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Suppressor of Cytokine Signaling (SOCS)-3 Protein Interacts with the Insulin-like Growth Factor-I Receptor

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SOCS proteins are a class of proteins that are negative regulators of cytokine receptor signaling via the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway. In a yeast two-hybrid screen of a human fetal brain library, we have previously identified SOCS-2 as a binding partner of the activated IGF-I receptor (IGFIR). To test whether or not SOCS-3 also binds to the IGFIR, we cloned human SOCS-3 by reverse transcription-polymerase chain reaction from human skeletal muscle mRNA. SOCS-3 mRNA was expressed in many human fetal and adult tissues and in some human cancer cell lines (Hela, A549 pulmonary adenocarcinoma and G361 human melanoma). We found that human SOCS-3 protein interacts directly with the cytoplasmic domains of the activated IGFIR and the insulin receptor (IR) in the yeast two-hybrid assay. In GST-SOCS-3 pull-down experiments using IGFIR from mammalian cells and in immunoprecipitation experiments in which IGFIR and FLAG-SOCS-3 were transiently expressed in human embryonic kidney 293 cells, we found that SOCS-3 interacts constitutively with IGFIR *in vitro* and in intact cells. Unlike SOCS-2, hSOCS-3 was phosphorylated on tyrosines in response to IGF-I addition to 293 cells. We conclude that SOCS-3 binds to the IGFIR and may be a direct substrate for the receptor tyrosine kinase.

Key Words: **yeast two-hybrid; phospho-SOCS-3; insulin receptor.**

A family of proteins having a similar domain structure has recently been described and include CIS (cytokine-inducible SH2-containing protein) and SOCS-1 to SOCS-7 (1–3). This common domain structure consists of an N-terminal region of variable length and amino acid composition, a central SH2 domain, and a C-terminal domain containing a SOCS box. Several of the members of this family have been shown to be induced by cytokines and to function in a negative feedback loop to inhibit JAK/STAT signaling pathways emanating from cytokine receptors.

In a yeast two-hybrid screen of a human fetal brain library using the cytoplasmic domain of the IGF-I receptor as bait (4–6), we recently cloned a fragment of SOCS-2 (7). GST-SOCS-2 fusion protein also bound to the activated IGF-I receptor from mouse fibroblasts overexpressing the IGF-I receptor, and in transient transfection experiments in 293 human embryonic kidney cells, SOCS-2 associated with the IGF-I receptor after treatment of the cells with IGF-I. These results raised the possibility that some members of the SOCS family may have more diverse functions than simply functioning as negative regulators of cytokine receptor signaling. In this paper we have cloned skeletal muscle SOCS-3 and examined its interaction with the IGF-I receptor.

Mouse and human SOCS-3 were originally cloned by comparing CIS and SOCS-1 with EST data bases and by cloning from a human activated Jurkat library (8–10). SOCS-3 is induced by a variety of cytokines in cell lines, mouse bone marrow cells, and mouse liver. Moreover, expression of SOCS-3 in myeloid leukemia M1 cells inhibits the stimulation of differentiation and apoptosis by IL-6 and LIF (9–13). Thus, in these reports, evidence was provided that SOCS-3 along with CIS and SOCS-1 are negative regulators of cytokine signaling pathways.

More recently, it was reported that SOCS-3 may play a role in signaling by a number of other cytokine receptors. IL-2 induced SOCS-3 expression in T cell lines and peripheral blood lymphocytes (14). In lymphocytes overexpressing SOCS-3, IL-2-induced phosphorylation of STAT5b was inhibited and IL-2 and IL-3 stimulation of cell proliferation was inhibited. In 3T3-F442A fibroblasts, growth hormone stimulated a rapid, transient increase in SOCS-3 (15). Induction of SOCS-3 was also

