Integration of Jak-Stat and AP-1 Signaling Pathways at the Vasoactive Intestinal Peptide Cytokine Response Element Regulates Ciliary Neurotrophic Factor-dependent Transcription*

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Ciliary neurotrophic factor (CNTF)-dependent induction of expression of the neuropeptide vasoactive intestinal peptide (VIP) gene is mediated by a 180-base pair cytokine response element (CyRE) in the VIP promoter. To elucidate the molecular mechanisms mediating the transcriptional activation by CNTF, intracellular signaling to the CyRE has been studied in a neuroblastoma cell line. It has been shown previously that CNTF induces Stat proteins to bind to a site within the CyRE. CNTF also induces a second protein to bind to a C/EBP-like site within the CyRE. In this report, we show that this inducible CyRE binding protein is composed of the AP-1 proteins c-Fos, JunB, and JunD. These proteins bind to a non-canonical AP-1 site located near the previously characterized C/EBP site. The serine/threonine kinase inhibitor H7 prevents CNTF-dependent induction of AP-1 binding and CyRE-mediated transcription, suggesting that an H7-sensitive kinase is important to mediating CNTF effects on VIP transcription. The integration at the VIP CyRE of the Jak-Stat and AP-1 signaling pathways with other pre-existing proteins provides a cellular mechanism for cell- and cytokine-specific signaling.

Ciliary neurotrophic factor (CNTF) is a member of the neuropoietic cytokine family, which includes interleukin-6, leukaemia inhibitory factor (LIF), oncostatin M, interleukin-11, and cardiotrophin-1 (1–4). These structurally related cytokines utilize a common signal transducing subunit, gp130, leading to tyrosine phosphorylation of a number of intracellular substrates (10, 11). The transmembrane components of the CNTF receptor, gp130 and LIFRβ, have no intrinsic kinase activity (9, 12) but associate with the Jak/Tyk tyrosine kinases (13–15). Activation of these kinases by ligand-induced receptor dimerization is thought to initiate signal transduction and induction of gene expression (13, 16, 17).

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The abbreviations used are: CNTF, ciliary neurotrophic factor; VIP, vasoactive intestinal peptide; gp, base pair(s); CyRE, cytokine response element; LIF, leukemia inhibitory factor; MAPK, mitogen-activated protein kinase; PCR, polymerase chain reaction; EMSA, electrophoretic mobility shift assay; CRE, cAMP response element.
the CyRE is composed of AP-1 proteins and that AP-1 activation represents an H7-sensitive nuclear signaling pathway required for CyRE-mediated transcriptional activation by CNTF.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture reagents were obtained from Life Technologies, Inc. (Gaithersburg, MD), fetal bovine serum was from Sigma, and cultured media were from Life Technologies, Inc. Recombinant human CNTF was a gift from Regeneron Pharmaceuticals (Tarrytown, NY). Oligonucleotides encoding the consensus sites for the transcription factors AP-1, NF-kB, and CREB were purchased from Promega (Madison, WI). The remaining oligonucleotides were synthesized in our laboratory on an Applied Biosystems 380B DNA synthesizer. Antisera to c-Jun, c-Fos, fosB, and an epitope common to all Jun family members were from Santa Cruz Biotechnology (Santa Cruz, CA), Antisera to JunB and JunD were a gift of Dr. Rodrigo Bravo (Bristol Myers Squibb, NJ). H7 was purchased from Calbiochem (La Jolla, CA).

**Cell Culture and Transfection**—NBFL cells were maintained and transfected as described previously (31). Cells were transfected by the calcium phosphate co-precipitation method. Each 10-cm plate received 20 μg of luciferase reporter plasmid and 3 μg of RSV CAT plasmid. Cytokine and/or inhibitors were added 24 h after transfection; cells were harvested 6 or 36 h later and assayed for luciferase (35) and CAT (36) activities. Nuclear protein extract was normalized to CAT activity to control for transfection efficiency.

