Negative regulation of gp130 signalling mediated through tyrosine-757 is not dependent on the recruitment of SHP2

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Cytokines of the interleukin-6 family utilize the shared cytokine receptor gp130 in the formation of active signalling complexes. Tyrosine-757 (Y757) on this receptor is critical for negative regulation of gp130-mediated signalling. Two signalling regulators, suppressor of cytokine signalling 3 (SOCS3) and Src homology 2 domain-containing tyrosine phosphatase-2 (SHP2), are recruited to Y757 following receptor activation; however, the relative contribution made by each of these in down-regulating gp130 signalling is not known. In the present study, we show the design of a mutant gp130 receptor that can recruit SHP2, but not SOCS3. This receptor maintains the critical Y757 residue, but contains mutations in other surrounding residues which are also important for interactions with the Src homology 2 domains

of SOCS3 and SHP2. Cells transfected with a chimaeric receptor containing the SHP2-selective gp130 intracellular domain showed an enhanced response to cytokine stimulation, which was similar to that shown by a chimaeric gp130 receptor mutant carrying a Y757F point mutation that failed to recruit either SOCS3 or SHP2. These results demonstrate that the recruitment of SHP2 alone is not sufficient for Y757-dependent negative regulation of gp130 signalling and that this activity must therefore be dependent on SOCS3.

Key words: cytokine, interleukin-6 signalling, suppressor of cytokine signalling.

INTRODUCTION

Cytokines of the interleukin (IL)-6 family, which includes IL-6, IL-11, leukaemia inhibitory factor, oncostatin M, ciliary neurotrophic factor, cardiotrophin-1 and cardiotrophin-like cytokine, share the common co-receptor gp130 (see [1] for a review). Signalling by these cytokines results in activation of the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway and transcription of downstream target genes. The IL-6-like cytokines mediate a wide variety of overlapping and unique biological responses across many different tissue systems and are important for the normal growth and differentiation of haematopoietic cells, bone, neurons, muscle, heart, adipose tissue, liver and testes [2].

Specific tyrosine residues in the cytoplasmic domain of gp130, which are transiently phosphorylated during signalling, serve as docking sites for Src homology 2 domain (SH2 domain)-containing proteins, which either propagate the signal or are involved in negative regulation. Activation of the JAK-STAT pathway requires the recruitment of STAT proteins, most notably STAT3, to one of four potential phosphorylated YXXQ motifs within the gp130 cytoplasmic domain [3]. On the other hand, the YSTV tyrosine-phosphorylated motif surrounding tyrosine-757 (Y757) in mouse gp130, corresponding to Y759 in human gp130, is a recruitment site for the signalling regulators SH2 domain-containing tyrosine phosphatase-2 (SHP2) and suppressor of cytokine signalling 3 (SOCS3) [3–6]. SHP2 is a ubiquitously expressed protein tyrosine phosphatase that is involved in the signalling pathways of a variety of growth factors, usually as a positive regulator of signalling [7]. SOCS3 is a member of the SOCS protein family and can

potently inhibit signalling by a number of cytokines *in vitro*, including those of the IL-6 family [5,8–10].

Two types of signals appear to occur after binding of SHP2 and SOCS3 to Y757. The first is positive signalling, resulting from the phosphorylation of gp130-associated SHP2 and subsequent activation of the Ras/Raf/mitogen-activated protein kinase signal transduction pathway [3,4]. The biological role of mitogen-activated protein kinase activation in response to gp130 signalling remains unclear, although it may play a role in the proliferative responses of some cells [4,11]. The second is negative signalling, which suppresses activation of the JAK-STAT pathway and depends on the recruitment of SOCS3 and/or SHP2 to Y757 [5,6].

The negative signal relayed through Y757 plays a key role in attenuating cytokine responses mediated by gp130. Disruption of SOCS3 and SHP2 recruitment, effected by the substitution of Y757 with phenylalanine [Tyr⁷⁵⁷ → Phe (Y757F)], was shown to result in an enhancement of downstream signalling mediated by STAT3 [5,6,12–15]. These studies showed that cytokine treatment induced prolonged STAT3 phosphorylation and a higher level of STAT3-mediated gene expression in transfected cells expressing mutant Y757F gp130 receptors compared with cells expressing Y757 receptors. Dysregulation of gp130 signalling was also observed in engineered mice containing a replacement of their endogenous gp130 genes by human gp130, bearing a Y759F mutation [16]. These mice displayed disease pathologies such as splenomegaly, lymphadenopathy and an enhanced acute-phase response typical of an increased response to IL-6. These studies highlight the important role that Y757 plays in mediating down-regulation of gp130 signalling; however, they do not address whether it is the recruitment of one or both of SOCS3 and SHP2 that is responsible.

