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Fibroblast Growth Factor and Cyclic AMP (cAMP) Synergistically Activate Gene Expression at a cAMP Response Element

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Growth factors and cyclic AMP (cAMP) are known to activate distinct intracellular signaling pathways. Fibroblast growth factor (FGF) activates *ras*-dependent kinase cascades, resulting in the activation of MAP kinases, whereas cAMP activates protein kinase A. In this study, we report that growth factors and cAMP act synergistically to stimulate proenkephalin gene expression. Positive synergy between growth factor- and cAMP-activated signaling pathways on gene expression has not been previously reported, and we suggest that these synergistic interactions represent a useful model for analyzing interactions between these pathways. Transfection and mutational studies indicate that both FGF-dependent gene activation and cAMP-dependent gene activation require cAMP response element 2 (CRE-2), a previously characterized cAMP-dependent regulatory element. Furthermore, multiple copies of this element are sufficient to confer FGF regulation upon a minimal promoter, indicating that FGF and cAMP signaling converge upon transcription factors acting at CRE-2. Among many different ATF/AP-1 factors tested, two factors, ATF-3 and c-Jun, stimulate proenkephalin transcription in an FGF- or Ras-dependent fashion. Finally, we show that ATF-3 and c-Jun form heterodimeric complexes in SK-N-MC cells and that the levels of both proteins are increased in response to FGF but not cAMP. Together, these results indicate that growth factor- and cAMP-dependent signaling pathways converge at CRE-2 to synergistically stimulate gene expression and that ATF-3 and c-Jun regulate proenkephalin transcription in response to both growth factor- and cAMP-dependent intracellular signaling pathways.

Growth factors such as fibroblast growth factor (FGF) are known to initiate receptor autophosphorylation on tyrosine residues and the binding of SH2 domain proteins, which in turn activate various effectors such as c-Ras (49, 58). Growth factor signaling downstream of Ras is known to involve activation of a protein kinase cascade including c-Raf, MEK, and mitogen-activated protein (MAP) kinases (26, 62, 64). In contrast, many neurotransmitters and hormones are known to stimulate cyclic AMP (cAMP) production and the activation of protein kinase A (PKA). Numerous studies indicate that growth factors and cAMP stimulate distinct intracellular signaling pathways, yet it has been long known that cAMP can stimulate (18, 53) or inhibit (16, 53, 65) growth and proliferation in a cell-specific manner. In a similar fashion, although neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) are synthesized and released from central nervous system neurons together with neurotransmitters (5, 66), little is known regarding their interaction and cumulative effects on target cells. Several recent studies suggest that inhibitory cross talk between the cAMP and growth factor pathways may occur via cAMP-dependent phosphorylation of c-Raf and the subsequent down regulation of MEK and MAP kinase activities (16, 28, 54, 65). However, it is currently unclear how cAMP may act to stimulate the mitogenic and neurotrophic responses of growth factors. In this study, we identify and characterize a novel positive synergy between growth factor- and cAMP-dependent signaling pathways on neurotransmitter gene expression.

The regulation of neurotransmitter expression is an important determinant of neural phenotype and signaling. Multiple cell-cell signaling events involving neurotransmitters, cytokines, and growth factors are known to regulate transmitter gene expression, and this process is thought to represent an important control point in the regulation of neural signaling (4, 46, 55). Neurotransmitters and growth factors are known to regulate expression of the gene encoding proenkephalin, the precursor to the opioid peptides Met- and Leu-enkephalin (1). During development, proenkephalin is expressed in nondifferentiated cells of diverse mesodermal lineage, and in the adult, expression is found in postmitotic neurons and various proliferating cells, including immune cells (68), male and female reproductive cells (38, 39, 52), and glial cells (44, 57). Although the growth factors and cell-cell interactions involved in regulating proenkephalin expression in proliferating cells are not well defined, it has been shown that growth factors present in serum stimulate proenkephalin expression in mesenchymal progenitor cells (37, 50). Growth and neurotrophic factors may also play an important role in the induction of proenkephalin gene expression in the developing and adult nervous system. For example, FGF is known to regulate the differentiation of central nervous system progenitor cells to neurons and glia (3), and in the adult, proenkephalin RNA expression is rapidly induced in the hippocampus (60), where combinations of growth factors, cytokines, and neurotransmitters are released in response to neural stimulation. Little is currently known regarding the role of growth and neurotrophic factors and their interactions with other signaling pathways in the regulation of neurotransmitter phenotype and signaling.

Transcription of proenkephalin is activated by multiple intracellular signaling pathways (13, 14, 48) which activate transcription via an inducible enhancer composed of multiple-

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cooperative DNA elements (11, 13, 14). Mutational analysis of this enhancer indicates that the most critical region is cAMP response element 2 (CRE-2), which binds AP-1 and ATF/CREB transcription factors (12, 35, 40, 60). Although the precise identities of the factors interacting with CRE-2 are currently unknown, multiple factors, including c-Jun, ATF-3, CREB, and JunD (12, 35, 40), are known to bind the CRE-2 site and to activate transcription, while others, such as JunB, repress expression (10, 40).

In this article, we report that FGF- and Ras-dependent pathways act synergistically with the cAMP-dependent pathway to activate proenkephalin gene expression in a human neuroblastoma cell line. Mutational analysis indicates that FGF and Ras activation requires CRE-2, and multiple copies of this element confer FGF regulation upon a minimal promoter, suggesting that growth factor- and cAMP-dependent pathways converge upon transcription factors acting at CRE-2. Furthermore, we demonstrate by using transfection assays that both ATF-3 and c-Jun, but not other ATF/AP-1 factors, stimulate proenkephalin transcription in an FGF- or Ras-dependent fashion at CRE-2. Together, these results indicate that growth factor- and cAMP-activated signaling pathways converge synergistically to stimulate proenkephalin gene expression at a well-defined CRE and suggest a role for an ATF-3/c-Jun heterodimeric complex in this response.

MATERIALS AND METHODS

Materials. Forskolin, 3-isobutyl-1-methyl-xanthine (IBMX), chlorophenylthioadenosine 3′,5′-cyclic monophosphate (cpt-cAMP), and *N*-butyryl coenzyme A were from Sigma. Basic FGF, fetal calf serum (FCS), and mammalian cell culture media were from GIBCO BRL. Protein A–Sepharose CL-4B beads were from Pharmacia. Rabbit reticulocyte lysates were from Promega. [^{35}S]methionine and $^{32}P_i$ were from ICN. [^{3}H]chloramphenicol was from NEN Research Products. c-Jun and JunB antibodies were from Oncogene Science. CREB antibody was a gift from David Ginty.

Cell culture. SK-N-MC neuroblastoma cells and JEG cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (vol/vol) FCS. Primary cultures of rat cortical type I astrocytes were isolated from postnatal day 1 to 2 rat pups, and cultured in DMEM-F12 with 10% (vol/vol) FCS as previously described (44).

Plasmid and DNA constructions. The following proenkephalin promoter-chloramphenicol acetyltransferase (CAT) constructs have been previously described: pENKAT-12 (13), pENKCRE-1, pENKCRE-2 (48), pENKM88, pENKM89, pENKM95 (14), pRSVATF-3 (12), pRSVJunB, pRSVJunD (40), pRSVCREB (27), pRSVc-fos (45), pMTCαNeo (63), pRSVLuc (43), oncogenic Ras (56), Ha-Ras (Asn-17) (21), and pGEXATF-3 (12). The multicopy CAT reporter constructs pENKCRE-1, pENKCRE-2, and pENKCRE-1+2 each contain six copies of oligonucleotides encoding ENKCRE-1, ENKCRE-2, and ENKCRE-1+2, respectively, fused to pENKAT-Δ84 as previously constructed (48) (depicted in Fig. 3a). pRSVc-Jun was constructed by inserting a 1,200-bp *Sac*I-*Eco*RV fragment containing the entire coding region of human c-Jun (7) into pRSV-SG cut with *Bgl*II (filled in) and *Sac*I. pGEXJunD was constructed by in-frame cloning of a PCR-amplified fragment of JunD (containing the amino-terminal 100 amino acids of JunD with 5′-*Eco*RI-*Hin*dIII-3′ restriction endonuclease sites) into pGEX-KG (29). The fragment was amplified by using the following 34-mer and 33-mer primers: 5′-TTAGCGAATTCTGATGGAAACGCCCTTCTATGGC-3′ and 5′-ACTCGGAAGCTTGGCTCAGTACGCCGGGACCT G-3′.

