STAT6 and Ets-1 Form a Stable Complex That Modulates Socs-1 Expression by Interleukin-4 in Keratinocytes*

Julia Travagli†, Martine Letourneur, Jacques Bertoglio, and Josiane Pierre§

From the INSERM U461, Faculté de pharmacie, 5 Rue J. B. Clément, 92296-Chatenay-Malabry, France

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Suppressor of cytokine signaling (SOCS)-1 is selectively and rapidly induced by appropriate agonists and modulates cytokine responses by interfering with the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway. On the basis of the observation that interleukin (IL)-4 up-regulates Socs-1 in the keratinocyte HaCaT cell line, we investigated which sequences of the 5′-Socs-1 gene are responsive to IL-4. We therefore have cloned the 5′-flanking region of this gene, and by promoter analysis we identified a functional IL-4-responsive element located at nucleotide (−684/−570) upstream from the transcription initiation site, whose presence and integrity are necessary to ensure IL-4 responsiveness. This element contains three STAT6 and one Ets consensus binding sequences of which specific mutations abolished IL-4 responsiveness either partially or totally. We also report that Ets-1 physically interacted with STAT6. Exogenous expression of Ets-1 in conjunction with STAT6 activation strongly inhibited expression of a Socs-1 promoter-luciferase reporter. Collectively, our data demonstrated the involvement of STAT6 and Ets, via a composite DNA element, in the IL-4 regulation of Socs-1 gene expression in keratinocytes.

Interleukin (IL)-4 is a pleotropic cytokine, which displays a variety of biological responses by binding to high affinity receptor complexes. IL-4 receptors are expressed on a wide variety of cells of hematopoietic or non-hematopoietic origin. Among the latter, epidermal keratinocytes, which represent the major cell type of the skin, have been shown to be IL-4-responsive cells.

Many studies have focused on the mechanisms by which IL-4 exerts its action. The Jak-STAT pathway has been shown to be activated by IL-4. Activation of JAK1 and JAK2 or 3, depending on the cell lines, is pivotal for the activation of downstream signaling events including the recruitment and rapid tyrosine phosphorylation of STAT6 (1–4). STAT6 can dimerize and translocate into the nucleus where it regulates IL-4 target genes. STAT6-deficient mice underlined the importance of this factor in gene expression and Th cell differentiation, and indicated that most IL-4-mediated functions are lost in the absence of STAT6 (5–7). In addition, the use of ectopic expression of STAT6 variants in cells have also emphasized its role in transcription regulation. The IL-4-induced expression of IL-13 Rα2 receptor chain and eotaxin-3 have been shown clearly to depend on STAT6 activation (8–10).

Responsiveness to cytokines depends upon a balance of positive and negative regulators. Among these regulators SOCS (suppressor of cytokine signaling) proteins have been described as negatively regulating the JAK/STAT pathway. There are at least eight members of the SOCS family, each of which contains a central SH2 domain, an amino-terminal domain of variable length and divergent sequence, and a carboxyl-terminal 40-amino acid module that is known as the SOCS-box (11, 12). Generally, these proteins are not highly expressed in unstimulated tissues but are induced by stimulation. SOCS proteins regulate the magnitude and duration of responses triggered by various cytokines by inhibiting their signal transduction pathway in a classical negative feedback loop (13–15). At the molecular level, SOCS proteins have been shown to bind directly to cytokine receptors or to the catalytic domain of JAK kinases and therefore impede the recruitment and phosphorylation of STAT (16).

Among the SOCS protein family, SOCS-1 was independently identified in three laboratories (17–19). Although the role of SOCS-1 in modulating cytokine signaling has been extensively studied, less is known about the mechanisms that control Socs-1 gene expression. Numerous studies have implicated STAT protein family members in the positive regulation of Socs gene expression (20, 21). More recently, after erythropoietin stimulation a positive and negative regulation of Socs-1 and Socs-3 gene expression has been reported implicating STAT5 in erythropoietin-responsive cell lines (22). Socs-1 expression is also controlled through translational repression (23, 24). In addition, transcriptional silencing of Socs-1 gene by hypermethylation was frequently observed in certain cancer cells (25, 26).

