Transforming Growth Factor-β and Ciliary Neurotrophic Factor
Synergistically Induce Vasoactive Intestinal Peptide Gene
Expression through the Cooperation of Smad, STAT, and AP-1 Sites*

Richard L. Pitts, Shuibang Wang, Elizabeth A. Jones, and Aviva J. Symes‡

From the Department of Pharmacology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814

The cytokine ciliary neurotrophic factor (CNTF) and transforming growth factor-β (TGF-β) both induce transcription of the vasoactive intestinal peptide (VIP) gene through a 180-base pair cytokine response element (CyRE) in the VIP promoter. While CNTF induces STAT and AP-1 proteins to bind to cognate sites in the VIP CyRE, the mechanism through which TGF-β acts to induce VIP gene transcription is not known. Here we show that Smad3 and Smad4 proteins can bind to two distinct sites within the VIP CyRE. These sites are absolutely required for the induction of VIP CyRE transcription by TGF-β. TGF-β induces endogenous Smad-containing complexes to bind to these sites in human neuroblastoma cells. CNTF and TGF-β synergize to induce VIP mRNA expression and transcription through the VIP CyRE. This synergy is dependent on the Smad, STAT, and AP-1 sites, suggesting that these two independent cytokine pathways synergize through the cooperation of pathway-specific transcription factors binding to distinct sites within the VIP CyRE.

Transforming growth factor-β (TGF-β)1 and ciliary neurotrophic factor (CNTF) have many functions in the developing and mature nervous system. These two unrelated cytokines mediate their effects through separate and distinct signaling cascades. CNTF, a member of the gp130 cytokine family, utilizes a multimeric receptor structure consisting of a GPI-linked ligand binding subunit, CNTFR-α, and two related transmembrane signal-transducing subunits, gp130 and leukemia inhibitory factor (LIF) receptor-β (1–4). Neither of these transmembrane components have intrinsic kinase activity; instead, they associate with the Jak/Tyk tyrosine kinases (5–7). Activation of these kinases by ligand-induced receptor multimerization is thought to initiate signal transduction and activation of gene expression (5, 8, 9). Cytokine stimulation induces STAT proteins to “dock” onto the receptor, enabling their own tyrosine phosphorylation (8, 10–12). Subsequently, STAT proteins translocate to the nucleus and bind to STAT sites in regulated genes to provide a rapid means of activating gene transcription (reviewed in Ref. 13). Various other signaling moieties are also activated by CNTF including the Ras-mitogen-activated protein kinase pathway (14–17), SHP-2 tyrosine phosphatase (18), phosphatidylinositol 3-kinase (15, 19), and components of the AP-1 transcription factor family (20, 21).

TGF-β signals through TGF-β type I (TβR-I) and type II receptors (TβR-II), which possess intrinsic serine-threonine kinase activity (for reviews of TGF-β signal transduction, see Refs. 22 and 23). The TGF-β/TβR-II complex recruits and then phosphorylates TβR-I to initiate signaling. The activated TβR-I phosphorylates the receptor-regulated Smad proteins, Smad2 and Smad3. Phosphorylated Smads dissociate from the receptor, complex with the co-Smad Smad4, and translocate to the nucleus. Smad bind to specific Smad sites in genomic regulatory regions. However, their DNA binding affinity is weak (24), and they usually complex with other classes of transcription factor to establish strong DNA binding to induce gene transcription of regulated genes (25).

Despite their differences, TGF-β and CNTF share some functional similarity. Both cytokines have neurotrophic actions, enhancing the survival of various populations of neurons in the central and peripheral nervous system (26–32). In addition, TGF-β enhances CNTF-mediated survival of cultured ciliary neurons, suggesting that TGF-β may act together with CNTF in specific cell populations (33). While these two cytokines sometimes share similar functions, the mechanism through which they may cooperate has not been investigated. We have previously shown that CNTF and activin, a TGF-β-related cytokine, independently induce VIP gene expression through a 180-bp element in the VIP promoter termed the cytokine response element (CyRE) (34). CNTF induces VIP gene expression through the induction of STAT and AP-1 proteins to bind to distinct sites within the CyRE (20, 35). These cytokine-induced proteins interact with other noninduced proteins to bring about a robust activation of VIP transcription through combinatorial interactions (36). While CNTF induces VIP gene expression ~50-fold, activin has a smaller effect on VIP mRNA, inducing it ~2-fold (34). However, co-treatment of CNTF and activin leads to a synergistic activation of VIP transcription mediated through the VIP CyRE. TGF-β also synergizes with CNTF to induce CyRE-directed transcription (34). However, TGF-β does not induce either STAT or AP-1 proteins to bind to the CyRE (34), and we do not know the molecular mechanisms through which TGF-β and related cytokines induce VIP gene expression. Understanding the TGF-β-initiated signaling pathways that regulate the VIP gene will allow us to investigate the mechanisms through which these two independent cytokine signals synergize to regulate neuropeptide gene expression. In