Northern (RNA) analysis. Total RNA was isolated from primary cultures of rat cerebral cortex type I astrocytes and separated by electrophoresis on 1.5% (wt/vol) denaturing agarose gels as previously described (44). Proenkephalin mRNA was detected by hybridization against a ^{32}P-labeled 1.1-kb *Eco*RI-*Hin*dIII fragment of the rat proenkephalin cDNA (67) as previously described (44).

Transient transfections and CAT assay. Transient transfection of SK-N-MC cells was performed as previously described (11, 12) with various amounts of expression plasmid and pRSVLuc as described in the figure legends. The total amount of DNA transfected was maintained at 25 μg with pGEM3. Following calcium phosphate transfections, cells were glycerol shocked and incubated for 16 h in medium containing 10% FCS. Cells were treated with forskolin (10 μM), IBMX (0.5 mM), or FGF (25 ng/ml) for 6 h. Cells were lysed, and CAT (11, 14) or luciferase (9) activity was determined as previously described.

Bacterial expression of GST fusion proteins and generation of antibodies. Glutathione *S*-transferase (GST) fusion proteins were expressed in *Escherichia coli*, induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and purified with glutathione-agarose beads as previously described (12, 59). Purified GST fusion proteins were used for the generation of specific polyclonal antisera in rabbits.

Cell labeling and immunoprecipitation. Cell labeling and immunoprecipitations were performed as previously described (41), with the following modifications. Prior to labeling experiments, 10^6 SK-N-MC cells were cultured in DMEM with 0.5% (vol/vol) FCS for 24 h, rinsed twice, and incubated with DMEM (minus methionine) containing 0.5% (vol/vol) FCS for 1 h. The cells were then labeled by the addition of 800 μCi of [^{35}S]methionine per ml of methionine-free medium or 1.5 mCi of $^{32}P_i$ per ml of phosphate-free medium for 4 h. Cells were lysed in 0.4 ml of either nondenaturing lysis buffer (50 mM Tris-HCl [pH 7.5], 0.6 mM NaCl, 1% [vol/vol] Triton X-100, 10 mM EDTA, 0.02% [wt/vol] sodium azide, 0.25 mM phenylmethylsulfonyl fluoride [PMSF], 1 μg [each] of aprotinin, leupeptin, and pepstatin per ml) or denaturing lysis buffer (50 mM Tris-HCl [pH 7.5], 0.5% [wt/vol] sodium dodecyl sulfate [SDS], 70 mM β-mercaptoethanol). Lysates were either mixed for 30 min at 4°C (nondenaturing lysis) or boiled for 10 min (denaturing lysis) before dilution with 4 volumes of either nondenaturing lysis buffer without NaCl (nondenaturing lysis) or RIPA buffer without SDS (10 mM Tris-HCl [pH 7.5], 1% [wt/vol] sodium deoxycholate, 1% [vol/vol] Nonidet P-40, 150 mM NaCl, 0.02% [wt/vol] sodium azide, 0.25 mM PMSF, 1 μg [each] of aprotinin, leupeptin, and pepstatin per ml) (denaturing lysis). Lysates were sequentially incubated at 4°C with 40 μl of protein A-Sepharose beads for 3 h, preimmune sera overnight, 40 μl of protein A-Sepharose beads for 3 h, specific antisera overnight, and 40 μl of protein A-Sepharose beads for 3 h. The beads were washed twice with buffer A (10 mM Tris-HCl [pH 7.5], 10 mM EDTA, 0.2% [vol/vol] Nonidet P-40, 0.25 mM PMSF) containing 150 mM NaCl, once with buffer A containing 500 mM NaCl, and once with buffer A (minus Nonidet P-40 and EDTA) before being boiled in denaturing lysis buffer for 10 min. For sequential immunoprecipitations, lysates were diluted with 4 volumes of RIPA buffer without SDS, incubated with specific secondary antisera overnight at 4°C, with 40 μl of protein A-Sepharose beads, and washed as described above. Immunoprecipitated proteins were separated by SDS–12% polyacrylamide gel electrophoresis (PAGE) and visualized by autoradiography with Kodak film.

In vitro transcription and translation. pBSATF-3 (12) and pBSc-Jun (7) were linearized with *Bam*HI and *Kpn*I, respectively, before mRNAs were transcribed in vitro with T3 RNA polymerase and proteins were translated in vitro with rabbit reticulocyte lysates in the presence of [^{35}S]methionine as recommended by Promega. Translation products were analyzed by SDS-PAGE. Coimmunoprecipitation of in vitro-translated proteins was performed as follows. Samples (2 μl) of [^{35}S]methionine-labeled in vitro-translated ATF-3 and c-Jun proteins were incubated at 37°C for 15 min, diluted with RIPA buffer, and incubated with specific antisera against ATF-3 or c-Jun at 4°C for 1 h. Immunoprecipitated proteins were incubated with 40 μl of protein A-Sepharose beads at 4°C before electrophoretic separation on SDS–12% PAGE gels and visualization by autoradiography with Kodak film.

Vaccinia virus expression. The complete coding regions of human ATF-3 and human c-Jun were amplified by PCR and cloned in front of the T7 promoter in the pTM3 expression vector (47) to produce pTMATF-3 and pTMc-Jun, respectively. PCRs were limited to 15 cycles by using 200 ng of RSVATF-3 or RSVc-Jun template to minimize PCR-induced mutations. To overexpress ATF-3 and c-Jun in mammalian cells as controls for DNA binding experiments, human JEG cells were transfected with 30 μg of pTMATF-3 or pTMc-Jun or 15 μg of each plasmid and then transfected cells were infected with a vaccinia virus strain expressing the T7 RNA polymerase (vTF7-3) as described previously (19). At 24 h after transfection, cells were harvested and whole-cell extracts were prepared.

DNA gel mobility shift assay. A double-stranded oligonucleotide spanning the proenkephalin CRE-2, GATCGGCCTG CGTCAGCTG, was labeled by using Moloney murine leukemia virus reverse transcriptase and [^{32}P]dCTP at 37°C for 1 h. SK-N-MC cells were maintained in DMEM containing 0.5% fetal bovine serum for 2 days. Two hours after FGF or forskolin treatment, cells were chilled to −80°C for 20 min and lysed in 200 μl of lysis buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 25% glycerol, 0.5 M KCl, 1.5 mM $MgCl_2$, 0.4 M EDTA, 0.5 mM dithiothreitol, 0.5 mM PMSF, 1 mM NaF, 5 μM microcystin). JEG cells overexpressing ATF-3 or c-Jun were lysed in the same lysis buffer. Protein concentration was determined by using the Bio-Rad assay, and DNA binding was carried out by using 5 μg of whole-cell extract, 2 ng of labeled CRE-2 oligonucleotide, 150 ng of poly(dI-dC), 50 mM KCl, and 1× buffer D (10 mM HEPES [pH 7.9], 10% glycerol, 0.1 mM EDTA, 2 mM $MgCl_2$, 2 mM dithiothreitol, 0.5 mM PMSF) in a final volume of 15 μl for 10 min at room temperature. Preincubations with antiserum were for 20 min prior to addition of ^{32}P-labeled CRE-2 probe. To resolve CRE-2 binding proteins, reaction mixtures were fractionated on 5% native polyacrylamide gels in 0.5× TBE buffer (45 mM Tris-borate, 1 mM EDTA) at 4°C at 200 V for 4 h.

