Interaction of STAT6 with its co-activator SRC-1/NCoA-1 is regulated by dephosphorylation of the latter via PP2A

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ABSTRACT

Regulation of gene expression represents a central issue in signal-regulated cellular responses. STAT6 is a critical mediator of IL-4 stimulated gene activation. To mediate this function, STAT6 recruits co-activator complexes. We have previously shown that STAT6 binds the PAS-B domain of the co-activator NCoA-1 via an LXXLL motif in its transactivation domain. Our recent finding that the PAS-B domain of NCoA-1 is also essential for co-activator complex formation points to an additional level of regulation of the co-activator assembly. In this study, we discovered that dephosphorylation of NCoA-1 is essential for the interaction with STAT6 and for IL-4-dependent transcriptional activation. PP2A dephosphorylates NCoA-1 and facilitates the activation of STAT6 target genes. Interestingly, simultaneous inhibition of phosphatase and cyclin-dependent kinases rescues the NCoA-1/STAT6 interaction. Moreover, arrest of cells at G1/S results in enhanced NCoA-1 phosphorylation. In summary, our results indicate that the interaction of NCoA-1 and STAT6 is dynamically regulated by the phosphatase PP2A and by cyclin-dependent kinases. This provides a mechanism for integrating transcriptional regulation by STAT6 with cell cycle progression.

INTRODUCTION

Interleukin-4 (IL-4) is a pleiotropic cytokine which regulates differentiation, proliferation and survival in a variety of cell types most notably in lymphocytes (1). Aberrant activation of IL-4 signaling is frequently associated with pathogenesis of leukaemia and lymphoma cells (2). IL-4 exerts its function mainly through activation of STAT6. IL-4 binding to its receptor results in phosphorylation of a specific tyrosine residue in STAT6 via Janus kinases. Phosphorylated STAT6 dimers bind to specific response elements in the promoter of IL-4 responsive genes and induce the assembly of the transcriptional machinery via the recruitment of specific co-activators (3).

Co-activators are essential for the transactivation potential of transcription factors and mediate the specificity for the promoters which are activated (4). The recruitment of transcriptional co-activators to target gene promoters and enhancers is a highly dynamic, sequential and ordered process (5). Co-activators function in multiprotein complexes which act as bridging factors to the general transcription machinery and modify histones to open the chromatin structure of the promoters. They possess structural domains, which provide surfaces for interactions with DNA-bound transcription factors and with other components of the multiprotein complexes. The p160/SRC/NCoA co-activator family contains three homologous members (6). They were first identified as transcriptional co-activators of nuclear receptors and subsequently found to be involved in transcriptional activation of several different transcription factors, like members of the STAT family (7–9) or NFκB (10,11). NCoA proteins are part of multiprotein complexes which contain co-activators with histone acetyltransferase activity (HAT), like NCoA-1, NCoA-3, p300, CBP and p/CAP and cofactors with histone methyltransferase activity (HMT), like CARM1 and PRMT1 (12). NCoA proteins also contain specific LXXLL motifs which mediate the interaction with nuclear receptors via the nuclear receptor interaction domain (NID), as well as with p300/ CBP through the so called CBP-interaction domain (CID), also called activation domain (AD1).

In previous studies, we found that NCoA-1 is recruited by the IL-4 activated transcription factor STAT6 (7). STAT6 directly contacts the PAS (Per/ARNT/Sim)
domain B of NCoA-1 via an LXXLL motif (where L is leucine and X is any amino acid) in its transactivation domain (7). The crystal structure of the NCoA-1 PAS-B domain in complex with the STAT6 LXXLL motif revealed that specific surface complementarities between the hydrophobic faces of the STAT6 LXXLL motif and of the NCoA-1 PAS-B domain almost exclusively define the binding specificity (13). Further characterization of the binding specificity revealed that the PAS-B domain is able to recognize the LXXLL motifs in its own CID/AD1 domain and also the LXXLL motifs in the CID/AD1 domain of NCoA-3 (14). Moreover, we demonstrate that the STAT6 and CID/AD1 motifs can compete for binding to the PAS-B domain. These findings indicate that the different interactions of NCoA-1 with STAT6, CBP and other NCoA family members are most likely dynamically regulated during the transcriptional activation process.

Several studies indicate that NCoA proteins are modified by post-translational modifications like phosphorylation, sumoylation, ubiquitination and methylation (15). Post-translational modifications may induce conformational changes and thereby influence the different interaction possibilities of co-activators (16). Induced post-translational modification thus lead to the integration of different signals in cellular transcriptional response (17).

