SOCS-3 Is an Insulin-induced Negative Regulator of Insulin Signaling*

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The SOCS proteins are induced by several cytokines and are involved in negative feedback loops. Here we demonstrate that in 3T3-L1 adipocytes, insulin, a hormone whose receptor does not belong to the cytokine receptor family, induces SOCS-3 expression but not CIS or SOCS-2. Using transfection of COS-7 cells, we show that insulin induction of SOCS-3 is enhanced upon Stat5B expression. Moreover, Stat5B from insulin-stimulated cells binds directly to a Stat element present in the SOCS-3 promoter. Once induced, SOCS-3 inhibits insulin activation of Stat5B without modifying the insulin receptor tyrosine kinase activity. Two pieces of evidence suggest that this negative regulation likely results from competition between SOCS-3 and Stat5B binding to the same insulin receptor motif. First, using a yeast two-hybrid system, we show that SOCS-3 binds to the insulin receptor at phosphotyrosine 960, which is precisely where Stat5B binds. Second, using confocal microscopy, we show that insulin induces translocation of SOCS-3 from an intracellular compartment to the cell membrane, leading to colocalization of SOCS-3 with the insulin receptor. This colocalization is dependent upon phosphorylation of insulin receptor tyrosine 960. Indeed, in cells expressing an insulin receptor mutant in which tyrosine 960 has been mutated to phenylalanine, insulin does not modify the cellular localization of SOCS-3. We have thus revealed an insulin target gene of which the expression is potentiated upon Stat5B activation. By inhibiting insulin-stimulated Stat5B, SOCS-3 appears to function as a negative regulator of insulin signaling.

The SOCS (suppressors of cytokine signaling) are a family of proteins initially characterized by their ability to negatively modulate cytokine signaling. The best described of these proteins are CIS (1) and SOCS-1 (also named SSI-1 (2) and JAB-1 (3)) to SOCS-7 (4, 5). They share a homologous structure, an N-terminal region of variable length, a central SH2 domain, and a C-terminal SOCS box. The SOCS are rapidly induced by various cytokines in a tissue-specific manner (4, 5). Once expressed, they participate in a negative feedback loop by inhibiting cytokine-mediated Jak-Stat activation. The function of the SOCS is assumed to be solely regulated at the transcriptional level. An IL-2-induced tyrosine phosphorylation of SOCS-3 has been described, but its role is undefined (6). The SOCS proteins inhibit the Jak-Stat pathway by various mechanisms. Through their SH2 domains they bind directly to tyrosine-phosphorylated cytokine receptors. For instance, CIS binds to the β chain of the IL-3 receptor and to the EPO receptor (1), whereas SOCS-1 binds to the kit receptor tyrosine kinase (7). These associations can lead to a competitive inhibition with SH2 domain-containing molecules (such as the Stats) or to the recruitment of other signaling molecules to the receptors. Indeed, SOCS-1 binds to SH3-containing proteins such as Grb-2 and Vav via a di-proline determinant, specific for SOCS-1 (7). Using its SH2 domain and an additional N-terminal 12-amino acid sequence, SOCS-1 binds to and inhibits the Jak tyrosine kinases (8, 9). More recently, it has been shown that the SOCS box binds to elongins B and C, but the resulting effect on SOCS stability is a matter of debate (10, 11). The vital function of the SOCS has been uncovered by the production and study of transgenic mice. SOCS-1 deletion causes perinatal lethality linked to excessive response to interferon γ (12–14). Transgene-mediated expression of SOCS-3 blocks fetal erythropoiesis, resulting in embryonic lethality, and SOCS-3 deletion results also in embryonic lethality associated with marked erythrocytosis (15).

