FEBS Letters 441 (1998) 88–92 FEBS 21225

Tumor-derived EMMPRIN (extracellular matrix metalloproteinase inducer) stimulates collagenase transcription through MAPK p38

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Received 25 October 1998

Abstract EMMPRIN (extracellular matrix metalloproteinase inducer) stimulates fibroblast metalloproteinases (MMP) 1, 2 and 3 (Kataoka et al. (1993) Cancer Res. 53, 3154–3158). Here we focus on MMP-1, showing that in lung tumors, MMP-1's cognate mRNA is strongly expressed in stromal fibroblasts adjacent to EMMPRIN-expressing tumor cells. In vitro, EMMPRIN upregulates MMP-1 mRNA expression in a concentration-dependent manner, with a peak accumulation at 24 h. The response is genistein-sensitive, suggesting it is dependent on tyrosine kinase activity. Analysis of tyrosine phosphorylation-dependent MAP kinases ERK 1/2, SAPK/JNK, and p38 showed that the activity of p38 but not that of the other 2 kinases was elevated in response to EMMPRIN. That p38 activity was required for EMMPRIN stimulation of MMP-1 was evident from results showing that the p38 inhibitor SB203580 blocked this response. This is the first available information regarding the mechanism by which tumor-associated molecules upregulate MMP synthesis in stromal fibroblasts.

Key words: Extracellular matrix metalloproteinase inducer; Metalloproteinase; MAP kinase; p38; Matrix metalloproteinase-1

1. Introduction

Tumor growth and metastasis require the breakdown of extracellular matrix, the components of which may be broken down collectively by matrix metalloproteinases. The regulation of these enzymes in tumorigenesis is poorly understood [1–3]. Although both tumor and stromal cells express MMPs localization studies show that fibroblasts are the primary source of MMPs in most tumors [4–8]. Our survey of human non-small cell lung carcinomas was consistent with this, thus supporting the notion that tumor-derived factors stimulate nearby fibroblasts to synthesize MMPs.

The purpose of the present study was to identify elements of the signaling pathway mediating extracellular matrix metalloproteinase inducer (EMMPRIN) stimulation of fibroblast MMP-1. Using chemical inhibitors of key signaling molecules, we determined that protein tyrosine kinases play an important role. More specifically, the activity of the p38 MAP kinase was required for the response. This is the first information regarding the mechanism by which tumor cells activate MMP-1 in adjacent fibroblasts, and demonstrates that p38 MAP kinase is an important regulator of MMP-1 gene expression.

2. Materials and methods

2.1. Materials

Samples of non-small cell lung carcinomas were collected at UCSF-affiliated hospitals (Moffitt-Long Hospital, UCSF/Mount Zion Hospital, and the Veterans Administration Hospital). Samples were frozen with or without fixation in 10% formalin. 16-Lu lung fibroblasts were purchased from the American Tissue Cell Culture facility (Bethesda, MD, USA). T7 and SP6 RNA polymerases, salmon sperm DNA, *Eco*RI restriction enzyme, random priming kits, and Trizol reagent were from Gibco. Probes used in Northern blots were produced by PCR using human cDNA as template. PCR products were cloned into TA cloning vector (Invitrogen). Acrylamide, Trizma base, boric acid, NaCl, tRNA, and sodium citrate were from Sigma. Ilford K5D emulsion was purchased from Polysciences. Kodak developer and fixative were used for in situ hybridization. Genistein, bisindolylmaleimide, PD98059, PP-1, KT5720, pertussis toxin, and SB203580 signal transduction inhibitors were from Calbiochem (La Jolla, CA, USA). Antibodies against p42/44, p38, and SAPK/JNK were purchased from New England Biolabs (Beverly, MA, USA).

2.2. Purification of EMMPRIN

EMMPRIN was purified from non-small cell lung carcinoma cell (either LX-1 or NCI H460) membranes. Homogenates from these cells were passed over immunoaffinity columns made with monoclonal [9] or polyclonal anti-EMMPRIN antibody (made with Affi-Gel 10, Bio-Rad). Rabbit anti-EMMPRIN polyclonal antiserum was generated against the synthetic peptide NH_2-DALPGQKTEEKVDSDDQW-GC-COOH [10]. The antibody recognized purified native EMMPRIN on Western blot (data not shown). For purification, lung tumor cell homogenates were loaded onto the column and the column was washed several times with 0.1 M HEPES buffer. EMMPRIN was eluted with 0.1 N acetic acid and concentrated in Centriprep and Centricon-10 columns (Amicon; Beverly, MA, USA).

