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Negative regulators of cytokine signal transduction

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Abstract. Enormous advances have been made over the last 10 years in unravelling cytokine signal transduction. This work has led to the recognition of the prime importance of Janus kinases (JAKs) and signal transducers and activators of transcription (STATs). More recently, the importance of negative regulators of this pathway has begun to be realised. There is now evidence for at least three families of proteins that inhibit JAK/STAT signalling. The supressors of cytokine signalling (SOCS), protein inhibitors of activated STATs (PIAS) and the SH2-containing phosphatase (SHP-1). This review describes some of the key features of SOCS proteins and contrasts their actions with other negative regulators, the PIAS proteins and SHP-1.

Key words. Cytokines; signal transduction; negative regulation.

Background

Four alpha-helical-bundle cytokines regulate a bewildering array of physiological processes. These range from key aspects of embryonic development such as implantation, decidualisation and the formation and maintenance of the nervous system, through to regulation of the production of at least nine distinct lineages of blood cells throughout adult life and the orchestration of the response of these cells to stresses such as infection and bleeding [1]. Ensuring that a cell response is appropriate requires that a cytokine is recognised by binding to a specific cell surface receptor, that the appropriate intracellular signal transduction cascade is initiated and that this cascade is switched off or tempered in a timely manner.

Despite their diverse biological roles, many common themes have emerged in cytokine signal transduction. Each of the cytokines binds to and induces dimerisation of members of the haemopoietin receptor family (fig. 1). These are defined by conserved sequence motifs in their extracellular domain and while they lack intrinsic kinase activity, cytokine-mediated receptor multimerisation leads to rapid tyrosine phosphorylation of a range of integral membrane and cytoplasmic proteins [2]. The current model for the crucial events in signal transduction has been reviewed in depth elsewhere [3–7].

The central mediators of phosphorylation following interaction of cytokines with their cognate receptors are the Janus kinases (JAKs). There are four members of the JAK family, JAK1, JAK2, JAK3 and TYK2, and each binds to the membrane-proximal region of the cytoplasmic domain of a specific subset of haemopoietin receptors. Upon receptor multimerisation, JAKs are brought into close proximity to each other leading to cross-phosphorylation and catalytic activation (figs 1, 2). Once activated, JAKs then phosphorylate a number of tyrosine residues in receptor cytoplasmic domains; in turn these provide docking sites for cytoplasmic proteins that contain SH2 or other phosphotyrosine binding motifs (fig. 1). Among such proteins are the signal transducers and activators of transcription (STATs). Once bound to haemopoietin receptors, STATs are themselves phosphorylated by JAKs, leading to dissociation from the receptor, and homo- or hetrodimerisation through a reciprocal SH2:phosphotyrosine interaction and migration to the nucleus (figs 1, 2). Within the nucleus, STAT dimers bind to recognition sequences in target genes to increase transcription. Among these target genes are a second wave of transcription factors [e.g. IRF-1 and CIITF in the case of interferon (IFN)-γ] that, with the STATs, orchestrate the changes in transcription necessary for a full biological response [8, 9]. The generation of knock-

out mice has, in the face of seemingly contradictory in vitro data, highlighted the importance of specific JAKs and STATs in mediating the response to specific cytokines.

The importance of negative regulation of signal transduction of the JAK/STAT pathway has long been suspected from an abundance of experiments in which cytokine levels have been experimentally elevated, either through injection of purified cytokine or by transgenesis, leading to derangement of normal physiological processes and disease. Recently, three protein families, the suppressors of cytokine signalling (SOCS), protein inhibitors of activated STATs (PIAS) and cytoplasmic phosphatses have been implicated in controlling cytokine signalling.

Discovery of SOCS proteins

The SOCS proteins were discovered by three groups at about the same time using quite different strategies [10–12]. The strategy developed by Robyn Starr and myself was based on the proposition that we could identify negative regulators of signal transduction by their ability to render cytokine-responsive M1 leukaemia cells insensitive to interleukin (IL)-6 [12]. To this end, we infected M1 cells with a retroviral cDNA library constructed from FDCP-1 cells and selected a single colony that failed to differentiate when stimulated by IL-6, from which we isolated the cDNA encoding SOCS-1 [12]. Kishimoto and colleagues identified the SOCS-1 protein based on its immunocross-reactivity to the SH2 domain of STAT3 and named it STAT-induced STAT inhibitor-1 (SSI-1) [11], while Yoshimura's group identified SOCS-1 as a protein capable of binding to JAK2 in a yeast two-hybrid screen and named it JAK-binding protein (JAB) [10].

When the predicted amino acid sequence of the SOCS-1 protein was compared to the database, the closest known relative to SOCS-1 was found to be CIS (cytokine inducible SH2-containing protein). CIS was also isolated by Yoshimura's group, this time using a screen to isolate genes whose transcription was induced following cytokine stimulation [13]. Like SOCS-1, CIS contained a central SH2 domain and a novel 40-amino-acid motif at the very C terminus that has been named the SOCS box (figs 1, 2) [12]. Further database searching has revealed the existence of six other mammalian SOCS proteins (SOCS-2 to SOCS-7) and a *Drosophila melanogaster* SOCS protein which appears most similar to SOCS-5 (figs 1, 2) [14, 15]. SOCS-1, SOCS-2, SOCS-3 and CIS show relatively low levels of sequence similarity, in the order of 25–30%. SOCS-4 and -5, and likewise, SOCS-6 and -7 have almost identical SH2 domains but divergent N-terminal domains [14]. A partial cDNA for SOCS-7 was also isolated independently on the basis of its capacity to bind to Nck and Ash in a yeast two-hybrid screen, and was called NAP-4 [16].