So far the induction of the SOCS seems to be restricted to activators of the cytokine receptor family, such as LIF, IL-2, IL-3, IL-6, interferon γ, GH, and leptin. The induction of the SOCS is thought to be mediated through Stat elements present in the promoters of the different SOCS (2, 16, 17). Although Stat activation is the hallmark of cytokines action, these transcription factors can be activated by other agents. Indeed, stimulation of serpentine receptors such as the angiotensin II receptor leads to the activation of Stat1, Stat2, and Stat3 (18). Tyrosine kinase receptors are also potential Stat activators. Stat1 and Stat3 belong to the signaling pathway of the epidermal growth factor, platelet-derived growth factor, and colony-stimulating factor-1 receptors (19). By performing a yeast two-hybrid screen using the insulin receptor as a probe we (20) and Chen et al. (21) show that Stat5B is a direct substrate of the insulin receptor. Insulin induces the tyrosine phosphorylation of Stat5B in transfected cells (20) but also in rhadomyosarcoma cells (22) and perfused rat liver (21). This tyrosine phos-

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phorylation of Stat5B leads to its activation, as measured by its ability to bind a cDNA probe corresponding to the Stat5 binding site from the β-casein promoter. Taken together, these studies bring at least the two following insights to insulin action. (i) Insulin can induce gene transcription by a signaling circuitry distinct from the classical mitogen-activated protein kinase- and phosphatidylinositol 3-kinase-dependent transcription factors; (ii) a direct signaling path linking the cell surface insulin receptor and the nucleus might control hormonial effects on gene expression.

Here we show that insulin induces the expression of SOCS-3 in 3T3-L1 adipocytes. In COS-7 cells, insulin-induced transcription of SOCS-3 is enhanced by Stat5B. Once expressed, SOCS-3 inhibits activation of Stat5B by insulin and appears to function as an inhibitor of insulin signaling. This inhibition is thought to be the result of a binding competition between Stat5B and SOCS-3 to the insulin receptor, since both molecules bind to the phosphorylated tyrosine 960 of the insulin receptor. This was observed by a yeast two-hybrid system and by confocal microscopy in intact cells. In conclusion, our study identifies SOCS-3 as an insulin-induced negative regulator of hormone signaling.

**EXPERIMENTAL PROCEDURES**

**Cells and Culture Conditions—**3T3-L1 fibroblasts were obtained from ATCC (Manassas, VA). They were grown and differentiated into adipocytes as described previously (23). Classically 80% of the cells were differentiated after 10 to 15 days. COS-7 fibroblasts were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum. 3T3-L1 and COS-7 were starved overnight in Dulbecco’s modified Eagle’s medium, 0.2% bovine serum albumin (w/v) before use.

**Northern Blot Analysis—**Total RNAs from 100-mm dishes were isolated with the TriZol reagent following the manufacturer’s instructions (Life Technologies, Inc.). 15 μg of total RNA was denatured in formamide and formaldehyde and subsequently separated by electrophoresis in formaldehyde-containing agarose gels. RNA was transferred to Hybond membranes (ICN, Costa Mesa, CA). Full-length SOCS-2, SOCS-3, and CIS cDNAs were used as probes. The probes were labeled with [α-32P]dCTP by random priming using the Rediprime kit (Amersham Pharmacia Biotech) and purified with the Probequant kit (Amersham Pharmacia Biotech). Hybridizations were performed at 42 °C in Denhardt’s, formamide 50%, 5 × saline/sodium phosphate/EDTA, 1% (w/v) SDS, and denatured salmon sperm DNA. Membranes were washed in 0.5 × SSC, 0.5% SDS at 42 °C for 30 min and subjected to autoradiography.

**Constructions and Transfection of Cells—**SOCSs cDNA subcloned in pEF have been described previously (9). The human insulin receptor cDNA from A. Ulrich (Max-Planck Institute, Gottingen, Germany) and subcloned in pCDNA3. Human Stat5B cDNA subcloned in pSx was a kind gift from W. Leonard (National Institutes of Health, Bethesda, MD). COS-7 cells were transfected by the DEAE-dextran procedure as described in Maniatis et al. (24). Cells were lysed for 36 or 48 h after transfection.

