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SOCS-1 Localizes to the Microtubule Organizing Complex-Associated 20S Proteasome

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The regulation of cytokine signaling is critical for controlling cellular proliferation and activation during an immune response. SOCS-1 is a potent inhibitor of Jak kinase activity and of signaling initiated by several cytokines. SOCS-1 protein levels are tightly regulated, and recent data suggest that SOCS-1 may regulate the protein levels of some signaling proteins by the ubiquitin proteasome pathway; however, the cellular mechanism by which SOCS-1 directs proteins for degradation is unknown. In this report, SOCS-1 is found to colocalize and biochemically copurify with the microtubule organizing complex (MTOC) and its associated 20S proteasome. The SOCS-1 SH2 domain is required for the localization of SOCS-1 to the MTOC. Overexpression of SOCS-1 targets Jak1 in an SH2-dependent manner to a perinuclear distribution resembling the MTOC-associated 20S proteasome. Analysis of MTOCs fractionated from SOCS-1-deficient cells demonstrates that SOCS-1 may function redundantly to regulate the localization of Jak1 to the MTOC. Nocodazole inhibits the protein turnover of SOCS-1, demonstrating that the minus-end transport of SOCS-1 to the MTOC-associated 20S proteasome is required to regulate SOCS-1 protein levels. These data link SOCS-1 directly with the proteasome pathway and suggest another function for the SH2 domain of SOCS-1 in the regulation of Jak/STAT signaling.

SOCS-1 is a member of a family of proteins which negatively regulate cytokine signaling in a classic feedback loop (reviewed in reference 1). SOCS-1 has been shown to be a potent inhibitor of Jak kinase activity and of several cytokine signaling pathways, including gamma interferon (IFN-γ) and interleukin 4 signaling (1, 2, 27, 30). Like all SOCS family members, SOCS-1 has a central SH2 domain and a conserved C-terminal domain termed the SOCS box. Structure-function analysis has demonstrated that the SOCS-1 SH2 domain and the 12 amino acids N terminal to the SH2 domain (extended SH2 subdomain) are required for efficient binding of SOCS-1 to the activation loop of activated Jak kinases (34, 35, 52). In addition, the 12 amino acids N terminal to the extended SH2 subdomain (kinase inhibitory region) function as a pseudo-Jak substrate and are required for efficient inhibition of Jak kinase activity (34, 52). Biochemical binding studies using overexpressed proteins have shown that the SOCS box of SOCS-1 interacts with the elongin BC complex, a component of the ubiquitin proteasome pathway that forms an E3 ligase with the von Hippel-Lindau protein (VHL) and Cul2 (VCB-Cul2) (18, 46, 53). Resembling a Skp1/Cul1/F-box (SCF) E3 ligase complex, this VCB-Cul2 complex ubiquitinates HIF1α and HIF2α to target them for degradation by the 26S proteasome (19, 46).

Recent data in overexpression systems have demonstrated that SOCS-1 can target several proteins, including Vav, TEL-Jak2, Jak2, and IRS1/IRS2, for ubiquitination and proteasomal degradation in a SOCS box-dependent manner (7, 9, 16, 37, 47). The levels of SOCS-1 itself are tightly regulated at both the transcriptional and posttranscriptional levels. SOCS-1 mRNA and protein are rapidly upregulated upon cytokine stimulation and subsequently downregulated by protein degradation (6, 38, 54). Interestingly, analysis of protein extracts from mice heterozygous for a genetic deletion of the SOCS box demonstrated that the SOCS-1 protein with the SOCS box deleted is expressed at significantly lower levels than in the wild-type protein (54). In addition, overexpression studies have shown that the SOCS box deletion mutant of SOCS-1 is no longer protected from proteasomal degradation (13, 34). However, Zhang et al. (53) and Chen et al. (6) have suggested that the elongin BC binding motif of SOCS-1 targets SOCS-1 for proteasomal degradation. Thus, the relationship between SOCS-1 and the ubiquitin proteasome pathway remains unclear.

The proteasome is a cylindrical multisubunit protein complex which consists of a core 20S particle and either a 19S (PA700) or an 11S (PA28) regulatory particle (reviewed in reference 11). The 19S particle is an ATP-dependent regulatory subunit that selects substrates targeted for degradation, prepares them for degradation, and facilitates their translocation into the core 20S particle. Unlike the 19S regulatory particle, the 11S particle increases the V_{max} for hydrolysis of specific peptides by the core 20S particle but it does not facilitate protein degradation, utilize ATP, or recognize substrates (14). Most proteins targeted for proteasomal degradation by the 20S proteasome are often covalently modified with a polyubiquitin chain. This ubiquitination is accomplished by a series of ubiquitin transferase enzymes: E1, E2, and E3. Subcellular localization and fractionation studies have shown that components

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of the ubiquitin proteasome pathway are associated with the microtubule organizing complex (MTOC), an organelle which nucleates the polymerization of microtubules from free tubulin subunits and anchors polymerized microtubules (15). Wigley et al. showed that components of the 26S proteasome pathway (the 20S core subunit, 19S regulatory subunit, and 11S regulatory subunit) colocalized and copurified with γ-tubulin (49), and Andersen et al. used a proteomics approach to demonstrate the cofractionation of several regulatory subunits of the 26S proteasome with previously characterized MTOC proteins such as pericentrin, γ-tubulin, and centrin (3). In addition, Skp1 and Cul1, components of the SCF E3 ligase complex, colocalize and copurify with the MTOC and are thought to regulate centrosome duplication by facilitating the proteolysis of factors required for steps of the centrosome cycle (10, 12).

In this report, immunofluorescence microscopy and subcellular fractionation were used to identify a cellular mechanism for SOCS-1 function. SOCS-1 was found to colocalize with the MTOC-associated 20S proteasome in 293T and COS cells in an SH2-dependent manner. With activated splenocytes, endogenous SOCS-1 was found to copurify with the MTOC, the MTOC-associated 20S proteasome, Jak1, and Vav. Overexpression of SOCS-1 with Jak1 demonstrated that SOCS-1 may target Jak1 in an SH2-dependent manner to a perinuclear location resembling the MTOC-associated 20S proteasome. Analysis of MTOCs fractionated from SOCS-1-deficient cells demonstrated that SOCS-1 may function redundantly to regulate the localization of Jak1 to the MTOC. Further, nocodazole, a microtubule-depolymerizing drug, delayed the protein turnover of SOCS-1, demonstrating that minus-end transport of SOCS-1 to the MTOC-associated 20S proteasome is required to regulate SOCS-1 protein levels. These data suggest a second function for the SH2 domain of SOCS-1 in the regulation of Jak/STAT signaling and identify a molecular link between SOCS-1 and protein degradation.

