Analysis of SOCS-3 Promoter Responses to Interferon γ*

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SOCS-3 (suppressor of cytokine signaling 3) is an intracellular protein that is selectively and rapidly induced by appropriate agonists and that modulates responses of immune cells to cytokines by interfering with the Janus kinase/signal transducer and activator of transcription (Jak/STAT) pathway. On the basis of the observations that interferon γ (IFNγ) up-regulates SOCS-3 gene and protein expression in primary mouse macrophages, J774 macrophage cell line and embryonal fibroblasts, we investigated which sequences of the 5′ SOCS-3 gene are responsive to IFNγ. By promoter deletion analysis we identified a functional IFNγ-responsive element, located at nucleotides −72/−64 upstream from the transcription initiation, whose presence and integrity is necessary to ensure responsiveness to IFNγ. This element contains a STAT consensus binding sequence (SOCS-3/STAT-binding element (SBE)) whose specific mutation totally abrogated the responsiveness to IFNγ. In contrast, discrete deletion of other 5′ regions of the SOCS-3 promoter did not substantially modify the inducibility by IFNγ. Electromobility shift assay analyses revealed that IFNγ promotes specific DNA binding activities to an oligonucleotide probe containing the SOCS-3/SBE sequence. Even though IFNγ triggered tyrosine phosphorylation of both STAT1 and STAT3 in macrophages and J774 cells, only STAT1 was appropriately activated and thus found to specifically bind to the SOCS-3/SBE oligonucleotide probe. Accordingly, IFNγ-induced SOCS-3 protein expression was not impaired in STAT3-deficient embryonal fibroblasts. Taken together, these results demonstrate that the induction of SOCS-3 by IFNγ depends upon the presence of a STAT-binding element in the SOCS-3 promoter that is specifically activated by STAT1.

Interferon γ (IFNγ)1 is a pluripotent cytokine involved in the regulation of nearly all the different phases of both innate and adaptive immune responses. Produced by activated T and natural killer cells, IFNγ has a crucial role in several processes, including host defense against viruses and microorganisms, cell proliferation, phagocyte activation, control of apoptosis, promotion of antigen processing and presentation, and T helper type 1 (Th1) differentiation (1). IFNγ has a number of activating properties on cells of the immune system but can also exert important immunosuppressive actions by modulating cellular responses to different cytokines and inflammatory stimuli (2). For example, under specific conditions, IFNγ may inhibit proinflammatory cytokine release by activated human peripheral blood mononuclear cells and polymorphonuclear neutrophils (3, 4) and up-regulates the release of cytokine antagonists such as IL-1Ra, type II IL-1 receptors, and IL-18BP from mononuclear phagocytes (5–9). The biologic activities of IFNγ are mainly mediated through the regulation of gene expression. This is achieved upon interaction of IFNγ with its specific cell surface receptor(s) and activation of different intracellular signaling cascades (10). It is well established that the immediate transcriptional responses induced by IFNγ are achieved primarily through the activation of the Jak/STAT signaling pathway (11). STAT1 plays a major role in mediating the immune and proinflammatory actions of IFNγ (12, 13), but also STAT3 and STAT5 can be activated by IFNγ in certain cell types (14–16).

One of the genes rapidly induced by IFNγ is SOCS-3 (suppressor of cytokine signaling 3) (17–22). SOCS-3 is a member of a family of intracellular proteins that negatively regulate responses of immune cells to cytokines by inhibiting the Jak/STAT pathway (23). Although in vitro overexpression studies have reported that the SOCS proteins are pleiotropic inhibitors of the Jak/STAT pathways activated by several cytokines, targeted gene disruption has shown that these molecules have more specific roles in vivo (20, 21, 24–28). In this regard, conditional gene targeting studies have recently suggested that SOCS-3 has a nonredundant function in macrophages and hepatocytes, in which it selectively regulates IL-6 and gp130 signaling (20, 21), in accordance with previous observations showing that SOCS-3 binds phosphorylated gp130 and regulates the subsequent activation of STAT3 induced by IL-6 (29–31). Furthermore, these recent reports revealed that SOCS-3 not only inhibits STAT3-dependent IL-6 signaling but also precludes IL-6 from eliciting STAT1-dependent responses, suggesting that this mediator has a critical role in the balance between these two pathways.