While SOCS proteins contain an SH2 domain and a C-terminal SOCS box, five other groups of proteins also

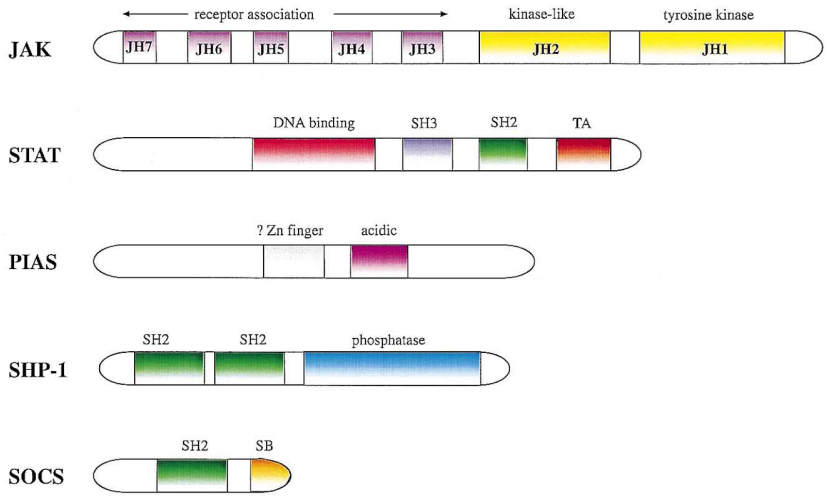

Figure 1. Schematic illustration of the structures of the key signalling molecules JAK and STAT and signal transduction inhibitors PIAS, SHP-1 and SOCS. Conserved protein domains are highlighted.

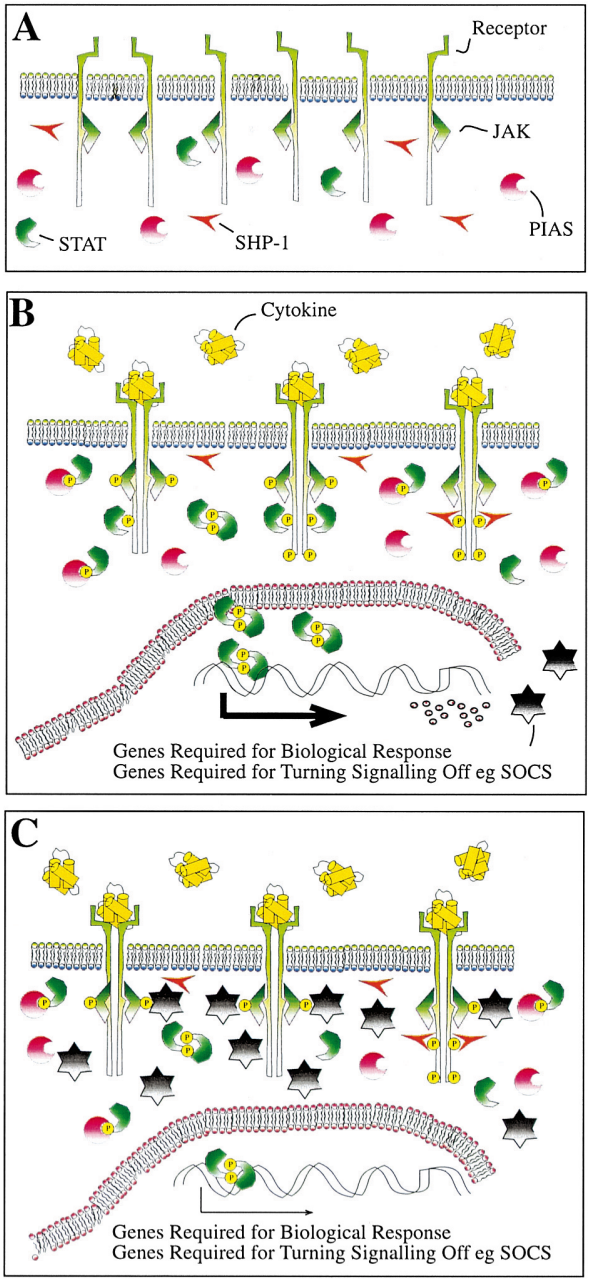

Figure 2. Speculative model of cytokine signalling. (*A*) Prior to cytokine stimulation, receptors are monomeric and associated JAK kinases are inactive. STAT remains monomeric, unphosphorylated and in the cytoplasm. PIAS and SHP-1 are present in the cytoplasm awaiting initiation of the signal transduction cascade. (*B*) At the height of a cytokine response, cytokine has induced receptor dimerisation, resulting in juxtaposition of JAKs and their activation by cross-phosphorylation. JAKs phosphorylate receptors and STATs. Phosphorylated JAKs and receptors provide docking sites for SHP-1 which dephosphorylates and inactivates JAK. Once phosphorylated, STATs are in equilibrium between formation of inactive complexes with PIAS and formation of active dimers which migrate to the nucleus and increase the rate of transcription of target genes. Among these genes are those required for mediating the biological effect of the cytokine and those, like the SOCS genes, which limit this biological response. (*C*) Once SOCS proteins are synthesised, they act back on the JAK/STAT pathway, in the case of SOCS-1, binding to and inactivating JAKs, and therefore dampening down the response to cytokine.

