Identification of Novel TGF-β/Smad Gene Targets in Dermal Fibroblasts using a Combined cDNA Microarray/Promoter Transactivation Approach*

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Despite major advances in the understanding of the intimate mechanisms of transforming growth factor-β (TGF-β) signaling through the Smad pathway, little progress has been made in the identification of direct target genes. In this report, using cDNA microarrays, we have focussed our attention on the characterization of extracellular matrix-related genes rapidly induced by TGF-β in human dermal fibroblasts and attempted to identify the ones whose up-regulation by TGF-β is Smad-mediated. For a gene to qualify as a direct Smad target, we postulated that it had to meet the following criteria: (1) rapid (30 min) and significant (at least 2-fold) elevation of steady-state mRNA levels upon TGF-β stimulation, (2) activation of the promoter by both exogenous TGF-β and co-transfected Smad3 expression vector, (3) up-regulation of promoter activity by TGF-β blocked by both dominant-negative Smad3 and inhibitory Smad7 expression vectors, and (4) promoter transactivation by TGF-β not possible in Smad2−/− mouse embryo fibroblasts. Using this stringent approach, we have identified COL1A2, COL3A1, COL6A1, COL6A3, and tissue inhibitor of metalloproteases-1 as definitive TGF-β/Smad3 targets. Extrapolation of this approach to other extracellular matrix-related gene promoters also identified COL1A1 and COL5A2, but not COL6A2, as novel Smad targets. Together, these results represent a significant step toward the identification of novel, early-induced Smad-dependent TGF-β target genes in fibroblasts.

Members of the TGF-β2 superfamily (activin, bone morphogenetic proteins, TGF-βs, and decapentaplegic) are multifunctional cytokines that control various aspects of cell growth and differentiation and play an essential role in embryonic development, tissue repair, or immune homeostasis (1, 2). In addition, TGF-β is the prototypic fibrogenic cytokine, enhancing extracellular matrix (ECM) gene expression and down-regulating that of matrix-degrading enzymes. Increased expression of TGF-β is often associated with fibrotic states and abnormal accumulation of ECM proteins in affected tissues (3–6). The TGF-β signal via serine/threonine kinase transmembrane receptors, which phosphorylate cytoplasmic mediators of the Smad family (7–9). The ligand-specific Smad1, Smad2, Smad3, and Smad5 interact directly with, and are phosphorylated by, activated TGF-β receptors type I. Smad1 and Smad5 are specific for bone morphogenetic proteins, whereas Smad2 and Smad3 can be activated by both TGF-β and activin receptors. Receptor-activated Smads are kept in the cytoplasm in the basal state bound to the protein SARA (Smad anchor for receptor activation) (10). Upon phosphorylation at their SSX domain carboxy-terminal motif, they are released from SARA and form heteromeric complexes with Smad4, a common mediator for all Smad pathways. The resulting Smad heterocomplexes are then translocated into the nucleus where they activate target genes, binding DNA either directly or in association with other transcription factors. Members of the third group of Smads, the inhibitory Smads, Smad6 and Smad7, prevent phosphorylation and/or nuclear translocation of receptor-associated Smads (7–9).

TGF-β also initiates other signaling pathways, such as the stress-activated protein kinase/c-Jun amino-terminal kinase (JNK) pathway (11). This intracellular signaling proceeds through sequential activation of a mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEKK1), a mitogen-activated protein kinase kinase (MKK4 or MKK7), and a mitogen-activated protein kinase, JNK. JNK then translocates into the nucleus where it phosphorylates several transcription factors including c-Jun, ATF-2, and Elk-1 (12), leading to specific transcriptional responses.

Although tremendous progress has been made over the past few years in the understanding of the molecular processes underlying TGF-β signal propagation by the Smad cascade, thus far, relatively few genuine mammalian Smad gene targets have been identified. Specifically, at present, only about a dozen genes are known to contain Smad-responsive regions, binding Smad complexes directly or indirectly. These genes include, in order of characterization as Smad targets, type VII collagen (COL7A1) (13), plasminogen activator inhibitor-1 (14), the cyclin-dependent kinase inhibitor p21 (15), JunB (16), and p21 (17), more recently, COL1A2 (17), c-Jun (18), immunoglobulin germ-line Cα (19), Smad7 (20), human germline IgA (21), platelet-derived growth factor-β (22), β5 integrin (23), apoCIII (24), and the cyclin-dependent kinase inhibitor p15 (25). Interestingly, to date, all Smad gene targets identified downstream of TGF-β are Smad3- not Smad2-, dependent.

