The balance between matrix metalloproteinases (MMPs) and their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), is pivotal in the remodeling of extracellular matrix. TGF-β has profound effects on extracellular matrix homeostasis, in part via its ability to alter this balance at the level of gene expression. The intracellular signaling pathways by which TGF-β mediates its actions include the Smad pathway, specific to the TGF-β superfamily, but also, for example, mitogen-activated protein kinase pathways; furthermore, cross-talk between the Smads and other signaling pathways modifies the TGF-β response. The reciprocal effect of TGF-β on the expression of Timp-1 and MMP-1 supports its role in matrix anabolism, yet the mechanisms by which TGF-β induces Timp-1 and represses induced MMP-1 have remained opaque. Here, we (i) investigate the mechanism(s) by which TGF-β1 induces expression of the Timp-1 gene and (ii) compare this with TGF-β1 repression of phorbol ester-induced MMP-1 expression. We report that the promoter-proximal activator protein 1 (AP1) site is essential for the response of both Timp-1 and MMP-1 to TGF-β (induction and repression, respectively). c-Fos, JunD, and c-Jun are essential for the induction of Timp-1 gene expression by TGF-β1, but these AP1 factors transactivate equally well from both Timp-1 and MMP-1 AP1 sites. Smad-containing complexes do not interact with the Timp-1 AP1 site, and overexpression of Smads does not substitute or potentiate the induction of the gene by TGF-β1; furthermore, Timp-1 is still induced by TGF-β1 in Smad knockout cell lines, although to varying extents. In contrast, Smads do interact with the MMP-1 AP1 site and mediate repression of induced MMP-1 gene expression by TGF-β1.

Timely breakdown and remodeling of the extracellular matrix (ECM) is an essential process in development, morphogenesis, and reproduction. ECM degradation is also associated with a variety of physiological and pathological processes such as joint destruction in the arthritides, wound healing, tumor metastasis, angiogenesis, and fibrosis (1). Pivotal to the turnover of ECM is the matrix metalloproteinase (MMP) family of enzymes; these enzymes have the capability, between them, of degrading the majority of the proteins that make up the ECM (2). The tissue inhibitors of metalloproteinases (TIMPs) protect ECM integrity by inhibiting MMPs (3). MMP-1, interstitial collagenase, is one of a subfamily of MMPs that can specifically degrade the collagen triple helix; hence, MMP-1 plays a central role in pathologies where collagen turnover is aberrant (4). As well as inhibiting most of the active MMPs, TIMP-1 is also reported to have diverse effects on cell growth and apoptosis (5).

Transforming growth factor-β (TGF-β) is a multifunctional growth factor controlling cell growth and differentiation and has marked effects on ECM homeostasis (6). This includes the induction of ECM gene expression and generally suppression of MMPs and induction of TIMPs to give a “synthetic” phenotype (7). Hence, TGF-β is associated, for example, with fibrosis in a number of diseases (8). TGF-β has previously been shown to repress the expression of MMP-1, induced by a variety of stimuli, in a number of cell types (9, 10). Conversely, TGF-β induces Timp-1 gene expression, often in synergy with other growth factors and cytokines (11, 12).

TGF-β signals via transmembrane receptors to intracellular mediators of the Smad family. Smad 2 and Smad 3 are receptor-specific Smads that are phosphorylated on serine residues by the type I TGF-β receptor. Upon phosphorylation, these Smads form heteromeric complexes with a common mediator Smad 4 and can then be translocated to the nucleus, where they regulate gene expression either directly or in association with a number of co-activators and co-repressors. An inhibitory Smad 7 blocks this cascade to prevent TGF-β-mediated alterations in gene expression (13). TGF-β can also signal through other pathways (e.g. mitogen-activated protein kinase pathways), although the mechanisms for activation of these pathways appear diverse (14, 15). Cross-talk between the Smad signaling cascade and other pathways also adds complexity to the system (16–18).

The proximal promoters of both MMP-1 and TIMP-1 genes contain an AP1 site, which, in each case, has been the focus for research on their regulation. In the murine Timp-1 gene, the AP1 site is located at −59 bp and has been shown to be impor-

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* This work was supported by the Biotechnology and Biological Sciences Research Council UK. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ Supported by the Arthritis Research Campaign UK.

¶ Supported by the Norfolk and Norwich Big C Appeal.

