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# p160/SRC/NCoA coactivators form complexes via specific interaction of their PAS-B domain with the CID/AD1 domain

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## ABSTRACT

**Transcriptional activation involves the ordered recruitment of coactivators via direct interactions between distinct binding domains and recognition motifs. The p160/SRC/NCoA coactivator family comprises three members (NCoA-1, -2 and -3), which are organized in multiprotein coactivator complexes. We had identified the PAS-B domain of NCoA-1 as an LXXLL motif binding domain. Here we show that NCoA family members are able to interact with other full-length NCoA proteins via their PAS-B domain and they specifically interact with the CBP-interaction domain (CID/AD1) of NCoA-1. Peptide competition, binding experiments and mutagenesis of LXXLL motifs point at distinct binding motif specificities of the NCoA PAS-B domains. NMR studies of different NCoA-1-PAS-B/LXXLL peptide complexes revealed similar although not identical binding sites for the CID/AD1 and STAT6 transactivation domain LXXLL motifs. In mechanistic studies, we found that overexpression of the PAS-B domain is able to disturb the binding of NCoA-1 to CBP in cells and that a CID/AD1 peptide competes with STAT6 for NCoA-1 *in vitro*. Moreover, the expression of an endogenous androgen receptor target gene is affected by the overexpression of the NCoA-1 or NCoA-3 PAS-B domains. Our study discloses a new, complementary mechanism for the current model of coactivator recruitment to target gene promoters.**

## INTRODUCTION

The recruitment of transcriptional coactivators to target gene promoters and enhancers by transcription factors is a highly dynamic, sequential and ordered process (1). Recruited coactivators modify histones and other coactivators, recruit secondary coactivators and function as bridging factors to the general transcription machinery. They are associated in multiprotein coactivator complexes. The composition of these complexes, as a consequence of association and dissociation of the involved coactivators, is influenced by conformational changes, e.g. after post-translational modifications. Different classes and families of coactivator proteins contribute to these complex processes and thus affect the regulation of gene expression. The p160/SRC/NCoA coactivator family contains three homologous members. NCoA-1 (Nuclear receptor coactivator 1), also called SRC-1, NCoA-2 also called SRC-2, TIF2 or GRIP1 and NCoA-3, also called p/CIP, RAC3, ACTR, AIB1 or TRAM-1 (2).

They were identified as transcriptional coactivators for nuclear receptors and it has been shown that they are also involved in transcriptional activation of several different transcription factors, like members of the STAT family (3–5) or NFκB (6,7).

The function of NCoA proteins is the recruitment of coactivators with histone acetyltransferase activity (HAT), like p300, CBP and p/CAF and cofactors with histone methyltransferase activity (HMT), like CARM1 and PRMT1. In addition, NCoA-1 and NCoA-3 possess weak intrinsic HAT activities at the C-terminal region (8,9). The ability of the NCoA family members to interact with and to coactivate the same nuclear receptors indicates that they have redundant functions. Nevertheless several publications and the different phenotypes of generated knockout mice suggest that they have also specific functions (10).

The three NCoA family members share common structural domains, which provide surfaces for interactions with DNA-bound transcription factors and with

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components of multiprotein coactivator complexes. The centrally located nuclear receptor interaction domain (NID) mediates the ligand-dependent direct interaction with different nuclear receptors. NCoAs thus coactivate together with associated coactivators hormone-induced transcription (11–13). Diverse groups of coactivators with different enzymatic activities are associated to and recruited by NCoA proteins through the interaction with two transcriptional activation domains, called AD1 and AD2. AD1 is also called CBP-interaction domain (CID), because it mediates the interaction with the general coactivators p300/CBP (9,12,14,15). The second transcriptional activation domain (AD2) is located at the C-terminus and is responsible for the interaction with the histone methyltransferases CARM1 and PRMT1 (16,17).

NCoA proteins contain specific LXXLL motifs (where L is leucine and X is any amino acid), which arrange the interaction with nuclear receptors via the NID (three motifs), as well as with p300/CBP through the CID/AD1 (two motifs). The α-helical LXXLL motif is a signature sequence which was identified in several coactivators (e.g. NCoA proteins, p300/CBP and RIP140) (11,12). It mediates the ligand-dependent recruitment of these proteins to nuclear receptors. Although LXXLL motifs themselves are responsible and sufficient for LXXLL-mediated interactions, also the flanking amino acids are important. They provide the specificity and affinity of these interactions and additional contacts to interaction partners (18–20). Several reports could show that only the flanking amino- or carboxyterminal amino acids are crucial for these interactions (18,21–25). Furthermore many coactivators contain multiple LXXLL motifs, which could allow cooperative interactions (26,27).

The most highly conserved region of NCoA proteins is the aminoterminal bHLH (basic helix-loop-helix) domain and PAS (Per/ARNT/Sim) domain region. In general bHLH domains are known as dimerization and DNA interaction surfaces for many transcription factors, while PAS domains are known to be involved in protein–protein interactions and contribute to the dimerization process mediated by the bHLH domains (28–30). NCoA proteins contain two PAS domain regions (PAS-A and PAS-B). The function of the bHLH/PAS region of the NCoA family members is still largely unknown. In previous studies we found that the PAS-B domain of NCoA-1 binds to an LXXLL motif in the transactivation domain of STAT6. This interaction is essential for STAT6-mediated transactivation (3,4). Thus our studies identified the PAS-B domain as an LXXLL motif recognition domain. The crystal structure of the NCoA-1 PAS-B domain in complex with the STAT6 LXXLL motif revealed several mechanistic differences in the LXXLL-motif-binding mode compared with previous, nuclear-receptor-based complexes (31). Specifically surface complementarity between the hydrophobic faces of the STAT6 LXXLL motif and of the NCoA-1 PAS-B domain almost exclusively defines the binding specificity. Several additional protein interactions of the aminoterminal region including the PAS domain had been described for NCoA proteins. NCoA-2 and NCoA-1 can contact the coactivators CoCoA and GAC63 via the aminoterminal region (32,33). Furthermore, it was postulated that NCoA family members are able to dimerize through their aminoterminal domains. First, gel filtration experiments showed an association of NCoA proteins in multiprotein complexes (34). Later it could be demonstrated that a pair of NCoA proteins is recruited to classical HRE- (hormone responsive element) containing late gene promoters. The bHLH/PAS domains are required for coactivation of these genes (35). Nevertheless a direct interaction of NCoA family members has not been detected in these studies *in vivo* or *in vitro*.

Since the PAS-B domain of NCoA-1 is able to recognize an LXXLL motif and NCoA proteins themselves exhibit several LXXLL motifs in their amino acid sequence, we hypothesized that the association of NCoA proteins can be mediated through an interaction of the PAS-B domain with the own LXXLL motifs. In this study we showed that NCoA proteins are able to interact with each other through the PAS-B domain and the CID/AD1. We demonstrated that the three members of the NCoA family use different binding motifs in the CID/AD1. Whereas NCoA-2 and NCoA-3 show similar binding motif specificities, NCoA-1 binds to different regions in the CID/AD1. Chemical shift perturbation mapping identified similar binding sites for the LXXLL motifs of the STAT6-TAD and the CID/AD1 domain. Furthermore, we were able to show that overexpression of the PAS-B domain or competition with a CID/AD1 peptide interfered with the binding of CBP or STAT6, respectively and affected the transcriptional activation mediated by nuclear receptors. Our data suggest a new mechanism for coactivator complex formation and recruitment of NCoA proteins to promoter sites.

## MATERIALS AND METHODS

### Cell culture and transient transfection methods

293T and HeLa cells were grown in Dulbeccos minimal essential medium (BioWhittacker) containing 10% fetal calf serum. 293T cells were transiently transfected by the standard calcium phosphate precipitation method. HeLa cells were transiently transfected with 'Superfect' transfection reagent (Qiagen) according to the manufacturer's instructions. LnCap cells were cultured in RPMI 1640 medium (BioWhittacker) supplemented with 10% fetal calf serum. Prior to hormone treatment cells were grown in phenol red-free medium supplemented with 10% charcoal-dextran-stripped fetal calf serum for at least 3 days. Cells were stimulated with 17β-estradiol (E2, $10^{-7}$ M) or dihydrotestosterone (DHT, $10^{-7}$ M) for 16 h. As a control cells were treated with vehicle (ethanol) in a corresponding dilution.