Abbreviations used: EPO, erythropoietin; EPOR, EPO receptor; IL, interleukin; JAK, Janus kinase; pY, phosphotyrosine; SH2 domain, Src homology 2 domain; SHP2, SH2 domain-containing tyrosine phosphatase-2; SOCS3, suppressor of cytokine signalling 3; STAT, signal transducer and activator of transcription; for brevity the one-letter system for amino acids has been used, Y757, for example, means Tyr⁷⁵⁷.

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The ligand-binding specificity of the SH2 domains from SHP2 and SOCS3 is similar [17], consistent with their shared use of specific tyrosine-phosphorylated docking sites on gp130 as well as the receptors for erythropoietin (EPO) and leptin [18–21]. Y985 on the leptin receptor appears to play the same role as Y757 on gp130 in that it acts as a shared recruitment site for SHP2 and SOCS3 and is also critical in mediating a negative signal, which dampens the intensity of downstream JAK-STAT signalling [19,21,22]. An understanding of the relative contributions of SOCS3 and SHP2 in suppressing gp130 signalling is therefore likely to have similar implications for signalling via the leptin receptor.

Dissection of the relative roles that SOCS3 and SHP2 play in negatively regulating gp130 signalling would be assisted greatly by mutant gp130 receptors which, on activation, will recruit selectively either SOCS3 or SHP2, but not both. The design of an SOCS3 or SHP2-selective gp130 receptor mutant is complicated by the shared use of the Y757 docking site and the overlap in specificity between the SH2 domains from SOCS3 and SHP2 [17]. Despite this, in characterizing the phosphopeptide-binding preferences of the SH2 domains from SOCS3 and SHP2 [17], we observed some differences in specificity. This led us to believe that the design of a selective phosphopeptide ligand, which would bind only one of SOCS3 or SHP2 with high affinity, would be possible. In the present study, we describe the design and functional analysis of a gp130 receptor mutant, which maintains the ability to associate with SHP2, but exhibits a significantly reduced capacity to recruit SOCS3. We demonstrate that this receptor shows an elevated responsiveness to cytokine, similar to that shown by a Y757F receptor mutant and, therefore, propose that the recruitment of SHP2 alone cannot effect significant negative regulation of gp130 signalling.

EXPERIMENTAL

Cytokines and antibodies

Recombinant human EPO was obtained from Janssen-Cilag Pty. (Sydney, Australia), anti-Flag antibody (M2) was from Sigma (St. Louis, MO, U.S.A.), anti-SHP2 antibody (C-18) was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), anti-phosphotyrosine (pY)-20 was from Transduction Laboratories (Lexington, KY, U.S.A.) and anti-Myc antibody (9E10) was from Roche Applied Science (Penzberg, Australia).

Recombinant protein production

cDNAs encoding full-length mouse SOCS3 or a fragment of human SHP2 (1–220) encoding the dual SH2 domains (amino acids 1–220) were cloned into a pET15b vector (Novagen, Madison, WI, U.S.A.) and expressed in BL21 DE3 pLysS *Escherichia coli* (Stratagene, La Jolla, CA, U.S.A.). Extraction, purification and refolding of the recombinant proteins were performed as described previously [17]. The amino acid sequences of the SH2 domain fragment from human and mouse SHP2 are identical, whereas human and mouse SOCS3 share 96 % sequence identity.

Phosphopeptide synthesis and binding assays

All phosphopeptides were synthesized on a Rink amide methylbenzhydrylamine resin (Novabiochem, Laufelfingen, Switzerland) using fluoren-9-ylmethoxycarbonyl amino acids and *O*-benzotriazol-1-yl-*N,N,N',N'*-tetramethyluronium hexafluorophosphate activation, and after cleavage/deprotection were purified by reverse-phase HPLC. Phosphopeptide-binding assays

measured the ability of soluble phosphopeptides to compete with an immobilized gp130-derived phosphopeptide [biotin-STASTVE(pY)STVVHSG] for binding to recombinant SOCS3 or SHP2 (1–220) constructs. These assays were performed on a BIAcore instrument (Biacore AB, Uppsala, Sweden) using conditions similar to those described previously [17].

