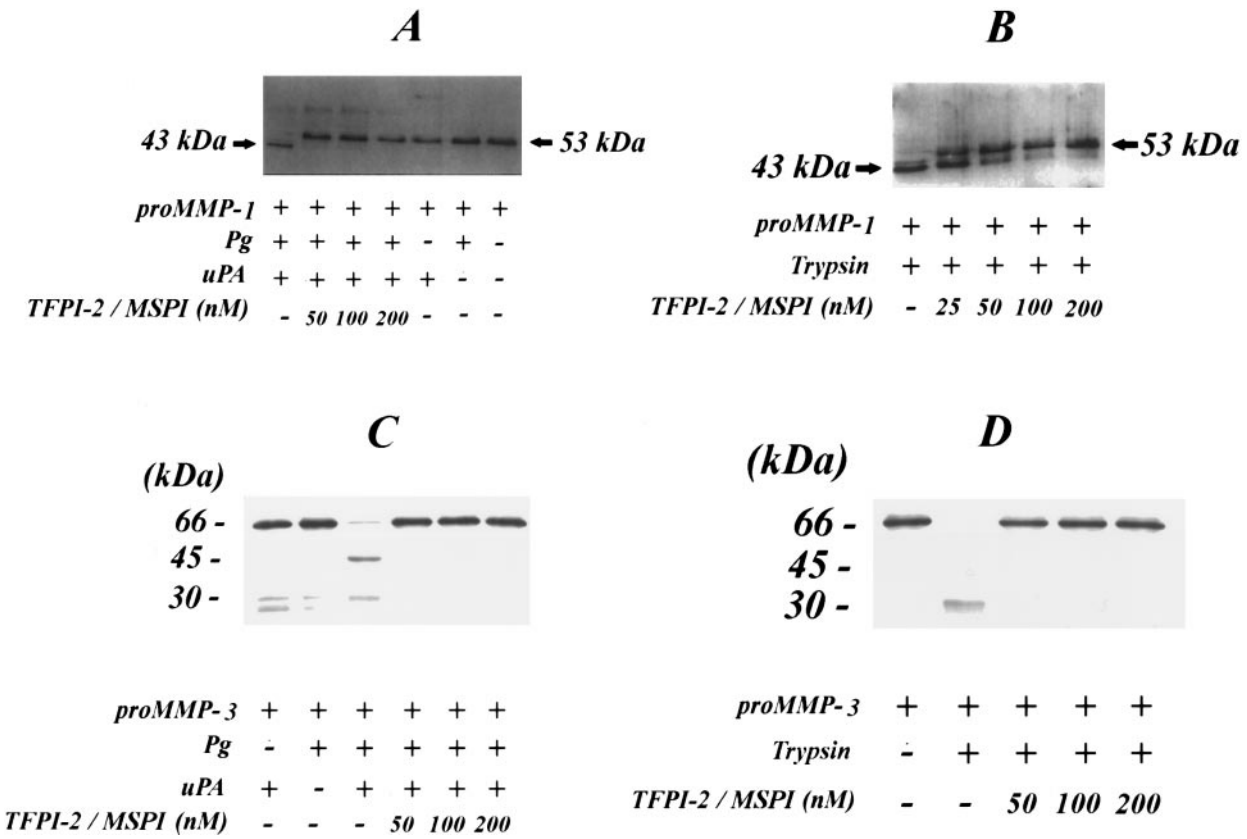

FIG. 1. TFPI-2/MSPI inhibited plasmin-or trypsin-mediated activation of proMMP-1 and proMMP-3. proMMP-1 (53-kDa) was incubated with uPA and Pg (A) or with 0.5 μg trypsin (B) in the presence (25 to 200 nM) or absence of recombinant TFPI-2/MSPI for 2 h at room temperature. The reactions were stopped with SDS–PAGE sample buffer, reduced with β-mercaptoethanol, electrophoresed on 12% acrylamide gels, and subjected to Western blotting with anti-MMP-1 antibodies. Similarly, proMMP-3 was incubated with uPA and Pg (C) or with trypsin (D) in the presence (50 to 200 nM) or absence of TFPI-2/MSPI, separated on 10% acrylamide gels by SDS–PAGE and processed for detecting the activated MMP-3 species by Western blotting with anti-MMP-3 antibodies.

