Endocrine Journal 1999, **46** (2), 253–261

Regulation of c-fos Gene Induction and Mitogenic Effect of Transforming Growth Factor-β1 in Rat Articular Chondrocyte

MAKOTO OSAKI, TOMOO TSUKAZAKI, AKIHIKO YONEKURA, YOUICHI MIYAZAKI, KATSURO IWASAKI†, HIROYUKI SHINDO AND SHUNICHI YAMASHITA*

Department of Orthopaedic Surgery, Nagasaki University School of Medicine, Nagasaki 852–8501, Japan
**Department of Nature Medicine, Atomic Bomb Disease Institute, Nagasaki University School of Medicine, Nagasaki 852–8523, Japan*

Abstract. We have previously reported that type I transforming growth factor beta (TGF-β1) is a potent stimulator of cell growth in articular chondrocytes. In this study, we examined the mechanism of TGF-β1 induced cellular proliferation by using cultured rat articular chondrocytes (CRAC). A time-course study of [^{3}H]thymidine incorporation upon TGF-β1 (1 ng/mL) or 10% fetal bovine serum stimulation revealed that TGF-β1 directly stimulates DNA synthesis in CRAC. Pretreatment with H7, an inhibitor for protein kinase C (PKC), completely blocks TGF-β1-induced proliferation. Since TGF-β1 has been shown to transduce signals through MAP kinase cascades, we investigated the induction of several protooncogenes by Northern blotting. TGF-β1 addition causes an immediate and transient induction of c-fos but not myc or jun mRNA. Furthermore, this c-fos expression is not inhibited by cycloheximide, but is completely abolished by pretreatment with TPA, so that the c-fos gene is a direct target of TGF-β1 signalling and PKC is involved in this c-fos induction. To refine our understanding of TGF-β1 regulation of the c-fos promoter region, we performed chloramphenicol acetyltransferase (CAT) assays. A serial deletion analysis of the c-fos promoter region reveals a TGF-β1 responsive element in a region between −403 and −329 bp upstream of the transcription initiation site. We attempted gel shift assays on this response element with CRAC nuclear extracts. Although this region contains a sis-inducible binding element, we fail to detect specific DNA-protein complexes. Our results, however, suggest that TGF-β1 acts as a primary mitogen in CRAC and this mitogenic activity requires PKC activation. Finally, the subsequent induction of c-fos expression occurs through an as yet unidentified transactivation mechanism.

Key words: Articular chondrocyte, TGF-β1, c-fos gene

(*Endocrine Journal* **46**: 253–261, 1999)

TRANSFORMING growth factor beta (TGF-β) has multifunctional effects on cell growth, differentiation, morphogenesis and production of extracellular matrix [1]. It is widely recognized that TGFβ inhibits the proliferation of epithelial and endothelial cells. Since disruption of growth inhibition by TGFβ can lead to cancer development, the majority of previous studies have focused on identification of target molecules involved in signalling or cell cycle control. In contrast to epithelial and endothelial cells, TGF-β1 has been shown to act as a stimulator of cell growth in certain other cell types [2–8]. We previously reported that TGF-β1 potentiates DNA synthesis and differentiation of cultured rat articular chondrocytes (CRAC) [5, 6], but the mechanism of TGF-β1 growth stimulation remains a poorly understood yet important aspect of TGF-β biology.

Current evidence indicates that TGF-β1 receptor

Received: September 17, 1998
Accepted: January 5, 1999
Correspondence to: Shunichi YAMASHITA, M.D. Professor and Chairman, Department of Nature Medicine, Atomic Bomb Disease Institute, Nagasaki University School of Medicine, 1–12–1 Sakamoto, Nagasaki 852–8523, Japan
† Prof. Iwasaki died on 13 June, 1996

superfamily members transduce signals via members of the the growing family of Smad proteins [9, 10]. For example, Smad2 is phosphorylated by the type I receptor, TβRI. Phosphorylated Smad2 then forms a heterometric complex with Smad4, together they translocate into the nucleus, which results in activation of target gene transcription. Recall, however, that prior to the identification of Smads, a body of evidence implicated the MAP kinase cascade in TGF-β1 signalling [7, 11–17]. While the activation of the MAPK cascade is unlikely to be a common event in all cell types, TGF-β1 can activate three different MAPK subfamilies: the classical Ras-Raf-MEK-MAPK, MEKKs mediated JNKs and p38 MAPK. Recently another MAPKKK family, TAK1, was also shown to play a role in TGF-β1 signalling [18]. These previous experiments implicating various MAPK cascades have identified which MAPK was activated by TGF-β1, but no report has analyzed the regulation of direct target gene activation.

In this study, we first excluded the possibility that the mitogenic effect of TGF-β1 on CRAC was due to newly synthesized growth factors other than TGF-β1. We also confirmed the involvement of PKC in this stimulatory effect of TGF-β1. Further, we investigated which protooncogene was activated by TGF-β1 and found immediate and transient induction of c-fos mRNA. Because there is no report investigating regulation of the transactivation of c-fos gene by TGF-β1, we focused on the identification of a TGF-β1 responsive element in the c-fos promotor.