MATERIALS AND METHODS

Plasmids. The green fluorescent protein (GFP)–SOCS-1 chimeric construct was generated by subcloning the SOCS-1 open reading frame into pEGFP-C3 (Clontech). PCR primers were used to generate XhoI (5′-GCCGCCCTCGAG ATGGTAGCACGCAACCAGGTC-3′) and EcoRI (5′GGCGAATTCTCAGA TCTGGAAGGG-3′) restriction sites at the N terminus and C terminus, respectively. The Xpress–SOCS-1 wild-type and mutant expression plasmids were generated as previously described (6). Briefly, SOCS-1ΔN, SOCS-1ΔC, and SOCS-1ΔSH2 deletion mutants were generated by PCR mutagenesis to remove amino acids 1 to 76, 168 to 212, and 85 to 158, respectively, and cloned into pCDNA3.1-HisA (Clontech). SOCS-1(RK) was generated with the following primer to convert Arg105 to Lys: 5′-GGCACCTTCTTGGTGAAAGACAGTC GCCAACGG-3′. Xpress-Jak1 was cloned by subcloning the Jak1 open reading frame into pCDNA3.1-HisB (Clontech). PCR primers were used to generate EcoRI restriction sites 5′ and 3′ of the start and stop codons, respectively.

Reagents and antibodies. The following antibodies were used at the indicated dilutions for immunofluorescence: anti-γ-tubulin monoclonal antibody GTU-88 (1:500; Sigma), anti-20S α-subunit from *Methanosarcina themophila* (1:500; Calbiochem catalog no. 539153), anti-Xpress (1:1000; Invitrogen), anti-Flag monoclonal antibody M2 (1:1,000; Sigma), anti-mouse antibody–Alexa Fluor 488 (1:500; Molecular Probes), anti-mouse antibody–Alexa Fluor 594 (1:500; Molecular Probes), and anti-rabbit antibody–Alexa Fluor 568 (1:500; Molecular Probes). The following antibodies were used for Western blotting: monoclonal anti-SOCS-1 4H1 antibody (gift of Doug Hilton, WEHI Australia), rabbit polyclonal anti-SOCS-3 antibody (generated from glutathione *S*-transferase–SOCS-3 by J. Losman and P. Rothman), polyclonal rabbit anti-Vav1 antibody (gift of Steven Greenberg, Columbia University), rabbit polyclonal anti-Jak1 antibody (Santa Cruz Biotechnology catalog no. HR-785), polyclonal rabbit anti-20S α1-subunit antibody from human (Calbiochem catalog no. 539145), polyclonal rabbit anti-PA700 subunit 10B antibody (Calbiochem catalog no. 539147), mouse anti-Hsp70 (Calbiochem catalog no. 386037), polyclonal rabbit anti-Cul2 antibody (Zymed catalog no. 51-1800), anti-STAT1(Santa Cruz Biotechnology catalog no. sc-346), anti-STAT1(pY701) (Cell Signaling catalog no. 9171), anti-STAT6 (Santa Cruz Biotechnology catalog no. sc-981), and anti-GFP monoclonal antibody 11E5 (1:1,000; Molecular Probes). Phorbol myristate acetate (PMA), ionomycin, lipopolysaccharide (LPS), DAPI (4′,6′-diamidino-2-phenylindole), cytochalasin D, and nocodazole were obtained from Sigma. IFN-γ was obtained from R&D Systems.

Immunofluorescence. COS-7 cells were cultured in a 12-well dish with poly-L-lysine-coated coverslips (BD Pharmingen). Cells were transfected with Lipofectamine (Invitrogen) according to the manufacturer's protocol. Cells were harvested 24 h posttransfection and fixed in either 4% paraformaldehyde for 15 min at 25°C or 100% ice-cold methanol for 10 min at −20°C. Cells fixed in methanol were washed with methanol by serial dilution with 1× phosphate-buffered saline (PBS). Cells were stained as previously described (48, 49). Briefly, cells were washed three times with 1× PBS following fixation; blocked with mouse, rabbit, and/or goat immunoglobulin G for 30 min at 25°C; incubated with primary antibody for 30 min at 25°C (or at 4°C overnight); washed three times with 1× PBS, incubated with secondary antibody for 30 min at 25°C; washed five times with 1× PBS, and mounted onto a slide with anti-fade fixant (1× PBS, 10% glycerol, 100 mM *N*-propyl gallate). Epifluoresent images were captured with a Nikon Eclipse E600 camera. Confocal images were captured with a Zeiss LSM 410.

Cell culture. Cells were cultured and transfected as previously described (6, 27). Briefly, 293T cells were cultured in Dulbecco modified Eagle medium containing 10% fetal calf serum (Sigma) and 1% penicillin-streptomycin (Invitrogen). COS cells were cultured in RPMI medium containing 10% fetal calf serum and 1% penicillin-streptomycin. Primary thymocytes and splenocytes were harvested as previously described (6) and stimulated with 50 ng of PMA per ml plus 500 ng of ionomycin per ml and 1 μg of LPS per ml plus 20 ng of IFN-γ per ml, respectively.

To analyze the half-life of GFP and GFP–SOCS-1, 293T cells were transfected and 2 days posttransfection were treated with 100 μg of cycloheximide per ml for 0, 3, and 6 h in the absence or presence of 10 μM nocodazole. Bands were quantified with NIH Imager 1.62 software as described for centrosome purifications (6).

Centrosome purification. Centrosomes were prepared according to the method of Moudjou and Bornens (31). Briefly, 293T cells were transfected by calcium phosphate precipitation as described previously (6). Fresh medium was added approximately 8 h posttransfection. Twenty-four hours posttransfection, cells were treated with 0.2 μM nocodazole and 1 μg of cytochalasin D per ml for 1 h at 37°C. Cells were harvested and washed in Tris-buffered saline (TBS). Cells were subsequently washed in 0.1×TBS–8% sucrose and lysed by resuspending them in 2 ml of 0.1× TBS–8% sucrose and 8 ml of lysis buffer (1 mM HEPES [pH 7.2], 0.5% NP-40, 0.5 mM $MgCl_2$, 0.1% β-mercaptoethanol, a 10-μg/ml concentration of protease inhibitors [leupeptin, pepstatin, and aprotinin], and 1 mM phenylmethylsulfonyl fluoride). For primary splenocytes and thymocytes, cells were harvested from 13 4-week-old mice and stimulated with either 1-μg/ml LPS plus 20-ng/ml IFN-γ or 50-ng/ml PMA plus 500-ng/ml ionomycin at 37°C for 4 h, followed by treatment with 0.2 μM nocodazole and 1-μg/ml cytochalasin D for 1 h at 37°C. The cells were harvested, washed in TBS at 4°C, washed in 0.1× TBS–8% sucrose at 4°C, and lysed in 0.4 ml of 0.1% 0.1× TBS–8% sucrose and 1.6 ml of lysis buffer at 4°C. Depending on the lysis volume, the lysate was passed five times through either a 10- or 2-ml serological pipette and spun at 2,500 × *g* for 10 min. The supernatant was filtered through a 40-μm-pore-size nylon membrane. HEPES buffer (pH 7.2) and DNase were added to final concentrations of 10 mM and 1 μg/ml, respectively. The lysate was placed on ice for 30 min, and 30 μl was saved as whole-cell extract. The lysate was transferred to an ultracentrifuge tube, underlaid with a 60% sucrose solution (10 mM PIPES [pH 7.2]–0.1% Triton X-100–0.1% β-mercaptoethanol–60% [wt/wt] sucrose, with a 1-ml cushion for 10 ml of lysate and with a 100-μl cushion for 1 ml of lysate), and spun at 10,000 × *g* for 30 min at 25°C in a Beckman TLS-55 rotor in a Beckman TL-100 ultracentrifuge. The upper 80% of the supernatant above the 60% sucrose cushion was removed and discarded. The remaining supernatant and sucrose cushion were gently vortexed, and 30 μl of this "60% cushion" was saved for Western blotting. Approximately 200 μl of the 60% cushion was loaded onto a 2.2-ml discontinuous sucrose gradient (40, 50, and 70% sucrose; 0.6, 0.6, and 1 ml) and spun at 120,000 × *g* for 1 h at 25°C in a Beckman TLS-55 rotor in a Beckman TL-100 ultracentrifuge. Fractions of 200 μl were collected (from the top to the bottom), diluted with 1 ml of 10 mM PIPES (pH 7.2), and spun for 10 min at 13,000 rpm at 25°C in a microcentrifuge. All of the supernatant except

