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Phosphoproteome Profiling of Transforming Growth Factor (TGF)-β Signaling: Abrogation of TGFβ1-dependent Phosphorylation of Transcription Factor-II-I (TFII-I) Enhances Cooperation of TFII-I and Smad3 in Transcription[D]

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Transforming growth factor-β (TGFβ) signaling involves activation of a number of signaling pathways, several of which are controlled by phosphorylation events. Here, we describe a phosphoproteome profiling of MCF-7 human breast epithelial cells treated with TGFβ1. We identified 32 proteins that change their phosphorylation upon treatment with TGFβ1; 26 of these proteins are novel targets of TGFβ1. We show that Smad2 and Smad3 have different effects on the dynamics of TGFβ1-induced protein phosphorylation. The identified proteins belong to nine functional groups, e.g., proteins regulating RNA processing, cytoskeletal rearrangements, and proteasomal degradation. To evaluate the proteomics findings, we explored the functional importance of TGFβ1-dependent phosphorylation of one of the targets, i.e., transcription factor-II-I (TFII-I). We confirmed that TGFβ1 stimulated TFII-I phosphorylation at serine residues 371 and 743. Abrogation of the phosphorylation by replacement of Ser371 and Ser743 with alanine residues resulted in enhanced complex formation between TFII-I and Smad3, and enhanced cooperation between TFII-I and Smad3 in transcriptional regulation, as evaluated by a microarray-based measurement of expression of endogenous cyclin D2, cyclin D3, and E2F2 genes, and by a luciferase reporter assay. Thus, TGFβ1-dependent phosphorylation of TFII-I may modulate TGFβ signaling at the transcriptional level.

INTRODUCTION

Transforming growth factor-β (TGFβ) isoforms are members of a family of polypeptide growth factors that regulate embryonal development as well as normal and pathological processes in adult multicellular organisms (reviewed by Derynck *et al*., 2001; Chang *et al*., 2002; Wakefield and Roberts, 2002; Shi and Massagué, 2003). TGFβ binds to cell surface receptors with intrinsic serine/threonine kinase domains, and triggers activation of receptor kinases, followed by phosphorylation of cytoplasmic substrates. Genetic and molecular biological studies identified Smad proteins as crucial components of TGFβ signaling. TGFβ receptor-dependent phosphorylation of Smad2 and Smad3 is the triggering event for following interactions of Smad2 and Smad3 with different proteins, including the common mediator Smad4. Smad2- and Smad3-containing protein complexes are translocated to the nucleus, where they regulate gene expression. Despite structural homology, Smad2 and Smad3 show different activities in functional assays (Derynck *et al*., 2001; Piek *et al*., 2001; Chang *et al*., 2002; Wakefield and Roberts, 2002; Shi and Massagué, 2003). One of the explanations of such differences is the ability of Smad3 to bind DNA directly, whereas Smad2 interacts with gene promoters via other DNA-binding proteins (reviewed by Derynck *et al*., 2001; Wakefield and Roberts, 2002; Shi and Massagué, 2003). A number of Smad-independent pathways are also affected by TGFβ, e.g., mitogen-activated protein (MAP) kinases, phosphoinositide 3-kinase, and ion channels (Mulder, 2000; Wakefield and Roberts, 2002).

TGFβ has a dual role in tumorigenesis, inhibiting tumor growth at early stages and promoting tumorigenesis at later stages of cancer progression. Potent growth-inhibitory and proapoptotic activities of TGFβ contribute to suppression of tumor growth, whereas proangiogenic and immunosuppressive effects of TGFβ, as well as stimulation of tumor cell invasiveness, contribute to promotion of tumorigenesis (Derynck *et al*., 2001; Souchelnytskyi, 2002a; Wakefield and Roberts, 2002). Studies of selected signaling pathways or activities have provided insights into some mechanisms behind this dual role of TGFβ. However, a comprehensive

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analysis of the role of TGFβ in cancer should also include unbiased approaches, such as global proteome profiling.

Protein phosphorylation is one of the most important regulatory modifications, which occurs in signal transduction via several types of transmembrane receptors, as well as in several intracellular signaling pathways (Manning *et al*., 2002). Thus, global analysis of phosphorylation pattern is of crucial importance for the understanding of signaling processes. Development of methods for global phosphoproteome profiling is one of the major challenges of proteomics. Techniques using phospho-site-specific antibodies, chemical modifications of phosphorylated residues, immobilized metal affinity chromatography (IMAC), and metabolic labeling of cells with radioactive [^{32}P]orthophosphate have been used with various degree of success (Figeys, 2003; Patterson and Aebersold, 2003). Among these techniques, metabolic labeling with [^{32}P]orthophosphate provides the most comprehensive and direct detection of phosphorylated proteins, compared with indirect detection with antibodies, purification of phosphoproteins and phosphopeptides by IMAC, or to purification of chemically modified phosphopeptides of proteins.

Here, we report a phosphoproteome profiling of TGFβ1-dependent protein phosphorylation in MCF-7 cells stably transfected with Flag-Smad2, myc-Smad3, or empty vector. Phosphoproteome profiling was performed using two-dimensional gel electrophoresis, computer-assisted image analysis and matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI TOF MS). We studied short- and long-term changes in protein phosphorylation, and we investigated the impact of increased expression levels of Smad2 and Smad3 on the phosphorylation. One of the identified targets, transcription factor-II-I (TFII-I), is known to facilitate interactions of the basal transcriptional machinery with specific transcription factors and is a convergence point for several signaling pathways in the regulation of transcription (Novina *et al*., 1998). Our data suggest that TGFβ1-induced phosphorylation of TFII-I modulates TGFβ1/Smad3-dependent transcriptional regulation.

MATERIALS AND METHODS

Cells and Reagents

MCF-7 cells were obtained from American Type Culture Collection (Manassas, VA), and cells were cultured in DMEM supplemented with 10% of fetal bovine serum (Sigma-Aldrich, St. Louis, MO). To generate stably transfected cells, MCF-7 cells were transfected with empty pMEP4 vector (MCF-7-vector cells), Flag-Smad2 (MCF-7-Smad2 cells), or myc-Smad3 (MCF-7-Smad3 cells) in pMEP4 vectors. Transfected cells were selected by culturing cells in the presence of hygromycin. To generate cells stably expressing wild type or Ser371,743Ala mutant of TFII-I, MCF-7 cells were transfected with respective constructs and were selected in the presence of G418 (Geneticin).

Treatment of Cells

To induce expression of Flag-Smad2 and myc-Smad3, stably transfected MCF-7 cells were incubated with $CdCl_2$ (5 μM) for 5 h. TGFβ1 (5 ng/ml) was added to cells 1, 4, or 24 h before lysis. Six hours before lysis, [^{32}P]orthophosphate (0.1 mCi/ml; GE Healthcare, Uppsala, Sweden) was added to culture media. Cells were solubilized directly in the buffer for sample preparation.

Sample Preparation

Cells were lysed in 1% Triton X-100, 40 mM Tris-HCl, pH 8.0, 65 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, 10 mM aprotinin, and complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Lysates were clarified by centrifugation, and samples for two-dimensional gel electrophoresis (2D-GE) were prepared using PlusOne 2D Clean-Up kit according to the manufacturer's recommendations (GE Healthcare). Samples were dissolved in buffer for 2D-GE (9.8 M urea, 2% CHAPS, 65 mM DTT, 0.5% IPG buffer, pH 3–10). Samples were aliquoted and stored at –70°C before use.

Two-dimensional Gel Electrophoresis

Samples (75 μg of protein) were subjected to isoelectric focusing (IEF) using IPGDry strips with immobilized pH gradient, pH range 3–10, 24 cm, linear (GE Healthcare). Samples were loaded by the in-gel rehydration technique, with active loading during the last 3 h. IEF was performed in an IPGphor (GE Healthcare) according to the following protocol: rehydration 10 h; 50 V, 3 h; 1000 V, 1 h; 8000 V, 10 h or to 50,000 Vh. After IEF, strips were equilibrated in 50 mM Tris-HCl, pH 8.8, 6 M urea, 2% SDS, 30% glycerol with 1% DTT for 10 min, and then for 10 min in the same buffer without DTT but with 4% iodoacetamide. Equilibrated strips were placed on top of 12% polyacrylamide gels and fixed with 0.5% agarose in a concentrating buffer (62.5 mM Tris-HCl, pH 6.8, 0.1% SDS). The SDS-PAGE was performed in a Dalt-II (GE Healthcare), following the manufacturer's recommendations (constant power 100 W, run for 6–8 h). Gels were fixed in 10% acetic acid and 20% methanol for 10–12 h. Proteins were detected by silver staining as described previously (Shevchenko *et al*., 1996; Kanamoto *et al*., 2002). Gels were dried, and then they were exposed and scanned in a FujiX2000 PhosphorImager (Fuji, Tokyo, Japan). The pH gradient of the first-dimension electrophoresis was evaluated as proposed by the manufacturer of strips. Totally, 48 gels with samples from four experiments were prepared and subjected to analysis.

Gel Analysis

Silver-stained gels were scanned in an ImageScanner with the MagicScan32 software and analyzed by calculation of volumes of spots by the ImageMaster 2D Elite software (GE Healthcare). Radioactively labeled gels were exposed in a FujiX2000 PhosphorImager and scanned using the AIDA software (Raytest IMG, Sprockhoefel, Germany). Scanning files of gels were imported into the ImageMaster 2D Elite software, and images were analyzed. The linearity of data transfer from the PhosphorImager (AIDA software) to the ImageMaster 2D Elite software was controlled in separate experiments with various fixed amounts of radioactivity. Gels that did not show deviations in pattern of protein migration were used to generate master gels of the proteome of cells treated or not with TGFβ1. Normalization was performed to a total volume of radioactivity in all matched spots. Proteins changing their phosphorylation after treatment with TGFβ1 were considered for identification. Statistical significance of changes was evaluated using the ImageMaster 2D Elite software.

Protein Identification

TGFβ1-regulated protein spots were excised from the gels, destained, and subjected to in-gel digestion with trypsin (modified, sequence grade porcine; Promega, Madison, WI) as described previously (Hellman, 2000; Kanamoto *et al*., 2002). Tryptic peptides were concentrated and desalted on a "nano-column" (Gobom *et al*., 1999). Peptides were eluted with acetonitrile, containing the α-cyano-4-hydroxycinnamic acid as a matrix, and were applied directly onto the metal target and analyzed by MALDI TOF MS on a Bruker Autoflex (Bruker Daltonics, Bremen, Germany). Peptide spectra were internally calibrated using known autolytic peptides from trypsin. To identify proteins, we performed searches in the NCBInr sequence database using Mascot (www.matrixscience.com) and ProFound (http://65.219.84.5/service/prowl/profound.html) search engines. One miscut and partial oxidation of methionine were allowed. Probability of identification was evaluated according to the "P" value (Probability Based Mowse Score; for searches via Mascot), and according to probability value, "Z" value, and sequence coverage (for searches using ProFound). Correspondence of experimental values of pI and molecular mass of proteins to the theoretical values was also considered for identification.

Low-Density Microarray Experiment

Low-density microarray experiment was performed with RNA extracted from MCF-7 cells stably transfected with wild-type TFII-I and Ser371,743Ala mutant of TFII-I. Because pcDNA3 vector containing TFII-I constructs provides neomycin resistance, the cells were selected and cultured in the presence of G418. RNA was extracted using a RNAsy minikit (QIAGEN, Valencia, CA), and RNA without any signs of degradation was used. Preparation of probes (GEArray Probe synthesis kit, SuperArray Bioscience, Frederick, MD), and hybridization with membranes of a Human Cell Cycle Gene array (HS-001-4 GE array Q series; SuperArray Bioscience) were performed, as recommended by the manufacturer. Membranes after hybridization were exposed in a FujiX2000 PhosphorImager, and images were analyzed using the dedicated ScanAlyze software (SuperArray Bioscience).