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seen in livers of mice treated with GH (16). In CHO cells transfected with the GH receptor and SOCS cDNAs, SOCS-1 and SOCS-3 blocked GH-induced transactivation of the GH-responsive serine protease inhibitor 2.1 gene promoter (15). *In vivo* treatment of mice with leukemia inhibitory factor (LIF) resulted in a ninefold increase in SOCS-3 mRNA in the pituitary (17). LIF also stimulated SOCS-3 mRNA expression in Akt-20 mouse pituitary cells and stable overexpression of SOCS-3 in these cells inhibited basal and LIF-stimulated increase in proopiomelanocortin mRNA and adrenocorticotrophin hormone secretion. Also, SOCS-3 appears to be the main negative regulator of prolactin-mediated JAK2/STAT5 pathway in postpartum mouse mammary glands (18). Peripheral leptin administration to leptin deficient mice (ob/ob) rapidly induced SOCS-3 mRNA in the hypothalamus but had no effect on CIS, SOCS-1, and SOCS-2 (19). In mammalian cell lines, SOCS-3, but not CIS or SOCS-2, blocked leptin-induced signal transduction. Peripheral ciliary neurotrophic factor (CNTF) administration to ob/ob mice rapidly induced SOCS-3 mRNA in areas of the hypothalamus that are both overlapping and distinct from those in which SOCS-3 was induced by leptin (20). Transient expression of SOCS-3 but not CIS or SOCS-2 inhibited CNTF induced signal transduction in astrocytes. SOCS-3 is essential in the regulation of fetal liver erythropoiesis in the mouse (21). SOCS-3 was highly expressed in erythroid lineage cells during embryonic development. SOCS-3 knockout mice exhibited embryonic lethality at day 12–16 associated with marked erythrocytosis. Conversely, SOCS-3 transgene mediated expression blocked fetal erythropoiesis and was also lethal.

Thus, there is evidence that SOCS-3 is induced by a number of ligands that signal through the cytokine family of receptors, and, in turn, SOCS-3 negatively regulates signaling from these receptors. In this paper, we provide evidence that SOCS-3 also interacts with the IGF-I receptor and may be a substrate of the receptor tyrosine kinase.

MATERIALS AND METHODS

Materials. The two-hybrid expression plasmids and EGY48 yeast strains were gifts from Dr. Roger Brent and have been described previously (4). Human embryonic kidney 293 cells were purchased from American Type Culture Collection. NIH-3T3 cells overexpressing IGFIR (NWTc43 cells) were obtained from Dr. Derek LeRoith. The monoclonal antibody to the IGFIR (αIR-3) was purified from ascites fluid by protein G affinity chromatography and coupled to Affi-Gel Hz hydrazide gel (Bio-Rad). The antibodies to the phosphotyrosine (sc-7020-HRP), IGFIR (sc-713), and the HRP conjugated secondary antibodies used for immunoblotting were purchased from Santa Cruz Biotechnology. The FLAG fusion protein expression system and anti-FLAG M2 and M5 monoclonal antibodies were purchased from Eastman Kodak. The oligonucleotides and primers were synthesized using an ABI DNA synthesizer.

Cell culture. 293 cells were grown in Dulbecco's modified Eagle medium with 2200 mG/L sodium bicarbonate (Biofluids) supplemented with 10% fetal bovine serum plus penicillin (50 units/ml) and streptomycin (50 μg/ml) in an atmosphere of 5% CO_2. NIH 3T3 cells overexpressing human IGFIR (NWTc43) were maintained in similar medium supplemented with 500 μg/ml geneticin (G418 sulfate).

Plasmid constructions. Human skeletal muscle mRNA (Clontech) was reverse transcribed using oligo (dT) primer and the resultant product was used for amplification of SOCS-3 cDNA using primers 5′-TGTGCGGAGGCCGCGAAGCAGCTGCAG-3′, and 5′-CATGCCCTTTGCGCCCTTTACCCCTTAAAGCGG-3′ based on the SOCS-3 sequence reported by Minamoto *et al.* (9). The full-length human SOCS-3 cDNA coding sequence was subsequently amplified using this PCR product and nested primers 5′-CATAGAATTCATGGTCACCCACAGCAAGTTTCCCGCC-3′ and 5′-ATCACTCGAGTTAAAGCGGGGCATCGTACTGGTCCAG-3′. The full-length SOCS-3 cDNA fragment was cloned into a pCR2.1 TA cloning vector (Invitrogen), sequenced, and then subcloned into expression plasmids to generate GEX-SOCS-3 and FLAG-SOCS-3. GST-SOCS-3 (1–44), GST-SOCS-3 (SH2) and GST-SOCS-3 (141–225) were generated using PCR amplified SOCS-3 fragments. The pcDNA-IGFIR mammalian expression vector has been previously described (4).

Two-hybrid assay. The yeast cultures, preparation of various yeast selection media and yeast transformations were carried out as described (4). The transformants were selected for growth on media lacking tryptophan, uracil, and histidine, and containing glucose as the carbon source. In the second step, interactors were selected by plating onto synthetic selective media containing galactose and X-gal and lacking leucine. The expression of the reporter genes was scored as described earlier (4).

