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SOCS-1 and SOCS-3 Block Insulin Signaling by Ubiquitin-mediated Degradation of IRS1 and IRS2*

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Liangyou Rui‡§, Minsheng Yuan‡, Daniel Frantz‡, Steven Shoelson‡, and Morris F. White‡¶‖

From the ¶Howard Hughes Medical Institute, ‡Joslin Diabetes Center, Harvard Medical School, Boston, Massachusetts 02215

Inflammation associates with peripheral insulin resistance, which dysregulates nutrient homeostasis and leads to diabetes. Inflammation induces the expression of SOCS proteins. We show that SOCS1 or SOCS3 targeted IRS1 and IRS2, two critical signaling molecules for insulin action, for ubiquitin-mediated degradation. SOCS1 or SOCS3 bound both recombinant and endogenous IRS1 and IRS2 and promoted their ubiquitination and subsequent degradation in multiple cell types. Mutations in the conserved SOCS box of SOCS1 abrogated its interaction with the elongin BC ubiquitin-ligase complex without affecting its binding to IRS1 or IRS2. The SOCS1 mutants also failed to promote the ubiquitination and degradation of either IRS1 or IRS2. Adenoviral-mediated expression of SOCS1 in mouse liver dramatically reduced hepatic IRS1 and IRS2 protein levels and caused glucose intolerance; by contrast, expression of the SOCS1 mutants had no effect. Thus, SOCS-mediated degradation of IRS proteins, presumably via the elongin BC ubiquitin-ligase, might be a general mechanism of inflammation-induced insulin resistance, providing a target for therapy.

Insulin and insulin-like growth factors exert many biological effects through receptor-mediated tyrosine phosphorylation of insulin receptor substrates (IRS[1] proteins), including IRS1, IRS2, IRS3, and IRS4 (1, 2). IRS proteins coordinate multiple signals through the PI 3-kinase → Pkb/Akt and the Grb2/Sos → Ras cascades (1, 2). In mice, IRS1 mediates the effects of insulin and IGF1 on somatic cell growth, whereas IRS2 is essential for nutrient homeostasis (3, 4). Mice lacking IRS1 or IRS2 display peripheral insulin resistance, which is a major determinant of type 2 diabetes (5–10). However, $IRS1^{-/-}$ mice never develop diabetes because of life-long compensatory hyperinsulinemia, whereas disruption of IRS2 causes severe glucose intolerance and diabetes (4). Dysregulation of IRS2 is especially problematic, because it regulates transcription factors in β-cells that are essential for glucose sensing and insulin secretion (1, 11). IRS proteins also integrate heterologous signals that negatively regulate the insulin-signaling cascade. Proinflammatory cytokines or insulin activate the c-Jun N-terminal kinase (JNK), which promotes serine phosphorylation of IRS1 and IRS2 that inhibits coupling to the activated insulin receptor (12). In addition, IRS proteins are decreased in people and rodents with diabetes (13–16). Our recent studies reveal that IRS proteins are ubiquitinated and subsequently degraded by the 26 S proteasome during insulin stimulation or during cellular stress, but the mechanisms that recruit ubiquitin-ligases to the IRS proteins are unknown (17, 18).

Insulin resistance is a common consequence of physiological stress, owing at least in part to the production of proinflammatory cytokines during infection or injury, pregnancy, growth and aging, or chronic obesity (19–22). Many proinflammatory cytokines up-regulate suppressors of cytokine signaling (SOCS) proteins, including eight isoforms that contain an NH_2-terminal SH2 domain and a COOH-terminal SOCS box (23, 24). SOCS proteins bind via their SH2 domains to activated cytokine receptors or their associated Janus kinases as part of a negative feedback loop to attenuate cytokine signaling (24–26). They also bind to the elongin BC-containing E3 ubiquitin-ligase complex via the conserved SOCS box (27–29). SOCS1 was shown recently to promote the ubiquitination and degradation of vav and JAK2 in a SOCS box-dependent fashion (30–32). Several reports suggest that SOCS1, SOCS3, or SOCS6 also inhibit heterologous pathways, including insulin receptor signaling (33–35). Consequently, disruption of SOCS1 in mice increases insulin sensitivity (36). In this report, we showed that SOCS1/3 promoted the ubiquitination and degradation of both IRS1 and IRS2. The elongin BC binding motif in SOCS1 and SOCS3 was required for the ubiquitination and degradation of IRS1 and IRS2, revealing a mechanism to inhibit insulin action and promote glucose intolerance during infection, inflammation, or metabolic stress.

