Regulation of the Transforming Growth Factor β-responsive Transcription Factor CTF-1 by Calcineurin and Calcium/Calmodulin-dependent Protein Kinase IV*

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Transforming growth factor β (TGF-β) is a pluripotent peptide hormone that regulates various cellular activities, including growth, differentiation, and extracellular matrix protein gene expression. We previously showed that TGF-β induces the transcriptional activation domain (TAD) of CTF-1, the prototypic member of the CTF/NF-I family of transcription factors. This induction correlates with the proposed role of CTF/NF-I binding sites in collagen gene induction by TGF-β. However, the mechanisms of TGF-β signal transduction remain poorly understood. Here, we analyzed the role of free calcium signaling in the induction of CTF-1 transcriptional activity by TGF-β. We found that TGF-β stimulates calcium influx and mediates an increase of the cytoplasmic calcium concentration in NIH3T3 cells. TGF-β induction of CTF-1 is inhibited in cells pretreated with thapsigargin, which depletes the endoplasmic reticulum calcium stores, thus further arguing for the potential relevance of calcium mobilization in TGF-β action. Consistent with this possibility, expression of a constitutively active form of the calcium/calmodulin-dependent phosphatase calcineurin or of the calcium/calmodulin-dependent kinase IV (ΔCaMKIV) specifically abrogates calcineurin- and CaMKIV-dependent phosphatase calcineurin or of the calcium/calmodulin-dependent form of the calcium/calmodulin-dependent kinase IV (ΔCaMKIV) specifically induces the CTF-1 TAD and the endogenous mouse CTF/NF-I proteins. Both calcineurin- and ΔCaMKIV-mediated induction require the previously identified TGF-β-responsive domain of CTF-1. The immunosuppressants cyclosporin A and FK506 abolish calcineurin-mediated induction of CTF-1 activity. However, TGF-β still induces the CTF-1 TAD in cells treated with these compounds or in cells overexpressing both calcineurin and ΔCaMKIV, suggesting that other calcium-sensitive enzymes might mediate TGF-β action. These results identify CTF/NF-I as a novel calcium signaling pathway-responsive transcription factor and further suggest multiple molecular mechanisms for the induction of CTF/NF-I transcriptional activity by growth factors.

Members of the transforming growth factor β (TGF-β) family are small peptide hormones that regulate growth, proliferation, differentiation, apoptosis, wound healing, and gene expression of responsive cells in a variety of tissues and organisms (reviewed in Ref. 1). The extreme diversity of TGF-β actions is further complicated by the fact that TGF-β often elicits opposite responses, depending on the cell type and the assay conditions. The importance of TGF-β in cell physiology and pathophysiology is emphasized by the identification of several fibrotic disorders that may arise from increased TGF-β sensitivity such as arthritis (2), as well as by the increased tumorigenicity of several epithelial cells that have lost TGF-β responsiveness (3).

TGF-β binds specific receptors at the cell surface, which are often composed of two dimers of distinct transmembrane serine/threonine kinase receptor chains referred to as type I (TβRI) and type II (TβRII) TGF-β receptors. Following ligand binding, TβRII is thought to phosphorylate and activate the TβRI kinase, which in turn propagates the signal to downstream substrates. Genetic and biochemical analyses in nematode, fruit fly, and primate cells led to the identification of several putative TGF-β signal transducers, including various proteins interacting with the TGF-β receptor complex and a family of related factors collectively referred to as the Smad proteins (1, 4). Some of the Smads were shown to influence known aspects of TGF-β signaling, which led to the proposal that they may act as bona fide TGF-β effectors. For example, Smad3 and Smad4 are necessary and sufficient for specific TGF-β-mediated growth and transcriptional responses in Mv1Lu cells (5), whereas Smad2 participates in the transcriptional induction of the Mix.2 gene, a well known target of the activin-like members of the TGF-β superfamily (6). Thus, individual Smads appear to play a pivotal role in relaying the signals of the various TGF-β ligands. Nevertheless, their specific functions remain unknown.

