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p300/CBP is required for transcriptional induction by interleukin-4 and interacts with Stat6

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ABSTRACT

Interleukin-4 (IL-4) induces tyrosine phosphorylation of the latent transcription factor Stat6, which mediates the transcriptional responses of IL-4. The transactivation domain of Stat6 has recently been mapped to the C-terminal region of Stat6. We have investigated the mechanism by which Stat6, through its transactivation domain, induces transcription. Previous studies have shown that diverse regulated transcription factors interact with coactivators such as p300 and CBP. We report that Stat6 used the interaction with p300/CBP to exert its stimulatory effects. Overexpression of p300/CBP increased IL-4-induced transcription of Stat6 activated reporter genes. The requirement of p300/CBP for Stat6-mediated transactivation is shown by coexpression of the adenovirus E1A protein. E1A repressed the IL-4-induced reporter gene activity, while mutants of E1A, which do not interact with p300/CBP, failed to block the IL-4-induced response. In addition, we found that the minimal transactivation domain of Stat6, when fused to the GAL4 DNA-binding domain, was repressed by E1A, whereas the fusion protein p300-VP16 increased the transcriptional activity. In two-hybrid protein interaction assays in mammalian cells, we mapped the interaction domain of CBP to a C-terminal region between amino acids 1850 and 2176, a region distinct from the interaction domain of CBP with Stat1, Stat2 or Stat5. Finally, we show that antibodies raised against p300 coimmunoprecipitated Stat6 and p300 from transfected COS7 cells and antibodies against Stat6 coimmunprecipitated endogenous Stat6 and CBP from Ba/F3 cells. Our data suggest that the transactivation domain of Stat6 makes contact with the basal transcription machinery by binding to p300/CBP.

INTRODUCTION

Stat6 is a member of the STAT (signal transducers and activators of transcription) family of transcription factors that are activated by various cytokines. Binding of the cytokine to its receptor at the cell surface induces receptor dimerization and activation of the cytoplasmic receptor associated Janus kinases (JAK). The JAK then phosphorylate a specific tyrosine residue, residue 641 in Stat6 (1), causing STAT dimerization, translocation to the nucleus and leading to specific DNA-binding to promoter sequences. This results in transactivation of the target genes (reviewed in 2,3). The different STAT molecules share significant homology in structure and function. Immediately upstream of the phosphorylated tyrosine residue, there is an SH2 domain which is involved in the recognition of the activated receptors and in the mediation of STAT dimerization. The DNA-binding domain (DBD) of Stat6 lies between amino acid residues 268 and 448 (4). The transactivation domain (TAD) of Stat6 has recently been mapped to a proline-rich region in the C-terminus of the protein. This domain alone is able to activate the transcription of a reporter gene when fused to the heterologous GAL4-DBD (5,6). Stat6 is activated in response to Interleukin-4 (IL-4) and IL-13 and it mediates many transcriptional responses of IL-4 (7,8). Stat6 DNA-binding sites have been identified in the promoter regions of several IL-4-inducible genes, including CD23, major histocompatibility complex class II, Ig class ε and γ1, the milk protein β-casein and the 3β-hydroxysteroid dehydrogenase (3β-HSD) type 1 (6,9–13). Studies performed with Stat6-deficient mice have confirmed that Stat6 plays an essential role in IL-4 and IL-13 signaling (14,15).

The CREB-binding protein (CBP) was first identified as a co-activator for the cAMP response element binding protein (CREB) (16), while p300 was cloned as a protein associated with the adenoviral E1A protein (17). CBP and p300 are two ubiquitously expressed nuclear proteins, they share sequence homology and have overlapping functions (18–20). This is emphasized by the similarities in the phenotype of p300 (–/–), CBP (–/–) and compound heterozygous knockouts (21). Although it was recently demonstrated that p300 and CBP have distinct roles in retinoic acid induced differentiation and transcription (22), there is no evidence, to date, suggesting that the other transcription factors specifically require CBP or p300. Therefore, p300/CBP will be used throughout the remainder of this manuscript when discussing properties of both p300 and CBP. It was shown that p300/CBP do not bind to DNA by themselves, but can be recruited to promoter elements by interaction with different sequence-specific DNA-binding factors. They act as coactivators for several classes of transcription factors such as c-Jun (23,24), c-Fos (25), NF-κB

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(26,27), Sap-1a (28), pp90RSK (29), p53 (30,31), several members of the nuclear receptor superfamily (32–39) and different steroid-receptor coactivators (40–43). Recently it has been shown that Stat1, Stat2 and Stat5 also interact with p300/CBP (40,44,45). It appears that p300/CBP stimulate the transcription of target genes by their intrinsic histone acetyltransferase activity (46,47) and by their association with another histone acetyltransferase, p/CAF (48). In addition, p300/CBP can act as co-activators or adapters by bridging transcription factors to basal transcription machinery, e.g. TFIIB, TBP and RNA helicase A (49–51). The adenovirus protein E1A binds to p300/CBP, which is essential for its transforming and mitogenic activity. E1A suppresses several transcription control elements by binding directly and sequestering p300/CBP (17). Because different pathways may interfere with each other by competing for limiting amounts of the coactivators p300/CBP, these proteins CBP/p300 serve as integrators of multiple signal transduction pathways within the nucleus (29,43,52).

In this study we have investigated whether Stat6 interacts *in vivo* with p300/CBP. We report that p300/CBP are coactivators for Stat6 and that they are associated with Stat6 *in vivo*. This study provides further insight into the mechanisms between the binding of Stat6 to its response element and the transmission of the signal to the basal transcription apparatus.