EXPERIMENTAL PROCEDURES

Reagents—Protein A-agarose was purchased from Repligen, and aprotinin and leupeptin were from Sigma. The enhanced chemiluminescence (ECL) detection system was purchased from Amersham Biosciences. FuGENE 6 was purchased from Roche Molecular Biochemicals. Monoclonal anti-hemagglutinin (HA), anti-Myc antibodies, and anti-actin were purchased from Santa Cruz Inc. Polyclonal anti-IRS1 antibodies were raised against the PTB domain (JD#228, used at a dilution of 1:100 for immunoprecipitation) or the full-length rat IRS1 (JD#159, used at a dilution of 1:15,000 for immunoblotting). Polyclonal antibodies against IRS2 were also prepared in rabbits immunized with full-length rat IRS2.

HEK293 Cell Transfection, Immunoprecipitation, and Immunoblotting—Plasmids encoding different proteins were transiently cotransfected into HEK293 cells using FuGENE 6 according to manufacturer's instruction. Twenty-four hours after transfection, the cells were deprived of serum overnight and then treated with insulin (100 nM) for 10

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§ Current address: Dept. of Physiology, University of Michigan Medical School, Ann Arbor, MI 48109.

‖ To whom correspondence should be addressed: Howard Hughes Medical Inst., Joslin Diabetes Center, 1 Joslin Place, Boston, MA 02215. Tel.: 617-732-2578; Fax: 617-732-2593; E-mail: whitemor@joslab.harvard.edu.

[1] The abbreviations used are: IRS, insulin receptor substrate; SOCS, suppressors of cytokine signaling; SH2, Src homology 2; E3, ubiquitin-protein isopeptide ligase; PI, phosphatidylinositol; HA, hemagglutinin; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon.

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min. In parallel experiments, cell extracts were prepared 48 h after transfection without starvation. Methods for preparation of cell extracts, immunoblotting, and immunoprecipitation were described previously (17).

Adenovirus Construction and Injection into Mice—Mutant SOCS1 (ΔS1) lacking the SOCS box (residues 167–212), or with two point mutations ($S^{LC\rightarrow PF}$, L175P, C179F) that disrupt the elongin C binding site were prepared by PCR-based mutagenesis. SOCS1, ΔS1, or $S^{LC\rightarrow PF}$ was tagged with HA at its COOH terminus and ligated into a transfer vector (pshuttle-CMV) at the *Bam*HI and *Eco*RI sites. SOCS1 adenovirus was generated by homologous recombination in bacteria according to the manufacturer's instructions. C57BL/6 male mice (Jackson Laboratory) were maintained on a 12-h light/dark cycle with free access to water and food and handled in accordance with Joslin Diabetes Center Animal Care and Use Committee protocols.

In some experiments, primary hepatocytes were isolated from mice and cultured for 24 h as described previously (14). The cells were infected with control or SOCS1 adenovirus (multiplicity of infection of 40). Forty-eight hours after infection, cell extracts were prepared for immunoprecipitation and immunoblotting as described previously (17).

Mice were infected with control adenovirus or adenovirus containing wild type or mutant SOCS1 via tail vein injection (10^{11} viral particles per animal). Blood glucose and Serum insulin levels were measured 10 or 28 days after infection, as described previously (3). Liver or muscle extracts (2 mg of protein) were prepared for immunoprecipitation and immunoblotting as described previously (3).

RESULTS

SOCS Proteins Associate with IRS1 and IRS2—The interaction of IRS1 and IRS2 with SOCS proteins was examined in HEK293 cells prepared by transient transfection with HA-tagged SOCS1, the insulin receptor, and either IRS1 or IRS2. The effect of insulin to stimulate association of SOCS1 with IRS2 was revealed in anti-HA immunoprecipitates. Before insulin stimulation, IRS2 was detected in SOCS1 immunoprecipitates, and the association increased significantly after 10 min of insulin stimulation (Fig. 1*A*). Similarly, IRS1 was coimmunoprecipitated with SOCS1, and insulin increased 2-fold the association between SOCS1 and IRS1 (Fig. 1*A*). IRS1 and IRS2 were also associated with SOCS2 or SOCS3 in similar coimmunoprecipitation experiments (data not shown).

