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TIF1β/KAP-1 Is a Coactivator of the Orphan Nuclear Receptor NGFI-B/Nur77*[S]

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In efforts to define mechanisms of transcriptional activation by the orphan nuclear receptor NGFI-B (Nur77), we identified TIF1β by mass spectrometry within a nuclear protein complex containing NGFI-B. TIF1β, also known as KAP-1 (KRAB domain-associated protein) or KRIP-1, acts as a transcriptional corepressor for many transcription factors, in particular for the Krüppel-associated box domain-containing zinc finger transcription factors. TIF1β is also an intrinsic component of two chromatin remodeling and histone deacetylase complexes, the N-CoR1 and nucleosome remodeling and deacetylation complexes. In contrast to these activities, we report that TIF1β is a coactivator of NGFI-B and that it is as potent as the SRC coactivators in this context. Using pull-down assays and immunoprecipitation, we showed that TIF1β interacts directly with NGFI-B and with other Nur family members. NGFI-B is an important mediator of hypothalamic corticotropin-releasing hormone (CRH) activation of proopiomelanocortin (POMC) transcription, and TIF1β enhances transcription mediated through the NGFI-B target, the Nur response element (NurRE). The NurRE binds Nur factor dimers and is responsive to signaling pathways. In keeping with the role of NGFI-B as mediator of CRH signaling, we found that TIF1β is recruited to the POMC promoter following CRH stimulation and that TIF1β potentiates CRH and protein kinase A signaling through the NurRE; it acts synergistically with the SRC2 coactivator. However, the actions of TIF1β and SRC2 were mapped to different NGFI-B AF-1 subdomains. Taken together, these results indicate that TIF1β is an important coactivator of NGFI-B-dependent transcription.

NGFI-B (also known as Nur77, TR3, and NAK-1) is a transcription factor belonging to the superfamily of nuclear receptors (NRs).[4] NGFI-B is closely related to Nurr1 (Nur-related factor 1) (RNR-1, TINUR, and HZF-3) and NOR-1 (neuron-derived orphan receptor 1) (MINOR) (1–3), together forming a distinct subfamily, the Nur factors. NGFI-B and NOR-1 are constitutively expressed in some regions of the brain as well as in peripheral tissues (3–5). In contrast, the Nurr1 expression pattern is more restricted in the central nervous system. The Nur factors are immediate early response genes that share a well conserved DNA binding domain and ligand binding domain but a poorly conserved N-terminal A/B region (6). Nur subfamily members are important physiological regulators implicated at multiple levels of the hypothalamo-pituitary-adrenal axis. This axis mediates the stress response via secretion of adrenocorticotropic hormone (ACTH) and induction of adrenal glucocorticoid synthesis. ACTH is derived from the processing of the proopiomelanocortin (POMC) precursor, and it is under the control of hypothalamic corticotropin-releasing hormone (CRH). At the hypothalamic level, CRH-producing neurons exhibit induced Nur factors after stress (7, 8), and these may in turn regulate CRH gene transcription (9). In pituitary corticotroph cells, CRH activates POMC gene transcription (10, 11). Upon binding to its receptor CRHR-1 on corticotrophs (12), CRH induces a signaling cascade that ultimately leads to increased POMC gene transcription. CRH increases cAMP levels, followed by activation of the protein kinase A (PKA) and mitogen-activated protein kinase pathways (13–15). Nur factors regulate the POMC promoter via the Nur response element (NurRE) that binds homodimers or heterodimers of Nur factors containing at least NGFI-B (16, 17). NGFI-B was shown to be an important mediator of CRH action on POMC transcription through the NurRE (17, 18). The molecular events involved in CRH activation include dephosphorylation of Ser^{316} of NGFI-B, which allows dimer binding to the NurRE and recruitment of SRC2 and Rb (18, 19). SRC/p160 coactivators enhance transcription in part by their intrinsic histone acetyltransferase activity and by recruitment of CBP/p300 and coactivators that contain other enzymatic activities (20, 21) such as the histone methyltransferase CARM-1.

Finally, glucocorticoids exert a negative feedback on POMC gene transcription. The NurRE activity is subject to glucocorticoid receptor (GR) *trans*-repression, involving direct interactions between NGFI-B and GR (17, 22, 23). In order to identify hormone responsive regulators that might

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1 and Fig. 1.

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[4] The abbreviations used are: NR, nuclear receptor; ACTH, adrenocorticotropic hormone; ChIP, chromatin immunoprecipitation; CRH, corticotropin-releasing hormone; GR, glucocorticoid receptor; GST, glutathione *S*-transferase; KRAB, Krüppel-associated box; NBRE, NGFI-B response element; NurRE, Nur response element; PKA, protein kinase A; POMC, proopiomelanocortin; Rb, retinoblastoma; siRNA, small interfering RNA; SRC, steroid receptor coactivator; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; Dex, dexamethasone; NuRD, nucleosome remodeling and deacetylation complex.

be involved in CRH activation and glucocorticoid repression of POMC transcription through the NurRE, we investigated CRH-dependent NGFI-B-associated proteins that may serve a transcription co-regulatory function in POMC-expressing cells. We thus report the identification and the characterization of TIF1β as a novel CRH-dependent coactivator of NGFI-B.

The transcriptional intermediary factors are part of a large family of transcriptional co-regulatory proteins that are involved in several developmental and physiological processes (24–28). The TIF1 (transcriptional intermediary factor 1) family includes TIF1α (29), TIF1β (27), TIF1δ (26), TIF1γ (30), and Bonus (24).

TIF1β, also known as KAP-1 (KRAB domain-associated protein) (31) or KRIP-1 (32), is proposed to act as the universal transcriptional corepressor for the Krüppel-associated box (KRAB) domain-containing zinc finger transcription factors, such as KOX1 (33), ZNF133, ZNF140 (31), and Kid-1 (32), through a conserved interaction between variants of the KRAB domain and TIF1β (34). For transcriptional repression, TIF1β recruits different enzymatic activities through direct protein-protein interactions, including 1) the nucleosome remodeling and deacetylation complex (NuRD/Mi-2α) (35) and 2) the histone methyltransferase SETDB1, which specifically methylates Lys^9 of histone H3 (36). TIF1β is also an intrinsic component of the N-CoR-1 complex containing both histone deacetylase and ATP-dependent SWI/SNF activities (37). Finally, TIF1β interacts directly with endogenous members of the HP1 (heterochromatin protein 1) family (27, 38, 39). These data support a role for TIF1β in the epigenetic regulation of transcription via the initiation and maintenance of heterochromatin structures. In agreement with this general repressor function, TIF1β was shown to be localized in heterochromatin; however, it was also observed in euchromatin (38). Consistent with this presence in active euchromatin, TIF1β was found to be a coactivator of CCAAT/enhancer-binding protein-β and GR for the induction of the α1-acid glycoprotein gene (40).

In this study, we show that TIF1β enhances POMC gene transcription through Nur orphan NRs. In particular, TIF1β is recruited to the POMC promoter under CRH stimulation, and it enhances Nur-dependent CRH and PKA responses. We further show that the action of TIF1β on NGFI-B-dependent transcription may involve synergistic activation with SRC2, a coactivator of a different structural class that has been implicated in the action of many NRs.

