Mutational analyses of the SOCS proteins suggest a dual domain requirement but distinct mechanisms for inhibition of LIF and IL-6 signal transduction

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SOCS-1 (suppressor of cytokine signaling-1) is a representative of a family of negative regulators of cytokine signaling (SOCS-1 to SOCS-7 and CIS) characterized by a highly conserved C-terminal SOCS box preceded by an SH2 domain. This study comprehensively examined the ability of several SOCS family members to negatively regulate the gp130 signaling pathway. SOCS-1 and SOCS-3 inhibited both interleukin-6 (IL-6)- and leukemia inhibitory factor (LIF)-induced macrophage differentiation of murine monocytic leukemic M1 cells and LIF induction of a Stat3-responsive reporter construct in 293T fibroblasts. Deletion of amino acids 51–78 in the N-terminal region of SOCS-1 prevented inhibition of LIF signaling. The SOCS-1 and SOCS-3 N-terminal regions were functionally interchangeable, but this did not extend to other SOCS family members. Mutation of SH2 domains abrogated the ability of both SOCS-1 and SOCS-3 to inhibit LIF signal transduction. Unlike SOCS-1, SOCS-3 was unable to inhibit JAK kinase activity *in vitro*, suggesting that SOCS-1 and SOCS-3 act on the JAK–STAT pathway in different ways. Thus, although inhibition of signaling by SOCS-1 and SOCS-3 requires both the SH2 and N-terminal domains, their mechanisms of action appear to be biochemically different.

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Introduction

Cytokine stimulation involves specific recognition of receptors expressed at the cell surface, leading to receptor oligomerization and activation of an intracellular cascade of signaling molecules. The JAK–STAT pathway is activated in a rapid, transient manner and has been shown to be critical in many biological responses to cytokines. Negative regulation of this response is paramount to maintaining appropriate control of cytokine responses and, with the exception of the phosphatase SHP-1, is poorly understood.

Recent studies have identified a new family of negative regulators of cytokine signaling called SOCS (Starr *et al.*, 1997). The prototype of this family, SOCS-1 or suppressor of cytokine signaling-1, was identified by retroviral expression of a cDNA library derived from the factor-dependent hemopoietic cell line, FDCP-1 (Rayner and Gonda, 1994), in the murine monocytic leukemic cell line, M1. Selection for cells which had lost the ability to differentiate in response to interleukin-6 (IL-6) resulted in the recovery of a cDNA encoding a novel 212 amino acid protein (Starr *et al.*, 1997). SOCS-1 was found to be distantly related to CIS (cytokine-inducible SH2-containing protein; Yoshimura *et al.*, 1995), the most striking homology being in a 40 amino acid domain located in the C-terminal region of both proteins. This novel domain has been termed the ‘SOCS box’, and database searches have since identified a large number of additional proteins containing a C-terminal SOCS box (Starr *et al.*, 1997; Hilton *et al.*, 1998). These proteins have been classified further on the basis of the structural domains located N-terminal to the SOCS box. Six other proteins (SOCS-2, -3, -4, -5, -6 and -7), like CIS and SOCS-1, contain an SH2 (*src* homology-2) domain preceded by an N-terminal region of variable length and limited homology (Hilton *et al.*, 1998). To date, little work has been done to investigate the functional relationships within this group of SH2-containing SOCS proteins.

SOCS-1 was discovered independently on the basis of its ability to interact with the kinase domain of JAK2 (Janus kinase 2) and was named JAB (JAK-binding protein; Endo *et al.*, 1997). It was also discovered because of antigenic similarity of the SOCS-1 SH2 domain to a sequence motif in the Stat3 SH2 domain, and was referred to as SSI-1 or STAT-induced STAT inhibitor-1 (Naka *et al.*, 1997). Transcription of the *SOCS-1* gene is induced in response to a number of cytokines (Starr *et al.*, 1997), and the protein has been shown to interact with and inhibit the tyrosine phosphorylation of each member of the JAK family of protein tyrosine kinases (Endo *et al.*, 1997; Naka *et al.*, 1997; Ohya *et al.*, 1997). It has also been demonstrated to inhibit TEC tyrosine kinase activity (Ohya *et al.*, 1997), suggesting that its spectrum of activity may extend beyond that of the JAK family. There is some evidence that transcription of the *SOCS-1* gene is regulated by Stat3 (Naka *et al.*, 1997) in a manner similar to the transcriptional regulation of CIS by Stat5 (Matsumoto *et al.*, 1997). The initial data therefore suggest that SOCS-1, at least, is part of a classical negative feedback loop. In contrast to SOCS-1, CIS appears to negatively regulate cytokine signaling by competing with Stat-5 for binding to phosphotyrosine residues within the erythropoietin and IL-3 receptor cytoplasmic domains (Yoshimura *et al.*, 1995). It is therefore of interest to determine whether other SOCS proteins (SOCS-2 to SOCS-7) can negatively

regulate cytokine signal transduction, and if so, to characterize their mechanism of action.

This study examines the ability of SOCS family members to negatively regulate IL-6 signal transduction in M1 cells and further delineates the functional domains within SOCS proteins required for inhibition of signaling by IL-6 and leukemia inhibitory factor (LIF).

Results

Assays of cytokine signaling

To assess the ability of SOCS proteins to inhibit signal transduction we have utilized two systems. (i) In soft agar, unstimulated M1 cells form large compact colonies of undifferentiated blast cells. When cells are incubated in the presence of IL-6 or LIF, the colonies are dispersed, with a halo of differentiating macrophages migrating out from the central core. At high concentrations of cytokine, the number of colonies observed is markedly reduced, a phenomenon referred to as clonal suppression (Metcalf, 1989). As described previously, when assessed by migration in agar, clonal suppression or morphological differentiation, M1 cells that constitutively express SOCS-1 are unable to respond to either IL-6 or LIF (Starr *et al.*, 1997). These cells retain the ability to differentiate in response to dexamethasone, indicating that SOCS-1 inhibition is specific to cytokine signaling (Starr *et al.*, 1997). (ii) 293T is a human fibroblast line expressing endogenous LIF receptors. Cells were transiently transfected with a LIF-responsive reporter construct, in which an element of the α -2 macroglobulin acute phase protein promoter was placed upstream of the luciferase-coding region (Endo *et al.*, 1997). As has been reported previously (Masuhara *et al.*, 1997), LIF stimulation resulted in a clear increase in luciferase activity. Co-expression of a β -galactosidase reporter construct under a constitutive promoter (S α - β -gal) was used to control for transfection efficiency, and luciferase activity was normalized against β -galactosidase activity.