The association between SOCS1 and IRS1 or IRS2 was also demonstrated in isolated mouse hepatocytes that were infected in tissue culture with an adenovirus containing HA-tagged SOCS1. Two days after infection, SOCS1 was detected in IRS1 or IRS2 immunoprecipitates by immunoblotting with anti-HA (Fig. 1*B*). This association was detected even though the levels of IRS1 and IRS2 were significantly reduced in the hepatocytes expressing SOCS1 (Fig. 1*B*). Moreover, SOCS1 also bound to hepatic IRS1 and IRS2 when it was expressed in mouse liver by adenovirus-mediated gene transfer (Fig. 1*C*). Thus, SOCS proteins associate with IRS1 or IRS2 in various cell backgrounds and might promote degradation of the IRS proteins.

SOCS1 and SOCS3 Promote Degradation of IRS1 and IRS2—The specificity of SOCS-induced degradation of IRS1 and IRS2 was investigated in HEK293 cells expressing the insulin receptor, IRS2, and either SOCS1, SOCS2, or SOCS3. Forty-eight hours after transfection, recombinant IRS2 was easily detected by immunoblotting in the absence of SOCS expression; however, coexpression of either SOCS1 or SOCS3 dramatically reduced IRS2 levels (Fig. 2*A*). By contrast, SOCS2 had no effect on the expression of IRS2, and actin levels were not affected by the expression of SOCS1, SOCS2, or SOCSS3 (Fig. 2*A*). SOCS1 also caused a dose-dependent reduction of IRS1 without affecting the level of actin or the insulin receptor (Fig. 2*B*); similar results were observed upon SOCS3 expression (data not shown). Thus, SOCS1 and SOCS3 might promote the specific degradation of IRS1 and IRS2, whereas SOCS2 has no effect.

The reduction of IRS protein levels by SOCS1 and SOCS3 was validated in other cell systems. SOCS1 or SOCS3 reduced

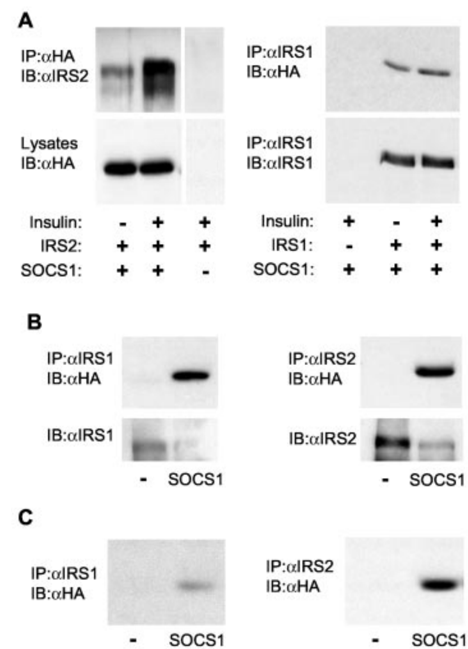

FIG. 1. **SOCS1 binds to both IRS1 and IRS2.** *A*, HA-tagged SOCS1 and insulin receptor were transiently coexpressed in HEK293 cells with either IRS1 or IRS2. Twenty-four hours after transfection, the cells were deprived of serum overnight and then treated with insulin (100 nM) for 10 min. Proteins in lysates were immunoprecipitated (*IP*) and immunoblotted (*IB*) with the indicated antibodies. *B*, primary hepatocytes culture was infected with control or SOCS1 adenovirus (MOI of 40 plaque-forming units per cell). Forty-eight hours after infection, cell extracts were immunoprecipitated (*IP*) with αIRS1 or αIRS2 and immunoblotted (*IB*) with αHA, αIRS1, or αIRS2 as indicated. *C*, C57BL/6 male mice (6 weeks old) were infected with either control or SOCS1 adenovirus via tail vein injection (10^{11} viral particles per animal). Ten days after infection, liver extracts (2 mg proteins) were immunoprecipitated (*IP*) with αIRS1 or αIRS2 and immunoblotted (*IB*) with αHA to detect SOCS1.

endogenous IRS1 and IRS2 levels in human MCF7 breast cancer cells; activation of the PI 3-kinase during insulin stimulation was also reduced (data not shown). Proinflammatory cytokines including IL-6, TNFα, IL-1β, or IFNγ promote the expression of SOCS1 and SOCS3 in various cells and tissues, including 3T3-L1 adipocytes (24, 37–43). Consequently, treatment of 3T3-L1 adipocytes with TNFα or IFNγ reduced the levels of IRS1 and IRS2 without reducing the level of the p85 regulatory subunit of the PI 3-kinase (p85) (Fig. 2*C*). Thus, the loss of IRS1 and IRS2 protein in various cellular backgrounds correlates with the expression of SOCS1 or/and SOCS3.

