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Tyrosine Dephosphorylation of Nuclear Proteins Mimics Transforming Growth Factor β1 Stimulation of α2(I) Collagen Gene Expression†

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Transforming growth factor β1 (TGF-β1) exerts a positive effect on the transcription of genes coding for several extracellular matrix-related products, including collagen I. We have previously identified a strong TGF-β1-responsive element (TbRE) in the upstream promoter sequence of the α2(I) collagen (*COL1A2*) gene. Our experiments have shown that TGF-β1 stimulates *COL1A2* transcription by increasing binding of an Sp1-containing complex (TbRC) to the TbRE. They have also suggested that the change occurs via posttranslational modification of a protein(s) directly or indirectly interacting with Sp1. Here, we provide evidence showing that tyrosine dephosphorylation of nuclear proteins mimics the stimulation of *COL1A2* transcription by the TGF-β1-activated signaling pathway. Preincubation of nuclear extracts with protein tyrosine phosphatase (PTPase) but not with protein phosphatase type 2A (PP2A), a serine/threonine phosphatase, enhanced binding of the TbRC to the same degree as culturing cells in TGF-β1. Consistent with these in vitro findings, genistein, a tyrosine kinase inhibitor, led to markedly increased *COL1A2* gene expression, whereas sodium orthovanadate, a tyrosine phosphatase inhibitor, decreased it substantially. These results were supported by transfection experiments showing that genistein and sodium orthovanadate have opposite effects on TbRE-mediated transcription. Moreover, nuclear proteins isolated from genistein-treated cells were found to interact with the TbRE significantly more than those from untreated cells. Furthermore, pretreatment of cells with sodium orthovanadate virtually abrogated nuclear protein binding to the TbRE, but not to a neighboring *cis*-acting element unresponsive to TGF-β1. The results of this study, therefore, provide the first correlation between tyrosine dephosphorylation, increased binding of a transcriptional complex, and TGF-β1 stimulation of gene expression.

Transforming growth factor β1 (TGF-β1) is the prototype of a family of polypeptides that orchestrate a plethora of biological processes in the developing vertebrate embryo and in the adult organism (21). Among others, one of the functions of TGF-β1 is to maintain the dynamic balance between the synthesis and degradation of the extracellular matrix (21). This role of TGF-β is critical for a variety of physiologic activities, including morphogenesis, growth, tissue remodeling, and wound healing (24). It follows that altered TGF-β expression is probably involved in the genesis and progression of several conditions, such as fibrotic disorders of the lung, skin, kidney, and liver (2). TGF-β1 contributes to matrix physiology by modulating cell proliferation, growth, and differentiation, while concomitantly regulating the expression of several matrix-related gene products (21). The regulatory role of TGF-β1 includes stimulating the synthesis of structural components and cognate receptors as well as inhibiting the production of matrix metalloproteinases and inducing their inhibitors (21).

Some of the TGF-β1 action on gene expression is exerted at the transcriptional level. TGF-β1-responsive elements (TbREs) of several matrix-related genes have been characterized; in a few instances, the mechanisms mediating the response have also been described (10, 14, 27). The first of these descriptions focused on how TGF-β1 inhibits expression of the rat transin/stromelysin gene. In their original report, Kerr et al. (14) showed that TGF-β1 induces the formation and binding of a Fos-containing complex which recognizes the TGF-β1-inhibitory promoter element. Interestingly, similar TGF-β1-inhibitory promoter elements lacking an AP-1 consensus sequence are present in several other metalloproteinase-coding genes known to be downregulated by TGF-β1 (14). A different mechanism was later shown to operate in the TGF-β1-induced upregulation of the genes coding for plasminogen activator inhibitor type 1 (*PAI-1*) and α2(I) collagen (*COL1A2*) (10, 27). According to the first of these descriptions, TGF-β1 treatment of cultured HepG2 cells resulted in a marked increase in the binding of an apparently novel 100-kDa protein to the cognate recognition site (27). The increase is likely to be caused by a posttranslational modification, since pretreatment of the cells with cycloheximide had no effect on the TGF-β1-induced effect on nuclear protein binding to the *cis*-acting sequence (27). At about the same time, we reported a similar mechanism operating in the stimulation of the human *COL1A2* gene (10). We in fact found that exposure of cultured fibroblasts to TGF-β1 leads to posttranslational modification and, thus, to increased binding of the nuclear protein complex to the cognate TbRE. Moreover, we demonstrated that Sp1, or an immunologically related factor, is a component of the TbRE-bound complex (TbRC) but not the target of the modification (10). Apparently, this posttranslational event involves an Sp1-interacting component(s) of the TbRC (10). It should be noted that earlier work by others had suggested that an NF1 binding site mediates the transcriptional activation by TGF-β1 of the mouse *COL1A2* gene (25). However, unpublished data from our laboratory and recent work by Chang and Goldberg (3) concur in excluding

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the involvement of NF1 and its alleged binding site in the *COL1A2* response to TGF-β1.