The abbreviations used are: ECM, extracellular matrix; AP1, activator protein 1; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; MMP, matrix metalloproteinase; PMA, phorbol 12-myristate 13-acetate; TGF-β, transforming growth factor-β; TIE, TGF-β inhibitory element; TIMP, tissue inhibitor of metalloproteinases (Timp-1, mouse gene; TIMP-1, human gene).
Regulation of Timp-1 and MMP-1 by TGF-β

Experimental Procedures

Cell Culture—Murine C3H10T1/2 fibroblasts, Swiss 3T3 cells, and human skin fibroblasts were routinely cultured in minimal essential medium with Earle’s salts and 1-glutamine (2 mM) (Invitrogen) containing 10% fetal bovine serum (Invitrogen), 1% nonessential amino acids, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 20 units/mL nystatin. Serum-free conditions used minimal essential medium containing 0.1% bovine serum albumin with the above antibiotics. AP1 knockout cells (c-Jun−/−, c-Fos−/−, and Fra1−/−), a kind gift from Professor E. Wagner (University of Vienna) and Dr. P. Angel (University of Heidelberg); Jun n−/− (32), a kind gift from Professor M. Yaniv and Dr. J. Weitzman (Pasteur Institute, Paris, France)) and Smad knockout cells (Smad 2−/−, Smad 3−/−, and Smad 4−/−, a kind gift from Dr. E. Bettigeri (NCI, National Institutes of Health, Bethesda, MD); Smad 4−/−, a kind gift from Professor T. Mak (University of Tokyo)) were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum with 1% nonessential amino acids, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 20 units/mL nystatin. Serum-free conditions used Dulbecco’s modified Eagle’s medium containing 0.5% bovine serum albumin with the above antibiotics.

Reporter Constructs and Expression Plasmids—Constructs using Timp-1 promoter driving luciferase expression were in pGL2-basic (Promega); point mutations altered the wild-type AP1 site (5′-TGGATGAGA-3′) to the Timp-1 consensus AP1 site (5′-TGGATGCA-3′) or a nonfunctional mutant AP1 site (5′-GCTGAATGTA-3′). Constructs using the MMP-1 promoter were in pGL3-basic (Promega) and were a kind gift from Prof. C. E. Brinckerhoff (Dartmouth Medical School) (33).

The AP1 expression plasmids in pCMV were described by Harrison et al. (34) and were a kind gift of Dr. P. R. Dobner (University of Massachusetts). Smad expression plasmids in pCMV were described by Wicks et al. (18) and were from the laboratory of Dr. A. Chantry (University of East Anglia).

All mutagenesis was performed using the QuikChange method (Stratagene). All mutations were verified by sequencing.

p5TF-Lux is an artificial promoter consisting of the plasminogen activator inhibitor-1 TGF-β-responsive promoter and three repeats of the AP1c site (35).

Transient Transfection—Cells were seeded in six-well plates at a density of 8850 cells/cm² and grown overnight in medium containing 10% fetal calf serum at 37 °C in a 5% CO2 atmosphere. Cells were transfected overnight in serum-containing medium with 1 µg/well reporter plasmid using FuGene 6 (Roche Molecular Biochemicals) according to the manufacturer’s instructions. The following day, cells were washed in Hank’s balanced salts solution and incubated in serum-free medium overnight. Cells were then stimulated with phorbol 12-myristate 13-acetate (PMA; 10−7 M) or TGF-β1 (2 ng/ml; R&D Systems) or both together for varying times as shown, prior to harvest. Harvest and assay were according to the manufacturer’s instructions (luciferase activity; Promega).

For co-transfection with expression constructs, an additional ≤ 1 µg/well expression vector(s) was included, keeping the total DNA to 2 µg/well using empty vector. For co-transfection with oligonucleotides, an additional 1 µg/well of oligonucleotide (wild-type or mutant control) was included in the transfection.

