Mechanism of STAT3 Activation by Insulin-like Growth Factor I Receptor*

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Recent evidence indicates that STAT proteins can be activated by a variety of receptor and non-receptor protein-tyrosine kinases. Unlike cytokine-induced activation of STATs, where JAKs are known to play a pivotal role in phosphorylating STATs, the mechanism for receptor protein-tyrosine kinase-mediated activation of STATs remains elusive. In this study, we investigated the activation of STAT proteins by the insulin-like growth factor I receptor (IGF-IR) in vitro and in vivo and assessed the role of JAKs in the process of activation. We found that STAT3, but not STAT5, was activated in response to IGF-I in 293T cells cotransfected with IGF-IR and STAT expression vectors. Moreover, tyrosine phosphorylation of STAT3, JAK1, and JAK2 was increased upon IGF-I stimulation of endogenous IGF-IR in 293T cells transfected with the respective STAT or JAK expression vector. Supporting the observation in 293T cells, endogenous STAT3 was tyrosine-phosphorylated upon IGF-I stimulation in the muscle cell line C2C12 as well as in various embryonic and adult mouse organs during different stages of development. Dominant-negative JAK1 or JAK2 was able to block the IGF-IR-mediated tyrosine phosphorylation of STAT3 in 293T cells. A newly identified family of proteins called SOCS (suppressor of cytokine signaling), including SOCS1, SOCS2, SOCS3 and CIS, was able to inhibit the IGF-I-induced STAT3 activation as well with varying degrees of potency, in which SOCS1 and SOCS3 appeared to have the higher inhibitory ability. Inhibition of STAT3 activation by SOCS could be overcome by overexpression of native JAK1 and JAK2. We conclude that IGF-IR/IGF-I is able to mediate activation of STAT3 in vitro and in vivo and that JAKs are essential for the process of activation.

The insulin-like growth factor I receptor (IGF-IR)1 belongs to the subfamily of type II receptor protein-tyrosine kinases. It is ubiquitously expressed in human tissues and has been implicated in diverse biological processes such as mitogenesis, cellular transformation, cell survival, and cell differentiation (1–5). One of the important functions of IGF-IR is its role in organogenesis of various organs during early embryonic development. It continues to play a critical role in mature organs as well. It has been shown that 95% of igf-I−/− mice die immediately following birth. Mice with an igf-Ir gene disruption also die at birth with respiratory failure and exhibit a severe growth deficiency (45% of the normal size) (6). The igf-Ir−/− mouse embryos display generalized organ hypoplasia, delayed ossification, and developmental abnormalities in the central nervous system and epidermis. Furthermore, abnormalities in renal development were found in igf-I−/− mice that survived past birth, i.e. the number of nephrons per kidney was reduced by ~20% in igf-I−/− mice (6). In adult animals, IGF-IR is known to play an important role in organ regeneration (such as in neurons and liver) as well as in compensatory kidney growth after renal failure. The function of IGF-IR in other organs in general is less clear. In addition, IGF-IR has been shown to have cell-transforming potential and has been implicated in tumor development. NIH3T3 cells overexpressing native IGF-IR form colonies in soft agar in response to IGF-I (7). Fusion of the 5′-truncated β-subunit of IGF-IR to a retroviral group-specific antigen sequence was shown to activate its oncogenic potential (8). Using an IGF-IR null mouse cell line called R−, it was demonstrated that IGF-IR is essential for transformation by a number of oncogenes, including SV40 large T antigen and ras (9). More important, IGF-IR is widely implicated in the development and/or progression of a variety of human tumors, including breast cancer (5, 9, 10). Introduction of antisense oligonucleotides, antisense mRNA expression plasmids, or dominant-negative mutants of IGF-IR into various cancer cell lines has been repeatedly shown to reverse the transformed phenotype in vitro and to inhibit tumorigenicity and metastasis in vivo (11–14). In addition, IGF-I is known to promote survival in many cell types in response to a range of stresses, including growth factor withdrawal, chemotherapeutic agents, and UV irradiation (15–17).