IL-4 induced transcriptional activation by STAT6 involves multiple serine/threonine kinase pathways in addition to tyrosine phosphorylation (18–20). STAT6 was shown to be phosphorylated on serine residues in response to IL-4 stimulation (21,22). Although the phosphorylation sites of STAT6 are mapped mainly to its transactivation domain, the role of the serine phosphorylation is still unclear. Both positive and negative effects on the transcriptional activation have been described (21,22). An implication of protein phosphatases in the regulation of STAT6 serine phosphorylation has also been described (23). All these studies do not address the effect of serine/threonine kinase and phosphatase pathways on the STAT6/coactivator assembly. In this study, we analyze how complex formation of STAT6 with its co-activator NCoA-1 is affected by phosphorylation pathways. We report here that the phosphorylation state of NCoA-1 but not that of STAT6 is essential for their interaction. NCoA-1 is dephosphorylated by PP2A. PP2A enhances the transcriptional activation by STAT6 and is essential for the expression of IL-4 induced target genes. Combined inhibitor treatment experiments indicate that phosphorylation of NCoA-1 by cell cycle regulated kinases and dephosphorylation by PP2A regulate its interaction potential with STAT6.

**MATERIALS AND METHODS**

**Cell culture, transient transfection and metabolic labeling**

293T cells were grown in Dulbecco’s minimal essential medium (DMEM) containing 10% fetal calf serum. A549, BJAB, Ramos and THP-1 cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. 293T cells were transiently transfected using polyethyleneimine. siRNA (10 nM) was transfected in 293T cells by using SilentFect reagent (Bio-Rad). For transfection of Ramos with siRNA (40 nM) cells were electroporated using the Gene Pulser Xcell electroporation system (Bio-Rad). Analysis was performed 48 h after transfection. Cells were starved in medium containing 1% fetal calf serum 24 h before IL-4 stimulation (10 ng/ml). For stimulation with 17β-estradiol (E2, 10^{-7} M), cells were grown in phenol red-free medium supplemented with 10% charcoal-dextran-stripped fetal calf serum for 2 days prior to treatment. For the P^{32} metabolic labeling 293T cells were seeded at 30% confluency. They were transiently transfected with 1.5 μg of NCoA-1 expression vector 24 h later. Another day later, they were washed twice with PBS followed by a 2-h incubation in phosphate-free medium. After renewal of the media, 0.1 μCi P^{32} were added per 6 cm dish. Two hours later, IL-4 and inhibitors were added as indicated for another 2 h. For the metabolic labeling of BJAB cells, 2 × 10^7 cells were washed in phosphate-free medium and incubated in phosphate-free medium for 2 h. After renewal of the medium, 0.5 μCi P^{32} were added per sample and 2 h later IL-4 and inhibitors were added as indicated. After cell lysis STAT6 and NCoA-1 were precipitated and analyzed by SDS–PAGE and autoradiography.

**Recombinant plasmids, constructs and siRNA**

The GST fusion constructs containing different fragments with amino acids numbers given are constructed or derived as described. The GST-STAT6 TAD fusion construct (642–847) was constructed by cloning the PCR amplified fragment in frame in the pGEX-2T vector. GST fusion constructs containing the estrogen receptor activation domain (312–595) was constructed by cloning the cDNA Fragment in frame in the pGEX-2T vector. pGEX-expression vector containing the hNCoA-1 fragment (260–370) has been described earlier (14). GST fusion constructs containing the P2A A protein and C fragment were derived from Richard Karas, Boston and has been described (24). The expression vector for human estrogen receptor (pRSV-hER) and the LacZ expression plasmid (pCH110) were used previously (14). Expression vectors for hNCoA-1 (pSG-SRC-1e), were provided by Markus Heim, Basel and was described (25).

The STAT6 reporter plasmid N4(STAT6-RE)3 LUC was described before (7). The estrogen responsive element (ERE) luciferase reporter construct (pGL2-ERE TK-luc) was obtained from L. Klein-Hitpass and has been described (25).

Twenty-seven-mer siRNAs targeting PP2A were purchased from Integrated DNA Technologies (Coralville). Sequences are available upon request.
Co-immunoprecipitation assays and western blotting

Co-immunoprecipitation and western blotting of endogenous proteins from Ramos or BJAB cells and proteins from transiently transfected 293T cells was performed as described previously (7). Nuclear and cytoplasmic fractionation was performed as described in (26). Antibodies against the following different antigens were used: GFP (Clontech), SRC-1 (M341, Santa Cruz), SRC-1 (1135, Santa Cruz), SRC-2 (OBT1796, AbD Serotec), NCoA-3 (M397, Santa Cruz), PP2A (clone 1D6, Upstate), STAT6 (M20 for IP, M200 for Co-IP, Santa Cruz), STAT6 (clone 23, Transduction Laboratories), Phospho-STAT6 (9361, Cell Signaling), unspecific IgG (Santa Cruz), Sin3a (K20, Santa Cruz) and Tubulin (Clone B-5-1-2, Sigma). Visualization was performed with the ECL western blotting Detection System (Pierce).