Northern hybridization. Multiple tissue human poly(A) RNA blots were purchased from Clontech and hybridized to a ^{32}P-labeled full-length SOCS-3 cDNA. Labeling of the probe and hybridization conditions were described previously (7).

In vitro binding assay. The GST fusions of full-length and different truncated SOCS-3 proteins were expressed in the BL21 *Escherichia coli* strain. Bacterial lysates containing GST-SOCS-3 or GST alone were bound to glutathione-Sepharose beads at room temperature for 1 h. The washed beads were then incubated overnight at 4°C with cell lysates derived from subconfluent monolayers of NWT c43 cells that were serum starved for 24 h and lysed prior to or after IGF-I (20 nM) stimulation for 30 min (6). After incubation with the lysates, the beads were washed 4 times with cold lysis buffer, boiled in Laemmli SDS sample buffer containing 100 mM DTT and resolved by SDS–PAGE. The proteins were transferred to nitrocellulose and immunoblotted with primary and secondary antibodies described in the text and figure legends. Proteins were visualized using ECL (Pierce).

Binding studies in intact cells. 293 cells were cotransfected with the FLAG-SOCS-3 and IGFIR plasmids or empty vector using LipofectAMINE PLUS reagent (Life Technologies). After 36 h in growth medium the transfectants were serum starved for 16 h and then lysed either prior to or after stimulation with IGF-I (20 nM) for 40 min (7). Immunoprecipitates obtained after 8 h incubation of lysates at 4°C with anti-FLAG (M2 affinity gel) or anti-IGFIR (αIR-3-Affi-Gel Hz) were washed 4 times with cold lysis buffer without detergent, resolved by SDS–PAGE under reducing conditions and transferred to nitrocellulose. Blots were incubated with appropriate primary and secondary antibodies (given in text or figure legend). Proteins were visualized using ECL.

RESULTS AND DISCUSSION

Cloning of Human SOCS-3

We have cloned SOCS-3 from human skeletal muscle mRNA (GenBank Accession No. AF159854) by RT-

PCR using primers based on the published nucleotide sequence of SOCS-3 (9). Human skeletal muscle SOCS-3 contains 225 amino acids and its sequence is 99% identical to that reported for SOCS-3 derived from an activated Jurkat cDNA library (9). These SOCS-3 sequences differ by two amino acid residues in the central SH2 domain. In skeletal muscle SOCS-3 there is threonine in place of alanine at residue 81 and histidine instead of tyrosine at position 125. Human skeletal muscle SOCS-3 is identical to Mo7e cell SOCS-3 cloned by Masuhara *et al.* (10). Like other SOCS family members (CIS and SOCS-1 to SOCS-7), SOCS-3 cDNA contains a similar domain structure with a central SH2 domain, a C-terminal SOCS box region containing two SOCS box motifs and a nonhomologous amino terminal region.

Expression of Human SOCS-3 in Tissues and Cell Lines

To examine the tissue distribution of SOCS-3 mRNA, both fetal and adult human multiple tissue poly(A) RNA blots were hybridized with a ^{32}P-labeled full-length SOCS-3 cDNA. A 3.0-kb mRNA was present in all the fetal and adult tissues examined with a large variation in expression in different tissues (Fig. 1). SOCS-3 mRNA was highly abundant in fetal lung, liver, kidney (Fig. 1A) and adult lung and peripheral blood leukocytes (Fig. 1B). Adult heart and thymus had moderate levels of expression. Compared to our results, Starr *et al.* (8) found relatively higher level of SOCS-3 mRNA in mouse spleen and Minamoto *et al.* (9) observed higher expression of SOCS-3 mRNA in human placenta and skeletal muscle and lower expression in thymus. The mRNA expression pattern of SOCS-3 in eight different cancer lines is shown in Fig. 2. SOCS-3 mRNA was highly abundant in Hela cell S-3, A549 lung adenocarcinoma and G361 melanoma cell lines whereas SOCS-2 was abundant in the K562 chronic mylogenous leukemia cell line. Interestingly, a low molecular weight mRNA (2.0 kb) for SOCS-2 was prominent in the cell lines whereas a predominant 5.0-kb message was found in the human tissues (7). Thus the distribution of SOCS-2 and SOCS-3 mRNA in cell lines is different, suggesting a different function for these two SOCS family members.

