The SOCS Box of SOCS-1 Accelerates Ubiquitin-dependent Proteolysis of TEL-JAK2

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Shintaro Kamizono‡‡, Toshikatsu Hanada‡, Hideo Yasukawa‡, Shigeru Minoguchi‡, Reiko Kato‡, Mayu Minoguchi‡, Kimihiko Hattori‡, Shigetsugu Hatakeyama‡, Masayoshi Yada*, Sumiyo Morita†, Toshio Kitamura†, Hirohisa Kato†, Kei-ichi Nakayama†, and Akihiko Yoshimura‡‡

From the ‡Institute of Life Science, Kurume University, Aikawa-machi 2432-3, Kurume 820-0861, the Department of Pediatrics, Faculty of Medicine, Kurume University, Kurume 830, the Departments of Molecular and Cellular Biology and Immunology, Medical Institute of Bioregulation, Kyushu University, Fukuoka 812-8582, and the Department of Hematopoietic Factors, Institute of Medical Science, University of Tokyo, 108-8639, Japan

Fusion of the TEL gene on 12p13 to the JAK2 tyrosine kinase gene on 9p24 has been found in human leukemia. TEL-mediated oligomerization of JAK2 results in constitutive activation of the tyrosine kinase (JH1) domain and confers cytokine-independent proliferation on interleukin-3-dependent Ba/F3 cells. Forced expression of the JAK inhibitor gene SOCS1/JAB/SSI-1 induced apoptosis of TEL-JAK2-transformed Ba/F3 cells. This suppression of TEL-JAK2 activity was dependent on SOCS box-mediated proteasomal degradation of TEL-JAK2 rather than on kinase inhibition. Degradation of JAK2 depended on its phosphorylation and its high affinity binding with SOCS1 through the kinase inhibitory region and the SH2 domain. It has been demonstrated that von Hippel-Lindau disease (VHL) tumor-suppressor gene product possesses the SOCS box that forms a complex with Elongin B and C and Cullin-2, and it functions as a ubiquitin ligase. The SOCS box of SOCS1/JAB has also been shown to interact with Elongins; however, ubiquitin ligase activity has not been demonstrated. We found that the SOCS box interacted with Cullin-2 and promoted ubiquitination of TEL-JAK2. Furthermore, overexpression of dominant negative Cullin-2 suppressed SOCS1-dependent TEL-JAK2 degradation. Our study demonstrates the substrate-specific E3 ubiquitin-ligase-like activity of SOCS1 for activated JAK2 and may provide a novel strategy for the suppression of oncogenic tyrosine kinases.

Cytokines induce the activation of the JAK family tyrosine kinases (JAKs) and the subsequent recruitment of various signaling proteins to the receptor complex, including the STAT family of transcription factors. Constitutive activation of the JAK/STAT pathway has been found in many leukemic cell lines, including cells transformed with Bcr-Abl (1, 2), as well as in human T-cell lymphotrophic virus-1-transformed T cells (3, 4). A constitutively activated form of STAT5 conferred factor-independent growth on Ba/F3 cells (5), and that of STAT3 has also been shown to function as an oncogene (6). Moreover, a constitutively activated JAK kinase generated by chromosome translocation between the TEL gene on 12p13 and the JAK2 gene on 9p24 has been shown to be associated with human leukemia (7, 8). TEL, a subset of the ETS family of transcription factors, contains a conserved oligomerization domain, known as the PNT domain, in the N-terminal region. Like other TEL-tyrosine kinase fusion proteins such as the TEL-PDGF receptor β chain and TEL-Abl, the JAK2 tyrosine kinase domain is constitutively activated by oligomerization mediated by the PNT domain. Stable expression of TEL-JAK2 confers factor-independent growth on IL-3-dependent Ba/F3 cells and induces myeloproliferative and T-cell lymphoproliferative diseases in mice (9).

The JAK/STAT pathway is regulated by several mechanisms, including dephosphorylation by protein phosphatases and degradation by the ubiquitin/proteasome system (see review; Yasukawa et al. (10)). The CIS family (also referred to as the SOCS or SSI family) has been shown to play an important role in regulating cytokine signal transduction. CIS1, the first member of this family to be cloned, suppresses STAT5 activation by binding to cytokine receptors (11, 12). The second family member found, JAB/SOCS1/SSI-1, directly binds to the JAK2 kinase (JH1) domain, thereby inhibiting tyrosine kinase activity (13–15). Mutational analysis and biochemical characterization revealed a novel type of inhibition of JAK2 tyrosine kinase activity through the two independent binding sites of SOCS1/JAB: the N-terminal kinase inhibitory region binds to the catalytic groove of JH1, and the SH2 domain binds to the phosphorylated tyrosine residue Tyr-1007 in the activation loop (16, 17). Gene disruption studies have suggested that one of the major physiological functions of SOCS1 is the negative regulation of the IFNγ/STAT1 pathway (18, 19).