RESULTS

FGF and cAMP act in synergy to stimulate proenkephalin gene expression. In agreement with previous studies (44, 57), primary cultures of type I astrocytes prepared from rat cerebral cortex tissue expressed proenkephalin mRNA (Fig. 1A). Treatment of type I astrocytes with either 10 μM forskolin or 25 ng of FGF per ml for 4 h resulted in only small changes in the level of proenkephalin mRNA. However, in the presence of both forskolin and FGF, proenkephalin mRNA levels were increased beyond the sum of the individual effects (Fig. 1A), suggesting that growth factor- and cAMP-stimulated pathways

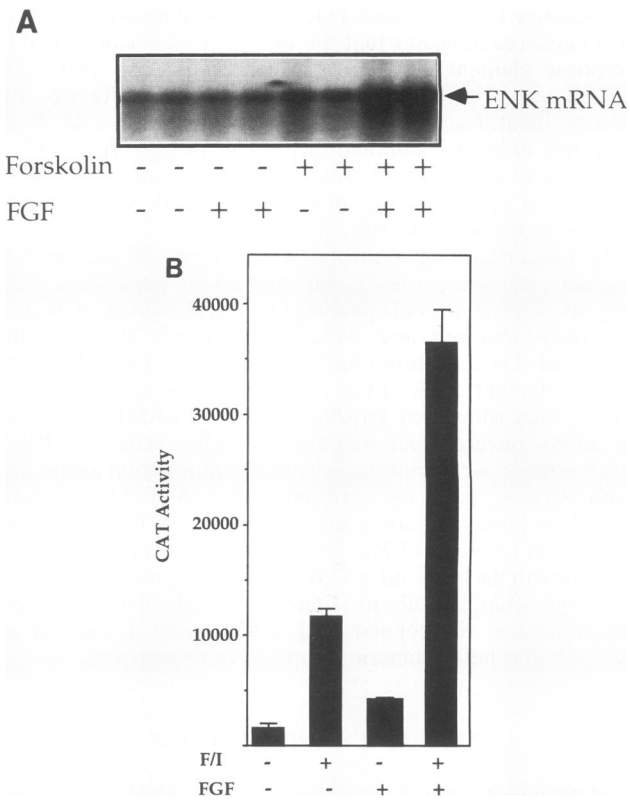

FIG. 1. (A) FGF and forskolin act synergistically to stimulate proenkephalin mRNA levels in primary cultures of rat cerebral cortex type I astrocytes. Total cytoplasmic RNA (20 μg per lane) was isolated from astrocytes treated for 4 h with either 10 μM forskolin and 0.5 mM IBMX or 25 ng of basic FGF (bFGF) per ml. The arrow indicates the position of the 1.45-kb proenkephalin RNA transcript. The proenkephalin probe was a ^{32}P-labeled 1.1-kb *Eco*RI-*Hin*dIII fragment of the rat proenkephalin cDNA (67). To control for variations in RNA loading and transfer, the membrane was rehybridized with a cyclophylin probe (data not shown), which indicated that equal amounts of RNA were present in each lane. (B) FGF and cAMP act in synergy to stimulate expression of a proenkephalin-CAT fusion gene in the human neuroblastoma cell line SK-N-MC. SK-N-MC cells were cotransfected with 2.5 μg of pENKAT-12 reporter gene and 2 μg of pRSVLuciferase. At 18 h posttransfection, cells were treated with 10 μM forskolin and 0.5 mM IBMX (F/I) or 25 ng of bFGF per ml (FGF) as indicated for 6 h. The total amount of DNA was maintained at 25 μg with pGEM-3. CAT activities were normalized to the level of pRSVLuciferase expression. The mean CAT activities ± standard deviations (SD) for three independent experiments using duplicate plates are indicated.

interact synergistically to activate proenkephalin gene expression.

To further characterize the effects of growth factors and cAMP on the activation of proenkephalin gene expression, we introduced a proenkephalin promoter-CAT fusion gene, pENKAT-12 (13), into human SK-N-MC neuroblastoma cells and examined the effects of activation of FGF- and cAMP-dependent signaling pathways on CAT expression. These cells express proenkephalin and have served as a model system for examination of cAMP-dependent gene regulation (12, 22). As shown in Fig. 1B, forskolin in the presence of the phosphodiesterase inhibitor IBMX produces a 13-fold increase in CAT activity (Fig. 1B, lane 2). Treatment with FGF stimulates CAT expression threefold (Fig. 1B, lane 3). As described above for primary cultures of rat astrocytes, FGF and forskolin added

