An LXXLL Motif in the Transactivation Domain of STAT6 Mediates Recruitment of NCoA-1/SRC-1*

Received for publication, April 12, 2002, and in revised form, July 9, 2002
Published, JBC Papers in Press, July 22, 2002, DOI 10.1074/jbc.M203556200

Claudia M. Litterst and Edith Pfitzner‡
Georg-Speyer-Haus, Institute for Biomedical Research, Paul-Ehrlich-Straße 42-44, 60596 Frankfurt, Germany

Signal transducer and activator of transcription 6 (STAT6) regulates transcriptional activation in response to interleukin-4 (IL-4)-induced tyrosine phosphorylation by direct interaction with coactivators. The CREB-binding protein and the nuclear coactivator 1 (NCoA-1), a member of the p160/steroid receptor coactivator family, bind independently to specific regions of STAT6 and act as coactivators. In this study we show that an LXXLL motif in the STAT6 transactivation domain mediates the interaction with NCoA-1. Peptides representing this motif as well as antibodies generated against this motif inhibited STAT6/NCoA-1 interaction in glutathione S-transferase pulldown assays. Peptides derived from the STAT6 transactivation domain adjacent to the LXXLL motif as well as antibodies against these peptides showed no inhibitory effect. Mutagenesis of the LXXLL motif eliminated the STAT6/NCoA-1 interaction in vitro and in vivo, supporting the specific role of this motif in NCoA-1 binding. Importantly, mutagenesis of the STAT-LXXLL motif strongly diminished the IL-4-regulated activation of the endogenous STAT6 target gene eotaxin-3. Taken together, these results indicate that the STAT6-LXXLL-binding motif mediates the interaction with NCoA-1 in transcriptional activation and represents a new potential drug target for the inhibition of the STAT6 transcription function in allergic diseases.

STAT6† belongs to the family of transcription factors that transmit signals from activated cytokine receptors to the nucleus. Following their obligatory tyrosine phosphorylation exerted by JAK kinases, STATs dimerize and move to the nucleus where they modulate transcription through binding to specific DNA sequence elements (1–3). STAT proteins share the same structure and functional domains, although they have distinct regulatory functions in the organism (4). STAT6 becomes activated in response to IL-4 and IL-13 and mediates most of the gene expression regulated by these cytokines. IL-4 regulates immune and anti-inflammatory responses. It promotes the differentiation of T helper (Th) precursors toward the Th2 lineage while inhibiting Th1 development. Furthermore, IL-4 triggers immunoglobulin class switching to the IgE isotype in B-cells. STAT6-deficient mice have defects in IL-4-mediated Th2 development and immunoglobulin class switching to IgE, demonstrating the essential role of STAT6 in these functions (5, 6). Recent studies (7, 8) have shown that the chemokine eotaxin-3 can be up-regulated by IL-4 and IL-13 in endothelial cells and dermal fibroblasts in a STAT6-dependent manner. Eotaxin-3 is a member of the family of CC chemokines, which are known to be potent chemoattractants for eosinophils. A STAT6-response element was identified in the promoter of the eotaxin-3 gene, characterizing this chemokine as a novel STAT6 target gene (8).

Control of gene expression is a dynamic and complex process. Gene transcription involves assembly of multiple transcription factors at the distal enhancer region and the basal transcriptional machinery, including RNA polymerase II, at the core promoter of target genes (9). According to the current model, gene expression involves the sequential assembly of an array of coregulatory proteins, including coactivators that are part of the chromatin-modifying complexes, possessing histone acetyltransferase activities (10). Coactivator complexes are recruited via specific protein/protein interactions, often mediated by specific interaction motifs in the transactivation domains of DNA-bound transcription factors or other coactivators.

The transactivation domain (TAD) of STAT6 was characterized as a modular, proline-rich region in the carboxyl terminus of the protein (11, 12). Two distinct transactivation functions, which cooperate in transcriptional activation, have been mapped to this domain (13). In earlier studies we reported that STAT6 recruits the coactivators p300/CBP and NCoA-1 via direct interaction with specific parts of its transactivation domain (14, 15). Both coactivators are required for transcriptional activation by IL-4 (14, 15). p300 and the CREB-binding protein CBP are large, highly related, multifunctional coactivators that share many structural and functional attributes and are referred to collectively as p300/CBP (16). p300/CBP function as coactivators for many DNA-binding transcriptional activator proteins, including nuclear receptors and other signal-regulated activators (17). p300/CBP have been shown to associate with other coactivators such as pCAF (18) and members of the NCoA coactivator family also called the p160/steroid receptor coactivator (SRC) family (19, 20), thereby providing a platform for a variety of proteins participating in gene expression (16). The NCoA coactivators were identified as nuclear receptor-binding proteins, which enhance transcriptional activation by ligand-induced transcription factors (21). Three homologous factors, termed NCoA-1, also called SRC-1 (19, 22); NCoA-2, also called TIF2 or GRIP1 (23, 24); and NCoA-3, also called pCIP, ACTR, or AIB1 (25–27), have been identified by

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Georg-Speyer-Haus, Institute for Biomedical Research, Paul-Ehrlich-Straße 42-44, 60596 Frankfurt, Germany. Tel.: 49-69-63395187; Fax: 49-69-63395297; E-mail: c.pfitzner@em.uni-frankfurt.de.

‡ The abbreviations used are: STAT6, signal transducer and activator of transcription 6; GST, glutathione S-transferase; IL, interleukin; CBP, CREB-binding protein; NCoA, nuclear coactivator; SRC, steroid receptor coactivator; TAD, transactivation activation domain; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ELISA, enzyme-linked immunosorbent assay; RAR, retinoic acid receptor; DBD, DNA binding domain; GFP, green fluorescent protein; RT, reverse transcriptase; Th, T helper.