In this study, we investigated the mechanisms regulating SOCS-3 expression by IFNγ in mouse peritoneal macrophages, J774 mouse macrophage cell line, and embryonal fibroblasts. Analysis of ∼7-kb of the genomic 5′-flanking region of the

Received for publication, August 14, 2003, and in revised form, January 14, 2004. Published, JBC Papers in Press, January 23, 2004, DOI 10.1074/jbc.M308999200
mouse SOCS-3 gene revealed that the most proximal STAT-Binding site present in the SOCS-3 promoter is necessary for IFN-γ-induced transcriptional activity. Deletion or point mutation of this STAT-Binding element (SBE) completely abrogated IFN-γ action. Accordingly, IFN-γ promoted specific DNA binding activities to an oligonucleotide corresponding to the SOCS-3/SBE sequence that exclusively contained STAT1. Any contribution of STAT3 was excluded, as demonstrated by the immobility of IFN-γ-activated STAT3 to bind the SOCS-3/SBE sequence and by the evidence that IFN-γ fully retains the capacity of inducing SOCS-3 expression in STAT3−/− embryonic fibroblasts. These data further reinforce the finding that induction of SOCS-3 expression by IFN-γ is STAT1-dependent. On the basis of these observations, it is conceivable that defining the molecular mechanism and the signaling pathway responsible for the induction of SOCS-3 gene expression in response to IFN-γ might help in clarifying how IFN-γ can modulate cellular responses to different cytokines.

EXPERIMENTAL PROCEDURES

Cell Culture—C57BL/6J mice, purchased from Harlan Italy (Correzzana, Milan, Italy), were injected with 1 ml of 4% thioglycollate 4 days before harvesting peritoneal elicited (PEM) cells. Red cells were removed by hypotonic lysis, and macrophages were seeded in 6-well plates in RPMI supplemented with 10% heat-inactivated low endotoxin fetal bovine serum (0.01 endotoxin units/ml; Biochrom, Berlin, Germany), 2 mM ultraglutamine 1,100 units/ml penicillin, 100 mg/ml streptomycin (Biowhittaker, Verviers, Belgium) and let adhered overnight. Mouse macrophage cell line J774 (kindly provided by Dr. V. Kruys, Université Libre de Brussels, Brussels, Belgium) was cultured in Dulbecco’s modified Eagle’s medium (from Biowhittaker Europe, Verviers, Belgium) supplemented with 5% heat-inactivated low endotoxin fetal bovine serum and passed twice weekly. Immortalized STAT3 wild type (flxed/fixed, fl/fl) and STAT3−/− (deleted/deleted, Δ/Δ) MEFs, generated as previously described (32), were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and passed every 3 days.

Construction of Reporter Plasmids—The 5′ genomic region of murine SOCS-3 was cloned from 129 genomic library (33) in Bluestrip (Stratagene, La Jolla, CA). Cloning of the XhoI/NcoI fragment into pBS was followed by orientation verification, restriction enzyme digestion, and subcloning of a ~7-kb construct into the KpnI/BamHI sites of the promoterless luciferase reporter vector pGL2basic (Promega, Madison, WI), generating construct 1. The SOCS-3 genomic fragment cloned in construct 1 spans from −6298 to +884 relative to the transcription initiation, which is defined as +1 (GenBank® accession number AF314501) and contains the untranslated exon 1 and the fragment of exon 2 upstream the ATG. Truncated forms of SOCS-3 promoter were generated by restriction enzyme digestion of construct 1 as follows: construct 2, deleted the KpnI fragment; construct 3, deleted the KpnI/Nhel fragment; construct 4, deleted the XhoI/Spel fragment; construct 5, deleted the KpnI/SacII fragment. Construct 6 was generated by KOD Plus 2° PCR (Toyobo, Tokyo, Japan) and spans −50 to +968 nucleotides. 3′-deleted construct 7 was generated by removing the proximal SacII/NcoI fragment from construct 1. A mutated form of the −72/−64 STAT-binding element in construct 5 was produced by substituting the TTTCAGGAA sequence with TTTCAGGTT by site-directed mutagenesis.

