SOC5 (suppressor of cytokine signaling) proteins have been shown to be negative regulators of cytokine receptor signaling via the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway. We have cloned a member of this family (hSOCS-2) by utilizing the insulin-like growth factor 1 receptor (IGF-IR) cytoplasmic domain as bait in a yeast two-hybrid screen of a human fetal brain library. The hSOCS-2 protein interacted strongly with the activated IGF-IR and not with a kinase negative mutant receptor in the two-hybrid assay. Mutation of receptor tyrosines 950, 1250, 1251, and 1316 to phenylalanine or deletion of the COOH-terminal 93 amino acids did not result in decreased interaction of the receptor with hSOCS-2 protein. hSOCS-1 protein also interacted strongly with IGF-IR in the two-hybrid assay. Glutathione S-transferase-hSOCS-2 associated with activated IGF-IR in lysates of mouse fibroblasts overexpressing IGF-IR. Human embryonic kidney cells (293) were transiently transfected with vectors containing IGF-IR and FLAG epitope-tagged hSOCS-2. After IGF-I stimulation, activated IGF-IR was found in anti-FLAG immunoprecipitates and, conversely, FLAG-hSOCS-2 was found in anti-IGF-IR immunoprecipitates. Thus, hSOCS-2 interacted with IGF-IR both in vitro and in vivo. hSOCS-2 mRNA was expressed in many human fetal and adult tissues with particularly high abundance in fetal kidney and adult heart, skeletal muscle, pancreas, and liver. These results raise the possibility that SOCS proteins may also play a regulatory role in IGF-1 receptor signaling.

A family of proteins has recently been reported to function in a negative feedback loop to regulate signaling by cytokine receptors via the JAK/STAT (signal transducer and activator of transcription) pathway (1–9). The first member of this family to be reported was mouse CIS (cytokine inducible SH2-containing protein) (1). Upon binding of ligand to cytokine receptors, receptor-associated JAKs become activated and phosphorylate tyrosine residues on the membrane distal portion of the receptor (10). Signaling molecules which subsequently bind to these phosphotyrosine containing motifs on the receptor include members of the STAT family. STATs are phosphorylated by cytokine receptor-associated JAKs, form dimers, and travel to the nucleus where they activate transcription. CIS was isolated as a cytokine responsive immediately early gene in mouse hematopoietic cells (1, 2). CIS mRNA encodes a polypeptide of 257 amino acids that contains an SH2 domain in the middle of the molecule. Expression of CIS in IL-3-dependent hematopoietic cell lines reduced the growth rate of the transformants, suggesting a negative role of CIS in signal transduction. The CIS protein associated with tyrosine-phosphorylated erythropoietin (EPO) receptor and the tyrosine-phosphorylated β chain of the IL-3 receptor, presumably by binding of the CIS SH2 domain to phosphotyrosine containing motifs in the receptors. A mutant IL-2 receptor that failed to activate STAT5 could not induce CIS, suggesting that STAT5 was important for cytokine induction of CIS. Indeed, upstream of the transcription initiation site in the CIS promoter are four potential STAT5-binding sites. Expression of STAT5 and the EPO receptor in HEK293 cells conferred EPO-dependent activation of the CIS promoter. In these cells, EPO-dependent tyrosine phosphorylation of STAT5 was suppressed when CIS was coexpressed. Taken together, these results provide evidence for a negative feedback loop in which CIS is induced by the cytokine and then binds to the cytokine receptor, preventing the activation of STAT by JAKs (1, 2). Subsequently, three CIS-related proteins were described and these proteins have been designated as SSI (STAT-induced STAT inhibitor) or SOCS (suppressor of cytokine signaling) proteins 1–3 (3–7, 9). Together with CIS, SSI/SOCS proteins share a common domain structure consisting of an NH2-terminal region of variable length, a central SH2 domain and a COOH-terminal motif, termed the SOCS box, of unknown function.