GST pull-down experiments

For GST pull-down experiments, 5 μg of GST or GST-fusion protein coupled to glutathione sepharose beads were used. Of extracts, 200–1000 μg from cells treated with the inhibitors as indicated, were incubated with the coupled GST-fusion proteins over night in NETN buffer (20 mM Tris–HCl pH 8, 100 mM NaCl, 1 mM EDTA, 0.2% NP-40, 10% Glycerin with complete protease inhibitors from Roche and phosphatase inhibitors from Sigma). The amount and integrity of bound GST proteins was controlled after SDS–PAGE in each experiment by Coomassie blue staining. The binding of the interacting proteins was detected by western blotting.

Phosphatase assays

The phosphatase activity in cell extracts prepared in NETN-Buffer without EDTA and phosphatase inhibitors was analyzed in a 96-well plates with 10 NETN-Buffer without EDTA and phosphatase inhibitors were used: GFP (Clontech), SRC-1 (M341, Santa Cruz), SRC-1 (1135, Santa Cruz), SRC-2 (OBT1796, AbD Serotec), NCoA-3 (M397, Santa Cruz), PP2A (clone 1D6, Upstate), STAT6 (M20 for IP, M200 for Co-IP, Santa Cruz), STAT6 (clone 23, Transduction Laboratories), Phospho-STAT6 (9361, Cell Signaling), unspecific IgG (Santa Cruz), Sin3a (K20, Santa Cruz) and Tubulin (Clone B-5-1-2, Sigma). Visualization was performed with the ECL western blotting Detection System (Pierce).

Preparation of RNA and real time-PCR

Extraction of total cellular RNA and cDNA synthesis was performed as described earlier with the modification that the RevertAidTM First Strand cDNA Synthesis Kit (Fermentas) was used (14). For quantification of specific transcripts, the i-Cycler MyiQ Single Color Real-Time PCR Detection System (Bio-Rad) was used. Amplification of samples was performed in triplicates. mRNA levels were normalized against endogenous 18S RNA. The following primers for the specific amplification of the transcripts were used: 18S RNA: 5’-CGGGCTACCA CATCCAGGA-3’; 5’-CCAAATTACGCGCTCGA AA-3’ (27); Ig-epsilon 5’-CACATCCACAGG CACAA AT-3’; 5’-ATGCCCGGTCCGGAAGTAGT-3’; TARC 5’-CAGGATGGCAATCGTTTTTGTAACGTGC-3’; 5’-CCTCACTGTGGCTCTTCCCTGCCA-3’.

Electrophoretic mobility-shift assay

293T cells, which do not contain functional STAT6 were transiently transfected with STAT6 expression plasmid (2 μg/10-cm dish). The cells were stimulated after 24 h post-transfection with IL-4 (10 ng/ml) in the presence of different concentrations of calyculin A for 30 min. Preparation of whole-cell extracts and mobility-shift assay was performed as described before (8).

RESULTS

Phosphatases are essential for the regulation of the STAT6 and NCoA-1 interaction

Previous studies demonstrate that serine/threonine kinases influence transcriptional activation by STAT6 (18,20). In addition, the phosphatase PP2A has been described to regulate IL-4-mediated STAT6 signaling (23). In order to investigate whether serine/threonine phosphorylation affects the interaction of STAT6 with its co-activator NCoA-1, co-immunoprecipitation experiments were performed in 293T cells treated with inhibitors specific for serine/threonine kinases and phosphatases known to be involved in transcriptional regulation by IL-4. NCoA-1 was coprecipitated with GFP tagged STAT6 but not with the control antibodies (IgG) confirming complex formation of the proteins (Figure 1, left panel). The interaction was not enhanced by IL-4 treatment of the cells as shown in previous studies (7), (8). Treatment of the cells with kinase inhibitors did not affect the STAT6/NCoA-1 interaction. Only calyculin A is a PP1/PP2A phosphatase inhibitor abrogated the STAT6/NCoA-1 interaction. Immunoprecipitation of GFP-STAT6 protein was not affected by inhibitor treatment (Figure 1, right panel). These experiments clearly demonstrate that phosphatases regulate the STAT6/NCoA-1 complex formation.

Both STAT6 and NCoA-1 are modified by phosphatases

We next investigated whether inhibition of phosphatases results in a modification of STAT6 and NCoA-1. For this, we treated various IL-4 responsive cell lines with different concentrations of the phosphatase inhibitors okadaic
acid and calyculin A. The electrophoretic mobility of endogenous STAT6 and NCoA-1 was analyzed by western blotting with specific antibodies. The mobility of both STAT6 and NCoA-1 was retarded in A549 cells treated with either of the phosphatase inhibitors (Figure 2A). Similar results were observed in the cell lines Ramos B and THP-1. These results indicate that not only STAT6 (23), but also NCoA-1 is modified by phosphatases. The degree of modification varied between the analyzed cell lines and phosphatase inhibitors as estimated by the mobility shift of the proteins and obviously depends on kinase pathways, which are currently active in the different cell lines.