2.3. In situ hybridization

Frozen sections of tumor and normal lung were probed with sense and antisense ^{35}S-labeled ribonucleoprobes corresponding to EMMPRIN, MMP-1 and MMP-2, and in situ hybridization was carried out as described [11]. Briefly, 6-μm thick tissue sections were fixed in 4% paraformaldehyde and treated with proteinase K and acetic anhydride prior to hybridization. The slides were incubated overnight at 55°C, then washed with 0.1×SSC at 62°C for 2 h. After dehydration they were dipped in emulsion and exposed for 2–3 weeks prior to developing. EMMPRIN, MMP-1 and MMP-2 were localized with riboprobes synthesized from nucleotides 73–403, 825–1091, and 1409–1747, respectively, of their cognate cDNAs [10,12,13]. All probes were cloned into pTA3 cloning vector [11].

2.4. Cell culture and Northern blot

Human lung fibroblasts (16-Lu and 13-Lu) and human fetal colon fibroblasts (CCD-18) were grown in Dulbecco's modified Eagle's

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PII: S0014-5793(98)01474-4

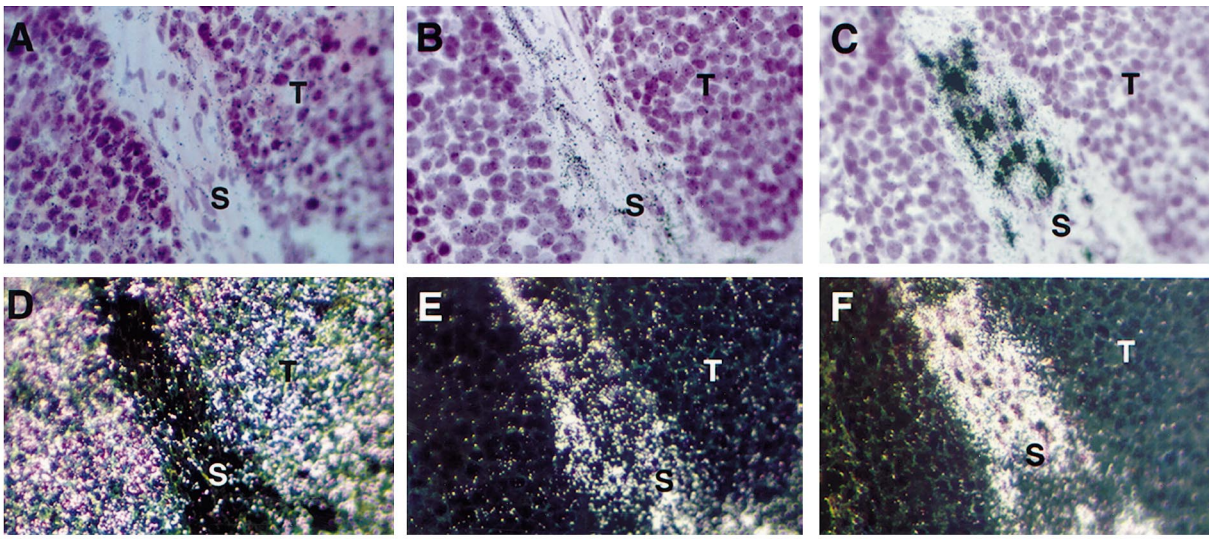

Fig. 1. In situ hybridization of NSCLC, brightfield and darkfield views. EMMPRIN probe (A, D) is strongly localized to tumor (T), whereas both MMP-2 (B, E) and MMP-1 (C, F) mRNAs are localized to stromal cells (S) between tumor islands. Magnification 100×.

(DME) medium/F12 (50:50) with essential amino acids (Gibco), supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells were grown to confluence in 6-well plates prior to starvation in serum-free medium. After 30 h the cells were treated with EMMPRIN or vehicle control. RNA was collected with Trizol reagent according to the manufacturer's instructions.

Total RNA (5–10 μg) was separated on a 1% agarose/formaldehyde gel and transferred to nylon (GeneScreen) in 10× SSC buffer. The RNA was fixed by UV crosslinking (Stratalinker 1800, Stratagene, Cambridge, MA, USA). The MMP-1 probe was excised from TA cloning vector with *Eco*RI and labeled with ^{32}P-dCTP by random priming. The GAPDH cDNA probe was made as described [11] and used as an internal control. Hybridization was performed with ExpressHyb (Clonetics). After 1 h the membranes were washed with 1× SSC at room temperature for 10 min 2× and 1× SSC/1% SDS at 50°C for 30 min. The membranes were exposed overnight at −80°C before developing. Quantification of data was by densitometry.

