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Dimer-Specific Potentiation of NGFI-B (Nur77) Transcriptional Activity by the Protein Kinase A Pathway and AF-1-Dependent Coactivator Recruitment

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The NGFI-B (Nur77) subfamily of orphan nuclear receptors (NRs), which also includes Nurr1 and NOR1, bind the NurRE regulatory element as either homo- or heterodimers formed between subfamily members. These NRs mediate the activation of pituitary proopiomelanocortin (POMC) gene transcription by the hypothalamic hormone corticotropin-releasing hormone (CRH), an important link between neuronal and endocrine components of the hypothalamo-pituitary-adrenal axis. CRH effects on POMC transcription do not require de novo protein synthesis. We now show that CRH signals activate Nur factors through the cyclic AMP/protein kinase A (PKA) pathway. CRH and PKA rapidly increase nuclear DNA binding activity of NGFI-B dimers but not monomers. Accordingly, CRH- or PKA-activated Nur factors enhance dimer (but not monomer) target response elements. We also show that p160/SRC coactivators are recruited to Nur dimers (but not to monomers) and that coactivator recruitment to the NurRE is enhanced in response to CRH. Moreover, PKA- and coactivator-induced potentiation of NGFI-B activity are primarily exerted through the N-terminal AF-1 domain of NGFI-B. The TIF2 (SRC-2) glutamine-rich domain is required for this activity. Taken together, these results indicate that Nur factors behave as endpoint effectors of the PKA signaling pathway acting through dimers and AF-1-dependent recruitment of coactivators.

NGFI-B (also known as Nur77, TR3, and NAK-1) is a transcription factor belonging to the superfamily of nuclear receptors (NRs) (14). NGFI-B was cloned as an immediate-early gene that could be induced by serum in mouse fibroblasts (26, 40) and was also identified as a NGF-inducible gene in differentiating rat PC12 cells (52). NGFI-B is constitutively expressed in various peripheral tissues and in some regions of the brain (41, 55, 81). NGFI-B is closely related to Nurr1 (RNR-1, TINUR, and HZF-3) and NOR-1 (MINOR), together forming a distinct subfamily (the Nur factors) of orphan NRs (41, 55). The DNA-binding domains (DBDs) of NGFI-B, Nurr1, and NOR-1 are highly homologous, while their ligand-binding domains (LBDs) are moderately homologous and their N termini are not homologous (17). Nurr1 is predominantly brain specific (41), and genetic inactivation of Nurr1 results in impaired development and maintenance of midbrain dopaminergic neurons (5, 69, 89). The expression pattern of NOR-1 is similar to that of NGFI-B, and NOR-1 and NGFI-B appear to play partly redundant roles in T cells and adrenal glands (8, 19).

NGFI-B was implicated at multiple levels of the hypothalamo-pituitary-adrenal (HPA) axis that ultimately controls adrenal glucocorticoid synthesis through the pituitary hormone adrenocorticotropin (ACTH). ACTH is processed from a precursor, proopiomelanocortin (POMC), which is itself under control of the hypothalamic corticotropin-releasing hormone (CRH). NGFI-B expression is strongly induced by a variety of stress stimuli in CRH-producing neurons of the hypothalamus where it may be implicated in CRH gene transcription (30, 53, 57). In the anterior pituitary gland, the stimulatory effect of CRH and the negative-feedback regulation of POMC transcription by glucocorticoids (at least, in part) appear to be mediated through NGFI-B (53, 62, 63). In the adrenals, ACTH treatment induces NGFI-B, leading to enhancement of steroid-21α-hydroxylase gene transcription (12, 84), a rate-limiting enzyme in steroidogenesis. NGFI-B also appears to be an important regulator of apoptosis in different cells. In T cells, it was shown to be essential for apoptosis of self-reactive immature thymocytes following stimulation of the T-cell receptor (TCR) (8, 47, 85). In response to apoptotic stimuli, NGFI-B translocates from the nucleus to mitochondria to induce cytochrome *c* release and apoptosis of LNCaP human prostate cancer cells (43).

NGFI-B, Nurr1, and NOR-1 were first shown to activate transcription and to bind DNA as monomers on a target site, the NGFI-B-responsive element (NBRE), that consists of an octanucleotide AAAGGTCA containing the canonical nuclear receptor hexanucleotide binding motif preceded by two adenines (8, 58, 83). In addition, NGFI-B and Nurr1 (but not NOR-1) can heterodimerize with RXR and activate transcription through a DR-5 element in a 9-*cis* retinoic acid-dependent manner (20, 61, 90). We reported that Nur family members can bind DNA and activate transcription as dimers (48, 62). The transcriptional target of these dimers, the Nur-responsive element (NurRE), consists of everted repeats of the octanucleotides AAAT(G/A)(C/T)CA which are related to the NBRE. Whereas the naturally occurring NurRE from the POMC pro-

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moter is preferentially bound and activated in the presence of NGFI-B, a synthetic consensus NurRE sequence (consisting in two perfect NBRE half-sites) does not exhibit this preference and is equally responsive to all three Nur factors (48).

NGFI-B is known to be a phosphoprotein, and increasing evidence suggests that the transcriptional activity of NGFI-B is tightly regulated by phosphorylation events. The N terminus of NGFI-B expressed in COS cells is heavily phosphorylated, which is primarily responsible for its transactivating activity, while its C terminus is almost devoid of phosphorylation (11). NGFI-B is rapidly modified posttranslationally in PC12 cells, mainly by phosphorylation on serine residues (18, 25). Interestingly, the extent of these phosphorylations depends on the nature of the stimulus, resulting in differential regulation of its transcriptional activity (25, 34). Indeed, while membrane depolarization induces the transcriptional activity of NGFI-B, nerve growth factor (NGF) treatment represses this activation presumably through phosphorylation at Ser316 which impairs NGFI-B binding to the NBRE (27, 34). Although this serine can be phosphorylated in vitro by protein kinase A (PKA) and $pp90^{rsk}$ (11, 27), the nature of the kinase phosphorylating Ser316 in vivo is still unclear. It might be NGFI-B kinase I, a NGF-induced cytosolic kinase that phosphorylates Ser316 in PC12 cells and which is very similar or identical to Fos kinase and $pp90^{rsk2}$ (28, 72). The Akt (PKB) kinase has also been shown to target Ser316 in vivo and in vitro in a phosphatidylinositol 3-kinase-dependent manner, thus inhibiting DNA binding and transcriptional activity of NGFI-B and resulting in suppression on NGFI-B-induced apoptosis in fibroblasts and TCR-mediated cell death in T-cell hybridomas (50, 60). NGF induces additional phosphorylation at Ser105 by the Trk/Ras/mitogen-activated protein kinase (MAPK) pathway and results in nuclear export of NGFI-B (35). ACTH also regulates NGFI-B phosphorylation status in adrenal-derived Y1 cells. Indeed, ACTH treatment produces dephosphorylation of Ser316, thus allowing for DNA binding of NGFI-B; in addition, ACTH action results in hyperphosphorylation of the N terminus, which may lead to enhancement of transcriptional potency (12, 46).

The transcriptional activity of many NR dimers is dependent on binding of a ligand to the C-terminal LBD and the molecular mechanism of ligand-induced transcriptional activity requires recruitment of coactivator protein to the C-terminal AF-2 activation domain. A large group of NR coactivators have been identified (reviewed in references 16, 51, and 67). One of the best-studied classes of coactivators is the p160 family of coactivators that includes SRC-1/NCoA-1, SRC-2/TIF2/GRIP1/NCoA-2, and SRC-3/pCIP/ACTR/AIB-1. The p160 coactivators mediate ligand-dependent transactivation through recruitment at the AF-2 domain. In addition, these coactivators interact with the carboxy-terminal region of CBP/p300 (33, 86), another transcriptional cofactor which itself interacts with NRs and which appears to play the role of a transcriptional integrator (23, 45). Until recently, it was thought that AF-2-dependent recruitment of coactivators was sufficient to account for ligand-dependent activity; however, recent results have also implicated the N-terminal AF-1 domain in the process (1, 29, 56, 82). Most notably, coactivator recruitment following phosphorylation events in the AF-1 domain has been shown to be important in ligand-independent transcriptional activation by NRs (24, 74). Although most NRs studied for coactivator dependence activate transcription as dimers, a similar paradigm was proposed for SF-1 acting as a monomeric activator of transcription (88). Also, it was recently demonstrated that Cam kinase (CamK) IV modulates recruitment of cofactors ASC-2 and SMRT by both NGFI-B monomers and dimers (70). However, the p160 coactivators do not appear to be involved in transcription activation by NGFI-B monomers (6).