### Recombinant plasmids and constructs

The GST fusion constructs containing different coactivator fragments (with amino acid numbers given) were constructed by cloning the PCR amplified fragments in frame in different pGEX-expression vectors: hNCoA-1 (260–370); hNCoA-2 (268–379), and hNCoA-3 (265–375) into the pGEX-5X-1 vector, hNCoA-1 (1–370), and

mCBP (2058–2130) into the pGEX-2-TEV vector (containing a Tobacco-Etch-Virus (TEV) protease cleavage site); hNCoA-1 (257–385) in the modified pET16b vector containing a TEV protease cleavage site (31). The expression vector pCS2 + MT encoding for fusion proteins with 6× Myc-tag and the construct of this plasmids encoding rNCoA-1 (569–805), rNCoA-1 (804–1032) and rNCoA-1 (1258–1442) were provided by L. Klein-Hitpass. All expression vectors encoding for 6× Myc-tag fusions of hNCoA-1 (1–370), hNCoA-1 (260–370), hNCoA-1 (901–970), hNCoA-1 (901–937), hNCoA-1 (938–970), hNCoA-3 (265–375) and hNCoA-3 (1022–1092) as well as the construct encoding for hSTAT6 (792–847) were generated by inserting the corresponding PCR fragment in frame into the pCS2 + MT vector. Mutations were introduced in the hNCoA-1 (901–970) construct by site-directed mutagenesis according to the manufacturer's protocol (Stratagene). Expression vectors for mNCoA-1, hNCoA-1 (pSG-SRC-1e), hNCoA-2 (pSG5-TIF2) and hNCoA-3 (pcDNA3.1-AIB1) were kindly provided by J. Torchia, M. Parker and H. Gronemeyer, respectively and have been described previously (36,37). The expression vector for CFP (pECFP-C1) was purchased from Clontech, BD Bioscience. The expression vector encoding YFP-tagged rNCoA-1 (213–462) was generated by insertion of the corresponding PCR fragment in frame into the pEYFP-C1 vector (Clontech, BD Bioscience). The expression vector containing FLAG-tagged residues mCBP (2058–2130) was generated by introducing the PCR fragment in frame before an internal ribosome entry site (IRES) and GFP into pMX-FLAG-IRES-GFP vector (a gift form T. Kitamura). The estrogen responsive element (ERE) luciferase reporter construct (pGL2-ERE TK-luc) was obtained from L. Klein-Hitpass. The expression vector for human estrogen receptor (pRSV-hER) and the *LacZ* expression plasmid (pCH110) were described previously (38,39). Lentiviral expression vectors containing residues hNCoA-1 (260–370) and hNCoA-3 (265–375) were generated by PCR and insertion of fragments before an IRES and GFP of pVIG (40). The packaging plasmid used for lentivirus production spΔ2 and pHIT-G were described previously (41,42). Insertion of all PCR generated fragments was verified by digestion and DNA sequence analysis.

### Expression, purification and digestion of recombinant proteins

Expression of the GST fusion proteins was performed in *Escherichia coli* BL 21 (DE3) pLysS. The GST fusion proteins were purified with glutathione Sepharose beads (Amersham Biosciences). Cleavage of GST fusion proteins expressed in pGEX-2-TEV vector was performed at room temperature for 18 h with TEV protease. GST-tag and TEV protease were removed by glutathione Sepharose and Ni-NTA Agarose beads (Qiagen).

### GST pulldown experiments

Recombinant cDNAs in the pCS2 + MT, pSG5 and pcDNA3.1 expression plasmids were transcribed and translated *in vitro* by reticulocyte lysate (Promega) in the presence of [$^{35}$S]methionine according to manufacturer's instructions. GST pulldown assays were performed as described previously (3). For competition experiments, radioactive-labeled proteins were pre-incubated with peptides for at least 15 min. 10 μg GST or GST fusion proteins were used in the competition assays in the absence or presence of corresponding peptides as indicated. The amount and integrity of bound GST proteins was estimated after SDS–PAGE in each experiment by Coomassie staining. The binding of the *in vitro* transcribed/translated proteins was detected by fluorography. Quantification of bound radioactive-labeled proteins was performed with a liquid scintillation analyzer (Canberra Packard). Relative radioactivity (cpm, counts per minute) was determined by normalizing against 10% of the *in vitro* transcribed/translated proteins.

### Peptides

Peptides representing the LXXLL motif 1 (KYSQTSHKL VQLLTTTAEQQL), motif 2 (SLTERHKILHRLLQEG SPSDI), motif 3 (KESKDHQLLRYLLDKDEKDLR), motif 4 (EDQCISSQLDELLCPPTTVEG), motif 5 (EGR NDEKALLEQLVSFLSGKD) of human NCoA-1 and the human STAT6 motif (LLPPTEQDLTKLLLEG QGESG) were synthesized by the peptide synthesis facility of the Department of NMR-based Structural Biology at the Max-Planck-Institute for Biophysical Chemistry.

### NMR spectroscopy

NMR spectra were recorded from samples containing 0.5 mM $^{15}$N- or $^{15}$N/$^{13}$C-labelled NCoA-1 PAS-B domain fragment 257–385 that had been crystallized before in complex with the STAT6 (794–814) peptide (31). NMR experiments were carried out at 298 K on DRX Bruker Avance spectrometers equipped with z-gradient cryoprobe and operating at 600 and 800 MHz. All spectra were processed using NMRPipe (43) and analyzed with Sparky (http://www.cgl.ucsf.edu/home/sparky/) and CARA (http://www.nmr.ch/). $^{1}$H-$^{15}$N HSQC experiments were recorded on $^{15}$N-labelled NCoA-1 sample in its free form and in presence of a 2-fold excess of peptide (STAT6 (794–814), motif 4 and motif 5 (Figure 3C)). NCoA-1 backbone resonance ($^{1}H^{N}$, $^{1}H^{\alpha}$, $^{15}$N, $^{13}C^{\alpha}$ and $^{13}$C') assignment was carried out on a $^{15}$N/$^{13}$C-labelled sample of the NCoA-1/STAT6 complex using conventional triple resonance experiments: HNCA, HNCO, HN(CA)CO and $^{1}$H-$^{15}$N HSQC-NOESY. The chemical shift mapping of the NCoA-1 binding site for the STAT6, motif 4 and motif 5 peptides was performed by comparing $^{1}$H-$^{15}$N HSQC spectra of NCoA-1 in its free form and in complex with the different peptides. The amide chemical shift perturbations ($\Delta\delta$) were calculated as $\Delta\delta = |\Delta\delta^{15}N|/5 + |\Delta\delta^{1}H|$(44).

### Co-immunoprecipitation assays and western blotting

293T cells were transiently transfected with the corresponding recombinant cDNA containing expression vectors before co-immunoprecipitation was performed as indicated. For LnCap cells, co-immunoprecipitation was performed on the endogenous protein level.

Co-immunoprecipitation and western blotting was performed as described previously (3). The following antibodies were used for co-immunoprecipitation: GFP antibody (Clontech), SRC-1 antibody (M341, Santa Cruz), SRC-2 antibody (OBT1796, AbD Serotec), NCoA-3 antibody (M397, Santa Cruz), unspecific IgG antibody (Santa Cruz) and anti-FLAG M2 Affinity Gel (Sigma). For western blotting, SRC-1 (1135, Biomol), SRC-2 (OBT1796, AbD Serotec), NCoA-3 (M397, Santa Cruz) or c-Myc antibody (Cell signaling) were used. Visualization was performed with the ECL plus western blotting Detection System (Amersham Biosciences).

### Luciferase assays

Before transfection, HeLa cells were grown for at least 3 days in hormone-free media. Cells were seeded in 12-well plates, and 1 day after, transiently transfected with 1.25 μg luciferase reporter plasmid, the indicated amount of expression vectors and 0.0125 μg of *LacZ* expression plasmid for normalization. The total amount of DNA was adjusted with the corresponding empty vector. Twenty-four hours after transfection, cells were treated with $10^{-7}$ M 17β-estradiol (E2) for 16 h. Cells were harvested and luciferase and β-galactosidase activities were assayed according to the manufacturer's protocol (Promega). Luciferase activities were normalized to the *LacZ* expression. The average of three independent experiments with standard deviation is shown.

### Viral transduction and flow cytometry

For production of lentivirus, 293T cells were transiently transfected with lentiviral vectors together with the packaging plasmids spΔ2 and pHIT-G. Medium was replaced after 16 h and after an additional 24 h, virus supernatants were collected. LnCap cells ($3 \times 10^5$) were transduced in the presence of 4 μg/ml polybrene (Sigma) with a MOI (multiplicity of infection) of 0.2–2. After two rounds of transduction, determination of lentiviral transduction efficiency was performed by flow cytometry on the basis of GFP co-expression on the FACS Calibur and FACS Scan (Beckton Dickinson) with Cell QuestPro software (Beckton Dickinson).