contain C-terminal SOCS boxes but have different N-terminal protein domains [14]. In mouse and human, there are at least four ASB proteins (ASB-1 to ASB-4) which have ankyrin repeats in front of their SOCS box, at least three SSB proteins (SSB-1 to SSB-3) which have SPRY domains, at least two WSB proteins (WSB-1 and WSB-2) which both have eight WD-40 repeats, and two highly related proteins which have a GTPase domain in addition to a C-terminal SOCS box [14]. The function of these proteins is unknown and there is no evidence that they play any role in regulating cytokine signal transduction.

Regulation of SOCS gene transcription and protein production

As described above, CIS, the first member of the SOCS protein family to be described, was isolated from a cDNA library prepared from mRNA from Ba/F3 cells stimulated through the erythropoietin (EPO) receptor and subtracted against a library prepared from unstimulated cells. Random clones from the cDNA library enriched for cytokine-inducible genes were sequenced and one of these contained an SH2 domain and was named cytokine-inducible SH2-containing protein (CIS) [13]. Transcription of the CIS gene is virtually undetectable in unstimulated cells, but is induced rapidly, within 30 min, upon stimulation by a range of cytokines [13]. Given the structural similarity between CIS and other SOCS family members it did not take a great leap of imagination to ask whether transcription of these genes was also regulated by cytokine. Again for SOCS-1, SOCS-2 and SOCS-3, there is ample evidence that in vitro stimulation of many cell types by different cytokines and injection of cytokines in vivo results in increased mRNA expression [11, 12, 14, 17–19]. There have been relatively few studies examining the production of native SOCS proteins; however, where this has occurred, it appears that the kinetics of mRNA production mirror the production of the protein (J.-G. Zhang and D. J. Hilton, unpublished observation). This suggests perhaps that the SOCS proteins are very short-lived.

There is no truly clear picture emerging about which cytokines induce expression of which SOCS genes; however, a number of conclusions can be made tentatively.

1) In many cell lines, Northern blot analyses show SOCS-1, -2, -3 and CIS expression to be induced by the same cytokine—notably IL-6 and growth hormone (GH) in the liver following cytokine injection [12, 17], granulocyte-macrophage-colony-stimulating factor (GM-CSF) stimulation of UT-7 cells, EPO stimulation

of F36E cells [20] and GH and leukaemia inhibitory factor (LIF) stimulation of 3T3-F442A adipocyte cells [17]. In the latter cases, it appears that SOCS-3 is the predominant family member induced.

2) Under certain situations there appears to be a level of specificity observed in the types of SOCS proteins induced by a particular cytokine; however, again there is no clear picture emerging. In M1 cells stimulated by IL-6, SOCS-1 and CIS, but not SOCS-2 or SOCS-3 are induced [12]. In M1 cells stimulated by IFN-γ, SOCS-1 is induced but not SOCS-2, SOCS-3 or CIS, but in NIH-3T3 cells, IFN-γ stimulates SOCS-1 and SOCS-3, but not SOCS-2 or CIS [21, 22]. By RT-PCR, while SOCS-1, SOCS-2, SOCS-3 and CIS are all expressed in the hypothalamus, only the expression of SOCS-3 is enhanced by leptin, and this is clearly apparent also by in situ hybridisation [18]. Likewise, IL-10 appears to be able to preferentially induce SOCS-3 expression in human monocytes [19].

3) Again, where compared in the same study, the kinetics of induction of SOCS-1, SOCS-2, SOCS-3 and CIS appear to be similar, being induced within 15–30 min of cytokine stimulation; however, expression of the SOCS-1 and SOCS-3 genes generally appears to be more transient than that of SOCS-2 or CIS [12, 17].

4) Although only studied in a limited way, no compelling evidence has yet been obtained to show that expression of SOCS-4, SOCS-5, SOCS-6 or SOCS-7 is induced by cytokine [14, 20].

Given that SOCS proteins are produced as part of a normal cell response to cytokine, it is useful to ask which signal transduction pathways are responsible for their increased expression. The promoter for CIS contains four potential STAT5-binding sites. When driving a reporter construct, the region of the CIS promoter containing the STAT5-binding sites is required for cytokine. Moreover, in cells expressing truncated STAT5 proteins lacking the C-terminal transactivation domain, which act in a dominant negative manner, the capacity of cytokine to induce CIS expression is diminshed [23]. Likewise, M1 cells expressing a dominant negative form of STAT3 show a diminished induction of SOCS-1 expression following IL-6 or LIF stimulation [11]. Perhaps the most direct evidence for the importance of STAT5 in CIS expression comes from examination of STAT5a/STAT5b double-knockout mice. These mice fail to show expression of CIS in the ovary, a prolactin-responsive organ [24].

