NCoA-1/SRC-1 Is an Essential Coactivator of STAT5 That Binds to the FDL Motif in the α-Helical Region of the STAT5 Transactivation Domain*

Signal transducer and activator of transcription 5 (STAT5) is a transcription factor that activates prolactin (PRL)-dependent gene expression in the mammary gland. For the activation of its target genes, STAT5 recruits coactivators like p300 and the CREB-binding protein (CBP). In this study we analyzed the function of p300/CBP-associated members of the p160/SRC/NCoA family in STAT5-mediated transactivation of β-casein expression. We found that only one of them, NCoA-1, acts as a coactivator for both STAT5a and STAT5b. The two coactivators p300/CBP and NCoA-1 cooperatively enhance STAT5α-mediated transactivation. For NCoA-1-dependent coactivation of STAT5, both the activation domain 1 and the amino-terminal bHLH/PAS domain are required. The amino-terminal region mediates the interaction with STAT5a in cells. A motif of three amino acids in an α-helical region of the STAT5α-transactivation domain is essential for the binding of NCoA-1 and for the transcriptional activity of STAT5a. Moreover we observed that NCoA-1 is involved in the synergistic action of the glucocorticoid receptor and STAT5a on the β-casein promoter. These findings support a model in which STAT5, in concert with the glucocorticoid receptor, recruits a multifunctional coactivator complex to initiate the PRL-dependent transcription.

STAT51 is an important regulating factor in the differentiation of the mammary gland (1). It mediates the prolactin-induced differentiation of lobular alveolar end buds and the production of milk proteins during pregnancy (2). Several STAT5-target genes have been identified, which are up-regulated in response to prolactin, including β-casein, β-lactoglobulin, whey acidic protein, and cytokine-induced sequence (CIS) (3–5).

There are two STAT5 genes in mammals, STAT5a and STAT5b (1, 6). Both proteins are more than 95% homologous and they form homo- and heterodimers after phosphorylation and activation. Although both isoforms have overlapping roles in cytokine responses, specific functions for STAT5a and STAT5b have also been observed (5). One reason for the different biological effects is a cell type-specific expression pattern of both isoforms. Moreover, it has been postulated that structural differences in the DNA binding affinity of the different STAT5 dimers might affect gene regulation (7). Due to the higher expression level of the STAT5a gene in mammary epithelial cells, STAT5a protein seems to be the main regulator in mammary gland functions (5). In contrast to that STAT5b mediates growth hormone-dependent sexual dimorphism of body growth and liver metabolism (8).

During transcriptional activation of target genes, dimerized STAT5 proteins bind to GAS elements in the promoter region and recruit coactivators to the promoter. These coactivators are able to promote the transcriptional initiation process through enhancement of DNA accessibility for general transcription factors by chromatin remodeling. Moreover, they promote the formation of the preinitiation complex by recruitment of components from the RNA polymerase holoenzyme.

STAT5a and STAT5b contain an α-helical region in their transactivation domain, which is essential for the transcriptional activity and for the proteasome-dependent turnover of the tyrosine-phosphorylated proteins (9). The hydrophobic residues 751 and 753 have been identified to be crucial for the transcriptional activity of STAT5a (10), but it is not known so far which proteins are targeted to this region. The transactivation domain of STAT5 proteins exhibits a weak transactivation potential, when tested as GAL4-STAT5 carboxyl-terminal fusion proteins (11). However, STAT5 acts as a strong activator of its target genes, for instance the β-casein gene. It is conceivable that the action of STAT5 is supported by other transcription factors, since the GAS sites in the promoter of target genes are in close vicinity to the DNA binding sites of other transcription factors.

p300 and CBP (collectively termed as p300/CBP) represent common factors required for different transcription factors (12). They possess intrinsic histone acetyl transferase (HAT) activity (13, 14). In previous studies we have shown that p300/CBP bind to STAT5 and coactivate STAT5-mediated transactivation of β-casein expression (15).

p300/CBP have been shown to serve as a platform for the assembly of multiprotein complexes, containing various transcription factors and coactivators (12, 16). According to the current model of transcriptional regulation, different preformed coactivator complexes exist, which differ in their composition of various coactivator family members (17). These subcomplexes are primed for recruitment by a specific transcription factor (16, 18, 19). In some cases, the interaction of coactivators is regulated by secondary modifications (20, 21). p300/CBP associate with members of the p160/SRC/NCoA-
Coactivation of STAT5 by NCoA-1/SRC-1

Gene by recruitment of a coactivator complex consisting of different NCoA family members interacts with many transcription factors. For example, they have been reported to be associated with nuclear hormone receptors like estrogen and progesterone receptor, AP1, NF-kB, and the STAT proteins STAT1, STAT3, and STAT6 (27–30). Functional studies with knockout mice have revealed the involvement of p160SRC/NCoA-proteins interact with steroid hormone receptors (34–36). For example, the knockout mice lacking NCoA-1 and NCoA-3 exhibit defects in the development of the mammary gland. This phenotype was explained by the requirement for steroid receptor transactivation (34, 36). However, it should be kept in mind that the activity of other transcription factors important for mammary gland development, including STAT5, might also be diminished.

