

Transcriptional Activation by STAT6 Requires the Direct Interaction with NCoA-1*

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Signal transducer and activator of transcription 6 (STAT6) is a transcription factor that is activated by interleukin-4 (IL-4)-induced tyrosine phosphorylation and mediates most of the IL-4-induced gene expression. Transcriptional activation by STAT6 requires the interaction with coactivators like p300 and the CREB-binding protein (CBP). In this study we have investigated the function of the CBP-associated members of the p160/steroid receptor coactivator family in the transcriptional activation by STAT6. We found that only one of them, NCoA-1, acts as a coactivator for STAT6 and interacts directly with the transactivation domain of STAT6. The N-terminal part of NCoA-1 interacts with the far C-terminal part of the STAT6 transactivation domain but does not interact with the other members of the STAT family. This domain of NCoA-1 has a strong inhibitory effect on STAT6-mediated transactivation when overexpressed in cells, illustrating the importance of NCoA-1 for STAT6-mediated transactivation. In addition, we showed that both coactivators CBP and NCoA-1 bind independently to specific regions within the STAT6 transactivation domain. Our results suggest that multiple contacts between NCoA-1, CBP, and STAT6 are required for transcriptional activation. These findings provide new mechanistic insights into how STAT6 can recruit coactivators required for IL-4-dependent transactivation.

STAT¹ proteins are transcription factors that transmit signals from activated cytokine receptors to the nucleus. Following their obligatory tyrosine phosphorylation exerted by JAK kinases, STATs dimerize and move to the nucleus where they modulate transcription through specific DNA sequence elements (1–3). Thus far, seven mammalian STATs have been identified. They share the same structure and functional domains. The N-terminal portion mediates cooperative binding to multiple DNA sites (4, 5). The region that determines the DNA-binding site specificity is located between amino acids 400 and 500 (6). The STAT-SH2 domain mediates association with the activated receptor (7, 8) and dimerization via reciprocal SH2-phosphotyrosine interactions (9, 10). The C-terminal

part constitutes the transactivation domain (2, 11). Although STAT3 and STAT5 are expressed in most cell types and activated by a variety of cytokines and growth factors, other STAT proteins play specific roles in host defenses (2).

In the present study we focused on STAT6, which is activated in response to IL-4 and IL-13, another cytokine that binds to the α chain of the IL-4 receptor (12). IL-4 regulates immune and anti-inflammatory responses. It promotes the differentiation of T helper precursors toward the Th2 lineage while inhibiting Th1 development. Furthermore, IL-4 stimulation of B-cells triggers Ig class switching to IgE isotype. This recombination is thought to be initiated following the transcriptional activation of the germline (GL) ϵ promoter, which leads to the generation of the sterile ϵ transcript (13, 14). STAT6-deficient mice have defects in IL-4-mediated functions including Th2 development, induction of CD23 and major histocompatibility complex class II expression, and immunoglobulin class switching to IgE, demonstrating the essential role of STAT6 in these IL-4-induced functions (15, 16). STAT6-binding sites have been identified in the promoter regions of several IL-4-responsive genes. They are best characterized in the Ig GL ϵ promoter, which contains a composite binding element for STAT6 and the CAAT/enhancer-binding protein (17). The transactivation domain of STAT6 was characterized as a modular, proline-rich region in the C terminus of the protein. The structure of this domain is quite different from that of the other members of the STAT family (18, 19). Recently studies have mapped two distinct transactivation functions in this domain that cooperate in transcriptional activation (20).

Activation of transcription in general requires the recruitment of transcriptional coactivators that are part of the chromatin modifying complexes possessing histone acetyltransferase activities and serve as a bridge to the basal transcriptional apparatus (21). In previous studies we demonstrated that the functionally conserved coactivators p300 and CREB-binding protein (CBP) are recruited by STAT6 and are required for transcriptional activation by IL-4 (22). p300/CBP are also recruited by different classes of transcription factors, including nuclear receptors, AP-1, p53, p65 subunit of NF κ B, and STAT1, STAT2, and STAT5 (23, 24). p300/CBP possesses intrinsic histone acetyltransferase activity and associates with other histone acetyltransferases (HATs) like p/CAF and members of the p160/steroid receptor coactivator (SRC) family (23).

The p160/SRC coactivator family, also called the NCoA coactivator family, was identified as nuclear receptor-binding proteins, which enhance transcriptional activation, by these ligand-induced transcription factors (25). Three homologous factors, termed NCoA-1, also called SRC-1 (26, 27); NCoA-2, also called TIF2 or GRIP1 (28, 29); and NCoA-3, also called p/CIP, ACTR, or AIB1 (30–32), were identified by several groups. NCoA factors can associate with p300/CBP (27, 33). It has been shown that these factors are also involved in transcriptional activation by AP-1, p53, serum response factor,

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¹ The abbreviations used are: STAT, signal transducer and activator of transcription; IL, interleukin; GL, germline; CREB, cAMP responsive element-binding protein; CBP, CREB-binding protein; HAT, histone acetyltransferase; SRC, steroid receptor coactivator; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; TAD, transactivation domain; PAS, Per-Arnt-Sim; NID, nuclear receptor interaction domain; bHLH, basic helix loop helix; AD, activation domain; DBD, DNA-binding domain; NCoA, nuclear receptor coactivator.

NF κ B, and STAT1 (30, 34–38). They contain conserved domains for interaction with downstream effectors such as HATs like p300/CBP, p/CAF, and protein methyltransferases (39–41). In addition, two members of this family, NCoA-1 and NCoA-3, contain moderate intrinsic HAT activity (42). Several studies have postulated a selectivity in the use of specific coactivators and HAT activities required for the function of distinct classes of transcription factors, *e.g.* p300 and NCoA-3 are required for STAT1, whereas p/CAF and NCoA-1 are dispensable for this transcription factor (40, 43). One important question concerning the function of NCoA coactivators focuses on whether or not the different NCoA cofactors fulfill redundant functions. All three family members possess similar properties in terms of interaction with nuclear receptors and enhancement of nuclear receptor transcriptional activation. However, several reports suggest that their activities are not completely redundant (30, 35).

Because NCoA coactivators are associated with p300/CBP, which in turn coactivates STAT6, we investigated their influence on STAT6 transactivation. We tested whether STAT6-mediated transactivation requires the activity of specific coactivators. In this paper we demonstrate that NCoA-1, but not the other members of the NCoA coactivator family, acts as a coactivator of STAT6. We found a direct interaction of STAT6 and NCoA-1 in cells and *in vitro*. Overexpression of the STAT6-interacting domain of NCoA-1 inhibits transactivation by STAT6 in a transdominant manner, demonstrating the importance of NCoA-1 for STAT6 transactivation. Additionally, we showed that CBP and NCoA-1 bind independently to specific parts of the STAT6 transactivation domain. The analysis of NCoA-1 mutants in which different functional domains were deleted demonstrates that coactivation by NCoA-1 requires its activation domain 1 and the STAT6 interaction region. Our results show that STAT6 enhances transcription by directly contacting at least two different coactivators (CBP and NCoA-1) with its modular transactivation domain.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—HepG2 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 2 mM L-glutamine, and penicillin/streptomycin. They were transfected with Superfect transfection reagent (Qiagen) according to the manufacturer's instructions. In a typical transfection experiment 1.5×10^5 cells were transfected with 2 μ g of luciferase reporter plasmid, the indicated amounts of expression vectors, and 0.025 μ g of SV40 promoter-driven *LacZ* expression vector to control the transfection efficiency. After 1 day the cells were induced with IL-4 (10 ng/ml) and lysed after a further 16 h of incubation. The murine pre-B-cell line Ba/F3-IL-4R, stably transfected with the human IL-4R α and γ chains (44), was cultured in RPMI medium containing 10% fetal calf serum, 2 mM L-glutamine, penicillin/streptomycin, and 5% supernatant of murine IL-3-overproducing WEHI cells as previously described (44). These cells (5×10^6) were transfected by electroporation with a Bio-Rad gene pulser at 350 V/960 microfarads. In a typical transfection experiment 5 μ g of luciferase reporter construct, the indicated amount of expression vectors, and 1 μ g of SV40-*LacZ* control plasmid was used. The total amount of DNA was adjusted to 20 μ g with pBSK. 4 h later the cells were aliquoted into two fractions; one was treated with murine IL-4 (10 ng/ml), and the other was left untreated. 20 h later the cells were harvested. 293T cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 2 mM L-glutamine, and penicillin/streptomycin. These cells were transfected by the calcium phosphate precipitation method. Luciferase and β -galactosidase activities were assayed as recommended by the manufacturer (Promega). Luciferase activities were normalized to the *LacZ* expression. At least three independent experiments were performed.