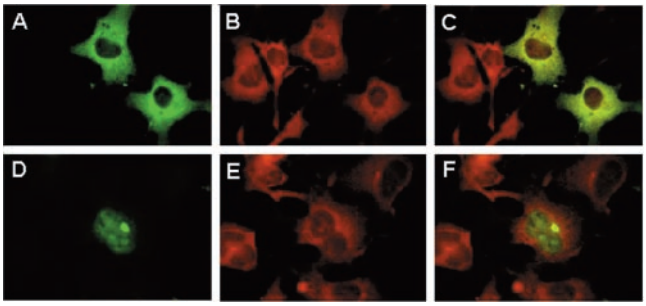

FIG. 1. Expression of GFP and GFP–SOCS-1 in COS cells. COS cells were transfected with pEGFP (A to C) or GFP–SOCS-1 (D to F) and visualized by fluorescence microscopy. Cells were visualized for GFP (A, D) or were stained with anti-γ-tubulin followed by anti-mouse antibody–Alexa Fluor 594 to visualize the MTOC (B, E). GFP fluorescence and Alexa Fluor 594 fluorescence were merged (C, F).

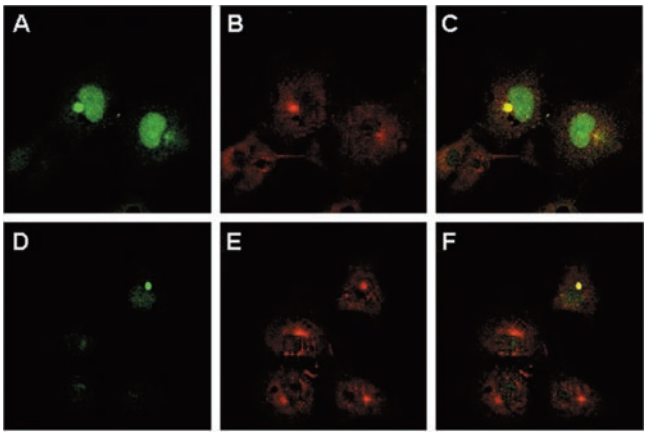

FIG. 2. Colocalization of GFP–SOCS-1 and Xpress–SOCS-1 with 20S proteasome. COS cells were transfected with GFP–SOCS-1 (A to C) or Xpress–SOCS-1 (D to F) and visualized by confocal microscopy. Cells were visualized for GFP (A), stained with anti-Xpress followed by anti-mouse antibody–Alexa Fluor 488 (D), or stained with anti-20S proteasome α-subunit followed by anti-rabbit antibody–Alexa Fluor 568 (B, E). Green (GFP or Alexa Fluor 488) and red (Alexa Fluor 568) images were merged to visualize colocalization (C, F).

approximately 30 μl was removed and discarded. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer was added, and the fractions were resolved on a denaturing 12% polyacrylamide gel and subjected to Western blotting with the antibodies indicated above.

To obtain percentages of fractionated SOCS-1 cells, blots were scanned with an Agfa Arcus 1200 scanner and saved as TIFF files. Subsequently, TIFF files were analyzed with the NIH Imager 1.62 gel analysis software. Briefly, "gel plotting macros" are installed on the application program. The first lane of the blot to be analyzed is marked with the square wave tool. Subsequent lanes are marked with the same square wave defined for the first lane. After all the lanes are marked, the bands for each lane are plotted as histogram files. Areas under the histograms are calculated for the bands corresponding to SOCS-1. The sum total of all the areas for each lane on the sucrose gradient represents the total SOCS-1 present on the gradient. The area for each lane is normalized to the total SOCS-1 on the gradient and multiplied by 100 to obtain the percentages of fractionated cells.

RESULTS

SOCS-1 colocalizes with the MTOC-associated 20S proteasome. In order to identify a molecular function for SOCS-1, a GFP–SOCS-1 chimeric protein was generated. The GFP–SOCS-1 chimeric protein suppressed interleukin 4-induced STAT6 transcription of a luciferase reporter as efficiently as a smaller epitope-tagged SOCS-1 (6), indicating that the fusion of SOCS-1 to GFP does not affect its ability to inhibit Jak/STAT signaling (B. Q. Vuong and P. B. Rothman, unpublished data). To examine the subcellular localization of SOCS-1, COS cells were transiently transfected with either the control GFP construct or the GFP–SOCS-1 chimera and visualized under a fluorescence microscope. COS cells transfected with the GFP construct showed a diffuse cytoplasmic localization with GFP (Fig. 1A), as previously reported (48). However, when cells were transfected with the GFP–SOCS-1 construct, the GFP fluorescence was found predominantly in the nucleus, with less fluorescence in the cytoplasm (Fig. 1D). In addition, approximately 60% of the cells showed a bright perinuclear spot (Fig. 1D). This bright perinuclear fluorescence did not depend on the level of expression of GFP–SOCS-1, as cells which had either a bright or dull total GFP fluorescence had a perinuclear spot (data available on request.) The SOCS-1 perinuclear fluorescence found in COS cells was also observed in 293T and Jurkat cells (Vuong and Rothman, unpublished data; and data not shown). In addition, this perinuclear staining pattern was found when a smaller epitope-tagged SOCS-1 (Xpress–SOCS-1) was used for immunofluorescence (Fig. 2D). Some GFP–SOCS-1 was found to localize in the nuclei of transfected COS, 293T, and Jurkat cells (Fig. 1D) (Vuong and Rothman, unpublished data; and data not shown), as previously described (4).

To determine whether the observed GFP–SOCS-1 perinuclear fluorescence colocalizes with the MTOC, COS cells transiently transfected with GFP or GFP–SOCS-1 were stained with antibodies to γ-tubulin, an established marker for MTOCs (10, 49). As shown in Fig. 1F, GFP–SOCS-1 colocalized with γ-tubulin in COS cells. While some GFP could be found colocalizing with γ-tubulin, most of the staining was found diffusely in the cytoplasm (Fig. 1C). In addition, the area of GFP–SOCS-1 fluorescence was significantly larger than the area of γ-tubulin staining, suggesting that SOCS-1 may localize to the MTOC as part of a large complex of proteins. Thus, these data show that SOCS-1 localizes to the MTOC.