**Plasmid—**CyLuc contains the entire 180-bp CyRE fused to ΔEVSuc (92). mCy2Luc was constructed by PCR site-directed mutagenesis as described by Ho et al. (37) with the oligonucleotides 5′-GGTAAC-TGGATTAGAAATACATCAGAAGCTCCGG-3′ and 5′-CCTGCTAT-GCTTAAATGTTATTCTTACTACGTTACCC-3′. These oligonucleotides were paired with oligonucleotide A1 or A4 (32), with CyLuc as a template, to create new fragments. The fragments were gel-purified and used as template in a subsequent PCR, with oligonucleotides A1 and A4 as primers, to create mCy2Luc. CyLucG3Luc was constructed by PCR amplification of CyLuc with the primers 5′-CCGGTGACTCCATAAAGG-GATGTACTTGATTAAGCCAGGACTCCTGGA-3′ and A4. The resultant 180-bp fragments were digested with KpnI and PstI gel purified, and ligated into KpnPstI-digested ΔEVSuc (32) to create plasmids containing the mutant sites. Both plasmids were sequenced to confirm their fidelity.

**Electrophoretic Mobility Shift Assay (EMSA)—**Synthetic complementary oligonucleotides with GGG or GATC overhangs were annealed and labeled with [α-32P]dCTP using Superscript reverse transcriptase (Life Technologies, Inc.) or Klenow fragment. AP-1 oligonucleotide had no overhang and was therefore labeled with [γ-32P]ATP using T4 polynucleotide kinase. Nuclear extracts were isolated and binding reactions were performed as described previously (32). Nuclear extracts (approximately 15 μg of protein) were incubated with 0.5 ng of labeled probe (approximately 200,000 cpm) for 20 min at room temperature before electrophoretic separation on a 5% non-denaturing polyacrylamide gel (37.5:1) in 0.5 × Tris-borate-EDTA at 200 V. Antibodies, when used, were added 10 min prior to the addition of the probe. The following pairs of complementary oligonucleotides were used in DNA mobility shift assays (mutated residues are underlined): NF-κB, 5′-AGTTGAGGGG-ACCTTTCAGGCGG-3′ and 3′-TCATACTCCCCCTGGAAAGGTTCCGGG-5′; CyB, 5′-GGGAAATATGTATAGCATAG3′ and 3′-TTTTATCTAAT-TCTGATGCGG-5′; mCyB, 5′-GGGAAATACTACATGAGGCCC-3′ and 3′-TTTTATCTAAT-TCTGATGCGG-5′; m2CyB, 5′-GAGTTGAGGGG-ACCTTTCAGGCGG-3′ and 3′-TTTTATCTAAT-TCTGATGCGG-5′; m3CyB, 5′-GGGAAATATGTATAGCATAG3′ and 3′-TTTTATCTAAT-TCTGATGCGG-5′. The rapid induction and protein synthesis dependence of the induction suggested that the protein complex may be composed of immediate early gene products such as AP-1 proteins.

To test the hypothesis that AP-1 proteins are present in CyB complex III, the CyB DNA-protein complexes were competed with excess unlabeled consensus AP-1 binding sites. Complex IV was competed by excess unlabeled AP-1, CRE, and CyB oligonucleotides (Fig. 1). In contrast, the uninduced complex III is competed only by excess cold CyB oligonucleotide (Fig. 1). These results suggest that complex III and complex IV are composed of distinct nuclear proteins and that proteins that bind to AP-1 and CRE oligonucleotides also bind to the CyB site.

**CyB site binds two protein complexes from NBFL cells. The more slowly migrating DNA-protein complex (referred to previously as complex III) is present in nuclear extract from untreated NBFL cells. The faster migrating complex (complex IV) is only present in nuclear extracts prepared from NBFL cells that have been treated with CNTF, LIF, or OM. Neither of these nuclear protein complexes is recognized by antibodies against known C/EBP proteins. Complex IV is bound to the CyB probe within 1 h of CNTF treatment. This induction is inhibited by the protein synthesis inhibitor cycloheximide.**