SOCS1 Expression in Liver Promotes Degradation of IRS1 and IRS2—Male C57BL/6 mice were infected by tail vein injection with control or SOCS1 adenovirus. Ten days after infection, recombinant SOCS1 was easily detected in liver lysates (Fig. 3*A*). Consistent with the cell-based experiments described above, immunoblotting revealed that hepatic IRS1 and IRS2 proteins were dramatically reduced during adenoviral-mediated expression of SOCS1 (Fig. 3*A*). By contrast, insulin receptor and p85 were not decreased (Fig. 3*B*). Moreover, 28 days after infection when SOCS1 was no longer detected in liver, IRS1 and IRS2 levels returned to normal (Fig. 3*A*). Since the liver is the major, if not the exclusive, site of gene expression upon injection of adenovirus vectors (44), SOCS1 was not detected in muscle extracts and IRS1 and IRS2 levels were not reduced in muscle (Fig. 3*C*).

The Role of Elongin BC in IRS Protein Degradation—Sequence analysis reveals an elongin C binding motif in the SOCS box of SOCS1 and SOCS3 (45). Elongin C forms a stable complex with elongin B, which assembles an E3 ubiquitin-

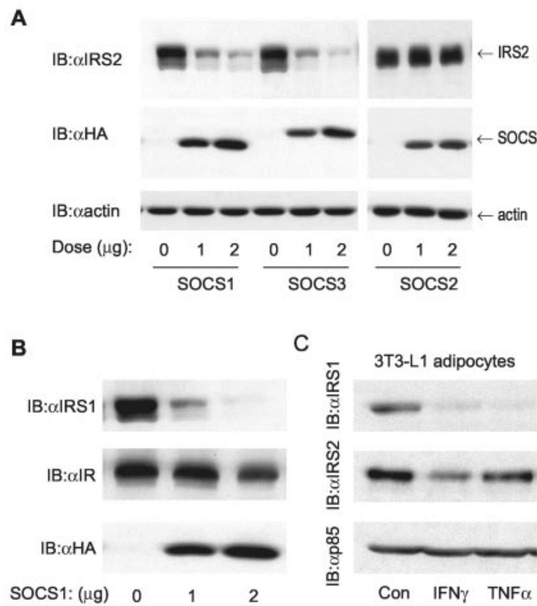

FIG. 2. **SOCS1 and SOCS3 promote specifically degradation of IRS1 and IRS2.** IRS2 (*A*) or IRS1 (*B*) were coexpressed transiently in HEK293 cells with insulin receptor and HA-tagged SOCS1. Forty-eight hours after transfection, cell extracts were immunoblotted (*IB*) individually with antibodies against IRS1, IRS2, insulin receptor (*IR*), HA, or actin as indicated. *C*, 3T3-L1 adipocytes were deprived of serum overnight and treated for 8 h with murine IFNγ (10 ng/ml) or TNFα (30 ng/ml). Proteins (40 μg) in cell extracts were immunoblotted (*IB*) with αIRS1, αIRS2, or αp85 antibodies.

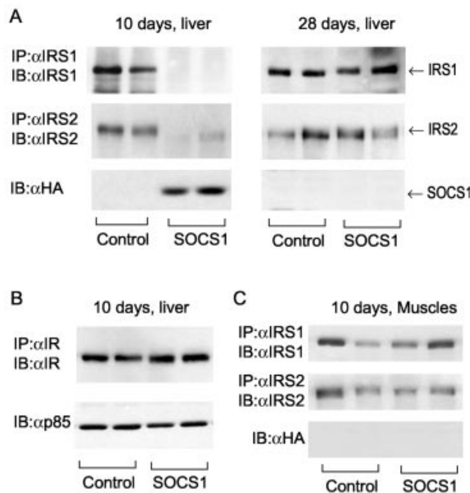

FIG. 3. **SOCS1 promotes degradation of IRS1 and IRS2 in mouse liver.** C57BL/6 male mice (6 weeks old) were infected for 10 or 28 days with either control or SOCS1 adenoviruses via tail vein injection (10^{11} viral particles per animal). Liver (2 mg of protein) or muscle (1 mg of protein) extracts were immunoprecipitated (*IP*) and immunoblotted (*IB*) with αIRS1, αIRS2, or anti-insulin receptor antibodies as indicated. Proteins in liver (200 μg) or muscle (100 μg) extracts were immunoblotted with αp85 or αHA as indicated. Each lane represents different mouse.