In order to confirm that phosphatase inhibitor treatment of cells promotes the phosphorylation of STAT6 and NCoA-1, we conducted metabolic labeling experiments with radioactive P32-ATP. These experiments were performed in BJAB cells which contain a high amount of NCoA-1. Cells were treated with IL-4, calyculin A or with calyculin A in the presence of IL-4 or the broad-spectrum kinase inhibitor staurosporine during labeling to find out whether the phosphorylation is inducible and reversible. STAT6 and NCoA-1 were precipitated from cell lysates and subsequently analyzed by SDS–PAGE and autoradiography (Figure 2B).

Calyculin A treatment caused a stronger phosphorylation of both STAT6 and NCoA-1. IL-4 treatment moderately enhanced the phosphorylation level of STAT6, but not that of NCoA-1. Cotreatment of the cells with the phosphatase inhibitor calyculin A and the kinase inhibitor staurosporine prevented the phosphorylation of both proteins. Thus these results confirmed that both STAT6 and NCoA-1 are modified by dynamic phosphorylation and dephosphorylation.

NCoA-1 but not STAT6 hyperphosphorylation inhibits STAT6/NCoA-1 interaction

So far our results indicate that both STAT6 and NCoA-1 are modified by phosphorylation. We wondered whether the modification of STAT6 or NCoA-1 or the modification of both proteins regulates their interaction. To address this, we performed GST pull-down experiments with bait proteins containing the different minimal interaction domains. The prey proteins were derived from extracts of inhibitor treated cells (experimental design shown in Figure 3D). GST fusion proteins possessing the STAT6 transactivation domain (GST-STAT6 TAD) or the NCoA-1 PAS domain (GST-NCoA-1 PAS) were incubated with lysates from Ramos B cells treated with IL-4, calyculin A or calyculin A in the presence of staurosporine. The binding of the interacting partner protein was analyzed by western blotting with specific antibodies.
antibodies against NCoA-1 or STAT6, respectively (Figure 3A and B).

No effect on the interaction was observed when extracts of the treated cells were incubated with GST-NCoA-1 PAS protein (Figure 3A). However, calyculin A treatment abolished the interaction of cellular NCoA-1 with GST-STAT6 TAD (Figure 3B). The in vitro interaction of NCoA-1 with the estrogen receptor (GST-ER E/F) was not affected or even slightly enhanced by the treatment, thus excluding unspecific inhibition by degradation of the cell extract. This result clearly shows that phosphorylation of NCoA-1, but not that of STAT6 abolishes the interaction (Figure 3D). Therefore active phosphatases are required to sustain the interaction potential of NCoA-1. The inhibitory effect of the phosphatase inhibitor was rescued by kinase inhibitor treatment, hence supporting that the interaction of NCoA-1 with STAT6 is regulated by phosphorylation. IL-4 treatment had no significant effect on the STAT6/NCoA interaction regardless of what direction was analyzed. Our observation, that the inhibitory effect of calyculin A on the STAT6/NCoA-1 interaction can be reverted was confirmed in coimmunoprecipitation experiments at the level of the endogenous proteins (Figure 3C).

PP2A dephosphorylates NCoA-1 and regulates the interaction of STAT6 and NCoA-1

Calyculin A inhibits the phosphatases PP1 and PP2A at low concentration (28). Both phosphatases have been described to regulate the function of NCoA-3 (29). Inhibition experiments with tautomycin, which at low concentration selectively inhibits PP1, but PP2A did not affect the STAT6/NCoA-1 interaction (data not shown). This indicates that PP1 does not dephosphorylate the critical NCoA-1 sites. In order to find out if PP2A is involved in the regulation of the STAT6/NCoA-1 interaction, we first analyzed whether NCoA-1 is a substrate of PP2A. For this, the PP2A catalytic subunit was precipitated from cell extract and incubated with P32 labeled NCoA-1 from extracts of metabolically labeled cells. NCoA-1 phosphorylation state was determined after specific precipitation by SDS–PAGE and autoradiography. To inhibit cellular phosphatases, cells were treated with calyculin A during labeling. In addition, calyculin A was included in one reaction (Figure 4A, last lane) to determine the specificity of the reaction. The experiments were performed with substrate derived from NCoA-1 over expressing 293T cells (left panel), but also with endogenous NCoA-1 from BJAB cells as substrate (right panel).

Phosphatase activity was efficiently precipitated from both cell lines with specific antibodies against the catalytic domain of PP2A, but not with the control IgG (Figure 4A, lower panel). Incubation of the precipitated PP2A holoenzyme with extract from labeled cells resulted in a decreased amount of radioactive labeled NCoA-1 (Figure 4, upper panel). The dephosphorylation of NCoA-1 was inhibited when calyculin A was included in the reaction (last lane). Thus PP2A holoenzyme recovered from different cell extracts is able to dephosphorylate antibodies against NCoA-1 or STAT6, respectively (Figure 3A and B).