The expression of the β-casein gene in mammary epithelial cells in vitro requires, in addition to prolactin stimulation, treatment with glucocorticoids (37). Glucocorticoids act through the glucocorticoid receptor (GR). GR has been shown to synergize with STAT5 in β-casein gene activation and physically associates with STAT5 (38). The molecular mechanism of the cooperative effect exerted by GR was reported to be due to a stabilization of DNA-binding and a prolonged phosphorylation of STAT5 (39). It is not yet clear if coactivators are required for the synergistic actions. Interestingly, GR recruits coactivators such as p300/CBP and members of the p160/SRC/NCoA family for activation of its target genes. Therefore it might be possible that GR supports coactivator recruitment of STAT5.

In this study we analyzed the function of p160SRC/NCoA factors for STAT5-mediated transactivation of β-casein expression. Our results clearly demonstrate that NCoA-1, and not the other members of the p160SRC/NCoA-family, acts as a coactivator for both STAT5α and STAT5b. We observed an interaction of STAT5 and NCoA-1 in cells. The analysis of NCoA-1 mutants in which functional domains were deleted, revealed that coactivation by NCoA-1 requires its activation domain 1 and the amino-terminal bHLH/PAS domain. This amino-terminal region mediates the interaction with STAT5α in cells. Two hydrophobic residues in the α-helical region of the STAT5α-transactivation domain are essential for the binding of NCoA-1 in cells and for the transcriptional activity of STAT5α. Our results demonstrate that STAT5α transactivates the β-casein gene by recruitment of a coactivator complex consisting of p300/CBP and NCoA-1. This complex is also involved in the synergistic action of GR and STAT5 on the β-casein promoter.

**EXPERIMENTAL PROCEDURES**

Plasmids—The luciferase reporter constructs pGL3-β-case-LUC, which contains the β-casein gene promoter (−344 to −1) was obtained by insertion of the corresponding promoter fragments into pGL3-basic (Promega). The estrogen responsive element (ERE) luciferase reporter construct pGL2-ERE TK-luc was obtained from Ludger Klein-Hitpass (Universitätsklinikum, Essen, Germany). The vector encoding for the NCoA-1 sequence residues 1–361 fused to 6×myc-tag was provided by Ludger Klein-Hitpass (Universitätsklinikum, Essen, Germany). The expression vector encoding residues 361–1442 of rat SRC-1/NCoA-1 was generated by insertion of the corresponding fragment, obtained by PCR, into the Xhol/XbaI sites of pCS2+ (Clontech). The plasmid pCMV-GAL4-DBD encoding the DNA binding and dimerization domain (GAL4-DBD) of yeast GAL4 (residues 1–147) and the reporter gene construct (GAL4-RE3)Luciferase were described previously (41). The GAL4 fusion constructs comprising STAT5α-TAD (residues 722–794) was generated by insertion of the corresponding inserts EcoRI and Nhel sites of pGAL4-DBD (Clontech). The expression vector encoding Guclocorticoid receptor has been described previously (45). The expression vector for GFP-STAT5α has been generated by insertion of the cDNA of murine STAT5α, obtained by PCR into the EcoRI and Xmal sites of pECPF-C1 (Clontech). GFP-STAT5α derivatives, which encode for a deletion mutant of STAT5α lacking amino acids 750–768, and a mutated variant, possessing alanines at residues 751–753, were generated by replacing of the BssHII-XbaI fragment from GFP-STAT5α by the the BssHII-XbaI fragment of the corresponding pXM-STAT5α mutant vectors. Insertion of all constructs was verified by digestion. Sequences of insertions generated by PCR were confirmed by DNA sequencing.

Cell Culture, Transfections, and Luciferase Assays—HeLa cells, 293 cells, and 293T cells were maintained in Dulbecco’s modified Eagle’s medium (Bio Whittaker) containing 10% fetal calf serum (Invitrogen), 2 mM glucose, and penicillin/streptomycin, 10 ng/ml insulin (Sigma-Aldrich Chemie GmbH). EGF, and 5 μg/ml insulin (Sigma-Aldrich Chemie GmbH). EGF was omitted during the transfections. HeLa cells were transfected with the Superfect transfection reagent (Qiagen) according to the manufacturer’s instructions. 293 cells were transfected by the calcium-phosphate precipitation method. HC11 cells were transfected with the FuGENE 6 reagent (Roche Applied Science) according to the manufacturer’s instructions. The total amount of DNA was adjusted with the corresponding empty vector. 16 h before collecting the cells, 5 μg/mL PRL (from sheep pituitary gland, Sigma-Aldrich Chemie GmbH) and 10−7 M dexamethasone (Sigma-Aldrich Chemie GmbH) were applied to the cells or not. 48 h after transfection, the cells were harvested. Luciferase and β-galactosidase activities were assayed as recommended by the manufacturer (Promega). For HeLa and 293 cells luciferase activities were normalized to the β-galactosidase activity. In the case of the HC11 cells, luciferase activities were normalized according to protein concentrations because of the high endogenous β-galactosidase activities. The average of three (HeLa, 293) and six (HC11) independent experiments with standard deviations is shown.