Despite the fundamental role played by TGF-β in ECM remodeling and as a fibrogenic factor, little is known about the
role of Smad signaling in ECM gene expression. In this report, we have used complementary techniques, differential hybridization of cDNA microarrays together with precise gene promoter analyses, to search for novel fibroblast Smad targets. This approach allowed us to characterize six novel Smad targets, COL1A1, COL3A1, COL5A2, COL6A1, COL6A3, and TIMP-1, and to propose an additional list of 49 immediate-early TGF-β target genes whose activation by TGF-β is rapid and does not require either protein neo-synthesis or JNK activity, therefore representing potential novel Smad targets.

MATERIALS AND METHODS

Cell Cultures and Reagents—Human dermal fibroblasts and Smad3α−/− mouse embryonic fibroblasts (26, 27) were a kind gift from Drs. A. B. Roberts and C. Deng, National Cancer Institute, NIH, Bethesda, MD, were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, and antibiotics (100 units/ml penicillin, 50 μg/ml streptomycin-G, and 0.25 μg/ml Fungizone™). Human recombinant TGF-β1 was from R & D Systems, Inc. (Minneapolis, MN). Curcumin and cycloheximide were purchased from Sigma.

Differential Hybridization of Atlas™ Human cDNA Expression Arrays—Total RNA from control and TGF-β-treated fibroblasts was obtained using an RNeasy kit (Qiagen) and treated with DNase I to avoid genomic DNA contamination of reverse transcription reactions. Radioactive cDNA synthesis was carried out as described in the Atlas™ cDNA expression array user manual (CLONTECH, San Diego, CA). Equal amounts of 32P-labeled cDNAs (10⁷ cpm) from control and TGF-β-treated fibroblast RNA samples were hybridized in parallel to Atlas™ human cell interaction cDNA expression arrays (catalog number 7746–1; CLONTECH) for 18 h at 68 °C. The filters were then washed four times in 2× SSC and 1% SDS for 30 min at 68 °C and twice in 0.1× SSC and 0.5% SDS at 68 °C, according to the manufacturer’s protocol. Membranes were then exposed to Eastman Kodak Co. phosphoimager screens for 3 days. Hybridization signals were quantified with a Storm 840 phosphorimagier using ImageQuant software (Amersham Pharmacia Biotech) and normalized against glyceraldehyde-3-phosphate dehydrogenase mRNA levels in the same samples. Significant modulation of gene expression was set arbitrarily to 2-fold.

Plasmid Constructs—Several ECM gene promoter/reporter gene constructs were used to examine the role played by TGF-β and Smad3 in their transcriptional activity. The human COL1A2, COL6A1, COL6A2, COL7A1, and decorin promoter constructs have been described previously (28–32). Human COL6A3 promoter was cloned recently in our laboratory.2 Human COL1A1 (33), mouse COL3A1 (34), human COL5A2 (35), and human TIMP-1 (36) promoter constructs were kind gifts from Drs. Sergio A. Jimenez (Thomas Jefferson University, Philadelphia, PA). Benoit de Crombrugghe (M. D. Anderson Cancer Center, Houston, TX), Francesco Ramirez (Mount Sinai Medical Center, New York, NY), and Dylan R. Edwards (University of Calgary, Calgary, Alberta, Canada). Full-length Smad3 and carboxy-terminally truncated dominant-negative Smad3 (Smad3ΔC) expression vectors (kind gifts from Drs. Anita B. Roberts and Rober J. Lechleider, National Cancer Institute, NIH, Bethesda, MD and Rik Derynck, University of California at San Francisco, San Francisco, CA, respectively) have been described previously (37, 38). Smad7 expression vector (39) was a kind gift from Dr. Peter ten Dijke, Netherlands Kanker Instituut, Amsterdam, Netherlands.

Transient Cell Transfection and CAT Reporter Assays—Transient cell transfections were performed with the calcium phosphate/DNA co-precipitation procedure. CAT activity was measured using [14C]chloramphenicol as a substrate (40) followed by thin layer chromatography and quantitation with a phosphorimagier.

