Suppressor of Cytokine Signaling-1 Inhibits VAV Function through Protein Degradation*

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Suppressor of cytokine signaling-1 (SOCS1) is an inducible Src homology 2 (SH2)-containing protein that negatively regulates cytokine and growth factor signaling required during thymic development. Recent evidence indicates that SOCS1 interacts with elongins B and C, which are components of a ubiquitin ligase complex, VCB (VHL/elonginC/B), based on the VHL (von Hippel Lindau) tumor suppressor protein. SOCS1 has previously been shown to operate as an inhibitor of Janus kinases. Here we show that SOCS1 has the distinct function of targeting the hematopoietic specific guanine nucleotide exchange factor, VAV, for ubiquitin-mediated protein degradation. VAV and SOCS1 form a protein complex through interactions between the VAV NH₂-terminal regulatory region and the SH2 domain of SOCS1 in a phosphotyrosine-independent manner. SOCS1 decreases the steady state levels of cotransfected VAV and onco-VAV and reduces the focus forming activity of onco-VAV. SOCS1 stimulates the polyubiquitination of VAV proteins in vitro, which was stabilized by proteasomal inhibitors. These results suggest that SOCS1 programs VAV degradation by acting as a substrate-specific recognition component of a VCB-like ubiquitin ligase complex.

The suppressor of cytokine signaling (SOCS)³ family is composed of eight related SH2-containing proteins and represents a class of adapter molecules that negatively regulate diverse cytokine signaling pathways (1–4). SOCS1 is expressed in the thymus and cells of hematopoietic origin (1, 5). SOCS1 is part of an autoregulatory loop in which SOCS1 induction, following cytokine or growth factor receptor stimulation, attenuates Janus kinase (JAK) activity (1–3, 5). Consistent with this model, targeted disruption of the SOCS1 locus in mice gives rise to a syndrome of perinatal lethality and thymic atrophy resulting from unbridled interferon-y signaling (6–8). SOCS1 inhibits JAK by binding, via its SH2 domain, to the positive regulatory tyrosine in the kinase domain activation helix (2, 9). Although SOCS1 also binds to the receptor tyrosine kinases Kit and Flt3, it does not suppress the kinase activity of these receptors (5). Nevertheless, SOCS1 potently blocks Kit- and Flt3-induced proliferation, suggesting that SOCS1 may modulate signaling through a mechanism distinct from kinase inhibition (5).

In addition to its interaction with receptor and non-receptor tyrosine kinases, SOCS1 binds to the hematopoietic-specific guanine nucleotide exchange factor, VAV (5). VAV contains several modular protein domains including an NH₂-terminal calponin homology (CH), an acidic region, a pleckstrin homology (DH) domain, a pleckstrin homology (PH) domain, a cysteine-rich region, and two SH3 domains flanking an SH2 domain (reviewed in Ref. 10 and references therein). VAV converts inactive Rac-GDP into active Rac-GTP, which in turn regulates cytoskeletal reorganization and the activation of the c-jun NH₂-terminal kinase (JNK) and p38HOG. VAV exchange factor activity is stimulated by tyrosine phosphorylation following antigen and cytokine receptor activation. VAV activity is also enhanced by its relocalization to the plasma membrane through interaction of its pleckstrin homology domain with phosphatidylinositol 3,4,5-trisphosphate. Deletion of the VAV NH₂-terminal sequences renders VAV oncogenic (11). VAV is negatively regulated by its NH₂-terminal sequences (12) through an unknown mechanism. Here, we provide evidence that SOCS1 binds to the NH₂ terminus of VAV and stimulates its ubiquitin-dependent degradation.

EXPERIMENTAL PROCEDURES

Cells, Plasmids, and Antibodies—NIH 3T3, COS7, and 293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies, Inc.) supplemented with 5% fetal bovine serum (Hyclone). Plasmid pCMV-VAV was constructed by inserting murine VAV cDNA in the vector pFlag-CMV-5b (Kodak). pEF-VAV and pEF-onco-VAV were gifts from Dr. A. Altman (San Diego). pMT3-SOCS1 expresses hemagglutinin (HA) epitope-tagged murine SOCS1 from the CMV promoter (5). A point mutation, R105K, within the SH2 domain of SOCS1 abolishes its ability to bind phosphotyrosine residues (5). pEF-SOCS1 was provided by T. Kishimoto (13). pEF-FlagCis, pEF-FlagSOCS2, and pEF-FlagSOCS3 were from D. Hilton (14). RasV12 was expressed from the plasmid pT22 (15). The HA-ubiquitin construct in pMT123 was from D. Bohmann (16). HA-SOCS1 was detected using either a rabbit polyclonal anti-serum raised against SOCS1 (aa 1–172) (5) or an anti-HA monoclonal antibody 12CA5 as indicated in the figures. Rabbit polyclonal anti-VAV antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Flag monoclonal antibody M2 was from Sigma.