Materials and Methods

Materials

Sprague-Dawley rats were purchased from Charles River Laboratories (Shizuoka, Japan). Fetal bovine serum (FBS) and Dulbecco's Modified Essential Medium (DMEM) were purchased from Gibco Oriental Co. (Tokyo, Japan). Cell culture plates were from Becton Dickinson Co. (Rutherford, NJ), Falcon (Lincoln Park, NY), Corning (Corning, NY), and Costar (Cambridge, MA). Human recombinant TGF-β1 was purchased from King Jozo (Tokyo). Alpha-[^{32}P]deoxy-CTP (3,000 Ci/nmol), [methyl-^{3}H] thymidine (5 Ci/nmol), D-threo[dichloroacetyl-1-^{14}C] chloramphenicol (50 nCi/nmol), Hybond-N nylon membrane, random prime labeling kit and Thermo Sequenase core sequencing kit were all purchased from Amersham (Tokyo). X-ray films were obtained from Eastman Kodak (Rochester, NY). The RNA extraction kit Isogen, scintillant Scintisol EX-H and WST-1 cell counting kit which is a modified method of an MMT assay were purchased from Wako (Tokyo). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO) and Takara Shuzou Co. (Kyoto, Japan). The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation at our institution.

Culture of articular chondrocytes

Articular chondrocytes from 5-week-old male rats were prepared as described previously [5]. Briefly, sliced articular cartilage was digested with trypsin/EDTA for 1 h and 0.2% collagenase for 4 h. The chondrocytes were cultured in a DMEM supplemented with 10% FBS, 100 IU/mL penicillin and 100 ng/mL streptomycin, in 5% CO_2 environment and fed at three days of culture. Five days later, after reaching confluence, the cells were passaged with trypsin/EDTA and seeded in culture dishes. After reaching subconfluence again (approximately 3 days of culture), the cell culture medium was replaced with serum-free medium for 24 h and the cultured cells were subjected to several treatments, unless otherwise stated.

[^{3}H] Thymidine incorporation assay and cell proliferation assay

DNA synthesis in chondrocytes was evaluated by [^{3}H]thymidine incorporation as described previously [5]. Briefly, monolayer chondrocytes seeded in a 24-well plate at a density of 1×10^{3} cells were cultured for 48 h with culture medium and then serum-starved for 48 h. The cells were then treated with TGF-β1, IGF-I or FBS in the presence of 1 μCi/mL [^{3}H] thymidine. At the end of labeling, cells were rinsed three times with ice-cold PBS, fixed with 10% trichloroacetic acid (TCA), rinsed twice with 5% TCA, and lysed with 0.7 mL/well 3% SDS. The lysate was added to 6 mL scintillant and incorporation of [^{3}H]thymidine was measured in a liquid scintillation counter (Hewlett Packard). Cell proliferation

was studied by the WST-1 method, which is a modified MTT assay. Isolated chondrocytes spreaded in 96-well plate were serum-starved for 24 h and then incubated with TPA or TGF-β1 in the presence or absence of H7 for 48 h. The cells were lysed with attached lysis buffer and absorbance at a wave length of 405 nm was measured.

RNA preparation and Northern blot analysis

Total RNA was extracted from cultured rat articular chondrocytes with an Isogen RNA extraction kit. A total of 25 μg RNA was denatured by incubation with 50% formamide at 65°C, and electrophoresed in 1.0% agarose gel containing formaldehyde. After electrophoresis, RNA was transferred to the Hybond-N nylon membrane. Northern blot analyses and cDNA labelings of human c-fos cDNA (2 kb fragment), v-myc cDNA (1.5 kb fragment), c-jun cDNA (1.1 kb fragment) and cyclophilin were performed as described previously [5]. The membranes were washed in 1 × SSC (0.15 M NaCl, 15 mM sodium citrate, pH 7.0) containing 0.1% SDS at room temperature (2 × for 10 min) and in 0.1 × SSC −0.1% SDS at 60°C (2 × for 20 min). Autoradiographic results were obtained after exposing the film to intensifying screens at −70°C for 24 h.

c-fos/CAT expression vectors

A fragment including the upstream flanking region (UFR) of the c-fos gene from position −727 to position +10 was obtained from pc-fos (human)-1 (ATCC 41042) by PCR and was subcloned into pCAT-Basic-Vector with KpnI and BglII sites (Promega, Madison, WI). A series of deletion mutants of human c-fos-CAT were constructed by digesting with unique restriction enzymes followed by fill-in with T4 DNA polymerase and ligation with T4 DNA ligase. The sequences of these constructs were confirmed by direct sequencing (Hitachi SQ5500).

DNA transfection and CAT assay

By the lipofectin method, 3 μg of CAT plasmid and 1 μg of β-gal plasmid were transiently transfected into cultured rat articular chondrocytes. After 12 h of transfection, the cells were incubated with or without 5 ng/mL of TGF-β1 for 36 h. The cells were then harvested with PBS, suspended in 150 μL tris-HCl (pH 7.5), and disrupted by five freeze-thaw cycles prior to sonication. CAT assays were performed as described previously (19). Transfection efficiencies were normalized by co-transfected β-gal activities.

