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Acetylation by Histone Acetyltransferase CREB-binding Protein/p300 of STAT6 Is Required for Transcriptional Activation of the 15-Lipoxygenase-1 Gene*

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Interleukin-4 (IL-4) induces expression of reticulocyte-type 15-lipoxygenase-1 (15-LOX-1) in various mammalian cells via the Janus kinase/signal transducer and activator of transcription 6 (STAT6) signaling system. We studied the mechanism of 15-LOX-1 induction in A549 lung epithelial cells and found that genistein, a potent tyrosine kinase inhibitor, prevented phopsphorylation of STAT6, its binding to the 15-LOX-1 promoter, and the expression of catalytically active enzyme. In contrast, cycloheximide did not prevent 15-LOX-1 induction. Surprisingly, we found that IL-4 up-regulated the histone acetyltransferase activity of CREB-binding protein (CBP)/p300, which is responsible for acetylation of nuclear histones and STAT6. The acetylation of both proteins appears to be essential for the IL-4-induced signal transduction cascade, because inhibition of CBP/p300 by the viral wild-type E1A oncoprotein abrogated acetylation of both histones and STAT6 and strongly suppressed transcriptional activation of the 15-LOX-1 gene. Moreover, we found that the inhibition by sodium butyrate of histone deacetylases, which apparently suppress 15-LOX-1 gene transcription, synergistically enhanced the IL-4-stimulated 15-LOX-1 expression. These data suggest that both phosphorylation and acetylation of STAT6 as well as acetylation of nuclear histones are involved in transcriptional activation of the 15-LOX-1 gene, although these reactions follow differential kinetics. STAT6 phosphorylation proceeds within the first hour of IL-4 stimulation. In contrast, CBP/p300-mediated acetylation requires 9–11 h, and similar kinetics were observed for the expression of the active enzyme. Thus, our results suggest that in the absence of IL-4, nuclear histones may be bound to regulatory elements of the 15-LOX-1 gene, preventing its transcription. IL-4 stimulation causes rapid phosphorylation of STAT6, but its binding to the promoter appears to be prevented by nonacetylated histones. After 9–11 h, when histones become acetylated, STAT6 binding sites may be demasked so that the phosphorylated and acetylated transcription factor can bind to activate gene transcription.

Lipoxygenases constitute a family of widely distributed nonheme-containing enzymes, which dioxygenate polyenoic fatty acids to their corresponding hydroperoxide derivatives (1, 2). Among the members of the lipoxygenase family, the reticulocyte-type 15-lipoxygenase (15-LOX-1)[1] is of particular interest because of its ability to oxygenate complex substrates, such as phospholipids (3), biomembranes (4), and lipoproteins (5). The enzyme has been implicated in the programmed breakdown of mitochondria during red blood cell maturation (6), in the development of fiber cells in the eye lens (7), and recently in actin polymerization during phagocytosis of apoptotic cells (8). 15-LOX-1 is also expressed in lipid-laden macrophages of atherosclerotic lesions (9) and in human bronchial epithelial cells (10).

Expression of the 15-LOX-1 gene is highly regulated. In young rabbit reticulocytes, a regulatory protein, which binds to a repetitive sequence element in the 3′-untranslated region of the 15-LOX mRNA, prevents its translation (11). In human monocytes (12), alveolar macrophages (13), A549 lung epithelial carcinoma cells (14), human tracheo-bronchial epithelial cells (15), human colorectal carcinoma HTB 38 cells (16), and Caco-2 cells (17), it is up-regulated by interleukin-4 (IL-4), IL-13, or both. Recently, it has been demonstrated that IL-4 also induces the expression of peroxisome proliferator-activated receptor-γ and transcription of the *CD36* gene (18).

IL-4 binding to its cell surface receptor leads to tyrosine phosphorylation of the intracellular part of the IL-4 receptor and to an activation of Janus kinases 1 and 3 (19). These kinases may be involved in phosphorylation of signal transducer and activator of transcription 6 (STAT6) (19). After phosphorylation, STAT6 dimerizes and translocates to the nucleus, where it may bind to a particular sequence elements (20) in the promoter of IL-4-responsible genes. The involvement of STAT6 in IL-4-induced 15-LOX-1 expression was underlined by the fact that no induction of 12/15-LOX-1 activity was observed in macrophages from homozygous STAT6-deficient mice (21). Unfortunately, the detailed mechanism by which STAT6 acts as a transcriptional activator is far from clear. Mutagenesis studies suggested that the biological activity of the transcription factor requires an intact carboxyl terminus (22). Moreover, several STAT proteins are known to recruit coactivators possessing

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[1] The abbreviations used are: 15-LOX-1, reticulocyte-type 15-lipoxygenase; IL-4, interleukin 4; STAT6, signal transducer and activator of transcription 6; HAT, histone acetyltransferases; HDAC, histone deacetylase; CREB, cAMP response element-binding protein; CBP, CREB-binding protein; wtE1A, wild-type E1A oncoprotein; E1AmCBP, mutant for CBP binding domain; EMSA, electrophoretic mobility shift assay; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RT, reverse transcription.

histone acetyltransferase (HAT) activity for the stimulation of gene expression (23, 24). For instance, STAT6 was shown to interact with the CREB-binding protein (CBP) and an associated protein, named p300, which exhibit HAT activity (25). This interaction required an intact carboxyl-terminal region of STAT6 (26).

In the present study, we have investigated the mechanism of IL-4-induced expression of the 15-LOX-1 gene in A549 cells and found that acetylation of histone proteins and STAT6 is required for transcriptional activation of this particular gene. From our data, it may be concluded that the acetylation of histones, which block STAT6 binding at the 15-LOX-1 promoter if they are present as nonacetylated proteins, enables promoter binding of phosphorylated and acetylated STAT6, which in turn may lead to transcriptional activation of the 15-LOX gene.

MATERIALS AND METHODS

Culture Medium and Reagents—A549 cells (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Recombinant IL-4 (human) was purchased from Biomol Research Laboratory (Hamburg, Germany). Anti-STAT6 and anti-acetyl antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-histone H3 and anti-acetylhistone H3 were from Upstate Biotechnology (Lake Placid, NY). Anti-15-LOX-1 antibody was purchased from Cayman Chemicals (Ann Arbor, MI). Genistein, cycloheximide, and sodium butyrate were supplied by Sigma.