* This work was supported by National Institutes of Health Grant R29 NS-35839 and Uniformed Services University of the Health Sciences intramural support (to A. J. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Dept. of Pharmacology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Rd., Bethesda, MD 20814. Tel.: 301-295-3223; Fax: 301-295-3220; E-mail: Asymes@usuhs.mil.

1 The abbreviations used are: TGF, transforming growth factor; CNTF, ciliary neurotrophic factor; LIF, leukemia inhibitory factor; TβR-I and -II, TGF-β type I and II receptor, respectively; CyRE, cytokine response element; STAT, signal transducers and activators of transcription; bp, base pairs; EMSA, electrophoretic mobility shift assay; GST, glutathione S-transferase; VIP, vasoactive intestinal peptide; SBE, Smad-binding element.

19966 This paper is available on line at http://www.jbc.org
this paper, we show that there are two Smad binding sites within the CyRE, distinct from the AP-1 and STAT sites, that are critical to the TGF-β regulation of VIP gene expression. We further show that the Smad, STAT, and AP-1 sites all contribute to the synergistic interaction between CNTF and TGF-β in the induction of VIP gene expression.

RESULTS

We have previously shown that TGF-β induction of VIP gene transcription is mediated through the VIP CyRE. To determine which regions within the 180-bp CyRE are important for the TGF-β-mediated transcriptional induction, we transfected NBFL neuroblastoma cells with a series of CyRE deletion constructs. These plasmids contain various regions of the CyRE upstream of a basal RSV promoter driving expression of the luciferase reporter gene (35). As described previously, luciferase activity in cells transfected with the full-length Cy1luc was assayed for luciferase activity (41) and normalized to β-galactosidase activity in cells transfected with either Cy1mS4luc or Cy1mg17luc. This plasmid, Cy1mg17luc, was constructed by polymerase chain reaction site-directed mutagenesis with GGG overhangs and labeled with [α-32P]dCTP using Moloney murine leukemia virus reverse transcriptase (Promega, WI). When used, competitor oligonucleotides or antibodies were incubated with the nuclear extracts for 10 min at room temperature prior to the addition of probe.

Electrophoretic Mobility Shift Assay (EMSA)—EMSAs were performed as described previously (35). NBFL cells were grown to confluence and serum-starved overnight before treatment with TGF-β for the times indicated. GST-Smad fusion proteins were prepared as described (37). Nuclear extracts were prepared, and binding reactions performed as previously described (35). Synthetic complementary oligonucleotides with GGG overhangs were annealed and labeled with [α-32P]dCTP using Mo1oey murine leukemia virus reverse transcriptase (Promega, WI). When used, competitor oligonucleotides or antibodies were incubated with the nuclear extracts for 10 min at room temperature prior to the addition of probe.

Cell Culture and Transfection—NBFL cells were maintained and transfected as described previously (40). Cells were plated at 1.5 × 10^5 cells/well in six-well plates and transfected overnight by calcium phosphate precipitation. Each well received 1 μg of luciferase reporter construct, 0.5 μg of EF-β-galactosidase, and 2.5 μg of carrier DNA. Cytokines were added in serum-free medium, 6 h after the DNA precipitate was removed, for 40 h before cell harvesting. Samples were assayed for luciferase activity (41) and β-galactosidase activity (Galec8tLight Plus kit, Tropix Inc, MA). Luciferase activity was normalized to β-galactosidase activity to control for transfection efficiency.

**Experimental Procedures**

Materials—Cell culture reagents were obtained from Mediatech (Herndon, VA), fetal bovine/horse serum from Life Technologies, Inc., and culture plates from Costar (Corning, NY). Recombinant human CNTF was a gift from Regeneron Pharmaceuticals (Tarrytown, NY), and TGF-β was purchased from R & D Systems (Minneapolis, MN). Oligonucleotides were synthesized on a PE Applied Biosystems 394 synthesizer by the Uniformed Services University of the Health Sciences in-house oligonucleotide facility. Anti-Smad2/3 antiserum was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). The Gal4-Smad fusion plasmids, pG5E1Bluc, and bacterial expression vectors for GST-Smad4 and GST-Smad3ΔC were obtained from Dr. R. Lechleider (Department of Pharmacology, Uniformed Services University of the Health Sciences) (37).

