The Transcriptional Co-activator p/CIP (NCoA-3) Is Up-regulated by STAT6 and Serves as a Positive Regulator of Transcriptional Activation by STAT6*

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Transcriptional activation by signal transducer and activator of transcription 6 (STAT6) has been shown to require the direct interaction not only with co-activators such as p300 and cAMP-responsive element-binding protein-binding protein (CBP) but also with nuclear co-activator 1, a member of the p160/steroid receptor co-activator family. Among the p160/steroid receptor co-activators, only p/CIP (nuclear co-activator 3) has been shown to be up-regulated by interleukin (IL)-4 in B cells through a STAT6-dependent mechanism using GeneChip analysis. In this study, we have investigated the function of p/CIP in the transcriptional activation by STAT6. We found that p/CIP indirectly interacted with STAT6 via p300, and overexpression of the CBP-interacting domain of p/CIP (p/CIP947–1084) prevented the interaction of p/CIP with STAT6 by blocking the binding of p/CIP to p300. Whereas expression of p/CIP947–1084 resulted in a marked reduction of STAT6-mediated transactivation, overexpression of wild type p/CIP resulted in significant enhancement of it. In addition, p/CIP947–1084 markedly reduced CD23 expression on B cells stimulated with IL-4, whereas overexpression of wild type p/CIP enhanced it. Chromatin immunoprecipitations demonstrate that IL-4 increases the interaction of p/CIP with the murine immunoglobulin heavy chain germ line epsilon promoter in B cells. These results suggest that p/CIP positively regulates STAT6 transcriptional activation through formation of a STAT6, p300/ CBP, and p/CIP complex.

STAT6 (1), a member of the family of signal transducer and activator of transcription (STAT),1 is known to be the common signal transducer of interleukin (IL)-4 receptor α chain and mediates IL-4- and IL-13-induced responses (2, 3). IL-4 and IL-13 are considered to be crucial cytokines for the development of type II allergic diseases (4), because IL-4 can promote immunoglobulin class-switching to the IgE isotype in B cells (5), and IL-4 and IL-13 mediate the induction of Th2 differentiation (6, 7). STAT6-deficient mice are defective in IL-4- and IL-13-induced Th2 cell differentiation and IL-4-induced class switching B cells to IgE and IgG1 (8–10). In addition, STAT6-deficient mice are protected from antigen-induced airway hyper-responsiveness and mucus production (11) and also from IL-13-induced airway responsiveness (12).

IL-4 stimulation results in activation of Janus kinase 1 and 3 tyrosine kinases, which in turn phosphorylate tyrosine residues in the cytoplasmic domain of the IL-4 receptor α. Then STAT6 is recruited to the intracellular domain of the receptor and interacts with the phosphorytrosine residues via its SH2 domain. Subsequently, STAT6 is phosphorylated by Janus kinase kinases, dimerizes, and translocates to the nucleus where it modulates transcription through binding to STAT6-response elements (13, 14). STAT6-binding elements were identified in the Iε promoter (15), the CD23 promoter (16), the IL-4 promoter (17), and other enhancer elements. Recently, we compared gene expression profiles of IL-4- and lipopolysaccharide-stimulated B cells obtained from STAT6+/+ and STAT6−/− mice using GeneChips analysis (18). Statistical analysis revealed that 31 of 70 known genes were expressed at higher levels, and 39 of 70 genes were expressed at lower levels in B cells from STAT6+/+ mice in comparison with B cells from STAT6−/− mice. This suggests that STAT6 acts as a positive and negative regulator of gene expression in IL-4-mediated gene expression.

Among the genes that were found to be up-regulated through a STAT6-dependent mechanism, the present study focuses on the function of p300/cAMP-responsive element-binding protein-binding protein (CBP) co-integrating protein (p/CIP) in transcriptional activation by STAT6. p/CIP, also called nuclear receptor co-activator-3 (NCoA-3), ACTR, or AIB1, is known to be one of the p160/SRC co-activator family members (19–21). The transcriptional co-activators CBP and p300 possess intrinsic histone acetyltransferase (HAT) activity that causes changes in chromatin structure facilitating access of nuclear factors, recruitment of RNA polymerase II, and the basal transcription machinery to DNA (22, 23). The NCoA/p160/SRC co-activators contribute to transcriptional activation mainly by recruitment of CBP/p300 to transcription factors (24–27). p/CIP (NCoA-3) has been demonstrated to enhance ligand-dependent transcription by several nuclear receptors and other CBP-dependent transcription factors such as STAT1 bound to the GAS promoter (19).

