A novel transcriptional factor with Ser/Thr kinase activity involved in the transforming growth factor (TGF)- β signalling pathway

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Transforming growth factor- β (TGF- β) shows a variety of biological activities in various organs or cells. Recently some factors such as Smads (Sma and Mad proteins) and TGF- β activating kinase 1 ('TAK1') have been characterized as signalling molecules downstream of TGF- β . Several TGF- β response elements have been identified such as cAMP response element, Smad binding element, and recognition sites for activating protein-1 and stimulating protein-1 in various gene promoters. We also reported a TGF- β response element in the human C-type natriuretic peptide (CNP) gene promoter. In this paper, we report on a novel factor which regulates the TGF- β response promoter. This factor, named TSF1 (TGF- β stimulated factor 1), possessed DNA-binding ability and activated the TGF- β responsive CNP promoter or vascular endothelial growth

factor gene promoter which possesses a sequence element analogous to the TGF- β responsive GC-rich element of the CNP promoter. TSF1 did not directly activate a Smads-dependent promoter from plasminogen activator inhibitor 1 gene, but it showed enhancement in co-operation with Smad3 and Smad4. Interestingly, this factor had the structural features of a Ser/Thr kinase and actually exhibited protein kinase activity. TSF1 mRNA as well as its protein level were stimulated by TGF- β treatment. Thus, TSF1 is a unique factor with two biological functions, transcriptional regulation and protein phosphorylation, that may be involved in TGF- β signals.

Key words: C-type natriuretic peptide, transfection, vascular endothelial growth factor.

INTRODUCTION

The transforming growth factor- β (TGF- β) family regulates many biological events such as cell proliferation, differentiation, and cell death [1–3]. However, details of the TGF- β signalling mechanism in each case are not fully understood. Recent studies have shown that Ser/Thr kinases such as the TGF- β activating kinase 1 (TAK1) and p38 cascade are involved in this TGF- β signalling pathway [4,5]. There are many reports showing that TGF- β regulates several transcription factors such as Smads (Sma and Mad proteins) [6], CREB (cAMP-response element binding protein) [7], activating transcription factor 2 (ATF2, identical to CRE-BP1) [8], AP-1 (activating protein-1) [9], SP-1 (stimulating protein-1) [10,11] and CTF/NF-1 (CAAT-binding transcription factor/nuclear factor-1) [12]. We have also studied transcriptional regulation of the human C-type natriuretic peptide (CNP), the third member of the mammalian natriuretic peptide gene by TGF- β in some cell lines, such as rat pituitary-derived GH3, and identified a GC-rich sequence in the proximal promoter region of the human CNP gene as a novel TGF- β responsive element [13,14]. To explore the transcriptional regulatory mechanism of TGF- β further, we tried to identify the nuclear factors bound to the TGF- β responsive element in the human CNP gene promoter. Several candidates were cloned by Southwestern screening, using the GC-rich oligonucleotide probe derived from the TGF- β responsive element in the CNP gene,

and the properties of one factor (TSC22, TGF- β stimulated clone 22) have already been reported [14]. In this paper, we report on another factor with unique properties. It has the characteristics of a transcription factor (DNA-binding ability and transcriptional activation) and may be involved in the expression of some TGF- β responsive genes such as CNP. Interestingly, it also was found to have the structural features of a Ser/Thr kinase and exhibited genuine protein kinase activity. Moreover, its gene expression was regulated by TGF- β . Therefore, we named this factor TSF1 (TGF- β stimulated factor 1). Cross-talk between TGF- β signalling mediators such as Smads and other kinase pathways has been studied [15]. However, how these signals work functionally in cells remains complex. In this manuscript, we show the very unique characteristics of TSF1 as a transcriptional regulatory factor, which can be involved in the TGF- β signalling pathway.

EXPERIMENTAL

Southwestern screening and cDNA cloning

A λ -ZAPII phage cDNA expression library (5×10^5 plaque-forming units with an average insert size of 1.2 kb) constructed from GH3-derived polyadenylated [poly(A)⁺] RNA was subjected to Southwestern screening as described previously [14]. Briefly, phage plaques were incubated for 4 h at 42 °C and then

Abbreviations used: TGF- β , transforming growth factor- β ; CNP, C-type natriuretic peptide; CREB, cAMP-response element binding protein; TSF1, TGF- β stimulated factor 1; Smads, Sma and Mad proteins; SBE, Smad binding element; AP-1, activating protein-1; SP-1, stimulating protein-1; CTF, CAAT-binding transcription factor; NF-1, nuclear factor-1; RACE, rapid amplification of cDNA ends; HA, influenza virus haemagglutinin epitope; MBP, myelin basic protein; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FBS, foetal bovine serum; VEGF, vascular endothelial growth factor; ATF2, activating transcription factor 2; TAK, TGF- β activating kinase; GST, glutathione S-transferase; EST, expressed sequence tag; poly(A)⁺, polyadenylated; MAP, mitogen activated protein; PAI-1, plasminogen activator inhibitor-1.

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The nucleotide sequence data reported for the rat, mouse and human will appear in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession numbers D86220, AB022179 and AB020739 respectively.