Plasmid construction and cell lines

Flag-tagged EPO receptor (EPOR)/gp130 chimaeric construct in pEF-FLAG-S, described previously [6], was used as a template for the construction of the receptor mutants. Briefly, a *SpeI* fragment was subcloned initially into the pOCUS-2 vector (Novagen). Mutagenesis was then performed using a whole-plasmid PCR method similar to that of Fisher and Pei [23], with modifications as described in [24]. Using bidirectional sequencing to confirm the presence of the mutations, the *BlpI*–*BamHI* fragment from the parent EPOR/gp130-pEF-FLAG-S plasmid was replaced with the corresponding fragments from the mutated constructs in pOCUS-2. Mammalian expression constructs encoding N-terminal Flag-tagged mouse SOCS3 and Myc-tagged human SHP2 were generated by cloning *AscI*–*MluI* cDNA fragments in-frame with the IL-3 signal and tag sequences of pEF-FLAG-S or pEF-MYC-I respectively.

Cell culture and transient transfection

HEK-293T cells were transfected using FuGENE 6 transfection reagent (Roche Applied Science) and maintained in Dulbecco's modified Eagle's medium containing 10 % (v/v) foetal calf serum, 62.5 µg/l penicillin and 100 µg/ml streptomycin.

Immunoprecipitation and Western blotting

HEK-293T cells were seeded into 6-well plates at 2.5×10^5 cells/well and, following transfection, were maintained in serum-containing medium for 48 h, followed by serum-free medium for a further 48 h. Cells were then incubated for 15 min in the medium alone, or in the medium containing 85 ng/ml EPO, and then lysed in RIPA buffer [50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 0.5 % (w/v) sodium deoxycholate, 1 % (v/v) Nonidet P40 and 0.1 % (w/v) SDS], supplemented with Complete protease inhibitor cocktail (Roche Applied Science) and 1 mM sodium orthovanadate. Proteins were immunoprecipitated with anti-SHP2 antibody and Protein G–Sepharose and separated by SDS/PAGE under reducing conditions. Protein was immunoblotted with anti-pY antibody. Membranes were stripped and reprobed with anti-Myc antibody to determine SHP2 loading.

Luciferase reporter assay

HEK-293T cells were seeded on 24-well plates at 5×10^4 cells/well and transfected transiently with cDNA constructs encoding EPOR/gp130 receptor, the leukaemia inhibitory factor-responsive reporter construct APRE-luc [25] and a β -galactosidase reporter construct under the control of a constitutive promoter (Sra- β -gal [25]). Cells were incubated overnight with a medium containing 85 ng/ml EPO and lysed in reporter lysis buffer (Promega, Madison, WI, U.S.A.). Lysates were treated with Luciferase substrate (Promega) and luciferase activity was determined using an ML3000 luminometer (Dynatech Laboratories, Chantilly, VA, U.S.A.). Luciferase activity was normalized against β -galactosidase activity to correct for differences in transfection efficiency. To study the effect of SOCS3 and SHP2

co-transfection, assays were performed as described above, but assays included titrated amounts of FLAG-tagged SOCS3 or Myc-tagged SHP2 DNA (0–200 ng). Expression levels of SOCS3 and receptor were determined by Western-blot analysis using anti-FLAG antibody, whereas SHP2 levels were detected with anti-Myc antibody.