Socs-1 expression is up-regulated by numerous cytokines and growth factors, among which is IL-4 (17, 27), and is also able to inhibit IL-4 signal transduction (28). In this work, we report on the mechanism by which IL-4 regulates Socs-1 expression in human keratinocytes. The establishment of cell lines expressing either a gain-of-function or a transcriptional dominant negative form of STAT6 allowed us to investigate the role of STAT6 in the regulation of Socs-1 mRNA expression. Moreover, we have cloned the 5′-flanking region of Socs gene and defined its promoter region which contains three STAT6-binding element (SBE). We then provide evidence that STAT6 binding to its distal SBE in the Socs-1 promoter was critical for IL-4-mediated Socs-1 expression. Furthermore, we report that the distal SBE is a composite element, which allows the bind-
ing of both STAT6 and Ets transcription factors. These two transcription factors are associated within the cells and transient transfection indicated a cooperation between these two unrelated transcription factors. Altogether these data argue for a novel transcription factor interaction mechanism that may account for SOCS-1 mRNA regulation of expression.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The human keratinocyte cell line HaCaT (29) was cultured in Dulbecco’s modified Eagle’s medium supplemented with antibiotics (50 μg/ml penicillin, 50 μg/ml streptomycin), with 1 mM sodium pyruvate, and 10% fetal calf serum.

**Cotransfection Activities, and Reporter**—Human recombinant IL-4, kindly provided by Dr. P. Ferrara (Sanofi, France), was added to the culture medium at a final concentration of 10 ng/ml. Anti-STAT6 (S-20), anti-Ets-1(2) (C-275), and anti-RNA pol II (N-20) polyclonal antibodies were from Santa Cruz. Anti-Phospho-STAT6 (TyR-641) polyclonal antibody was from Cell Signaling Technology. Anti-FLAG M2 monoclonal antibody was from Sigma. Anti-ETS antisera directed against Ets-1 (number 8), Ets-2 (number 41), and Ets-2 (number 38) were a generous gift of Dr. J. Gysdela. cDNA corresponding to hemagglutinin (HA)-pS1-Ets-1 was a generous gift of Dr. F. Soncin.

**PCR Analysis**—Total RNA was extracted with Trizol (Invitrogen) as described by the manufacturer and quantified at 280 nm. Total RNA was reverse-transcribed using a (dT) oligomer primer and 2 units of avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) and then used as template for PCR. The specific primers corresponding to the region +912/+1241 of the SOCS-1 cDNA were 5′-CAGCCACCTTC-GGCAACTTCC (forward) and 5′-GCCGACACGTCGAAAGAGCA (reverse). PCRs were carried out with 1 unit of Taq polymerase (Qiagen, Carlsbad, CA) and 5% of Me2SO during 35 cycles of successive incubations at 94 °C (for 30 s), at 55 °C (for 1 min), and at 72 °C (for 1 min). PCR products were run on a 1.5% agarose gel. Real time PCR was performed using a Light Cycler. 1 μl of reverse transcription was amplified with the SOCS-1-specific primers and 28S specific primers. Results obtained with SOCS-1-specific primers were normalized against 28S.

**Plasmids Constructs**—The construct encoding FLAG-SOCS-1 was from pCMV-Tag1-STAT6 (8). The pCMV-Tag1-STAT6 was cut with NheI and HpaI and inserted into the same site in pIRESneo vector (Clontech). The double mutant STAT6VT was prepared by introducing point mutations at 439 and 443 of the first exon was end-labeled with 32P-dCTP and hybridized to the total reaction mixture and run on a 6% polyacrylamide 7.5% urea sequencing gel in 1× TBE (89 mM Tris base, 89 mM boric acid, and 1.9 mM EDTA). Detection was achieved by autoradiography on a phosphorfluorimagier (Storm 840).

**Immunoprecipitation, Coimmunoprecipitation, and Western Blotting**—After stimulation for 30 min with IL-4 (10 ng/ml), cells were lysed in a buffer containing 50 mM Tris-HCl, pH 7.5, 1% Triton X-100 for immunoprecipitation (1% Nonidet P-40 for commumunoprecipitation), 50 mM NaCl, 50 mM NaF, 10 mM Na3PO4, 5 mM EDTA, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml leupeptin. The cell lysates were clarified by centrifugation. 500 μg of total protein was incubated with appropriate antibodies overnight and with protein G-Sepharose for 1 h at 4 °C. The immunoprecipitates were separated through 8% SDS-PAGE, proteins were transferred to Hybond-C extra membrane (Amersham Biosciences). Membranes were probed with the appropriate antibodies. This was followed by incubation with appropriate horseradish peroxidase-conjugated secondary antibody. Detection was achieved by enhanced chemiluminescence assay (Amersham Biosciences).