Recombinant Plasmids and Constructs—The reporter genes (GAL4-RE)3TK LUC, N4(STAT-RE)3 LUC, and *LacZ* expression plasmid (pCH110) have been described previously (19). The reporter gene Ige-TK LUC, containing nucleotides –111 to –62 of the human C ϵ promoter, was kindly provided by Andre Zimmer (Albert-Ludwigs-University, Freiburg, Germany). The expression vectors for hSTAT6 (pXM-

Stat6), hSTAT6 residues 1–792 in the pXM vector, GAL4-DBD, and the derivatives containing residues 677–792, 677–847, and 792–846 of STAT6 have been previously described (19). pcDNA3-STAT1 and pBSK-STAT2 were kindly provided by Markus Heim (University Hospital, Basel, Switzerland). pSG5-hSTAT3 and pXmMSTAT4 have been described previously (45, 46). pSG6-mSTAT4 was generated by insertion of the *KpnI* fragment of pXM-mSTAT4 into the *KpnI* sites of pSG6. pBSK-hSTAT5A and pBSK-mSTAT5B were kindly provided by Fabrice Gouillieux (University of Picardie-Jules Verne, Amiens, France). pSG6-hSTAT6 was generated by insertion of the *EcoRI/NotI* fragment of pXM-hSTAT6 into the *EcoRI/NotI* sites of pSG6. The expression vectors for murine NCoA-1, NCoA-2, and NCoA-3 (pCMV-NCoA-1/SRC-1, pCMV-NCoA-2, and pCMV-NCoA-3/p/CIP) were kindly provided by Joe Torchia (University of Western Ontario, London, Canada). The expression vectors for full-length rat SRC-1/NCoA-1 (pSG6-SRC-1a) and rat SRC-1/NCoA-1 residues 569–804 fused to six Myc tags were provided by Ludger Klein-Hitpass (Universitätsklinikum, Essen, Germany). The series of expression vectors for murine NCoA-1 residues 1–462, 100–462, and 213–462 fused to six Myc tags were generated by inserting each corresponding region (generated by digestion or polymerase chain reaction) in frame into the *StuI/XbaI* and *XhoI/XbaI* sites, respectively, of pCS2+. The fragments containing the NCoA-1 sequence residues 1–571 and 1–787 of pSG6-SRC-1/NCoA-1 were subcloned into the *XbaI/EcoRI* and *XbaI/BamHI* sites of pSG6. The constructs containing the NCoA-1 sequence residues 361–571 and 313–462 were obtained by insertion of the *EcoRI* and *PvuII/XbaI* fragments of pCMV-SRC-1/NCoA-1 into the *EcoRI* and *EcoRV/XbaI* sites of pcDNA3.1/His (Invitrogen). The construct containing the NCoA-1 sequence residues 1–361 was obtained by deletion of the *EcoRI* fragment of pSG6-SRC-1/NCoA-1. The expression vectors for GST fusion proteins pGEX-AHK and pGEX-KGK were kindly provided by Thorsten Heinzel (Georg-Speyer-Haus, Frankfurt, Germany). The GST fusion construct containing residues 1–787 of NCoA-1 was generated by insertion of the corresponding *BamHI* fragment of pCMV-SRC-1/NCoA-1 into the *BamHI* site of pGEX-KGK. The GST fusion constructs containing residues 677–792, 677–847, and 792–847 of human STAT6 were generated by subcloning of the corresponding insert from the series of GAL4-DBD-STAT6 vectors, generated by digestion with *EcoRI/NheI*, in frame in the *EcoRI/XbaI* sites of pGEX-AHK. Insertion of all constructs was verified by digestion. Inserts generated by polymerase chain reaction were additionally verified by sequencing analysis. The SRC-1/NCoA-1 deletion constructs pSG5-SRC1e- Δ AD1, pSG5-SRC1e- Δ AD2 (Δ 1053–1123), pSG5-SRC1e-1–1240, and pSG5-SRC1e Δ PAS (381–1399) have been described previously (47, 48). The full-length pSG5-SRC1e was obtained by insertion of the *NheI/BglII* fragment of pSG5-SRC1e Δ PAS (381–1399) into the *NheI/BglII* sites of pSG5-SRC1e(Δ 1053–1123).

GST *In Vitro* Binding Assays—Recombinant cDNAs in the pSG6, pBSK, pCMXPL2, or pCS2+ expression vectors were transcribed and translated *in vitro* in reticulocyte lysates (Promega) in the presence of [³⁵S]methionine according to the manufacturer's instructions. GST or GST fusion proteins were expressed in *Escherichia coli* and purified with glutathione-Sepharose beads (Amersham Pharmacia Biotech). For binding assays, GST fusions or GST alone (1.5–5 μ g) bound to glutathione-Sepharose beads were incubated with labeled proteins in 200 μ l of binding buffer as described previously (24). After extensive washing bound proteins were eluted and separated on SDS-polyacrylamide gels. Radiolabeled proteins were visualized by fluorography. Amounts and integrity of bound proteins were estimated on SDS-PAGE by Coomassie staining.

Coimmunoprecipitation Assay—293T cells were transfected with 8 μ g of pXM-Stat6 or pXM-STAT6 Δ 792 and 8 μ g of pSG6-SRC-1/NCoA-1 per 10-cm dish. 2 days after transfection the cells were lysed in NETN buffer (20 mM Tris/HCl, pH 8, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.2% Nonidet P-40, and complete protease inhibitor; Roche Molecular Biochemicals). Cleared cell lysates were incubated with 2 μ g of STAT6 antibody (Zymed Laboratories Inc.), SRC-1 antibody (M341, Santa Cruz) or 5 μ l of rabbit serum for 2 h and for a further hour with protein A/G-agarose beads as described previously (24). The immunoprecipitates were separated by SDS-PAGE, and Western blots were analyzed with SRC-1 antibody (C20, Santa Cruz) and STAT6 antibody (Transduction Laboratories).

RESULTS

NCoA-1 Enhances the IL-4-induced Transcription by STAT6—Previous studies characterized the transactivation domain (TAD) of STAT6 (18–20) and identified autonomously transactivating elements. We have recently demonstrated that

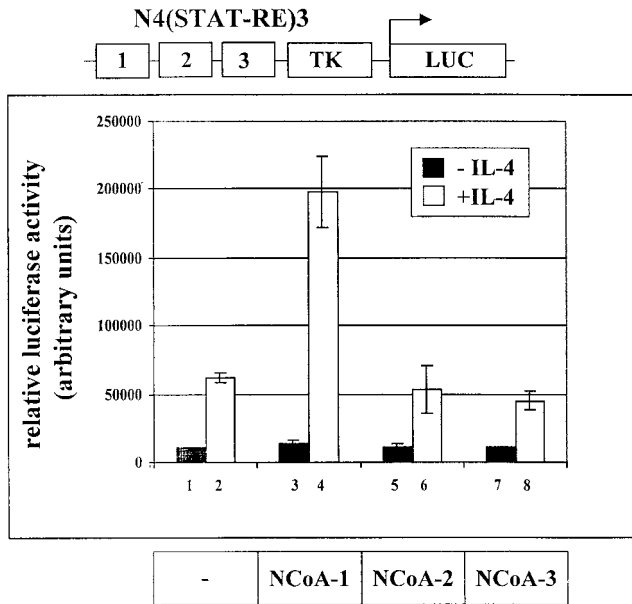


FIG. 1. NCoA-1, but not NCoA-2 and NCoA-3, enhances IL-4-induced transcription by STAT6. HepG2 cells were transfected with N4(STAT-RE)3 LUC reporter plasmid (2 μ g), STAT6 expression vector (25 ng), NCoA-1, NCoA-2, NCoA-3, or empty expression vectors (200 ng) along with SV40-LacZ expression plasmid (25 ng) as a transfection control, as indicated. Transfected cells were either treated with IL-4 (10 ng/ml) for 16 h (white bars) or left untreated (black bars). Cell extracts were prepared, and luciferase activities were determined. The relative luciferase activities are normalized to β -galactosidase activities. The average values with standard deviations from three independent experiments are given.