2.5. Stimulation of fibroblasts with purified EMMPRIN

16-Lu, CCD-18, and 13-Lu fibroblasts were stimulated with purified EMMPRIN (1–3 μg/ml) for 24 h prior to RNA extraction. In time course experiments total RNA from 16-Lu cells was extracted after 2, 6, 12, 24, and 48 h. To assess concentration-dependence, one tenth of the dose (0.3 μg) used in these experiments was added to 16-Lu fibroblasts and RNA was collected at 24 h.

2.6. Inhibition of EMMPRIN stimulation by signal transduction inhibitors

16-Lu fibroblasts were stimulated with EMMPRIN in the presence of various signal transduction inhibitors used at saturating concentration [14–17]. Genistein (67 μM), bisindolylmaleimide I (10 nM), PD98059 (10–100 μM), PP-1 (30 μM), KT5720 (0.5 μM), pertussis toxin (100 ng/ml), and SB203580 (1–100 μM) were added to cells in serum-free medium one hour before and for the duration of EMMPRIN exposure. RNA was collected after 12 h and analyzed for the presence of MMP-1 and GAPDH RNA.

2.7. Immunoblotting analysis of Erk1/2, p38, and SAPK/JNK MAP kinases

16-Lu fibroblasts were stimulated with EMMPRIN for 0, 15, 30, and 60 min prior to harvesting of total cellular protein. For each time point equal amounts of protein were electrophoresed under reducing conditions. Identical gels were run simultaneously and the proteins then transferred to PVDF membrane (Immobilon-P, Millipore, Bed-

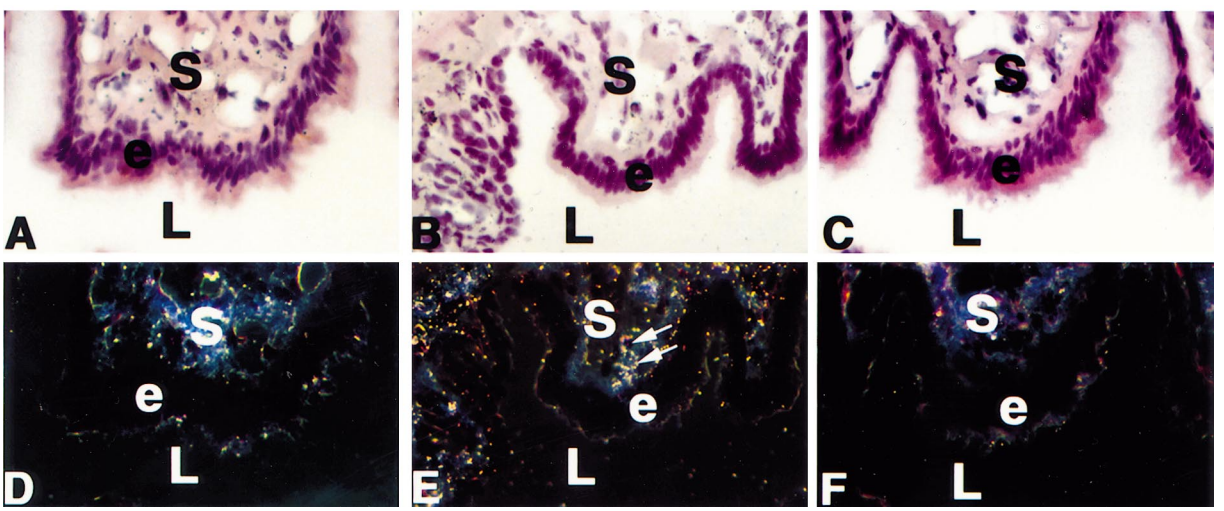

Fig. 2. In situ hybridization of normal bronchial epithelium, brightfield and darkfield views. EMMPRIN (A, D) and MMP-1 (C, F) probes reveal little to no signal, whereas MMP-2 (B, E) shows low intensity signal in subepithelial fibroblasts (white arrows in E). S = stroma, L = lumen, e = epithelium. Magnification 100×.