In contrast to cytokine receptors which do not have intrinsic tyrosine kinase activity but utilize JAKs for receptor phosphorylation and phosphorylation of downstream signaling molecules such as STATs, the insulin-like growth factor I (IGF-I) receptor is a member of the tyrosine kinase family of growth factor receptors (11, 12). The IGF-I receptor is important for cellular growth, differentiation, and inhibition of apoptosis. Binding of IGF-I or IGF-II to the IGF-I receptor results in receptor autophosphorylation. Receptor autophosphorylation amplifies the tyrosine kinase activity of the receptor and creates binding motifs for downstream signaling molecules. The IGF-I receptor and the closely related insulin receptor utilize a family of large docking proteins (insulin receptor substrate,
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IRS) (13). IRS proteins bind to phosphorytrosine motifs on the receptor and, in turn, are phosphorylated on multiple tyrosine residues, creating binding motifs for the regulatory subunit of phosphoinositide 3-kinase (p85), the adapter proteins Grb-2 and Nck, and the tyrosine phosphatase, Syp. The adapter protein She also binds directly to the IGF-I receptor (14) and insulin receptor (15), providing an alternative pathway for the activation of Ras via Grb-2 and SOS. Thus, the major signaling pathways utilized by the IGF-I receptor are those in which Ras and phosphoinositide 3-kinase play an important role. We now report that a member of the SOCS family (SOCS-2) also interacts with the IGF-I receptor. SOCS-2 was cloned by a yeast two-hybrid screen of a human fetal brain library using the IGF-I receptor as bait. GST-hSOCS-2 binds to the activated IGF-I receptor from mouse fibroblasts and hSOCS-2 associates with the activated IGF-I receptor in vivo after transient transfection of human embryonic kidney 293 cells with IGF-I receptor and hSOCS-2 plasmids. These results raise the possibility that SOCS proteins may also play a regulatory role in IGF-I receptor signaling.

EXPERIMENTAL PROCEDURES

Materials—The human fetal brain activation domain fusion cDNA library, yeast strain EGY48, and two-hybrid expression plasmids were obtained from Dr. Roger Brent and have been previously described (14, 16). NIH 3T3 cells overexpressing the IGF-IR (NWTc43 cells) were obtained from Dr. Derek LeRoith. Human transformed primary embryonal kidney cells (293) were obtained from American Type Culture Collection. The antibody to the IGF-IR (number 4803) used for immunoblotting has been previously described (14, 16). The FLAG fusion protein expression system and anti-FLAG M2 monoclonal antibody were purchased from Eastman Kodak. The LexA monoclonal antibody was purchased from CLONTECH. The HA (12CA5) monoclonal antibody was obtained from Boehringer Mannheim. The monoclonal antibody to the IGF-I receptor (aI3) was purified from ascites fluid by protein G affinity chromatography and coupled to Reacti-Gel (Pierce). All oligonucleotides and primers were synthesized using an ABI DNA synthesizer. Other reagents were purchased from commercial sources as indicated in the text or figure legends.

Plasmid Constructions—The LexA-IGF-IR and LexA-IGF-IR (KR) (kinase negative receptor) bait hybrid plasmid constructs used in these studies have been previously described (14, 16). All mutants derived from the LexA-IGF-IR fusion protein were generated either by truncation or site-directed mutagenesis using PCR. The sequences of the mutant PCR fragments were verified by manual dideoxy or automatic ABI prism DNA sequencing. The full-length SOCS-2 cDNA was constructed by cloning the overlapping clones 63 and 7-10 as described (18). The SOCS-1 cDNA was obtained by PCR using cosmid clone 356d7 (accession number AC002286) as template. The GEX-hSOCS-2 plasmid was constructed by introducing the coding sequence for amino acids 29–198 of hSOCS-2 into the vector GEX-4T-1 (Pharmacia Biotech Inc.). The GST fusion protein was expressed in the BL21 Escherichia coli strain and purified according to the manufacturer’s protocol. The cDNA residues 29–198 of hSOCS-2 were also subcloned in the pFLAG-CMV-2 mammalian expression vector (Eastman Kodak) to generate FLAG-hSOCS-2. The full-length human IGF-IR cDNA (provided by Dr. Derek LeRoith) was subcloned into pcDNA mammalian expression vector (Invitrogen) for the construct pcDNA-IGF-IR.