**Electrophoretic Mobility Shift Assay—**After appropriate treatment, cells were rinsed twice with Buffer A (10 mM Hepes, pH 7.5, 10 mM Na3PO4, 100 mM NaF, 2 mM Na2VO4, 0.1 mM EGTA, 1 mM EDTA), then scraped into Buffer B (Buffer A supplemented with 10 mM KCl, 1 mM DTT, 10 μg/ml aprotinin, 1 mM PMSF, 10 μg/ml leupeptin). 0.75% Triton X-100 was added. Cells were vortexed for 10 s and spun for 2 min. Cytosolic extracts were used for immunoblotting. Nuclei were collected and lysed by a 15-min incubation in Buffer C (10 mM Hepes, pH 7.5, 400 mM NaCl, 10 mM Na3PO4, 100 mM NaF, 2 mM Na2VO4, 1 mM EGTA, 1 mM EDTA, 10 mM KCl, 1 mM dithiothreitol, 10 μg/ml aprotinin, 1 mM pheynethylsulfonfluoride, 10 μg/ml leupeptin). After a 15-min spin, nuclear extracts were quantified for protein content. 10 μg of extract were diluted in 8 μl Hepes, pH 7.5, 1 mM dithiothreitol, 60 mM KCl, 2 mM EDTA, 0.1 μg/ml bovine serum albumin, 10% (v/v) glycerol, 0.05% (v/v) Nonidet P-40, 0.5% (v/v) Ficoll 0.04 mg/ml poly(dI-dC) (Amersham Pharmacia Biotech), 4 mM spermidine, and 0.05 mg/ml salmon sperm for 15 min at 4 °C. The DNA binding assay was initiated by addition of 3 × 108 cpm of the DNA-labeled probe. After 30 min at 30 °C, samples were loaded on a 5% acrylamide gel. After electrophoresis, the gel was dried and subjected to autoradiography.

For β-casein, the sequence of the probe is AGATTTCTCGAGAT-TCAACC. CTAAAACATCCAGAAAGACGCGCC is the 102 to 79 sequence of the SOCS-3 promoter containing the −95 to −87 putative Stat binding element (in bold). GGCGGTTCCGAGATCGGGG is the 78 to 55 sequence of the SOCS-3 promoter containing the −72 to −64 putative Stat binding element (in bold).

**Yeast Two-Hybrid System—**p-Lex-IR, amino acids 944 to 1343 of the insulin receptor β-subunit, the various insulin receptor mutants, and the controls (pLex-lamin, pAct-IRS1, and pAct-raf) were previously described (25). L40 yeast (MAT-a, trpl, his3, lys2::Lex-A HIS3, ura3::Lex-A-lacZ) were grown at 30 °C in yeast extract/peptone/dextrose medium. 2% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) glucose or in synthetic complete (SC) medium lacking the appropriate auxotrophic amino acids. Yeasts were transformed as described previously with the appropriate plasmids and plated in SC plates lacking tryptophan and leucine to select for the expression of pLex-9 and pAct-2 (25). After 48 h, double transformants were patched in SC plates lacking leucine, tryptophan, and histidine to test for histidine prototrophy. Other patches were performed in SC plates lacking leucine and tryptophan. After 2 days, the β-galactosidase assay was performed by a color filter assay using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as described previously (25).

**Fluorescent Staining and Confocal Microscopy—**COS-7 cells transfected with pCDNA3-HIR, HIRY960F, and pEF-SOCS-3 and grown on coverslips were incubated in serum-free media with 1% (w/v) yeast extract, 2% (w/v) bacto-peptone, and 2% (w/v) glucose or in synthetic complete (SC) medium lacking the appropriate auxotrophic amino acids. Yeasts were transformed as described previously with the appropriate plasmids and plated in SC plates lacking tryptophan and leucine to select for the expression of pLex-9 and pAct-2 (25). After 48 h, double transformants were patched in SC plates lacking leucine, tryptophan, and histidine to test for histidine prototrophy. Other patches were performed in SC plates lacking leucine and tryptophan. After 2 days, the β-galactosidase assay was performed by a color filter assay using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as described previously (25).

**RESULTS**

**Insulin Induces SOCS-3 mRNA Expression in 3T3-L1 Adipocytes—**Serum-starved 3T3-L1 adipocytes were treated with GH (96 nM) for 2 h or with insulin (100 nM) for 30 min, 2, or 6 h. At the end of the incubation, RNA was extracted and analyzed by Northern blotting using labeled probes for CIS, SOCS-2, or SOCS-3 (Fig. 1). Of all the SOCS tested, only SOCS-3 showed a detectable basal level. A 120-min growth hormone treatment of 3T3-L1 adipocytes led to an increase in the mRNA of CIS, SOCS-2, and SOCS-3. The effect of insulin on SOCS expression is different. Indeed, insulin did not modify the levels of CIS and SOCS-2 mRNA but induced SOCS-3 mRNA expression. This increase was barely detectable after 30 min but reached values 2.7 and 4.3 times above basal after 2 and 6 h of treatment, respectively.