Immunoprecipitations and Western Blots—293T cells were transiently transfected with the indicated expression vectors and treated for 30 min with 5 μg/ml PRL and/or 10−7 M dexamethasone or left untreated. Cells were lysed in NETN buffer (0.2% Nonidet P-40, 20 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 10% glycerol), supplemented with complete protease inhibitor (Roche Applied Science) and phosphatase inhibitor mixture I and II (Sigma). Immunoprecipitations were performed with 2.5 μl of anti-GFP antisem (Clontech) as indicated for the Western blot analysis. The total amount of DNA was adjusted with the corresponding empty vector. 16 h with protein A/G-Sepharose beads (Pierce). Immobilized immunoprecipitates were then incubated for further 1.5 h at 4°C with protein A/G-Sepharose beads (Pierce). Immobilized immunoprecipitates were then washed three times with NETN buffer and separated by SDS-PAGE. Western blots were performed with anti-estrogen receptor antibodies, BD Biosciences, anti-NCoA-1 (Upstate Biotechnologies) and anti-Myc antibody (BD Pharmingen) and developed with ECL (Amersham Biosciences).

Chromatin Immunoprecipitations (ChIP) Assays—ChIP experiments were performed according to the method of Barre et al. (46). HC11 cells were grown to high confluency and kept for 2 days in EGF-free RPMI medium containing 2% fetal calf serum derived serum. Cells were stimulated with prolactin (5 μg/ml) and dexamethasone (0.1 μM) for 30 min, washed twice with PBS and cross-linked with 1% formaldehyde at room temperature for 10 min. To harvest HC11 cells the plates were rinsed with ice-cold PBS and then scraped. Cells were collected by centrifugation, washed in PBS and then resuspended in 1 ml Tris-EDTA buffer (1% SDS, 50 mM EDTA, 0.5% Nla3 I), supplemented with complete protease inhibitor (Roche Applied Science) and phosphatase inhibitor mixture I and II (Sigma) and sonicated three times for 15 s each at 50% amplitude Branson Sonifier W-250D. Supernatants were then recovered by centrifugation at 12,000 rpm for...
10 min at 4°C, diluted 3–5 times in dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1) and subjected to one round of immunoclearing for 2 h at 4°C with 2 μg of sheared salmon sperm DNA, 2.5 μg of control IgG (sc-2027, Santa Cruz Biotechnology) and 20 μl of protein A/G-Sepharose beads (Pierce). Immunoprecipitation was performed with the following antibodies: Stat5α and Stat5b were coimmunoprecipitated with the following antibodies: STAT5a and STAT5b was performed with the following antibodies: STAT5a and STAT5b (PA-ST5a and PA-ST5b, R&D Systems; 2 μg each), NCoA/SRC-1 (M-341, Santa Cruz Biotechnology; 4 μg). After overnight incubation 2 μg of sheared salmon sperm DNA and 25 μl of protein A/G-Sepharose beads (50% slurry) were further added for 1 h at 4°C. Immunoprecipitates were washed sequentially for 5 min each in TSE I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl), TSE II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 500 mM NaCl) and buffer III (0.25 μ LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1). Bead precipitates were then washed three times with TE buffer and eluted two times with 1% SDS, 0.1 M NaHCO3. Eluates were pooled and heated at 65°C overnight to reverse the formaldehyde cross-linking. Supernatants were then incubated for 1 h at 45°C with protease inhibitor K (80 μg each), and DNA was precipitated using classical procedures. For PCR, 5 μl from a 50-μl DNA preparation were used for 30 cycles of amplifications. The following primers were used for STAT5 binding: region = 243/–1 of the CIS promoter (according to GenBank™ accession no. NT 039477) 5′-CGC- CCGAGTTTTCCGAAGT3′ (forward primer), 5′-AAAGGTGTG- GTGGCAGAAGCT-3′ (backward primer), control region of the cis promoter = 3549/–3660 5′-GTGAACAGCAGAAACTCTCA3′ (forward primer), 5′-GACTTCCATGTGAGGCAGCA-3′ (backward primer). Primers used for real-time PCR of CIS amplification were already described by (47): region = 188/–104, 5′-GTCGAAAGACTTAGACGCAGG-5′ (forward primer), 5′-TCGCCGAGCCCTACCTT-3′ (backward primer).