Effect of SOCS proteins on LIF and IL-6 signaling

To determine whether other SOCS family members had similar activity to SOCS-1 in regulating IL-6 signaling, cDNAs encoding N-terminally Flag-tagged versions of SOCS-2, SOCS-3, CIS and SOCS-5 (Figure 1) were stably transfected into M1 cells, and protein expression was assessed by immunoprecipitation and Western blot analysis using anti-Flag antibodies. Several clones expressing each protein were isolated; expression of SOCS-1 was found to be lower than SOCS-3 and CIS, with SOCS-2 protein levels consistently high. SOCS-5 was also expressed, but at relatively low levels (Figure 2B).

Constitutive expression of SOCS-3, like that of SOCS-1, inhibited all IL-6- and LIF-induced effects, including clonal suppression, changes in cell morphology and up-regulation of surface markers, whereas M1 cells expressing SOCS-2 or SOCS-5 were reduced 3- to 10-fold in their sensitivity to IL-6 and LIF (Figure 2A, data not shown). Expression of CIS had no effect on either IL-6- or LIF-induced differentiation or clonal suppression (Figure 2A, data not shown).

Similar results were found using 293T cells. Again, SOCS-1 and SOCS-3 completely abolished the LIF-

induced activation of luciferase; SOCS-5 partially inhibited the LIF response, while SOCS-2, CIS and SOCS-6 were unable to inhibit the response (Figure 3). Appropriate expression of the various SOCS proteins was confirmed by Western blot analysis using anti-Flag antibodies (Figure 3).

SOCS proteins that inhibit M1 cell differentiation block STAT3 tyrosine phosphorylation

To understand further the mechanism by which SOCS-1 and SOCS-3 were able to inhibit M1 cell differentiation, we examined LIF-induced Stat3 tyrosine phosphorylation in M1 cells expressing the various SOCS proteins. Stat3 tyrosine phosphorylation has been implicated previously in IL-6-induced differentiation of M1 cells, both by the use of dominant-negative Stat3 constructs and by specific tyrosine mutations within the IL-6 signaling chain gp130, which block recruitment of Stat3 to the receptor complex (Nakajima *et al.*, 1996; Yamanaka *et al.*, 1996).

In each instance, Stat3 tyrosine phosphorylation correlated inversely with the ability of the expressed SOCS protein to inhibit M1 cell differentiation. Stat3 was phosphorylated rapidly in response to LIF in M1 cells which either did not express SOCS proteins or expressed SOCS-2 or CIS, while LIF-induced Stat3 tyrosine phosphorylation was inhibited in M1 cells expressing either SOCS-1 or SOCS-3 (Figure 4). This suggests that the ability of both SOCS-1 and SOCS-3 to inhibit M1 differentiation may be mediated through inhibition of the JAK-STAT pathway.

JAK1 activity is critical for LIF signal transduction in 293T cells and is blocked by SOCS-1 but not SOCS-3

Increasing evidence suggests that JAK1 is the critical JAK kinase involved in signaling through the IL-6 family of receptors. JAK1 knockout mice exhibit defective responses to IL-6 and LIF (Rodig *et al.*, 1998), as do somatic cell mutants lacking functional JAK1 (Guschin *et al.*, 1995). To address which JAK kinases were required for LIF signaling in 293T cells, we examined the ability of mutant JAK proteins lacking a functional kinase domain to inhibit LIF-induced activation of the APRE-luc reporter gene (Figure 5). Transient expression of mutant JAK1 (kinase dead) resulted in a dominant-negative effect, markedly reducing LIF-induced luciferase activity (to 3% of control values). Expression of mutant JAK2 (kinase dead) partially blocked signaling (to 69% of control values), whilst expression of mutant JAK3 (kinase dead) had no effect on LIF-induced luciferase activity (97% of control values). Expression of wild-type JAK1 and JAK2 enhanced LIF-induced transcription of the APRE, whilst expression of wild-type JAK3 had no effect (Figure 5). Comparable protein expression of the various JAK constructs was confirmed by Western analysis (Figure 5). These results suggest that in 293T cells, JAK1, and to a lesser extent, JAK2, is required for LIF-induced activation of Stat3.

While SOCS-1 has been implicated directly in inhibiting members of the JAK kinase family, the mechanism of SOCS-3 action is unknown. Given the importance of JAK1 and JAK2 in IL-6 and LIF signal transduction (Figure 5; Guschin *et al.*, 1995; Rodig *et al.*, 1998), the ability of SOCS-1 and SOCS-3 to inhibit JAK kinase activity directly was examined. Overexpression of JAK protein results in a constitutively active kinase, presumably

Fig. 1. Schematic diagram of the SOCS proteins analysed in this study. The red flag indicates either an N- or C-terminal Flag epitope. The SH2 domains are cross-hatched, and SOCS boxes are stippled. Domain swaps are indicated by SOCS-specific colors: CIS, pink; SOCS-1, yellow; SOCS-2, blue; SOCS-3, green; SOCS-5, orange; SOCS-6, purple.

because the high level of expression allows dimerization and activation to take place in the absence of ligand stimulation. JAK1 or JAK2 were transiently expressed with or without Flag-tagged SOCS-1 or SOCS-3. JAK proteins were immunoprecipitated using specific antibodies, and intrinsic kinase activity (or autophosphorylation) assessed using an *in vitro* kinase assay. Co-expression of SOCS-1 inhibited both JAK1 and JAK2 kinase activity (Figure 6). Interestingly, co-expression of SOCS-3 did not inhibit either JAK1 or JAK2 kinase activity and in fact enhanced JAK1 kinase activity (Figure 6). However, SOCS-3 enhancement of JAK1 activity appeared restricted to co-expression in 293T cells as, in COS cells, SOCS-3 neither inhibited nor enhanced JAK1 activity (data not shown). This suggests that although SOCS-1 and SOCS-3 act on the JAK-STAT pathway, they may do so through different mechanisms.