Recent work has shown that the 20S proteasome and components of the ubiquitin proteasome pathway are associated with the MTOC and may play a role in regulating cell cycle proteins and centrosome duplication (10, 23, 49). In addition, components of the SCF complex, Skp1 and Cul1, have been found to colocalize and copurify with the MTOC (10, 12). Immunostaining COS cells with antibodies to the 20S proteasome α-subunit and γ-tubulin demonstrated the specific colocalization of the 20S proteasome with the MTOC (data not shown), as previously described (49). Because SOCS-1 has been shown to bind to components of the ubiquitin proteasome pathway to target itself or its binding partners for ubiquitin-mediated proteolysis (6, 7, 9, 16, 18, 37, 47, 53), we sought to determine if the observed SOCS-1 localization to the MTOC corresponded with colocalization of SOCS-1 with the MTOC-associated 20S proteasome. As shown in Fig. 2, confocal microscopy of COS cells transiently transfected with GFP–SOCS-1 or an Xpress–SOCS-1 construct showed that SOCS-1 colocalizes with the 20S proteasome (Fig. 2C and F).

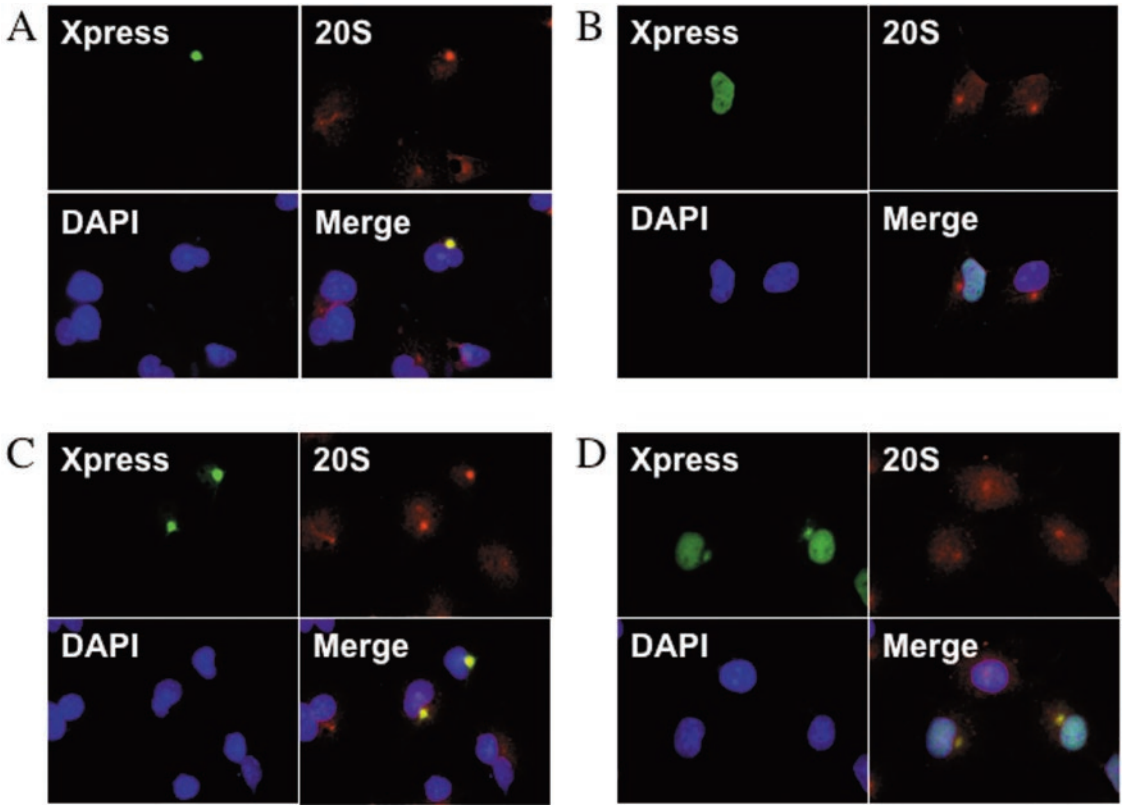

FIG. 3. The SH2 domain of SOCS-1 is required for its localization with the 20S proteasome. COS cells were transfected with Xpress-tagged SOCS-1ΔN (A), SOCS-1ΔSH2 (B), SOCS-1ΔSB (C), or SOCS-1(RK) (D) and stained with anti-Xpress followed by anti-mouse antibody–Alexa Fluor 488, anti-20Sα followed by anti-rabbit antibody–Alexa Fluor 568, and DAPI.

Thus, overexpressed SOCS-1 colocalizes with the MTOC-associated 20S proteasome.

SH2 domain of SOCS-1 is required for its localization to 20S proteasome. To determine which domain of SOCS-1 is required for its localization to the MTOC-associated 20S proteasome, Xpress-tagged SOCS-1 deletion mutant proteins were transfected into COS cells and analyzed for their subcellular localization. Immunofluorescence analysis of an N-terminal deletion mutant (SOCS-1ΔN), an SH2 deletion mutant (SOCS-1ΔSH2), and a SOCS box deletion mutant (SOCS-1ΔSB) of SOCS-1 showed that the SOCS-1 SH2 domain is required for targeting SOCS-1 to the MTOC-associated 20S proteasome (Fig. 3A to C). Surprisingly, SOCS-1ΔSB or a SOCS-1 mutant which does not bind the elongin BC complex (with the mutation L175P and C179F, respectively) still localized to the MTOC (Fig. 3C) (18, 53; B. Vuong and P. Rothman, unpublished data). These data indicate that binding of elongin BC to SOCS-1 is not required for the localization of SOCS-1 to the MTOC-associated 20S proteasome. The mutation of arginine-105 to lysine [SOCS-1(RK)] or of RDS (amino acids 105 to 107) to KEC in the FLVRDS motif of the SOCS-1 SH2 domain did not alter its localization to the MTOC-associated 20S proteasome (Fig. 3D) (B. Vuong and P. Rothman, unpublished data). Thus, the SH2 domain but not the SOCS box of SOCS-1 is required for localization of SOCS-1 to the MTOC-associated 20S proteasome in a putative phosphotyrosine-independent manner.

SOCS-1 copurifies with MTOC. MTOCs and their associated proteins have been biochemically purified by the technique of Moudjou and Bornens (31). This method has been used to identify the association of the 26S proteasome and components of the SCF complex with the MTOC (3, 10, 49). To complement the immunofluorescence data, MTOCs were purified from 293T cells transiently transfected with GFP or GFP–SOCS-1 (Fig. 4A). Enrichment of γ-tubulin was found in fraction 7 of the sucrose gradient (Fig. 4A), which corresponds to the interface of 50 and 70% sucrose, as previously described (49). When the blot was reprobed for GFP, more than 90% of the GFP was found to fractionate in fractions 1 to 3; however, GFP–SOCS-1 was found to fractionate predominately in fraction 7. Similarly, analysis of MTOCs from 293T cells expressing an Xpress-tagged SOCS-1 protein demonstrated that a smaller epitope-tagged SOCS-1 can cofractionate to the same fractions that are enriched for γ-tubulin on the sucrose gradient (Fig. 4B). Consistent with the immunofluorescence data, purification of MTOCs from SOCS-1ΔSH2-expressing cells showed that more than 80% of this SOCS-1 mutant did not copurify with the MTOC (Fig. 4B); however, purified MTOCs from cells expressing SOCS-1(RK), which cannot bind and inhibit Jak kinases (34, 35, 52), demonstrated that this mutant protein still copurifies with the MTOC (Fig. 4C). These data confirm the importance of the SH2 domain in regulating the localization of SOCS-1 to the MTOC-associated proteasome and sug-