Mature IGF-IR is a tetrameric type II receptor protein-tyrosine kinase consisting of two ligand-binding α-subunits and two transmembrane β-subunits. The binding of ligand to IGF-IR results in its conformational change and cross-phosphorylation between the β-subunits of the receptor complex, leading to phosphorylation of additional tyrosine residues and further activation of the protein-tyrosine kinase activity (18, 19). Phosphorylation of various tyrosine residues also creates binding sites on the receptor for its immediate downstream signaling molecules, which typically contain phosphotyrosine-binding or SH2 domains (20–23). The juxtamembrane tyrosine (Tyr850) in the context of the sequence NPXY is known to be the
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major binding site for insulin receptor substrate-1 and -2 and Shc, which are the major substrates for IGF-IR (24). Through binding and phosphorylation of insulin receptor substrate-1 and -2 and their subsequent recruitment of Grb2 and the regulatory subunit p85 of the phosphatidylinositol 3-kinase, the two major signaling pathways, viz. the Ras/MAPK pathway and the phosphatidylinositol 3-kinase pathway, are activated. Signaling by the MAPK pathway, which includes sequential activation of a cascade of serine/threonine protein kinases, plays an important role in promoting cell growth and in regulating gene expression (20, 21). The phosphatidylinositol 3-kinase pathway is known to be involved in various functions, including cell growth and cell survival (25, 26).

STAT proteins include STAT1–4, -5a, -5b, and -6 and have been shown to play an important role in cytokine signaling (27, 28). These proteins are tyrosine-phosphorylated by JAKs following the binding of cytokine to its receptor. Four members of the JAK family have been identified so far: JAK-1, JAK-2, JAK-3, and TYK-2 (29). JAK-1, JAK-2, and TYK-2 are ubiquitous (29), whereas JAK-3 is expressed only in T lymphocytes (29). Recent studies showed that STAT proteins can also be activated by a variety of receptor and non-receptor protein-tyrosine kinases (27, 28). Upon tyrosine phosphorylation, STAT proteins form homo- or heterodimers through intermolecular interactions of the SH2 domains with the phosphorylated tyrosines and rapidly translocate to the nucleus and induce gene expression. Thus far, >40 different polypeptide ligands have been shown to cause STAT activation. They include growth factors such as epidermal growth factor (EGF) (27, 28), platelet-derived growth factor (PDGF) (27, 28), colony-stimulating factor-1 (27, 28), and insulin (28, 30). Recent evidence has demonstrated the necessity of STAT3 in cell growth and transformation. STAT3, but not STAT1, -5, or -6, has been shown to be activated by Src and Fps protein-tyrosine kinases in NIH3T3 and Rat-1 cells, respectively (31). Using oncogenic src and an NIH3T3 cell system, Turkson et al. (32) and Bromberg et al. (33) showed that STAT3 is required for Src-induced cell transformation. Using a group-specific antigen/IGF-IR fusion receptor and an EGF receptor/Ros chimera, we have demonstrated that activation of STAT3 is essential for the establishment and maintenance of cell transformation by these receptor protein-tyrosine kinases (34). A growing number of tumor-derived cell lines as well as human tumors have been reported to contain constitutively activated STAT proteins, particularly STAT3, which was observed to be activated in five out of nine breast carcinoma cell lines and in a high proportion of head and neck cancers examined (31). Recently, it was shown that a mutation that causes constitutive dimerization of STAT3 was able to activate its oncogenic potential (35).

In mouse development, the mRNA of STAT3 could be detected within early post-implantation stages, and STAT3 was activated during this early development (36). Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality, indicating that STAT3 plays an important role in embryonic development (37). Using the approach of tissue-specific gene knockout, STAT3 was shown to play an anti-apoptotic role in T cells and in monocytes during their differentiation (38).

The JAK/STAT signaling pathways are tightly regulated processes, but little is known about how these signals are turned off. The family of SOCS proteins, which includes SOCS1–7 and CIS, has recently been isolated and shown to act as negative regulators of cytokine-induced signaling (39). SOCS proteins appear to switch off JAK/STAT signaling by at least two mechanisms. SOCS1 and SOCS3 have been shown to bind to activated JAKs and inhibit their catalytic activity, although their precise mechanism is not clear (39). In contrast, CIS appears to bind directly to phosphorylated receptors and to compete with signaling intermediates including STAT5 for binding to the receptor (39). So far, the understanding of the function of SOCS proteins has been derived from using a cytokine system as the experimental model. To date, there is only one report demonstrating the involvement of the SOCS protein in growth factor-mediated signal transduction, viz. suppression of the Steel factor-dependent proliferation of SOCS1 (40).