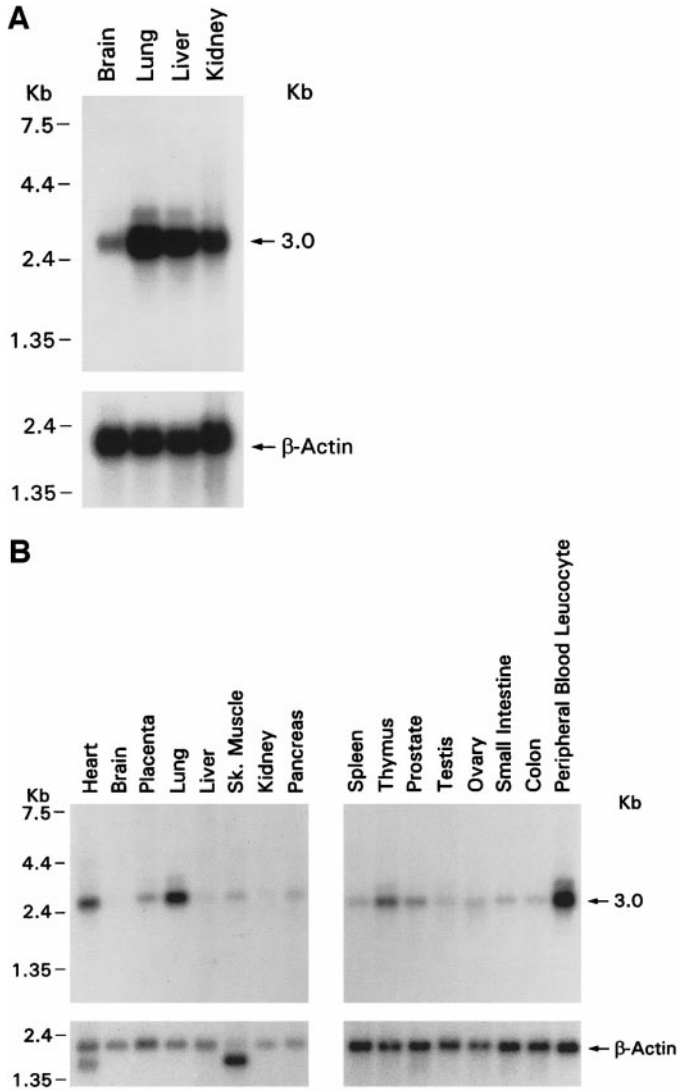

FIG. 1. Tissue distribution of human SOCS-3 mRNA in different fetal (A) and adult tissues (B). Multiple tissue Northern blots were hybridized to a ^{32}P-labeled full-length cDNA of SOCS-3 under high stringency condition. The blots were washed using high stringency conditions and autoradiographed overnight. Tissues are denoted above each lane and the position of the size markers (Kb) is shown on the left of these blots. A 3.0-Kb mRNA is present in most of the fetal and adult tissues. These blots were stripped and reprobed with control β-actin cDNA (shown at the bottom of each blot).

Identification of SOCS-3 as an IGFIR Interacting Protein

To test whether SOCS-3 is a new binding partner of the IGF-I receptor, we carried out a yeast two-hybrid assay using human SOCS-3 and the cytoplasmic domain of the IGF-I receptor. The LexA-IGF-I receptor hybrid described earlier (4) was used as bait and the full-length SOCS-3 protein was fused with the B42 activation domain by inserting the SOCS-3 cDNA sequence into JG4-5 activation domain hybrid vector. Coexpression of this plasmid with the LexA-IGFIR hybrid resulted in strong galactose dependent activation of both the *lacZ* and *LEU* reporter genes (Table 1). This interaction was not seen when SOCS-3 activation hybrid was coexpressed with a control LexA-bicoid hybrid and when LexA-IGFIR was expressed alone, indicating specificity of this interaction. The SOCS-3 activation hybrid did not interact with a kinase negative IGFIR in which lysine 1003 is replaced by arginine (4). These results indicate that SOCS-3 interacts with the IGFIR