the activity of the STAT6 TAD is enhanced by the coactivators p300 and CBP and observed an interaction of STAT6 with p300 and CBP *in vivo* (22). The region between amino acids 1850–2176 was characterized as the STAT6-interacting domain of CBP. This domain also mediates the interaction of p300/CBP with the NCoA family of nuclear receptor coactivators (33), which together form a large coactivator complex. To investigate whether the NCoA coactivators are involved in the transactivation by STAT6, transient transfection assays in the IL-4-responsive liver cell line HepG2 were carried out. The cells were transfected with a luciferase reporter construct containing multimerized STAT6 response elements and expression vectors encoding STAT6 and the coactivators NCoA-1, NCoA-2, and NCoA-3, respectively. After transfection, the cells were treated with IL-4 or left untreated. Induction with IL-4 led to a 6-fold enhancement of basal reporter gene expression (Fig. 1, lanes 1 and 2). This induction was further enhanced up to 20-fold when NCoA-1 was cotransfected (lanes 3 and 4), whereas cotransfection of NCoA-2 or NCoA-3, respectively, had no effect (lanes 5–8). These results indicate that NCoA-1 is a coactivator of STAT6, whereas the other members of the NCoA family, NCoA-2 and NCoA-3, do not seem to be involved in transactivation by STAT6.

The Transactivation Domain of STAT6 Interacts with NCoA-1, but Not with NCoA-2 and NCoA-3, *In Vitro*—The results from our transfection experiments suggested that NCoA-1 has, in contrast to the other CBP-associated NCoA-family members, a specific function in STAT6-mediated transcriptional activation. To analyze whether NCoA-1 can directly interact with the transactivation domain of STAT6, GST pull-down experiments were performed with NCoA-1 and the two other related members of the NCoA family. Equal amounts of GST and GST-STAT6-TAD containing the STAT6 transactivation domain fused to GST were incubated with *in vitro* synthe-

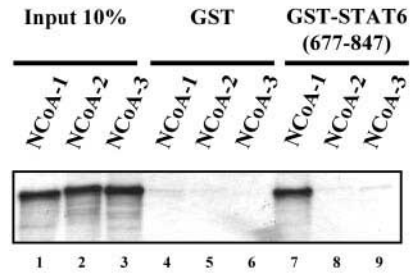


FIG. 2. The transactivation domain of STAT6 specifically interacts with NCoA-1 *in vitro*. GST or GST STAT6 TAD fusion protein were purified from *E. coli*, bound to glutathione-Sepharose, incubated with NCoA-1, NCoA-2, or NCoA-3, respectively, transcribed, and translated *in vitro* in the presence of methionine. Material bound to the glutathione-Sepharose was recovered from the binding reaction, washed extensively, and analyzed by SDS-PAGE and fluorography (lanes 4–9). Input control in lanes 1–3 reflects 10% of the total amount of [35 S]methionine-labeled protein used in the experiments.

sized, 35 S-labeled coactivators. NCoA-1 strongly bound to GST-STAT6 TAD (Fig. 2, lane 7). In contrast, NCoA-2 and NCoA-3 failed to interact with STAT6 TAD (lanes 8 and 9). No binding was observed with GST alone (lanes 4–6). These findings are consistent with the transient transfection experiments shown in Fig. 1, where NCoA-1, but not NCoA-2 and NCoA-3, enhanced STAT6 transactivation potential. Taken together, only NCoA-1 interacts with STAT6 and serves as a specific coactivator for STAT6.

NCoA-1 Strongly Enhances the Transactivation Potential of the C-terminal Part of STAT6 Transactivation Domain—Previous studies have characterized the transactivation domain of STAT6 as a modular domain with different transactivation functions that mediates transcriptional activation when fused to the heterologous GAL4-DNA-binding domain (18–20). We investigated whether NCoA-1 enhances the transactivation potential of STAT6 by contacting a specific part of the STAT6 transactivation domain. Two GAL4 fusion proteins, possessing either the N-terminal part of the STAT6 transactivation domain (amino acids 677–791) or the far C-terminal part of the STAT6 transactivation domain (amino acids 792–847) (Fig. 3A) were used to analyze whether NCoA-1 is recruited to these domains. The GAL4-STAT6-TAD constructs and a luciferase reporter construct containing three GAL4 response elements in its promoter region were transiently transfected into HepG2, with or without cotransfection of the coactivator NCoA-1. Expression of the isolated GAL4-DBD did not result in a significant enhancement of the luciferase reporter gene activity, which was also not affected by cotransfection of NCoA-1 (Fig. 3B, lanes 1 and 2). GAL4-STAT6 (677–791) strongly induced the reporter gene expression (lane 3). Cotransfection of NCoA-1 led to a slight enhancement (lane 4). GAL4-STAT6 (792–847) did not significantly induce the reporter gene activity, indicating that this domain has only a minor transactivation potential when fused to the GAL4 DBD. Surprisingly, cotransfection of NCoA-1 strongly enhanced the transactivation function of this far C-terminal part of the STAT6 TAD to a level as high as the N-terminal transactivation domain of STAT6 (compare lanes 6 and 4). To validate this finding and to exclude the possibility that the strong effect of NCoA-1 on the transactivation activity of GAL4-STAT6 (792–847) was cell type-specific, we repeated the experiments in the pre-B-cell line Ba/F3-IL-4R (Fig. 2C). Again, GAL4-DBD alone had no significant transactivation capacity, and this was not changed by coexpression of NCoA-1 (Fig. 3C, lanes 1 and 2). GAL4-STAT6 (677–791) strongly induced reporter gene activity (lane 3), but in contrast to our results in HepG2 cells, this was not further enhanced by coexpression of NCoA-1 (lane 4). GAL4-STAT6 (792–847) did not