RESULTS

Design of an SHP2-selective phosphopeptide ligand

Our previous studies [6,17] have shown that the SH2 domains from SOCS3 and SHP2 both bind with high affinity to a phosphopeptide derived from the mouse gp130 docking site surrounding Y757 (corresponding to Y759 in human gp130). Relative to other SH2 domain-containing proteins, SOCS3 and SHP2 recognize extended phosphopeptide ligands spanning eight amino acids, i.e. from the second residue upstream to the fifth residue downstream from pY [6]. They also share a similar preference for the identity of amino acids found at the upstream pY – 2 and downstream pY + 1 and pY + 3 positions in phosphopeptide ligands [17]. Given the more restricted consensus phosphopeptide-binding motif of SOCS3 [pY-(S/A/V/Y/F)-hydrophobic-(V/I/L)-hydrophobic-(H/V/I/Y)] relative to that of SHP2 [pY-(S/T/A/V/I)-X-(V/I/L)-X-(W/F)] [17], we chose to design a phosphopeptide ligand which was capable of binding SHP2 but would no longer bind SOCS3. Our aim in designing these analogues was to identify a phosphopeptide that exhibited minimal binding to SOCS3, while displaying a binding affinity for SHP2 similar to that observed for the gp130-derived phosphopeptide centred on Y757. To do this, we exploited subtle differences in the specificity of SHP2 versus SOCS3 at each of the non-pY positions between pY + 1 and pY + 5. The designed phosphopeptide, VE(pY)IKLDW, bound to a recombinant fragment of SHP2 containing both SH2 domains with a 14-fold higher affinity when compared with the corresponding gp130-derived phosphopeptide VE(pY)STVVH, whereas binding to recombinant SOCS3 was reduced by 560-fold (Table 1). A further substitution of the pY – 2 residue to alanine, known to reduce the affinity of phosphopeptide binding to both SOCS3 and SHP2 [6,17], resulted in a phosphopeptide ligand with the desired characteristics. This phosphopeptide, AE(pY)IKLDW, bound to SHP2 with an affinity similar to that of the gp130-derived phosphopeptide, whereas the affinity for binding to SOCS3 was decreased approx. by 900-fold (Figure 1). The drastic change in selectivity for binding to SHP2 over SOCS3 was a consequence of small gains made for each of the six substitutions in the AE(pY)IKLDW phosphopeptide sequence. This is readily apparent from our subsequent analysis of a series of gp130-derived phosphopeptide analogues in which individual residues were replaced with the corresponding amino acid from the SHP2-selective sequence (Table 1).

Design of an SHP2-selective gp130 receptor

To determine whether SHP2 recruitment was sufficient to mediate some or the entire negative signal associated with Y757 in the mouse gp130 receptor, a series of chimaeric receptor constructs were prepared. Each of these constructs consisted of a FLAG-tagged EPOR extracellular domain and a gp130-derived intracellular domain as described previously [6]. A total of four constructs were prepared, each differing in the amino acid sequence surrounding the SOCS3/SHP2-binding site at Y757 (Figure 2). The receptors contained either the wild-type gp130

Table 1 Affinities for phosphopeptides binding to SOCS3 and SHP2(1–220)

Peptide	IC ₅₀ (μM)	
	SOCS3 binding	SHP2(1–220) binding
VE(pY)STVVH*	0.48	2.6
VE(pY) IKLDW	270 (560)	0.18 (0.069)
AE (pY) IKLDW	440 (920)	0.76 (0.29)
VE(pY)ITVVH	1.3 (2.7)	1.7 (0.65)
VE(pY) SKVVH	1.7 (3.5)	7.7 (3.0)
VE(pY)STLVH	1.7 (3.5)	3.2 (1.2)
VE(pY)STVVW	1.6 (3.3)	1.3 (0.50)

* Phosphopeptide corresponding to mouse gp130(755–762). Residues in bold highlight differences with the gp130-derived sequence. Values in parentheses are the ratio of IC₅₀ analogue/IC₅₀ gp130(755–762).

Figure 1 Selective binding of designed phosphopeptide to SHP2

A competitive binding assay was used to compare the affinities of the gp130-derived phosphopeptide VE(pY)STVVH with that of the designed phosphopeptide AE(pY)IKLDW for binding to SHP2(1–220) and SOCS3.

intracellular domain (EPOR/gp130^{Y757}), the SHP2-selective motif (EPOR/gp130^{SHP2}Y757) or the corresponding constructs containing a phenylalanine residue in place of Y757 (EPOR/gp130^{F757} and EPOR/gp130^{SHP2}F757). Whereas the wild-type-like EPOR/gp130^{Y757} receptor contained a functional SOCS3/SHP2-docking site at Y757, the SHP2-selective EPOR/gp130^{SHP2}Y757 receptor was predicted to recruit only SHP2 following EPO-mediated receptor activation. In contrast, the receptor constructs containing phenylalanine substitutions at position 757 (EPOR/gp130^{F757} and EPOR/gp130^{SHP2}F757) were not expected to recruit either SOCS3 or SHP2 in response to EPO stimulation.

Figure 2 Chimaeric EPOR/gp130 receptor constructs

Each construct comprised the extracellular region of mouse EPOR (amino acids 25–249) and the intracellular region of mouse gp130 (amino acids 618–917). The four constructs differed in the amino acid sequences surrounding residue 757 within the gp130 intracellular domain, i.e. the docking site for SOCS3 and SHP2. EPOR/gp130^{Y757} contained the wild-type gp130 sequence in this region, whereas for EPOR/gp130^{shpY757}, amino acids 755–762 were replaced with the SHP2-selective motif AEYIKLDW. The receptor constructs EPOR/gp130^{F757} and EPOR/gp130^{shpF757} contained the corresponding Y → F substitutions at position 757.