Immunoprecipitation and Western Blots—Immunoprecipitations were performed by incubating the nuclear extracts with 2 μg of the primary antibody for 1 h, and the immune complexes were bound to protein A-agarose. The beads were then washed three times with radioimmunoprecipitation assay buffer (1% Nonidet P-40, 0.5% deoxycholate, and 0.1% SDS dissolved in phosphate-buffered saline (PBS) along with phenylmethylsulfonyl fluoride, leupeptin, and pepstatin as protease inhibitors), and the immune complex was released by SDS sample buffer. After separation of proteins by SDS-polyacrylamide gel electrophoresis (PAGE), they were transferred onto Immobilon nitrocellulose membranes (Millipore) by semi-dry blotting. The membranes were then probed with various antibodies and developed using the ECL detection system (Amersham Pharmacia Biotech). For the detection of acetyl-STAT6 among abundantly present acetylhistone proteins a double immunoprecipitation strategy was used. The first immunoprecipitation was performed with anti-acetylhistone H3 antibody. Subsequently, proteins were extracted with elution buffer (1% SDS and 0.1 M $NaHCO_3$) from the immunoprecipitate and subjected to a second immunoprecipitation in radioimmunoprecipitation assay buffer using anti-STAT6 antibody. The so obtained immunoprecipitate was electrophoresed, blotted on a nylon membrane, and probed with an antibody raised against acetylated proteins (Santa Cruz Biotechnology, Heidelberg, Germany). This anti-acetyl antibody apparently exhibits higher affinity for acetyl-STAT6 than for acetylhistone H3, giving a more intense band of acetyl-STAT6 than that of acetylhistone H3.

Chromatin Immunoprecipitation—Formaldehyde was added to the IL-4-treated cells at a final concentration of 1% and incubated for 20 min at room temperature. The reaction was stopped by the addition of glycine to a final concentration of 0.125 M. The cells were washed with cold PBS and harvested. The soluble chromatin was prepared according to the method of Dignam *et al.* (27) and sonicated at maximal power for 30 s twice to shear the genomic DNA. Immunoprecipitations were performed with anti-histone, anti-acetylhistone, and anti-STAT6 antibodies. Cross-linking was reversed in the immunoprecipitate complexes by the addition of NaCl to a final concentration of 200 mM and incubation at 65 °C for 6 h. The DNA was purified by proteinase K treatment (150 μg/ml) for 1 h, followed by phenol-chloroform extraction and precipitation by ethanol. The polymerase chain reaction (PCR) analysis was performed for the presence of the 15-LOX-1 promoter using specific primers. The extract aliquoted before the immunoprecipitations was used to prepare control input genomic DNA, which was also used for PCR analysis. For Western blotting, protein was directly denatured by electrophoresis sample buffer and applied to SDS-PAGE.

Electrophoretic Mobility Shift Assay—Double-stranded oligonucleotide containing the STAT6 binding element, present at −963 base pairs counted from the start of transcription of the human 15-LOX-1 gene, was used as probe in the gel shift assays. The assays were performed with nuclear extracts from IL-4-treated cells as described previously (25). The reaction mixture was electrophoresed on 6% PAGE and visualized by autoradiography.

Transfections and Plasmids—Transfections were performed using Transfectase reagent (Life Technologies, Inc.). 1.5 μg of each mammalian expression plasmid containing wild-type E1A oncoprotein (wtE1A) and a mutant for the CBP binding domain (E1AmCBP), which were kind gifts from Dr. A. Hecht, Freiburg, Germany, were cotransfected together with 0.1 μg of control plasmid PRSVLACZ to normalize for transfection efficiency. A titration was performed with varying amounts of wtE1A plasmid to determine the quantity of DNA required for maximal transfection efficiency.

DNA Affinity Chromatography—A 1-kilobase fragment of the 15-LOX-1 promoter proximal to the coding sequence was amplified by PCR. 100 pmol of this DNA were end-labeled with biotin 16-dUTP. The end-labeled DNA was purified and bound to streptavidin-coated magnetic beads (Roche Molecular Biochemicals). The beads were washed with PBS and incubated with the nuclear protein extracts and 10 μg of poly(dI-dC), a nonspecific competitor of DNA, in electrophoretic mobility shift assay (EMSA) buffer for 1 h at 4 °C. The beads were washed three times with PBS, and proteins were eluted with buffer containing 2 M NaCl. The eluted protein was desalted and analyzed by SDS-PAGE. Silver staining was performed to visualize the proteins.

In Vitro HAT Assay—Filter binding assays were performed as described (28) with minor modifications. Core histones were isolated from acid-solubilized nuclear proteins after trichloroacetic acid-acetone precipitation. 3.3 mg/ml histones were acetylated in a reaction buffer containing 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10% glycerol, and 1 mM proteinase inhibitor phenylmethylsulfonyl fluoride. [^{3}H]Acetyl-CoA (Amersham Pharmacia Biotech) and 6 μg of protein extract for 30–60 min at 30 °C. The reaction mixture was spotted onto P81 phosphocellulose paper (Upstate Biotechnology) and washed for 30 min with 0.2 M carbonate buffer, pH 9.2. The filter paper was dried and used for liquid scintillation counting. Similar experiments were performed using nonradioactive acetyl-CoA. The reaction mixture was denatured and loaded onto SDS-PAGE. The Western blot was probed with anti-acetylhistone H3 antibodies and developed using the ECL detection system.