**CyB probe within 1 h of CNTF treatment.**

**FIG. 1.** Competition of inducible binding to the CyB probe. DNA mobility shift assay with nuclear extracts prepared from NBFL cells treated for 1 h with CNTF (25 ng/ml). Arrows indicate complex III, whose binding to the CyB probe is unaffected by CNTF treatment, and complex IV, which is inducible to bind to the CyB probe by CNTF treatment (33). Competing unlabeled oligonucleotides were present at 10 and 100-fold molar excess. Complex IV is competed by CyB, CRE, and AP-1 oligonucleotides, but the uninduced complex III is only competed by CyB.
specific to JunB and JunD were added to the AP-1 binding reaction, the more slowly migrating complex (“supershift”) was formed (Fig. 4). The addition of c-Fos antisera to the m3CyB EMSA inhibited complex IV binding. Antisera to c-Jun and fos-B did not affect complex IV binding to the m3CyB probe. Similar results were obtained when the native CyB site was used as a probe (data not shown). Thus, CNTF induces an AP-1 protein complex to bind to the CyB site within the VIP CyRE. This AP-1 complex contains c-Fos, JunB, and JunD.

To characterize the differences between m3CyB and a consensus AP-1 site, the binding of CNTF-induced complex IV to both probes was examined. Complex IV binding to m3CyB was competed with equal efficiency by itself, AP-1, or CRE-unlabeled oligonucleotide. In contrast, CNTF-induced AP-1 binding to the consensus AP-1 site was most strongly competed by unlabeled AP-1 and most weakly by m3CyB (Fig. 4B). These data suggest that AP-1 proteins have a lower affinity for the m3CyB site than for a typical AP-1 site. The mobilities of the CNTF-induced AP-1 complex to the AP-1 consensus site, the CyB, and the m3CyB probe were identical (data not shown). Therefore, the m3CyB mutation was used to assess the importance of the AP-1 complex to CNTF-mediated transcriptional activation through the CyRE. Mutation of the CyB AP-1 sequence within the CyRE reduced CNTF-mediated induction of luciferase by 50% compared with the native CyRE contained in the Cy1luc reporter (Fig. 5). These data demonstrate that the AP-1 site within the CyB region of the CyRE is required for CNTF-dependent transcriptional activation mediated by the CyRE and further suggest that AP-1 protein binding to this site is important to this activation. However, a luciferase reporter containing three multimerized copies of the CyB site directing transcription from a basal promoter was not induced by CNTF (data not shown). Therefore, the CyB AP-1 site appears to act in concert with other sites within the CyRE to mediate the full CNTF-dependent transcriptional activation mediated by the CyRE.

We next sought to determine whether the AP-1 site within the CyRE was important to the CNTF-dependent activation of transcription mediated by the 180-base pair CyRE linked to the luciferase reporter. A luciferase reporter plasmid was constructed that had three base pairs of the CyB AP-1 site mutated to produce the m2CyB site (Fig. 2) within the context of the CyRE (m2CyBluc). The m2CyB mutation did not bind the CNTF-induced AP-1 proteins (Fig. 2) or compete for binding of these proteins to the CyB probe in competition assays (data not shown). However, the m2CyB mutation did not alter the binding of the non-inducible complex III proteins (Fig. 2). Therefore, the m2CyB mutation was used to assess the importance of the AP-1-like CyB site was a location at which the H7 sensitivity of
CNTF-dependent VIP transcriptional activation was mediated, we determined whether AP-1 induction and binding to the CyRE was inhibited by H7 treatment. We first examined the kinetics and sensitivity of CNTF-mediated induction of mRNA encoding the immediate early genes c-fos and jun-B, which comprise the AP-1 proteins binding to the CyB site. H7 treatment abolished CNTF-mediated c-fos mRNA induction and significantly attenuated jun-B mRNA induction, also delaying its induction (Fig. 6A). H7 pretreatment of NBFL cells also attenuated CNTF-dependent AP-1 binding (Fig. 6). H7 treatment alone did not induce binding to the AP-1 site. Similar results were obtained using the CyB site as a probe (data not shown). Thus, H7 inhibits the CNTF-mediated induction of c-fos and jun-B mRNA and formation of the AP-1 complex, suggesting that the CyB AP-1 site is a possible location through which H7 inhibits the CNTF-mediated induction of VIP mRNA.