### Preparation of RNA and real time-PCR

Extraction of total cellular RNA was performed using TriFast™ (Peqlab) and Rneasy Mini Kit (Qiagen) according to the manufacturer's instructions. cDNA was obtained by reverse transcription of 4 μg of total cellular RNA using Omniscript reverse transcriptase (Qiagen) according to the manufacturer's protocol. For quantification of specific transcripts, the i-Cycler MyiQ Single Color Real-Time PCR Detection System (Biorad) and the ABsolute QPCR SYBR Green Mix (Abgene) were used. Amplification of samples was performed in triplicates. mRNA levels were normalized against endogenous 18S mRNA. The following primers for the specific amplification of different transcripts were used: 18S RNA: 5′-CGGCTACCACATCCAAGGA-3′; 5′-CCAATTACAGGGCCTCGAAA-3′ (40); PSA: 5′-GGCAGCATTGAACCAGAGGAG-3′; 5′-GCATGAACTTGGTCACCTTCTG-3′ (45); hNCoA-1 PAS-B domain: 5′-TCAGGGCAGAGAACCATCTTAT-3′; 5′-CATGATGAAAGGTTGCATGTCT-3′; hNCoA-3 PAS-B domain: 5′-ATGGTCCCAGAAACGTCACTAT-3′; 5′-AAGGAAGTGGGTTGAGACAAAG-3′

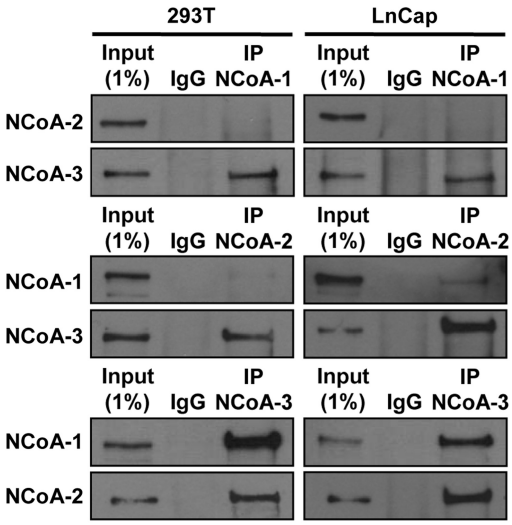


**Figure 1.** NCoA proteins interact in cells. Whole cell extracts of 293T and LnCap cells were prepared and immunoprecipitation (IP) was performed with an anti-NCoA-1-specific, an anti-NCoA-2-specific, an anti-NCoA-3-specific or an unspecific antibody (IgG). All samples were analyzed by SDS–PAGE and western blotting with specific antibodies against NCoA-1, NCoA-2 or NCoA-3, vice versa to immunoprecipitation. As a control 1% of the cell lysates were analyzed in parallel (Input 1%).

## RESULTS

### Interaction of NCoA proteins in cells

Previous studies demonstrated that members of the SRC/NCoA family can form heteromultimeric complexes with each other (34). In addition, heterodimerization of NCoA proteins was proposed as a mechanism for the recruitment of distinct pairs of NCoAs to certain promoters (35). In order to clarify how the interaction of these coactivators takes place, complex formation of NCoA family members was first analyzed by co-immunoprecipitation experiments with whole cell lysates of 293T and LnCap cells. In both cell lines NCoA-3 could be co-immunoprecipitated with NCoA-1 and NCoA-2 (Figure 1, upper and middle lanes) and vice versa NCoA-1 and NCoA-2 co-precipitated with NCoA-3 (lower lanes). In contrast NCoA-1 showed no or only a very weak co-immunoprecipitation with NCoA-2 (upper and middle lanes). The control antibodies (IgG) showed no precipitation at all. These experiments clearly demonstrated an interaction of the endogenous NCoA coactivators in two different cell lines but no interaction of NCoA-1 and NCoA-2.

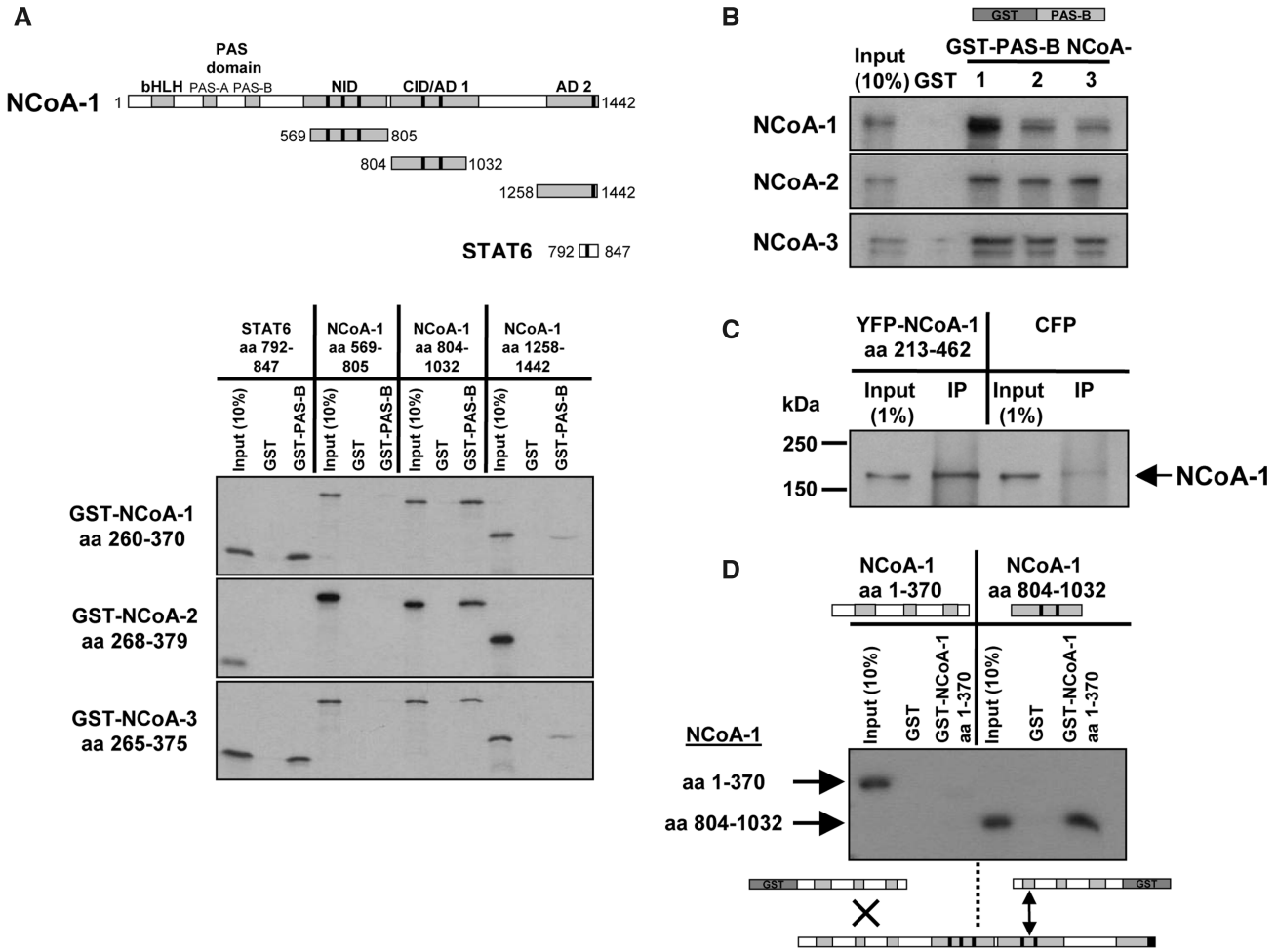


**Figure 2.** Interaction of NCoA proteins through the PAS-B domains and CID/AD1. (**A**) Schematic representation of NCoA-1, different NCoA-1 constructs and the STAT6-TAD fragment. bHLH domain, PAS domain, NID [three LXXLL motifs (black bars)], CID/AD1 (two LXXLL motifs) and AD2 (one LXXLL motif) are indicated. The STAT6 peptide comprises the NCoA-1 interacting LXXLL motif (black bar). The terminal amino acids of each construct are shown. The NCoA-1 fragments representing different regions containing LXXLL motifs were *in vitro* transcribed/translated and [$^{35}$S]methionine-labeled. A STAT6 fragment which contains the NCoA-1 interacting LXXLL motif was used as a control. After incubation with GST or GST fusion proteins of NCoA-1, NCoA-2 or NCoA-3 PAS-B domains, bound to glutathione Sepharose beads, proteins were eluted and analyzed by SDS–PAGE and fluorography. As an input control 10% of the radioactive-labeled proteins were analyzed in parallel. (**B**) Experiments were performed with the radioactive-labeled full-length NCoA-1, NCoA-2 and NCoA-3 as described before. (**C**) 293T cells were transiently transfected with expression vectors encoding either a YFP fusion protein of NCoA-1 aa 213–462, containing the PAS-B domain, or CFP and full-length NCoA-1 (each 3 μg). Cell extracts were prepared and immunoprecipitation (IP) was performed with an anti-GFP antibody, recognizing CFP and YFP. Proteins were analyzed by SDS–PAGE and western blotting with an anti-NCoA-1 antibody. As a control 1% of the cell lysates were analyzed in parallel (Input 1%). (**D**) The GST pulldown assay was performed with the GST fusion protein comprising the aminoterminal region of NCoA-1 (aa 1–370) and the [$^{35}$S]methionine-labeled fragments of NCoA-1 containing the aminoterminal region or the CID/AD1 (aa 804–1032).