Plasmids—Details of Cy1luc, the Cyluc deletion series, 3′G3 luc, and 3′G2 luc have all been described (35). The series of 3′-bp substitution mutants of Cy1luc was amplified from Cy1luc as described previously (36). The plasmid Cy1mS5 is identical to Cy1 mg11luc. Cy1 mg17luc was constructed by polymerase chain reaction site-directed mutagenesis (38) with the oligonucleotides 5′-CAATCCAGGAGAAAATTTCTCCATCATGAGTTGAAAACACTTAATCTC-3′ and 5′-GAATTAAGTTTCAAAATTGCGATGAATTTGTTTCCCAGATTG-3′. These oligonucleotides were paired, with either A1 or A4 (35), and Cy1luc as template to create new fragments. The fragments were gel-purified and used as template in a subsequent polymerase chain reaction with oligonucleotides A1 and A4 as primers to create Cy1mg17luc. This plasmid, Cy1mg17luc, was used as template with primers A1 and mS5 (36) to create Cy1mg17mg11luc. Cy1mg17mg11luc was then used as template DNA to construct the further mutated Cy1mg17mg11luc, Cy1mg17mg17luc, and Cy1mg17mg17luc using the CLONTECH Transformer site-directed mutagenesis kit (CLONTECH Transformer site-directed mutagenesis kit). The Gal4-Smad fusion plasmids, pG5E1Bluc, and bacterial expression vectors for GST-Smad4 and GST-Smad3ΔC have been described previously (37).

Cell Culture and Transfection—NBFL cells were maintained and transfected as described previously (40). Cells were plated at 1.5 × 10^5 cells/well in six-well plates and transfected overnight by calcium phosphate precipitation. Each well received 1 μg of luciferase reporter construct, 0.5 μg of EF-β-galactosidase, and 2.5 μg of carrier DNA. Cytokines were added in serum-free medium, 6 h after the DNA precipitate was removed, for 40 h before cell harvesting. Samples were assayed for luciferase activity (41) and β-galactosidase activity (Galec8tLight Plus kit, Tropix Inc, MA). Luciferase activity was normalized to β-galactosidase activity to control for transfection efficiency.

**RESULTS**

We have previously shown that TGF-β induction of VIP gene transcription is mediated through the VIP CyRE. To determine which regions within the 180-bp CyRE are important for the TGF-β-mediated transcriptional induction, we transfected NBFL neuroblastoma cells with a series of CyRE deletion constructs. These plasmids contain various regions of the CyRE upstream of a basal RSV promoter driving expression of the luciferase reporter gene (35). As described previously, luciferase activity in cells transfected with the full-length Cy1luc was induced after treatment with TGF-β ~4-fold (34). Deletion of 28 bp at the 3′-end of the CyRE either from the full-length construct or from two other plasmids with deleted 5′-ends reduced the transcriptional induction of CyRE luciferase reporter plasmids by over 60% (Fig. 1). However, deletion of up to 50 bp from the 5′-end of the CyRE did not significantly reduce the induction of luciferase activity driven by these plasmids in response to TGF-β. These data suggest that a region within the most 3′ 28 bp of the CyRE is important to the TGF-β-mediated induction of VIP CyRE transcription.

To examine more precisely the sequences within this 3′ region that contribute to the TGF-β induction of VIP transcription, we transfected NBFL cells with CyRE-luciferase plasmids containing a sequential series of 3′-bp mutations of the most 3′ 30 bp (Fig. 2). TGF-β was unable to induce transcriptional activity in cells transfected with either Cy1mS4luc or Cy1mS5luc (Fig. 2). These plasmids contain sequential mutations of the sequence GTCTGA, which, read inverted on the
minus strand, contains a CAGA box (TCAGAC). In other promoters, this CAGA motif can bind Smad proteins and mediate TGF-β-induced transcription (39, 45). The mutations present in Cy1mS7luc and Cy1mS8luc reduced but did not eliminate TGF-β-induced transcription (Fig. 2), suggesting that these mutated sequences may also contribute to the TGF-β induction of VIP CyRE transcription. Mutations in other regions within the 3′-end of the CyRE resulted in reduced unstimulated and TGF-β-stimulated luciferase activity but no significant alteration in the overall TGF-β induction (Fig. 2). The exception to this, the large TGF-β induction of Cy1mS6luc, was not reproducible. These data suggest that the sequence TCAGAC is critical to the ability of TGF-β to induce transcription through the VIP CyRE.