Two-hybrid Screening and Cloning of hSOCS-2—Routine yeast culture, preparation of various yeast selection media, and yeast transformations were carried out as described (17). The two-hybrid library screen was performed as described (16). In the first step, 0.5 μg of the library was transformed into EGY48 yeast containing LexA-IGF-IR plasmid and two reporter genes, lacZ and LEU2. 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 approximately 10^7 primary transformants on the same medium containing galactose and 5-bromo-4-chloro-3-indolyl β-D-galactoside and lacking leucine. About 80–100 clones having galactosidase-dependent, lacZ+, LEU2+ phenotypes were sorted by PCR and restriction digestion. This analysis identified eight distinct cDNA inserts. One of these cDNA fragments (clone 7-10) encoded amino acids 29–198 of human SOCS-2.

Subsequently, 5′-RACE PCR was used to clone the full-length hSOCS-2 cDNA from a human fetal brain cDNA library (Marathon ready, CLONTECH). Two gene specific primers, 5′-CCCTGGACATCTG-GAACATGATGTCGATCAG-3′ (hSOCS-2, 720–689, accession number AF037899) and 5′-GGTTGACATAAGTATAGCTCGAATCT-3′ (hSOCS-2, 659–629), designed from the complementary sequence of clone 7-10, were used in this amplification. The 5′ primer was provided by CLONTECH. Twelve RACE PCR fragments were cloned into pCR2.1 vector (Invitrogen) and sequenced. The largest clone (number 63), contains the 5′ sequence of hSOCS-2 cDNA.

Northern Hybridization—Multiple tissue human poly(A) RNA blots were obtained from CLONTECH and hybridized to ^32P-labeled clone 7-10 (accession number AF037899). The probe was labeled by random priming and separated from the free nucleotides by G-50 Sephadex chromatography. The blots were hybridized at 68 °C for 1 h after adding the labeled DNA probe at a concentration of 2 × 10^6 cpm/ml of hybridization buffer. Hybridization was followed by three to five high stringency washes and autoradiography.

In Vivo Binding Studies—GST-hSOCS-2(29–198) or glutathione S-transferase (GST) in bacterial lysates was bound to glutathione-Sepharose beads and the washed beads were incubated overnight at 4 °C with cell lysates derived from NWTc43 cells (NIH 3T3 cells overexpressing human IGF-IR). Subconfluent monolayers of these cells were serum starved for 24 h in Dulbecco’s modified Eagle’s media and lysed prior to or after IGF-I (20 nm) stimulation. Lysates were prepared as described previously (16). After incubation with the beads, the beads were washed 4 times with cold lysis buffer, boiled in Laemmli SDS sample buffer containing 100 mM dithiothreitol, and resolved by SDS-PAGE. The proteins were transferred to nitrocellulose and probed with primary and secondary antibodies described in the text and figure legends. Detection was with ECL (Amersham).

In Vivo Binding Studies—The human SOCS-2 (amino acids 29–198) cDNA was subcloned into the pFLAG-CMV-2 mammalian expression vector (FLAG-hSOCS-2). 293 cells were transfected with the vector using LipofectAMINE PLUS reagent (Life Technologies). For co-transfection experiments pcDNA-IGF-IR (or the pcDNA empty vector) was included. After 48 h in growth medium the cells were serum starved for 32 h and were then lysed either prior to or after stimulation with IGF-I (100 units/ml) at 37 °C for 10 min. Lysates were prepared as described (16). Cell lysates derived from NWTc43 cells (NIH 3T3 cells overexpressing human IGF-IR (NWTc43) were maintained in similar medium supplemented with 50 μg/ml Genetin (G418 sulfate).

RESULTS

Isolation of hSOCS-2 as an IGF-IR Interacting Protein—We have used the yeast two-hybrid system to identify proteins which interact with the cytoplasmic domain of the IGF-IR. A LexA DNA-binding vector containing the entire coding sequence of the cytoplasmic domain of the IGF-IR was used as bait to screen a human fetal brain cDNA library fused to the B42 activation domain. Using this system we detected positive interactors by galactose-dependent activation of two reporter genes, lacZ and LEU2. Clone 7-10 was one of eight distinct cDNAs, which included Grb10 (14), 14-3-3β (16), and p55y (18). Sequencing of clone 7-10 showed that it contains an open reading frame encoding a protein of 170 amino acids. A multiple tissue human poly(A) RNA blot probed with the NWTc43 cell lysate showed that the clone overlaps 768 bases between the NWTc43 cell lysate and the clone 7-10.