It is one of our goals to characterize in full the TGF-β1-induced cascade leading to *COL1A2* upregulation, information critical to our understanding of matrix remodeling and fibrotic conditions. Toward this end, we have begun studying the nature of the posttranslational modification(s) and the identity of the Sp1-interacting factor(s) targeted by it. Here, we describe several lines of evidence that establish a relationship between tyrosine dephosphorylation of a nuclear protein(s) and the TGF-β1-elicited change in TbRC binding. To the best of our knowledge, this is the first case in which dephosphorylation, in particular, tyrosine dephosphorylation, of a nuclear protein(s) has been associated with induced transcription. Aside from its novelty, the finding represents the first step toward delineation of the pathway that transduces the TGF-β1 signal from the cell surface to the *COL1A2* gene.

MATERIALS AND METHODS

Reagents. TGF-β1 was purchased from Collaborative Biomedical Products (Bedford, Mass.). Genistein and okadaic acid were obtained from Upstate Biotechnology Incorporated (Lake Placid, N.Y.). Sodium orthovanadate and 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7) were purchased from Sigma Chemical Company (St. Louis, Mo.). Tyrosine-specific protein phosphatase (PTP-1B) was a generous gift from H.-C. Li (Mount Sinai School of Medicine, New York, N.Y.). The serine/threonine phosphatases protein phosphatase type 1 (PP1) and PP2A were purchased from Upstate Biotechnology Inc. Yersinia-derived protein tyrosine phosphatase (PTPase) was obtained from Boehringer Mannheim (Indianapolis, Ind.).

Cell culture and transfection assays. Primary human fetal skin fibroblasts (CF37) were grown in Dulbecco's modified Eagle's medium containing a high concentration of glucose (4.5 g/liter) supplemented with 10% fetal bovine serum (HyClone, Logan, Utah). Preparation and transfection of plasmid DNA into cells, by the calcium phosphate method, were performed as previously described (1). Five hours after transfection, fibroblasts were treated with 10% glycerol for 90 s and then placed in medium containing 3.5% fetal bovine serum. Eighteen hours later, protein kinase or phosphatase inhibitors were added at the concentrations indicated below. The concentration of inhibitors was optimized experimentally within a range close to that used in other systems (20). Cells were harvested 8 h later and subjected to luciferase and chloramphenicol acetyltransferase (CAT) assays as previously described (10). Transcriptional activity of each of the chimeric constructs was normalized against a cotransfected simian virus 40 luciferase vector. The Mann-Whitney U test was used to determine the statistical value of all the functional data. Viability of the cells was estimated by the trypan blue (0.4% in phosphate-buffered saline [PBS]) exclusion test; in all cases, cellular viability was more than 90%. To generate stable transfectants, the hygromycin gene under the control of the PGK-1 promoter was subcloned downstream of the CAT gene in the −378COL1A2/CAT construct (1). Stable transfectants were selected in the presence of 150 μg of hygromycin per ml. Individual hygromycin-resistant clones were amplified in 100-microwell plates and genotyped to estimate the number of integrated copies of the construct.

Chimeric plasmids. The chimeric plasmids containing upstream *COL1A2* promoter sequences linked to CAT have been previously described (1). The plasmid containing the three TbRE copies was constructed by sequential subcloning of the −340 to −183 fragment of *COL1A2* upstream of the thymidine kinase (TK) promoter in the pBLCAT2 vector (19). The integrity and orientation of the sequence were verified by DNA sequencing (28).

Northern (RNA) blot analysis and in vitro transcription assay. Confluent cultures of CF37 fibroblasts were washed twice with PBS and placed in Dulbecco's modified Eagle's medium containing 3.5% fetal bovine serum. Eighteen hours later, they were treated with genistein (50 μg/ml), H7 (80 μM), sodium orthovanadate (100 μM), or okadaic acid (100 nM), alone or in combination with TGF-β1 (2 ng/ml). After 4 h in culture, total RNA was extracted as described by Chomczynski and Sacchi (6) and used for Northern blot hybridization to probes for human *COL1A2* and glyceraldehyde-3-phosphate dehydrogenase (GAPD). Blots were washed under stringent conditions (0.1× SSC [0.15 M NaCl plus 0.015 M sodium citrate]–0.1% sodium dodecyl sulfate at 65°C) and exposed to X-ray film at −70°C with intensifying screens. Rates of *COL1A2* transcription were determined in control, sodium orthovanadate-treated, and genistein-treated cells according to a published protocol (29). GAPD cDNA and pBR322 vector DNA were used in parallel as controls. Quantitative data were obtained with the computer program Adobe Photoshop (Adobe Systems Incorporated, Mountain View, Calif.). All experiments were performed in triplicate.