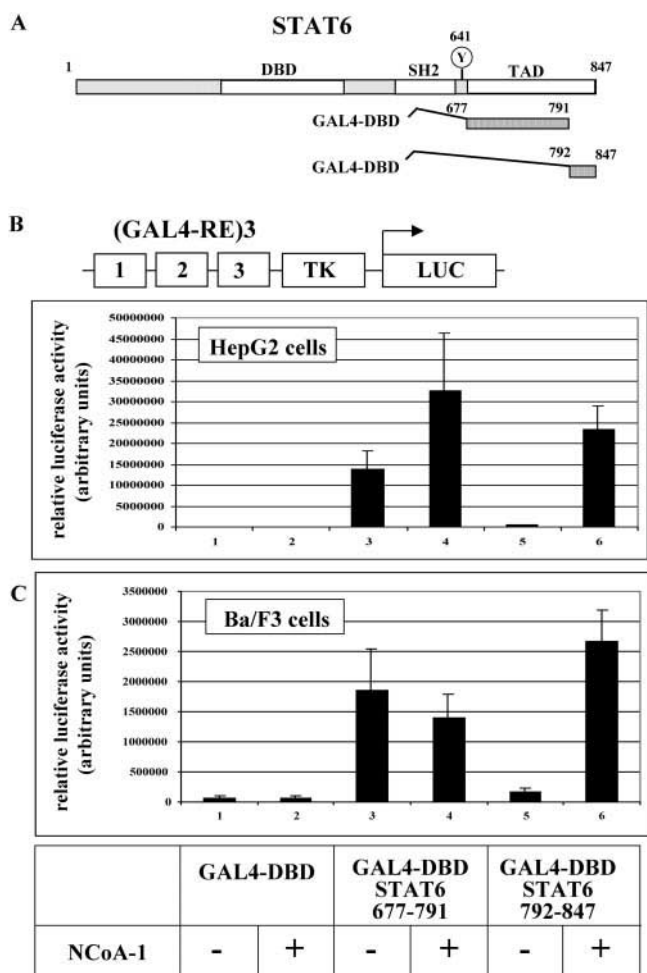


FIG. 3. The transactivation potential of the far C-terminal part of the STAT6 transactivation domain is strongly enhanced by NCoA-1. *A*, structure of human STAT6 and the fusion proteins of the GAL4-DBD with the STAT6 transactivation domain (TAD) are shown. The GAL4-DBD (amino acids 1–147) was fused to different regions of STAT6 TAD. Amino acid position, DBD, SH2 domain, TAD, and the cytokine-dependent phosphorylation site (Y) are indicated. *B*, the reporter plasmid (GAL4-RE)³TK LUC (2 μ g), expression plasmids encoding the GAL4-DBD or GAL4-DBD-STAT6-TAD fusion proteins (50 ng), NCoA-1 or empty expression plasmid (400 ng), and SV40-*LacZ* expression plasmid (25 ng) were transfected into HepG2 cells as indicated. *C*, Ba/F3 cells were transfected with the reporter plasmid (GAL4-RE)³TK LUC (5 μ g), the plasmids encoding the GAL4-DBD or GAL4-DBD-STAT6-TAD fusion proteins (50 ng), NCoA-1 (500 ng), or empty expression plasmid and SV40-*LacZ* expression plasmid (1 μ g) as indicated. *B* and *C*, relative luciferase activities were determined and normalized against β -galactosidase activities. The average values with standard deviations of three independent experiments are shown.

show significant transactivation activity by itself (lane 5). In the presence of cotransfected NCoA-1, GAL4-STAT6 (792–847) strongly enhanced the transcription (lane 6). These results confirm that NCoA-1 is a coactivator of the STAT6 transactivation domain. The strong coactivation effect on the far C-terminal part of the STAT6 TAD (amino acids 792–847), which has very low autonomous transactivation activity, suggests that NCoA-1 is recruited to this domain. The cell type-specific effect of NCoA-1 on the N-terminal transactivation domain of STAT6 (amino acids 677–791) might be due to an indirect recruitment of NCoA-1 to this domain by cell-specific factors.

The Far C-terminal Part of the STAT6 TAD Is Required for Full Transactivation Activity of STAT6—We compared the activity of full-length STAT6 and a truncated version lacking the far C-terminal part of the TAD (STAT6 Δ 792) to prove the

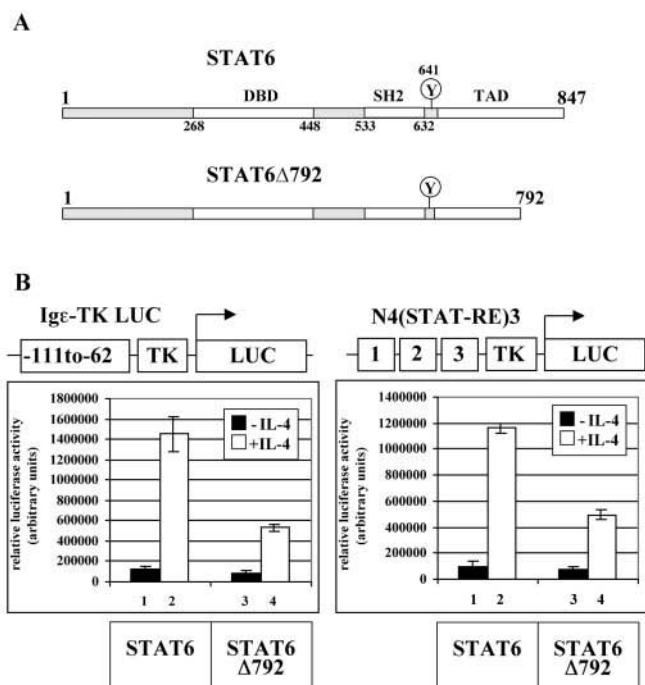


FIG. 4. The far C-terminal part of the STAT6 TAD contributes to the full transactivation activity of STAT6. *A*, schematic representation of STAT6 functional domains. Depicted are DBD, SH2 domain, TAD, and the cytokine-dependent phosphorylation site (Y). *B*, Ba/F3 cells were transfected with the N4(STAT-RE)³ LUC or the Ige-TK LUC reporter plasmid (5 μ g), the plasmid encoding full-length or truncated STAT6 (200 ng), and SV40-*LacZ* expression vector (1 μ g) as indicated. 4 h after transfection, the cells were treated with IL-4 or left untreated. 24 h post-transfection, luciferase activities were determined and normalized against β -galactosidase activities. The values represent the average from three independent experiments.

importance of this domain for STAT6 transactivation (Fig. 4A). To exclude promoter-specific effects we analyzed the transactivation potential of these proteins on two different reporter constructs. The first contained part of the Ig GL ϵ promoter, including the relevant STAT6-binding site upstream of a minimal thymidine kinase promoter (Ige-TK LUC), and the second contained multimerized STAT6 binding sites (N4(STAT-RE)³ LUC) (Fig. 4B). Ba/F3-IL-4R cells were transfected with the vectors expressing full-length or truncated STAT6 and the reporter plasmids. After transfection, the cells were treated with IL-4 or left untreated. Western blotting confirmed similar expression of wild type and mutant STAT6 (data not shown). As expected, the full-length STAT6 strongly induced expression of both reporter constructs upon IL-4 treatment (Fig. 4B, lanes 1 and 2). However, the transactivation capacity of the truncated version was markedly reduced (lanes 3 and 4). Only 40% of the full-length STAT6-induced activity was obtained. These results indicate that although the C-terminal part of STAT6 has no or very low activity when fused to the GAL4-DBD (Fig. 3), it delivers more than 50% of the STAT6 transactivation capacity in its normal molecular context.

NCoA-1 and the Far C-terminal Region of STAT6 Interact *In Vivo*—Transfection experiments showed that NCoA-1 strongly enhanced the transactivation potential of the far C-terminal STAT6 TAD. To examine a possible interaction between NCoA-1 and this region of STAT6, *in vivo* coimmunoprecipitation experiments were carried out. 293T cells were transfected with expression vectors encoding full-length STAT6 or STAT6 Δ 792, lacking the far C-terminal part of the TAD, and NCoA-1. Whole cell extracts were prepared, and coimmunoprecipitation experiments were performed with the indicated antibodies. Immunoprecipitates were analyzed by Western blot-

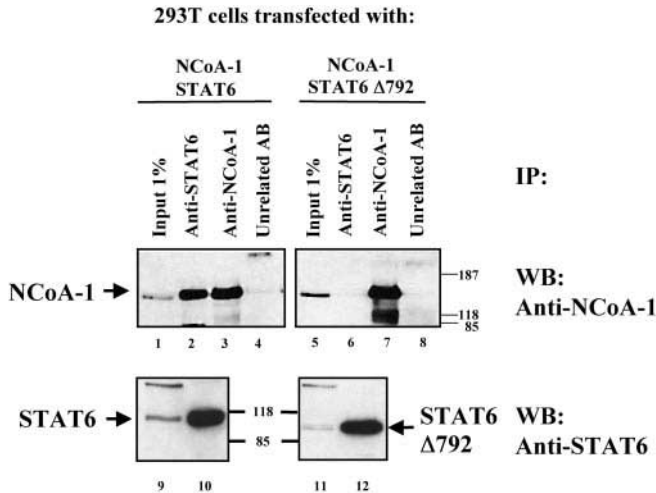


FIG. 5. **NCoA-1 coimmunoprecipitates with STAT6.** 293T cells were transfected with expression vector encoding full-length or truncated STAT6 along with NCoA-1 expression vector. Whole cell extracts were prepared and immunoprecipitated (IP) with STAT6-specific antibody, NCoA-1-specific antibody, or unrelated antibody. All samples were analyzed by SDS-PAGE and Western blotting with antisera against NCoA-1 (upper panels) and STAT6 (lower panels). An aliquot of the cell lysate corresponding to 1% of the material used for the assay was analyzed in parallel (lanes 1, 5, 9, and 11).