While each of these lines of evidence points toward the importance of STATs in the induction of SOCS expression, there is little evidence to suggest that there is a one-to-one correspondence between the activation of a particular STAT and the expression of a particular SOCS. Rather, it is more likely that STAT proteins will be capable of increasing the expression of several SOCS proteins and, reciprocally, SOCS protein expression may be increased by more than one STAT. The most definitive experiments are likely to involve analysis of the expression of various SOCS proteins in response to physiological levels of cytokine in mice lacking one or more STAT proteins.

Overexpression studies show SOCS proteins inhibit cytokine signal transduction

A great deal of work has been carried out examining the function of SOCS genes in enforced expression systems, in vitro using cell lines and in vivo using transgenic animals. In every system tested to date, SOCS-1 has been found to inhibit cytokine signalling. Notably, SOCS-1 profoundly inhibits IL-6, LIF, oncostatin M, IFN-γ, IFN-α, IFN-β, thrombopoeitin (TPO) and GH signalling [10–12, 15, 17, 21, 22]. SOCS-3 actions appear to parallel that of SOCS-1 in many respects. Enforced SOCS-3 expression can inhibit IFN-α, IFN-β and IFN-γ signalling, though not as well as SOCS-1 [22; J. Fenner and P. Hetzog, personal communication]. In contrast, SOCS-3 appears to be a more potent inhibitor of leptin and GH signalling. CIS has also been found to inhibit signalling though its action is not as widespread or potent as either SOCS-1 or SOCS-3, being restricted to cytokines such as EPO and IL-3 [13, 25]. SOCS-2 in contrast has been found to be weakly able to inhibit IL-6 signalling, but where tested shows no activity in other assays [15]. There have been few studies of the biological effect of expression of SOCS-4, SOCS-5, SOCS-6 or SOCS-7; however, there is some evidence, again from reporter construct assays, that SOCS-5 but not SOCS-6 may be a weak inhibitor of IL-6 signalling [15].

While useful in showing that SOCS proteins can act as inhibitors of signalling, these studies must be interpreted with extreme caution for many reasons, the most important of which are: (i) the expression levels of SOCS proteins, in most cases, are likely to be much higher than normally achieved, and (ii) enforced expression is temporally inappropriate. As mentioned above, a key feature of SOCS protein expression is inducibility by cytokine. In the simplest situation, when cytokine stimulates a cell for the first time, little or no SOCS will be present, allowing signalling to proceed for a time until it is inhibited by SOCS produced as a result of activation of STATs.

Analysis of SOCS knockout mice

In general, it is likely that in vitro studies of protein action suggest far broader roles than occur physiologically. In a sense, these studies show what a protein can do, rather than what it does do under physiological circumstances. This is likely to be the case for the SOCS proteins, as it was for STAT1. In vitro, STAT1 appears to be activated in response to a bewildering array of cytokines and growth factors, yet the real clue to its physiologically important role in IFN signalling came from the study of knockout mice [26, 27].

Unlike the dramatic phenotype observed for SOCS-1-deficient mice, unpublished observations of the CIS knockout mice suggest that it has no obvious phenotype (S. N. Ihle and A. Yoshimura, unpublished observations).

SOCS-1 (SOCS-1 $-/-$) knockout mice have been generated by two laboratories, with similar results [12, 28]. SOCS-1 $-/-$ mice develop normally through embryogenesis and are born in the expected Mendelian frequency. For the first few days of life, SOCS-1 $-/-$ mice are indistinguishable from their littermates; however, after a week they fail to gain weight and become moribund between 2 and 3 weeks of age, with none remaining healthy enough to wean. There appear to be three major cellular abnormalities in SOCS-1 $-/-$ mice. (i) Mice are severely lymphopaenic, with a dramatic (10- to 30-fold) reduction in all thymic subsets and a profound loss of pre-B and mature B cells, with little reduction in pro-B cells. Other haemopoietic cells appear relatively normal. (ii) Severe fatty degeneration of the liver. (iii) Macrophage infiltration of several organs including the liver, heart, lung and skin. Clearly, lymphoid cells are not required for mouse survival in clean animal rooms and the morbidity of the SOCS-1 $-/-$ mice is more likely to be due to their liver disease.

Naka, Kishimoto and colleagues have noted that the lymphopaenia is due to increased apoptosis and have shown that these cells contain elevated levels of the pro-apoptotic protein Bax [28]. They speculate that SOCS-1 may be able to regulate the apoptotic pathways directly or indirectly through signalling pathways other than the JAK/STAT pathway. There are perhaps more parsimonious hypotheses. While many cytokines acting in the haemopoietin system act via the JAK/STAT pathway to prolong survival, others, such as IFN-γ, are themselves proapoptotic. An alternative explanation for the phenotype of the SOCS-1 $-/-$ mice is that they are hyper-responsive to a cytokine such as IFN-γ.