**Transfection Experiments**—For transient transfection, cells were seeded at 4 × 105 cells/well in 6-well dishes 24 h before transfection. The different plasmid DNA and the pCMV-galactosidase were adjusted to 3 μg of DNA and 6 μl of jet-PEITM solution (Polyplus transfection, Illkirch, France). After 24 h incubation, cells were supplemented with fresh serum-free culture medium and stimulated with IL-4 or not stimulated. Cells were lysed 16 h later in reporter lysis buffer (Promega). Luciferase gene expression was monitored using a commercial kit (Promega) and photon counting (MicroLumat Plus 96V, Berthold, Nasau, NH). Each transfection experiment was performed in triplicate.

For stable transfection experiments, HaCaT cells were transfected with pIRES plasmid containing the different cDNA coding for the different STAT6 variants and then cultured under 500 μg/ml G418 selective conditions for at least 1 month. Resistant cells were used for further experiments.

**Chromatin Immunoprecipitation Assay**—Chromatin immunoprecipitation experiments were performed essentially as previously described (31). DNA for PCR, 5 μl from 25 μl of IP was prepared for 35 cycles of amplification. The following primers, region –825/+439 of the SOCS-1 promoter 5′-AGGCGGAGATCCAGGTCCAGA (forward) and 5′-AGGAGCCGACAGGAGGTGTC (reverse), were used after anti-STAT6 or anti-Ets-1/2 immunoprecipitation and region –165/+52 of the Socs-1 promoter, was used after anti-RNA pol II immunoprecipitation, primers 5′-TCCGAGAAGGAGCGAAGGAC (forward) and 5′-GGCCGCTCTC-GCGATGCTC (reverse).

**Electrophoretic Mobility Shift Assays (EMSA)**—HaCaT cells were stimulated with IL-4 during 30 min, scraped in phosphate-buffered saline supplemented with 1 mM Na3VO4, and pelleted. Nuclear extracts, radiolabeled double-strand oligonucleotides, were prepared, and EMSA experiments were performed as described previously (33). The probes used in EMSA are listed in Table I. Supershift experiments were performed using the anti-STAT6 or with anti-Ets-specific antibodies.

**Oligonucleotide Pull-down Assays**—Streptavidin-agarose beads were incubated overnight with 5 μg of biotin-labeled oligonucleotides corresponding to the distal specific probes in binding buffer (20 mM HEPES, pH 7.5, 2.5 mM MgCl2, 40 mM KCl, 3 mM EDTA, 1 mM dithiothreitol, and 40% glycerol). Total cell extracts (1 mg of protein) were added for 90 min at 4 °C. Beads were washed in binding buffer and in TNE buffer (50 mM Tris-HCl, pH 8, 140 mM NaCl, 5 mM EDTA) and then resuspended in SDS-PAGE loading buffer and analyzed by SDS-PAGE. After electrophoresis, proteins were transferred to Hybond C extra membrane and then probed with the appropriate antibodies.

**RESULTS**

**Transcriptional Regulation of SOCS-1 mRNA by IL-4 Stimulation**—To analyze whether IL-4 enhances SOCS-1 gene expression in keratinocytes, we first performed reverse transcription (RT)-PCR to determine its transcript level. HaCaT cells were stimulated with IL-4 (10 ng/ml) for increasing periods of time. As shown in Fig. 1A (top), a low level of expression of SOCS-1 was observed in unstimulated cells; the expression of SOCS-1 appeared after 30 min of stimulation, peaked at 2 h, and declined thereafter to the basal level in 8 h. The level of 28S mRNA expression, used as RNA loading control, was not modified by IL-4 treatment (Fig. 1A, middle). We also examined whether IL-4 could regulate the expression of two other mem-
bers of the Socs family. In unstimulated cells no SOCS-3 mRNA was detected, and no modulation of its expression was detected after IL-4 stimulation (data not shown). Comparatively, a relatively high level of SOCS-2 mRNA was observed in unstimulated cells, which was not modified by cytokine treatment (Fig. 1A, bottom). To quantify SOCS-1 mRNA expression after IL-4 stimulation we used quantitative real time RT-PCR. As shown in Fig. 1B, we observed the same time course of activation and determined a 120-fold induction of SOCS-1 mRNA expression above the basal level after 2 h of IL-4 stimulation.