Figure 3. NCoA-1 dephosphorylation is essential for its interaction with STAT6. (A and B) Ramos B cells were stimulated with IL-4 (10 ng/ml) or treated with calyculin A (10 nM) or with calyculin A in combination with staurosporine (200 nM) for 2h. Cells were lysed in NETN buffer and interaction of endogenous proteins were analyzed in GST pull-down experiments with coupled fusion proteins expressing the PAS domain of NCoA-1, GST-NCoA-1 Pas (A) or the transactivation domain of STAT6, GST-STAT6-TAD (B) or the activation domain 2 of the estrogen receptor, GST-ER E/F. Interaction of STAT6 (A) and NCoA-1 (B) was analyzed by western blotting with specific antibodies. (C) Treatment of the cells as in A. STAT6 was immunoprecipitated and co-precipitated NCoA-1 was detected after western blotting with specific antibodies. The efficiency of the STAT6 precipitation was examined by reprobing with a STAT6 specific antibody. The amount of NCoA-1 in two percent of the cell lysates was analyzed in parallel (In). (D) Schematic model of the design and the results of the pull-down experiments performed in (A) (left panel) and in (B) (right panel).
NCoA-1 indicating that NCoA-1 is a bona fide substrate of PP2A. To further confirm the relationship between PP2A and its substrate NCoA-1, we investigated whether NCoA proteins can interact with PP2A. Co-immunoprecipitation experiments clearly showed that endogenous NCoA-1 and NCoA-3, but not NCoA-2 were able to bind to the catalytic domain of PP2A (Figure 4B). Efficiency of the precipitation is shown in (Supplementary Figure S1). Pull-down experiments with GST fusion proteins containing the catalytic subunit (GST-PP2A/C) or the scaffold A subunit (GST-PP2A/A) of PP2A confirmed that both NCoA-1 and NCoA-3 specifically interacted from BJAB cells by specific antibodies against the different NCoAs (NC). Coprecipitated PP2A was detected by western blotting with an antibody against the catalytic subunit of PP2A. As controls two percent of the cell lysates were analyzed in parallel (In) and unspecific binding was analyzed by precipitation with IgG. (C) Interaction of NCoA proteins with the A and C subunits of PP2A. Lysates of BJAB cells were incubated with coupled GST-fusion proteins expressing the A subunit of PP2A (labeled A) or the C subunit of PP2A (labeled C) or with GST as control. Bound NCoA proteins were detected by western blotting with specific antibodies.

Figure 4. NCoA-1 is dephosphorylated by PP2A. (A) Dephosphorylation of NCoA-1 by PP2A. 293T cells transfected with 3μg of NCoA-1 expression vector were metabolically labeled 24h after transfection with 0.5mCi P32. Endogenous phosphatases were inactivated prior lysis by treatment with calyculin A (10nM) for 10min. For the labeling of BJAB cells, 6×10⁷ cells were used. For the dephosphorylation assay PP2A immunoprecipitated out of unlabeled cells was used. As controls, a precipitation with an unspecific antibody (second lane) and a precipitation of PP2A under treatment with calyculin A (10nM) (last lane) were used. The dephosphorylation reaction was carried out for 1 h at 37°C under shaking. NCoA-1 was immunoprecipitated out of the lysates and phosphorylation was visualized in autoradiography after SDS-PAGE (upper lanes). The amount of NCoA-1 was determined by western blotting (lower lanes). The phosphatase activity of the immunoprecipitated PP2A was determined in parallel (bar chart). (B) Interaction of PP2A with the NCoA family members. Endogenous NCoA-1, NCoA-2 and NCoA-3 were immunoprecipitated from BJAB cells by specific antibodies against the different NCoAs (NC). Coprecipitated PP2A was detected by western blotting with an antibody against the catalytic subunit of PP2A. As controls two percent of the cell lysates were analyzed in parallel (In) and unspecific binding was analyzed by precipitation with IgG. (C) Interaction of NCoA proteins with the A and C subunits of PP2A. Lysates of BJAB cells were incubated with coupled GST-fusion proteins expressing the A subunit of PP2A (labeled A) or the C subunit of PP2A (labeled C) or with GST as control. Bound NCoA proteins were detected by western blotting with specific antibodies.

Phosphatase activity is essential for IL-4 induced transcriptional activation

To investigate the biological importance of the NCoA-1 dephosphorylation, we analyzed the effect of phosphatase inhibition and PP2A knockdown on IL-4 induced transcriptional activation. Both okadaic acid and calyculin A inhibited the IL-4 induced transcription of an STAT6 luciferase reporter construct in a dose-dependent manner (Figure 6A, upper panel). In contrast, to this an enhancement of the activity of an estrogen responsive reporter was observed at low-inhibitor concentration (Figure 6A, lower panel). At high concentration, the estrogen-activated transcription was only slightly inhibited. These results support that dephosphorylation of NCoA-1 is essential for STAT6-mediated transcriptional activation, but is a disadvantage for the transcriptional activation by the estrogen receptor. This is in concordance with our finding that inhibition of phosphatases has a specific inhibitory effect on the STAT6/NCoA-1 interaction but slightly enhances the ER/NCoA-1 interaction (Figure 3B).