ting with NCoA-1- and STAT6-specific antisera. A large amount of NCoA-1 was precipitated with NCoA-1-specific antibodies (Fig. 5, lanes 3 and 7). Unrelated antibodies did not precipitate NCoA-1 at all (lanes 4 and 8). STAT6-specific antibodies coimmunoprecipitated similar amounts of NCoA-1 as the NCoA-1-specific antibodies did (lane 2). This was only observed when full-length STAT6 was cotransfected into the cells. When the truncated form, STAT6 Δ 792, was present in the cells, no NCoA-1 could be coimmunoprecipitated (compare lanes 2 and 6), although equal amounts of wild type and truncated STAT6 were precipitated in both reactions (compare lane 10 and 12). In summary, these experiments clearly demonstrate that NCoA-1 and STAT6 strongly interact *in vivo* and that this interaction depends on the far C-terminal region of STAT6.

Distinct Parts of the STAT6 Transactivation Domain Interact with NCoA-1 and CBP *in Vitro*—Our previous studies showed that p300/CBP, which can also bind to NCoA-1, is recruited to the STAT6 TAD *in vivo* (22). We performed GST pull-down experiments to analyze whether both coactivators NCoA-1 and CBP can independently interact with the STAT6 TAD. Various GST-STAT6 fusion constructs representing the whole TAD (amino acids 677–847), the main N-terminal TAD (amino acids 677–791), and the far C-terminal region (amino acids 792–847), which strongly responds to NCoA-1, were tested for interaction with NCoA-1 and CBP (Fig. 6A). As expected, NCoA-1 strongly interacts with GST STAT6 containing the whole TAD (Fig. 6B, lane 2). The same strong interaction was observed with the GST fusion protein representing only the far C-terminal part of STAT6 TAD (lane 4), whereas the GST fusion protein representing the N-terminal STAT6 TAD failed to bind to NCoA-1 (lane 3). This result confirms our hypothesis that a direct interaction between NCoA-1 and the far C-terminal part of the STAT6 TAD is responsible for the potent coactivator function of NCoA-1. In the next set of experiments we tested whether CBP is also able to bind to a specific region on the STAT6 TAD *in vitro*. An *in vitro* translated GAL4 fusion protein containing the STAT6 interaction region amino acids 1678–2441 of CBP, as it was mapped in mammalian two-hybrid experiments (22), was tested for interaction with the

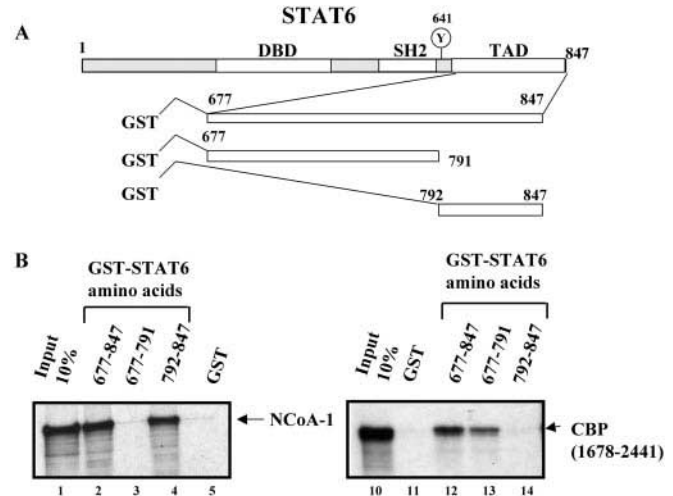


FIG. 6. **STAT6 contacts NCoA-1 and CBP via different parts of its transactivation domain.** A, structure of human STAT6 and the GST-STAT6 TAD fusion proteins. B, NCoA-1 and GAL4-CBP fusion protein comprising amino acids 1678–2441 of CBP were labeled with [³⁵S]methionine by *in vitro* translation and incubated with glutathione-Sepharose bound GST or GST STAT6 fusion proteins as indicated. Specifically bound material was eluted from the glutathione-Sepharose and resolved by SDS-PAGE. An aliquot of the reticulocyte lysate corresponding to 10% of the material used for the assay was analyzed in parallel (lanes 1 and 10). Radioactive labeled protein was visualized by fluorography.

different GST-STAT6-TAD fusion proteins. GAL4-CBP (1678–2441) bound to the whole STAT6 TAD (lane 8) as well as to the main N-terminal TAD (lane 9). In contrast, no interaction was observed with the C-terminal part of STAT6 TAD or GST alone (lanes 10 and 6). These results clearly demonstrate that NCoA-1 and CBP have distinct binding sites in STAT6, CBP binding between amino acids 677–792, whereas NCoA-1 binds to the amino acids 792–847 of STAT6.

The Transactivation Domain of STAT6 Interacts Directly with Amino Acids 213–462 of NCoA-1—NCoA-1 contains several structural and functional domains (Fig. 7A). To investigate which domain in NCoA-1 mediates the interaction with STAT6, we tested various fragments of NCoA-1 for interaction with STAT6 in pull-down experiments (Fig. 7). We used the GST STAT6 TAD fusion protein comprising residues 677–847 to analyze the binding to various fragments of *in vitro* translated labeled NCoA-1. We observed specific interaction of an N-terminal fragment spanning amino acids 1–781 (Fig. 7B, lane 11) and the full-length NCoA-1 protein (lane 15) with GST-STAT6 TAD but no interaction with GST alone (lanes 6–10). The fragments containing the nuclear receptor interaction domain (lane 12), the p300/CBP interaction domain (lane 13), and the C-terminal transactivation domain (lane 14) failed to interact. To further narrow down the STAT6-binding site in NCoA-1, we dissected the N-terminal region of NCoA-1 and tested different fragments in pull-down experiments (Fig. 7C). The divided parts (1–361 and 361–571) of the shortest N-terminal interacting fragment (1–571) failed to interact with STAT6 TAD (Fig. 7C, lanes 6 and 9), indicating that the binding site is around amino acid 361. The minimal fragment of NCoA-1 that was still able to interact with GST STAT6 encompassed the region from amino acids 213 to 462 (lane 18), which comprises the PerArnt-Sim (PAS) domain B and part of the serine/threonine rich domain. Further deletion of the N terminus (fragment 313–462, lane 21) or the C terminus (fragment 1–361, lane 6) abolished the binding. Strong interaction was also observed when GST STAT6 was incubated with purified histidine-tagged NCoA-1 (213–462) (data not shown), confirming that the inter-