Electrophoretic mobility shift assay

After incubation for 24 h in serum-free medium, monolayer subconfluent chondrocytes were either stimulated with 5 ng/mL TGF-β1 or treated with equal volumes of the vehicle (5 mM HCl). After 30 min, the cells were harvested and nuclear extracts were prepared by the method of Digman *et al.* [20]. A DNA fragment was isolated from c-fos UFR by digestion with EcoNI (position −324) and SacII (position −403) and labeled with T4 polynucleotide kinase in the presence of [^{32}P]-γATP. Gel mobility shift assays were performed as described previously [19]. Briefly, binding mixtures containing 25 μl (10 μg) of nuclear extract and 10 fmol of a ^{32}P-labeled DNA fragment were incubated at 4°C for 60 min. These mixtures were then subjected to electrophoretic separation at 4°C on 6% nondenaturing polyacrylamide gels at 200 V for 1–3 h under TBE (0.05 M Tris base, 0.05 M borbic acid, and 1 mM EDTA-Na) buffer. After electrophoresis, the gels were dried and subjected to autoradiography.

Results

DNA synthesis and proliferation of rat articular chondrocytes

To distinguish whether the mitogenic activity of TGF-β1 is direct or not, CRAC were serum-starved for 48 h and stimulated with TGF-β1 (1 ng/mL) or 10% FBS, and incorporated [^{3}H]thymidine was monitored every three hours until 36 h after stimulation. IGF-I (25 ng/mL), another growth factor, was also used in parallel control experiments. If TGF-β1-induced DNA synthesis is mediated through other newly synthesized factors, TGF-β1-induced incorporation of [^{3}H]thymidine should be delayed significantly compared to incorporation induced by FBS. The addition of FBS resulted in a rapid increase in the [^{3}H]thymidine uptake from 18 h and reached maximum levels after 27 h (Fig. 1). Simi-

larly, stimulation by TGF-β1 also potentiated DNA synthesis dramatically from 21 h after stimulation, and uptake continued until 36 h. In contrast, IGF-I showed only a modest increase. These results suggest that TGF-β1 stimulates DNA synthesis directly, without the requirement of secondary factors, although the effect of TGF-β1 treatment was delayed three hours compared with that of FBS. To confirm that the enhancement of DNA synthesis reflects actual cell proliferation, we evaluated the numbers of cells by a modified MTT assay. As expected, TGF-β1 increased the number of cells to 2.3 times the control (Fig. 2). Since TGF-β1 transduces signals via the PKC pathway in some cells, we compared the effect of TPA with that of TGF-β1 on CRAC cell growth. We also used equivalent amounts of H7, an inhibitor of PKC, to distinguish whether the stimulatory effect of TGF-β1 is mediated by PKC. Incubation with TPA alone increased the number of cells to approximately twice the control, and preincubation with H7 completely inhibited TPA-induced cell growth. Similarly, H7 also completely abolished TGF-β1-stimulated cell growth. These results indicate that TGF-β1 directly stimulates cell growth of chondrocytes, probably through PKC activation.

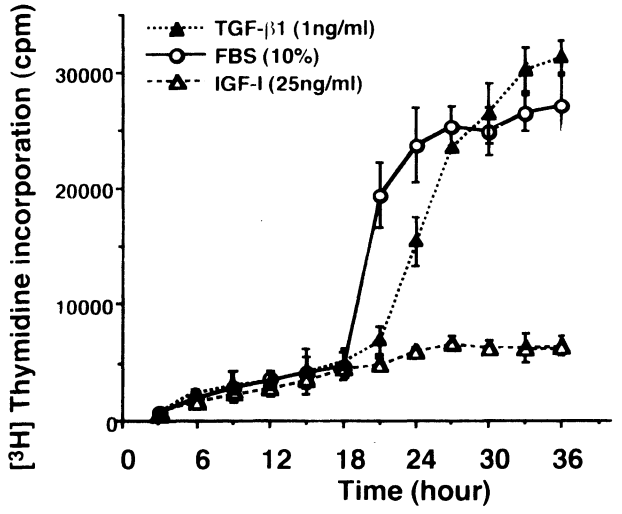

Fig. 1. Time course study of DNA synthesis induced by TGF-β1, FBS and IGF-I in URAC. Subconfluent URAC in 24-well plates was serum-starved for 48 h and incubated with [^{3}H]thymidine together with TGF-β1 (1 ng/mL), FBS (10%) or IGF-I (25 ng/mL). Incorporated [^{3}H]thymidine was monitored every three hours until 36 h after stimulation. The data are the means±SD of triplicates from a representative experiment.