Recently, a possible role for calcium in TGF-β action has been proposed (7, 8). For instance, TβRI was shown to interact with FKBP12 (9), the prototype of the FKBP class of immunophilins (see Ref. 10 for review). FKBP12 is of interest, as it has the potential to inhibit the activity of the calcium-dependent protein phosphatase calcineurin (11). In addition, FKBP12 and calcineurin may regulate the function of the inositol 1,4,5-triphosphate and ryanodine receptors (IP₃R and RyR), the major endoplasmic reticulum (ER) receptor channels controlling calcium stores (12, 13). However, whether calcineurin and

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FIG. 1. TGF-β regulates calcium dynamics in NIH3T3 cells. A, TGF-β stimulates calcium influx. 7 × 10^4 serum-starved NIH3T3 cells growing in 24-well plates were washed twice with PSS buffer containing 0.12 mM CaCl_2 and then stimulated with ethanol carrier or 5 ng/ml of human TGF-β for the indicated times at 37 °C. The cells were subsequently labeled with 1 μCi of ^45^CaCl_2 at 37 °C for 5 min in the continued presence or absence of TGF-β, as indicated. The cells were washed four times with cold PSS buffer containing 1.2 mM CaCl_2, lysed in 1% SDS, and the ^45^CaCl_2 content of the lysate was measured by liquid scintillation counting. For each time point, bars represent the mean ± standard deviation of three independent influx experiments done in quadruplicate. Results are expressed as percentage of specific ^45^CaCl_2 influx relative to that obtained in the absence of TGF-β, which corresponds to a mean of 344 cpm /min and was arbitrarily set to 100. The star indicates a statistically significant value (p < 0.05, Student’s t test). B, TGF-β leads to an increase in the cytoplasmic free calcium concentration ([Ca^{2+}]_c) of NIH3T3 cells. 3 × 10^4 serum-starved NIH3T3 cells growing on glass coverslips were loaded with fura-2 and subsequently processed for fura-2 fluorescence, as described under “Materials and Methods.” TGF-β was added 0.5 min after the beginning of the recordings, as indicated by the arrow. The trace represents measurements of [Ca^{2+}]_c recorded from a single field of about five cells and is representative of four independent experiments with similar results. C, extracellular calcium is required for the TGF-β-dependent increase of [Ca^{2+}]_c. Fura-2-loaded NIH3T3 cells received 10 mM EGTA and 5 ng/ml TGF-β 1 and 2 min after the beginning of the recordings respectively, as indicated by the arrows. The trace represents measurements of [Ca^{2+}]_c in the presence of both agents, recorded from a single field of about five cells. The experiment was repeated twice with identical results.

other known calcium-dependent phosphatases or kinases mediate TGF-β regulation remains to be demonstrated.

We have previously shown that TGF-β specifically induces the activity of the transcriptional activation domain (TAD) of CTF-1, the prototypic member of the CTF/NF-I family of proline-rich transcription factors (14). Interestingly, the TGF-β-responsive domain of the CTF-1 TAD mediates histone H3 interaction and alters nucleosomal structure, suggesting that TGF-β may induce gene expression by chromatin remodeling. In addition, the TGF-β-responsive domain mediates tumor necrosis factor α (TNF-α) repression of basal and TGF-β-induced CTF-1 transcriptional activity (15). This antagonistic regulation of CTF-1 activity by TGF-β and TNF-α correlates with the opposing effects of the two growth factors on the regulation of some collagen genes (16, 17), which may be relevant for the process of wound healing.

Here, we analyzed the relevance of calcium signaling in the induction of CTF-1 transcriptional activity by TGF-β. We show that TGF-β stimulates calcium influx and mediates an increase in the free cytoplasmic calcium concentration in NIH3T3 cells. TGF-β induction of the CTF-1 TAD is inhibited in cells pretreated with thapsigargin, which interferes with calcium homeostasis, while expression of constitutive forms of calcineurin or of the calcium/calmodulin-dependent kinase IV (CaMKIV) induces CTF-1 transcriptional activity. Thus, calcium signaling may be relevant for the regulation of CTF-1 by extracellular stimuli.