In view of the accumulating evidence implicating IGF-IR and STAT3 in embryonic development, cell transformation, and tumor development, we set out to investigate the functional interaction between IGF-IR and STAT3 in vitro and in vivo. We observed that STAT3 is activated upon IGF-I stimulation in cells transfected with IGF-IR and STAT3. Following in vivo injection of IGF-I, STAT3 activation was detected in various tissues of mice in different developmental stages. Our results suggest that STAT3 is a physiological signaling molecule of IGF-IR. Using SOCS proteins and dominant-negative mutants of JAKs, we found that IGF-I-induced STAT3 activation requires Janus kinases.

EXPERIMENTAL PROCEDURES

Cell Culture—293T cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. C2C12 cells were grown in Dulbecco's modified Eagle's medium containing 15% fetal bovine serum and 0.5% chicken embryo extract.

DNA Transfection—Transfections were carried out by the standard calcium phosphate method (34). Cells were seeded at 70% confluency/60-mm diameter dish in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum at 18 h prior to transfection. Total DNA for transfection was 100–1000 ng of the appropriate plasmids/dish plus 5 μg of calf thymus DNA. Transfection was terminated 15 h later by removing the medium and washing once with fresh medium. The cells were then maintained in the same medium for 24 h before being starved overnight in serum-free medium for growth factor stimulation.

Plasmids and Antibodies Used—phEFIGF-IR was generated by removing the full-length IGF-IR insert from pMXIGF-IR (7) and cloning it into the human elongation factor promoter-based plasmid. Expression plasmids containing CIS and SOCS1–3 were kindly provided by Drs. Tom W. Wheeler and Henry Sadowski with the help of Tom Moran at the HybriDoma Core Facility of the Mount Sinai School of Medicine by using phosphorylated STAT5 peptide as the antigen. Anti-phospho-STAT3 and anti-phospho-p42/44 MAPK antibodies were purchased from New England Biolabs Inc. Anti-STAT3, anti-STAT5a, and anti-STAT5b antibodies were purchased from Santa Cruz Biotechnology, Inc. Anti-phosphotyrosine antibody (RC20) was purchased from Transduction Laboratories. Anti-JAK1 and anti-JAK2 antibodies were purchased from Upstate Biotechnology, Inc. Anti-FLAG antibody (M2) was purchased from Sigma.

Protein Analysis and Electrophoretic Mobility Shift Assay—Protein extraction, immunoprecipitation, and Western blotting were done according to published procedures (34). Seventeen-day pregnant DBA mice were injected with 0.2 ml of PBS or PBS containing 2 μg/ml IGF-I via the tail vein. Forty minutes later, the mice were killed, and embryos were removed for sampling of various organs. One-week-old mice were injected intraperitoneally with 0.2 ml of PBS or PBS containing 20 μg/ml IGF-I. Thirty minutes later, the mice were killed, and proteins were extracted from various organs. Adult mice were injected with 0.2 ml of PBS or PBS containing 2 μg/ml IGF-I via the tail vein for 1 h and were killed 30 min later. Protein extraction and analysis were done as for newborn mice. The tissue samples were homogenized in a Dounce homogenizer in radio-immune precipitation assay buffer. The protein concentration of the cleared lysate was determined. Two milligrams of each tissue lysate was immunoprecipitated with anti-STAT3 antibody and subjected to gel electrophoresis and sequential Western blotting with anti-phospho-
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RESULTS