Nuclear Extracts—Confluent cells at a density of ~2 × 10^6/mm² dish were washed and incubated in medium containing 0.1% bovine serum albumin overnight. Cells were then treated with TGF-β1 (2 ng/ml), PMA (10−7 M), or both together for 3 h. Cells were then rinsed twice with phosphate-buffered saline, 0.1% Nonidet P-40 for 30 s. After centrifugation at 10,000 × g for 10 s, pellets were resuspended in 1 ml of phosphate-buffered saline, 0.1% Nonidet P-40 and then resuspended in 3 volumes of high salt buffer (25 mM HEPES, pH 7.8, 500 mM KCl, 0.5 mM MgSO4, 1 mM diethiothreitol) containing 1× Complete protease inhibitors (Roche Molecular Biochemicals). Samples were in-

M. C. Hall, D. A. Young, J. G. Waters, A. D. Rowan, A. Chantry, D. R. Edwards, and I. M. Clark, unpublished observation.
cubated on ice for 20 min with occasional vortex and then centrifuged at 1,000 × g for 2 min at 4 °C. Supernatant was then divided into aliquots, frozen on dry ice, and stored at −80 °C.

Protein concentration in the nuclear extract was determined by Bradford assay (Bio-Rad) and aliquots, frozen on dry ice, and stored at −80 °C. Protein concentration in the nuclear extract was determined by Bradford assay (Bio-Rad) and was typically 2–5 μg of protein/μl.

Probe Labeling—Oligonucleotides for electrophoretic mobility shift assay (EMSA) were synthesized by MWG-Biotech. Timp-1 AP1 site, 5′-AGCTGGATGAGTAATGCG-3′; MMP-1 AP1 site, 5′-AGCTGGATGAGTAATGCG-3′; mutant AP1 site, 5′-AGCTGGATGAGTAATGCGG-3′; S4BE, 5′-GATCTCGAGAGCCAGACAAAAAGCCAGACTTTAGCCAGACAC-3′; S4BEmut, 5′-GATCTCGAGAGCTACAAAAGCTGATCTCGAGAGCCAGACAC-3′. Double-stranded probes were labeled with [32P]dCTP using Klenow fill-in, whereas single-stranded probes were labeled with [γ-32P]ATP using T4 polynucleotide kinase. Labeling reactions were followed by phenol/chloroform extraction and purification through a Sephadex G50 spin column.

EMSA—Nuclear extracts (~2 μg), 1 μg of poly(dl-dC), and radiolabeled probe (~30,000 cpm) were incubated in 1× binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5 mM dithiothreitol, 5 mM MgCl2, and 5% glycerol) with or without competitor DNA for 20 min at room temperature in a total volume of 10 μl. For antibody supershift analyses, 2 μg of the appropriate antibody (anti-c-Fos, sc-52-Gx; anti-c-Jun, sc-45-Gx; anti-FosB, sc-48-Gx; anti-Fra1, sc183x; anti-Fra2, sc-604x; anti-JunB, sc-46-Gx; anti-JunD, sc-74-Gx; anti-Smad4, sc-7966x (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti-Smad2/3, S66220 (Cruz Biotechnology, Inc., Santa Cruz, CA) and anti-Smad2/3, S66220 were labeled with [32P]dCTP using Klenow fill-in, whereas single-stranded probes were labeled with [γ-32P]ATP using T4 polynucleotide kinase. Labeling reactions were followed by phenol/chloroform extraction and purification through a Sephadex G50 spin column. The labeled probe was then mixed with a small amount of nuclear extract and 5% glycerol and incubated at room temperature for 20 min with occasional vortex and then centrifuged at 1,000 × g of poly(dl-dC), and radiolabeled probe (~30,000 cpm) were incubated in 1× binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5 mM dithiothreitol, 5 mM MgCl2, and 5% glycerol) with or without competitor DNA for 20 min at room temperature in a total volume of 10 μl. For antibody supershift analyses, 2 μg of the appropriate antibody (anti-c-Fos, sc-52-Gx; anti-c-Jun, sc-45-Gx; anti-FosB, sc-48-Gx; anti-Fra1, sc183x; anti-Fra2, sc-604x; anti-JunB, sc-46-Gx; anti-JunD, sc-74-Gx; anti-Smad4, sc-7966x) were labeled with [32P]dCTP using Klenow fill-in, whereas single-stranded probes were labeled with [γ-32P]ATP using T4 polynucleotide kinase. Labeling reactions were followed by phenol/chloroform extraction and purification through a Sephadex G50 spin column. The labeled probe was then mixed with a small amount of nuclear extract and 5% glycerol and incubated at room temperature for 20 min with occasional vortex and then centrifuged at 1,000 × g for 2 min at 4 °C. Supernatant was then divided into aliquots, frozen on dry ice, and stored at −80 °C.