Amino acids 51–80 are critical for SOCS-1 function

Previous work has suggested that the interaction between SOCS-1 and the JAK family of protein tyrosine kinases is mediated through binding of the SOCS-1 SH2 domain to a phosphorylated tyrosine residue within the JAK kinase domain (JH1). In these studies, JAK2 was constitutively activated through overexpression in COS cells or yeast (Endo *et al.*, 1997; Naka *et al.*, 1997; Ohya *et al.*, 1997). To investigate the structural basis of SOCS-1 action in a

ligand-inducible system, a series of SOCS-1 deletion mutants was constructed, in which either the N-terminal region (S1Δ1-76), the C-terminal SOCS box (S1Δ170-212) or both the N-terminal region and the SOCS box (S1Δ1-76&170-212) were deleted. In addition, a construct was created in which the SH2 domain and the SOCS box were both deleted, leaving just the N-terminal 81 amino acids (S1Δ82-212; Figure 1).

The SOCS-1 deletion mutants were transiently expressed in the 293T reporter system and tested for their ability to inhibit LIF-induced luciferase activity. Expression of the mutant lacking the N-terminal region (S1Δ1-76), or lacking both the N-terminal region and the SOCS box (S1Δ1-76&170-212), did not block LIF induction of luciferase activity, whereas the mutant lacking the SOCS box (S1Δ170-212) was fully active and completely blocked luciferase activity (Figure 7A). In addition, a protein lacking both the SH2 domain and SOCS box (S1Δ82-212) was unable to inhibit LIF-induced luciferase activity, suggesting that the N-terminal region alone was insufficient to mediate this action. Protein expression of the various SOCS proteins was confirmed by Western blot using anti-Flag antibodies. The low-level expression of S1Δ1-76 and S1Δ1-76&170-212 was observed consistently in 293T cells, making assessment of the activity of these constructs difficult. Deletion mutants were therefore stably expressed in M1 cells and several independent

Fig. 2. (A) Effect of SOCS proteins on M1 cell differentiation. Semi-solid agar cultures of parental M1 cells (control) and M1 cells expressing SOCS-1, SOCS-2, SOCS-3, SOCS-5 or CIS, showing the percentage of colonies that differentiated (dispersed) in response to a titration of 1 µg/ml mIL-6. Two independent clones are shown for each construct, and a dashed line represents the control untransfected cell line. (B) SOCS protein expression levels. Equal numbers of parental M1 cells and M1 cells expressing SOCS-1, SOCS-2, SOCS-3, CIS or SOCS-5 were lysed and analyzed by immunoprecipitation and Western blot using anti-Flag antibody. Arrows indicate the migration of the Flag-tagged SOCS proteins.

transfectants obtained for each construct. Consistent with the 293T reporter assay, constitutive expression of the mutant either lacking the N-terminal region (S1Δ1-76) or lacking both the N-terminal region and the SOCS box (S1Δ1-76&170-212) did not block IL-6- or LIF-induced differentiation. In contrast, cells expressing either the mutant lacking the SOCS box (S1Δ170-212) or full-length SOCS-1 fully blocked IL-6- and LIF-induced differentiation (Figure 8A, data not shown). Analysis with anti-Flag antibodies confirmed expression of S1Δ1-76, S1Δ1-76&170-212 and S1Δ170-212 at equivalent levels to cells expressing full-length SOCS-1 (Figure 8B). These results indicated that although neither alone was sufficient, the combination of the SOCS-1 N-terminal region and SH2 domain was critical for SOCS-1-inhibition of LIF and IL-6 signaling.

The luciferase reporter system was utilized further to analyze a series of smaller truncations within the N-terminal region of SOCS-1. Deletion of the N-terminal 10, 20, 30, 40 or 50 amino acids did not alter the ability of SOCS-1 to inhibit LIF-induced luciferase activity (Figure 7B and data not shown). However, deletion of the N-terminal 60 amino acids completely abolished SOCS-1 inhibitory action (Figure 7B). To narrow down the critical

Fig. 3. Effect of SOCS proteins on LIF-induction of the Stat3 reporter gene. Upper panel: levels of transiently expressed SOCS-1 (S1), SOCS-2 (S2), SOCS-3 (S3), CIS, SOCS-5 (S5) and SOCS-6 (S6) proteins from a representative experiment were determined by Western blot with anti-Flag antibody. Lower panel: 293T cells were transiently transfected with either vector alone or cDNAs expressing the various SOCS proteins in the presence of the APRE-luc and Srα-β-gal reporter genes. Cells were incubated in the presence (+) or absence (-) of 10 ng/ml hLIF overnight and cell extracts prepared. Luciferase activity from triplicate samples was determined and normalized against β-galactosidase activity.

Fig. 4. Stat3 tyrosine phosphorylation in M1 cells expressing the various SOCS proteins. (A) Upper panel: untransfected M1 cells and M1 cells stably expressing SOCS-1 or SOCS-2 were incubated with 5×10⁵ U/ml mLIF for the times indicated, lysed, and Stat3 proteins immunoprecipitated with anti-Stat3 antibody. Phosphorylated Stat3 protein was detected by Western blot with antibody specific to tyrosine-phosphorylated Stat3 (anti-phosphoSTAT3; New England Biolabs Inc., Beverly, MA). Lower panel: blots were stripped and re-probed with anti-Stat3 antibody. (B) Untransfected M1 cells and M1 cells stably expressing SOCS-3 or CIS were analyzed as in (A).

region, three additional mutants were generated in which amino acids 50-60, 60-70 or 70-78 were deleted. When tested in the 293T reporter assay, none of these constructs was able to inhibit LIF-induced luciferase activity (Figure 7B). The 30 amino acids directly N-terminal to

Fig. 5. JAK1 is critical for LIF signal transduction in 293T cells. Upper panel: levels of transiently expressed JAK1, kinase-dead JAK1 (JAK1-KD), JAK2, kinase-dead JAK2 (JAK2-KD), JAK3 and kinase-dead JAK3 (JAK3-KD) proteins from a representative experiment were determined by Western blot with anti-JAK1 (Transduction Laboratories, Lexington, KY), JAK2 and JAK3 (Santa Cruz) antibody, respectively. Lower panel: 293T cells were transiently transfected with either vector alone or cDNAs expressing the various JAK proteins in the presence of the APRE-luc and Sr α - β -gal reporter genes. Cells were incubated in the presence (+) or absence (-) of 10 ng/ml hLIF overnight and cell extracts prepared. Luciferase activity from triplicate samples was determined and normalized against β -galactosidase activity.