MATERIALS AND METHODS

Plasmids—pG5BCAT, pGCT-387-3xAAd, pCATΔ55, and p3TP-Luc have been described previously (14, 18). The CMV-driven mammalian expression vectors for the CTF-1 fusion protein GAL 399–499 and its derivatives GAL 399–486, GAL 486–499, GAL 479–499 (3xM), the CTF-2 fusion vector GAL 399–430 and GAL-AP2 were derived after subcloning appropriate fragments of the corresponding SV40-based vectors (14), in pCMV5 (19). The untagged version of ΔCNA, a deletion mutant of the mouse CNα calcineurin catalytic subunit lacking the autoinhibitory and the calmodulin-binding domains, was expressed from the previously described SRE-driven vector (pSRE-ΔCNA-Mi, Ref. 20). ΔCNA was tagged at the C terminus with the hemagglutinin HA1 epitope after subcloning a 1.3-kilobase pair cDNA fragment encoding the first 398 amino acids of the protein in pCMV-HA, a modified pCMV5 vector to yield pCMV-ΔCNA-HA. (The addition of the tag had no detectable effect on ΔCNA activity, hence pSRE-ΔCNA-Mi and pCMV-ΔCNA-HA were used interchangeably in these studies). The ΔCNA mutant used in Fig. 2 was constructed in pCMV-HA using overlap-extension PCR mutagenesis and pSRE-ΔCNA-Mi as template. It contains several point mutations that convert codons 349, 350, 356, and 357 of the CNα cDNA in glutamate, thereby abolishing CNB binding, as shown previously (E mutant in Ref. 21), as well as a carboxy-terminal HA tag, as above. A 0.5-kilobase pair cDNA fragment encoding the regulatory B subunit of calcineurin was cloned from total rat brain RNA by reverse transcription PCR and appropriate primers designed after the published sequence (GeneBank™ accession number L03554), digested with Asp-718 and EcoR1, and inserted between the cohesive sites of pCMV5 to yield pCMV-CNB. Oligonucleotide-mediated PCR
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mutagenesis of an RSV-driven expression vector encoding a truncated 313-amino acid-long catalytic domain of the mouse calmodulin-dependent protein kinase IV (ΔCaMKIV, which lacks the calmodulin binding and the autoinhibitory domains of wild-type CaMKIV, Ref. 22) was used to construct a catalytically inactive ΔCaMKIV kinase variant, after replacement of lysine 75 by glutamate (23). The SRα-based mammalian expression vectors for the constitutive and the catalytically inactive variants of the γ isomorph of the CaMKII kinase have been described previously (24). The CMV-CaMKI expression vector was constructed after subcloning of a 1.3-kilobase pair HI-RI fragment encoding the complete rat cDNA sequence (from pGEX-3T-ATG, Ref. 25) in the cohesive sites of pCDNA3 (Invitrogen). Correct PCR amplification of all recombinant clones was confirmed by dyeoxy DNA sequencing.

Cell Lines and Transfections—NIH3T3 cells were grown in standard DMEM medium supplemented with 10% donor calf serum (Life Technologies, Inc.) and antibiotics. Cells were transiently transfected by electroporation, essentially as described (14). Briefly, 4.5 × 10⁶ cells were mixed with 70 μg of total plasmid DNA and pulsed once at 960 microfarads and 250 V at room temperature, according to the instructions of the electroporator manufacturer (Bio-Rad). The contents of one pulse cuvette were split in two, and cells were plated in DMEM plus 0.5% donor calf serum for 3–5 h. Cultures were then induced for a period of 15 h with either ethanol vehicle or 5 ng/ml of human TGF-β or 10 ng/ml of human TGF-β (Nacalai Tesque, Kyoto, Japan). Cells were lysed in 1 × reporter lysis buffer (Promega Corp.); CAT and luciferase activities were determined using standard procedures and normalized according to β-galactosidase activity from a co-transfected internal control plasmid (CMVβgal, CLONTECH). As ΔCaMKIV slightly (∼2 × ) induces CMVβgal activity, CAT activities in the experiments involving ΔCaMKIV were normalized according to the total protein concentration.

