Unbiased identification of substrates for the Epac1-inducible E3 ubiquitin ligase component SOCS-3

Jamie J.L. Williams and Timothy M. Palmer

Institute of Cardiovascular and Medical Sciences, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

Abstract

The anti-inflammatory effects of the prototypical second messenger cAMP have been extensively documented in multiple cell types. One mechanism by which these effects are achieved is via Epac1 (exchange protein directly activated by cAMP 1)-dependent induction of SOCS-3 (suppressor of cytokine signalling 3), which binds and inhibits specific class I cytokine receptors. One important aspect of SOCS-3 functionality is its role as the specificity determinant within an E3 ubiquitin ligase complex which targets cellular substrates for polyubiquitylation and proteasomal degradation. In the present review, we describe key inhibitory processes that serve to reduce cytokine receptor signalling, focusing primarily on SOCS protein function and regulation. We also outline a strategy we have developed to identify novel ubiquitylated substrates for the Epac1-inducible SOCS-3 E3 ubiquitin ligase complex following purification of the ubiquitinome. It is anticipated that identifying substrates for the Epac1-regulated SOCS-3 E3 ubiquitin ligase, and assessment of their functional significance, may pinpoint new sites for therapeutic intervention that would achieve therapeutic efficacy of cAMP-elevating drugs while minimizing the adverse effects usually associated with these agents.

Regulating the JAK/STAT pathway

Cytokines control many important biological responses, including haemopoiesis, inflammation and T-cell differentiation and expansion. Several cytokine receptors, including gp130 [glycoprotein 130, the signal transduction component required for IL (interleukin)-6 responses], ObR (the leptin receptor) and IFNGR (IFNγ (interferon γ) receptor), activate receptor-associated JAKs (Janus kinases), which then tyrosine-phosphorylate and activate STAT (signal transducer and activator of transcription) proteins. These dimerize and translocate to the nucleus, where they function as transcription factors by binding to specific promoter elements and recruiting transcriptional co-activators [1,2].

Because chronic activation of this pathway can initiate and perpetuate chronic inflammatory diseases as well as certain forms of cancer [3], several inhibitory mechanisms operate at different levels to ensure that signalling responses are transient in nature. Negative regulation occurs through multiple routes within both extracellular and intracellular domains via inhibitory and degradative mechanisms. For example, sgp130 (soluble gp130) can trap circulating soluble IL6Ra (IL-6 receptor α)/IL-6 complexes and thus quench inappropriate trans-signalling [4]. Importantly, intracellular signalling intermediates can be made functionally mute by PTPs (protein tyrosine phosphatases), and also via direct inhibition by PIASs (protein inhibitors of activated STAT) and SOCS (suppressor of cytokine signalling) proteins, as described below.

Protein tyrosine phosphatases

Whereas SHP (Src homology 2 domain-containing protein tyrosine phosphatase)-2 can activate the ERK1/2 (extracellular-signal-regulated kinase 1/2) pathway downstream of gp130 by functioning as an adaptor, it can also negatively regulate the JAK/STAT pathway via its PTP activity against gp130, JAK, and STAT proteins. SHP-2 is ubiquitously expressed and consists of two N-terminal SH2 (Src homology 2) domains and a C-terminal catalytic phosphatase domain. In its inactive state, the SH2 domains bind to the phosphatase domain to block substrate interaction. This inhibitory action is removed upon tyrosine phosphorylation by cytokine-activated JAKs. Other phosphatases might also have roles in JAK/STAT regulation such as PTPsC, PTP1B, CD45, SHP-1 and nuclear-localized phosphatase TC-PTP (T-cell PTP) [1,5].