placed in contact with Hybond-C nitrocellulose membranes (Amersham, Little Chalfont, Bucks., U.K.) previously soaked in 20 mM isopropyl- β -D-thiogalactopyranoside ('IPTG') for 3 h at 37 °C. Next, the membranes with lifted-up plaques were soaked in binding buffer [10 mM Tris/HCl (pH 8.0), 10% (v/v) glycerol, 0.05% (w/v) Nonidet P-40, 1.25 mM dithiothreitol, 50 mM NaCl and 1 mM EDTA] containing 6 M guanidine hydrochloride for 30 min at 4 °C. The concentration of guanidine hydrochloride was then gradually lowered by sequential dilution with binding buffer and finally the membranes were soaked in binding buffer without guanidine. The membranes were blocked with binding buffer containing 5% (w/v) non-fat dried milk for 60 min at room temperature, washed with the binding buffer, and then probed for 2 h at 4 °C in the same buffer containing 10 μ g/ml calf thymus DNA with a multimer of the 32 P-labelled (10⁶ c.p.m./ml) double-stranded A1 oligonucleotide that contains one of the two GC-rich cis elements of the CNP promoter and linker sequence (sense, 5'-gatCCGGGCGGCCCGGTGGG-3', and antisense, 5'-gatCCCCACCGGGCGGCCCGG-3'; linker sequence in small letters). After washing with the binding buffer at 4 °C, the membranes were subjected to autoradiography. Positive plaques were picked up and processed to the second and third screening with 32 P-labelled double-stranded A1 (5'-CCGGGCGGCCCGGTGGG-3') and mutated A1 (A1M, 5'-CCGGGCGAAACGGTGGG-3') oligonucleotide probes. Clones that were positive to the A1 probe and negative to the A1M probe were finally selected. Positive phagemid clones in Southwestern screening were isolated and treated with a helper phage R-408 (Stratagene, La Jolla, CA, U.S.A.) to recover cDNA clones as Bluescript plasmids for plasmid rescue. DNA sequencing was performed on both strands by the dideoxy chain-termination method with a Bcabeast sequencing kit (Takara, Tokyo, Japan) in an ALFred DNA sequencer (Pharmacia, Uppsala, Sweden). The sequence of one of the positive clones (F5-2), later named TSF1, showed no endogenous initiation codon and therefore seemed to be a partial sequence. To obtain the 5' portion of rat TSF1 cDNA, the rapid amplification of cDNA ends (RACE) technique was performed using a Marathon cDNA Amplification Kit (Clontech Laboratories, Palo Alto, CA, U.S.A.) with GH3-derived poly(A)⁺ RNA using a TSF1 gene-specific antisense primer GSP1 (5'-TTCAGG-TGCCCGGTAGGAGATGG-3') and nested antisense primer GSP2 (5'-TAGAGCTAGGCCTGGCGAGATC-3') with the adapter-specific primer in the kit. Amplified fragments were isolated by agarose gel electrophoresis and subcloned into Bluescript SK(−) vector (Stratagene). Several clones were sequenced to obtain the cDNA sequence. Finally, a full length cDNA of TSF1 was inserted into the Bluescript SK(−) vector as a pBSTSF1. The murine TSF1 clone was obtained by PCR from mouse liver RNA using primers designed from the full open reading frame of rat TSF1. Cloning of human TSF1 was done by sequence walking in the expressed sequence tag (EST) data base. Finally, actual cDNA of human TSF1 was cloned from a human fetal kidney cDNA library (Clontech Laboratories) by PCR using primers designed from EST sequences (sense primer 1, 5'-CTCACCTGGACGAGCTCTTCGGTAGCC-3', and antisense primer 2, 5'-AGATGCAGGACTGAGATGATTGTCC-TGACCC-3'). Sequences of murine and human TSF1 clones were verified by sequencing several clones.

Construction of plasmids

To construct glutathione S-transferase (GST)-fused TSF1, PCR was carried out using primers (sense primer N1, 5'-AAGAGG-GATCCGAGAATTCATGGCCATGCACTGTGTGTCTGT-

3', and antisense primer N6, 5'-GCTCACTGGTCGACTCA-GATTTGGGTGGTGTGCTGGCCAGG-3'). PCR products were digested by *Bam*HI and *Sal*I, and ligated to the pGEX-KG expression vector [16] also digested by *Bam*HI and *Sal*I. Histidine (His)-tagged TSF1 expression vector, pQETSF1, was obtained by the insertion of a *Bam*HI/*Sal*I digested PCR fragment into a pQE11 vector (Qiagen GmbH, Hilden, Germany). Construction of the KM mutant (Lys⁴⁹ → Met) vector, pBSKMTSF1, was performed with a QuickChange site-directed mutagenesis kit (Stratagene) using complement primers (5'-CTTCTACGCCCT-GATGCGAATCCTGTGTC-3' and 5'-GACACAGGATTCG-CATCAGGGCGTAGAAG-3') and pBSTSF1 as a template. To the obtained His-tagged KM mutant, pQEKMTSF1, a *Hind*III/*Sma*I digested fragment from pBSKMTSF1 was inserted into a pQE11 vector. A *Bam*HI/*Sal*I digested PCR fragment, amplified with sense primer N2 (5'-GCCTCAGGATCCACGAATTCAA-AGAACGAGGTGCTAAGCATGAA-3') and antisense primer N6, was inserted into a pQE11 vector to obtain the His-tagged Δ 4 mutant vector, pQE Δ 4TSF1. PCR fragments amplified from pBSTSF1 with primer N3 (5'-ACTGAGACGCCATGGGCC-ATGCAC-3') and M13-40 primer (Pharmacia) were digested with *Nco*I, blunted, further digested with *Hind*III, and then ligated into blunted *Bam*HI and *Hind*III sites of the pCG-N-BL [17] vector to obtain influenza virus haemagglutinin epitope (HA)-tagged TSF1 mammalian expression vectors, pHATSF1. A *Ban*III/*Sma*I fragment of pBSKMTSF1 was inserted into the *Ban*III/*Sma*I site of pHATSF1 to obtain pHAKMTSF1. Amplified fragments using sense and antisense primer sets and pBSTSF1 as template were digested with *Bam*HI and *Sal*I, and inserted into the *Bam*HI/*Sal*I site of the pCG-N-BL vector to generate expression vectors for HA-tagged TSF1 mutants. Primer sets used were: sense primer N1 and antisense primer N4 (5'-TAGCACCTGTCGACTACAAAGAGTAAGCCATGAGG-CGAAG-3') for pH Δ 1TSF1, sense primer N1 and antisense primer N5 (5'-ACCGCTGGGTGCGACTAGTGCTGTAGAG-CTAGGGCCTGGCG-3') for pH Δ 2TSF1, and sense primer N2 and antisense primer N5 for pH Δ 3TSF1. To generate pH Δ 4TSF1, a *Bam*HI/*Xba*I fragment from pQE Δ 4TSF1 containing Δ 4TSF1 was blunted, digested with *Hind*III, and then inserted into a pCG-N-BL vector. To construct human VEGF(−88/+54)-LUC, an amplified fragment using a primer set (sense, 5'-GCCCCCGGGGCGGCCGGGGCGGGGTC-C-3', and antisense, 5'-TGGTGCTAGCCCCAGCGCCAC-GACCTCCGAGCTAC-3') and VEGF-LUC vector as a template was digested with *Sma*I and *Nhe*I, and then ligated into the *Sma*I/*Nhe*I site of the pGL-B luciferase vector (Promega, Madison, WI, U.S.A.). VEGF(−88/+54) mut-LUC was constructed in the same way except that a primer set was used for the mutated promoter (sense, 5'-GCCCCCGGGGCGGTACCTAGGGCGG-GGTCC-3', and antisense, 5'-TGGTGCTAGCCCCAGCGC-CACGACCTCCGAGCTAC-3'). All constructs were verified by sequencing. CNP4LUC (luciferase driven by the shortest CNP promoter with full activity), CNP4mutLUC (four bases were mutated in the shortest CNP promoter of CNP4LUC) and pEF-GAL (elongation factor 1 promoter-driven β -galactosidase expression vector) were constructed as reported previously [14]. VEGF-LUC, a human vascular endothelial growth factor (VEGF) promoter (−1041 to +89)-driven luciferase vector, was from Dr. T. Yoshioka (Shionogi Research Laboratories, Osaka, Japan). p800-Luc [a plasminogen activator inhibitor-1 (PAI-1) promoter-driven luciferase] was a gift from Dr. H. Shibuya (National Institute for Basic Biology, Okazaki, Japan) [5], and Smad-expression vectors, pactEF-Smad3 and pactEF-Smad4, and pactEF empty vector were donated by Dr. K. Okazaki (Bioengineering Research Institute, Suita, Japan).