SHP2-selective EPOR/gp130^{shpY757} receptor mediates ligand-dependent SHP2 association and phosphorylation

Given that the EPOR/gp130^{shpY757} receptor contained a number of mutations in the vicinity of Y757 relative to the wild-type gp130 sequence, it was important to demonstrate that this tyrosine residue would still be phosphorylated in response to EPO treatment. To do this, we compared the ability of each of the chimaeric gp130 receptors to recruit and phosphorylate SHP2 in transiently transfected HEK-293T cells, given that this activity is associated with phosphorylated Y757 of the gp130 receptor [3,4,14]. Activation of SHP2 was not detectable in HEK-293T cells unless overexpressed in conjunction with either JAK1 or JAK2 (results not shown). Accordingly, cells were transfected with constructs encoding one of the chimaeric receptors, SHP2 and JAK2. Transfected cells were then treated for 15 min with or without EPO and lysed. Analysis of the phosphorylation state of SHP2 was performed by immunoprecipitating SHP2 followed by blotting with an anti-pY antibody (Figure 3A). This showed that EPO-induced SHP2 phosphorylation occurred in cells transfected with the EPOR/gp130^{Y757} and EPOR/gp130^{shpY757} receptors, but not in receptor constructs carrying a phenylalanine substitution at position 757. The expression levels of each of the receptors were shown to be similar by Western-blot analysis of cell extracts with an anti-FLAG antibody (Figure 3B). Moreover, the intensity of the phosphorylated SHP2 band, when corrected for total SHP2 levels (Figure 3A, lower panel), was similar for EPOR/gp130^{Y757}

Figure 3 SHP2 is phosphorylated in response to activation of EPOR/gp130^{shpY757} receptor

HEK-293T cells were transfected transiently with 0.5 μ g of JAK2 and 0.5 μ g of Myc-tagged SHP2, or 0.5 μ g of JAK2, 0.5 μ g of Myc-tagged SHP2 and 1 μ g of EPOR/gp130 receptor construct. EPOR/gp130 receptor constructs were (i) EPOR/gp130^{Y757} (Y757), (ii) EPOR/gp130^{F757} (F757), (iii) EPOR/gp130^{shpY757} (shpY757) and (iv) EPOR/gp130^{shpF757} (shpF757). Transfected cells were treated with or without 85 ng/ml EPO in medium for 15 min and lysed. (A) Myc-tagged SHP2 was immunoprecipitated from cell lysates with an anti-Myc antibody and bound proteins were separated by SDS/PAGE. Immunoblotting was performed sequentially with an anti-pY antibody, followed by reprobing with anti-Myc antibody to compare Myc-tagged SHP2 expression levels. (B) Receptor expression levels were compared by separating an equal amount of sample of each cell extract by SDS/PAGE and immunoblotting with an anti-FLAG antibody.

and EPOR/gp130^{shpY757} receptors, indicating that the activation of SHP2 by these two receptors occurred to a similar extent. Thus phosphorylation of SHP2 was induced by the activation of the SHP2-selective receptor, which depends on Y757, indicating that Y757 must be phosphorylated in response to EPO treatment.

Loss of SOCS3 association leads to enhanced gp130-induced gene expression

To assess whether SHP2 recruitment plays a role in negatively regulating gp130 signalling, a reporter gene assay was performed in HEK-293T cells transfected with each of the EPOR/gp130 chimaeric receptors. The chimaeric receptor system allowed us to activate the gp130 signalling pathway independently of endogenous gp130 receptors. The reporter construct, in which an element of the α_2 -macroglobulin acute-phase protein promoter was placed upstream of the luciferase-coding region [25], produces luciferase in response to gp130 signalling as described previously [6].

Stimulation of transfected cells with EPO resulted in a strong induction of luciferase activity for each of the four receptor constructs, whereas Western blotting of cell extracts confirmed that similar levels of each receptor were expressed (Figure 4). As observed previously by us and other groups [6,12–15], the receptor construct containing the Y757F point mutation (EPOR/gp130^{F757}) showed enhanced induction of luciferase activity relative to the receptor with the wild-type gp130 sequence (EPOR/gp130^{Y757}). The ability of SHP2 to transmit some or all of the Y757-associated negative signal seems to be ruled out by the response of EPOR/gp130^{shpY757}-transfected cells. EPO treatment of these cells resulted in the same magnitude of enhanced reporter gene expression as seen for EPOR/gp130^{F757}-transfected cells. Moreover, no further increase in reporter gene expression was seen for cells expressing the EPOR/gp130^{shpF757} receptor. Taken together, these results suggest that the recruitment of SHP2 to gp130 is unable to suppress downstream STAT-mediated gene expression.