EXPERIMENTAL PROCEDURES

Plasmids—The reporter plasmids were constructed in pXP1-luc (41) containing the minimal POMC promoter (−34 to +63 bp). The −480 POMC promoter with mutations of the NurRE and NBRE regulatory elements were described previously (42). The luciferase reporter plasmid containing three copies of the NurRE, the NBRE, or the Tpit/PitxRE regulatory elements was described (16, 43). CMX-NGFI-B, CMX-Nurr1, CMX-NOR-1, and CMX-SRC2 expression vectors have been previously generated (18, 44). Several deletions (ΔC3, ΔN1, ΔN3, and ΔN5) of CMX-NGFI-B expression vectors were described previously and shown to be expressed at similar levels (18). The NGFI-B N-terminal mutant ΔN7 was deleted between amino acids 74 and 174; it is expressed at similar levels as wild-type NGFI-B (supplemental Fig. 1). The plasmids pCGN-TIF1β, pcDNA3-FLAG-HA-TIF1β, and pCDNA3-FLAG-HA-TIF1β 6KR are generous gifts from Dr. Muriel Aubry (45). To generate the 3× FLAG-NGFI-B-V5-His expression plasmid, we modified the $pLNCX_2$ (BD Biosciences) plasmid by insertion of an oligonucleotide containing the 3× FLAG and the restriction sites BglII and HindIII in each end (AGATCTCCACCATGGACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGACTACAAGGATGACGATGACAAG) and an oligonucleotide containing the V5-His (GGATCCGGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCGATTCTACGCGTACCGGTCATCATCACCATCACCATTGAAAGCTT). This insertion included a BamHI site for subsequent cloning of NGFI-B coding sequence (NM_010444). The NGFI-B-V5-His expression plasmid was produced as described above but without the 5′ 3× FLAG sequence. The expression plasmids for FLAG-tagged TIF1β (NM_011588), Pelp1 (NM_029231), and Mybbp1a (NM_016776) were generated as described above but without the 3′ V5-His sequence. We generated expression plasmids for 3× FLAG-tagged Mta2 (NM_011842), p66β (NM_139304), SRC2 (46), and E47 (AF352579) with a 3× FLAG insertion (GGATCCGACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGACTACAAGGATGACGATGACAAGTGAAAGCTT).

Cell Culture and Transfection—AtT-20 and HEK293T cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and penicillin/streptomycin. AtT-20 cells were transfected by lipofection with Lipofectamine (Invitrogen), as previously described (17). Each transfection (in duplicates) was repeated at least three times.

Generation of Retrovirus-infected AtT-20 Cells—The Eco-Pack-2 293 cells (Clontech, Mountain View, CA) were plated (5×10^6) in a 10-cm plate and transfected by the calcium phosphate coprecipitation method with 10 μg of expression plasmid $pLNCX_2$-3×FLAG-NGFI-B-V5-His for 8 h. 16 h later, the medium containing the retrovirus was filtered with a 0.22-μm filter; diluted in Dulbecco's modified Eagle's medium, 10% fetal bovine serum with penicillin/streptomycin and 1 μg/ml Polybrene (Sigma); and added to AtT-20 cells. This infection procedure was repeated three times/day for 2 days, and infected AtT-20 cells were cultured with 400 μg/ml neomycin. The growing neomycin-resistant colonies were pooled together to generate a retrovirus-infected population of about 5,000 independent colonies.

Nuclear Extract Preparation and FLAG Purification Procedure—Nuclear extracts used for affinity FLAG purification of flagged proteins were prepared with 4×10^9 cells AtT-20 cells. The cells were washed twice with cold phosphate-buffered saline and collected in a 50-ml tube. The cells were then centrifuged and resuspended in 10 volumes of buffer A (20 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM DTT, and the protease inhibitors leupeptin, aprotinin, and pepstatin (10 μg/ml each)) and centrifuged immediately. The cytoplasmic extract was prepared by resuspending the cell pellet in 2 volumes of buffer A and leaving it at 4 °C for 10 min with gentle mixing. The nuclei

were collected by centrifugation, washed once with 1 volume of buffer A, and extracted with the addition of 1 volume of buffer HS (20 mM Hepes, pH 7.9, 500 mM KCl, 10% glycerol, 3 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM PMSF, 1 mM DTT, and the protease inhibitors leupeptin, aprotinin, and pepstatin (10 μg/ml each)). The nuclei were allowed to extract for 30 min at 4 °C with continuous gentle mixing. The extract was centrifuged, and the protein concentration of the supernatant was determined by a Bradford assay (Bio-Rad). The nuclear extracts were precleared at 4 °C using IgG-Sepharose 6 fast flow beads (Amersham Biosciences), A/G-agarose beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and agarose IgG beads (Sigma) for 1 h. The extracts were then diluted with 1 volume of IP 2× buffer (20 mM Hepes, pH 7.9, 10 mM KCl, 10% glycerol, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM PMSF, 1 mM DTT, 0.2% Tween 20, and the protease inhibitors leupeptin, aprotinin, and pepstatin (10 μg/ml each)), and the supernatant was immunoprecipitated for 3 h at 4 °C with α-FLAG M2 or nonimmune IgG cross-linked to agarose beads (Sigma). Immunoprecipitates were washed twice with IP buffer (20 mM Hepes, pH 7.9, 125 mM KCl, 5% glycerol, 1.5 mM $MgCl_2$, 0.1 mM EDTA, and 0.1% Tween 20) and twice with IP buffer containing 300 mM KCl instead of 125 mM KCl. The eluate was obtained by incubating the bead with 500 μg/ml of 3× FLAG peptide.

Mass Spectrometry Analysis—The eluate was resolved on an 8% SDS-polyacrylamide gel, and the gel was silver-stained. The protein bands were excised from the gel and subjected to reduction, alkylation, and in-gel tryptic digestion, as previously described (47). The resulting tryptic peptides were purified and identified by MALDI-TOF mass spectrometry. For the proteins indicated in Fig. 2, different peptides were identified, as indicated in supplemental Table 1.

Coimmunoprecipitation Assays and Western Blot Analysis—HEK293T cells (3×10^6) were plated in a 10-cm plate and transfected by the calcium phosphate coprecipitation method with 5 μg of expression plasmids for tagged polypeptides for 48 h. The cells were then washed twice with cold phosphate-buffered saline and then centrifuged and resuspended in 5 volumes of buffer A (20 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.5 mM PMSF, 1 mM DTT, and the protease inhibitors leupeptin, aprotinin, and pepstatin (10 μg/ml each)) and centrifuged immediately. The cell pellet was resuspend in 2 volumes of buffer A, and cells were allowed to swell on ice for 10 min before the addition of 3 volumes of buffer B (20 mM Hepes, pH 7.9, 500 mM KCl, 10% glycerol, 3 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM PMSF, 1 mM DTT, and the protease inhibitors leupeptin, aprotinin, and pepstatin (10 μg/ml each)). Nuclei were allowed to extract for 30 min at 4 °C with continuous gentle mixing. The extract was centrifuged, and the protein concentration of the supernatant was determined by a Bradford assay (Bio-Rad). The extracts were precleared at 4 °C using IgG Sepharose 6 fast flow beads (Amersham Biosciences), A/G-agarose beads (Santa Cruz Biotechnology), and agarose IgG beads (Sigma), and the supernatant was immunoprecipitated for 2 h at 4 °C with α-FLAG M2 cross-linked to agarose beads (Sigma). Immunoprecipitates were washed twice with buffer C (20 mM Hepes, pH 7.9, 125 mM KCl, 5% glycerol, 1.5 mM $MgCl_2$, 0.1 mM EDTA, and 0.1% Tween 20) and twice with buffer D (buffer C with 300 mM KCl). After SDS-PAGE, Western blots were revealed with antibodies against V5 (R960-25; Invitrogen), FLAG M2 (Sigma), hemagglutinin 12CA5 (ab16918; Abcam). Immunodetection was done with horseradish peroxidase-conjugated anti-rabbit (or mouse) IgG (Sigma), and revelation was performed with the ECL+ reagent (Amersham Biosciences) as described by the manufacturer.