proMMP-3 activation assays with HT-1080 fibrosarcoma cells and HeLa cells with or without uPA charging. Earlier studies had shown that uPA receptors on HT-1080 fibrosarcoma cells are already saturated with endogenous uPA (21), but the uPA receptors on HeLa cells are free for binding to exogenous uPA (19). Incubation of the 66-kDa proMMP-3 with HT-1080 cells resulted in the formation of 45- and 30-kDa polypeptides but only in the presence of Pg (Fig. 2A). Similar results were noted with uPA-charged HeLa cells (data not shown). These results suggest that Pg is required for the activation of proMMP-3 by tumor cells. To assess whether cell-surface uPA is required for the activation of proMMP-3, we incubated proMMP-3 with HeLa cells before or after charging with uPA. Only the uPA-charged HeLa cells converted the 66-kDa proMMP-3 to the 30-kDa activated MMP-3 polypeptides (Fig. 2B). These results suggest that cell-surface uPA is required for the activation of proMMP-3 by HT-1080 fibrosarcoma cells and HeLa cells.

TFPI-2/MSPI inhibited the activation of proMMP-3 by tumor cells. The results described above suggest that Pg activation by cell-surface uPA resulted in the conversion of the 66- kDa proMMP-3 to the 45- and

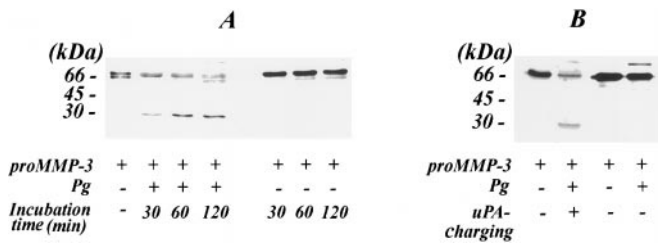

FIG. 2. Plasminogen (Pg) and cell-surface urokinase-type plasminogen activator (uPA) were essential for the activation of proMMP-3 by tumor cells. HT-1080 fibrosarcoma cells (A) and HeLa cells with or without uPA charging (B) were incubated with proMMP-3 for the indicated periods (A) or for 30 min (B) at room temperature with end-over-end rotation. The supernatants were collected, subjected to SDS–PAGE with 10% acrylamide gels under reducing conditions, and assayed for MMP-3 activated species by Western blotting.

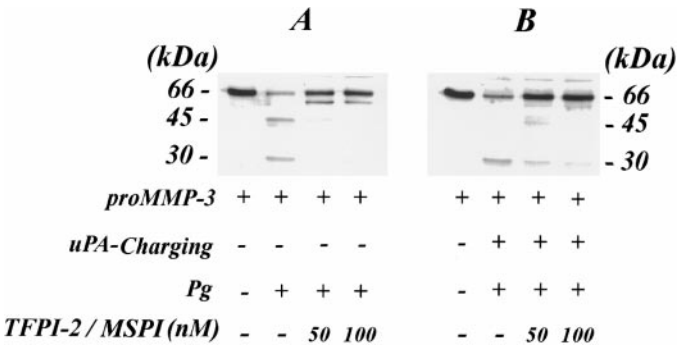

FIG. 3. TFPI-2/MSPI inhibits plasminogen-mediated proMMP-3 activation by tumor cells. HT-1080 fibrosarcoma cells (A) or urokinase-type plasminogen activator (uPA)-charged HeLa cells (B) were incubated with proMMP-3 with or without recombinant TFPI-2/MSPI for 30 min at room temperature with end-over-end rotation. The supernatants were separated by SDS–PAGE with 10% acrylamide gels under reducing conditions. Activated MMP-3 species were detected by Western blotting.

30-kDa activated MMP-3 polypeptides by HT-1080 fibrosarcoma cells and uPA-charged HeLa cells. To determine whether TFPI-2/MSPI inhibits Pg-mediated activation of proMMP-3 by tumor cells, we incubated proMMP-3 with HT-1080 cells or uPA charged HeLa cells in the presence of 50 or 100 nM inhibitor. TFPI-2/MSPI inhibited the conversion of the 66-kDa proMMP-3 to the 45- and 30-kDa polypeptides by HT-1080 fibrosarcoma cells (Fig. 3A) or uPA-charged HeLa cells (Fig. 3B). These results suggest that TFPI-2/MSPI inhibited proMMP-3 activation by tumor cells.