Because p300/CBP enhances STAT6-mediated transactivation through direct association (29) and p/CIP can interact with CBP/p300 (19), p/CIP may serve as a co-activator via p300/CBP protein in STAT6-dependent transcriptional activation. Here, we demonstrate that p/CIP protein expression is dramatically up-regulated by IL-4 in primary B cells at 4 h, with a peak expression at 24 h post-stimulation. Furthermore, the interaction of p/CIP with p300 bound to STAT6 can potentiate IL-4-
mediated promoter transactivation, and the interference of p/CIP binding to p300 by expression of the CBP-interacting domain of p/CIP results in a strong down-regulation of IL-4-mediated gene transcription. In addition, p/CIP interaction with the IL-4-inducible immunoglobulin heavy chain I epsilon promoter is increased in IL-4-stimulated B cells. This study suggests that p/CIP functions as a co-activator for STAT6-mediated activation of gene transcription through interaction with p300/CBP in a positive feedback loop.

MATERIALS AND METHODS

Primary Culture of B Cells and Cell Culture—Naïve splenic B cells from BALB/c (Charles River) mice were purified to >95% purity by means of negative selection using MACS® magnetic beads (Miltenyi Biotec, Auburn, CA) specific for anti-CD43 antibody as described previously (30). Isolated B cells were cultured at 2 × 10^6 cell/ml in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 1% sodium pyruvate, 1% nonessential amino acid, 50 μM 2-mercaptoethanol, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. 293T human embryonic kidney epithelial cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 1 mM sodium pyruvate, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. The murine B cell line M12.4.1 was maintained in RPMI 1640 supplemented with 10% FBS, 10 mM glucose, 2 mM L-glutamine, 10 mM HEPES, 100 units/ml of penicillin, and 100 μg/ml of streptomycin.

Plasmid Constructs—The STAT6 response element reporter construct was described previously (31). pRL-TK containing Renilla luciferase was purchased from Promega. HA-tagged p300 subcloned in pCMV was kindly donated by Dr. Bing-Feng Lu (Yale University). Xpress-tagged p/CIP was constructed by PCR amplification of the p/CIP end and 5'-CGGGGATCCACCATGGATTATAAAG-3' containing BamHI site at the 5'-end and 5'-GGCGCTCGAGTCAT-3' containing KpnI at the 5'-end and 5'-GGCGATATCGTCAGCAGTATTTCTGATCGG-3' containing BamHI at the 5'-end and 5'-GGCGATATCGTCAGCAGTATTTCTGATCGG-3' containing EcoRI and XhoI and ligated into pcDNA 3.1 (Invitrogen) which contains Xpress tag. Isolated B cells were cultured at 2 × 10^6 cells/ml. The transfected cells were lysed in cell lysis buffer described above. Thirty micrograms of whole cell lysate were used for SDS-PAGE, and Western blot was carried out with the indicated antibodies.

Luciferase Assays—293T cells were transfected using the calcium phosphate co-precipitation method. The cells were plated at a density of 2.5 × 10^6 in 12-well plates 24 h before transfection. For transfection, 0.1 μg of STAT6 response element reporter construct and 0.3 μg of STAT6 were used in combination with 0.1–1 μg of either Xpress-tagged p/CIP or FLAG-tagged p/CIP along with the packaging vector pCL-Eco. After 2 days of culture, the supernatants were collected and filtered through a 0.45-μm filter. M12.4.1 cells were plated at 2 × 10^6 cells/ml in 24-well plates and were incubated with virus-containing medium supernatant in the presence of polybrene for 24 h. This procedure was repeated once. Then the medium was changed to fresh culture medium for M12.4.1 cells and cultured for 2 more days before use.