Superose 6 Gel Filtration—Size fractionation of protein complexes was carried out on an AKTA fast protein liquid chromatography apparatus with a Superose 6 10/30 column (Amersham Biosciences). Nuclear extracts as previously described (2 mg) were submitted to gel filtration fractionation, and fractions of 500 μl were collected. 80 μl of each fraction were subjected to SDS-PAGE and analyzed by Western immunoblotting with specific antibodies. Molecular size standards were apoferritin (443 kDa) and thyroglobulin (669 kDa) (Amersham Biosciences). After SDS-PAGE, Western blots were revealed with antibodies against Nurr1 (N83220; BD Biosciences), NGFI-B (18), Tpit (48), NeuroD1 (49), TIF1β (ab10483; Abcam), Mybbp1a (M9600–04; U.S. Biological), and SRC2 3C11 (3Ti-3C11; Euromedex).

Chromatin Immunoprecipitation and Quantitative PCR—AtT-20 cells were treated for 30 min with 10^{-7} M CRH and/or 10^{-7} M dexamethasone (Dex) and prepared for chromatin immunoprecipitation (ChIP) as described (19) with 5 μg of antibodies against TIF1β (mouse monoclonal 1Tb3 (38)) and matched nonimmune IgG (Sigma). Similar enrichments were obtained with rabbit polyclonal antibody against TIF1β (PF64) (25).

siRNA and Reverse Transcription-Quantitative PCR—AtT-20 cells (6×10^5) were plated in 6-well plates and transfected with 50 nmol of siRNA against TIF1β (ON-TARGET plus SMART pool or ON-TARGET plus set of four siRNAs; Dharmacon) or with a nontarget siRNA (Dharmacon) using Lipofectamine (Invitrogen). Two days after transfection, total RNA and whole cell extracts from AtT-20 cells were performed for reverse transcription-quantitative PCR analysis and protein expression analysis, respectively. Total RNA was prepared with the RNAeasy columns (Qiagen) according to the manufacturer's instructions. Reverse transcription was performed with 1 μg of this RNA using oligo$(dT)_{12-18}$ (Invitrogen) and SuperScript II RT (Invitrogen). The cDNA was then used for quantitative real time PCR (MX 3005; Stratagene, La Jolla, CA) with the SYBR Green kit (Qiagen, Valencia, CA). The primers used were as follows: TIF1β sense, GGATGTTCCAGGGGAGGAATGGAG; TIF1β antisense, CCAGGCTGCTCCATGGAGAATG; POMC sense, TGGAAGATGCCGAGATTCTGCTACAGT; POMC antisense, GATGCAAGCCAGCAGGTTGCTCTC; β-actin sense, TGAACCCTAAGGCCAACCGTGAAA; β-actin antisense, GTCCATCACAATGCCTGTGGTA. For the whole cell extracts, AtT-20 cells were harvested in cold phosphate-buffered saline and extracted at 4 °C for 30 min in a buffer containing 20 mM Tris, pH 8, 300 mM NaCl, 10% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.1% Nonidet P-40, 20 mM phenylmethylsulfonyl fluoride, and protease inhibitors. After centrifugation, 25 μg of protein extracts were subjected to SDS-PAGE and Western blot analysis with antibodies against TIF1β

(ab10483; Abcam) and DM1a α-tubulin (sc-32293; Santa Cruz Biotechnology).

Recombinant Protein Production and Pull-down Assays—The TIF1β-GST and empty-GST was provided by M. Aubry. Recombinant protein production and pull-down assays were done as previously described (19).

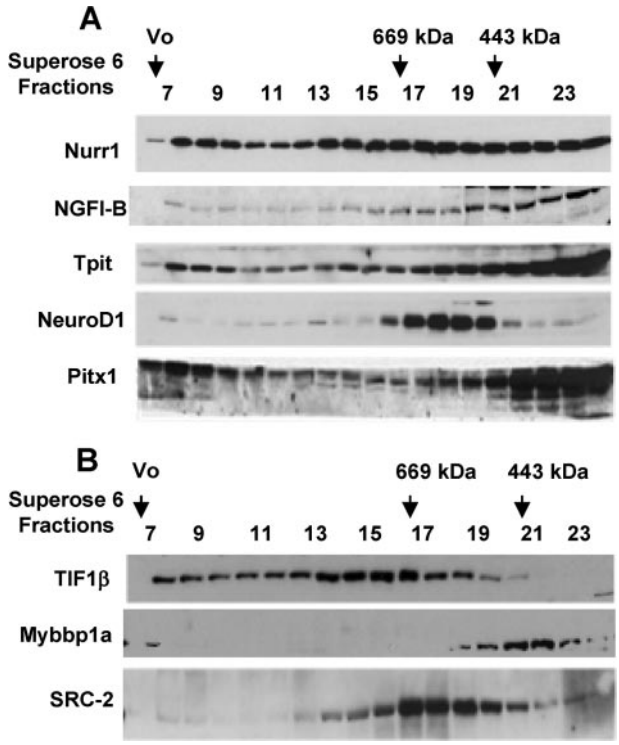

FIGURE 1. **The orphan nuclear receptors NGFI-B and Nurr1 are part of high molecular weight protein complexes.** *A* and *B*, nuclear extracts from AtT-20 cells were subjected to size fractionation using Superose 6 gel filtration. Fractions were analyzed by Western blot using specific antibodies, as indicated on the *left* of each *panel*. Fraction numbers and size standards are indicated at the *top*. *Vo*, void volume; *443 kDa*, apoferritin; *669 kDa*, thyroglobulin. Data are representative of at least two different experiments.

RESULTS

Several Transcriptional Regulatory Proteins Interact with NGFI-B—We have previously shown that SRC2 and Rb enhance the activity of the POMC promoter, and a part of this action was ascribed to the interactions with the orphan nuclear receptor NGFI-B (19). These coactivators are probably recruited as part of larger coactivator complexes. Thus, we used gel filtration of AtT-20 nuclear extracts on a Superose 6 column to test whether NGFI-B and Nurr1 are present within high molecular weight protein complexes (Fig. 1*A*). NGFI-B and Nurr1 were detected by Western blot in many fractions ranging in size from ≥1 MDa to free protein. A similar profile was obtained for Tpit, another target of CRH signaling (18). In contrast, other POMC transcription factors, NeuroD1 and Pitx1, exhibited different profiles. Indeed, NeuroD1 is exclusively present in a ~500-kDa protein complex, whereas Pitx1 is mainly found in its free form. The profiles were similar in CRH-treated AtT-20 cells (data not shown). These findings suggest that NGFI-B and Nurr1 are present in high molecular weight protein complexes, which supports the hypothesis of an association with multiple protein complexes in AtT-20 cells.