**Stat5B Is Involved in Insulin-induced SOCS-3 mRNA Expression—**We (20) and others (21, 22, 26) show that insulin induces the phosphorylation and the activation of Stat transcription factors. However, the genes induced by insulin in a Stat5B-dependent fashion are not known. We tested whether SOCS-3 could be such a gene. As a first step, we verified that Stat5 was activated by insulin in 3T3-L1 adipocytes. Cells were treated with insulin and then thereafter, cytosolic and nuclear extracts were isolated. EMSAs were performed using a previously characterized cDNA probe containing a Stat5 binding site present in the promoter of the β-casein gene. In parallel, Stat5B was immunoprecipitated, and its level of tyrosine phosphorylation was analyzed by Western blot with antibody to phosphotyrosine. As observed in Fig. 2, insulin activates
Stat5B DNA binding activity in 3T3-L1 adipocytes, as detected by a specific shift of the cDNA probe that is displaced by an excess of unlabeled probe. In parallel, insulin induces an increase in Stat5B tyrosine phosphorylation. These data indicate that insulin activates Stat5B in 3T3-L1 adipocytes. We then tested if Stat5B was involved in the induction of SOCS-3 mRNA by insulin. To do so, we used COS-7 cells as a model system since they do not express detectable levels of endogenous Stat5B protein (27). COS-7 cells were transfected with plasmids coding for insulin receptor and with or without pSX-Stat5B. Cells were then treated or not for 2 h with insulin, RNA was extracted, and Northern analyses were performed using a cDNA probe for SOCS-3 (Fig. 3). In COS-7 cells expressing only the insulin receptor, insulin induces a weak increase in SOCS-3 mRNA (1.4-fold over basal). By comparison, in cells expressing both insulin receptor and Stat5B, insulin induces a 2.9-fold stimulation of SOCS-3 expression over basal. These data indicate that, in COS-7 cells, Stat5B enhances insulin-induced SOCS-3 transcription.

Recently, a 2.9-kilobase murine promoter of SOCS-3 has been isolated (17). A sequence located at -99 to -60 has been found to be essential for LIF transactivation of this SOCS-3 promoter. This sequence contains two putative Stat elements (TT(N )5AA, where N is a nucleoside) found at -95 to -87 and at -72 to -64. We investigated whether insulin was able to induce an electrophoretic mobility shift of the cDNA probes corresponding to these two putative Stat binding sites and whether this shift was dependent upon Stat5B. COS-7 cells were transiently transfected with plasmids encoding the insulin receptor and Stat5B. Cells were treated or not with insulin for 15 or 30 min, and nuclear extracts were isolated. EMSA were performed using probes corresponding to the -95 to -87 or the -72 to -64 sequence of the SOCS-3 promoter (Fig. 4). No insulin-stimulated electrophoretic shift was detected using the probe corresponding to the -95 to -87 sequence of the SOCS-3 promoter, whatever condition tested. However, an insulin-stimulated shift was observed when the -72 to -64 cDNA probe was used with nuclear extracts from cells expressing both the insulin receptor and Stat5B. This shift was specific since it disappears in the presence of an excess of unlabeled probe. The mobility shift was dependent upon Stat5B expression since no specific DNA binding activity was observed when nuclear extracts from cells expressing only the insulin receptor were utilized. These observations suggest that insulin induces SOCS-3 expression directly through Stat5B stimulation and binding of Stat5B to the SOCS-3 promoter.

SOCS-3 Inhibits Insulin-induced Stimulation of Stat5B—According to the emerging picture, the SOCS proteins are usually part of a negative feedback loop in which they inhibit activation of the Stats by cytokines. The insulin receptor is thought to directly activate Stat5B upon binding the transcription factor and phosphorylating it. Therefore, we were interested in examining the action of SOCS-3 on the stimulation of Stat5B by insulin. COS-7 cells were transfected with plasmids encoding the insulin receptor and Stat5B in the presence or...
SOCS-3 has been very difficult, if not impossible, to visualize using a previous report. Direct interactions between insulin extract (data not shown). This is not surprising considering the precipitation of the insulin receptor and SOCS-3 using COS-7 cell lines. We were not able to show co-immunoprecipitation whether SOCS-3 could bind to the insulin receptor at the same site as Stat5B does. The results shown are representative of experiments performed at least two times with identical results.