Gel Mobility Shift Assays—For gel shift analysis of the endogenous CTF/NF-I or of the transiently expressed GAL4 fusion proteins, cells were lysed in extraction buffer (20 mM Tris, pH 7.5, 70% glycerol, 500 mM KCl, 1 mM dithiothreitol, and protease inhibitors) as described by Martinez et al. (26). Whole-cell lysates were normalized for protein concentration and incubated with end-labeled double-stranded DNA probes containing either the high-affinity CTF/NF-I binding site found within the first 50 base pairs of the Adenovirus origin of replication (27) or the 17-base pair GAL4 binding site (28). Protein-DNA complexes were separated from free probe on native polyacrylamide gels and revealed by autoradiography.

**Calcium Influx and Fura-2 Fluorescence Studies**—For 45Ca²⁺ influx studies, 7 × 10⁴ exponentially growing NIH3T3 cells were plated in 24-well plates and incubated in standard DMEM plus 0.5% donor calf serum overnight. Cells were washed twice with 0.5 ml of PBS buffer (145 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 10 mM glucose, 5 mM Hepes, pH 7.4) containing 0.12 mM CaCl₂, and they were then induced with 5 ng/ml of TGF-β or ethanol carrier in 0.2 ml of the same buffer at 37°C. The cells were labeled with 1 μCi of 45Ca²⁺/well at 37°C for the last 5 min of the incubation in the presence or absence of the growth factor. The assay was terminated by aspirating the labeling medium and rinsing the cells 4 × with 0.5 ml of cold PBS buffer containing 1.2 mM CaCl₂. The cells were then trypsinized, lysed in 0.25 ml of 1% SDS, and the content of the lysate was measured by liquid scintillation counting (Packard Tri Carb 4640).

The cytoplasmic free calcium concentration ([Ca²⁺]c) was measured using fura-2 fluorescence, essentially as described (29). Briefly, 3 × 10⁵ serum-starved NIH3T3 cells plated on glass coverslips were washed twice with PBS buffer containing 1.2 mM CaCl₂ and were subsequently loaded with 5 μM fura-2/AM (Molecular Probes) for 40 min in the same buffer at room temperature and in the dark. The excess of fura-2 was then removed by washing twice with PBS, 1.2 mM CaCl₂, and the coverslip was inserted into a thermostatted chamber on a Nikon Diaphot inverted epifluorescence microscope, which is part of a PhoCal single cell fluorescence analyzer (Life Science Resources, Cambridge, UK). The cells were illuminated with alternating light of 340 and 380 nm from a rotating filter wheel. Emission was monitored at 510 nm from a field of about five cells, and the data were analyzed using PhoCal software. Calibrations were performed by treating the cells with 10 μM ionomycin plus 10 μM calcium to obtain the maximal signal, followed by the addition of 10 mM EGTA to obtain the minimal signal. Background fluorescence, obtained by quenching the signal with 1 mM MnCl₂, was subtracted from the signals. [Ca²⁺], was calculated based on the equation of Grynkiewicz et al. (30).
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A

G5BCAT+GAL 399-499

G5BCAT+GAL-AP2

B

GAL 399-499

GAL-AP2

ΔCNA (WT) + CNB
ΔCNA (MUT) + CNB

ΔCNA (WT) + CNB + TGF-β
ΔCNA (MUT) + CNB + TGF-β

ΔCNA (WT) + CNB
ΔCNA (MUT) + CNB

ΔCNA (WT) + CNB + TGF-β
ΔCNA (MUT) + CNB + TGF-β

C

normalized CAT activity

GAL 399-499

GAL-AP2

ΔCNA (WT)

ΔCNA (MUT)

ΔCNA (WT) + CNB
ΔCNA (MUT) + CNB

ΔCNA (WT) + CNB + TGF-β
ΔCNA (MUT) + CNB + TGF-β

GAL 399-499

GAL-AP2

vector

ΔCNA (W.T.)