In order to identify such NGFI-B-associated proteins, we produced AtT-20 cells expressing a tagged version of NGFI-B at levels that are similar to endogenous NGFI-B (Fig. 2*A*). NGFI-B-associated proteins were purified from AtT-20 nuclear extracts using a FLAG-M2 affinity column followed by elution with FLAG peptides (Fig. 2*B*). This eluate was resolved by SDS-PAGE, and the interacting proteins were revealed by silver staining and analyzed by mass spectrometry (Fig. 2*C*). A list of tryptic peptides identified by mass spectrometry is provided in supplemental Table 1. All bands identified on Fig. 2*C* were only present in the eluate from FLAG-NGFI-B-V5-His but not mock-transfected AtT-20 cells, except the three lower bands (EF-1α, β-actin, and TMOD3) (data not shown). Different subunits of known multiprotein complexes were identified, such as BAF57 and BAF155 of the SWI/SNF complex and Chd4/Mi-2β, MTA2, and p66β of the Mi-2β/NuRD repression complex. These proteins were present at similar levels in control and forskolin-treated AtT-20 cells (data not shown). In contrast, some proteins appeared more abundant in treated compared with control cells (Fig. 2*D*): Mybbp1a, Pelp1 (MNAR), and the transcriptional coregulator TIF1β were among this group. We did not detect the previously characterized NGFI-B coactivators

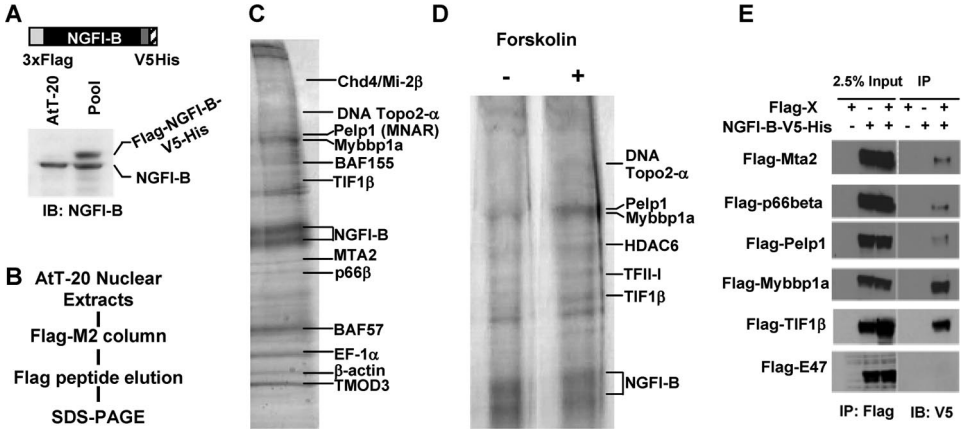

FIGURE 2. **Proteins associated with NGFI-B in AtT-20 nuclear extracts.** *A*, comparison of NGFI-B and FLAG-NGFI-B-V5-His levels in pools of infected and control AtT-20 cells. Western blotting with antibodies against NGFI-B was performed on whole cell extracts of the indicated cells. *B*, purification scheme used to purify NGFI-B-interacting proteins from AtT-20 nuclear extracts. *C*, identification of NGFI-B-associated proteins. The eluate from the FLAG-M2 column was subjected to SDS-PAGE, followed by silver staining. Gel slices were excised and digested with trypsin. The identity of the indicated bands was determined by peptide mass fingerprinting using MALDI-TOF analysis. *D*, comparison of NGFI-B-associated proteins in forskolin-treated compared with control AtT-20 cells. *E*, confirmation of the interactions found by mass spectrometry by coimmunoprecipitation of NGFI-B-V5-His with FLAG-tagged polypeptides. HEK293T cells were transfected with expression vectors, as indicated on the *left*, and NGFI-B-V5-His was revealed by immunoblotting (*IB*) using anti-V5 after immunoprecipitation (*IP*) of the FLAG-tagged polypeptides (*Flag-X*). Data are representative of at least two different experiments.

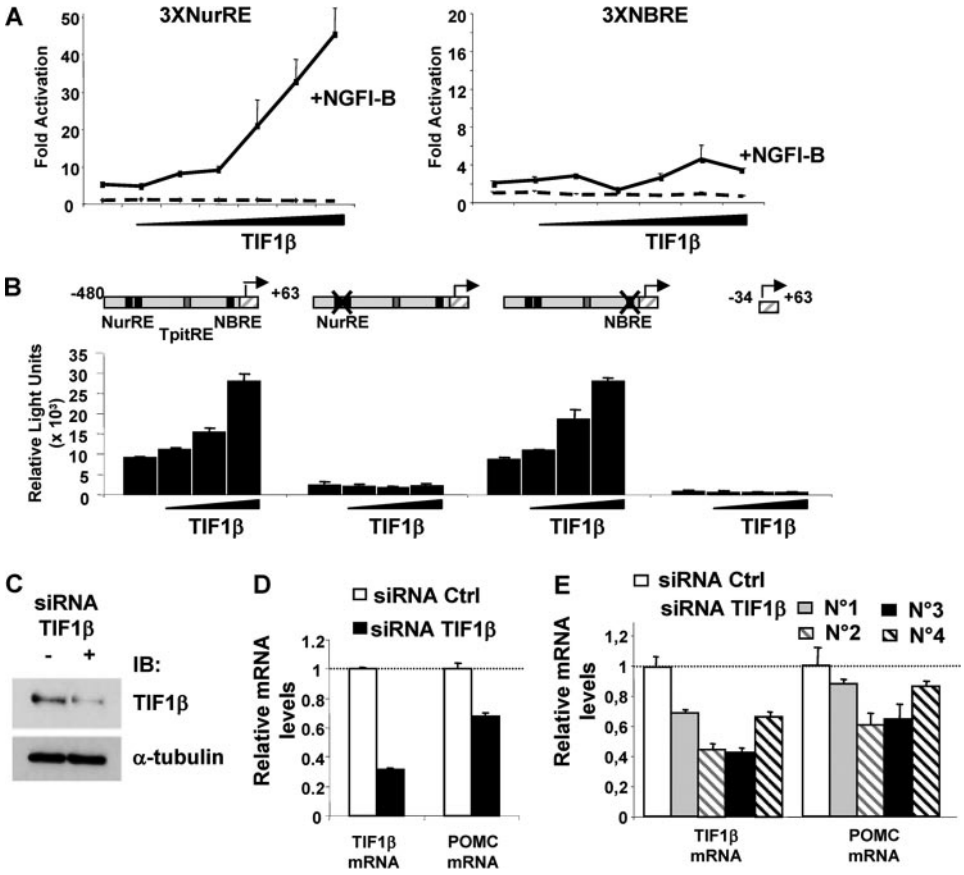

FIGURE 3. **TIF1β enhances POMC gene transcription and NGFI-B-dependent transcription.** *A*, the NurRE is much more responsive to TIF1β enhancement of NGFI-B-dependent activity than the NBRE in AtT-20 cells. Increasing amounts of TIF1β expression plasmid (10, 25, 50, 100, 200, and 300 ng) enhance NGFI-B-dependent activity of the NurRE reporter with NGFI-B (*left*) and, to a lesser extent, of the NBRE reporter (*right*). *B*, TIF1β enhances POMC gene transcription through the NurRE but not the NBRE. Increasing amounts of TIF1β expression vector (100, 200, and 300 ng) were transfected with different POMC promoter constructs fused to the luciferase reporter gene in POMC-expressing AtT-20 cells. The intact promoter construct (−480 to +63 bp) and a promoter mutation in the NBRE regulatory element were similarly responsive to TIF1β, whereas a NurRE mutant and minimal promoters were unresponsive. *C*, Western blot analysis (*IB*) of TIF1β and α-tubulin expression in AtT-20 cell extracts transfected with a pool of four siRNAs against TIF1β (50 nM); α-tubulin was used as loading control. *D*, endogenous TIF1β is important for basal POMC mRNA expression in AtT-20 cells. Transfection of a pool of four siRNAs against TIF1β (50 nM) decreased endogenous mRNA levels of TIF1β and POMC compared with β-actin mRNA in AtT-20 cells. Endogenous mRNAs were quantitated by reverse transcription-quantitative PCR. *E*, four individual siRNAs against TIF1β exhibit correlation between knockdown of TIF1β and POMC mRNAs. Data represent the means ± S.E. of three experiments each performed in duplicates (except in *E*; performed twice).