Antibodies—Anti-p/CIP (F-2), anti-p300 (N-15), anti-STAT6 (M-20), anti-HA-probe (Y-11), and anti-His-probe (H-15) were purchased from Santa Cruz Biotecnology, Inc. Anti-STAT6 and anti-p/CIP were from Sigma, anti-Xpress mouse monoclonal IgG1 antibody was from Invitrogen, and anti-phosphotyrosine monoclonal mouse IgG2b antibody (4G10) was from Upstate Biotechnology, Inc. For flow cytometry, phycoerythrin-conjugated anti-CD23 and anti-CD16/CD32 (FcIII/II receptor) antibodies (Fc block) were purchased from PharMingen.

Co-immunoprecipitation, SDS-PAGE, and Western Blot—For co-immunoprecipitation assay, transfected 293T cells were cultured in the presence or absence of human IL-4 during the last 24 h of culture. They were then washed with ice-cold phosphate-buffered saline at 40 h post-transfection and lysed in cell lysis buffer (50 mM Tris, pH 8.0, 0.5% Nonidet P-40, 10% glycerol, 0.1 mM EDTA, 100 mM NaCl, 50 mM NaF, 1 mM Na3VO4, 1 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 3 μg/ml aprotinin, and 2 μg/ml pepstatin. The cell extracts (1 mg) were preclayed for 1 h followed by the addition of either protein A- or protein G-Sepharose at 4 °C for 2 h. Cleared extracts were immunoprecipitated with the indicated antibodies for 2 h followed by the addition of 30 μl of either protein A- or protein G-Sepharose at 4 °C for 2 h. The immunoprecipitates were washed in cell lysis buffer four times and separated by SDS-PAGE. The proteins were transferred to nitrocellulose membranes and analyzed by Western blot with the indicated antibodies diluted in TBS-T buffer (10 mM Tris, pH 7.5, 150 mM NaCl, and 1% Tween 20) supplemented with milk powder (5%), whereas only bovine serum albumin (1%) was added for phosphotyrosine antibody. The proteins were visualized using the ECL system (Amersham Biosciences). For straight Western blotting, cultured splenic B cells were stimulated with murine IL-4 for various durations of time and M12.4.1 cells infected with pMIG, pMIG-p/CIP, or pMIG-p/CIP were lysed in cell lysis buffer described above. Thirty micrograms of whole cell lysate were used for SDS-PAGE, and Western blot was carried out with the indicated antibodies.
an IL-4–stimulated transcriptional response by both p/CIP and p300. To determine whether IL-4 also induced p/CIP (NCoA-3) protein among the p160/SRC family members, only p/CIP (NCoA-3) mRNA is up-regulated by IL-4 through a STAT6-dependent mechanism in murine primary B cells (18), whereas NCoA-1 recently demonstrated by GeneChip expression analysis that, mRNA is up-regulated by IL-4 through a STAT6-dependent mechanism in murine primary B cells (18), whereas NCoA-1 recently demonstrated by GeneChip expression analysis that,

**RESULTS**

**Induction of p/CIP by IL-4 in Splenic B Cells**—We have recently demonstrated by GeneChip expression analysis that, among the p160/SRC family members, only p/CIP (NCoA-3) mRNA is up-regulated by IL-4 through a STAT6-dependent mechanism in murine primary B cells (18), whereas NCoA-1 and NCoA-2 mRNA are not up-regulated (data not shown). To determine whether IL-4 also induced p/CIP (NCoA-3) protein levels, we carried out Western blotting analysis using whole