The Journal of Biological Chemistry Vol. 277, No. 39, Issue of September 27, pp. 36052–36060, 2002
Printed in U.S.A.

http://www.jbc.org/
several laboratories. Subsequently a number of transcription factors have been shown to recruit these coactivators. It has been proposed that distinct classes of transcription factors selectively use specific coactivators and histone acetyltransferase activities for their function (28, 29). In accordance with this model, we have observed a specific role of NCoA-1, but not NCoA-2 or NCoA-3, for STAT6-mediated transactivation (15). Only NCoA-1 acts as a coactivator of STAT6. STAT6 directly contacts NCoA-1 via a small carboxy-terminal part of its transactivation domain. A region between amino acids 213–462 in NCoA-1, which comprises the B part of the Per-Arnt-Sim domain and part of a serine/threonine region, mediates the interaction with STAT6. Overexpression of the STAT6-interacting domain of NCoA-1 inhibits transactivation by STAT6 in a transdominant manner, demonstrating the importance of NCoA-1 for STAT6 transactivation. In the present study we characterize the 56-amino acid spanning interaction domain in the STAT6-TAD that mediates the recruitment of NCoA-1. We have identified an LXXLL motif (where L is leucine and X is any amino acid) with a predicted α-helical structure in the shortest NCoA-1-interacting fragment of the STAT6-TAD. The α-helical LXXLL motif was originally found in a variety of coactivators, e.g. NCoA/p160/SRC members, p300/CBP, RIP-140. It is described as a signature motif, which mediates the recruitment of these proteins by the nuclear hormone receptors (30, 31). Specific LXXLL motifs of NCoAs mediate the ligand-dependent interaction with nuclear receptors, as well as interaction with p300/CBP. In this study we demonstrate that STAT6 recruits NCoA-1 via an LXXLL motif in its transactivation domain. We show that a peptide containing the LXXLL motif of STAT6, as well as antibodies raised against this peptide, are potent inhibitors of the interaction with NCoA-1 in vitro. Furthermore, mutagenesis of the STAT6-LXXLL motif abolishes the binding of NCoA-1 in vitro and in vivo. A STAT6 variant, in which the LXXLL motif is mutated, is less active in induction of a STAT6-dependent reporter gene. Moreover, the LXXLL mutant of STAT6 shows impaired induction of eotaxin-3 expression, indicating that recruitment of NCoA-1 by the STAT6-LXXLL motif is essential for full transactivation of this endogenous target gene. The LXXLL motif of STAT6 shows no sequence homology to the known coactivator motifs. A peptide containing the STAT6-LXXLL motif does not interfere with nuclear receptor/coactivator interactions, suggesting that this motif might represent a specific target for STAT6 inhibition. Our data indicate that NCoA-1 does not only use its own LXXLL motifs to contact DNA-bound factors and coactivators, but can also be recruited itself by an LXXLL motif of DNA-bound transcription factors, such as STAT6.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Transfections, and Luciferase Assays—**293T cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 2 mM l-glutamine, penicillin/streptomycin. Transfections were performed by the calcium phosphate precipitation method. The following day, cells were induced with human IL-4 (10 ng/ml, Peprotech) or left untreated as indicated and lysed after a further 16 h of incubation. The human lung epithelial cell line NCI-H2292 was grown in RPMI medium containing 10% fetal calf serum, 2 mM l-glutamine, penicillin/streptomycin. Cells were transfected with FuGENE 6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer’s instructions. Cells were induced with human IL-4 (10 ng/ml) or left untreated and were harvested 40 h after transfection for luciferase assays. Luciferase and β-galactosidase activities were assayed as recommended by the manufacturer (Promega). Luciferase activities were normalized to the lacZ expression. At least three independent experiments were performed.

**Plasmids—**The reporter genes (GAL4-RE3)TK LUC, N4/STAT-RE3 LUC, the lacZ expression plasmid (pCH110), the expression vectors for h-STAT6 (pX-M-STAT6), and GAL4-DNA binding domain have been described previously (12). The expression vector for mutant STAT6 with the point mutations L802A,L805A (pX-M-STAT6-mut) was generated from pX-M-STAT6 by site-directed mutagenesis according to the protocol of Stratagene. The expression vectors encoding GFP-tagged wild type or mutant STAT6 variants were obtained by insertion of the corresponding STAT6 gene fragment into the XhoI and KpnI sites of ECFP-C1 vector (CLONTECH). Expression vectors for murine NCoA-1 was kindly provided by Joe Torchia (University of Western Ontario, London, Canada). The expression vectors for the GAL4-DNA binding domain fusion proteins containing residues 729–947 of wild type and mutant STAT6 (L802A,L805A) were generated by insertion of PCR fragments encoding the corresponding sequences into the BamHI site of pCMV-GAL4-DBD. GAL4-SX1 was purchased from Amersham Biosciences. The expression vectors for wild type and mutant STAT6 (L802A,L805A) residues 642–847 fused to GST were generated by insertion of the corresponding region (generated by PCR) in-frame into the BamH1/XhoI sites of pGEX-5X-1. The expression vector for GST-RAR has been described previously (32). To generate a bacterial expression vector for residues 213–462 of NCoA-1 fused to an amino- and carboxy-terminal histidine tag, the PCR fragment encoding the corresponding region was inserted into the BamH1/XhoI sites of pET30C (Novagen). Baculovirus expression vector for full-length GST- and histidine-tagged STAT6 was generated by introducing the GST sequence in the NdeI site of pFAST-BacHTb-STAT6 expression vector which contains full-length STAT6 sequences downstream of His6-pPA2. Baculovirus expression vector was purchased from Invitrogen. All constructs were verified by digestion. Inserts generated by PCR were additionally verified by sequence alignment.

**Generation of STAT6 Peptides and Antibodies—**Peptides comprising sequences 794–814 (peptide 1), 817–830 (peptide 2), and 830–847 (peptide 3) of STAT6 were obtained from Eurogentec. Antibodies against the peptides were generated by Eurogentec according to standard procedures. Crude end bleedings after three booster injections were used as antisera.