The main physiological regulators of corticotrope cells and POMC transcription are hypothalamic CRH (which stimulates POMC transcription) and glucocorticoids (which repress POMC expression), thereby exerting negative feedback on the HPA axis (21). Upon binding its receptor on corticotrope cells, CRH induces a rapid increase in cyclic AMP (cAMP) levels (39) followed by activation of PKA (66). CRH induction of the cAMP pathway also results in increased intracellular Ca^{2+} levels through an enhancement of the activities of L- and P-type Ca^{2+} channels, which are known to be modulated by PKA (37, 38). Previous work has shown that CRH and cAMP stimulate POMC gene transcription and that this induction is rapid and transient and does not require de novo protein synthesis (21, 22). However, whereas glucocorticoids are known to exert their transcriptional repression through the glucocorticoid receptor (GR) (15, 63; C. Martens, M. H. Maira, S. Bilodeau, Y. Gauthier, and J. Drouin, unpublished data), the nature of factors mediating the nuclear events elicited by cAMP and resulting in POMC transcription remain elusive. Classically, cAMP- and Ca^{2+}-induced transcription is mediated through responsive elements, the cAMP-responsive element (CRE) and Ca-responsive element (CaRE), respectively, that are both variations of the TGACGTCA palindromic sequence (42). However, no such sequence is found in the rat POMC promoter. Instead, the POMC NurRE was shown to be important for CRH-induced transcription in POMC-expressing AtT-20 cells (62) and to confer strong transcriptional responsiveness to both CRH and NGFI-B.

We now report that the cAMP/PKA pathway elicited by CRH in AtT-20 cells sharply enhances DNA binding activity of Nur dimers, coactivator recruitment to NurRE (Nur dimers), and NurRE-dependent transcription. In contrast, these effects are not exerted on the NBRE target sequence of Nur monomers. The dependence on Nur dimers for cAMP/PKA-dependent NGFI-B activity was not previously recognized. Both PKA and SRC coactivator effects are mediated through the N-terminal AF-1 domains of NGFI-B and Nurr1. The present work defines the action of the PKA pathway through the orphan NRs of the Nur subfamily and highlights the roles of dimerization and of the N-terminal AF-1 domain for transcriptional activity, potentiation by PKA, and coactivator recruitment.

MATERIALS AND METHODS

Plasmids and oligonucleotides. The various reporter plasmids were constructed in pXP1-luc (54) containing the minimal (positions −34 to +63) POMC promoter. The −480 POMC promoter was previously described (73). NBRE (5′-GATCCTCGTGCGAAAAGGTCAAGCGCTA-3′) or $NurRE_{POMC}$ (5′-GATCGTGATATTTACCTCCAAATGCCA-3′) oligonucleotides containing plasmids were cloned as described previously (48). The same oligonucleotides were used in gel shift experiments; the $NurRE_{CON}$ (5′-GATCCGTGACCTTTATTCTCAAAGGTCA-3′) was also used in the electrophoretic mobility shift assay

(EMSA). CMX-Nurr1 and CMX-NGFI-B expression vectors contain complete cDNA sequences for rat proteins cloned into pCMX (77). NGFI-B N-terminal mutants have deletions between amino acids (aa) 3 to 36, 3 to 74, 3 to 174, or 3 to 214, and the ΔC mutants have deletions that end at the amino acid position indicated in Fig. 6A. ΔC Nurr1 mutant has a C-terminal deletion that ends at aa 352, and Nurr1 N-terminal mutants have internal deletion between aa 72 and 162 and between aa 160 and 236. Full-length SRC-1 expression vector was described previously (75). Full-length TIF2 (SRC-2) was provided by P. Chambon and has been described elsewhere (79). Full-length TIF2 was subcloned in the pCMX vector within the *Kpn*I and *Bam*HI sites. The various TIF2 mutants have been described elsewhere (3). Full-length CBP expression vector was described previously (9).

Cell culture and transfections. CV-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine fetal serum and maintained at 37°C in 5% CO_2. AtT-20 D16v cells were grown in the same conditions, except that charcoal-stripped fetal bovine serum was used. CV-1 cells were transfected using the calcium phosphate coprecipitation method, whereas AtT-20 cells were transfected by lipofection using lipofectamine (Gibco BRL), as previously described (63). Results are presented as the means of 3 to 10 experiments performed in duplicate. The plasmid pRSV-GH was used as an internal control for transfection efficiency.

EMSA. The EMSAs were performed as described previously (48). Binding reactions were performed in a volume of 20 μl containing gel shift binding buffer (10 mM Tris-HCl [pH 8.0], 40 mM KCl, 1 mM dithiothreitol [DTT], 6% glycerol, 0.05% NP-40), 500 ng of poly(dI-dC), and about 5 μg of cell or nuclear extracts. We used 50,000 cpm per reaction (~20 fmol) of double-stranded oligonucleotide probes, end labeled the probes by filling in with Klenow fragment in the presence of [α-^{32}P]dATP, and purified the probes on a G-25 Sephadex column. The reaction mixtures were incubated for 10 min at 25°C prior to loading. The samples were separated by electrophoresis using 5% polyacrylamide gels (acrylamide-bisacrylamide 29:1) in 0.5× Tris-borate-EDTA (TBE) at 25°C for 2 to 2.5 h. For supershift experiments, the antibodies were preincubated with the nuclear extracts for 30 min at 25°C prior to addition of the probe.

Preparation of AtT-20 cell extracts. After CRH or forskolin treatment, approximately 4×10^7 AtT-20 cells were washed once with cold phosphate-buffered saline (PBS) and then harvested with cold PBS containing 1 mM EDTA. The cells were then centrifuged and then resuspended in 500 μl of buffer A (10 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.1 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 1 mM DTT, and the protease inhibitors leupeptin, aprotinin, and pepstatin [10 μg/ml each]). Cells were allowed to swell on ice for 15 min before addition of 50 μl of NP-40 followed by vigorous vortexing. After centrifugation, the supernatant (cytosolic fraction) was set aside, and the nuclear pellet was resuspended in 100 μl of buffer B (10 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.4 M NaCl, 5% glycerol, 0,5 mM PMSF, 1 mM DTT, and the protease inhibitors [10 μg/ml each]) and shaken vigorously at 4°C for 30 min. The extract was then centrifuged, and the protein concentration of the supernatant (nuclear fraction) was estimated by Bradford assay.

Biotinylated-DNA affinity precipitation assay. AtT-20 whole-cell extracts (500 μg) were incubated for 10 min at 4°C with gel shift binding buffer (see above), and 20 μg of poly(dI-dC) in a final volume of 1 ml. Preclearing was performed by adding 50 μl of a 50% slurry (washed twice with PBS) of streptavidin-coated agarose beads (Pierce) and incubating the mixture for 15 min with gentle rocking at 4°C. After centrifugation, the supernatant was incubated with 1 μg of a double-stranded biotinylated NurRE probe for a time period ranging from 4 h to overnight at 4°C with gentle rocking. Then 50 μl of streptavidin-coated agarose beads (50% slurry) was added, followed by a further 2-h incubation at 4°C. Beads containing bound complexes were recovered by centrifugation and washed twice with Tris-EDTA (100 mM NaCl), twice with gel shift binding buffer, and once with PBS, before being subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis.

Western blotting. Western blotting of 25 μg of nuclear extract or 50 μg of cytosolic extract was performed using an anti-NGFI-B antibody from Geneka, or an anti-NGFI-B antibody produced in this laboratory (see Fig. 2E), anti-Nurr1 antibody (sc-991; Santa Cruz), anti-NGFI-B/Nurr1 antibody (sc-990; Santa Cruz), anti-NGFI-B (NGFI-B phosphorylated on Ser316) (kindly provided by Y Hirata) or monoclonal anti-TIF2 (generously provided by P. Chambon) and horseradish peroxidase-conjugated anti-rabbit (or mouse) immunoglobulin G (IgG) (Sigma). Revelation was performed by chemiluminescence as described by the manufacturer (ECL+plus; Amersham Pharmacia).

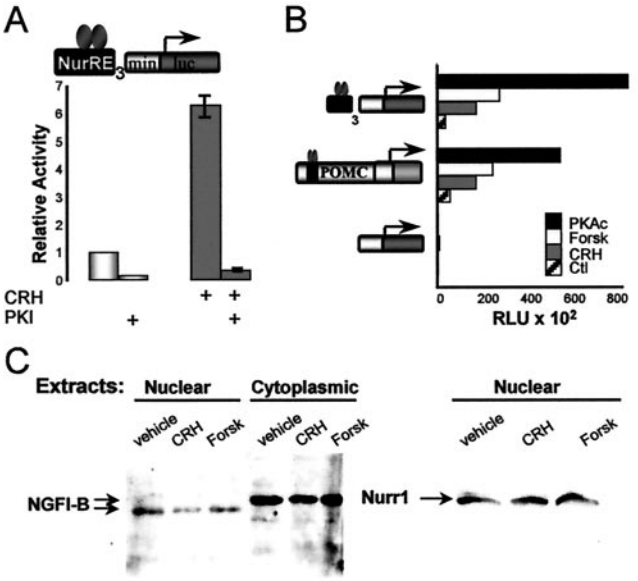


FIG. 1. The CRH/cAMP/PKA pathway strongly enhances NurRE-dependent activity without affecting NGFI-B protein levels. (A) The effects of PKI, the peptidic inhibitor of PKA, on basal activity (white bars) and CRH-induced (10^{-7} M CRH) activity (shaded bars) was assessed by lipofection into AtT-20 cells of a reporter plasmid consisting of three copies of the NurRE sequence cloned upstream of the luciferase (luc) gene. An expression vector for PKI (100 ng) was cotransfected as indicated. (B) The effects of CRH or activators of the cAMP/PKA pathway were tested on transcription driven by the minimal POMC promoter (min) (positions −36 to +63), the intact POMC promoter (positions −480 to +63), or the NurRE reporter. AtT-20 cells were transfected by lipofection with reporter plasmids alone (control [Ctl]) or with PKAc (50 ng). Where indicated, transfected AtT-20 cells were treated with CRH (10^{-7} M) or forskolin (Forsk) (10^{-5} M) 4 h prior to harvesting. RLU, relative light units. (C) NGFI-B and Nurr1 protein levels were determined by Western blotting using either anti-NGFI-B (Geneka) or anti-Nurr1 (N-20; Santa Cruz) antibodies. AtT-20 cells were treated for 30 min with either CRH (10^{-7} M) or forskolin (Forsk) (10^{-5} M), and both nuclear (25 μg) and cytosolic (50 μg) extracts were prepared and analyzed by SDS-PAGE.