DISCUSSION

The activities of MMP-1 and MMP-3 are regulated extracellularly by at least two factors, first by their specific inhibitors called tissue inhibitors of metalloproteases or TIMPs (22) and second by the factors that control the activation of their proenzyme forms (3–9). The exact mechanisms by which the proMMP-1 and proMMP-3 proenzymes are activated *in vivo* are unclear but the proenzymes can be activated by factors that induce conformational changes, limited proteolysis or by autocatalysis. Of these factors, the best characterized is the serine protease plasmin which is generated from Pg by uPA or tissue-type plasminogen activator (23). In the present communication we provide evidence that the newly identified plasmin inhibitor TFPI-2/MSPI inhibits the activation of proMMP-1 and proMMP-3 by tumor cells.

Of the MMPs, MMP-3 has been proposed to play a central role in the regulation of connective tissue turnover. MMP-3, in addition to degrading multiple connective tissue components, has the unique characteristic of participating in the activation of at least two other proMMPs, one being the partially activated 43-kDa MMP-1, a product of proMMP-1 through initial cleavage by plasmin, trypsin or plasma kallikrein (24), and the other being proMMP-9 (25). Our findings suggest that the activation of proMMP-3 on the tumor cell membrane required the presence of plasmin or both Pg and uPA, and that TFPI-2/MSPI inhibited the Pg-mediated activation of proMMP-3 by tumor cells. In addition to plasmin, proMMP-3 can be activated by a number of serine proteases including trypsin, chymotrypsin, and plasma kallikrein (26, 27). Interestingly, TFPI-2/MSPI inhibits plasmin, trypsin, chymotrypsin, and plasma kallikrein with nanomolar affinity (16). Moreover, we have shown here that TFPI-2/MSPI inhibits the trypsin- and plasmin-mediated activation of both proMMP-1 and proMMP-3 as well as the proMMP-3 activation by HT-1080 cells or uPA-charged HeLa cells. From these results, we speculate that TFPI-2/MSPI can function in the negative regulation of MMP-1-, MMP-3-, and MMP-9-catalyzed matrix proteolysis (Fig. 4).

Several lines of evidence suggest that TFPI-2/MSPI is available for regulating proMMP-1 and proMMP-3 activation *in vivo.* We and others have shown that TFPI-2/MSPI and two additional glycosylation variants of this inhibitor are produced by human foreskin fibroblasts, neonatal keratinocytes (11, 12, 14), and endothelial cells derived from human umbilical vein (13), saphenous vein and dermal microvessels (15). The inhibitors also have been found in placenta (28, 29). Interestingly, more than 90% of the cell-secreted TFPI-2/MSPI and its glycosylation variants associate with the ECM (11–15) through arginine-mediated ionic interactions (C. N. Rao *et al.*, manuscript submitted). Our work revealed that the ECM-bound TFPI-2/MSPIs were functional and that recombinant TFPI-2/MSPI inhibited plasmin that was bound to HT-1080 cells as well as matrix degradation and invasion by these cells, thus suggesting the possibility that the TFPI-2/MSPI functions at the cell:ECM interface (17). Results from

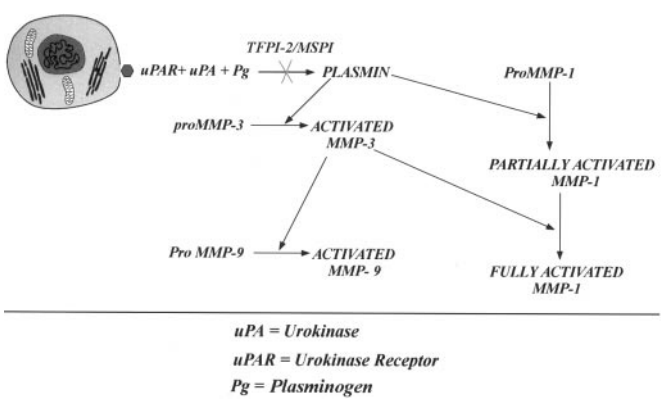

FIG. 4. Schematic representation of the site of action of TFPI-2/MSPI in the activation of proMMP-1, proMMP-3, and proMMP-9 by tumor cells.

the current study reported here indicate yet another pathway by which TFPI-2/MSPI influence connective tissue degradation; i. e. by regulating the activation of proMMP-1 and proMMP-3.