**GST Pulldown Assays—**Recombinant cDNAs of NCoA-1 in the pcMPL2 or NCoA-1 amino acids 213–462 in pET30C expression vector were transcribed and translated in <i>vitro</i> in reticulocyte lysates (Promega) in the presence of [35S]methionine according to the manufacturer’s instructions. GST and other GST-STAT6-TAD fusion proteins were expressed in <i>E. coli</i>. GST-histidine-tagged full-length STAT6 fusion protein was expressed in S9 insect cells, infected with recombinant baculovirus. All GST proteins were purified with glutathione-Sepharose beads (Amersham Biosciences). For binding assays, GST fusions or GST alone (1.5–5 μg) bound to glutathione-Sepharose beads were incubated with radiolabeled or histidine-tagged proteins in 200 μl of binding buffer as described previously (33). Ligand-dependent interaction of GST-retinoic acid receptor α and NCoA-1 was investigated in the presence of all-trans-retinoic acid (10−6 μM). For competition assay, binding was performed in the presence of antisera or peptides as indicated. After extensive washing, bound proteins were eluted and separated on SDS-PAGE. Radiolabeled proteins were visualized by fluorography. Histidine-tagged proteins were detected by Western blot using an anti-histidine tag. Antibody reactivity of bound proteins were estimated after SDS-PAGE by Coomassie staining.

**Common precipitation Assays—**293T cells were transfected with 3 μg of expression vectors encoding GFP-tagged wild type or mutant STAT6 variants, and 7 μg of expression vector for NCoA-1 per 10 cm-dish. 2 days after transfection the cells were lysed in NETN buffer as described previously (15). Cleared lysates were incubated with 2 ml of anti-GFP antisera (CLONTECH) for 2 h and for another hour with protein A/G-agarose beads. The immunoprecipitated proteins were separated by SDS-PAGE and analyzed by Western blotting with STAT6 antibody (BD PharMingen and Transduction Laboratories) and SRC-1 antibody (clone 1135, Upstate Biotechnology, Inc.).

**Preparation of Whole Cell Extracts and Electrophoretic Mobility Shift Assays—**Transfected cells were induced with IL-4 (50 ng/ml) for 20 min. They were then washed and supplied with fresh medium. Whole cell extracts were prepared after different time points as described previously (12). A double-stranded oligonucleotide probe containing the STAT6-binding site in the murine Ce germ line promoter between positions −117 and −97 was end-labeled using [γ-32P]ATP and T4 polynucleotide kinase (New England Biolabs). Unlabeled 23–39P]ATP was separated from the labeled probe via gel migration through a MicroSpin™ G-25 column (Amersham Biosciences). The nucleoprotein binding reaction was performed as described previously (32) using 8 μg of whole cell extracts and 20,000 cpm (corresponding to 0.5 ng of the labeled probe).

**Preparation of RNA and RT-PCR—**Total RNA was prepared using Trisaf reagent (Pqlab) according to the manufacturer’s instructions.
The cDNAs were obtained by reverse transcription of total RNA (2 µg) using Omniscript reverse transcriptase (Qiagen) and random hexamer primers. Amplification of eotaxin-3 cDNA was performed with specific primers (8) and Taq polymerase (Invitrogen) according to the manufacturer's instructions. As a control, the cDNA of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified with specific primers (34). For both genes an initial denaturation for 4 min at 94 °C and subsequent 30–35 cycles of PCR were performed. Each cycle consisted of a denaturation period (30 s at 94 °C), an annealing phase (35 s at 60 °C for eotaxin-3 and at 65 °C for GAPDH), and an extension period (35 s at 72 °C). PCR products were separated by agarose gel electrophoresis, and the DNA was stained with ethidium bromide.

ELISA—To test the affinities of the rabbit antisera to STAT6, a GST 96-well detection module from Amersham Biosciences was used according to the manufacturer's instructions. GST-STAT6-TAD was bound to the plates, which were precoated with GST antibody. After washing, rabbit antisera were added and detected using peroxidase-coupled anti-rabbit antibody (Amersham Biosciences). Human eotaxin-3 was measured by ELISA according to a standard streptavidin-horseradish peroxidase assay protocol. Briefly, eotaxin-3 was captured with an anti-human eotaxin-3 antibody and detected with biotinylated anti-human eotaxin-3 antibody (PeproTech) and streptavidin-horseradish peroxidase (Jackson ImmunoResearch Laboratories). As a control, Dulbecco's modified Eagle's medium was analyzed in parallel and used as reference. This value was subtracted from sample values.

RESULTS

A 21-Amino Acid Peptide Contains the STAT6/NCoA-1 Interaction Motif—We have shown recently that the p160/SRC family member NCoA-1 is a specific coactivator of STAT6. STAT6 recruits NCoA-1 via its TAD (15). The NCoA-1-binding site is located at the carboxy-terminal region between amino acids 792 and 847 of the STAT6-TAD (15). To identify interaction motifs, which mediate the recruitment of NCoA-1, we designed three peptides that cover the NCoA-1-binding site in STAT6-TAD (Fig. 1A), and we investigated whether they would act as competitors for the interaction in GST-pulldown experiments. A GST fusion protein containing the TAD of STAT6 was coupled to glutathione-Sepharose and incubated with [35S]-labeled NCoA-1 in the presence or absence of the different peptides. As expected, NCoA-1 bound strongly to GST-STAT6-TAD (Fig. 1B, lane 3) but not to GST (lane 2). Peptide 1 efficiently blocked the interaction between STAT6 and NCoA-1 (lane 4). In contrast, peptide 2 and peptide 3 had no effect on the binding of STAT6 and NCoA-1 (lanes 5 and 6). These results suggest that peptide 1 contains an essential motif for the interaction with NCoA-1 and could therefore act as a competitor for binding.

To determine the concentration of peptide 1 required for efficient inhibition of the STAT6/NCoA-1 binding, a similar experiment was performed with decreasing amounts of peptide 1. For these experiments we used histidine-tagged fusion proteins comprising the STAT6 interaction domain of NCoA-1 defined in our former studies (15) and GST fusion proteins containing the full-length STAT6 sequence. The GST-STAT6 fusion also contains a histidine tag allowing estimation of both protein concentrations by Western blotting. GST or GST-STAT6 were incubated with NCoA-1 (amino acids 213–462) and decreasing amounts of peptide 1. Precipitated proteins were subjected to Western blotting with an antibody recognizing the histidine tag. NCoA-1 bound strongly to GST-STAT6 but not GST alone (Fig. 1C, lanes 2 and 3). The highest amount of peptide 1 (0.28 µm) completely abolished this interaction (lane 4), while at a lower peptide concentration (28 µm) a small amount of bound NCoA-1 could still be detected. The lowest concentration of peptide 1 (2.8 µm, lane 6), which represents a 50-fold molar excess over the bound GST-STAT6, was still able to compete for binding of NCoA-1 (compare lanes 3 and 6). These results indicate that peptide 1 represents a sequence of STAT6 that is essential for the interaction with NCoA-1 and can therefore act as a competitor in a concentration-dependent manner.