Cloning of the 5′-Flanking Region of Human Socs-1 Gene—We therefore undertook the cloning of the promoter region of the human Socs-1 gene. Dot plot comparison of human versus mouse sequences indicated that putative promoter elements are located on the human sequence (accession number Z46940) between 25,800 and 26,650 base position (23). The DNA sequence was subjected to computational analysis (MatInspector Software) (34). Putative binding sites for STAT6 (3 sites), Ets (2 sites), Sp1 (4 sites), IRF-1, AP-1, and GATA-1 were found in the proximal region of the promoter. The sequence and the DNA boxes are shown in Fig. 2. Alignment of the 5′-flanking region containing the 3xSTAT6 binding sites of the human and mouse Socs-1 genes revealed a 78% sequence identity in this part of the promoter (not shown).

The transcriptional start site was determined by computational analysis (TSSW using Softberry Software) and predicted to be located at nucleotide −16 from the first nucleotide of the previously described start of the first exon (accession number NM_003745). The real transcriptional site was determined by primer extension analysis carried out with RNA preparation from IL-4-stimulated cells using a 20-nucleotide-labeled primer. Two initiation sites were determined at nucleotide −20 and −16 from the start of the first exon (Fig. 3). This latter site seems to be the major initiation transcription site. DNA sequencing analysis revealed that the Socs-1 gene contains a TATA box located −30 nucleotide from the most proximal start of transcription (Fig. 2).

Our next aim was to determine whether RNA polymerase II could occupy the transcriptional initiation site after IL-4 stimulation. Chromatin immunoprecipitation experiments showed that RNA pol II was indeed recruited to the Socs-1 promoter after IL-4 stimulation of HaCaT cells (Fig. 4A).

Sequence analysis of the putative promoter revealed the existence of three STAT6-binding element (TTCN4GAA) lo-
STAT6 was recruited to this Socs-1 promoter region after IL-4 stimulation. The time course study indicated that the occupancy of the Socs-1 promoter by STAT6 was detectable as soon as 15 min after IL-4 stimulation (Fig. 4B).

To identify the elements that are important for IL-4-stimulated transcription of the Socs-1 promoter, two DNA fragments were generated by PCR with two 5′-primers surrounding the three STAT6 binding sites one upstream (Fig. 2, (1)) and the other downstream (Fig. 2, (2)). The 3′-primer corresponds to the previously described start of the first exon (Fig. 2, (3)). We used genomic HaCaT DNA as the template for the Socs-1 promoter cloning. We have therefore cloned these two DNA fragments, one of ~780 bp (PGL3-P1) and the other of ~580 bp (PGL3-P2) into the promoterless PGL3 basic, a luciferase reporter plasmid. Transient transfection in HaCaT cells of these two promoter constructs indicated that the activity of P1 promoter was indeed enhanced by IL-4, whereas the expression of P2 was not modified by IL-4 stimulation. These results suggested that IL-4REs are located between positions −587 and −747 (Fig. 5).

Implication of STAT6 in the Regulation of the Promoter—We have previously described that the tyrosine phosphorylation of STAT6 occurs after IL-4 stimulation of HaCaT cells (8). To further understand the implication of STAT6 in the regulation of either endogenous SOCS-1 mRNA or PGL3-P1 luciferase reporter plasmid expression, we developed HaCaT cells stably expressing gain-of-function (STAT6VT) or transcriptional dominant negative forms of STAT6 (STAT6ΔC) (30, 35). The constructs of different STAT6 variants are depicted in Fig. 6A. As shown in Fig. 6B, immunoprecipitation of STAT6 followed by Western blotting with anti-phospho-STAT6 confirmed that IL-4 induced the phosphorylation of STAT6 in HaCaT cells (lanes 1–2). The phosphorylation of exogenous STAT6 examined after anti-FLAG immunoprecipitation followed by Western blotting with anti-phospho-STAT6, indicated that these proteins are indeed phosphorylated after IL-4 stimulation (Fig. 6, lanes 6 and 10 compared with lanes 5 and 9). The phosphorylation of endogenous STAT6 was not affected by expression of STAT6ΔC (Fig. 6, lane 4 compared with lane 2). In STAT6VT-expressing cells after IL-4 stimulation and immunoprecipitation with anti-STAT6 antibodies, we observed an enhanced tyrosine phosphorylation of STAT6, which probably results from phosphorylation of the exogenous STAT6 in addition to the endogenous STAT6 (Fig. 6, lane 8 compared with lane 2).
The level of SOCS-1 mRNA expression was further characterized in the different cell lines by RT-PCR (Fig. 7A). Real-time quantitative PCR demonstrated that expression of SOCS-1 mRNA was increased by 3-fold in STAT6VT-expressing cells as compared with the parental cell line, whereas in STAT6/H9004C the expression was lowered by 4-fold (Fig. 7B).