High Performance Liquid Chromatography Analysis—A549 cells were cocultured in the presence of 670 pM of IL-4 for 24 h. The cells were trypsinized, washed, and resuspended in 500 μl of PBS. After addition of arachidonic acid (100 μM), the reaction was allowed to proceed at 37 °C for 20 min. Reduction of hydroperoxy fatty acids to their corresponding hydroxy derivatives was achieved by the addition of a molar excess of sodium borohydride. The reaction mixture was acidified to pH 3.0, and lipids were extracted with an equal volume of ethyl acetate. A defined amount of 13-hydroxyoctadecaenoic acid, which is absent in cells, was added as an internal standard before extraction. High performance liquid chromatography analysis was performed on a Supelcosil column (250 × 4.6 mm, 5 μm) using *n*-hexane/2-propanol/acetic acid (100:2:0.1 v/v/v) as a mobile phase at a flow rate of 1 ml/min. 15-Hydroxyeicosatetraenoic acid and 13-hydroxyoctadecaenoic acid were detected and quantified at 235 nm. Similar experiments were performed with A549 cells transfected with wtE1A and E1AmCBP oncoproteins.

RESULTS

Induction of 15-LOX-1 Expression in A549 Human Lung Epithelial Cells—A549 cells were cultured for various periods in the presence of 670 pM IL-4. The cells were harvested, and the lysates were analyzed for the expression of 15-LOX-1 mRNA by reverse transcription (RT)-PCR using 15-LOX-1-specific primers. The highest mRNA concentration was detected after a 12-h incubation period (Fig. 1*A*). After longer incubation periods, the mRNA levels dropped perceptibly. Similar kinetics were observed when the expression of the 15-LOX-1 protein was followed by Western blot analysis (data not shown). To find out whether IL-4 has to be present during the entire incubation period or whether a single cytokine stimulus may be sufficient to induce 15-LOX-1 expression, the following experiment was carried out. A549 cells were exposed to IL-4 for various periods, the cytokine was washed away, and incubation was resumed for a total of 24 h. Finally, the expression of 15-LOX-1 mRNA was analyzed by RT-PCR. As shown in Fig. 1*B*, 15-LOX-1 expression in A549 cells required a minimum of 11 h of continuous exposure to IL-4. These data indicate that a single IL-4

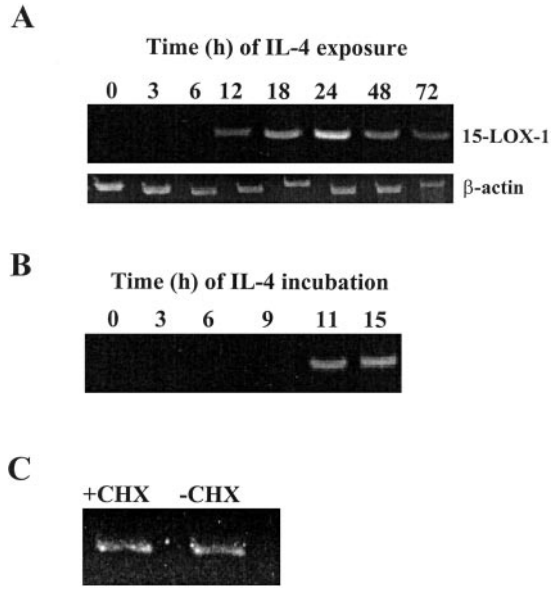

FIG. 1. **IL-4-mediated 15-LOX-1 induction is delayed and requires continuous exposure to IL-4.** *A*, A549 cells were incubated with 670 pM IL-4 in serum-free medium for different periods (0–72 h), the cells were harvested, and total RNA was extracted. Semiquantitative RT-PCR was performed to detect 15-LOX-1. β-Actin RT-PCR was used for the normalization of 15-LOX-1 expression. *B*, A549 cells were exposed to IL-4 (670 pM) for varying periods (0–11 h). IL-4 was removed by washing thrice with PBS, and incubation was resumed for a total of 24 h; then the cells were lysed for RNA extraction. 15-LOX-1 expression was assayed by RT-PCR. *C*, cells were exposed to cycloheximide (10 μg/ml) along with IL-4 (670 pM) for 12 h. 15-LOX-1 expression was assayed by RT-PCR. *−CHX*, IL-4 treatment alone in the absence of cycloheximde; *+CHX*, presence of cycloheximide along with IL-4.

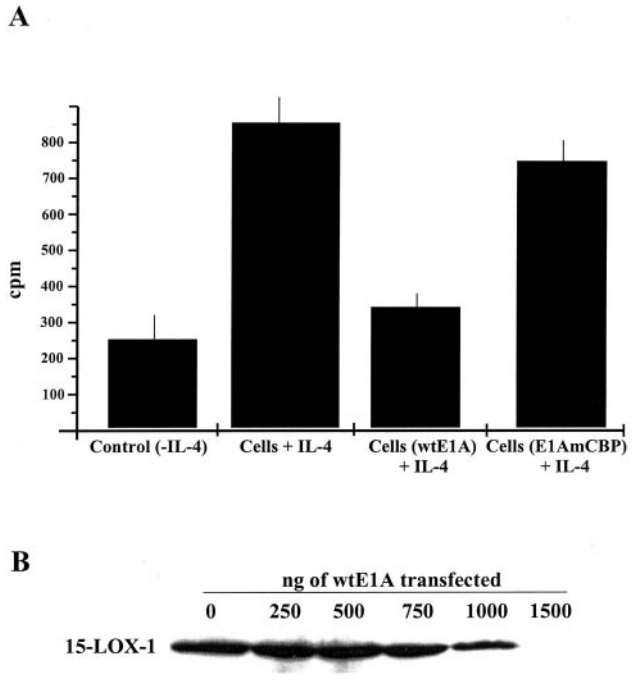

FIG. 2. **IL-4-induced up-regulation of histone acetyl transferase activity is due to the activation of CBP/p300.** *A*, A549 cells were exposed to IL-4 for 3 h, and cell lysates were used to measure the histone acetyltransferase activity as described under "Materials and Methods." Cells were either untransfected or transfected with the wtE1A or with its antagonizing mutant E1AmCBP. *B*, titration of the transfection efficiency was performed by using varying amounts of wtE1A (0–1.5 μg) and probing the cell lysates with anti 15-LOX-1 antibody.

stimulus is not sufficient to up-regulate expression of the 15-LOX-1 mRNA. Moreover, it was concluded that the IL-4-induced intracellular signal transduction cascade leading to 15-LOX-1 expression is a time-requiring process and may involve yet unidentified regulatory elements (21, 29). To find out whether IL-4-induced 15-LOX-1 expression involves *de novo* synthesis of STAT6-dependent regulatory proteins, additional transcription factors, or both, experiments were carried out in the presence of cycloheximide, a protein synthesis inhibitor. As shown in Fig. 1*C*, cycloheximide did not affect the activation of 15-LOX-1.