Luciferase Reporter Assay

Reporter assays with CAGA(12)-luc, ARE-luc, and SRE-luc reporters were performed as described previously (Yakymovych *et al*., 2001). COS7 cells were used for this assay because they express relatively lower levels of TFII-I, compared with other cells. COS7 cells are responsive to treatment with TGFβ, albeit to a lower degree, compared with some other cells, e.g., Mv1Lu. 293T cells were used, because they are responsive to TGFβ and allow efficient expression of proteins.

Immunoprecipitation and Immunoblotting

Immunoprecipitation and immunoblotting experiments were performed as described previously (Yakymovych *et al*., 2001). Cells were transfected and treated as indicated in the text.

DNA-Precipitation Assay

DNA-precipitation assay was performed with nuclear extracts from cells transiently transfected and treated as indicated in the text. Preparation of the CAGA(4x) probe, incubations of nuclear extracts, and electrophoresis were performed as described previously (Kurisaki *et al*., 2003).

Two-dimensional Phosphopeptide Mapping

MCF-7 or COS7 cells were treated as indicated in the text. Metabolic labeling of cells with [^{32}P]orthophosphate (GE Healthcare) was performed as described previously (Yakymovych *et al*., 2001). Radioactively labeled TFII-I was subjected to digestion with trypsin (modified, sequence grade porcine; Promega), and the tryptic digest was separated on thin-layer cellulose plates by electrophoresis and chromatography as described previously (Yakymovych *et al*., 2001). Plates were exposed in a FLA-3000 PhosphorImager (Fuji), and images were analyzed. Phosphopeptides of interest were subjected to phosphoamino acid analysis and to Edman degradation, as described earlier (Yakymovych *et al*., 2001).

RESULTS

Phosphoproteome Profiling of TGFβ1 Signaling in MCF-7 Cells

We explored changes in protein phosphorylation in MCF-7 cells stably transfected with inducible Flag-Smad2, myc-Smad3, or empty pMEP4 vector, in response to TGFβ1 stimulation (Figures 1, A and B, and 2; Table 1; and Supplemental Figures A and B). MCF-7 cells are often used as a model in studies of signaling in human breast epithelial cells, including TGFβ-dependent signaling, with >2000 publications referred in the MedLine. These publications suggest that processes studied in MCF-7 cells may be approximated to in vivo. To assess quick and long-term changes in protein phosphorylation, each of the cell lines was analyzed after incubation with TGFβ1 for 1, 4, and 24 h and was compared with nontreated cells. Expression of myc-Smad3 and Flag-Smad2 was induced by pretreatment of stably transfected cells with $CdCl_2$; high levels of expression of Smad proteins were sustained during the time of treatment with TGFβ1 (Figure 1, C and D). myc-Smad3 and Flag-Smad2 were phosphorylated on their C-terminal serine residues upon TGFβ1 treatment, suggesting that the initiation of TGFβ signaling in stably transfected cells is not affected (Figure 1E). Samples from MCF-7 cells were subjected to 2D-GE; in an average 2D gel, we detected 1400 protein spots after silver staining. Silver-stained protein spots were distributed in all areas of the pH gradient (pH 3–10), and an approximately even distribution was found for proteins in the range of molecular masses from 14 to 100 kDa (Figure 1A). Four hundred forty-one phosphoprotein spots of ^{32}P-labeled proteins were identified (Figure 1B). Most of the phosphorylated proteins were found in the part of the gel corresponding to pI values lower than 6.5, probably due to introduction of negative charges into protein molecules by phosphorylation. We found inter-gel variations for <8% of detected protein spots, suggesting a high reproducibility in generation of the 2D gels (Supplemental Figure A; our unpublished data).

Eighty-nine phosphoprotein spots, which showed TGFβ1-dependent differences in phosphorylation of >30% of incorporated radioactivity between at least two experimental conditions, were considered for identification; for example, if differences in protein phosphorylation were observed between experimental conditions corresponding to 0 and 1 h or between 0 and 4 h, or between 0 and 24 h, or between 4 and 24 h, or between 1 and 4 h, or between 1 and 24 h of TGFβ stimulation. After analysis of silver-stained gels, 47 phosphoprotein spots were selected for identification; the other 42 TGFβ1-regulated phosphoproteins were not suitable for mass spectrometry analysis because they were undetectable by silver staining or comigrated with abundant silver-stained spots. Selected protein spots were subjected to identification by peptide mass fingerprinting, as described in *Materials and Methods*. Ten of the selected phosphoprotein spots did not provide peptide spectra of high quality and will not be discussed. Thus, 32 proteins were identified with high confidence in 37 spots; some of the proteins were identified in multiple spots (Table 1).

Smad2 and Smad3 Have Different Effects on Protein Phosphorylation

In total, 111 phosphorylation profiles for identified proteins were obtained. We observed phosphorylations of the 32 proteins in a time- and/or Smad-dependent patterns that were specific for each protein (Figure 2 and Supplemental Figure B). Moreover, we observed an oscillating character of phosphorylation for many proteins.

We found that expression of Flag-Smad2 or myc-Smad3 strongly affected protein phosphorylation, compared with phosphorylation profiles of the same proteins in vector-transfected cells. Expression of Flag-Smad2 or myc-Smad3 would enhance Smad2- or Smad3-dependent phosphorylation of proteins upon treatment of cells with TGFβ. Different roles of Smad2 and Smad3 in transcriptional regulation have been shown (Piek *et al*., 2001), and our data indicate that Smads have also different effects on phosphorylation. We identified 5 groups of proteins, which differed with respect to how Smad expression affected their phosphorylation (Figure 2). The first group contains eleven proteins which were phosphorylated in vector-transfected cells with different time dependence than in Smad-transfected cells, whereas there were no pronounced differences between the same proteins from Flag-Smad2- and myc-Smad3-transfected cells (Figure 2, represented by breast cancer antigen NY BR-75, spot P22; Table 1, spots P2, P3, P4, P9, P17, P18, P22, P27, P28, P24, and P34; and Supplemental Figures B and C). The second group contains eight proteins with phosphorylation patterns similar for vector- and Flag-Smad2-transfected cells, but different for myc-Smad3-transfected cells (Figure 2, represented by Rab GDI, spot P15; Table 1, spots P7, P14, P15, P19, P20, P21, P32, and P35). The third group contains eight proteins with patterns of phosphorylation similar in vector- and myc-Smad3-transfected cells, but different in Flag-Smad2-transfected cells (Figure 2, represented by enolase-1, spot P23; and Table 1, spots P1, P5, P6, P29, P12, P23, P26, and P31). A fourth group contains two proteins that showed similar phosphorylation patterns in all three types of cells (Figure 2, represented by keratin 8, spot P30; and Table 1, ribonucleoprotein K, spot P8), and the fifth group consists of six proteins with phosphorylation patterns different in each of the cell types (Figure 2, represented by Ret finger protein, spot P16, and by HSP gp96 precursor, spot P25; Table 1, spots P10.1, P10.2, P10.3, P11, P13, P16, P25, and P33; and Supplemental Figures B and C). Thus, Smad2 and Smad3 affected TGFβ1-dependent protein phosphorylation of certain proteins differentially. This finding supports the notion that Smad2 and Smad3 have specific and various roles in transcriptional regulation and degradation processes (Piek *et al*., 2001; Shi and Massagué, 2003) and extends it to regulation of protein phosphorylation. The molecular mechanisms behind the effect of Smad2 and Smad3 on protein phosphorylation may be related to different interacting partners of these Smads and have to be explored further.

Table 1. Identified proteins which change their phosphorylation upon TGFβ1 treatment

No.[a]	Protein identity[b]	Accession no.[b]	Probability[b]	Estimated Z[b]	Sequence coverage, %	Theoretical value		Experimental value	
						pI	M_r (kDa)	pI	M_r (kDa)
P1	Eukaryotic translation elongation factor 1δ	AAH08012	1.0e+000	2.37	17	4.9	31.2	4.7	38
P2	Eukaryotic translation elongation factor 1δ	AAH08012	1.0e+000	2.43	22	5.0	31.3	4.8	38
P3	Eukaryotic translation elongation factor 1β	NP 001950	1.0e+000	1.68	20	4.5	24.9	4.2	32
P4	Acidic ribosomal phosphoprotein P2	NP_000995	1.0e+000	2.40	71	4.4	11.7	4.1	17
P5	60S ribosomal phosphoprotein P0	NP_000993	1.0e+000	2.31	29	5.7	34.4	5.6	40
P6	Heterogenous nuclear ribonucleoprotein C (C1/C2)	AAH08423	1.0e+000	1.09	12	5.0	33.6	4.9	45
P7	Heterogenous nuclear ribonucleoprotein C (C1/C2)	AAH08364	1.0e+000	2.37	18	5.0	32.4	4.9	42
P8	Heterogeneous nuclear ribonucleoprotein K	NP_112553	1.0e+000	2.35	32	5.1	51.3	5.0	80
P9	Heterogeneous nuclear ribonucleoprotein K	NP_112553	1.0e+000	2.34	33	5.2	51.3	5.3	80
P10.1	General transcription factor II-I	NP_127494	5.4e-001	0.28	6	6.4–8.4	108–112	6.6	110
P10.2	General transcription factor II-I	NP_127494	1.0e+000	1.90	5	6.4–8.4	108–112	6.7	110
P10.3	General transcription factor II-I	NP_127494	1.0e+000	2.41	6	6.4–8.4	108–112	6.8	110
P11	RAD23 homolog B, HHR23B	NP_002865	1.0e+000	2.42	24	4.8	43.2	4.5	60
P12	Proteasome subunit, α type, 3	NP_002779	1.0e+000	2.28	26	5.2	28.6	5.0	30
P13	26S proteasome subunit p40.5	NP_002808	1.0e+000	2.43	14	5.5	43.2	5.7	46
P14	Ras-GTPase-activating protein SH3-domain-binding protein	NP_005745	1.0e+000	2.39	21	5.4	52.2	5.4	80
P15	Rab GDP dissociation inhibitor, β	NP_001485	1.0e+000	2.34	29	5.9	51.1	6.2	55
P16	dJ25J6.4, Ret finger protein	CAB55434	9.7e-001	0.76	16	6.2	29.6	5.8	40
P17	Tumor necrosis factor α-induced protein 2	XP_007258	5.5e-001	0.44	8	5.8	50.1	6.3	55
P18	Cofilin1 (nonmuscle)	NP_005498	1.0e+000	2.29	37	8.5	18.7	6.9	20
P19	Putative human HLA class II associated protein I	NP_006296	1.0e+000	1.25	19	4.0	28.7	3.8	30
P20	Melanoma antigen gp 75	CAA35820	1.0e+000	1.68	20	4.7	28,5	4.6	27
P21	Serologically defined colon cancer antigen 28	XP_035147.1	1.0e+000	2.28	34	6.7	33.4	6.2	40
P22	Serologically defined breast cancer antigen NY-BR-75	AAG48263	6.0e-001	1.34	13	6.7	36.4	6.5	41
P23	Enolase 1	NP_001419	1.0e+000	1.44	14	7.0	49.5	4.6	55
P24	Glucose 6-phosphate dehydrogenase	1QKIH	1.0e+000	2.31	22	6.3	59.5	6.2	60
P25	HSP gp96 precursor	AAK74072	1.0e+000	2.34	20	4.7	90.4	4.5	120
P26	HSP 90-α (HSP86)	P07900	1.0e+000	1.96	18	4.9	85.1	4.7	100
P27	HSP 27	NP_001531	1.0e+000	2.43	32	6.0	22.8	5.6	28
P28	BiP protein	AAF13605	1.0e+000	2.43	21	5.2	71.0	4.8	90
P29	Keratin, 67K type II cytoskeletal, human	A22940	1.0e+000	2.23	14	6.0	65.6	5.5	65
P30	Keratin 8, type II cytoskeletal	P05787	1.0e+000	2.38	28	5.5	53.7	5.5	60
P31	Keratin 10, type I, cytoskeletal	P13645	9.7e-001	1.51	14	5.2	59.8	5.4	55
P32	keratin 18, cytoskeletal, human (fragment)	S06889	1.0e+000	1.76	16	5.3	47.3	5.2	52
P33	Keratin-like protein, human (fragment)	I38025	1.0e+000	2.43	18	5.5	28.5	5.0	31
P34	Hypothetical protein	XP_002623	8.4e-001	0.44	8	7.1	16.1	6.6	17
P35	Hypothetical protein	XP_018191	1.0e+000	2.05	27	4.4	49.0	4.0	70

[a] Selected protein spots from ^{32}P-labeled gels.