ΔCNA (W.T.) + CNB
ΔCNA (W.T.) + CNB + TGF-β

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Fig. 3. Transcription factor CTF-1 mediates calcineurin induction in NIH3T3 fibroblasts. A, synergistic activation of the CTF-1 TAD upon co-expression of calcineurin A and B, NIH3T3 cells were transfected with G5BCAT, CMVβgal, and CMV-driven expression vectors encoding GAL 399–499 (left panel) or GAL-AP2 (right panel). Cells additionally received vectors expressing the wild-type or mutated mouse calcineurin catalytic subunit truncation mutant (ΔCNA/WT) and ΔCNA(MUT), see “Materials and Methods.” (20, 21), the wild-type rat calcineurin regulatory B subunit (CNB), or equimolar amounts of the parental empty vector, as indicated. Five hours post-transfection, cells received 5 ng/ml TGF-β or were left untreated, as indicated. CAT activities were determined 18 h later and normalized to β-galactosidase activity. The mean values of at least three independent experiments are presented, expressed as normalized CAT activity relative to that obtained with GAL 399–499 in the absence of stimuli, which was set to 100.  B, NIH3T3 cells were either transfected with expression vectors coding for GAL 399–499, GAL-AP2, and/or ΔCNA and CNB (lanes 2–10) or mock-transfected (lane 1), as in part A. Five hours post-transfection, cells received 5 ng/ml TGF-β or were left untreated, as indicated. The cells were collected after overnight incubation, and equal protein amounts of whole cell lysates were analyzed for GAL4 DNA binding activity in a gel mobility shift assay, as described under “Materials and Methods.” The relevant GAL4 fusion-DNA complexes are indicated by arrows, whereas the closed circle indicates complexes that may result from proteolytic degradation products of the GAL4 fusion proteins. C, the immunosuppressants FK506 and cyclosporin A abolish calcineurin-mediated induction of the CTF-1 TAD. NIH3T3 cells were transfected with G5BCAT, CMVβgal, and CMV-driven expression vectors for either GAL 399–499 or GAL-AP2 and/or wild-type ΔCNA, as indicated. Five hours post-transfection, cells were stimulated with MeSO (DMSO), 2 μM/ml FK506 (Fujiwasa USA Inc.) or 1 μM/ml of cyclosporin A (CsA, Novartis Pharma Inc.), and 20 min later they received 5 ng/ml TGF-β or carrier, as indicated. After overnight incubation in the presence of these agents, the cells were collected and CAT activities were determined and normalized to β-galactosidase activity. The mean values of at least three independent experiments are expressed, as normalized CAT activity relative to that obtained with GAL 399–499 in the absence of stimuli, which was set to 100.
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The enzymatic activity of calcineurin can be inhibited by the immunosuppressive drugs FK506 and cyclosporin A (CsA) complexed with their respective cellular receptors, such as the immunophilins FKBP12 and cyclophilin A respectively, but not by a rapamycin-FKBP12 complex (11). We thus tested the effect of these three immunosuppressants on GAL 399–499 activation by ΔCaMKIV and TGF-β (Fig. 3C). GAL 399–499 still mediated TGF-β induction in the presence of FK506, whereas a slightly reduced TGF-β induction was consistently observed in CsA-treated NIH3T3 fibroblasts. Significantly, treatment of the cells with either FK506 or CsA eliminated the ability of ΔCaMKIV to induce basal GAL 399–499 activity (Fig. 3C). In contrast, rapamycin had no effect on GAL 399–499 regulation, whereas GAL-AP2 and GAL-Oct2 activities were unaffected by treatment with either immunosuppressant (Fig. 3C and data not shown). Thus, the enzymatic activity of calcineurin is required for GAL 399–499 induction.