c-fos gene expression

From the above experiments, we speculated that the MAPK cascade acting downstream of PKC might be involved in TGF-β1-induced cell growth of CRAC. To investigate the involvement of the MAPK cascade, we studied the expression of several protooncogenes; c-fos, myc and jun, all of which could be target genes of the MAPK cascade. For this purpose, serum-deprived cells were treated with TGF-β1 and the mRNA expression of these target genes was assayed by Northern blotting. Interestingly, expression of c-fos mRNA was induced starting at 15 min post-stimulation and reached maximum levels after 30 min (Fig. 3). Subsequently, the expression decreased gradually and returned to basal levels after 4 h of stimulation. Expression of c-jun was also enhanced by TGF-β1 stimulation, but peak expression was observed after 2 h. In contrast, c-myc expression decreased upon TGF-β1 addition with minimum levels at 1 h after ligand stimulation,

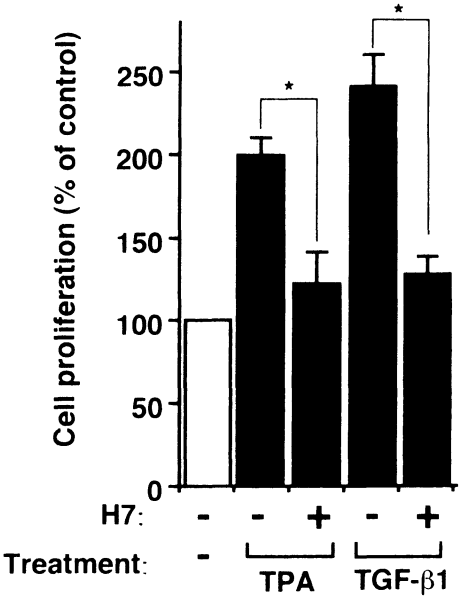

Fig. 2. Abolition of TPA- or TGF-β1- induced cell growth by H7. URAC serum starved for 24 h was cultured for 48 h in the presence or absence of TPA (100 nM) or TGF-β1 (5 ng/mL) and the number of cells was calculated by the WST-1 method after 48 h of stimulation. In parallel, Some CRAC were incubated with H7 (50 μM) together with the above reagents. Results are shown as means±SD of triplicates from a representative experiment. *Significant at $P<0.001$.

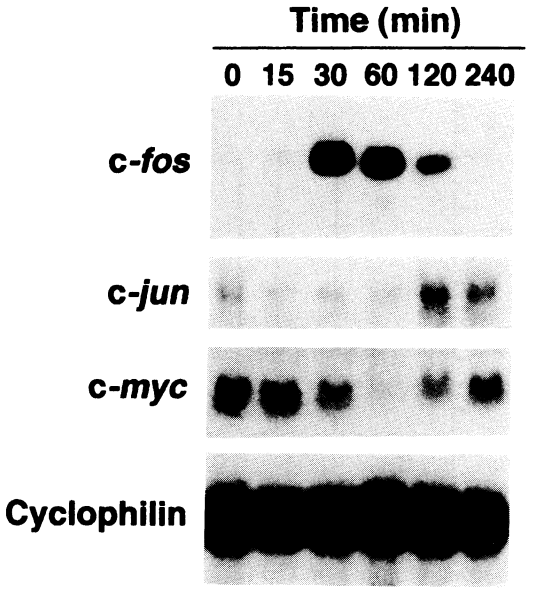

Fig. 3. Time-dependent expression of c-fos, myc and jun mRNA in response to TGF-β1. URAC was serum starved for 24 h and incubated with 5 ng/mL TGF-β1. Total RNA was then extracted at the time indicated and Northern blotting was performed first with c-fos probe followed by rehybridization with jun, myc and cyclophilin.

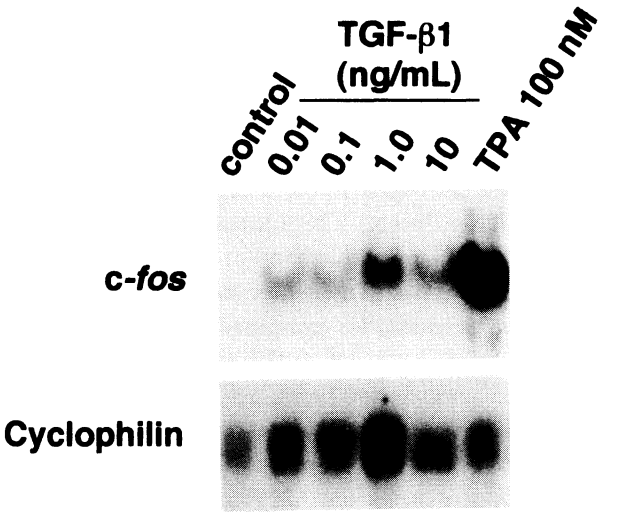

Fig. 4. c-fos mRNA expression induced by different concentrations of TGF-β1. URAC was serum freed for 24h and stimulated with the indicated concentrations of TGF-β1 for 30 min. Expressions of c-fos and cyclophilin were analyzed by Northern blotting.