Transfection and Foci Formation Assays—293T cells were transfected by the calcium phosphate method (5). COS7 cells were transfected respectively; JAK, Janus kinase; JNK, c-jun NH₂-terminal kinase; CMV, cytomegalovirus; DMEM, Dulbecco’s modified Eagle’s medium; PAGE, polyacrylamide gel electrophoresis; aa, amino acid; GST, glutathione S-transferase.
fected with Fugene 6 (Roche Molecular Biochemicals) according to the manufacturer’s instructions. After 36–48 h, cells were lysed and subjected to immunoprecipitation and Western blotting as described earlier (5). For co-precipitation assays, 1.5 x 10^7 NIH 3T3 cells in 100-mm dishes were transfected 24 h later by calcium phosphate method with plasmids expressing onco-VAV in the presence or absence of SOCS1 along with 5 μg of mouse genomic DNA as carrier. The cells were fixed 10–14 days post-transfection in 10% formaldehyde for 10 min and stained overnight with 0.02% Giemsa stain.

**Pulse-Chase Experiments**—COS7 cells transfected with VAV or onco-VAV in the presence or absence of SOCS1 were washed 36–48 h after transfection and incubated in Cys- and Met-free DMEM (ICN). After 30 min, the medium was replaced with Cys, Met-free DMEM containing 2.5% dialysed calf serum and 100 μCi/ml Trans35S-labelTM (ICN, Specific activity 1175 Ci/mmol) to label the newly synthesized proteins. Following 20 min of labeling, the dishes were washed once in phosphate-buffered saline and incubated in chase medium (DMEM containing 10% fetal calf serum and 10-fold molar excess of Cys and Met). At the end of the indicated chase period, the cells were washed and lysed. The immunoprecipitated VAV or onco-VAV proteins, separated by SDS-PAGE, were detected by autoradiography and quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**In Vivo Ubiquitination of VAV**—In vivo ubiquitination was performed essentially as described by Marti et al. (17). COS7 cells were cotransfected with plasmids expressing VAV, HA-SOCS1, and HA-ubiquitin. After 36–48 h, the cells were washed, lysed in SDS lysis buffer (50 mM Tris, pH 7.4, 1% SDS, and 1 mM dithiothreitol), boiled, and diluted 10-fold before immunoprecipitation with anti-VAV antibody. Proteasomal inhibitor epoxomycin (Affinity, Exeter, UK) was added during the final 8 h before lysis. An aliquot of the diluted lysate was used to monitor the ubiquitination of total cellular proteins and the expression of SOCS1. Ubiquitinated VAV was detected with anti-HA antibody.

**RESULTS**

**SOCS1 Binds to the NH2 Terminus of VAV**—To identify proteins that interact with SOCS1, we used the full-length murine SOCS1 cDNA as a bait to screen a cDNA library in the yeast two-hybrid system and isolated a clone encoding the NH2-terminal region of VAV (5). To determine which region of SOCS1 is involved in this interaction, the NH2 terminus of SOCS1 (aa 1–79), the central SH2 domain (aa 64–172) or the COOH terminus (aa 168–212) were expressed as LexA fusion proteins along with VP16-VAV in yeast. The isolated SOCS1 NH2-terminal region of SOCS1 was sufficient to mediate VAV binding (not shown). To confirm the VAV-SOCS1 interaction in mammalian cells, HA-tagged SOCS1 was cotransfected with full-length murine VAV cDNA into 293T cells, and communoprecipitation was analyzed by Western blotting. VAV was detected in SOCS1 immune complexes derived from cells transfected with both cDNAs but not from cells transfected with VAV or HA-SOCS1 alone (Fig. 1A). Similarly, SOCS1 was detected in VAV immunoprecipitates (data not shown). We then tested whether SOCS1 could bind to onco-VAV, an activated form of VAV that lacks the first 67 residues of the wild-type protein (11). SOCS1 coprecipitated onco-VAV, indicating that the NH2-terminal sequences deleted in onco-VAV are not required for the SOCS1 interaction. To determine whether the VAV acidic domain was sufficient for SOCS1 binding, GST fusion proteins containing the entire NH2 terminus or the minimal acidic domain (aa 116–199) were expressed and used as affinity reagents. Both constructs were capable of binding to SOCS1 from cell lysates, whereas the entire NH2-terminal polypeptide (aa 1–199) failed to bind to a control protein (Fig. 1C). These results were recapitulated using the yeast two-hybrid assay in which the acidic domain of VAV (aa 116–199) interacted with SOCS1 (data not shown).