SRC2 and Rb in these experiments. This may be due to low expression levels of these proteins in AtT-20 cells or to lower stability of their association with the NGFI-B complex.

We confirmed these interactions by reverse coimmunoprecipitation in HEK293T cells that overexpressed the different FLAG-tagged polypeptides (FLAG-X) identified by mass spectrometry with V5-His-tagged NGFI-B. This experiment confirmed the interactions between NGFI-B-V5-His and the different proteins tested; in contrast, the basic helix-loop-helix factor E47 used as control did not co-immunoprecipitate with NGFI-B (Fig. 2*E*). The purification of these different proteins with NGFI-B suggests that they are part of large protein complexes similar to those described above (Fig. 1*A*). Indeed, we found that TIF1β has a similar molecular weight distribution as Nurr1 and NGFI-B in AtT-20 nuclear extracts (Fig. 1*B*) in agreement with the hypothesis that they may act together. It is also possible that NGFI-B is a part of protein complexes of different molecular weights, and that could relate to different profiles on Superose 6, such as the SRC2 profile (Fig. 1*B*).

NurRE-dependent Transcription Is Enhanced by TIF1β—Previous analyses of POMC promoter sequences responsive to both CRH and NGFI-B identified the NurRE as target (17). The dimer-binding NurRE is much more responsive than the monomer-binding NBRE to NGFI-B (16, 18), particularly to signal-activated NGFI-B (17). The putative action of TIF1β on NGFI-B activity was tested directly using NurRE and NBRE reporters in AtT-20 cells (Fig. 3*A*). Expression vector for NGFI-B or empty vector was cotransfected in AtT-20 with increasing amounts of TIF1β expression plasmid using the NBRE-Luc (*right*) or NurRE-Luc (*left*) reporter. Without NGFI-B, TIF1β had no effect on NurRE and NBRE, but in its presence, we observed that TIF1β behaved as a strong coactivator of NGFI-B dimers acting on the NurRE compared with the NBRE (Fig. 3*A*).

TIF1β Enhances POMC Gene Transcription—Since the NurRE was previously shown to be important for POMC expression and responsiveness to NGFI-B (17), we tested whether TIF1β could regulate POMC promoter activity in AtT-20 cells (Fig. 3*B*). Whereas the minimal promoter was not affected (Fig. 3*B*, *right*), we observed that the intact POMC promoter was indeed activated by increasing amounts of TIF1β (*left*). Similar results were obtained in HEK293T and CV1 cells (data not shown). Mutagenesis of the POMC promoter NBRE-like sequence did not affect the ability of TIF1β to enhance POMC transcription. In contrast, mutagenesis of the NurRE abolished enhancement by TIF1β (*middle*). The *in vivo* importance of endogenous TIF1β protein in POMC transcription was assessed directly using a pool of four siRNAs against TIF1β in AtT-20 cells. The knock-down of TIF1β was ascertained by Western blot (Fig. 3*C*) and correlated with a reduction of POMC mRNA levels assessed by reverse transcription-quantitative PCR (Fig. 3*D*). Further, each siRNA from the pool resulted in varying degrees of TIF1β mRNA knockdowns that are directly correlated with their effect on POMC mRNA levels (Fig. 3*E*). Thus, basal POMC expression is partly dependent on TIF1β acting through the NurRE regulatory element.

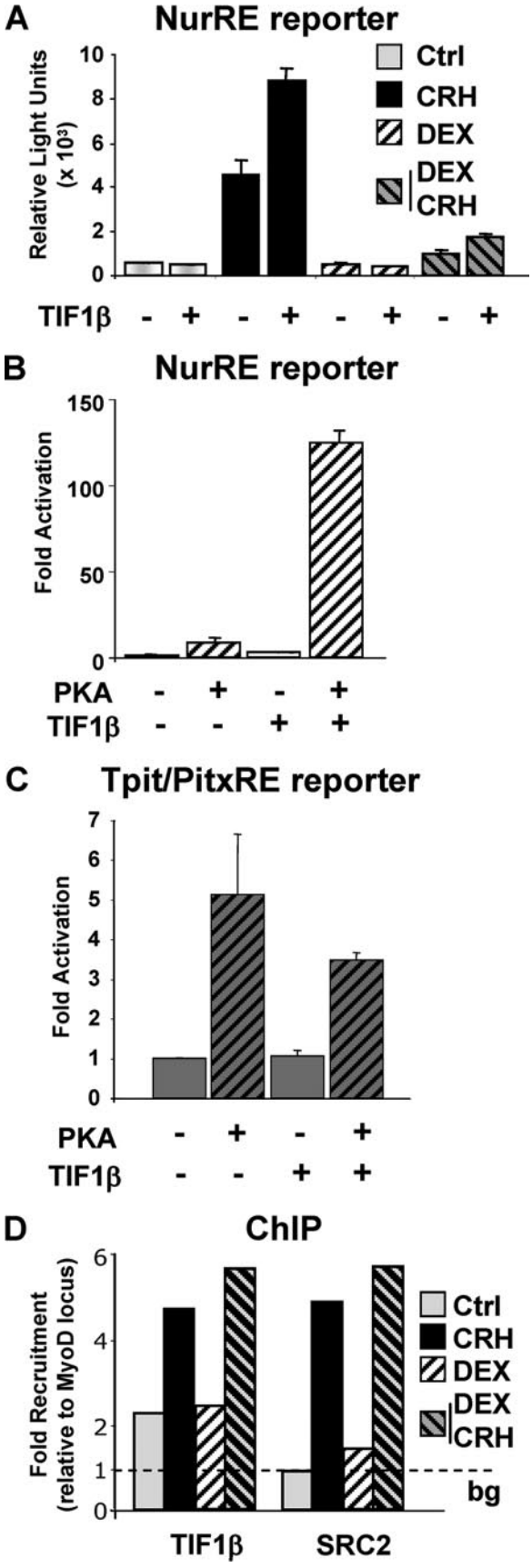

FIGURE 4. **TIF1β enhances CRH and PKA responses.** *A*, TIF1β enhances CRH response of the NurRE reporter. Transfection of AtT-20 cells with an expression vector for TIF1β (100 ng) enhances CRH activation of the NurRE reporter but does not modulate basal activity or Dex repression. *B* and *C*, TIF1β potentiates PKA stimulation of the NurRE reporter (*B*) but not the Tpit/PitxRE reporter (*C*). AtT-20 cells were transfected with expression vectors for the catalytic subunit of PKA (50 ng) and/or TIF1β (200 ng) to assess their effect on NurRE (*B*) and Tpit/PitxRE (*C*) reporters. *D*, chromatin immunoprecipitation was performed in AtT-20 cells treated with or without 10^{-7} M CRH and/or 10^{-7} M Dex to show recruitment of TIF1β and SRC2 to the POMC promoter. Recruitment is shown for the POMC promoter relative to the MyoD exon 1. Data represent the means ± S.E. of three experiments each performed in duplicates. *bg*, background signal obtained with nonimmune IgG; *Ctrl*, control.

TIF1β Potentiates Both CRH and PKA Signaling through the NurRE but Not the Tpit/PitxRE—The NurRE and the cognate Nurs factors participate in basal activity as well as in CRH responsiveness of the POMC promoter (16, 17). Consequently, we tested the ability of TIF1β to enhance the stimulatory effect of CRH on Nur-dependent transcription in AtT-20 cells. As shown in Fig. 4*A*, the activity of the NurRE reporter was activated by CRH, and this activation was reversed by Dex, a synthetic glucocorticoid. The overexpression of TIF1β alone did not affect basal reporter activity but potentiated both CRH and Dex/CRH responses.