Tyrosine Phosphorylation of STAT3 in Mouse Tissues in Response to IGF-I Stimulation—To determine whether STAT3 is a downstream signaling molecule of IGF-I and its receptor in vivo, we examined tyrosine phosphorylation of STAT3 in different tissues after injection of IGF-I into mice at different stages of development. Tyrosine phosphorylation of STAT3 was detected in response to IGF-I in liver, kidney, intestine, and muscle in 17-day-old embryos (Fig. 1A). No significant phosphorylation of STAT3 was observed in brain and heart. Although the abundance of STAT3 varied among tissues, its increased phosphorylation was not due to the varying protein amount of STAT3 (Fig. 1A). In 1-week-old mice, STAT3 tyrosine phosphorylation was detected in lung, kidney, spleen, and thymus in response to injected IGF-I (Fig. 1B). A similar pattern of STAT3 activation was observed in response to IGF-I in adults compared with newborn mice with the exception of the thymus (Fig. 1C). Despite a relatively high expression level of IGF-IR in brain, no receptor tyrosine phosphorylation could be detected in response to IGF-I injection (Fig. 1, B and C, lower panels). This could be due to failure of the injected IGF-I to pass through the blood-brain barrier. The IGF-IR expression level was relatively lower in adult liver in comparison with that in 1-week-old pups. Both the expression level and extent of activation of STAT3 in response to IGF-I in liver were higher in embryonic mice than in newborn and adult mice. The kidney had the highest level of IGF-IR expression among the adult tissues examined. Taken together, our data indicate that IGF-I and its receptor are able to mediate activation of STAT3 in a number of tissues throughout mouse development.

STAT3, but Not STAT5, Is Activated by IGF-I—We have shown previously that insulin and the insulin receptor are able to mediate the activation of STAT5 in vitro and in vivo (30). Since IGF-IR and the insulin receptor are the two most closely related receptor protein-tyrosine kinases, we tested if IGF-IR can also mediate the activation of STAT5 in addition to STAT3. For this purpose, plasmids encoding IGF-IR and control vector, IGF-IR and STAT3, or IGF-IR and STAT5α/STAT5b were transiently transfected, respectively, into 293T cells. The lysates from unstimulated or stimulated cells were analyzed by Western blotting with antibodies specific to tyrosine-phosphorylated STAT3 or STAT5, respectively (Fig. 2, top panels). IGF-I was detected by immunoprecipitation with specific antibody, followed by blotting with anti-phosphotyrosine antibody (Fig. 2, A–C, bottom panels). Activation of IGF-IR by IGF-I led to a significant increase in the tyrosine phosphorylation of STAT3 (Fig. 1A). The increased tyrosine phosphorylation was correlated with the increased DNA-binding ability of STAT3 using the high affinity M67 DNA-binding site (SIE) deoxylguanucleotide. No significant tyrosine phosphorylation of STAT5α was observed with or without IGF-I stimulation (Fig. 2B). There was a relatively high background level of STAT5b tyrosine phosphorylation; however, reproducibly, no significant increase was detected in response to IGF-I (Fig. 2C). We conclude that IGF-IR mediates the activation of STAT3, but not that of STAT5. Furthermore, STAT3 was activated upon IGF-I stimulation of endogenous IGF-IR in 293T cells transfected only with the STAT3 expression plasmid (Fig. 2D). Supporting the observation of 17-day embryonic muscle, STAT3 was tyrosine-phosphorylated upon stimulation of the muscle cell line C2C12, in which neither STAT3 nor IGF-IR was overexpressed (Fig. 2E)