IL-4 Up-regulates Acetyltransferases in A549 Cells—Activation of cellular acetyltransferases may constitute an additional regulatory element in the intracellular signal transduction cascade (24). Acetylation of histones causes conformational changes of nuclear proteins, leading to demasking of potential transcription factor binding sites, so that the transcription factor may bind to the promoter of target genes. Because histone acetylation has recently been implicated in the induction of 15-LOX-1 expression in CaCo-2 cells (31), we investigated the effect of IL-4 on HAT activity in our cellular model. For this purpose, cells were exposed to IL-4 (670 pM final concentration) for 3 h, and the cell lysates were assayed for HAT activity. We found that IL-4 significantly up-regulated HAT activity even after a relatively short incubation periods (Fig. 2). The HAT activity is the sum of several catalytic processes and involves the activity of various proteins. One of these enzymes is the transactivating protein CBP/p300, which exhibits strong HAT activity. To find out whether CBP/p300 is involved in IL-4-induced up-regulation of acetyltransferase activity in A549 cells, we transfected the cells with the viral oncoprotein wtE1A, which has been identified as an endogenous inhibitor of CBP/p300. After IL-4 treatment, the transfected cells exhibited significantly reduced HAT activity (Fig. 2). When different amounts of cDNA were transfected, the suppression was found to be dose-dependent (Fig. 2*B*). However, this suppression of IL-4-induced HAT activity could be reversed by transfecting the cells with E1AmCBP, a mutant of E1A protein incapable of binding to CBP/p300. These data indicate that the augmented acetyltransferase activity is mainly due to activation of CBP/p300.

The acetylation degree of nuclear histones depends on cellular HAT activity but also on the activity state of histone deacetylases (HDACs). In resting cells, there appears to be a steady state of acetylating and deacetylating events and inhibitors, or activators of either process may shift the equilibrium in either direction. Recently, it has been reported that activation of HDAC may cause alterations in the chromatin state and may inhibit gene transcription (30). If our working hypothesis (CBP/p300-catalyzed histone acetylation is important for IL-4 induced 15-LOX-1 expression) is true, inhibitors of cellular HDAC are likely to act synergistically to IL-4 or may even be capable of inducing 15-LOX-1 expression in the absence of IL-4. To test this conclusion, A549 cells were incubated with suboptimal doses (335 pM) of IL-4 in the presence of sodium butyrate, and the expression of 15-LOX was assayed by Western blotting. From Fig. 3 it can be seen that IL-4 at suboptimal concentrations did not induce 15-LOX-1 expression. However, in the presence of sodium butyrate, a strong LOX signal was observed. Interestingly, sodium butyrate did also induce 15-LOX-1 expression in the absence of IL-4. Similar results have recently been reported for other cellular systems (31).

In Vitro Binding of Transcription Factors to the 15-LOX-1 Promoter and Role of Tyrosine Phosphorylation—Because histone acetylation may be important for binding of transcription factors to the promoter of the 15-LOX-1 gene, we carried out transcription factor binding assays *in vitro*. A549 cells were incubated with IL-4 for different periods; then nuclear extracts

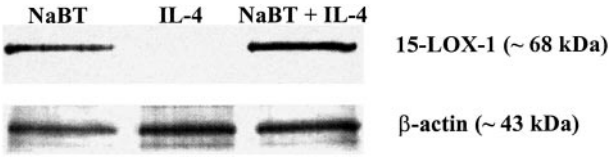

FIG. 3. **IL-4 synergistically up-regulates the expression of 15 LOX-1 by sodium butyrate.** Cells were cultured for 24 h in the presence of a suboptimal dose of IL-4 (330 pM) alone, 2 mM sodium butyrate (*NaBT*) alone, or of both together. The cells lysates were then analyzed for 15-LOX-1 expression using a specific antibody. The amount of protein was normalized by Western blotting with β-actin antibody.

were prepared, and binding studies of nuclear proteins to the 15-LOX-1 promoter were performed. In cells that were cultured in the absence of IL-4, we did not detect any promoter-binding proteins (Fig. 4*A, first lane*). In contrast, a variety of 15-LOX-1 promoter-binding proteins were present in the nucleus of IL-4-treated cells (Fig. 4*A, second* and *third lanes*). As expected, STAT6 was one of the major components, and its identity was confirmed by Western blots using commercially available anti-STAT6 antibodies and by EMSA (data not shown). Interestingly, the pattern of the binding proteins was very similar when cells were treated with IL-4 for 1 or 12 h (Fig. 4*A, second* and *third lanes*). These data indicate that under *in vitro* conditions, the transcription factors including STAT6 are capable of binding to the immobilized 15-LOX-1 promoter and that 1 h incubation is sufficient for maximal *in vitro* binding. Combining these data (rapid *in vitro* binding) with the results shown in Fig. 1 (delayed 15-LOX-1 expression), one may conclude that *in vivo* the binding of phosphorylated STAT6 may be prevented. Alternatively, coactivators exhibiting a prolonged time dependence may be required for transcriptional regulation of the 15-LOX-1 gene.

It has been reported for other cell types that tyrosine phosphorylation is involved in IL-4- and IL-13-induced 15-LOX-1 expression (29). Thus, we examined the effect of genistein, a potent tyrosine kinase inhibitor, on protein phosphorylation and on the binding activity of nuclear proteins to the 15-LOX-1 promoter. A549 cells were treated with genistein (25 μg/ml) for 30 min. After washing away the inhibitor, 670 pM IL-4 was added, and the cells were cultured for additional 12 h. Subsequently, the nuclear extracts were analyzed for the presence of 15-LOX-1 promoter-binding proteins. From Fig. 4*A, fourth lane*, it can be seen that genistein completely blocked the binding of proteins to the promoter. Surprisingly, genistein did also abrogate STAT6 acetylation (Fig. 4*B*). These data suggests that tyrosine phosphorylation in A549 cells may be a prerequisite for STAT6 acetylation and STAT6 binding to the 15-LOX-1 promoter.