Six additional CIS/SOCS/SSI family members were cloned from a database search (20–22). In this family, the SH2 domain and the C-terminal region of about 40 amino acids, referred to as the SOCS box, are conserved. The database search also revealed that a similar SOCS box is present in several proteins containing ankyrin-like repeats, Ras-like GTPases, or WD40 domains (20, 22). The SOCS box has been implicated in protein stability or degradation of associated molecules, because it was found to interact with the Elongin B and Elongin...
C (Elongin B, C) complex, which may recruit Cullin-2 (Cul-2), Rbx1, and the E2 ubiquitin-conjugating enzyme (23–25). Therefore, CIS family members are hypothesized to function as E3-like ubiquitin-ligase complexes against target molecules from an analogy with the von Hippel-Lindau (VHL) tumor-suppressor gene product. However, no evidence in support of this hypothesis has yet been reported. Kamura et al. (23) reported that the protein levels of full-length Jak2 were not affected by coexpression of SOCS1, and, rather, that coexpression of Elongin B, C stabilized the SOCS1 protein. Furthermore, it has been shown that the SOCS box is not essential for the inhibition of cytokine-induced Jak/Stat activation by SOCS1 (16, 17, 23, 26). Therefore, the role of the SOCS box of SOCS1 still remains to be elucidated.

To suppress the oncogenic potential of activated tyrosine kinases, we introduced the SOCS1 gene into Ba/F3 cells transformed with TEL-JAK2 using a retrovirus system. Overexpression of SOCS1 could efficiently suppress the transforming potential of TEL-JAK2. However, simple inhibition of kinase activity by SOCS1 could not explain the suppression of TEL-JAK2. We found that SOCS1 promoted ubiquitin-proteasome-dependent degradation of TEL-JAK2 and full-length JAK2, and that this process required the C-terminal SOCS box of SOCS1 as well as the phosphorylation of Jak2.

### EXPERIMENTAL PROCEDURES

**Cells and Transfection**—Murine IL-3-dependent Ba/F3 cells were maintained in RPMI medium supplemented with 10% fetal calf serum and 10% conditioned medium from WEHI-3B cells as a source of IL-3. Ba/F3 cells were transformed with pCDNA3-TEL-JAK2 or pCDNA3-Bcr-Abl as described previously (11). After selection with G418 (1 mg/ml), cells that could grow without IL-3 were subsequently selected. Transient transfection and the luciferase assay in 293 cells have been described previously (20).

**cDNA Construction**—Deletion, substitution, and chimeric mutants were generated by standard PCR methods as described previously (16, 27). Some of the mutants and wild-type SOCS1 were subcloned into a pMX-IRES-EGFP vector (28). For swapping of the SOCS box, the SOCS boxes of SOCS1 (codoen 167–212), CIS3/SOCS3 (codoen 180–225), and CIS1 (codoen 213–257) were interchanged by introducing an Sp1 site at the joint. All constructs contained an N-terminal Myc- or FLAG-tag (13). For TEL-JAK2 fusion, the human TEL (codoen 1–162) part was obtained by PCR and fused to the mouse JAK2 JH1 domain (13, 14). The TEL-JAK2 fusion, the human TEL (codoen 1–162) part was obtained by PCR and fused to the mouse JAK2 JH1 domain (13, 14).

**Retrovirus Production and Infection**—Retroviruses were produced by transient transfection of the PLAT-E packaging cell line with cDNAs in pMX-IRES-EGFP (28). Forty-eight hours after transfection, the culture supernatant was harvested and stored at −80 °C. BF/TEL-JAK or BF/Bcr-Abl cells (2 × 10⁵ cells) were infected with appropriately diluted PLAT-E supernatants containing 10 μg/ml Polybrene for 24 h. After being washed, cells were further cultured in an RPMI medium for an additional 24 or 48 h. Then, aliquots of cells (1 × 10⁶) were analyzed using a Coulter EPICS-XL flow cytometer. All experiments were performed in the absence of IL-3.