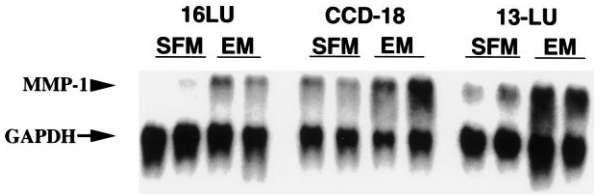

Fig. 3. EMMPRIN upregulation of MMP-1 mRNA in three fibroblast cell lines. With each shown in duplicate, EMMPRIN (EM) or serum-free medium (SFM) was administered to 16-Lu, CCD-18, and 13-Lu fibroblasts for 24 h, then total RNA extracted for Northern blotting. EMMPRIN upregulates steady state MMP-1 mRNA in all 3 cell lines. GAPDH is shown as an internal control.

ford, MA, USA). After blocking with 5% (w/v) non-fat dry milk, the blots were incubated for one hour with antibodies directed against phosphorylated and non-phosphorylated forms of Erk/p44/42, SAPK/JNK, and p38. Blots were washed 5 min 3× with Tris-buffered saline/0.1% Tween-20 and incubated with horseradish peroxidase-conjugated secondary antibody (1:2000) for one hour. The membranes were washed as above, and the bands detected by chemiluminescence after 30 s–1 min exposures.

3. Results

3.1. EMMPRIN mRNA is in tumor cells adjacent to MMP-1 and -2 expressing fibroblasts

Rapidly frozen NSCLC (6 samples) and normal lung (3 samples) specimens were examined for RNA expression of EMMPRIN, MMP-1, and MMP-2. In both squamous cell ($n=4$) and adenocarcinoma ($n=2$) histologic subtypes, carcinoma cells uniformly expressed high levels of EMMPRIN (Fig. 1A and D) and adjacent stromal fibroblasts strongly expressed MMP-1 and MMP-2 (Fig. 1B, C, E, F). The latter is consistent with previous studies reporting elevated metalloproteinase expression in lung and head/neck tumors [18–20].

Normal lung samples did not contain detectable levels of EMMPRIN and MMP-1 mRNAs (Fig. 2A, D, C, F). MMP-2 was weakly expressed in subepithelial fibroblasts (Fig. 2B and E).

3.2. EMMPRIN stimulates MMP-1 steady state mRNA in multiple fibroblast cell lines

Fibroblast cell lines from both adult lung (16-Lu and 13-Lu) and fetal colon (CCD-18) showed increased MMP-1 RNA in response to EMMPRIN (Fig. 3), although the degree of stimulation differed.

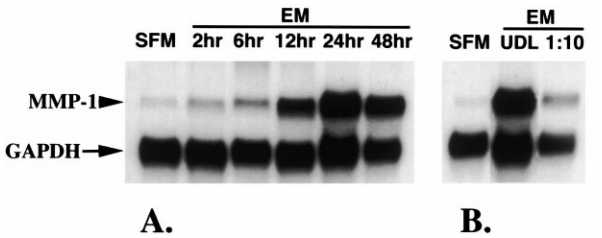

Fig. 4. Time course and concentration dependence of MMP-1 upregulation. A: EMMPRIN (1–3 μg/ml) was administered to 16-Lu fibroblasts for 2, 6, 12, 24, or 48 h, then total RNA collected. MMP-1 steady state mRNA begins to rise at 2 h and peaks at 24 h (B). EMMPRIN was administered at 3 μg/ml (undiluted, UDL) or 0.3 μg/ml (1:10) for 24 h before RNA collection. Effect on MMP-1 mRNA is concentration-dependent.

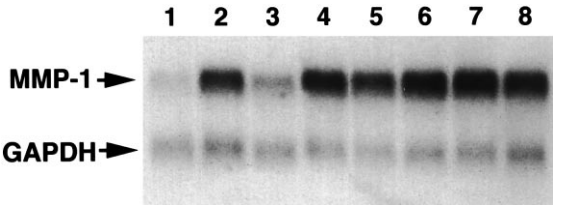

Fig. 5. Effect of signal transduction inhibitors on EMMPRIN stimulation of MMP-1. EMMPRIN (3 μg/ml) was co-administered with various inhibitors for 10 h prior to total RNA collection. 1: Serum-free medium; 2: EMMPRIN alone; 3–8: EMMPRIN plus (3) genistein, (4) bisindolylmaleimide, (5) PP-1, (6) PD98059, (7) KT5720, (8) pertussis toxin. Only genistein shows reduction in MMP-1 steady state mRNA.