It is well known that IL-4-induced intracellular signal transduction cascade bifurcates in various cellular systems and may initiate the Janus kinase/STAT6 pathway, the mitogen-activated protein kinase/protein kinase C route, or both (32–34). Because we found that the protein kinase C inhibitor bisindolylmaleamide and calphostin C failed to inhibit IL-4-induced 15-LOX-1 expression (data not shown), this pathway may not be relevant for the regulatory mechanism in A549 cells. These data confirm earlier observations in a different cellular model (35).

In Vivo Binding of STAT6 and Histones to the 15-LOX-1 Promoter—From Fig. 4 it was concluded that under *in vitro* conditions, phosphorylated STAT6 is capable of binding to the 15-LOX-1 promoter. The next series of experiments were aimed at addressing the question of whether such a binding may actually occur *in vivo*. For this purpose, A549 cells were cultured in the presence of IL-4 for various periods, and DNA-

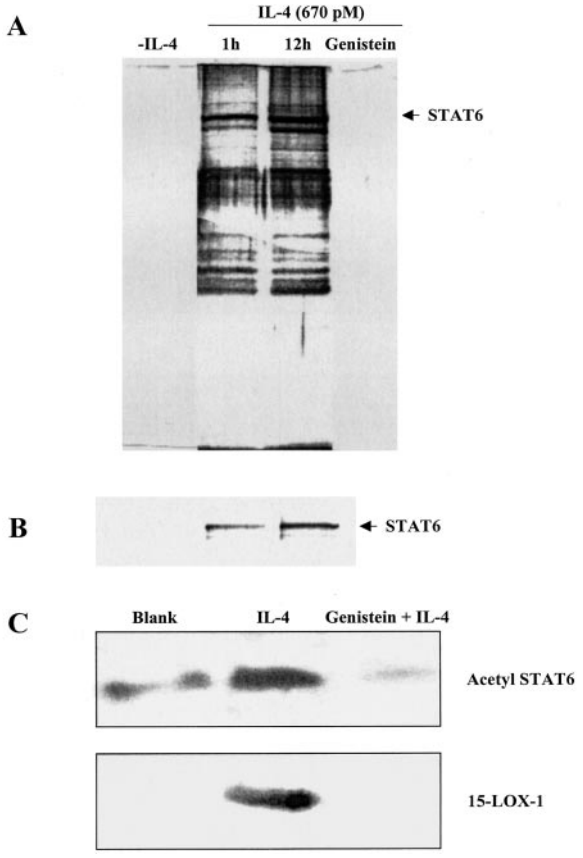

FIG. 4. ***In vitro* binding of transcription factors is dependent on tyrosine phosphorylation.** *A*, A549 cells were cultured for 1 and 12 h in the absence or presence of IL-4 (670 pM). Before starting the incubation, one batch of cells was pretreated with genistein (25 μg/ml) for 15 min. After the incubation period, cells were harvested, nuclear extracts were prepared, and DNA binding assays were performed as described under "Materials and Methods." *First lane*, cells incubated for 12 h in the absence of IL-4; *second lane*, cells incubated for 1 h in the presence of IL-4; *third lane*, cells incubated for 12 h in the presence of IL-4; *fourth lane*, genistein-pretreated cells incubated for 12 h in the presence of IL-4. *B*, A549 cells were cultured for 12 h in the absence or presence of IL-4 (670 pM). Before starting the incubation, one batch of cells was pretreated with genistein (25 μg/ml) for 15 min. After terminating the incubation, cells were harvested, and DNA binding was performed as described under "Materials and Methods." *C*, After immunoprecipitation, the proteins were analyzed by Western blotting for acetyl-STAT6 and 15-LOX-1.

binding proteins were cross-linked to the nucleic acid by formaldehyde treatment; then STAT6 was immunoprecipitated with a specific antibody, and the cross-linked DNA was analyzed by PCR using 15-LOX-1 promoter-specific primers. We found that the earliest binding of STAT6 was detected after 11 h of IL-4 exposure (Fig. 5, *upper row*). These data were somewhat surprising, because both STAT6 phosphorylation and its *in vitro* binding were rapid processes. Thus, it was concluded that the binding of STAT6 to the 15-LOX-1 promoter *in vivo* was inhibited during early phases of the incubation period. Similar *in vivo* binding studies were performed using anti-histone H3 (Fig. 5, *second row*) and anti-acetylhistone H3 antibodies (Fig. 5, *third row*). Here we observed that nonacetylated histones are bound at the early phases of the induction process. In contrast, STAT6 and acetylated histones were bound exclusively at later stages. These data indicate an inverse correlation between the binding of nonacetylated histone and the activation of the 15-LOX-1 gene. At early time points when histones are bound to the promoter (Fig. 5), we did not observe any 15-LOX-1 expression (Fig. 1). In contrast, after long-term incubations (≥11 h), when we observed promoter

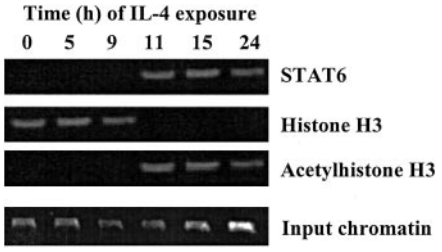

FIG. 5. **Differential kinetics of *in vivo* binding of STAT6, histone, and acetylated histone to the 15-LOX-1 promoter.** A549 cells were exposed to IL-4 (670 pM) for the periods indicated and then treated with formaldehyde to cross-link DNA binding proteins to the DNA (see "Materials and Methods"). The protein-nucleic acid complexes were immunoprecipitated with anti-STAT6, anti-histone H3, and anti-acetylhistone H3 antibodies. The cross-linked DNA was purified and analyzed by PCR for the presence of 15-LOX-1 promoter DNA. An aliquot of the complexes was removed before the immunoprecipitations and was similarly processed and used as a control for the PCR reaction. This DNA is referred to as input chromatin.

binding of acetylated histones and STAT6, the 15-LOX-1 mRNA was also expressed.