RESULTS AND DISCUSSION

Effects of TGF-β on Fibroblast ECM-related Gene Expression Profiles as Measured by cDNA Microarray Analysis—The technique of differential hybridization of cDNA expression arrays was used to identify differences in the expression pattern of 265 known ECM-related genes between control and TGF-β-treated fibroblasts. Because Smad activation and nuclear translocation occurs within minutes and Smad-DNA complexes can be observed as early as 10 min after TGF-β addition into fibroblast culture medium (13), we focused our attention on early time points, to determine which genes are activated rapidly by TGF-β, as opposed to secondary gene activation that may involve protein/transcription factor neo-synthesis. At each of the time points tested (30, 60, 120, and 240 min), RNA was extracted from both control and TGF-β-treated fibroblast cultures, and differential hybridization of cDNA arrays was performed. Among the 265 genes whose probe sets are represented onto the Atlas™ cell interactions cDNA arrays used in these experiments, 77 of them showed no significant hybridization signal in either control or TGF-β-treated cultures at any of the time points tested (not shown). Among the 188 genes detected, 90 showed no or little alteration in their expression levels upon TGF-β treatment. The remaining 98 genes, modulated by TGF-β, were classified into clusters, based upon the temporal profile of their activation (Fig. 1). Clusters 1–3 contain 58 genes whose expression is strongly up-regulated 30 min after TGF-β addition and keeps increasing with time (cluster 1), reaches a plateau (cluster 2), or returns rapidly to basal level (cluster 3). Clusters 4–6 comprise genes whose expression is delayed; their expression is not noticeably up-regulated by TGF-β at the 30-min time point and then follows various patterns of temporal regulation. The complete list of genes contained within these clusters is provided in Table I.

Because our main aim was to identify immediate-early targets of the Smad pathway, genes induced as early as 30 min post-TGF-β addition to the cultures were further studied in experiments in which on-going protein synthesis is blocked by cycloheximide. Analysis of gene expression by differential hybridization of cDNA arrays indicated that, at the 30-min time point, a similar set of 58 genes was induced by TGF-β in the presence or absence of cycloheximide, consistent with a transcriptional response not requiring on-going protein synthesis, such as expected from direct Smad targets. It should be noted that a broad increase in gene expression induced by cycloheximide alone was also observed (not shown), a phenomenon that has been previously described (41).

In the presence of curcumin, an inhibitor of JNK activity (42), only three genes among the 58 identified above and belonging to clusters 1–3 were not stimulated by TGF-β after 30 min, fibronectin, perlecain, and closely related low density lipoprotein receptor (43). Interestingly, it has have been shown previously that fibronectin gene activation by TGF-β is a JNK-

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2 R. Z. Zhang and M.-L. Chu, manuscript in preparation.
### Table I

List of genes classified in clusters according to their induction kinetics by TGF-β

90/198 expressed genes derived from cDNA microarray (Atlas™ cell interaction array, Clontech catalog number 7746-1) analysis were significantly (over 2-fold) upregulated by TGF-β in two independent experiments. They are classified in six clusters according to the kinetics of their induction (see Fig. 1). GenBank™ accession numbers, gene names, and categories for classification are provided, as supplied by Clontech. Note that clusters 1–3 contain genes whose expression is significantly upregulated by TGF-β after 30 min.