[b] NCBInr sequence identification numbers. Probability, Z-value, coverage and theoretical pI and M_r were obtained from the ProFound search. The calculation of experimental pI and M_r was based on migration of proteins on a 2D gel.

The identified proteins can be divided into nine groups according to their functions in cells. Six proteins that are components of the cytoskeleton or that regulate actin filament formation were identified (cofilin1, keratin 67K type II, keratin 8 type II, keratin 10 type I, keratin 18 and keratin-like protein; spots P18, P29, P30, P31, P32, and P33, respectively). Four proteins involved in protein translation form the second group (eukaryotic translation elongation factors 1δ and 1β, acidic ribosomal phosphoprotein P2 and 60S ribosomal phosphoprotein P0; spots P1, P2, P3, P4, and P5, respec-

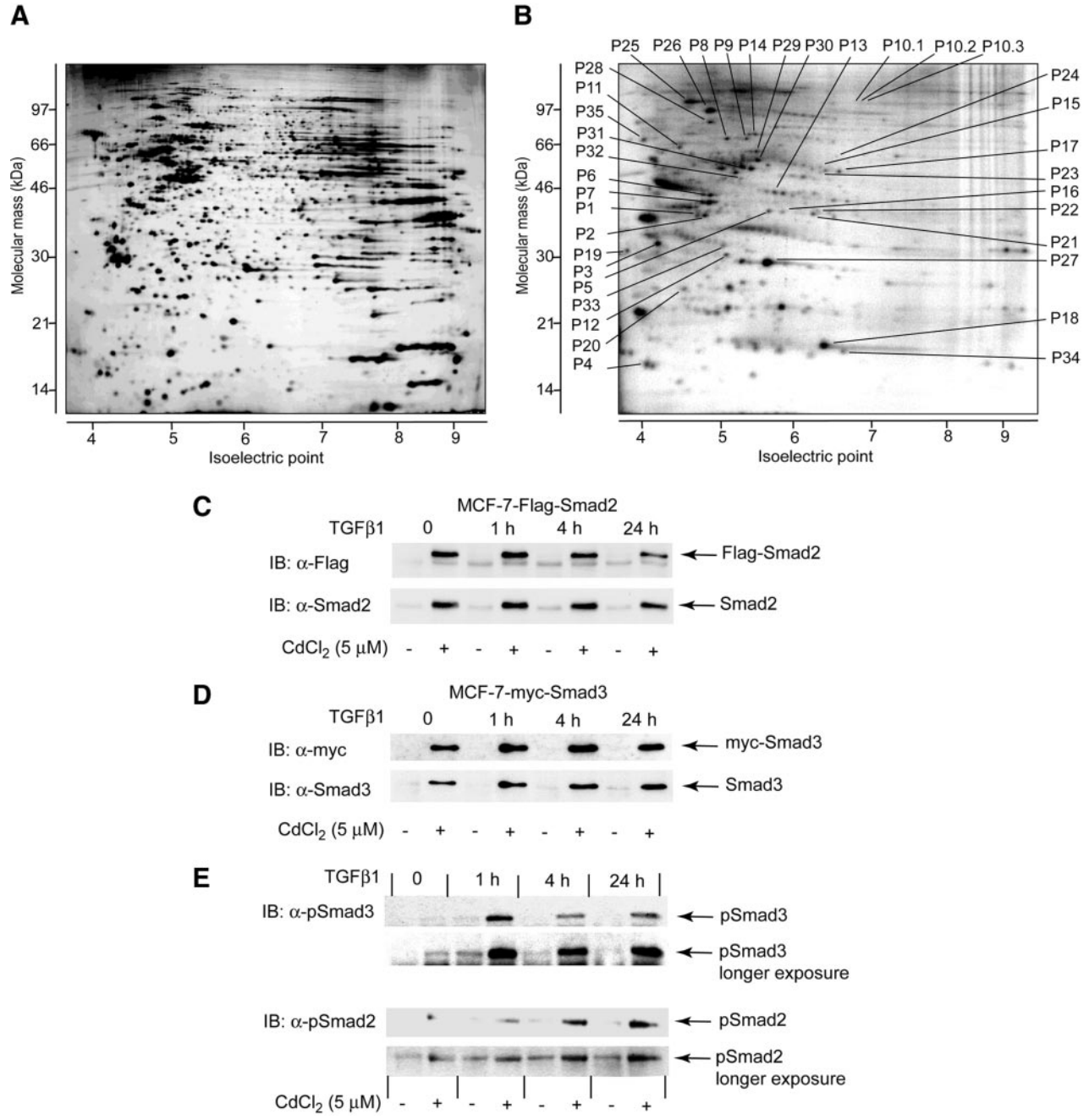

Figure 1. Two-dimensional maps of phosphorylated proteins in MCF-7 cells. Images of a silver-stained gel (A) and a gel with ^{32}P-labeled proteins (B). Shown images are of gels that were obtained from vector-transfected MCF-7 cells nontreated with TGFβ1 (also see Supplemental Figure A for images of gels representative for all experimental conditions). Migration positions of spots containing proteins that changed phosphorylation level upon treatment with TGFβ1 cells transfected with the empty vector, Flag-Smad2, or myc-Smad, are shown. Annotation of spots and proteins that were identified in the spots by peptide mass fingerprinting, is the same as in Table 1. The pH gradient of the first-dimension electrophoresis is shown at the bottom of the gels. Migration positions of molecular mass markers for the second-dimension SDS-PAGE is shown on the side of the gels. P1 to P35 indicate migration positions of identified proteins. (C and D) Expression levels of Flag-Smad2 and myc-Smad3 in stably transfected cells were found to be sustained during the time cells were incubated with TGFβ1. Expression of Flag-Smad2 (C) was monitored by immunoblotting of total cells extracts with anti-Flag antibodies and antibodies to endogenous Smad2 protein. Expression of myc-Smad3 (D) was monitored by immunoblotting of total cell extracts with anti-myc antibodies and antibodies to endogenous Smad3 protein. (E) Phosphorylation of Smad2 and Smad3 was monitored by immunoblotting of total cell extracts with specific antibodies that recognize phosphorylated C-terminal serine residues in respective Smad proteins. C-terminal serine residues are phosphorylated by activated type I TGFβ receptor. Phosphorylation of endogenous Smad2 and Smad3 are indicated in panels with longer exposure. Expressions of total Smad2 and Smad3 proteins are shown in C and D. Cells were treated with TGFβ1 and preincubated with $CdCl_2$ (5 μM), as indicated. Migration positions of Smad proteins are shown by arrows.

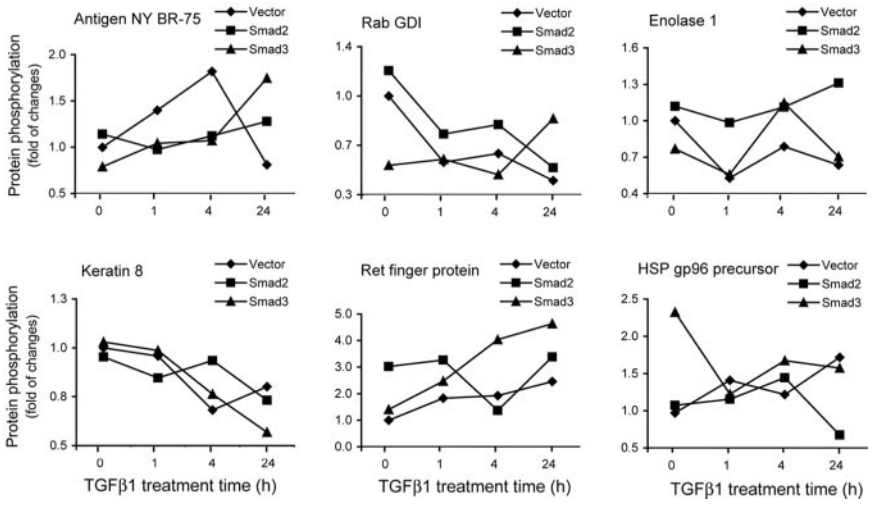

Figure 2. Clustering of phosphoproteins regulated by TGFβ1. Clustering of phosphorylated proteins according to an effect of the increased expressions of Flag-Smad2 or myc-Smad3. Five identified clusters are represented by phosphorylation profiles of breast cancer antigen NY-BR-75 (spot P22; group 1), RabGDI (spot P15; group 2), enolase 1 (spot P23; group 3), keratin 8 (spot P30; group 4), Ret finger protein (spot P16; group 5), and HSP gp96 precursor (spot P25; group 5). To calculate fold of changes, the radioactivity values of the same spot in various experimental conditions were compared with the radioactivity value of spot in the vector-transfected cells without TGFβ1 treatment. Radioactivity values are the average of four experiments. Average values of incorporated radioactivity and statistical significance of changes were calculated using the ImageMaster 2D Elite software. For annotation of proteins see Figure 1 and Table 1; phosphorylation profiles of all identified proteins are presented in Supplemental Figure B. Ret finger protein (spot P16) and HSP gp96 precursor (spot P25) represent the same cluster of proteins which showed phosphorylation patterns different in each of the cell types.

tively). Two other groups are formed by proteins regulating RNA processing (hnRNP C1/C2 and hnRNP K, identified in spots P6, P7, P8, and P9) and proteins with chaperoning functions (HSP gp96 precursor, HSP90 α, HSP27, and BiP, identified in spots P25, P26, P27, and P28). Three proteins involved in regulation of proteasomal degradation (Rad23 homolog B, proteasome subunit α3 and 26S proteasome subunit p40.5; spots P11, P12, and P13), and five proteins with functions in various signaling processes (TFII-I, RasGAP, Rab GDI, Ret finger protein and TNF-α-induced protein 2; spots P10, P14, P15, P16, and P17, respectively), were identified. Phosphorylation of enzymes regulating glycolysis (enolase 1, spot P23) and pentose cycle (glucose 6-phosphate dehydrogenase, spot P24) was found to be affected by TGFβ1. Three proteins that were identified as antigens (spots P20, P21, and P22) and HLA class II-associated protein I (spot P19) form the eighth group. The ninth group consists of two novel proteins with not yet attributed functional roles (spots P34 and P35). We did not observe common time-dependent changes for proteins within any of described functional groups. This is in agreement with various functional roles of phosphorylation, e.g., stimulatory or inhibitory. This also suggests that phosphorylation of every protein has to be explored in the context of its function.

We did not identify proteins known to change their phosphorylation upon TGFβ treatment, e.g., Smad2, Smad3, Erk kinase, or p38 kinase, despite that the phosphorylation of Smad2 and Smad3 was detectable in MCF-7 cells (Figure 1E; Fanayan *et al*., 2000; our unpublished data). Because we identified 32 proteins in 37 spots out of 89 protein spots that were regulated by TGFβ1, it is possible that some of the known targets are among the 52 nonidentified protein spots.