Expression of TSF1 in *Escherichia coli* and preparation of antibody

A bacteria strain, JM109, harbouring each recombinant expression vector, was grown until the D_{600} reached 0.5. Isopropyl- β -D-thiogalactopyranoside was added to 0.5 mM and the incubation was continued for 12 h at 25 °C. Bacteria were harvested and solubilized by sonication in 50 mM sodium phosphate buffer (pH 7.0) containing 300 mM NaCl. Cell lysates were centrifuged at 13000 g for 30 min, and the supernatants were collected as bacterial extracts. These extracts containing the respective GST-or His-tagged fusion proteins were purified using a GST purification module (Pharmacia) or TALON metal affinity resin (ClonTech Laboratories) according to the manufacturers' protocols. Purity was examined by SDS/PAGE (12.5% gel) stained with Coomassie Brilliant Blue, and the protein concentration was determined by a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, U.S.A.).

To prepare antibody against TSF1, a rabbit was initially immunized with purified His-tagged TSF1 (0.4 mg) emulsified in complete Freund's adjuvant. Immunization was repeated four times in the same manner every three weeks. Seven days after the last booster, the whole serum was collected and subjected to purification with an affinity column in which purified GST-TSF1 was immobilized. The specificity of the affinity-purified antibody (1.9 mg of protein/ml) was evaluated in Western blot analysis of GH3 cell extracts using preimmune-phase rabbit serum as a control, and the optimal dilution of the antibody in the typical Western analysis was 2500-fold.

Mammalian cell culture

Rat pituitary-derived GH3 cells and human cervical epithelial carcinoma-derived HeLa cells (both from A.T.C.C.) were cultured in Ham's F10 medium (Flow Laboratories, McLean, VA, U.S.A.) supplemented with 15% (v/v) horse serum and 2.5% (v/v) foetal bovine serum (FBS) and in Dulbecco's modified minimum essential medium (Gibco BRL, Gaithersburg, MD, U.S.A.) with 10% (v/v) FBS respectively. Human epidermoid carcinoma-derived A431 cells (A.T.C.C.) and murine calvaria-derived MC3T3E-1 cells (provided by Dr. H. Kodama, Ohu University, Koriyama, Japan) were cultured in α -minimal essential medium (Gibco BRL) with 10% (v/v) FBS. All cultures were at 37 °C under 5% (v/v) CO₂ atmosphere. In some experiments, recombinant human TGF- β 1 protein (PeproTech, Rock Hill, NJ, U.S.A.) was used to study the effect on gene expression.

Northern blot analysis

Cells were incubated in their respective media containing 0.5% (v/v) FBS for 24 h, and further incubated with TGF- β in low-serum medium. After incubation for 2–4 h, cells were harvested and total cellular RNA was prepared according to the method of Chomczynski and Sacchi [18]. Total cellular RNA (10 μ g per lane) was subjected to electrophoresis in a denatured agarose gel and transferred to a Hybond-N nylon membrane (Amersham). The membrane was hybridized overnight at 65 °C with a ³²P-labelled full-length TSF1 cDNA probe in the hybridization buffer solution [0.5 M sodium phosphate (pH 7.5), 10 mg/ml BSA, 1 mM EDTA, 7% (w/v) SDS], washed for 30 min at 65 °C in 0.1 $\times$ SSC (1 $\times$ SSC = 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) plus 0.1% (w/v) SDS, and subjected to autoradiography. The filters were successively reprobed with ³²P-labelled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the control. Rat multi-tissue blot was purchased from

ClonTech Laboratories and probed in a similar manner. To analyse the expression of TSF1 in human tissues, Human RNA Master Blots™ (ClonTech Laboratories) were subjected to hybridization with a ³²P-labelled full-length TSF1 cDNA probe according to the manufacturer's instructions.

Electrophoretic mobility shift assay (EMSA)

EMSA was essentially performed as described previously [14], with the same amount (0.1 μ g for each incubation mixture) of His-tagged recombinant wild-type or mutant TSF1 and a ³²P-labelled double-stranded A1 probe (10000 c.p.m.) from the human CNP promoter or a double-stranded human VEGF probe (5'-CCGGGGCGGGCCGGGGCGGGT-3'). For the competition experiments, a 100-fold excess amount of unlabelled A1, A1M (mutated A1) or VEGF oligonucleotide was usually added to the incubation mixture. Consensus oligonucleotides for well-characterized transcription factors (Promega) such as SP-1 (5'-ATTCGATCGGGGCGGGGCGAGC-3'), AP-1 (5'-CGCTTGATGAGTCAGCCGGAA-3') and AP-2 (5'-GATCGAACTGACCGCCCGCGGCCCGT-3') were also used as competitors. For absorption experiments with antibody, 1 μ g each of affinity-purified rabbit polyclonal antibody raised against recombinant His-tagged TSF1 or preimmune-phase rabbit antiserum was incubated with 1 μ g of GH3 nuclear extract, prepared according to the method described in [19], in the EMSA reaction mixture for 30 min prior to the addition of labelled probe.

Western blot analysis

Cells were lysed on ice with lysis buffer [50 mM Hepes (pH 7.5), 150 mM NaCl, 1% (w/v) Triton X-100, 10% (w/v) glycerol, 1 mM EDTA, and protease inhibitors] for 15 min and centrifuged at 27000 g for 10 min at 4 °C. Cell lysates containing 20 μ g of protein respectively were subjected to SDS/PAGE. After electrophoresis, protein was transferred to Hybond-C nitrocellulose membranes (Amersham) and probed with anti-(His-tagged TSF1) rabbit polyclonal antibody (1.9 mg/ml) at a dilution of 1:2500, or monoclonal antibody 12CA5 against the HA epitope (Boehringer Mannheim, Mannheim, Germany) at a dilution of 1:5000. The membrane was then incubated with alkaline phosphatase-conjugated anti-rabbit or anti-mouse IgG and further subjected to a ProtoBlot II detection system (Promega) for visualization.