Calcium/Calmodulin-dependent Protein Kinase IV Is a Potent Activator of the CTF-1 TAD—Although calcineurin induces the basal transcriptional activity of CTF-1, it has little effect on TGF-β induction per se (Fig. 3), suggesting that calcineurin may not be a direct intermediate of TGF-β action. ΔCNA may act indirectly when overexpressed, however, for example by increasing the activity of a calcium-sensitive component(s) of the TGF-β pathway. We therefore tested whether other known calcium-regulated signaling enzymes, such as specific calcium/calmodulin-dependent kinases (CaMKs, reviewed in Ref. 34), might be involved in the induction of GAL 399–499 transcriptional activity by TGF-β. Surprisingly, expression of a truncated catalytic subunit of CaMKIV, lacking both the autoinhibitory domain (TRD) and the calcium/calmodulin-binding domains (CaMKIV), was found to induce basal GAL 399–499 transcriptional activity more than 80-fold in NIH3T3 fibroblasts, and to a lesser extent in Mv1Lu, TA-1, and COS-7 cells (Fig. 4A, “ΔIV,” and data not shown). In contrast, expression of a catalytically inactive ΔCaMKIV mutant, or of the calcium/calmodulin-dependent protein kinases I and II (24, 25), failed to induce the CTF-1 TAD. In addition, ΔCaMKIV did not significantly affect GAL-DBD, GAL-AP2, GAL-Sp1, and GAL-Oct2 basal transcriptional activities, whereas it increased only slightly the expression of GAL-AP2.
levels of all GAL4 fusion proteins tested (Fig. 4A and data not shown). Thus, ΔCaMKIV specifically induces the activity of the CTF-1 transcriptional activation domain.

TGF-β induction of CTF-1 transcriptional activity is mediated by the TGF-β responsive domain (TRD), which resides in the last 20 carboxyl-terminal amino acids of the CTF-1 TAD (14). To test whether the CTF-1 TRD might be also required for ΔCaMKIV-mediated activation, we examined several previ-
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**Fig. 6. A working model for the induction of CTF-1 transcriptional activity by TGF-β.** Following stimulation of NIH3T3 cells with TGF-β, the growth factor induces influx of extracellular calcium ions, probably mediated by a putative receptor-operated channel (ROC). These calcium ions may serve as the primary signal for the release of stored calcium from the endoplasmic reticulum (ER) in the cytoplasm of the cells, mediated by an intracellular calcium channel (RyR/IP3 R). This leads to an increase in total cytoplasmic calcium concentration ([Ca2+]c), which can be prevented upon chelation of the extracellular calcium with EGTA. Increased [Ca2+]c leads to the activation of calcium-dependent signaling enzymes such as calcineurin (CN, pathway 1) and CaMKIV (pathway 2), which bind Ca2+ and/or Ca2+-complexed to calmodulin (CaM). These and other putative TGF-β-regulated cascades (pathways 3 and 4) relay the TGF-β signals further downstream in the nucleus, where CTF-1 activation occurs (probably through the phosphorylation of a CTF-1-interacting protein(s), such as histone H3), leading to CTF-1-dependent chromatin remodeling (14) and eventually to the transcriptional induction of TGF-β-responsive genes, such as collagen. FK506 complexed to FKBP12 binds CN and blocks its phosphatase activity, while thapsigargin inhibits the function of the ER calcium (SERCA) pumps and depletes the calcium pool, thereby blocking TGF-β action. The Ras/Raf pathway, which may mediate CTF-1 repression in response to TNF-α, thereby antagonizing TGF-β induction (15), is also indicated. Note that the intracellular localization of CN and CaMKIV is hypothetical in this scheme.