Immunoprecipitations were performed to obtain experimental evidence for a physical interaction between STAT6 and histones on IL-4 stimulation. To check for *in vivo* interaction, the cells were treated with 1% formaldehyde to cross-link the proteins, followed by immunoprecipitation in the protein extract, applying a dual immunoprecipitation strategy. After the first immunoprecipitation with the anti-acetylhistone H3 antibody, the protein was divided into 2 lots. For immunostaining of the acetylhistone H3, only 25% of the initial immunoprecipitate was used because of its abundance in the cell. The major fraction (75%) was used for the second round of immunoprecipitation with STAT6 antibody and Western blotting using an anti-acetyl antibody as probe. This tedious method was applied because no antibody against acetyl-STAT6 is currently available. This strategy and the differential cross-reactivity of the anti-acetyl antibody with different acetylated proteins do not allow a direct comparison of the relative amounts of STAT6 and histone H3. At 11 h after IL-4 stimulation, we observed that acetyl-STAT6 was bound to the acetylhistones, whereas at 4 h only a meager interaction was observed (Fig. 6*A*). This observation is in accordance with the data obtained from the chromatin immunoprecipitation experiments (Fig. 5). Moreover, after the reversal of cross-linking, the same immunoprecipitation was performed, and similar results were obtained. Here again, we observed increased binding of acetyl STAT6 to acetylhistone H3 during the time course of IL-4 treatment (Fig. 6*B, left panel*). When immunoprecipitation was carried out with an anti-STAT6 antibody, we did not observe any coprecipitation of acetylhistone H3 at 4 h of IL-4 exposure but a strong signal after 11 h (Fig. 6*B, right panel*). The chromatin immunoprecipitation experiments (Fig. 5) showed that after 11 h of IL-4 exposure, only acetylated histone H3 and acetyl-STAT6 were bound at the 15-LOX-1 promoter, and the increased binding of acetylhistone to STAT6 during the time course of IL-4 exposure (Fig. 6) is in line with these data. It may be concluded that IL-4-induced acetylation of chromatin-bound histones induces enhanced binding of STAT6, predominantly in its acetylated form, which also allows the STAT6 to bind to the promoter. This interaction of STAT6 with histones is interesting because this would stabilize the interactions with the chromatin itself.

The inverse *in vivo* promoter binding kinetics of nonacetylated histones and acetylated STAT6 to the 15-LOX-1 gene suggest the following scenario of events involved in IL-4-induced transcription of the 15-LOX-1 gene. Under basal conditions, nonacetylated histones are bound at the 15-LOX-1 promoter and block STAT6-sensitive binding sequences. However, when IL-4 is present for 11 h or longer, the histones become acetylated and open up STAT6 binding sites so that phosphorylated and acetylated transcription factors can bind to activate gene transcription.

Role of CBP/p300 as Coactivator in Transcriptional Up-regulation of 15 LOX-1 Gene Expression—If acetylation of STAT6 and nuclear histones is crucial for IL-4-induced transcription of the 15-LOX-1 gene, inhibition of acetylation was expected to block this process. It is known from the literature that CBP/p300, a transactivating protein with HAT activity, is capable of acetylating STAT6 (25). In fact, our transfection studies shown in Fig. 2 suggested that IL-4 treatment up-regulated acetyltransferase activity of CBP/p300 in A549 cells. If this up-regulation is somehow involved in IL-4-induced activation of 15-LOX-1 gene transcription, the viral oncoprotein E1A (inhibitor of CBP/p300 acetyltransferase activity) was expected to interrupt the IL-4-induced signal transduction cascade and subsequently to block 15-LOX-1 expression. To study the role of CBP/p300, we again transfected A549 cells with wtE1A and one E1A mutant, which differed from the wild type with respect to its functional properties. The mutant E1AmCBP, which lacks amino acids 64–68, the CBP binding region, acts as an E1A antagonist and does not inhibit the acetyltransferase activity of CBP/p300 (36). After transient transfection of A549 cells with the appropriate cDNA constructs, the cells were treated with IL-4 for 12 h. Cells were harvested and lysed, and acetylation of STAT6 was measured by Western blotting using an acetyl-specific antibody (Fig. 7*A*). In cells transfected with wtE1A, we did not observe any enhancement in STAT6 acetylation. Under these conditions, no 15-LOX-1 mRNA was detectable (data not shown). In contrast, cells transfected with the E1AmCBP mutant (E1A antagonist) showed a strong STAT6 acetylation signal, and 15-LOX-1 mRNA was detected.

The data shown in Figs. 6 and 7*A* indicate that STAT6 acetylation is up-regulated when the cells are stimulated with IL-4 and that the acetyltransferase activity of CBP/p300 is involved. To find out whether STAT6 acetylation is required for its binding to the 15-LOX-1 promoter, EMSAs were carried out (Fig. 7*B*). Cells transfected with wtE1A and with the noninhibitory mutant E1AmCBP were cultured in the presence of IL-4 for 12 h. The cells were then lysed, and EMSA was carried out with STAT6 binding consensus sequences. From Fig. 7*B* it can be seen that STAT6 binding occurred when cells were treated with IL-4, and similar results were obtained when cells were transfected with the E1A antagonist E1AmCBP, which cannot bind CBP/p300 and therefore is unable to inhibit the CBP/p300 acetylase activity. On the other hand, we did not observe any STAT6 binding when acetylation was prevented by transfecting the cells with wtE1A (Fig. 7*B*). While analyzing the 15-LOX activity as a measure for expression of the functional protein, we found that similar amounts of hydroxy fatty acids were formed in control cells and in cells transfected with E1AmCBP. In contrast, significantly reduced 15-LOX-1 activity was observed when the cells were transfected with wtE1A (Fig. 7*C*).