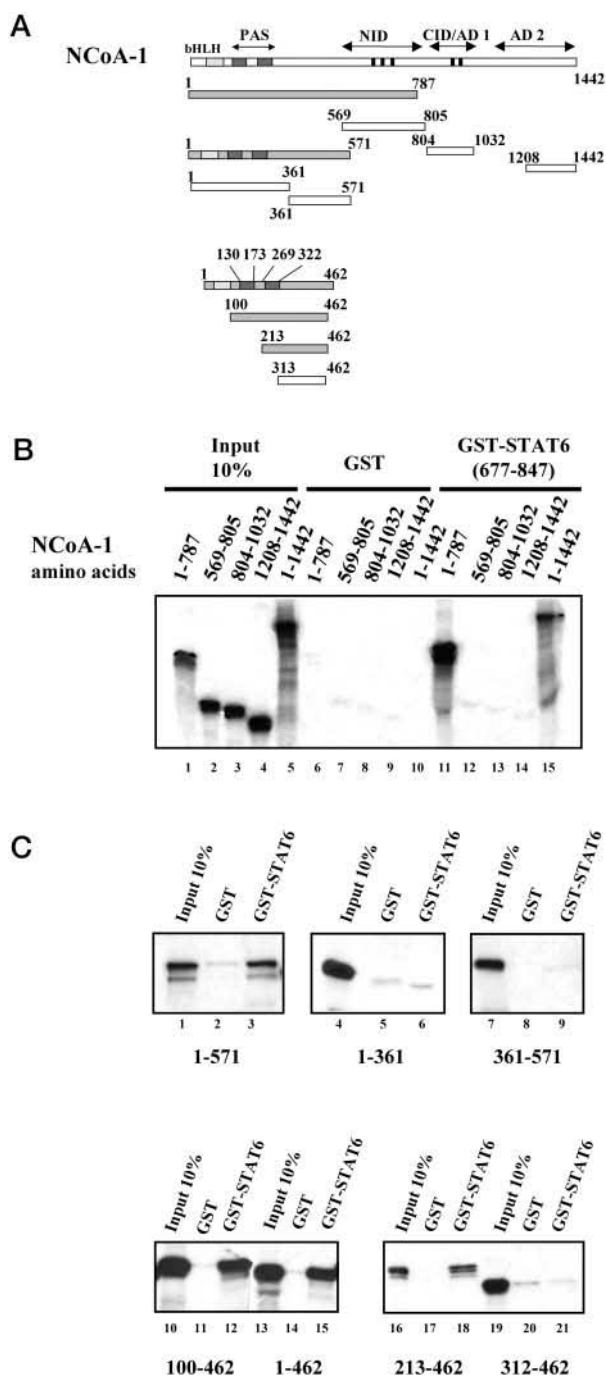


FIG. 7. The N-terminal part of NCoA-1 mediates interaction with STAT6. *A*, schematic representation of the NCoA-1 constructs. The full-length NCoA-1 and a series of NCoA-1 deletion mutants are shown. bHLH, PAS domain, NID, CBP interaction domain (CID)/AD1, and AD2 are indicated. The amino acid numbers for each construct are shown. NCoA-1 fragments binding to STAT6 are represented with striped rectangles, and nonbinding fragments are represented with white rectangles. *B* and *C*, NCoA-1 and a series of NCoA-1 fragments were *in vitro* translated in the presence of [³⁵S]methionine and incubated with GST alone or GST-STAT6-TAD fusion proteins bound to glutathione-Sepharose as indicated. Precipitated proteins were washed, eluted, and resolved by SDS-PAGE. Aliquots of the *in vitro* translated NCoA-1 deletion mutants corresponding to 10% of the material used for the interaction assay were analyzed in parallel (*B*, lanes 1–5; *C*, lanes 1, 4, 7, 10, and 16). Radioactive labeled protein was visualized by fluorography.

action between NCoA-1 and STAT6 is direct and does not depend on additional proteins.

Coactivation of STAT6-mediated Transactivation by NCoA-1

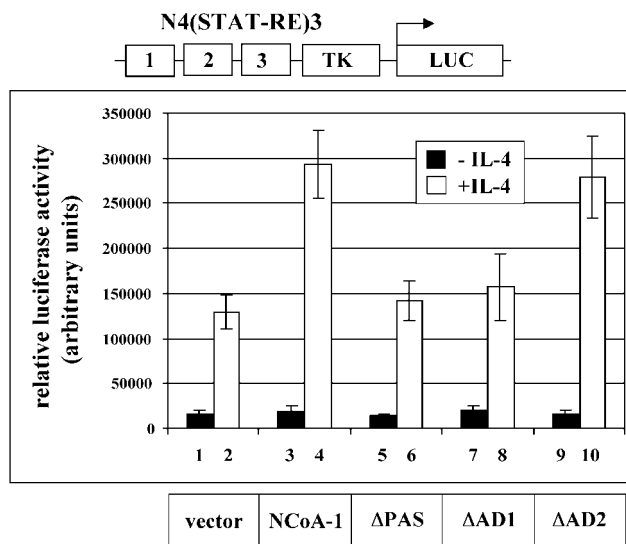


FIG. 8. The N-terminal part and the AD1 of NCoA-1 are important for coactivation of STAT6. HepG2 cells were transfected with N4(STAT-RE)3 LUC reporter plasmid (2 μg), STAT6 expression vector (25 ng), full-length NCoA-1 or NCoA-1 deletion mutants (ΔPAS, ΔAD1, ΔAD2), or empty expression vector (200 ng) along with SV40-LacZ expression plasmid (50 ng) for transfection control, as indicated. Transfected cells were either treated with IL-4 (10 ng/ml) for 16 h (white bars) or left untreated (black bars). Cell extracts were prepared, and luciferase activities were determined. The relative luciferase activities are normalized to β-galactosidase activities. The average values with standard deviations from three independent experiments are given.

Requires the PAS Domain and the Activation Domain 1 of NCoA-1—To explore the domains of NCoA-1 required to enhance transactivation by STAT6, NCoA-1 mutants with distinct functional defects were tested for their coactivator function. As shown in Fig. 1, cotransfection of wild type NCoA-1 enhanced IL-4-induced transactivation by STAT6 (Fig. 8, compare lane 4 with lane 2). NCoA-1 mutant in which the STAT6 interaction domain was deleted (ΔPAS) failed to enhance transactivation by STAT6 (lane 6). The NCoA-1 mutant in which the activation domain 1 was deleted (ΔAD1) was also no longer functional in coactivating STAT6 transactivation (lane 8). In contrast, the deletion mutant of the second activation domain of NCoA-1 (ΔAD2) was still able to enhance STAT6 transactivation like wild type NCoA-1 (lane 10). These results indicate that NCoA-1 needs to interact with STAT6 and to recruit additional factors like CBP via its activation domain 1 to coactivate the STAT6 activity.

Only STAT6 Interacts with the N-terminal Region of NCoA-1—To analyze whether the interaction with NCoA-1 is specific for STAT6 or could also be observed with other structurally related members of the STAT family, pull-down experiments with NCoA-1 and the various STAT proteins were performed. We created a GST fusion protein with the N-terminal part of NCoA-1 and analyzed the binding of *in vitro* synthesized STATs. Only STAT6 interacted strongly with the N-terminal region of NCoA-1 (Fig. 9, lane 21), whereas the other STATs showed no significant binding. This confirms that the characterized interaction with NCoA-1 is a specific feature of the STAT6 TAD.

The N-terminal Region of NCoA-1 Has a Dominant Negative Effect on Transcriptional Activation by STAT6 but Has No Effect on the Transactivation by RAR/RXR—To confirm the relevance of the interaction with NCoA-1 to STAT6 transactivation, we investigated whether overexpression of the N-terminal domain of NCoA-1 (Fig. 10A) could influence the transcriptional activation capacity of STAT6. Therefore, we expressed the N-terminal fragment (amino acids 1–571) or, as