Figure 4 Activation of EPOR/gp130^{shpY757} receptor results in enhanced acute-phase gene induction

HEK-293T cells were co-transfected with cDNAs expressing either EPOR/gp130^{Y757} (Y757), EPOR/gp130^{F757} (F757), EPOR/gp130^{shpY757} (shpY757) or EPOR/gp130^{shpF757} (shpF757), in addition to APRE-luc and β -gal reporter genes, or with the reporter constructs alone. Cells were incubated overnight in the presence or absence of EPO and cell extracts were prepared. (A) Luciferase activity from quadruplicate samples was determined and normalized against β -gal activity. The ratio of normalized luciferase activity for EPO-treated versus untreated cells (fold induction of luciferase activity) was calculated for each of the EPOR/gp130 receptors. (B) EPOR/gp130 protein levels in cell extracts were determined by Western-blot analysis with anti-FLAG antibody.

Overexpressed SOCS3 shows a differential capacity to inhibit signalling by the EPOR/gp130 receptor mutants

We had observed previously that overexpression of SOCS3 could potentially suppress gp130-induced gene expression in HEK-293T cells transfected with the EPOR/gp130^{Y757} receptor [6]. Suppression was also observed for cells transfected with the EPOR/gp130^{F757} construct, although this necessitated a much higher level of SOCS3 overexpression. The relative ability of overexpressed SOCS3 to inhibit signalling by each of the different EPOR/gp130 chimaeric receptors was assessed by co-transfection of HEK-293T cells with increasing amounts of a cDNA expressing SOCS3, in addition to the reporter gene construct.

Overexpression of SOCS3 inhibited signalling by each of the different receptor constructs; however, the dose responsiveness to SOCS3 differed in each case (Figure 5). The wild-type-like EPOR/gp130^{Y757} receptor was most sensitive to the effects of transfected SOCS3 cDNA, whereas a greater amount of transfected SOCS3 cDNA was required to inhibit signalling by the other receptor constructs. These differences in sensitivity to SOCS3 were observed consistently over a number of experiments and, qualitatively, indicate the different affinities expected for SOCS3 binding to each of the receptors. The receptors containing a phenylalanine residue at position 757 were the least sensitive to SOCS3, as expected, based on the critical importance of the tyrosine residue for SH2 domain binding. The intermediate sensitivity of the EPOR/gp130^{shpY757} receptor to SOCS3 reflects, probably, the residual capacity of this receptor to engage SOCS3 in a Y757-dependent manner. Moreover, the difference in sensitivity to SOCS3 between the EPOR/gp130^{shpY757}

Figure 5 EPOR/gp130^{shpY757} receptor shows reduced sensitivity to overexpression of SOCS3

HEK-293T cells were co-transfected with cDNAs expressing EPOR/gp130 receptor, APRE-luc and β -gal reporter genes, in addition to increasing amounts of Flag-tagged SOCS3. Cells were incubated in the presence or absence of EPO and lysed. (A) Effect of increasing amounts of co-transfected SOCS3 on reporter gene expression. Relative luciferase activities represent the ratio of EPO-induced luciferase activity obtained in the presence versus absence of co-transfected SOCS3. (B) FLAG-tagged SOCS3 protein levels in cell lysates were determined by Western-blot analysis with anti-FLAG antibody.

and EPOR/gp130^{shpF757} receptors again suggests that Y757 is phosphorylated in the former receptor in response to cytokine treatment.

Overexpression of SHP2 does not result in significant suppression of gp130 signalling

In addition to SOCS3, we also tested whether overexpression of SHP2 could alter gp130-induced gene expression. In contrast with SOCS3, overexpression of SHP2 had a limited effect on reporter gene expression (Figure 6). Luciferase production in EPO-treated HEK-293T cells expressing any of the EPOR/gp130 constructs was mostly unaffected by co-transfection of up to 200 ng of SHP2 cDNA construct. As SHP2 is expressed constitutively in HEK-293T cells, Western-blot analysis of cell lysates was used to confirm that SHP2 was being produced in excess of background endogenous levels (Figure 6C). At the highest levels of co-transfected SHP2, we found that some inhibition of reporter gene expression was observed for the EPOR/gp130^{shpY757} receptor; however, this was relatively small compared with the inhibitory activity of SOCS3.