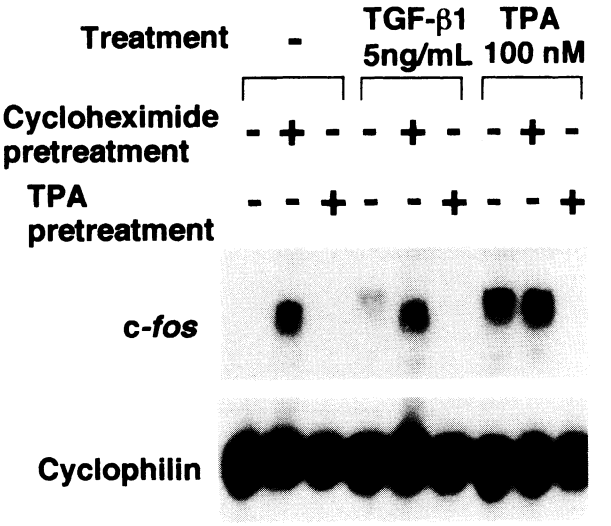

Fig. 5. Effect of pretreatment with TPA or cycloheximide on c-fos gene expression. Serum-starved URAC was pretreated with TPA (100 nM) or cycloheximide (1 ng/mL) for 1 h and then incubated with TGF-β1 (5 ng/mL) or TPA (100 nM) for 30 min. Expressions of c-fos and cyclophilin were visualized by Northern blotting.

and returned to pre-stimulation levels at 4 h. These results indicate that unlike to c-myc or c-jun, c-fos is a direct target gene transactivated by TGF-β1. We then varied the conditions of TGF-β1 stimulation and studied the effects on c-fos mRNA expression. The expression of c-fos was induced from 0.01 ng/mL TGFβ and remained constant even with a high TGF-β1 concentration (Fig. 4). To clarify the role of PKC in c-fos mRNA induction, CRAC were preteated with TPA for 30 min and then stimulated by TGF-β1 for 30 min. Pretreatment with TPA completely abolished TGF-β1-induced expression of c-fos mRNA (Fig. 5). We also checked for the requirement of new protein synthesis for c-fos induction by employing cycloheximide (1 ng/mL), an inhibitor of protein synthesis. Preteatment with cycloheximide augmented TGF-β1-induced c-fos mRNA expression.

CAT activity by TGF-β1

To investigate the mechanism of c-fos induction by TGF-β1, we attempted to identify the TGF-β1 responsive element in the 5′ UFR of the c-fos gene by using standard CAT transactivation assays. A fragment containing the 5′ UFR of c-fos from position −727 to +10 was subcloned into pCAT-Basic-Vector. CRAC were transiently transfected with this reporter construct together with a βGAL plasmid and

cultured with or without TGF-β1. We first examined CAT activity due to incubation with various concentrations of TGF-β1. Consistent with the result of c-fos mRNA induction, CAT activity was stimulated by TGF-β1 from concentrations as low as 0.01 ng/mL and showed a stable doubling of the control even at higher doses. This indicates the presence of a TGF-β1 responsive element in this region of the promotor (Fig. 6). To further localize the TGF-β1-responsive element, we examined six different deletion constructs between −727 and −70. Deletion from the 5′ end to −403 resulted in a decrease of the basal level transactivation, but these mutants maintained TGF-β1 responsiveness (Fig. 7). Deletion constructs further to −329 of this promotor, however, lost responsiveness to TGF-β1. To confirm the necessity of the region between −403 and −329 for TGF-β1-induced activation, we further studied several internal deletion mutants and found a TGF-β1 responsive sis-element present between −403 and −329.

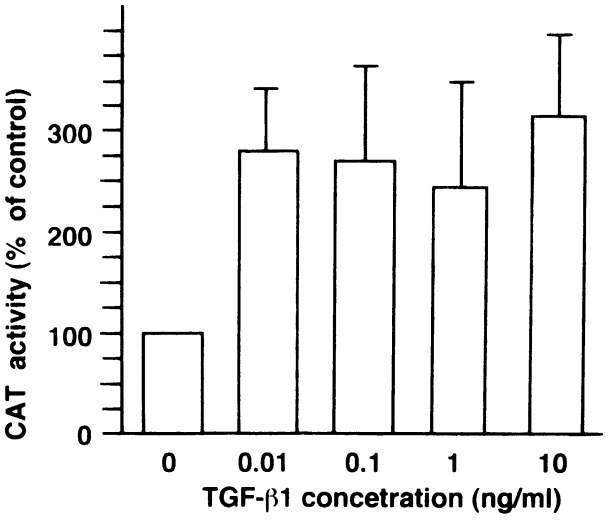

Fig. 6. c-fos (−727 to +10)/CAT activity with different concentrations of TGF-β1 in CRAC. c-fos(−727)/CAT, containing UFR of c-fos gene extending from position −727 to +10, was transiently transfected by the liposome method. After 12 h of transfection, the cells were treated with the indicated concentrations of TGF-β1 for a further 36 h, and then CAT activity was measured as described in Materials and Methods. CAT activity was normalized to β-galactosidase and was expressed as a percentage of the control without TGF-β1-treatment and represents the means±SD of three experiments.