Six proteins out of the 32 identified have previously been reported to be affected by TGFβ. TGFβ1-dependent regulation of expression of keratin 10 type I, keratin 67K type II, and enolase 1 was described in Mv1Lu cells (Kanamoto *et al*., 2002). The levels of HSP27 and cofilin were found to be affected by TGFβ in fibroblasts (Bratt *et al*., 2001) and by glucose 6-phosphate dehydrogenase in human endothelial cells (Lomnytska *et al*., 2004). However, TGFβ-dependent phosphorylation of these six proteins has not been described. The other twenty-six identified proteins are novel targets of TGFβ1, which may provide insights into TGFβ regulation of cytoskeletal rearrangements, trafficking, proteasomal degradation and RNA processing (Table 1).

TFII-I Is Phosphorylated on Ser371 and Ser743 upon TGFβ1 Treatment of MCF-7 Cells

We selected one of the novel targets, transcription factor-II-I (TFII-I; spots P10.1, P10.2, and P10.3), for an exploration of the functional importance of the observed TGFβ1-dependent phosphorylation. TFII-I was identified in three phosphoprotein spots of similar molecular mass, suggesting at least three sites of phosphorylation of TFII-I (spots P10.1, P10.2, and P10.3; Figure 3A).

To confirm our findings, we transfected MCF-7 cells with a myc-TFII-I construct and investigated the effect of TGFβ1 on TFII-I phosphorylation (Figure 3B). We found that incubation of cells with TGFβ1, increased myc-TFII-I phosphorylation (Figure 3B). Phosphorylation of TFII-I was increased already after 1 h of TGFβ1 treatment of MCF-7-vector cells, compared with nontreated cells. TFII-I phosphorylation further increased after 4 h of treatment but then slightly decreased after 24 h of incubation of cells with TGFβ (Figure 3B).

We then further investigated phosphorylation of endogenous TFII-I. In accordance with the results obtained using proteomics technique, we observed that in vector-transfected cells TGFβ1 induced increase of phosphorylation already after 1 h and especially 4 h (2.1-fold, as strongest induction) of treatment, followed by slight decrease of phosphorylation after 24 h, compared with nontreated cells. TFII-I phosphorylation was increased in MCF-7-Flag-Smad2 cells even in absence of treatment with ligand to 1.3-fold, compared with vector-transfected nontreated cells (Figure 3C). Incubation of MCF-7-Flag-Smad2 cells with TGFβ resulted in further increase of TFII-I phosphorylation, although the fold of increase of TFII-I phosphorylation was relatively low (1.5-fold, as the strongest induction, and compared with nontreated cells). Incubation with TGFβ1 increased the phosphorylation of TFII-I in myc-Smad3-transfected cells as well, e.g., 1.87-fold after 1 h, 1.97-fold after 4 h, and 1.75-fold after 24 h, compared with nontreated cells

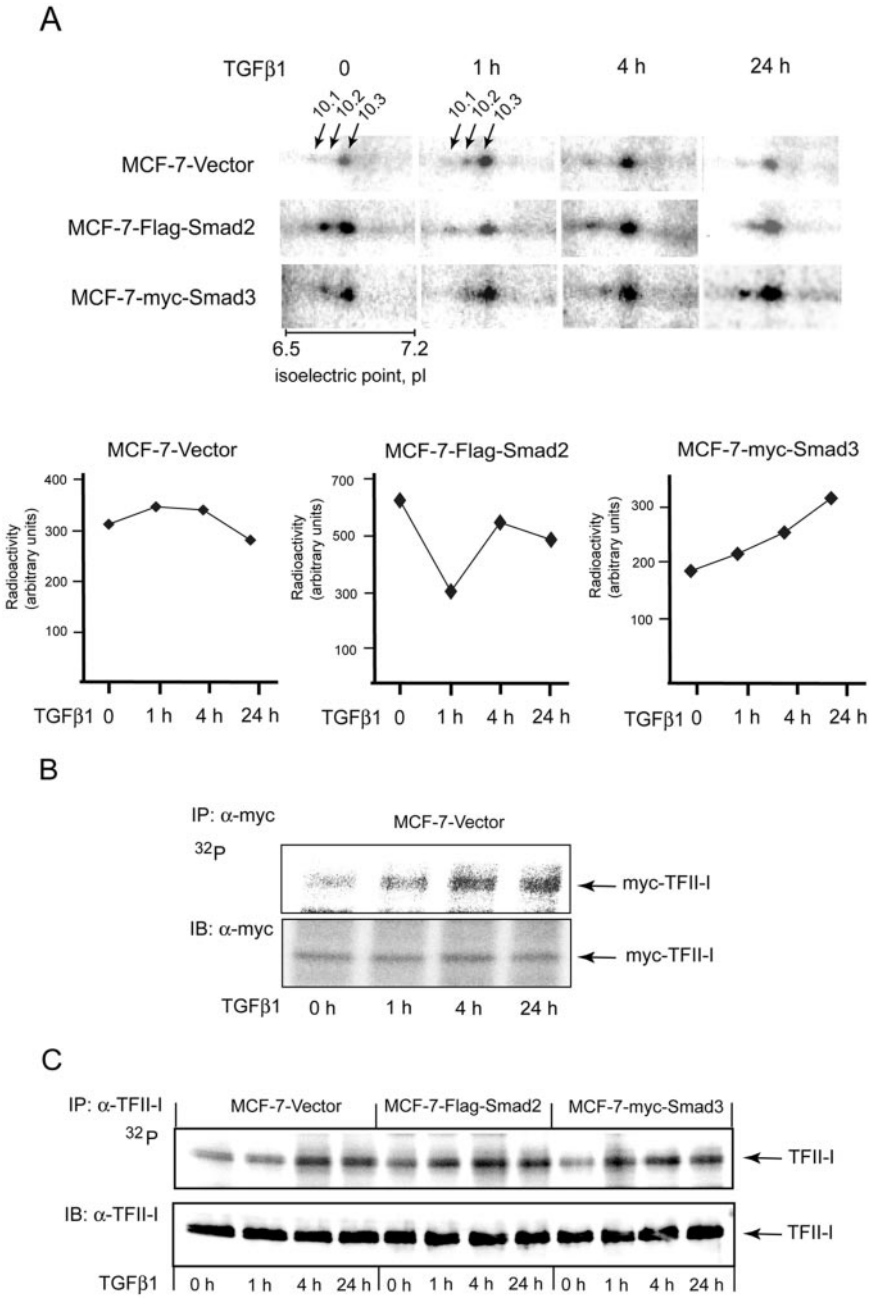

Figure 3. TFII-I is phosphorylated upon treatment of cells with TGFβ1. (A) Phosphoprotein spots in which TFII-I was identified are shown in selected areas of images of gels with ^{32}P-labeled proteins, which were prepared from MCF-7-vector, MCF-7-Flag-Smad2, and MCF7-myc-Smad3 cells. Migration positions of TFII-I-containing spots P10.1, P10.2, and P10.3 are indicated and are shown by arrows in two panels. Due to the presentation of gel images at similar intensities, borders for some of the software-designated spots may not be clearly recognized in panels. pH gradient is shown on the bottom of one of the images. Type of cells and time of treatment with TGFβ1 are shown on the side and on the top of the images, respectively. Quantification of total radioactivity in all three TFII-I-containing spots (summarized total activity in P10.1, P10.2, and P10.3 spots for one experimental condition) in cells treated with TGFβ1, or not, as indicted, is shown below the images. Note the difference in total radioactivity at 0-h time point. TGFβ1 treatment and cell lines are indicated. (B) Total phosphorylation of TFII-I was analyzed by immunoprecipitation of ^{32}P-labeled myc-TFII-I with anti-myc antibodies. Phosphorylation of transfected myc-TFII-I in MCF-7-vector cells upon treatment of cells with TGFβ1 (5 ng/ml) for 1, 4, and 24 h is shown (top). Expression of myc-TFII-I was monitored by immunoblotting of the same membrane with anti-myc antibodies (B; bottom). (C) Phosphorylation of endogenous TFII-I in MCF-7-vector, MCF-7-Flag-Smad2, and MCF-7-myc-Smad3 cells was evaluated by immunoprecipitation of TFII-I with antibodies specific to endogenous protein from cells metabolically labeled with [^{32}P]orthophosphate. Metabolic labeling of cells with [^{32}P]orthophosphate was performed, as described in the *Materials and Methods*. Expression of myc-TFII-I was monitored by immunoblotting of the same membrane with anti-myc antibodies. Migration position of ^{32}P-labeled and immunodetected myc-TFII-I are shown by arrows. Representative experiments out of three (B and C) performed are shown.

(Figure 3C). Thus, we confirmed TGFβ- and Smad-dependent phosphorylation of TFII-I (Figure 3, B and C).

It should be noted that one-dimensional gel electrophoresis does not allow an efficient separation of TFII-I with various levels of phosphorylation, as was observed in 2D-GE. Under closer examination, two bands of endogenous TFII-I could be detected (Supplemental Figure D). The two bands may represent two isoforms of TFII-I (Cheriyath and Roy, 2000). Phosphopeptide mapping confirmed that these two bands represent the same protein; the maps of the two forms of the endogenous protein were identical to each other, and to the map of transfected TFII-I (Figures 4 and 5 and Supplemental Figure D).

To identify the sites of TGFβ1-dependent phosphorylation of TFII-I, we performed phosphopeptide mapping of TFII-I (Figure 4). We analyzed both phosphoprotein bands which were observed in the 1D gel (Figure 3C). Phosphopeptide maps of both endogenous proteins and phosphopeptide maps of TFII-I expressed in MCF-7 or COS7 cells were identical, strongly suggesting that this is the same protein (Figures 4 and 5 and Supplemental Figure D). We found multiple phosphorylation sites in TFII-I in the basal state; this is in agreement with the reported role of TFII-I as a convergence point for multiple kinases in regulation of transcription (Kim *et al*., 1998; Novina *et al*., 1998; Kim and Cochran, 2000; Egloff and Desiderio, 2001; Kim and Cochran, 2001; Roy, 2001Casteel *et al*., 2002; Cheriyath *et al*., 2002). We found that treatment of cells with TGFβ1 led to appearance of two new phosphopeptides (Figure 4). These phosphopeptides were also observed in maps of TFII-I obtained from Flag-Smad2- or myc-Smad3-tansfected cells, even if the cells were not treated with TGFβ1 (Figure 4; our unpub-