Reverse Transcription-PCR—RNA was isolated from monolayer cultures using Trizol (Invitrogen). Quantitative reverse transcription-PCR was performed using the Applied Biosystems ABI Prism 7700 sequence detection system (TaqMan®) as described (37).

RESULTS

Deletion Analysis of the Timp-1 Promoter—Prior to studying the response of the Timp-1 promoter to TGF-β1, the transient transfection protocol was optimized to enable us to assay the response of the promoter-reporter constructs at early time points. Previous studies at the level of steady-state mRNA for Timp-1 have suggested that there is an early primary response to TGF-β1, followed by a later secondary response, which may be mediated by or in conjunction with TGF-β1-induced autocrine factors (12). Using luciferase as a reporter, transgene expression was robust at a 6-h time point, and this was therefore used throughout the studies of the Timp-1 promoter.

A -925/+47 Timp-1 promoter construct reiterates the response of the endogenous Timp-1 gene (12) to PMA and TGF-β1, demonstrating a significant induction by each factor alone and an augmented response to both factors together (Fig. 1). Deletion from the 5′ end of this construct demonstrated that, whereas upstream sequences may impact on the level of induction (or indeed on basal expression), this pattern of expression is maintained in a -62/+47 Timp-1 promoter construct; how-
ever, induction is lost in a −50/+47 construct in which an AP1 site at −59/−53 is absent. This construct loses TGF-β1 inducibility and the synergism between TGF-β1 and PMA, but it maintains a low level of PMA inducibility (−1.5-fold). This demonstrates that the −59/−53 AP1 site is critical for TGF-β1 induction of the Timp-1 gene. This is confirmed by an inactive binding mutation in this AP1 site in the context of the −223/+47 construct, whereby PMA and TGF-β1 induction are markedly reduced compared with wild-type (and the synergism between the two is lost) although not completely abolished. It should also be noted that the pattern of response of the Timp-1 promoter to TGF-β1 and PMA is replicated in other cell lines (e.g. Swiss 3T3).

Protein Binding to the Timp-1 AP1 Site—EMSA and supershift analysis was used to probe protein binding to the Timp-1 AP1 site under PMA and TGF-β1 stimulation (Fig. 2). At this 3-h time point, the AP1 site is bound in unstimulated nuclear extracts; upon TGF-β1 or PMA stimulation, binding to this site increases, although the mobility of the complex remains unaltered. Treatment with both factors together increases binding further. Specificity of binding was ascertained by competition with cold self and mutant oligonucleotides (data not shown). Supershift/antibody blocking analysis using antibodies against components of the AP1-binding complex under each condition. Nuclear extracts from cells induced with PMA or PMA plus TGF-β1 appear to contain all of the Fos and Jun family members assayed. Stimulation with TGF-β1 alone gave a similar pattern of response except for the absence of a supershift with the anti-Fra1 antibody; this indicates that TGF-β1 alone does not induce Fra1 binding to this oligonucleotide.

When these experiments were repeated using an oligonucleotide containing the MMP-1 consensus AP1 sequence, identical results were obtained (data not shown).

Overexpression of AP1 Factors Transactivates the Timp-1 Promoter—In order to assess the role of differing Fos and Jun family members in activating transcription from the Timp-1 promoter, co-transfection experiments were performed. The −95/+47 Timp-1 luciferase construct was transiently transfected into C3H10T1/2 cells with combinations of expression constructs for c-Fos, Fra-1, Fra-2, FosB, c-Jun, JunD, and JunB; empty vector was used as a control (Fig. 3). Expression and function of these factors was assessed by EMSA (data not shown). Alone, none of the Jun family members could transactivate the Timp-1 promoter, whereas c-Fos and, to a lesser extent, FosB, do transactivate, presumably in combination with endogenous Jun factors expressed in these cells. The combination of c-Fos with c-Jun or JunD gives the most potent induction of the Timp-1 promoter construct, −4-fold above c-Fos alone and 8-fold above control.

Substitution of the wild-type AP1 (5′-TGAGTAA-3′) site in the −95/+47 for the MMP-1 consensus API site (5′-TGAGTACA-3′) (i.e. an A to C transversion) did not alter the pattern of response to these AP1 family members (data not shown). Furthermore, using a −125/+60 MMP-1 luciferase construct, transiently transfected into C3H10T1/2 cells or Swiss 3T3 cells (in which PMA-induced MMP-1 expression either is not or is repressed by TGF-β1, respectively; see below), with AP1 factors, gave the same pattern of response (data not shown).