3.3. EMMPRIN stimulation of MMP-1 mRNA peaks at 24 h and is dose dependent

A kinetic analysis of EMMPRIN-induced MMP-1 mRNA showed a peak intensity of MMP-1 steady state mRNA at 24 h (Fig. 4A). The response at 24 h was concentration-dependent (Fig. 4B).

3.4. EMMPRIN activates protein tyrosine kinases

A survey of the effects of various signal transduction inhibitors showed a sensitivity of EMMPRIN signaling to the protein tyrosine kinase inhibitor genistein (Fig. 5). In contrast, bisindolylmaleimide (PKC inhibitor), PD98059 (MEK1/2 inhibitor), KT5720 (PKA inhibitor), PP-1 (Src kinase inhibitor), and pertussis toxin (G protein inhibitor) did not inhibit the response. Because protein tyrosine kinases play an important

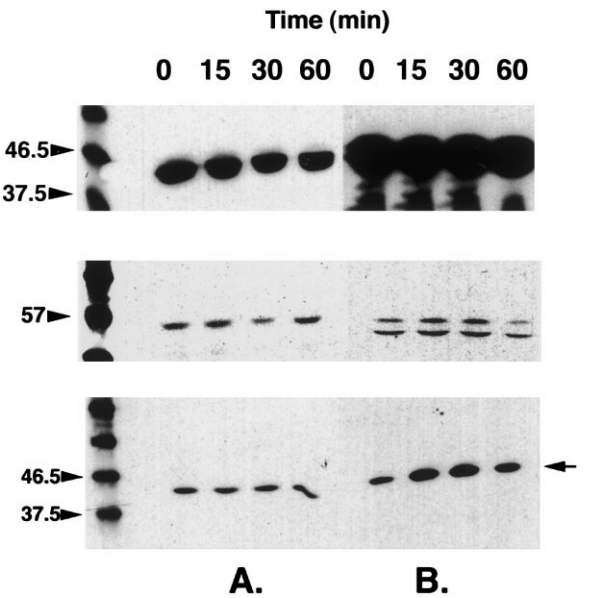

Fig. 6. ERK, SAPK/JNK, and p38 immunoblotting. Total cellular protein from 16-Lu cells stimulated with EMMPRIN (3 μg/ml) for 0, 15, 30 and 60 min was analyzed by 10% SDS-PAGE and transferred to membrane prior to immunoblotting. Reactions with antibodies directed against the non-phosphorylated (A) and phosphorylated (B) forms of 3 MAP kinases are shown. Phosphorylation of p38 at 15 min (lower panel, B) is detected. ERK1/2 (upper panel, B) and JNK/SAPK (middle panel, B) do not show increased phosphorylation. Reactions with antibodies detecting non-phosphorylated MAP kinases (upper, middle, and lower panels, A) are included as controls. Biotinylated markers are on the left of each panel.

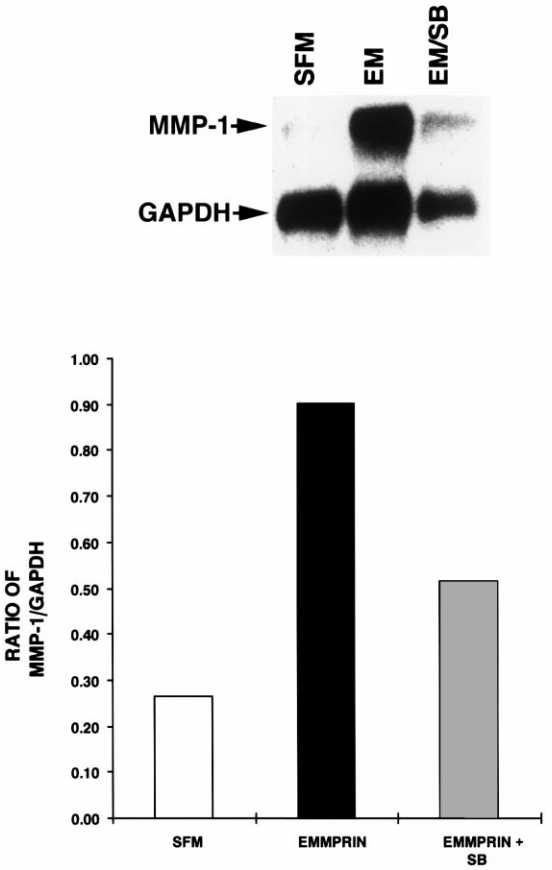

Fig. 7. SB203580 inhibits EMMPRIN upregulation of MMP-1. The specific p38 inhibitor, SB2035580 (10 μM), was preadministered 1 h and co-administered with EMMPRIN for 10 h prior to total RNA collection. The graph compares MMP-1/GAPDH signal intensity and indicates a 45% inhibition by SB203580. SFM = serum-free medium; EM = EMMPRIN alone; EM/SB = EMMPRIN with SB203580.

role in MAP kinase activation, we investigated the potential role of MAP kinases.