Gel mobility shift assay

Based on the results of the c-fos/CAT assay, we postulated the formation of some ligand-dependent complex formation involving transactivators and the −403 to −329 TGF-β1 response element. We therefore performed gel shift assays by using this

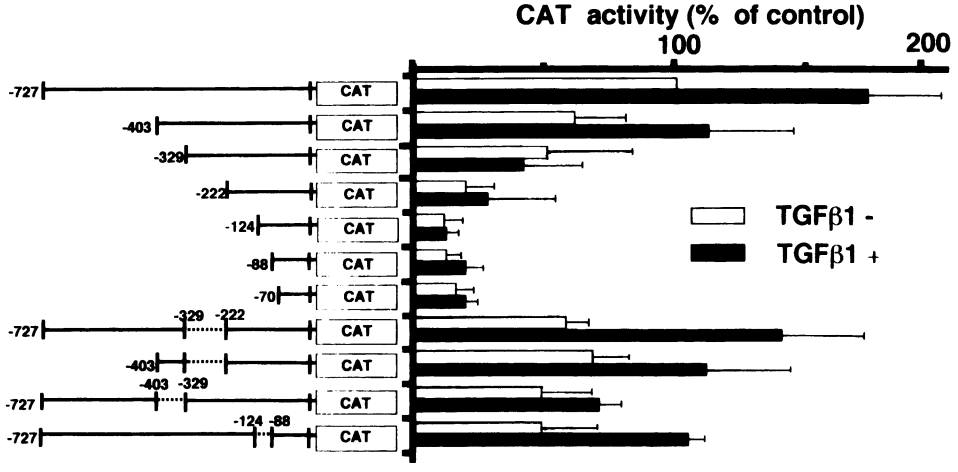

Fig. 7. Mapping of TGFβ responsive element on c-fos promotor by CAT assay. To map TGFβ-responsive element on the c-fos gene, six mutants that deleted from 5′ until −70 and internal deletion mutants between −403 and −88 were made as shown in the left panel. These constructs were transiently transfected with βGAL plasmid and incubated with or without 5 ng/mL of TGF-β1. CAT activity was normalized to β-galactosidase and was expressed as a percentage of control (−727 to −10) without TGF-β1-treatment and represents the means±SD of five experiments.

promotor region, but we could not detect any protein-DNA complexes despite many trials with a variety of conditions (data not shown).

Discussion

In the present study we examined the molecular mechanism of the mitogenic effect of TGF-β1 by using cultured rat articular chondrocytes. We demonstrated that the mitogenic effect of TGF-β1 is direct and we excluded the possibility that secondarily produced growth factors are required to stimulate cell growth. It has been demonstrated, in an embryo-derived cell line, that TGF-β1 induces mRNA and protein expression of PDGF. This PDGF induction causes cell growth by autocrine and/or paracrine mechanisms [8], so that the mitogenicity of TGF-β1 in this cell line is delayed relative to stimulation with serum or other growth factors. Our results indicate that there is a minor lag in time-dependent uptake of [^{3}H]thymidine between TGF and FBS. This suggests that there is no involvement of secondary growth factors.

The PKC activation by TGF-β1 has been demonstrated in a variety of cells including chondrocytes [7]. By using H7, an inhibitor of PKC activity, we demonstrated that the mitogenic effect of TGF-β1 is mediated, at least in part, through the activation of PKC. H7 is known to preferentially inhibit the PKC activity at low concentrations but when used at higher concentrations this reagent inhibits PKA activity in addition to PKC [21]. We therefore used H7 only at lower concentrations, consistent with amounts used in other experiments aimed at blocking only PKC activity. That cell growth is completely inhibited by H7 does not provide direct evidence of the involvement of PKC in TGF-β1 signalling. However, together with our c-fos induction data, these results strongly support the theory of the direct involvement of PKC in TGF-β1 mitogenicity in rat articular chondrocytes.

Previous experiments have demonstrated that TGF-β1 induces the expression of several protooncogenes [2–4, 22]. In some cell types, expression of c-fos, myc or Jun family members is induced immediately in response to TGF-β1. We demonstrated that among these protooncogenes, only c-fos is induced immediately after TGF-β1 treatment. TGF-β1 also induces c-jun, but this activation was delayed, suggesting an indirect induction perhaps by secondary growth factors. In our experiments with cycloheximide, we also demonstrated that c-fos induction does not require new protein synthesis. Furthermore, induction of the c-fos gene appears to be mediated through PKC activation. Taken together, these results indicate that induction of c-fos mRNA expression is due to direct signalling from TGF-β1 receptors and requires PKC activity. Similarly, immediate and transient induction of c-fos expression by TGF-β1 has been demonstrated in rat osteoblasts and EL2 cells [3, 4]. Specifically, in experiments with anti c-fos antisense oligonucleotides, c-fos expression appeared to be essential for osteoblast cell growth [4], but the precise mechanism of induction has not yet been elucidated. We therefore focused on promotor analysis of the c-fos gene to clarify the mechanism of TGF-β1 mitogenicity in chondrocytes. The expression of c-fos is regulated by several regulatory elements in the 5′ flanking region, including SRE, SIE and AP-1 binding sites, cAMP, estrogen and vitamin D response elements [23–28]. Also, MAPK, PKC, signal transducer and activators of transcription (STATs), phospholipase C and nuclear receptor super-families are known to transactivate through these elements. From a serial analysis of deletion mutants of c-fos/CAT assay, we demonstrated that the −403 to −329 region of the c-fos promotor contains a TGF-β1 responsive element, but we could not detect any DNA-protein complexes in gel mobility shift assays even though we used a 70 bp responsive fragment as a probe. Our gel mobility shift assays were likely performed correctly as separate experiments with other regions for probes were able to show complex formation under identical conditions.