Multiple sites within the 180-bp CyRE or sites outside the CyRE in the VIP promoter are potential sites of H7 inhibition. To determine whether H7 inhibited the CNTF-mediated induction of transcription through the VIP CyRE, NBFL cells transfected with the CyRE-luciferase reporter Cy1luc were treated with H7, and the effect on CNTF induction was assessed. H7 completely inhibited CNTF induction of luciferase activity mediated by Cy1luc (Fig. 7) without significantly reducing luciferase activity driven by RSVluc (data not shown). To ascertain whether H7-mediated repression of CNTF-dependent transcriptional activation was mediated by the CyB AP-1 site, NBFL cells were transfected with the m2CyBluc reporter, and the effect of H7 on CNTF-induced transcription was assessed. If H7 acted through the CyB AP-1 site, then mutation of this site should abrogate the H7 inhibition. Similar to its effect on the native CyRE, however, H7 completely inhibited CNTF induction of transcription mediated by a CyRE containing a mutation at the CyB AP-1 site (m2CyBluc; Fig. 7). This indicates that the ability of H7 to inhibit CNTF-dependent transcriptional activation depends on other sites within the CyRE.

As serine-threonine phosphorylation has been shown to affect the ability of STAT proteins to activate transcription (40), H7 could influence transcriptional activation by CNTF by altering serine-threonine phosphorylation of STAT proteins. We have shown that STAT proteins contribute to the CNTF-de-
a non-canonical AP-1 site. The two nucleotides that distinguish the CyB AP-1 and canonical AP-1 sites have been shown by mutational analysis to be critical for AP-1 binding (42) although AP-1 proteins can bind to this sequence in other genes (43). The sequence difference between the CyB AP-1 site and the canonical AP-1 site may be reflected in the differences in affinities of AP-1 proteins for these sites (Fig. 4).

There are two general cellular mechanisms by which CNTF may activate AP-1. Expression of the Fos-Jun components of the AP-1 complex may be induced, or phosphorylation of Fos and Jun proteins by CNTF-activated kinases may lead to a more transcriptionally active AP-1 complex. In NBF1 cells, we have shown that CNTF induces c-fos and JunB mRNA (Fig. 6A and Ref. 30) and that CNTF induction of AP-1 binding is protein synthesis-dependent (33). The pathways that lead to induction of IEG transcription are complex and involve multiple pre-existing transcription factors such as STATs, serum response factor, and CREB (44–48). STAT proteins themselves appear to interact with the c-fos promoter to contribute to c-fos induction by CNTF (19). Similar pathways may activate transcription of the Jun-B gene (49) in response to CNTF or related cytokines.

The intracellular signaling pathways that lead to regulation of AP-1 activity have been partially characterized (50). The MAP kinases, JNK, ERK, and FRK increase c-Fos and c-Jun synthesis and phosphorylation, thereby increasing AP-1 binding and activation (51–53). These kinases are, therefore, candidate molecules for mediating the CNTF-induced activation of AP-1 in NBF1 cells. While it has been shown that neurotrophic cytokines activate ERK 1 and 2 in several different cell types (10, 25, 26), it is not known whether other MAPKs are activated by these cytokines and act to regulate gene expression. Consistent with the involvement of MAPKs in CNTF gene regulation, we have previously shown that CNTF activates Ras, an upstream regulator of MAPKs, in NBF1 cells (24). CNTF-mediated induction of AP-1 binding is also partially sensitive to H7, consistent with the role of MAPK or other serine-threonine kinases in AP-1 activation in response to CNTF.