DISCUSSION

The involvement of SHP2 in gp130-mediated signal transduction was first described by Boulton et al. [26]. Using chimaeric receptors, Stahl et al. [3] identified Y759 of human gp130 to be crucial for SHP2 phosphorylation, and it was shown subsequently

Figure 6 Overexpression of SHP2 has no effect on gp130-induced gene expression

HEK-293T cells were co-transfected with cDNAs expressing EPOR/gp130 receptor, APRE-luc and $Sr\alpha$ - β -gal reporter genes, in addition to increasing amounts of Myc-tagged SHP2. Cells were incubated in the presence or absence of EPO and lysed. (A) Effect of increasing amounts of co-transfected SHP2 on reporter gene expression. Relative luciferase activities represent the ratio of EPO-induced luciferase activity obtained in the presence versus absence of co-transfected SHP2. (B) Myc-tagged SHP2 protein levels in cell lysates were determined by Western-blot analysis with anti-Myc antibody. (C) Total SHP2 protein levels for cells transfected with the EPOR/gp130^{Y757} receptor.

that an immobilized phosphopeptide spanning the sequence surrounding this tyrosine residue could pull down SHP2 from cell lysates [4]. Whereas the gp130-dependent phosphorylation of SHP2 was shown to activate the Ras/MAPK signalling pathway [4], a second role was also proposed for SHP2 as a negative regulator of gp130 signalling [12–16]. This proposal was based on studies showing that substitution of Y759 by phenylalanine led to an enhancement of downstream JAK-STAT signalling. However, these studies were performed before the identification of this same tyrosine residue as a docking site for SOCS3 [5,6]. Overexpression of SOCS3 but, as we have shown, not overexpression of SHP2, leads to potent inhibition of gp130-mediated signalling and this activity is dependent on a functional binding site at Y757 in mouse gp130, corresponding to Y759 in human gp130 [5,6]. This implies that at least part of the negative signal mediated via Y757 can result from the actions of SOCS3, hence requiring that the role of SHP2 in suppressing gp130 signalling be reinvestigated.

A dissection of the relative roles that SOCS3 and SHP2 play in negatively regulating gp130 signalling is complicated by several factors. Deletion of either the SOCS3 or SHP2 genes in mice results in embryonic death, thus making it difficult to study the

individual contributions these genes make to gp130 signalling *in vivo* [27–29]. *In vitro* studies are hampered by the shared use of Y757 as the sole gp130-binding site, as substitution of this tyrosine residue results in the failure of gp130 to associate with either SOCS3 or SHP2. We have taken a more subtle mutagenesis approach by substituting residues flanking the critical Y757. The substitution of six amino acids surrounding the SOCS3/SHP2 docking site within a chimaeric EPOR/gp130 receptor resulted in a chimaeric receptor, which retained the capacity to recruit SHP2 to Y757, but not SOCS3.

Given that the SHP2-selective EPOR/gp130^{shpY757} receptor contained a number of amino acid changes in the sequence surrounding Y757, it was important to verify that this tyrosine residue was still phosphorylated in response to cytokine stimulation. We did this by demonstrating that EPO treatment of the EPOR/gp130^{shpY757} receptor resulted in the phosphorylation of SHP2. As gp130-induced activation of SHP2 requires recruitment to phosphorylated Y757 [3,4,14], and this activity was not observed for the EPOR/gp130^{shpF757} receptor, this result demonstrated that Y757 within the SHP2-selective EPOR/gp130^{shpY757} receptor must be phosphorylated in response to EPO treatment.

Despite being capable of recruiting SHP2, the SHP2-selective EPOR/gp130^{shpY757} receptor showed enhanced gp130-induced reporter gene expression relative to the receptor construct with the wild-type gp130 intracellular domain. Moreover, the magnitude of this enhancement was the same as that shown by the receptor containing a Y757F point mutation. This result suggests that the recruitment of SHP2 to Y757 is not sufficient to dampen downstream JAK-STAT signalling and that this activity is probably dependent on the recruitment of SOCS3.