Fig. 6. JAK kinase autophosphorylation is inhibited by SOCS-1 but not SOCS-3. 293T cells were transiently transfected with vector alone or cDNAs expressing either Flag-tagged SOCS-1 or SOCS-3 in the presence (+) or absence (-) of cDNA expressing JAK1 or Flag-tagged JAK2. (A) JAK kinase activity. After 48 h, cells were lysed and JAK1 and JAK2 proteins immunoprecipitated using specific anti-JAK antibodies. Immunoprecipitates were then subjected to an *in vitro* kinase assay. (B) Immunoprecipitates from (A) containing either JAK1 or JAK2 protein were split prior to kinase assay and analyzed by Western blot with anti-JAK1 and anti-Flag antibody, respectively. (C) SOCS protein expression levels. Lysates from (A) were analyzed by Western blot with anti-Flag antibody.

the SOCS-1 SH2 domain therefore appear critical for inhibition of LIF signal transduction, whilst the first 50 amino acids are dispensable for SOCS-1 action.

To define the critical residues within the N-terminal region required for SOCS-1 activity, a series of point

mutations was assessed for their effect on SOCS-1 function. These mutations either replaced individual residues with alanine or resulted in a charge reversal. The following mutations had no effect when proteins were assayed using the 293T reporter system: P51A, G52A, D53A, D53R, T54A, H55A, R57A, R57E, T58A, R60A and R70E (data not shown; Figure 7C). Despite expression at equivalent levels to wild-type SOCS-1, mutation of F56A, F59A or D64R resulted in a non-functional SOCS-1 protein (Figure 7C).

The SOCS-1 and SOCS-3 N-terminal domains are functionally interchangeable

As described above, we have demonstrated functional differences between individual SOCS proteins in their ability to inhibit biological activity. Further, we have identified critical regions in the N-terminal region of SOCS-1 that are crucial for this response. To determine whether the SOCS protein domains are interchangeable, a series of chimeric proteins was created. The N-terminal region of SOCS-1 was replaced with the N-terminal domain of SOCS-2 (S2/1/1), SOCS-3 (S3/1/1), CIS (C/1/1), SOCS-5 (S5/1/1) or SOCS-6 (S6/1/1). These constructs were Flag-tagged, transiently expressed in 293T cells and LIF induction of luciferase activity assayed. The SOCS-1 chimeric protein containing the N-terminal region of SOCS-3 was able to inhibit LIF induction of luciferase activity to the same level as wild-type SOCS-1, whereas the N-terminal regions of SOCS-2 or CIS could not functionally replace the N-terminal region of SOCS-1 (Figure 7D). Consistent with the levels of wild-type SOCS-5 and SOCS-6 protein, chimeric proteins S5/1/1 and S6/1/1 were expressed at low levels, making assessment of the activity of these constructs difficult (Figure 7D). A series of chimeric proteins was also created in which the SH2 domain of SOCS-1 was replaced with the SH2 domain of SOCS-2 (S1/2/1), SOCS-3 (S1/3/1), CIS (S1/C/1), SOCS-5 (S1/5/1) or SOCS-6 (S1/6/1). Little or no inhibition of LIF-induced luciferase activity was observed with chimeric proteins containing other SOCS SH2 domains (Figure 7D).

To determine whether the functional similarity between the SOCS-1 and SOCS-3 N-terminal regions was unidirectional, a series of SOCS-3 chimeric proteins was also created. The N-terminal region of SOCS-3 was replaced with the N-terminal region of either SOCS-1 (S1/3/3), SOCS-2 (S2/3/3), CIS (C/3/3), SOCS-5 (S5/3/3) or SOCS-6 (S6/3/3). Consistent with the ability of the SOCS-3 N-terminal region to functionally replace the N-terminal region of SOCS-1, the converse was also observed. The SOCS-3 chimeric protein containing the N-terminal region of SOCS-1 was able to inhibit LIF induction of luciferase activity, whereas the N-terminal regions of SOCS-2 or CIS were unable to functionally replace the N-terminal region of SOCS-3 (Figure 9A). Chimeric proteins were also created in which the SH2 domain of SOCS-3 was replaced with the SH2 domain of SOCS-1 (S3/1/3), SOCS-2 (S3/2/3), CIS (S3/C/3), SOCS-5 (S3/5/3) or SOCS-6 (S3/6/3). In contrast to the equivalent SOCS-1 chimeric proteins, the SH2 domain of SOCS-1, and, to a slightly lesser extent, that of SOCS-2 and CIS, was able to replace completely the SOCS-3 SH2 domain (Figure 9B).

Sequence alignment of the N-terminal regions of

Fig. 7. Mutational analysis of SOCS-1. Upper panels: levels of transiently expressed SOCS-1 mutant proteins from representative experiments were determined by Western blot with anti-Flag antibody. Lower panels: 293T cells were transiently transfected with vector alone or cDNAs expressing the various SOCS-1 mutant proteins in the presence of the APRE-luc and S α - β -gal reporter genes. Cells were incubated in the presence (+) or absence (-) of 10 ng/ml hLIF overnight and cell extracts prepared. Luciferase activity from triplicate samples was determined and normalized against β -galactosidase activity. (A) 293T cells were transiently transfected with full-length SOCS-1 or SOCS-1 deletion mutants S1 Δ 82-212 (lacking both the SH2 domain and the SOCS box), S1 Δ 170-212 (lacking the SOCS box), S1 Δ 1-76&170-212 (lacking the N-terminal region and the SOCS box) and S1 Δ 1-76 (lacking the N-terminal region). (B) 293T cells were transiently transfected with full-length SOCS-1 or SOCS-1 deletion mutants S1 Δ 1-30, S1 Δ 1-40, S1 Δ 1-50, S1 Δ 1-60, S1 Δ 1-70, S1 Δ 50-60, S1 Δ 60-70 and S1 Δ 70-78. (C) 293T cells were transiently transfected with full-length SOCS-1 or SOCS-1 point mutants S1-D53R, S1-R57E, S1-D64R, S1-R70E, S1-P51A, S1-F56A, S1-F59A and S1-G52A. (D) 293T cells were transiently transfected with full-length SOCS-1 or SOCS-1 N-terminus or SH2 domain swap mutants S2/1/1, S3/1/1, C/1/1, S5/1/1, S6/1/1, S1/2/1, S1/3/1, S1/C/1, S1/5/1 and S1/6/1.