The −403 to −329 region contains SIE elements which are direct targets of STAT family members. Despite the involvement of PKC in TGF-β1 signalling in CRAC, this region does not contain either AP-1 binding sites or SREs. This finding is difficult to interrupt, but we think that transcriptional activation of the c-fos gene by TGF-β1 may be regulated by other unknown pathways besides STAT or the classical PKC-MAPK pathway. Indeed, recent evidence indicates that TGF-β1 receptor-regulated Smad3 and the common Smad, Smad4 bind DNA directly [29]. Since DNA binding motifs of Smad3 and Smad4

seem to be not conserved [29], it is possible that some Smads activated by TGF-β1 signalling are involved in c-fos gene activation. These Smads may bind the promotor region which we identified, but these DNA-protein complexes may be transient and unstable. Further study is required to clarify this signalling mechanism.

Our results demonstrate that TGF-β1 stimulates DNA synthesis and cell growth in cultured articular chondrocytes, and rapidly augments c-fos mRNA levels. Furthermore, the 5′ promotor of c-fos gene might contain a novel TGF-β1-specific stimulatory response element, which is mediated by PKC activation but independent of the involvement of SRE. A direct link between TGF-β1 action on the one hand and c-fos gene induction and growth of articular chondrocytes on the other, should broaden our understanding of the physiology and pathophysiology of articular chondrocytes.

References

1. Hoodless PA, Wrana JL (1998) Mechanism and function of signaling by the TGF beta superfamily. *Curr Top Microbiol Immunol* 228: 235–272.
2. Liboi E, Pelosi E, Di FP, Gallinari P, Petrini M, Sposi NM, Testa U, Rossi GB, Peschle C (1987) The EL2 rat fibroblasts line: differential effects of growth factors (EGF, PDGF, FGF, TPA and TGF beta) on cell proliferation and c-fos expression. *Ann N Y Acad Sci* 511: 318–328.
3. Liboi E, Di FP, Gallinari P, Testa U, Rossi GB, Peschle C (1988) TGF beta induces a sustained c-fos expression associated with stimulation or inhibition of cell growth in EL2 or NIH 3T3 fibroblasts. *Biochem Biophys Res Commun* 151: 298–305.
4. Machwate M, Jullienne A, Moukhtar M, Lomri A, Marie PJ (1995) c-fos protooncogene is involved in the mitogenic effect of transforming growth factor-beta in osteoblastic cells. *Mol Endocrinol* 9: 187–198.
5. Tsukazaki T, Usa T, Matsumoto T, Enomoto H, Ohtsuru A, Namba H, Iwasaki K, Yamashita S (1994) Effect of transforming growth factor-beta on the insulin-like growth factor-I autocrine/paracrine axis in cultured rat articular chondrocytes. *Exp Cell Res* 215: 9–16.
6. Tsukazaki T, Ohtsuru A, Namba H, Oda J, Motomura K, Osaki M, Kiriyama T, Iwasaki K, Yamashita S (1996) Parathyroid hormone-related protein (PTHrP) actiion in rat articular chondrocyte: comparison of PTH(1-34), PTHrP(1-34), PTHrP(1-141), PTHrP(100-114) and antisense oligonucleotides against PTHrP. *J Endcrinol* 150: 359–368.
7. Sylvia VL, Mackey S, Schwartz Z, Schuman L, Gomez R, Boyan BD (1994) Regulation of protein kinase C by transforming growth factor beta 1 in rat costochondral chondrocyte cultures. *J Bone Min Res* 9: 1477–1487.
8. Leof EB, Proper JA, Goustin AS, Shipley GD, DiCorleto PE, Moses HL (1986) Induction of c-sis mRNA and activity similar to platelet-derived growth factor by transforming growth factor beta: a proposed model for indirect mitogenesis involving autocrine activity. *Proc Natl Acad Sci USA* 83: 2453–2457.
9. Wrana JL, Attisano L (1996) MAD-related proteins in TGF-beta signalling. *Trends Genet* 12: 493–496.
10. Attisano L, Wrana JL (1998) Mads and Smads in TGF beta signalling. *Curr Opin Cell Biol* 10: 188–194.
11. Wang W, Zhou G, Hu M, Yao Z, Tan TH (1997) Activation of the hematopoietic progenitor kinase-1 (HPK1)-dependent, stress-activated c-Jun N-terminal kinase (JNK) pathway by transforming growth factor beta (TGF-beta)-activated kinase (TAK1), a kinase mediator of TGF beta signal transduction. *J Biol Chem* 272: 22771–22775.
12. Reimann T, Hempel U, Krautwald S, Axmann A, Scheibe R, Seidel D, Wenzel KW (1997) Transforming growth factor-beta1 induces activation of Ras, Raf-1, MEK and MAPK in rat hepatic stellate cells. *Febs Lett* 403: 57–60.
13. Huwiler A, Pfeilschifter J (1994) Transforming growth factor beta 2 stimulates acute and chronic activation of the mitogen-activated protein kinase cascade in rat renal mesangial cells. *Febs Lett* 354: 255–258.
14. Hartsough MT, Mulder KM (1995) Transforming growth factor beta activation of p44mapk in proliferating cultures of epithelial cells. *J Biol Chem* 270: 7117–7124.
15. Frey RS, Mulder KM (1997) Involvement of extracellular signal-regulated kinase 2 and stress- activated protein kinase/Jun N-terminal kinase activation by transforming growth factor beta in the negative growth control of breast cancer cells. *Cancer Res* 57: 628–633.
16. Axmann A, Seidel D, Reimann T, Hempel U, Wenzel KW (1998) Transforming growth factor-beta1-induced activation of the Raf-MEK-MAPK signaling pathway in rat lung fibroblasts via a PKC-dependent