Subsequently, RACE PCR was used to clone the full-length hSOCS-2 cDNA from a human fetal brain cDNA library (Marathon ready, CLONTECH). Two gene specific primers, 5′-CCCTGGACATCTGGAAACATGATGTCGATCAG-3′ (hSOCS-2, 720–689, accession number AF037899) and 5′-GGTTGACATAAGTATAGCTCGAATCT-3′ (hSOCS-2, 659–629), designed from the complementary sequence of clone 7-10, were used in this amplification. The 5′ primer was provided by CLONTECH. Twelve RACE PCR fragments were cloned into pCR2.1 vector (Invitrogen) and sequenced. The largest clone (number 63), contains the 5′ sequence of hSOCS-2 cDNA.
Fig. 1. Identification of hSOCS-2 from a human fetal brain cDNA library. Panel A, schematic diagram of the cloning strategy of hSOCS-2. Clone 7-10 was obtained by a two-hybrid screen of a human fetal brain cDNA library using cytoplasmic domain of the IGF-IR as bait. This clone contained the coding sequence residues 29–198 and 1033 base pairs of the 3'-untranslated region. Clone number 63 was derived by 5'-RACE of human fetal brain cDNAs and contained 317 base pairs of the 5'-untranslated region and the coding sequence residues 1–114 of hSOCS-2. The regions of the coding sequence are highlighted as follows: amino terminal region 1–47 are shown in (light gray), central SH2 domain is shown in (black), and the COOH-terminal SOCS box/motif is shown in (dark gray). The untranslated regions are shown in white. Panel B, the deduced amino acid sequence of hSOCS-2 protein. Different regions of this protein are shaded as described in the legend to panel A.

5'-end of clone 7-10 (Fig. 1A). These overlapping clones (numbers 63 and 7-10) represent a 1947-base pair cDNA that contains an open reading frame spanning nucleotides 318 to 914 and encodes a protein of 198 amino acids (Fig. 1B). The presumed initiation codon is similar, but not identical to, a Kozak consensus sequence (19). Data base analysis done at that time indicated that this protein is identical to hSOCS-2 (6). The hSOCS-2 protein contains a 47-residue amino-terminal region, a central SH2 domain, and a COOH-terminal SOCS box (20).

Characterization of the Interaction of the IGF-IR with SOCS Proteins in the Yeast Two-hybrid System—We used the yeast two-hybrid system to characterize the interaction of SOCS proteins with the IGF-IR. To determine if the full-length SOCS-2 protein interacts with the IGF-IR, the cDNA encoding the full-length protein was constructed using a primer overlapping a sequence common to clones numbers 63 and 7-10 and the product was inserted into the activation domain hybrid plasmid (AD-SOCS-2(FL)). Co-expression of this plasmid with the IGF-IR bait in which lysine 1003 is changed to arginine (Table II) resulted in reporter gene activation (Table I). Neither reporter gene was activated when the kinase negative bait was co-expressed with the AD-SOCS-2(FL) construct (AD-SOCS-1(FL)). Co-expression of this plasmid with the kinase negative bait in which lysine 1003 is changed to arginine (Table II) did not result in reporter gene activation (Table I).

To determine if other members of the SOCS family interact with the IGF-IR, an activation domain hybrid containing the complete coding sequence of SOCS-1 was constructed (3). When this construct (AD-SOCS-1(FL)) was co-expressed with the wild-type IGF-IR bait, the reporter genes were expressed at high levels. Again, co-expression with the kinase negative bait did not result in reporter gene activation (Table I).

We have previously shown that the yeast two-hybrid system can be used to map the sites of interaction of the IGF-IR with IRS-1, Shc, and 14-3-3 proteins (14, 16). To identify the site of interaction of the IGF-IR with SOCS-2, we coexpressed the full-length protein with a series of mutant receptor baits. These included mutants in which tyrosines 950, 1250, 1251, and 1316 were mutated to phenylalanine, either alone or in combination, and constructs in which the carboxyl-terminal portions of the receptor was deleted. Mutation of the tyrosine residues to phenylalanine, either alone (data not shown) or in combination (i.e. 4F), had little effect on binding of the receptor (Table I). Deletion of the carboxyl-terminal 93 amino acids (i.e. 1244Y) increased binding slightly (Table I). These data indicate that tyrosine 950 and the carboxyl-terminal region of the receptor containing tyrosines 1250, 1251, and 1316 are not required for interaction with SOCS-2 and suggest that the interaction occurs through either the kinase domain or the juxtamembrane region of the receptor.