It has been described that high concentration of calyculin A (80nM) affects the DNA-binding of STAT6 (23). To rule out that impaired STAT6 DNA-binding
accounts for the inhibition of transcriptional activation, we assessed the DNA-binding activity of STAT6 in the calyculin A treated cells by electrophoretic mobility shift assay (Supplementary Figure S3A). STAT6 DNA binding was not affected at the concentration that we used in our reporter-gene assays. We could also exclude an effect of calyculin A treatment on the tyrosine phosphorylation or stability of STAT6 (Supplementary Figure 3B). The phosphorylation of the C-terminal domain of RNA polymerase II was enhanced by treatment with phosphatase inhibitors, which should have even a positive effect on general transcription (Supplementary Figure 3C). All these experiments confirmed our hypothesis that inhibition of the NCoA-1 dephosphorylation disturbs its interaction with STAT6 and thereby decreases STAT6-mediated transcriptional activation.

To confirm the specific function of PP2A in transcriptional activation in response to IL-4, we analyzed the effect of PP2A over expression and knockdown in luciferase reporter-gene assays. The amount of PP2A in cells transiently transfected with expression-vector coding for the catalytic domain of PP2A or with PP2A specific siRNA was analyzed by western blotting (Supplementary Figure S4). Overexpression of PP2A resulted in 2-fold increase of the IL-4 stimulated activity of the STAT6-reporter construct (Figure 6B). Transfection of the PP2A specific siRNA reduced the reporter activity by 50%. Although treatment with the phosphatase inhibitors okadaic acid and calyculin A had a much stronger effect on transcriptional activation, we propose inhibition of NCoA-1 dephosphorylation as the major factor for the inhibition of IL-4 activated transcription. This is supported by the observation that 50% reduction of PP2A or NCoA-1 by siRNA resulted in comparable reporter gene activity (Supplementary Figure 5).

Moreover, we analyzed the importance of PP2A for the induction of endogenous IL-4 target genes. For this, Ramos cells were transfected with siRNA against PP2A or as a control with scrambled (scr) siRNA. The RNA amount of the IL-4 responsive genes Ig epsilon and TARC was measured in response to IL-4 treatment.

PP2A-protein level and the tyrosine phosphorylation of STAT6 were determined by western blotting (Supplementary Figure 6). Strong induction of both STAT6 target genes was detected after IL-4 treatment of the cells. This induction was inhibited by PP2A depletion (Figure 6C). The inhibitory effect on target-gene expression by PP2A siRNAs was comparable to the inhibition by low amounts (2 nM) of calyculin A. In summary, our results confirmed that PP2A is an essential regulator for IL-4 induced gene expression.

**Interaction of STAT6 and NCoA-1 is inversely regulated by phosphatases and cell cycle-dependent kinases**

Our previous results indicate that the phosphorylation status of NCoA-1 is dynamically regulated and that dephosphorylation is required for binding to STAT6 as well as for STAT6-mediated transcriptional regulation (Figures 2B and 3). Short-term treatment of cells with IL-4 did not affect the STAT6/NCoA-1 interaction (Figures 1 and 3). However, we observed a slightly increased nuclear localization of PP2A after 8 h IL-4 treatment (Supplementary Figure S7). In order to further characterize the mechanism how NCoA-1 phosphorylation is regulated in cells, we analyzed which kinase pathway is responsible for NCoA-1 phosphorylation. 293T cells transfected with NCoA-1 were metabolically labeled in the presence of specific kinase inhibitors. Okadaic acid and calyculin A treatment strongly enhanced the phosphorylation level of NCoA-1 (Figure 7A, upper panel) and thus confirmed our experiments in the B cell line (Figure 2B). In contrast, none of the kinase inhibitors had an effect on the NCoA-1 phosphorylation level, when applied alone. However, when roscovitine, a chemical inhibitor of cdk2, cdk1 and cdk5 (30) was applied in combination with calyculin A, the phosphorylation of NCoA-1 was dramatically decreased, even stronger than by staurosporine (Figure 7A, lower panel). We next tested if roscovitine can also rescue the STAT6/NCoA-1 interaction when cells were treated with calyculin A. Both pull-down GST (Figure 7B, lower panel) and co-immunoprecipitation experiments in Ramos cells...
(Figure 7C) showed that roscovitine treatment can restore the interaction potential of NCoA-1. Treatment with roscovitine alone had no effect on the interaction of NCoA-1 with STAT6 or the estrogen receptor as determined in GST pull-down experiments (Figure 7B, upper panel).