Biochemical mechanism of SOCS protein action

To date, every experiment involving identification of proteins with which SOCS proteins might interact has involved overexpression of at least one and in most cases both the proteins of interest. These studies have involved yeast two-hybrid analysis, transient overexpression of epitope-tagged proteins in COS and 293 cells and stable overexpression of proteins in a variety of cell lines. Not a single experiment has involved analysis of SOCS proteins produced at normal levels in cells stimulated by cytokine.

There is little doubt that overexpression of SOCS proteins inhibits the JAK/STAT pathway. How this occurs is more problematic. Overexpression of JAKs leads to their phosphorylation and activation independent of cytokine. In this setting, SOCS-1 is capable of interacting with all four members of the JAK family, JAK1, JAK2, JAK3 and TYK2, and inhibiting their phosphorylation and catalytic activity [10, 11, 15]. Similarly, overexpressed SOCS-1 can inhibit IFN-γ-induced phosphorylation of JAK1, JAK2 and TYK2 in NIH-3T3 cells [21]. While the capacity of SOCS-1 to bind and inhibit the activity of members of the JAK family is very reassuring given the capacity of SOCS-1 to inhibit signalling by a spectrum of cytokines, the interaction with JAKs can hardly be said to be specific. In similar overexpression systems, SOCS-1 has been shown to interact with kit, the fibroblast growth factor (FGF) receptor, PYK2 and Tec [13], raising the possibility that the in vitro systems are capable of detecting promiscuous interactions with no physiological relevance, or that SOCS-1 may have a far broader role than inhibition of the JAK/STAT pathway.

Similarly broad interactions have been attributed to SOCS-3. Using the yeast two-hybrid system, SOCS-3 has been observed to interact with the JAK2 kinase domain, the FGF receptor, PYK2 and active kit [20]. SOCS-3 has also been found to interact with the JAK2 kinase domain in glutathione S-transferase (GST) pull-down experiments [20]. In a 293 cell overexpression system, we have been unable to show any inhibition of JAK1 or JAK2 phosphorylation or kinase activity by SOCS-3 despite the activity of SOCS-1 in parallel assays and the capacity of SOCS-3 to block LIF and IL-6 signalling in 293 cells [15]. With all the caveats in place, it might be argued that while SOCS-1 might act through direct interaction with JAKs and inhibition of their catalytic activity, SOCS-3 may block the JAK/STAT pathway at a different point. Where remains unclear.

Like SOCS-3, the evidence points toward CIS inhibiting signalling via a mechanism other than inhibiting JAK kinase. Yoshimura and colleagues have shown that CIS is capable of interacting with the EPO receptor and βc chain of the IL-3 receptor in a phosphorylation-dependent manner [13, 25]. This has led to the speculation that CIS may act by competing with STAT molecules for binding to phosphorylated receptor cytoplasmic domains, thereby preventing their phosphorylation and

subsequent activation. Mayeux and colleagues have also shown interaction of CIS with the EPO receptor in a tyrosine-phosphorylation-dependent manner in a number of cells [29]. Further, they have shown that mutation of a second tyrosine (Y401) to phenylalanine in the EPO receptor cytoplasmic domain abolished binding of CIS, but left intact STAT activation through Y343, a tyrosine to which CIS cannot bind. This suggests that CIS may act by a mechanism other than competition with STATs for receptor binding [29]. Evidence that SOCS proteins may act, in part, by targeting signalling molecules for degradation is discussed below.

The N-terminal and SH2 domains of the SOCS proteins are required for inhibiting signalling

Structurally, SOCS proteins can be recognised as having an SH2 domain, a C-terminal 40-amino-acid domain termed the SOCS box which is shared with other protein families of unknown function including the ASBs, SSBs, WSBs and a subclass of small GTPases. The N-terminal domains of the SOCS proteins are highly variable in length and amino acid composition. An obvious question that has begun to be answered is which parts of the SOCS proteins are necessary for inhibiting signal transduction.

Simple truncation experiments using SOCS-1 have shown that in an overexpression system, the SH2 domain alone is capable of binding to the kinase domain of JAKs; however, it does not efficiently inhibit kinase activity [13, 15, 30]. Nor does the SH2 domain of SOCS-1 alone inhibit the biological effects of LIF and IL-6 in M1 or 293 cells using a reporter assay [15, 30]. The importance of the SH2 domain for SOCS function is emphasised by the inactivity of SOCS-1 and SOCS-3 proteins that have point mutations in conserved SH2 domain residues [15]. If the SH2 domain alone is necessary but not sufficient for inhibiting signalling, what else is required?