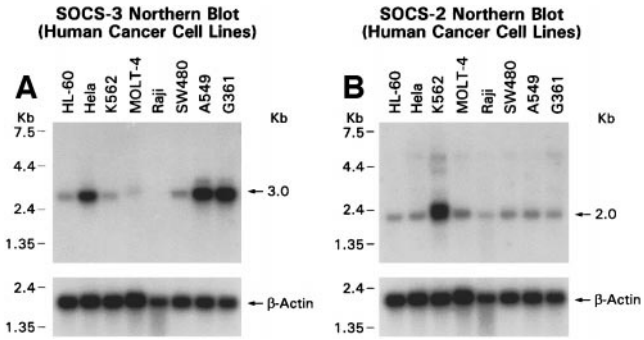

FIG. 2. Analysis of mRNA expression of SOCS-3 and SOCS-2 in eight different human cancer cell lines. The blot was hybridized with ^{32}P-labeled full-length cDNA of SOCS-3 under high stringency conditions. This blot was then stripped and reprobed with ^{32}P-labeled N-terminal fragment of SOCS-2, clone 63 (3). Subsequently, this blot was again stripped and reprobed with β-actin cDNA (shown at the bottom of the blot).

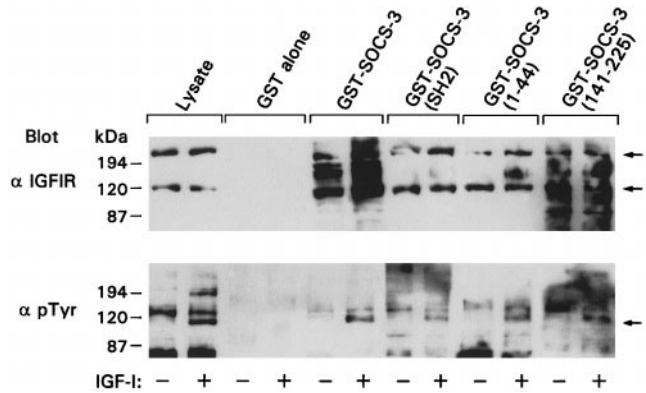

FIG. 3. *In vitro* binding of SOCS-3 to the IGFIR. NIH 3T3 cells overexpressing IGFIR (NWTc43) were stimulated or not stimulated with 20 nM IGF-I for 30 min and lysed at 4°C. Lysates were incubated with GST-fusion protein beads containing either full-length SOCS-3, the amino terminal fragment of SOCS-3, the central SH2 domain of SOCS-3, or carboxy terminal fragment containing the SOCS boxes. Beads containing GST alone were used as a negative control. Coprecipitating protein complexes were washed four times with cold lysis buffer, resolved by SDS–PAGE and immunoblotted with a receptor antibody (upper panel) or with a phosphotyrosine antibody (lower panel).

in the yeast two-hybrid system and this interaction is dependent upon autophosphorylation of the receptor. SOCS-3 also interacted with the insulin receptor. Recently, Emanuelli *et al.* (22) also reported the interaction of SOCS-3 with the insulin receptor using the yeast two-hybrid system.

In Vitro Association of SOCS-3 with the IGFIR

We investigated the association of SOCS-3 with the IGFIR *in vitro* using a GST-SOCS-3 fusion protein and IGFIR solubilized from NIH 3T3 cells overexpressing the receptor (Fig. 3). After incubation of the cell lysates with the fusion proteins, the bound proteins were resolved by SDS–PAGE and analyzed by immunoblotting with an antiserum directed against the carboxyl terminus of the human IGFIR (sc 713). The IGFIR from NWTc43 cells bound to the GST-SOCS-3 beads in an IGF-I independent fashion (Fig. 3, upper panel). No binding was seen when the lysates were incubated with resin containing GST alone. Immunoblotting of a duplicate blot with phosphotyrosine antibody confirmed that the receptor was phosphorylated on tyrosine in lysates from IGF-I treated cells (Fig. 3, lower panel). These data indicate that SOCS-3 binds to the IGFIR in an *in vitro* system and that, unlike the yeast two-hybrid results, binding is constitutive. It is possible that the conformation of the cytoplasmic portion of the receptor is different in the holoreceptor compared to its conformation when expressed as a fragment in yeast. Cohney *et al.* (14) also observed that although binding of SOCS-3 to JAK1 was IL-2-dependent there was also a low level of constitutive binding.