We then examined the rest of the CyRE sequence to look for other regions that might have similarity to the CAGA site at the 3′-end of the CyRE. Interestingly, we found one other region, toward the center of the CyRE, that also contained the core CAGA sequence. This sequence (TCCAGACAT) is located 1250 bp upstream of the transcription start site. To determine whether Smad proteins could bind to these CAGA-containing sequences from the VIP CyRE, we incubated purified GST-Smad fusion with probes from these regions, P17 and P11 (Table I). EMSA analysis indicated that GST-Smad3 bound to the CAGA-containing probes, P17 and P11, and to a control SBE but not to an adjacent, non-CAGA-containing probe, P18 (Fig. 3C). Binding of GST-Smad3 to either P17 or P11 was specifically competed by a 100-fold molar excess of nonlabeled wild type oligonucleotide but not by the same amount of these sequences with 3-bp mutations in their CAGA boxes (Fig. 3D). GST-Smad4 bound very weakly to either P17 or P11, despite strong binding to the control SBE (Fig. 3E). Truncated GST-Smad4ΔC, without the C-terminal MH2 domain, did not bind more strongly than full-length GST-Smad4 (data not shown), suggesting that it was not the presence of the MH2 domain that inhibited Smad4 binding to the CyRE sequences. Taken together, these data show that two sites within the VIP CyRE are able strongly and specifically to bind Smad3 but that these same sites bind Smad4 only weakly.

To assess the role of these two CyRE Smad binding sites in mediating TGF-β inducibility of CyRE-directed transcription, we examined the ability of TGF-β to induce transcription of luciferase reporter plasmids containing mutations in either or both of these Smad sites. Identical mutations to those that eliminated the ability of Smad proteins to bind to P17 or P11 were introduced into the wild type Cy1luc plasmid. Transfection of these plasmids into NBFL cells indicated that mutation of either P17 or P11 attenuated the ability of TGF-β to induce CyRE-mediated transcription (Fig. 4). Mutation of both P17 and P11 completely eliminated the ability of TGF-β to induce CyRE-driven transcription. Thus, while each CyRE Smad binding site contributes, both sites are necessary to TGF-β induction of CyRE-driven transcription.

To determine whether TGF-β induces endogenous proteins within NBFL cells to bind to these Smad sites, we prepared nuclear extract from untreated and TGF-β-treated NBFL cells. After 1 h of treatment, TGF-β strongly induced NBFL nuclear protein binding to probes containing the CyRE Smad binding sequences (Fig. 5). The probe, P21, is a truncated version of P17. The TGF-β-induced p21-binding complex is competed specifically by a 100-fold molar excess of unlabeled probe and also by a similar molar excess of P17 and P11. It is not competed by oligonucleotides with mutations in their CAGA boxes that were unable to bind GST-Smad proteins (Fig. 5A). These data suggest that in NBFL cells, TGF-β activates Smad proteins to translocate to the nucleus and bind to Smad binding sites within the VIP CyRE. Confirmation of the composition of these TGF-β-induced binding complexes was obtained by demonstrating that an antibody recognizing Smad2 and Smad3 was able to interfere with binding of these complexes to the P21 and P11 probes (Fig. 5). Thus, taken together, our data show that TGF-β induces Smad proteins to bind to two sites with the VIP CyRE and that this binding is critical for TGF-β induction of VIP transcription.

We first demonstrated the VIP CyRE to be a response ele-
ment for the gp130 cytokines (in particular for CNTF) (35) and subsequently that CNTF synergized with activin to induce VIP mRNA (34). Since activin and TGF-β utilize very similar pathways to regulate gene expression, we wanted to confirm that CNTF would also synergize with TGF-β to induce VIP mRNA.