Expression of hSOCS-2 mRNA in Fetal and Adult Tissues—We examined the tissue distribution of hSOCS-2 mRNAs by Northern blot analysis using human multiple tissue poly(A) RNA blots and probing with the 32P-labeled amino-terminal fragment number 63 (nucleotides 1–658; accession number AF037989) (Fig. 1A). A 5.0-kb mRNA species was present in all fetal and adult tissues examined (Fig. 2, A–C). This mRNA was most abundant in fetal kidney and adult heart, skeletal muscle, pancreas, and liver. A prominent 5.0-kb band also seen in adult kidney, thymus, prostate, testis, small intestine, and colon. Interestingly, additionally, smaller mRNA species (the most prominent being 2.0 and 2.8 kb) were also observed in adult tissues but not in the fetal tissues examined. Thus hSOCS-2 mRNA expression appears to be under both developmental and tissue specific controls.

In Vivo Association of IGF-IR and hSOCS-2 in 293 Cells—To demonstrate that the interaction of the IGF-IR and hSOCS-2 occurs in vivo we utilized transient transfection of human embryonic kidney cells (293). An expression vector that directed the synthesis of hSOCS-2 (amino acids 29–198) fused to an amino-terminal FLAG epitope tag was co-transfected with pcDNA-IGF-IR expression plasmid into 293 cells. After 48 h in growth medium, transfectants were serum starved for 32 h and then lysed either prior to or after IGF-I stimulation for 40 min. Extracts were immunoprecipitated with IGF-IR antibody (aI3) beads; coimmunoprecipitated proteins were resolved by SDS-PAGE and immunoblotted with either FLAG M2 monoclonal antibody or IGF-IR antibody (number 4803). Receptor expression in the 293 cells transfected with receptor was confirmed by the immunoblotting the lysates with receptor antibody (Fig. 4A, top panel, lanes 3, 4, 6, and 7). A low level of endogenous receptor expression was seen in the untransfected cells (lane 1). IGF-IR antibody immunoprecipitated a protein
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Table I
Interaction of the IGF-IR with SOCS proteins in the yeast two-hybrid system

| Bait         | Prey          | LEU2 | lacZ (relative activity) |
|--------------|---------------|------|--------------------------|
|              |               | Glu  | Gal                      |
|              |               | +4   | ± S.D.                   |
| WT           | SOCS-2(FL)    | 0    | 1.00                     |
| WT           | SOCS-2(29–198) | 0 | 0.63 ± 0.19              |
| KR           | SOCS-2(FL)    | 0    | 0.00                     |
| WT           | SOCS-1(FL)    | 0    | 1.53 ± 0.11              |
| KR           | SOCS-1(FL)    | 0    | 0.00                     |
| 4F           | SOCS-2(FL)    | 0    | 0.73 ± 0.09              |
| 1244Y        | SOCS-2(FL)    | 0    | 1.46 ± 0.11              |
| WT           | None          | 0    | 0.00                     |
| WT           | IRS-1(2–516)  | 0    | 0.23 ± 0.06              |

hSOCS-2 is Not Phosphorylated on Tyrosines as a Consequence of Its Interaction with IGF-IR—We have also analyzed the phosphorylation status of hSOCS-2 in 293 cells. Sequence analysis of hSOCS-2 indicated it has a YVQM motif (amino acids 129–132, Fig. 1B), a possible target of IGF-IR tyrosine kinase. IGF-IR substrates which contain this motif include IRS-1 and -2 and Shc (11, 14). Using immunoblot analysis with a phosphotyrosine antibody (Fig. 4A, lower two panels) confirmed the ligand-dependent autophosphorylation of the receptor and the association of the activated receptor with hSOCS-2 protein (lane 7).