DISCUSSION

Acetylation of proteins is a common principle to modify their biological activity. It influences protein properties and thus may alter protein-protein interaction, DNA recognition, and protein stability. Histones were the first proteins that were identified as targets for protein acetylation. Although there are several lines of experimental evidence suggesting the importance of histone acetylation in the transcription of a variety of genes (37), its precise role in nucleosome remodeling is still

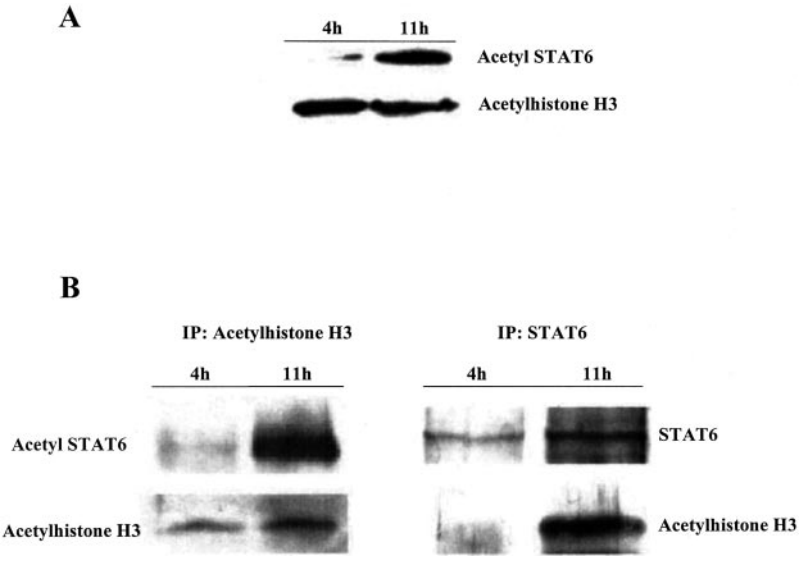

FIG. 6. **Kinetics of STAT6 and histone acetylation in A549 cells.** *A*, cells were exposed to IL-4 for 4 and 11 h, and DNA-binding proteins were cross-linked to the DNA by formaldehyde treatment. Protein-nucleic acid complexes were first immunoprecipitated with an anti-acetylhistone antibody, and the proteins were eluted with 100 μl of elution buffer (1% SDS and 0.1 M $NaHCO_3$). Cross-linking was reversed before the electrophoretic separation. A small fraction (25 μl) was analyzed by Western blotting using anti-acetylhistone H3 antibody as probe (*bottom row*). The major fraction (75 μl) was subjected to a second immunoprecipitation with anti-STAT6 antibody and the resulting immunoprecipitate was probed with antibody against acetyl residues (*top row*). *B*, cells were incubated with IL-4 for 4 and 11 h, and cross-linking with formaldehyde was carried out. After disruption of cells by sonication, cross-linking was reversed as described under "Materials and Methods." Proteins were first immunoprecipitated with anti-acetylhistone H3 antibody. The precipitate was reconstituted in 100 μl of elution buffer, of which 25 μl were used for Western blotting and probed with anti-acetylhistone H3 antibody (*left panel, bottom row*). The remaining 75 μl were then subjected to a second immunoprecipitation with the anti-STAT6 antibody. Proteins were recovered by elution and analyzed by Western blotting using the anti-acetyl antibody (*left panel, top row*). Next, an inverse immunoprecipitation strategy was applied. After reversal of cross-linking, proteins were immunoprecipitated first with the anti-STAT6 antibody, and the so obtained precipitate was probed with anti-STAT6 and anti-acetylhistone H3 antibodies (*right panel*).

elusive. Several families of histone acetyltransferases (PCAF/GCN5, p300/CBP, TAF250, SRC1, MOZ) have been characterized in the past, and recently even non-histone nucleic acid-binding proteins, such as HMG-1, p53, and GATA1, have been identified as acetylation substrates (38, 39). The consequence of acetylation of these regulatory proteins depends on the internal sites of acetylation. For instance, HMG-1 is acetylated at its DNA binding site, which results in the disruption of its DNA binding capabilities (38). In contrast, other transcription factors such as p53, GATA1, and E2F1 are acetylated outside their DNA binding site, and this results in stimulation of DNA binding (39). Sequence alignments have indicated that STAT6 contains several potential acetylation sites, and its acetylation has already been reported (25). However, this process was independent of IL-4 stimulation. The data presented in this study clearly indicate that IL-4-induced transcription of the 15-LOX-1 gene requires up-regulation of STAT6 acetylation, which is mainly due to activation of the acetyltransferase activity of CBP/p300. It should, however, be stressed that the acetylation degree of cellular proteins is a result of acetylating and deacetylating processes. Thus, an increase in the acetylation degree of STAT6 can be achieved either by activation of acetyltransferases (CBP/p300) or by inhibition of deacetylases. Deacetylases have been shown to occur in the nucleus and appear to play an important role in transcriptional repression (40). Whether they are recruited by nuclear hormone receptors bound to certain nuclear corepressors is not clear (41). Our findings–that sodium butyrate (nonspecific inhibitor of cellular HDAC) alone is capable of inducing 15-LOX-1 expression in A549 cells–suggest that transcriptional repression of the 15-LOX gene in resting cells may be due to a preponderance of deacetylating processes over acetyltransferases. It would be of particular interest to elucidate whether it is a general principle for transcriptional repression of the 15-LOX-1 gene in mammalian cells. Recently, Kamitani *et al*. (31) have observed that treatment of colorectal cell line Caco-2 with sodium butyrate and other histone deacetylase inhibitors causes an up-regulation of 15 LOX-1 expression and found that this up-regulation is linked to the state of histone acetylation. Moreover, the question of whether similar mechanisms may be involved in transcriptional activation of other IL-4-inducible genes remains to be investigated in the future.