The absence of various doses of pEF-SOCS-3 (expressing an epitope-tagged flag-SOCS-3). Cells were stimulated for 15 min with insulin, and thereafter, cytoplasm and nuclear extracts were isolated. Western blots were performed to verify that the quantity of insulin receptor and Stat5B were equivalent in all conditions and that SOCS-3 was expressed (Fig. 5). We found that insulin induced a shift of the probe, which disappeared in the presence of an excess of unlabeled probe. Expression of SOCS-3 inhibited the activation of Stat5B by insulin in a dose-dependent manner, as shown by the decrease in the intensity of the specific shift.

**SOCS-3 Does Not Alter Insulin Receptor Tyrosine Phosphorylation**—The inhibitory action of SOCS-3 on insulin stimulation of Stat5B could be due to a direct intervention of SOCS-3 on the receptor phosphorylation and kinase activity. Therefore, we investigated the effect of SOCS-3 expression on insulin receptor phosphorylation. COS-7 cells were transfected with plasmids encoding insulin receptor in the presence or absence of SOCS-3. Cells were stimulated with insulin for 15 min and lysed. The expression level of insulin receptor and SOCS-3 was assessed by a Western blot using antibodies to insulin receptor or to the flag epitope. Insulin receptor was immunoprecipitated and revealed by Western blotting with antibody to phosphotyrosine (Fig. 6). As expected, insulin induced tyrosine phosphorylation of its receptor β-subunit. Although it was expressed (lower panel), SOCS-3 did not alter tyrosine phosphorylation of insulin receptor, whose expression was identical in all conditions. These data indicate that the inhibition exerted by SOCS-3 on insulin-induced activation of Stat5B is not linked to an effect on insulin receptor tyrosine phosphorylation.

**SOCS-3 Associates with the Insulin Receptor**—As SOCS-3 inhibited insulin-induced Stat5B activation without noticeable modification of insulin receptor kinase activity, we investigated whether SOCS-3 could bind to the insulin receptor at the same site as Stat5B does. We were not able to show co-immunoprecipitation of the insulin receptor and SOCS-3 using COS-7 cell extract (data not shown). This is not surprising considering previous reports. Indeed, direct interactions between insulin receptor and its bona fide partners (such as IRS, Shc, and Stat) have been very difficult, if not impossible, to visualize using a co-immunoprecipitation approach. In contrast, alternative approaches such as the yeast two-hybrid system has been used with success (20, 28–33). Therefore, we studied the interaction of SOCS-3 with the insulin receptor by means of the yeast two-hybrid system. Full-length SOCS-3 was subcloned in frame with the activation domain of Gal-4. The other hybrid consisted of the insulin receptor fused to the cDNA binding domain of LexA. The two hybrids were expressed in L40 yeast. The association between both proteins led to transactivation of the two reporter genes (LacZ and His3), measured as described previously (25). Controls were performed using pLex-lamin, pAct-raf, and pAct-IRS-1. Several previously described mutants of the insulin receptor (25) were also tested for their interaction with SOCS-3. As observed in Table I, SOCS-3 interacts with the insulin receptor, as reflected by transactivation of the two reporter genes in L40 yeast (row 1). The interaction has a strength comparable with the one observed
SOCS-3 interaction with insulin receptor in a yeast two-hybrid assay

L40 yeasts were co-transformed with the plasmids encoding the various pAct and pLex constructs. Wild type (WT) and several mutants of the insulin receptor (IR) have been used. KD is a kinase-deficient mutant where lysine 1018 has been mutated into alanine. The position of the tyrosine (Y) residue mutated in phenylalanine (F) residue is indicated. Mutation R71K of SOCS-3 abolished its SH2 domain function. Interaction between the insulin receptor and SOCS-3 is evaluated by measuring the expression (+) or not (0) of the two receptor genes, as specified under “Experimental Procedures.”