RESULTS

The CRH/cAMP/PKA pathway strongly enhances NurRE-dependent activity. Previous studies from our laboratory have identified the NurRE as a major mediator of CRH action on the POMC promoter (62). We demonstrated that CRH induces NurRE-dependent transcription and that a dominant-negative mutant of NGFI-B can abrogate this activation. Since cAMP is known to mediate CRH-induced signaling, we tested whether inhibition of this second messenger pathway would also abrogate CRH effects on NurRE-dependent transcription. AtT-20 cells were transfected with a luciferase reporter plasmid driven by three copies of the NurRE, and as expected, this construct is responsive to CRH treatment (Fig. 1A). However, coexpression of PKI (a peptidic inhibitor of PKA) repressed basal activity of this reporter and completely abrogated CRH-induced activation (Fig. 1A). We then tested whether the NurRE is also responsive to direct activation by the cAMP/PKA pathway. Both NurRE and intact POMC promoter-driven reporters were activated by CRH or forskolin (an adenylate cyclase activator) treatment, as well as by coexpression

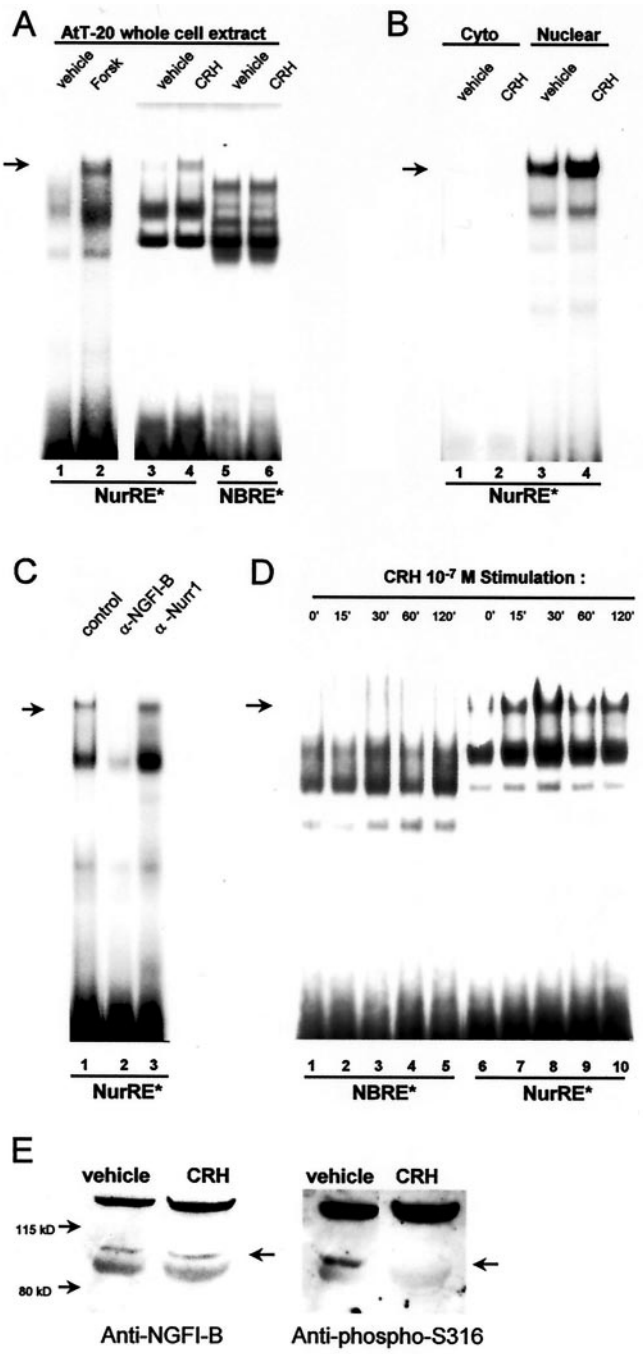


FIG. 2. The CRH/cAMP/PKA pathway transiently enhances NGFI-B binding to the NurRE. (A) NurRE binding activity is increased in whole-cell extracts of AtT-20 cells treated for 30 min with 10^{-5} M forskolin (Forsk) (lane 2) or 10^{-7} M CRH (lane 4). These treatments result in a new complex (arrow) in gel retardation experiments using the NurRE probe. This new complex comigrates with NGFI-B dimers as previously characterized (48, 62). (B) CRH treatment (10^{-7} M) of AtT-20 cells increases nuclear binding to the NurRE probe (lanes 3 and 4), whereas cytoplasmic (Cyto) extracts from the same cells (lanes 1 and 2) do not show significant binding. (C) The NurRE binding activity of AtT-20 cells nuclear extracts is impaired by preincubation with an anti-NGFI-B antibody (α-NGFI-B) (provided by J. Milbrandt) (lane 2) but not with an anti-Nurr1 antibody (α-Nurr1) (provided by T. Perlmann) (lane 3). These antisera were previously shown to block binding of cognate antigens in EMSA (48). The lower band may be monomeric binding or proteolytic degradation. (D) Time course of CRH stimulation (10^{-7} M) of NurRE binding activity in nuclear extracts of AtT-20 cells (lanes 6 to 10). The binding activity of the same extract was also tested using a NBRE probe (lanes 1 to 5). (E) Western blot analysis of NGFI-B phospho-Ser316 (arrow) in whole-cell extracts of AtT-20 cells (right blot) treated (30 min) with vehicle and CRH (10^{-7} M). The same blot was also analyzed for control using an anti-NGFI-B antiserum produced in rabbit (left blot). The positions of molecular mass markers (in kilodaltons) are indicated to the left of the blot.

of the catalytic subunit of PKA (Fig. 1B). However, these treatments had no effect on the minimal POMC promoter.

A simple explanation for the effects of the CRH/cAMP/PKA pathway on NurRE-dependent transcription would be an induction of Nur factor gene transcription. Indeed, we reported that CRH induces mRNA levels of all three Nur factors (48). However, this could not account for the acute CRH effect on POMC transcription, since we previously demonstrated that it did not require de novo protein synthesis (22). We therefore tested whether CRH or forskolin treatment affected Nur protein levels by Western blotting. After a 30-min treatment with CRH or forskolin, which corresponds to the peak of POMC responsiveness, neither NGFI-B nor Nurr1 levels were upregulated (Fig. 1C). Similar results were obtained after 1-h treatments and with a variety of antibodies against NGFI-B and Nurr1 (data not shown). Interestingly, the NGFI-B antibody revealed a protein of slower migration in cytosolic extracts, suggesting that as seen in PC12 and Y1 cells (34, 46), NGFI-B is also present in the cytoplasm in an hyperphosphorylated form (Fig. 1C).

The CRH/cAMP/PKA pathway transiently enhances NGFI-B binding to the NurRE. We then tested if the CRH-induced transcriptional activation of the NurRE reporter correlated with an increase in NurRE binding. Whole-cell extracts from AtT-20 cells were subjected to EMSAs. Pretreatment with 10^{-5} M forskolin induced the binding of a slowly migrating complex compared to vehicle treatment (Fig. 2A, lanes 1 and 2). This complex was also induced in cells pretreated with 10^{-7} M CRH (Fig. 2A, lanes 3 and 4). The same extracts did not reveal any difference in DNA binding and activity when assessed with a NBRE probe for Nur factor monomers (Fig. 2A, lanes 5 and 6). Since our Western blot analysis revealed the presence of NGFI-B in both cytoplasm and nucleus, we performed gel retardation assays using extracts from both compartments. Little or no bound complex was detected with cytosolic extracts (Fig. 2B, lanes 1 and 2), even after long exposure or when using greater amounts of extract (data not shown). However, nuclear extracts revealed the presence of the CRH-inducible slowly migrating complexes (Fig. 2B, lanes 3 and 4). To assess which members of the Nur subfamily are present in this complex, we performed experiments using antibodies previously shown to block binding of either NGFI-B or Nurr1 (48). The addition of anti-NGFI-B, but not of anti-Nurr1, to the nuclear extracts completely blocked the binding of the slower-migrating complex (Fig. 2C), indicating that NGFI-B is present in the complex. We then performed time course analyses to determine the kinetics of this CRH-enhanced binding of NGFI-B. Binding of the slower-migrating complex to the NurRE probe was rapidly induced, reaching a maximum after 30 min of treatment with 10^{-7} M CRH and