Kinase assay

To measure *in vitro* kinase activity for recombinant protein, each protein (typically 1 pmol of protein in each reaction) was incubated with 5 μ Ci [γ -³²P]ATP in 50 mM Hepes buffer (pH 7.5) containing 5 mM MgCl₂ and 5 mM MnCl₂ using 10 pmol of myelin basic protein (MBP; Sigma, St. Louis, MO, U.S.A.), histone H1 (Boehringer Mannheim) or recombinant His-tagged human Elk transcriptional activation domain (278–428 amino acids) as a substrate. After 10 min at 30 °C, the samples were subjected to SDS/PAGE and then to autoradiography. For the *in vivo* kinase assay within cells, we used immunoprecipitation with another anti-TSF1 polyclonal antibody against a synthetic peptide, Cys-Leu-Ser-Gln-Leu-Glu-Ala-Leu-Gln-Pro-Pro-Ala-Pro-Gly-Gln-His-Thr-Thr-Gln-Ile, corresponding to the C-terminal sequence of TSF1. Cells were lysed, as described in the Western analysis procedure, and then precipitated with the antibody against the C-terminus of TSF1 or control preimmune-phase antiserum. Immunoprecipitates were then subjected to the kinase assay using MBP as a substrate as described in the *in vitro*

assay. Phosphorylated proteins were separated by SDS/PAGE and visualized by autoradiography. Anti-HA antibody instead of anti-TSF1 was used for immunoprecipitation of transfected cells.

Expression of TSF1 in eukaryotic cells and reporter assays

Cells were seeded into a 6-well dish at a density that gave 40 to 50% confluency on the next day. Typically, after 18 to 24 h, cells were transfected using FuGENE6 (Boehringer Mannheim) with 0.5 µg of reporter plasmid, 0.5 µg of TSF1 expression plasmid and 0.5 µg of pEF-GAL as a reference plasmid to normalize transfection efficiency. At 48 h after transfection, cells were lysed in the lysis buffer compatible with β-galactosidase and luciferase (Promega), and subjected to assays for the respective reporter molecules as described previously [14]. To examine the effect on the CNP promoter, the CNP4LUC or CNP4mutLUC reporter, together with either pHATSF1 (pCG-N-BL used for the control) or one of the other HA-tagged mutant expression vectors were transfected into GH3 cells. To examine the effect on the VEGF promoter, VEGF-LUC [or VEGF(−88/+57)LUC] reporter and pHATSF1 (or either of the other HA-tagged mutant expression vectors) were transfected into A431 cells. A431 cells transfected with expression vectors were also subjected to Western blot analysis using anti-HA antibody for confirming equivalent levels of mutant expression. To examine the effect on the PAI-1 promoter, p800-Luc reporter plasmid and pHATSF1 (or pHAKMTSF1) were transfected into MC3T3E-1 cells with pactEF-Smad3 plus pactEF-Smad4 (pactEF for control) in the presence or absence of 20 ng/ml TGF-β. Luciferase activity was normalized with β-galactosidase activity in each transfection. Results from at least three independent experiments were expressed as means ± S.D. of relative luciferase activity.

RESULTS

Cloning, molecular features and other characteristics of TSF1

Southwestern screening of a rat GH3 cDNA library, constructed in the λ-ZAPII vector, with a ³²P-labelled A1 probe corresponding to the GC-rich TGF-β responsive element in the human CNP gene was carried out as described previously [14]. One clone, designated as F5-2, among several positives was plasmid-rescued and then subjected to DNA sequence analysis. The sequence obtained showed that it fused to β-galactosidase in frame with its own termination codon, but did not contain its own initiation codon. Therefore, full-length cDNA was further cloned by a 5'-RACE technique with poly(A)⁺ RNA from GH3 cells. The full-length sequence of the F5-2 clone was 1.4 kb and contained an open reading frame coding for 305 amino acids. The sequence originally cloned as a β-galactosidase-fusion starts at His⁷⁴ (Figure 1A). *In vitro* translation analysis of full-length F5-2 revealed translation products of approx. 34 kDa, consistent with the molecular mass of 34407 Da calculated from its amino acid sequence (results not shown). A BLAST search in the Genbank® database showed its similarity with several kinase domains. The highest score was obtained with the hypothetical 30.4 kDa Ser/Thr kinase of the SPAC3H1.13 gene in *Schizosaccharomyces pombe*. PROSITE profile search revealed that it contains a protein kinase domain profile in amino acids 20–293, also suggesting its protein kinase feature. PROSITE pattern search suggested a Ser/Thr protein kinase active-site signature in amino acids 144–156. Although this factor was originally cloned according to its DNA-binding ability, no motifs characteristic of a transcription factor, for example, in an acidic domain or proline-rich domain, was revealed by computer motif search using the PROSITE or BLOCKS programs. However, there is a

Figure 1 Comparison of rat, mouse and human TSF1 amino acid sequences and distribution of its mRNA in rat tissues

(A) Amino acids in the mouse and human sequences are indicated only when they differ from the other sequences. The sequence corresponding to the Ser/Thr protein kinase active-site signature proposed by the PROSITE pattern search is double underlined. The putative leucine zipper-like motif preceded by basic amino acid residues (bZip) sequence is underlined with the broken line and the helix-turn-helix sequence is indicated by a straight line above the sequence. Theoretical phosphorylation sites by casein kinase II, cAMP- and cGMP-dependent kinase, and protein kinase C are shown by *, **, and #, respectively. L/I-x-x-LL sequences are shown by a ● below the sequence. EST clones used to identify the human sequence are shown by two-way arrows below the sequence. (B) A rat multi-tissue blot was probed by a ³²P-labelled rat TSF1 full-length cDNA probe or GAPDH (G3PDH) probe for the control, as described in the Experimental section.

Table 1 Wide distribution of TSF1 mRNA in human tissues

Distribution of TSF1 mRNA in human tissues was examined using human RNA master blots. The filter was incubated with a ^{32}P -labelled human TSF1 cDNA probe, and after washing at high stringency, the filter was analysed by a phosphorimager to calculate the relative intensity.