Surprisingly, in overexpression systems, SOCS-1 and SOCS-3 proteins lacking the SOCS box but containing intact N-terminal and SH2 domains appear to inhibit signal transduction well [15, 30]. This suggests that the N-terminal region of the SOCS proteins plays an important functional role. Indeed, truncation and deletion mutants of the SOCS-1 and SOCS-3 proteins demonstrate that in both cases a 20- to 30-amino-acid motif that juxtaposes the SH2 domain is the only part of the protein that is required, along with the SH2 domain, for activity [15, 30]. Within this 30-residue domain, there are clearly conserved residues present in SOCS-1, SOCS-3 and SOCS-5 but not other SOCS proteins. The production of chimaeras in which the N-terminal regions of SOCS-1, SOCS-2, SOCS-3, SOCS-5, SOCS-6 and CIS are placed on the backbone of either SOCS-1 or SOCS-3 shows that the SOCS-1 and SOCS-3 N-terminal domains are interchangeable but cannot be functionally replaced by the N-terminal region of other SOCS proteins. This observation reinforces the functional overlap observed between SOCS-1 and SOCS-3 in many in vitro experimental situations [15].

While biochemical data point tentatively toward the JAKs as the targets for SOCS-1 inhibition of signalling and it is clear that the N-terminal and SH2 domains of SOCS-1 are required for its activity, the molecular basis by which kinase activity is inhibited remains unclear.

The SOCS box mediates binding of SOCS, ASB, SSB and WSB proteins to elongin B and elongin C

Overexpression studies in 293 cells demonstrate that the SOCS-1 and SOCS-3 proteins lacking the SOCS box work as effectively as intact proteins in inhibiting IL-6 signalling [15]. Similar results have been observed in M1 cells, though while Nicholson and colleagues have shown that complete inhibition of signalling is observed when a mutant lacking the SOCS box is expressed [15], Kishimoto and colleagues suggest this mutant is only partially active. This discrepancy might be explained by differences in the level of protein expression [30].

Clearly the SOCS box does not play an indispensable role in the inhibition of cytokine signalling by SOCS-1 and SOCS-3. This is hardly surprising given the plethora of divergent proteins that contain a SOCS box at the C terminus, many of which will likely have no role in regulating cytokine signalling. Indeed, a general role for the SOCS box in regulating protein turnover has recently been established. Two separate groups have recently shown that the SOCS box interacts with a complex of elongin B and elongin C [31, 32]. In an elegant study, Conaway and colleagues noted the similarity between the sequence of the SOCS box and the regions of the Von Hippel-Lindau tumour suppressor protein and elongin A known to interact with elongin B and C [31]. This group then went on to show, using a variety of systems, that the SOCS box in SOCS, ASB, WSB and small GTPase proteins mediated their interaction with elongin B and C. Nicola and colleagues approached the problem by generating SOCS box affinity columns and asking which proteins in a cell lysate were capable of interaction with the column [1]. Again the answer was elongin B and C and again this appeared to be a general feature of SOCS boxes from all SOCS, ASB, SSB and WSB proteins tested [32].

There appears to be little dispute that the SOCS box interacts with elongin B and C; however, the consequences of this interaction are open to question. Elongin B is a 118-amino-acid protein composed pri-

marily of an N-terminal ubiquitin-like domain; elongin C is slightly smaller and shares homology with Skp1. In addition to interaction with proteins containing a SOCS box, the elongin B and C complex has also been shown to interact with a putative E3 ubiquitin ligase, cullin-2 (cul2). One possibility, therefore, is that activated JAK kinases are recognised by SOCS-1, which also binds to elongin B and C. Then, either through interaction of the UBL sequence of elongin B or ubiquitination of the SOCS or JAK proteins, the protein complex is delivered to the proteasome, where the SOCS-box-containing proteins and associated signalling molecules are degraded [32].

This model has some parallels with the phosphoprotein-ubiquitin-ligase complex (PULC) system. In this case, proteins like the cyclins Cln1 and Cln 2 are phosphorylated and bound by adaptor proteins such as cdc4, which also contain N-terminal motifs called F boxes. The F box mediates interaction with Skp-1, an elongin C homologue. In these systems, therefore, the SOCS and F box may be playing an analogous role. Skp1 then interacts with E2 and E3 ubiquitin ligases, the latter homologous to the cullins, resulting in ubiquitination of the phosphorylated substrates and their targeting for proteosomal degradation.

The model described above, in which the SOCS box couples the SOCS proteins and the signalling proteins to which they are bound to proteosomal degradation is consistent with preliminary experiments in which SOCS-3 produced following cytokine stimulation of J774 cells has been shown to have a short half-life (J.-G. Zhang and D. J. Hilton, unpublished observations) which may be extended by treatment of cells with proteasome inhibitors. In this situation, it is not clear whether SOCS-3 is itself ubiquitinated or whether it is targeted to the proteasome via interaction with the ubiquitin-like domain of elongin B. In overexpression systems, ubiquitination of CIS is detectable when it is expressed in COS cells and like the situation for native SOCS-3, its half-life is increased by proteasome inhibitors. Mayeux and colleagues further showed that activation of the EPO receptor and the half-life of CIS/EPO receptor complexes are prolonged by inhibitors of the proteasome pathway [29]. This supports the contention that SOCS proteins may not just be degraded by the proteasome themselves, but also might act as adaptors, resulting in degradation of the signalling proteins to which they bind. Evidence exists that JAKs and STATs are also degraded by the same system and indeed, JAK2 can be co-immunoprecipitated in a complex containing SOCS-1, elongin B and elongin C [31].