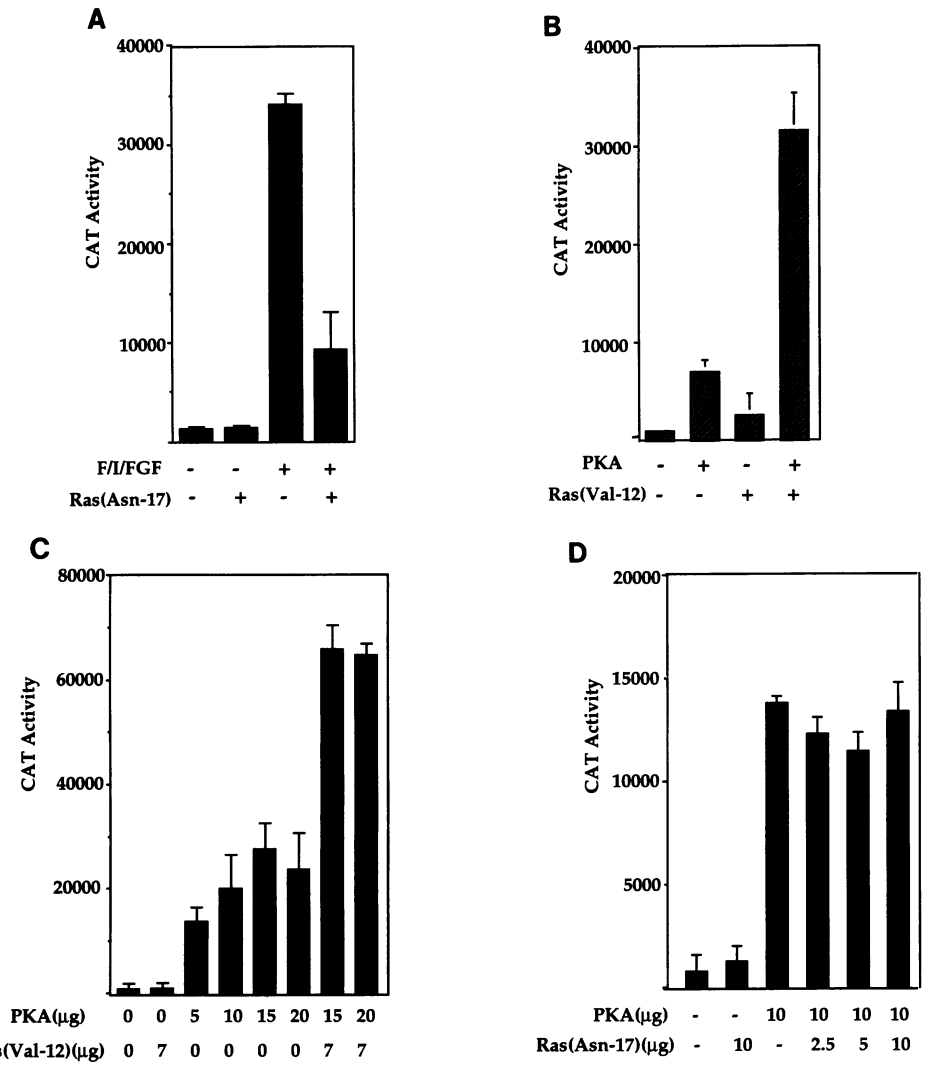

FIG. 2. FGF stimulates proenkephalin gene expression via a Ras-dependent pathway. (A) FGF stimulation is blocked by a dominant inhibitory Ha-Ras (Asn-17) mutant. SK-N-MC cells were cotransfected with 2.5 μg of pENKAT-12 and 2 μg of pRSVLuciferase in the presence or absence of 10 μg of a plasmid expressing a dominant inhibitory Ha-Ras mutant, Ha-Ras (Asn-17). At 24 h posttransfection cells were treated with 10 μM forskolin and 25 ng of bFGF per ml for 6 h and harvested, and CAT and luciferase activities were determined. The mean CAT activities ± SD for three independent experiments using duplicate plates are indicated. (B) Oncogenic Ras, Ha-Ras (Val-12), activates proenkephalin gene expression in a cAMP-dependent fashion in SK-N-MC cells. SK-N-MC cells were cotransfected with 2.5 μg of pENKAT-12 in the presence of 2 μg of pMTCneo (catalytic subunit of PKA), or 7 μg of pEJ-6.6, expressing Ha-Ras (Val-12), as indicated. At 24 h posttransfection cells were harvested, and CAT and luciferase activities were determined. The mean CAT activities ± SD for three independent experiments using duplicate plates are indicated. (C) Ras stimulates proenkephalin gene expression when the cAMP pathway is maximally stimulated. SK-N-MC cells were cotransfected with 2.5 μg of pENKAT-12 and 2 μg of pRSVLuciferase in the presence of increasing amounts of pMTCneo (as indicated by the number below each lane). Under conditions of maximal PKA activation, oncogenic *ras* (7 μg of pEJ-6.6) further stimulates proenkephalin gene expression. At 24 h posttransfection cells were harvested, and CAT and luciferase activities were determined. The mean CAT activities ± SD for three independent experiments using duplicate plates are indicated. (D) Expression of the dominant inhibitory *ras* [Ha-Ras (Asn-17)] has little effect on the activation of proenkephalin gene expression by PKA in SK-N-MC cells. SK-N-MC cells were cotransfected with 2.5 μg of pENKAT-12, 2 μg of pRSVLuciferase, 10 μg of pMTCneo, and increasing amounts of the dominant inhibitory Ha-Ras (Asn-17) as indicated below each lane. The total amount of DNA was maintained at 25 μg with pGEM-3. CAT activities were normalized to the level of pRSVLuciferase expression. The mean CAT activities ± SD for three independent experiments using duplicate plates are indicated.

together to SK-N-MC cells act synergistically, resulting in approximately 50-fold induction of CAT activity (Fig. 1B, lane 4). These studies suggest that SK-N-MC cells serve as an appropriate model for further characterization of the signaling pathways and transcription factors mediating the actions of FGF and cAMP on proenkephalin gene expression.

FGF acts through a Ras-dependent signaling pathway. To examine the role of Ras in FGF-dependent signaling, a dominant negative mutant of Ras, Ha-Ras (Asn-17), was cotransfected together with pENKAT-12 into SK-N-MC cells. This Ras allele encodes a p21 protein that binds GDP with an affinity higher than that for GTP (21), and it appears to compete with the endogenous cellular $p21^{ras}$ for upstream activators while lacking the capacity to propagate a signal (20), thus inhibiting activation of cellular $p21^{ras}$ in proportion to the relative level of expression. Expression of Ha-Ras (Asn-17)

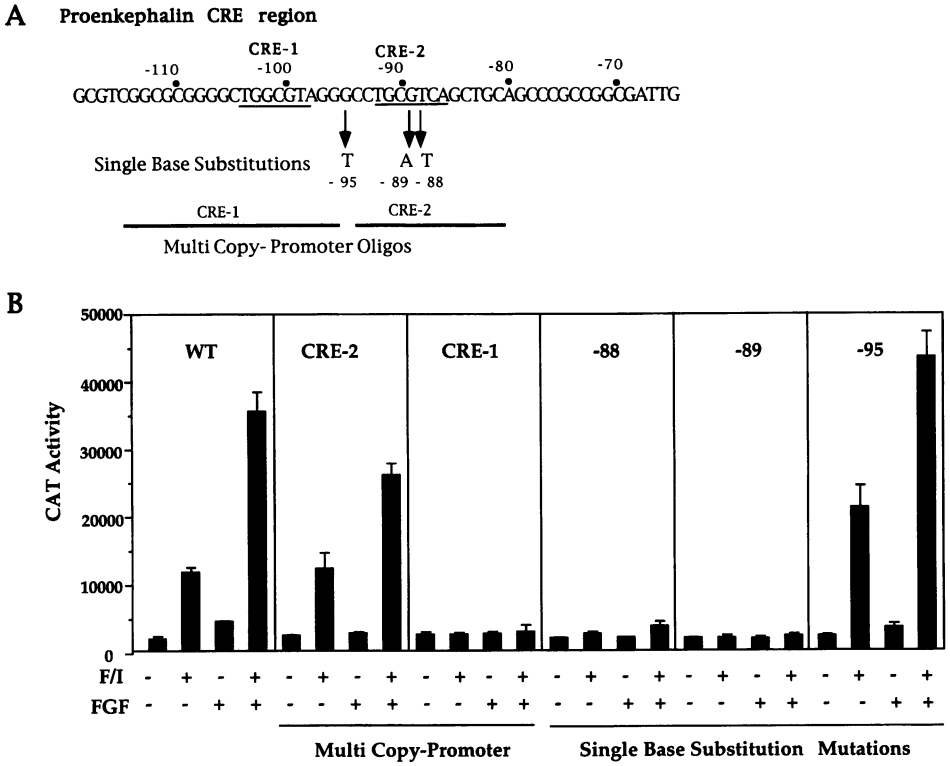

FIG. 3. FGF stimulation requires the CRE-2 element located within the proenkephalin cAMP-inducible enhancer. (A) Nucleotide sequences spanning the proenkephalin cAMP-inducible enhancer region (bases −114 to −80 relative to the transcription start site). CRE-1 and CRE-2 are underlined. Regions covered by oligonucleotides (oligos) used in the multicopy CAT constructs are shown as solid bars below the sequence. Single-base-substitution mutations are indicated by arrows. (B) Mapping of the DNA elements conferring FGF-dependent gene activation. Panels indicate CAT expression after transfection of SK-N-MC cells with plasmids carrying a wild-type promoter/enhancer and various promoter/enhancer mutations after the indicated treatments with forskolin and FGF. SK-N-MC cells were transfected with 2 μg of pRSVLuciferase and 2.5 μg of each of the indicated proenkephalin-CAT plasmids, treated for 6 h in the presence (+) or absence (−) of 10 μM forskolin, 0.5 mM IBMX, and 25 ng of bFGF per ml, harvested, and CAT and luciferase activities were determined. The panel marked WT represents transfection with pENKAT-12; the panels marked CRE-1 and CRE-2 represent transfection with a minimal proenkephalin promoter (Δ80) containing multiple copies of the CRE-1 or CRE-2 oligonucleotide shown in Fig. 3A. The panels labeled −88, −89, and −95 represent transfection with plasmids carrying single-base-substitution mutations located within CRE-2 (−88 and −89) or between CRE-2 and CRE-1 element (−95). At 18 h posttransfection, cells were treated with forskolin, IBMX, and bFGF for 6 h and harvested, and CAT and luciferase activities determined. All CAT activities were normalized to the level of pRSVLuciferase expression. The mean CAT activities ± SD for three independent experiments using duplicate plates are indicated.