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**Fig. 3. Interference of the interaction of p/CIP and p300 and the formation of a STAT6/p300/p/CIP complex by expression of the CBP-interacting domain of p/CIP (p/CIP<sub>p27-1104</sub>).** A, 293T cells were transfected with expression plasmids encoding Xpress-tagged p/CIP (10 μg) and HA-tagged p300 (10 μg) together with 5 or 10 μg of FLAG-tagged p/CIP<sub>p27-1104</sub>, or empty vector. Whole cell lysates (WCL) prepared at 40 h post-transfection were immunoprecipitated (IP) with anti-HA antibody (lanes 2, 4, and 6) or control antibody (lanes 1, 3, and 5) and separated by SDS-PAGE, transferred to nitrocellulose membrane, probed with anti-Xpress antibody to detect p/CIP, and reprobed with anti-HA antibodies to detect p300. Reciprocal immunoprecipitations were performed with anti-Xpress antibody (lanes 8–10) or control antibody (lanes 7, 9, and 11), followed by Western blotting with anti-HA antibody and reprobed with anti-Xpress antibody. Lysates from each transfection also were run on SDS-PAGE and blotting with anti-FLAG, anti-HA, and anti-Xpress antibodies to detect p/CIP and p300 and p/CIP. B, 293T cells were transfected with expression vectors encoding Xpress-tagged p/CIP (10 μg) and STAT6 (5 μg) together with 1 or 10 μg of FLAG-tagged p/CIP<sub>p27-1104</sub> or empty vector. Whole cell lysates were immunoprecipitated with anti-STAT6 antibody (lanes 2, 4, and 6) or control antibody (lanes 1, 3, and 5), followed by Western blotting with anti-Xpress antibody to detect p/CIP and reprobed with anti-STAT6. Lysates from each transfection were also run on SDS-PAGE and blotted with anti-Xpress and anti-FLAG antibodies to detect p/CIP and p/CIP<sub>p27-1104</sub>.

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diffected cells were stimulated with 10 ng/ml IL-4 for 24 h, after which cell extracts were prepared and assayed using a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions. Luciferase activity was normalized based on Renilla luciferase activity (relative luciferase activity). This experiment was repeated at least four times.

**Flow Cytometry**—Transduced M12.4.1 cells (2 × 10<sup>6</sup>/ml) were stimulated with IL-4 (kindly provided by W. Paul, National Institutes of Health) for 48 h. The cells were washed in cold phosphate-buffered saline with 1% FBS and stained with phycoerythrin-conjugated anti-mouse CD23 antibody in the presence of anti-Fc receptor antibody for 30 min on ice. The cells were washed in cold phosphate-buffered saline with 1% FBS and analyzed on a FACScan instrument. The mean fluorescence intensity of CD23 expression in GFP-transfected cells was assessed.

**Chromatin Immunoprecipitation**—Chromatin immunoprecipitation assays were performed based on the protocol described by Yu et al. (32). 15 × 10<sup>7</sup>/10 ml transduced M12.4.1 cells, after 4 h of IL-4 stimulation, were formaldehyde cross-linked by the addition of fixing solution (0.1 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 50 mM HEPES, pH 8.0, and 27% formaldehyde) directly into the medium to achieve a final concentration of 1% and incubated for 30 min at 37 °C. Formaldehyde was quenched with 0.125 mM glycine. The cells were washed, suspended in IP buffer (5 mM Pipes, pH 8.0, 85 mM NaCl, and 0.5% Nonidet P-40) containing protease and phosphatase inhibitors, pelleted, and resuspended in SDS lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris-HCl, pH 8.1) with protease and phosphatase inhibitors. The lysates were subsequently subjected to sonication to reduce DNA length to between 200 and 1,000 bp. The samples were diluted 10-fold by using dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, and 167 mM NaCl) and precleared by incubating with protein A beads overnight. Anti-STAT6 antibody or anti-His probe were added, and immunoprecipitation was done overnight at 4 °C with rotation. Immunocomplexes were collected with protein A agarose beads and eluted after extensive washing, and the cross-links were reversed by heating at 65 °C. Samples were subjected to proteinase K treatment. DNA was recovered by phenol-chloroform extraction, ethanol-precipitated, and used as a template for PCR (5-fold serial dilutions) using 1 epsilon promoter-specific primers (5'-CTAGAAGAGGCCTCACCTG-3' and 5'-GCGACAGTGTCTTTATTCG-3') and proteasome-specific primers (5'-TTTGAACTGGAGCTGCTAG-3' and 5'-ATGC-3'). The general conditions for PCR were the following: 95 °C for 5 min, 95 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min, and 72 °C for 5 min for 37 cycles. All PCRs were amplified in 25-μl mixtures containing 1× PCR buffer, 280 mM concentrations of each primer, 200 μM deoxynucleoside triphosphate, 1.5 mM MgSO<sub>4</sub>, and 1 unit of DNA platinum Taq polymerase (Sigma). PCR products were analyzed on a 2% agarose gel.
cell lysates obtained from mouse splenic B cells stimulated with IL-4. Low levels of p/CIP protein were detected in resting B cells. After IL-4 stimulation, the amount of p/CIP protein was dramatically increased at 4 h and peaked at 24 h, whereas there was little or no increase in p300 and STAT6 protein expression (Fig. 1).