SOCS-1, -3, -4 and -5 suggested some limited homology within these regions (Figure 9D). To investigate further the functional similarity between the SOCS-1 and SOCS-3 N-terminal regions, SOCS-3 constructs were generated that contained similar mutations in the SOCS-3 N-terminal region (S3-L22A, S3-F25A and S3-E30R) (Figure 9D). Mutation of L22A or E30R had no effect when proteins were assayed using the 293T reporter system whereas, like the analogous mutation in SOCS-1, mutation of F25A resulted in a non-functional SOCS-3 protein (Figure 9C).

SOCS-1 and SOCS-3 act through distinct mechanisms

The above data in both M1 cells and 293T cells indicated that whilst the N-terminal region was critical for SOCS-1 and SOCS-3 function, the SH2 domain was also required. Previous work has shown that mutation of the invariant arginine within the highly conserved FLVRES motif disables SH2 binding to phosphotyrosine (Mayer *et al.*, 1992; Waksman *et al.*, 1992). To confirm that the SH2 domain was required for activity, analogous point

Fig. 8. Expression of SOCS-1 deletion mutants in M1 cells. **(A)** Effect of SOCS proteins on M1 cell differentiation. Semi-solid agar cultures of parental M1 cells (control) and M1 cells expressing SOCS-1, S1Δ1-76&170-212 (lacking both the N-terminal region and the SOCS box), S1Δ1-76 (lacking the N-terminal region) and S1Δ170-212 (lacking the SOCS box) showing the percentage of colonies that differentiated (dispersed) in response to a titration of 1 μg/ml mIL-6. Two independent clones are shown for each construct, and a dashed line represents the control untransfected cell line. **(B)** Protein expression levels. Equal numbers of parental M1 cells (M1) and M1 cells expressing SOCS-1 (S1), S1Δ1-76&170-212, S1Δ1-76 and S1Δ170-212 were lysed and analyzed by immunoprecipitation and Western blot using anti-Flag antibody.

mutations were made in SOCS-1 and SOCS-3. These constructs were then transiently expressed in the 293T reporter system. SOCS-1 containing a mutated SH2 domain (S1-R105K) was unable to inhibit LIF-induced luciferase activity (Figure 10), confirming a critical role for the SH2 domain in SOCS-1 function. Interestingly, mutation of the SOCS-3 SH2 domain (S3-R71K) did not greatly alter the ability of SOCS-3 to inhibit LIF signaling (Figure 10). To investigate further the role of the SOCS-3 SH2 domain, a construct was generated which contained three point mutations; R71K, D72E and S73C (S3-SH2mut). In contrast to S3-R71K, this construct showed only weak inhibition of LIF signaling (Figure 10). This suggests that although the SH2 domains of SOCS-1 and SOCS-3 are required for inhibition of LIF signal transduction, differences exist between the two proteins.

The N-terminal region and SH2 domain of SOCS-1 contribute to inhibition of JAK kinase activity

Previous work has shown that the SH2 domain of SOCS-1 can interact with the kinase domain (JH1) of JAK2 (Endo *et al.*, 1997). This makes it likely that the SH2 domain is required for binding to a phosphotyrosine residue within the JH1 domain of an active JAK kinase and that this interaction is at least partially responsible for blocking intrinsic JAK activity.

To investigate this model, Flag-tagged JAK1 or JAK2

was transiently expressed in 293T or COS cells with or without the SOCS-1 deletion mutants, and kinase activity was analyzed using an *in vitro* kinase assay. Co-expression of the SOCS-1 mutant that lacked the SOCS box (S1Δ170-212) inhibited JAK1 *in vitro* kinase activity to the same extent as full-length SOCS-1. In contrast, co-expression of SOCS mutants which lacked either the N-terminus (S1Δ1-76), the N-terminus and the SOCS box (S1Δ1-76&170-212) or the SH2 domain and the SOCS box (S1Δ82-212) were unable to inhibit JAK1 *in vitro* kinase activity (Figure 11A). Consistent with the Stat3 reporter assay and the M1 cell differentiation data, this suggests that both the N-terminal region and the SH2 domain of SOCS-1 are required for inhibition of JAK1 kinase activity.

Interestingly, the results differed with respect to JAK2 *in vitro* kinase activity. Co-expression of any of the SOCS-1 deletion mutants resulted in inhibition of JAK2 autophosphorylation (Figure 11D). Therefore, while both the SOCS-1 N-terminus and SH2 domain were required for inhibition of JAK1 kinase activity, either of these two domains alone were sufficient for inhibition of JAK2 kinase activity *in vitro*.

If the SOCS-1 N-terminus contributes to inhibition of JAK kinase activity, it may do so by direct interaction with JAK. To investigate this, full-length SOCS-1 or the SOCS-1 mutant lacking both the SH2 domain and the SOCS box (S1Δ82-212) were co-expressed with or without Flag-tagged JAK1, mutant JAK1 (kinase dead) or JAK2 in 293T cells. JAK proteins were immunoprecipitated and the immunoprecipitates analyzed by Western blot for the presence of the SOCS proteins. Full-length SOCS-1 co-immunoprecipitated with both JAK1 and JAK2, but not with mutant JAK1 (kinase dead), in cells overexpressing the respective JAK proteins, thus confirming that the SOCS-1-JAK1 interaction is phosphorylation dependent (Figure 12A). The SOCS-1 mutant lacking both the SH2 domain and the SOCS box (S1Δ82-212) co-immunoprecipitated with JAK2 weakly in comparison with full-length SOCS-1. However, this association was not observed with JAK1 (Figure 12A). These data suggest that the N-terminal region of SOCS-1 can, under certain conditions, associate independently with JAK and may contribute to inhibition of kinase activity.

Discussion

A total of eight SOCS proteins have now been identified that contain a C-terminal SOCS box and an SH2 domain preceded by an N-terminal region of variable length (Hilton *et al.*, 1998). Several of these proteins have been found to regulate cytokine signal transduction negatively, SOCS-1 through direct inhibition of the JAK family of protein tyrosine kinases (Endo *et al.*, 1997; Naka *et al.*, 1997) and CIS by competing with Stat5 for phosphotyrosine residues within receptor subunits (Yoshimura *et al.*, 1995). However, very little is known regarding the action of other related SOCS proteins or the structural requirements for SOCS protein function.