**In Vitro Kinase Assay**—An in vitro kinase assay for TEL-JAK2 was performed as described previously (16). Briefly, FLAG-tagged TEL-

**Fig. 1.** Suppression of TEL-JAK2-dependent growth of Ba/F3 cells by SOCS1. A, BF/TEL-JAK cells were infected with retrovirus carrying IRES-EGFP alone (Control) or wild-type SOCS1. The numbers of EGFP-positive cells were scored by flow cytometry on the indicated day after infection. Cells were cultured in the absence of IL-3. B and C, BF/TEL-JAK cells were infected with a control virus (open square and lane 1) or a retrovirus carrying wild-type SOCS1 (WT; closed circle and lane 2), or a DC40 mutant SOCS1 (DC40) (closed square and lane 3). BF/Bcr-Abl cells were also infected with a retrovirus carrying WT-SOCS1 (open circle). The mean and standard error shown are from three independent experiments. C, a DNA fragmentation assay was performed 24 h after infection. Infection efficiency (I.E.), which is the percentage of EGFP-positive cells, is shown at the bottom. M, DNA size marker.

**Fig. 2.** Expression levels of TEL-JAK2 after wild-type and mutant SOCS1 infection. BF/TEL-JAK or BF/Bcr-Abl cells were infected with retrovirus carrying the indicated cDNAs. One day after infection, infection efficiency (I.E.) was determined as the EGFP-positive fraction. Then, the cells were lysed and immunoblotted with the indicated antibodies. The phosphorylated forms of TEL-JAK2 and Bcr-Abl detected with anti-PY (αPY) antibody were determined from their molecular size. The intensity of the bands was quantified by a densitometer and normalized with that of control virus-infected cells.
Fig. 3. Effect of WT and dC40-SOCS1 on TEL-JAK2 stability in 293 cells and in vitro kinase activity. 293 cells were transfected with pCDNA3 carrying TEL-JAK2 (0.1 μg), pCDNA3 carrying Myc-tagged WT-SOCS1 (WT) or dC40-SOCS1 (dC40) (0.001, 0.01, 0.1, and 1.0 μg), β-galactosidase (0.1 μg), and the APRE reporter gene (0.5 μg) that can monitor STAT3 and STAT5 activity. The cell lysate (30 μg of protein/lane) was prepared and subjected to immunoblotting (A) with the indicated antibodies and a luciferase assay (B). The values of β-galactosidase activity as an indicator of transfection efficiency are listed in A. The membrane was reprobed with anti-STAT5 (αSTAT5) to show equal loading of the samples, and the intensity of the bands was quantified by a densitometer. The relative ratio (TJ/S ratio) of the band intensity of TEL-JAK2 versus that of STAT5 is shown in A. In C, FLAG-tagged TEL-JAK2 expressed in 293 cells was purified by anti-FLAG antibody-conjugated protein G-Sepharose. After incubation with 293 cell lysates containing WT- or dC40-SOCS1 for 1 h at 4 °C, the beads were reacted with GST-YY as a substrate in the presence of 50 μM ATP for 5 min. The reaction mixture was separated by SDS-polyacrylamide gel electrophoresis and immunoblotted with αPY and αMyc antibodies. GST-YY was stained with Coomassie Brilliant Blue (CBB).

JAK2 expressed in 293 cells (3.0 μg/transfection) grown in 10-cm dishes was immunoprecipitated with anti-FLAG antibody in 60 μl (50% v/v) of protein G-Sepharose. Then the resin was incubated with 1 ml of cell extracts from 293 cells transiently expressing wild-type SOCS1 (WT) or a deletion mutant lacking 40 amino acids at the C terminus (dC40) at 4 °C for 1 h. After being washed twice with kinase reaction buffer (50 mM Hepes-buffer, pH 7.5, 50 mM NaCl, 5 mM MgCl2, 5 mM MnCl2, 10 mM dithiothreitol, and 10 μM Na3VO4), the beads were resuspended in 20 μl of kinase reaction buffer containing the substrate polypeptide, GST-EPOR cytoplasmic domain (GST-YY) (16) (0.1 mg/ml), and ATP (50 μM) and incubated at 30 °C for 5 min. Kinase activity was analyzed by immunoblotting of GST-YY with anti-phosphotyrosine (4G10).