MATERIALS AND METHODS

Cell culture and transfection

HeLa cells, COS7 cells and 293 cells were cultured in Dulbecco's modified Eagles' medium (DMEM) supplemented with 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin and 50 µg/ml streptomycin sulfate. COS7 cells were plated at a density of 500 000 cells per 10 cm dish or 100 000 cells in 6-well plates. HeLa and 293 cells were plated at a density of 500 000 cells per 10 cm dish or 200 000 cells in 6-well plates. Cells were transfected by either the calcium phosphate precipitation technique or with the ExGen 500 reagent (Euromedex) according to the manufacturer's instructions. One day after transfection, the cells were treated, where indicated, for 12 h with 10 ng/ml IL-4. IL-4 was purchased from R&D Systems (Minneapolis, MN). Two days after transfection cells were harvested, washed twice in cold PBS, and lysed in 25 mM glycylglycine, pH 7.8, 1 mM dithiothreitol (DTT), 15 mM $MgSO_4$, 4 mM EGTA and 1% Triton for 10 min at 4°C. Samples were centrifuged for 5 min at 14 000 r.p.m. Cleared supernatant were used for luciferase and β-galactosidase assays. For β-galactosidase determination, 20 µl of extract were added to 200 µl of reaction buffer containing 100 mM Na-phosphate, pH 8.0, 1 mM $MgCl_2$ and 1× Galacton (Tropix, Bredford, MA) and incubated for 30 min at room temperature. Measurements were made by injecting 300 µl of accelerator solution (10% Emerald luminescent amplifier and 0.2 N NaOH) and the samples were counted for 20 s in the luminometer 953 (Berthold, Pforzheim, Germany). Luciferase activities (100 µl extracts and 300 µl accelerator solution) were quantified in the same apparatus and normalized to the β-galactosidase activities. All experiments were repeated at least three times. Typical results and the average of three independent experiments with standard deviation are shown. The relative luciferase activities are given as arbitrary units.

Plasmids

The reporter genes: β-casein luciferase, (GAL4)3TK luciferase, N4(STAT-RE)3 luciferase, cytokine inducible sequence luciferase (CIS-LUC), oncostatin M luciferase (OSM-LUC) and the expression vectors pXM-hStat6, pXM-IL-4Rα, CMX-GAL4 and GAL4-Stat6 were described previously by Moriggl *et al.* (6). The reporter gene 3β-HSD type 2 luciferase contained the sequences from –496 to +53 of the human 3β-hydroxysteroid dehydrogenase (3β-HSD) type 2 gene. This fragment was amplified by PCR with primer containing *Xba*I linkers and cloned into the *Nhe*I site of the pGL3 vector. Expression vector encoding E1A 12S or E1A 12S mutants were described by Bannister *et al.* (25). p300 and p300-VP16 were provided by R. Eckner and mCBP expression vector were provided by R. Goodman. The series of GAL4-CBP fusion proteins were obtained by inserting each corresponding region (obtained by restriction enzyme digestion or by PCR) into the required site of CMX-GAL4 to preserve the reading frame. Orientation and insertion of all constructs was confirmed by digestion and DNA sequencing. pcDNA3-VP16 was generated by amplifying VP16 (amino acids 411–489) with primers containing additional sequences. The 5'-primer contained the following additional sequences: a unique *Hin*dIII site, a Kozak sequence, a start codon, the sequence encoding the nuclear localization signal (NLS) from SV40 large T antigen (amino acids 126–132), an *Eco*RI site, an *Eco*RV site and a *Bam*HI site all in the same reading frame as VP16. The downstream primer contained a stop codon and a unique *Xba*I site. The PCR fragment containing the VP16 sequence was cloned as *Hin*dIII–*Xba*I fragment into the *Hin*dIII and *Xba*I sites of pcDNA3. Stat6-VP16 fusion proteins were generated by PCR amplification of the Stat6 sequence, corresponding to amino acids residues 2–268, 269–448, 449–641 and 642–847, with downstream primers containing a unique *Eco*RI site and upstream primers containing either a unique *Bgl*II or *Bam*HI site. The digested fragments were cloned into *Bam*HI and *Eco*RI sites of pcDNA3-VP16. The control reporter vector CMV-βGal was drived from Stratagene (La Jolla, CA). 1 µg of the control reporter vector CMV-βGal was transfected per 10 cm plate.

Coimmunoprecipitation

COS7 cells were transfected with expression plasmids encoding Stat6 and p300. One day after transfection, the cells were treated with 10 ng/ml IL-4 for 1 h prior to harvesting. Whole cell extracts were prepared and immunoprecipitation was carried out with p300-specific monoclonal antibodies (Upstate Biotechnology, Lake Placid, NY) or with antibodies against GAL4-DBD as a control (Santa Cruz biotechnology, Santa Cruz, CA). The immunoprecipitates were separated by SDS–PAGE and the western blots were analyzed using Stat6-specific antibodies (Santa Cruz biotechnology, Santa Cruz, CA). The murine pre-B-cell line Ba/F3 was cultured in RRMI medium containing 4% Wehi supernatant, 10% FCS, 2 mM L-glutamine and penicillin–streptomycin (10 U/ml and 10 µg/ml, respectively). Before induction, Ba/F3 cells were starved in medium with 3% horse serum for 3 h in the absence of Wehi supernatant and then stimulated with 100 U/ml of mIL-4 for 15 min prior to harvesting. Whole cell extracts were prepared and immunoprecipitation was carried out with Stat6-specific monoclonal antibodies (Transduction Laboratories, Lexington, KY) or

with antibodies against GAL4-DBD as a control (Santa Cruz biotechnology, Santa Cruz, CA). The immunoprecipitates were separated by SDS–PAGE and the western blots were analyzed using CBP-specific antibodies (Santa Cruz biotechnology, Santa Cruz, CA).