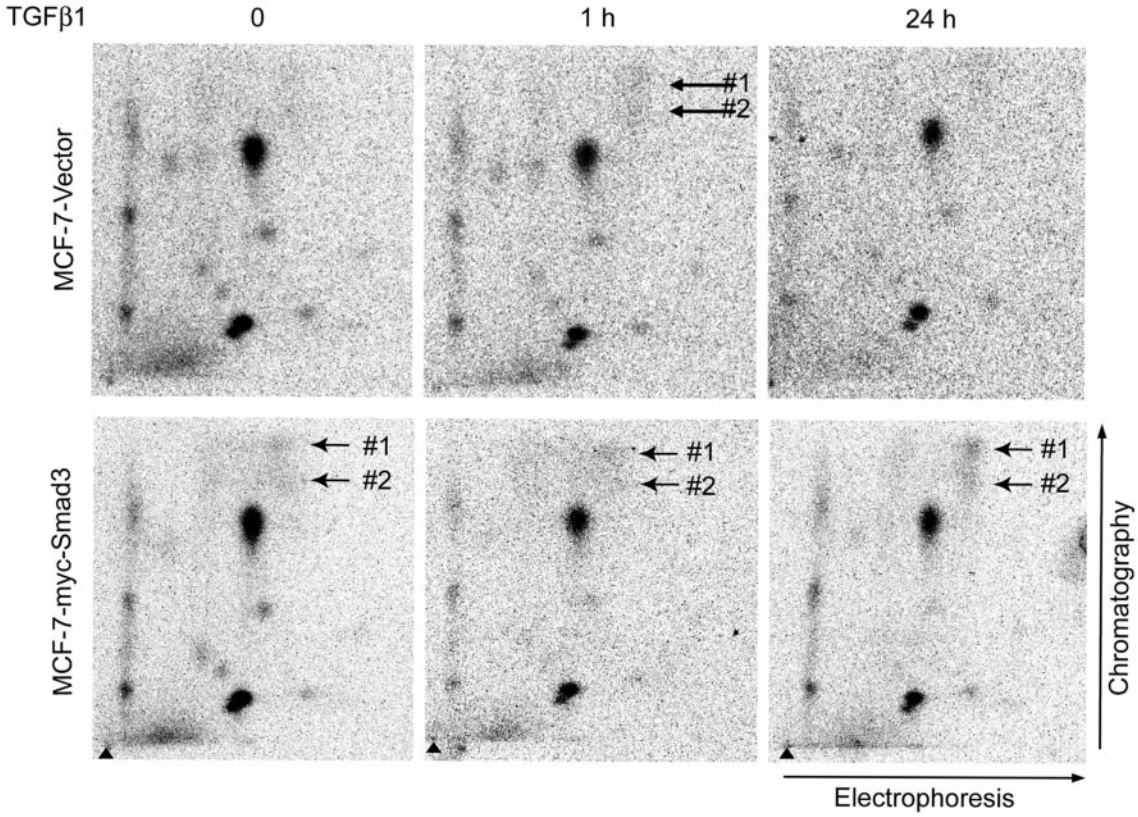

Figure 4. TGFβ1-induced appearance of two phosphopeptides in phosphopeptide maps of endogenous TFII-I. Endogenous TFII-I from vector-transfected MCF-7 cells or MCF-7-myc-Smad3 cells, which were treated or not with TGFβ1 (5 ng/ml), was subjected to two-dimensional phosphopeptide mapping. Arrows show migration position of phosphopeptides that occurred after treatment of cells with TGFβ1. Triangles show sample application points. Directions of electrophoresis and chromatography are shown at the sides of the maps. Cell lines are indicated at the side of the maps, and time of treatment of cells with TGFβ1 is indicated on the top of the maps. Representative maps out of three performed experiments are shown.

lished data). Thus, the appearance of new phosphopeptides correlated with the pattern of TFII-I phosphorylation, which was observed in 2D gels.

To identify the sites of phosphorylation of TFII-I, the TGFβ1-dependent phosphopeptides were eluted from the plates and subjected to phosphoamino acid analysis and radiochemical sequencing using Edman degradation (Figure 5, A–C). We found that both phosphopeptides contained phosphoserine residues, which were located at position three (Figure 5, B and C). In silico digestion of TFII-I with trypsin predicted nine peptides with a serine residue at the third position (Supplemental Figure E). The predicted migration of potential candidates in 2D phosphopeptide maps, suggested that peptides containing Ser371 and Ser743 best corresponded to the observed phosphopeptides. These two peptides have similar sequences and differ only by one amino acid residue (Figure 5C); the presence of a tyrosine residue versus a phenylalanine residue may explain the slight difference in migration of the peptides in chromatography, and almost identical migration in electrophoresis (Figure 5A). We transfected TFII-I constructs with the serine residues 371 and 743 mutated to alanine residues into MCF-7 cells and performed phosphopeptide mapping of TFII-I; as predicted, the TGFβ1-dependent phosphopeptides were not detected in the maps of the Ser371,743Ala mutant of TFII-I (Figure 5D). We also confirmed phosphorylation at Ser371 and Ser743 of TFII-I expressed in COS7 cells, which were used for functional evaluation of TFII-I phosphorylation (Figure 5E). Thus, we have identified Ser371 and Ser743 in TFII-I as sites of TGFβ1-induced phosphorylation.

Abrogation of TFII-I Phosphorylation on Ser371 and Ser743 Affects TGFβ1-dependent Regulation of Cyclin D2, Cyclin D3, and E2F2 Gene Expression

TFII-I is a unique transcription factor because it provides a link between the basal transcriptional machinery and specific transcription factors (Roy, 2001). To explore whether phosphorylation of TFII-I affects transcriptional responses to TGFβ1, we performed a low-density microarray analysis of MCF-7 cells stably transfected with wild-type and mutated TFII-I and treated or not with TGFβ1. We used two cell clones for the phosphorylation-deficient mutant of TFII-I, one with the high level and the second with the low level of TFII-I expression (Figure 6A). Human Cell Cycle Gene Array Q series allows evaluation of expression of 96 genes involved in the cell cycle regulation and response to DNA damage. We found that expressions of mRNA of cyclin D2, cyclin D3, and E2F2 were regulated in a TFII-I-dependent manner (Figure 6, B–D, top). TGFβ1 increased expression of these three genes, and transfection of wild-type TFII-I enhanced the effect of TGFβ1. Transfection of MCF-7 cells with the Ser371,743,Ala TFII-I mutant resulted in an enhancement of *cyclin D2*, *cyclin D3*, and *E2F2* gene expression, compared

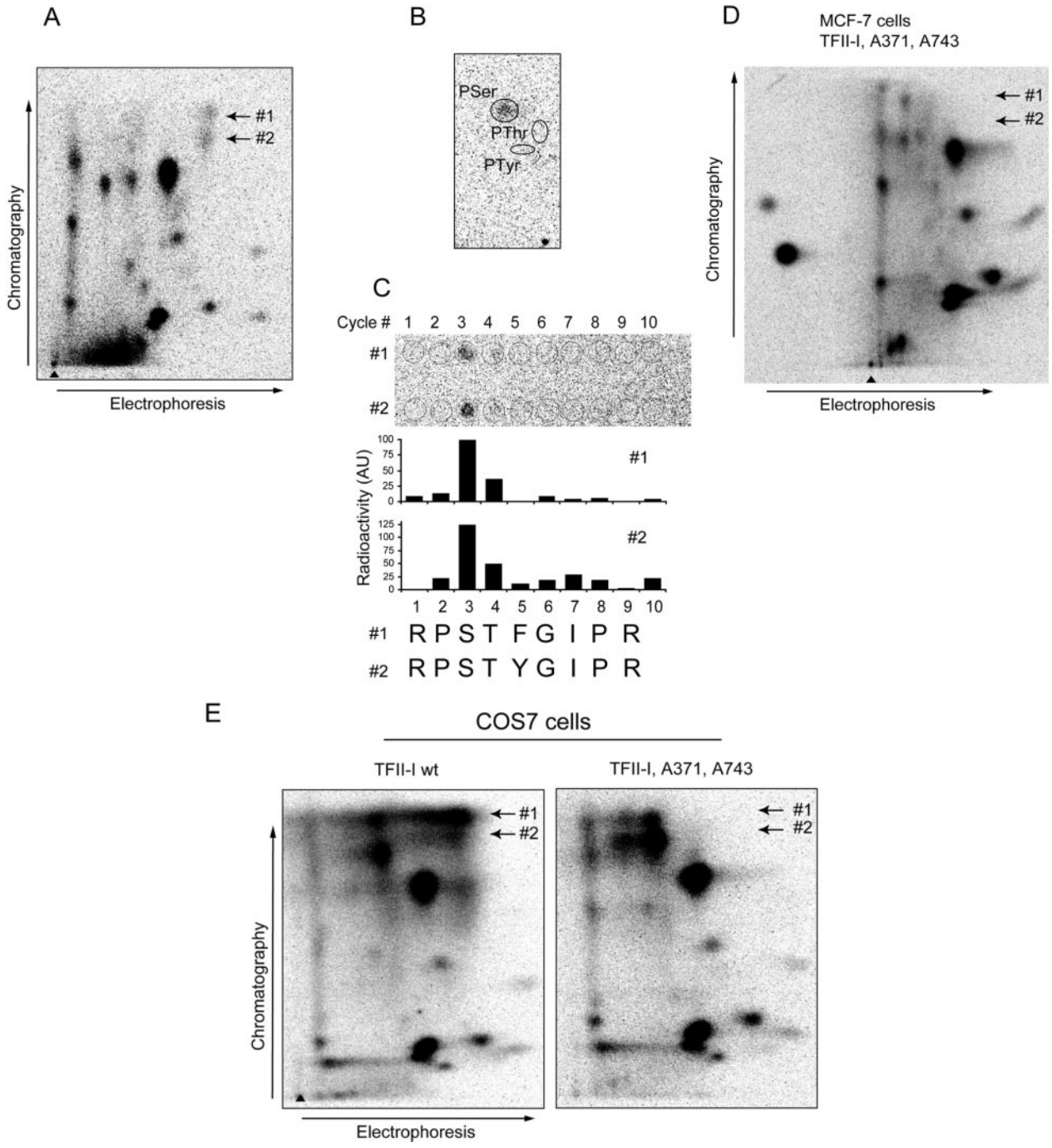

Figure 5. TFII-I is phosphorylated at serine residues 371 and 743 upon treatment of cells with TGFβ1. (A) Two TGFβ1-induced phosphopeptides, #1 and #2, were identified in a phosphopeptide map of transfected myc-TFII-I that was immunoprecipitated from MCF7-vector cells metabolically labeled with [^{32}P]orthophosphate. Cells were treated with TGFβ1 (5 ng/ml) for 4 h. Migration positions of the peptides are shown by arrows. (B) The phosphopeptides shown in A were eluted and subjected to phosphoamino acid analysis. For both peptides, radioactivity was comigrating with phospho-serine (pSer), and no radioactivity was comigrating with phospho-threonine (pThr) or phospho-tyrosine (pTyr). Migration positions of pSer, pThr, and pTyr are indicated. (C) Radioactivities in both TGFβ1-dependent phosphopeptides were released in the third cycle of radiochemical sequencing (C; top). Localizations of applied fractions are shown by open cycles, and number of cycles is shown (top). Quantification of the signals presented (top of panels) is shown (middle). Cycles of radiochemical degradation are aligned with sequences of two candidate peptides (C; bottom). (D) Phosphopeptide mapping of myc-TFII-I with serine residues 371 and 743 replaced by alanine residues did not show phosphopeptides #1 and #2. TFII-I mutant Ser371,743Ala was transfected in MCF-7 cells, and metabolic labeling, immunoprecipitation, and phosphopeptide mapping were performed as for wild-type myc-TFII-I. Expected migration positions for phosphopeptides #1 and #2 are shown by arrows. (E) Mutation of serine residues 371 and 743 abrogated appearance of two phosphopeptides migrating at identical positions as TGFβ1-dependent phosphopeptides, in myc-TFII-I expressed in COS7 cells. Cells transfected with wild-type or mutant myc-TFII-I were metabolically labeled with [^{32}P]orthophosphate and were incubated with 8-Br-cGMP to activate cotransfected PKG. myc-TFII-I was immunoprecipitated with anti-tag antibodies, and phosphopeptide mapping was performed. Migration positions of phosphopeptides #1 and #2 are shown by arrows.