inhibited FGF-forskolin-stimulated CAT expression at least fivefold (Fig. 2A), suggesting a role for p21^{ras} in FGF signaling and gene activation. We also examined the effect of oncogenic Ras (Val-12) on proenkephalin gene expression. As shown in Fig. 2B, Ras alone, like FGF, produced only a small increase in CAT expression (two- to threefold). However, in a fashion similar to that of the effects of FGF and forskolin, an activated Ras gene coexpressed with the catalytic subunit of PKA (35) strongly stimulated the proenkephalin promoter (compare Fig. 2B with Fig. 1B). These results indicate that FGF stimulates proenkephalin transcription through a Ras-dependent signaling pathway in SK-N-MC cells and that both FGF and Ras act synergistically with forskolin and PKA. To determine whether Ras acted simply to further stimulate PKA activity, we measured the effect of Ras cotransfection under conditions in which the effects of PKA were maximized. As shown in Fig. 2C, Ras stimulated CAT expression at saturating doses of PKA (Fig. 2C), suggesting that Ras and PKA act via distinct intracellular signaling pathways. In addition, dominant negative Ras (Asn-17) blocked FGF-stimulated (Fig. 2A) but not PKA-stimulated CAT expression (Fig. 2D; compare the third bar from left with the fourth and fifth bars), further supporting the validity of a model in which two different signaling pathways converge to regulate proenkephalin gene expression.

FGF stimulates transcription via CRE-2. To test whether distinct DNA elements mediate FGF and cAMP signaling, we analyzed the effects of FGF and cAMP on plasmids that had multiple copies of either CRE-2 or CRE-1 (Fig. 3A) inserted upstream of a proenkephalin promoter truncated at position −80. The proenkephalin-CAT fusion gene, pENKAT-80, lacks the previously defined cAMP-, tetradecanoyl phorbol acetate (TPA)-, and Ca^{2+}-responsive enhancer (13, 14, 48), and it is not responsive to intracellular signaling pathways. As shown in Fig. 3B, multiple copies of CRE-2 but not CRE-1 reconstitute activation by FGF and cAMP. Furthermore, point mutations within CRE-2 (T to A at position −88 and G to A at position −89) that block cAMP activation (14) also block activation by FGF. These results indicate that both FGF and cAMP activate proenkephalin gene expression through CRE-2, and they suggest that both FGF-Ras- and cAMP-PKA-dependent sig-

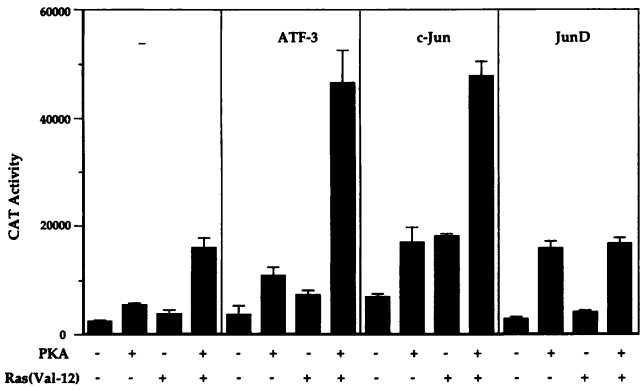

FIG. 4. ATF-3 and c-Jun stimulate FGF-dependent gene activation. SK-N-MC cells were cotransfected with 2.5 μg of pENKAT-12, 2 μg of pRSVLuciferase, and 6 μg of plasmids expressing ATF-3 (pRSVATF-3), c-Jun (pRSVcJun), and JunD (pRSVJunD) in the presence (+) or absence (−) of 1 μg of a plasmid expressing the catalytic subunit of PKA (pMTCneo) or 7 μg of pEJ-6.6 Ha-Ras (Val-12) as indicated. At 18 h posttransfection cells were harvested, and CAT and luciferase activities were determined. The total amount of DNA was maintained at 25 μg with pGEM-3. CAT activities were normalized to the level of pRSVLuciferase expression. The mean CAT activities ± SD for three independent experiments using duplicate plates are indicated.

naling pathways converge to modify the binding or activity of proteins interacting at this site.

FGF and Ras stimulate ATF-3- and c-Jun-dependent gene expression. Previous studies have suggested the involvement of several different AP-1 (14, 40, 60) and ATF/CREB-like proteins (12, 35) in the regulation of proenkephalin gene expression. To characterize the role of these factors in FGF-Ras-dependent gene activation, plasmids directing the expression of ATF-3, c-Jun, JunD, JunB, c-Fos, and CREB from a Rous sarcoma virus promoter were cotransfected into SK-N-MC cells. We assayed the abilities of the different factors to

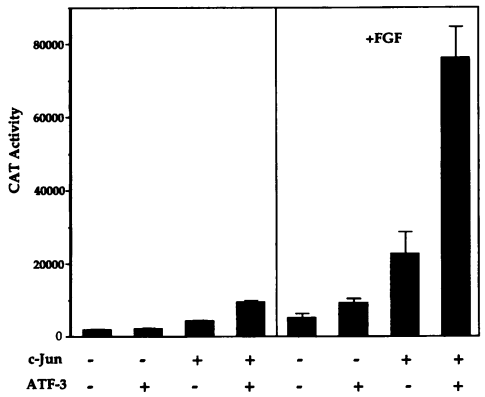

FIG. 5. ATF-3 and c-Jun act synergistically to stimulate FGF-dependent gene expression. SK-N-MC cells were transfected with 2.5 μg of pENK12 and 6 μg of RSVATF-3 or 2.5 μg of RSVc-Jun, treated for 6 h in the presence (+) or absence (−) of 25 ng of bFGF per ml, and harvested, and CAT and luciferase activities were determined. As described in the text, 2 μg of pRSVLuciferase was included in all transfections and the total amount of DNA was maintained at 25 μg with pGEM-3. CAT activities were normalized to the level of pRSV-Luciferase expression. The mean CAT activities ± SD for three independent experiments using duplicate plates are indicated.

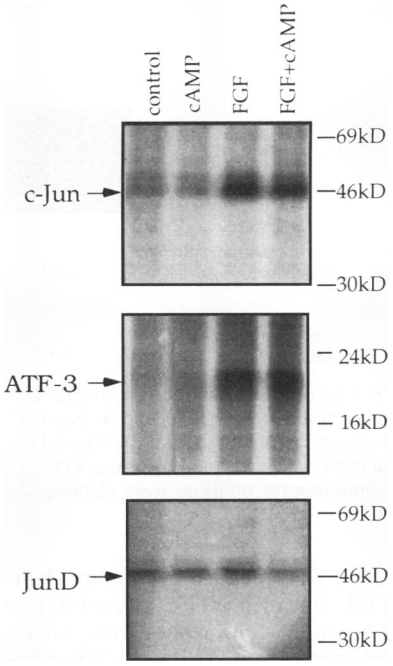

FIG. 6. Characterization of AP-1/CRE binding proteins present in SK-N-MC cells. SK-N-MC cells were treated with either 100 μM cpt-cAMP, 25 ng of bFGF per ml, or both, as indicated, for 4 h. At the time of drug treatment cells were also labeled with [^{35}S]methionine. After 4 h of drug treatment and exposure to [^{35}S]methionine, cells were harvested, and cell extracts were prepared under denaturing conditions (0.5% SDS; see Materials and Methods). Cell extracts were immunoprecipitated by using antibodies specific for c-Jun, ATF-3, JunD, CREB, and JunB (i.e., αc-Jun, αATF-3, αCREB, αJunB, and αJunD). The products were electrophoresed on a 12% denaturing polyacrylamide gel and exposed to autoradiography. Results for c-Jun, ATF-3, and JunD only are shown.