In a complementary experiment, 293 cells were co-transfected with the plasmids expressing FLAG-SOCS-2 and IGF-IR and the cell extracts were immunoprecipitated with anti-FLAG M2-Sepharose beads (Fig. 4B). Immunoblotting was performed with either the FLAG M2 antibody (upper panel) or the receptor antibody (lower panel). The FLAG antibody coprecipitated IGF-IR when the cells were stimulated with IGF-I (lower panel, lane 7) but not in unstimulated cells (lane 6) or in cells containing the empty FLAG vector (lower panel, lane 5). These experiments demonstrate that the association of hSOCS-2 with the activated IGF-IR occurs in vivo.

Discussion

In this study, we cloned hSOCS-2 in a yeast two-hybrid screen of a human fetal brain library using the IGF-I receptor as bait. Human SOCS-2 has also recently been cloned from an activated Jurkat cDNA library (6) and by a combination of EST data base search and RACE-PCR using poly(A) + RNA from Mo7e cells (9). Besides two-hybrid interaction, we have demonstrated in vitro and in vivo interaction of the IGF-1 receptor and hSOCS-2. This interaction depends upon IGF-I receptor activation in fibroblasts and human embryonic kidney 293 cells overexpressing the IGF-1 receptor. In the yeast two-hybrid assay the interaction is also dependent upon receptor autophosphorylation because kinase inactive receptor did not interact with the hSOCS-2. No other binding partner of SOCS-2 has been reported; specifically, SOCS-2 does not bind to JAK-2 (9).

Several earlier reports indicated that expression of some but not all SOCS mRNAs are inducible by cytokines and STATs (3–6). A wide range of the cytokine superfamily members, including interleukin-3, interleukin-4, interleukin-6, leukemia inhibitory factor, erythropoietin, granulocyte macrophage colony stimulatory factor (1, 3–5, 8, 9), and growth hormone (21), induce transcriptional activation of one or more of the SOCS or CIS genes in hematopoietic cells or murine liver through activation of the JAK/STAT signaling pathway. Thus, SOCS genes may function as part of an intracellular negative feedback loop, inhibiting either JAK activity or STAT phosphorylation and thereby suppressing cytokine signal transduction. Peripheral leptin administration rapidly induced SOCS-3 mRNA in the hypothalamus but had no effect on CIS, SOCS-1, or SOCS-2 (22). In mammalian cell lines, SOCS-3, but not SOCS-2 or CIS, blocked leptin-induced signal transduction. The suppression of cytokine signal transduction by SOCS-2 is not clear. In a preliminary study, hSOCS-2 was shown to inhibit leukemia inhibitory factor-mediated differentiation and growth arrest of myeloid leukemia M1 cells (6). However, in a similar experiment utilizing M1 cells, Masuhara et al. (9) observed inhibition of leukemia inhibitory factor-induced differentiation and growth arrest by SOCS-3 but not SOCS-2.

Although SOCS family members exhibit a similar domain structure with a central SH2 domain and a COOH-terminal SOCS box, the comparison of the amino acid sequences among family members shows that CIS, SOCS-1, SOCS-2, and SOCS-3 are only distantly related (3). A search of DNA data bases for amino acid sequences corresponding to conserved residues in the SOCS box identified four additional members of the family (SOCS-4 to SOCS-7) (20). Full-length cDNAs have not been isolated for all of these new members of the SOCS family. The fact that SOCS family members are not closely related at the level of protein sequence may point to different functions among family members.

Tissue expression of mRNA is different among SOCS family members. SOCS-1 and SOCS-3 mRNA expression is most prominent in thymus, spleen, and lung whereas expression was more widespread for CIS and SOCS-2 (3). We found that hSOCS-2 mRNA was most abundant in adult heart, skeletal muscle, pancreas, and liver with intermediate amounts in kidney, thymus, prostate, testes, small intestine, and colon. Although Minamoto et al. (96) also found that hSOCS-2 mRNA was expressed in many tissues their results differed from ours for a number of tissues. Compared with our results, Minamoto et al. (6) found relatively lower levels of SOCS-2 mRNA in liver, skeletal muscle, pancreas, and thymus, and higher levels of...
The different patterns of tissue expression for different SOCS family members suggest that different SOCS proteins may have different functions.