Transient Transfection and Dual Luciferase Assay—J774 (4 × 105 cells/well) were seeded in 12-well plates and transfected 24 h later with SuperFect transfection reagent (Qiagen). 3 μg of each SOCS-3 promoter/luciferase construct were mixed at a 50:1 ratio with the Renilla reniformis luciferase activity construct (Promega) and incubated with 7.5 μl of SuperFect transfection reagent according to the manufacturer’s instructions. 12 h before stimulation the cells were split into equal aliquots and replated. The cells were then treated with 100 units/ml IFN-γ (PeproTech, London, UK) or left untreated as a control, for the time indicated. After stimulation, the cells were harvested, washed twice with phosphate-buffered saline, and lysed in 30 μl of Passive lysis buffer (Promega) followed by two freeze-thaw cycles. Luciferase assays of both firefly and Renilla reniformis luciferases were performed using a dual luciferase reporter assay system (Promega) according to the manufacturer’s instructions, and the enzymatic activities of both luciferases were quantified using a Packard Lumicount Microplate Lumimeter (Packard Instrument Co., Meriden, CT). The values of firefly luciferase activity were divided for the R. reniformis luciferase activity, to normalize for differences caused by unequal transfection efficiency.

Western Blot—The cells were seeded the day before stimulation for: 1.5 × 106 J774, 6.5 × 105 P2M and 0.75 × 106 MEFs in 6-well plates. After stimulation for the times indicated with 100 units/ml IFN-γ or 200 units/ml IL-6 (PeproTech), whole or nuclear cell lysates were prepared, and immunoblot analysis was performed as previously described (34) using the following primary Abs: anti-NH2 terminus SOCS-3 (Immuno-Biological Laboratories, Tokyo, Japan) diluted at 5 μg/ml, anti-phosphoysotrine STAT3, anti-STAT3, anti-phosphoysotrine STAT1, and anti-p38 mitogen-activated protein kinase (Cell Signaling Technology, Beverly, MA) diluted as recommended by the manufacturer; anti-STAT2 diluted at 2 μg/ml and anti-lamin A/C (1/100 diluted) (Santa Cruz Biotechnology, Santa Cruz, CA) and/or (1/2000) (Sigma). Antibody binding was detected by using horseradish peroxidase-conjugated anti-rabbit IgG (1/5000 diluted) (Amersham Biosciences) and was revealed using ECL (Amersham Biosciences). In selected experiments, quantitative analysis of STAT3 phosphorylation was carried out utilizing the Odyssey Infrared Imager (Li-Cor). In the latter case, antibody binding was detected by using Alexa Fluor 680-conjugated goat anti-rabbit IgG (1/5000 diluted) (Molecular Probes, Eugene, OR) or IRDye 800-conjugated goat anti-mouse IgG (1/2000 diluted) (Rockland, Gilbertsville, PA), according to the manufacturer’s instructions.

Northern Blot—After stimulation with IFN-γ, J774 were harvested at different times, and total RNA was extracted by the guanidinium isothiocyanate method and processed for Northern blot analysis, as already described (35). Northern blot analysis was performed on 15 μg of RNA/lane. Specific SOCS-3 mRNA was detected by autoradiography after Northern blot hybridization with the cDNA probe encoding SOCS-3, labeled by the Ready-to-go kit (Amersham Biosciences).

