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Independent Expression and Cellular Processing of M_r 72,000 Type IV Collagenase and Interstitial Collagenase in Human Tumorigenic Cell Lines

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ABSTRACT

The regulation of M_r 72,000 type IV collagenase and interstitial collagenase expression was studied *in vitro*. Three tumorigenic human cell lines were used, together with human fetal lung fibroblasts as a nontumorigenic control. M_r 72,000 type IV collagenase was expressed constitutively by all four cell lines, whereas only A2058 melanoma cells exhibited constitutive expression of interstitial collagenase. Treatment of cells with transforming growth factor β1 (TGF-β1) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) revealed an opposite pattern of regulation of these two metalloproteinases. Specifically, TPA increased interstitial collagenase mRNA levels in each cell line and decreased type IV collagenase mRNA levels in control fibroblasts and the tumorigenic cell lines, HT-1080 and A2058. TGF-β1 treatment increased type IV collagenase mRNA levels in each cell line and decreased interstitial collagenase mRNA levels in A2058 melanoma cells.

Interstitial collagenase mRNA induction was accompanied in all cell lines by elevated interstitial procollagenase in the conditioned medium, as detected by zymography. Changes in M_r 72,000 type IV collagenase expression revealed a more complex pattern of regulation. TPA and TGF-β1 treatment of HT-1080 cells resulted in the appearance of two bands of gelatinolytic activity with a molecular weight of approximately 62,000 and 59,000. The M_r 62,000 species was also induced by TGF-β1 treatment of A2058 cells. Addition of affinity-purified radiolabeled M_r 72,000 type IV procollagenase to TPA-treated HT-1080 cells demonstrated that both species were products of the M_r 72,000 proenzyme and that exogenous proenzyme could be processed by these cells. Western blot analysis with specific antipeptide antibodies revealed that both the M_r 62,000 and 59,000 species were derived from the M_r 72,000 proenzyme by amino-terminal cleavage. There was no evidence for cellular processing of either interstitial procollagenase or the M_r 92,000 type IV procollagenase. These results demonstrate that the M_r 72,000 type IV collagenase is under the control of different regulatory elements from interstitial collagenase, at the level of both mRNA expression and cellular processing, and that this processing appears to be the result of a phorbol ester and TGF-β1-inducible cellular activation mechanism. The ratio of active enzyme species to latent M_r 72,000 proenzyme may provide a better correlation with invasive potential than overall levels of this widely expressed metalloproteinase.

INTRODUCTION

Matrix metalloproteinases (EC 3.4.24.7) are now known to constitute a family of structurally related zymogens, capable of degrading a wide variety of extracellular matrix components, including interstitial and basement membrane collagens and glycoproteins (1–7). These metalloproteinases include interstitial collagenase (1, 2), stromelysin (3, 4, 7), transin, the rat homologue of stromelysin (8, 9), stromelysin-2 (6), Pump-1 (6, 10), M_r 72,000 type IV collagenase (5, 11, 12), and the more recently described M_r 92,000 type IV collagenase (13). Each is synthesized and secreted as a latent proenzyme requiring the removal of an 80-amino acid amino-terminal domain in order to attain activity (3, 5, 14).

The synthesis, secretion, and activation of these zymogens, together with the synthesis and secretion of their inhibitors, TIMP-1[2] (15, 16) and TIMP-2 (17–19), are key regulatory steps in the remodeling of extracellular matrix that occurs in both normal and pathological processes such as wound healing, angiogenesis, rheumatoid arthritis, and tumor invasion (7, 20). However, it is unclear to what extent the expression of the different matrix metalloproteinases is coordinately regulated. Previous studies have demonstrated a parallel induction of interstitial collagenase and stromelysin in fibroblasts following treatment with TPA (21–23), bFGF (24), and heat shock (25), although, in a recent study, these two metalloproteinases were found to be noncoordinately regulated (26). There are also data demonstrating the independent regulation of M_r 72,000 type IV collagenase secretion by TGF-β (27), although there has been no such analysis of M_r 72,000 type IV collagenase mRNA expression.

Another unresolved area is the physiological mechanism of activation of matrix metalloproteinases, which are secreted as latent proenzymes. M_r 72,000 type IV procollagenase can be activated *in vitro* by treatment with the organomercurial, p-APMA, which induces the autoproteolytic removal of an 80-amino acid amino-terminal domain and yields a M_r 62,000 active enzyme (14). Plasmin (28) and stromelysin (29) have been shown to activate interstitial collagenase *in vitro*, also by removal of an amino-terminal domain, and *in vivo* mechanisms based on this ability have been proposed. However, neither plasmin nor stromelysin appears to be an effective activator of the M_r 72,000 type IV procollagenase, and its physiological activation mechanism remains a focus of much current research (30).

In the current study the expression of M_r 72,000 type IV collagenase and interstitial collagenase was studied in human cell lines of varying invasive potential. Specifically, the extent of constitutive expression, the effect of chemical and cytokine modulation, and the secretion and activation of these two metalloproteinases were compared. Three tumorigenic cell lines and one nontumorigenic control were selected, ranging in invasive potential from the highly invasive HT-1080 fibrosarcoma cells to the poorly invasive nontumorigenic WI-38 fibroblasts (31). Four agents known to modulate the expression of interstitial collagenase mRNA were also selected. IL-1 has been shown to induce interstitial collagenase mRNA and protein in human synovial and foreskin fibroblasts (32, 33). TPA has been shown to induce interstitial collagenase mRNA and protein in rabbit synovial fibroblasts (22, 34, 35) and human skin fibroblasts (36, 37) as well as interstitial collagenase protein and secreted activity in human monocytic U937 tumor cells (38). TGF-β, a negative regulator, blocks EGF and bFGF induction of interstitial collagenase mRNA in human fetal lung fibroblasts

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[2] The abbreviations used are: TIMP, tissue inhibitor of metalloproteinases; p-APMA, *p*-aminophenylmercuric acetate; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-1α, interleukin 1α; SDS, sodium dodecyl sulfate; TGF-β1, transforming growth factor β1; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; cDNA, complementary DNA.