Although EPO-dependent phosphorylation of SHP2 confirmed the association of SHP2 with the EPOR/gp130^{shpY757} receptor, despite numerous attempts, we were unable to detect EPO-induced association of SOCS3 with the EPOR/gp130^{Y757} receptor in co-immunoprecipitation experiments. The reason for this is not known, but it may be because inhibition of gp130 signalling by co-transfected SOCS3 resulted in very low levels of Y757 phosphorylation, thus limiting the amount of receptor-associated SOCS3 that could be detected by subsequent immunoprecipitation. However, strong evidence for a reduced association between SOCS3 and the EPOR/gp130^{shpY757} receptor came from an analysis of the inhibitory activity of overexpressed SOCS3. When compared with the EPOR/gp130^{Y757} receptor, the EPOR/gp130^{shpY757} receptor showed a reduced sensitivity to inhibition of reporter gene expression by SOCS3. In fact, the sensitivity of the various receptors to SOCS3 decreased in the order EPOR/gp130^{Y757} > EPOR/gp130^{shpY757} > EPOR/gp130^{F757} $\approx$ EPOR/gp130^{shpF757}. This order correlates with the predicted order [6] of affinities for SOCS3 binding to the peptide motifs surrounding residue 757, i.e. VE(pY)STVVH > AE(pY)IKLDW > VEFSTVVH > AEFIKLDW. (Table 1). These results demonstrate that, as predicted, the ability of SOCS3 to associate with the EPOR/gp130^{shpY757} receptor is reduced relative to the EPOR/gp130^{Y757} receptor.

Recently, Lehmann et al. [30] investigated the relative contributions that SOCS3 and SHP2 make to regulating IL-6 signalling. Whilst they found that SOCS3 is essential for negative regulation, they proposed, in contrast with our work, that SHP2 also contributes directly to the negative regulation of gp130 signalling. This conclusion, which is different from ours, must be a consequence of the different experimental approaches that were used in their work compared with ours. Whereas we used mutant gp130 receptors with differing SOCS3/SHP2 selectivities, Lehmann et al. [30] studied gp130 signalling in cells

overexpressing dominant-negative SHP2 mutants, or in mouse fibroblasts lacking exon 3 of SHP2. In both these cases, a mutant SHP2 molecule competes with SOCS3 for binding to Y757 of gp130. This is true for Δ exon 3 SHP2, as this exon only codes for the first 65 amino acids of SHP2, leaving the C-terminal SH2 domain intact, which we have shown can still bind Y757 [17]. In fact, given that the N-terminal SH2 domain of SHP2 is involved in negatively regulating the phosphatase activity of SHP2 [31], it is not clear whether removal of part of this domain would reduce or enhance the signalling activity of SHP2. There was no such competition between SHP2 and SOCS3 for receptor binding in our system, as the EPOR/gp130^{SHP Y757} receptor had been engineered to bind only SHP2. Lehmann et al. [30] also measured the effects on signalling of artificial 'receptor targeted' SHP2 constructs, such as a gp130-SHP2 chimaeric protein and a membrane-anchored SHP2. Clearly, the behaviour of such hybrid SHP2 constructs forced into proximity of gp130 may be quite different from that of wild-type SHP2. Relative contributions of SOCS3 and SHP2 to the negative regulation of leptin signalling have been assessed in two other studies [21,32]. Leptin receptor and gp130 are related cytokine receptors and leptin also induces the expression of SOCS3, which in turn attenuates signalling [33]. As with gp130, SOCS3 and SHP2 are recruited to the same tyrosine residue in the leptin receptor, and similar to our findings here for gp130, it was found that only SOCS3 was involved in attenuating leptin signalling [21,32].

Results presented here suggest that the recruitment of SHP2 to gp130 is not sufficient to inhibit downstream JAK-STAT signalling, and that the negative signal associated with Y757 on this receptor must therefore be due to the recruitment of SOCS3. Although a gp130 receptor mutant with the reciprocal specificity, i.e. engineered to bind only SOCS3 and not SHP2, might have provided additional results to support this conclusion, the design of a SOCS3-selective ligand has been more challenging and is the subject of ongoing work. Finally, given the range of disease pathologies exhibited by gp130 knock-in mice harbouring a non-functional SOCS3/SHP2 docking site [16,34], it would be interesting to see if the gp130 receptor mutants described here could be used to distinguish further which of these pathologies is due to excessive STAT signalling (loss of SOCS3 binding) versus a failure to activate the Ras/mitogen-activated protein kinase pathway (loss of SHP2 binding).

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