It is already known that IL-4 promotes the proliferation of T lymphocytes by down regulation of p27Kip1 (31).
It has also been described that NCoA-1 (SRC-1) is phosphorylated by cyclinA/cdk2 and that this is essential for its interaction with the progesterone receptor and for progesterone-dependent activation of target genes (32), indicating a cell cycle-dependent regulation of NCoA-1 by phosphorylation. We therefore analyzed whether the phosphorylation of NCoA-1 and its interaction with STAT6 is regulated during the cell cycle. Cell-cycle profiles of arrested cells were determined by FACS analysis (Supplementary Figure S8). Enhanced NCoA-1 phosphorylation was observed when cells are arrested at G1 and S phase, but not at G2 phase (Figure 7D). NCoA-1 derived from extracts of cells arrested at distinct phases of the cell cycle was able to bind to GST-STAT6 fusion proteins (Figure 7E). However, the inhibition of the STAT6/NCoA-1 interaction by calyculin A was prevented if cells were arrested at the G2 phase by colcemide (Figure 7F). These experiments indicate that the interaction of NCoA-1/STAT6 is regulated by kinases which are active at G1/S phase. Inactivation of these
discussed interaction under conditions where phosphatases are inhibited. Hence, our results support a dynamic regulation of the NCoA-1 phosphorylation in which the phosphatase PP2A is the dominant regulator.

**DISCUSSION**

The potential importance of serine/threonine phosphorylation pathways in IL-4 induced transcriptional regulation has been suggested, but the actual regulators and target proteins are still largely unknown. We demonstrated here that the function of NCoA-1 to act as a co-activator of STAT6 is regulated by serine/threonine phosphorylation. Dephosphorylation of NCoA-1 by PP2A is essential for its specific interaction with STAT6. Regulated phosphorylation and dephosphorylation of NCoA-1 might enable interactions of NCoA-1 with distinct transcription factors. Co-activators can thus function as integrators, which coordinate transcriptional regulation by different signaling pathways (17). The interaction of NCoA-1 with the progesterone receptor e.g. requires NCoA-1 phosphorylation (32). In accordance with this we observed a slight enhancement of the NCoA-1 interaction with the estrogen receptor, another member of the nuclear receptor family when NCoA-1 dephosphorylation is inhibited (Figure 3B). Functional regulation by phosphatases has only been described for NCoA-3 (SRC-3). However, in that study, PP1 and PP2A affect the transcriptional co-regulatory activity in steroid receptor-signaling negatively (29).

Our studies revealed that IL-4 induced transcriptional activation is regulated at an additional level by NCoA-1 phosphorylation. It has been postulated that signal-induced gene regulation by IL-4 requires serine phosphorylation pathways in addition to the JAK kinase-mediated tyrosine phosphorylation of STAT6. Both direct and indirect mechanisms for this regulation have been described. IL-4 and IL-13 induce phosphorylation of distinct serine and threonine residues in the transactivation domain of STAT6 (19), (21), and this can result in an inhibition of the STAT6 DNA binding (22). Activation of p38 MAPK by CD40 stimulation enhances transcriptional activation by STAT6 without affecting STAT6 phosphorylation (20). Although the mechanism for this is still unclear, it was suggested that p38 MAPK-mediated phosphorylation somehow regulates the interaction of STAT6 with co-regulators. Induced phosphorylation of the RNA Polymerase II C-terminal domain has also been proposed as a mechanism how serine kinases can modify IL-4-dependent gene regulation (18). Although we observed a slightly enhanced serine phosphorylation of STAT6 in response to IL-4 stimulation (Figure 2B), this had no effect on the interaction of STAT6 with NCoA-1. In addition, different serine/threonine kinase pathways described to modulate IL-4 signaling are obviously not involved in the regulation of the STAT6/NCoA-1 interaction (Figure 1). Hence, the regulation of the STAT6/NCoA-1 interaction seems not to be involved in the formerly described examples where IL-4 induced transcriptional activation are regulated by serine/threonine phosphorylation (18,19,21,22).

We identified a phosphatase pathway and specifically PP2A as the major regulator of the STAT6/NCoA-1 interaction, which is also important for IL-4 responsive transcriptional activation. PP2A has already been described to catalyze the dephosphorylation of STAT6 and thereby regulate the DNA binding of STAT6 (23). However in this work, extremely high concentrations of the PP1/PP2A inhibitor calyculin A were used (80 nM). At such concentration, PP1 is mainly inhibited. We did not observe an inhibition of the STAT6/NCoA-1 interaction by using tautomycin a more specific PP1 inhibitor (data not shown). However, we detect an enhanced phosphorylation of both STAT6 and NCoA-1 by using much lower concentration of the inhibitor calyculin A (10 nM) (Figure 2B). At that concentration, no inhibitory effect on the STAT6 DNA binding could be observed. We therefore postulate that the reduction of the IL-4 induced gene expression by PP2A inhibition does not result from the inhibition of the STAT6 DNA-binding activity but from the inhibition of the STAT6/NCoA-1 interaction which does not take place when NCoA-1 dephosphorylation is inhibited. In addition, it has been described that PP2A directly regulates the activity of the JAK/STAT pathway by affecting Janus kinases (33,34). However, since we did not observe an effect on STAT6 tyrosine phosphorylation either at low-inhibitor concentration (Supplementary Figure S3) or by PP2A knockdown (Supplementary Figure S6), such a mechanism could be ruled out in the case of IL-4-mediated gene regulation. Until now, we cannot exclude that dephosphorylation of other coactivators or co-activator multiprotein complex formation are also regulated by PP2A-mediated dephosphorylation. This has to be analyzed in further studies.