Here we have shown that there is a high degree of functional specificity among the SOCS proteins. SOCS-3, like SOCS-1, was found to inhibit IL-6 and LIF signal transduction profoundly, while SOCS-5 appeared partially to inhibit IL-6 and LIF signaling. SOCS-2, SOCS-6 and

Fig. 9. Mutational analysis of SOCS-3. Upper panels: levels of transiently expressed SOCS-3 mutant proteins from representative experiments were determined by Western blot with anti-Flag antibody. Lower panels: 293T cells were transiently transfected with vector alone or cDNAs expressing the various SOCS-3 mutant proteins in the presence of the APRE-luc and $Sr\alpha$ - β -gal reporter genes. Cells were incubated in the presence (+) or absence (-) of 10 ng/ml hLIF overnight and cell extracts prepared. Luciferase activity from triplicate samples was determined and normalized against β -galactosidase activity. (A) 293T cells were transiently transfected with full-length SOCS-3 or SOCS-3 N-terminus swap mutants S1/3/3, S2/3/3, C/3/3, S5/3/3 and S6/3/3. (B) 293T cells were transiently transfected with full-length SOCS-3 or SOCS-3 SH2 domain swap mutants S3/1/3, S3/2/3, S3/C/3, S3/5/3 and S3/6/3. (C) 293T cells were transiently transfected with full-length SOCS-3 or SOCS-3 point mutants S3-L22A, S3-F25A and S3-E30R. (D) Sequence alignment between the N-terminal regions immediately preceding the SH2 domains of SOCS-1, SOCS-3, SOCS-4, SOCS-5 and a *Drosophila melongaster* homolog of SOCS-5 (Dros.). Alignments were carried out by inspection, and gaps (indicated by dashes) introduced to improve sequence similarity. Residues shaded in black indicate identity, residues shaded in gray indicate similarity and residues shaded in light gray indicate conservation between both SOCS-1 and SOCS-3 or SOCS-4 and SOCS-5. The sequence for the *Drosophila* SOCS-5 homolog was obtained from DDBJ/EMBL/GenBank accession No. AC005131.

Fig. 10. The SOCS-1 and SOCS-3 SH2 domains are required for inhibition of LIF signaling. Wild-type SOCS-1 (S1), SOCS-2 (S2), SOCS-3 (S3), CIS or SOCS proteins containing either a single point mutation in the SH2 domain (S1-R105K, S2-R73K, S3-R71K and CIS-R107K) or three point mutations in the SH2 domain (S3-SH2mut) were transiently expressed in 293T cells in the presence of the APRE-luc and $Sr\alpha$ - β -gal reporter genes. Upper panels: protein expression levels from representative experiments were determined by Western blot with anti-Flag antibody. Lower panels: 293T cells transiently transfected with cDNAs expressing wild-type SOCS proteins or various SOCS SH2 mutant proteins were incubated in the presence (+) or absence (-) of 10 ng/ml hLIF overnight and cell extracts prepared. Luciferase activity from triplicate samples was determined and normalized against β -galactosidase activity.

CIS showed little or no activity. Functional analysis of SOCS-1 revealed two distinct domains that were required for protein function; in addition to the SH2 domain, the 28 amino acids immediately N-terminal of the SH2 domain were also critical for function. Mutational analysis of SOCS-3 further suggested that the requirement for the N-terminal region might extend to other members of the SOCS family. Interestingly, a *Drosophila* homolog of SOCS-5 retains sequence identity both in the SH2 domain and in the 20 amino acids immediately preceding it (Figure 9D). This evolutionary conservation of the N-terminal region implies a conserved function within the SOCS family proteins.

At least 20 proteins have now been identified which contain a C-terminal SOCS box (Hilton *et al.*, 1998). Somewhat unexpectedly, deletion of either the SOCS-1 or SOCS-3 SOCS box did not alter the ability of these proteins to inhibit LIF induction of luciferase activity in 293T cells or LIF- and IL-6-induced macrophage differentiation of M1 cells (Figures 7 and 8; unpublished data). Whether this region is also dispensable in a physiological setting is unclear and currently is being addressed.

In vitro, both the SOCS-1 N-terminus and SH2 domain were required to inhibit JAK1 kinase activity. This is in agreement with LIF induction of M1 cell differentiation and transcription of the Stat3 reporter gene, i.e. that both domains are functionally required to inhibit LIF and IL-6 signaling. In contrast, either the SOCS-1 N-terminus or

Fig. 11. The N-terminus and SH2 domain of SOCS-1 contribute to inhibition of JAK1 and JAK2 kinase activity. COS or 293T cells were transiently transfected with vector alone (–) or cDNAs expressing either Flag-tagged SOCS-1 (S1) or the SOCS-1 deletion mutants S1Δ170–212, S1Δ1–76&170–212, S1Δ1–76 and S1Δ82–212 in the presence of cDNA expressing either Flag-tagged JAK1 (+) or JAK2 (+). (A) JAK1 kinase activity. (B) Immunoprecipitates from (A) were split prior to kinase assay and analyzed by Western blot with anti-Flag antibody (JAK1 proteins). (C) Protein expression levels. Lysates from (A) were analyzed by Western blot with anti-Flag antibody (SOCS-1 proteins). (D) JAK2 kinase activity. (E) Immunoprecipitates from (D) were split prior to kinase assay and analyzed by Western blot with anti-Flag antibody (JAK2 proteins). (F) Protein expression levels. Lysates from (D) were analyzed by immunoprecipitation and Western blot with anti-Flag antibody (SOCS-1 proteins; left panel) and Western blot with either anti-Flag (SOCS-1 proteins; center panel) or anti-myc antibody (Stat1 and Stat5; right panel).

Fig. 12. The N-terminus of SOCS-1 associates with JAK2. 293T cells were transiently transfected with either vector alone (–), cDNA expressing Flag-tagged SOCS-1 (SOCS-1) or cDNA expressing Flag-tagged N-terminus (S1Δ82–212) in the presence of cDNA expressing either Flag-tagged JAK1 (+), kinase-dead JAK1 (JAK1-KD) (+) or JAK2 (+). (A) SOCS-1 association with JAK1 and JAK2. After 48 h, cells were lysed and JAK proteins immunoprecipitated using specific anti-JAK antibodies. Immunoprecipitates were then analyzed for the presence of either full-length SOCS-1 or SOCS-1 N-terminus (S1Δ82–212) by Western blot with anti-Flag antibodies. (B and C) Protein expression levels. Lysates from (A) were analyzed by Western blot with anti-Flag antibody. (B) JAK proteins, (C) SOCS-1 proteins.