	Relative intensity of TSF1 mRNA (arbitrary units)
Heart	81
Aorta	60
Colon	70
Testis	101
Ovary	109
Pancreas	95
Pituitary gland	109
Adrenal gland	81
Kidney	131
Liver	78
Spleen	55
Lung	39
Fetal brain	20
Human DNA	1
Yeast total RNA	3

leucine zipper-like motif preceded by basic amino acid residues (putative bZip) in amino acids 86–128, and an overlapping helix-turn-helix structure in amino acids 106–130; both are found in many transcription factors [20]. Yeast SPAC3H1.13 Ser/Thr kinase does not have such a leucine zipper-like motif or helix-turn-helix structure, suggesting that the F5-2 clone belongs to a family distinct from the SPAC3H1.13 kinase. PROSITE pattern search also suggested other motifs: a cAMP- and cGMP-dependent protein kinase phosphorylation site (Thr¹⁰⁵), two casein kinase II phosphorylation sites (Ser³² and Ser²⁸⁸) and two protein kinase C phosphorylation sites (Ser¹⁹⁷ and Ser²⁵⁸). In addition, the sequences of the L/I-x-x-L-L motif such as the 'NR box' were found in the C-terminus. This motif has been found in transcriptional co-activators and seemed to mediate the binding of nuclear receptors [21,22]. Therefore, it remains possible that F5-2 could interact with other nuclear factors. As described later, both mRNA and protein levels of this factor were stimulated by TGF- β , thus we named this factor TGF- β stimulated factor 1.

The full-length mouse TSF1 clone was obtained by PCR using a primer set based on the rat TSF1 sequence using mouse kidney RNA as a template. The human TSF1 sequence was obtained by computer search in the human EST database, and the cDNA was cloned from a human fetal kidney cDNA library. Comparison of the three amino acid sequences revealed that TSF1 is highly conserved among the rat, mouse and human forms (97.7%, 95.1% and 93.8% identical in amino acids between rat and mouse, rat and human, and mouse and human respectively), and especially the amino acids involved in the motifs mentioned above, which are almost completely identical, suggesting the essential functions of TSF1 in these animals.

The tissue distribution pattern in rat showed the ubiquitous expression of TSF1 including the brain (Figure 1B). Results from human multi-tissue blots revealed higher expression of TSF1 also in the human pituitary gland (Table 1), consistent with its existence in rat pituitary-derived GH3 cells.

Presuming that TSF1 has an effect on the TGF- β responsive element in the CNP promoter, the expression of TSF1 itself might be regulated by TGF- β . This led us to examine the

Figure 2 TSF1 expression is enhanced by TGF- β treatment

(A) GH3 cells were incubated in low-serum medium (0.5% FBS) for 24 h and then treated with 100 ng/ml TGF- β 1 in the same medium. After incubation for 0, 2 or 4 h, total cellular RNA was isolated and subjected to Northern blot analysis using a ^{32}P -labelled TSF1 cDNA probe or GAPDH probe for the control. (B) GH3 cells (top panel) or HeLa cells (bottom panel) were treated with TGF- β 1 as described above, and then cell lysates were subjected to Western blot analysis with a polyclonal antibody against recombinant His-tagged TSF1.

possibility of TGF- β -dependent expression of TSF1. When GH3 cells were treated with TGF- β , the expression of TSF1 mRNA was rapidly augmented (approx. 3-fold in 2 h) (Figure 2A). Furthermore, Western blot analysis showed an increase in the TSF1 protein level with TGF- β treatment (Figure 2B, top panel). The increase in the TSF1 protein level by TGF- β was also observed in HeLa cells (Figure 2B, bottom panel). Therefore, the expression of TSF1 itself could be regulated by TGF- β .

Protein kinase activity of TSF1

As TSF1 had the structural features of a Ser/Thr kinase, we tested whether or not it possessed actual kinase activity. First, GST-fused TSF1 was prepared and subjected to an *in vitro* kinase assay with several proteins generally used as kinase substrates (Figure 3A). TSF1 exhibited an autophosphorylation pattern. It also phosphorylated MBP and histone H1, both of which are often used as substrates for Ser/Thr kinases, as well as the recombinant His-tagged Elk1 activation domain. The kinase activity was also detected in the protein immunoprecipitated from GH3 cell lysates with anti-TSF1 antibody (Figure 3B).

Figure 3 *In vitro* and *in vivo* kinase assays of TSF1

(A) Recombinant TSF1 expressed and purified from bacteria was examined for its protein kinase activity *in vitro*. TSF1 exhibited autophosphorylation and phosphorylation of MBP, histone H1 and recombinant Elk1 activation domain. **(B)** GH3 cell extracts were subjected to immunoprecipitation with polyclonal antibody against the C-terminal peptide of TSF1 or control preimmune-phase antiserum. The immune complexes were subjected to kinase assay using MBP as a substrate.

However, the activation of the kinase activity of TSF1 with TGF- β treatment was not observed in GH3 cells (results not shown). The KM mutant of TSF1, in which Lys⁴⁹ corresponding to a putative ATP-binding site was substituted by Met, and the Δ 4 mutant, from which amino acids 1–85 were deleted, lacked kinase activity as expected in both *in vitro* (results not shown) and *in vivo* assays (Figure 4). These results indicate that TSF1 can function as a genuine protein kinase.

DNA-binding properties of TSF1

We cloned TSF1 as a DNA-binding factor. The original clone we obtained by Southwestern screening corresponded to amino acids 74–305 that were fused to the C-terminus of β -galactosidase. Therefore, we examined whether the full-length TSF1 could bind to DNA using recombinant His-tagged TSF1 and the KM mutant. As shown in Figure 5(A), His-tagged wild-type TSF1 exhibited a single shifted band with a ³²P-labelled double-stranded A1 probe (5'-CCGGGCGGCCCGGTGGG-3'), that represents the TGF- β responsive element in the CNP gene [14], in the EMSA. The shifted band was lost in the competition with an excess of unlabelled A1 probe but not with an excess of mutated A1 (A1M, 5'-CCGGGCGAAACGGTGGG-3') probe. Competition with consensus oligonucleotide probes for binding

Figure 4 Western blot analysis of HA-tagged TSF1 mutant proteins in A431 cells and their kinase activity

The cell lysates at 48 h after transfection with various HA-tagged TSF1 expression vectors, as shown in the top panel, were subjected to Western blot analysis using anti-HA monoclonal antibody (middle panel) or kinase assay after immunoprecipitation with anti-HA antibody as in Figure 3 (bottom panel).