RESULTS

P300/CBP enhance the activation of transcription by Stat6

P300 is a protein with intrinsic histone acetyltransferase activity and has been found to be a crucial contributor to activation by diverse classes of exogenously regulated transcription factors. In order to investigate whether the p300 coactivator is also involved in the transcriptional regulation exerted by Stat6, 293 cells were transfected with an expression vector for Stat6 and a luciferase reporter construct containing three copies of the Stat6 response element from the Ig heavy-chain ε promoter linked to the TK promoter N4(STAT-RE)3LUC. IL-4 treatment of the cells caused a 4-fold stimulation of the reporter gene (Fig. 1A, lanes 1 and 2). Cotransfection of p300 expression vector further increased the IL-4-dependent transactivation up to 17-fold (lanes 3 and 4), whereas p300 had no significant effect on the basal activity of the Stat6 responsive promoter.

To investigate whether p300-mediated coactivation is dependent on a particular Stat6 responsive promoter, we have further analyzed the effect of p300 on the IL-4 induction of four different cytokine-inducible promoter constructs. The β-casein gene promoter, the 3β-HSD type 2 gene promoter, the cytokine-inducible SH2-containing protein (CIS) gene promoter and the oncostatin M (OSM) gene promoter were used in transfection assays. The β-casein gene was originally identified as a prolactin-inducible Stat5 responsive gene, but it was subsequently shown to be also induced by IL-4 via Stat6 (6). The 3β-HSD type 2 promoter also contains Stat6 responsive elements (S.Gingras and J.Simard, manuscript in preparation). The CIS and OSM were previously identified as immediate early cytokine-responsive genes, which contain Stat5 responsive elements in their promoter sequences (53,54), but can also be activated by Stat6 (54). The β-casein-luciferase construct was stimulated nearly 4-fold upon IL-4 treatment, whereas coexpression of p300 further enhanced the IL-4-induced activity of the β-casein reporter gene up to 38-fold (Fig. 1B). The 3β-HSD type 2 luciferase gene construct was stimulated 3-fold upon IL-4 treatment, coexpression of p300 further enhanced the IL-4 induced activity up to 11-fold (Fig. 1C). Interestingly, although no effect of IL-4 was observed on the CIS-luciferase and OSM-luciferase reporter constructs in 293 cells, co-transfection of p300 enhanced the activity in an IL-4-dependent manner (Fig. 1D and E). The fact that p300 induced the basal activities of these promoters suggests that ubiquitous transcription factors, which bind to these promoters and are responsible for the constitutive expression, are also able to contact p300/CBP. In summary, these results indicate that p300 acts as a cofactor in the Stat6-mediated transcriptional activation.

E1A inhibits IL-4-induced transactivation by Stat6

The adenovirus E1A protein has been shown to inhibit the induction of transcription, which is dependent on its ability to bind and sequester p300/CBP (20,55). To further demonstrate the involvement of p300/CBP in Stat6-mediated induction of

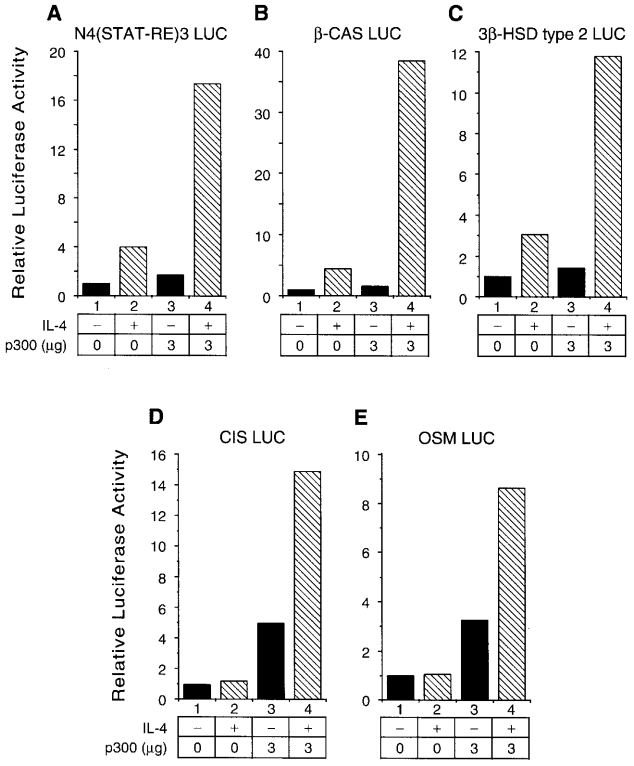

Figure 1. IL-4-induced transcriptional activation by Stat6 is enhanced by p300. The luciferase reporter gene constructs containing either (**A**) N4(STAT-RE)3, (**B**) the β-casein, (**C**) 3β-HSD type 2, (**D**) the cytokine inducible sequence or (**E**) OSM gene promoter (1 μg) were transfected in 293 cells with plasmids expressing hStat6 (1 μg) and IL-4Rα (1 μg) and expression vectors encoding p300 (3 μg), when indicated. Transfected cells were either untreated (filled bars) or treated for 12 h with h-IL-4 (10 ng/ml) (striped bars). All experiments were repeated at least three times. Typical results are shown.