ligase complex (28). Thus, elongin BC ubiquitin-ligase might mediate ubiquitination and degradation of IRS1 and IRS2 by SOCS1 or SOCS3. The SOCS box (residues 167–212) was deleted (ΔS1), and ΔS1 was coexpressed with IRS2 in HEK293 cells. SOCS1 and ΔS1 were expressed to approximately equal levels (Fig. 4*A*). As expected, IRS2 was degraded in cells expressing SOCS1, but not degraded in cells expressing ΔS1 (Fig. 4*A*). To validate a specific role for elongin BC binding site, two conserved residues essential for elongin BC binding, Leu^{175} and Cys^{179}, were replaced with Pro and Phe ($S^{LC\rightarrow PF}$) (27).

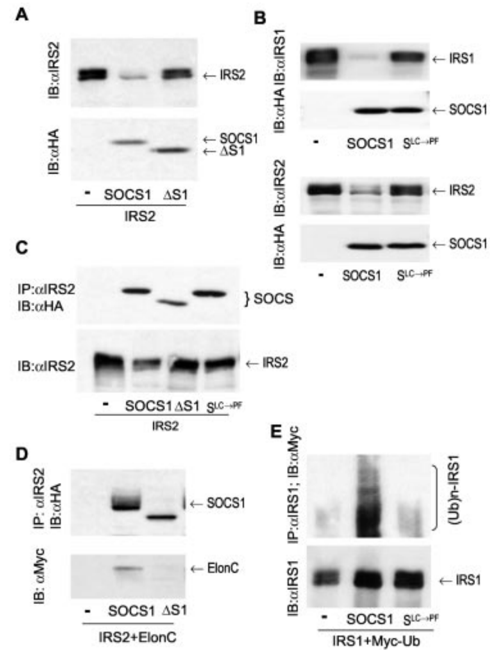

FIG. 4. **SOCS1 targets IRS1 and IRS2 for ubiquitination and degradation via an elongin BC ubiquitin-ligase.** *A* and *B*, IRS2 or IRS1 was coexpressed in HEK293 cells with HA-tagged SOCS1, ΔS1, or $S^{LC\rightarrow PF}$ as indicated. Cell extracts (60 μg of protein) were immunoblotted (*IB*) with αIRS2, αIRS1, or αHA. *C*, extracts from cells coexpressing IRS2 with SOCS1, ΔS1 or $S^{LC\rightarrow PF}$ were immunoprecipitated (*IP*) with αIRS2 and immunoblotted (*IB*) with αHA or αIRS2. *D*, IRS2 was coexpressed with Myc-tagged elongin C in the presence or absence of SOCS1 or ΔS1 in HEK293 cells. Cell lysates were immunoprecipitated (*IP*) with αIRS2 and immunoblotted (*IB*) with αHA or αMyc to detect SOCS1 or elongin C, respectively. *E*, IRS1- and Myc-tagged ubiquitin were coexpressed in HEK293 cells with either SOCS1 or $S^{LC\rightarrow PF}$. A similar amount of IRS1 proteins in cell extracts was immunoprecipitated (*IP*) with αIRS1 and immunoblotted (*IB*) with αMyc. The same blot was reprobed with αIRS1.

SOCS1 or $S^{LC\rightarrow PF}$ was expressed to equal levels in HEK293 cells together with the insulin receptor and either IRS1 or IRS2. $S^{LC\rightarrow PF}$ weakly degraded IRS1 or IRS2 by comparison to SOCS1 (Fig. 4*B*). Importantly, the mutations in the SOCS box did not prevent binding of SOCS1 to IRS1 or IRS2 (Fig. 4*C*).