Expression of Timp-1 in AP1 Knockout Cells—In order to verify the data gained from overexpression of AP1 factors, a Timp-1 promoter construct was transiently transfected into cell lines deleted for one of c-Fos, c-Jun, JunD, or fra-1, and cells were stimulated with either PMA or TGF-β1 (Fig. 4). In these experiments, a −925/+47 Timp-1 luciferase plasmid was used, since many of the AP1 knockout cell lines transfected poorly, and this construct gives a higher level of expression. As a control vector, p3TP-Lux was used; this is an artificial construct consisting of three copies of the MMP-1 AP1 site and one copy of the TGF-β1-responsive PAI-1 promoter. p3TP-Lux is known to be both AP1- and Smad-responsive. All constructs were co-transfected with pRSmad and CAT expression was used to normalize the data for transfection efficiency. It should be noted that the +/+ cells in the c-Fos and c-Jun experiments are Swiss 3T3 cells, since cells from wild-type littermates of the knockouts were not available. Conversely, +/+ cells in the JunD and Fra-1 experiments were from wild-type littermates.

![Fig. 2. Electrophoretic mobility shift assay on an AP1 oligonucleotide. C3H10T1/2 marine fibroblasts were serum-starved for 24 h and then stimulated with TGF-β (2 ng/ml), PMA (10−7 M), or both together. Nuclear extracts were harvested at t = 3 h. EMSA was performed on the Timp-1 AP1 site oligonucleotide in the absence or presence of antibodies to members of the Fos and Jun families (2 µg per binding reaction). Lane 1, control; lane 2, TGF-β1; lane 3, PMA; lane 4, TGF-β1 + PMA.](image-url)
Variability in response of these "wild-type" cells to TGF-β1 and PMA is apparent, and this reinforces the need for a PMA- and TGF-β1-inducible control plasmid such as 3TP-Lux.

The data show that c-Fos, c-Jun, and JunD are each necessary, and Fra-1 is not necessary for TGF-β1-induction of the Timp-1 gene, although PMA still induces expression in the absence of c-Fos. Interestingly, c-Jun is also necessary for induction of 3TP-Lux by either TGF-β1 or PMA, and JunD is necessary for induction by PMA. These data underscore the importance of c-Fos, c-Jun, and JunD as AP1 factors that are necessary for induction by PMA. 

EMSMA analysis on overlapping oligonucleotides across the −62/+47 region shows no obvious alterations in protein binding after TGF-β1 or PMA stimulation other than on the AP1 site above (data not shown).

TGF-β1 Represses PMA-induced Expression from the MMP-1 Promoter—It has been previously reported that TGF-β1 represses the expression of MMP-1 when induced by a variety of factors (e.g. PMA, interleukin-1, and tumor necrosis factor-α) (9, 10). In order to reiterate this in our model system, a construct containing −517/+60 of the human MMP-1 promoter in a luciferase vector was transiently transfected into both murine Swiss 3T3 cells and primary human skin fibroblasts. In both cases, PMA potently induces expression from this construct, and TGF-β1 represses this induction although, at the doses used, not back to control levels (see Fig. 5, A and B). TGF-β1 alone does not significantly repress basal expression of MMP-1. These data agree with data published in other cell lines, and we have confirmed that the same response is seen with either 8- or 24-h stimulation (data not shown). It should be noted that TGF-β1 does not repress PMA-induced expression from the MMP-1 promoter in C3H10T1/2 cells.

A shorter MMP-1 promoter construct, −153/+60, shows an identical pattern of response to −517/+60. This suggests that the putative TIE at −245 is not involved in the repression of PMA-induced MMP-1 expression. A construct of −80/+60, containing the proximal AP1 site at −72, gives very low levels of expression, but the TGF-β1 repression of PMA-induced expression is still apparent; therefore, elements within the −80/+60 region must be responsible for this effect of TGF-β1 (Fig. 5A).