Tyrosine Phosphorylation of JAK in Response to IGF-I Stimulation, and STAT3 Activation by IGF-I Requires JAK Activity—Previous studies have demonstrated that JAK1 and JAK2 can be activated in NIH3T3 cells overexpressing IGF-IR (41). To explore the role of JAKs in IGF-I-induced STAT3 activation, we examined the tyrosine phosphorylation of transiently expressed JAK1 or JAK2 in 293T cells in response to IGF-I stimulation of endogenous IGF-IR. Increased JAK1 and JAK2 tyrosine phosphorylation was detected upon IGF-I stimulation, confirming the published observations in NIH3T3 cells. Dominant-negative JAK1 and JAK2 mutants are inactive in their catalytic activity and, when coexpressed with the wild type, were able to work dominant-negatively against the IGF-I-induced tyrosine phosphorylation of wild-type JAKs (Fig. 3). The dominant-negative JAK mutants had no effect on the activation of IGF-IR. To further investigate whether STAT3 activation by IGF-I requires JAK activity, the dominant-negative JAK mutants were used to test their effect on STAT3 activation upon IGF-I stimulation. The degree of tyrosine phosphorylation of STAT3 was decreased by coexpression of either dominant-negative JAK1 or JAK2, and the inhibition occurred in a...
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Dose-dependent manner (Fig. 4). At the highest dose (0.4 μg), either dominant-negative JAK mutant was able to completely block the activation of STAT3. Conversely, cotransfection of either JAK1 or JAK2 resulted in the augmentation of the activation of STAT3 by IGF-1 (data not shown). Using the kinase inhibitor AG490, which has previously been shown to inhibit JAK activity (42, 43), we observed a dose-dependent inhibition of STAT3 phosphorylation that was completely abolished at 100 nM (data not shown). By contrast, no significant inhibition of the tyrosine phosphorylation of STAT3 was observed with the Src family kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butylyl)pyrazolo[3,4-d]pyrimidine (PP2) (44) at a concentration up to 50 μM (data not shown). We conclude that IGF-1/IGF-IR-mediated tyrosine phosphorylation of STAT3 requires JAK activity.

SOCS Proteins Inhibit STAT3 Activation, but Have No Effect on the Receptor Itself or on Receptor-mediated MAPK Activation—SOCS proteins have recently been reported to suppress the kinase activity of Janus kinase family members. To check if SOCS can block STAT3 activation, we measured the STAT3 phosphorylation following IGF-1 stimulation in 293T cells transiently transfected with IGF-IR and with STAT3 together with control or different SOCS expression vectors. Coexpression of SOCS proteins did not affect the induction of IGF-IR phosphorylation or subsequent MAPK activation (Fig. 5). However, the tyrosine phosphorylation of STAT3 was significantly inhibited by SOCS protein expression (Fig. 5). The inhibition was more pronounced with the expression of SOCS1 or SOCS3 in comparison with CIS or SOCS2. Our results indicate that SOCS proteins have no effect on the receptor kinase activity, but are potent inhibitors of IGF-IR-mediated STAT3 activation. Our observations of the effects of SOCS and dominant-negative JAKs strongly suggest that JAK(s) plays a critical role in IGF-IR-mediated activation of STAT3.

STAT3 Activation by JAKs Can Be Inhibited by Overexpressing SOCS1, and Inhibition of STAT3 Activation by SOCS1 Can Be Rescued by Overexpressing JAKs—To further understand the role of JAKs and the effect of SOCS on IGF-1-mediated STAT3 activation, we performed the following experiments. 293T cells were transfected with IGF-IR, STAT3, and a constant amount of JAK1 or JAK2 with an increasing amount of SOCS1. We observed that SOCS1 potently inhibited STAT3 activation by IGF-IR and JAK1 (Fig. 6A). By contrast, SOCS1 inhibition of STAT3 activation by IGF-IR and JAK2 was not as effective, although notable inhibition was observed at the highest dose of SOCS1, suggesting that SOCS1 is a more effective inhibitor of JAK1 than JAK2 (Fig. 6B). Overexpression of either JAK1 or JAK2 could overcome the SOCS1 inhibition of IGF-1-induced STAT3 activation (Fig. 6C). Our results further confirm the important role of JAKs in IGF-1-induced STAT3 activation. The results here are consistent with those obtained with dominant-negative JAK1 and JAK2 and the kinase inhibitors described above and suggest that both JAKs are involved in the STAT3 activation.