In vitro dephosphorylation of nuclear extracts. Control and TGF-β1-treated nuclear extracts were dephosphorylated with calf intestinal phosphatase (5 U/5 μg of protein) (CIP) (New England BioLabs, Beverly, Mass.) at room temperature for 30 min in binding buffer containing 5 mM $MgCl_2$. CIP-treated nuclear extracts were subsequently used for electrophoretic mobility shift assay (EMSA) as described above; similar experiments were performed with PTPase, PP1, and PP2A.

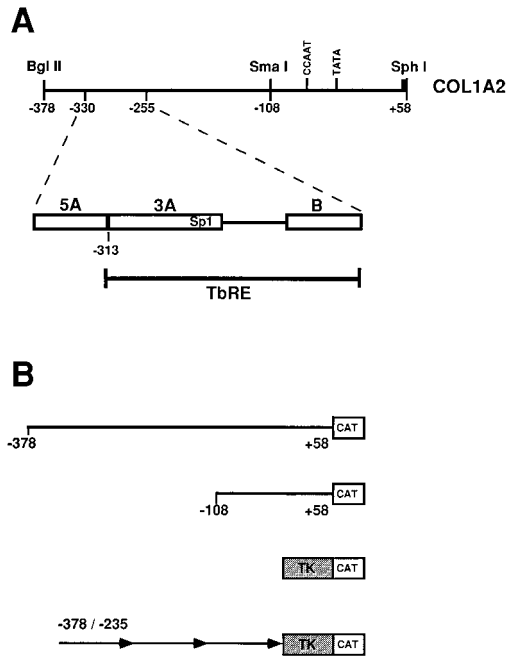

FIG. 1. Schematic representation of the *COL1A2* promoter (A) and of the constructs used in the transient and stable transfections (B [top and bottom, respectively]). The diagram includes the relative positions of the Sp1 binding site in the TbRE, the CCAAT and TATA boxes, and the first exon of the gene (black box).

DNA-protein binding assays. Nuclear extracts were prepared from control cells and fibroblasts treated with TGF-β1, sodium orthovanadate, okadaic acid, H7, or genistein. Confluent cells were maintained for 18 h in Dulbecco's modified Eagle's medium containing 3.5% fetal bovine serum prior to the addition of either TGF-β1 or the inhibitors of protein kinases and phosphatases at the concentrations described above. Cells were harvested 1 h later and used to prepare nuclear extracts according to our previously published protocol (31). Nuclear extracts were in turn used for the EMSA with *COL1A2* oligonucleotides spanning from −314 to −183 (TbRE) and from −330 to −296 (see Fig. 1A, box 5A), as well as with a high-affinity Sp1 recognition sequence (10). Oligonucleotides were end labeled with [α-^{32}P]dCTP by using the Klenow fragment of DNA polymerase (26).

RESULTS

In vitro dephosphorylation of nuclear proteins enhances binding to the TbRE. We have previously shown that an upstream promoter sequence (−340 to −183) of the *COL1A2* gene contains a strong TbRE (Fig. 1A) (10). We have also provided evidence indicating that Sp1, or an immunologically related factor, binds to the 5′ half of TbRE (Fig. 1A, box 3A). We were, however, unable to characterize the factor(s) interacting with box B, the 3′ half of the TbRE. In that study, we also correlated TGF-β1 stimulation of *COL1A2* gene transcription with a rapid, i.e., protein synthesis-independent, increase in the binding of the TbRC, the nuclear complex that interacts with the TbRE. However, there was no difference in the binding to box 3A alone or to a high-affinity Sp1 recognition sequence of nuclear proteins from TGF-β1-stimulated cells compared with untreated fibroblasts. Therefore, we concluded that a TbRC component other than Sp1, but interacting with Sp1, is modified as a result of TGF-β1 treatment. We further argued that the modification causes enhanced affinity

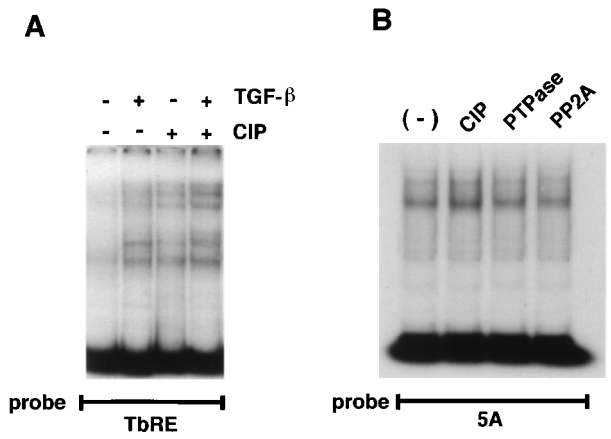

FIG. 2. Induction of TbRC binding with nuclear extracts treated in vitro with CIP. (A) The 131-bp TbRE probe was incubated with nuclear extracts from CF37 human fibroblasts cultured without (−) and with (+) TGF-β1; some of the extracts were preincubated with CIP prior to binding. (B) The 34-bp box 5A probe was incubated with nuclear proteins purified from CF37 fibroblasts cultured without TGF-β1 and left untreated (−) or preincubated in vitro with CIP, PTPase, or PP2A. The same amount of nuclear proteins (5 μg) was used in each of the samples shown in the autoradiograms.