Interaction of p/CIP with STAT6 Depends on p300/CBP—Previous studies have shown that among the p160 co-activator family, only NCoA-1, but not NCoA-2 and NCoA-3, directly bind to STAT6 and enhance the transactivation by STAT6 (28). Because transcriptional activation by STAT6 requires interactions with p300/CBP and p/CIP can interact with p300/CBP, we hypothesized that the up-regulated p/CIP might potentiate the transcriptional activity of STAT6 through p300/CBP. To analyze whether p/CIP interacts with STAT6 via p300, the protein interaction between STAT6 and p/CIP was investigated by co-immunoprecipitation experiments. 293T cells were transfected with expression vectors encoding STAT6 and Xpress-tagged p/CIP with or without p300, and 24 h later the cells were stimulated with IL-4 for 24 h. Whole cell lysates were prepared, and co-immunoprecipitation assays were carried out with anti-STAT6 monoclonal antibody. Immunoprecipitates were analyzed by Western blotting with anti-Xpress antibody. As shown in Fig. 2, anti-STAT6 antibodies co-immunoprecipitated a small amount of p/CIP irrespective of IL-4 stimulation (lanes 3, 4). Co-transfection with p300 strongly enhances the amount of p/CIP co-immunoprecipitated with anti-STAT6 (lanes 7, 8). Because no significant change in the amount of p/CIP was found in whole cell lysate (lanes 3, 4, 7, and 8), the interaction of p/CIP to STAT6 seems to be mediated via p300. The small amounts of p/CIP co-immunoprecipitated with anti-STAT6 in 293T cells not transfected with p300 (lanes 3 and 4) were due to the presence of endogenous p300 (lanes 3 and 4). No p/CIP co-immunoprecipitation was seen using control antibody instead of anti-STAT6 for immunoprecipitation (data not shown).

Next, we investigated whether the CBP-binding domain of p/CIP (p/CIP947–1084) would act as a competitor for the interaction of p/CIP and p300 in co-immunoprecipitation experiments. 293T cells were transfected with expression vector encoding Xpress-tagged p/CIP and HA-tagged p300 along with increasing amounts of FLAG-tagged p/CIP947–1084 expression vector.
Co-immunoprecipitation was reciprocally carried out with either anti-Xpress or anti-HA antibody, and the proteins were analyzed by Western blotting with anti-HA or anti-Xpress antibodies, respectively. As expected, p/CIP co-immunoprecipitated with anti-p300 (Fig. 3A, lanes 1–6) and vice versa. The amount of p300 that co-immunoprecipitated with anti-p/CIP (lanes 7–12) decreased dose-dependently when p/CIP947–1084 was co-transfected, although equal amounts of p300 and p/CIP were expressed (lanes 13–15). This indicates that p/CIP947–1084 can inhibit the interaction of p300 and p/CIP.

Furthermore, we investigated whether p/CIP947–1084 also prevents the formation of the STAT6/p300/p/CIP complex. 293T cells were transfected with expression vector encoding Xpress-tagged p/CIP and STAT6 along with increasing amounts of FLAG-tagged p/CIP947–1084 expression vector. Co-immunoprecipitation was carried out with anti-STAT6 antibody, and precipitates were analyzed by Western blotting with anti-Xpress. p/CIP co-immunoprecipitated with STAT6 decreased by co-transfection with p/CIP947–1084 in a dose-dependent manner (Fig. 3B, lanes 1–6). Similar amounts of STAT6 were precipitated in each lane, and p/CIP was expressed at similar levels in all lysates (lanes 2, 4, 6, and 7–9). This result suggests that p/CIP947–1084 prevents the formation of a STAT6/p300/p/CIP complex.