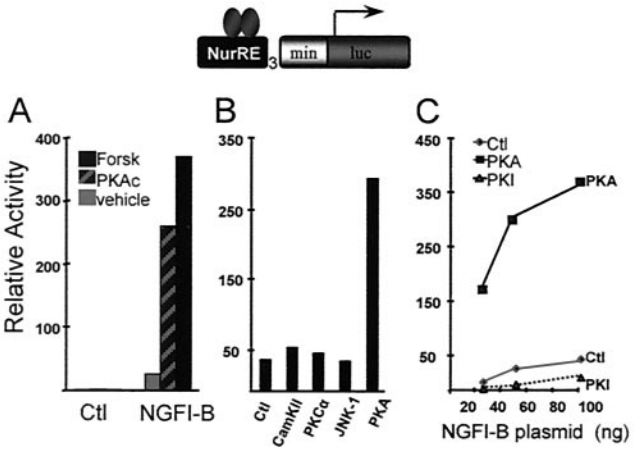


FIG. 3. Specificity of PKA potentiation of NGFI-B activity. (A) NGFI-B-dependent transcription assessed with a NurRE reporter is increased by coexpression of the catalytic subunit of PKA (100 ng of PKAc expression vector) or by forskolin (Forsk) (10^{-5} M) treatment of CV-1 cells. Ctl, control. (B) Effects of different regulatory kinases on NGFI-B-dependent transcription using the NurRE reporter. Each kinase was overexpressed in transfected cells using appropriate expression vectors (76). (C) NGFI-B-dependent transcription is enhanced by PKA and reduced by coexpression of PKA inhibitor, PKI, in CV-1 cells.

then decreasing to near basal levels after 1 h (Fig. 2D, lanes 6 to 10). This result correlates very well with the rapid induction of both cAMP levels and POMC transcription rate, which are maximal within 15 and 30 min of CRH treatment (21, 39). Interestingly, CRH treatment did not enhance the binding of any of the complexes bound to NBRE probe (Fig. 2D, lanes 1 to 5), indicating that CRH specifically modulates NGFI-B when bound to the dimeric NurRE element.

Prior work in adrenal cells had shown dephosphorylation of NGFI-B at Ser316 in response to ACTH, as well as an inhibitory effect of phosphorylation on this residue for DNA binding (12, 27, 46). We used a NGFI-B Ser316 phospho-specific antibody in Western blot experiments to show that CRH induced dephosphorylation of this residue in AtT-20 cells (Fig. 2E). Thus, restoration of NGFI-B's ability to bind DNA may be one of the first effects of CRH. Taken together, these results suggest that in pituitary corticotropes, the CRH/cAMP/PKA pathway enhances NGFI-B transcriptional activity by modulating its DNA binding activity, particularly dimer binding to the NurRE.

PKA specifically potentiates NurRE-dependent NGFI-B transcriptional activity. Since our results suggested that the CRH/cAMP/PKA pathway exerts its effects through preexisting proteins, we tested whether PKA would modulate NGFI-B transcriptional activity in an heterologous context. A NurRE-driven reporter was cotransfected with a CMV-NGFI-B expression plasmid or the empty vector in CV-1 cells. As expected, NGFI-B enhanced NurRE-dependent transcription. Both coexpression of PKA and treatment with forskolin potentiated NGFI-B transcriptional activity (Fig. 3A). PKA also enhanced the transcriptional activity of Nurr1 and NOR-1 (Fig. 4), suggesting a common mechanism of action for PKA on all three Nur factors. PKA and forskolin had no effect on the NurRE reporter alone, and they did not affect the activation of a DR-5 reporter by a CMV-RXRα plasmid, indicating that they were not acting on the cytomegalovirus (CMV) promoter of the expression plasmid (data not shown). In order to assess the specificity of PKA action on NGFI-B transcriptional activity, we coexpressed NGFI-B with a variety of kinases (CamK II, JNK-1, and PKCα), and none of these enhanced NurRE-dependent NGFI-B transcriptional activity (Fig. 3B). We then tested if endogenous PKA activity modulated the transcriptional activity of NGFI-B. Indeed, under conditions where PKA enhanced NGFI-B activity, coexpression of NGFI-B and PKI resulted in approximately 50% inhibition of NGFI-B transcriptional activity (Fig. 3C). Similar results were obtained with Nurr1 and NOR-1, whereas PKI had no effect on CMV-RXRα activity (data not shown).

Since gel retardation experiments suggested specific PKA action on NGFI-B dimers (Fig. 2), we then tested whether PKA would affect NBRE-dependent activity of monomeric NGFI-B. CV-1 (or COS-1) cells were transfected with a limiting amount of NGFI-B expression plasmid and increasing amounts of PKA along with reporter plasmids. In conditions where coexpression of PKA induced a 10-fold increase in NGFI-B transcriptional activity on the NurRE reporter (Fig. 4A), PKA has no significant impact on NBRE-driven transcription (Fig. 4B). Similar results were obtained for Nurr1 (Fig. 4C) and NOR-1 (Fig. 4D). To examine the possibility that this specificity is a reflection of a differential effect of PKA on monomer versus dimer binding, we cotransfected COS cells with PKA and NGFI-B expression plasmids and performed gel retardation essays using whole-cell extracts. As predicted, whereas cotransfection of PKA with NGFI-B resulted in a sharp increase in dimer binding on the NurRE, it had no effect on monomer binding on the NBRE (Fig. 4E). Thus, these results suggest that PKA specifically potentiates NurRE-dependent NGFI-B transcriptional activity by increasing dimer binding to the NurRE.

NurRE-dependent transcription (but not NBRE-dependent transcription) is enhanced by SRCs. The current paradigm for NR activation of transcription stipulates that NRs recruit coactivator complexes to activate target promoters. However, a previous report concluded that Nurr1 monomers did not recruit the p160/SRC coactivator SRC-1, since Nurr1-dependent activation of a NBRE reporter was not stimulated by this coactivator (6). Given the specificity of PKA action on Nur dimers (but not monomers), we tested whether NurRE-dependent activity of NGFI-B, Nurr1, or NOR-1 would exhibit such a preference for coactivator recruitment. Expression vectors for Nur factors were cotransfected in AtT-20 cells with or without SRC-1 expression plasmid using the NBRE-Luc or NurRE-Luc reporters and SRC-1 did not enhance Nur factors-dependent activity on the NBRE-Luc reporter (Fig. 5A) as previously reported (6). In striking contrast, SRC-1 enhanced the transcriptional activity of all three Nur factors using the NurRE reporter up to 10-fold (Fig. 5A). Thus, the ability of SRC-1 to enhance Nur-dependent transcription appears to be restricted to dimers of these factors. We then tested the ability of other p160 coactivators to enhance NGFI-B-dependent transcription (Fig. 5B). Cotransfection experiments conducted in CV-1 cells using either SRC-1, SRC-2 (TIF2), or SRC-3 indicated that all three p160 coactivators enhance NGFI-B-dependent transcription in a dose-dependent manner. Since

SRC-1 is able to interact with the C terminus of CBP/p300 (86), we tested whether CBP and SRC-1 can act together to enhance Nur factor-dependent transcription. Expression vectors for CBP, SRC-1, or both were cotransfected with vectors for Nur factors in AtT-20 cells. On its own, CBP only slightly enhanced Nur factor-dependent activity by comparison to SRC-1, but together, they acted synergistically to enhance the activities of all three Nur factors (Fig. 5C). Dimerization of the Nur factors also appeared important for CBP action, since no CBP-dependent enhancement was observed with the NBRE reporter (data not shown) (6). These data are consistent with the current model of corecruitment of SRC and CBP coactivators by transcriptionally active NRs (51).

The similarities between coactivator- and PKA-induced enhancement of NGFI-B transcriptional activity prompted us to assess if activation of the cAMP pathway results in increased recruitment of SRC family members. We performed an affinity precipitation assay using a biotinylated NurRE probe that was incubated with control or CRH-stimulated AtT-20 whole-cell extracts. Complexes bound to streptavidin-agarose beads were analyzed by Western blotting for the presence of Nur factors and TIF2 (SRC-2). CRH treatment significantly increased the recruitment of TIF2 (SRC-2) in the NurRE-bound protein complexes that also showed increased Nur factors (antibody used recognized NGFI-B and Nurr1 [Fig. 5D, compare lanes 2 and 1 and lanes 4 and 3]).