To confirm that the region of STAT6 present in peptide 1 mediates the interaction with NCoA-1, we raised antibodies against the different peptides shown in Fig. 1A and tested their ability to block the STAT6/NCoA-1 interaction. Two rabbits (a and b) were immunized with each peptide. We performed GST-pulldown assays with the GST-STAT6-TAD and the radioactively labeled STAT6 interaction domain of NCoA-1 comprising amino acids 213–462 in the presence or absence of antiserum. The binding of GST-STAT6-TAD to NCoA-1 was not affected by the addition of a preimmune serum to the binding reaction (Fig. 1D, compare lane 3 to 4). Antisera 1a and 1b, which were raised against peptide 1, efficiently blocked the STAT6/NCoA-1 interaction (lanes 5 and 6). In contrast, antisera raised against peptides 2 and 3 did not significantly affect the binding (lanes 7–10). All antisera had similar binding affinities to STAT6, as determined by ELISA (Fig. 1E). The blocking capacity of antisera 1a and 1b indicates that these antibodies cover the specific interaction motif in STAT6, which is then no longer available for NCoA-1. In summary, the experiments suggest that the region of STAT6 spanning amino acids 794–814 harbors a protein interaction motif which mediates the recruitment of NCoA-1.

Mutagenesis of the STAT-LXXLL Motif Abrogates NCoA-1 Binding in Vitro—Sequence analyses of peptide 1 revealed a sequence matching the LXXLL consensus sequence (Fig. 2A).

LXXLL motifs mediate protein/protein interactions and were originally found in a variety of coactivators. In order to test whether the LXXLL motif of STAT6 is essential for the recruitment of NCoA-1, we mutated the sequence by replacing leucines 802 and 805 with alanine (Fig. 2A). GST fusion proteins containing the wild type or mutant TAD of STAT6 (amino acids 642–847) were tested for their binding to NCoA-1 in GST pulldown assays. Equal amounts of the GST fusion proteins or GST alone were incubated with [35S]-labeled NCoA-1 (amino acids 213–462). As shown before, wild type GST-STAT6-TAD bound NCoA-1 strongly (Fig. 2B, lane 3). Point mutations in the LXXLL motif completely abolished the binding of GST-STAT6-TAD to NCoA-1 (lane 4). Although the two point mutations alter the migration of the STAT6 fusion protein in SDS-PAGE, both wild type and mutant proteins were expressed in similar amounts (Fig. 2C, lanes 2 and 3). This excludes the possibility that the difference in binding was due to a difference in protein input. The results from these experiments clearly demonstrate that the LXXLL motif of STAT6 is essential for the interaction with NCoA-1 in vitro.

Mutations in the LXXLL Motif Abolish NCoA-1 Recruitment in Vivo—To prove the function of the STAT-LXXLL motif in NCoA-1 recruitment in vivo, fusion of wild type and mutant STAT-TAD (amino acids 792–847) with the GAL4-DNA binding domain were analyzed in transactivation studies (Fig. 3A). 293T cells were transiently transfected with the different GAL4-DNA binding domain fusions, a GAL4 reporter, and the NCoA-1 expression vector or control vector as indicated. Expression of GAL4-DNA binding domain alone did not result in marked expression of the luciferase gene neither in the presence nor in the absence of exogenous NCoA-1 (Fig. 3B, lanes 1 and 2). The GAL4 DNA binding domain fusion with wild type STAT6 amino acids 792–847 strongly enhanced luciferase expression (lane 3). This induction was increased 2-fold by coexpression of NCoA-1 (lane 4). The mutant GAL4 STAT6 fusion displayed only 20% of the wild type transactivation potential (compare lanes 5 and 3). Furthermore, NCoA-1 could not stimulate the transactivation potential of the mutant GAL4-STAT6 fusion (lane 6). To verify these results, we repeated the exper-
ments in the lung epithelial cell line NCI-H292. As observed in 293T cells basal transactivation by the GAL4-DNA binding domain alone was not affected by coexpression of NCoA-1 (Fig. 3, lanes 1 and 2). In contrast to what we observed in 293T position, DBD, Src homology 2 (SH2) domain, TAD, and the cytokine-dependent phosphorylation site (Y) are indicated.

**FIG. 1.** Peptide 1 is a potent inhibitor of the interaction between STAT6 and NCoA-1 in vitro. A, the structure of human STAT6, the GST-STAT-TAD fusion protein, and peptides derived from the TAD and antisera raised against the different peptides. Amino acid

**FIG. 2.** Point mutations in the LXXLL motif of STAT6-TAD abolish the interaction with NCoA-1 in vitro. A, the sequences of peptide 1 and the LXXLL motif. Alanine mutations introduced into the sequence are labeled with asterisks. B, GST or GST fusion proteins containing residues 642–847 of wild type (WT) or mutant (mut) STAT6 were incubated with 35S-labeled NCoA-1 fragment, comprising residues 213–462. Material bound to glutathione-Sepharose was separated by SDS-PAGE. Radiolabeled protein was detected by fluorography. C, a quarter of each of the reactions was separately analyzed by SDS-PAGE and Coomassie staining in order to detect the GST fusion proteins.