In A549 cells, the expression of the 15-LOX-1 gene may be inhibited under resting conditions, because nonacetylated histones formed by HDAC are bound to the 15-LOX-1 promoter. This mode of transcriptional repression has been reported for a variety of inducible genes and appears to be well characterized (37). Histone binding to genomic DNA forms a condensed nucleosomal structure, and there is no possibility for the binding of specific transcription factors. The acetylation of histones opens the chromatin, making it accessible to transcription factors for binding to the promoter of relevant genes. The stability of the interaction between the transcription factors and the chromatin could be modulated by the interaction between the histones and the transcription factors. These interactions lead to the formation of a more stable structure and also help in the establishment of the correct chromatin confirmation. Originally, it was postulated that phosphorylation of STAT6 would be sufficient to allow its binding to the 15-LOX-1 promoter. However, our data indicate that this may not be true in this case. In A549 cells, IL-4 induces STAT6 acetylation in addition to phosphorylation, and both reactions appear to be required for translational activation of the 15-LOX-1 gene. This conclusion may be drawn from the following experimental data: (i) IL-4 increases the activity of cellular acetyltransferases (Fig. 2), particularly of CBP/p300, which is capable of acetylating STAT6 (Fig. 6); (ii) the viral oncoprotein wtE1A, an inhibitor

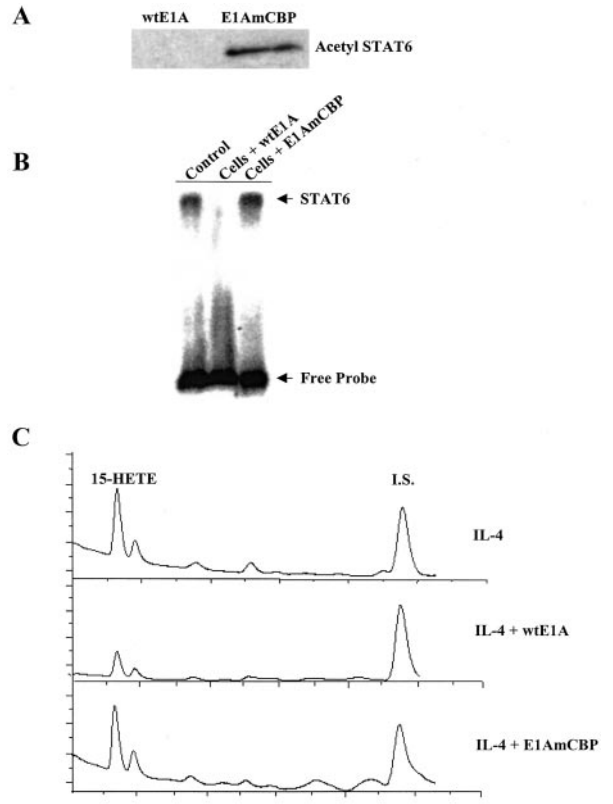

FIG. 7. **STAT6 acetylation by CBP/p300 is required for promoter binding.** *A*, A549 cells were transfected with wtE1A plasmid and mutant form E1AmCBP, in which the CBP binding domain has been deleted. Cells were then exposed to IL-4 for 11 h, and the nuclear extracts were analyzed by Western blotting for the presence of acetylated STAT6. *B*, EMSA was performed with the protein extracts from the above-mentioned transfected cells and STAT6 binding element obtained from the 15-LOX-1 promoter. *C*, A549 cells transfected with wtE1A and E1AmCBP as well as untransfected controls were cultured in the presence of IL-4 for 24 h. The cell lysates were incubated with exogenous arachidonic acid, and 15-LOX-1 activity was assayed by straight phase-high performance liquid chromatography as described under "Materials and Methods." 13-Hydroxyoctadecaenoic acid, which cannot be formed from arachidonic acid, was used as an internal standard (*I.S.*). *15-HETE*, 15-hydroxyeicosatetraenoic acid.

of acetyltransferase activity of CBP/p300, prevented STAT6 acetylation and expression of the functional enzyme; in contrast, its noninhibitory mutant E1AmCBP was unable to do so (Fig. 7*A*); (iii) inhibition of STAT6 acetylation by wtE1A prevented STAT6 binding to the 15-LOX-1 promoter (Fig. 7*B*); in contrast, the E1AmCBP mutant did not inhibit the CBP/p300 acetylase activity and also did not prevent STAT6 binding; and (iv) the histone deacetylase inhibitor sodium butyrate synergistically induced the IL-4-stimulated 15-LOX-1 expression.

Because further acetylation of STAT6 by CBP/p300 acetyltransferases takes place inside the nucleus, tyrosine-phosphorylated STAT6, which is inevitably required for homodimerization and subsequent nuclear translocation, becomes an essential precursor. This is strongly supported by the inhibition of STAT6 acetylation by genistein (Fig. 4, *A* and *B*). No effect of wtE1A oncoprotein on the tyrosine phosphorylation of STAT6 was observed, as checked with the anti-phospho-STAT6 antibody in a Western blot (results not shown).

Phosphorylation of transcription factors is a rapid process, and our *in vitro* binding assays indicated that phosphorylated STAT6 quickly binds to the naked 15-LOX-1 promoter. On the other hand, *in vivo* expression of the 15-LOX-1 mRNA in intact A459 cells requires at least 11 h, and similar observations have been reported for other cytokines (29). This obvious discrepancy, together with the results presented in Fig. 5, indicated that *in vivo* the binding of phosphorylated STAT6 to the 15-LOX-1 promoter appears to be inhibited during the first 11 h of IL-4 treatment. Although the detailed mechanism of the inhibitory processes is still unclear, our data suggest that the binding of nonacetylated histones may be involved. Acetylation of histones, which appears to be a delayed process in A549 cells, may overcome this inhibitory process, so that acetylated STAT6 can bind to the 15-LOX-1 promoter. For the time being, the reasons for the delayed acetylation of histones and STAT6 remain obscure. Although the cellular acetyltransferase activity is up-regulated within 1 h after IL-4 exposure, acetylated histones and acetylated STAT6 could only be detected after a 9-h incubation period.

In summary, it can be concluded from our data that the mechanism of transcriptional activation of the 15-LOX-1 gene by IL-4 does not follow the conventional activation pathway of IL-4-inducible genes. Acetylation of both histone H3 and STAT6 is essentially required for transcriptional activation of the 15-LOX-1 gene, and this acetylation is mainly due to the acetyltransferase activity of PCB/p300. Moreover, inhibition of histone deacetylases by sodium butyrate synergistically enhanced the IL-4-induced activation of 15-LOX-1.

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