stimulate proenkephalin transcription in a Ras-dependent fashion. JunB, c-Fos, and CREB expression had no effect on Ras-stimulated gene expression (data not shown). However, expression of either ATF-3 or c-Jun strongly stimulated Ras-dependent activation of proenkephalin gene expression (Fig. 4). Although both JunD and ATF-3 stimulate PKA-dependent gene expression (12, 40), JunD is not further stimulated by Ras, demonstrating a highly selective reception of Ras signaling among different CRE-2 binding proteins. The profile of ATF-3 stimulation is remarkably similar to the profile of Ras and PKA stimulation seen in SK-N-MC cells, whereas the profile of c-Jun activation differs in that Ras stimulation does not require cAMP. Stimulation of c-Jun transcriptional activity by Ras signaling pathways has been previously reported (6, 8, 36, 51), and it is thought to involve changes in c-Jun phosphorylation which regulate both DNA binding and transcriptional activation. Since Ras activation of ATF-3 transcriptional activity had not been previously observed, we set out to further characterize the role of ATF-3 and c-Jun in Ras-cAMP signaling at CRE-2.

Because ATF-3 is known to heterodimerize with c-Jun (31, 33, 34) and to bind CRE-2 (31) and since both ATF-3 and c-Jun stimulate Ras-dependent transcription, we next carried out cotransfection experiments to examine the effects of coexpressing ATF-3 and c-Jun. As shown in Fig. 5, although basal activation is low (Fig. 5), cotransfection of both ATF-3 and c-Jun produces an effect larger than the sum of the two

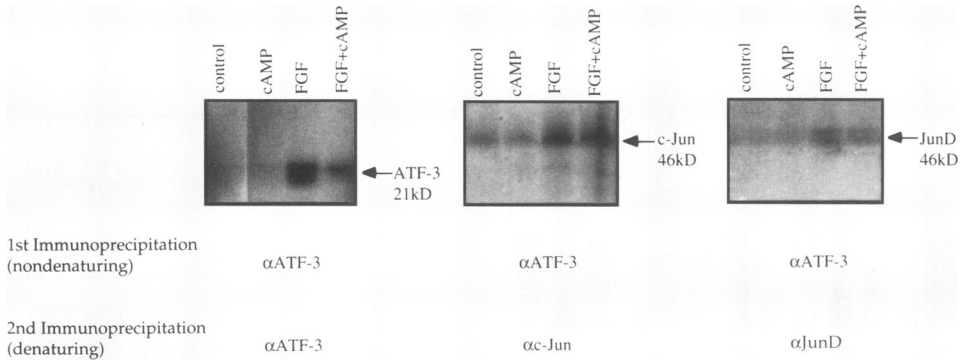

FIG. 7. ATF-3 is complexed with c-Jun and JunD in SK-N-MC cells. SK-N-MC cells were treated with cpt-cAMP or bFGF and labeled with [^{35}S]methionine as described in the legend to Fig. 6. Cell extracts were prepared under nondenaturing conditions and then immunoprecipitated with an ATF-3-specific antibody (αATF-3). The ATF-3 immunoprecipitates were boiled in denaturing buffer to release complexes, and a second immunoprecipitation was performed by using ATF-3-, c-Jun-, and JunD-specific antibodies (i.e., αc-Jun, αATF-3, and αJunD). Products of these two sequential immunoprecipitations were electrophoresed on a 12% denaturing polyacrylamide gel and subjected to autoradiography.

individual effects. This effect is much more clearly seen in the presence of FGF, in which ATF-3 and c-Jun produce individual effects of 6- and 18-fold, respectively, whereas when cells are transfected with both ATF-3 and c-Jun, a 53-fold increase in CAT expression is observed (Fig. 5; compare lanes 6, 7, and 8). These studies demonstrate that expression of both ATF-3 and c-Jun in SK-N-MC cells stimulates the ability of FGF and Ras to activate proenkephalin transcription, and they suggest that this complex may mediate cAMP- and Ras-dependent gene activation at CRE-2.

Characterization of AP-1/CRE binding proteins present in SK-N-MC cells. To characterize CRE-2 binding proteins present in SK-N-MC cells and to determine whether FGF alters the levels of CRE-2 binding proteins, we labeled SK-N-MC cells with [^{35}S]methionine and analyzed the synthesis of a number of different AP-1/CRE binding proteins. Cells were labeled for 4 h with [^{35}S]methionine and treated with either cpt-cAMP, FGF, or both for 4 h. Denatured cellular lysates were prepared and immunoprecipitated with antibodies specific for either ATF-3, c-Jun, JunD (Fig. 6), JunB, or CREB (data not shown). JunB was not detected, and low levels of CREB which did not change in response to FGF or cpt-cAMP treatment were detected (data not shown). Control or untreated cells contained detectable levels of ATF-3, c-Jun, and JunD (Fig. 6). Treatment of SK-N-MC cells for 4 h with FGF alone or with cpt-cAMP significantly increased the levels of ATF-3 and c-Jun but had little effect on JunD levels. In contrast, cAMP treatment had no detectable effect on the levels of any of the proteins examined.

FGF increases levels of ATF-3/c-Jun heterodimers. Having demonstrated that ATF-3 and c-Jun are coinduced by FGF and that the action of the two proteins together is synergistic, we next asked whether ATF-3 stably associates with c-Jun or JunD in vivo. SK-N-MC cells were labeled with [^{35}S]methionine and treated with cAMP, FGF, or FGF plus cAMP for 4 h. Sequential immunoprecipitations were carried out by use of an ATF-3-specific antibody under nondenaturing conditions followed by immunoprecipitation under denaturing conditions using ATF-3-, c-Jun-, or JunD-specific antibodies (Fig. 7). FGF but not cAMP increased the levels of ATF-3/c-Jun and ATF-3/JunD complexes (Fig. 7). ATF-3/CREB and ATF-3/JunB complexes were not detected (data not shown). As shown in Fig. 8A, the ATF-3 antibody recognized both ATF-3 homodimers (Fig. 8A, lane 1) and ATF-3/c-Jun heterodimers (Fig. 8A, lane 3). In contrast, the c-Jun antibody efficiently immunoprecipitated c-Jun homodimers (Fig. 8A, lane 4) but would not recognize the c-Jun/ATF-3 heterodimers (Fig. 8A, lane 6). As shown in Fig. 8B through D, FGF treatment led to an increase in total c-Jun levels as detected under denaturing conditions. However, the fraction of c-Jun complexed with ATF-3 (Fig. 8D) increased with FGF treatment, whereas the fraction not complexed with ATF-3 (Fig. 8C) decreased.

We next examined the ability of ATF-3 and c-Jun to bind the proenkephalin CRE-2 in JEG cells overexpressing ATF-3 and c-Jun and in SK-N-MC cells before and after treatment with FGF and cAMP. As shown in Fig. 8E, overexpression of ATF-3 and c-Jun produced by using vaccinia virus in human JEG cells resulted in a CRE-2 binding complex with mobility intermediate between those of complexes observed when either protein was expressed alone in JEG cells (Fig. 8E; compare the first three lanes). Treatment of SK-N-MC cells with FGF or FGF plus cAMP but not cAMP alone resulted in the induction of a DNA binding complex with mobility identical to that of the ATF-3/c-Jun heterodimeric complex observed in JEG cells. Attempts to supershift or block DNA binding of the ATF-3/c-Jun heterodimer in overexpressing JEG cells or in FGF-treated SK-N-MC cells resulted in only a partial block with the antibodies currently available, although each antibody effectively blocked homodimer DNA binding. Treatment of SK-N-MC extracts with a CREB-specific antiserum blocked formation of a CRE-2 binding complex but did not block the FGF-induced CRE-2 binding complex (Fig. 8E, last four lanes at the right). Taken together, these results suggest that FGF increases the levels of c-Jun complexed with ATF-3 and that this heterodimer may play an important role in activation of proenkephalin transcription by FGF and cAMP.