SH2 domain alone were sufficient to inhibit JAK2 kinase activity. This may indicate that SOCS-1 has a differential ability to inhibit JAK1 and JAK2, or may simply result from variations in protein expression levels. Co-immunoprecipitation of the SOCS-1 N-terminal region with JAK2 suggested that the N-terminal region represented an independent binding domain. If the N-terminus and SH2 domains interact with JAK and both contribute to inhibition of kinase activity, this may involve either a sequential interaction with JAK or co-operative binding between the two domains and JAK. SOCS-1 was also identified by its ability to associate with TEC protein tyrosine kinase, the authors demonstrating that the N-terminal region was able to mediate this interaction (Ohya *et al.*, 1997). These data document a protein–protein interaction via the N-terminus and further suggest that SOCS-1 action may not be restricted to the JAK family of protein tyrosine kinases.

Recently, a second group of SH2-containing proteins (Grb10, Grb7 and Grb14) has been identified, in which a 50 amino acid region immediately upstream of the SH2 domain is also important for protein function (He *et al.*, 1998). Certain parallels can be drawn between the two groups of proteins; both SOCS-1 and Grb10 interact with tyrosine kinases and, in each, two analogous domains are required for function. Our observations and those of He *et al.* (1998) may advance a more general model for protein function in which both the SH2 domain and a defined region upstream are required for full functional activity.

Although SOCS-1 and SOCS-3 clearly were able to negatively regulate IL-6 and LIF signal transduction via the JAK–STAT pathway, some subtle differences appeared to exist between the actions of the two proteins. SOCS-3

may block JAK association with receptor subunits or block JAK access to substrate molecules such as Stat3. Alternatively, SOCS-3 may parallel the action of CIS by binding to receptor phosphotyrosine residues and blocking Stat3 recruitment to the receptor. The inability of SOCS-3 to inhibit either JAK1 or JAK2 *in vitro* kinase activity suggests that SOCS-3 does not act by directly inhibiting JAK kinase activity. Although difficult to explain, the apparent SOCS-3 enhancement of JAK1 kinase activity could result from either SOCS-3 interaction with a JAK1 inhibitor or stabilization of the JAK1 dimer. It remains possible that post-translational modification of SOCS-3 is required before it can inhibit JAK activity and that this does not occur in transient expression systems in the absence of ligand stimulation. Experiments are currently underway to investigate SOCS-3 association with various components of the IL-6 and LIF signaling pathways.

Mutagenesis of the SH2 domains provided further evidence for the differential activity of SOCS-1 and SOCS-3. In contrast to SOCS-1, mutation of the conserved arginine in the SOCS-3 SH2 domain did not alter the ability of SOCS-3 to negatively regulate the Stat3-responsive reporter gene. Additional conservative mutations within the SH2 phosphotyrosine-binding loop were required before a dramatic effect on SOCS-3 function was observed, suggesting that the SH2 domains of SOCS-1 and SOCS-3 may confer different binding affinities. Although there appear to be distinct differences between the mechanism of SOCS-1 and SOCS-3 inhibition of IL-6 and LIF signaling, the N-terminal region, and to some extent the SH2 domains are interchangeable. This raises the interesting possibility that by swapping the SOCS-1 and SOCS-3 N-termini, we in fact changed the site or mechanism of protein action. Further experiments are needed to address this possibility.

Materials and methods

SOCS expression vectors

The cDNAs encoding mouse SOCS-1, SOCS-2, SOCS-3, SOCS-5 and SOCS-6 have been described previously (Starr *et al.*, 1997; Hilton *et al.*, 1998). Constructs encoding SOCS and CIS proteins with an N-terminal Flag epitope tag (DYKDDDDK) were generated by PCR to give fragments with in-frame *MluI* restriction enzyme sites at both the N- and C-termini and were subcloned into the mammalian expression vector pEF-FLAG-I (found at <http://www.wehi.edu.au/willson> vectors) (Figure 1).

SOCS-1 deletion mutants

SOCS-1 deletion mutants were generated by PCR to give fragments with in-frame *AscI* and *MluI* restriction enzyme sites at the N- and C-termini, respectively, and subcloned into pEF-FLAG-I to give proteins with an N-terminal Flag epitope tag (Figure 1).

Domain swap mutants

To facilitate the synthesis of hybrid mouse SOCS-1 cDNAs, an *XhoI* site was introduced at the boundary between the N-terminal region and SH2 domain of the SOCS-1 cDNA. Two nucleotide changes were introduced using the PCR-based technique, splicing by overlap extension (Horton *et al.*, 1989), and the PCR fragment was cloned into the *KpnI* and *SacI* sites of pBluescript SK II (+) (Stratagene). To facilitate cloning of the DNA fragments into pEF-FLAG-I, an in-frame *AscI* restriction enzyme site was introduced one amino acid after the predicted translational start site, and an *MluI* site was inserted immediately before the stop codon of the mouse SOCS-1 cDNA. Since the C-to-G nucleotide alteration leads to a D-to-E amino acid substitution at position 76, the altered SOCS-1 cDNA was cloned into the *MluI* site of pEF-FLAG-I and shown to have identical activity to wild-type SOCS-1 in the luciferase

assay (data not shown). Hybrid cDNAs, in which the SH2 domain of the SOCS-1 sequence was replaced by homologous regions of mouse CIS, SOCS-2, -3, -5 or -6, were synthesized from PCR-generated restriction fragments (SH2 domain fragments were cloned in as *XhoI*-*NotI* fragments). Hybrid cDNAs, in which the N-terminal region of the SOCS-1 sequence was replaced with homologous regions of mouse CIS, SOCS-2, -3, -5 or -6 were generated using the PCR-based technique, splicing by overlap extension. All hybrid cDNAs were then cloned into the *MluI* site of pEF-FLAG-I in order to express mouse SOCS-1 domain swap mutant proteins with an N-terminal Flag epitope tag (Figure 1).