3.5. *EMMPRIN stimulates phosphorylation of p38 but not other MAP kinases*

EMMPRIN stimulated phosphorylation of p38 but not Erk1/2 or JNK (Fig. 6). Anti-phospho-p38, which detects dual phosphorylation of p38 at Tyr-182 and Thr-180, showed enhancement after 15- and 30-min exposure to EMMPRIN, declining at 60 min (Fig. 6, lower panel). No increased signal was detected with the phospho-Erk/p44/p42 or phospho-SAPK/JNK antibodies (Fig. 6, upper and middle panels).

3.6. *SB203580 inhibits EMMPRIN upregulation of MMP-1 mRNA*

To determine whether the observed stimulation of p38 is required for EMMPRIN upregulation of MMP-1, we tested the effect on the response of the p38 inhibitor SB203580. When SB203580 was co-administered (at saturating doses) with EMMPRIN, the response decreased by ~45% (Fig. 7A and B).

4. Discussion

Our results show that the tumor-associated protein EMMPRIN stimulates metalloproteinase-1 mRNA in fibroblasts via a p38-dependent signaling pathway. These results are important in light of the fact that metalloproteinase activity has been shown to be necessary for tumor growth [21]. It seems likely that signaling mechanisms by which tumors increase local metalloproteinase activity may constitute novel anti-tumor drug targets.

MMP-1 is a member of the matrix metalloproteinase family, zinc-dependent metalloenzymes that contribute importantly to both normal tissue remodeling [22–25] and pathological processes [26–29]. As a type I collagenase, it has the unique ability to cleave fibrillar collagen [2]. Although synthesis of metalloproteinases shows a general, albeit differential, sensitivity to growth factors and cytokines [30,31], and specific tyrosine kinase- [14], protein kinase C- [14], protein kinase A- [32], and Src- [33] dependent mechanisms have been identified, the control of these enzymes by extracellular stimuli is largely unknown.

We focused on the control of MMPs by the tumor-associated protein EMMPRIN. Results of a pharmacological screen showed that tyrosine kinases, but not PKA, PKC, or Src are required for EMMPRIN induction of MMP-1. Since tyrosine kinases are integrally involved in MAP kinase signaling pathways, we subsequently examined the effects of inhibitors of these pathways, specifically the MEK1/2 inhibitor PD98059 and the p38 inhibitor SB203580. The latter significantly attenuated the response to EMMPRIN, indicating a role for p38 activation in the induction of MMP-1. Consistent with this, p38 was the only one of three major MAP kinases (ERK1/2, SAPK/JNK, and p38) that underwent phosphorylation (an index of activation) in response to EMMPRIN. At saturating doses of the p38 inhibitor, MMP-1 induction was decreased by only ~45%. Clearly, although p38 plays an important role in EMMPRIN induction of MMP-1, it does not account entirely for its upregulation. Potential contributors to this induction may include less well-described MAP kinase pathways [34] or posttranscriptional mechanisms (e.g. prolongation of RNA half life).

Interestingly, p38 has recently been implicated in the induction of another metalloproteinase, MMP-9, by phorbol ester [15]. Originally described by Han et al. [35], p38 is generally regarded as a stress-activated enzyme and has been shown to have downstream effects on transcription factor activation [36], actin filament rearrangement [37], and matrix degradation ([15] and this report). As such, p38 eventually may be recognized to play a variety of important roles in the general process of tissue remodeling.

The identity of signaling molecules linking p38 and MMP-1 transcription remains unknown. Clues are provided, however, by information regarding known response elements on the MMP-1 promoter. There is a well-described AP-1 binding site at −72/−67 [38] that is responsive to a variety of stimuli including phorbol esters, TGF-β, cAMP and TNF-α [39,40]. AP-1 is activated by a variety of signaling cascades, some of which are p38-dependent [36]. Studies to determine the locus of the EMMPRIN response element associated with the MMP-1 gene are in progress.

Acknowledgements: We thank Wilfred Raymond and George Caughey for their assistance with EMMPRIN purification, and Marianne Gallup for help with the preparation of figures. This work was supported by grants P01 HL43762 (CB) and R01 HL24136 (CB).

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