Analysis of ATF-3 and c-Jun phosphorylation in SK-N-MC cells. Previous work has shown that c-Jun is regulated both at the level of gene induction and at the posttranslational level by phosphorylation (6, 51). We next analyzed whether activation of the cAMP or FGF signaling pathway increased phosphorylation of ATF-3 or c-Jun in SK-N-MC cells. As shown in Fig. 9, both ATF-3 and c-Jun were phosphorylated in unstimulated SK-N-MC cells labeled with $^{32}P_i$ for 2 h. Treatment of SK-N-MC cells with either cAMP or FGF for 15 min resulted in a small but reproducible (three separate experiments) increase in the phosphorylation of ATF-3 and c-Jun (Fig. 9, lanes 2 and 3). Although further phosphopeptide analysis of

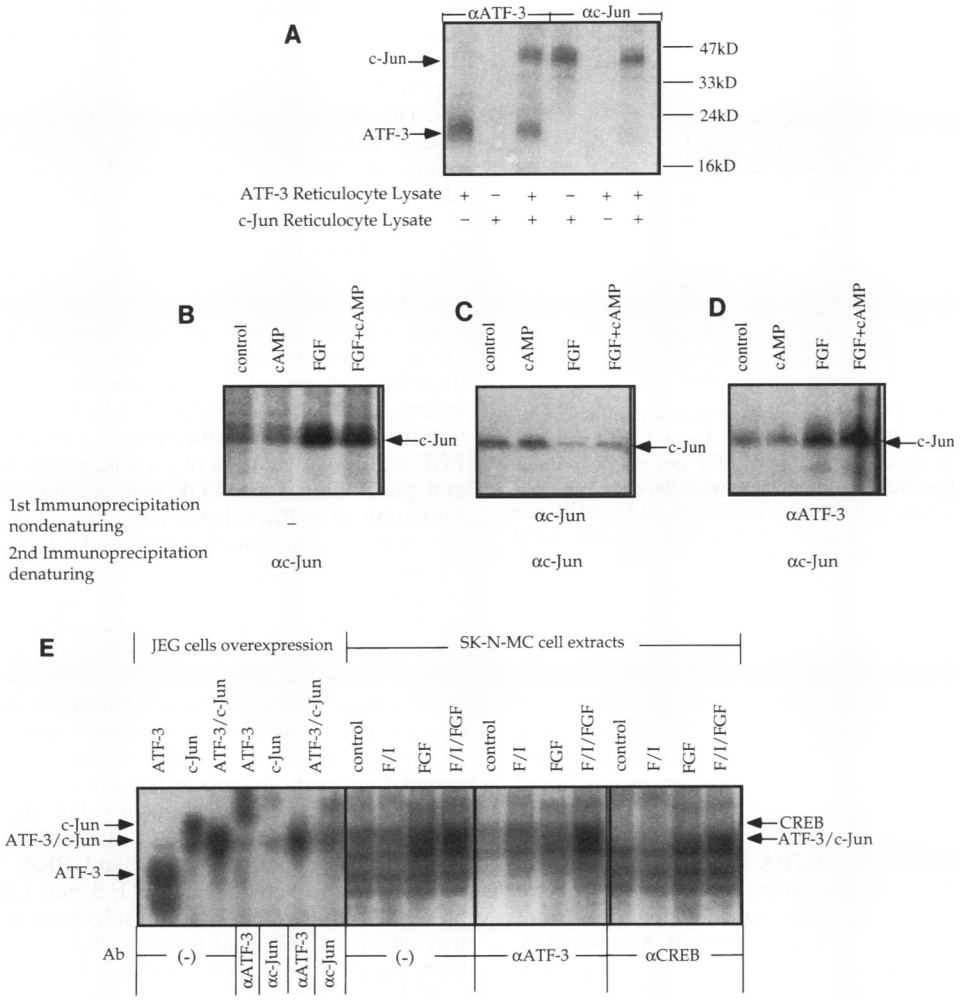

FIG. 8. FGF increases the level of ATF-3/c-Jun complexes in SK-N-MC cells. (A) ATF-3 and c-Jun antibody characterization. Samples (2 μl) of [^{35}S]methionine-labeled ATF-3 and c-Jun protein produced by in vitro translation using rabbit reticulocyte lysates were mixed and incubated at 37°C for 15 min to allow complex formation. A total of 2 μl of each protein or 4 μl of the ATF-3 and c-Jun mixture was immunoprecipitated with ATF-3- (left panel) or c-Jun (right panel)-specific antibodies (i.e., αATF-3 and αc-Jun), and immunoprecipitated proteins were analyzed by 12% PAGE and autoradiography. (B) FGF increases c-Jun protein levels in SK-N-MC cells. SK-N-MC cells were treated with cAMP and FGF and labeled with [^{35}S]methionine as described in the legend to Fig. 6. Total levels of c-Jun protein were analyzed by denaturing immunoprecipitation with c-Jun-specific antibodies (αc-Jun) as described in the legend to Fig. 6. (C) FGF decreases the level of c-Jun homodimer in SK-N-MC cells. SK-N-MC cells were treated with cpt-cAMP or bFGF and labeled with [^{35}S]methionine as described in the legend to Fig. 6. Cell extracts were prepared and immunoprecipitated first with a c-Jun-specific antibody (i.e., αc-Jun) under nondenaturing conditions. Immunoprecipitates were then boiled to release complexes, and a second immunoprecipitation, using c-Jun-specific antibodies (i.e., αc-Jun), was performed under denaturing conditions. The products were analyzed by SDS–12% PAGE and exposed to X-ray film overnight. (D) FGF increases the level of ATF-3/c-Jun complexes in SK-N-MC cells. Experimental procedures were as described for panel C, except that cell extracts were immunoprecipitated first with an ATF-3-specific antibody (i.e., αATF-3) under nondenaturing conditions and then were immunoprecipitated under denaturing conditions with c-Jun-specific antibodies (i.e., αc-Jun). (E) FGF but not cAMP induces a CRE-2 DNA binding complex identical in mobility to the ATF-3/c-Jun heterodimer. Gel shift assays were performed by using ATF-3 and c-Jun prepared from JEG cells transfected with pTMATF-3 and pTMc-Jun and infected with a T7 RNA polymerase-expressing vaccinia virus strain (vTF7-3) as described previously (19) or from SK-N-MC cells treated with 10 μM forskolin (F), 0.5 mM IBMX (I), and 25 ng of FGF per ml. Arrows indicate the CRE-2 DNA complexes formed with 2 μg of whole-cell extract prepared from JEG cells transfected with pTMATF-3, pTMc-Jun, or both pTMATF-3 and pTMc-Jun. Lanes 4 through 7 show the effects of preincubation with ATF-3- and c-Jun-specific antibodies. Lanes 8 through 11 show CRE-2 DNA binding complexes present in SK-N-MC cells before and after treatment with forskolin, FGF, or both for 2 h. Lanes 12 through 15 and 16 through 19 show the effects of pretreatment of extracts with ATF-3- and CREB-specific antibodies, respectively. Arrows indicate the positions of the CRE-2 complex induced by FGF but not by cAMP and of the CREB-specific complex.

the sites phosphorylated on ATF-3 and c-Jun needs to be carried out, these results are consistent with the idea that changes in the phosphorylation of ATF-3 or c-Jun play a regulatory role in the induction of proenkephalin transcription by FGF and cAMP.