It seems likely that the binding of CIS to the EPO receptor and IL-3 receptor β subunit and the binding of SOCS-1 to JAK2 is explained by the SOCS SH2 domain binding to a phosphotyrosine motif in the receptors (1, 2, 5). However, these phosphotyrosine motifs have not been identified. Our finding that hSOCS-2 binds only to the autophosphorylated IGF-I receptor is consistent with hSOCS-2 SH2 domain binding to a phosphotyrosine containing motif in the receptor. However, mutation of tyrosines 950, 1250, 1251, and 1316 to phenylalanine did not result in a decrease in the interaction of hSOCS-2 with the IGF-I receptor. In the case of binding of SOCS-1 to JAK2 there is evidence for additional involvement of other regions of the SOCS-1 protein (4). Although it could be demonstrated that SOCS-1 SH2 domain bound to JAK2, full inhibition of JAK2 kinase was not achieved by a SOCS-1 construct lacking both the COOH- and NH2-terminal regions. In the case of the binding of SOCS-1 to Tec, a cytoplasmic tyrosine kinase, the COOH-terminal region, and the SH2 domain were not required for interaction, nor was kinase activity of Tec required (7). These results suggest that the interaction of SOCS-1 and Tec does not utilize either the SH2 domain of SOCS-1 or phosphotyrosine motifs in Tec.

Although both CIS and SOCS-1 have been shown to inhibit the JAK/STAT pathway, the mechanism of inhibition appears to be different for each of the regulatory proteins. CIS associates with the tyrosine-phosphorylated EPO receptor and the phosphorylated β subunit of the IL-3 receptor (1). Binding of CIS does not inhibit the phosphorylation of the EPO receptor or the IL-3 receptor β subunit. CIS does not interact with JAK-2 (4). In Ba/F3 lymphoid cells expressing the EPO receptor and CIS under the control of dexamethasone, induction of CIS expression resulted in a decrease in the phosphorylation of STAT5 in response to EPO (2). Therefore, one model for the inhibitory action of CIS is that the binding of CIS to the phosphorylated EPO receptor prevents the binding of STAT5 to the receptor, resulting in decreased tyrosine phosphorylation of

**Fig. 2.** Analysis of mRNA expression hSOCS-2 in fetal and adult human tissues. Tissue distribution of hSOCS-2 mRNAs was examined in different fetal (panel A) and adult tissues (panels B and C). Multiple tissue Northern blots were hybridized to a 32P-labeled DNA of clone number 63 (nucleotides 1–658; accession number AF037989). The different tissues are denoted above each lane. These blots were stripped and subsequently reprobed with β-actin cDNA. The position of the β-actin marker (kb) is shown on the left of the blots. The positions of the mRNAs for hSOCS-2 are indicated by the arrows (right) in each blot.

**Fig. 3.** In vitro binding of hSOCS-2 to the IGF-IR. Panel A, NIH 3T3 cells overexpressing IGF-IR (NWTc43) were stimulated with 20 nM IGF-I and lysed at the times indicated. Extracts were incubated with GST-Sepharose beads or GST-hSOCS-2 Sepharose beads overnight at 4 °C, washed, and the material bound to the beads was analyzed by SDS-PAGE and immunoblotting with anti-IGF-IR antibody. Lane 1 shows 20 μl of the whole cell extract. The position of the 98-kDa marker is shown on the left and the position of the β-subunit of IGF-IR is indicated (arrow). Panel B, a similar experiment was performed as described in panel A except that immunoblotting was performed with a phosphotyrosine antibody (Upstate Biotechnology). Lanes 1 and 2 show the whole cell lysates (20 μl). Lysates were incubated with GST-SOCS-2 in lanes 3 and 4 and GST alone in lane 5.
the bottom cells were co-transfected with expression plasmids (respectively) to assess the phosphorylation status of IGF-IR and FLAG-hSOCS-2, and reprobed with phosphotyrosine antibody (Upstate Biotechnology) immunoprecipitation is shown in lanes 1–4 (analyzed by SDS-PAGE and immunoblotting with antibodies indicated). In summary, we have provided evidence for interaction of hSOCS-2 with the activated IGF-I receptor. We are conducting experiments to investigate the role of SOCS-2 in IGF-I receptor signaling. The distant relatedness of SOCS family members, variation in tissue mRNA expression, and differences in mechanism of action of two family members (CIS and SOCS-1), suggest that SOCS proteins may have diverse functions.

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