The interaction of VAV and SOCS1 in yeast suggested that the interaction did not require phosphoryrosine modification as has been reported for other SH2-containing proteins (18, 19).

Consistent with this finding, VAV was coprecipitated with a mutant SOCS1 (Fig. 1A, lane 6) that is unable to bind to tyrosine phosphorylated proteins as a result of R105K substitution in the phosphotyrosine-binding pocket of the SH2 domain (5). These data demonstrated that SOCS1 SH2 binds to the acidic region of VAV. Bacterially expressed GST-VAV fusion proteins encoding VAV amino acids 1–199, 116–199, or 168–212 were incubated with lysates from untransfected 293T cells, 293T cells transfected with SOCS1, or a control protein, Lc. The GST fusion proteins recovered on a glutathione-Sepharose affinity matrix were probed for SOCS1 or Lc using specific antibodies.

**SOCS1 Destabilizes VAV Protein**—During the course of these experiments, we observed that the steady state levels of transfected VAV and onco-VAV were diminished when coexpressed with SOCS1. This effect was more pronounced in 293T cells and was dependent on the dose of cotransfected SOCS1 plasmid (Fig. 3A and data not shown). The capacity of SOCS1 to alter VAV protein expression was specific because other SOCS family proteins, SOCS2 or SOCS3, had no effect on the level of VAV expression (Fig. 3B). To assess the effects of SOCS1 on VAV protein turnover, COS7 cells were cotransfected with VAV in the presence or absence of SOCS1. Metabolically 35S-labeled VAV and onco-VAV immunoprecipitated after various chase times were separated by SDS-PAGE.
and visualized by fluorography (Fig. 3C). We observed that onco-VAV had an extended half-life (5.2 h) compared with VAV (1.4 h) and that in both cases SOCS1 increased the rate of degradation of VAV proteins. In the presence of SOCS1, the measured half-life of onco-VAV was 1.2 h and of VAV was 0.5 h.

Ubiquitination of VAV Is Increased by SOCS1—A number of signaling proteins have been shown to be down-regulated through proteolytic degradation pathways involving the ubiquitin and the 26S proteasome machinery (20). To test for whether SOCS1 might influence ubiquitination of VAV, we expressed HA-tagged ubiquitin together with VAV and SOCS1 in COS7 cells. Western blotting the immunoprecipitated VAV with anti-HA antibody showed that co-expression of SOCS1 results in a marked increase of VAV ubiquitination (Fig. 4A, top panel). Probing the whole cell lysate with anti-HA antibody revealed comparable levels of total ubiquitinated proteins in cells expressing SOCS1 and ubiquitin (Fig. 4A, bottom panel). Similar results were obtained for onco-VAV (data not shown). Further, the addition of the proteasomal inhibitor epoxomycin resulted in the accumulation of ubiquitinated VAV (Fig. 4B). These results indicate that SOCS1 couples VAV to the ubiquitination machinery and targets the ubiquitinated VAV to proteasomal degradation.

DISCUSSION

In this report we ascribe a new mechanism whereby the SOCS1 protein negatively regulates the VAV guanine nucleotide exchange factor by facilitating its degradation. This inhibitory function is distinct from its role as an inhibitor of the JAK family of tyrosine kinases. Insight into the mechanism by
which SOCS1 targets VAV for ubiquitination and destruction comes from the recent finding that SOCS1 and SOCS3 interact with the elongin BC complex (21, 22). Elongin BC also interacts with the tumor suppressor protein VHL to form an E3 ubiquitin ligase complex called VCB (YHL/elonginC/B) (23, 24). VHL binds to elongin C through an intermolecular four-helical bundle structure formed from one helix of elongin C and the three helices donated by VHL (25). The VHL a-helices that bind to elongin C are present as homologous structures contained within the COOH-terminal SOCS box of SOCS1 and all other SOCS family proteins (4, 21, 25). VHL functions as an adapter molecule recruiting specific proteins fated for ubiquitin-mediated degradation such as the hypoxia-inducible factor, HIF-1α, to the VCB E3 ligase (26). We propose that different SOCS box proteins have a function similar to VHL and recruit distinct sets of protein substrates such as VAV to the VCB-like E3 ligase complexes, where the substrates are ubiquitinated and targeted to proteasomal degradation. In support of this notion, another SOCS family protein, CIS, appears to recruit signaling molecules such as VAV via dedi-

in this report, we have provided evidence for a novel function of SOCS1, to recruit signaling molecules such as VAV via dedicated protein-protein interaction domains for ubiquitin-medi-

ated protein destruction. Validation of this model will require in vitro reconstitution of the ubiquitin ligase activity of the VAV-SOCS-VCB-like complex using recombinant components.

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