RESULTS

NCoA-1 Acts as a Coactivator for Transcriptional Activation by STAT5a and STAT5b—Both STAT5α and STAT5b, mediate prolactin action in mammary epithelial cells, e.g. the expression of the β-casein gene (6, 48). In previous studies we demonstrate, that the coactivators p300 and CBP enhance transcriptional activation of the β-casein promoter by STAT5(15). Moreover, we observed an interaction of p300 with the STAT5 transactivation domain in vivo. Both p300 and CBP are associated with members of the p160/SRC/NCoA-family (22, 23). Two of these coactivators, NCoA-1 also called SRC-1 and NCoA-3 also called AIB-1, have been described to be involved in the regulation of mammary gland development and breast carcinogenesis (34, 36, 49). Therefore, we investigated whether members of the p160/SRC/NCoA-family are also involved in the transactivation of the β-casein gene, mediated by STAT5α and STAT5b. To address this question HeLa cells were transiently transfected with the prolactin receptor, STAT5α, and the β-casein luciferase reporter plasmids. This heterologous cell system allows study of prolactin-induced signaling and transcription normally restricted to mammary epithelial cells (15). A 10-fold enhancement of reporter gene activity was obtained upon prolactin treatment under these conditions (Fig. 1A, lane 1). When various amounts of NCoA-1, -2, or -3 were cotransfected, only NCoA-1 was able to enhance STAT5α-mediated transactivation. Increasing amounts of NCoA-1-enhanced transcriptional activation by STAT5α in a linear dose-dependent manner. Cotransfection of 200 ng of NCoA-1 enhanced the activation 5-fold, relative to the level without exogenous coactivator (lanes 1 and 4). Thus, NCoA-1 is a limiting coactivator for STAT5α-mediated transactivation, whereas the other family members NCoA-2 and NCoA-3 may not be.

STAT5α and STAT5b differ mainly in the amino acid sequence of the transactivation domain, the region, which contacts transcriptional coactivators. We wondered whether STAT5b would also show a preferential recruitment of NCoA-1 with respect to p160/SRC/NCoA-coactivators. When HeLa cells were transfected with STAT5b under the same conditions, a 12-fold activation of reporter gene expression in response to prolactin was observed (Fig. 1B, lane 1). Remarkably, coexpression of NCoA-1 led to a strong dose-dependent increase of the reporter gene activity. With the highest amount of NCoA-1, a 5-fold enhancement was observed. In contrast coexpression of NCoA-2 had no effect on STAT5b action. A low level of NCoA-3 could slightly stimulate STAT5b-mediated transactivation (Fig. 1B, lane 8), whereas higher amounts did not increase it any further (lanes 9 and 10).

Previous studies have shown that all p160/SRC/NCoA coactivators enhance the activity of the estrogen receptor (ER) (27, 28, 50, 51). To control our experimental settings, we analyzed the effect of the various coactivators on the activity of the ER. HeLa cells were transfected with expression vectors for ER, the indicated amounts of the coactivators along with the pGL2ERE tk-luc reporter plasmid. The transcription activation mediated by ER was strongly induced (8.5-fold) in the presence of estradiol (E2) (Fig. 1C). Coexpression of all three coactivators resulted in a strong stimulation of the ER activity in a concentration-dependent manner. The highest amount of NCoA-1 induced a 5.8-fold enhancement. Coexpression of NCoA-2 and NCoA-3 resulted in a comparable enhancement. A 5.6-fold and 5.4-fold, respectively, increase of the ER-mediated transactivation was observed. This result indicates that all members of the p160/SRC/NCoA-family coactivators are able to enhance transcription in our experimental settings and confirms our previous observation that NCoA-1 has a specific role in the STAT5-mediated transactivation.

NCoA-1 Enhances STAT5-induced Transcriptional Activation in Mammary Epithelial Cells—To prove these findings in a more physiological system, we repeated these experiments in the nontumorigenic mammary epithelial cell line HC11. Upon prolactin treatment, we observed a 4.5-fold increase of reporter gene activity in cells, which were transfected with the β-casein luciferase reporter plasmids and expression vectors for the prolactin receptor and STAT5α (Fig. 2). After cotransfection of the three coactivators, only NCoA-1 was able to enhance STAT5α-mediated activation of the β-casein promoter in response to prolactin, with a 3.5-fold activation relative to the level with STAT5α alone (Fig. 2). Thus, we could confirm our findings in the mammary epithelial cell line HC11. NCoA-1, but not NCoA-2 or -3, is a coactivator for STAT5.