transcription, we studied whether E1A was able to repress the transcriptional induction by activated Stat6. For these experiments, transient transfection assays were carried out in COS7 cells to avoid any effects from endogenous E1A present in adenovirus transformed 293 cells. COS7 cells transfected with an expression vector for Stat6 and the N4(STAT-RE)3 luciferase reporter construct were treated with IL-4. Cotransfection of increasing amounts of an expression vector for E1A 12S resulted in a dose-dependent inhibition of the observed IL-4 induction (Fig. 2A). The basal activity of the Stat6 responsive reporter in untreated cells was not inhibited by E1A (Fig. 2A, lanes 3 and 5), indicating that E1A specifically inhibits Stat6-mediated transcriptional induction.

To determine whether the inhibitory effect of E1A was due to competition for limited amounts of p300/CBP, we characterized the domains of E1A 12S involved in the suppression of Stat6-induced transcription. The deletion mutant ΔCR1 of E1A lacks the p300 and the Rb binding functions, whereas the mutant ΔCR2 lacks the Rb binding function, but retains its p300 binding function. In addition, more specific mutants of the CR1 domain were analyzed: the E1A-mut CBP is unable to bind p300, but is still able to bind Rb; the E1A-mut Rb is not affected in its ability to interact with p300/CBP, but is unable to bind Rb (25). The structures of these molecules are schematically shown in Figure 2B. The expression of the ΔCR1 mutant

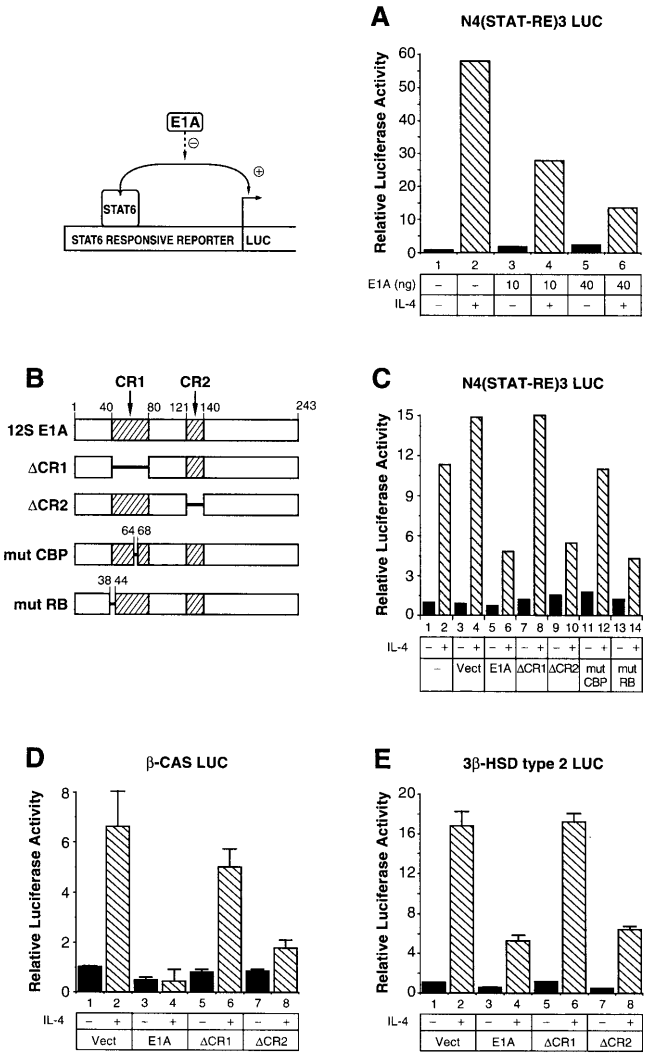

Figure 2. E1A suppresses IL-4-induced transactivation by Stat6. (**A**) COS7 cells were transfected with an expression vector encoding hStat6 (2.5 μg) and a luciferase reporter gene (2 μg) containing three GAS elements with a 4 bp spacing in front of the thymidine kinase promoter [N4(STAT-RE)3 LUC]. The indicated amount of expression plasmid encoding E1A was cotransfected as indicated. (**B**) The schematic structures of E1A and the E1A mutants (ΔCR1, ΔCR2, mut CBP and mut RB) used in this study are shown. (**C**) COS7 cells were transfected with expression vectors encoding hStat6 (2.5 μg) and the N4(STAT-RE)3 luciferase reporter (2 μg). The expression vector encoding E1A or E1A mutants were cotransfected as indicated. (**D**) HeLa cells were transfected with expression vectors encoding hStat6 (0.5 μg), IL-4Rα (0.5 μg) and the β-casein luciferase reporter (0.5 μg). The expression vector encoding E1A or E1A mutants were cotransfected as indicated. In each panel, the transfected cells were either untreated or treated for 16 h with h-IL-4 (10 ng/ml) as indicated. (**E**) The experiment was performed as in (D) except that the 3β-HSD type 2 luciferase reporter was used. All experiments were repeated at least three times. Typical results and the average of three independent experiments with standard deviation are shown.

did not interfere with Stat6-induced transcription (Fig. 2C, lanes 7 and 8), while the ΔCR2 mutant inhibited IL-4-induced transcription (lanes 9 and 10) to a similar extent as wild type E1A (lanes 5 and 6). The E1A-mut CBP did not affect IL-4 inducibility of the reporter construct (lanes 11 and 12), whereas the E1A-mut Rb was as effective as wild type E1A in repressing