The P1 and P2 promoter reporter plasmids were transiently transfected in all the above described HaCaT cell lines and assayed for luciferase activity. As shown in Fig. 7C, the response to IL-4 was dramatically enhanced in STAT6VT-expressing cells as compared with its expression in wild-type cells (18-fold as compared with 5-fold). Almost no expression of PGL3-P1 was observed in STAT6/H9004C-expressing cells after IL-4 stimulation. Altogether these data emphasized the predominant role of STAT6 in regulation of PGL3-P1 expression.

We then focused on the promoter elements that might account for IL-4 regulation of the P1 promoter. In the above characterized cells, we therefore used EMSA experiments to examine the nature of the protein-DNA complexes on the three potential SBEs within the promoter of Socs-1 gene. This binding was examined for each individual element. As shown in Fig. 8A, IL-4 induced the formation of one SBE-specific protein-DNA complex (Fig. 8, C1) on the proximal (pSBE) and on the median SBE (mSBE) probes in nuclear extracts from IL-4-stimulated cells as compared with extracts from unstimulated cells. The same experiment was carried out with nuclear extracts from STAT6VT- or STAT6/H9004C-expressing cells. An enhanced DNA-protein complex was observed in STAT6VT-expressing cells upon IL-4 stimulation, thus paralleling the increase of STAT6 phosphorylation described above (Fig. 8B). The expression of STAT6ΔC in these cells led to the formation of two complexes (C1 and C1*), one migrating at the same level as the endogenous STAT6-DNA complex (C1), the other (C1*) corresponding to STAT6ΔC-DNA complex (Fig. 8C). The presence of STAT6 in these DNA-protein complexes was demonstrated by supershift experiments with STAT6-specific antibodies.

In contrast, IL-4 induced formation of two distinct SBE-specific protein complexes (C1 and C2) on the probe corresponding to the distal SBE (dSBE), one migrating (C1) as the above described DNA-protein complex observed with the other probes and the other corresponding to a slower migrating DNA complex (C2). These two DNA-protein complexes were further supershifted by anti-STAT6 antibodies indicating that they both contain STAT6 (Fig. 8A). These two complexes could result from different degrees of STAT6 multimerization or to the presence of additional polypeptides. As shown in Fig. 8B the same DNA-protein complexes were observed with the nuclear extracts derived from STAT6VT-expressing cells, the intensity of the slower migrating complexes (C1) just being reinforced. In comparison nuclear extracts derived from STAT6/H9004C-expressing cells yielded a more complicated profile. In fact, each band was split into two species thus confirming the presence of STAT6 in the two protein-DNA complexes (Fig. 8C). In nuclear extracts from nonstimulated cells we observed a low intensity DNA-protein complex (Fig. 8C, *), which migrated slightly faster than the IL-4-stimulated protein-DNA complex. This complex did not contain STAT6, because it was not supershifted by STAT6 antibodies.

The Distal SBE Binds STAT6 and Ets—IL-4 induces formation of two SBE-specific protein-DNA complexes (C1 and C2) on the dSBE regulatory element within the Socs-1 promoter. The
Fig. 7. Effect of expression of STAT6 variants on endogenous SOCS-1 mRNA, PGL3-P1, or PGL3-P2 expression. HaCaT expressing STAT6 variants were either stimulated or not with IL-4 for 2 h. The effect on the SOCS-1 mRNA expression was investigated either by RT-PCR (A) or real time RT-PCR (B) as described in the legend to Fig. 1. C, expression of PGL3-P1 or PGL3-P2 was also examined after IL-4 stimulation in the different cell lines. The results are expressed as the fold increase in relative light units to the level in unstimulated HaCaT cells. The data are given as means of three independent experiments.