Analysis of VIP mRNA expression in NBFL cells by Northern blotting showed that TGF-β treatment alone induced VIP mRNA in a dose-dependent manner. 1 ng/ml TGF-β induced VIP mRNA 2.8-fold, and 10 ng/ml led to a 12.4-fold induction of VIP mRNA (Fig. 6). As we had previously observed with activin, the TGF-β induction is significantly less robust than that elicited by CNTF, which produced a 55-fold induction in VIP mRNA. When NBFL cells were treated with CNTF together with TGF-β, VIP mRNA was markedly induced: 147-fold by CNTF with 1 ng/ml TGF-β and 257-fold by CNTF together with 10 ng/ml TGF-β (Fig. 6). Thus, TGF-β acts synergistically with CNTF to induce VIP mRNA.

TGF-β and CNTF both induce VIP gene transcription through inducing proteins to bind to specific sequences within the VIP CyRE, yet they activate different transcription factors. We wanted to assess the contribution of sites important to either the CNTF or TGF-β pathways to the synergistic signaling of these two independent cytokines. We therefore introduced additional mutations at the STAT and/or AP-1 sites into the Cy1luc reporter with both Smad sites mutated and compared the activity of all of these mutated luciferase reporters to the wild type Cy1luc plasmid. NBFL cells transfected with Cy1luc produced the synergistic effect of CNTF and TGF-β on VIP mRNA, mediating strong inducibility by CNTF, less by TGF-β, and very marked synergistic signaling by cotreatment with the two cytokines (Fig. 7). In cells transfected with Cy1mg17mg11 (containing mutations of both CyRE Smad sites), TGF-β was no longer able to induce luciferase activity, as shown previously (Fig. 4). Interestingly, the CNTF-induced
transcription driven by Cy1mg17mg11 was reduced by 60% in comparison with Cy1luc, and there was no significant difference in transcriptional induction after cotreatment with both cytokines from that of CNTF alone (Fig. 7). Transcriptional activity driven by a luciferase reporter with mutations in both Smad sites and the AP-1 site (Cy1mg17mg11mg2) was not induced by TGF-β but still retained CNTF induction. The synergy between TGF-β and CNTF was no longer evident.

Plasmids containing mutations of the Smad and STAT sites (Cy1mg17mg11mg3) or of the Smad, STAT, and AP-1 sites (Cy1mg17mg11mg3mg2) did not respond to stimulation by either cytokine alone or together. These data suggest that CNTF and TGF-β synergistically induce CyRE transcription through stimulating a combination of Smad, STAT, and AP-1 proteins.

CNTF and TGF-β stimulate very different signaling pathways to activate gene expression. However, the possibility of cross-talk between these pathways exists at many different levels. Smad proteins, while initially phosphorylated by the receptor, may also be phosphorylated by cytoplasmic kinases, such as the extracellular signal-regulated kinases (37, 46). To investigate whether CNTF signaling may contribute to Smad activation, independent of any potential Smad-DNA binding effects, we utilized Gal4-Smad fusion proteins. Expression vectors for Gal4-Smad fusion proteins were co-transfected with a reporter containing multimerized Gal4 DNA binding sites, pG5-E1B-luc. As previously published (37, 47), TGF-β induced transcriptional activation mediated by Gal4-Smad2, Gal4-Smad3, and Gal4-Smad4 but not by Gal4 alone (Fig. 8A). TGF-β induction of Gal4-Smad3 transcriptional activity was the most robust. CNTF did not induce transcription by any Gal4-Smad fusion proteins. However, CNTF and TGF-β co-treatment significantly induced Gal4-Smad3 fusion proteins. However, CNTF signals alone do not induce Smad transcriptional activation, CNTF may enhance TGF-β's activation of Smad3. However, CNTF and TGF-β co-treatment did not enhance TGF-β's induction of the multimerized SBE luciferase reporter, SBE luc (Fig. 8D). These data suggest that CNTF does not significantly alter the TGF-β activation of endogenous Smad proteins. Thus, the effects of CNTF signals on TGF-β induction of Smad transcriptional activity are minimal.

To determine whether TGF-β may affect CNTF activated pathways, we examined the effects of cytokine cotreatment on luciferase reporters driven by multimerized STAT (G3) or AP-1 (G2) sites. We have previously shown that CNTF does not induce transcription driven by multimerized AP-1 sites and
The information encoded within the VIP CyRE therefore allows signaling by these cytokine classes to interact by the cooperation of transcription factors binding to DNA within a relatively small region.