The CRH signaling pathway involves a rapid increase in cAMP levels (14), followed by activation of PKA. Previous work had identified NGFI-B and Tpit as end point effectors of PKA signaling, through the NurRE and Tpit/PitxRE, respectively (18, 50). We tested whether TIF1β modulates PKA-dependent transcription using the NurRE reporter in AtT-20 cells. PKA stimulation alone had a strong effect on NGFI-B-dependent transcription, and this effect was markedly potentiated by TIF1β (Fig. 4*B*). Similar results were obtained in CV-1 and 293T cells (data not shown). In contrast, TIF1β did not change the activity of the Tpit/PitxRE reporter with or without PKA (Fig. 4*C*). Thus, TIF1β potentiates both CRH and PKA signaling through the NurRE but not the Tpit/PitxRE.

TIF1β Is Recruited to the POMC Promoter—We next assessed recruitment of TIF1β to the POMC promoter upon treatment of AtT-20 cells with CRH, Dex, and CRH + Dex by ChIP technique. In basal conditions, TIF1β was present at the promoter but only slightly above background. The recruitment of TIF1β was enhanced on the POMC promoter upon CRH and Dex/CRH treatments (Fig. 4*D*, *left*). Treatment with Dex, which represses POMC transcription (10, 51, 52), did not affect TIF1β recruitment to the POMC promoter, in accordance with previous data showing that Dex does not affect NGFI-B recruitment either (23). These results support a model in which TIF1β is recruited to the POMC promoter following CRH stimulation to enhance POMC transcription; this model is also supported by the greater amount of TIF1β associated with NGFI-B in extracts from forskolin-treated cells (Fig. 2*D*).

The Nur Factors Are Targets of TIF1β and Directly Bind TIF1β—To test whether all three members of the Nur subfamily are targets of TIF1β, we investigated the effect of TIF1β on NGFI-B, Nurr1, and NOR-1. Putative potentiation by TIF1β was assessed using luciferase reporters containing three copies of either the POMC gene NurRE ($NurRE_{POMC}$) (Fig. 5*A*) or a consensus NurRE ($NurRE_{CON}$) (Fig. 5*B*). We previously showed that the $NuRE_{POMC}$ has a preference for NGFI-B-containing dimers, whereas the $NurRE_{CON}$ does not exhibit a pref-

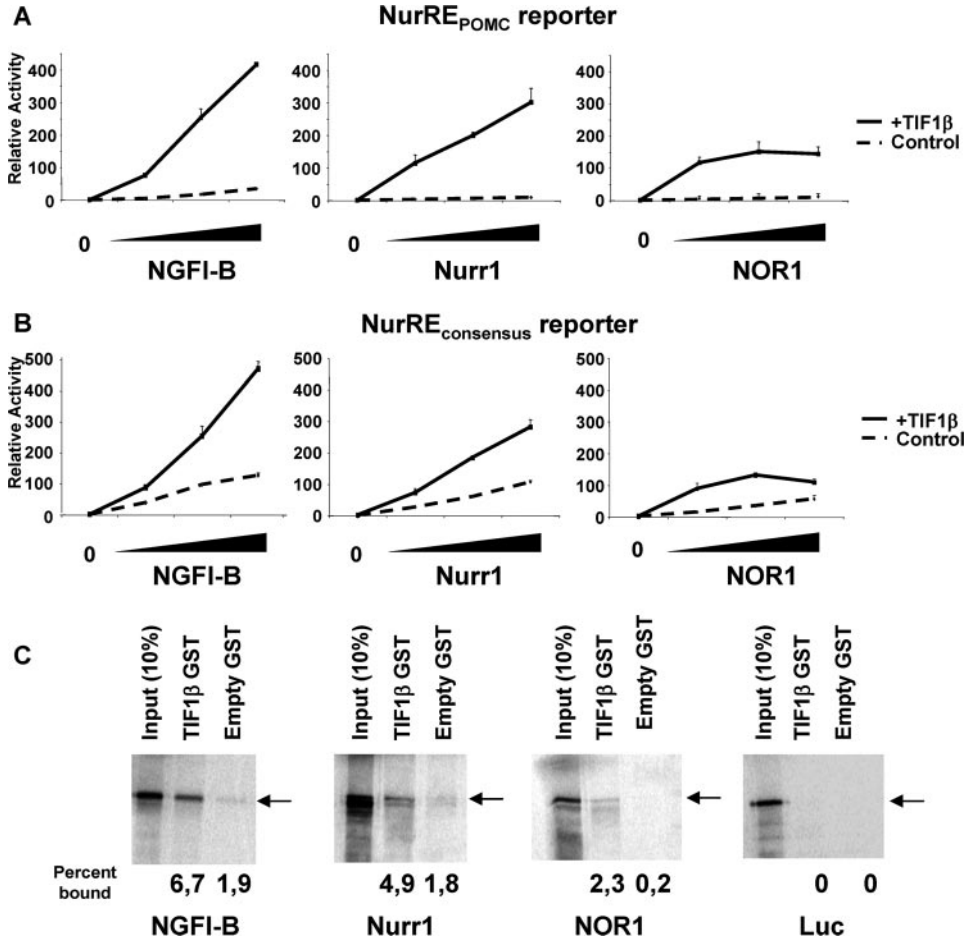

FIGURE 5. **The three Nur factors are targets of TIF1β.** *A*, TIF1β coactivates preferentially NGFI-B on a NurRE reporter. AtT-20 cells were transfected with TIF1β expression vector (200 ng) and increasing amounts of expression vectors for each Nur factor (5, 10, and 20 ng) along with the NurRE$_{POMC}$ (*A*) or the NurRE$_{CONSENSUS}$ reporter (*B*). Data represent the means ± S.E. of three experiments, each performed in duplicates. *C*, the abilities of TIF1β and Nur factors to interact *in vitro* were assessed by using a pull-down assay. Fusion proteins consisting of GST and TIF1β or empty vector (as control) were bound to glutathione-Sepharose beads, and either *in vitro* translated Nurr1, Nur77, NOR-1, or luciferase (as control) was tested for interaction. *Input lanes* contain 10% of the pull-down sample, and the percentage of input that was retained in each pull-down sample is indicated *below* each *lane*. The *arrow* indicates full-size protein. Data are representative of two different experiments.

erence (16). TIF1β potentiated activity dependent on all three Nur factors on both reporters; the lower potentiation of NOR-1-dependent activity is consistent with prior data (16).

We showed interactions between TIF1β and NGFI-B (Fig. 2*E*). We therefore investigated the direct protein-protein interaction by using an *in vitro* pull-down assay (Fig. 5*C*), in which a resin-bound GST-TIF1β fusion protein was tested for interaction with *in vitro* translated NGFI-B, Nurr1, NOR-1, or luciferase. The three Nur factors bound specifically the GST-TIF1β column but not the empty vector-GST. Thus, all three Nur factors interact *in vitro* with TIF1β.

TIF1β Enhances NGFI-B-dependent Transcription through the N-terminal AF-1A Domain—We previously showed that SRC coactivators exert their effects through the AF-1 N-terminal domain of NGFI-B (18). The AF-1 was subdivided into two regions comprised between amino acids 20 and 36 (AF-1A) and between amino acids 74 and 174 (AF-1B); both subdomains were found to contribute to SRC coactivator potentiation, but AF-1B mediated the PKA stimulatory effect (18). Expression vectors for NGFI-B and several deletions of NGFI-B were cotransfected in AtT-20 with increasing amounts of TIF1β expression plasmid and the NurRE-Luc reporter (Fig. 6*A*). Enhancement of NGFI-B-dependent activity by TIF1β was partially affected by deletion of NGFI-B N-terminal sequences between amino acids 3 and 13 (deletion ΔN1). Complete loss of activity was observed upon deletion of the AF-1A subdomain (ΔN3). To support the interpretation that the AF-1A subdomain is entirely responsible for the action of TIF1β, we created an internal deletion that only removed the AF-1B subdomain (mutant ΔN7). This ΔN7 mutant is fully competent to respond to TIF1β (Fig. 6*A*). Similar results were obtained in HEK293T and CV1 cells (data not shown). These results indicate that the AF-1A domain of NGFI-B is necessary and sufficient for TIF1β potentiation.