Point Mutations in −517/+60 Confirm the Role of the AP1 Site in TGF-β1-mediated Repression of MMP-1—Since previous data suggest that in MMP-1 promoter constructs extending further 5′ than −321 (18), the AP1 site at −72 may contribute less to expression of the transgene, functionally inactivating point mutations in both TIE and AP1 motifs were made in the context of −517/+60. Fig. 5B shows that mutation of the TIE does not prevent TGF-β1 repression of PMA-induced expression. However, the inactivating mutation in the AP1 motif, in the presence or absence of the TIE mutation, actually leads to a further induction of PMA-induced gene expression by TGF-β1. Interestingly, the TIE mutation increases absolute levels of expression, whereas the AP1 mutation decreases absolute levels. Exchanging the consensus AP1 sequence in −517/+60 for the Timp-1 AP1 sequence does not alter the pattern of expression; moreover, transient transfection of a −95/+47 Timp-1 construct containing the MMP-1 AP1 site into C3H10T1/2 or Swiss 3T3 cells did not alter the pattern of TGF-β1 and PMA induction seen in the same construct containing the wild-type Timp-1 AP1 motif (data not shown).

Overexpression of Smads 2, 3, and 4 Does Not Potentiate TGF-β1 Induction of Timp-1 Promoter—In order to probe the role of the Smad signaling pathway in the response of the Timp-1 gene to TGF-β1, expression vectors for Smads 2, 3, 4, and 7 were co-transfected into C3H10T1/2 with either the −95/+47 Timp-1 promoter construct or the Smad-responsive 3TP-Lux, using empty vector as a control. Cells were then stimulated for 6 h with TGF-β1. Fig. 6A shows that the 3TP-
Lux construct behaves in a Smad-responsive manner as expected; TGF-β induces expression of luciferase, and this is further induced by the addition of either Smad 2, 3, or 4 alone. Combinations of these Smads yield even higher levels of expression. The Smad dependence of this response is underlined by co-transfection of the inhibitory Smad 7, which potently blocks TGF-β induction of 3TP-Lux. In comparison with this, TGF-β induction of the Timp-1 promoter is not potentiated by Smad 2, 3, or 4 alone, with Smads 3 and 4 acting in a repressive fashion; combinations of Smads 2 and 3 or 4 acting in a repressive fashion; combinations of Smads 2 and 3 or 4 have no effect, whereas Smads 3 and 4 or Smads 2, 3, and 4 potently repress TGF-β1 stimulation of the Timp-1 construct (Fig. 5B). Furthermore, Smad 7 does not repress TGF-β1-stimulated Timp-1 expression. Together, these data suggest that the response of the Timp-1 gene to TGF-β is not Smad-dependent.

Conversely, co-expression of Smad 7 with the −517/+60 MMP-1 construct blocks the TGF-β1-mediated repression of PMA-induced luciferase expression from the MMP-1 promoter (Fig. 6C). This suggests that the effect of TGF-β1 on the MMP-1 gene is Smad-dependent.

Timp-1 Expression Is Induced by TGF-β1 in Smad Knockout Cell Lines—In our hands, Smad knockout cell lines and their wild-type partner lines proved difficult to transfect reproducibly. Hence, Smad 2, Smad 3, or Smad 4 knockout cells and their wild-type partners were stimulated with TGF-β1, PMA, or both together, and expression of the endogenous Timp-1 gene was assessed by quantitative reverse transcription-PCR using the Taqman system. All three knockout cell lines retain some TGF-β1 inducibility, although this is at a reduced level compared with wild types (data not shown). All three knockout cell lines also retain the synergism in Timp-1 induction with PMA and TGF-β1 together. Interpretation of these data is clouded by the fact that wild-type cell lines show wide variation in their response to TGF-β1 in both these experiments and, for example, in Fig. 4.
A Smad-binding Oligonucleotide Blocks TGF-β1-mediated Repression of the MMP-1 Promoter but Not Induction of the Timp-1 Promoter—In order to reinforce the overexpression data above, cells were co-transfected with the 517/60 MMP-1 promoter or −95/47 Timp-1 promoter construct with either the Smad-binding oligonucleotide, S4BE, containing three Smad-binding sites, or a mutant oligonucleotide S4BEmut, where the Smad-binding sites were functionally mutated. Binding of Smads to S4BE and the absence of binding to the S4BEmut were demonstrated using EMSA (see Fig. 8; data not shown). Fig. 7 shows that wild-type oligonucleotide does not alter the pattern of TGF-β1 or PMA induction of the Timp-1 gene, whereas the S4BEmut has no effect. Again, this suggests that whereas Smads do mediate TGF-β1 repression of the MMP-1 gene, they are not involved in TGF-β1 induction of the Timp-1 gene.