Fig. 2. IGF-I-induced activation of STAT3, -5a, and -5b. One-hundred nanograms of phEFIGF-IR and 200 ng of pcSTAT3, pcSTAT5a, pcSTAT5b, or control vector pcCMV DNA (A–C) or pcSTAT3 alone (D) (as indicate in the boxes) were cotransfected into 293T cells at 60% confluence by the calcium phosphate method. After 24 h, cells were serum-starved for 12 h and then were stimulated with IGF-I (50 ng/ml) for 15 min or were left unstimulated. Protein was extracted using radiomune precipitation assay buffer. Twenty micrograms of cell lysate was directly Western-blotted with anti-phospho-STAT3 antibody (A, upper panel), anti-phospho-STAT5 antibody (B and C, upper panels), or anti-STAT3 antibody (A, second panel; and D, lower panel), or anti-STAT5a antibody (B, second panel), or anti-STAT5b antibody (C, second panel). In the third panels, 5 μg of total cellular extract from each transfection was used for DNA binding by electrophoretic mobility shift assay using the M67 serum inducible element (SIE) probe containing the binding site for STAT3 (A) or the β-casein promoter sequence containing the binding site for STAT5 (B and C). Arrows indicate the DNA and protein complex. In the lower panels, 500 μg of total cellular extract was immunoprecipitated (IP) with anti-IGF receptor antibody (anti-IGFR), separated by SDS-polyacrylamide gel electrophoresis, and Western-blotted with anti-Tyr(P) antibody. N.S. indicates the nonspecific background band. C2C12 cells were serum-starved for 24 h and then were stimulated with IGF-I (50 ng/ml) for 15 min or were left unstimulated. Fifty micrograms of each cell lysate was directly Western-blotted with anti-phospho-STAT3 antibody (B, upper panel), and the same filter was stripped and reprobed with anti-STAT3 antibody (second panel). Five-hundred micrograms of cell lysate was immunoprecipitated with anti-IGF-IR antibody and Western-blotted with anti-Tyr(P) antibody (E, third panel), and the same filter was stripped and reprobed with anti-IGF-IR antibody to monitor the protein level (bottom panel). IB, immunoblotted.
DISCUSSION

In this study, we show that IGF-I and its receptor are able to induce activation of STAT3 in vivo and in vitro. Our results indicate that the in vivo protocol of IGF-I injection is effective in activating IGF-IR and its downstream signaling. A similar method has been used successfully to study substrate tyrosine phosphorylation in neonatal mice (45) as well as STAT5 tyrosine phosphorylation and nuclear translocation in mouse liver in response to EGF stimulation (46). We have chosen DBA mice here since we have previously demonstrated STAT5 activation by perfusion of insulin in liver (30). In the current protocol, each mouse was injected with 200 μl of saline or a 10^3 physiological concentration of IGF-I (2 μg/ml) through the tail vein, which presumably would be diluted to the approximately physiological concentration in vivo. The physiological concentration of IGF-I in the mouse is ~200 ng/ml (47). Under similar stimulation with insulin, no phosphorylation of STAT3 was detected in spleen or kidney; therefore, the observed STAT3 activation by IGF-I was unlikely due to cross-activation of the insulin receptor. STAT3 was activated by IGF-I in many fetal and adult tissues, particularly in kidney, lung, pancreas, and thymus, suggesting that STAT3 plays a role in IGF-IR signaling in these organs during different stages of mouse development and in adult life. Our data also suggest that STAT3 signaling may be more important for fetal as opposed to adult liver and that the opposite is true for kidney, although its precise role in those organs is unclear.

The role of STATs in receptor protein-tyrosine kinase signaling and the mechanism of their activation are not well understood. Tyrosine phosphorylation of JAKs in response to EGF, PDGF, insulin, and IGF-I among others has been observed (29, 41). However, the role of JAKs in receptor tyrosine kinase-mediated signaling is still unclear. EGF has been shown to be able to lead to activation of STAT1, -3, and -5 (41, 46), although only STAT3 (but not STAT1) activation is thought to play a significant role in EGF-induced cell growth in vitro (48). Using a pharmacological inhibitor of Src kinases and a dominant-negative mutant of Src or JAK expression vectors, it was shown that STAT activation by EGF requires Src kinase activity, which was also shown to be able to regulate JAK activity (49). By contrast, the PDGF receptor was found to be able to activate STAT1, -3, and -6 in the absence of detectable Src activation (50). This was shown by using the PDGF receptor mutant that was impaired in binding to Src. Furthermore, using cell-free...
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Fig. 5. SOCS proteins inhibit STAT3 activation but have no effect on the receptor itself or receptor-mediated MAPK activation. Subconfluent 293T cells were cotransfected with pEFIGF-IR (200 ng) and pcSTAT3 (400 ng) together with either control empty vector or one of the FLAG-tagged SOCS expression vectors (2 μg). Cells were serum-starved and stimulated with IGF-I as described in the legend to Fig. 3. Cell lysates were directly analyzed for tyrosine phosphorylation of STAT3 (upper panel), STAT3 protein amount (second panel), or SOCS protein expression levels (third panel) by Western blotting with the antibody indicated below the respective panels. In addition, the lysates were immunoprecipitated with anti-IGF-IR antibody (anti-IGFR) and Western-blotted with anti-Tyr(P) antibody to demonstrate tyrosine phosphorylation of IGF-IR (fourth panel). MAPK phosphorylation was assessed by directly Western blotting total cell lysates with anti-phospho-MAPK antibody (lower panel). IB, immunoblotted.