CRH/PKA Signaling Is Mediated through the N-terminal AF-1 Domain—The CRH signaling pathway involves activation of PKA. In order to gain further insight into mechanisms of PKA- or coactivator-induced transcriptional activity of NGFI-B, we used the series of deletion mutants in transfection experiments that included PKA stimulation using an expression vector for the catalytic subunit of PKA (Fig. 6*B*). PKA stimulation on its own had a strong effect on NGFI-B-dependent transcription, and this effect was potentiated by TIF1β (Fig. 6*B*, *WT NGFI-B*).

Deletion between amino acids 17 and 74 of the N-terminal domain of NGFI-B (deletion ΔN3) resulted in loss of the TIF1β-dependent enhancement (Fig. 6*A*), but this deletion did not abolish the effect of PKA. Indeed, deletion of the two AF-1 subdomains (deletion ΔN5) was needed to abolish the PKA effect; however, the PKA-enhanced activity of mutant ΔN3 was not increased by TIF1β, in agreement with the interpretation that TIF1β can only act on the AF-1A domain. This activity is thus probably mediated through SRC coactivators, as previously described (18). In agreement with this, the ΔN7 mutant that is only deleted of subdomain AF-1B was as sensitive to PKA and TIF1β as wild-type NGFI-B (Fig. 6*B*). These results suggest that both basal and PKA-stimulated TIF1β activities are mediated through the AF-1A domain, whereas prior work had localized PKA potentiation of SRC2-dependent activity to the NGFI-B AF-1B subdomain (18).

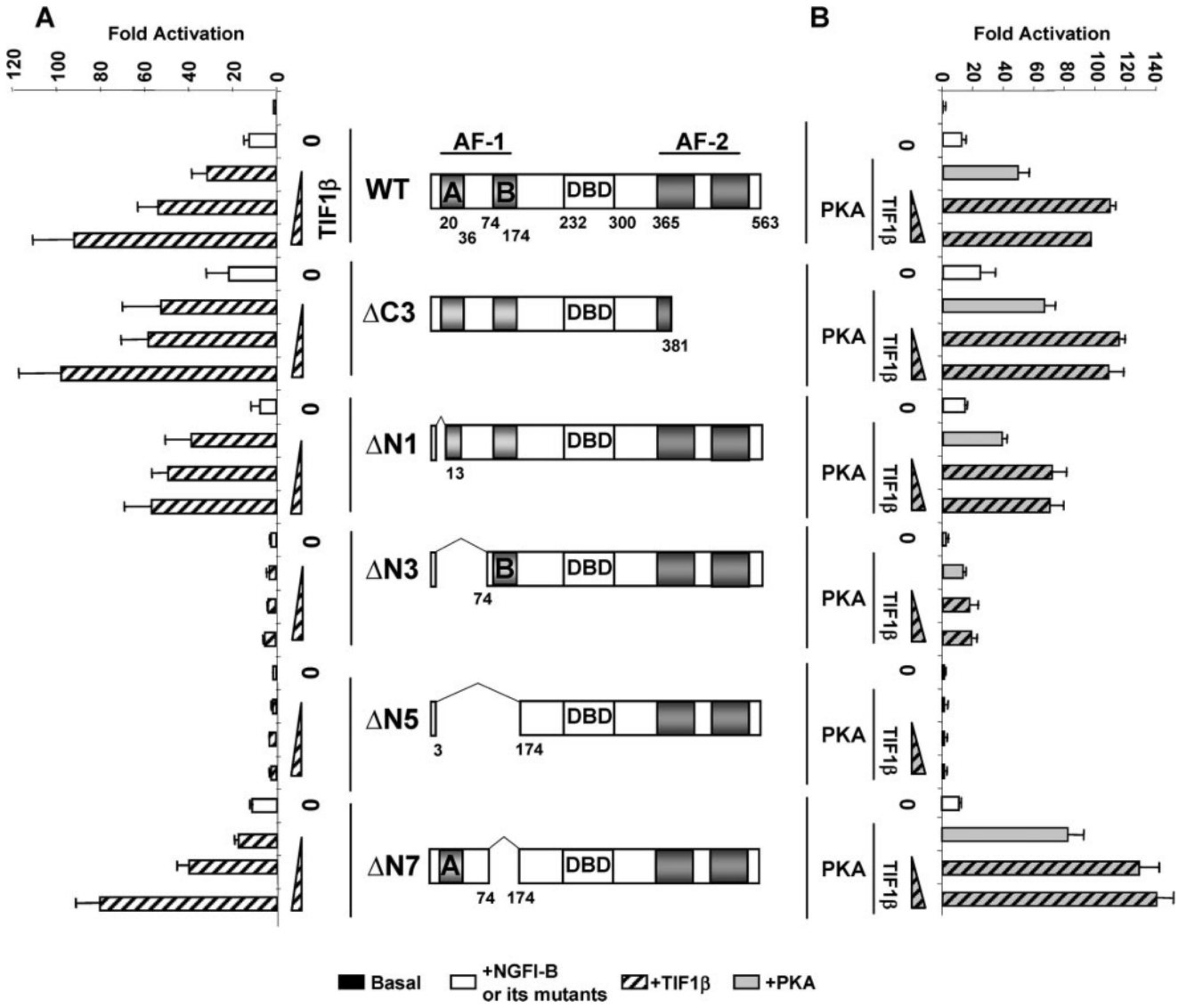

FIGURE 6. **The N-terminal half of the NGFI-B AF-1 domain is required for TIF1β activity.** *A*, mapping of TIF1β-dependent activities to the NGFI-B AF-1 domain. The *middle panel* presents schematic structures of NGFI-B and its deletion mutants used in this work. Increasing amounts of TIF1β expression plasmid were cotransfected with the series of NGFI-B mutants and NurRE reporter in AtT-20 cells. *B*, TIF1β potentiation of NGFI-B and its mutants assessed using NurRE reporter with PKA expression vector (50 ng). Results presented as -fold activation represent means ± S.E. of at least three experiments performed in duplicates. *DBD*, DNA binding domain.

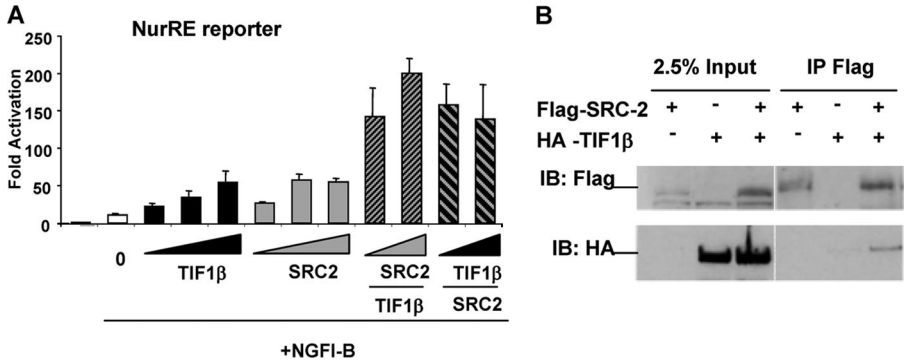

FIGURE 7. **Synergistic action of TIF1β and SRC2 on NGFI-B-dependent transcription.** *A*, AtT-20 cells were transfected with the NurRE reporter and with SRC2 or TIF1β expression vectors (100, 200, and 300 ng) or with SRC2 and TIF1β together (200 ng of SRC2 with 100 or 200 ng of TIF1β or 200 ng of TIF1β with 100 or 200 ng of SRC2). Data represent the means ± S.E. of three experiments, each performed in duplicates. *B*, coimmunoprecipitation of HA-TIF1β with FLAG-tagged SRC2. HEK293T cells were transfected with expression vectors for HA-TIF1β or FLAG-SRC2, as indicated. HA-TIF1β and FLAG-SRC2 were revealed by immunoblotting (*IB*) with anti-HA and anti-FLAG, respectively, after immunoprecipitation (*IP*) of FLAG-SRC2. The figure presents a representative experiment of three that we performed.