(24) and EGF induction of transin mRNA in rat fibroblasts (9), and it directly suppresses interstitial collagenase synthesis and secretion in human gingival fibroblasts (27). Retinoic acid, also a negative regulator of this enzyme, has been reported to decrease interstitial collagenase mRNA and protein levels in both rabbit synovial fibroblasts (34) and human skin fibroblasts (39).

We report here the differential expression and regulation of M_r 72,000 type IV collagenase and interstitial collagenase, at the level of both mRNA and secreted activity. We also demonstrate specific amino-terminal processing of the M_r 72,000 latent proenzyme. This processing appears to be the result of a phorbol ester- and cytokine-inducible cellular activation mechanism.

MATERIALS AND METHODS

Growth Factors and Reagents. Human recombinant IL-1α and porcine TGF-β1 were obtained from R & D Systems (Minneapolis, MN). TGF-β1 was held at −70°C as a 5-μg/ml stock in 4 mM HCl containing 1 mg/ml of bovine serum albumin. TPA and all-*trans*-retinoic acid were obtained from Sigma Chemical Company (St. Louis, MO) and dissolved in dimethyl sulfoxide and 95% (v/v) ethanol, respectively. Final concentrations of these solvents in culture medium were 0.01% and 0.1% (v/v), respectively. Aprotinin and ϵ-amino-*n*-caproic acid were also obtained from Sigma. TIMP-2 was isolated from A2058 human melanoma cells and purified as previously described (17). All other chemicals were commercially available analytical grade reagents.

Cell Culture. WI-38 (human embryonic lung fibroblasts), HT-1080 (human fibrosarcoma cells), and HT-144 (human melanoma cells) were obtained from American Type Culture Collection (Rockville, MD). A2058 (human melanoma cells) were derived from a brain metastasis (40). All cells were grown to 80% confluence in DMEM (Gibco Laboratories, Grand Island, NY), supplemented with 10% (v/v) fetal bovine serum (Hyclone Laboratories, Logan, UT) and 25 μg/ml of Gentamycin (Gibco). The medium was then replaced with DMEM supplemented with 0.5% (v/v) ITS$^+$ (Collaborative Research, Inc., Bedford, MA) and 25 μg/ml of Gentamycin. The medium was changed after 4 h, and the culture was continued for a further 20 h prior to the addition of IL-1α (100 pg/ml) (41), TPA (10 ng/ml) (34), TGF-β1 (5 ng/ml) (24), or retinoic acid (0.3 μg/ml) (34). These concentrations were selected on the basis of previous studies on the modulation of interstitial collagenase and were below levels believed to cause nonspecific or cytotoxic effects. Conditioned medium was collected after 24 h. Fresh medium with modulating agent was added after 24 h to allow the collection of a further 24-h conditioned medium. Conditioned medium was centrifuged at 1000 × *g* for 5 min to remove cells and debris and then stored at −20°C.

RNA Isolation and Northern Blot Analysis. Total cytoplasmic RNA was isolated as described by Gough (42) after 24 h of treatment. Yields, as determined by spectroscopic analysis, were approximately 10 to 15 μg/10^6 cells. Aliquots (5 μg) of RNA were applied to formaldehyde/1% (w/v) agarose gels, electrophoretically separated (43), and transferred onto Nytran filters (Schleicher & Schuell, Keene, NH). Following UV cross-linking (Stratalinker 2400; Stratagene, La Jolla, CA), RNA was hybridized to a ^{32}P-labeled probe as described by Church and Gilbert (44). After extensive washing at 65°C (44), filters were exposed to X-OMAT AR X-ray film at −70°C. A cDNA probe for M_r 72,000 type IV collagenase (1.1 kilobase) was derived from a clone obtained by screening a human placental cDNA library.[3] A full-length cDNA probe for interstitial collagenase (2.2 kilobases) was derived from a clone obtained by screening an A2058 human melanoma cDNA library.[4] The identity of both probes was confirmed by direct sequence analysis. The probe for rat GAPDH was as described by Fort *et al.* (45). Probes were labeled with α-[^{32}P]dCTP (6000 Ci/mmol; NEN Research Products, Boston, MA) using a random primer labeling kit (Bethesda Research Laboratories, Gaithersburg, MD). Radiolabeled bands were quantitated from X-ray film by laser densitometry (Ultroscan 2202; LKB Bromma, Uppsala, Sweden). A series of short exposures were used to compensate for differences in message intensity and to ensure that image intensity fell within the linear range of the densitometer.

Gelatin Zymography. Gelatinolytic activity was assayed by the method of Heussen and Dowdle (46) as adapted for a minigel format. Aliquots of conditioned medium (5 μl) were mixed with sample buffer and applied directly, without prior heating or reduction, to 9% (w/v) acrylamide gels containing 1 mg/ml of gelatin (ICN Biochemicals, Cleveland, OH). After removal of SDS from the gel by incubation in 2.5% (v/v) Triton X-100 for 30 min, the gels were incubated at 37°C for 6 to 14 h in 50 mM Tris-HCl, pH 7.6, containing 0.2 M NaCl, 5 mM $CaCl_2$, and 0.02% (w/v) Brij-35. Gels were stained for 3 h in 30% methanol/10% glacial acetic acid containing 0.5% (w/v) Coomassie Brilliant Blue G-250 and destained in the same solution without dye. The gelatinolytic activity of each collagenase was evident as a clear band against the blue background of stained gelatin.