The N termini of both Nurr1 and NGFI-B mediate PKA potentiation and coactivator recruitment. In order to gain further insight into the mechanisms of basal and PKA- or coactivator-induced transcriptional activity of dimeric NGFI-B, we used a series of truncation mutants (Fig. 6A) in transfection experiments where we tested either their intrinsic transcriptional activity (Fig. 6B), the potentiation effect of PKA (Fig. 6C), or the enhancement by p160 coactivators (Fig. 6D). Deletion of the last 57 aa (ΔC2) resulted in a 60% decrease in transcriptional activity, suggesting the presence of an AF-2 activating domain in this vicinity. However, further deletion of aa 380 to 563 restored the activity to wild-type levels (Fig. 6B). Similar results were reported for NBRE-dependent activity of NGFI-B (11, 59), suggesting the presence of a repressor domain between aa 380 and 506, which encompasses the LBD (Fig. 6A). Progressive deletion of the NGFI-B N terminus completely abrogated NurRE-dependent transactivation and revealed the presence of two important domains together forming the AF-1 domain of NGFI-B (Fig. 6B). A first domain is located between aa 16 and 36, deletion of which can account for a 75% decrease in activity. A second region is located between aa 74 and 124, since the remaining activity was completely lost upon deletion of this region (Fig. 6B). Defective intracellular localization cannot account for the decreased activity of the N-terminal mutants, since all proteins were shown to be located in the nucleus (11, 59). Also, they all appeared to be expressed at similar levels (11, 59) (Fig. 6F and G).

We then tested the effects of PKA on the activity of these mutants. Neither deletion of the entire C terminus nor elimination of aa 3 to 74 of NGFI-B affected PKA potentiation. However, deletion between aa 74 and 124 resulted in a sharp decrease in PKA responsiveness (Fig. 6C). Further deletion of the N terminus had no significant effect. This clearly indicates

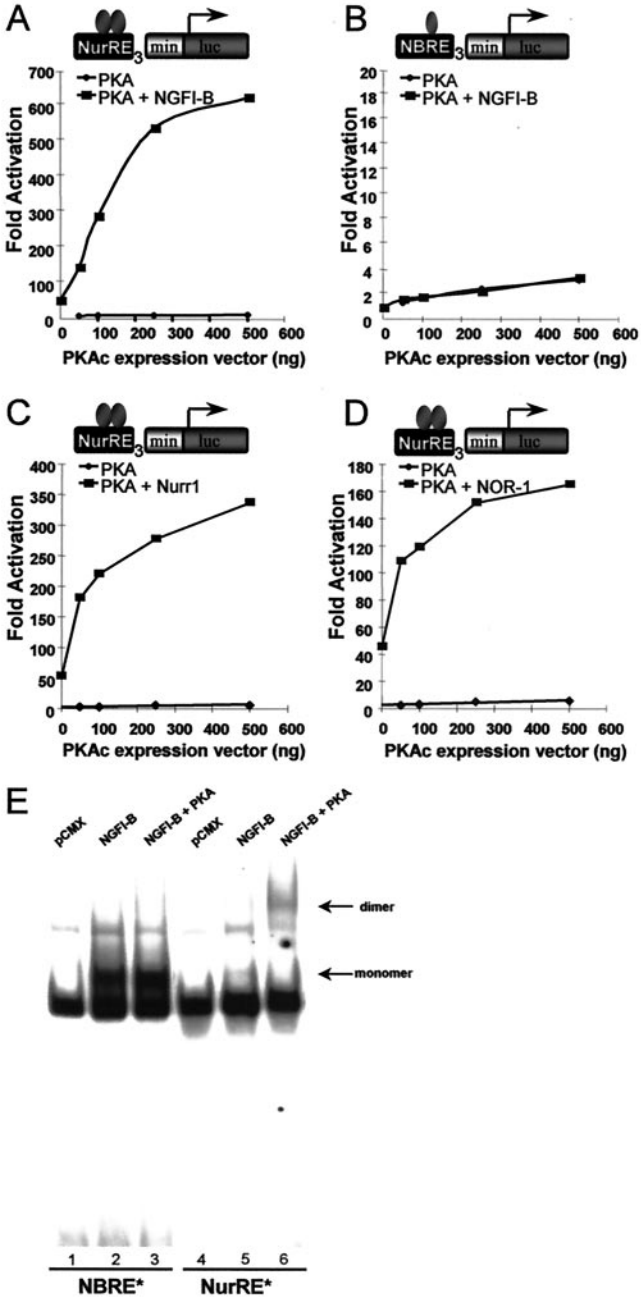


FIG. 4. PKA-dependent enhancement of NGFI-B activity selectively targets dimer-binding NurRE, but not monomer-binding NBRE. (A) Effects of increasing amounts of PKAc expression vector on NGFI-B-induced activity (25 ng of NGFI-B) of NurRE reporter transfected in CV-1 cells. (B) Effects of increasing amounts of PKAc expression vector on NGFI-B-dependent activity (25 ng of NGFI-B) of NBRE reporter. (C and D) Effects of increasing amounts of PKAc expression vector on Nurr1- and NOR-1-dependent activation of NurRE, respectively. (E) NurRE and NBRE binding activity present in whole-cell extracts from COS cells transfected with expression vectors for NGFI-B alone (lanes 2 and 5) or together with PKAc (lanes 3 and 6).

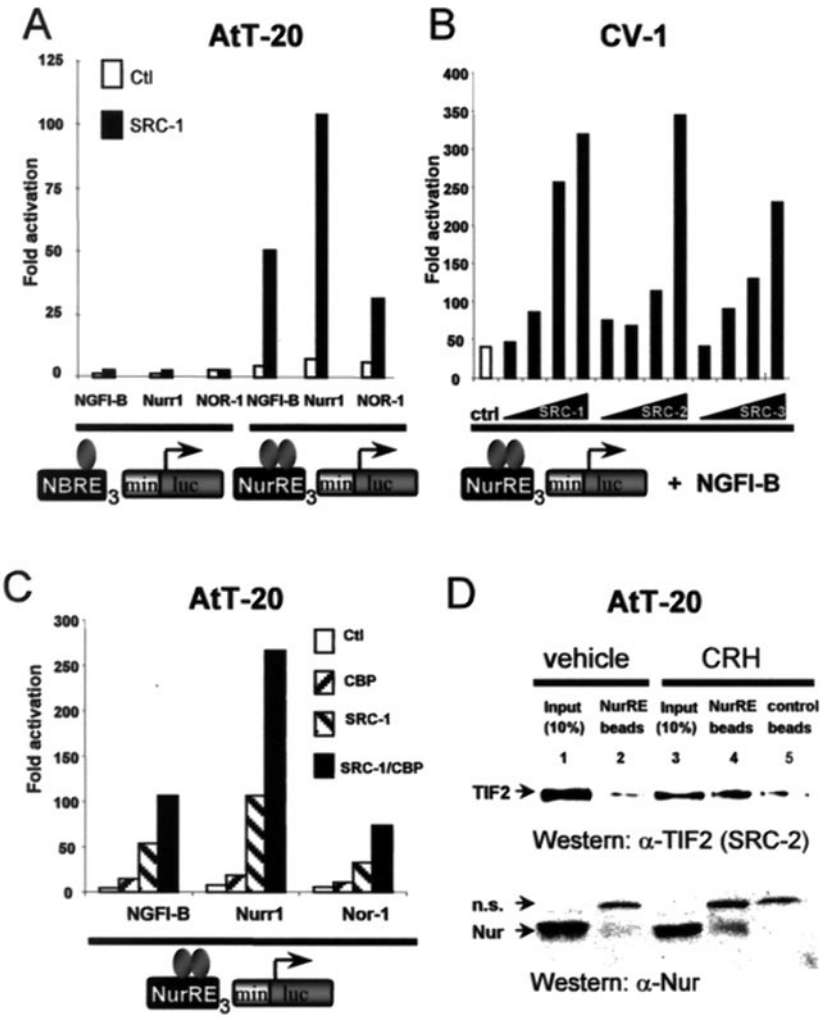
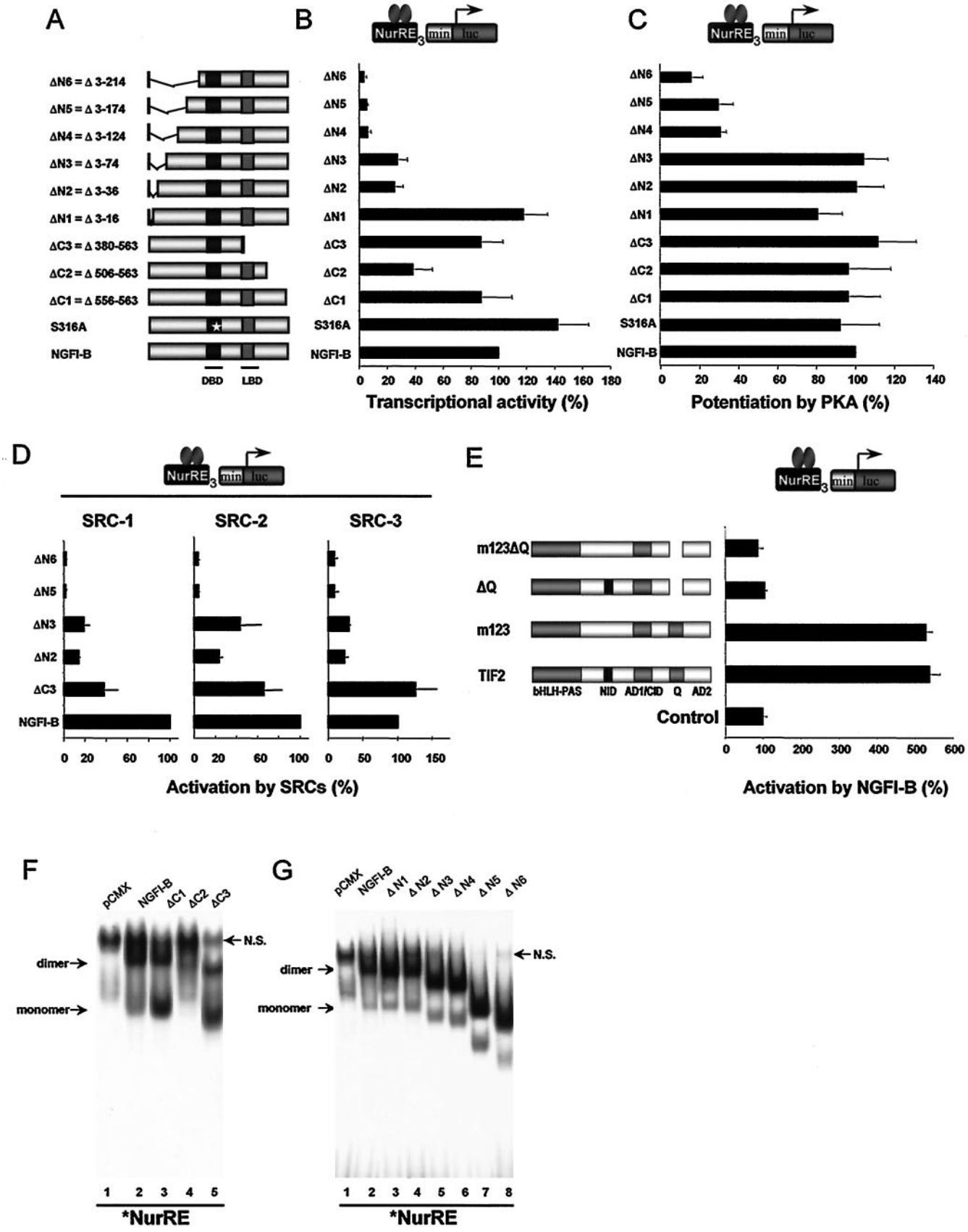