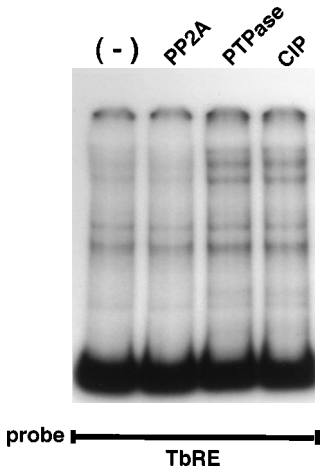

FIG. 3. Induction of TbRC binding by in vitro treatment with phosphatases. The TbRE probe was incubated with 5 μg of CF37 nuclear extracts pretreated without phosphatases (−) or with PP2A, PTPase, or CIP. The same amount of nuclear proteins (5 μg) was used in each of the lanes.

of the whole TbRC, which is in turn translated into stimulation of *COL1A2* transcription (10). It is, however, unclear whether the change in TbRC binding observed in the EMSA is due to an increased affinity of prebound complexes or to an increased number of complexes binding to the TbRE. For the sake of simplicity, we will nevertheless refer to this phenomenon throughout the report as affinity.

In the present study, we investigated the nature of the TGF-β1-induced modification responsible for *COL1A2* gene upregulation via the TbRC. As a first step, we examined how treatment of nuclear extracts with a nonspecific phosphatase affects binding of the protein complex. To this end, nuclear extracts purified from untreated and TGF-β1-treated fibroblasts were incubated with CIP prior to binding to the TbRE. As a control, we included binding of the same nuclear proteins to box 5A, a *cis*-acting element contiguous to TbRE and unresponsive to TGF-β1 (Fig. 1A) (10, 11). Retarded bands in the CIP-preincubated–TGF-β1-untreated sample were markedly more intense than those of the TGF-β1-untreated sample; in addition, they were almost as intense as those from the TGF-β1-treated cells (Fig. 2A). The EMSA results therefore documented that nuclear protein dephosphorylation in vitro and TGF-β1 treatment of cultured fibroblasts have similar effects on the affinity of TbRC. Indeed, binding of the TbRC from the TGF-β1-treated cells was further augmented when the nuclear extract was preincubated with CIP (Fig. 2A). In all cases, the increase was most evident, and most often seen, with the slower migrating bands of the TbRC. These are the same bands affected by competition with the Sp1 recognition sequence and incubation with Sp1 antisera (10). The specificity of the CIP effect on TbRC binding was corroborated by the finding that all nuclear extracts interacted in the same way with box 5A, the *cis*-acting element immediately upstream of the TbRE and unresponsive to TGF-β1 (Fig. 2B).

In the next experiment, we employed specific reagents in order to discriminate between the consequences of dephosphorylating serine/threonine (PP2A) residues and tyrosine (PTPase) residues. Unlike PP2A, preincubation of nuclear proteins with PTPase yielded an EMSA pattern comparable to that of the sample preincubated with CIP (Fig. 3). Incidentally, other tyrosine (PTP-1B) and serine/threonine (PP1) phosphatases yielded the same results as PTPase and PP2A (data not shown). Finally, neither of the two specific phosphatases affected the binding of nuclear proteins to box 5A (Fig. 2B). On the basis of these data, we concluded that tyrosine dephosphorylation changes TbRC affinity in a manner qualitatively comparable to that of the TGF-β1-activated signal pathway.

Tyrosine dephosphorylation stimulates TbRE-mediated *COL1A2* gene transcription. Our conclusion that tyrosine dephosphorylation may stimulate *COL1A2* transcription in the same way as the signaling pathway induced by TGF-β1 was correlative. To provide more direct support for it, we examined *COL1A2* expression in fibroblasts cultured with a variety of phosphatase and kinase inhibitors. Consistent with the in vitro binding data, genistein, a tyrosine kinase inhibitor, and sodium orthovanadate, a tyrosine phosphatase inhibitor, had opposite effects on α2(I) collagen mRNA accumulation (Fig. 4A). Genistein increased the mRNA steady-state level by about 2.7-fold, whereas sodium orthovanadate reduced it by approximately 2.5-fold (Table 1). Results similar to those obtained with sodium orthovanadate were obtained with phenyl arsine oxide, another tyrosine phosphatase inhibitor (data not shown). Sodium orthovanadate was also an effective antagonist of TGF-β1 stimulation, whereas genistein added only mini-