TABLE 1

SOCS-3 Interacts with the IGFIR and Insulin Receptor in the Yeast Two-Hybrid System

Bait	Prey	LEU2 Glu	LEU2 Gal	*lacZ* (relative activity) mean ± SD
IGFIR	None	0	0	0
IGFIR	SOCS-2 (control)	0	+4	1.00
IGFIR	SOCS-3	0	+4	0.880 ± 0.011
IGFIR(KR)	SOCS-3	0	0	0.010 ± 0.002
IR	SOCS-3	0	+4	1.318 ± 0.020
bicoid (control)	SOCS-3	0	0	0.008 ± 0.002

Note. LexA-IGFIR or control LexA-bicoid baits are shown in the left column and activation domain (Ad)-SOCS-3 and control Ad-SOCS-2 preys are given in the second column. The plates were read at 72 h and the scoring system was as described in Dey *et al.* (14). Immunoblotting with HA antibody indicated that the prey constructs were expressed at approximately equal levels.

Binding of SOCS-3 Fragments to the IGFIR

We delineated the regions of SOCS-3 that interacted with the IGFIR using GST-fusion proteins containing different regions of SOCS-3 (Fig. 3). Sepharose beads containing either GST-SOCS-3 (1–44), GST-SOCS-3 (SH2), or GST-SOCS-3 (141–225) bound equally to the basal and IGF-I stimulated IGFIR. Collectively, these data demonstrate that SOCS-3 has three distinct domains which interact with the IGFIR.

Sasaki *et al.* (23) recently mapped the portions of SOCS-3 N- and C-termini that are involved in binding to the kinase domain of JAK2. They found that deletion of more than 44 amino acids from the C-terminus (to residue 181) abolished the ability of SOCS-3 to interact with the kinase domain of JAK2 or with a phosphopeptide from the kinase domain (pY1007). They inter-

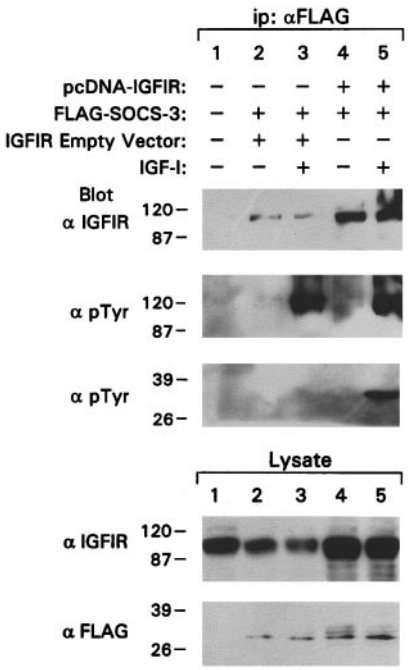

FIG. 4. *In vivo* association of SOCS-3 with the IGFIR. Human embryonic kidney (293) cells were cotransfected with the expression plasmids (lanes 2 to 5) shown at the top of the figure or untransfected (lane 1). After 36 h in growth medium the cells were serum starved for 24 h and subsequently lysed prior to or after IGF-I stimulation for 40 min. FLAG-SOCS-3 was immunoprecipitated from cellular lysates by FLAG antibody, the immunocomplexes were resolved by SDS–PAGE (reducing conditions, gradient gel) and were immunoblotted with a receptor antibody (first panel), and a phosphotyrosine antibody (second and third panels). In the lower two panels, IGFIR and FLAG-SOCS-3 protein expression was verified in the lysates (20 μl and 5 μl respectively) used for the immunoprecipitations shown in the upper panels.

preted this result to mean that the greater than 44 amino acid deletion disrupted the function of the SH2 domain which ends at residue 141 based on sequence alignment. We interpret our results to indicate that GST-SOCS-3 (141–225) binds directly to the receptor. Sasaki *et al.* (23) also examined amino terminal deletion mutants of SOCS-3. Mutants with deletions shorter than 21 amino acids retained the ability to inhibit Epo- and LIF-induced STAT3 or STAT5 activation; further deletion of the N-terminal sequence caused loss of activity. Thus, 24 amino acids N-terminal to the predicted SH2 domain were necessary for the activity of SOCS-3. Similar to the findings for SOCS-1, this 24 amino acid region was found to consist of 12 amino acid sequence immediately N-terminal to the SH2 domain termed the extended SH2 domain (ESS) and an additional N-terminal 12 amino acid sequence termed the kinase inhibitory region (KIR). Thus, Sasaki *et al.* (23) also found that N-terminal, SH2, and C-terminal portions of SOCS-3 were important for interaction with JAK-2. However, in contrast to our results, interaction of SOCS-3 with JAK2 required phosphorylation of JAK-2.