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REFERENCES

1. Powell, W. C., and Matrisian, L. M. (1996) *Curr. Topics Microbiol. Immunol.* **213,** 1–21.
2. Nagase, H. (1997) *Biol. Chem.* **378,** 151–160.
3. Suzuki, K., Enghild, J. J., Morodomi, T., Salvesen, G., and Nagase, H. (1990) *Biochemistry* **29,** 10261–10270.
4. Nagase, H., Enghild, J. J., Suzuki, K., and Salvesen, G. (1990) *Biochemistry* **29,** 5783–5789.
5. Eeckhout, Y., and Vaes, G. (1977) *Biochem. J.* **166,** 21–31.
6. Werb, Z., Mainardi, C. L., Vater, C. A., and Harris, E. D. (1977) *N. Engl. J. Med.* **296,** 1017–1023.
7. He, C., Wilhelm, S. M., Pentland, A. P., Marmer, B. L., Grant, G. A., Eisen, A. Z., and Goldberg, G. I. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 2632–2636.
8. Gavrilovic, J., and Murphy, G. (1989) *Cell Biol. Int. Rep.* **13,** 367–375.
9. Murphy, G., Atkinson, S., Ward, R., Gavrilovic, and Reynolds, J. J. (1992) *Ann. N.Y. Acad. Sci.* **667,** 1–13.
10. Sprecher, C. A., Kisiel, W., Mathewes, S., and Foster, D. C. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 3353–3357.
11. Rao, C. N., Peavey, C. L., Liu, Y. Y., Lapiere, J. C., and Woodley, D. T. (1995) *J. Invest. Dermatol.* **104,** 379–383.
12. Rao, C. N., Liu, Y. Y., Peavey, C. L., and Woodley, D. T. (1995b) *Arch. Biochem. Biophys.* **317,** 311–314.
13. Rao, C. N, Gomez, D. E., Woodley, D. T., and Thorgeirsson, U. P. (1995) *Arch. Biochem. Biophys.* **319,** 55–62.
14. Rao, C. N., Prasad Reddy, Liu, Y. Y., O'Toole, E. A. O., Reeder, D. J., Foster, D. C., Kisiel, W., and Woodley, D. T. (1996) *Arch. Biochem. Biophys.* **335,** 82–92.
15. Lino, M., Foster, D. C., and Kisiel, W. (1998) *Arterioscler. Thromb. Vasc. Biol.* **18,** 40–46.
16. Peterson, L. C., Sprecher, C. A., Foster, D. C., Blumburg, H., Hamamoto, T., and Kisiel, W. (1996) *Biochemistry* **35,** 266–272.
17. Rao, C. N., Liu, Y. Y., Krishna, C., Stack, S. M., Kisiel, W., Foster, D. C., and Woodley, D. T. (1998) *Int. J. Cancer* **67,** 749–756.
18. Sanchez-Lopez, R., Nicholson, R., Gensel, M-C., Matrisian, L. M., and Breathnach, R. (1988) *J. Biol. Chem.* **263,** 11892–11899.
19. Ossowski, L. (1988) *J. Cell. Biol.* **107,** 2437–2445.
20. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
21. Stephens, R. W., Pollanen, J., Tapiovaara, H., Leung, P. C., Sim, P., Salonen, E. M., Ronne, E., Behrendt, N., Dano, K., and Vaheri, A. (1989) *J. Cell Biol.* **108,** 1987–1997.
22. Gomez, D. E., Alonso, D. F., Yoshiji, H., and Thorgeirsson, U. P. (1997) *Eur. J. Cell Biol.* **74,** 111–122.
23. Vassalli, J. D., Sappino, A. P., and Belin, D. (1991) *J. Clin. Invest.* **88,** 1067–1072.
24. Ito, Y., and Nagase, H. (1988) *Arch. Biochem. Biophys.* **267,** 211–216.
25. Okada, Y., Enghild, J. J., and Nagase, H. (1992) *J. Biol. Chem.* **267,** 3581–3584.
26. Okada, Y., Harris, E. D., Jr., and Nagase, H. (1988) *Biochem. J.* **254,** 731–714.
27. Chin, J. R., Murphy, G., and Werb, Z. (1988) *J. Biol. Chem.* **260,** 12307–12376.
28. Butzow, R., Huhtala, M. L., Bohn, H., Virtenin, I., and Seppala, M. (1988) *Biochem. Biophys. Res. Commun.* **150,** 483–490.
29. Kisiel, W., Sprecher, C. A., and Foster, D. C. (1994) *Blood* **84,** 4384–4385.