sites of well-characterized transcription factors was also examined. Probes for SP-1 (a GC-rich sequence) and AP-1 sites did not compete effectively. An AP-2 probe (another GC-rich sequence) shows some competition, but much weaker than the A1 probe. These results were consistent with our previous observations [13]. This indicates that full-length TSF1 has DNA-binding ability in a sequence-specific manner. The kinase-defective KM mutant also exhibited a specific shifted band as did wild-type TSF1 (Figure 5B), suggesting that its DNA-binding ability is independent of kinase activity. This was supported by the observation that the His-tagged Δ 4 mutant, which lacks kinase activity, also showed a shifted band. Interestingly, the KM mutant seemed to bind more strongly to the probe than did the wild-type. The Δ 4 mutant lacks the N-terminal 84 amino acids and therefore has a similar structure to that obtained from Southwestern screening. Thus, the N-terminal third of TSF1 is not required for its DNA-binding ability. We also analysed the character of TSF1 in GH3 cells using anti-TSF1 antibody. Although we expected the super-shifted band with this antibody, this antibody specifically absorbed the shifted band (Figure 5C). This result showed that TSF1 is involved in the shifted band caused by the GH3 cell nuclear extract. The expression of the VEGF gene has been reported to be regulated by TGF- β [23]. Gille et al. [24] identified a GC-rich element (5'-CCGGGCGGCCCGGGGGCGGGGT-3') in the proximal promoter region of the VEGF gene that is involved in both constitutive and TGF- α -induced VEGF gene expression. The GC-rich element also participates in gene regulation by mitogen activated protein (MAP) kinase [25]. Compared with the GC-rich element in the CNP promoter, the element found in the

Figure 5 DNA-binding properties of TSF1

(A) His-tagged TSF1 exhibits a shifted band in an EMSA with a ³²P-labelled A1 probe derived from the GC-rich sequence in the CNP promoter. The shifted band competes with a 100-fold excess of unlabelled A1 probe but not with the mutated A1M probe nor with probes for SP-1 (SP1) and AP-1 (AP1). (B) Kinase-deficient TSF1 mutants, KM and $\Delta 4$, also bind to the A1 probe. (C) Nuclear extracts from GH3 cells were analysed in the presence of anti-TSF1 polyclonal antibody (α -TSF1) or preimmune-phase antisera (PIS). (D) Alignment of GC-rich sequences derived from the CNP proximal promoter and VEGF proximal promoter. Probe sequences are indicated by a solid line (CNP A1 probe) and a broken line (VEGF probe) with arrowheads. Mutation sites used in the VEGF (–88/+54)mutLUC reporter plasmid are also shown. (E) Both His-tagged wild-type and the KM mutant of TSF1 exhibit a shifted band in EMSA with a ³²P-labelled GC-rich VEGF probe. The shifted band competes with a 100-fold excess of unlabelled probe.

VEGF promoter shows very high similarity; 12 out of the 14 bases are identical when –85 to –72 of the VEGF promoter is aligned with –53 to –40 of the CNP promoter (Figure 5D). Therefore, we wondered whether TSF1 could bind to the GC-rich element of the VEGF promoter. Both His-tagged wild-type and KM mutant of TSF1 exhibited a single shifted band with the ³²P-labelled double-stranded VEGF probe (Figure 5E), indicating that TSF1 could also bind to the GC-rich element of the VEGF promoter. Again, the KM mutant seemed to bind more strongly than the wild-type.

Activation of CNP and VEGF promoter function by TSF1

As indicated above, TSF1 could bind to the GC-rich elements in both the CNP and VEGF proximal promoter regions. Next, we tested whether or not TSF1 could regulate the transcription function of these promoters. The CNP promoter function was tested in GH3 cells as described previously [13,14]. Co-transfection of the HA-tagged TSF1 expression vector pHATSF1 and CNP4LUC reporter plasmid, which contains the shortest and fully active CNP promoter (positions –56 to +73), together with pEF-GAL (a reference β -galactosidase reporter plasmid) exhibited about 6-fold augmentation of relative luciferase activity, while TGF- β treatment without pHATSF1 usually showed 2–3-fold augmentation (Table 2). pHATSF1 did not affect luciferase activity of CNP4mutLUC which has four base mutations in the CNP promoter sequence in CNP4LUC. Next,

Table 2 Effect of TSF1 on CNP promoter function

GH3 cells were transfected with pHATSF1 (or pCG-N-BL empty vector for control and TGF- β treatment) together with CNP4LUC or CNP4mutLUC reporter plasmid and pEF-GAL reference vector as described in the Experimental section. After 24 h, the medium was exchanged with low-serum medium (0.5% FBS) in the presence or absence of recombinant TGF- β 1. At 48 h after transfection, the cells were lysed and subjected to assays for luciferase and β -galactosidase. Luciferase activity was normalized to the β -galactosidase activity and expressed as a value relative to the control (pCG-N-BL without TGF- β 1). Results from three independent experiments are expressed as means $\pm$ S.D. Significant differences (*, $P < 0.05$ or **, $P < 0.01$) from the control by Student's t test are shown.

Reporter plasmid (0.5 μ g of each)	Co-transfected (0.5 μ g of each)	TGF- β	Relative luciferase activity
CNP4LUC	pCG-N-BL	–	1.0 $\pm$ 0.1
	pCG-N-BL	+	2.0 $\pm$ 0.2*
	pHATSF1	–	6.0 $\pm$ 0.8**
CNP4mutLUC	pCG-N-BL	–	0.23 $\pm$ 0.01
	pHATSF1	–	0.19 $\pm$ 0.05

VEGF promoter function was studied in A431 cells as described [24]. TSF1 activated the TGF- β responsive VEGF promoter (positions –1041 to +89) in a dose-dependent manner (Table 3). TSF1 also activated the VEGF proximal promoter (–88 to +54) function, but not the mutated VEGF promoter, indicating

Table 3 Effect of TSF1 on VEGF promoter function

A431 cells were co-transfected with the VEGF promoter-driven reporter construct [VEGF-LUC or VEGF(−88/+54)LUC], TSF1 expression vector plasmid (pHATSF1, pHAKMTSF1, or pCG-N-BL for control) and pEF-GAL reference vector. At 48 h after transfection, the cells were lysed and subjected to reporter assays. Luciferase activity was normalized to the β -galactosidase activity and expressed as a value relative to the control (pCG-N-BL, 0.9 μ g). Results from three independent experiments are expressed as means $\pm$ S.D. Significant differences (*, $P < 0.05$ or **, $P < 0.01$) from the control of each set (with pCG-N-BL only) by Student's *t* test are shown.