mechanism. *Biochem Biophys Res Commun* 249: 456–460.

17. Atfi A, Djelloul S, Chastre E, Davis R, Gespach C (1997) Evidence for a role of Rho-like GTPases and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) in transforming growth factor beta-mediated signaling. *J Biol Chem* 272: 1429–1432.
18. Yamaguchi K, Shirakabe K, Shibuya H, Irie K, Oishi I, Ueno N, Taniguchi T, Nishida E, Matsumoto K (1995) Identification of a member of the MAPKKK family as a potential mediator of TGF-beta signal transduction. *Science* 270: 2008–2011.
19. Usa T, Tsukazaki T, Namba H, Ohtsuru A, Kimura H, Villadolid MC, Nagataki S, Yamashita S (1994) Correlation between suppression of c-myc and antiproliferative effect of transforming growth factor-beta 1 in thyroid carcinoma cell growth. *Endocrinology* 135: 1378–1384.
20. Gilman MZ (1988) The c-fos serum response element responds to protein kinase C-dependent and -independent signals but not to cyclic AMP. *Genes Develop* 2: 394–402.
21. Ido M, Asao T, Sakurai M, Inagaki M, Saito M, Hidaka H (1986) An inhibitor of protein kinase C, 1-(5-isoquinolinylsulfonyl)-2- methylpiperazine(H-7) inhibits TPA-induced reduction of vincristine uptake from P388 murine leukemic cell. *Leuk Res* 10: 1063–1069.
22. Li L, Hu JS, Olson EN (1990) Different members of the jun proto-oncogene family exhibit distinct patterns of expression in response to type beta transforming growth factor. *J Biol Chem* 265: 1556–1562.
23. Whitmarsh AJ, Shore P, Sharrocks AD, Davis RJ (1995) Integration of MAP kinase signal transduction pathways at the serum response element. *Science* 269: 403–407.
24. Weisz A, Rosales R (1990) Identification of an estrogen response element upstream of the human c-fos gene that binds the estrogen receptor and the AP-1 transcription factor. *Nucleic Acids Research* 18: 5097–5106.
25. Wagner BJ, Hayes TE, Hoban CJ, Cochran BH (1990) The SIF binding element confers sis/PDGF inducibility onto the c-fos promoter. *EMBO J* 9: 4477–4484.
26. Sadoshima J, Izumo S (1993) Mechanical stretch rapidly activates multiple signal transduction pathways in cardiac myocytes: potential involvement of an autocrine/paracrine mechanism. *Embo J* 12: 1681–1692.
27. Fisch TM, Prywes R, Roeder RG (1987) c-fos sequence necessary for basal expression and induction by epidermal growth factor, 12-O-tetradecanoyl phorbol-13-acetate and the calcium ionophore. *Mol Cell Biol* 7: 3490–3502.
28. Candeliere GA, Jurutka PW, Haussler MR, St AR (1996) A composite element binding the vitamin D receptor, retinoid X receptor alpha, and a member of the CTF/NF-1 family of transcription factors mediates the vitamin D responsiveness of the c-fos promoter. *Mole Cell Biol* 16: 584–592.
29. Labbe E, Silvestri C, Hoodless PA, Wrana JL, Attisano L (1998) Smad2 and Smad3 positively and negatively regulate TGF beta-dependent transcription through the forkhead DNA-binding protein FAST2. *Mol Cell* 2: 109–120.