The inability of ΔS1 and $S^{LC\rightarrow PF}$ to promote degradation of IRS1 and IRS2 might be caused by the absence of an elongin BC-based E3 ubiquitin-ligase. To test this possibility, IRS2 immunoprecipitates were prepared from HEK293 cell extracts expressing recombinant IRS2, Myc-tagged elongin C, and either SOCS1 or ΔS1. IRS2 immunoprecipitates from these transfected cells contained SOCS1 and ΔS1. However, coexpression of SOCS1, but not ΔS1, promoted the association of elongin C with IRS2 (Fig. 4*D*). These results support the hypothesis that SOCS1 has distinct binding sites for the ubiquitin-ligase complex and for IRS proteins and recruits elongin BC ubiquitin-ligase onto IRS1 or IRS2.

Since the elongin BC binding motif in SOCS1 associates with an E3 ubiquitin-ligase, we investigated whether IRS1 was ubiquitinated in HEK293 cells transiently cotransfected with IRS1, Myc-tagged ubiquitin, and either SOCS1 or $S^{LC\rightarrow PF}$. A similar amount of immunopurified recombinant IRS1 proteins were immunoblotted with anti-Myc to measure the levels of IRS1 ubiquitination. SOCS1, but not $S^{LC\rightarrow PF}$, promoted ubiquitination of IRS1 (Fig. 4*E*). SOCS1, but not $S^{LC\rightarrow PF}$, also promoted ubiquitination of IRS2 (data not shown). Consistent with the degradation of IRS proteins by the ubiquitin/proteasome system, proteasome inhibitors (lactacystin or MG132) block insulin-induced reduction of both IRS1 and IRS2 (17). Insulin promotes expression of SOCS3 (33).

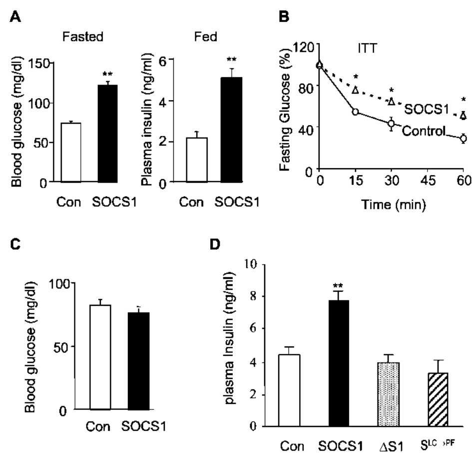

FIG. 5. **SOCS1 induces insulin resistance by promoting elongin BC ligase-mediated degradation of hepatic IRS1 and IRS2.** *A–C*, C57BL/6 male mice (6 weeks old) were infected with either control (*Con*) or SOCS1 adenovirus via tail vein injection (10^{11} viral particles per animal). *A*, fasting (15 h) blood glucose and fed plasma insulin levels 10 days after infection ($n = 6$ in each group). *B*, insulin tolerance tests (*ITT*) (0.7 IU/kg) were performed on mice ($n = 6$ in each group) 10 days after infection. The results were expressed as percentage of initial blood glucose concentration. *C*, fasting blood glucose 28 days after infection ($n = 6$ in each group). *D*, fed plasma insulin levels in mice (9 weeks old, $n = 7$ in each group) infected for 6 days with adenovirus encoding SOCS1, ΔS1, or $S^{LC\rightarrow PF}$. Values are the mean ± S.E. *, $p < 0.005$ *versus* control; **, $p < 0.001$.

The Role of Hepatic SOCS1 in Glucose Homeostasis—Previous reports suggest that the binding of SOCS proteins to the activated insulin receptor inhibits signal transduction (33, 34); however, our data suggest that SOCS-mediated degradation of IRS1 and IRS2 might be the critical step for insulin resistance. To investigate these possibilities, male C57BL/6 mice were infected with control or SOCS1 adenoviruses via a tail vein injection, and blood glucose and plasma insulin levels were monitored and insulin tolerances tests were performed to estimate insulin sensitivity. Ten days after viral infection, SOCS1 adenovirus-infected mice displayed fasting hyperglycemia, which was consistent with the loss of hepatic IRS1 and IRS2 (Fig. 5*A*). By contrast during random feeding, blood glucose levels were indistinguishable in SOCS1 mice (140 ± 7 mg/dl) and control mice (150 ± 6 mg/dl), because of significant compensatory hyperinsulinemia (Fig. 5*A*). Consistent with these results, SOCS1 mice were relatively resistant to intraperitoneal insulin injections compared with control virus infection (Fig. 5*B*). When SOCS1 expression subsided 28 days after infection, hepatic IRS1 and IRS2 returned to normal levels (Fig. 3*A*), and the fasting blood glucose and insulin returned to the normal range (Fig. 5*C*).