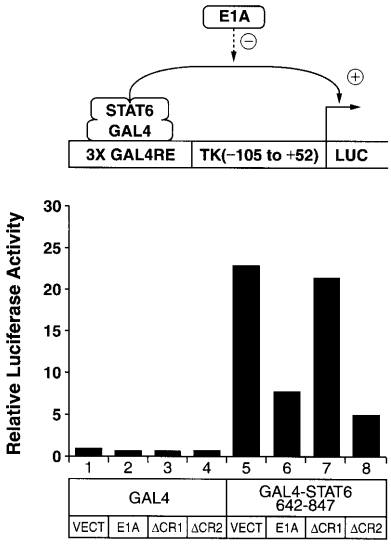

Figure 3. E1A suppresses the induction of transcription by Stat6 C-terminus. The reporter plasmid (GAL4)3TK-luciferase (0.5 μg) was transfected in COS-7 cells with plasmids expressing the transactivation domain of hStat6 (amino acids 642–847) fused to the GAL4 DBD (0.5 μg) or a plasmid encoding the GAL4 DBD alone. The expression vector encoding E1A or E1A mutants (0.1 μg) were cotransfected as indicated. All experiments were repeated at least three times.

IL-4-induced luciferase activity (lanes 13 and 14). This indicates that the repression by different E1A proteins correlates with their ability to bind to p300/CBP. These results were supported by cotransfection experiments in HeLa cells, where the effects of E1A and mutants thereof were analyzed on IL-4-responsive promoters: the β-casein and the 3β-HSD type 2 reporter genes (Fig. 2D and E).

The transactivation activity of the C-terminus of Stat6 is repressed by E1A

The TAD of Stat6 (amino acids 642–847) is autonomously active when fused to the heterologous GAL4-DBD (6). To determine if the C-terminal domain of Stat6 mediates transcriptional activation by interacting with p300/CBP, we analyzed whether E1A is able to repress the activity of a GAL4-Stat6 fusion protein as it represses the full-length Stat6. Expression vectors for GAL4-Stat6 (amino acids 642–847) and the (GAL4)3TK-luciferase reporter were transfected into HeLa cells. GAL4-Stat6 (amino acids 642–847) increased the activity of the reporter construct and this activation was dependent upon the Stat6 C-terminal domain (Fig. 3 compare lanes 1 and 5). Cotransfection of expression vector encoding wild-type E1A inhibited transcriptional activation by GAL4-Stat6. The CR1 domain, but not the CR2 domain, of E1A was necessary for this inhibition (lanes 7 and 8). These results demonstrate that E1A specifically represses the transcriptional activation function of the Stat6 TAD independently of the DBD.

P300/CBP interacts with the transactivation domain of Stat6

Since p300/CBP act as Stat6 coactivators, we investigated the potential interaction of these proteins in a mammalian two-hybrid assay. The GAL4-Stat6 (amino acids 642–847) fusion

construct was transfected with the (GAL4)3TK-luciferase reporter in HeLa cells. GAL4-Stat6 (amino acids 642–847) activated the transcriptional activity of the reporter construct (Fig. 4, lane 5), the addition of increasing amounts of a p300-VP16 expression vector, encoding p300 fused to the TAD of VP16 significantly enhanced the activity of the reporter gene (Fig. 4, lanes 6 and 7). Transfection of p300-VP16 in the presence of a control expression vector, encoding only the GAL4-DBD had no effect on the reporter construct activity (Fig. 4, lanes 2 and 3). In order to determine if the C-terminal TAD of Stat6 was responsible for the interaction with p300/CBP, smaller fragments of Stat6 C-terminus were fused to the GAL4-DBD. The deletion-mutants of the TAD tested were able to transactivate the GAL4 reporter plasmid when transfected into HeLa cells and they were still able to interact with p300/CBP (Fig. 4, lanes 8–16). These results indicate that the TAD of Stat6 interacts with p300 and that the transactivation function of the Stat6 C-terminus correlates with its ability to interact with p300/CBP.

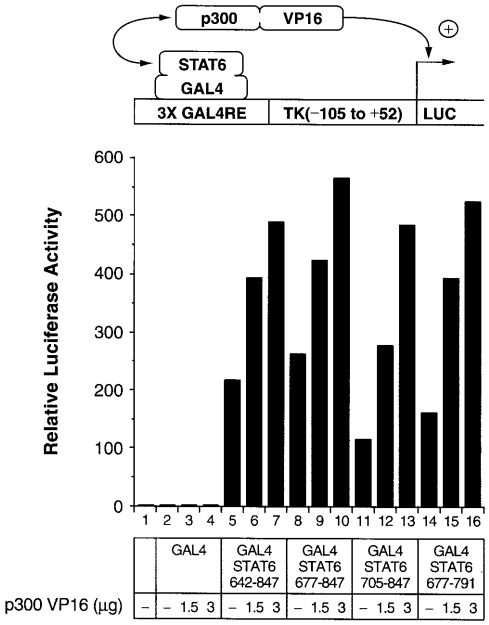

Figure 4. p300 interacts with the transactivation domain of Stat6 in the mammalian two-hybrid assay. The reporter plasmid (GAL4)3TK-luciferase (2.5 μg) was transfected in HeLa cells with plasmids expressing the C-terminal transactivation domain of hStat6 (amino acids 642–847, 677–847, 705–847 or 677–791) fused to the GAL4 DBD (1 μg) or a plasmid encoding the GAL4 DBD alone or in combination with increasing amounts of the expression vector encoding p300 fused to the VP16 transactivation domain as indicated. All experiments were repeated at least three times.