Inhibition of serine-threonine kinases by H7 may not only inhibit AP-1 activation but may regulate the Jak-Stat pathway directly. Serine-threonine phosphorylation of STATs can increase the ability of these proteins to activate transcription and bind DNA (54). STAT proteins contain a MAPK phosphorylation site and demonstrate ligand-dependent association with MAPK that is required for interferon β-dependent activation of STATs (55). Therefore, H7 may attenuate CNTF-dependent transcription by inhibiting MAPK phosphorylation of Stat proteins and limiting their ability to activate transcription. In NBF1 cells, H7 does not affect CNTF-induced Stat binding detected by EMSA (32), but serine-threonine phosphorylation of Stat3 may enhance its ability to activate transcription without a change in the amount of Stat binding (54).

The sensitivity of CNTF-induction of VIP mRNA to the serine/threonine kinase inhibitor H7 (24) supports a role of kinases, such as MAPKs or protein kinase C, in CNTF-mediated induction of VIP gene expression. We show here that this H7 sensitivity of CNTF-dependent induction of VIP mRNA in NBF1 cells is mediated, at least in part, through the CyRE (Fig. 7). H7 does not appear to inhibit transcription one of the CyRE sites (STAT and CyB AP-1 sites) that we have shown to bind inducible proteins (Stat1, Stat3, and AP-1) in response to CNTF. Instead, H7 may be acting at a point further upstream in CNTF-mediated nuclear signaling, inhibiting a kinase or kinases which have effects on multiple regions of the CyRE. If H7 sensitive kinases are acting through both the AP-1 and Stat sites, then the transcriptional activity remaining when one site
is mutated would be abolished by H7 effects at the other. If these effects are mediated by two separate H7-sensitive kinases, then use of more specific kinase inhibitors than H7 may assist in clarifying this issue. Alternatively, H7-sensitive kinases may affect proteins binding to regions of the CyRE other than the AP-1 and STAT sites, affecting activity of a transcription factor we have not yet identified.

The mechanism by which Jak-Stat and AP-1 intracellular signals integrate to regulate CNTF transcription through the CyRE is not known. AP-1 and STAT transcription factors, which appear to be required for full CNTF-dependent transcription by the CyRE, may directly interact to activate transcription (56). A direct interaction between c-Jun and a STAT protein (Stat3β) binding to adjacent AP-1 and STAT sites in the IL6-responsive element of the α2 macroglobulin gene promoter has been postulated to result in synergistic transcriptional activation (56). Direct protein-protein interaction between STATs and AP-1 proteins could stabilize protein-DNA binding complex formation and contribute to CNTF-mediated transcriptional activation in NBFL cells. However, there is approximately 100 bp separating the Stat and AP-1 sites with many other proteins binding to intermediate sites (32, 41). Therefore, the integration of these two signaling pathways may be through complex formation with other basal or cell-specific factors.

The composition of the AP-1 complex with different members of the Fos/Jun family may have important biological consequences (57, 58). Jun family members have similar DNA binding and dimerization domains but differ in their activation domains (59, 60). JunB and JunD are weaker transcriptional activators than c-Jun and may antagonize its function (59, 61, 62). The JunB gene is often activated during differentiation, in contrast to the c-Jun gene that is induced during proliferation and cell death (57, 58, 63). Fos and JunB genes are often co-induced in the nervous system in response to a variety of extracellular signals (64, 65). It has been previously shown that neuroepoietic cytokines induce the c-Fos and JunB genes in several types of transformed and primary cells (7, 23, 27, 30, 66). In NBFL cells, cytokine treatment induced an AP-1 complex that contained c-Fos, JunB, and JunD. CNTF does not produce a proliferative response in NBFL cells but induces several neuroepoietic genes that mimic the cytokine-mediated differentiation of sympathetic neurons (31, 67). Thus, induction of AP-1 proteins (JunB, in particular) in NBFL cells by CNTF is another example of the participation of this transcriptional activator in differentiation-related process.

A model summarizing CNTF-induced signaling to the VIP CyRE is shown in Fig. 8. AP-1 and Stat proteins are induced by CNTF treatment to bind to sites in the VIP CyRE. Stat proteins also bind to the promoters of immediate early genes and may activate AP-1 proteins (JunB, in particular) in NBFL cells by CNTF.

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