To date, however, there is little evidence that removal of the SOCS box increases the half-life of the protein, as might be expected if this motif couples proteins to degradation. Indeed, both Kishimoto's and Conway's groups provide evidence that deletion of the SOCS box may decrease the half-life of SOCS-1 [30, 31]. In neither study, however, are the data overwhelming. In one case, no difference is seen in the degradation when the proteins are expressed in COS cells on their own; however, when co-expressed with elongin B and C, the proteins without the SOCS box have an extended half-life [31]. Whether this reflects the normal role of the SOCS box or whether, in this system, the excessive production of free elongin B and C is competing with elongin B and C bound to SOCS proteins to extend the latter's half-life is not clear.

The question as to the fate of SOCS-box-containing proteins upon interaction with elongin C remains open and may only be addressed when the effect of deletion of the SOCS box is studied in a more physiological setting. If the SOCS box targets SOCS proteins and bound signalling molecules for proteasomal degradation, then this may be important in contributing to the mechanism by which cytokine signal transduction might be inhibited in settings in which SOCS proteins are expressed at physiological levels. The importance of the degradation of signal inhibitors may then be masked in situations where SOCS protein expression is grossly elevated. On the other hand, even when produced at normal levels, SOCS proteins may inhibit cytokine signal transduction perfectly well, the role of proteasomal degradation being to rid the cell of SOCS protein, allowing the cell to once again respond to cytokine. In this case, removing the SOCS box from a SOCS protein may render a cell hypo-responsive to a cytokine for a longer period.

Protein inhibitors of activated STATs

PIAS-1 was the first member of this family to be identified by Shuai and colleagues [33, 34]. PIAS-1 was discovered in yeast two-hybrid screens for proteins capable of interaction with the C-terminal transcriptional activation domain of STAT1 [33]. In DNA database comparisons, PIAS-1 was found to be almost identical to a previously characterised protein named Gu/RNA helicase II binding protein (GuBP). Moreover, cDNAs for three other PIAS family members were also identified in the EST database. These were termed PIAS-3, PIAS-x and PIAS-y. The PIAS proteins share about 50% amino acid identity; however, they have few well-defined protein motifs [33].

PIAS-1 and PIAS-3, as their names suggest, are inhibitors of STAT1 and STAT3, respectively. PIAS-1 preferentially binds STAT1 and to a lesser extent STAT3, while the reciprocal is true for PIAS-3. Interaction of PIAS proteins and STATs in cells requires prior

stimulation by cytokine, which leads to phosphorylation of STATs on their conserved C-terminal tyrosine [33, 34]. Indeed, by complementing Stark and Kerr's STAT1-deficient IFN-unresponsive mutant cell lines with wild-type STAT1 or a STAT1 mutant in which tyrosine 701 was mutated to phenylalanine, the critical importance of phosphorylation of this residue to the interaction with PIAS-1 was established [34]. Given the lack of canonical SH2 or PDB domains, the molecular basis of the interaction of PIAS proteins with tyrosine phosphate is not clear. Irrespective of mechanism, it is clear that interaction of PIAS proteins with STATs prevents their interaction with DNA, either directly or by preventing formation of STAT dimers.

Unlike SOCS proteins, expression of PIAS family members does not appear to be regulated by cytokines themselves, nor does the inhibition of STATs by PIAS lead to a cessation in signalling and therefore production of phosphorylated STAT. Rather, PIAS proteins are constitutively expressed. In this sense, PIAS proteins do not act to form a negative feedback loop, but rather may act like a buffer, titrating the amount of activated STAT that is available within particular cells following cytokine stimulation.

SHP-1 and motheaten

Of the three families of negative regulators I have described in this review, the story of the phosphatase SHP-1 is by far the most intriguing. Understanding of the biological role of SHP-1 may be largely credited to Schulz and colleagues at the Jackson Laboratories in Maine, who in the mid 1960s identified a novel mouse mutant they named motheaten. This group has also recently reviewed the biological and biochemical role of SHP-1 in some depth [35]. The basic model for SHP-1 action is that interaction of a variety of ligands with their cognate receptors results in activation of tyrosine kinases, whether intrinsic to the receptor or cytoplasmic (e.g. src family kinases and JAKs). Kinase activation, in turn, leads to phosphorylation of tyrosine residues in receptor cytoplasmic domains and creation of docking sites for localisation of the phosphatase SHP-1. Once localised, SHP-1 then catalyses the dephosphorylation of tyrosines critical to the activation of kinases, reducting their catalytic activity.

Homozygous motheaten (*me*/*me*) mice develop a multilineage haemopoietic disease that includes polycythaemia, accumulation of macrophages and neutrophils in the lung, causing a fatal pneumonitis, and in the skin, causing the characteristic motheaten appearance of the coat [35]. Motheaten mice also have lymphoid abnormalities, including a decrease in the number of B cell progenitors and natural killer cells and a premature thymic involution leading to immunodeficiency, followed by a polyclonal expansion in mature B cells leading to autoimmune disease [35].