| Row No. | pLex | pAct | Colony color | Growth his |
|---------|------|------|--------------|------------|
| 1       | IR WT | SOCS-3 | +            | +          |
| 2       | IR KD | SOCS-3 | 0            | 0          |
| 3       | IR Y960F | SOCS-3 | 0            | 0          |
| 4       | IR Y1146F | SOCS-3 | +            | +          |
| 5       | IR Y1150F | SOCS-3 | +            | +          |
| 6       | IR Y1151F | SOCS-3 | +            | +          |
| 7       | IR Y1154F | SOCS-3 | +            | +          |
| 8       | IR Y1154F | SOCS-3 | +            | +          |
| 9       | IR ACT | SOCS-3 | +            | +          |
| 10      | Lamin | SOCS-3 | 0            | 0          |
| 11      | IR WT | SOCS-3 R71K | 0            | 0          |
| 12      | IR WT | IRS-1 | +            | -          |
| 13      | IR WT | Raf   | 0            | 0          |

Discussion

Insulin modulates cellular metabolism, growth, and differentiation by modulating the activity and the intracellular localization of several proteins. Insulin is also known to exert some of its actions through modulation of gene transcription. Several genes have been identified as being insulin-responsive. For example, insulin induces fatty acid synthase, leptin, and β-casein and inhibits phosphoenolpyruvate carboxykinase transcription. The identification of specific “insulin response elements” in the promoters of insulin-responsive genes has been disappointing despite major efforts of several laboratories. These difficulties probably reflect the fact that insulin triggers several transcription factors with different DNA binding activities. The best characterized pathways controlled by insulin, which lead to gene transcription, are the ones involving the mitogen-activated protein kinase and phosphatidylinositol 3-kinase cascades. Mitogen-activated protein kinase phosphatases and activates the serum response element-binding proteins p62TCF and p67SRF, whereas it phosphorylates and inhibits peroxisome proliferator-activated receptor γ (36); phosphatidylinositol 3-kinase phosphatases and inhibits FKHR1 through protein kinase B activation (37). More recently, we and others have shown that insulin stimulates Stat transcription factors and that Stats might be part of a newly identified insulin signaling circuitry (20–22, 26). The key observations are as follows. (i) Stat5B binds to the insulin receptor, as shown by a yeast two-hybrid assay. This interaction involves Stat5B SH2 domain and insulin receptor tyrosine 960. (ii) The insulin receptor induces the tyrosine phosphorylation of Stat5B in vitro and of Stat3 and Stat5B in cultured cells. (iii) Insulin stimulates Stat3 and Stat5 DNA binding activities, and (iv) insulin transactivates a reporter gene containing Stat binding sites. However, to the best of our knowledge no reports on insulin target genes, which transcription is regulated by Stat, have been published.

Here we show that insulin, via its receptor with intrinsic tyrosine kinase activity, induces SOCS-3 expression in 3T3-L1 adipocytes. This induction seems to be specific since the hormone does not modify CIS or SOCS-2 expression. So far, the majority of the cytokines induce several members of the SOCS family. However, a SOCS-3 specific induction has also been observed in hypothalamic from leptin and ciliary neurotrophic factor-treated mice (38, 39). Although the biological meaning of this SOCS-3-specific production is unknown, it is intriguing to note that the three molecules able to specifically induce SOCS-3 are recognized sialic factors.

In COS-7 cells we have shown that insulin induces a weak increase in SOCS-3 mRNA. This effect is potentiated by Stat5B expression, suggesting that insulin induces SOCS-3 expression through Stat5B activation. Although insulin-induced SOCS-3
expression is potentiated by Stat5B, we cannot exclude the possibility that Stat3 or other transcription factors or transcriptional co-activators stimulated by insulin also play a role in this mechanism. The low level of insulin-induced SOCS-3 expression in COS-7 cells, not transfected with Stat5B (1.3-fold over basal), could be due to the low level of endogenous Stat3 (40).