Electromobility Shift Assay—J774 cells/well treated with IFN-γ, 20 × 105 PEM, and 5 × 105 MEF were seeded in 60-mm culture dishes overnight. After IFN-γ or IL-6 stimulation for the times indicated, the cells were harvested, and the nuclear extracts were prepared as described previously (36). Protein-DNA complexes were detected by EMSA analysis as previously described (19). 5 μg of nuclear extracts were incubated with a 25,000-fold double-stranded oligonucleotide probe containing the STAT-binding element (SOCS-3/SBE) located at −72/−64 nucleotides of the promoter of the SOCS-3 gene (5′-CATTTCCAGGAA-TGCGGGGGC-3′) or with the mutated form of this sequence (5′-CATTTCCAGGTTTGCGGGGGC-3′) for 15 min. Supershift experiments were performed by incubating extracts with 2 μg of anti-STAT1, anti-STAT3, or anti-CRE Abs (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min at room temperature before adding the labeled probe. The reaction mixtures were then subjected to electrophoresis in a 5% non-denaturing polyacrylamide gel, dried, and analyzed in an Instant Imager (Packard Instruments, Meriden, CT).

RESULTS AND DISCUSSION

Induction of SOCS-3 Gene and Protein Expression by IFN-γ—We initially investigated the ability of IFN-γ to induce SOCS-3 protein expression in the mouse macrophage cell line J774 and PEM. SOCS-3 protein expression was not detectable in resting J774 and PEM cultures (Fig. 1, A and B, medium). The addition of 100 units/ml IFN-γ to each cell culture induced high levels of SOCS-3 protein expression, which was maximal within 3 h, remained stable for up to 6 h and declined thereafter (Fig. 1, A and B). Northern blot analysis indicated that barely detectable levels of SOCS-3 mRNA were present under non stimulated conditions in J774 cells (Fig. 1C). Following IFN-γ stimulation SOCS-3 mRNA was rapidly induced, reaching maximum levels within 1 h (Fig. 1C). These results extend at the protein level previous observations showing that IFN-γ is able to transiently induce SOCS-3 mRNA in different cell types (17, 18, 20, 37–39).

Responses of SOCS-3 Reporter Gene to IFN-γ—To identify the region(s) responsive to IFN-γ in the SOCS-3 gene, we carried out a functional analysis of the SOCS-3 promoter. The murine SOCS-3 gene structure has been previously determined (40) and comprises an untranslated exon 1 (+1 to 299) separated from exon 2 (starting at +856) by an intron (+300 to +855). The main transcription site identified has been referred to as +1, and the translation initiation site for murine SOCS-3 has been
identified in exon 2 at +946 (Fig. 2) (18). A series of 5' deletions of the mouse SOCS-3 promoter/reporter constructs (schematically represented in Fig. 2) were generated as described under “Experimental Procedures.” Construct 1 spans the −6298 to +884 5’ genomic region of mouse SOCS-3 linked to the luciferase reporter in pGL2Basic vector. The 5’ truncations of construct 1 are: construct 2, nucleotides −3980 to +884; construct 3, nucleotides −2661 to +884; construct 4, nucleotides −2056 to +884; construct 5, nucleotides −456 to +884; and construct 6, nucleotides −50 to +968. The 3’ truncation of construct 1 is: construct 7, nucleotides −6298 to −457. Basal and IFNγ-induced luciferase activity were assayed after transient transfection of J774 cells with the different constructs together with pRL-null, used to normalize transfection efficiency. Luciferase activity was expressed as fold induction by IFNγ. pRL-null was normalized to the corresponding Renilla luciferase activity. Re sponsiveness to IFNγ was calculated as the ratio of normalized luciferase activity of stimulated over unstimulated cells. The mean values with S.D. as indicated are from four independent experiments.