### NCoA proteins interact via a direct binding of their PAS-B domains to the CID/AD1 domain

Our own studies on the coactivator function of NCoA-1 in transcriptional regulation by STAT6 demonstrated that the PAS-B domain of NCoA-1 recognizes an LXXLL motif in the STAT6 transactivation domain (3,4,31). To investigate if the PAS-B domains of NCoA family members are able to interact with their own LXXLL motifs, we used different fragments of NCoA-1-spanning regions with LXXLL motifs and as a control a fragment of STAT6 containing the NCoA-1 interacting LXXLL motif (Figure 2A) and analyzed the binding in GST pulldown experiments. All GST fusion proteins of the PAS-B domains of NCoA-1, NCoA-2 and NCoA-3 revealed a specific interaction with the construct containing two LXXLL motifs in the CID/AD1 *in vitro* (Figure 2A). The other domains of NCoA-1 which also contain LXXLL motifs showed almost no interaction with PAS-B domain fusion proteins (NID) or only a weak interaction with the transcriptional activation domain 2 (AD2) in the case of NCoA-1 and NCoA-3. No binding of radioactive-labeled constructs to GST alone was detected. In addition to the already described interaction of the STAT6 construct with the PAS-B domain of NCoA-1, also an interaction with the PAS-B domain of NCoA-3 could be observed. These results indicate that the NCoA proteins are able to interact *in vitro* via their PAS-B domain specifically with the CID/AD1, which contains

two LXXLL motifs. In contrast to former results, the PAS-B domain of NCoA-3 is also able to interact with the STAT6 LXXLL motif.

We next tested whether the PAS-B domains of the NCoA proteins are able to interact with the full-length proteins of NCoA family members. All [$^{35}$S]-labeled full-length NCoA proteins were bound by the PAS-B domains of the different NCoA family members *in vitro* (Figure 2B). The NCoA-1 PAS-B domain showed a stronger interaction with full-length NCoA-1 compared to the interaction of the other NCoA proteins. Thus the interaction of the PAS-B domain that we observed before is not restricted to NCoA-1 and functions also for binding of all three full-length coactivators, although obviously with different affinities.

To investigate if the PAS-B domain of NCoA-1 is able to bind full-length NCoA-1 also *in vivo*, co-immunoprecipitation experiments were carried out. 293T cells were transiently co-transfected with an expression construct encoding a YFP fusion protein of NCoA-1 comprising amino acids 213–462 and a full-length NCoA-1. Immunoprecipitation with an anti-GFP antibody co-precipitated full-length NCoA-1 only when the YFP fusion protein containing the PAS-B domain of NCoA-1 was co-transfected, thus supporting our *in vitro* interaction studies, showing that the isolated PAS-B domain is sufficient to bind full-length NCoA-1 (Figure 2C, left panel). No precipitation was observed in the presence of the CFP control.

Since the bHLH/PAS region in the aminoterminus of NCoA family members was suggested to be a dimerization domain which should mediate multimerization of NCoA proteins (29,46) we next compared the interaction potential of the aminoterminus and the CID/AD1. Whereas the CID/AD1 (aa 804–1032) strongly bound to the GST fusion protein of NCoA-1 comprising amino acids 1–370 (Figure 2D, right panel) we could not detect an interaction of the aminoterminal region with itself (Figure 2D, left panel). Thus these results support our hypothesis that complex formation of NCoA family members is attributed to an interaction between the PAS-B domain and the CID/AD1, but not to a dimerization of NCoA proteins through the aminoterminal bHLH/PAS domain region.

### Distinct functions of the LXXLL motifs in the interaction of the NCoA PAS-B with the CID/AD1 domain

The fragment which we tested for interaction with the PAS-B domain of NCoA proteins contains two LXXLL motifs and the complete region which mediates the interaction with CBP (amino acids 926–960; Figure 3A) (47). We wondered whether NCoA proteins and CBP bind to the same motifs, overlapping motifs or different motifs in the CID/AD1. To ascertain this, we first narrowed down the CID/AD1 fragment to a region which contains the known binding motifs and then analyzed distinct parts of the restricted region. A minimal fragment comprising amino acids 901–970, which contains both LXXLL motifs and the minimal CBP-interaction motif was tested for interaction with the PAS-B domains of all three NCoA family members and a fusion protein containing the NCoA-1 interaction surface of CBP, which contains the CBP amino acids 2058–2130. All three NCoA PAS-B domain constructs and the CBP fusion protein strongly interacted with the restricted CID/AD1 spanning amino acids 901–970 (Figure 3B).

Two fragments comprising the amino acids 901–937 and 938–970 of the CID/AD1 (Figure 3A) were used to further analyze the binding specificity of the different NCoA family members and CBP. These fragments contain either the aminoterminal region with both LXXLL motifs or the carboxyterminal part without an LXXLL motif. The PAS-B domain of NCoA-1 interacts with the aminoterminal region at a level that was comparable to the interaction with the intact fragment (Figure 3B). In contrast, the PAS-B domains of NCoA-2 and NCoA-3 showed a strongly reduced interaction. The carboxyterminal fragment (aa 938–970) interacted only with the NCoA-1 PAS-B domain. However this interaction was much weaker compared to the interaction of the aminoterminal part (aa 901–937) or the complete region (aa 901–970) (Figure 3B). The CBP-GST fusion interacted with none of the shortened fragments.

To further examine the difference between NCoA-1 and NCoA-3 we repeated the experiment with a fragment representing the minimal CID/AD1 region of NCoA-3 (aa 1022–1092) (Figure 3A). Only the NCoA-1 PAS-B domain and the CBP construct strongly bound to the CID/AD1 fragment of NCoA-3 (Figure 3B). The GST PAS-B domain fusions of NCoA-2 and NCoA-3 showed no interaction at all. Taken together these results indicate that the PAS-B domains of the NCoA family members and CBP recognize distinct binding sites in the CID/AD1.

To examine the contribution of individual LXXLL motifs to the interaction we tested whether a single LXXLL motif-containing peptide is able to compete with the interaction between the PAS-B domains of NCoA-1, NCoA-2 and NCoA-3 and the CID/AD1 of NCoA-1. Peptides were designed to cover each LXXLL motif with the neighboring eight amino acids at both sites in the CID/AD1 of NCoA-1 (motif 4 and motif 5, Figure 3C). As a control we used a peptide that contains the LXXLL motif of STAT6, which was shown to abolish the interaction between NCoA-1 and the STAT6 transactivation domain (4). The peptide representing the LXXLL motif 4 and motif 5 blocked almost completely the interaction of the NCoA-1 PAS-B domain with the CID/AD1 at the highest concentration (280 μM, Figure 3C, lane 4 and 7). But also lower concentrations showed inhibitory effects (lanes 5 and 8). The peptide representing the STAT6 motif blocked the interaction only at the highest concentration (lane 10). In contrast to the interaction of the NCoA-1 PAS-B domain, the interaction of the NCoA-2 and NCoA-3 PAS-B domain was only slightly affected in competition experiments at the highest concentration with LXXLL motif 5 (lanes 19 and 31). Also the STAT6 motif did not inhibit these interactions efficiently (lanes 22–24 and 34–36). To test whether the observed competition effects are specific for the LXXLL motifs of the CID/AD1, we performed similar experiments with peptides representing the three LXXLL motifs located in the NID domain of

NCoA-1 (Supplementary Data 1). These peptides representing motifs 1, 2 and 3 showed no inhibitory effect on the binding of the CID/AD1 to the PAS-B domains of NCoA-1 or NCoA-2 and NCoA-3 at any given concentration. In summary, our results indicate that the PAS-B domains of the NCoA coactivators recognize specific motifs in the CID/AD1, which comprise both LXXLL motifs, but obviously also different binding structures. The amino acid context of the LXXLL motifs seems to define the binding specificity. In addition, different interaction modes might be used by the PAS-B domains of the different NCoA family members.

To examine directly the importance of each LXXLL motif for the NCoA PAS-B-CID/AD1 interaction, we mutated the sequence of the LXXLL motifs. We replaced leucine 913 and 916 of motif 4 (MutM4), leucine 933 and 936 of motif 5 (MutM5) or all four leucine residues together (MutM4 + 5) with alanine in the restricted region of the NCoA-1 CID/AD1 (aa 901–970) (Figure 3D). The interaction of the NCoA-1 PAS-B domain with the CID/AD1 was not affected through the point mutations in LXXLL motif 4 and only slightly affected through the mutation of motif 5 or both motifs (Figure 3E and F). In contrast the interactions of the PAS-B domains of NCoA-2 and NCoA-3 were clearly reduced through mutation of motif 4 and strongly inhibited through mutation of motif 5. Mutation of both motifs nearly abolished the interaction. The binding of the CBP fusion protein to the CID/AD1 fragment was nearly not affected by point mutations in LXXLL motif 4, whereas mutations in motif 5 or both motifs strongly diminish the contact. This was expected since only motif 5 is part of the CBP binding region (Figure 3A). These results underline that the LXXLL motifs obviously act differentially in the coactivator interactions. Both LXXLL motifs contribute to the interaction of the PAS-B domains of NCoA-2 and NCoA-3, although motif 5 seems to have the more important function. The binding of CBP requires only motif 5 and the leucines in both motifs are obviously not essential for the interaction of the NCoA-1 PAS-B domain.