Sequence of the distal probe was analyzed with MatInspector version 2.2 (34) and found to contain binding sites for other transcription factors. A potential Sp1 binding site was evidenced a few bases 5' of the dSBE and an EBS, which is included in the SBE. By using competition with specific Sp1 probes and/or antibodies, we ruled out the presence of Sp1 in the two complexes observed on the SBE probe (data not shown). Competition with oligonucleotides containing a high affinity EBS (36) abrogated the C2 complex, whereas its mutated counterpart had no effect (data not shown). These data strongly suggested that at least the C2 complex contains a transcription factor able to bind to an EBS. To further characterize this transcription factor, supershift experiments and/or inhibition of binding assays were performed with specific antisera. Anti-STAT6 antibodies supershifted both C1 and C2 complexes confirming that both complexes contain STAT6 (Fig. 9A). Inhibition of binding by antisera specific for Ets-1 and/or Ets-2 revealed that the slower migrating inducible C2 complex may contain both Ets proteins (Fig. 9A), whereas the faster migrating C1 complex was unaffected and therefore only contains STAT6. Antisera against other members of the Ets family failed to affect the formation of the C2 complex (data not shown). Migration of the single complex, evidenced on the median or proximal SBE of Socs-1 promoter, was not modified by an interaction with antisera specific for Ets-1 and Ets-2 (data not shown), indicating that Ets was not present in this complex. The presence of Ets on the Socs-1 promoter chromatin region was further examined by chromatin immunoprecipitation. As shown in Fig. 9B, the presence of Ets is detectable on the IL-4RE after IL-4 stimulation, because a PCR product was only observed after IL-4 stimulation and immunoprecipitation with antibodies directed against Ets 1/2.

Using DNA affinity purification we further identified the factors able to interact with biotinylated dSBE. Western blotting confirmed that IL-4 stimulation induced not only STAT6 but also Ets-1 binding to this oligonucleotide (Fig. 9C). The same experiment was also performed in HA-p51Ets-1 protein-overexpressing cells and as shown in Fig. 9C (lanes 3 and 4), we also observed Ets-1 protein binding, which was revealed by anti-HA antibodies. In unstimulated cell extracts, we observed the binding of Ets-1 to the biotinylated oligonucleotide indicating that the in vitro Ets protein is able to bind to this site in absence of stimulation. Taken together, these results strongly supported the fact that Ets1 and STAT6 formed DNA-protein complex on the STAT6 distal probe in response to IL-4 in HaCaT cells.

Interactions of STAT6 and Ets-1 in Response to IL-4—Identification of an IL-4-induced STAT6-Ets DNA-binding complex prompted us to investigate whether these proteins might interact in the absence of DNA. When HaCaT cell extracts from either unstimulated or IL-4-stimulated cells were immunoprecipitated with two different STAT6 antibodies, directed either against the transactivation or the DNA binding domain of this protein, and/or with anti-Ets-1 antibodies directed against the DNA binding sites, no communoprecipitation was detected. This might be due in part to the fact that epitopes recognized by these antibodies are hidden and therefore implicated in the protein-protein interaction. To eliminate this, we have transfected cDNAs that encode an HA-p51Ets-1 and a FLAG-STAT6 protein and performed immunoprecipitation of ectopic proteins with antibodies directed against the tags. Immunoprecipitation of STAT6 pulled down low amounts of Ets in cellular extracts of unstimulated cells (Fig. 9D, left). The level of communoprecipitated proteins increased after IL-4 stimulation. The same result was observed in the reciprocal experiment (Fig. 9D, right). These observations indicated that STAT6 and Ets might form a complex that corresponds to one of the protein-DNA complexes (C2) observed on the dSBE and could be observed either in presence or absence of DNA.

Transcriptional Activity of Socs-1 Promoter Variants—The Socs-1 promoter contains three STAT6 binding sites (37), which are necessary for IL-4 regulation of the transcription of the gene. In fact, when this DNA element (−747/+52) was cloned upstream the luciferase gene in pGL3 basic vector (PGL3-P1), we observed an ~5-fold activation of the promoter after IL-4 stimulation of these cells (Fig. 5). To rule out a possible cooperation (inhibition or enhancement) of the DNA element located between the STAT6 binding sites and the transcriptional initiation site, we have subcloned the region between −747 and −434 into a minimum plasmid (pTATAluc) to give rise to pGL3-IL-4RE. As shown in Fig. 10 we observed an ~15-fold enhancement of luciferase activity after IL-4 stimulation, which corresponds to a 3-fold increase as compared with the whole 5'-flanking promoter region. We then examined individual or combined mutations of each STAT6 binding site (Table I). Mutations of either pSBE or mSBE led to promoters that are still able to respond to IL-4, but the level of luciferase activity is decreased by 50% (Fig. 10). Another additional 50% decrease was observed when the proximal and median STAT6 (mut mpSBE) binding sites were both mutated. The resulting construct still showed IL-4 inducibility.