**Immunoprecipitation and Western Blot Analysis—**Immunoprecipitation and immunoblotting were performed as described previously (11). Anti-JAK2 JH1 (αJAK2) rabbit polyclonal antibody, anti-Myc (αMyc) monoclonal and polyclonal antibodies, and anti-phosphotyrosine (αPY, αG10) antibodies have been described previously (16). For pulse-chase experiments, 293 cells (1 × 106) grown in 10-cm dishes were transfected with TEL-JAK2 (2.0 μg of plasmid) and WT or dC40 cDNA (0.02 μg). After 18 h, the cells were pulse-labeled with Tran35S-label (ICN) at a concentration of 150 μCi/ml for 15 min and then scraped. After being divided into four parts, cells were replated into 3.5-cm dishes. Following the indicated chase periods, cells were lysed and immunoprecipitated with anti-JAK2 antibody followed by protein A-Sepharose, separated on SDS-polyacrylamide gel electrophoresis, exposed, and quantified by a BAS-2000 imaging system (Fuji). To see the effect of proteasome inhibitors, 293 cells transfected with TEL-JAK2 and WT-SOCS1 were treated with lactacystin or MG132 (25 μM each) for 30 min before labeling. The drugs were maintained throughout the pulse-chase period. For the cycloheximide treatment experiment, 293 cells (1 × 106) were transfected with 2.0 μg of TEL-JAK2 plus WT or mutant SOCS1 (0.02 μg). Eighteen hours after transfection, cells were trypanized and divided into four parts. After a 5-h incubation period, cells were attached to the dishes and then treated with 50 μg/ml cycloheximide for the indicated periods. The cell extracts were prepared and immunoblotted with anti-JAK2, anti-STAT5, and anti-Myc antibodies as described (16). Band intensity was quantified by a densitometer as described (16).

**RESULTS**

**SOCS Box-dependent Suppression of TEL-JAK2 Transforming Activity by SOCS1**—To determine whether the JAK inhibitor SOCS1 can suppress oncogenic tyrosine kinases, SOCS1 cDNA was introduced into Ba/F3 cells transformed with either TEL-JAK2 (BF/TEL-JAK) or p210 Bcr-Abl (BF/Bcr-Abl) together with enhanced green fluorescence protein (EGFP) using the bicistronic retrovirus vector pMX-IRES-EGFP (28). Because the infected cells expressed both EGFP and Myc-tagged SOCS1, the percentage of infected cells was determined as the EGFP-positive rate by flow cytometry. The same virus was shown to induce apoptosis of parental Ba/F3 cells in the presence of IL-3 (28). As shown in Fig. 1 (A and B), the population of wild-type SOCS1 (WT)-infected BF/TEL-JAK cells decreased markedly, suggesting that WT-infected cells disappeared, while the infected cells continued to grow without apoptotic cell death. Indeed, WT-infected BF/TEL-JAK cells underwent apoptosis characterized by DNA fragmentation (Fig. 1C, lane 2). Similar effects were observed even in the presence of IL-3 (data not shown). WT did not affect the growth of BF/Bcr-Abl cells (Fig. 1B), which is consistent with the observation that SOCS1 did not inhibit Bcr-Abl tyrosine kinase.
activity (data not shown). Thus, the inhibitory effect of SOCS1 was shown to be specific to JAKs.

We have shown that the N-terminal kinase inhibitory region and the SH2 domain, but not the C-terminal SOCS box, are essential for SOCS1 to inhibit JAK kinase activity in vitro and in vivo (16). However, unexpectedly, the dC40 mutant lacking the entire SOCS box could not suppress the growth of BF/TEL-JAK2 cells (Fig. 1, B and C). To clarify the reason for this discrepancy, we examined STAT5 tyrosine phosphorylation in BF/TEL-JAK2 cells after infection (Fig. 2; infection efficiency is listed as I.E.). Infection with WT and dC40, but not with a control virus, resulted in a decrease in the tyrosine phosphorylation of STAT5 (Fig. 2, aPY-STAT5). Reduction of STAT5 phosphorylation by WT was more profound than that by dC40. Tyrosine phosphorylation of TEL-JAK2 was partially reduced by dC40, suggesting that dC40 could reduce TEL-JAK2 kinase activity but was not sufficient to induce apoptosis of BF/TEL-JAK2 cells at the expression levels obtained by the retrovirus system. It should be noted that the infection efficiency of the WT virus to BF/TEL-JAK2 judged by flow cytometry was less than 55%, whereas those of dC40 and control viruses were more than 75%, even though virus titers were similar when assayed with NIH-3T3 cells. This is presumably because WT virus-infected cells die rapidly after infection. Thus, the infection efficiency of the WT virus will be underestimated.

To confirm a similar kinase inhibitory activity of WT and dC40, we performed an in vitro kinase assay using the recombinant protein of the GST-tagged erythropoietin receptor (EPOR) cytoplasmic domain as substrate (16). Consistently, with the previous study using GST-JH1 as a constitutively activated tyrosine kinase domain, our results indicate that although the SOCS box of SOCS1 is not sufficient to suppress the oncogenic potential of TEL-JAK2 and that the C-terminal SOCS box is necessary for complete suppression of the oncogenic potential of TEL-JAK2 by reducing the TEL-JAK2 protein level.