NCoA-1 and STAT5 Interact in Cells and Occupy the Cytokine-induced Sequence (CIS) Promoter after Hormone Stimulation—The stimulating effect of NCoA-1 on STAT5-mediated transactivation prompted us to investigate whether these proteins might interact in cells. For this purpose, 293T cells were transiently transfected with the prolactin receptor along with NCoA-1 and GFP-STAT5α or GFP as a control. The GFP-fusion allowed efficient immunoprecipitation by an anti-GFP antiserum, but did not affect the activation and the transactivation potential of STAT5 (data not shown). After transfection cells were treated with prolactin. Cell lysates were immunoprecipitated with anti-GFP antiserum, and lysates and immunoprecipitates were then analyzed by immunoblotting with anti-NCoA-1 and anti-STAT5α antibody (Fig. 3). The level of NCoA-1 in the lysates of cells transfected with GFP and GFP-STAT5α was the same (lanes 1 and 3). NCoA-1 did not nonspecifically bind to GFP alone (lane 2). However, NCoA-1 was coimmunoprecipitated with GFP-STAT5α (lane 4). Therefore, NCoA-1 and STAT5a form a complex in cells. To directly investigate STAT5 and NCoA-1 binding to a prolactin regulated promoter in vitro, chromatin immunoprecipitation assays were performed. Expression of the endogenous β-casein gene in HC11 cells requires in addition to prolactin stimulation signals from cell-cell contacts as well as
Fig. 1. NCoA-1, but not NCoA-2 and NCoA-3, enhances prolactin-induced transcription by STAT5a as well as STAT5b. A and B. HeLa cells were transfected with the β-casein-luciferase reporter construct (1.25 μg), the plasmids encoding the prolactin receptor (12.5 ng), STAT5a (A) or STAT5b (B) (12.5 ng), SV40-LacZ plasmid (12.5 ng) and various amounts of the plasmids encoding NCoA-1, -2, and -3 or the empty expression plasmid as indicated. Transfected cells were treated with PRL for 16 h (+PRL) or left untreated (−PRL). Cell extracts were prepared, and luciferase activities were determined. The relative luciferase activities were normalized to the β-galactosidase activities. The average of three independent experiments with S.D. is shown. C, the pGL2-ERE -TK-luc reporter plasmid (1.25 μg) and the expression vector for the estrogen receptor (12.5 ng) were transfected into HeLa cells along with 50 or 100 ng of the plasmids encoding NCoA-1, -2, and -3 or the empty expression plasmid as indicated. Cells were treated with 10^-7 M estradiol (E2) or left untreated. The luciferase assay was performed as described above.
of the 9-fold and NCoA-1 antibodies precipitate 5-fold of the amount (AD2), full-length NCoA-1 or H9004/HC11 cells were transfected with the β-casein luciferase reporter gene, prolactin receptor, STAT5a and combinations of NCoA-1 and/or p300 expression vectors. At low levels (50 ng) both coactivators were unable to enhance STAT5a-induced transcription significantly (Fig. 5, compare lanes 1–3). However, coactivation was observed by simultaneous coexpression of NCoA-1 and p300 (Fig. 5, lane 4). In conclusion, the combination of NCoA-1 and p300 had a cooperative effect on STAT5 transactivation.

The Amino-terminal Domain of NCoA-1 Mediates Binding to STAT5—Since we observed that the amino-terminal domain of NCoA-1 (residues 1–381) is required for the stimulation of STAT5 transactivation, we wondered whether this region might contain the binding site for STAT5. Therefore, interaction studies were performed with full length NCoA-1, a NCoA-1 deletion mutant lacking the region from amino acids 1 to 361 (NCoA-1ΔPAS) and a Myc-tagged NCoA-1 fragment comprising amino acids 1–361(Fig. 6A). The constructs encoding these proteins were transfected into 293T cells along with the expression vectors for GFP-STAT5a or GFP and the prolactin receptor. Cells were treated with prolactin and cell lysates were subjected to immunoprecipitation using anti-GFP antiserum. The precipitated proteins were analyzed by Western blot with anti-NCoA-1 or anti-Myc antibody and anti-STAT5 antibody. As observed before, full-length NCoA-1 was precipitated with STAT5 (Fig. 6B, lane 2). However, NCoA-1 lacking the amino-terminal region failed to interact with STAT5 (lane 4). In contrast, the amino-terminal region was coprecipitated with GFP-STAT5a (Fig. 6C, lane 4). Nearly no unspecific binding to GFP alone was observed (lane 2). This experiment clearly shows that STAT5 binds to the amino-terminal region of NCoA-1. A region between amino acids 1 and 361 of NCoA-1 is sufficient for the interaction to STAT5. In former experiments we were unable to detect an interaction between NCoA-1 and STAT5, when we used bacterially expressed GST-NCoA-1 protein containing the amino-terminal part of NCoA-1 and in vitro translated STAT5 protein (33). The lack of detectable interaction between purified proteins in GST pull-down assays indicates that the binding between STAT5 and NCoA-1 might be dependent on the presence of additional proteins or on secondary modifications of STAT5 or NCoA-1.

NCoA-1 Acts on the STAT5a Transactivation Domain—The β-casein promoter fragment, which we used in our studies, contains several binding sites for other transcription factors next to the GAS-element. Previous studies have shown that STAT5, C/EBPβ, and GR synergistically activate β-casein gene expression (38, 53). In addition, GR is coactivated by NCoA-1 (54). Therefore, we wanted to confirm that the observed stimulatory effect of NCoA-1 is the result of coactivation of STAT5, and not due to the stimulation of other factors, which bind to the β-casein promoter. We used the heterologous GAL4-system for these studies. Previous studies have shown that the transactivation domains of STAT5a and STAT5b, when fused to the DNA-binding domain (DBD) of GAL4, can stimulate transactivation of a reporter gene construct containing GAL4 binding
sites in the promoter region (11). GAL4 fusion constructs were transfected into HeLa cells together with NCoA-1, -2, or -3 and NCoA-1 as indicated. Cells were treated with prolactin. Cell lysates were subjected to immunoprecipitation using anti-GFP antiserum. The immunoprecipitates and 1% of the cell lysates were analyzed by SDS-PAGE and Western blot using anti-NCoA-1 (upper panel) and anti-STAT5a (lower panel) antibodies. B, soluble chromatin was prepared from HC11 cells treated with PRL and dexamethasone for 30 min (right panel) or left untreated (left panel). Immunoprecipitation of cross-linked chromatin was performed with antibodies directed against STAT5a and STAT5b, NCoA-1/SRC-1 or control IgG (control). The final extraction was amplified using primers that cover the STAT5 binding sites. Products of the semiquantitative RT-PCR is shown after agarose-gel electrophoresis and ethidium bromide staining. C, position of the primers used for semiquantitative (−243/−1) and real-time (−188/−104) RT-PCR of the STAT5 binding regions and upstream region (−3489/−3660) in the CIS gene are given. Gray boxes represent STAT5 binding sites, the first nucleotide of the GAS element is given.
proteins were analyzed by Western blot with anti-NCoA-1 and anti-STAT5a antibody (Fig. 8B). As expected from our former experiments, high amounts of NCoA-1 were coprecipitated with GFP-STAT5a (lane 4). However, only minimal amounts of NCoA-1 could be coprecipitated in the presence of either STAT5a mutants (lanes 6 and 8), although both mutants were expressed at the same level as the wild-type GFP-STAT5a (compare lane 11 with 13 and 15). Taken together these results show that the α-helical region, in particular the amino acids 751–753 of STAT5, mediate the interaction with NCoA-1.