The phosphorylation of both STAT6 and NCoA-1 is enhanced when PP2A is inhibited by calyculin A. Our results indicate that only the phosphorylation of NCoA-1 is essential for the regulation of the STAT6/NCoA-1 interaction. This is in agreement with the finding that IL-4 treatment which slightly enhances STAT6 phosphorylation, but not NCoA-1 phosphorylation does not affect the STAT6/NCoA-1 interaction either. Transcriptional regulation requires the specific interaction of transcription factors and co-activators. The members of NCoA co-activator family are involved in transcriptional activation by several transcription factors, which are themselves controlled by distinct signaling pathways. Several studies already showed that NCoA proteins are post-translationally modified and that such modifications can disturb protein interaction and regulate the cellular localization, stability and conformation of NCoA proteins (15). In the case of NCoA-3, the best studied family member, a phosphorylation-dependent mono- and polyubiquitination has been identified, which is essential for transcriptional regulation by nuclear hormone receptors and regulates co-activator degradation during the transcriptional activation process (35). The retarded electrophoretic mobility of NCoA-1 that we detected after calyculin A treatment could also be caused by mono-ubiquitination. However, we did not
detect an enhanced degradation of NCoA-1 after calyculin A treatment (Figure 2) or by knockdown of PP2A (Supplementary Figure S4). In preliminary experiments, we could also not detect enhanced ubiquitination or sumoylation (data not shown).

Although the phosphorylation of NCoA-1 is not regulated in response to short-term IL-4 stimulation our results advise to a dynamic phosphorylation and dephosphorylation process, whereas the dephosphorylation by PP2A seems to be stronger. This is corroborated by the finding that sole inhibition of kinases by staurosporin or roscovitine had no significant effect on the NCoA-1 phosphorylation or its interaction with STAT6. Only simultaneous inhibition of kinases and phosphatases shows a significant effect (Figure 7). Kinases active during G1/S phase obviously mediate NCoA-1 phosphorylation and thereby regulate its co-activator function for STAT6 (Figure 7). Other kinase described to be involved in IL-4 signaling do not play a role in this process. It is already known that IL-4 alone or in combination with CD40 stimulation promotes the proliferation of lymphocytes by upregulation of cyclin D3 and cdk6 and down regulation of p27 (31,36). Cdk2 which is active during G1/S has been described to phosphorylate NCoA-1 and thereby enhances its co-activator function for progesterone receptor (32). Our finding indicates that phosphorylation by cdk2 also impedes the co-activator function of NCoA-1 for STAT6. We would therefore expect that IL-4 can further modulate transcriptional regulation by altering the phosphorylation state of NCoA-1 via regulating cycle-dependent kinase activity. Several examples for cell-cycle-dependent regulation of nuclear hormone receptor-driven transcriptional activation had already been described (37). Different mechanisms are involved including the function of cyclins as bridging factors. A recruitment of cyclin D1 by a STAT3/NCoA complex which prevents the recruitment of CBP and RNA polymerase II has also been described (38). The function of cyclins in STAT6-mediated gene regulation has to be determined in future experiments. It will also be important to investigate if transcriptional activation by STAT6 is modulated by cell cycle. Such a regulation could be important in fast proliferating cells like leukemia cells which have a different cell-cycle status.

Our results show an essential function of PP2A in STAT6 regulated transcriptional activation. PP2A dephosphorylates NCoA-1 and thereby regulates its interaction potential with STAT6 as shown in the model in Figure 8. Cdk2 phosphorylates NCoA-1, when it is active during the cell cycle. It is not yet clear how the activity of PP2A is regulated in this process. Our preliminary experiments indicate that long-term IL-4 treatment enhances a transient localization of PP2A into the nucleus (Supplementary Figure 7). The amount of PP2A that can dephosphorylate nuclear NCoA-1 can thus be regulated by IL-4. However, we expect that specific regulatory subunits of PP2A will have an even more important function in controlling the PP2A activity. Although we detected a direct interaction of NCoA-1 with the catalytic subunit of PP2A in vitro (Figure 4C) regulatory subunits of PP2A will be essential for its regulation in the cellular context (39). Our results point to an additional level of regulation for STAT6-activated genes by co-activator phosphorylation. This may have an important function in specific cell types in the context of the activated signaling pathways and transcription factors and can have therapeutic impact.

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