Western Blot Analysis. Aliquots of conditioned medium were concentrated 10-fold using an Ultrafree-MC filter (Millipore Products Division, Bedford, MA) and 10 μl were applied to 10 to 20% (w/v) acrylamide gradient gels (Enprotech, Hyde Park, MA). Following electrophoresis, samples were electroblotted onto Immobilon P membranes (Millipore) at 30 V overnight at 4°C. Peptides corresponding to Regions 1 to 17 and 75 to 94 of the M_r 72,000 type IV procollagenase were synthesized on a Biosearch 9600 peptide synthesizer (Biosearch, Inc., Nevato, CA). Rabbit polyclonal antibodies to these peptides were prepared and affinity purified as previously described (47). A polyclonal antibody was also raised against purified native M_r 72,000 type IV procollagenase. Rabbit primary antibodies were detected with a goat anti-rabbit immunoglobulin G-horseradish peroxidase complex and substrate kit (Bio-Rad Laboratories, Richmond, CA). Prestained high-molecular-weight markers (Bethesda Research Laboratories) were run on each gel.

Biosynthetic Labeling and Affinity Purification of M_r 72,000 Type IV Collagenase. WI-38 fibroblasts were cultured for 20 h in serum-free DMEM containing 1 μg/ml of cold methionine and 50 μCi/ml of [^{35}S]methionine (Amersham Corporation, Arlington Heights, IL). The medium was collected, and the M_r 72,000 type IV collagenase was purified by gelatin-Sepharose (Sigma) affinity chromatography. The affinity resin was washed extensively in 50 mM Tris-HCl, pH 7.6, containing 0.5 M NaCl, 5 mM $CaCl_2$, and 0.02% (w/v) Brij-35, and the bound proenzyme was eluted in the same buffer containing 10% (v/v) dimethyl sulfoxide. The radiolabeled M_r 72,000 proenzyme was precipitated with an equal volume of a saturated solution of ammonium sulfate, resuspended in phosphate-buffered saline, and dialyzed against the same. Purified radiolabeled M_r 72,000 type IV collagenase was added to HT-1080 cells (30,000 cpm/ml) cultured in the absence or presence of 10 ng/ml of TPA as described. Aliquots of 24-h-conditioned medium were analyzed by electrophoresis on 10 to 20% acrylamide gradient gels (Enprotech) in the presence of SDS and processed for fluorography with Autofluor (National Diagnostics, Manville, NJ) according to the manufacturer's instructions.

RESULTS

Differential Regulation of M_r 72,000 Type IV and Interstitial Collagenase mRNA Expression. Northern blot analysis of total cytoplasmic RNA obtained after 24 h of chemical/cytokine treatment revealed marked differences in the steady-state levels of M_r 72,000 type IV collagenase and interstitial collagenase mRNAs (Fig. 1). The relative abundance of each mRNA is given in Table 1, which shows absolute values obtained by laser densitometry. The exposures used to obtain these data were shorter than those shown in Fig. 1 to ensure that readings fell

[3] A. T. Levy, L. A. Liotta, and W. G. Stetler-Stevenson, manuscript submitted for publication.

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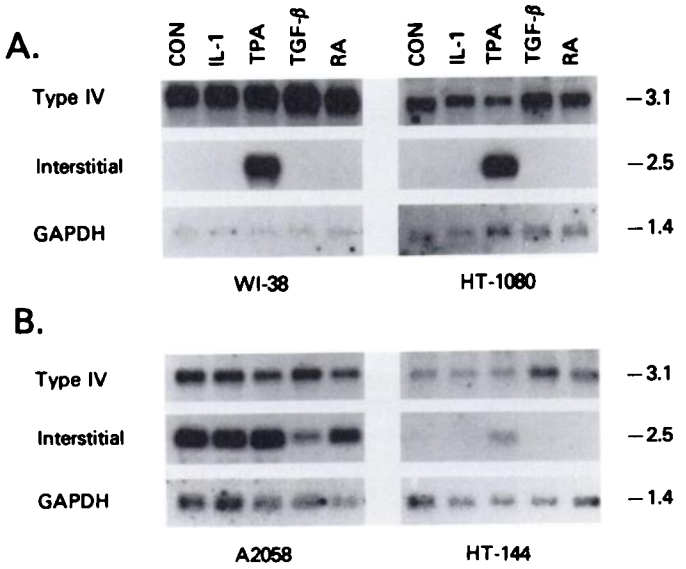

Fig. 1. Northern blot analysis of M_r 72,000 type IV collagenase and interstitial collagenase mRNA expression in WI-38 and HT-1080 cells (*A*) and A2058 and HT-144 melanoma cells (*B*). Cells were treated with IL-1α (100 pg/ml), TPA (10 ng/ml), TGF-β1 (5 ng/ml), or retinoic acid (*RA*) (0.3 μg/ml) for 24 h. *CON*, untreated. Aliquots of total cytoplasmic RNA (5 μg) were electrophoresed and blotted as described in "Materials and Methods." RNA was hybridized to ^{32}P-labeled probes specific for M_r 72,000 type IV collagenase, interstitial collagenase, and GAPDH. The resulting autoradiograph is shown with the approximate sizes given in kilobases. X-ray films were exposed to the blot for 2 days (interstitial), 3 days (type IV), 6 days (type IV, HT-144), and 14 days (GAPDH).

Table 1 *Quantitation of Northern blot analysis by laser densitometry*

Blots were exposed to XAR film for shorter periods than those shown in Fig. 1 to ensure that image intensity fell within the linear range. The bands were quantitated by laser densitometry.