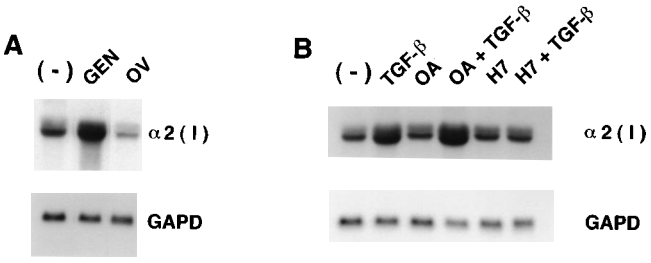

FIG. 4. Effect of kinase and phosphatase inhibitors on α2(I) collagen mRNA accumulation. (A) Northern analysis of RNA purified from control cells (−) and from fibroblasts cultured with genistein (GEN) and sodium orthovanadate (OV) and hybridized to the *COL1A2* (top) and GAPD (bottom) probes. (B) Northern analysis of RNA purified from untreated cells and from CF37 fibroblasts cultured with TGF-β1 or with okadaic acid (OA) or H7 alone or in combination with TGF-β1.

TABLE 1. Kinase and phosphatase inhibitors and *COL1A2* gene expression[a]

Inhibitor	mRNA level (Northern analysis)		Gene transcription result (run-on assay)
	−TGF-β1	+TGF-β1	
None	1 ± 0.05	2.1 ± 0.3[b]	1 ± 0.1
Genistein	2.7 ± 0.4[b]	3.0 ± 0.3[b,c]	2.3 ± 0.2[b]
Sodium orthovanadate	0.35 ± 0.1[b]	0.4 ± 0.1[b]	0.5 ± 0.1[b]
H7	0.9 ± 0.3	1.1 ± 0.2	
Okadaic acid	1.2 ± 0.1	2.8 ± 0.2[b,c]	
Staurosporine	1.1 ± 0.3	2.3 ± 0.2[b]	

[a] Results are expressed relative to those obtained from control CF37 cells after normalization against GAPD values. Each value (average ± SD) represents the results of five (Northern) or three (run-on) independent experiments.
[b] Values are statistically different from those for the controls.
[c] Values are statistically greater than those for cells treated only with TGF-β1 (Mann-Whitney U test, $P < 0.05$).

mally to *COL1A2* upregulation (Table 1). The latter result may conceivably reflect plateauing of the *COL1A2* response to TGF-β1 induction. The specificity of the variations in *COL1A2* gene expression was substantiated by the invariant level of GAPD mRNA under the different experimental conditions (Fig. 4A).

We then compared *COL1A2* transcription in nuclei from untreated cells with that in nuclei from fibroblasts cultured with genistein or sodium orthovanadate (Fig. 5). The nuclear run-on assay documented that the rates of *COL1A2* transcription in genistein-treated and in sodium orthovanadate-treated cells were 2.3-fold higher and twofold lower than in untreated fibroblasts, respectively (Table 1). The results of these experiments were, therefore, consistent with the notion that preventing tyrosine dephosphorylation specifically and negatively affects *COL1A2* transcription.

The first step in the TGF-β1 signal transduction pathway involves serine/threonine phosphorylation of one of its receptors (4, 32). We therefore investigated whether or not serine/threonine kinase and phosphatase inhibitors alter the basal level or the TGF-β1-stimulated accumulation of α2(I) collagen mRNA (Fig. 4B). As expected, we found that neither H7, a serine/threonine kinase inhibitor, nor okadaic acid, a serine/threonine phosphatase inhibitor, affects basal expression of the gene, as judged by the nearly equal levels of α2(I) collagen mRNA in these and untreated cells (Table 1). In contrast, H7 effectively counteracted TGF-β1 induction of *COL1A2* expression, probably by inhibiting phosphorylation of the receptors (Table 1). Okadaic acid somewhat augmented TGF-β1 stimulation of *COL1A2* expression, perhaps by inhibiting serine/threonine dephosphorylation of the cytokine-activated TGF-β1 receptors (Table 1). Additional experiments with staurosporine, a protein kinase C inhibitor, showed that this compound had no effect on either basal or TGF-β1-stimulated *COL1A2* expression (Table 1).

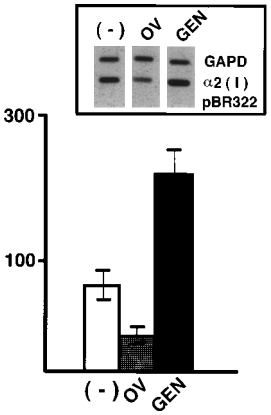

FIG. 5. Effect of kinase and phosphatase inhibitors on *COL1A2* transcription. Nuclear run-on assay with nuclei from control cells (−) and from CF37 fibroblasts cultured with sodium orthovanadate or genistein; labeled products were hybridized to filter-bound GAPD or *COL1A2* cloned DNA or control pBR322 plasmid. The results of three independent assays (average ± standard deviation [SD]) are summarized in the histograms below the autoradiograms as percentages of the control values, after normalization against GAPD values.