To further probe the means through which the NCoA-1 PAS-B domain recognizes the different LXXLL motifs, we performed $^{1}H$-$^{15}N$ HSQC monitored chemical shift mapping upon STAT6, motif 4 and motif 5 peptide binding. We recorded and compared $^{1}H$-$^{15}N$ HSQC spectra of the $^{15}N$-labeled PAS-B domain protein in presence of saturating concentrations of the motif 4 and 5 peptides (Figure 3G) as well as the STAT6 peptide. We were able to assign 88% of the 117 observable resonances on the $^{1}H$-$^{15}N$ HSQC spectrum (out of 124 expected). The same level of assignment was obtained for the $^{1}H$-$^{15}N$ HSQC spectra of the different NCoA-1/peptide complexes. All three complexes exhibit strong chemical shift perturbations for the same parts of the protein, namely the $\beta B$ strand and $\alpha C$, $\alpha D$ helices (Figure 3G and Supplementary Data 3). These secondary structure elements form part of the hydrophobic cleft in which the STAT6 peptide binds in the NCoA-1/STAT6 complex X-ray structure (31). Other significant chemical shift perturbations were observed on other parts of the protein, especially upon STAT6 peptide binding. These shifts are probably generated by long-range perturbations. Upon binding of each of the three peptides, HN resonances of the same set of NCoA-1 residues are affected with a similar $\Delta\delta$ pattern. This observation reveals that all three peptides bind to the same site, namely the hydrophobic cleft as observed in the NCoA-1/STAT6 X-ray structure. Nevertheless a closer inspection of the $\Delta\delta$ values shows also differences between the $\Delta\delta$ patterns of the three peptides (Supplementary Data 2). The motif 4 and motif 5 peptides generate very similar chemical shift perturbation patterns upon binding whereas the chemical shift perturbations generated upon STAT6 binding show clear differences.

Using isothermal titration calorimetry (ITC) the $K_d$ of the STAT6 peptide for binding to the NCoA-1 PAS-B domain has been determined before to be about 1 μM (31). Trials to measure the $K_d$ values of the motif 4 and motif 5 peptides for binding to the NCoA-1 PAS-B domain with ITC and fluorescence titration failed. With both methods interpretable saturation curves could not be obtained. Measurement of the affinities of the three peptides using chemical shift perturbations of affected residues in the NCoA-1 PAS-B domain were also not feasible due to an exchange rate in the fast to intermediate timescale (data not shown). Thus a direct comparison of the affinities of the three motifs to the NCoA-1 PAS-B domain was not possible. However, we expect from these approaches, that motifs 4 and 5 have an at least 10–50-fold lower affinity compared to the STAT6 motif. We assume that both LXXLL motifs and amino acids next to the motifs of the CID/AD1 are particularly important for higher affinity binding. This would be in agreement with our results from the qualitative analysis of the binding (Figure 3).

### Competition between the interaction domains of NCoA-1, CBP and STAT6

To analyze the influence of the identified interactions on the formation and composition of coactivator complexes, we first examined if the interaction of the PAS-B domain and the CID/AD1 is able to affect the interaction of NCoA-1 with the coactivator CBP. This could be very important for the recruitment of coactivator complexes and transcriptional activation mediated by transcription factors like the nuclear receptors. For this purpose 293T cells were transiently transfected with an expression construct encoding a FLAG-tagged CBP fragment (aa 2058–2130) comprising the region which is necessary for the interaction with NCoA-1 (47) together with an expression vector of the Myc-tagged CID/AD1 fragment of NCoA-1 (aa 804–1032). To test for competition, the cells were additionally transfected with a 30-fold excess of a construct encoding the PAS-B domain of NCoA-1 (aa 213–462). Co-immunoprecipitation experiments were performed with a specific antibody recognizing the FLAG-tag and analyzed by SDS–PAGE and western blotting with an anti-Myc-tag specific antibody recognizing the fragment of the NCoA-1 CID/AD1. The fragment of the CID/AD1 of NCoA-1 (aa 804–1032) could be co-immunoprecipitated with the FLAG-tagged CBP

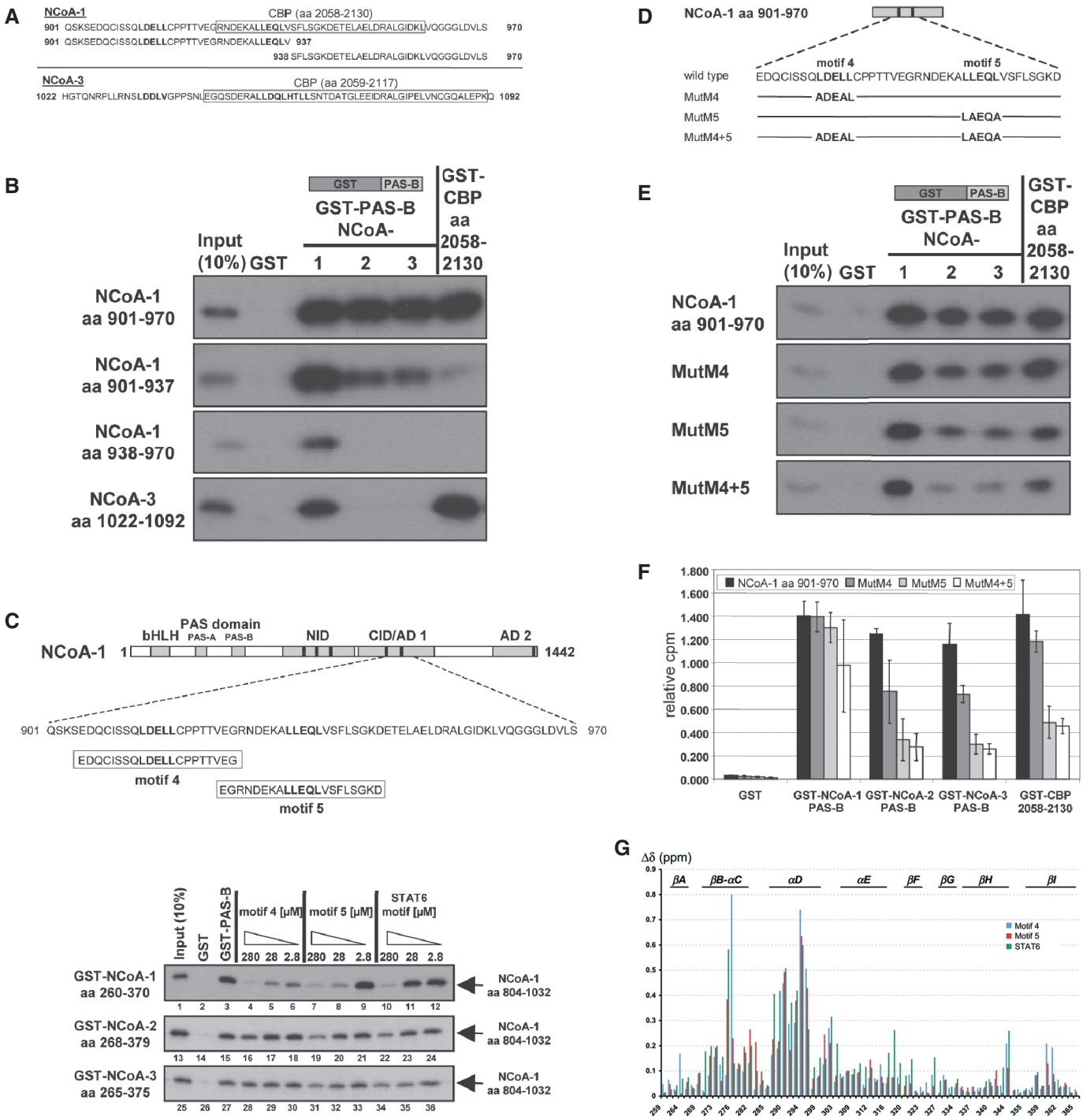


**Figure 3.** LXXLL motifs differentially contribute to the interaction of the PAS-B domains and the CID/AD1 of NCoA-1. (**A**) Sequence alignment of the fragments in the CID/AD1 of NCoA-1 and NCoA-3. LXXLL motifs are printed in bold. The regions of NCoA-1 and NCoA-3 which mediate the interaction with CBP are indicated in boxes (NCoA-1 aa 926–960 and NCoA-3 aa 1045–1091). Terminal amino acids of each construct are shown. Sequences of NCoA-1 and NCoA-3 correspond to the protein ID's AAI11534 and AAC51677, respectively. (**B**) Fragments of NCoA-1 comprising amino acids 901–970, 901–937 or 938–970 and NCoA-3 comprising amino acids 1022–1092 of the CID/AD1 were *in vitro* transcribed/translated and [$^{35}$S]methionine-labeled. The fragments were incubated with GST alone or GST fusion proteins of the PAS-B domains of all three NCoA family members or a fusion protein of the CBP NCoA interacting region (aa 2058–2130) bound to glutathione Sepharose. Precipitated proteins were analyzed by SDS–PAGE and fluorography. 10% of radioactive-labeled material used for interaction assays was analyzed in parallel (Input). (**C**) Structure of NCoA-1. Different functional domains (grey boxes) and LXXLL motifs (black bars) are indicated. Peptides derived from the CID/AD1 used in competition experiments are shown. The sequences representing LXXLL motif 4 and motif 5 are printed in bold. GST pulldown assays were performed as described in B, with the NCoA PAS-B domains and the [$^{35}$S]methionine-labeled fragment of the NCoA-1 CID/AD1 in absence or presence of 280, 28 or 2.8 μM of each peptide. (**D**) Schematic representation of the NCoA-1 CID/AD1 sequence. LXXLL motifs 4 and 5 are printed in bold. Point mutations of leucines to alanines are depicted for motif 4 (MutM4), motif 5 (MutM5) and both motifs (MutM4 + 5). (**E**) Experiments were performed as described in B with the radioactive-labeled fragments containing residues 901–970 of wild type or

fragment containing the NCoA-1 interacting region (Figure 4A, lane 4). There was no immunoprecipiation with the FLAG-tag control (lane 2). Co-transfection of the YFP fusion protein of the NCoA-1 PAS-B domain led to a strong and almost complete inhibition of the interaction between the CID/AD1 and the CBP fragment (lane 6), whereas overexpression of CFP showed no effect (lane 4). This experiment demonstrated that overexpression of the NCoA-1 PAS-B domain is able to disturb the interaction of the NCoA-1 CID/AD1 with CBP.