	WI-38	HT-1080	A2058	HT-144
Type IV				
Control	0.81[a] (1.00)[b]	1.51 (1.00)	1.30 (1.00)	1.72 (1.00)
IL-1	0.68 (0.86)	1.03 (0.67)	0.94 (0.59)	0.64 (0.61)
TPA	0.70 (0.79)	0.50 (0.25)	0.39 (0.41)	1.00 (0.97)
TGF-β	1.82 (1.47)	2.51 (1.48)	1.22 (1.47)	3.87 (3.37)
RA[c]	0.96 (0.68)	1.51 (1.35)	0.35 (1.18)	3.37 (1.86)
GAPDH				
Control	0.23	0.86	1.49	1.35
IL-1	0.22	0.88	1.82	0.83
TPA	0.25	1.14	1.08	0.81
TGF-β	0.35	0.96	0.94	0.90
RA	0.40	0.63	0.34	1.42
Interstitial				
Control			3.23 (1.00)	
IL-1			3.29 (0.83)	
TPA			4.09 (1.74)	
TGF-β			0.32 (0.16)	
RA			1.29 (1.75)	

[a] Each band was scanned in three positions, and the peak areas were averaged to give the values presented. Units of peak area are arbitrary.

[b] Numbers in parentheses, values derived by first normalizing peak areas to the corresponding values for GAPDH and subsequently expressed as ratios to an untreated control value of 1.00.

[c] RA, retinoic acid.

within the linear range of the densitometer. Values for collagenase mRNA were normalized against GAPDH mRNA, and modulations induced by chemical agents or cytokines were expressed as a ratio against an untreated control value of 1.00.

These data reveal several important differences between M_r 72,000 type IV collagenase and interstitial collagenase mRNA expression. Most notably, all of the cell lines tested, tumorigenic and nontumorigenic, showed basal levels of type IV collagenase mRNA expression, whereas only A2058 melanoma cells showed constitutive levels of interstitial collagenase mRNA. Also, whereas TPA treatment caused a 1.7-fold increase in interstitial collagenase mRNA in A2058 cells and a dramatic induction of this mRNA in the other three cell lines, the same treatment down-regulated M_r 72,000 type IV collagenase mRNA expression in three of the four cell lines, with a 4-fold decrease in HT-1080 fibrosarcoma cells. TGF-β1 treatment of A2058 cells resulted in a 6-fold decrease in interstitial collagenase mRNA, consistent with previous studies showing TGF-β1 suppression of EGF induction of interstitial collagenase mRNA (24). In marked contrast, the same treatment caused a modest increase in the expression of type IV collagenase mRNA in all four cell lines.

IL-1α negatively regulated type IV collagenase mRNA expression in the three tumorigenic cell lines and to a lesser extent in WI-38 fibroblasts. Somewhat surprisingly IL-1α failed to increase or induce interstitial collagenase mRNA in any of the four cell lines tested. Retinoic acid treatment resulted in differing effects, decreasing type IV collagenase mRNA expression in WI-38 fibroblasts while increasing the expression of this mRNA in HT-144 melanoma cells (Table 1). It is also important to note that the modulations of M_r 72,000 type IV collagenase mRNA were small, especially when compared with the dramatic inductions of interstitial collagenase mRNA. This may be related to the constitutive nature of its expression.

Independent Regulation of Secreted Collagenases. Concomitant changes in the secreted levels of M_r 72,000 type IV collagenase and interstitial collagenase were studied by quantitative gelatin zymography (46). Preliminary experiments demonstrated that most of the M_r 72,000 type IV collagenase and interstitial collagenase activity was secreted, and only small amounts were associated with the cell membrane or cell lysate (data not shown). Thus, serum-free conditioned media were used to monitor changes in collagenase activity. Interstitial collagenase exhibits poorer gelatinolytic activity than does the M_r 72,000 type IV collagenase and was underrepresented in the zymographic assay. Therefore separate zymograms were run for each collagenase, with 10-fold-concentrated conditioned medium being used for the detection of interstitial collagenase.

Zymograms of conditioned medium taken from cells after 24 h of chemical or cytokine treatment showed fundamental differences between M_r 72,000 type IV collagenase and interstitial collagenase expression. Excellent correlation was found to exist between interstitial collagenase mRNA and interstitial collagenase secretion as monitored by functional gelatinolytic activity. This metalloproteinase was detected only in WI-38, HT-1080, and HT-144 cell-conditioned media following TPA treatment and constitutively in A2058 conditioned medium (Fig. 2*A*). These results are identical to those found at the mRNA level. Only the M_r 54,000 interstitial procollagenase was noted, and no lower molecular weight activated forms were detected.

Changes in the secretion of the M_r 72,000 type IV procollagenase were more complex. All four cell lines produced and secreted this metalloproteinase constitutively. Untreated A2058 melanoma cells produced only a single form of the M_r 72,000 type IV collagenase, which has previously been identified as the M_r 72,000 latent proenzyme (14). This latent enzyme was also the only species of gelatinolytic activity detected in the conditioned medium of WI-38 fibroblasts and HT-144 melanoma cells. The latent form is active in the zymographic assay because of its partial and reversible denaturation by SDS (27, 48). As with the modulations of mRNA expression, chemical/cytokine-induced changes in the levels of secreted enzyme were small. TGF-β1 treatment resulted in an increased secretion of this