SOCS1 Promotes Proteasome-dependent Degradation of TEL-JAK2—We clarified the mechanism of reduction of the protein level of TEL-JAK2 by coexpression of SOCS1 using a transient expression system in 293 cells. As shown in Fig. 3A, WT, but not dC40-SOCS1, also reduced the level of TEL-JAK2 in a dose-dependent manner in 293 cells (Fig. 3A, aJAK2). Thus, SOCS1 reduced the TEL-JAK2 protein level SOCS-box-dependently not only in Ba/F3 cells but also in 293 cells. Consequently, WT suppressed the TEL-JAK2-mediated STAT activation 50 times more efficiently than dC40 (Fig. 3B). To confirm a similar kinase inhibitory activity of WT and dC40, we performed an in vitro kinase assay using the recombinant protein of the GST-tagged erythropoietin receptor (ERPO) cytoplasmic domain as substrate (16). Consistently, with the previous study using GST-JH1 as a constitutively activated kinase, WT and dC40 could similarly suppress the in vitro kinase activity of TEL-JAK2 (Fig. 3C, lanes 3 and 4). These results indicate that, although the SOCS box of SOCS1 is not necessary for kinase inhibition, the inhibitory effect of WT-SOCS1 was strongly enhanced by inducing degradation of TEL-JAK2 (Fig. 3, A and B).

The half-life of TEL-JAK2 was examined in a metabolic pulse labeling and chase experiment (Fig. 4A). The half-life of TEL-JAK2 was over 60 min, but coexpression of WT accelerated the decay of TEL-JAK2, reducing its half-life to less than 30 min. dC40 did not affect the half-life of TEL-JAK2. Accelerated degradation of TEL-JAK2 in the presence of WT, but not dC40, was also observed after cells were treated with a protein synthesis inhibitor, cycloheximide (Fig. 4B). Normalized levels of TEL-JAK2 are shown as the TJ/S ratio against the levels of JAK2 and STAT5, which is a very stable protein (Fig. 4B).

As shown in Fig. 4 (A and C), the rapid degradation of TEL-JAK2 by coexpression of SOCS1 was significantly delayed by treatment of the cells with two proteasome inhibitors, lactocystin and MG132. This is presumably because WT infected cells die rapidly after infection. Thus, the infection efficiency of the WT virus will be underestimated. More drastically, we noticed that the protein levels of TEL-JAK2 decreased in WT-infected cells but not in dC40-infected cells (Fig. 2, aJAK2). WT-SOCS1 did not affect the protein level of Bcr-Abl (Fig. 2, aAbl). We found a 70–80% decrease of TEL-JAK2 and PY-STAT5 levels in WT virus-infected cells, whereas the infection efficiency was only 50%. This is probably because of the underestimation of the infection efficiency of WT-infected cells. These data suggest that kinase inhibition by SOCS1 is not sufficient to suppress the oncogenic potential of TEL-JAK2 and that the C-terminal SOCS box is necessary for complete suppression of the oncogenic potential of TEL-JAK2 by reducing the TEL-JAK2 protein level.

The half-life of TEL-JAK2 was examined in a metabolic pulse labeling and chase experiment (Fig. 4A). The half-life of TEL-JAK2 was over 60 min, but coexpression of WT accelerated the decay of TEL-JAK2, reducing its half-life to less than 30 min. dC40 did not affect the half-life of TEL-JAK2. Accelerated degradation of TEL-JAK2 in the presence of WT, but not dC40, was also observed after cells were treated with a protein synthesis inhibitor, cycloheximide (Fig. 4B). Normalized levels of TEL-JAK2 are shown as the TJ/S ratio against the levels of JAK2 and STAT5, which is a very stable protein (Fig. 4B).
tacystin and MG132. These data suggest that SOCS1 promotes proteasome-dependent degradation of TEL-JAK2.