We next examined whether the deletion or mutation of this region would also affect the transactivation potential of STAT5 and the coactivation by NCoA-1. HeLa cells were transiently transfected with the β-casein-luciferase reporter and expression vectors for STAT5 variants and the prolactin receptor along with NCoA-1 or empty vector (Fig. 8C). Wild-type STAT5 led to a 4.5-fold induction of luciferase expression in response to prolactin (lane 1). This activity was enhanced 12-fold in the presence of NCoA-1 (lane 2). The STAT5 deletion mutant lacking the α-helix displayed a very weak induction of luciferase expression (lane 3), and coexpression of NCoA-1 only slightly increased its activity (3-fold) (lane 4). The STAT5 variant possessing point mutations in the α-helical region was also not very active (lane 5). This transactivation was modestly enhanced in the presence of NCoA-1 (6-fold) (lane 6). These data clearly indicate that both mutants show strongly impaired transactivation activity, which is due to a less efficient recruitment of NCoA-1.

NCoA-1 Enhances the Synergistic Activation of the β-casein Promoter by GR and STAT5—The GR and STAT5 synergistically activate the transcription of the β-casein promoter (38). Since GR is known to bind NCoA-1, we wondered whether NCoA-1 might contribute to the cooperative action of GR and STAT5. To address this question, reporter gene experiments were again carried out. HeLa cells were transfected with the β-casein reporter gene and expression vectors for the prolactin receptor, STAT5a and various combinations of GR and NCoA-1. After transfection cells were treated with prolactin and/or the glucocorticoid dexamethasone. As expected, cotransfection of NCoA-1 led to a 9-fold enhancement of the prolactin-induced STAT5-mediated transactivation (Fig. 9, lanes 1 and 2). If cells were cotransfected with GR, a slight enhancement of prolactin-induced luciferase expression was observed (lane 3). Activation of GR by dexamethasone resulted in a further 12-fold enhancement of the STAT5-mediated activity. This has been previously
FIG. 6. STAT5a binds to the amino-terminal region of NCoA-1. A, full-length NCoA-1, a deletion mutant lacking the PAS domain and a Myc-tagged construct expressing the amino-terminal region of NCoA-1 (amino acids 1–361) is shown. B and C, 293T cells were transfected with expression vectors encoding prolactin receptor, GFP, or GFP-STAT5a alone with the various NCoA-1 constructs. Cells were treated with prolactin and lysed. The lysates were subjected to immunoprecipitation using anti-GFP-antiserum. The immunoprecipitates and 1% of the cell lysates were analyzed by SDS-PAGE and Western blot using anti-NCoA-1 (B) or anti-Myc (C) and anti-STAT5 antibodies. Asterisk marks the heavy chain of the antibodies used for precipitation.

FIG. 7. NCoA-1 acts as a coactivator of the STAT5a transactivation domain. HeLa cells were transfected with the (GAL4-RE)3TK-luciferase (1.25 μg), expression plasmid encoding the GAL4-DBD and GAL4-DBD-STAT5a-TAD fusion protein (25 ng), along with NCoA-1, -2, or -3 or empty expression plasmid (400 ng) and SV40-LacZ (12.5 ng) as indicated. 48 h after transfection, luciferase activities were determined. The average of three independent experiments with S.D. is shown.
described to result from the synergistic action of the GR and STAT5 on the β-casein promoter (37). Simultaneous coexpression of GR and NCoA-1 led to a dramatic increase (60-fold) of the STAT5-mediated transactivation (compare lanes 1 and 4), which was unequivocally greater than the sum of the single effects of GR and NCoA-1 (9- and 12-fold). This result clearly

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erase activities were determined and normalized according to the
p300/CBP are required for prolactin-dependent STAT5-mediated
transactivation of the β-casein expression in HeLa as well as in mammary epithelial cells (Figs. 1 and 2). In addition, NCoA-1 was able to act as a coactivator of the STAT5 transactivation domain in the heterologous GAL4-system (Fig. 7). In contrast, NCoA-2 and NCoA-3 failed to enhance STAT5 activity, both on the β-casein promoter and in the heterologous GAL4-system. The inability of NCoA-2 and NCoA-3 does not result from a different expression level, because all coactivators similarly enhance the transactivation of the ER, when transfected in the same way. However, we cannot totally rule out that NCoA-2 and NCoA-3 are required for STAT5 activity at all. They might be implicated in the expression of other STAT5 target genes in other cell types.