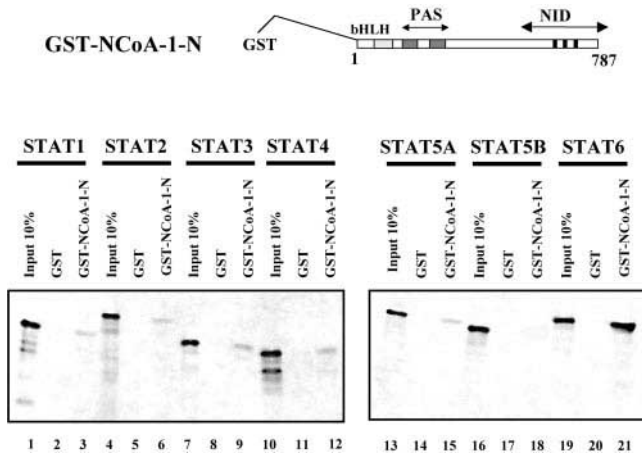


FIG. 9. STAT6, but not the other members of the STAT family, interacts with the N-terminal region of NCoA-1. GST and GST fusion proteins comprising the N-terminal half (GST-NCOA-1-N) of NCoA-1 were bound to glutathione-Sepharose and incubated with the various STAT proteins, which were labeled by *in vitro* translation in the presence of [³⁵S]methionine. Bound proteins were resolved by SDS-PAGE. Radioactive proteins were visualized by fluorography. Aliquots of the *in vitro* translated proteins corresponding to 10% of the material used for the assay were analyzed in parallel (lanes 1, 4, 7, 10, 13, 16, and 19).

a control, the nuclear receptor interaction domain (NID) of NCoA-1 (amino acids 569–805) in Ba/F3-IL-4R cells and analyzed their effect on the activity of the STAT6-responsive Ige-TK luciferase reporter construct. Upon induction with IL-4 a strong stimulation of the luciferase reporter gene was observed (Fig. 10B, lanes 1 and 2). Coexpression of the N-terminal NCoA-1 constructs containing the STAT6 interaction region abolished this activation (lanes 3 and 4). In contrast, coexpression of the fragments harboring the NID did not show any marked effect on the STAT6-mediated stimulation (lanes 5 and 6). To exclude the possibility that overexpression of the N-terminal part of NCoA-1 leads to general inhibition of the transactivation by transcription factors, we investigated its influence on the nuclear receptors RAR and RXR. The coactivator function of NCoA-1 for the nuclear receptors has been characterized previously by several groups. Blocking of the nuclear receptor interaction domain of NCoA-1 by specific antibodies leads to inhibition of RAR/RXR function (30). We used a luciferase reporter containing part of the RAR β promoter, which possesses two response elements for RAR/RXR, upstream of a minimal promoter (β -RE-2-TK LUC). Ba/F3-IL-4R cells were transfected with the β -RE-2-TK LUC reporter, expression vectors for RAR and RXR, and vectors encoding the N-terminal fragment and NID domain of NCoA-1 by electroporation. After transfection, the cells were induced with retinoic acid or left untreated, and luciferase activity in cell lysates was determined. Treatment with retinoic acid led to strong induction of luciferase expression (Fig. 10C, lanes 1 and 2). Coexpression of the N-terminal part of NCoA-1 did not influence the transactivation by RAR/RXR (lanes 3 and 4), whereas overexpression of the NID domain led to a reduction of the RAR/RXR-induced transactivation (lanes 5 and 6). In summary, these experiments indicate that overexpression of the N-terminal domain of NCoA-1, which possesses the STAT6 interaction domain, specifically inhibits STAT6 function, whereas it does not influence the transactivation capacity of the nuclear receptors RAR/RXR.

DISCUSSION

In previous studies, we have shown that p300 and CBP, which coactivate a variety of transcription factors, are required

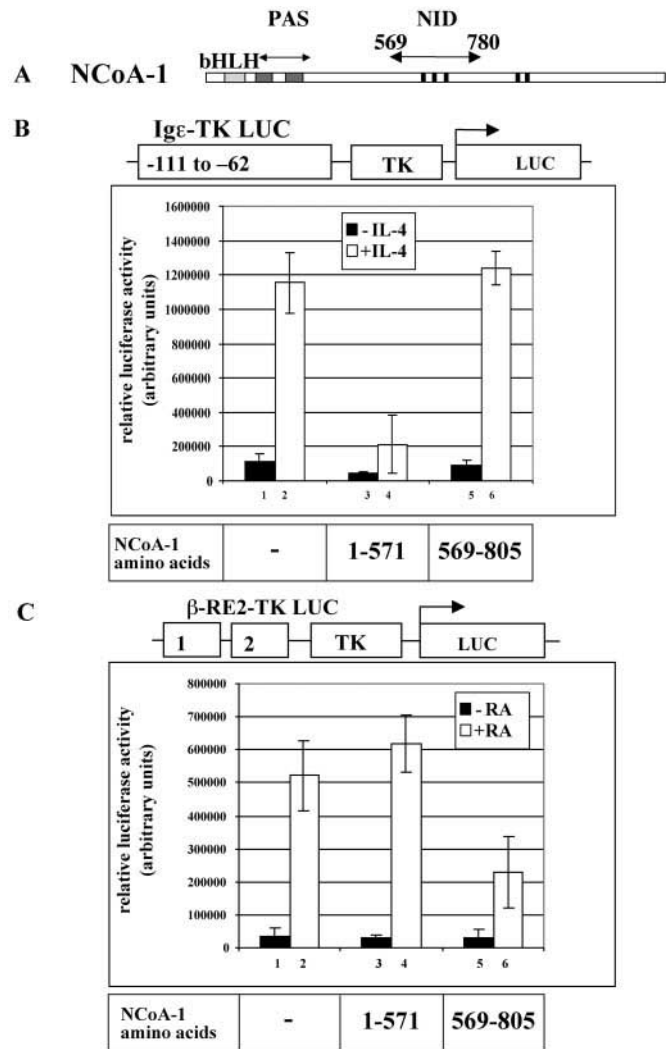


FIG. 10. The N-terminal region of NCoA-1 has a dominant negative effect on transcriptional activation by STAT6. A, structure of NCoA-1. Depicted are functional and structural domains bHLH, PAS domain, and NID. B, Ba/F3 cells were transfected with 200 ng of STAT6 expression vector, 3 μ g of expression vector encoding the indicated fragments of NCoA-1 or empty vector, along with 5 μ g of Ige-TK LUC reporter plasmid and 1 μ g of SV40-LacZ expression vector. 4 h after transfection cells were treated with IL-4 or left untreated. C, Ba/F3 cells were transfected with expression vectors encoding RAR and RXR (200 ng) and the indicated fragments of NCoA-1 (3 μ g), along with the β -RE-2-TK LUC reporter plasmid (5 μ g) and SV40-LacZ expression vector (1 μ g). 4 h after transfection cells were treated with 10^{-6} M retinoic acid or left untreated. B and C, 20 h after transfection cells were harvested, and luciferase activities were determined and normalized against β -galactosidase activities. The values represent the averages from three independent experiments.

for IL-4-induced STAT6-mediated transactivation (22). p300/CBP have been shown to associate with other coactivators like p/CAF (49) and members of the NCoA coactivator family also called p160/SRC-1 family (27, 33), thus providing a platform for a variety of proteins playing a role in gene expression (23). Different classes of transcription factors require specific components of the p300/CBP/NCoA coactivator complex as well as their HAT activities, suggesting the existence of distinct multiprotein coactivator complexes (40). In this report, we investigated whether the NCoA coactivators are involved in transcriptional activation by STAT6.

We demonstrated that STAT6 uses NCoA-1 as a coactivator for transcriptional activation but not the closely related family members, NCoA-2 and NCoA-3, which have also been reported to interact with p300/CBP. This suggests that these

coactivators are either functionally different or are not limiting in the cells that we used in our study. Indeed, our *in vitro* interaction studies argued for the first possibility. Only NCoA-1 was able to interact directly with STAT6, supporting the idea that NCoA-1 has a specific function in STAT6-mediated transactivation.