Since NCoA-1 is recruited by STAT6 via a direct interaction of its PAS-B domain with the LXXLL motif in the STAT6-TAD we wondered whether the binding of the CID/AD1 is able to inhibit this interaction. *In vitro* competition assays with bacterially expressed NCoA-1 CID/AD1 peptides (aa 901–970) and GST fusion proteins of the NCoA-1 PAS-B domain and radioactive-labeled STAT6 fragment (aa 792–846) were performed. The interaction of the NCoA-1 PAS-B domain with the STAT6-TAD fragment was diminished in the presence of the CID/AD1 peptide (Figure 4B). The quantitative analysis revealed a more than 50% reduction of the interaction (Figure 4C). This result indicates that the interaction of the PAS-B domain and the CID/AD1 could not only affect the binding of the coactivator CBP, but is also able to influence the interaction and recruitment of NCoA-1 by transcription factors like STAT6.

### Different effects of the PAS-B domains on the transcriptional activation by nuclear receptors

Since overexpression of the PAS-B domain affects the interaction of NCoA-1 with CBP as shown before, we investigated the role of the PAS-B domain-CID/AD1 interaction on the formation of coactivator complexes in transcriptional activation. Particularly the postulated recruitment of NCoA protein pairs to certain target gene promoters of nuclear receptors could be concerned (35). We first analyzed whether the overexpression of the isolated PAS-B domain and the CID/AD1 might influence transcriptional activation by the estrogen receptor in luciferase reporter assays. Co-expression of increasing amounts of the NCoA-1 PAS-B domain (aa 260–370) had no effect on the transcriptional activation mediated by the estrogen receptor in comparison to the control (Figure 5A). Only a slight decrease in transactivation could be observed with the fragment of the PAS-B domain of NCoA-3 (aa 265–375). The CID/AD1 fragment (aa 804–1032) strongly inhibited the estrogen-dependent transactivation, even at the lowest amount (50 ng). These results suggest that interactions via the CID/AD1 are more essential for the recruitment of coactivator complexes than the PAS-B domain in luciferase reporter assays with the used promoter construct.

The comparably low effects of the PAS-B domains that we observed in luciferase reporter assays could be a result of the differential recruitment of single or pairs of NCoA proteins to certain promoters. Previous studies postulated that pairs of NCoA-1 and NCoA-3 or NCoA-2 and NCoA-3 are recruited to the promoter of the PSA (prostate specific antigen) gene, which is a natural target gene of the androgen receptor (AR) in LnCap prostate carcinoma cells (35). We therefore examined next whether overexpression of the PAS-B domains influences the expression of PSA. For this we stably expressed the PAS-B domains of NCoA-1 (aa 260–370) or NCoA-3 (aa 265–375), corresponding to the fragments used in the reporter assays, by lentiviral transduction in the LnCap cells. As a control, cells were transduced with the empty vector (pVIG-Flag). The transduction efficiency was confirmed by FACS analysis on the basis of GFP co-expression, and overexpression of the PAS-B domain fragments was measured by quantitative RT-PCR. Expression of PSA was measured by quantitative RT-PCR in transduced LnCap cells grown in the absence of androgen for at least 3 days, followed by treatment with DHT for 16 h (Figure 5B). Overexpression of the PAS-B domains of NCoA-1 and NCoA-3 had contrary consequences on the activation of the PSA gene. Whereas the overexpression of the PAS-B domain of NCoA-1 (pVIG-Flag-NCoA-1 aa 260–370) enhanced expression of PSA, overexpression of the NCoA-3 PAS-B domain (pVIG-Flag-NCoA-3 aa 265–375) reduced PSA expression. The effects on the expression of PSA were confirmed with independently transduced LnCap cells (data not shown). Our results indicate that interactions via the PAS-B domains of both NCoA family members NCoA-1 and NCoA-3 are involved in the transcriptional regulation of an endogenous AR target gene that had been postulated to be dependent on the recruitment of two NCoA proteins.

## DISCUSSION

The NCoA protein family of coactivators mediates many different interactions with transcription factors and other coactivators essential for transcriptional regulation. Previous studies postulated that NCoA proteins are associated in high molecular weight complexes (34) and that dimerization of NCoA proteins is involved in their recruitment to specific nuclear receptor target gene promoters (35).

We demonstrated here that the PAS-B domains of all NCoA family members alone are sufficient to interact with all full-length NCoA proteins (Figure 2B). These results clearly indicate that the PAS-B domain is not only able to mediate the interaction with STAT6 as we had shown before (4), but also allows the assembly with members of

mutant (MutM4, MutM5 or MutM4 + 5) NCoA-1. (**F**) Quantification of bound radioactive-labeled fragments of wild type or mutant NCoA-1 CID/AD1 (aa 901–970) to GST and GST fusion proteins. Relative radioactivity (cpm; counts per minute) was determined by normalization against an aliquot representing 10% of the radioactive-labeled material. The average of three independent experiments with standard deviation is shown. (**G**) Chemical shift perturbation plot on NCoA-1 sequence upon peptides binding. $^{1}H$-$^{15}N$ HSQC titration data for the addition of LXXLL peptides to the $^{15}N$ -NCoA-1 PAS-B domain $\Delta\delta$ values (calculated as described in Materials and Methods) generated upon motif 4 (blue bars), motif 5 (red bars) and STAT6 peptide (green bars) binding are plotted against the NCoA-1 PAS-B domain sequence.

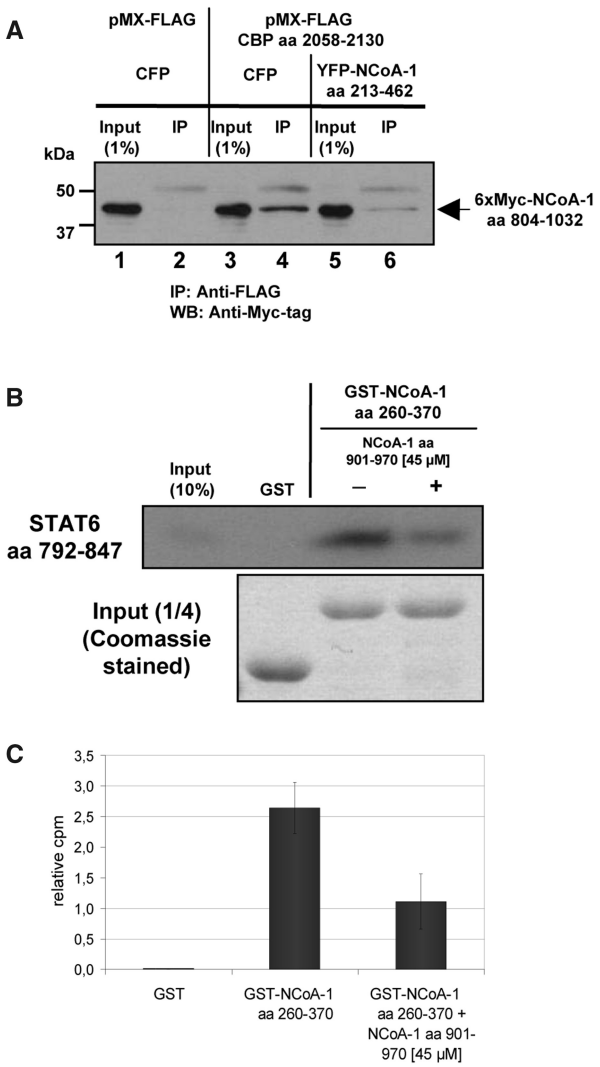


**Figure 4.** Overexpression of the NCoA PAS-B domain or competition with the CID/AD1 inhibits contact of NCoA-1 to CBP or the STAT6-TAD. (**A**) 293T cells were transiently transfected with expression vectors encoding a FLAG-tagged CBP fragment (aa 2058–2130) or the empty FLAG-tag vector (pMX-FLAG) (each 1 µg), a 6× Myc-tagged CID/AD1 fragment of NCoA-1 (aa 804–1032) (0,1 µg) along with a vector containing a YFP fusion protein encoding NCoA-1 aa 213–462 or CFP as a control (each 3 µg). Whole cell extracts were prepared and co-immunoprecipitation was performed with a specific anti-FLAG-tag antibody. Precipitated proteins were analyzed by SDS–PAGE, followed by western blotting with an anti-Myc-tag antibody. Aliquots corresponding to 1% of the used cell lysates were analyzed in parallel (Input 1%). (**B**) GST or the GST fusion protein of NCoA-1 PAS-B domain (aa 260–370) were bound to glutathione Sepharose beads and incubated with a [$^{35}$S]methionine-labeled fragment of the STAT6-TAD (aa 792–847) in absence or presence of 45 µM of a purified NCoA-1 CID/AD1 peptide (aa 901–970). After washing and elution, precipitated proteins were analyzed by SDS–PAGE and fluorography. The CID/AD1 fragment was bacterially expressed, digested and purified as described in Materials and Methods. An aliquot representing 10% of the *in vitro* transcribed/translated fragment was analyzed in parallel (Input). A quarter of each binding reaction was separately analyzed by SDS–PAGE and Coomassie staining in order to prove same amounts of GST or GST fusion proteins. (**C**) Quantification of bound STAT6-TAD fragment to GST or GST fusion protein of the NCoA-1 PAS-B domain in absence or presence of the CID/AD1 peptide. The relative radioactivity (cpm; counts per minute) was determined by normalization against 10% of the *in vitro* transcribed/translated protein. The average of three independent experiments with standard deviation is shown.