| Accession number | Definition | Accession number | Definition | Accession number | Definition |
|------------------|------------|------------------|------------|------------------|------------|
| **Cluster 1**    |            | **Cluster 2**    |            | **Cluster 3**    |            |
| M59911           | integrin alpha-3 | X55525         | collagen type I pro-alpha-2 | X592761       | fibronectin |
| X04429           | plasminogen activator inhibitor-1 | X14420         | collagen type III pro-alpha-1 | X02322        | MMP-3;stromelysin-1 |
| M15518           | tissue-type plasminogen activator precursor | X18579        | collagen type VI alpha-1 | X20180        | decorin |
| M30269           | nidogen | X52022          | collagen type VI alpha-3 | X13916        | low-density lipoprotein receptor-related protein 1 precursor |
| J03040           | SPAR | U77493         | notch2 | M74088         | APC |
| U43901           | laminin 37-kDa receptor | Y11306        | beta-catenin hTGF-4 | M65062        | IGFBP5 |
| L16306           | versican (isoform, V1, V2, V3) | L20471        | emmprin | M65062        | IGFBP5 |
| X41766           | metalloprotease disintegrin cystein-rich protein precursor | X75208        | tyrosine-protein kinase receptor | M65062        | IGFBP5 |
| **Cytoplasmic regulators and effectors** |            | **Cluster 4**    |            | **Cluster 5**    |            |
| J03210           | TRK E | X57766         | MMP-2 | M33294        | MMP-11 |
| M31159           | IGFBP3 | X57527         | collagen type VII alpha-1 | M59911        | integrin alpha-3 |
| M855289          | HSPG/perlecan | D26512        | MMP-14 (MT1-MMP) | D50477        | MMP-16 (MT3-MMP) |
| U16306           | versican | X05232        | MMP-3 | X02134        | TIMP-1 |
| L25080           | rhoB | Z20183        | TIMP-3 | X13916        | low-density lipoprotein receptor-related protein 1 precursor |
| X61587           | rhoG | L16785         | e-myc-transcription factor | X49911        | zyxin+zyxin-2 |
| **Cluster 6**    |            | **Cell-matrix interaction** |            | **Cell-matrix interaction** |            |
| M14219           | decorin | M93642         | collagen type XVI alpha-1 | M93642        | collagen type XVI alpha-1 |
| M13395           | leukocyte adhesion protein beta subunit | M36174         | cadherin-6 | M61916        | laminin B1 |
| X05232           | MMP-3 | X76565         | tenascin-C | X14787        | thrombospondin 1 precursor |
| D26512           | MMP-14 (MT1-MMP) | X14787       | thrombospondin 1 precursor | L12350        | thrombospondin 2 precursor |
| D50477           | MMP-16 (MT3-MMP) | X05231        | MMP-1; collagenase-1 | X89576        | MMP-17 (MT4-MMP) |
| X20134           | TIMP-1 | M93642         | collagen type XVI alpha-1 | M61916        | laminin B1 |
| Z20183           | TIMP-3 | X76565         | tenascin-C | X14787        | thrombospondin 1 precursor |
| X13916           | low-density lipoprotein receptor-related protein 1 precursor | M93642         | collagen type XVI alpha-1 | M61916        | laminin B1 |

**Clustering Criteria:**

- **Cluster 1:** Cell-Cell interaction
- **Cluster 2:** Cell-matrix interaction
- **Cluster 3:** Cell-matrix interaction
- **Cluster 4:** Cell-matrix interaction
- **Cluster 5:** Cell-matrix interaction
- **Cluster 6:** Cell-matrix interaction

**Note:**

- Accession numbers are as supplied by Clontech.
- GenBank™ accession numbers are provided for each gene.
- Gene names and categories for classification are also provided.
- The table includes genes whose expression is significantly upregulated by TGF-β after 30 min.
FIG. 2. Exogenous TGF-β and Smad3 overexpression transactivate the COL1A2, COL3A1, COL6A1, COL6A3, and TIMP-1 promoters. A, human dermal fibroblast cultures were transfected with COL1A1, COL3A1, COL6A1, COL6A3, or TIMP-1 promoter/CAT reporter constructs. 3 h later, TGF-β (10 ng/ml) was added to the medium, and the incubation was continued for 24 h, at which time CAT activity was determined. B, empty pcDNA3 or Smad3 expression vectors were co-transfected with the same promoter/reporter constructs as in panel A. 24 h later CAT activity was determined. Fold induction of promoter activities by TGF-β (panel A) or Smad3 (panel B) is shown as the mean ± S.D. of at least three independent experiments performed in duplicate.

FIG. 3. Overexpression of dominant-negative Smad3 or Smad7 prevents COL1A2, COL3A1, COL6A1, COL6A3, and TIMP-1 promoter up-regulation by TGF-β. Human dermal fibroblast cultures were co-transfected with COL1A1, COL3A1, COL6A1, COL6A3, or TIMP-1 promoter/CAT reporter constructs, together with empty pcDNA3, Smad3ΔC, or Smad7 expression vectors, as indicated. 3 h later, TGF-β (10 ng/ml) was added (+) or not (−) to the medium, and incubations were continued for 24 h before CAT activity was determined. Relative promoter activities (mean ± S.D.) of at least three independent experiments performed in duplicate are shown in the form of bar graphs.