A further 5’ removal of 404 nucleotides resulted in the complete loss of responsiveness to IFNγ. Indeed, stimulation with IFNγ caused no further increase of luciferase activity in J774 cells transfected with construct 6 (−50/+968 nucleotides) as compared with J774 transfected with pGL2Basic alone (1.4 ± 0.08 and 1.3 ± 0.1-fold, respectively) (Fig. 3), indicating that the region from nucleotides −50 to +968 is likely not involved in promoter activity. Collectively, these data show that the promoter region mediating IFNγ induction is restricted within nucleotides −452 to −50. To further confirm these data, we assayed IFNγ-induced luciferase activity after transfecting J774 with construct 7 (−6298/−453 nucleotides) carrying a 3’ deletion of the full-length promoter that removes the proximal 1381 nucleotides, where the minimal IFNγ-responsive elements are located. Removal of this region reduced luciferase activity of IFNγ-stimulated versus untreated cells to 1.9 ±

**Fig. 2.** Graphic representation of the ~7-kb genomic 5’-flanking region of murine SOCS-3 and of different SOCS-3 promoter reporter constructs. Exon 1 (+1/299) and exon 2 (+858/+946) and the start codon ATG are shown on top. A diagram of the each pGL2Basic-derived construct linking the firefly luciferase reporter gene with different portions of the mouse SOCS-3 promoter, is represented. The numbers refer to the 5’ terminal nucleotides included in each construct with respect to the transcription start site of the above sequence.
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1-fold, indicating that the sequences located upstream nucleotide −454 do not significantly contribute to IFNγ responsiveness.

Previous analysis of the region encompassing −454 to −50 nucleotides revealed the presence of two putative STAT-binding elements (TTXγAA, where X is any nucleotide), found at −95 to −87 and at −72 to −64 (40). The −72/−64 proximal STAT consensus element (TTCGAGGA) had been shown to be essential for leukemia inhibitory factor-induced SOCS-3 promoter transactivation in ACTH-secreting corticotroph AtT-20 cells (40) and to be involved in insulin-induced SOCS-3 expression (41). Therefore, in subsequent experiments, we focused on this SOCS-3/SBE. To analyze whether SOCS-3/SBE was necessary for SOCS-3 induction by IFNγ, we generated a mutated form of this sequence by site-directed mutagenesis, introducing AA → TT substitution of −65/−64 nucleotides into the −454/ +884 nucleotides promoter of construct 5 (Fig. 4, Construct 5mut), thus destroying the specific TTCGAGGA. Luciferase activity induced by IFNγ was then assayed in J774 cells transiently transfected with the luciferase expression plasmid carrying the mutated form of the −454/+884 SOCS-3 promoter (construct 5mut) and was compared with that obtained with the −454/+884 wild type promoter (construct 5) (Fig. 4). Luciferase activity in response to IFNγ was completely abrogated in the presence of mutant STAT-binding sequence at any time assayed (Fig. 4).

Taken together, our SOCS-3 promoter analysis indicated that (i) the region from nucleotides −50 to +968 (construct 6) is likely not involved in promoter activity; (ii) the 5′ truncated constructs 1–5 display similar inducibility by IFNγ, indicating that the region upstream nucleotide −454 is not responsible for IFNγ-induced SOCS-3 promoter activity; (iii) the region responsive to IFNγ is localized at nucleotides −454 to −50, as indicated by the complete loss of responsiveness to IFNγ upon its removal (in construct 6 and construct 7); and (iv) within the region from −454 to −50, a STAT-binding element from nucleotides −72 to −64 is fully responsible for the induction of the SOCS-3 reporter gene in response to IFNγ.