position, DBD, Src homology 2 (SH2) domain, TAD, and the cytokine-dependent phosphorylation site (Y) are indicated. B, GST or the GST-STAT6-TAD fusion proteins were purified from E. coli, bound to glutathione-Sepharose, and incubated with the 35S-labeled NCoA-1 in the presence of 100 µg of peptides as indicated. Material bound to the glutathione-Sepharose was recovered from the binding reaction, extensively washed, and analyzed by SDS-PAGE and fluorography (lanes 2–6). Input control in lane 1 reflects 10% of the total amount of 35S-labeled protein used in the experiments. C, GST-STAT6 was purified from Sf9 insect cells infected with baculovirus expression vector for GST and histidine-tagged full-length STAT6. GST or GST-STAT6 was bound to glutathione-Sepharose and incubated with the histidine-tagged NCoA-1 fusion protein comprising residues 213–462, purified from E. coli. Binding was carried out in the presence of 0.28 mM (lane 4), 28 µM (lane 5), and 2.8 µM (lane 6) peptide 1 as indicated. Precipitated proteins were washed, eluted, and resolved by SDS-PAGE. An aliquot of the NCoA-1 fragment corresponding to 50% of the material used for the interaction assay was analyzed in parallel (lane 1). Histidine fusion proteins were visualized by Western blot analysis with an antibody recognizing the histidine tag. D, GST or GST-STAT6-TAD fusion protein, bound to glutathione-Sepharose, was incubated with 35S-labeled histidine-NCoA-1 fusion protein, comprising residues 213–462. In samples 4–8 the binding reaction was carried out in the presence of 10 µl of the different rabbit antisera (AB 1a, AB 1b, AB 2a, AB 2b, AB 3a, and AB 3b) or preimmune sera as indicated. Bound proteins were analyzed by SDS-PAGE and fluorography (lanes 2–8). As a control, 10% of the 35S-labeled protein used in the experiments was analyzed in parallel (lane 1). E, the rabbit sera were analyzed for binding to STAT6 in ELISA. GST-STAT-TAD fusion protein was immobilized on plates that were coated with GST antibodies. The rabbit antisera were added to the wells containing GST-STAT-TAD (black bars) or buffer as a control (white bars). Bound antibodies were detected using peroxidase-coupled anti-rabbit antibody. As a control, the binding of secondary antibody to the plates was analyzed in parallel (lanes 13 and 14). aa, amino acids.
cells, the wild type GAL4-STAT6 fusion did not activate transcription when transfected alone indicating that NCoA-1 is more limiting in this cell line (lane 3). Cotransfection of NCoA-1 dramatically stimulated the transactivation potential of wild type GAL4-STAT6 (lane 4). In contrast, NCoA-1 could not enhance transactivation by the mutant GAL4-STAT6 fusion (lane 6). Together these data support the proposal that the LXXLL motif of STAT6 is required for recruitment of NCoA-1 in vivo and for NCoA-1-mediated transcriptional activation.

The LXXLL Motif of STAT6 Is Required for Full Transactivation but Is Not Involved in DNA Binding—In order to study the function of the LXXLL motif in IL-4-mediated transactivation, we introduced the L802A,L805A mutation into the full-length STAT6 sequence (Fig. 4A).

We first investigated whether the mutation would disrupt recruitment of NCoA-1 by full-length STAT6 protein. Coimmunoprecipitation experiments were performed with both STAT6 variants and NCoA-1. We used GFP-tagged STAT6 versions, which can be efficiently immunoprecipitated by anti-GFP serum. 293T cells were transfected with expression vectors for the different STAT6 variants together with NCoA-1. Both STAT6 proteins were immunoprecipitated from cell lysates. The presence of NCoA-1 in the protein complexes was analyzed by Western blot. NCoA-1 was coimmunoprecipitated with wild type STAT6 protein (Fig. 4B, lane 6) but not by the mutant STAT6 (lane 8). Western blot performed with STAT6-specific antibodies indicated that there were comparable amounts of both STAT6 proteins precipitated (lanes 2 and 4). These data show that the mutant STAT6 version is not at all capable of forming a complex with NCoA-1 in cells.

Next we tested whether such a mutation would affect the activation or the DNA-binding ability of STAT6. 293T cells were reported to contain a defective STAT6 (11, 35). We therefore used this cell line to study the function of the STAT6-L802A,L805A mutant without interference from endogenous STAT6. Wild type and mutant STAT6 were transfected into 293T cells, and DNA binding activity was analyzed by electrophoretic mobility shift assays with cell lysates. To determine the DNA binding stability of wild type and the STAT6-L802A,L805A variant cells were treated with IL-4 for 20 min, after which the medium was replaced with cytokine-free medium. Cells were harvested at various time points thereafter. Extracts from mock-transfected cells showed no STAT6-DNA complex confirming that the endogenous defective STAT6 cannot bind to DNA (Fig. 4C, lanes 1–4). Both wild type and the STAT6 variant specifically bound to the probe, and this binding did not decrease even after 2 h (lane 5–12). No difference in complex formation was observed between wild type and the STAT6 variant indicating that the L802A,L805A mutations do not influence activation by tyrosine phosphorylation or DNA binding of STAT6.

To evaluate the transactivation function of the STAT6 variant, 293T cells were transfected with either wild type or variant STAT6 together with a luciferase reporter construct driven by multimerized STAT6-response elements. Luciferase expression was investigated in cell lysates after stimulation with IL-4. As expected, endogenous STAT6 was not able to induce luciferase expression of the reporter plasmid (Fig. 4D, lanes 1 and 2). Transfection of wild type STAT6 strongly enhanced the reporter activity after IL-4 stimulation (lane 4), whereas the STAT6 variant displayed only 50% of the wild type activity (lane 6). Similar amounts of wild type and variant STAT6 were expressed in the transfected cells as proven by Western blotting (Fig. 4E, compare lanes 2 and 3). This rules out the possibility that the distinct transactivation potentials result from a difference in STAT6 variant expression. Instead these results clearly show that the LXXLL motif is required for full STAT6-mediated transactivation and indicate that NCoA-1 recruitment by this motif mediates 50% of the transactivation potential of STAT6.
The STAT6-LXXLL Motif Contributes to IL-4-regulated Eotaxin-3 Expression—Because transcriptional activity of a reporter gene driven by multimerized binding sites does not really reflect the transcriptional regulation of chromatin-packaged genes, the contribution of the STAT6-LXXLL motif for transactivation of an endogenous target gene was analyzed. 293T cells have been described to express the chemokine eotaxin-3 in an IL-4-dependent manner only after transfection with functional STAT6. Thus we determined eotaxin-3 expression in 293T cells transiently transfected with either wild type or mutant STAT6 after IL-4 stimulation (Fig. 5). In accordance with a previous report (35), we could detect IL-4-enhanced eotaxin-3 mRNA expression by RT-PCR only after transfection of STAT6 (Fig. 5A, lane 4). Transfection of the STAT6-L802A,L805A variant also resulted in eotaxin-3 expression in IL-4-treated cells but to a lower extent (compare lanes 4 and 6).