**Phosphorylation of the JH1 Domain Is Necessary for SOCS1-mediated Degradation of JAK2**—Previously, Kamura et al. (20) reported that SOCS1 overexpression did not affect wild-type JAK2 protein stability. We tried to resolve this discrepancy.
between TEL-JAK2 and full-length JAK2. As shown in Fig. 5A, full-length JAK2 was much less tyrosine-phosphorylated than TEL-JAK2 when expressed alone in 293 cells. Therefore, we suspect that the phosphorylation of the JH1 domain is necessary for SOCS1-mediated degradation. Because glutathione S-transferase (GST) is a dimer, the JH1 domain fused to GST (GST-JH1) is another constitutively activated form of the JAK2 tyrosine kinase domain (16). Like TEL-JAK2, GST-JH1 was markedly decreased in its expression level when coexpressed with WT, but not with dC40 (Fig. 5B). Pulse-chase experiments revealed that WT-SOCS1 also shortened the half-life of GST-JH1 (data not shown). Moreover, the protein level of the phosphorylation-deficient mutant (FF) of GST-JH1 was not affected by SOCS1 (Fig. 5B). Therefore, reduction in the GST-JH1 pro-

**Fig. 7.** The effect of SOCS box swapping on TEL-JAK2 degradation. A, schematic structures of SOCS1, CIS1, and SOCS3 and their chimeric constructs are shown on the left. 293 cells transfected with TEL-JAK2 and each construct were incubated with 50 μg/ml cycloheximide for the indicated periods and immunoblotted with αJAK2, αSTAT5, and αMYC antibodies. The TJ/S ratio is listed. B, TEL-JAK2 (0.1 μg) and WT or indicated mutant SOCS constructs (0, 0.01, or 0.1 μg of plasmids) were transfected into 293 cells. Total cell lysates were blotted with the indicated antibodies.

**Fig. 8.** SOCS1 promotes the ubiquitination of activated JAK2. 293 cells were transfected with 1 μg TEL-JAK2 (A) or GST-JH1 (B) and 0.5 μg of HA-ubiquitin/pCDNA3 together with a 0.01-μg control vector (lane 1) or plasmids carrying dC40 (lane 2) or WT-SOCS1 (lane 3). To keep the levels of TEL-JAK2, relatively low amounts of SOCS1 plasmid (0.01 μg) were used. The cell extracts were precipitated with αJAK2 (A) or GSH-Sepharose (B) and then blotted with anti-HA (αHA) and αGST antibodies. Total cell lysates (TCL) were also blotted with the indicated antibodies.
tein level by SOCS1 was dependent on tyrosine phosphorylation or the activation of the JH1 domain.

Next, we examined whether SOCS1 promotes the degradation of activated full-length JAK2. To achieve the activation of JAK2, JAK2 was fused to gyrase B (Gyr-JAK2), and Gyr-JAK2 was dimerized by coumermycin (29). As shown in Fig. 6A, coumermycin treatment enhanced the tyrosine phosphorylation of Gyr-JAK2. Without coumermycin, WT-SOCS1 did not affect the protein levels of JAK2 (Fig. 6B, left). However, WT, but not dC40, induced the degradation of Gyr-JAK2 in the presence of coumermycin (Fig. 6B, Cmou. (+), oJAK2). These data suggest that the SOCS1-SOCS box can potentially induce the degradation of full-length JAK2 but that this process requires tyrosine phosphorylation (or activation) of JAK2.

The SOCS Box of SOCS1 Can Be Replaced with That of CIS1 but Not That of SOCS3—To examine the functional redundancy of the SH2 domain and the SOCS box for TEL-JAK2 degradation, we constructed chimeric mutants among CIS1, CIS3/SOCS3, and SOCS1 (Fig. 7). The SH2 domain mutant R105E-SOCS1 exhibited a lesser effect on TEL-JAK2 protein stability. Thus, tight binding of SOCS1 to the JH1 domain through the SH2 domain is necessary for the degradation of TEL-JAK2. CIS1, which does not bind to JAK2, did not induce TEL-JAK2 degradation, although CIS1 itself was unstable compared with SOCS1 and SOCS3 (see aMYC blot). SOCS3, which also suppresses JAK2 signaling (27), did not induce the degradation of TEL-JAK2. As shown in Fig. 7 (A and B), the mutant SOCS1 whose SOCS box was replaced with that of CIS1 (1/C) reduced the TEL-JAK2 level, whereas the mutant replaced with the SOCS box of SOCS3 (1/3) did not. 1/C induced the degradation of TEL-JAK2 more strongly than did WT-SOCS1. This indicates that the SOCS box of SOCS1 can be replaced with that of CIS1 but not with that of SOCS3. On the other hand, the SOCS3 mutant whose SOCS box was replaced with that of SOCS1 (3/1) did not affect TEL-JAK2 stability. SOCS3 and 3/1 bound to the JH1 domain, but their affinity was much lower than that of WT-SOCS1 or 1/C (27). All these observations were confirmed when different amounts of wild-type or mutant SOCS/CIS genes were expressed (Fig. 7B). These data suggest that the particular SOCS box and its tight binding or proper orientation to the JH1 domain are necessary for the promotion of the degradation of TEL-JAK2.