To verify that SOCS1-induced glucose intolerance was caused by ubiquitin-mediated degradation of hepatic IRS proteins, SOCS1, ΔS1, or $S^{LC\rightarrow PF}$ were expressed in mouse liver by adenovirus-mediated gene delivery. As expected, only wild-type SOCS1 expression caused compensatory hyperinsulinemia, whereas insulin levels were normal in mice expressing ΔS1 or $S^{LC\rightarrow PF}$ (Fig. 5*D*). Taken together, these results show that SOCS1 caused insulin resistance and glucose intolerance because of degradation of hepatic IRS1 and IRS2, rather than by disrupting the assembly of signaling complexes around the insulin receptor or the IRS proteins.

DISCUSSION

Insulin resistance is not only the driving force for type 2 diabetes, but also associated with multiple other diseases, including hypertension, obesity, infertility, and neurodegeneration (5–10). Proinflammatory cytokines are believed to play a critical role in insulin resistance during obesity and stress. In this study, we provided evidence showing that SOCS-promoted degradation of IRS proteins might mediate cytokine-induced insulin resistance. Previous reports suggest that SOCS1, SOCS3, and SOCS6 block assembly of signaling complexes that mediate insulin action by binding to tyrosine-phosphorylated sites in the insulin receptor or in the insulin receptor substrates, IRS1 or IRS2 (33, 34, 36). However, our work reveals that binding is not sufficient, as SOCS-mediated ubiquitination of IRS1 or IRS2 is a required step for inhibition of insulin action in various cell lines and mouse liver. This mechanism is consistent with the function of SOCS1 and SOCS3 as adapter molecules linking tyrosine-phosphorylated proteins to an elongin BC-based E3 ubiquitin-ligase (27). SOCS1 mutants lacking binding site for elongin BC E3 bind IRS1 and IRS2, while failing to mediate their degradation or causing glucose intolerance in mice. SOCS1/3 are likely to promote insulin resistance by targeting IRS1 and IRS2 for degradation rather than by inhibiting insulin receptor function or by competing with other SH2 proteins for binding to the IRS proteins.

During mechanical or thermal injury, or microbial infection, ordinary metabolic regulation by insulin is suspended to mobilize glucose and other nutrients for defense and repair. Proinflammatory cytokines produced under these conditions, including TNFα, interleukin-1, and IFNγ, inhibit the insulin-signaling cascade and promote glucose intolerance (19). TNFα, IFNγ, or IL-6 up-regulate SOCS proteins in various cell and tissues during the inflammatory response, including liver and pancreatic β-cells. SOCS3 is elevated for up to 10 days after thermal injury, which might contribute to the life-threatening catabolic state (22). SOCS3 is elevated in insulin target tissues of obese mice, which correlates with reduced levels of IRS1 and IRS2 and insulin resistance (13, 14, 33). The induction of SOCS proteins might be a general mechanism to attenuate insulin signaling when nutrients are required for repair or defense. Moreover, the high energy demands of pregnancy or adolescence might be ensured in part by SOCS-mediated insulin resistance induced by prolactin or growth hormone.

Our experiments with mice following adenoviral-mediated expression of SOCS1 in liver confirm that hepatic SOCS1 degrades IRS1 and IRS2, resulting in glucose intolerance. Diabetes does not emerge during the experimental interval, because glucose tolerance is managed by compensatory hyperinsulinemia. If SOCS proteins are also up-regulated in pancreatic islets, compensatory insulin secretion might be compromised, because the IRS2 branch of the insulin/IGF signaling pathway is essential for β-cell function (11). Moreover, localized inflammation at pancreatic islets might promote SOCS-mediated degradation of IRS2 in β-cells, contributing to type 1 diabetes.

In summary, we showed that SOCS1 and SOCS3 have distinct binding sites for IRS1 or IRS2 and for elongin BC ubiquitin-ligase. SOCS1/3 promote ubiquitination and degradation of IRS1 and IRS2 in both cultured cells and animal tissues, contributing to insulin resistance. The binding of elongin BC ubiquitin-ligase is required for SOCS1-promoted degradation of IRS proteins and glucose intolerance. Drugs that induce expression of IRS proteins or protect them from degradation might have value for the treatment of the insulin resistance syndromes and diabetes.

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