Stat6 transactivation domain interacts with amino acids 1850–2176 of CBP

Our experiments so far indicate that p300/CBP act as coactivators in the transactivation process mediated by Stat6 and directly interact with the TAD of Stat6. To delineate the domain of CBP required for the interaction with Stat6, several fusion proteins were constructed and analyzed in the mammalian two-hybrid assay. Different domains of CBP were fused to the GAL4-DBD and distinct portions of Stat6 were fused to the potent TAD of VP16 (schematized in Fig. 5A and B, respectively). As previously reported, GAL4-CBP1–452 and GAL4-CBP452–721 have transcription activity (28,56), but their transcriptional activity was not modulated by any of the Stat6-VP16 fusion proteins (Fig. 5C). Importantly, the Stat6-642–847-VP16 fusion protein stimulated specifically the reporter activity when cotransfected with GAL-CBP-1678–2441, but not when GAL4-DBD alone or GAL4-DBD fused to other CBP N-terminal regions were cotransfected. Furthermore, other regions of Stat6 fused to the VP16-TAD or the VP16-TAD alone failed to interact with any GAL4-CBP constructs. To further narrow the Stat6 interaction domain of CBP, other GAL4-CBP fusion proteins were analyzed in transfection experiments. Neither GAL4-CBP1460–1891 nor GAL4-CBP1891–2441 interacts with Stat6-TAD suggesting that the Stat6 interaction domain of CBP spanned amino acid 1891. In fact, the smallest segment of CBP, which was still able to interact with Stat6-TAD, is amino acids 1850–2176. Further deletion at the N-terminus, from 1850 to 1891, or at the C-terminus, from 2176 to 2039, abolished the interaction with the Stat6-TAD.

Stat6 interacts with p300/CBP *in vivo*

To confirm the direct interaction between Stat6 and p300/CBP, COS7 cells were transfected with expression vectors encoding Stat6 and p300 and coimmunoprecipitation experiments were performed. Transfected cells were either treated with IL-4 (Fig. 6A, lanes 1–4) or were untreated (lanes 5–8). Whole cell extracts were prepared and proteins were immunoprecipitated with p300-specific antibodies (Fig. 6A, lanes 2 and 6) or with an unrelated antibody (lanes 4 and 8). The immunoprecipitates were analyzed by western blotting with an antiserum specific for Stat6. Figure 6A shows that p300-specific antibodies coimmunoprecipitate a small amount of Stat6. This interaction is independent of the activation state of Stat6, since IL-4-activated as well as the latent form of Stat6 can be immunoprecipitated. When control antibodies with specificity for the GAL4-DBD were used in the immunoprecipitation reaction, no Stat6 was detected. To investigate further whether endogenous CBP and Stat6 interact, we performed additional coimmunoprecipitation experiments using extracts from the murine pre-B-cell line Ba/F3. We used this cell line because it expresses high amounts of Stat6 and IL-4 receptor chains and several studies on IL-4-dependent gene regulation were performed in this cell line. Whole cell extracts from IL-4-induced Ba/F3 cells were immunoprecipitated with Stat6-specific antibodies (Fig. 6B, lane 3) or with unrelated antibodies (lane 1). The immunoprecipitates were analyzed by western blotting with an antiserum specific for the N-terminus of CBP. These antibodies recognized several protein species in Ba/F3 cell extract which presumably correspond to full-length CBP as well as proteolytic degradation products of CBP (lane 1). Stat6-specific antibodies (lane 2) but not unrelated antibodies against the GAL4-DBD (lane 2) coprecipitated only full-length CBP. These results further confirm the presence of specific complexes of p300/CBP and Stat6 *in vivo* and suggest that endogenous p300/CBP and Stat6 interact in Ba/F3 cells.

DISCUSSION

The aim of this study was to determine whether p300/CBP is critical for Stat6-induced transcription. Our data show that