In contrast when the distal binding site was mutated, preventing the binding of both STAT6 and Ets proteins, no IL-4-induced luciferase activity was observed. A partial restoration of IL-4 responsiveness was observed in the mutant (mut dEBS), which allowed only the binding of STAT6. EMSA analysis with
were used in supershift experiments. Anti-STAT6 antibodies observed in the nuclear extracts from unstimulated cells. *, indicates the low intensity band pressing HaCaT cells (B) or IL-4-stimulated HaCaT cells (C-expressing cells and analyzed by EMSA. The result was almost complete suppression of IL-4 responsiveness of the PGL3-IL-4RE vector gradually decreased. The net induction was lower than the level of endogenous SOCS-1 mRNA expression observed and quantified by real time PCR in the cells after IL-4 stimulation. In fact, we have cloned the proximal promoter region of SOCS-1 gene, and we can not formally exclude that additional distal enhancers may be implicated in the regulation of endogenous SOCS-1 transcription in the context of chromatin.

In the present study, we isolated and identified 800 bp of the 5'-flanking region of the human SOCS-1 gene to gain insight into its induction following keratinocyte stimulation by IL-4. This sequence allowed us to characterize the human SOCS-1 promoter region and identified a DNA region involved in the regulation of this gene by IL-4.

Mapping of the transcriptional start by primer extension demonstrated the presence of a major start site located at −18 bp relative to the first exon. The 5'-flanking region of the human SOCS-1 gene contains a TATA box located 30-bp upstream from the transcription initiation site. We found that the 5'-flanking region including up to −790 bp relative to the transcriptional initiation site contains all of the elements necessary to achieve basal promoter activity. The IL-4RE is, in contrast, located between −747 and −587, relative to the transcription initiation site, and contains three STAT6 binding sites (named proximal, median, and distal SBE). We have then linked this IL-4RE (−747 to −587) to a minimal luciferase reporter plasmid (pTATA luc). This construct shows a better responsiveness to IL-4 than the whole 5'-flanking region, suggesting that the region (−587 to +52) might contain negative regulatory elements. The level of SOCS-1 promoter induction was lower than the level of endogenous SOCS-1 mRNA expression observed and quantified by real time PCR in the cells after IL-4 stimulation. In fact, we have cloned the proximal promoter region of SOCS-1 gene, and we can not formally exclude that additional distal enhancers may be implicated in the regulation of endogenous SOCS-1 transcription in the context of chromatin.

By EMSA, we demonstrated that one single DNA-protein complex, which is supershifted by anti-STAT6 antibodies, was observed with the probes corresponding to the proximal and the median SBE. In contrast the distal probe gives rise, with nuclear extracts from IL-4-stimulated cells, to two bands in EMSA that are both supershifted with anti-STAT6 antibodies. Computational analysis of this promoter region revealed, in the distal probe, the presence of putative binding sites for Sp1 and Ets family transcription factor. By using either competitive studies with specific probes for Sp1 or Ets or displacement experiments with different antibodies, we demonstrated that Ets factors, Ets-1 and Ets-2, are also present in the slower migrating DNA-protein complexes observed in the distal SBE probe. We therefore evidenced a physical and functional association between Ets-1 and STAT6. Interactions between these two unrelated transcription factor families resulted in the formation of a composite protein-DNA complex on the distal probe. During the completion of this work a recent paper described characterization of the human SOCS-1 gene promoter. In this study the authors described the fixation of STAT6 on the three SBE, and in addition they claimed that an unknown complex was also observed on the distal SBE probe. They suggest that this complex could be implicated in the regulation of the basal level of SOCS-1 gene expression.
Mutations of either proximal, median SBE, or both sites in the pGL3-IL-4RE lead to a significantly reduced IL-4-induced luciferase activity in comparison with the wild-type construct. In contrast, mutation of the STAT6 consensus sequence of the distal SBE, which prevents binding of both STAT6 and Ets transcription factors, leads to a dramatic loss of IL-4 responsiveness of the construct. The importance of the Ets-binding element within the distal SBE was further determined by an other mutation (mut dEBS), which preserves the binding of STAT6 but prevents the binding of Ets. However, EMSA analysis indicated that the binding of STAT6 to this mutated sequence is dramatically lowered as compared with the binding observed on the wild-type distal probe. It is unclear whether the lower inducibility of the mut dEBS construct is because of reduced affinity of STAT6 or to abrogation of Ets binding.