FIG. 5. All three p160/SRC coactivators enhance transcription dependent on NurRE (dimer) but not NBRE (monomer). (A) The activity of NGFI-B, Nurr1, or NOR-1 expression vector (25 ng) was assessed in AtT-20 cells using 200 ng of NBRE reporter or NurRE reporter in the presence or absence of SRC-1 expression vector (1 μg). Data are expressed as the ratios of luciferase activity in the presence of the indicated Nur factor to that in the absence of the indicated Nur factor. Ctl, control. (B) Activity of NGFI-B (control [ctrl]) (100 ng) is enhanced by increasing amounts (100, 250, 500, and 1,000 ng) of SRC-1, SRC-2 (TIF2), and SRC-3 expression vector cotransfected with NurRE reporter (1 μg) in CV-1 cells. Data are expressed as fold activation compared to the activity of reporter in the absence of NGFI-B or SRCs. (C) Nur factor expression vectors (25 ng) were cotransfected in AtT-20 cells with vectors (1 μg) for SRC-1, CBP, or both together with the NurRE reporter (300 ng). (D) NGFI-B and coactivator recruitment to the NurRE revealed in affinity precipitation assay using an antibody against Nur factors (α-Nur) (NGFI-B/Nurr1; Santa Cruz) and a monoclonal TIF2 (SRC-2) antibody. AtT-20 cell extracts (500 μg) treated with either vehicle (control) or CRH (10^{-7} M) for 30 min were subjected to affinity precipitation using a biotinylated NurRE probe and streptavidin-coated agarose beads. n.s., nonspecific.

that most of the effects of PKA are mediated by the region between aa 74 and 124 of NGFI-B.

Dephosphorylation of NGFI-B Ser316 appears to have a permissive role for DNA binding (12, 27, 46) (Fig. 2). A Ser316Ala NGFI-B mutant (which cannot be phosphorylated) might therefore be expected to have higher basal activity than NGFI-B. This was indeed observed (Fig. 6B), and consistent with an effect of PKA on the NGFI-B N terminus, the activity of this mutant was enhanced by PKA (Fig. 6C).

We then performed a similar analysis with the three p160 coactivators. Recruitment or enhancement of SRC-1, but not SRC-2 or SRC-3, was affected by deletion of the C terminus of NGFI-B (Fig. 6D) or Nurr1 (data not shown). Progressive deletion of the N-terminal domain of NGFI-B resulted in a complete loss of SRC-dependent enhancement. Deletion of NGFI-B aa 3 to 36 resulted in an initial decrease of SRC-dependent activity for all three SRCs, and further deletion to position 74 did not further affect activity (Fig. 6D). This localization correlates well with the identification of NBRE-dependent (11, 59) and NurRE-dependent (Fig. 6B) AF-1 activity within the region between aa 16 and 36. The remaining SRC-dependent activity was completely lost upon deletion of sequences between aa 74 and 174. Again, this region was also involved in NBRE- and NurRE-dependent NGFI-B activity (Fig. 6B). The transcriptional activity of AF-1 sequences is thought to depend on physical interaction with coactivators.


A
ΔN6 = Δ 3-214
ΔN5 = Δ 3-174
ΔN4 = Δ 3-124
ΔN3 = Δ 3-74
ΔN2 = Δ 3-36
ΔN1 = Δ 3-16
ΔC3 = Δ 380-563
ΔC2 = Δ 506-563
ΔC1 = Δ 556-563
S316A
NGFI-B
DBD
LBD
B
NurRE3 min luc
Transcriptional activity (%)
C
Potentiation by PKA (%)
D
SRC-1
SRC-2
SRC-3
Activation by SRCs (%)
E
m123ΔQ
ΔQ
m123
TIF2
bHLH-PAS NID AD1/CID Q AD2
Control
Activation by NGFI-B (%)
F
pCMX
NGFI-B
ΔC1
ΔC2
ΔC3
N.S.
dimer
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dimer
monomer
*NurRE

The putative interaction of SRC coactivators with the NGFI-B AF-1 domains was ascertained in recent work showing direct interactions in pulldown assays (80).

Distinct SRC regions mediate the AF-1 and AF-2 activities of NRs. In order to define the domains of TIF2 required for NGFI-B coactivation, we used a series of TIF2 mutants known to specifically impair interaction with either the AF-1 or AF-2 domain of estrogen receptor alpha (ERα) (3). Indeed, the TIF2m123 mutant containing alanine substitutions of critical leucines in all three LxxLL motifs of the NR-interacting domain (NID) abrogates stimulation of the ligand-dependent AF-2 function, while the TIF2ΔQ deletion mutant lacking the Q-rich domain impairs the ligand-independent AF-1-mediated transcriptional activity of ERα (3, 79). In transient-transfection experiments, the TIF2m123 mutant was as efficient as wild-type TIF2 in enhancing NGFI-B transcriptional activity, whereas mutant TIF2ΔQ abrogated TIF2-mediated enhancement (Fig. 6E). As expected, the double mutant TIF2m123ΔQ was also deficient in enhancing NGFI-B-dependent activity (Fig. 6E). These results clearly implicate the AF-1-interacting Q-rich domain of TIF2 in NGFI-B coactivation.

Since PKA and SRCs only potentiated the activities of NGFI-B dimers on the NurRE (and not NBRE), the loss of activity by NGFI-B mutants could reflect impaired dimerization. In order to determine which region of NGFI-B is important for dimer binding on the NurRE, we transfected COS cells with expression plasmids for each truncation mutant, and gel retardation experiments were performed using whole-cell extracts. Two major complexes bind the NurRE in extracts from cells transfected with wild-type NGFI-B, the slower-migrating one being predominant and consisting of NGFI-B homodimers (Fig. 6F, lane 2) (48, 62). The extreme C terminus appears to play some role in dimer binding, since deletion of the last 7 aa resulted in approximately equal binding for both complexes, which was also observed upon deletion of the entire C terminus (Fig. 6F, lanes 3 and 4). Curiously, the mutant lacking the last 57 aa reproducibly exhibited only the slower-migrating complex. However, the results of Western blot analysis did not support the conclusion that this mutant is expressed at a level lower than those of other NGFI-B proteins (data not shown); inappropriate folding or sorting may account for the difference in DNA binding activity. All N-terminal mutants exhibited predominantly dimeric binding (Fig. 6G), indicating that the loss of basal and PKA-induced transcriptional activity is not a direct consequence of an impaired ability to form dimers on the NurRE. Taken together, these results suggest that the N terminus modulates the transactivation activities of NGFI-B, while the DBD and the extreme C terminus appear to contribute to dimer binding to the NurRE.

The results of a similar structure-function analysis reveal that Nurr1 behaves slightly differently than NGFI-B. Indeed, deletion of the C terminus retained only 20% of the activity of wild-type Nurr1 (Fig. 7B), indicating that it plays a prominent role in NurRE-dependent Nurr1 transactivation, which was not the case for NGFI-B. However, an internal deletion of aa 72 to 162 which removes all but the first 3 aa of the corresponding region defined as the AF-1 of NGFI-B also abrogated most of the transcriptional activity (Fig. 7B), thus ascribing a major role for the AF-1 domain of Nurr1 in NurRE-dependent transcription. Deletion of aa 160 to 236 had no effect on Nurr1 transactivation abilities. As seen for NGFI-B, only the mutant lacking the AF-1 domain showed impaired PKA potentiation (Fig. 7C), although it is still somewhat responsive to PKA, suggesting that other regions are responsible for part of the PKA effect.