STAT5 and NCoA-1 strongly interact in intact cells as shown by coimmunoprecipitations (Fig. 3). In addition, chromatin immunoprecipitation assays show that both, STAT5 as well as NCoA-1 bind after stimulation in HC11 cells to the promoter of the CIS gene. This indicates that NCoA-1 is involved in the regulation of an endogenous STAT5 target gene. However, no interaction was observed between bacterially expressed purified proteins and in vitro translated protein (33). These results suggest that the interaction might be dependent on secondary modifications. It has already been described that the interaction of p160/SRC/NCoA coactivators with nuclear receptors can be regulated by phosphorylation as well as acetylation (20, 57, 58). We are currently analyzing which secondary modification of NCoA-1 is a prerequisite for STAT5 interaction.

p160/SRC/NCoA coactivators possess different conserved domains responsible for the interaction with other transcription factors, as well as domains required for the recruitment of downstream effectors (24). We localized the interaction domain for binding of STAT5 to a region between residues 1 and 361, which comprises the bHLH and PAS domain. 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 as well as dimerization (59). The region 1–361 has been shown to interact with the transcriptional activators TEF4 and p53 in vitro (44, 60). Since the amino-terminal region of NCoA-1 has to serve as an interaction domain for different activators, secondary modifications of this domain might regulate the interactions of NCoA-1 with its different binding partners.

According to the current model of transcriptional activation, coactivators function as chromatin modifying enzymes and as bridging factors to recruit components of the basal transcription machinery to the respective promoter. NCoA-1 possesses various effector domains. The AD1 domain mediates the interaction with the coactivators p300/CBP and p/CAF. The AD2 domain harbors a weak histone acetyltransferase activity (26) and contacts the methyltransferases CARM1 and PRMT1 (61, 62). In addition, the first 93 amino acids of NCoA-1 were also found to exhibit a weak transcriptional 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 are required for coactivation of STAT5. The results we obtained from these experiments indicate that the amino-terminal part of NCoA-1 and the AD1 domain are essential for coactivation of STAT5 (Fig. 4). The amino terminus of NCoA-1 is responsible for binding to STAT5 (Fig. 6). However, in addition to its function in contacting STAT5, this domain could have a role in recruiting effector proteins via its transactivation function. The AD1 appears to mediate coactivation of STAT5 by interaction with p300/CBP or other AD1-binding proteins like

\[
\text{relative luciferase activity}
\]

FIG. 9. NCoA-1 is able to enhance the cooperative action of GR and STAT5a. HeLa cells were transfected with the β-casein-luciferase reporter construct (1.25 μg), the plasmids encoding the prolactin receptor (12.5 ng), STAT5a (12.5 ng), the SV40-LacZ plasmid (12.5 ng), and the plasmids encoding NCoA-1 (150 ng), GR (6.25 ng) or the empty expression plasmids (–) as indicated. Transfected cells were treated with prolactin and/or dexamethasone for 16 h or left untreated. Luciferase activities were determined and normalized according to the β-galactosidase activities. The average of three independent experiments with S.D. is shown.

shows that NCoA-1 strongly enhances the synergistic transcriptional activation of the β-casein promoter by STAT5 and GR, and suggests that NCoA-1 is involved in the synergy between STAT5 and GR.

DISCUSSION

Previous studies have shown that STAT5 controls the proliferation and differentiation of mammary alveolar epithelium (2, 5, 55). Several STAT5 target genes, which are required for milk production, have been identified, including whey acidic protein, β-casein, and CIS (3, 5, 6, 48).

For activation of its target genes, STAT5 has to recruit transcriptional coactivators. These multifunctional proteins enable the binding of general transcription factors by chromatin remodeling and promote the formation of preinitiation complex by recruitment of general transcription factors. In previous studies, we have shown that the universal coactivators p300/CBP are required for prolactin-dependent STAT5-mediated transactivation of β-casein expression (15). P300/CBP have been shown to associate with other coactivators including the members of the p160/SRC/NCoA family (12, 23, 25), thus providing a platform for a variety of proteins playing a role in gene expression. Different classes of transcription factors require specific components of a p300/CBP coactivator complex as well as their HAT activities (16, 56), suggesting the existence of distinct preformed multiprotein complexes. In this study we analyzed the role of the p300/CBP-associated p160/SRC/NCoA coactivators in STAT5 action.