To evaluate the difference in eotaxin-3 induction by wild type and mutant STAT6 on the protein level, secreted eotaxin-3 was measured in cell culture supernatants by ELISA. As expected from the RT-PCR results, mock-transfected cells did not secrete detectable levels of eotaxin-3, even after cytokine treatment (Fig. 5B, lanes 1 and 2), whereas cells transfected with wild type STAT6 produced high amounts of eotaxin-3 upon cytokine treatment (lane 4). Transfection of the STAT6-L802A,L805A
variant also enabled cells to produce eotaxin-3 but again at a reduced level. Only 50% of wild type expression was achieved (compare lanes 4 and 6). To confirm that the difference in eotaxin-3 expression was not due to a variance in STAT6 expression level, Western blot analysis was performed with cell lysates. As shown in Fig. 5C, both wild type and variant STAT6 were expressed in similar amounts. These data correspond nicely to the results obtained from transfection experiments with an artificial STAT6 reporter gene confirming that the STAT6-mediated transactivation is impaired when the LXXLL motif is mutated. The LXXLL motif hence contributes substantially to STAT6-mediated transactivation.

Peptide 1 Comprising the STAT-LXXLL Motif Does Not Influence RAR/NCoA-1 Interaction—The functional characterization of the STAT6-LXXLL suggests that it is the most important motif for the interaction with NCoA-1. Thus we wondered whether this motif would be structurally and functionally related to the LXXLL motifs that are present in NCoA-1 itself. These motifs form an α-helical structure with a hydrophobic face built by the conserved leucines. To obtain initial hints about the structure, we performed a protein two-dimensional structure prediction of the sequence spanning the LXXLL motif using DNAsis software, as depicted in Fig. 6A. To activate RAR, ligand was added (10⁻⁶ M all-trans-retinoic acid (ATRA)) (lanes 4 and 5). In samples 3 and 5 the binding reaction was carried out in the presence of 50 μg of peptide 1. Bound proteins were analyzed by SDS-PAGE and fluorography (lanes 2–8). As a control, 10% of the 35S-labeled protein used in the experiments was analyzed in parallel (lane 7).
that the STAT6-LXXLL motif is not related to the NCoA-1-LXXLL motifs.

**DISCUSSION**

The aim of this study was the identification of essential motifs in the transactivation domain of STAT6 to get new insights into the transactivation mechanism of STAT6 and to identify targets to inhibit the STAT6 transactivation function. Transcriptional activation requires the recruitment of coactivators by DNA-bound transcription factors. This often occurs via protein/protein interaction mediated by distinct structural motifs within the transactivation domains of the DNA-bound transcription factors and the coactivator interaction domains (36). In previous studies (14, 15) we have shown that at least two coactivators, p300/CPB and NCoA-1, are involved in STAT6-mediated transactivation. Both coactivators bind independently to different parts of the STAT6 transactivation domain. NCoA-1 binds between residues 792 and 847 of the STAT6-TAD.

In this study we have further characterized this interaction domain. Peptides spanning different parts of the STAT6 interaction domain and antibodies raised against these peptides were used to find potential interaction motifs. A peptide representing the first part of the STAT6 interaction domain (amino acids 794–814) efficiently competed for NCoA-1 binding. Moreover, antibodies recognizing this region in STAT6 efficiently blocked NCoA-1 binding, indicating that the NCoA-1 interaction motif lies between amino acids 794 and 814 of STAT6. Importantly, antibodies that bind very closely adjacent to the STAT6-binding motif were not able to block NCoA-1 binding, confirming that a particular motif has to be masked to prevent NCoA-1 binding. These experiments showed that it is possible to target the STAT6/NCoA-1 interaction very specifically either by masking the binding domain of NCoA-1 with peptides representing the STAT6-binding motif or by covering the STAT6-binding motif with an antibody. The inhibitory peptides and antibodies identified here will be valuable tools for further *in vivo* studies, e.g. microinjection experiments to investigate whether blocking the NCoA-1 recruitment will inhibit STAT6-mediated transactivation. In addition, the inhibitory peptide can present the starting point for the development of small molecules that block the STAT6/NCoA-1 interaction.

The sequence of peptide 1 contains an LXXLL motif (where L is a leucine and X any amino acid). This motif was originally identified in transcriptional coactivators and mediates the interaction of these proteins with nuclear hormone receptors (31). LXXLL motifs are necessary and sufficient for the binding of these coactivators to ligand-bound nuclear hormone receptors, and they are required for coactivation of transcription. Here we demonstrate that the LXXLL motif in STAT6 is involved in the interaction of STAT6 with its coactivator NCoA-1. Point mutations (L802A,L805A) in the LXXLL motif (Fig. 2A) completely abolished the interaction of GST-STAT6-TAD to NCoA-1 (Fig. 2B), demonstrating the crucial role of the LXXLL motif for binding in *vitro*. Furthermore, we observed that the STAT6-LXXLL motif is absolutely required to recruit NCoA-1 to the promoter and to enhance transcription by the GAL4-STAT6 fusion domain transactivation of STAT6-TAD (amino acids 792–947). The transcriptional potential of this GAL4 fusion seems to be based entirely on NCoA-1, which is present at different levels in 293T and NCI-H292 cells (data not shown). In 293T cells, which express high NCoA-1 levels, GAL4-STAT6-TAD strongly enhanced transcription (Fig. 3B). In NCI-H292 cells, which express a much lower level, NCoA-1 seemed to be a limiting factor, leading to a weak transactivation potential (Fig. 3C). Cotransfection of NCoA-1 dramatically amplified transactivation by GAL4-STAT6-TAD. Recruitment of NCoA-1 to the GAL4-STAT6-TAD was strictly dependent on the intact LXXLL motif, because mutations of the motif completely abolished the transactivation potential of the GAL4-STAT6-TAD fusion in both cell lines, and this could not be rescued by coexpression of NCoA-1 (Fig. 3, B and C).