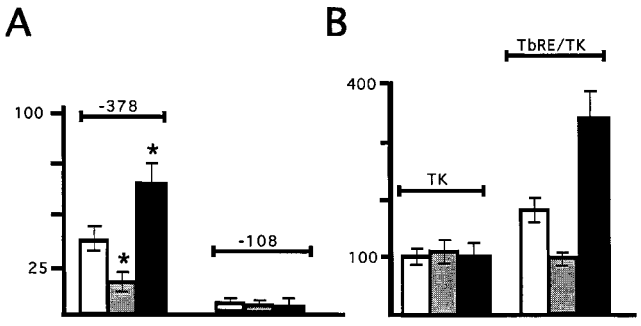

FIG. 6. Effects of kinase and phosphatase inhibitors on transient, TbRE-mediated transcription. (A) Transient-transfection assays with constructs containing the −378 to +58 (TbRE-plus) or the −108 to +58 (TbRE-minus) promoter segment of the *COL1A2* gene linked to the CAT gene. Values are normalized against a cotransfected control and expressed relative to the activity of the simian virus 40 promoter-driven plasmid. Each value (average ± SD) includes the results from five independent experiments. The asterisk indicates that the values are statistically different from those for the controls (Mann-Whitney U test, $P < 0.05$). (B) Transient-transfection assays with a chimeric construct containing the TbRE upstream of the TK promoter driving CAT gene expression. Values are expressed relative to that of the parental pBLCAT2 (TK) plasmid in untreated (−) cells (assumed to be 100%); they represent the average of five independent tests ± SD. □, untreated cells; ▨, cultured with sodium orthovanadate; ■, cultured with genistein.

The data presented above established a causal relationship between a specific posttranslational modification, i.e., tyrosine dephosphorylation, and a specific transcriptional response, i.e., increased *COL1A2* expression, in intact cells. Furthermore, the in vitro binding assays described above suggested that tyrosine dephosphorylation may directly or indirectly act upon the transcriptional complex that interacts with the TbRE. To more firmly link the conclusions from the experiments done in vitro and with intact cells, we examined the consequences of blocking or inducing tyrosine dephosphorylation on TbRE-mediated transcription. To this end, we performed transient and stable transfection assays with the TbRE operating within the context of its own promoter sequence or that of a heterologous promoter.

In the transient-transfection assays, we used chimeric constructs with (−378COL1A2/CAT) and without (−108COL1A2/CAT) the TbRE (Fig. 1B). Like the endogenous *COL1A2* gene, sodium orthovanadate and genistein had opposite effects on transient expression of the TbRE-containing construct (Fig. 6A). The differences in CAT gene expression, about twofold in either direction, are statistically significant and qualitatively comparable to the range of responses elicited in the same construct by TGF-β1 and the antagonist tumor necrosis factor alpha (10, 11). Moreover, two independent clones with stably integrated copies of the −378COL1A2/CAT construct replicated the behavior of the transiently transfected construct and

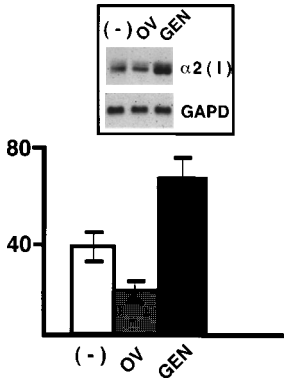

FIG. 7. Effect of kinase and phosphatase inhibitors on stable, TbRE-mediated transcription. The histograms summarize the values obtained with two different cellular clones harboring stably integrated copies of the −378COL1A2/CAT plasmid. The values are expressed as arbitrary units of CAT activity normalized for the number of viable cells. Each value (average ± SD) represents the results from three independent experiments. (Top) Representative Northern analysis showing the expression of the endogenous *COL1A2* and GAPD genes in one of the two cellular clones.

of the endogenous gene (Fig. 7). In these tests, we could not unfortunately use a mutated, i.e., TGF-β1-unresponsive, TbRE because the integrity of this element is essential for high *COL1A2* promoter activity (10, 13). Indeed, a −378 construct harboring a mutant TbRE sequence has been shown to be as active as −108COL1A2/CAT, the TbRE-minus negative control (Fig. 6A) (30). Because of this problem, we resorted to using a chimeric construct in which three copies of the TbRE had been inserted in front of the TK promoter driving the CAT gene (Fig. 1B). Unlike the parental plasmid without the TbRE sequence, expression of the CAT gene in this chimeric promoter was inhibited by sodium orthovanadate and stimulated by genistein (Fig. 6B). Thus, the TbRE is by itself sufficient to render transcription from the TK promoter sensitive to the level of tyrosine phosphorylation within the cells. Altogether, the cell culture and transfection experiments associated tyrosine dephosphorylation with *COL1A2* inducibility via the TbRE.