SOCS1-SOCS Box Promotes the Ubiquitination of TEL-JAK2, and Dominant Negative Cul-2 Inhibits TEL-JAK2 Degradation—Because proteasome inhibitors suppressed SOCS1-mediated degradation of TEL-JAK2, ubiquitination of TEL-JAK2 may be involved in the degradation process. The SOCS box is similar to the BC box of VHL protein, which interacts with the Elongin B,C complex. The VHL-Elongin B,C (VBC) complex further recruits Cul-2 and Rbx-1 as subunits of ubiquitin ligase. Rbx-1 has a RING-finger motif with which E2 ubiquitin-conjugating enzymes are suggested to interact. Kamura et al. (23) and Zhang et al. (24) demonstrated that the SOCS1-SOCS box binds to the Elongin B,C complex. Therefore, the SOCS box has been hypothesized to form a complex with Cul-2 and Rbx-1 and function as an E3 ubiquitin ligase (25). However, neither the existence of this complex nor the SOCS box-dependent ubiquitination of the target molecule has been demonstrated to date.

First, we examined the ubiquitination of TEL-JAK2 and GST-JH1 using HA-ubiquitin. As shown in Fig. 8 (A and B), the ubiquitination of TEL-JAK2 and GST-JH1 was markedly enhanced when they were coexpressed with WT-SOCS1, whereas the dC40 mutant did not induce their ubiquitination. Next, we examined the interaction between the SOCS box and Cul-2 (Fig. 9A). HA-tagged Cul-2 was coprecipitated with WT, but not with dC40, in 293 cells. We also confirmed the binding of the Elongin B,C complex with WT-SOCS1 (data not shown). Thus, similarly to VHL, the SOCS1-SOCS box can interact with the Elongin B,C complex and Cul-2. Overexpression of wild-type Cul-2 (WT-CUL2) accelerated SOCS1-induced TEL-JAK2 degradation (Fig. 9, A and B), whereas SOCS1-induced degradation of TEL-JAK2 was almost completely blocked when mutant Cul-2 containing R452C substitution (R452C-CUL2) (0.5 μg) together with 0.01, 0.1, or 1.0 μg of WT-SOCS1 plasmids. The cell lysates were immunoblotted with the indicated antibodies. The TJS ratio is listed. C, 293 cells transfected with TEL-JAK2 and WT-SOCS1, together with either WT-Cul-2 or R452C mutant Cul-2, were incubated with 50 μg/ml cycloheximide for the indicated periods and immunoblotted with αJAK2 or αSTAT5 antibodies. The TJS ratio is listed.
DISCUSSION

It has been demonstrated that the SOCS boxes of VHL and SOCS1 interact with the Elongin B,C complex. Although the VHL-SOCS box is proposed to recruit Cul-2 and Rbx-1 and function as an E3 enzyme subunit, the function of the SOCS box of the CIS/SOCS family has not been resolved. In this paper, we demonstrate that the SOCS box of SOCS1 is critically involved in the ubiquitin/proteasome-dependent degradation of TEL-JAK2 and phosphorylated JAK2.

By analogy with the VHL complex, the SOCS boxes of SOCS1 and CIS1 were suggested to be involved in the ubiquitin/proteasome-dependent degradation of JAK2 and the EPO receptor, respectively (25, 32). Indeed, CIS1 itself is shown to be ubiquitinated and degraded very rapidly (32). Because the tyrosine phosphorylation of the EPO receptor in response to EPO was sustained by the treatment of cells with proteasome inhibitors, we proposed the possibility that the phosphorylated EPO receptor-CIS1 complex becomes a target of the proteasome. Zhang et al. (24) also suggested that the SOCS box of SOCS3 leads the SOCS3 protein to the degradation pathway, because SOCS3 degradation was blocked by proteasome inhibitors. In this case, the SOCS box may be involved in ubiquitination of SOCS3 itself. However, there is no direct evidence that SOCS box-containing proteins have an E3-like ubiquitin-transfer activity with the target molecule. Kamura et al. (23) reported that the Elongin B,C complex increased the stability of SOCS1 and that SOCS1 overexpression did not induce the degradation of JAK2.