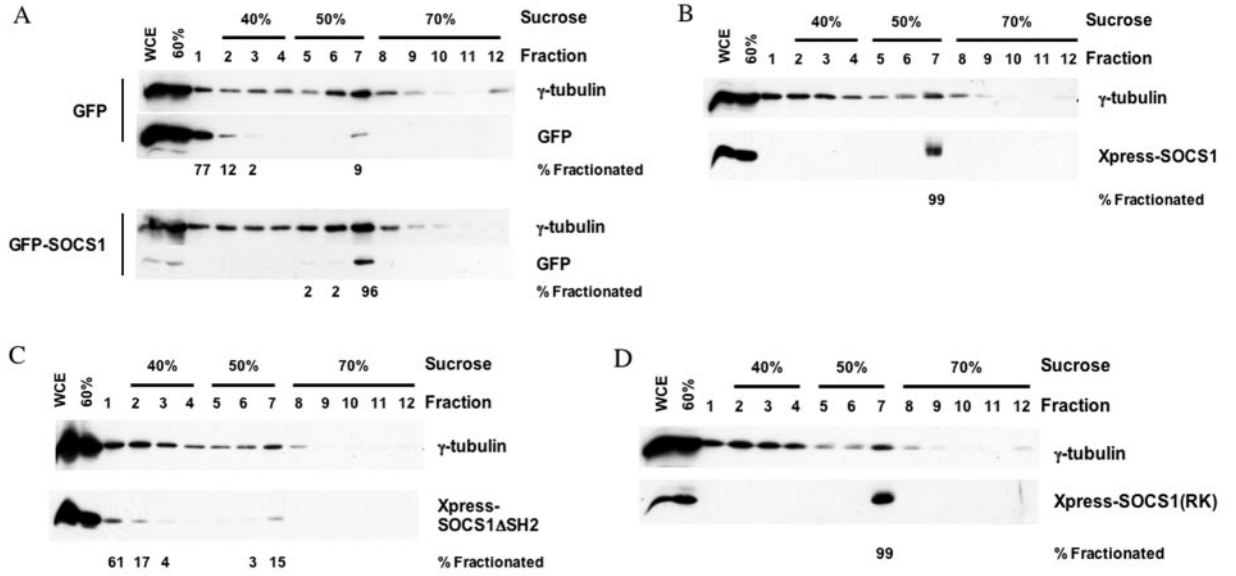

FIG. 4. SOCS-1 biochemically copurifies with the MTOC. MTOCs were purified on a discontinuous sucrose gradient from 293T cells transiently transfected with GFP and GFP–SOCS-1 (A) or Xpress-tagged SOCS-1 (B), SOCS-1ΔSH2 (C), and SOCS-1(RK) (D). MTOCs were purified as described in Materials and Methods and subjected to Western blotting with antibodies against the indicated proteins. "WCE" and "60%" refer to 30-μl samples of the whole-cell extract and the 60% sucrose cushion, respectively. Values for "% Fractionated" were calculated as described in Materials and Methods and indicate the amount of fractionated protein relative to the total protein in all of the fractions.

gest a potential phosphotyrosine-independent mechanism of SOCS-1 function.

Identification of endogenous SOCS-1 subcellular localization. SOCS-1 mRNA and protein are rapidly upregulated upon IFN-γ, LPS, and PMA-ionomycin stimulation (6, 22, 33, 38). Attempts to localize endogenous, cytokine-stimulated SOCS-1 by immunofluorescence techniques with mouse embryonic fibroblasts derived from wild-type or *socs-1*$^{-/-}$ mice stimulated with IFN-γ, LPS, or LPS plus IFN-γ have been unsuccessful, likely due to the low cellular levels of SOCS-1 (Vuong and Rothman, unpublished data). To determine if endogenous, cytokine-induced SOCS-1 localized to the MTOC, biochemical purification of MTOCs was utilized. Total splenocytes from BALB/c mice were stimulated for 4 h with LPS and IFN-γ to induce maximal SOCS-1 expression, and MTOCs were subsequently purified. As shown in Fig. 5, endogenous SOCS-1 copurified with γ-tubulin from LPS–IFN-γ-stimulated splenocytes. SOCS-1 was also found to copurify with γ-tubulin in BALB/c thymocytes stimulated with PMA and ionomycin for 4 h and in Abl-transformed pre-B cells, which have constitutive expression of SOCS-1 mRNA and protein (B. Vuong, A. Limnander, and P. Rothman, unpublished data). SOCS-1 protein biochemically enriched from LPS–IFN-γ-stimulated splenocytes was found only in fractions enriched for γ-tubulin (fractions 6, 7, and 8 in Fig. 5). Consistent with previously published results, the 20S proteasome, PA700 (ATP-dependent 19S regulatory subunit of the 20S proteasome), and Hsp70 cofractionated with γ-tubulin in MTOCs purified from LPS–IFN-γ-stimulated splenocytes and PMA-ionomycin-stimulated thymocytes (Fig. 5) (49; Vuong and Rothman, unpublished data). Cul2, a component of the VCB-Cul2 E3 ligase that has been shown to coimmunoprecipitate with SOCS-1 (16, 17, 46), and a protein related to Cul1 which has been found to copurify with the MTOC (10, 12) also cofractionated with the MTOC and SOCS-1 (Fig. 5).

To control for the specific localization of SOCS-1 and its associated proteins to the MTOC-associated proteasome, MTOCs purified from LPS–IFN-γ-stimulated splenocytes and PMA-ionomycin-stimulated thymocytes were examined for SOCS-3, another SOCS family member that has significant sequence homology to SOCS-1 and has been shown to inhibit several cytokine signaling pathways and Jak2 kinase activity (1, 39). Western blot analysis revealed that SOCS-3 does not copurify with γ-tubulin from MTOCs derived from LPS–IFN-γ-stimulated splenocytes or PMA-ionomycin-stimulated thymocytes (Fig. 5) (Vuong and Rothman, unpublished data). These data are consistent with immunofluorescence data relating to overexpression of SOCS-3 in COS cells (Vuong and Rothman, unpublished data) and previously published results on SOCS-3 localization (4). Thus, although SOCS-3 may be an effective inhibitor of cytokine signaling and Jak kinase activity, it does not localize to the same subcellular compartment or purify with the same subcellular fraction as SOCS-1. These data reiterate the functional differences between SOCS-1 and SOCS-3's abilities to negatively regulate cytokine signaling.