Unlike SOCS and PIAS proteins, which, from the preliminary and relatively superficial in vitro analyses performed to date, appear to be primarily inhibitors of the JAK/STAT pathway, the tyrosine phosphatase SHP-1 regulates several different signalling pathways [35]. There is evidence that SHP-1 is a key negative regulator of the activity of the tyrosine kinase c-fms, which acts as the macrophage-colony-stimulating factor (M-CSF = CSF-1) receptor [36]. Likewise, through an elegant genetic experiment, SHP-1 appears to negatively regulate a close relative of c-fms, the receptor for stem cell factor, c-kit. Bernstein and colleagues crossed mice harbouring the *me* allele of SHP-1 with mice bearing a dominant negative allele (W^v) of the stem cell factor receptor, c-kit [37]. The latter have a deficit in mast cells, anaemia and defects in germ cell function and melanocyte migration and a reduced viability when the W^v allele is homozygous. The combination of W^v and *me* reduced the lethalty associated with both genes and ameliorated the deficit in mast cells, but not the anaemia associated with W^v [37]. In lymphoid cells, there is evidence that SHP-1 regulates signalling from antigen receptors and Fc receptors, perhaps by dephosphorylation of members of the src kinase family, lyn, lck and fyn [35].

Evidence has also accumulated that SHP-1 regulates cytokine signalling, presumably through dephosphorylation and inactivation of JAK following cytokine stimulation and, indeed, SHP-1 has been observed to interact with JAK2 [38]. There are enhanced proliferative responses of bone marrow progenitor cells from *me*/*me* mice to GM-CSF, granulocyte-colony-stimulating factor and EPO, which may in part explain the increased numbers of monocytes, neutrophils and erythrocytes [39, 40]. Likewise, there is evidence that SHP-1 controls responsiveness to IFNs [41, 42]. The most interesting data in this area come from analysis of the EPO receptor. Expression of a mutant EPO receptor lacking a C-terminal tyrosine that is known to be a docking site for SHP-1 renders cells hypersensitive to EPO [43], consistent with the hypersensitivity of CFU-E from motheaten mice to EPO [44] and the capacity of SHP-1 to interact with the C-terminal end of the EPO receptor and attenuate JAK2 signalling [45]. Further, a Finnish family harbouring a deletion of the C-terminal region of the EPO receptor, again including the docking site for SHP-1, exhibit a profound polycythaemia, perhaps in part explaining their remarkable achievements in cross-country skiing [46]. This has now been observed for other families [47]. Why motheaten mice should be anaemic, while Olympic cross-country skiers are not, is uncertain; however, the anaemia in these

animals may be a secondary effect of removal of red blood cells due to the presence of high circulating autoantibody levels or excessive numbers of activated macrophages [35]. SHP-1, like SOCS proteins, acts in a classic negative feedback loop. SHP-1 action is dependent on docking sites created by phosphorylation of receptors by activated kinases and, in turn, SHP-1 catalyses the dephosphorylation and inactivation of kinases such as JAK. A major difference between SHP-1 and SOCS-1 is temporal. Production of SOCS proteins is dependent on transcription initiated by activated STATs and thus negative regulation occurs at least 30 min after cytokine stimulation. SHP-1, however, appears to be present constitutively, and is therefore available to regulate signalling as soon as kinases are active, an event that occurs rapidly (within a minute) of cytokine binding to receptor.

Summary and perspectives

The data to date suggest that SOCS proteins are an important element in a classic negative feedback loop that regulates JAK/STAT signal transduction initiated by many cytokines. Interaction of cytokines with their receptors leads to rapid activation of JAKs and phosphorylation of receptors and STAT molecules. Phosphorylated STATs then dimerise and migrate to the nucleus where they increase transcription of not only the genes important in *mediating* the biological effects of the cytokine but also genes encoding the SOCS proteins which *limit* the biological effect of the cytokine. Once produced, the SOCS proteins inhibit signal transduction, SOCS-1 by inactivating JAKs and CIS, possibly through binding to phosphorylated cytokine receptors. In turn, inhibition of the JAK/STAT pathway leads to a decrease in the expression of SOCS proteins which, coupled with possible degradation of the SOCS proteins via the proteasome, relieves the inhibition allowing cells to once again respond to cytokine.

SHP-1, like SOCS proteins, acts as part of a negative feedback loop, but because it is present constitutively rather than being induced by cytokine, SHP-1 acts in a more acute fashion. PIAS proteins, in contrast, appear to act more as buffers, regulating the amount of activated STAT available for enhancing the transcription of target genes.

The physiological effect of SOCS proteins is unlikely to be as black and white as turning signalling off. Rather, it is more likely that SOCS proteins may determine the threshold level of cytokine to which a cell will meaningfully respond or the steady-state level and duration of such a response. An intriguing additional role of SOCS proteins produced in response to one cytokine may be to make the cell refractory to stimulation by a second cytokine. Preliminary in vitro evidence for this possibility comes from the demonstration that IL-10, which inhibits the biological action of IFN-α and IFN-γ, produces SOCS-3 and results in decreased capacity of IFNs to activate STAT1 [19]. As with the JAKs and STATs, it is likely that precise physiological roles of the SOCS proteins will be determined from careful study of mice harbouring null mutations and more subtle mutations in SOCS genes rather than trying to divine their role from contrived and often ambiguous in vitro experiments.

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