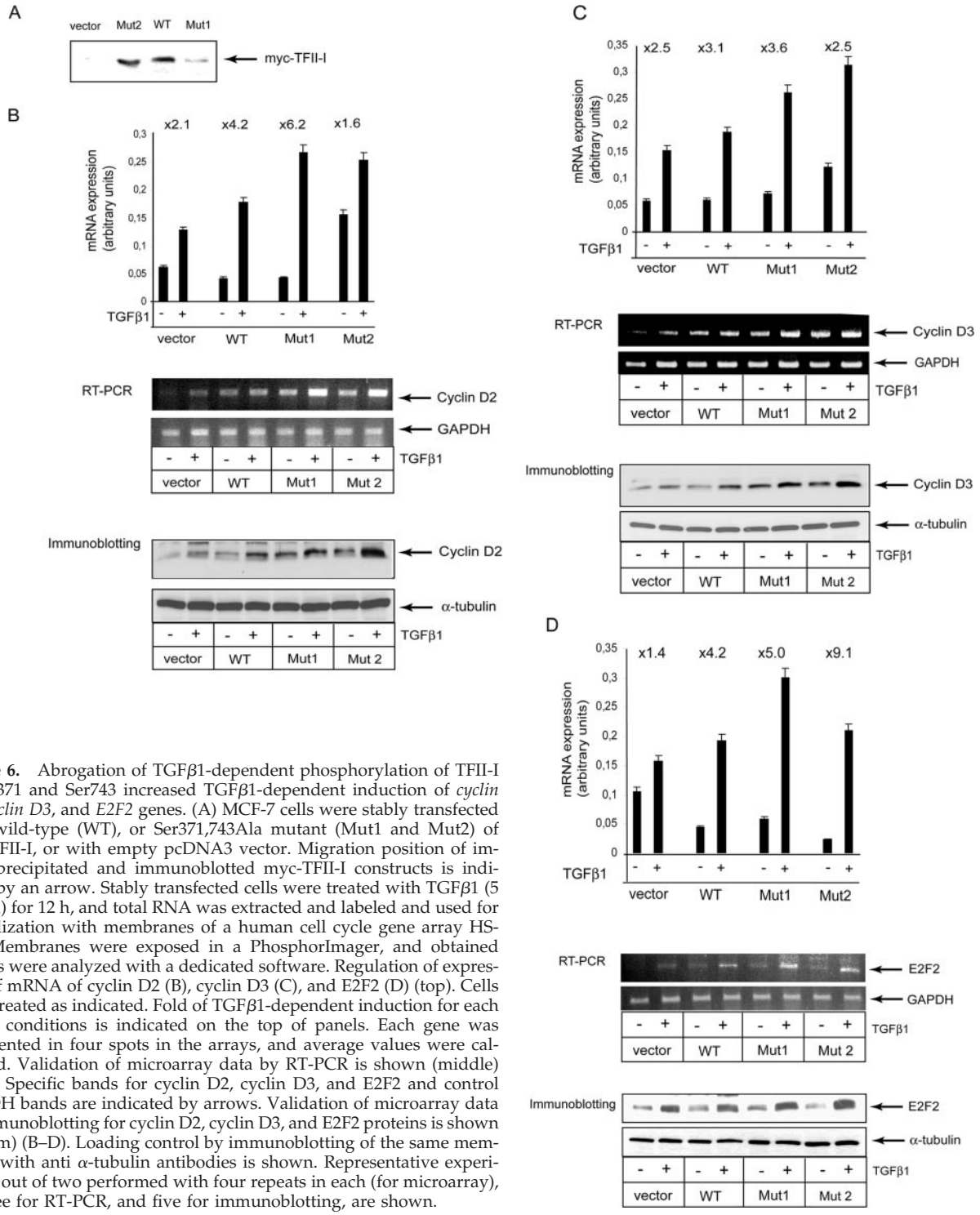

Figure 6. Abrogation of TGFβ1-dependent phosphorylation of TFII-I at Ser371 and Ser743 increased TGFβ1-dependent induction of *cyclin D2*, *cyclin D3*, and *E2F2* genes. (A) MCF-7 cells were stably transfected with wild-type (WT), or Ser371,743Ala mutant (Mut1 and Mut2) of myc-TFII-I, or with empty pcDNA3 vector. Migration position of immunoprecipitated and immunoblotted myc-TFII-I constructs is indicated by an arrow. Stably transfected cells were treated with TGFβ1 (5 ng/ml) for 12 h, and total RNA was extracted and labeled and used for hybridization with membranes of a human cell cycle gene array HS-001. Membranes were exposed in a PhosphorImager, and obtained images were analyzed with a dedicated software. Regulation of expression of mRNA of cyclin D2 (B), cyclin D3 (C), and E2F2 (D) (top). Cells were treated as indicated. Fold of TGFβ1-dependent induction for each of the conditions is indicated on the top of panels. Each gene was represented in four spots in the arrays, and average values were calculated. Validation of microarray data by RT-PCR is shown (middle) (B–D). Specific bands for cyclin D2, cyclin D3, and E2F2 and control GAPDH bands are indicated by arrows. Validation of microarray data by immunoblotting for cyclin D2, cyclin D3, and E2F2 proteins is shown (bottom) (B–D). Loading control by immunoblotting of the same membrane with anti α-tubulin antibodies is shown. Representative experiments out of two performed with four repeats in each (for microarray), of three for RT-PCR, and five for immunoblotting, are shown.

with cells transfected with wild-type TFII-I (Figure 6, B–D). The higher expression level of mutated TFII-I (Mut 2) also led to ligand-independent increase in transcriptional activation of *cyclin D2* and *cyclin D3* genes, compared with cells expressing mutated TFII-I at the lower level. This is in agreement with the role of TFII-I phosphorylation in regulation of these genes. The basal level of E2F2 expression in cells transfected with mutant TFII-I at high level was lower, compared with other cells. However, the induction after TGFβ1 stimulation was almost twofold higher, compared with cells transfected with wild-type TFII-I (Figure 6D). Microarray data were confirmed using reverse transcription (RT)-PCR with specific primers (Figure 6, B–D, middle). Moreover, the similar pattern of regulation by TGFβ and TFII-I expression was observed for cyclin D2, cyclin D3, and E2F2 proteins, as evaluated by immunoblotting with specific antibodies (Figure 6, B-D, bottom). Microarray, RT-PCR and immunoblotting experiments clearly indicate that TFII-I and

its phosphorylation at Ser371 and Ser 743 modulate TGFβ-dependent expression of selected genes (Figure 6, B-D).

Importantly, we found that TGFβ1-dependent regulation of a number of other genes was not affected by transfection of TFII-I wild-type or mutant (Supplemental Figures F and G). We found also that TGFβ1 regulated in the stably transfected cells its known target genes, e.g., *cyclin D1*, *cyclin F*, *cyclin G2*, *cdc2*, *cdk2*, *E2F2*, *E2F3*, and *GADD45A* (Supplemental Figure G). This suggests that TFII-I modulates TGFβ1-dependent transcriptional regulation selectively and does not have a general effect. It also suggests that initiation of the TGFβ signaling pathway, at least on the level of receptors and Smad activation, is not affected by transfection of wild-type or mutant TFII-I. Thus, we found that substitution of the phosphorylatable serine residues 371 and 743 in TFII-I to alanine residues, modulated TGFβ1-dependent transcription of endogenous cyclin D2, cyclin D3, and E2F2 genes in MCF-7 cells.

Abrogation of TFII-I Phosphorylation on Ser371 and Ser743 Increases TFII-I Cooperation with Smad3 in Transcriptional Activation

To explore further the importance of TFII-I phosphorylation for transcriptional responses to TGFβ1, we performed a luciferase reporter assay with the TGFβ-responsive CAGA(12)-luc reporter. This reporter contains a minimal promoter and multiple CAGA boxes (Dennler *et al*., 1998). The minimal promoter contains the initiator (Inr) sequence of the adenovirus major late promoter, which is a binding site for TFII-I; the CAGA sequence is a specific binding site for Smad3 and Smad4 (Roy *et al*., 1997; Dennler *et al*., 1998; Roy, 2001). Because Smad2 requires another protein to mediate binding to a promoter DNA, use of the CAGA-containing reporter provided a possibility to correlate transcriptional activity with direct binding to DNA of activated Smad3. Thus, we explored whether TFII-I influenced Smad3-dependent activation of CAGA(12)-luc reporter. We used COS7 cells because they express TFII-I at a lower level than other cells, and therefore have a low background when mutated TFII-I is analyzed in transfection experiments (Casteel *et al*., 2002). We observed that wild-type TFII-I cooperated with Smad3 in activation of the CAGA(12)-luc reporter (Figure 7A). Transfection of TFII-I alone did not affect the activation of CAGA(12)-luc reporter. The abrogation of TGFβ1-dependent phosphorylation of TFII-I resulted in a further increase of cooperation, as shown by the transfection of the Ser371,743Ala mutated TFII-I with Smad3; the mutated TFII-I consistently increased Smad3-dependent induction of the reporter 2.0 fold, whereas the wild-type TFII-I increased Smad3-dependent transcription 1.3-fold, compared with TGFβ1-treated cells transfected with Smad3 only. Our results suggest that the phosphorylation at serine residues 371 and 743 may inhibit the cooperation between Smad3 and TFII-I in regulation of transcription.

To explore role of endogenous Smad3 and TFII-I in activation of CAGA(12)-luc reporter, we used small interfering RNA (siRNA) specific to Smad3 and TFII-I (Figure 7, B and C, insets). We found that inhibition of Smad3 expression strongly blocked activation of the reporter (Figure 7B). Transfection of wild-type or mutant TFII-I did not affect luciferase activity. Because activation of CAGA(12)-luc reporter is dependent on Smad3 binding to the CAGA elements in the promoter, experiment with Smad3 siRNA indicated that TFII-I, endogenous or transfected, cannot modulate reporter activity without Smad3. Interestingly, down-regulation of TFII-I with specific siRNA decreased TGFβ- and Smad3-dependent induction of CAGA(12)-luc reporter (Figure 7C). This suggests that endogenous TFII-I is required for efficient Smad3-dependent activation of the reporter. In contrast to Smad3, Smad2 does not bind to DNA directly and does not interact with CAGA elements. As expected, expression of Smad2 with wild-type or mutant TFII-I did not affected TGFβ-dependent induction of CAGA(12)-luc reporter (Figure 7D). However, when we tested Smad2-responsive ARE-luc reporter, Ser371,743Ala mutant of TFII-I cooperated with Smad2 (Figure 7E). Smad2 binds to the ARE promoter via FAST protein, and expression of both proteins may affect responsiveness of ARE-luc reporter, compared with Smad3-dependent CAGA(12)-luc. Experiments with Smad-independent SRE-luc reporter confirmed that TFII-I does not affect transcriptional regulation in a nonspecific way (Figure 7F). Notably, transfection of TFII-I had a marginal effect on activation of SRE-luc reporter by epidermal growth factor (Figure 7F). Thus, reporter assays showed that TFII-I cooperates with Smad3 via forming a complex with Smad3 and not via a Smad-independent mechanism.

To explore whether TFII-I affects binding of Smad3 to CAGA elements, we performed a DNA precipitation assay (Figure 8A). We observed a strong binding of Smad3 to the CAGA-probe upon expression of the wild-type and Ser371,743Ala mutant TFII-I. For cells transfected with the Ser371,743Ala mutant TFII-I, Smad3 binding was further enhanced upon treatment of cells with TGFβ1 (Figure 8A). TGFβ1 had a marginal effect on Smad3 binding upon expression of wild-type TFII-I. This suggests that direct binding to the CAGA elements is not sufficient for the maximal Smad3-dependent transcriptional activation, and other ligand-induced events are required, e.g., phosphorylation of Smad3 and interaction with other proteins. Smad3 transfected alone showed weak binding to the CAGA probe. This binding was enhanced upon treatment with TGFβ1. Because TFII-I did not affect the stability of Smad3, our results suggest that TFII-I enhanced binding of Smad3 to the CAGA box, and abrogation of TGFβ1-dependent phosphorylation of TFII-I enhanced further Smad3 binding.

To explore whether Smad3 and TFII-I could form a complex, we performed a coimmunoprecipitation assay of Smad3 cotransfected with wild-type or mutant myc-TFII-I. We observed Smad3 in a complex with wild-type TFII-I upon treatment of cells with TGFβ1 (Figure 8B). Mutant TFII-I formed a complex with Smad3 even in the absence of treatment with ligand. Ligand-independent complex formation between wild-type TFII-I and Smad3 was weak (our unpublished data), and the complex formation was enhanced after addition of TGFβ1 (Figure 8B). Whether interaction between TFII-I and Smad3 is direct, remains to be investigated. Because TFII-I cooperated with Smad3 in transcriptional regulation, a preservation of the complex may increase the transcriptional cooperation between TFII-I and Smad3, whereas TGFβ1-dependent phosphorylation of TFII-I at Ser371 and Ser743 may restrain this transcriptional cooperation.