SOCS-1 targets Jak1 to MTOC-associated 20S proteasome. SOCS-1 has been shown to bind and inhibit Jak kinases (8, 32, 35) and to target Vav, Jak2, TEL-Jak2, and IRS1/IRS2 for ubiquitination and proteasomal degradation (7, 9, 16, 37, 47). To determine whether SOCS-1 can target Jak1 and Vav1 to the MTOC-associated 20S proteasome, MTOCs fractionated from LPS–IFN-γ-stimulated splenocytes were examined for Jak1 and Vav1 cofractionation. As shown in Fig. 5, both Jak1 and Vav1 were found to cofractionate with the MTOC and its associated proteins following LPS–IFN-γ stimulation. Thus, endogenous, activation-induced SOCS-1 copurifies with the

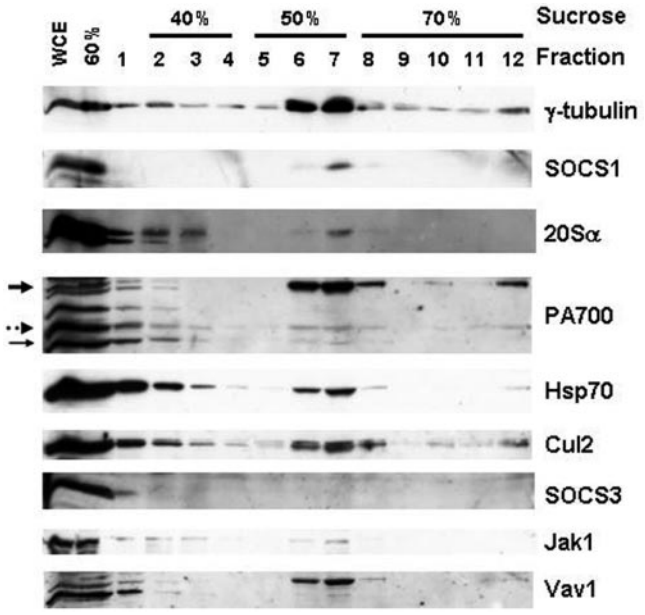

FIG. 5. Activation-induced, endogenous SOCS-1 copurifies with the MTOC. Total splenocytes from BALB/c mice were stimulated with 1 μg of LPS per ml plus 200 ng of IFN-γ per ml for 4 h. MTOCs were purified as described in Materials and Methods and subjected to Western blotting with antibodies against the indicated proteins. "WCE" and "60%" refer to 30-μl samples of the whole-cell extract and the 60% sucrose cushion, respectively. ➡, ⇢, and → denote the 90, 46, and 42 kDa, respectively, isoforms of PA700 subunit 10B.

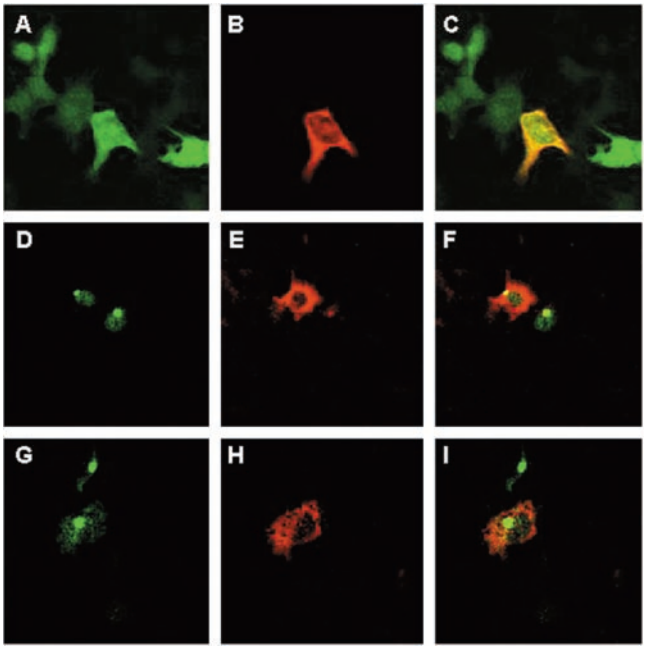

FIG. 6. SOCS-1 targets Jak1 to a perinuclear distribution. GFP (A-C), GFP–SOCS-1 (D-F), or GFP–SOCS-1RK (G-I) were transiently transfected with Xpress-Jak1 in 293T cells and visualized by confocal microscopy. Cells were fixed and visualized for GFP fluorescence (A, D, G) or stained with anti-Xpress (B, E, H) antibodies followed by anti-mouse antibody–Alexa Fluor 594. GFP fluorescence and Alexa Fluor 594 fluorescence were merged (C, F, I).

MTOC and may function to target proteins such as Jak1 and Vav1 for degradation by the MTOC-associated proteasome.

To determine if overexpressed SOCS-1 can target Jak kinases to the MTOC-associated proteasome, GFP, GFP–SOCS-1, or GFP–SOCS-1(RK) was cotransfected with wild-type Xpress-tagged Jak1 in 293T cells and subsequently processed for immunofluorescence. Confocal microscopy revealed that both GFP and Jak1 have a diffuse cellular localization (Fig. 6A to C). While GFP could be found in both the nucleus and the cytoplasm, Jak1 was found predominantly in the cytoplasm (Fig. 6A and B). In contrast, when GFP–SOCS-1 was cotransfected with Xpress-Jak1, a fraction of the Jak1 protein in the cytoplasm was localized to the MTOC. In some cells, almost all of the Jak1 protein colocalized with the MTOC-associated 20S proteasome (Fig. 6D to F). When GFP–SOCS-1(RK) was coexpressed with Jak1, Jak1 staining was found diffusely localized in the cytoplasm even though GFP–SOCS-1(RK) localized to the MTOC-associated 20S proteasome (Fig. 6G to I). These data suggest that SOCS-1 can target Jak1 for proteolysis at the MTOC-associated 20S proteasome and suggest a novel role for the SOCS-1 SH2 domain in the regulation of Jak/STAT signaling.

To examine if SOCS-1 was required to regulate the localization of Jak1 to the MTOC, MTOCs were purified from IFN-γ–LPS-stimulated splenocytes of *ifng*$^{-/-}$ *SOCS-1*$^{-/-}$ and *ifng*$^{-/-}$ *socs*$^{+/+}$ littermate mice. As shown in Fig. 7, fractionation of MTOCs from *ifng*$^{-/-}$ *socs*$^{+/+}$ mice resulted in the cofractionation of approximately 45% of the total Jak1 with γ-tubulin. However, fractionation of MTOCs from *ifng*$^{-/-}$ *socs1*$^{-/-}$ mice demonstrated that approximately 35% of the total Jak1 cofractionated with γ-tubulin. Similarly, a 25 to 50% reduction in the total amount of Jak1 fractionating with the MTOC was found in SOCS-1-deficient mouse embryonic fibroblasts and Abl-transformed fetal liver pre-B cells compared to the amount in wild-type cells (Vuong and Rothman, unpublished results). These data are consistent with the observed subcellular localization of Jak1 in Fig. 6, which demonstrates that, in a cell with high levels of Jak1 and SOCS-1, only a fraction of the total cellular Jak1 colocalizes with SOCS-1 at a perinuclear location. Thus, although SOCS-1 can target Jak1 to the MTOC, SOCS-1 is partly dispensable in regulating the

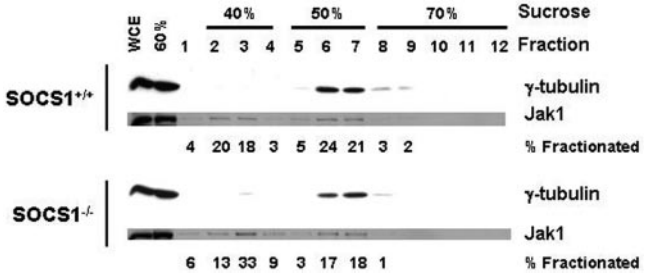

FIG. 7. SOCS-1-deficient cells have reduced Jak1 fractionating with the MTOC. Total splenocytes from two *ifng*$^{-/-}$ SOCS-1$^{-/-}$ and two *ifng*$^{-/-}$ *socs*$^{+/+}$ littermate mice were stimulated with 1-μg/ml LPS plus 200-ng/ml IFN-γ for 4 h. MTOCs were purified and analyzed as indicated in the legend to Fig. 5. Values for "% Fractionated" were calculated as described in Materials and Methods and indicate the amount of Ja999k1 in each fraction relative to the total Jak1 in all of the fractions.