**STAT1-dependent Induction of SOCS-3 by IFNγ—SOCS-3 induction has been shown to be strictly dependent on both STAT1 and STAT3 activation in leukemia inhibitory factor-stimulated AtT-20 cells (40) and on STAT3 activation in granulocyte colony-stimulating factor-activated granulocytes (42). Because our data indicated that the induction of the SOCS-3-reporter gene by IFNγ was dependent on a STAT-binding sequence, we determined which was/were the STAT(s) involved in the activation of the SOCS-3 promoter by IFNγ. As shown in Fig. 5A, analysis of whole cell lysates prepared from both J774 cells and PEM stimulated with IFNγ revealed maximal tyrosine phosphorylation of STAT1 and STAT3 within 20 min. Accordingly, EMSA performed with nuclear extracts from resting and IFNγ-stimulated J774 and PEM revealed strong DNA binding activities to the oligonucleotide probe containing the SOCS-3/SBE sequence only in activated macrophages (Fig. 5B, lanes 2 and 6). Importantly, disruption of the SOCS-3/SBE consensus sequence resulted in the complete loss of any DNA binding activities (Fig. 5B, lanes 4 and 8), suggesting that SOCS-3 induction by IFNγ relies on a direct and specific binding of STAT(s) to the SOCS-3/SBE sequence. Indeed, preincubation of nuclear extracts from IFNγ-treated J774 and PEM cells with Abs against STAT1, but not against STAT3, fully displaced the DNA-protein complex (Fig. 5C, lanes 3, 5, 9, and 11), demonstrating that the nuclear factor(s) binding to the SOCS-3/SBE motif is represented by STAT1 only. Nevertheless, the SOCS-3/SBE sequence remains perfectly able to bind tyrosine-phosphorylated STAT3, as observed, for instance, in J774 cells or PEM stimulated with IL-6 (Fig. 5D). Three distinct SOCS-3/SBE-binding complexes (A, B and C) were in fact detected in nuclear extracts from IL-6-treated macrophages, the most abundant ones being complexes A and C in nuclear lysates of IL-6-stimulated J774 cells (Fig. 5D, lane 3) and complex A in PEM-derived nuclear lysates (Fig. 5D, lane 10). The presence of these three specific DNA-binding complexes is consistent with the formation of STAT1/STAT3 homo- and heterodimers, as revealed by the incubation with Abs against STAT1 and/or STAT3, which abolished the formation of complexes A (STAT3 homodimers) and C (STAT1 homodimers) in J774 cells and PEM (Fig. 5D, lanes 4–6 and 11–13) and complex B (STAT1/STAT3 heterodimers) in J774 cells (Fig. 5D, lanes 4–6). The very faint complex B in IL-6-activated PEM lysates was displaced neither by anti-STAT1 nor by anti-STAT3 Abs (Fig. 5D, lanes 11–13). Taken together, these data not only show that, at least in response to IL-6, STAT3 can bind to the SOCS-3/SBE element, but also exclude the possibility that the lack of binding of the IFNγ-activated STAT3 to the SOCS-3/SBE probe is due to the selectivity of this DNA sequence for STAT1.

To clarify why the IFNγ-activated STAT3 does not bind the SOCS-3/SBE oligonucleotide, we initially compared the relative ability of IFNγ and IL-6 to induce STAT3 activation/nuclear translocation in both J774 cells and PEM and then determined whether and how the latter phenomena correlated with cytokine-induced SOCS-3/SBE binding activities. Western blot experiments, depicted in Fig. 6A, demonstrate that the amount of tyrosine-phosphorylated STAT3 present in nuclear lysates of IL-6-treated cells is much higher than that induced by IFNγ. In
contrast, tyrosine phosphorylation of STAT1 triggered by IFNγ was much stronger than that elicited by IL-6 (Fig. 6A). Furthermore, because STAT3 can be phosphorylated in a regulated and inducible manner on serine, other than tyrosine, residues (an event that can affect STAT3 function (43, 44)), we also analyzed whether IFNγ was defective in promoting STAT3 serine phosphorylation. However, as shown in Fig. 6A, this appeared not to be the case, even though the ability of IFNγ to induce STAT3 serine phosphorylation was, once again, very weak as compared with that determined by IL-6.