We have shown that a C-terminal truncated Smad3 protein can bind to two distinct sites within the VIP CyRE (Fig. 3). Mutation of both of these sites abolishes the ability of TGF-β to induce transcription through the VIP CyRE (Fig. 4). Both Smad sequences have strong homology to Smad binding sites in many other genes including the PAI and junB promoters (45, 48). From EMSA studies it appears that the sequence within P17, CCAGACA, has higher affinity for GST-Smad3ΔC than the sequence within P11, TCAGACT (data not shown). Additionally, we have shown that in NBFL cells TGF-β induces nuclear protein complexes to bind to these two Smad sites within the CyRE. These complexes are removed by antisera recognizing both Smad2 and Smad3 (Fig. 5). Smad2 does not bind to DNA (49), so these binding complexes probably contain Smad3. However, we cannot rule out the involvement of Smad2 in transcriptional activation of the VIP gene by TGF-β. GST-Smad4 can bind weakly to the two CyRE Smad sites. Although Smad4 can bind to sites with a CAGA-rich motif, similar to that to which the drosophila vestigial gene (24). Our results indicate a discrepancy between the affinity of Smad3 and Smad4 for the VIP CyRE Smad sites, suggesting that Smad3 and Smad4 proteins have different sequence specificity even within binding to CAGA box sites. Removing the C-terminal MH2 domain from Smad4 did not increase binding to the CyRE Smad sites, in contrast to the results of Jonk et al. (48), who show much improved binding of GST-Smad4ΔC over full-length GST-Smad4 to sites in the JunB promoter. Thus, Smad4 may require other proteins to assist its binding to the VIP CyRE.

Smad proteins bind DNA with low affinity (49). Therefore, to achieve high affinity interaction with specific DNA sequences, Smad proteins usually form complexes with other transcriptional co-factors or bind as multimeric proteins to repeats of the CAGA motif (50, 51). Cooperative binding of Smad proteins confers a greater level of specificity, conferring dependence on the specificity of promoter sequence of each gene and the availability of co-factors with which to bind. The distance between the two VIP CyRE Smad sites (85 bp) suggests that Smad proteins require other proteins to achieve high affinity binding. Thus, Smad3, possibly together with Smad2 and Smad4, may bind to each site in complex with an as yet unknown transcriptional co-factor. Indeed, in EMSA experiments, we have seen a larger TGF-β-induced nuclear protein complex binding to the longer P17 probe than binds to P21 (data not shown). However, our data also show that deleting or mutating only one Smad site significantly reduces the ability of TGF-β to induce transcription through the VIP CyRE (Figs. 1 and 4). Thus, one Smad site alone together with its adjacent sequence is not sufficient to confer full TGF-β induction of VIP transcription. The two Smad sites may functionally cooperate through the loop out of intervening sequences to form a greater transcriptional activating complex with which to recruit co-activators.

While the Smad sites within the VIP CyRE are critical for TGF-β-mediated induction of CyRE transcriptional activity, the AP-1 site is an additional site through which TGF-β may act. We have previously shown that mutation of the AP-1 site in the VIP CyRE reduces activin-mediated induction of CyRE-directed transcription −50% (34). However, our observation that mutation of the CyRE Smad sites eliminated TGF-β stimulation of CyRE transcription suggests that the Smad sites are more critical to the CyRE response to TGF-β than the AP-1.
site. Our data are in contrast to studies on the collagenase promoter, where mutations in the AP-1 site within the collagenase I promoter reduce TGF-β-mediated induction of this gene to a much greater extent than mutations in any or all of the Smad sites (52). Thus, the relative contributions of the AP-1 and Smad sites to transcriptional induction by TGF-β appear to be gene-specific.

One possible mechanism mediating the synergy between CNTF and TGF-β is the convergence of their signaling pathways to activate specific transcription factors. Our data suggest that some kinase activation by CNTF may contribute to Smad transcriptional activation when already activated by TGF-β (Fig. 8A). However, this effect is likely to be minimal due to the lack of synergistic signaling by CNTF and TGF-β in the activation of a transcriptional reporter composed of multimerized Smad sites (Fig. 8D). Thus, synergy between CNTF and TGF-β is not mediated solely through Smad proteins; nor can TGF-β synergize with CNTF in activation of a multimerized STAT reporter. Thus, the synergy between CNTF and TGF-β is not mediated by the action of one cytokine signaling directly to the transcription factor activated by the second cytokine.