Protein inhibitors of activated STAT

PIASs, as their name suggests, block the function of active/phosphorylated STATs, but they also interact with
a wide range of non-STAT proteins, most of which are transcription factors such as NF-κB (nuclear factor κB), and so have wider actions as transcriptional regulators [6]. Five PIAS family members (PIAS1, PIAS3, PIASα, PIASβ, and PIASγ) have been defined to date. All PIASs bind to active/tyrosine-phosphorylated nuclear-localized STAT proteins, although the inhibitory mechanism appears to be PIAS-specific. IFNγ-induced STAT1 and IL-6–induced STAT-dependent gene transcription are suppressed by PIAS1 and PIAS3 respectively via inhibition of transcription factor binding to target DNA. In contrast, PIASx and PIASγ inhibit gene transcription by acting as transcriptional co-repressors. All PIASs also have E3 SUMO (small ubiquitin-like modifier) ligase activity, enabling the reversible covalent attachment of SUMO to the ε-amino groups of lysine residues residing within a PXXKXD/E (Ψ is a hydrophobic residue) consensus sequence for SUMOylation on target proteins. This post-translational modification modulates cellular localization, function and protein–protein interactions, thus altering the composition and action of transcription factor complexes. Regulation by PIASs is complex, with certain regulatory roles being SUMOylation-independent and others relying on specific PIAS domains [7].

**Suppressor of cytokine signalling proteins**

Suppressor of cytokine signalling proteins SOCS proteins constitute a family of eight related proteins [CIS (cytokine-inducible SH2-domain-containing protein) and SOCS-1–SOCS-7], of which SOCS-1 and SOCS-3 have been characterized most intensively. These proteins were identified by their functional role as end-points in classical negative-feedback loops whereby cytokine-mediated activation of STAT proteins by JAKs triggers SOCS-1 and/or SOCS-3 induction, which can then inhibit signal propagation [1,8]. However, in addition to their well-characterized STAT-mediated classical negative-feedback role, it is now clear that both SOCS-1 and SOCS-3 can also be induced in trans via stimuli that do not activate the JAK/STAT pathway, thereby providing a mechanism by which otherwise distinct signalling pathways can negatively cross-regulate cytokine responsiveness. The list of inducers of SOCS proteins is growing and therefore the variety of SOCS-substrate recognition and regulation. Critically, regulation of SOCS proteins, as well as SH2-dependent SOCS/substrate recognition and regulation. Critically, the SOCS-box domain can potentially target SH2-domain-containing SOCS/substrate recognition and regulation. Critically, the SOCS-box domain can potentially target SH2-domain-containing SOCS-3 as the specificity determinant within an E3SOCS-3 ubiquitin ligase complex

Suggestions of E3 ligase functionality resulted from analysis of SOCS-3 which identified a conserved C-terminal domain, the ‘SOCS-box’, containing motifs also present in known E3 ligases such as vHL (von Hippel–Lindau), MuF1 and elongin A [16]. SOCS-box domains have been identified within over 70 human proteins conjugated to a variety of protein–protein interaction domains, including SH2 domains, WD40 repeats, SPRY domains, leucine-rich repeats and ankyrin repeats that are thought to add target specificity and diversity. E3 ligase functionality has been demonstrated for SOCS-1 and SOCS-3, but has yet to be confirmed for most other SOCS family members [17].

Structurally, each SOCS family member consists of a conserved central SH2 domain, an ESS (extended SH2 subdomain), a variable-length non-conserved N-terminal domain, and a 40-amino-acid C-terminal domain SOCS-box (Figure 1). The SOCS-box is considered to be a multifunctional region, as it can regulate stability and the cross-regulation of SOCS proteins, as well as SH2-dependent SOCS/substrate recognition and regulation. Critically, the SOCS-box domain can potentially target SH2-domain-bound partners for proteasomal degradation by directing formation of an E3 (elongin–cullin–SOCS) ubiquitin ligase complex (Figure 1) [18]. This is because the SOCS-box can recruit elongins B and C, which, together with Cul5 (cullin 5) and Rbx (RING-box) 2, form an E3 ubiquitin ligase complex. This complex associates with a ubiquitin-activating E1 enzyme and a ubiquitin-conjugating E2 enzyme to mediate the Lys48-linked polyubiquitylation and subsequent proteasomal degradation of specific SOCS-bound substrates. Mice in which a SOCS-box-deleted SOCS-3 has been ‘knocked in’ to replace endogenous SOCS-3 (termed SOCS3ΔSB/ΔSB mice) display prolonged STAT3 signalling intermediate to that of Epac (exchange protein directly activated by cAMP) 1, resulting in the diminution of IL-6 signalling in vascular endothelial cells and fibroblasts [10,11].