All together, the analysis with deletion mutants identified a major role for the AF-1 domains of Nur factors in basal, PKA-induced, and SRC enhancement of Nur dimer activity and strongly suggests a link between PKA potentiation and coactivator recruitment by Nur factor dimers.

DISCUSSION

Although the primary response to CRH leading to activation of the cAMP/PKA pathway and accumulation of intracellular Ca^{2+} has been well characterized (38, 39, 66), a wide variety of POMC gene response elements and transcriptional effectors have been proposed to mediate the nuclear events triggered by these second messengers. These include cFos (4), CREB (36), and PCRH-REB-1 (32). However, these proteins are probably not major factors relaying the effects of CRH, since their promoter target sequences do not appear to be essential for CRH responsiveness. Our laboratory has previously identified an element essential for CRH responsiveness in the POMC promoter and shown that it is a target for NGFI-B dimers (48, 62). The demonstration that a dominant-negative mutant of NGFI-B abolished CRH-dependent activity of both the NurRE and the intact POMC promoter (62) strongly suggested that this orphan NR was a major integrator of the signals elicited by CRH in pituitary corticotropes.

FIG. 6. Mapping of basal and PKA- and SRC-dependent activities of NGFI-B to the AF-1 domain. (A) Schematic representation of NGFI-B and deletion mutants used in this work. The amino acids deleted in the mutants are shown. (B) Activity of NGFI-B and its mutants expressed as a percentage of the NGFI-B activity assessed using the NurRE reporter cotransfected into CV-1 cells. Data are represented as means ± standard errors of the means (indicated by the error bars) of at least three experiments performed in duplicate. (C) PKA potentiation of NGFI-B and its mutant's activity assessed using the NurRE reporter. Results are presented as a percentage of the PKA-induced activity to the basal activity for each mutant, and the effect of PKA on wild-type NGFI-B-dependent transcription is set at 100%. (D) SRC coactivator enhancement of NGFI-B-dependent transcription assessed for wild-type and selected mutants using the NurRE reporter. Enhancement of wild-type NGFI-B-dependent transcription is set at 100%. (E) Transcriptional enhancement of NGFI-B-dependent activity by TIF2 and its mutants. CV-1 cells were transfected with NurRE reporter or with the NGFI-B expression vector alone or with TIF2 expression plasmid. Activity of NGFI-B alone was set at 100%. (F) Gel retardation experiments showing binding activity of NGFI-B and its C-terminal deletion mutants after transfection into COS cells. Nuclear extracts (5 μg) from transfected cells were assessed for NurRE probe binding activity. Dimer and monomer arrows indicate the positions of relevant complexes for intact NGFI-B. N.S., nonspecific. (G) DNA binding activity of NGFI-B and its N-terminal deletion mutants after transfection into COS1 cells. Nuclear extracts from these cells were used in gel shift assays using the NurRE probe. Similar results were obtained with in vitro-translated proteins (data not shown).

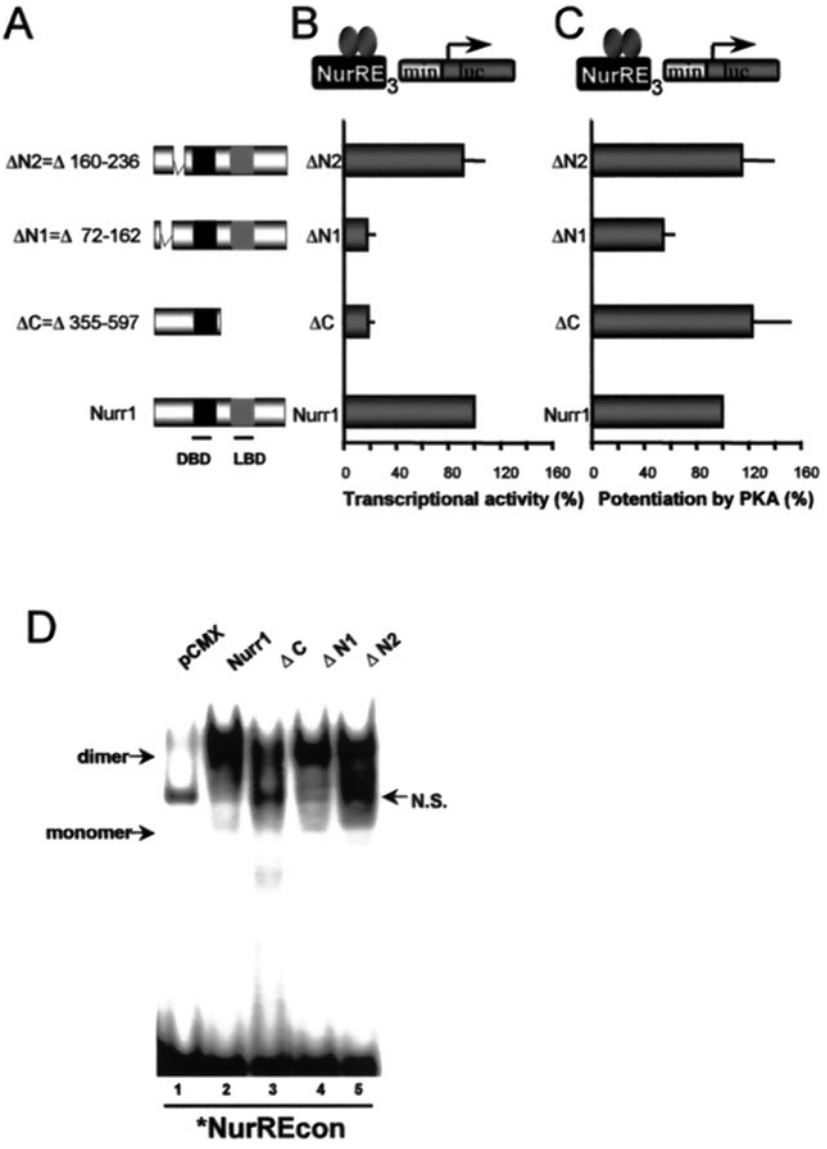

FIG. 7. Structure-function analysis of Nurr1. (A) Schematic representation of Nurr1 and its deletion mutants. (B) Activity of Nurr1 and its deletion mutants assessed by cotransfection into CV-1 cells using the NurRE reporter. Activity is expressed relative to that of Nurr1. (C) Potentiation of Nurr1-dependent activity by PKA overexpression assessed by cotransfection into CV-1 cells using the NurRE reporter. (D) DNA binding activity of Nurr1 and its deletion mutants assessed by gel mobility shift assays using nuclear extracts of transfected COS1 cells and the NuRE consensus probe as described previously (48). N.S., nonspecific.

We now report that the NurRE is highly responsive to activation of cAMP/PKA signaling and that inhibition of PKA abrogates its responsiveness to CRH (Fig. 1), thus confirming that the CRH/cAMP/PKA pathway signals in this system through activation of NurRE-dependent transcription by NGFI-B. NurRE responsiveness cannot be due to induction of NGFI-B mRNA transcription, since Nur subfamily protein levels were unaffected by CRH treatment. This is in agreement with our previous observation that CRH effects on POMC transcription do not require de novo protein synthesis (22). Rather, CRH enhances NurRE binding by a nuclear complex containing NGFI-B, and we showed that this binding peaks after 30 min of CRH treatment. This correlates very well with previous reports which had demonstrated that cAMP levels and POMC transcription reach their maximum within 15 to 30 min following CRH treatment (22, 39). These results further position NGFI-B as the major mediator of CRH signaling, although other mechanisms might also contribute to CRH responsiveness in certain conditions. Thus, signaling through NGFI-B may involve acute responses mediated through existing protein in addition to effects elicited through NGFI-B gene transcription. It is noteworthy that a variety of genes that are thought to be regulated by NGFI-B can also be induced by the cAMP/PKA pathway. These genes include the salivary-specific R15 gene (44), the steroidogenic p450c21 (21-hydroxylase) (7) and p450c17 (91) genes, and a variety of thyrocyte-specific genes (64). Therefore, it is tempting to propose that modula-

tion of NGFI-B transcriptional activity is a general mechanism by which the cAMP/PKA pathway may exert its effects.