| System | STAT3 Activation by IGF-IR |
|--------|-----------------------------|
| STAT3  | Activated by IGF-IR |
| JAKs   | Activated by IGF-IR |
| SOCS   | Inhibit STAT3 activation |

**Fig. 6. STAT3 activation by JAK can be inhibited by overexpressing SOCS1, and the inhibition can be rescued by overexpressing JAK.** In A and B, subconfluent 293T cells were cotransfected with pEFIGF-IR (200 ng), pcSTAT3 (400 ng), and wild-type JAK1 expression vector pRK5JAK1 (100 ng) (A) or wild-type JAK2 expression vector pRK5JAK2 (100 ng) (B) together with increasing amounts of SOCS1. Cells were serum-starved and stimulated with IGF-I as described in the legend to Fig. 3. Cell lysates were analyzed for tyrosine phosphorylation of STAT3 (A and B, upper panels), STAT3 protein amount (A and B, middle panels), and SOCS1 protein expression levels (A and B, lower panels). In C, 293T cells were cotransfected with the same amounts of pEFIGF-IR and pcSTAT3 plus either SOCS1 expression vector (2 μg) or the corresponding empty vector together with wild-type JAK1 (500 ng) or JAK2 (500 ng) expression vector as indicated. Cells were serum-starved and stimulated with IGF-I as before. Cell lysates were directly analyzed by Western blotting with anti-phospho-STAT3 antibody to determine STAT3 tyrosine phosphorylation. IB, immunoblotted.

STAT3 tyrosine phosphorylation by either dominant-negative JAK1 or JAK2 at high doses was most likely due to cross-inhibition of both JAKs by either of the dominant-negative mutants. Among the SOCS family proteins, SOCS2 was isolated using yeast two-hybrid screening for interacting proteins of the cytoplasmic domain of IGF-IR (53). Both SOCS1 and SOCS2 were shown to interact with IGF-IR (53). We show here that these negative regulators of JAKs are able to block STAT3 activation by IGF-IR. This further confirms the role of JAKs in IGF-I/IGF-IR-mediated activation of STAT3. Among the SOCS proteins tested, SOCS1 and SOCS3 appear to have greater inhibitory potency. This is consistent with the finding that both SOCS proteins are able to bind to activated JAKs and to inhibit their catalytic activity. It is also in agreement with our observation that the inhibition by SOCS1 can be overcome by overexpression of JAK1 or JAK2. These observations imply that either JAK1 or JAK2 is able to independently mediate the IGF-I/IGF-IR-induced activation of STAT3. Like the dominant-negative JAK mutants, each SOCS protein appears to play an inhibitory role in STAT3 activation, suggesting that these negative regulators of JAKs are able to block STAT3 activation by IGF-IR.

Several possibilities of STAT3 activation by IGF-IR via JAKs exist. First, the activated receptor associates with JAKs, which phosphorylate STAT3. The STAT3-binding site could be generated by activated IGF-IR or JAKs. Second, upon activation by IGF-IR, JAKs could directly phosphorylate STAT3. Third, JAKs could provide STAT3 recruitment sites in the receptor complex, as suggested for JAK2 in response to growth hormone receptor activation (54). Identification of the IGF-IR-interact-
ing site for JAKs and assessment of its effect on IGF-I-induced STAT3 activation should help to answer the questions.

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