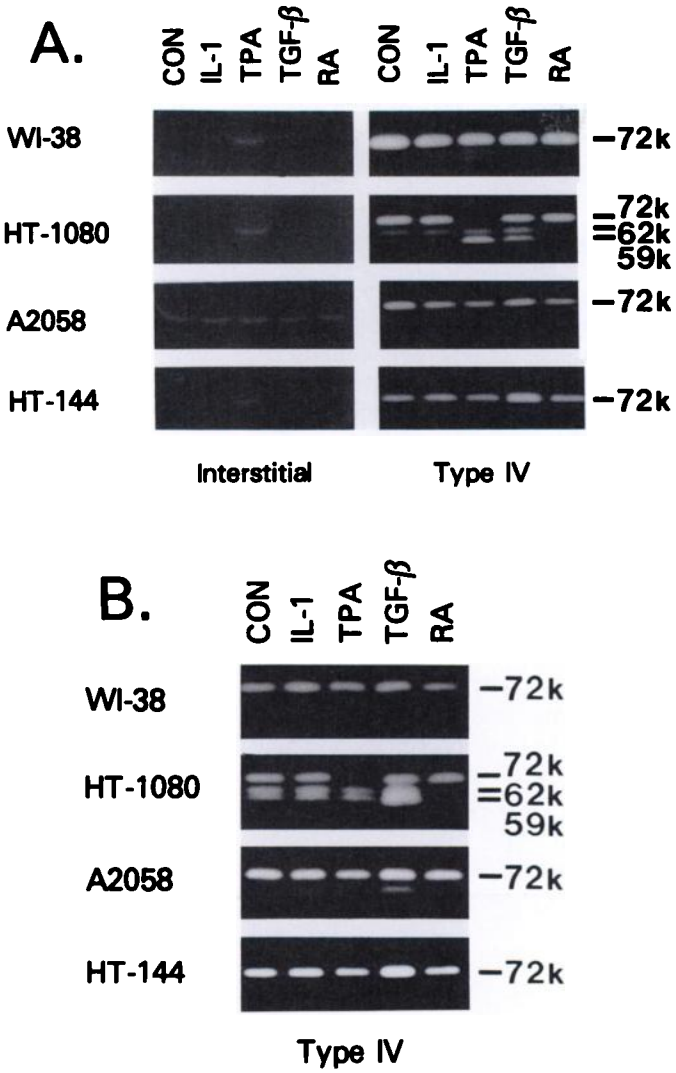

Fig. 2. Zymographic analysis of interstitial and M_r 72,000 type IV collagenase levels in serum-free conditioned medium. Cells were treated for 24 (*A*) and 48 (*B*) h with IL-1α (100 pg/ml), TPA (10 ng/ml), TGF-β1 (5 ng/ml), or retinoic acid (*RA*) (0.3 μg/ml), and aliquots (5 μl) of conditioned medium were electrophoresed on gelatin containing polyacrylamide gels in the presence of SDS. Following removal of the SDS, zymograms were incubated for 14 h at 37°C. The samples of conditioned medium used in the detection of interstitial collagenase were concentrated 10-fold prior to electrophoresis. Approximate molecular weights are given for the M_r 72,000 type IV collagenase and related bands. Only a single band of interstitial procollagenase was detected, with an approximate molecular weight of 54,000. *CON*, untreated.

proenzyme form into the culture medium of HT-144 cells, and TPA treatment reduced the amount of proenzyme secreted by A2058 cells (Fig. 2*A*). Both effects are in agreement with those demonstrated at the mRNA level.

More striking, however, was the presence of two bands of gelatinolytic activity of slightly lower molecular weight than that of the M_r 72,000 latent proenzyme. These were detectable in the conditioned medium of untreated HT-1080 fibrosarcoma cells. The larger of the two bands was assigned a molecular weight of 62,000 based on its comigration with the M_r 62,000 active enzyme produced by organomercurial treatment (as demonstrated in Fig. 4). The smaller of the two bands had an estimated molecular weight of 59,000. TPA treatment of these cells resulted in a marked decrease in the secretion of the M_r 72,000 proenzyme and an enhanced production of the M_r 62,000 and 59,000 species (Fig. 2*A*). TGF-β1 treatment also increased production of these two species, most notably by 48 h (Fig. 2*B*), without a concomitant reduction in the M_r 72,000 proenzyme. Additionally, TPA treatment of WI-38 fibroblasts resulted in the production of both M_r 62,000 and 59,000 species (Fig. 2*A*), and TGF-β1 treatment of A2058 melanoma cells led to the production of the M_r 62,000 species (Fig. 2*B*). Treatment of HT-1080 cells with retinoic acid suppressed the expression of the M_r 62,000 and 59,000 species. This is consistent with previous studies that have demonstrated the ability of retinoic acid to reduce net type IV collagenolytic activity and invasion through reconstituted basement membrane (49).

Characterization of Cytokine-induced Gelatinolytic Activities. The relation of the M_r 62,000 and 59,000 species to the M_r 72,000 latent proenzyme was examined by the addition of affinity-purified, biosynthetically [^{35}S]methionine-labeled, M_r 72,000 proenzyme to cultures of HT-1080 cells (30,000 cpm/ml). The HT-1080 conditioned medium was collected after 24 h of culture in the absence or presence of TPA (10 ng/ml). Analysis of the radiolabeled metalloproteinase by fluorography revealed that the TPA-treated cells had partially converted the M_r 72,000 proenzyme to the M_r 62,000 and 59,000 species. No conversion was evident in the conditioned medium from untreated cells (Fig. 3). Addition of the serine protease inhibitors, ϵ-amino caproic acid (50 μg/ml) and aprotinin (200 μg/ml), failed to block this conversion process (Fig. 3). This result demonstrates that the M_r 62,000 and 59,000 species are processed forms of the M_r 72,000 type IV procollagenase and that