Smad Binding to the Timp-1 or MMP-1 AP1 Motifs—Using the S4BE oligonucleotide in EMSA with nuclear extracts from C3H10T1/2 cells, five DNA-protein complexes were apparent (Fig. 8). Supershift analysis with antibodies against Smad 2/3 confirmed the S4BE oligonucleotide in EMSA with nuclear extracts from C3H10T1/2 or human skin fibroblasts shows an identical pattern of binding on human skin fibroblasts shows an identical pattern of binding to
Regulation of Timp-1 and MMP-1 by TGF-β

Fig. 8. Electrophoretic mobility shift assay on the S4BE oligonucleotide. C3H10T1/2 murine fibroblasts were serum-starved for 24 h and then stimulated with TGF-β (2 ng/ml), or TGF-β and PMA (10−7 M) together. Nuclear extracts were harvested at t = 3 h. EMSA was performed on the S4BE oligonucleotide in the absence or presence of antibodies to Smad2/3 or Smad4 (2 μg per binding reaction). T, TGF-β; TP, TGF-β + PMA. Shift (A-E) and supershift complexes are marked.

each oligonucleotide (as shown in Fig. 2). No supershift band could be seen in the additional presence of the anti-Smad 4 antibody (data not shown). However, using the labeled S4BE as probe, the MMP-1 AP1 oligonucleotide competes band A (the Smad-containing complex) at a 100-fold excess, whereas the Timp-1 AP1 oligonucleotide does not (Fig. 9). Intriguingly, the Timp-1 AP1 oligonucleotide competes band E, but because self-competition with cold S4BE shows this band as nonspecific, this finding is difficult to interpret (Fig. 9).

DISCUSSION

TGF-β has profound effects on extracellular matrix homeostasis, in part via its ability to alter the balance between proteinases and their inhibitors at the level of gene expression (7, 8). The intracellular signaling pathways by which TGF-β mediates its actions are diverse. The Smad pathway, specific to the TGF-β family, is probably of prime importance, but many reports implicate other pathways (e.g. mitogen-activated protein kinase pathways (14, 15)); furthermore, cross-talk between the Smads and other signaling pathways modifies the TGF-β response (16-18). The reciprocal effect of TGF-β on the expression of TIMP-1 and MMP-1 initially described by Edwards et al. (11) supports its role in matrix anabolism. The mechanisms by which TGF-β induces Timp-1 yet represses induced MMP-1 have remained opaque; hence, the current study sought to address this, with a focus on Timp-1 gene expression.

The promoter requirements for TGF-β-induction of the Timp-1 gene were ascertained using deletion mutants, demonstrating that the proximal (−59/−53) AP1 site plays a major role. Internal substitutions across −50 to +47, leaving the AP1 site intact in the context of −223/+47 Timp-1, failed to demonstrate further elements necessary for TGF-β induction (data not shown). This was reinforced by EMSA on overlapping oligonucleotides across −50 to +47, which showed no altered protein-DNA interactions. EMSA on an oligonucleotide containing the AP1 sequence demonstrated an increase in binding upon either TGF-β or PMA stimulation with the only significant difference between the two being the induction of Fra-1 containing complexes by PMA but not TGF-β. Analysis of the contribution of Fos and Jun family members using both overexpression and cell lines from AP1 knockout mice revealed a requirement for c-Fos, c-Jun, and JunD in TGF-β induction of Timp-1, whereas c-Fos was not essential for PMA induction of the gene. Smad co-expression experiments show that the Timp-1 gene is not Smad-responsive; nor is TGF-β-induction of the gene blocked by the inhibitory Smad 7. These facets are shown very clearly in the control plasmid 3TP-Lux. Finally, co-transfection of a Smad-binding oligonucleotide has no effect on TGF-β induction of the Timp-1 gene. From all of these data, it can be concluded that the induction of Timp-1 gene expression by TGF-β is AP1- but not Smad-dependent. This firm conclusion must be tempered by the data coming from Smad knockout cells, where induction of Timp-1 is still evident, but its magnitude is reduced, compared with wild-type cells. However, it should be reiterated that the magnitude of response to TGF-β1 varies among wild-type cell lines and also that the absence of Smads may have secondary consequences to cellular function that are separate from direct effects on Timp-1 expression.