Endogenous p/CIP Potentiates STAT6 Transcriptional Activity—To investigate whether the p/CIP co-activator is involved in the transcriptional regulation exerted by IL-4, 293T cells were transfected with an expression vector for STAT6 and a luciferase reporter construct containing four copies of the STAT6 response element from the Ig heavy chain/H9280 promoter linked to the minimal thymidine kinase promoter together with increasing amounts of wild type p/CIP and p/CIP947–1084. When stimulated with IL-4, a dramatic increase in luciferase activity was observed. Overexpression of wild type p/CIP further increased the IL-4-dependent transactivation without affecting the basal activity. Enhancement of luciferase activity by overexpression of p/CIP upon IL-4 stimulation was only about 40% (Fig. 4A), suggesting that p/CIP is expressed at nearly saturating levels in 293T cells. In contrast, overexpression of p/CIP947–1084 markedly suppressed IL-4-stimulated reporter gene activation, suggesting that endogenous p/CIP positively regulates transcriptional activation by STAT6.

Next, we investigated whether overexpression of p/CIP or p/CIP947–1084 affects the phosphorylation of STAT6. As shown
NCoA-3 Regulates Transcriptional Activation by STAT6

in the murine immunoglobulin heavy chain germ line characterized functional STAT6-binding sites that are present be recruited to IL-4-inducible promoters by STAT6. We char-

The interaction of p/CIP and STAT6 and the enhancement of transcription by p/CIP suggests that p/CIP may —

Promoter in Murine B Lymphoid Cells Stimulated with IL-4

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FIG. 6. p/CIP interacts with the I epsilon promoter in M12.4.1 B cells stimulated with IL-4. M12.4.1 B cells were infected with retrovirus encoding empty vector or His-tagged wild type p/CIP. Transduced cells were stimulated with IL-4 for 4 h or left unstimulated. Chromatin immunoprecipitation assay was performed by use of a polyclonal anti-His (p/CIP) or anti-STAT6 antibody. Shown are serial dilutions (1:5) of immunoprecipitation fractions (right panel) comparing association of either p/CIP or STAT6 to the I epsilon promoter and the proteasome gene. Serial dilutions of IP fractions with a nonspecific antibody (IgG) are also shown (right panel, third level).

DISCUSSION

In a previous study, we have shown by using GeneChip expression analysis that p/CIP, but not NCoA-1 and NCoA-2, is up-regulated by IL-4 in splenic B cells through a STAT6-dependent mechanism (18). However, the functional role of this finding remained uncertain. In the present study, we have provided new evidence suggesting that STAT6 requires p/CIP as a co-activator for transcriptional activation.

The p160/steroid receptor co-activator family, NCoA-1, NCoA-2, and NCoA-3 (p/CIP), is thought to be involved in transcriptional activation by various nuclear proteins such as p53, AP-1, NF-kB, retinoic acid receptor, STAT1, STAT3, and...
STAT6 (19, 28, 35–41). Their co-activator activity is mainly mediated by the recruitment of other co-activators such as p300 and CBP to transcription factors and the basal transcription machinery (24–27). p300/CBP and NCoA-1 are known to enhance STAT6 transcriptional activation through direct association with STAT6 (28, 29, 38). In one study, the authors argued that p/CIP does not seem to be required for STAT6-mediated transcriptional activation, because p/CIP is not able to directly bind to STAT6, and overexpression of p/CIP did not affect STAT6 transcriptional activation (28).