TIF1β Synergizes with the Coactivator SRC2 for NGFI-B-dependent Transcription—SRC2 was previously implicated in CRH/PKA-dependent activation of NGFI-B (18). Hence, we tested a putative collaboration between SRC2 and TIF1β using the NurRE reporter in AtT-20 cells. As shown in Fig. 7*A*, a limiting amount of SRC2 and TIF1β synergistically enhanced NGFI-B-dependent activity on the NurRE reporter, suggesting that TIF1β and SRC2 may be part of the same transcriptional complex. We next assessed a putative interaction between TIF1β and SRC2 *in vivo* using coimmunoprecipitation in

HEK293T cells. Immunoprecipitation of FLAG-tagged SRC2 brought down HA-TIF1β (Fig. 7*B*).

We wanted to verify that SRC2 and TIF1β are present at the POMC and whether their recruitment is increased by CRH. Using ChIP, we assessed recruitment of SRC2 and TIF1β to the POMC promoter (Fig. 4*B*). In basal conditions as well as following CRH activation and Dex repression, both TIF1β and SRC2 are recruited in parallel to the POMC promoter, in agreement with previous data (19). Thus, TIF1β and SRC2 may be part of a common transcriptional complex and thus synergistically enhance NGFI-B-dependent transcription. It is noteworthy that the combined action of TIF1β and SRC2 surpasses their individual action (Fig. 7*A*).

DISCUSSION

The present work documented a coactivator function for TIF1β and implicated this coactivator in hormone responsiveness mediated through NGFI-B and related NRs. Multiple experiments support the role of TIF1β as a hormone-responsive coactivator of NGFI-B, starting with the identification of this protein by mass spectrometry within protein complexes associated with NGFI-B. Not only was TIF1β identified within affinity-purified complexes, but further, the amount of TIF1β was shown to be enhanced by cAMP signaling (Fig. 2). We showed that TIF1β enhances transcription dependent on the POMC promoter and its NurRE (Fig. 3) and that this action potentiates responsiveness to the hypothalamic hormone CRH and to PKA (Fig. 4). These actions were mapped to the N-terminal half of the NGFI-B AF-1 domain (AF-1A; Fig. 6). The *in vivo* relevance of these interactions was supported by ChIP and siRNA knockdown experiments.

In the general context of NR-dependent activation of transcription, it is also noteworthy that we report coactivation by both TIF1β and the SRC2 coactivator that was previously implicated in the action of many NRs. We have shown recruitment of both coactivators in response to CRH at the POMC promoter, and we showed that both can be present within the same protein complexes in cells, thus providing a molecular basis for their synergistic coactivation of transcription dependent on NGFI-B and the NurRE. Interestingly, the activity of each coactivator was mapped to a different subregion of the NGFI-B AF-1 domain. Whereas SRC2 was previously shown to mediate the activator effect of CRH-dependent signaling through both NGFI-B and Tpit, TIF1β appears to be only involved in NGFI-B-dependent transcription. The involvement of two different coactivators in enhancement of NGFI-B-dependent transcription may increase the possibilities of signaling inputs into the control of target genes; it will ultimately be interesting to assess whether other NGFI-B-dependent target genes also use these two coactivators.

TIF1β as Coactivator—TIF1β was initially identified as the universal transcriptional co-repressor for the large family of vertebrate-specific KRAB domain-containing zinc finger transcription factors (53). TIF1β-mediated gene silencing involves the recruitment of histone deacetylases (37, 54), and of histone methyltransferases (36). Only one study had previously described TIF1β as a coactivator. Indeed, TIF1β was found to act as coactivator of the bZIP transcription factor CCAAT/enhancer-binding protein-β, which mediates together with GR the induction of the α1-acid glycoprotein gene by inflammatory cytokines and glucocorticoids (40).

The present work supports the function of TIF1β as a transcriptional activator of NGFI-B. It was previously shown that the AF-1 domain of NGFI-B mediates coactivator recruitment through direct protein-protein interaction (55). SRC1, -2, and -3 as well as Rb were shown to exert coactivator effects through the AF-1 N-terminal domain of NGFI-B (18, 19). Prior work (18) subdivided the AF-1 into two regions comprised between amino acids 20 and 36 (AF-1A) and between amino acids 74 and 174 (AF-1B) and indicated that SRC1, -2, and -3 were acting through both subdomains but that PKA signals were primarily targeting the AF-1B-dependent activity. In contrast, all TIF1β-dependent actions (basal and PKA-induced) on NGFI-B appeared to depend on the AF-1A subdomain (Fig. 6). Taken together, these data indicate that TIF1β acts on the AF-1A subdomain and SRCs act preferentially, but not exclusively, on the AF-1B subdomain.

It was suggested that sumoylation is important for the repressor activity of TIF1β (45). Sumoylation was found to stabilize the association of the bromodomain with the chromatin modifiers SETBD1 and the NurD complex, thereby promoting establishment of the silent gene expression state. When we tested whether the sumoylation sites of TIF1β have an impact on NurRE activation, we found the TIF1β sumoylation-deficient mutant to be unaffected in its ability to activate the NurRE reporter (data not shown). Transcriptional co-activation by TIF1β therefore appears independent of its sumoylation, but it may be modulated by other post-translational modification, such as phosphorylation (38).

Coactivator Synergism—We observed synergistic activation of NGFI-B-dependent transcription by TIF1β and SRC2 (Fig. 7*A*). When assessed separately for coactivation of NGFI-B dimers, TIF1β is of similar potency as SRC2. Both act on the NurRE target of NGFI-B dimers but not on the NBRE monomer target (Fig. 3*A*) (18). TIF1β may be recruited as part of a transcriptional complex together with SRC2 for NGFI-B-dependent transcription, as suggested by the fact that both can be co-immunoprecipitated (Fig. 7*B*) and that both are recruited to the POMC promoter following CRH activation (Fig. 4*D*). The data may be taken to suggest that TIF1β and SRC2 are co-recruited to NGFI-B dimers upon CRH stimulation. However, there are differences in the action of these two co-activators on NGFI-B and on different POMC promoter targets. Indeed, the two coactivators target different AF-1 subdomains. In addition, the CRH signaling pathway acting through PKA and mitogen-activated protein kinase targets SRC2 to both NGFI-B and Tpit through the NurRE and Tpit/PitxRE, respectively (50). In contrast, TIF1β potentiated PKA activation of NGFI-B-dependent transcription but not the activity of the Tpit/PitxRE with or without PKA (Fig. 4, *B* and *C*). SRC2 enhanced the intrinsic activity of Tpit on its cognate POMC target as well as its synergistic activity with Pitx1. These results underline functional differences in the mechanisms of action of these two co-activators and may reflect the involvement of multiple signals for CRH activation.

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