Following their accumulation, SOCS proteins can then bind to and terminate signalling from specific cytokine receptors [1,8]. A role for SOCS-3 in specifically terminating signalling from gp130, a signalling transducer utilized by IL-6, OSM and other class I cytokines, has been demonstrated by several studies, including the unrestricted agonist-stimulated activation of STAT3 seen in cell-specific conditional SOCS-3-deficient mice [12,13]. SOCS-3 terminates signalling by binding to JAK-phosphorylated receptors via a central SH2 domain, allowing it to interact with and inhibit adjacent JAKs via its KIR (kinase-inhibitory region), thereby preventing the recruitment and tyrosine phosphorylation of STATs [14]. SOCS-3 can also competitively block receptor recruitment of SHP-2 to Tyr59 of gp130, thus inhibiting activation of the Ras/Raf/MEK (mitogen-activated protein kinase/ERK kinase)/ERK1/2 pathway [15].
SOCS-3 interactors are necessarily ubiquitylated by SOCS-3. A good example of this is gp130, which is ubiquitylated instead by the RING E3 ubiquitin ligase c-Cbl following its recruitment via a SHP-2-dependent mechanism [25]. Also, ubiquitylation of target lysine residues by E3 ubiquitin ligases does not occur within predictable linear consensus sequences, making in silico prediction of potential substrates impossible [26].

Therefore, to gain a greater appreciation of the importance of SOCS-3-mediated ubiquitylation, quantitative proteome-wide approaches are necessary to obtain a more complete assessment of the cellular substrates of the ECS$^{SOCS-3}$ complex. The application of such approaches to identify and characterize cellular substrates for the SUMO family of ubiquitin-like molecules [27,28] is well established, but only a few studies have utilized the approach to identify substrates for specific E3 ubiquitin ligases (e.g. [29]). We have generated wild-type SOCS-3$^{+/+}$ and SOCS3$^{−/−}$ MEFs (murine embryonic fibroblasts) that stably express equivalent levels of a tandem-affinity-tagged ubiquitin transgene [30]. This allows for a facile two-step purification of the cellular ubiquitinome under denaturing conditions that preserve ubiquitylation status and minimize co-purification of ubiquitin-binding proteins. Using SILAC (stable isotopic labelling of amino acids in cell culture) in conjunction with LC (liquid chromatography)–MS/MS (tandem MS) analysis of the purified ubiquitinome from these cells, we have identified several ubiquitylated proteins present exclusively in SOCS-3$^{+/+}$ but not SOCS-3$^{−/−}$ MEFs, which suggest these may be new targets for ubiquitylation by the ECS$^{SOCS-3}$ complex. Importantly, although many of these proteins have not previously been demonstrated to be targets for SOCS-3, we have also been able to detect FAK1, a well-described substrate for SOCS-3-dependent ubiquitylation, thereby validating this approach.

Summary

We have generated and validated new tools to identify cellular targets for ubiquitylation by the cAMP/Epac1-regulated ECS$^{SOCS-3}$ E3 ubiquitin ligase complex. In addition to validating these interactions, it will clearly be critical to assess their significance in the context of cAMP’s functional effects. Several drugs currently on the market exert their therapeutic effects by globally elevating cAMP levels. These include phosphodiesterase inhibitors such as pentoxifylline, ibudilast and (most recently) the phosphodiesterase 4-selective inhibitor rofumilast. However, although efficacious, these drugs are limited by a range of side effects that include nausea, emesis, diarrhoea and arrhythmia, which arise, at least in part, from systemic activation of cAMP-dependent protein kinase and Epacs [31]. By identifying cellular substrates for the cAMP/Epac1-regulated ECS$^{SOCS-3}$ E3 ubiquitin ligase and assessing their functional significance, it may ultimately be possible to pinpoint new sites for therapeutic intervention that would achieve therapeutic efficacy while minimizing adverse drug effects.
References


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