CNTF and TGF-β activate pathway-specific transcription factors that translocate to the nucleus to activate gene transcription through the VIP CyRE. As STAT, Smad, and AP-1 pathways to activate specific transcription factors. Our data suggest that some kinase activation by CNTF may contribute to Smad transcriptional activation when already activated by TGF-β (Fig. 8A). However, this effect is likely to be minimal due to the lack of synergistic signaling by CNTF and TGF-β in the activation of a transcriptional reporter composed of multimerized Smad sites (Fig. 8D). Thus, synergy between CNTF and TGF-β is not mediated solely through Smad proteins; nor can TGF-β synergize with CNTF in activation of a multimerized STAT reporter. Thus, the synergy between CNTF and TGF-β is not mediated by the action of one cytokine signaling directly to the transcription factor activated by the second cytokine.

Acknowledgments—We thank Fern Murdock and Bob Lechleider and members of their laboratory for many helpful discussions and suggestions from Liliana Attisiano. We thank Regeneron Pharmaceuticals for supplying the CNTF.

REFERENCES

1. Ip, N. Y., Nye, S. H., Boulton, T. G., Davis, S., Taga, T., Li, Y., Birren, S. J., Yasukawa, K., Kishimoto, T., Anderson, D. J., Stahl, N., and Yancopoulos, G. D. (1993) Cell 70, 1121–1132
2. Davis, S., Aldrich, T. H., Stahl, N., Pan, L., Taga, T., Kishimoto, T., Ip, N. Y., and Yancopoulos, G. D. (1998) Science 285, 89–92
3. Davis, A., Graziani, R., Laufer, R., Cibello, G., and Pannese, G. (1995) J. Mol. Biol. 245, 795–808
4. Carpenter, L. R., Yancopoulos, G. D., and Stahl, N. (1998) Adv. Protein Chem. 52, 109–140
5. Gueugnin, D., Bonheur, V., Briscoe, B., Witsch, B., Bataller, D., Horn, F., Pellegrini, S., Yasukawa, K., Heinrich, P., Stahl, N., and Koller, K. (1995) EMBO J. 14, 1241–1249
6. Nakanishi, M., Witschit, B. A., Yoshida, K., Silvervogeno, O., Yasukawa, K., Ile, J. N., Kishimoto, T., and Taga, T. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2285–2289
7. Stahl, N., Boulton, T. G., Parguegula, T., N. Y., Davis, S., Witsch, B. A., Queitzer, F. W., Silvervogeno, O., Barbieri, G., Pellegrini, S., Ile, J. N., and Yancopoulos, G. D. (1998) Science 283, 92–95
8. Stahl, N., Farragulla, T. J., Boulton, T., Zhou, Z., Darnell, J., and Yancopolous, G. D. (1995) Science 269, 1349–1353
9. Stahl, N., and Yancopoulos, G. D. (1994) J. Neurobiol. 25, 1454–1466
10. Bonni, A., Frank, D. A., Schindler, C., and Greenberg, M. E. (1993) Science 262, 1575–1579
11. Luttrell, C., Wegenka, U. M., Yuan, J., Buschmann, J., Schindler, C., and Ziemiecki, A., Harpur, A. G., Willks, A. F., Yasukawa, K., Taga, T., Kishimoto, T., Barbieri, G., Pellegrini, S., Sendtner, M., Heinrich, P. C., and Horn, F. (1994) Science 263, 89–92
12. Zhong, Z., Wen, Z., and Darnell, J. E. (1994) Science 264, 95–98
13. Heinrich, P. C., Behrman, I., Muller-Newen, G., Schaper, F., and Graeve, L. (1999) Biochem. J. 334, 313–314
14. Schwarzschild, M. A., Dauer, W. T., Lewis, E. S., Hamill, L. K., Fink, J. S., and Hyman, S. E. (1994) J. Neurochem. 63, 1246–1254
15. Boulton, T. G., Stahl, N., and Yancopoulos, G. D. (1994) J. Biol. Chem. 269, 1648–1655
16. Schiemann, W. P., and Nathan, N. M. (1994) J. Biol. Chem. 269, 6376–6382
17. Giordano, V., De Falco, G., Chiari, R., Quinto, I., Pelicci, P. G., Bartholomew, L., Hyman, S. E. (1994) Mol. Cell. Biol. 14, 36013–36020
18. Krieglstein, K., Farkas, L., and Unsicker, K. (1998) Eur. J. Neurosci. 10, 2746–2750
19. Poulsen, J. T., Armanini, M. P., Klein, R. D., Hynes, M. A., Phillips, H. S., and Rosenthal, A. (1994) Neuron 13, 1245–1252
20. Magal, E., Lewis, J. C., Oudega, M., and Vonar, S. (1993) Neuron 4, 779–782
21. Sendtner, M., Arakawa, Y., Stockli, K. A., Kreuzthe, G. W., and Toonen, H. (1991) J. Cell Sci. (Suppl.) 15, 103–109
22. Richardson, P. M. (1994) Pharmcol. Ther. 63, 187–198
23. Krieglstein, K., Pfeifer, L., and Unsicker, K. (1994) J. Neurobiol. 17, 51–59
24. Jones, E. A., Conover, J., and Symes, A. J. (2001) J. Biol. Chem. 276, 36015–36020
25. DeCeuster, M. P., Parks, W. T., Frank, C. J., Castagnino, P., Battaro, D. P., Roberts, A. B., and Lechleider, R. J. (1998) Genes Dev. 12, 1587–1592
26. Ho, S. N., Hunt, H. D., Hort, R. M., Pullen, J. K., and Pease, L. R. (1989) Gene (Amst.) 77, 51–60
27. Zawel, L., Dai, J. L., Buckhaults, P., Zhou, S., Kitsler, K. W., Vogelstein, B., and Kern, S. E. (1998) Mol. Cell. Biol. 1, 611–617
28. Symes, A. J., Rao, M. S., Lewis, E. S., Landis, S. C., Hyman, S. E., and Fink, J. S. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 572–576
29. Brasier, A. R., Tate, J. E., and Habener, J. F. (1989) BioTechniques 7, 1116–1122
30. Symes, A. J., Rajan, P., Conover, J., and Symes, A. J. (2001) J. Biol. Chem. 276, 8068–8075
31. Tsukada, T., Horovitch, S. J., Montminy, M., Mandel, G., and Goodman, R. H. (1985) DNA 4, 293–300
32. Daniels, P. E., Foras-Fetter, S., Brown, M. A., Cavalletta, L., Douglass, J., Milner, R. J., and Sutcliffe, J. G. (1988) DNA 7, 261–267
33. Denoller, S., Ihle, S., Vivien, D., ten Dijke, P., Huet, S., and Gauthier, J. M. (1998) EMBO J. 17, 3091–3100
TGF-β and CNTF Synergistically Induce VIP Gene Transcription