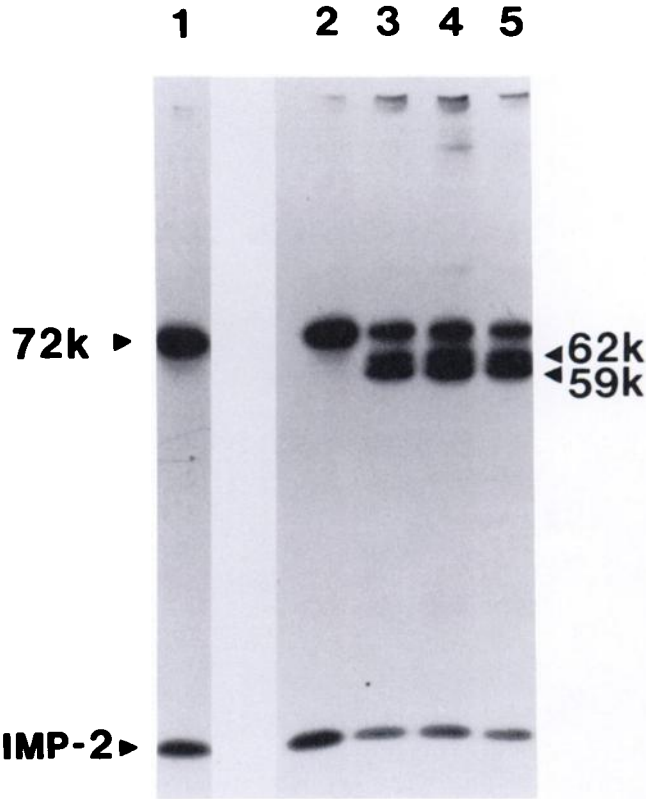

Fig. 3. Conversion of exogenous M_r 72,000 procollagenase to lower molecular weight species. Affinity-purified, biosynthetically [^{35}S]methionine-radiolabeled, M_r 72,000 type IV procollagenase was added to HT-1080 fibrosarcoma cells (30,000 cpm/ml) incubated in the absence or presence of TPA (10 ng/ml). Conditioned medium was collected after 24 h, analyzed by polyacrylamide gradient gel electrophoresis, and processed for fluorography. The resulting autoradiograph is shown. *Lane 1*, original affinity-purified, radiolabeled material; *Lane 2*, conditioned medium from untreated cells; *Lane 3*, conditioned medium from TPA-treated cells; *Lane 4*, as *Lane 3*, in the presence of 200 μg/ml of aprotinin; *Lane 5*, as *Lane 3*, in the presence of 50 μg/ml of ϵ-amino-*N*-caproic acid.

the cellular processing of this metalloproteinase probably occurs extracellularly or at the cell surface. The conversion process does not appear to be dependent on soluble serine protease activity (*i.e.*, plasmin).

The M_r 62,000 and 59,000 processed forms of the M_r 72,000 proenzyme were further analyzed by gelatin zymography and Western transfer. Treatment of the M_r 72,000 latent proenzyme with p-APMA has been shown to induce the autocatalytic removal of an 80-amino acid amino-terminal domain, resulting in conversion to a M_r 62,000 active enzyme (14). When conditioned media were analyzed by zymography, with and without p-APMA pretreatment, the M_r 62,000 activated form of the enzyme was evident in the p-APMA-treated WI-38 and A2058 conditioned media and comigrated with the M_r 62,000 species in the conditioned medium from TPA-treated HT-1080 cells (Fig. 4*A*). The M_r 62,000 and 59,000 species were unaffected by p-APMA treatment.

The M_r 92,000 type IV collagenase was also detected in the conditioned medium of HT-1080 cells and was increased by TPA treatment (Fig. 4*A*). The identity of this band has been confirmed by amino-terminal sequence analysis and recognition by anti-peptide antibodies with specificity for the amino-terminus (data not shown). p-APMA activation of the M_r 92,000 type IV collagenase was seen in the TPA-treated HT-1080 cell-conditioned medium, with partial conversion to a M_r 82,000 band. Notably, TPA treatment of the cells did not in itself result in processing of this metalloproteinase indicating that the induced cellular processing is specific for the M_r 72,000 type IV procollagenase. Inhibition studies demonstrated that all metalloproteinase activities could be inhibited by EDTA and by the recently isolated TIMP-2 (Fig. 4*B*). TIMP-2 appeared to inhibit the M_r 72,000 type IV collagenase more efficiently than the M_r 92,000 type IV collagenase. This may reflect the apparent preferential interaction of TIMP-2 with the M_r 72,000 enzyme as previously reported (17, 19).

Western blot analysis using a polyclonal antibody raised against purified native M_r 72,000 type IV procollagenase revealed the presence of both the M_r 72,000 proenzyme and the M_r 62,000 and 59,000 species in conditioned medium from TPA-treated HT-1080 cells and M_r 72,000 proenzyme in conditioned medium from untreated HT-1080 cells (Fig. 5). The polyclonal antibody did not cross-react with the M_r 92,000 type IV procollagenase but did detect a M_r 54,000 band, most probably interstitial procollagenase induced by TPA treatment. The antibody also recognized a protein with an approximate molecular weight of 30,000, which may be Pump-1. Neither the