To establish whether the low amount of nuclear STAT3 detected in IFNγ-treated samples (Fig. 6A) reflects an impaired nuclear translocation or a reduced STAT3 activation, we compared the levels of tyrosine-phosphorylated STAT3 in cytoplasmic and nuclear fractions of cells treated with either IFNγ or IL-6 in the same experiment. Quantitative analysis of these data revealed that following IFNγ stimulation, total tyrosine-phosphorylated STAT3 is approximately four times, whereas nuclear tyrosine-phosphorylated STAT3 is eight times, lower than those induced by IL-6 treatment (Fig. 6B). Altogether, these data made clear that, compared with IL-6, IFNγ proves to be a very poor inducer of both tyrosine phosphorylation and nuclear translocation of STAT3, raising the possibility that the nuclear levels of IFNγ-activated STAT3 are not high enough to
To examine additional reasons for the lack of DNA binding activity by IFNγ-activated STAT3 to the SOCS-3/SBE oligonucleotide, we further considered the possibility that IFNγ could somehow block the ability of STAT3 to bind to the SOCS-3/SBE sequence. To verify this hypothesis, J774 cells were stimulated with IL-6, alone or in combination with IFNγ, and STAT3 activation was analyzed by EMSA and Western blot. As shown in Fig. 8A, IL-6-induced complex A (consisting of STAT3 homodimers; see Fig. 5A) turned out to be completely withdrawn from binding to the SOCS-3/SBE probe when the cells are stimulated with IL-6 together with IFNγ (Fig. 8A, lane 4), despite similar levels of STAT1 activation (Fig. 8A and B). However, under the same experimental conditions, nuclear translocation of tyrosine/serine-phosphorylated STAT3 triggered by IL-6 is not modified by IFNγ (Fig. 8B), indicating that the lack of STAT3 binding to the SOCS-3/SBE is not due to a defective activation of the upstream signaling pathway leading to STAT3 activation and nuclear translocation. Furthermore, the addition of equal amounts of nuclear extracts from IFNγ-activated cells to nuclear extracts from IL-6-activated cells did not modify IL-6-dependent STAT3 DNA binding activity (Fig. 8C, lane 4). This result demonstrates that the disruption of the IL-6-induced STAT3-DNA complex determined by IFNγ is not caused by the elevated amounts of STAT1 competing for the same sequence.

How IFNγ prevents IL-6-activated STAT3 from binding to SOCS-3/SBE probe remains to be addressed. One of the mechanisms able to affect the binding of a transcription factor to its DNA recognition sequence is represented by its physical interaction with other different transcription factors, an event that has been described to occur in several situations and that has become a commonly recognized model of action through which gene regulation can be both inhibited and activated. In this context, STAT3 has been reported to be able to interact with
different partners, for instance NF-κB p65 homodimers, c-Jun, and GRIM-19, that can either promote or prevent STAT3-dependent transcriptional activity (45–51). Based on these observations, it is tempting to speculate that STAT3, once activated by IFN-γ, is prevented from binding to the SOCS-3/SBE oligonucleotide because it might interact with other transcriptional factors activated at the same time by IFN-γ.

To convincingly exclude any STAT3 involvement in the IFN-γ-dependent induction of SOCS-3, we investigated the ability of IFN-γ to induce SOCS-3 protein expression in mouse embryonic fibroblasts deleted of STAT3 (32). Besides hematopoietic cells, fibroblasts are also targets of IFN-γ. Furthermore, there is increasing evidence that fibroblasts exert an important role in the orchestration of immune response (52). As shown in Fig. 9A, in wild type MEFs (STAT3 fl/fl) IFN-γ activates STAT1 and STAT3 tyrosine phosphorylation, whereas in STAT3-deficient MEFs (STAT3−/−) only STAT1 tyrosine phosphorylation is activated by IFN-γ. Gel shift analysis showed that IFN-γ induced DNA binding activities to the SOCS-3/SBE probe both in wild type and STAT3 ΔΔ MEFs (Fig. 9B). Under these experimental conditions, SOCS3 protein expression is induced by IFN-γ in wild type and ΔΔ MEFs with similar kinetics and at comparable levels (Fig. 9C), indicating that STAT3 activation does not take part in the mechanisms of SOCS-3 induction triggered by IFN-γ.