Tyrosine dephosphorylation modulates TbRC binding affinity in intact cells. We have shown that in vitro treatment of nuclear extracts with PTPase augments binding of TbRC to the cognate site. We have also correlated exposure of intact cells to genistein with increased expression of the *COL1A2* gene, as well as with upregulation of TbRE-mediated transcription in transient and stable transfectants. To establish a causal relationship between the in vitro data and the intact-cell findings, we compared the affinities of nuclear proteins purified from fibroblasts cultured in the presence of kinase inhibitors and in the presence of phosphatase inhibitors for the TbRE sequence.

The results of the binding assays with nuclear extracts from cells treated with the inhibitors were entirely consistent with those of protein dephosphorylation in vitro and gene expression in intact cells (Fig. 8). First, nuclear proteins from genistein-treated cells bound to the TbRE more strongly than those from untreated cells. Second, culturing fibroblasts in the presence of sodium orthovanadate virtually abolished TbRC binding. Third, neither H7 nor okadaic acid treatment of intact cells led to changes in the interaction between nuclear proteins and the TbRE. Lastly, none of the compounds altered binding to a high-affinity Sp1 oligonucleotide, thus reemphasizing that Sp1 is not directly targeted by the modification (Fig. 8A). In addition, in vitro treatment with PTPase, but not with PP2A, of nuclear proteins purified from cells cultured in the presence of sodium orthovanadate restored binding of the TbRC (Fig. 8B). On the basis of these findings, we concluded that tyrosine dephosphorylation of a nuclear protein(s) directly or indirectly interacting with the TbRC stimulates *COL1A2* transcription.

DISCUSSION

The first step in the signal transduction pathway of TGF-β1 involves dimerization of receptors I and II, concomitantly with serine/threonine phosphorylation of receptor I (4, 32). This phosphorylation allows receptor I to propagate the signal to downstream substrates. Emerging evidence indicates that the signal transduction pathway from TGF-β1 is not a simple and linear cascade, but rather a complex branching system with activation, and sometimes inactivation, of discrete sets of kinases (33). Very little is, however, known about the subsequent steps of the TGF-β1-induced cascade. For example, TGF-β1 activates distinct signal transduction pathways which lead to either stimulation or inhibition of epithelial cell growth (33). Moreover, the multiplicity of TGF-β1-elicited effects include increased expression of c-*fos* and *junB* (18); phosphorylation of CREB and several other uncharacterized nuclear proteins (16, 17); and blocking the cell cycle-dependent phosphorylation of RB, the retinoblastoma susceptibility gene product (8).

It is well established that part of the TGF-β1 stimulation of collagen I expression occurs at the transcriptional level via a mechanism that does not require de novo protein synthesis (9, 23). We have previously shown that TGF-β1 upregulates the *COL1A2* gene by enhancing the binding of an upstream complex through the posttranslational modification of its component(s) (10). The activity of transcription factors is often regulated by phosphorylation; in most instances, phosphorylation enhances transcription and dephosphorylation decreases it (12). For example, dephosphorylation has been shown to activate T-cell early genes, including several transcription factor-encoding genes, through the cytosolic action of a serine/threonine phosphatase, namely, the calcium- and calmodulin-dependent calcineurin enzyme (7). Results presented in this report constitute an apparent exception to the established paradigm. They in fact indicate that tyrosine dephosphorylation of a nuclear protein(s) is involved in transcriptional stimulation.

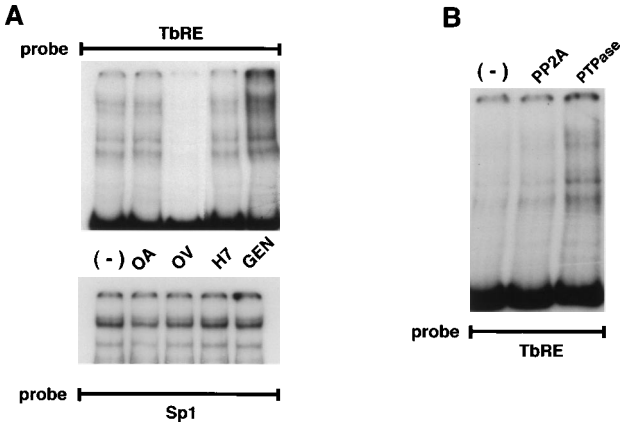

FIG. 8. TbRC binding in cells treated with phosphatase and kinase inhibitors. (A) The TbRE (upper panel) and Sp1 (lower panel) probes were incubated with 5 μg of nuclear extracts purified from control (−) cells and from fibroblasts cultured in the presence of sodium orthovanadate (OV), okadaic acid (OA), genistein (GEN), or H7. (B) Five micrograms of nuclear proteins from sodium orthovanadate cultures were treated with PP2A, PTPase, or neither (−) prior to incubation with the TbRE probe.