**Effects of TGF-β and Smad3 Overexpression on ECM Promoter/CAT Reporter Gene Constructs**—We next tried to determine whether the rapid elevation of steady-state mRNA levels observed for several ECM-related genes upon TGF-β stimulation, as observed using differential cDNA array hybridization, resulted from transcriptional activation at the level of their promoter regions. We focused our attention on the 5′ regulatory regions of COL1A2, COL3A1, COL6A1, COL6A3, and TIMP-1 genes, which all belong to clusters 1 and 2, corresponding to genes whose expression is enhanced at least two times by TGF-β within 30 min. In a first set of experiments, TGF-β responsiveness was examined. All promoter constructs tested responded to exogenous addition of TGF-β by a 3–5-fold elevation of their activity (Fig. 2A). As a first approach to determine whether these promoters were sensitive to Smad activation downstream of TGF-β, co-transfection experiments were performed in which each ECM promoter/CAT reporter construct was co-transfected with a Smad3 expression vector. As shown in Fig. 2B, Smad3 overexpression led to significant up-regulation of each of the promoters tested, indicating that the Smad pathway may be involved in the TGF-β effect.
TGF-β, where both COL6A1 and COL6A3 are coordinately regulated and are direct Smad targets, whereas COL6A2 is not. These data differ slightly from previous observations indicating specific up-regulation of COL6A3 but not COL6A1 or COL6A2 by TGF-β, when mRNA steady-state levels were detected after 48 h of stimulation (46).

mRNA steady-state levels of COL1A1, which encodes the α1(1) chain of type I collagen, have been shown previously to be elevated by TGF-β (47). The corresponding promoter was found to be up-regulated by both exogenous addition of TGF-β and co-transfection of a Smad3 expression vector (not shown). In addition, its activation by TGF-β was blocked by both dominant-negative Smad3 and Smad7 overexpression (not shown), indicating that the COL1A1 promoter is also a Smad target. Interestingly, these data corroborate the previously described coordinate regulation of COL1A1 and COL1A2 (48–50) and indicating that the COL1A1 promoter is also a Smad target. These data differ slightly from previous observations indicating that the COL1A1 promoter is also a Smad target.

Absence of COL1A1, COL1A2, COL3A1, COL5A1, COL6A3, and TIMP-1 Promoter Transactivation by TGF-β in Smad3+/− Mouse Embryo Fibroblasts—To ascertain the role played by Smad3 in the transactivation of the ECM-related promoters identified above, transient cell transfection experiments were carried out using either wild-type or Smad3+/− mouse embryo fibroblasts (26, 27). As expected from the data presented above in which either dominant-negative Smad3 or inhibitory Smad7 expression vectors blocked TGF-β-driven transactivation of the COL1A1, COL1A2, COL3A1, COL5A2, COL6A1, COL6A3, and TIMP-1 promoters in Smad3+/− mouse embryo fibroblasts. On the other hand, all promoters add their activity significantly increased by TGF-β in the corresponding wild-type mouse embryo fibroblasts (not shown). These results confirm the role played by Smad3 to mediate TGF-β transactivation of these ECM promoters.

The implication of TGF-β in fibrotic processes has long been suspected (3–6). The demonstration that several genes encoding fibrillar collagens, COL1A1, COL1A2, COL3A1, and COL5A2, are up-regulated by TGF-β acting directly through the Smad pathway indicates that the latter is likely to play a key role in the development of tissue fibrosis. It also suggests that therapeutic approaches directed toward the Smad cascade may prove useful in the treatment of fibrotic disorders.

In conclusion, using cell matrix interaction-specific commercial cDNA microarrays we have identified 58 immediate-early targets for TGF-β. Only three of these 58 genes had their activation blocked by curcumin, a selective inhibitor of JNK, a signaling pathway alternative to the Smad cascade downstream of the TGF-β receptors. These data suggest that the JNK pathway downstream of the TGF-β receptors likely affects very few early ECM-related target genes as compared with the Smad pathway. Using promoter/reporter gene constructs to analyze the transcriptional responsiveness to the TGF-β/Smad pathway of several genes identified by differential hybridization of cDNA arrays, we have formally identified six novel TGF-β/Smad immediate-early gene targets, namely COL1A1, COL3A1, COL5A2, COL6A1, COL6A3, and TIMP-1. Together with the identification of 49 other immediate-early TGF-β gene targets, this study represents a major leap forward in the identification of Smad/TGF-β targets.

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