Recently, Auernhammer et al. (17) have characterized the SOCS-3 promoter. They have isolated a region, located at $-99$ to $-60$ that was found to be responsible for LIF-induced SOCS-3 promoter transactivation. This sequence contains two putative Stat binding elements at $-72$ to $-64$ and at $-95$ to $-87$. We found that insulin stimulated a shift of the $-72$ to $-64$ probe but not of the $-95$ to $-87$ probe. This shift was dependent upon Stat5B expression. The $-72$ to $-64$ sequence is a TTCCAGGA element that had been characterized by the authors as a Stat1/Stat3 binding element following LIF stimulation. However, this site is a better match for Stat5, for which the consensus DNA binding sequence is TTCC(A/T)GGAA (compared with Stat1 TTCC(G/C)GGAA or to Stat3 TTCC(G = C)GGAA) (19). Taken together, our data would indicate that after insulin receptor stimulation, Stat5B binds directly to the SOCS-3 promoter. We have thus identified a straight cell membrane-to-nucleus connection, since the cell surface insulin receptor, through the transcription factor Stat5B, impinges directly on SOCS-3 expression.

Another key observation of our work is that, once expressed, SOCS-3 inhibits the activation of Stat5B by insulin. Generally speaking, SOCS proteins inhibit cytokine signaling through Jak inhibition. Therefore, our findings were surprising since the insulin receptor kinase is thought to activate Stat5B directly, independently of Jak (21, 22). Moreover, in agreement with a previous study (2), we have been unable to observe a SOCS-3 effect on insulin receptor kinase activity. Therefore, we would like to propose that Stat5B inhibition by SOCS-3 happens through competition between SOCS-3 and Stat5B for binding to insulin receptor tyrosine 960. This hypothesis is based on two key observations. First, using a yeast two-hybrid assay, we found that SOCS-3 binds directly to the insulin receptor. This interaction involves tyrosine 960 of the insulin receptor and the SH2 domain of SOCS-3. Second, using confocal microscopy, we showed that in intact cells, insulin induces translocation of SOCS-3 from the cytoplasm to the cell membrane. This leads to a colocalization of SOCS-3 with the insulin receptor. In agreement with the results gathered from the yeast two-hybrid system, the colocalization is dependent upon phosphorylation of insulin receptor tyrosine 960. Indeed, insulin does not modify the localization of SOCS-3 in cells expressing the insulin receptor mutant Y960F. To the best of our knowledge, this is the first observation in intact cells of a hormone-regulated intracellular movement of a SOCS protein. The molecular mechanism that triggers this translocation remains to be defined.

Although SOCS-3 is best known to inhibit Jaks, this is not the first observation of an association between SOCS-3 and a transmembrane receptor. Indeed, SOCS-3 binds to the tyrosine-phosphorylated IL-2/β receptor (6) and to the growth hormone receptor (41, 42). An association between a receptor tyrosine kinase and SOCS has previously been observed. SOCS-1 binds to the tyrosine-phosphorylated kit receptor (7), whereas SOCS-1 and SOCS-2 bind to the insulin-like growth factor-I receptor, a protein homologous to the insulin receptor (43).
the insulin-like growth factor-I receptor, the mechanism of interaction is different, since these SOCS do not bind to tyrosine 950 of the insulin-like growth factor-I receptor, which corresponds to tyrosine 960 of the insulin receptor. Furthermore, the function and mechanism of association are unknown.

The inhibition of insulin-activated Stat5b by SOCS-3 characterizes SOCS-3 as a newly identified modulator of insulin signaling. Indeed, by blocking Stat5b, SOCS-3 will prevent the expression of the genes that are induced by insulin in a Stat5b-dependent manner. SOCS-3 is also a known inhibitor of Jak that can be activated by insulin (44). Insulin is known to tightly regulate its signaling by various negative feedback loops (45, 46). These control circuits are necessary to maintain adequate order in the multiple signaling pathways activated by the hormone and, hence, to allow a final coordinated cellular response. Insulin induction of SOCS-3 could be a means of attenuating such a concerted action. One can also envision SOCS-3 as a "shuttle" for various signaling pathways in order to coordinate cross-talk. For instance, tumor necrosis factor α, a molecule thought to be involved in insulin resistance, has been shown independently to induce SOCS-3 (4) and to inhibit insulin-induced STAT5 activation (47). GH, a molecule known to lead to a state of this manuscript.

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