diously constructed GAL-CTF1 fusions (14) for ΔCaMKIV responsiveness in NIH3T3 cells (Fig. 4B). As expected, the DNA-binding domain of GAL4 (GAL-DBD) did not mediate ΔCaMKIV activation in this assay, while the activity of GAL 399–499 was potently induced by ΔCaMKIV. Interestingly, deletion of the CTF-1 TRD abolished kinase induction (Fig. 4B, GAL 399–498), whereas its direct fusion to GAL-DBD was sufficient to confer full ΔCaMKIV responsiveness (GAL 486–499). A derivative mutated in all phosphorylation acceptor sites in the TRD could still be efficiently induced by ΔCaMKIV (Fig. 4B, GAL 479–499 3xM), as it is induced by TGF-β (14). In contrast, the CTF-2 TAD, which naturally lacks a TRD as a result of alternative splicing events (37), conferred little, if any, ΔCaMKIV- and TGF-β-mediated induction (Fig. 4B, GAL 399–430, and Ref. 14). TGF-β was still able to increase the CaMKIV-activated levels of all TGF-β-responsive GAL-CTF derivatives (for instance GAL 486–499 and GAL 479–499 3xM, Fig. 4B), even though the ability of TGF-β to induce GAL 399–499 activity was reduced in cells overexpressing ΔCaMKIV (from 8.8- to 1.8-fold, Fig. 4A). Thus, we conclude that the TGF-β-responsive domain mediates the ΔCaMKIV-dependent transcriptional activation of CTF-1. Furthermore, similarly to TGF-β and calcineurin regulation, ΔCaMKIV regulation is confined to specific CTF/NF-I species, such as CTF-1 but not CTF-2. Finally, our results indicate that TRD activation may occur in the absence of ΔCaMKIV-mediated phosphorylation, implying that other ΔCaMKIV-regulated proteins must be involved in this regulatory process.

**Mouse CTF/NF-I Proteins Mediate Calcineurin- and CaMKIV-regulated Transcriptional Activation—**To address the possibility that the endogenous CTF/NF-I polypeptides might be also induced by calcineurin and CaMKIV, we used a reporter promoter containing three high-affinity CTF/NF-I binding sites in front of the α-globin TATA box and the cat gene. As shown previously (15), this CTF/NF-I-responsive promoter is induced by TGF-β in transiently transfected NIH3T3 fibroblasts (Fig. 5A, pcAT ΔA78–3xAd). Interestingly, expression of either ΔCNA or ΔCaMKIV significantly induced basal CTF/NF-I transcriptional activity (Fig. 5A). ΔCNA had no effect on CTF/NF-I induction by TGF-β, whereas TGF-β induction was increased in cells expressing ΔCaMKIV. In contrast, neither enzyme had any effect on the activity of a control promoter lacking CTF/ NF-I binding sites or on the activity and TGF-β induction of a distinct TGF-β-responsive promoter (Fig. 5A, pcAT ΔA55 and p3TP-Lux). Gel mobility shift analysis of the endogenous CTF/ NF-I proteins indicated that ΔCNA and ΔCaMKIV did not significantly affect CTF/NF-I binding activity and/or expression levels (Fig. 5B). Thus, these data indicate that the transcriptional activity of endogenous mouse CTF/NF-I polypeptides is induced by expression of constitutive calcineurin and CaMKIV, which suggests that CTF/NF-I is a relevant transcriptional target for these calcium-regulated enzymes in vivo. However, as for the GAL4 fusion proteins (Fig. 4), ΔCaMKIV did not prevent CTF/NF-I induction by TGF-β, suggesting that CTF/NF-I is unlikely to be directly involved in this regulatory process.

**DISCUSSION**

Growth factors regulate gene expression by modulating the activity of target transcription factors through signal transduction cascades. Stimulation of collagen synthesis by mesenchymal cells is an important aspect of TGF-β regulatory action for tissue remodeling, wound healing, and the pathogenesis of some fibrotic disorders (1, 2). A role for CTF/NF-I in collagen synthesis was initially proposed by Rossi et al. (16), who suggested that induction of the human collagen α1(I) promoter by TGF-β might involve CTF/NF-I binding sites (16). Indeed, TGF-β was subsequently demonstrated to induce the transcriptional activity of CTF-1, the prototypic member of the CTF/ NF-I family (14). Recently, a possible role for calcium in TGF-action was suggested, as the TGF-β type 1 receptor was shown to interact with FKBP12, a protein involved in the regulation of calcium homeostasis (9). Here, we investigated a possible role of free calcium signaling in the induction of CTF-1 transcriptional activity by TGF-β.