Serine residues 371 and 743 are located in a sequence context suggesting that they can be phosphorylated by cGMP-dependent protein kinase (PKG). In agreement with this prediction, site-directed mutagenesis of both serine residues abrogated TFII-I phosphorylation by PKG (Casteel *et al*., 2002). Thus, we explored whether inhibition of PKG would block TGFβ1-dependent phosphorylation of TFII-I. To exclude an influence of basal phosphorylation of TFII-I, we performed two-dimensional phosphopeptide mapping of ^{32}P-labeled TFII-I, because it allows evaluation of phosphorylation of specific peptides. We found that pretreatment

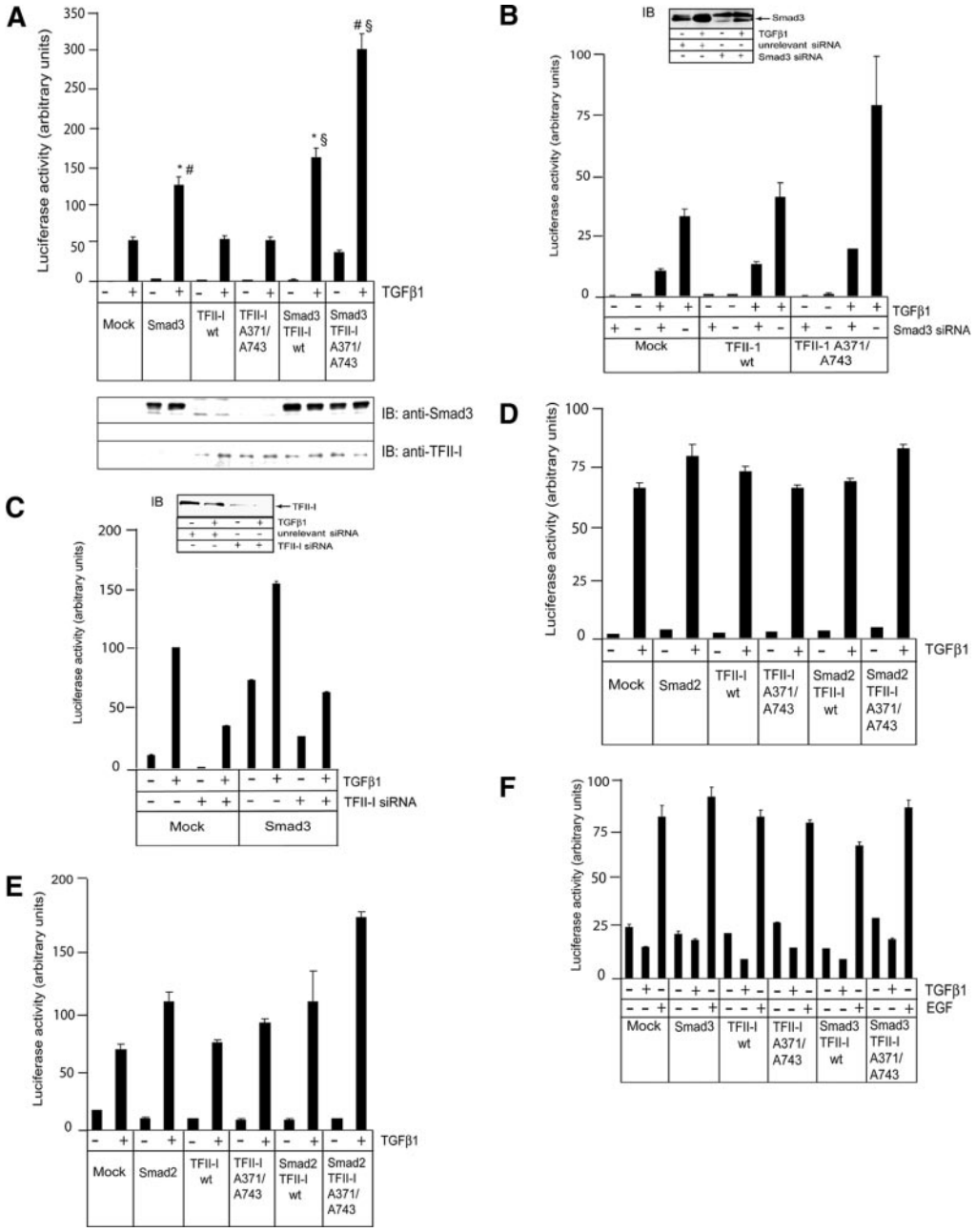

Figure 7. Abrogation of TGFβ1-dependent phosphorylation of TFII-I at Ser371 and Ser743 increased TFII-I-dependent cooperation with Smad3 in transcriptional regulation. (A) CAGA(12)-luc luciferase assay was performed using COS7 cells. Cells were transfected with myc-Smad3 (Smad3) and TFII-I wild-type (TFII-I wt) and mutant (TFII-I A341/A743) constructs, as indicated. Cells were treated with TGFβ1 (5 ng/ml), as indicated, and luciferase activity was measured after 18 h. Expression of Smad3 and TFII-I proteins was evaluated by immunoblotting of total cell extracts used for luciferase assay. Antibodies specific to Smad3 and TFII-I were used, as indicated. A representative experiment out of three performed, is shown. *$p < 0.05$, cells transfected with Smad3 and TFII-I wild-type, compared with Smad3 transfected cells. $^{\#}p < 0.001$, cells transfected with Smad3 and mutant TFII-I, compared with Smad3 transfected cells. $^{\S}p < 0.005$, cells transfected with Smad3 and TFII-I wild-type, compared with cells transfected with Smad3 and mutant TFII-I. (B) Smad3 siRNA strongly inhibited activation of CAGA(12)-luc reporter. COS7 cells were transfected with Smad3-specific siRNA, nonspecific siRNA, wild-type or mutant TFII-I, and treated with TGFβ1 (5 ng/ml), as indicated. Inserts shows down-regulation of Smad3 expression by siRNA. (C) Endogenous TFII-I is required for Smad3-dependent activation of CAGA(12)-luc reporter. COS7 cells were transfected with TFII-I specific siRNA, nonspecific siRNA, Smad3, and treated with TGFβ1 (5 ng/ml), as indicated. Inset shows down-regulation of endogenous TFII-I by siRNA. (D) Smad2 and TFII-I do not affect TGFβ-dependent induction of CAGA(12)-luc reporter. 293T cells were transfected, as described in A, except Smad2 was transfected instead of Smad3. Cells were treated and luciferase activity was measured, as described in A. (E) Ser341,743Ala mutant of TFII-I cooperated with Smad2 in activation ARE-luc luciferase reporter. 293T cells were transfected with Smad2, wild-type and mutant TFII-I, and treated with TGFβ1 (5 ng/ml), as indicated. Luciferase activity was measured, as described in A. (F) TFII-I and TGFβ did not affect activation of SRE-luc reporter. 293T cells were transfected with Smad3, wild-type and mutant TFII-I, and treated with TGFβ1 (5 ng/ml) and EGF (1 ng/ml), as indicated. Luciferase activity in all assays (A–F) was measured as described in A. Transfection efficiency in all luciferase reporter assays (A–F) was normalized to β-galactosidase activity of cotransfected LacZ plasmid. Representative experiments out of three performed (A–F), are shown.

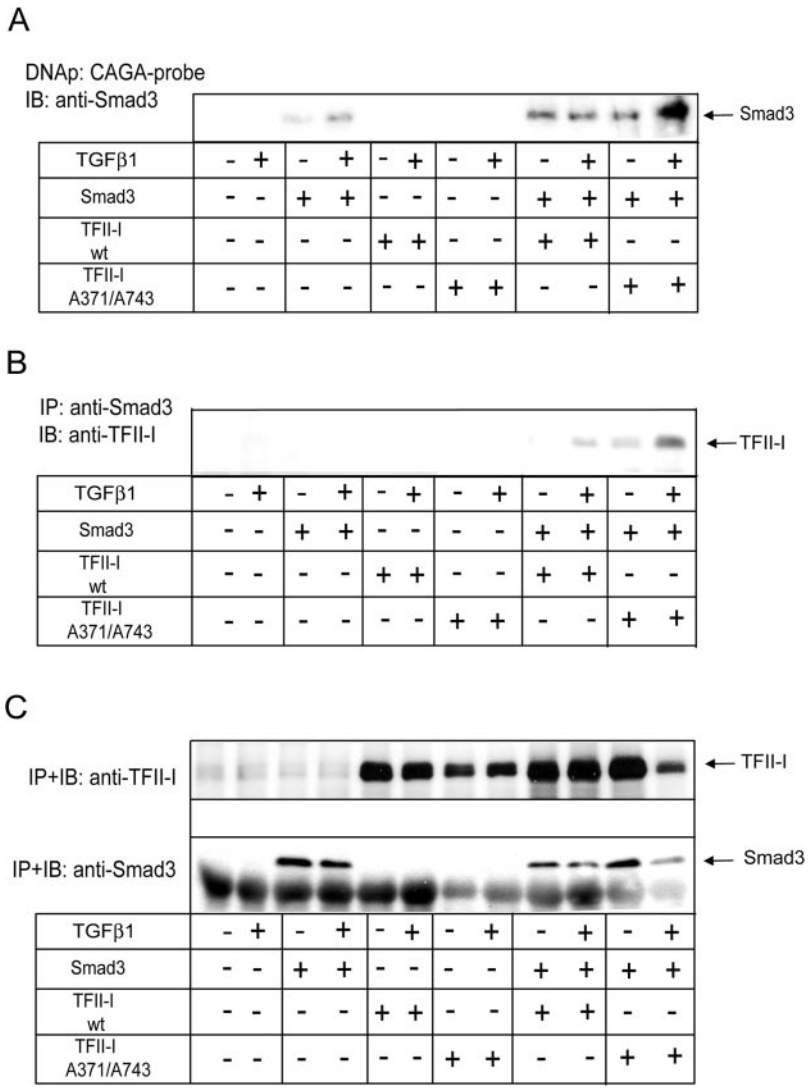

Figure 8. TFII-I transfection increased binding of Smad3 to the CAGA elements. (A) DNA precipitation assay with immobilized CAGA-probe showed increased binding of myc-Smad3 (Smad3) to the probe upon cotransfection of myc-TFII-I Ser371,743Ala mutant (TFII-I A371/A743) and treatment with TGFβ1, compared with binding of myc-Smad3 cotransfected with wild-type myc-TFII-I (TFII-I wt). COS7 cells were transfected with myc-Smad3 and TFII-I plasmids, and treated with TGFβ1 (5 ng/ml), as indicated. Proteins bound to CAGA probe were separated by SDS-PAGE, and transferred onto nitrocellulose membrane. Smad3 was detected by immunoblotting with anti-Smad3 antibody. The migration position of Smad3 is shown by an arrow. (B) Smad3 and TFII-I form a complex. COS7 cells were transfected with myc-Smad3, myc-TFII-I wild-type and Ser371/743Ala mutant, and empty vector, as described in B, and were treated with TGFβ1 (5 ng/ml), as indicated. Proteins immunoprecipitated with anti-Smad3 antibodies were immunoblotted with anti-TFII-I antibodies. The anti-Smad3 and anti-TFII-I antibodies used recognize also endogenous proteins. Migration position of TFII-I is shown by an arrow. (C) Expression of Smad3 and TFII-I in cells which were used in DNA-precipitation and coimmunoprecipitation assays. Transfection of Smad3 and TFII-I plasmids was performed in identical manners for the two assays, and transfected cells were divided and used for assays at the same time. Expression of Smad3 and TFII-I proteins was evaluated by immunoprecipitation and immunoblotting of Smad3 and TFII-I with respective specific antibodies which recognize epitopes also in endogenous proteins. Migration positions of Smad3 and TFII-I are shown by arrows. Representative experiments out of three (A–C) performed are shown.

of cells with the PKG-specific inhibitor K5823 decreased, but did not abolish, phosphorylation of TFII-I (Figure 9, A and B). Pretreatment of cells with the H7 inhibitor of broad specificity had stronger inhibitory effect (Figure 9C), but the mitogen-activated protein kinase kinase inhibitor PD098059 did not affect the phosphorylation (our unpublished data). Thus, PKG may contribute to the TGFβ1-dependent phosphorylation of TFII-I, but an involvement of other kinase(s) is also possible. This suggests that the same residues, Ser371 and Ser743, may be phosphorylated by various kinases.