DISCUSSION

Convergence of FGF- and cAMP-dependent signaling pathways at CRE-2. Numerous studies indicate that FGF and cAMP stimulate distinct intracellular signaling pathways (17,

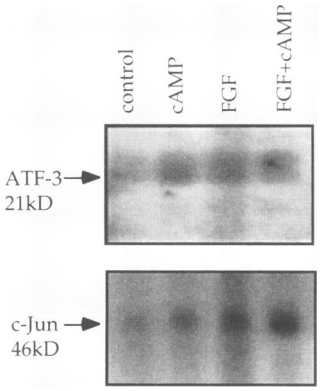

FIG. 9. Analysis of ATF-3 and c-Jun phosphorylation in SK-N-MC cells. SK-N-MC cells were labeled with $^{32}P_i$ for 2 h and treated for 15 min with either 100 μM cpt-cAMP, 25 ng of FGF per ml, or both, as indicated. At 15 min after drug treatment, cells were harvested and cell extracts were prepared under denaturing conditions (0.5% SDS; see Materials and Methods) in the presence of phosphatase inhibitors (50 mM NaF and 2 mM $NaVO_4$) and immunoprecipitated with ATF-3- and c-Jun-specific antibodies (i.e., αATF-3 and αc-Jun). Immunoprecipitated proteins were analyzed by 12% PAGE and exposed to X-ray film.

25, 61). In PC-12 cells, Ha-Ras (Asn-17) blocks both NGF- and FGF-induced neuronal differentiation as well as the induction of several immediate early genes (61). However, expression of Ha-Ras (Asn-17) did not block neurite outgrowth or immediate early gene induction by dibutyryl cAMP or TPA (61), suggesting that Ras, cAMP, and TPA signal through distinct intracellular pathways. NGF responses, including both neural differentiation and immediate early gene induction, are not affected in clonal PC-12 cell lines deficient in PKA activity (24, 25), again suggesting independent pathways. In agreement with the findings described above, our results also suggest that FGF and cAMP act via distinct signaling pathways, since (i) Ras inhibition has little effect on PKA-stimulated gene expression and (ii) Ras further stimulates gene expression under conditions of maximal PKA activation. Consistent with the independent nature of the Ras and PKA signaling pathways, several studies have shown that Ras and PKA regulate gene expression in a mutually antagonistic fashion (15, 23), and in one case, Ras appears to act through protein kinase C to down regulate PKA activity (23). Recent reports also suggest that inhibitory cross talk between the cAMP and growth factor signaling pathways may, at least in part, result from cAMP-dependent phosphorylation of c-Raf and down regulation of MAP kinase activity (16, 28, 54, 65).

ATF-3 and c-Jun stimulate FGF and cAMP signaling through CRE-2. DNA mapping experiments clearly demonstrate that both FGF and Ras act via CRE-2, located within the proenkephalin second messenger-inducible enhancer (14). This element is known to mediate gene activation via cAMP-, TPA-, and Ca^{2+}-dependent signaling pathways (13, 48) and to bind ATF/AP-1 complexes (12, 14, 35, 40, 60). These results suggest that proteins which bind to this DNA element may also mediate gene activation by FGF and Ras. To identify and characterize candidate transcription factors, we screened several different AP-1 and ATF/CREB proteins for their abilities to support FGF-dependent gene expression at CRE-2 (c-Jun, JunB, JunD, c-fos, CREB, and ATF-3). Two factors, ATF-3 and c-Jun, stimulated gene activation at the proenkephalin CRE-2 in a Ras- and FGF-dependent fashion. ATF-3 (32), also called liver regeneration factor 1 (LRF-1) (34), has been implicated in the induction of gene expression associated with the G_0-to-G_1 cell cycle transition (33) and hence might be expected to be regulated by growth factors, mitogens, and Ras (2). The previously demonstrated abilities of ATF-3 to bind to CRE-2 and to stimulate proenkephalin transcription in response to cAMP (12) and FGF/Ras (this study) in a fashion identical to that observed in SK-N-MC cells suggest an important role for this factor in both FGF and PKA signaling. c-Jun also stimulates proenkephalin gene expression at CRE-2 in a Ras-dependent fashion. However, unlike ATF-3 stimulation, Ras stimulation of c-Jun activity does not require PKA. This result agrees with previous reports that activated (oncogenic) Ha-Ras is a constitutive stimulator of both c-Jun activity and phosphorylation and that the normal c-Ha-Ras protein is a serum-dependent modulator of c-Jun's activity (6, 36).

What signals and modifications of c-Jun and ATF-3 underlie FGF and Ras regulation of proenkephalin transcription? Several possibilities exist. FGF may act simply to stimulate the formation of a PKA-dependent ATF-3/c-Jun complex. FGF increases the synthesis of ATF-3 and c-Jun in SK-N-MC cells, leading to increased levels of ATF-3/c-Jun protein complexes at 4 h post-FGF treatment. The fact that overexpression produced by cotransfection of either c-Jun or ATF-3 results in stimulated transcription suggests that the levels of these proteins in SK-N-MC cells are limiting. Conceivably, FGF-dependent increases in the levels of a limiting ATF-3/c-Jun complex could explain the observed positive synergy. However, this mechanism does not explain the Ras- and FGF-dependent stimulation seen in cotransfection experiments which employed constitutive expression of c-Jun and ATF-3 from the Rous sarcoma virus promoter. Instead, these experiments suggest that induction by Ras and PKA may also involve changes in the activities of ATF-3 and c-Jun protein complexes. Taken together, these results suggest that the stimulatory effects of FGF and Ras are likely to involve changes in both the abundance and activity of ATF-3/c-Jun complexes.

FGF- and Ras-induced changes in the transcriptional activity of ATF-3/c-Jun complexes are likely to involve protein phosphorylation of either ATF-3, c-Jun, or both proteins as a heterodimeric complex. In this model, synergy with the cAMP pathway may involve sequential phosphorylation events resulting in enhanced transcriptional activity, as appears to be the case for CREB (42). Ras has been shown to stimulate phosphorylation of a transcriptional activation domain located within the N-terminal region of c-Jun (6, 51), and this modification may regulate the transcriptional activity of the c-Jun/ATF-3 complex. Our findings that both proteins exist in SK-N-MC cells as a heterodimeric complex and that phosphorylation of this complex increases with cAMP and FGF treatment further suggest this possibility.

In contrast to the well-described inhibitory effects of cAMP on growth factor signaling, our results indicate that these pathways can also positively interact to stimulate gene expression at CRE-2. Positive synergy between growth factor- and cAMP-activated signaling pathways at the level of gene expression have not, to the best of our knowledge, been previously reported, and we suggest that this system represents a useful model for analyzing the interactions between these pathways. It is well known that in many differentiated cell types and tissues, hormones acting through cAMP-dependent mechanisms can stimulate cell proliferation (18). For example, thyrotropin stimulates the synthesis and secretion of thyroid hormones by thyrocytes and also controls cell growth. Overexpression of thyrotropin leads to thyroid hyperfunction and hyperplasia, and in canine thyrocytes, cAMP acts synergisti-

cally with other mitogens such as epidermal growth factor to stimulate DNA synthesis (18). It therefore seems likely that synergistic cAMP-growth factor interactions occur in many different cell types and serve to coordinate and regulate the cells' response to diverse growth and hormonal signals. It is tempting to speculate that in the nervous system, synergistic interactions between neurotrophic and neurotransmitter-stimulated pathways may be required to elicit and coordinate the long-term changes in signaling associated with neural plasticity.

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