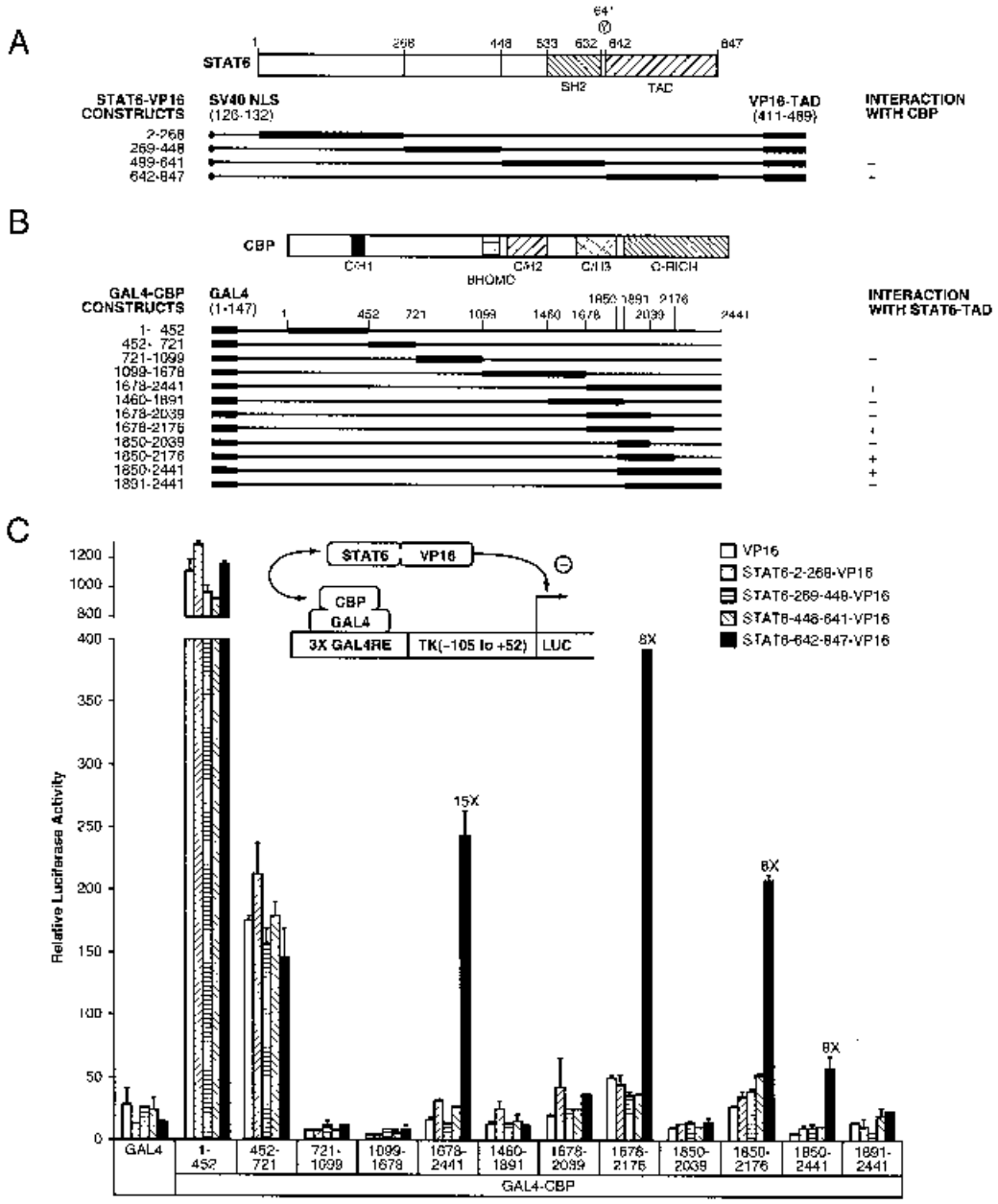

Figure 5. Stat6 transactivation domain interacts with the C-terminus of CBP in the mammalian two-hybrid assay. (**A**) Structure of the Stat6-VP16 fusion proteins. Different regions of Stat6 were fused with an NLS from SV40 large T antigen (amino acids 126–132) at the N-terminus and with the TAD of herpes simplex virus protein VP16 (amino acids 411–489) at the C-terminus. (**B**) Structure of GAL4-CBP fusion proteins. The yeast GAL4 DBD (amino acids 1–147) was fused to different regions of CBP. The different domains of CBP, such as cysteine/histidine (C/H) domains, the bromo domain and the glutamine (Q)-rich domain are indicated. (**C**) The reporter plasmid (GAL4)3TK-luciferase (0.25 μg) was transfected in COS7 cells with 0.25 μg of plasmids expressing GAL4-mCBP fusion protein and with 0.25 μg of expression vectors encoding Stat6-VP16 fusion protein as indicated. All experiments were repeated at least three times. The average of three independent experiments with standard deviation are shown.

overexpression of p300/CBP increases IL-4-induced, Stat6-mediated transcription. We have shown that IL-4 induction of transcription mediated by Stat6 is repressed by E1A. Our results also demonstrate that E1A was able to inhibit the transcriptional activity induced by the Stat6 TAD fused to a GAL4-DBD. Thus the inhibitory effect of E1A on Stat6-mediated transcription is specifically due to its ability to interfere with the transactivation function of Stat6. The involvement of p300/CBP in Stat6 transactivation is emphasized by the fact that the adenovirus protein E1A repression of Stat6-mediated transcription is dependent on its ability to bind to p300/CBP. In fact, mutants of E1A, unable to interact with p300/CBP, were also unable to inhibit Stat6-mediated transcription.

The functional interaction between Stat6 and p300/CBP was shown by two approaches: a mammalian two-hybrid assay and coimmunoprecipitation. First, the transcription activity of