A recent report by Verrecchia et al. (31) states that the Timp-1 gene is Smad-dependent, in disagreement with the majority of data in the current study. The following lines of evidence are presented: (i) TGF-β induces a greater than 2-fold induction of the gene within 30 min as assessed by cDNA microarray analysis, and this is not blocked by the protein synthesis inhibitor cycloheximide or the c-Jun N-terminal kinase inhibitor curcumin; (ii) a Timp-1 promoter construct driving CAT expression is induced by TGF-β at a 24-h time point, and this is blocked by dominant negative Smad 3 or by Smad 7; and (iii) co-expression of Smad 3 with the Timp-1 promoter construct mimics the effect of TGF-β, and no promoter transactivation is seen in Smad 3−/− cells. There are many differences in these data compared with the current study. (i) The microarray data in Verrecchia et al. (31) shows the TGF-β-induction of Timp-1 gene expression maximal at 30–60 min, remaining at this level at 4 h. In our hands, in both murine fibroblasts and human fibroblasts (dermal or lung), TGF-β induction of Timp-1 becomes maximal at 12–24 h, as assessed by Northern blot (12). (ii) The use of cycloheximide as a protein synthesis inhibitor in such studies is problematic, since it has been shown to augment the induction of immediate early genes such as c-fos and c-jun by growth factors (38) via the p38 mitogen-activated protein kinase pathway (39); indeed, Verrecchia et al. (31) state that cycloheximide treatment causes “a broad increase in gene expression . . . .” Using emetine as a protein synthesis inhibitor, the induction of Timp-1 by TGF-β is dependent on new protein synthesis (data not shown). (iii)
Inhibitors of the mitogen-activated protein kinase pathways such as curcumin (c-Jun N-terminal kinase), U0126 (extracellular signal-regulated kinase), or SB202190 (p38) all block the TGF-β induction of Timp-1 to varying extents in the C3H10T1/2 cells used in the current study (data not shown). (iv) The promoter studies in Verrecchia et al. (31) use an undisclosed length of murine promoter driving CAT expression in human cells measured at a 24-h time point with no serum starvation prior to TGF-β treatment (Timp-1 is serum-responsive [40]); the studies above use constructs driving luciferase expression measured at a 6-h time point to avoid potential secondary responses of the cells to TGF-β-induced growth factors. (v) Smad 3/5−/− cells, in our hands, were difficult to transfect; however, as discussed above, the endogenous Timp-1 gene is expressed in these cells is still induced by TGF-β, albeit at a reduced level.

The dependence of Timp-1 gene expression on AP1 factors has been described in other systems and with other inducing agents (e.g., Botelho et al. [41] ascribe the induction of Timp-1 by oncostatin M to an induction of c-Fos and a change in the major AP1-binding complex from c-Jun/c-Fos to JunD/c-Fos in HepG2 cells; Smart et al. [42] demonstrate that JunD, Fra2, and FosB associate with the Timp-1 AP1 site during hepatic stellate cell activation, of which JunD is functionally the most important. The current study indicates that c-Fos, Fra2, FosB, c-Jun, JunD, and JunB are all present in TGF-β-induced cell nuclear extracts and can bind the Timp-1 AP1 sequence on EMSA (presuming the specificity of antibodies used in supershift experiments). An overall increase in AP1 binding is observed upon TGF-β treatment compared with control. Whereas c-Fos, c-Jun, and JunD were shown to be necessary for Timp-1 induction using knockout cell lines, JunB and Fra2 have not been assessed in this manner, since cells were not available. It is possible that members of the cAMP-response element-binding protein family of transcription factors, which can heterodimerize with AP1 factors, are also important in the response of Timp-1 to TGF-β; indeed, ATF2 is a target of TGF-β signaling via both the Smad pathway and TAK1/p38 (43).

The AP1 motif in the Timp-1 promoter differs at a single base pair from the MMP-1 consensus (5′-TGAGTCA-3′ compared with 5′-TGAGTCA-3′), with the only known consequence being binding of an unknown single-stranded DNA-binding protein to the former but not the latter (23). However, substitution of the wild-type site for the consensus in a promoter construct dem-
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The Comparative Role of Activator Protein 1 and Smad Factors in the Regulation of Timp-1 and MMP-1 Gene Expression by Transforming Growth Factor-β1

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J. Biol. Chem. 2003, 278:10304-10313.
doi: 10.1074/jbc.M212334200 originally published online January 13, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M212334200

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