Three distinct lines of evidence support this conclusion. First, inhibiting tyrosine kinase in intact cells stimulates *COL1A2* transcription, with an increase in mRNA accumulation comparable to that elicited by TGF-β1. Conversely, blocking tyrosine phosphatase activity downregulates production of α2(I) collagen transcripts; furthermore, it counteracts the stimulation of *COL1A2* by TGF-β1. Second, binding to TbRE, but not to a neighboring *cis*-acting element, is stimulated by tyrosine dephosphorylation of nuclear proteins in vitro and in intact cells. Both the specificity and nature of the change closely resemble the behavior of nuclear extracts purified from TGF-β1-stimulated fibroblasts. Lastly, the TbRE sequence confers both TGF-β1 and genistein inducibility on the otherwise unresponsive TK promoter.

We have also demonstrated that inhibiting serine/threonine phosphorylation can counteract *COL1A2* upregulation by TGF-β1. However, the mechanism is different from that operating in the case of the tyrosine phosphatase inhibitor. Consistent with recent data from the PAI-1 and JunB genes, the serine/threonine kinase inhibitor H7 probably prevents TGF-β1-induced expression of *COL1A2* by acting upon the initial step of the signaling cascade, namely, the serine/threonine phosphorylation of the TGF-β1-receptor (4, 22, 32). This conclusion is supported by the different responses of fibroblasts treated with the tyrosine phosphatase inhibitor sodium orthovanadate and with H7. Addition of the former abolished TbRC binding and decreased α2(I) mRNA accumulation, whereas H7 had no noticeable effect on either TbRC binding or α2(I) mRNA accumulation. We also expected to observe increased *COL1A2* stimulation in cells simultaneously treated with TGF-β1 and the serine/threonine phosphatase inhibitor okadaic acid. It is, in fact, conceivable that by blocking serine/threonine phosphatases, enhanced phosphorylation of the receptor is maintained and, consequently, the TGF-β1 effect is sustained for a longer period of time than without it.

We have hypothesized that activation of the TbRC occurs through the posttranslational modification of a factor(s) interacting with Sp1 (10). Others have recently demonstrated that Sp1 activity can be indirectly modulated by RB, in that dephosphorylation of RB leads to the release of an inhibitor from Sp1 (5). There are also data implicating TGF-β1 in RB phosphorylation via activation of a serine/threonine phosphatase (15). However, the TGF-β1–RB–Sp1 connection seems not to apply to *COL1A2* stimulation. Others have already shown that inhibition of RB activity by overexpression of the T antigen of simian virus 40 does not alter TGF-β1-elicited upregulation of several matrix-related genes, including *COL1A2* (3, 18). Also, our unpublished data exclude RB involvement. We have in fact observed that preincubating nuclear extracts with anti-RB antibodies has no effect on TbRC binding or on CIP-induced TbRC affinity (data not shown). Additionally, we failed to note any change in the steady-state level of α2(I) collagen mRNA in cells overexpressing RB.

In conclusion, this report provides new insights into the mechanism underlying TGF-β1 modulation of collagen gene expression and, more generally, matrix remodeling. To the best of our knowledge, this is also the first study to implicate tyrosine dephosphorylation of a nuclear protein(s) in transcriptional stimulation. Our model predicts that TGF-β1 activates a signaling cascade which ultimately leads to tyrosine dephosphorylation of a nuclear component(s), increased binding of the TbRC, and transcriptional activation of the *COL1A2* gene. Although consistent with the evidence presented, this picture is far from being completely understood. For example, our data could not establish whether tyrosine dephosphorylation is the result of increased phosphatase activity or decreased kinase activity. They also could not determine if the posttranslational modification involves one or more of the TbRC components or a factor which affects, directly or indirectly, the TbRC. As a result, the details regarding translation of the protein modification into increased complex binding remain obscure. One possibility is that tyrosine dephosphorylation has a direct effect on the affinity of the prebound TbRC; alternatively, it may stimulate the formation of the TbRC by facilitating the assembly of one or more of its components. It is also formally possible that TGF-β1 acts on TbRC assembly, whereas inhibitors of tyrosine phosphorylation might influence TbRC affinity. Work in progress is aimed at clarifying these important points through the identification of the other TbRC components. Together with the characterization of the cytosolic steps, our work will ultimately contribute to the full elucidation of this particular signaling pathway activated by TGF-β1. This information will be relevant to our understanding of matrix formation and remodeling and, possibly, to the development of new therapies for fibrotic diseases.

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