Cross-talk between transcription factors has become a commonly recognized mode of gene regulation. Such interactions can either inhibit or activate transcription. For example in the case of STAT6 a physical interaction with NF-κB results either in synergistic activation or inhibition of IL-4-induced transcription depending on the physical properties of the two binding sites. In fact, when the two transcription binding sites overlap as in the E-selectin promoter a transcriptional repression has been observed (40). In contrast, STAT6 synergizes with activators of NF-κB to induce IL-4-responsive genes when the distance between the two sites is increased (41). On the other hand several Ets transcription factors interact with other regulators of gene expression and cell cycle progression. Ets-1 and/or Ets-2 and STAT6 are components of protein-DNA complexes. A. nuclear extracts from IL-4-stimulated cells were used untreated or after incubation with anti-STAT6 (lane 3), anti-Ets1/2 (lane 4), anti-Ets-1 (lane 5) and anti-Ets-2 (lane 6) antibodies. Radiolabeled dSBE probe of the Socs-1 promoter was incubated with the different nuclear extracts and analyzed by gel electrophoresis. B. soluble chromatin from HaCaT cells either stimulated or not with IL-4 for 30 min was immunoprecipitated with anti-Ets1/2 antibodies. PCR was performed by using oligonucleotides that cover the three STAT6 binding sites as described in the legend to Fig. 4. Input represents PCR amplification of total sample. C. affinity purification of DNA-binding proteins was performed with 5'-biotinylated dSBE probe and whole extracts from HaCaT cells left unstimulated (lane 1) or stimulated with IL-4 for 30 min (lane 2) or whole extracts from HA-Ets-1-expressing HaCaT cells left unstimulated (lane 3) or stimulated with IL-4 for 30 min (lane 4). Bound proteins were identified by Western blotting with anti-STAT6, anti-Ets-1, and anti-HA-specific antisera. D. HaCaT were transiently transfected with FLAG-STAT6 and HA-Ets-1. Cell lysates were immunoprecipitated with either anti-FLAG (left) or anti-HA (right) monoclonal antibodies as described under "Experimental Procedures." The immunoprecipitates were separated on SDS-PAGE, and the presence of STAT6 or Ets-1 was visualized by Western blotting with specific antibodies.
Absolutely required for activation of Socs-1. Overexpression of Ets-1 protein dramatically decreased the expression of PGL3-IL-4RE. These findings suggested at least four non-mutually exclusive hypotheses. First, overexpression of Ets could bind to SBE motifs without transactivation, and therefore compete with STAT6 for site occupancy. Thus the high level of Ets...
binding might prevent Socs-1 transcription. Second, Ets could induce an inhibitory gene product. Third, the STAT6 transcription domain may be linked to Ets-1 and therefore block its ability to activate transcription. The occupancy of the STAT6 transcription activation domain during interaction with Ets is suggested by the fact that antibodies directed to the STAT6 transcription activation domain were unable to coimmunoprecipitate Ets. Finally, Ets could compete with STAT6 for limited quantities of transcriptional coactivators such as CBP/p300, Src/NCoA, and/or other members of coactivator families. The p160/NcoA and CBP/p300 families of transcription coregulators have been shown to be specifically recruited by the transcription domain of STAT6 (47–49), and this interaction with these coactivators is absolutely required for maximal IL-4-regulated transcription. The CBP/p300 might be one of likely candidates for interaction, because it has been shown that CBP can bind to both STAT6 (48, 49) and Ets (50). Two Ets-1 binding sites have been described on CBP/p300, one region maps between amino acids 313 and 452 and the second one maps between amino acids 1449 and 1892 (50, 51). On the other hand, the smallest segment of CBP/p300, which was still able to interact with STAT6 transcription activation domain is between amino acids 1850 and 2176 (48). Thus, the domains of CBP, which interact with STAT6 and Ets are different and therefore may suggest that the binding of these two transcription factors would be cooperative in the recruitment of the coactivator. The loss of any contact points, such as mutation in the p- and/or mSBE reduces IL-4 induced Socs-1 promoter activity and loss of dSBE and EBS element abolished it. The presence of STAT6 alone is not sufficient to induce transcription of the Socs-1 gene, presumably because it is unable to promote the formation of an effective transcriptional complex. Recent study indicated that the binding of Ets in the context of chromatin is independent of any chromatin accessory factors. However, Ets binding itself is not sufficient for full activation of transcription (52). It has been suggested that the binding induces changes in chromatin and thereafter allows the recruitment of other coactivator or transcription factors, which act as enhancers of transcription.

In this paper, we have identified the importance of SBE and Ets elements in the activation of the human Socs-1 promoter by IL-4. Our novelty of these results is in the presence of such associations between two unrelated factors, Ets and STAT6. We also demonstrated that this interaction is implicated in the regulation of Socs-1 expression in human keratinocytes.

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