We analyzed the function of the LXXLL motif in transcriptional activation by full-length STAT6. These experiments were performed in 293T cells, which lack functional STAT6 and can therefore be used for analyzing the function of STAT6 variants. In addition, the ability of 293T cells to produce eotaxin-3 after IL-4 treatment allowed us to analyze the contribution of the STAT6-LXXLL motif to the transactivation of an endogenous target gene (8).

The L802A,L805A mutation did not affect the activation by tyrosine phosphorylation or the DNA binding of STAT6 after IL-4 treatment (Fig. 4B). However, the ability to activate transcription on an artificial reporter gene containing multimerized STAT6-binding sites (Fig. 4) as well as on the endogenous eotaxin-3 target gene (Fig. 5) was strongly reduced. The STAT6-L802A,L805A variant displayed in both assays a 50% reduced transactivation potential compared with wild type STAT6. Significantly, the finding that eotaxin-3 protein expression was also reduced by 50% in STAT6 variant-expressing cells confirmed that the recruitment of NCoA-1 by the STAT6-LXXLL motif is an essential step in the transcriptional activation of chromatin-packaged endogenous genes. IL-4-induced transcriptional activation of the eotaxin-3 promoter is highly dependent on STAT6-binding sites (8). In contrast to other STAT6 target genes, e.g. the Ce germ line gene, no activation or synergistic effects on transcription by NF-xB was detected. Our results so far demonstrate that the expression of eotaxin-3 is largely dependent on the recruitment of NCoA-1 by the STAT6-LXXLL motif. However, we could not predict that this is true for all IL-4-regulated genes, especially when they are already activated by tumor necrosis factor-α. It is possible that the role of NCoA-1 in STAT6-mediated transactivation varies depending on the specific promoter context as well as on the cellular context. Further studies have to be performed to investigate the importance of the LXXLL motif for other STAT6 target genes.

In previous studies we observed that the coactivator p300/CPB, which associates with NCoA-1 (19, 20), binds directly to a different region of the STAT6-TAD (amino acids 677–791) (15). Therefore NCoA-1 could still be recruited to the STAT6-LXXLL mutant via bridging to p300/CPB. In this case NCoA-1 would have even a greater role in STAT6 transactivation. To evaluate the exact contribution of NCoA-1 for STAT6 transactivation, the transcriptional potential of STAT6 has to be determined in cells lacking this coactivator.

LXXLL motifs are abundant in different coactivators. Many coactivators contain several LXXLL motifs. The p160/SRC family members contain three motifs, which mediate the interaction with the nuclear hormone receptors and two motifs, which mediate the p300/CPB interaction. p300/CPB itself contains two motifs (31). All motifs described so far form α-helical structures with a hydrophobic face, which is built by the conserved leucines. However, not all LXXLL motifs are functionally equivalent. Sequences flanking the LXXLL motif determine target specificity (37, 38). We analyzed whether the STAT6-LXXLL motif has any structural or functional relation to the NCoA-1-LXXLL motifs. From the structural prediction we assumed that the STAT6-LXXLL motif also forms an α-helix. However, this motif does not share any homologous residues amino- or carboxyl-terminally to the LXXLL core sequence with the motifs in the p160/SRC or p300/CPB coactivators (Fig. 6 and data not shown). Because positive as well as negative
cross-talk between STAT proteins and nuclear receptors has been described (39, 40), we tested whether the STAT6-LXXLL motif could compete with the NCoA-1-LXXLL motif for nuclear receptor binding. We demonstrated that peptide 1, which represents the STAT6-LXXLL motif and which was a potent inhibitor of the NCoA-1/retinoic acid receptor α (Fig. 6) or NCoA-1/estrogen receptor interaction (data not shown). This suggests that the STAT6-LXXLL motif is unique and has most likely no function in cross-talk. It can also be expected that the structure of the STAT6-LXXLL is unique. Structural analyses of the STAT6 peptide in complex with the STAT6 binding domain of NCoA-1, which we mapped to a region between amino acids 213 and 462 (15), are currently in progress.

The IL-4/STAT6 signaling pathway is an attractive target for the treatment of allergic diseases, because STAT6 is crucial for class switching to IgE, a pivotal factor in the pathogenesis of asthma. Several approaches have been made to inhibit this pathway as follows: oligonucleotides, which specifically inhibit binding of STAT6 to its response element (41, 42); intracellular peptides, which inhibit the binding of STAT6 to the receptor (43); antagonistic IL-4 mutants (44); and IL-4-receptor antagonists (45). We revealed here a novel approach to inhibit this pathway. The STAT6-LXXLL motif will be a new, attractive drug target. This motif is required for the STAT6-mediated expression of eotaxin-3, a potent chemotactant of eosinophils. The recruitment of eosinophils to sites of inflammation is a hallmark of allergic diseases. Therefore inhibition of eotaxin-3 would be a beneficial therapy. It might be feasible to inhibit the STAT6 signaling pathway by targeting the STAT6-LXXLL motif without affecting similar motifs. Moreover, small drug inhibitors can be developed based on the peptide characterized here that will be able to inhibit the STAT6/NCoA-1 interaction.

Acknowledgments—We thank Bernd Groner, Fabrice Gouillieux, Richard Moriggl, and Joe Tschirch for plasmids and Thorsten Heinzel, Cathrine Mahoney, and Barbara Schnierle for comments on the manuscript.