Our results demonstrate that NCoA-1 is a limiting coactivator of both STAT5a and STAT5b. NCoA-1 stimulates the STAT5-mediated transactivation of β-casein expression in HeLa as well as in mammary epithelial cells (Figs. 1 and 2). In addition, NCoA-1 was able to act as a coactivator of the STAT5 transactivation domain in the heterologous GAL4-system (Fig. 7). In contrast, NCoA-2 and NCoA-3 failed to enhance STAT5 activity, both on the β-casein promoter and in the heterologous GAL4-system. The inability of NCoA-2 and NCoA-3 does not result from a different expression level, because all coactivators similarly enhance the transactivation of the ER, when transfected in the same way. However, we cannot totally rule out that NCoA-2 and NCoA-3 are required for STAT5 activity at all. They might be implicated in the expression of other STAT5 target genes in other cell types.

STAT5 and NCoA-1 strongly interact in intact cells as shown by coimmunoprecipitations (Fig. 3). In addition, chromatin immunoprecipitation assays show that both, STAT5 as well as NCoA-1 bind after stimulation in HC11 cells to the promoter of the CIS gene. This indicates that NCoA-1 is involved in the regulation of an endogenous STAT5 target gene. However, no interaction was observed between bacterially expressed purified proteins and in vitro translated protein (33). These results suggest that the interaction might be dependent on secondary modifications. It has already been described that the interaction of p160/SRC/NCoA coactivators with nuclear receptors can be regulated by phosphorylation as well as acetylation (20, 57, 58). We are currently analyzing which secondary modification of NCoA-1 is a prerequisite for STAT5 interaction.

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p/CAF. The C-terminal transactivation domain is dispensable for the ability of NCoA-1 to enhance STAT5 activity, indicating that the recruitment of methyltransferases and the HAT activity of NCoA-1 are not required for coactivation of STAT5 in transient reporter assays. We found that two coactivators, p300/CAF and NCoA-1 are able to enhance STAT5 activity (Fig. 5). Both coactivators act cooperatively in the stimulation of STAT5 action when expressed together at low amounts. This implies that each of the two coactivators exerts at least one non-redundant function. Analogous to the model set up for the nuclear hormone receptors (63), NCoA-1 might stabilize the STAT5/p300/CAF coactivator complex. Consistent with this model, NCoA-1 binds to STAT5 and to p300/CAF via different regions. The model is also supported by the observation that NCoA-1-dependent stimulation of STAT5 activity requires the AD1 domain (Fig. 4).

The transactivation domain of STAT5a contains a small amphipathic helical region spanning from amino acids 751–762, which has been shown to be critical for the STAT5 transactivation potential and for the proteasome-dependent turnover of the tyrosine phosphorylated STAT5 (9). Residues Phe-751 and Leu-753 have been shown to be essential for the activity of the STAT5a transactivation domain (10). However, it was not yet known which proteins might interact with this region. In the present study we demonstrate that this region of STAT5 mediates the binding of NCoA-1. Deletion of the helical region or mutation of the critical residues 751–753 to alanine completely abolished the binding of NCoA-1 (Fig. 8). In accordance with previous studies we observed that the STAT5 mutants lacking the NCoA-1 binding site or possessing triple alanine mutations displayed a strong decrease in transcriptional activity, and were not coactivated by NCoA-1 as efficiently as wild-type STAT5. The minimal effect that NCoA-1 still exerted on these mutants might result from an indirect recruitment of NCoA-1 via the endogenous p300/CAF. In conclusion, these data suggest that the transcriptional activity of the helical region is based on the interaction with NCoA-1.

The transactivation domains of STAT5a and STAT5b harbor various differences in the amino acid sequence, which implies that these proteins differ in the coactivator recruitment. However, our data show that both proteins recruit p300/CAF and NCoA-1. Interestingly, a sequence analysis in silico revealed that the putative NCoA-1 binding site is also present in the STAT5b sequence (data not shown). The residues 751–753, which have been shown to be critical for binding of NCoA-1, are absolutely conserved. This suggests that the recruitment of NCoA-1 is an essential feature of both STAT5a and b transactivation domains.

Several studies have described both positive and negative cross-talk between STAT5 and several steroid receptors, including TR, ER, PR, AR, and PPARα (64–69). The mechanism of the hormonal cooperation or repression is not fully understood. Steroid receptors have been shown to effect nuclear translocation, phosphorylation and DNA-binding of STAT5. Additionally, steroid receptors might affect the recruitment of coactivators both in a positive and in a negative manner. In this study we analyzed the contribution of NCoA-1 to hormonal synergy of the lactogenic hormones dexmethasone and prolactin, which induce the production of milk proteins in HC11 cells. STAT5 and GR synergistically regulate the expression of the β-casein gene (Fig. 9). A prolonged phosphorylation and a stabilized DNA binding of STAT5 was shown to mediate the synergistic effect of GR. In addition, it has been postulated that GR might support STAT5 in coactivator recruitment, since STAT5 alone possesses a very weak transactivation domain. The data provided here suggest that NCoA-1 is involved in the synergistic action of GR and STAT5. We observed a strong enhancing effect of NCoA-1 on the cooperative action of GR and STAT5, which could be due to a concerted recruitment of NCoA-1. A recent study has shown that a deletion mutant of GR retaining the AF1 function but lacking AF2 is still able to engage in synergism with STAT5 (53). Since NCoA-1 is able to bind to the AF1 domain (54), these data are consistent with the possibility that NCoA-1 contributes to the cooperative action of STAT5 and GR.

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