Our results provide evidence for the potential relevance of calcium signaling in TGF-β action, as we found that (i) TGF-β induces calcium influx in NIH3T3 cells, (ii) it increases free cytoplasmic calcium concentration ([Ca2+]c) to about 150 nM, which is consistent with the requirements for the activation of CaMKIV.
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Calcium-dependent signaling enzymes (31), (iii) TGF-β is unable to increase [Ca\(^{2+}\)]\(_i\) in EGTA-pretreated cells, and (iv) TGF-β induction of CTF-1 transcriptional activity is prevented by thapsigargin, a compound that inhibits calcium signaling by specifically blocking calcium uptake in the ER (32, 33). Altogether, these results indicate that TGF-β stimulates calcium influx in NIH3T3 cells, thus leading to an increase in the cytoplasmatic calcium concentration that may in turn activate downstream calcium-dependent effectors such as calcineurin and CaMKIV (Fig. 6, pathways 1 and 2). Consistent with this possibility, constitutively active variants of CNA and CaMKIV specifically induce the basal transcriptional activity of both the CTF-1 TAD and the endogenous mouse CTF/NF-I proteins in NIH3T3 cells. However, these enzymes are unlikely to be directly involved in the TGF-β induction pathways, as (i) the calcineurin inhibitors FK506 and CsA do not inhibit TGF-β induction of the CTF-1 TAD, (ii) TGF-β still potentiates GAL 399–499 transcriptional activity in ΔCaMKIV and/or ΔCNA co-expressing cells, and (iii) NIH3T3 cells do not express significant levels of the CaMKIV protein, although they do express CNA and CNB mRNAs (as estimated by reverse transcription PCR).2 Our results rather imply that, if calcium signaling is indeed relevant for TGF-β induction of CTF-1, as suggested by the thapsigargin inhibitory effect, it may involve other calcium-regulated intermediates (Fig. 6, pathway 3). In any case, however, calcium signaling alone is unlikely to account for all of the TGF-β actions, as simple administration of calcium ionophores is unable to mimic TGF-β induction of CTF-1.2 Therefore, we postulate the existence of yet another, potentially calcium-dependent signaling pathways linking CTF-1 activity to cellular signaling by TGF-β (Fig. 6, pathway 4).

ΔCaMKIV action (and also calcineurin-mediated CTF-1 induction, results not shown) specifically targets the previously identified kinase and the catalytic activity of the kinase is required for this effect. This suggests that the kinase phosphorylates either the TRD itself or an TRD-interacting protein. However, phosphorylation-defective TRD variants still confer efficient TGF-β induction, as well as calcineurin- and ΔCaMKIV-mediated activation. Thus, these effects must be mediated by a protein(s) interacting with the TGF-β-responsive domain. One such protein could be histone H3, since it binds the CTF-1 TAD, and it has been proposed to be relevant for TGF-β induction (14). For instance, CaMKIV or a related kinase might regulate the interaction of the CTF-1 TAD with histone H3, in response to extracellular signaling. Consistent with this possibility, several growth factors and protein kinases have been found to induce histone H3 phosphorylation, and this effect has been correlated with changes in gene expression (38).

In summary, the results presented in this study identify CTF-1 as a novel molecular target of calcineurin and CaMKIV action, and they further argue for the potential relevance of calcium signaling in the regulation of extracellular matrix gene expression by TGF-β. Analysis of CTF/NF-I regulation might thus provide insights in the control mechanisms of wound healing and extracellular matrix production by TGF-β and may thus help understand the role of TGF-β in the pathogenesis of fibrotic disorders.

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