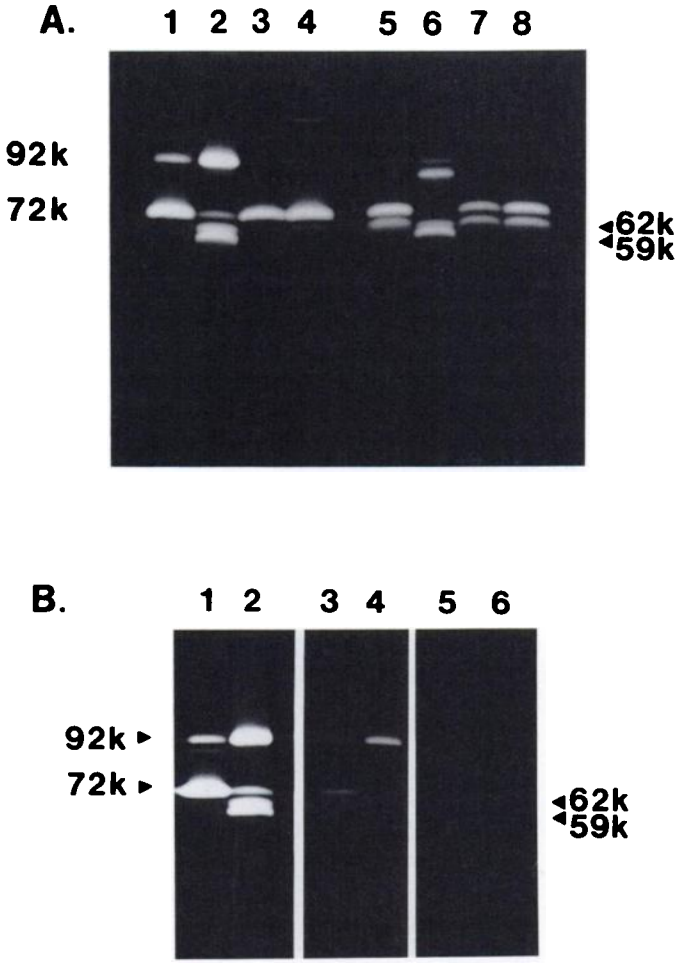

Fig. 4. Zymographic analysis of M_r 92,000 and 72,000 type IV collagenases in 24-h serum-free cell-conditioned medium. *A*, aliquots (5 μl) of conditioned medium from HT-1080 cells (*Lanes 1* and *5*), TPA-treated HT-1080 cells (10 ng/ml) (*Lanes 2* and *6*), WI-38 fibroblasts (*Lanes 3* and *7*), and A2058 cells (*Lanes 4* and *8*) incubated at 37°C for 30 min in the absence (*Lanes 1* to *4*) and presence (*Lanes 5* to *8*) of 10 mM p-APMA and electrophoresed on gelatin containing polyacrylamide gels in the presence of SDS. Following removal of the SDS, zymograms were incubated for 14 h at 37°C. *B*, conditioned medium from HT-1080 cells cultured for 24 h in the absence (*CON*, untreated control) (*Lanes 1, 3,* and *5*) and presence of TPA (10 ng/ml) (*Lanes 2, 4,* and *6*) and subjected to zymography as described. The zymogram was incubated in the absence (*Lanes 1* and *2*) or presence of TIMP-2 (10 mg/ml) (*Lanes 3* and *4*) or EDTA (10 mM) (*Lanes 5* and *6*) for 20 h at 37°C.

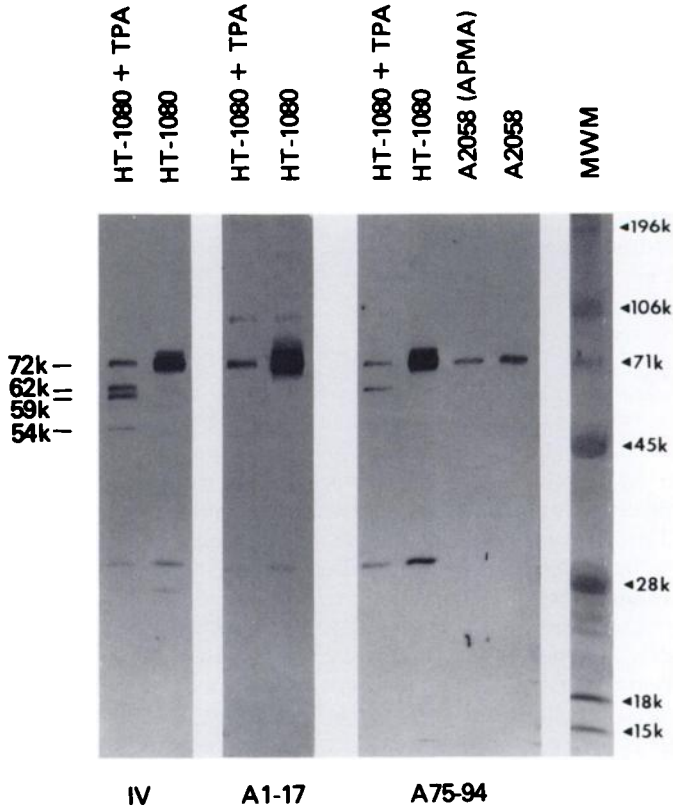

Fig. 5. Western blot analysis of M_r 72,000 type IV collagenase in 24-h serum-free conditioned medium from HT-1080 cells cultured in the absence or presence of TPA (10 ng/ml) and from untreated A2058 cells, with and without p-APMA activation. Aliquots of conditioned medium were electrophoresed on 10 to 20% (w/v) acrylamide gradient gels, electrophoretically transferred to Immobilon P membranes, and immunostained with a polyclonal antibody raised against purified native M_r 72,000 type IV procollagenase (IV) and affinity-purified antibodies to amino acyl Residues 1 to 17 and 75 to 94 of M_r 72,000 type IV procollagenase.

M_r 62,000 nor 59,000 species in the conditioned medium from TPA-treated HT-1080 cells was recognized by affinity-purified antibody (A1-17) to amino acyl Residues 1 to 17 of the M_r 72,000 proenzyme. This antibody recognized the M_r 72,000 proenzyme in the conditioned medium of both untreated and TPA-treated cells. An antibody (A75-94) to amino acyl Residues 75 to 94 recognized the M_r 72,000 proenzyme and the M_r 62,000 species in TPA-treated HT-1080 conditioned medium and the M_r 72,000 proenzyme and M_r 62,000 p-APMA-activated enzyme in A2058 conditioned medium (Fig. 5). These results indicate that the M_r 62,000 species has been processed by cleavage at the amino terminus in a manner similar to the M_r 62,000, p-APMA-generated, active enzyme. The M_r 59,000 species appears to have been further processed at the amino terminus, as demonstrated by the loss of immunoreactivity with antibodies raised against the amino-terminal domain of the M_r 62,000 active enzyme (A75-94).