Reporter plasmid (0.5 μ g of each)	Co-transfected		Relative luciferase activity
VEGF-LUC	pHATSF1	pCG-N-BL	
	0 μ g	0.9 μ g	1.0 $\pm$ 0.1
	0.6 μ g	0.3 μ g	1.6 $\pm$ 0.2*
	0.9 μ g	0 μ g	2.9 $\pm$ 0.6**
VEGF(−88/+54)LUC	pCG-N-BL (0.5 μ g)		1.0 $\pm$ 0.02
	pHATSF1 (0.5 μ g)		2.0 $\pm$ 0.2**
	pHAKMTSF1 (0.5 μ g)		2.0 $\pm$ 0.2**
	pCG-N-BL (0.5 μ g)		0.65 $\pm$ 0.08
VEGF(−88/+54)mutLUC	pHATSF1 (0.5 μ g)		0.71 $\pm$ 0.10
	pHAKMTSF1 (0.5 μ g)		0.81 $\pm$ 0.10

Table 4 Effects of TSF1 mutants on VEGF promoter function

A431 cells were co-transfected with the VEGF-LUC reporter, expression vector plasmids for wild-type and several mutants of TSF1 (pCG-N-BL for control) and the pEF-GAL reference vector. At 48 h after transfection, the cells were lysed and subjected to reporter assays. Luciferase activity was normalized to the β -galactosidase activity and expressed as a value relative to the control. Results from three independent experiments are expressed as means $\pm$ S.D. Significant differences (*, $P < 0.05$ or **, $P < 0.01$) from the control by Student's *t* test are shown.

Reporter plasmid (0.5 μ g)	TSF1 vector (0.5 μ g of each)	Relative luciferase activity
VEGF-LUC	Control	1.0 $\pm$ 0.2
	WT (1–305)	2.4 $\pm$ 0.2**
	KM (K ⁴⁹ M)	3.0 $\pm$ 0.5*
	Δ 1 (1–85)	0.56 $\pm$ 0.15**
	Δ 2 (1–188)	3.4 $\pm$ 0.35**
	Δ 3 (86–188)	1.0 $\pm$ 0.1**
	Δ 4 (86–305)	0.56 $\pm$ 0.1**

that the transactivation depends on the GC-rich sequence (Table 3). To examine functional domains in TSF1, several mutants were tested for their transactivation activities. The KM mutant activated the VEGF promoter (−88 to +54) function as well as the wild-type TSF1 in a sequence-dependent manner (Table 3). Four deletion mutants of TSF1, Δ 1 (amino acids 1–85), Δ 2 (amino acids 1–188), Δ 3 (amino acids 86–188), and Δ 4 (amino acids 86–305), were also examined as shown in Table 4. Among the four deletion mutants, only the Δ 2 mutant which lacks the C-terminal third of TSF1 showed transactivation of the VEGF promoter. The Δ 4 mutant which retained DNA-binding ability did not show any activation of promoter function. Similar transactivation patterns for these mutants were observed with the CNP promoter construct in GH3 cells (results not shown).

The expression of HA-tagged TSF1 and mutants was confirmed by Western blot analysis of cell lysates with anti-HA antibody and only wild-type TSF1 exhibited kinase activity (Figure 4). The results of co-transfection experiments indicate that TSF1 is directly capable of transactivation of both the CNP and VEGF promoters through GC-rich sequence elements. These

Table 5 TSF1 enhances Smads function on the PAI-1 promoter

MC3T3E-1 cells were transfected with pactEF-Smad3 (0.5 μ g) plus pactEF-Smad4 (0.5 μ g) or pactEF empty vector (1 μ g), together with pHATSF1 (0.5 μ g) or pCG-N-BL (0.5 μ g), in addition to the p800-Luc reporter plasmid (0.5 μ g) and pEF-GAL (0.5 μ g). After 24 h, the medium was changed to that with low serum (0.5% FBS) in the presence or absence of 20 ng/ml TGF- β 1. After another 24 h, the cells were lysed and subjected to reporter assays. Luciferase activity was normalized to the β -galactosidase activity and expressed as a value relative to the control (with pactEF empty vector and no TGF- β , line 1). Results from three independent experiments are expressed as means $\pm$ S.D. Significant differences from the control by Student's *t* test: *, $P < 0.01$ versus line 1; #, $P < 0.01$ versus line 5 (Smads only).

Reporter plasmid	Co-transfected			Relative luciferase activity
	pactEF-Smad3/4	pHATSF1	TGF- β	
p800-Luc	1	—	—	1.0 $\pm$ 0.1
	2	—	—	4.7 $\pm$ 0.7*
	3	—	+	0.56 $\pm$ 0.08
	4	—	+	3.6 $\pm$ 0.5*
	5	+	—	5.1 $\pm$ 0.7
	6	+	+	17.3 $\pm$ 0.3#
	7	+	—	13.6 $\pm$ 2.7#
	8	+	+	44.4 $\pm$ 3.8#

two TGF- β responsive promoters contain no typical SBE sequence, and were not activated by Smad3 and Smad4 (results not shown). Therefore, they seem to be regulated by factors independent of Smads. In other words, TSF1 can be one candidate for factors participating in the Smad-independent response to TGF- β . The N-terminal third and central portions of TSF1, which contain residues essential for Ser/Thr kinase activity, are required for its transcriptional regulatory function, and therefore for DNA-binding ability. On the other hand, the C-terminal third of TSF1 does not seem to be required for its transcriptional regulatory function. The kinase activity is independent of its transcriptional regulatory function, because the KM mutant transactivated the VEGF and CNP promoters as efficiently as the wild-type.

Effect of TSF1 on the Smad-dependent promoter function

Smads are known as mediators of TGF- β signalling. As TSF1 is capable of transactivation of TGF- β responsive promoters, the question arose of whether or not TSF1 affects the Smad-dependent promoter function. In many cases, the functions of Smads in the TGF- β signalling pathway have been tested using the PAI-1 promoter and MC3T3E-1 cell line due to sensitivity reasons [5]. We also used this system to examine the effect of TSF1. A 800 bp region of the PAI-1 promoter was responsive to TGF- β in MC3T3E-1 cells as shown in the transfection experiments (Table 5). TSF1 alone was negative for the PAI-1 promoter function. This was reasonable because there is one SP-1 element in the 800 bp region of the PAI-1 promoter but no other GC-rich sequences at all, as observed in the TGF- β responsive promoter region of the CNP or VEGF gene. Among the Smads family, Smad2 and Smad3 are known as R-Smad (receptor regulated Smad) specific to TGF- β signalling. Introducing Smad3 together with common Smad, Smad4, into the cells has been shown to lead to TGF- β signals [26]. When the TSF1 vector was co-transfected with Smad3 and Smad4 vectors, the transactivation signal by Smads was enhanced and the response to TGF- β still retained. These results indicated that TSF1 could not directly activate PAI-1 promoter activity, but could up-regulate the function of Smad3 and Smad4. The expression of A-Smads

(anti-Smads), Smad6 and Smad7, is known to be induced by TGF- β itself as negative feedback [27,28], and the expression of TSF1 itself was also stimulated by TGF- β (Figure 2). Therefore, it is possible that TSF1 is regulated by TGF- β to expand its signals.