its own coactivator family. Interaction via the PAS-B domain might therefore enable the homo- and heterodimerization of different pairs of NCoA proteins at certain promoters, as it has been suggested for the co-recruitment of NCoA-3 and -1 or NCoA-3 and -2 to estrogen receptor and androgen receptor regulated target gene promoters (35). In contrast to NCoA-3, NCoA-2 was not able to interact with NCoA-1, although it could interact with NCoA-3 (Figure 1). This result adds an additional level of complexity to the interaction of NCoA proteins which might be regulated by post-translational modification of the full-length proteins in cells.

The PAS-B domains of all NCoA proteins bind to the NCoA-1 CID/AD1 region which contains two LXXLL motifs, but not to the NID with three LXXLL motifs or the transcriptional activation domain 2 (AD2) with one LXXLL motif. This indicates that there is a functional redundancy of the NCoA coactivators, because all three NCoA proteins are able to interact with each other through the same domains. We were not able to show an intermolecular interaction mediated through the amino-terminal bHLH/PAS domains as postulated in previous studies (29) (Figure 2D). Therefore our results suggest that the described complex formation and association of NCoA family members (34,35) is mediated by the interaction of the PAS-B domain and the CID/AD1 and not by the dimerization through the bHLH and PAS domains. This is in contrast to other members of the bHLH/PAS family of proteins, like AhR and ARNT (30,48,49), which show dimerization through the bHLH/PAS domains. However there are already examples of bHLH/PAS protein interactions described, like the interaction of NCoA-1 with AhR, ARNT or HIF1α (50–52), in which the binding is not mediated through the association of both bHLH/PAS domain regions.

Although all NCoA proteins are able to bind via their PAS-B domain to the CID/AD1 of NCoA-1, only the NCoA-1 PAS-B domain can interact with the CID/AD1 of NCoA-3 (Figure 3B). This indicates that the PAS-B domains of NCoA-2 and NCoA-3 might have different binding specificities compared to the PAS-B domain of NCoA-1, which obviously uses different binding sites in the CID/AD1. Binding assays with the amino- and carboxyterminal fragments of the CID/AD1 (Figure 3B) and competition assays with peptides representing the single LXXLL motifs (Figure 3C) demonstrate that both LXXLL motifs are important for the interaction, but with different impact. In addition, the carboxyterminal fragment of the CID/AD1 (aa 938–970), which contains no LXXLL motifs, obviously binds independently and specifically to the PAS-B domain of NCoA-1. This indicates that the NCoA-1 PAS-B domain apparently has several contact sites within the CID/AD1 region.

Our results also demonstrate that an efficient interaction of the NCoA-2 and NCoA-3 PAS-B domains requires an intact CID/AD1 region comprising both the aminoterminal region with the LXXLL motifs and the carboxyterminal region. Splitting of this region strongly reduced the binding to the aminoterminal region and rather abolished the binding to the carboxyterminal fragment. This is in contrast to the NCoA-1 PAS-B domain (Figure 3B).

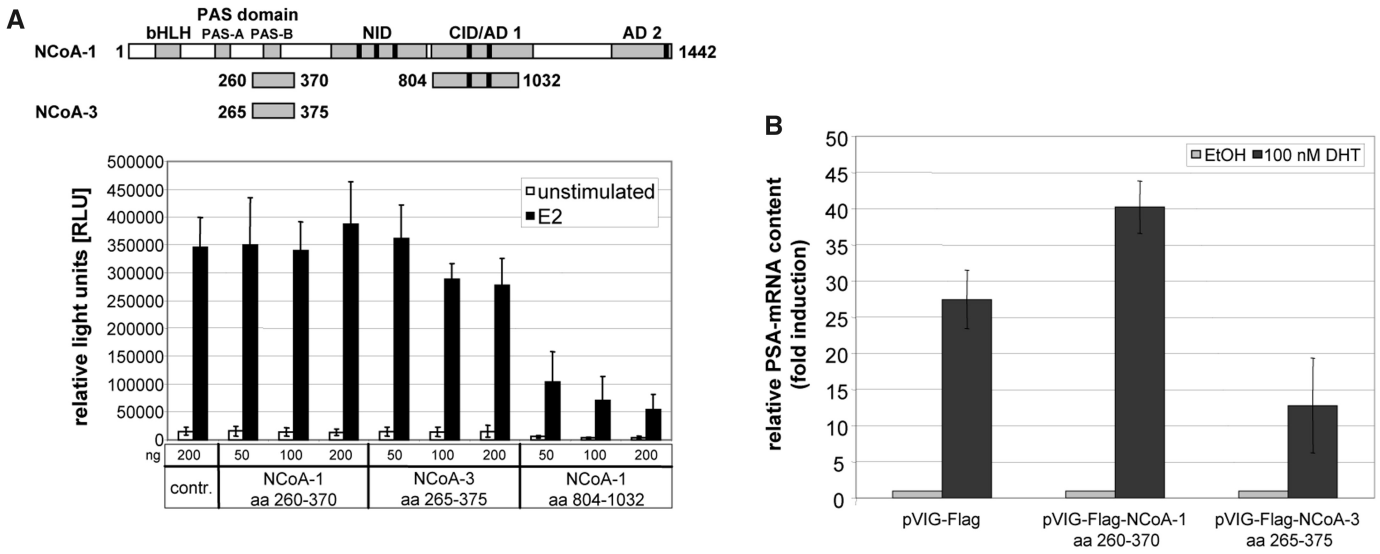


**Figure 5.** Overexpression of the NCoA-1 and NCoA-3 PAS-B domains or a CID/AD1 fragment differentially affects transcriptional activation by nuclear receptors. (**A**) Schematic structure of NCoA-1 and different NCoA protein constructs. Functional and structural domains of NCoA-1 are depicted. The bHLH domain, PAS domain, NID, CID/AD1, AD2 and corresponding LXXLL motifs (black bars) are indicated. Terminal amino acids of each construct are shown. HeLa cells were transfected with expression vectors for the luciferase reporter plasmid pGL2-ERE TK-luc (1.25 μg), the estrogen receptor (12.5 ng) and the *LacZ* expression plasmid (12.5 ng) along with expression plasmids for the PAS-B domains of NCoA-1 (aa 260–370) or NCoA-3 (aa 265–375) or the NCoA-1 CID/AD1 (aa 804–1032) (each 50, 100 or 200 ng) or the empty expression vector (200 ng; contr.) as indicated. Total amount of DNA was adjusted with the empty expression vector. Transfected cells were grown for at least 3 days without 17β-estradiol (E2) and then treated (black bars) or not treated (unstimulated; white bars) with E2 ($10^{-7}$ M) for 16 h. Cell extracts were prepared and luciferase and β-galactosidase activities were determined. Luciferase activities were normalized to the *LacZ* expression. The average of three independent experiments ± standard deviation is shown. (**B**) LnCap cells were transduced with lentiviruses encoding the PAS-B domain of NCoA-1 (pVIG-Flag-NCoA-1 aa 260–370), NCoA-3 (pVIG-Flag-NCoA-3 aa 265–375) or the empty vector (pVIG-Flag) alone. The transduction efficiency for the PAS-B domain of NCoA-1 (88–95%), NCoA-3 (32–54%) and the empty vector (92–95%) was confirmed by FACS analysis on the basis of GFP co-expression. Cells were grown for at least 3 days in absence of androgen, followed by treatment (dark grey bars) or no treatment [EtOH (vehicle; ethanol), light grey bars] with dihydrotestosterone (DHT; 100 nM) for 16 h. Total cellular RNA of transduced cells was extracted and transcribed to cDNA. Relative PSA (prostate specific antigen) expression was quantified via quantitative RT-PCR using specific primers. mRNA levels of PSA were normalized against endogenous 18S mRNA. A 12-fold overexpression of the NCoA-1 PAS-B domain and a 2-fold overexpression of the NCoA-3 PAS-B versus endogenous NCoA-1 or NCoA-3, respectively were also determined via quantitative RT-PCR with specific primers. A representative result of three independent transduction of LnCap cells is shown with standard derivation from triplicate RT-PCR measurements.