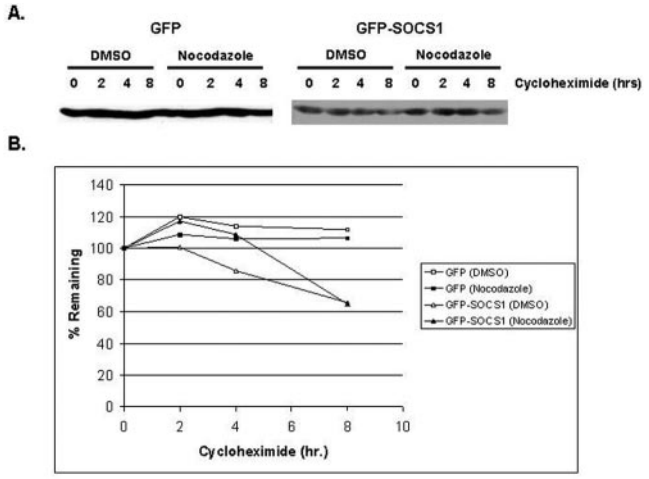

FIG. 8. Nocodazole prolongs the half-life of GFP–SOCS-1. (A) 293T cells were transiently transfected with GFP (left panel) or GFP–SOCS-1 (right panel) and treated with 100 μg of cycloheximide per ml in the presence of a carrier (DMSO) or 10 μM nocodazole for 0, 3, or 6 h. Forty micrograms of whole-cell extracts were run on an SDS–10% PAGE gel and subjected to Western blotting with an anti-GFP antibody. (B) Bands were quantified using NIH Imager 1.62. "% Remaining" reflects the band intensity of GFP or GFP–SOCS-1 at 0, 2, 4, or 8 h normalized to the band intensity at 0 h. The figure is representative of results of three independent experiments.

localization of Jak1 to the MTOC and another Jak1 binding protein may function redundantly to direct the subcellular localization of Jak1.

An intact microtubule network is required for the turnover of SOCS-1 protein. Transport in the minus-end direction of the microtubule cytoskeleton requires an intact microtubule network (20). Disruption of the microtubule network and microtubule-dependent transport can be achieved with nocodazole (10, 12). Previous work has demonstrated that the half-life of SOCS-1 protein can be prolonged with treatment of proteasomal inhibitors such as *N*-acetyl-Leu-Leu-norleucinal (LLnL) (6, 13, 43). To examine whether SOCS-1 protein levels are regulated by microtubule-dependent trafficking, 293T cells were transiently transfected with GFP or GFP–SOCS-1 and treated with cycloheximide (to inhibit protein synthesis) in the presence or absence of nocodazole. As shown in Fig. 8, the levels of GFP remain unaltered following treatment with cycloheximide and either the control carrier, dimethyl sulfoxide (DMSO), or nocodazole. However, the levels of GFP–SOCS-1 decreased dramatically in the presence of cycloheximide and DMSO, as previously described (6, 13, 18). The turnover of GFP–SOCS-1 can be delayed with nocodazole. These data demonstrate that the SOCS-1 protein levels are regulated by microtubule transport and provide additional evidence that SOCS-1 and possibly its binding partners are degraded at the MTOC-associated 20S proteasome.

DISCUSSION

In this report, we show that SOCS-1 localizes to the MTOC and the MTOC-associated 20S proteasome. This localization is dependent on the SOCS-1 SH2 domain and is confirmed by subcellular fractionation experiments. Although the SH2 domain is required for the observed SOCS-1 localization, an Arg-to-Lys or RDS-to-KEC mutation in the FLVRDS motif of the SH2 domain did not alter the SOCS-1 localization to the MTOC. Purification of MTOCs from LPS–IFN-γ-stimulated splenocytes demonstrated that endogenous, activation-induced SOCS-1 copurifies with the MTOC. Overexpression of SOCS-1 with Jak1 demonstrated that SOCS-1 could target Jak1 to the MTOC-associated 20S proteasome in an SH2-dependent manner. Analysis of MTOCs fractionated from SOCS-1-deficient cells demonstrated that SOCS-1 may function redundantly to regulate the localization of Jak1 to the MTOC. In addition, nocodazole can delay the protein turnover of SOCS-1. These data suggest that the SH2 domain of SOCS-1 can target itself or its associated proteins to the MTOC-associated proteasome and reveal a novel function of the SH2 domain of SOCS-1.

MTOCs fractionated from LPS–IFN-γ-stimulated splenocytes showed cofractionation of Jak1 and Vav1 with γ-tubulin, the 20S proteasome, and SOCS-1 but not with SOCS-3 (Fig. 5). Interestingly, only a fraction of the cellular Jak1, Vav1, and 20Sα subunit of the 20S proteasome copurified with endogenous SOCS-1 and MTOCs (Fig. 5, compare fractions 1 to 3 and fractions 6 to 8). Following cytokine stimulation, only a fraction of the total cellular Jak kinases are activated by tyrosine phosphorylation (27). Because SOCS-1 is thought to bind only to the phosphorylated forms of the Jak kinases but to bind to Vav1 in a phosphotyrosine-independent manner, it is not surprising to find that only a fraction of the cellular Jak1 copurifies with SOCS-1 and that most of the cellular Vav copurifies with SOCS-1 (7, 34, 52). Presumably, these SOCS-1-bound proteins are actively targeted for inactivation, dephosphorylation, ubiquitination, and/or degradation. Similarly, immunofluorescence microscopy and purification of MTOCs shows enrichment but not complete colocalization and copurification of γ-tubulin, Cul2, and the 20S proteasome with SOCS-1 (Fig. 1, 2, and 5). These data suggest that only a fraction of the cellular proteasome and ubiquitination ligases are associated with both SOCS-1 and the MTOC, consistent with previous published reports (10, 49). Thus, the localization of SOCS-1 and its binding partners to the MTOC-associated proteasome suggests that SOCS-1 functions as an E3 ligase to direct signaling components such as Jak1 and Vav to ubiquitin-mediated degradation by the MTOC-associated 20S proteasome.