DISCUSSION

The data presented demonstrate differential regulation of the M_r 72,000 type IV and interstitial collagenases. This is clearly shown in the noncoordinate expression of M_r 72,000 type IV collagenase and interstitial collagenase mRNAs. TPA treatment resulted in dramatic induction of interstitial collagenase mRNA in three cell lines and increased the constitutive levels of expression in the fourth. The same treatment decreased M_r 72,000 type IV collagenase mRNA levels in WI-38, HT-1080, and A2058 cells. Conversely, TGF-β1 treatment markedly decreased the constitutive levels of interstitial collagenase mRNA in A2058 cells and increased the expression of M_r 72,000 type IV collagenase mRNA in all four cell lines. The differential regulation of M_r 72,000 type IV and interstitial collagenase mRNA expression by TPA is consistent with the absence of TPA-responsive elements in the known 5′ promoter regions for the M_r 72,000 type IV collagenase gene (50). Such elements have been identified in the 5′-flanking region of the human interstitial collagenase gene (51). The M_r 92,000 type IV collagenase is also TPA inducible (13). Therefore the M_r 72,000 type IV collagenase gene may be unique among the currently characterized matrix metalloproteinases in its promoter, enhancer elements, and transcriptional regulation.

Induction of interstitial collagenase mRNA was paralleled by an increase in secreted enzyme. The enzyme was only secreted constitutively in A2058 cells, and there was no evidence of cellular processing or activation. By contrast, M_r 72,000 type IV procollagenase was secreted constitutively by all four cell lines and was processed to M_r 62,000 and 59,000 species following TPA treatment of WI-38 and HT-1080 cells by TGF-β1 treatment of HT-1080 and A2058 cells. This cellular processing of the M_r 72,000 type IV procollagenase is highly significant, since it occurs at the amino-terminal region which is known to be autoproteolytically removed during organomercurial activation (14). The cellular processing may therefore be the result of a natural activation process which, as demonstrated, is induced by both TPA and TGF-β1. Further determination of the specific activity of the M_r 62,000 and 59,000 processed forms will require purification of individual species by procedures that maintain native enzymatic activity and do not alter the latent state of the M_r 72,000 type IV procollagenase, which would result in autoproteolytic processing.

TPA treatment of HT-1080 cells decreased type IV collagenase mRNA and M_r 72,000 proenzyme levels and caused a transient increase in secretion of both M_r 62,000 and 59,000 species of the enzyme. Ultimately by 48 h, TPA treatment resulted in a decrease in secretion of both the M_r 72,000 proenzyme and the M_r 62,000 and 59,000 species. This correlates well with the recent observation that 48-h TPA treatment of HT-1080 cells substantially reduced net type IV collagenolytic activity (31).

Treatment with TGF-β1 resulted in increased M_r 72,000 type IV collagenase mRNA levels in all four cell lines tested. Enhanced levels of secretion were evident after 48 h in HT-1080, A2058, and HT-144 cells, although in the latter there was no evidence of cellular processing to lower molecular weight species. TGF-β1 did not, however, affect the levels of secreted M_r 72,000 type IV collagenase in WI-38 human fetal lung fibroblasts. This differs from the zymographic analysis by Overall *et al.* (27), which demonstrated that TGF-β1 increased the level of this metalloproteinase in the conditioned medium of human gingival fibroblasts, and may reflect differing responses to TGF-β1 in adult and fetal derived fibroblasts.

The role of TGF-β1 in the regulation of collagenase expression is of particular interest, since it has been demonstrated that TGF-β1 has a positive effect on the accumulation of extracellular matrix by increasing the production of extracellular matrix proteins and their receptors (52–54), by decreasing the amounts of various matrix-degrading proteinases (9, 27, 55), and by increasing the levels of proteinase inhibitors (24, 27, 55). Although these effects would be generally beneficial in the reduction of tumor invasiveness, the ability of TGF-β1 to cause a substantial and sustained increase in the secretion of type IV collagenase in HT-1080 fibrosarcoma cells should sound a cautionary note with respect to its potential usefulness as an antitumorigenic agent.

The nature of the cellular process of converting the M_r 72,000 proenzyme to the M_r 62,000 and 59,000 species is not known. It has been proposed that plasmin and plasminogen activator might process the M_r 72,000 latent proenzyme *in vivo* to yield an active form (56). However, the process of conversion, although apparently extracellular, did not prove susceptible to inhibition by aprotinin and ϵ-amino caproic acid. These serine protease inhibitors were effective in blocking the cell-mediated activation of latent TGF-β1 (57), a process believed to be mediated by the plasminogen activator/plasmin system.

The current study demonstrates the complex nature of collagenase regulation, even in well-established cell lines, and illustrates the difficulty of correlating collagenase mRNA or protein expression with tumorigenic or metastatic behavior. Nonmalignant WI-38 fibroblasts exhibited higher steady-state levels of M_r 72,000 type IV collagenase mRNA than did HT-1080 fibrosarcoma cells, whose highly invasive behavior has been well documented in *in vitro* assays (31, 58). On a per cell basis, WI-38 fibroblasts also secreted greater quantities of M_r 72,000 type IV collagenase than did HT-1080 cells, as measured by zymography. However, in WI-38 cells, all of this enzyme was in the form of the M_r 72,000 latent proenzyme, whereas a proportion of the M_r 72,000 type IV collagenase secreted by HT-1080 cells was consistently converted to the M_r 62,000 and 59,000 species. Thus the ratio of active enzyme species to latent proenzyme may provide a better correlation with invasive behavior than the overall level of expression of this widely expressed metalloproteinase. Accordingly the cellular activation of the M_r 72,000 type IV procollagenase and its inhibition by TIMPs are likely to be key regulatory controls of its activity. Transcriptional and translational controls may play a greater

role in the regulation of interstitial collagenase activity, where rapid induction of large quantities of enzyme may overwhelm the supply of TIMP.

The ratio of active to latent enzyme, the form of active enzyme, and the relative expression of TIMP-1 and TIMP-2 are likely to be the key determinants of tumorigenic/metastatic phenotype. In analyzing type IV collagenase activity, the authors of many previous studies have assayed net activity, often following trypsin or organomercurial activation. The recent identification of the M_r 92,000 type IV collagenase (13) and TIMP-2 (17), together with the description of cellular processing of secreted zymogens, now allows for a more detailed analysis of the type IV collagenolytic phenotype and a greater understanding of the role of type IV collagenolysis in tumor invasion and metastasis.

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