The C-terminal part of STAT6 contains an autonomous, modular TAD (18, 19, 50). The efficiency of this TAD seems to depend on the cell type, implying a complex transactivation mechanism and the involvement of cell type-specific factors (18, 19). The modular structure of the transactivation domain suggests that STAT6 recruits the transcription machinery by interactions with multiple partners. We investigated whether NCoA-1 can enhance transcription by contacting a specific part of the STAT6 TAD fused to the GAL4-DBD (Fig. 3). The N-terminal part of the STAT6 TAD (residues 677–792) showed a high transcriptional activation function. Coexpression of NCoA-1 slightly enhanced this activity in a cell type-specific manner, indicating that NCoA-1 might be recruited indirectly via other cell type-specific coactivators. In contrast, the C-terminal part of the TAD (residues 792–847) had a low transactivation potential *per se*, but this activity was strongly enhanced in the presence of exogenous NCoA-1. This result shows that NCoA-1 is the limiting mediator for transcriptional activation by this region. It directly interacted with this region (Fig. 6). Furthermore, interaction of NCoA-1 with the full-length STAT6 protein was dependent upon the presence of this region (Fig. 5). Taken together, these results showed that the direct binding of NCoA-1 led to a dramatic increase of the activity of the far C-terminal part of the STAT6 TAD.

Because the far C-terminal part of the STAT6 TAD has a negligible transactivation potential on its own, we wondered whether this part of the transactivation domain contributes to the full transactivation potential of STAT6. A truncated mutant of STAT6 lacking the NCoA-1-interacting part of the TAD showed a marked decrease in transactivation efficiency, up to 40%. This was observed both with a promoter consisting of multimerized STAT6 binding elements and with a promoter fragment of the natural GL ϵ promoter containing CAAT/enhancer-binding protein and NF- κ B-binding sites (Fig. 3). This result supports the importance of the NCoA-1-responsive part of the STAT6 TAD for optimal IL-4-induced STAT6-mediated transactivation.

We found that STAT6 recruited the two coactivators, CBP and NCoA-1, by direct contact with distinct parts of its TAD. The N-terminal part of the STAT6 TAD recruited CBP, whereas the very far C-terminal part directly contacted NCoA-1. Although the strength of the interaction cannot accurately be determined by pull-down experiments, our results suggest that more NCoA-1 than CBP is recruited by STAT6 (Fig. 6). We would therefore propose a model by which NCoA-1 binds to the far C-terminal part of the STAT6 transactivation domain, thereby recruiting CBP, which interacts directly with the N-terminal part of the STAT6 TAD. Multiple interactions of CBP, NCoA-1, and STAT6 would stabilize binding to the promoter. Additional experiments are required to verify this model.

NCoA-1, NCoA-2, and NCoA-3 possess different conserved domains responsible for the interaction with transcription factors like the nuclear hormone receptors, as well as interaction domains responsible for the recruitment of downstream effectors (39). At the N terminus, NCoA coactivators share a highly conserved basic helix loop helix (bHLH)/PAS domain accompanied by a serine/threonine-rich region. The bHLH domain functions as a DNA-binding and dimerization surface in many transcription factors. The PAS motif is also found in several

regulators and seems to play a role in protein-protein interactions and dimerization as well (51). We localized the region in NCoA-1 responsible for interaction with STAT6 to a region spanning amino acids 213–462, which comprises the B part of the PAS domain and a part of a serine/threonine-rich region (Fig. 7). Interestingly, neither the bHLH/PAS domain alone (residues 1–361) nor the serine/threonine-rich domain (361–571) was able to interact strongly with STAT6. These regions of NCoA-1 have already been shown to interact with other transcription factors. Residues 1–361 bind TEF4 and p53 *in vitro* (35, 48), and residues 361–568 interact with serum response factor (36). Because the presence of both the PAS-B domain and the serine/threonine-rich domain are necessary and sufficient for the interaction with STAT6 *in vitro* and *in vivo* (in yeast, data not shown), we have characterized a novel interaction surface of NCoA-1.

The current model of transcriptional activation proposes that coactivators function as bridging factors to recruit additional cofactors and the basal transcription machinery to the DNA-bound transcription factors and/or function as chromatin modifying enzymes. NCoA-1 possesses at least two effector domains. AD1 is able to interact with the coactivators p300/CBP and p/CAF (40). AD2 contains a weak histone acetyltransferase activity and contacts the methyltransferases CARM1 and PRMT1 (52, 53). In addition, the first 93 amino acids of NCoA-1 were also found to exhibit a weak transactivation function when fused to the GAL4-DNA-binding domain (54). Using NCoA-1 deletion mutants, we have analyzed which of the functional domains of NCoA-1 mediated coactivation of STAT6. The results we obtained from these experiments indicate that the N-terminal part of NCoA-1, as well as the AD1 domain was required for STAT6 coactivation. The N terminus of NCoA-1 was responsible for binding to STAT6. We therefore expected that deletion of this domain would abolish the coactivation function of NCoA-1. However, in addition to its function in contacting STAT6, this domain could have a role recruiting effector proteins via its weak transactivation function. The AD1 appears to mediate coactivation of STAT6 by the interaction with p300/CBP and/or other AD1 binding proteins like p/CAF. Recruited CBP could, in addition to its function in histone acetylation, stabilize the NCoA-1/STAT6 interaction because it binds to the STAT6 TAD at a region near to the NCoA-1-binding site (Fig. 6). The C-terminal AD2 domain can be deleted without affecting the ability of NCoA-1 to enhance STAT6 activity, indicating that recruitment of methyltransferases as well as the HAT activity of NCoA-1 are not required for coactivation of STAT6 in transient reporter assays.

Although the STAT family members are highly related in structure and function, we propose no common coactivation mechanism for different STATs. STAT1, STAT2, STAT3, STAT5, and STAT6 each contact different domains of p300/CBP (2, 22, 24, 55). Our studies of NCoA-1 showed that among the STAT proteins, only STAT6 was able to interact with the N-terminal part of NCoA-1 (Fig. 9). Sequence comparison of the transactivation domains of the most related STAT proteins (STAT6, STAT5A, STAT5B, and STAT4) reveals that the NCoA-1 interaction domain of STAT6 is located in a region that extends past the sequence end of the other STATs, supporting the idea that this interaction module is a specific feature of STAT6. We will not exclude the possibility that NCoA-1 has also a function in transcription activation by the other STAT proteins, but it may be recruited by another mechanism. We hypothesize that different STAT proteins recruit specific coactivator complexes with distinct content and structure, because of requirements for their respective promoter context.

Indeed, on the promoters of IL-4-responsive genes, like GL ϵ ,

GL γ 1, and CD23, the STAT6 response elements are close to binding sites of other transcription factors, e.g. NF- κ B, CAAT/enhancer-binding protein, and PU.1. These factors can act in a synergistic manner (17, 56–61). PU.1, NF- κ B subunit p65, and STAT6 have been shown to interact with different regions of CBP (22, 62–64). Also, NF- κ B p50 and STAT6 recruited NCoA-1 via different regions (38).

We speculate that the synergistic activation of IL-4-responsive genes occurs, in addition to cooperative DNA binding of the factors, as a result of a concerted recruitment of a common CBP/p300/NCoA-1 coactivator complex. Thus, p300/CBP and NCoA-1 would represent integrators of different signaling pathways that exert their function via multiple interaction surfaces. From our transient transfection experiments we would predict that the IL-4-regulated expression of the GL ϵ gene depends on a functional interaction between STAT6 and NCoA-1. It will be important to see whether the expression of the endogenous STAT6-activated genes (e.g. GL ϵ , GL γ 1, CD23, and major histocompatibility complex II) are dependent on this mechanism. We are currently addressing this question by the overexpression of the dominant negative acting interaction domain into stable cell lines.

Our data suggest that NCoA-1 is an essential requirement for STAT6 function. Overexpression of the N-terminal STAT6 interaction domain of NCoA-1 resulted in a dominant negative inhibition of the IL-4-induced STAT6-mediated transactivation of the GL ϵ promoter. In contrast, overexpression of this domain did not alter transactivation by the nuclear receptors RAR/RXR, indicating that expression of this domain does not *per se* inhibit transactivation. STAT6 has an essential role in IgE production and represents, therefore, an important target for therapeutic intervention in cases of allergic diseases. The identification of NCoA-1 as a key component in the transactivation by STAT6 and the characterization of its specific interaction domain open the possibility of developing new strategies to interfere with STAT6 function at the level of transcription activation.

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