DISCUSSION

Several TGF- β responsive transcription factors such as AP-1, SP-1, CTF/NF-1, ATF-2 and Smads were reported. Recently, the TGF- β signalling pathway through Smads has been intensively investigated [6,15]. Heterodimers or homodimers of members of the Smad family successively interact in response to TGF- β , and then the final Smad complex is translocated to the nucleus and binds to the SBE sequence (consensus, 5'-GTCTAGAC-3') of a target gene such as the PAI-1 promoter. However, as the fully active proximal promoter regions of CNP and VEGF do not have such SBE sequences, there should be a mediator other than the Smad family for the response to TGF- β . We have already identified the GC-rich element essential for both constitutive and TGF- β responsive transcription of the human CNP gene [14]. In this report, we have presented a novel transcription factor TSF1 that could up-regulate the transcription of the CNP promoter through the GC-rich element. TSF1 could also transactivate the VEGF proximal promoter, in which the GC-rich sequence highly analogous to that in the CNP promoter is found. On the other hand, there is no such GC-rich element in the TGF- β responsive PAI-1 promoter. This agrees with the finding that TSF1 could not directly activate the PAI-1 promoter transcription in MC3T3E-1 cells. To our surprise, co-expression of TSF1 with Smad3 and Smad4 greatly enhanced both constitutive and TGF- β responsive PAI-1 promoter activity. There are many reports showing that Smads interact with other factors such as CBP, c-Fos, TGIF (5'TG3' interacting factor), and a novel anchor protein called SARA (Smad anchor for receptor activation) [9,29–31]. Our results suggest that TSF1 and Smads independently function in the TGF- β signalling, but TSF1 affects Smads function. Smad-dependent signals have been shown to go through direct DNA binding of the Smad complex as well as through interaction with other transcription factors [15,32]. Similarly, TSF1 activates promoters, such as the CNP gene promoter, through direct DNA binding. The existence of the 'NR box' in the C-terminus suggests the possibility of the interaction of TSF1 with other nuclear factors such as Smads. However, more evidence is required to show the direct interaction of TSF1 with Smads.

Results from the BLAST homology search and PROSITE motif search of the TSF1 sequence revealed the possibility that TSF1 was a Ser/Thr kinase, and TSF1 exhibited protein kinase activity in both *in vitro* and *in vivo* kinase assays. The kinase activity of TSF1 is independent of its DNA-binding ability and transactivation activity as well. Knop and Martin [33] showed that kinase negative interleukin-1 receptor-associated kinase 1 ('IRAK1') could play a role as a signalling molecule under interleukin-1 stimuli. Our studies showed TSF1 worked functionally without its kinase ability in DNA binding and transactivation. A proto-oncogene product c-Abl is known to be a protein tyrosine kinase with DNA-binding activity [34]. It enhances some promoter functions by interacting with transcription factors or phosphorylating them [35], but no direct transactivation effect has been shown. To our knowledge, there is not yet a report on a Ser/Thr kinase with direct transactivation ability. Some kinases such as casein kinase II have been reported to interact with transcription factors [36], but how those kinases

regulate transcription machinery is still unclear. The endogenous substrate for TSF1 kinase has not yet been identified, and therefore the biological function of its kinase activity is still unknown. There are several putative phosphorylation sites in the TSF1 sequence itself. Thus, the function of TSF1 may be regulated by some kinases or may cross-talk with other kinase signalling cascades. The KM mutant, which showed no autophosphorylation pattern, seems to be augmented in DNA-binding ability. The wild-type TSF1 expressed in bacteria can be autophosphorylated, at least partially. The immunoprecipitated TSF1 protein from mammalian cells exhibited low autophosphorylation activity, suggesting that TSF1 in cells was already phosphorylated (Figure 3B). Thus, the DNA-binding ability of TSF1 may be negatively regulated by its kinase activity through its phosphorylated status. Similarly, CTF-1 has been shown to be regulated by TGF- β through calcineurin and calcium/calmodulin dependent kinase IV [37]. TGF- β has also been reported to induce protein phosphatase 1 activity in keratinocytes [38]. The functions of many transcription factors such as c-Jun, Elk1 and CREB have been reported to be regulated by phosphorylation [39–41]. In our preliminary study, TSF1 strongly enhanced the Elk1 signal, but not the c-Jun and CREB signals (results not shown), suggesting the possible involvement of TSF1 in the phosphorylation of some nuclear transcription factors. It could activate the MAP kinase pathway leading to Elk1 activation as much as in the TGF- β signalling pathway. As the MAP kinase pathway has been reported to be involved in one of the TGF- β signalling pathways [42,43], TSF1 may be one of the candidates that link the MAP kinase pathway to TGF- β signalling.

Recently, a novel kinase called PKL12 or Krcr (kinase related to cerevisiae and thaliana) has been identified [44,45]. TSF1 should be the same as this kinase. As both PKL12 and Krcr were identified according to their similarity or motif of the protein kinase family, the characterization was performed only as a protein kinase, and it was suggested to have limited biological function. On the other hand, we obtained the TSF1 clone by quite a different approach. We originally identified TSF1 as a novel DNA-binding factor and characterized its ability for transcriptional regulation as well as kinase activity. We also showed that TSF1 could be involved in Smad-independent TGF- β signalling.

In conclusion, TSF1 is a novel factor with unique molecular characteristics in Smad-independent TGF- β signal transduction pathways, because it directly transactivates the TGF- β responsive CNP and VEGF promoters, and its expression is regulated by TGF- β . TSF1 points to an approach to exploring the response to TGF- β which is not completely understood for Smads and TAK1. In addition, we are generating the TSF1-gene-deficient mouse to analyse the physiological functions of TSF1. We hope that our findings will promote the investigation of this novel protein kinase family.

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