It is notable that SOCS3 did not affect TEL-JAK2 degradation, because SOCS3 has a SOCS box and can bind to JH1. The SOCS3-SOCS box was not functional when fused to the SOCS1 N-terminal region and the SH2 domain (1/3 mutant in Fig. 7). We found that SOCS3 lacking a SOCS box is much more stable than wild-type SOCS3 in Ba/F3 cells (data not shown). This suggests that the SOCS box of SOCS3 may not regulate the protein levels of the target molecule but rather destabilize SOCS3 itself. Like CIS1, SOCS3 has been shown to be an unstable molecule that is degraded by the ubiquitin/proteasome system (24). Therefore, the SOCS box may be involved in inter- as well as intramolecular ubiquitin transfer, depending on the structure of the SOCS box. Our experiments do not exclude the possibility that SOCS-3 has another target besides JAKs or cytokine receptors.

Using constitutively active forms of JAK2, we demonstrated that the SOCS1-SOCS box could indeed induce ubiquitination and proteasome-dependent degradation of JAK2. Our study indicated that the SOCS-box-dependent degradation of JAK2 requires the phosphorylation of JAK2 and strong interaction with SOCS1. Confirming the result of Kamura et al. (23), SOCS1 did not induce the degradation of full-length JAK2 when these two molecules were simply expressed in 293 cells. This was probably due to the low efficiency of the phosphorylation of JAK2 molecules (see Fig. 5A). Similarly to TEL-JAK2, phosphorylated full-length JAK2 by forced dimerization was degraded by coexpression of SOCS1. Thus, acceleration of the degradation of SOCS1 is dependent on the activation (or phosphorylation) of JAK2.

It is still not clear whether this accelerated degradation of full-length JAK2 occurs under physiological conditions. Callus and Mathey-Prevot (33) reported that proteasome-dependent degradation is the major mechanism of the down-regulation of JAK2 after stimulation with IL-3 in Ba/F3 cells. SOCS1 or other CIS/SOCS members may be involved in this process. However, we and other researchers have reported that the SOCS box of SOCS1 has no apparent role in the suppression of cytokine-dependent signaling in 293 cells (16, 17). In these assays, SOCS1 was overexpressed by transient transfection before cytokine stimulation. We tried to see the effect of SOCS1 on the degradation of activated JAK2 in response to IFNγ using embryonic fibroblasts from wild-type and SOCS1+/− mice. We could confirm that IFNγ-induced JAK2 phosphorylation was prolonged in SOCS1+/− fibroblasts; however, we did not detect any decrease in the protein level of JAK2 in either cell types. This is probably because only a small fraction of JAK2 is phosphorylated in response to IFNγ. Further studies are necessary to address the role of the SOCS box on JAK ubiquitination and degradation in physiological conditions.

After completion of this study, De Sepulveda et al. (34) reported that SOCS-1 accelerated the ubiquitination and degradation of Vav. Vav and SOCS1 form a protein complex through interactions between the Vav N-terminal regulatory region and the SH2 domain of SOCS1 in a phosphotyrosine-independent manner. Thus, SOCS1 may induce the degradation of Vav when expressed at very high levels. It has not been reported whether or not the SOCS box of SOCS1 is necessary for the ubiquitination of Vav. Therefore, the molecular mechanism of the ubiquitination of Vav by SOCS1 is still unclear. Moreover, it has not been demonstrated that such phosphorylation-independent interaction can occur in physiological conditions. However, it would be interesting to determine whether SOCS1 can induce ubiquitination and degradation of phosphorylated signaling molecules other than JAKs. In future studies, it will be possible to verify the physiological role of the SOCS box of SOCS1 by introducing mutations in the SOCS box using a knock-in strategy in mice.

SOCS-box-mediated ubiquitination and degradation of activated JAK2 are reminiscent of c-Cbl-mediated ubiquitination and degradation of the activated receptor tyrosine kinases (35–37). Other groups as well as ours have shown that the c-Cbl RING-finger domain interacts with the E2 ubiquitin-conjugating enzyme, thereby accelerating the ubiquitination of the epidermal growth factor receptor or the PDGF receptor with which c-Cbl binds through its SH2 domain. Thus, ubiquitination and proteasome-dependent degradation of activated tyrosine kinases by a specific E3 complex may be a common mechanism in the down-regulation of tyrosine kinases. In addition, we have reported that c-Cbl suppressed EPO-induced STAT5 activation in collaboration with APS (an adaptor containing PH and SH2 domains) (38). Because Cbl family members possess the SH2 domain, multiple tyrosine phosphorylation sites, and proline-rich motifs that interact with SH3 domains, they can interact with many tyrosine-phosphorylated proteins as well as SH2- and SH3-containing proteins. Therefore, Cbl could also be involved in ubiquitin/proteasome-dependent degradation of activated cytokine receptors and their downstream signal transducers. Our study also provides a basis for the inhibition of oncogenic, constitutively active tyrosine kinases by ubiquitination and degradation using the SOCS box or the RING-finger domain.

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