The results presented in this work demonstrate that SOCS-3 induction by IFN-γ is achieved via activation of STAT1, which in turn binds to the SOCS-3/SBE element. Our data are apparently in contrast with recent works reporting, by microarrays analysis, that IFN-γ up-regulates SOCS-3 gene expression in STAT1-deficient mouse macrophages and hepatocytes (22, 37). However, the levels of SOCS-3 mRNA induced by IFN-γ in STAT1-deficient macrophages were significantly reduced as compared with macrophages derived from wild type mice (37). Furthermore, in STAT1−/− mouse hepatocytes, the induction of SOCS-3 mRNA by IFN-γ was enhanced and sustained, and it correlated with a prolonged IFN-γ-dependent STAT3 activation as compared with wild type cells (22). These observations suggest that, in the absence of STAT1, activation of the alternative STAT3-dependent pathway, leading to induction of SOCS-3 expression, is favored. Indeed, an increased and prolonged activation of STAT3 in the absence of STAT1 has been described in the brain of STAT1-deficient mice in response to IFNα (53). The observation that the SOCS-3/SBE sequence is not selective for recruiting activated STAT1 but is also able to bind tyrosine-phosphorylated STAT3 confirms previous observation (40) and indicates that, in the absence of STAT1, an
enhanced and prolonged STAT3 activation will ensure the expression of SOCS-3, an intracellular mediator whose absence has been shown to have profound influence on macrophage responses (20, 21, 28). It would be interesting to analyze whether in the absence of STAT1 the expression of STAT3-dependent genes is quantitatively enhanced in response to IFNγ, similarly to the switch to STAT1-dependent gene expression observed in response to IL-6 in cells lacking STAT3 (32).

Our result that the induction of SOCS-3 expression by IFNγ is STAT1-dependent is relevant in the context of the emerging concept that IFNγ/STAT1 and IL-6/STAT3 are mutually antagonists and negatively regulate each other through the induction of SOCS proteins (22). Indeed, although SOCS-1 and SOCS-3 are both induced by IFNγ and IL-6 and can both inhibit functional responses to each of these cytokines when overexpressed in vitro (54), studies conducted with mice in which SOCS-1 or SOCS-3 genes have been selectively inactivated indicated that their actions are more restricted and specific in vivo (20, 21, 24, 25, 28). It appears that SOCS-1 and SOCS-3 have complementary roles in regulating cytokine signaling; SOCS-3−/− cells show hyper-responsiveness to IL-6 but not to IFNγ (20, 21), whereas the opposite is true with SOCS-1−/− cells (24, 25). The biological consequences of SOCS-3 expression are not limited to the inhibition of STAT3-dependent IL-6 responses but are also responsible of preventing STAT1-dependent IL-6 alternative signaling pathway. In fact, the absence of STAT3 or of SOCS-3 has been found to be determinant for a prolonged activation of STAT1 in response to IL-6 and the subsequent induction of IFNγ-like responses (20, 21, 32). Our finding that SOCS-3 expression induced by IFNγ is achieved via STAT1 activation is relevant in that it points out that a STAT1-dependent pathway is utilized by IFNγ to prevent amplification of IFNγ-like responses triggered by IL-6. This consideration should be taken into account in studies aimed at selectively blocking one specific signaling pathway.

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J. Biol. Chem. 2004, 279:13746-13754.
doi: 10.1074/jbc.M308999200 originally published online January 23, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M308999200

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