SOCS-1 forms a complex with Cul5, Rbx1, elongin B, and elongin C and coimmunoprecipitates with Cul2 and Rbx1, components of the VCB-Cul2 complex (16, 17). The VCB-Cul2 complex regulates the ubiquitination and degradation of HIF1α and HIF2α, which act to upregulate hypoxia-responsive genes, such as VEGF (19). Interestingly, Skp1 and Cul1, components of the SCF E3 ligase, colocalize and copurify with the MTOC and are thought to regulate centrosome duplication by facilitating the proteolysis of factors which mediate steps of the centrosome cycle (10, 12). Analysis of MTOCs from LPS–IFN-γ-stimulated splenocytes demonstrated that Cul2 copurifies with γ-tubulin and SOCS-1 (Fig. 5). SOCS-1 has recently been shown to bind TRIM8, a RING finger protein, which dramatically increases the degradation of both SOCS-1 and TRIM8 (45). Toniato et al. suggest that SOCS-1 may form an E3 ligase complex with TRIM8, thereby increasing the protein turnover of both SOCS-1 and TRIM8 (45). Analysis of MTOCs from Abl-transformed pre-B cells showed the copurification of

TRIM8 in a SOCS-1-dependent manner (Vuong and Rothman, unpublished data). Thus, TRIM8 may link SOCS-1 and/or its binding partners to proteasomal degradation like c-Cbl, a RING finger E3 ligase that targets EGFR and PDGFR for ubiquitination and degradation (42).

SOCS-1 is a labile protein with a half-life estimated to be 1.5 h, which can be prolonged by treatment with proteasomal inhibitors such as LLnL (6, 13, 18, 43). SOCS-1 has been shown to bind to the elongin BC complex through its C-terminal SOCS box (6, 18, 53), but whether this association regulates SOCS-1 protein levels remains unclear. Kamura et al. (18) and Hanada et al. (13) suggest that the elongin BC association with SOCS-1 stabilizes the SOCS-1 protein, whereas Zhang et al. (53) and Chen et al. (6) suggest that this association accelerates SOCS-1 protein turnover. Interestingly, IFN-γ stimulation of mice heterozygous for a SOCS box deletion of SOCS-1 demonstrated that the wild-type SOCS-1 protein is expressed at higher levels than in the deletion mutant, suggesting that the SOCS box plays a role in stabilizing the SOCS-1 protein in vivo (54). The formation of functional E3 ligase complexes has been shown to stabilize several proteins. The interaction of VHL with the elongin BC complex stabilizes VHL (40), and the formation of the BRCA1/BARD1 heterodimeric E3 ligase stabilizes the BRCA1 and BARD1 proteins (51). Given the pivotal role of the SOCS box in regulating the SOCS-1 protein in vivo, it is not surprising to find that the SOCS-1 SH2 domain and not the SOCS box is required to target SOCS-1 to the MTOC-associated 20S proteasome (Fig. 3 and 4). Preliminary data using chimeric proteins suggest that the SH2 domain of SOCS-1 can confer instability to a heterologous protein (B. Vuong and P. Rothman, unpublished data). These results are consistent with the aforementioned data and suggest a novel role for the SOCS-1 SH2 domain in the regulation of SOCS-1 protein levels and the inhibition of cytokine signaling.

Although the SOCS-1 SH2 domain is required for the localization of SOCS-1 to the MTOC-associated 20S proteasome, SOCS-1(RK), which is unable to bind and inhibit Jak kinases (34, 35, 52), still localizes to the MTOC (Fig. 3 and 4). In addition, we have analyzed a SOCS-1(RDS→KEC) mutant which has been mutated in three amino acids that are required by other SH2 domain-containing proteins for efficient binding to phosphotyrosines (24). This SOCS-1 mutant still localizes to the MTOC-associated proteasome either by immunofluorescence analysis or sucrose gradient fractionation (B. Vuong and P. Rothman, unpublished data). It remains possible that the SH2 mutant SOCS proteins can still bind to phosphorylated and/or ubiquitinated substrates. The SOCS-1(RK) mutant can bind effectively to phosphorylated Vav and focal adhesion kinase (7, 26), demonstrating a nonclassical SH2 protein interaction that has been described for SAP/SLAM, Syk/integrin β, and Vav1/Mer (29, 36, 50). However, there are no published reports demonstrating SOCS-1 binding to ubiquitination motifs on specific proteins. SOCS-1 may therefore passively localize to the MTOC-associated proteasome by binding to ubiquitinated proteins such as Jak1 or Vav1 that are actively targeted for proteasomal degradation because of their ubiquitination. Figure 8 shows that a microtubule network is required for the efficient degradation of SOCS-1. Whether or not SOCS-1 or its ubiquitinated binding partners interact with the microtubule transport mechanism responsible for its MTOC localization remains unclear. The data presented here suggest that the interaction of the SOCS box of SOCS-1 with elongin BC functions to inhibit the degradation of SOCS-1 and that SOCS-1 functions as an adapter between elongin BC and SOCS-1 binding partners, including Jak1 and Vav. The SH2 domain of SOCS-1 may bind Vav and/or Jak1, while the SOCS box of SOCS-1 binds elongin BC, thereby targeting these signaling proteins for proteasomal degradation through the formation of an E3 ligase complex.

The colocalization of SOCS-1 and Jak1 does not occur in all cells, and in a cell expressing high levels of Jak1, there is only a fraction of the total Jak1 that colocalizes with SOCS-1 (Fig. 6). These data are consistent with the fractionation of total splenocytes for SOCS-1, Jak1, and Vav, which demonstrates that only a fraction of the total cellular Jak1 and Vav cofractionate with SOCS-1 (Fig. 5). In addition, analysis of MTOCs fractionated from SOCS-1-deficient cells demonstrates a 25% reduction in the amount of Jak1 that cofractionated with γ-tubulin compared to the amount of MTOCs that fractionated from wild-type cells (Fig. 7). Immunofluorescence analysis of untransfected cells indicates that the 20S proteasome predominately localizes with γ-tubulin and the MTOC; however, diffuse cytoplasmic staining of the 20S proteasome is also observed (49). These data suggest several different possibilities: (i) SOCS-1 may not be able to bind all of the Jak1, (ii) SOCS-1 targets Jak1 to proteasomal degradation outside of the MTOC, and/or (iii) another protein regulates the degradation and localization of Jak1. While it is unlikely that the MTOC-associated 20S proteasome is the sole site for JAK protein degradation in a SOCS-dependent manner, our data suggest that it is a functionally relevant site for the degradation of SOCS-1, JAK, and/or other SOCS-1-associated proteins.

Growing evidence suggests that SOCS-1 plays several roles in the regulation of cytokine signaling that include the inhibition of Jak kinases as well as the targeting of activated signaling molecules for ubiquitination and proteasomal degradation. Recent data suggest that degradation of activated signaling complexes is a common mechanism of terminating signal transduction (21, 44). Following ligand binding, signaling complexes of the β2-adrenergic receptor and epidermal growth factor receptor (EGFR) are internalized and targeted for degradation (25, 41). In addition, STAT3 is endocytosed with EGFR and accumulates in the perinuclear region following EGF treatment (5). These signaling complexes may travel on microtubule-dependent pathways to the MTOC, where they are subsequently degraded by the proteasome to terminate signaling. Interestingly, following T-cell activation, the MTOC reorganizes towards the site of T-cell and antigen-presenting cell interaction in an ITAM/Lck-dependent manner (28). Thus, SOCS-1 may function to downregulate cytokine signaling by targeting signaling complexes of cytokine receptors and activated signaling molecules such as Jaks and STATs for degradation at the MTOC-associated 20S proteasome.

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