46. Kretzschmar, M., Doody, J., Timokhina, I., and Massague, J. (1999) *Genes Dev.* 13, 804–816
47. de Caestecker, M. P., Yahata, T., Wang, D., Parks, W. T., Huang, S., Hill, C. S., Shiota, T., Roberts, A. B., and Lechleider, R. J. (2000) *J. Biol. Chem.* 275, 2115–2122
48. Jonk, L. J., Itoh, S., Heldin, C. H., ten Dijke, P., and Kruijer, W. (1998) *J. Biol. Chem.* 273, 21145–21152
49. Shi, Y., Wang, Y. F., Jayaraman, L., Yang, H., Massague, J., and Pavletich, N. P. (1998) *Cell* 94, 585–594
50. Johnson, K., Kirkpatrick, H., Comer, A., Hoffmann, P. M., and Laughon, A. (1999) *J. Biol. Chem.* 274, 20709–20716
51. Attisano, L., and Wrana, J. L. (2000) *Curr. Opin. Cell Biol.* 12, 235–243
52. Qing, J., Zhang, Y., and Derynck, R. (2000) *J. Biol. Chem.* 275, 38802–38812
53. Bannister, A. J., and Kouzarides, T. (1995) *EMBO J.* 14, 4758–4762
54. Feng, X. H., Zhang, Y., Wu, R. Y., and Derynck, R. (1998) *Genes Dev.* 12, 2153–2163
55. Horvai, A. E., Xu, L., Korzus, E., Brard, G., Kalafus, D., Mullen, T. M., Rose, D. W., Rosenfeld, M. G., and Glass, C. K. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 1074–1079
56. Janknecht, R., Wells, N. J., and Hunter, T. (1998) *Genes Dev.* 12, 2114–2119
57. Nakashima, K., Yanagisawa, M., Arakawa, H., Kimura, N., Hisatsune, T., Kawabata, M., Miyazeno, K., and Taga, T. (1999) *Science* 284, 479–482
58. Bottner, M., Kriegstein, K., and Unsicker, K. (2000) *J. Neurochem.* 75, 2227–2240