Furthermore, binding assays with single and double mutants of the LXXLL motifs in the CID/AD1 domain confirmed our assumption that both motifs contribute to the binding of the NCoA-2 and NCoA-3 PAS-B domains (Figure 3D–F). These studies also disclose that the leucines in both LXXLL motifs of the CID/AD1 are obviously not essential for the binding of the NCoA-1 PAS-B domain. This was a surprise because peptides representing specifically these motifs inhibited the binding of the NCoA-1 PAS-B domain to the CID/AD1 significantly (Figure 3C). Thus we assume that other amino acids than the leucines in these motifs provide the contact to the NCoA-1 PAS-B domain. Or the carboxy-terminal part of the CID/AD1 (aa 938–970) which weakly interacts independently with the NCoA-1 PAS-B domain (Figure 3B) stabilizes the binding of the mutants. All these results confirmed that the PAS-B domains of NCoA-2 and NCoA-3 might have different binding motif specificities for the CID/AD1 in comparison to the NCoA-1 PAS-B domain.

Preliminary NMR studies of the different NCoA-1-PAS-B/LXXLL peptide complexes, which were performed to get a closer insight into the binding mode of the LXXLL motifs to the NCoA-1 PAS-B domain, revealed similar strong chemical shift perturbations for all three complexes (Figure 3G). The same parts of the PAS-B hydrophobic cleft, previously shown to represent the binding side of the STAT6 peptide (31) (Supplementary Data 3) were affected upon binding of the STAT6, motif 4 and motif 5 peptides providing an unambiguous evidence that all three peptides bind to the same site in solution. Further NMR studies which will include the PAS-B domain of NCoA-3 as well as peptide fragments representing both LXXLL motifs together with the carboxyterminal part of the CID/AD1 will be essential to disclose the differences in the binding mode and to understand how the selectivity of the NCoA PAS-B domains is achieved. These studies will be conducted in the future.

The binding sites of the PAS-B domains overlap with the binding site of CBP [NCoA-1 aa 926–960, (47)]. This is supported by several findings. First, the CBP fragment, containing the NCoA-1 interaction site showed almost the same binding characteristics with the amino- and carboxy-terminal fragments of the NCoA-1 CID/AD1 as the NCoA-2 or NCoA-3 PAS-B domains did (Figure 3B). The NCoA-1 PAS-B domain and the CBP region interacted similar with the NCoA-3 CID/AD1 (Figure 3B).

Finally, mutation of the LXXLL motif 5 in the NCoA-1 CID/AD1 region affected the binding of the NCoA-2 and NCoA-3 PAS domains to nearly the same degree as the binding of the CBP fragment (Figure 3D–F). The structure analysis of the CBP NCoA interaction fragment in complex with the CID/AD1 of NCoA-3 (ACTR; Activator of thyroid and retinoic acid receptors) revealed that the CID/AD1 has little intrinsic structure but appears to undergo induced folding in complex with CBP (53,54). A recent report, which compared this structure with the structure of the CBP NCoA interaction fragment in complex with the NCoA-1 CID/AD1 found that the CID/AD1 adopt distinct conformations in both complexes (55). These studies suggest that the NCoA CID/AD1 domains have a degree of conformational flexibility, which might facilitate the rapid dissociation of complexes. It will therefore be interesting to see if the CID/AD1 adopts different conformation in the complex with CBP and the PAS-B domain.

We analyzed whether CBP and NCoA-1 compete for binding to the CID/AD1 region, which should affect the formation and recruitment of coactivator complexes and thus transcriptional activation. Overexpression of the PAS-B domain is able to inhibit the binding of the CBP NCoA interaction region to the CID/AD1 fragment in cells as shown by co-immunoprecipitation experiments (Figure 4A). The CID/AD1 fragment can also inhibit the recruitment of the NCoA-1 PAS-B domain by the STAT6-TAD *in vitro* (Figure 4B and C) indicating that binding of different proteins to the same subdomain can affect the coactivator assembly. The ordered recruitment of coactivators during the transcriptional activation process might allow such competitive interactions of different partners, since it could be regulated by allosteric changes induced within interacting partners (56). Multicomponent complexes of the NCoA coactivators (57) are not static entities but rather dynamic rearrangements that are related to their particular roles during different subreactions in transcription (58).

Overexpression of the CID/AD1 domain strongly reduced the estrogen-dependent transactivation after transient transfection, indicating that the CID/AD1 fragment inhibits the interaction between NCoA proteins and p300/CBP and thereby destabilizes the interaction between p300/CBP and the estrogen receptor (47,59,60). The marginal effects that we observed for the PAS-B domain fragments in transient assays might result from the promoter context dependent function of coactivators. The promoter region of the luciferase reporter construct might not require the co-recruitment of NCoA protein pairs via the PAS-B domain or transcriptional activation can be achieved through the recruitment of different coactivator complexes. In contrast, the stable overexpression of the NCoA-1 or NCoA-3 PAS-B domain influences the expression of the endogenous AR target gene PSA, which was postulated to display the co-recruitment of NCoA protein pairs (35), in different ways (Figure 5B). While the PAS-B domain fragment of NCoA-1 enhanced the expression of PSA, the NCoA-3 PAS-B domain fragment diminished transactivation. Although we could not finally elucidate which step in the coactivator assembly is modified, these results are probably a consequence of an affected coactivator recruitment and altered coactivator complex formation (data not shown).

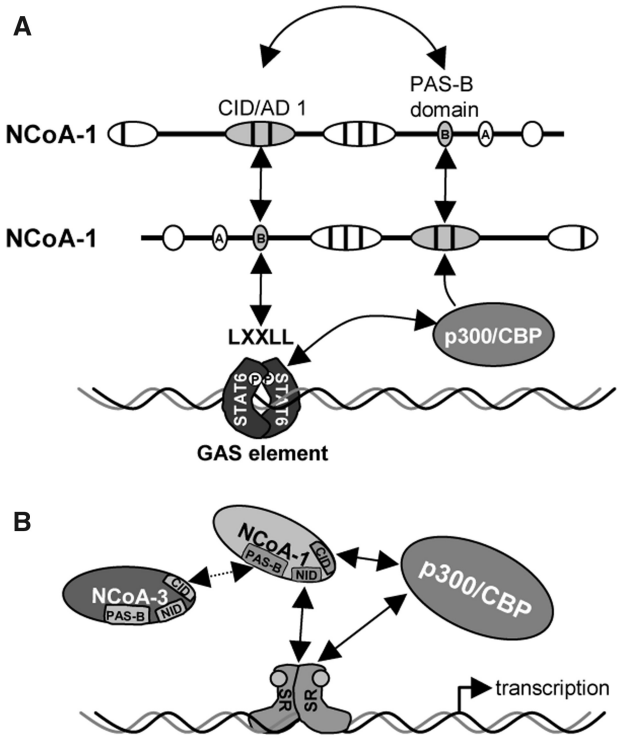


**Figure 6.** Models for the recruitment of NCoA proteins. (**A**) Model for the STAT6-mediated recruitment of NCoA-1. The contact of the PAS-B domain of NCoA-1 to the STAT6 LXXLL motif abolishes the intermolecular interaction of two NCoA-1 proteins. Interaction of p300/CBP with NCoA-1 stabilizes the contact of p300/CBP with STAT6 and enables transcriptional activation. (**B**) Model for the recruitment of pairs of NCoA proteins to steroid hormone receptor (SR) target gene promoters. The postulated co-recruitment of pairs of NCoA-1 and NCoA-3 is mediated through the PAS-B domain and the CID/AD1.

Taken together our results demonstrate for the first time that NCoA proteins are able to interact via the PAS-B domain and the CID/AD1. This allows intermolecular interactions, possibly regulated through post-translational modifications. These interactions obviously influence the formation and recruitment of coactivator complexes to promoter regions and thereby affect the transcriptional activation process. Our data suggest a new and complementary mechanism of the current model for the recruitment of NCoA proteins. We postulate two dynamic models of coactivator recruitment for the STAT6 and steroid hormone receptor mediated transcriptional activation (Figure 6). First, the interplay between the PAS-B domain and the CID/AD1 of NCoA proteins allows intermolecular and potentially also intramolecular interactions. The contact of the NCoA-1 PAS-B domain to the STAT6 LXXLL motif abolishes these interactions. Simultaneously, interaction of p300/CBP and the NCoA-1 CID/AD1 stabilizes the contact of STAT6 and p300/CBP (61) (Figure 6A). The second model comprises the steroid hormone receptor mediated transcriptional activation and is based on the postulated recruitment of NCoA protein

pairs to specific target gene promoters of the estrogen receptor and androgen receptor (35). In this model, the co-recruitment of two NCoA proteins is achieved through the interaction of the PAS-B domain and the CID/AD1 (Figure 6B). The postulated dimerization through the aminoterminal bHLH/PAS domain could not be detected in this work and therefore does not play a role in our model. Further structural analysis of the NCoA-1 PAS-B/CID/AD1 complex, which is currently underway, will help us to understand conformational changes involved in coactivator recruitment.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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