Our results suggest that PKA signaling regulates the transcriptional activity of NGFI-B (Fig. 3 and 4). Indeed, we demonstrated that stimulation of PKA signaling strongly enhanced Nur factor transcriptional activity and DNA binding activity to the NurRE. The potentiation effect of PKA appeared specific to Nur dimers and the NurRE (Fig. 3 and 4). Both dimer binding (Fig. 2 and 4E) and PKA effects are closely associated, as shown by activation of the PKA pathway (Fig. 4) or by its blockade (Fig. 1 and 3). This is supported by the observation that CRH does not induce NGFI-B binding to the NBRE (Fig. 2), and it does not significantly increase NBRE-dependent transcription (62). The first level at which PKA signaling may intervene is DNA binding, since the NGFI-B DBD is known to be dephosphorylated at Ser316 following activation (12, 46) (Fig. 2E) to allow protein-DNA interaction. The N terminus of NGFI-B was also shown to be hyperphosphorylated in response to some signals (12), and such action would be consistent with the involvement of the AF-1 in PKA action (Fig. 6C). However, it is doubtful that N-terminal phosphorylation plays a major role in dimer formation, since only C-terminal deletion affected NGFI-B dimers (Fig. 6F). Thus, PKA signaling may initially have a mostly permissive effect for DNA binding by inducing dephosphorylation of the DBD. In itself, this does not however explain the preference for dimer binding to DNA observed in nuclear extracts of cells treated with the constitutively active catalytic subunit of PKA (PKAc) or CRH (Fig. 2 and 4E). In any event, NGFI-B appears to be the endpoint mediator of PKA signaling initiated by the G-protein-coupled CRH receptor.

Until recently, Nur factor-dependent activation of transcription appeared to be very atypical in the NR superfamily, not only because of the lack of ligand but also because of the apparent independence from coactivator recruitment when activating as monomers (6) (Fig. 5). However, monomer activity per se may not account for Nur factor's inability to recruit p160 coactivators, since another NR acting on monomers, SF-1, does recruit these coactivators (10, 24, 31). Classically, NR-ligand binding is thought to induce conformational changes in the LBD that result in association between the AF-2 domain and coactivators, which in turn, contact the basal transcriptional machinery to increase transcription. Thus, it is of particular interest that coactivators of the p160/SRC and CBP/p300 families are specifically recruited by Nur factors acting as dimers following PKA activation. The specificity of dimer action may depend on the effects of the PKA pathway on the N terminus of NGFI-B and its ability to recruit coactivators. In this context, it is noteworthy that the NGFI-B AF-1, rather than AF-2, is involved in PKA dependence and SRC coactivator recruitment. Dimer specificity may result from stabilization of DNA-bound dimers (but not monomers) by AF-1-dependent interaction with the coactivators. Recent reports showing ligand-independent recruitment of coactivators to NRs through phosphorylation of the AF-1 domain have implicated the MAPK pathway (24, 74). The striking requirement for dimers also sets this mechanism apart from a recent study reporting both NBRE- and NurRE-dependent transcription enhancement by the coactivator ASC-2 (70).

Structure-function analysis indicated that the AF-1 domains

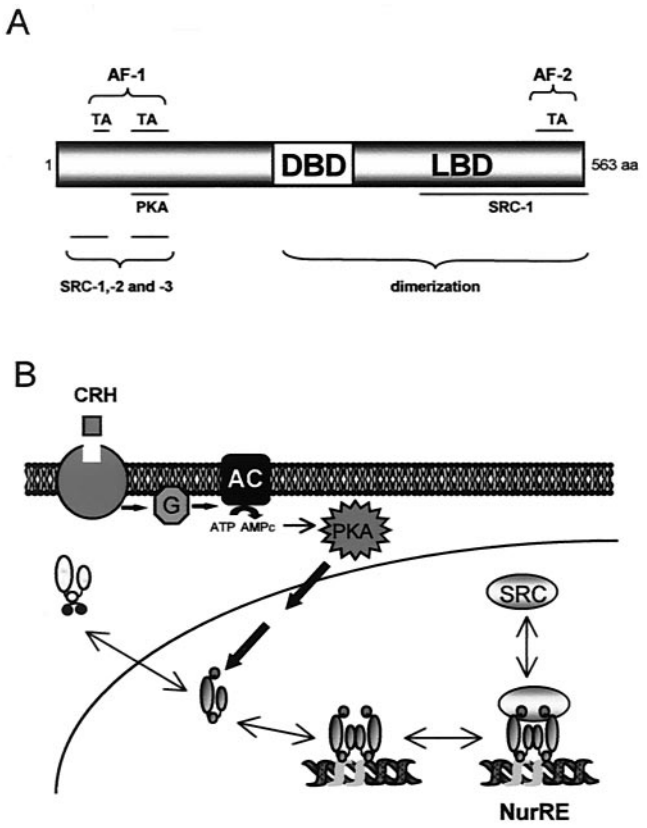

FIG. 8. Model of NGFI-B structure and activation mechanism. (A) Summary of structure-function data for NGFI-B dimer action on NurRE-dependent transcription. The N terminus contains a composite AF-1 domain where both subdomains (TA bars) confer part of basal transcriptional activity (TA) and SRC coactivator-dependent activity. Only the distal subdomain (aa 74 to 124) is required for PKA potentiation. Homodimerization appears to be mainly mediated through the DBD with some contribution by the C terminus. (B) Working model for action of CRH/cAMP/PKA pathway on NGFI-B transcriptional activity. Upon PKA activation, NGFI-B is dephosphorylated on the DBD (Fig. 2E), and the activity of its N-terminal AF-1 domain is enhanced, presumably by phosphorylation (12) and recruitment of SRC coactivators (Fig. 5). Both PKA action and SRC enhancement are preferentially exerted on DNA target of Nur factor dimers.

of NGFI-B and Nurr1 were absolutely required for NurRE-dependent basal and PKA-enhanced transcriptional activity (Fig. 6 and 7). A summary of our structure-function results for NGFI-B is presented in Fig. 8A. In this case, the AF-1 domain could be subdivided into two distinct regions: a region between aa 16 and 36 primarily involved in basal activity and a second region between aa 74 and 124 which conferred most, if not all, responsiveness to PKA. A deletion between aa 72 to 162 of Nurr1, which encompasses the corresponding AF-1 region of NGFI-B impaired both basal activity and PKA potentiation, suggesting a common role for the AF-1 domains of Nur factors. The Nurr1 C terminus may have a different role than NGFI-B. The stimulatory effects of SRC coactivators on NGFI-B activity were also mapped to the same AF-1 subdomains that are important for basal activity (Fig. 8A). Thus, only one NGFI-B AF-1 subdomain (aa 74 to 124) is targeted by both PKA signals and SRC coactivators. It is noteworthy that

only SRC-1, but not SRC-2 or SRC-3, showed some dependence on the C-terminal AF-2 domain of NGFI-B (Fig. 6D), possibly reflecting differential properties of SRC coactivators. The strong dependence of NGFI-B on its AF-1 function sets it apart from most NRs and may be taken as further indication of its role as the endpoint of a cytoplasmic signaling cascade such as the PKA pathway. This is further highlighted by the facts that only the AF-1-interacting Q-rich domain of TIF2 is important for transcriptional stimulation of NGFI-B activity (Fig. 6E) and that this domain is known to stimulate the ligand-independent activity of NRs following phosphorylation of their AF-1 domains (3, 74).

We do not know whether PKA itself or another downstream kinase is targeting NGFI-B. PKA was reported to modulate the activity of a variety of NRs. However, the transcriptional effects seem to vary depending on the receptor. Indeed, while PKA enhances binding and transcriptional activity of SF-1 (71), glucocorticoid receptor (GR) (65), and mineralocorticoid receptor (49), it has the opposite effect on HNF4 (78). This is interesting, since HNF4 has an A box similar to that of NGFI-B, and the negative effects of PKA have been attributed to phosphorylation of a serine (Ser134) that corresponds to Ser316 in the DBD of NGFI-B (78). This observation and the known negative effect of phosphorylated Ser316 on NBRE binding (27, 46) suggest that PKA itself might not be directly phosphorylating NGFI-B. An indirect effect of PKA is also supported by the fact that ACTH-induced PKA signaling results in hypophosphorylation of NGFI-B Ser316 in adrenals (12, 27). PKA might modulate the activity of phosphatases such as calcineurin; interestingly, the calcineurin inhibitor cyclosporine A is though to inhibit T-cell apoptosis by interfering with NGFI-B binding activity (87). Also, the phosphatase 1 and 2A inhibitor okadaic acid prevents dephosphorylation of Ser316 in ACTH-treated Y1 cells (46). In any case, phosphorylation of Ser316 could explain why the cytosolic form of NGFI-B present in AtT-20 cells did not bind the NurRE (Fig. 1 and 2).

In summary, we have shown (Fig. 8B) that CRH stimulation of cAMP/PKA signaling leads to dephosphorylation of NGFI-B Ser316 permitting DNA binding. PKA action also rapidly increases nuclear dimer (but not monomer) NGFI-B DNA binding activity, SRC coactivator recruitment to the AF-1 domain, and NGFI-B-dependent transcription. Although not tested in the present work, the coactivators themselves could be targets of CRH/PKA signaling. Such regulatory events have been well documented both for CBP and p160 coactivators (2, 13, 68). NGFI-B transcriptional enhancement is primarily exerted through the N-terminal AF-1 domain rather than the AF-2 domain, setting Nur factors apart from other NRs. The present work clearly establishes NGFI-B as an effector for the cAMP/PKA pathway. It also highlights the unique features of cAMP/PKA-dependent transcriptional activation by comparison to ligand-dependent NR action.

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