In contrast, we have shown here that p/CIP associates with STAT6 in vivo as detected by co-immunoprecipitation, and the amount of co-precipitated p/CIP is enhanced by overexpression of p300. This suggests that p/CIP indirectly associates with STAT6 via p300. Interestingly, expression of the CBP-interacting domain of p/CIP (amino acids 947–1084), which blocks the binding of p/CIP to p300, prevented the formation of a STAT6/p/CIP/p300 complex and resulted in a dramatic decrease in STAT6-mediated transcriptional activation. Also, IL-4-induced CD23 expression was markedly inhibited by expression of the CBP-interacting domain of p/CIP in M12.4.1 B cells. These results indicate that endogenous p/CIP functions as a STAT6 co-activator. In addition, overexpression of wild type p/CIP resulted in a subtle but statistically significant enhancement of STAT6-mediated transcriptional activation and also enhanced IL-4-induced CD23 expression in B cells. This is inconsistent with the previous report demonstrating that overexpression of p/CIP showed no enhancement of transcriptional activation by STAT6 (28). The precise reason for this discrepancy remains uncertain. One possibility is that endogenous p/CIP is expressed at amounts of nearly saturating to completely saturating in the various cell types used in the different studies. We found detectable but obviously not limiting amounts of endogenous p/CIP both in M12.4.1 B cells and 293T cells (data not shown). Thus, overexpression of p/CIP enhanced STAT6-mediated transcription about 40% in 293T cells and enhanced CD23 surface expression about 200% in B cells.

In mouse primary splenic B cells, a small amount of p/CIP protein was detected by Western blotting analysis in primary B cells before IL-4 stimulation. This is consistent with our previous report demonstrating that p/CIP mRNA was expressed in resting B cells obtained from either STAT6−/− or STAT6+/− mice. This suggests that p/CIP can co-activate STAT6-mediated transcriptional activation immediately after IL-4 stimulation. After IL-4 stimulation, the level of p/CIP expression increased at 4 h and peaked at 24 h, after which it decreased to prestimulation levels.

In contrast, little or no increase in p300 and STAT6 protein expression or tyrosine phosphorylation was observed, further supporting a model of specific enhancement of STAT6-mediated transcriptional activation by p/CIP. Recently, STAT6 activation has been shown to be maintained in the nucleus indefinitely by continuous cycling of STAT6, i.e. phosphorylation, nuclear import, DNA binding, deactivation, nuclear export, and reactivation, under continuous IL-4 stimulation. In contrast, the half-life of phosphorylated STAT6 and STAT6 DNA binding activity was less than 1 h in the absence of IL-4 stimulation (42). This is consistent with the results of the chromatin immunoprecipitation assays, in which there was little or no binding to the I epsilon promoter without IL-4 stimulation. However, in the IL-4-stimulated samples, either with endogenous STAT6 alone or in combination with exogenous p/CIP, we saw a significant increase in binding activity to the I epsilon promoter. Because we demonstrated a strong tripartite interaction between p/CIP, STAT6, and p300 upon IL-4 stimulation, leading to enhanced STAT6-mediated luciferase activity, these findings support an important positive regulatory role for this complex in transcriptional activation leading to isotype switching. Interestingly, upon IL-4 stimulation the binding level to the I epsilon promoter of endogenous STAT6 in combination with overexpressed p/CIP did not prove to be significantly higher than endogenous STAT6 in combination with just empty vector, suggesting that p/CIP does not affect the affinity of STAT6 for this promoter.

Taken together, we propose a model where p/CIP enhances STAT6-mediated transcriptional activation immediately after IL-4 receptor triggering as well as during ongoing IL-4 stimulation. Because p/CIP induction by IL-4 peaks at 24 h of stimulation and drops nearly to prestimulation levels thereafter, p/CIP may also serve to limit STAT6-mediated gene transcription to some extent.

The precise mechanism by which endogenous p/CIP enhances transcriptional activation by STAT6 remains uncertain. Although NCoA-1 is considered to contribute to transcriptional activation through further recruitment of p300/CBP to STAT6 (28) and histone acetylation through its intrinsic HAT activity (43), there has been no evidence that p/CIP has HAT activity or can directly associate with STAT6. Therefore, we propose a possible mechanism where p/CIP causes a conformational alteration in CBP holoprotein, resulting in modulation not only of interactions with other co-activators, such as P/CAF (CBP-associated factor) (44), that possess HAT activity or the basal transcriptional machinery such as RNA polymerase II (45–47). The interaction of p/CIP with CBP may also result in modulation of the intrinsic HAT activity of p300/CBP (48). This change in HAT activity would probably lead to altered luciferase activity (49, 50). HAT activity of the CBP co-activator complex enhanced by interaction with p/CIP through p300/CBP might result in further STAT6 transcriptional activation through chromatin remodeling and thereby allow the basal transcriptional machinery at the promoter of target genes (51, 52).

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