Interaction of SOCS-3 with the IGFIR in Intact Cells

To determine whether the IGFIR and SOCS-3 interact in intact cells, we utilized transient transfection of human embryonic kidney cells (293). Lysates were examined for the expression of IGFIR and the FLAG-SOCS-3 proteins (Fig. 4, lower panels). Lysates were immunoprecipitated with FLAG antibody beads, the beads washed and examined by western blotting with a receptor or a phosphotyrosine antibody (Fig. 4). FLAG antibody coimmunoprecipitated IGFIR from either when IGF-I-stimulated or unstimulated cells. FLAG-SOCS-3 fusion protein also coimmunoprecipitated the endogenous IGFIR of 293 cells in an IGF-I independent pattern. IGFIR was not immunoprecipitated in the absence of FLAG-SOCS-3 (i.e., in 293 cells alone), showing the specificity of this association. Furthermore, immunoblotting with phosphotyrosine antibody confirmed that SOCS-3 binds to the activated receptor. In a complementary immunoprecipitation experiment, IGFIR receptor antibody (αIR-3) coimmunoprecipitated FLAG-SOCS-3 fusion protein both in the presence and absence of IGF-I (Fig. 5). These results confirmed that association of SOCS-3 with the IGFIR occurs *in vivo* and SOCS-3 interacts with the both activated and basal receptor.

In 293 cells overexpressing IGFIR, FLAG-SOCS-3 was phosphorylated on tyrosine upon IGF-I stimulation of 293 cells overexpressing IGFIR (Fig. 4). This tyrosine phosphorylated SOCS-3 was only detected in the FLAG antibody immunoprecipitates and not in the

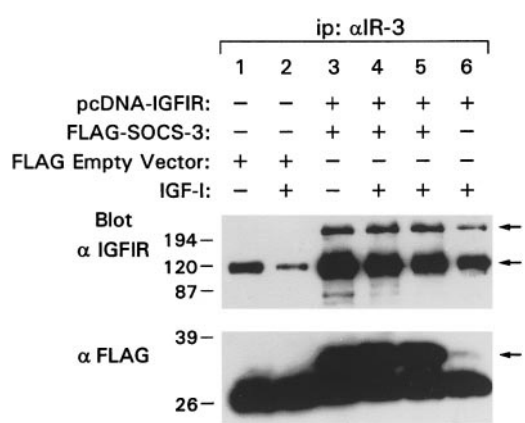

FIG. 5. *In vivo* association of SOCS-3 with the IGFIR. In a complementary experiment to the one shown in Fig. 5, extracts of 293 cells cotransfected with the indicated expression plasmids were immunoprecipitated with a receptor antibody (αIR-3). The coprecipitated materials were analyzed by SDS–PAGE (12% gel) under reducing conditions. Duplicate blots were immunoblotted with a receptor antibody (upper panel) or with a FLAG antibody (lower panel). The FLAG-SOCS3 band is indicated by an arrow.

lysates (data not shown). SOCS-3 phosphorylation on tyrosines was also not detected upon IGF-I stimulation of 293 cells containing only endogenous IGFIR. Therefore, SOCS-3 is phosphorylated on tyrosine upon IGF-I stimulation and may be a substrate of the IGFIR tyrosine kinase. Phosphorylation on tyrosine residues of SOCS-3 has also been reported following stimulation of a T cell line by IL-2 and following addition of Epo to Ba/F3 cells expressing the Epo receptor (14, 23). Our results raise the possibility that IGF-I dependent tyrosine phosphorylation of SOCS-3 may be involved in IGFIR signaling.

In summary, SOCS-3 cloned from human skeletal muscle mRNA encodes a protein which is identical to Mo7e cell SOCS-3 and differs by two amino residues from Jurkat T cell SOCS-3. SOCS-3 mRNA is widely expressed in fetal and adult tissues and in some human cancer cell lines. SOCS-3 interacts with activated IGFIR and IR in a yeast two-hybrid assay. SOCS-3 interacts constitutively with IGFIR *in vitro* and in intact cells. In cells overexpressing IGFIR and SOCS-3, SOCS-3 is phosphorylated on tyrosine residues after treating the cells with IGF-I, suggesting that SOCS-3 is a substrate for the receptor tyrosine kinase.

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