Point mutants

SOCS-1 and SOCS-3 point mutants were generated using the PCR-based technique, splicing by overlap extension. PCR fragments with in-frame *AscI* and *MluI* restriction sites at the N- and C-termini, respectively, were then subcloned into pEF-FLAG-I to give proteins with an N-terminal Flag epitope tag.

Construction of SH2 SOCS mutants

SH2 mutants in which the invariant arginine was replaced by lysine were generated using the PCR-based technique, splicing by overlap extension. The resulting constructs, SOCS-1-R105K, SOCS-2-R73K, SOCS-3-R71K and CIS-R107K encode SOCS-1, SOCS-2, SOCS-3 and CIS proteins with arginine to lysine amino acid substitutions at the equivalent position in the SH2 domain. A SOCS-3 construct was also generated containing two additional mutations in the SH2 domain (S3-SH2mut). This construct encodes a SOCS-3 protein containing an arginine to lysine (R71K), an aspartic acid to glutamic acid (D72E) and a serine to cysteine substitution (S73C). In-frame *AscI* and *MluI* sites were inserted at the N- and C-termini, respectively, and the PCR-generated fragments subcloned into pEF-FLAG-I.

All constructs were sequenced in their entirety before use.

JAK constructs

The kinase-dead mutants of JAK1 (JAK1-KD) and JAK3 (JAK3-KD) have been described previously (Briscoe *et al.*, 1996; Chen *et al.*, 1997). The kinase-dead JAK2 mutant (JAK2-KD) was generated by enzymatic digestion with *NdeI* and *BclI*, and blunt-end ligated together creating an in-frame stop codon, effectively deleting amino acids 934–2001 in the JAK2 JH1 domain.

Stable transfection of cell lines

The murine monocytic leukemic cell line, M1, was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. M1 cell derivatives were generated by electroporation with the pEF-FLAG-I expression vector carrying cDNAs encoding the various SOCS proteins and the pGKPUroA expression vector. Cells were selected with 20 µg/ml puromycin (Sigma, St Louis, MO). SOCS expression was determined by immunoprecipitation and Western blot with anti-Flag antibody (M2; Eastman Kodak Co., New Haven, CT). Several independently cloned cell lines were obtained for each construct.

Transient transfection of cell lines

293T cells were maintained in DMEM containing 10% fetal serum. SOCS-expressing cells were generated using FuGENE 6 transfection reagent (Boehringer Mannheim, Mannheim, Germany) with the pEF-FLAG-I expression vector carrying cDNA encoding the various SOCS proteins, according to the manufacturer's instructions, and harvested after 48 h. COS cells were maintained in RPMI containing 10% fetal calf serum. SOCS-expressing cells were generated by electrophoretic transfection. Cells were harvested after 48 h for analysis.

M1 cell colony assays

Cultures were performed in 35 mm plastic Petri dishes, as described previously (Metcalf, 1984). Briefly, 100 cells were cultured in 1 ml of 0.3% agar (Difco Laboratories, Detroit, MI) in DMEM containing 10% bovine serum. For each titration point, 0.1 ml of mIL-6 (titrated from 1 µg/ml) was added to the Petri dish prior to the addition of the cell suspension. Cultures were incubated at 37°C in a humidified incubator containing 10% CO₂ for 7 days. The dishes were then scored for the percentage of differentiated colonies, as judged by colonies with a halo of dispersed cells.

Luciferase assay

293T cells were plated into either 24-well plates at 1×10^5 cells/well or 6-well plates at 5×10^5 cells/well. The LIF-responsive promoter-luciferase reporter gene (APRE-luc) was a kind gift from A.Yoshimura and

has been described previously (Endo *et al.*, 1997). The positive control vector $\text{Src}\alpha$ - β -gal encoding the β -galactosidase gene has also been described (Ogilvy *et al.*, 1998). Plasmids of reporter genes with either pEF-FLAG-I or pEF-FLAG-SOCS constructs were introduced into cells using FuGENE 6 transfection reagent (Boehringer Mannheim) and harvested after 48 h. Cells were incubated overnight with or without 10 ng/ml recombinant human LIF (hLIF) (Amrad, Melbourne, Australia) prior to lysis with 40 μ l of Reporter Lysis Buffer (Promega, Madison, WI) containing protease inhibitors (Complete Cocktail tablets, Boehringer Mannheim). Lysates were then assayed for luciferase activity using Luciferase Assay Substrate (Promega) according to the manufacturer's instructions and analyzed using a ML3000 microtiter plate luminometer (Dynatech Laboratories Inc., Chantilly, VA). β -Galactosidase activity was measured by spectrophotometric assay as described (MacGregor *et al.*, 1992). SOCS protein expression was analyzed by immunoprecipitation and Western blot with anti-Flag antibodies.

Immunoprecipitation and Western analysis

M1 cells were grown in serum-free medium for 1–2 h prior to incubation with 5×10^5 U/ml recombinant mouse IL-6 (mIL-6) and lysed in KALB lysis buffer (Nicholson *et al.*, 1995) containing protease inhibitors (Complete Cocktail tablets), 1 mM Na_3VO_4 and 1 mM NaF. 293T cells were grown under the described conditions prior to cell lysis. Proteins were immunoprecipitated with anti-Stat3 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-JAK1 (Transduction Laboratories) or anti-JAK2 antibodies (UBI; Upstate Biotechnology Inc., Lake Placid, NY) and protein A-Sepharose (Pharmacia, Uppsala, Sweden) or anti-Flag antibody conjugated to Sepharose (M2; Eastman Kodak Co.) and separated on SDS-PAGE (Bio-Rad, Hercules, CA) under reducing conditions. Protein was then electrophoretically transferred to PVDF-Plus membranes (Micron Separations Inc., Westborough, MA). Membranes were blocked overnight in 10% skim milk and incubated with primary antibody for 2 h. Antibody binding was visualized with either peroxidase-conjugated anti-rabbit Ig (Silenus, Melbourne, Australia) or peroxidase-conjugated anti-mouse IgG, which specifically recognizes the immunoglobulin heavy chain (Jackson ImmunoResearch Laboratories Inc., West Grove, PA), and the enhanced chemiluminescence (ECL) system (Amersham, Little Chalfont, UK). To re-blot, the membrane was first stripped of antibodies by 62.5 mM Tris-HCl, pH 6.7, 2% SDS, 100 mM 2-mercaptoethanol.

In vitro kinase assay

In vitro kinase assays were performed as described previously (Nicholson *et al.*, 1995).

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