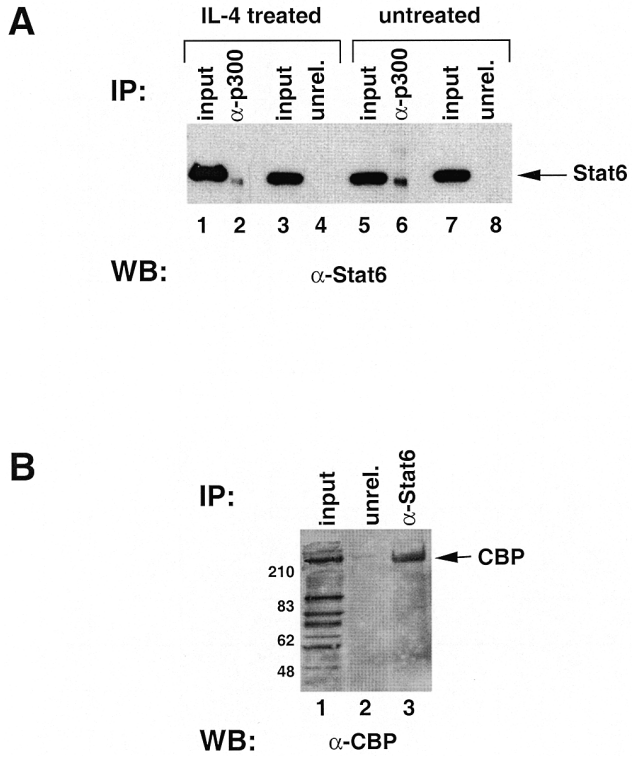

Figure 6. Stat6 coimmunoprecipitates with p300/CBP. (**A**) COS7 cells were transfected with expression vectors encoding p300 and Stat6. Cells were treated for 1 h with 10 ng/ml hIL-4 or left untreated as indicated, and whole cell extracts were prepared. One-tenth of each extract was loaded directly onto an SDS–polyacrylamide gel (lanes 1, 3, 5 and 7). The rest was immunoprecipitated with p300-specific antibodies (lanes 2 and 6), or with unrelated antibodies (lanes 4 and 8). All samples were analyzed by western blotting with an antiserum against the C-terminus of Stat6. (**B**) Ba/F3 cells were stimulated with 100 U/ml of mIL-4 for 15 min prior to preparing whole cell extracts. One-twentieth of the extracts was loaded as an input control directly onto the SDS–polyacrylamide gel (lane 1). The rest was immunoprecipitated with Stat6-specific antibodies (lane 3), or with unrelated antibodies (lane 2). All samples were analyzed by western blotting with an antiserum against the N-terminus of CBP.

Stat6-TAD fused to GAL4-DBD was increased by cotransfection of p300-VP16 using the two-hybrid assay. In the reciprocal assay, we have demonstrated that the Stat6 TAD was necessary and sufficient for the interaction with the domain spanning amino acids 1850–2176 of CBP fused to GAL4-DBD. Consequently, the C-terminus of Stat6 has transactivation activity that correlates with the ability to make contact with p300/CBP. The identified region of CBP interacting with Stat6 is different from the regions previously described for the binding of Stat1, Stat2 or Stat5 (40,44,45). Although the different STAT proteins belong to the same family due to the similar structure and mode of action, each member of the STAT protein family makes contact with p300/CBP in a unique fashion. This is in contrast to members of the nuclear receptor family which all interact with the same domain of p300/CBP. Interestingly, the domain of CBP, which interacts with Stat6 has already been reported to be the region of interaction with three proteins of the family of the steroid receptor coactivators (SRC), namely SRC-1, TIF-2 (GRIP-1, SRC-2) and p/CIP (RAC3, ACTR, AIB1, SRC-3) (35,40,42,43). From our data, we could not conclude whether the binding of Stat6 or SRC family members to p300/CBP would be exclusive or cooperative. Stat6 and p300/CBP might be part of a larger complex containing more proteins and we cannot exclude that other proteins might be involved in the interaction of Stat6 with basal transcription apparatus. Such a complex has already been described for the nuclear receptors and their coactivator SRC-1, which bind to each other and to p300/CBP simultaneously (40,41,57). Moreover, it should be noted that injection of an antibody against p/CIP entirely inhibited Stat-1-dependent transcriptional activation events (40), indicating that other proteins are closely associated with p300/CBP and such proteins also play an essential role in p300/CBP associated functions.

The results obtained from the mammalian two-hybrid assay are in agreement with the outcome of the coimmunoprecipitation assay. The mammalian two-hybrid assays demonstrate that the interaction between Stat6 and CBP occurs even in the absence of IL-4 treatment and, in accordance with that, coimmunoprecipitation of full-length Stat6 with p300 is also observed in the absence of IL-4. However, we do not believe that the interaction between Stat6 and CBP observed in the absence of IL-4 is relevant for transcriptional regulation by Stat6, because the phosphorylation of Stat6 is a prerequisite for its nuclear translocation and DNA-binding activity (1) while CBP in contrast is a nuclear protein (16). In addition, it is possible that the interaction of Stat6 with p300/CBP *per se* is not sufficient to cause transcriptional coactivation. This would be similar to the interaction described for Sap-1a, which can interact with CBP in an unphosphorylated form, but the phosphorylation of Sap-1a was necessary for coactivation by CBP. The authors suggested that the TAD of CBP are masked and phosphorylation of Sap-1a could induce a conformational change in CBP that allowed access to these domains (28).

P300/CBP possesses intrinsic histone acetyltransferase activity and acetylation of the histones is associated with chromatin decondensation. Histone acetyltransferase enzymes are also capable of acetylating components of the general RNA polymerase II machinery (58). In addition, p300/CBP interacts with components of the basal transcription machinery, suggesting that recruitment of the transcription machinery and post-transcriptional modification of certain components, as well as chromatin remodeling, may contribute to the molecular function of the coactivators. In this report, the effect of p300/CBP on Stat6-mediated induction of transcription in transient transfection is probably due to increased recruitment of the transcription machinery, while histone acetyltransferase is unlikely to play an important role.

In conclusion, our results demonstrate that p300/CBP are bona fide coactivators for Stat6, and that the ability of p300/CBP to form multi-protein complexes may provide a key to signal integration. Different transcription factors activated by diverse signaling pathways may interfere with one another by competing for limiting common cofactors. The results presented here suggest that IL-4 signaling may interfere with other signaling pathways or conversely that IL-4 responses could be inhibited by costimulation with other signals.

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