REFERENCES
1. Darnell, J. E. (1997) Science 277, 1630–1635
2. Hele, J. N. (1996) Cell 84, 331–334
3. Kaplan, M. H., Schindler, U., Smiley, S. T., and Grusby, M. J. (1996) Immunity 4, 313–319
4. Shinkai, A., Yoshise, H., Kikue, M., Sheji, E., Kagawa, S., Saito, A., Takeda, T., Imabeppu, S., Kato, Y., Hanai, N., Anazawa, H., Kuga, T., and Nishi, T. (1999) J. Immunol. 163, 1602–1610
5. Hoek, J., and Wieschlogler, M. (2001) J. Immunol. 167, 3216–3222
6. Hanphey, M., and Reinberg, D. (1999) Curr. Opin. Genet. & Dev. 9, 132–139
7. Chen, H., Tini, M., and Evans, R. M. (2001) Curr. Opin. Cell Biol. 13, 218–224
8. Lu, B., Reichel, M., Fisher, D. A., Smith, J. F., and Rothman, P. (1997) J. Immunol. 159, 1255–1264
9. Moriggl, R., Berchtold, S., Friedrich, K., Standke, G. J., Kammer, W., Hein, M., Wissler, M., Stocklin, E., Gouillieux, F., and Groner, B. (1997) Mol. Cell. Biol. 17, 3663–3678
10. Gouillieux, F., and Groner, B. (1999) Nucleic Acids Res. 27, 2722–2729
11. Lu, B., Reichel, M., Fisher, D. A., Smith, J. F., and Rothman, P. (1997) Curr. Opin. Cell Biol. 9, 1553–1557
12. Vo, N., and Goodman, R. H. (1991) J. Biol. Chem. 266, 13905–13908
13. Yang, X. J., Ogryzko, V. V., Nishikawa, J., Howard, B. H., and Nakatani, Y. (1999) Nature 382, 319–324
14. Kamei, Y., Xu, L., Heinzel, T., Torchia, J., Kurokawa, R., Glass, B., Lin, S. C., Heyman, R. A., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1996) Cell 85, 463–474
15. Yao, T. P., Ku, G., Zhou, N., Seully, R., and Livingston, D. M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10626–10631
16. Xu, L., Glass, C. K., and Rosenfeld, M. G. (1999) Curr. Opin. Genet. & Dev. 9, 140–147
17. Otake, S. A., Tsai, S. Y., Tsai, M. J., and O‘Malley, B. W. (1995) Science 270, 1354–1357
18. Voegel, J. J., Heine, M. J., Zechel, C., Champon, P., and Gronemeier, H. (1996) EMBO J. 15, 3667–3675
19. Hong, K., Kohli, K., Garabedian, M. J., and Stallcup, M. R. (1997) Mol. Cell. Biol. 17, 2735–2744
20. Torchia, J., Rose, D. W., Inostroza, J., Kamei, Y., Westin, S., Glass, C. K., and Rosenfeld, M. G. (1997) Nature 387, 677–684
21. Chen, H., Lin, R. J., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M. L., Nakatani, Y., and Evans, R. M. (1997) Cell 90, 569–580
22. Anzick, S. L., Kononen, J., Walker, R. L., Azorsa, D. O., Tanner, M. M., Guan, X. Y., Sauter, G., Kallioniemi, O. P., Trent, J. M., and Meltzer, P. S. (1997) Science 296, 965–968
23. Xue, L., Glass, C. K., and Rosenfeld, M. G. (1998) Cell 94, 793–797
24. Kurokawa, R., Kalafus, D., Ogliastro, M. H., Kioussi, C., Xu, L., Torchia, J., Rosenfeld, M. G., and Glass, C. K. (1998) Science 270, 700–703
25. McInerney, E. M., Rose, D. W., Flynn, S. E., Westin, S., Mullen, T. M., Krones, A., Inostroza, J., Torchia, J., Nolte, R. T., Assa-Munt, N., Milburn, M. B., Glass, C. K., and Rosenfeld, M. G. (1998) Genes Dev. 12, 3357–3368
26. Heery, D. M., Kalkhoven, E., Hoare, S., and Parker, M. G. (1997) Nature 387, 733–736
27. Pfitzner, E., Kiefel, J., Becker, P., Rolke, A., and Schule, R. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 5359–5364
28. Pfitzner, E., Jahne, R., Wissler, M., Stocklin, E., and Groner, B. (1998) Mol. Endocrinol. 12, 1582–1593
29. Wolf, M., Krahl, M. A., Manz, R. H., and Heinz, B. M. (1998) Blood 92, 1713–1720
30. Hoek, J., and Wieschlogler, M. (2001) J. Immunol. 166, 4507–4515
31. Chen, L. (1999) Curr. Opin. Struct. Biol. 9, 48–55
32. Chang, C., Norris, J. D., Gron, H., Paige, L. A., Hamilton, P. T., Kenan, D. J., Fowlkes, D., and McDonnell, D. P. (1999) Mol. Cell. Biol. 19, 8226–8239
33. Duy, X. P., Anderson, C. M., Ma, H. Hong, H., Uhl, R. M., Kushner, P. J., and Stallcup, M. R. (1998) Mol. Endocrinol. 12, 302–313
34. Stocklin, E., Wissler, M., Gouillieux, F., and Groner, B. (1996) Nature 383, 726–728
35. Bisla, A., Andreau, K., David, M., Sturm, M., Haake, M., Bertoglio, J., and Pallardy, M. (2000) FEBS Lett. 487, 229–233
36. Wang, L. H., Yang, X. Y., Kirken, R. A., Resau, J. H., and Farrar, W. L. (2000) Blood 95, 1249–1257
37. Stutz, A. M., Hoek, J., Natt, F., Cuenoud, B., and Wieschlogler, M. (2001) J. Biol. Chem. 276, 11759–11765
38. Stolzenberger, S., Haake, M., and Dusch, A. (2001) Eur. J. Biochem. 268, 4809–4814
39. Gronewald, S. M., Werthmann, A., Schnarr, B., Klein, C. E., Brocker, E. B., Mohrs, M., Bronnbacker, F., Seibald, W., and Dusch, A. (1998) J. Immunol. 160, 4904–4909
40. Tomkinson, A., Duce, C., Ciechlewicz, G., Pratt, J. C., Joetham, A., Shanafelt, M. C., Gundel, R., and Gelfand, E. W. (2001) J. Immunol. 166, 5792–5800
An LXXLL Motif in the Transactivation Domain of STAT6 Mediates Recruitment of NCoA-1/SRC-1
Claudia M. Litterst and Edith Pfizner

J. Biol. Chem. 2002, 277:36052-36060.
doi: 10.1074/jbc.M203556200 originally published online July 22, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M203556200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 45 references, 25 of which can be accessed free at http://www.jbc.org/content/277/39/36052.full.html#ref-list-1