DISCUSSION

We performed a phosphoproteome profiling of TGFβ1 signaling in MCF-7 cells to unveil novel regulatory targets of TGFβ1. 2D-GE and peptide mass fingerprinting using MALDI TOF MS in combination with ^{32}P metabolic labeling of cells provided reproducible generation of phosphoproteome maps and high efficiency of protein identification (Souchelnytskyi, 2002b; Figeys, 2003; Patterson and Aebersold, 2003). We identified 32 proteins that changed their phosphorylation upon TGFβ1 treatment of MCF-7 cells; 26 of these proteins are novel targets of TGFβ1 (Table 1, Figure 1, and Supplementary Figure A).

The most striking feature of the identified patterns of protein phosphorylation is their high dynamics, which complicates clustering of proteins according to the time dependence of phosphorylation (Supplemental Figure B). This is not a particularity of TGFβ signaling, because a highly dynamic protein phosphorylation was also observed in mouse fibroblasts stimulated with platelet-derived growth factor (Soskic *et al.*, 1999).

The second conclusion is that an increased expression of Smad3 or Smad2 influenced TGFβ1-regulated phosphorylation of proteins. Five types of responses were identified, depending on which Smad protein had an effect on protein phosphorylation (Figure 2). Smad3 can bind DNA directly, whereas Smad2 does not. Interaction of these Smad proteins with activated TGFβ receptors can also be modulated in different ways (Derynck *et al.*, 2001; Souchelnytskyi, 2002a; Wakefield and Roberts, 2002). Smad3 and Smad2 show different responses in a number of assays (Piek *et al.*, 2001), and ablation of their respective genes in mice resulted in different phenotypes (Chang *et al.*, 2002). Thus, Smad3 and Smad2 mediate different biological activities. Our data conforms to the notion that Smad3 and Smad2 have different signaling potential. Smad2 and Smad3 can bind different proteins, serving as docking molecules, or they could affect expres-

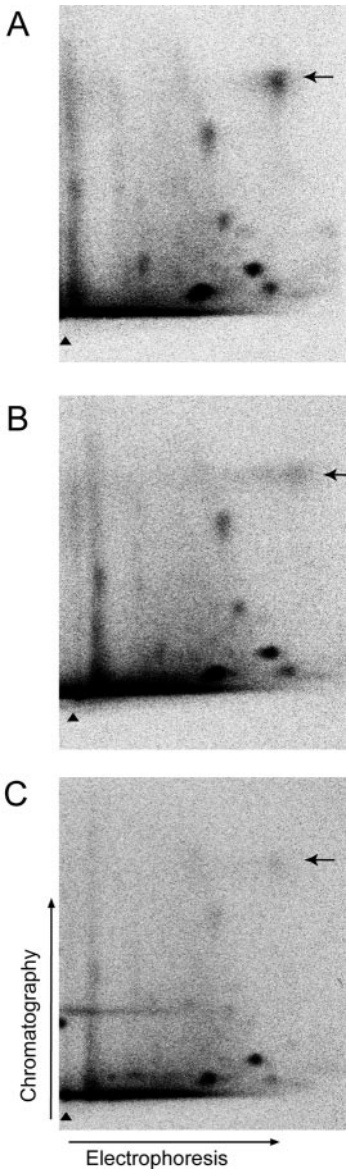

Figure 9. Inhibition of PKG decreases phosphorylation of TFII-I to moderate levels. COS7 cells were transfected with myc-Smad3, myc-TFII-I, and type I and type II TGFβ receptors plasmids. Cells were metabolically labeled with [^{32}P]orthophosphate and were pretreated with TGFβ1 (10 ng/ml; A) and the inhibitors K5823 (1 μM; B) and H7 (10 μM; C). Phosphorylated TFII-I was immunoprecipitated from cells and subjected to two-dimensional phosphopeptide mapping. Phosphopeptide maps of TFII-I from cells not treated with inhibitors (A) and from cells treated with K5823 (B) and H7 (C) are shown. Migration position of peptides phosphorylated in TGFβ1-dependent manner is shown by arrows. Strongly phosphorylated peptide #1 is shown (compare with Figure 5E). Pictures from a representative experiment out of two performed are shown.

sion of phosphorylated proteins in different ways. However, the mechanism of the described Smad dependency of protein phosphorylation remains to be explored.

TGFβ1-dependent changes of protein phosphorylation showed examples of relatively high amplitude (Figure 2 and Supplemental Figure B), compared with the amplitudes of protein expression (Bratt *et al*., 2001; Kanamoto *et al*., 2002). Thirty proteins out of 32 identified showed changed levels of phosphorylation of >50% (Figure 2 and Supplemental Figure B), whereas most of the changes in protein expression was ~30% (Bratt *et al*., 2001; Kanamoto *et al*., 2002). This confirms the high dynamics of protein phosphorylation induced by TGFβ1.

TGFβ, via phosphorylation and activation of Smad3 and Smad2, initiates signaling pathways that result in Smad-dependent regulation of gene transcription (Chang *et al*., 2002; Wakefield and Roberts, 2002; Shi and Massagué, 2003). We explored the importance of TGFβ1-dependent phosphorylation of TFII-I, which plays a particular role in linking the basal transcriptional machinery and specific transcription factors (Roy, 2001). TFII-I may be involved in Williams–Beuren syndrome and X-linked agammaglobulinaemia (Yang and Desiderio, 1997; Pérez Juardo *et al*., 1998; Novina *et al*., 1999). TFII-I binds to the initiator (Inr) element, E-box sites and sis-inducible element (SIE) (Grueneberg *et al*., 1997; Roy *et al*., 1997; Kim *et al*., 1998; Parker *et al*., 2001). TFII-I binding to the Inr element mediates activation of transcription from promoters lacking TATA box. Negative cooperation of TFII-I with c-Myc may involve binding of TFII-I to E-box elements (Roy *et al*., 1993), and activation of c-*fos* was dependent on TFII-I binding to SIE and SRE (Roy *et al*., 1997; Kim *et al*., 1998; Casteel *et al*., 2002). The observed TFII-I-dependent modulation of the effect of TGFβ1 on expression of *E2F2*, *cyclin D2*, and *cyclin D3* genes (Figure 6), is in agreement with the lack of TATA box sequences in promoters of these genes, and the presence of TFII-I- and Smad3 (CAGA)-binding elements (Brooks *et al*., 1996; Sears *et al*., 1997).

TFII-I functions are regulated by phosphorylation. TFII-I is phosphorylated predominantly on serine residues, but threonine and tyrosine phosphorylation were also identified (Yang and Desiderio, 1997; Novina *et al*., 1998). TFII-I phosphorylation did not affect binding of TFII-I to Inr element, but tyrosine and serine phosphorylation was required for transcriptional activation (Novina *et al*., 1998, 1999; Egloff *et al*., 2001; Kim and Cochran, 2001; Casteel *et al*., 2002; Cheriyath *et al*., 2002). The list of identified phosphorylation sites in TFII-I includes Tyr248, Tyr357, Ser371, Tyr462, Ser633, Ser627, and Ser743. Jak2, Src, and Btk kinase stimulate phosphorylation of Tyr248, which leads to an increased activation of c-*fos* (Grueneberg *et al*., 1997; Yang and Desiderio, 1997; Egloff *et al*., 2001; Kim and Cochran, 2001; Cheriyath *et al*., 2002). TFII-I phosphorylation at Ser633 and Ser627 by the Erk MAP kinase (Kim and Cochran, 2000), and phosphorylation at Ser371 and Ser743 by PKG (Casteel *et al*., 2002) also enhance the activation of c-*fos* transcription. Thus, TFII-I is a convergence point for various regulators of transcriptional activation, and the activity of TFII-I is regulated by phosphorylation.

We identified Ser371 and Ser743 as sites of TGFβ1-dependent phosphorylation; two-dimensional phosphopeptide mapping of wild-type and Ser371,743Ala mutant of TFII-I showed unambiguously specific phosphorylation of these two serine residues (Figures 3–5 and Supplemental Figures D and E). This phosphorylation was observed in endogenous and transfected TFII-I, and in two types of cells, COS7 and MCF-7. We also found that TFII-I in the basal state is phosphorylated on at least 11 amino acid residues. In agreement with a previous report (Novina *et al*., 1998), phosphopeptides that were subjected to phosphoamino acid analysis showed presence of phosphoserine and phosphothreonine in TFII-I in the basal state of phosphorylation (our unpublished data).

We found that a Ser371,743Ala mutant of TFII-I was a more potent coactivator of endogenous cyclin D2, cyclin D3,

and E2F2 genes, and a Smad3-responsive reporter, compared with the wild-type TFII-I (Figures 6 and 7 and Supplemental Figures F and G). Reporter assays also showed that TFII-I required Smad3 for the cooperation in transcriptional activation and that TFII-I did not act on TGFβ/Smad3-responsive reporters on its own (Figure 7).

TGFβ1-dependent phosphorylation of TFII-I may have an effect on the expression of genes containing the initiator (Inr) element, but not on genes containing the TATA-box promoter, as was shown for Mullerian inhibiting substance gene (Morikawa *et al*., 2000). This provides a mechanism for adaptation of TGFβ-induced gene expression to physiological status of cells. Notably, availability and activity of TFII-I phosphorylating kinase may define which genes and to which level they will be regulated by TGFβ. It is known that mitogen-treated cells are less responsive to growth inhibitory action of TGFβ (Derynck *et al*., 2001; Souchelnytskyi, 2002a; Wakefield and Roberts, 2002). We observed cooperative effect of TFII-I on expression of genes involved in cell cycle progression, e.g., cyclin D2, cyclin D3, and E2F2 (Figure 6). Stimulation of these genes would counteract TGFβ growth inhibitory action. Thus, enhanced activity of TFII-I kinase, e.g., PKG, may result in a strong TGFβ-dependent phosphorylation of TFII-I. This may lead to attenuation of growth inhibitory activity of TGFβ, whereas other activities may not be affected. It would be important to explore whether such modulation of TGFβ signaling contributes to the dual role of TGFβ in tumor progression, when abrogation of growth inhibitory action of TGFβ and stimulation of epithelial-mesenchymal transdifferentiation are thought to be crucial for TGFβ-promoted metastasis (Derynck *et al*., 2001; Souchelnytskyi, 2002a; Wakefield and Roberts, 2002).

Increased binding of Smad3 to the CAGA-element upon coexpression with the TFII-I Ser371,743Ala mutant (Figure 8B), compared with a coexpression of Smad3 with wild-type TFII-I, may provide an explanation for the stronger costimulation of CAGA(12)-luc reporter by the mutant TFII-I (Figure 7). TFII-I cooperated with TGFβ1 in transcriptional regulation of cyclin D2, cyclin D3, and E2F2 genes, and abrogation of the TGFβ1-dependent phosphorylation of TFII-I at serine residues 371 and 743 led to a higher transcriptional activity, compared with the wild-type phosphorylatable TFII-I (Figure 6). Thus, TGFβ1-induced phosphorylation of TFII-I may be an example of a feedback mechanism on the level of transcriptional regulation of a set of genes. This mechanism may include TGFβ-dependent stimulation of transcription via activation of Smad3 and simultaneous restrain of transcriptional activation via phosphorylation of TFII-I. Combination of such mechanisms may contribute to selective and fine-tuned activation of genes by TGFβ.

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