Regulation of interleukin-3-induced substrate phosphorylation and cell survival by SHP-2 (Src-homology protein tyrosine phosphatase 2)

Helen WHEADON, Christine EDMEAD and Melanie J. WELHAM¹

Department of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, U.K.

The cytosolic SHP-2 (Src homology protein tyrosine phosphatase 2) has previously been implicated in IL-3 (interleukin-3) signalling [Bone, Dechert, Jirik, Schrader and Welham (1997) *J. Biol. Chem.* **272**, 14470–14476; Craddock and Welham (1997) *J. Biol. Chem.* **272**, 29281–29289; Welham, Dechert, Leslie, Jirik and Schrader (1994) *J. Biol. Chem.* **269**, 23764–23768; Qu, Nguyen, Chen and Feng (2001) *Blood* **97**, 911–914]. To investigate the role of SHP-2 in IL-3 signalling in greater detail, we have inducibly expressed WT (wild-type) or two potentially substrate-trapping mutant forms of SHP-2, generated by mutation of Asp-425 to Ala (D425A) or Cyst-459 to Ser (C459S), in IL-3-dependent BaF/3 cells. Effects on IL-3-induced tyrosine phosphorylation, signal transduction and functional responses were examined. Expression of C459S SHP-2 protected the β -chain of the murine IL-3R (IL-3 receptor), the adaptor protein Gab2 (Grb2-associated binder 2), and a cytosolic protein of 48 kDa from tyrosine dephosphorylation, consistent with them being bona fide substrates of SHP-2 in IL-3 signalling. The tyrosine phosphorylation of a 135 kDa transmembrane protein was also protected upon expression of C459S SHP-2. We have identified the inhibitory immunoreceptor PECAM-1 (platelet

endothelial cell adhesion molecule-1)/CD31 (cluster determinant 31) as a component of this 135 kDa substrate and also show that IL-3 can induce tyrosine phosphorylation of PECAM-1. Expression of WT, C459S and D425A forms of SHP-2 had little effect on IL-3-driven proliferation or STAT5 (signal transduction and activators of transcription) phosphorylation or activation of protein kinase B. However, expression of WT SHP-2 increased ERK (extracellular-signal-regulated kinase) activation. Interestingly, expression of C459S SHP-2 decreased ERK activation at later times after IL-3 stimulation, but potentiated IL-3-induced activation of Jun N-terminal kinases. In addition, expression of C459S SHP-2 decreased cell survival in suboptimal IL-3 and upon IL-3 withdrawal. These findings indicate that SHP-2 plays an important role in mediating the anti-apoptotic effect of IL-3 and raises the possibility that PECAM-1 participates in the modulation of cytokine-induced signals.

Key words: Aic2A (β -chain of the murine IL-3R), Gab2, PECAM-1 (platelet endothelial cell adhesion molecule-1), SHP-2, substrate-trapping.

INTRODUCTION

Tyrosine-phosphorylation- and dephosphorylation-dependent events are critical for controlling growth, differentiation and cell survival. Interleukin-3 (IL-3) induces the activation of members of the Jak [1] and Src families of PTKs (protein tyrosine kinases) [2]. Once activated, these PTKs phosphorylate tyrosine residues on IL-3R (IL-3 receptor) and a number of cytoplasmic proteins. Signalling molecules are recruited to the receptor leading to activation of the STAT5 (where STAT stands for signal transduction and activators of transcription) [3], Ras/MAPK (where MAPK stands for mitogen-activated protein kinase; also referred to as ERK) [4] and phosphoinositide-3 kinase [5,6] pathways. Regulation of these cascades involves co-ordination of the interactions between adaptor proteins and signalling molecules [7]. One protein that has been implicated in such regulation of IL-3 signalling is the SHP-2 (Src homology protein tyrosine phosphatase 2) [8–10].

SHP-2 is a 68 kDa ubiquitously expressed PTP (protein tyrosine phosphatase) characterized by a single phosphatase domain at its C-terminus with two adjacent SH2 domains at its N-

terminus [11,12]. Binding of the N-terminal SH2 domain to the phosphatase domain maintains SHP-2 in an inactive conformation [13]. Activation occurs after engagement of the N-terminal SH2 domain by phosphotyrosine proteins, leading to relief of inhibition on the phosphatase domain. Both the SH2 and phosphatase domains contribute to functional specificity, as shown by domain-swapping experiments [14,15], and it is probable that the SH2 domains play a key role in targetting SHP-2 to its substrates. Generation of mice harbouring a deletion of the N-terminal SH2 domain of SHP-2 has demonstrated the requirement of SHP-2 during embryonic development and haemopoiesis [16–20].

SHP-2 can act as a positive mediator of cellular responses by promoting ERK1 (extracellular-signal-regulated kinase/mitogen-activated protein kinase 1) and ERK2 activation [21–27]. However, the mechanisms used by SHP-2 to mediate these effects are complex. It has been proposed that this may be because SHP-2 can act both as an adaptor protein (binding to proteins via its SH2 domains and tyrosine-phosphorylated residues) and through its phosphatase activity (via its ability to modulate the function of its substrates by dephosphorylation). The association of SHP-2 with adaptor proteins Gab1 (Grb2 associated binder 1) and Gab2

Abbreviations used: CD31, cluster determinant 31; DiOC₆, 3,3'-dihexyloxycarbocyanine iodide; IL, interleukin; IL-3R, IL-3 receptor; Aic2A, β -chain of the murine IL-3R; ERK, extracellular-signal-regulated kinase; Gab1, Grb2-associated binder 1; IP, immunoprecipitation; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; PECAM-1, platelet endothelial cell adhesion molecule-1; PTP, protein tyrosine phosphatase; SH2, Src-homology 2; PZR, protein zero related; SHP-2, Src-homology protein tyrosine phosphatase 2; STAT, signal transduction and activators of transcription; WT, wild-type; XTT, sodium 3'-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulphonic acid hydrate.

¹ To whom correspondence should be addressed (e-mail m.j.welham@bath.ac.uk).

has been implicated in SHP-2 regulation of ERK activation [28–31] and studies have indicated that Gab1 and Gab2 are also substrates for SHP-2 [28,29,32].

SHP-2 also associates with a wide range of growth factor and cytokine receptors [26,33,34], inhibitory receptors [35] and cellular proteins [26,36,37]. We have previously demonstrated that IL-3 induces the association of SHP-2 with the β -chain of the IL-3R [8], activation and tyrosine phosphorylation of SHP-2, which leads to co-precipitation of SHP-2 with Grb2 and PI3K (phosphoinositide 3-kinase) [9,10]. The association of SHP-2 with PI3K is mediated by Gab2 [29,32]. We have recently generated a series of SHP-2 mutants, based on the studies of Flint et al. [38], by mutation of Asp-425 to Ala (D425A) or Cys-459 to Ser (C459S), to generate catalytically inactive 'substrate-trapping' versions of SHP-2. Using these mutants *in vitro* enabled us to identify both the β -chain of the IL-3R and Gab2 as potential SHP-2 substrates [39]. These studies also highlighted the fact that proteins bound by the SH2 domains of SHP-2 were also potential substrates [39]. We have now extended these studies to examine substrates whose tyrosine phosphorylation is regulated by SHP-2 in IL-3-signalling using the tetracycline-inducible gene expression system to express WT (wild-type), C459S and D425A forms of SHP-2 in IL-3-dependent BaF/3 cells. We have also used these transfectants to investigate the biochemical and functional importance of SHP-2 in IL-3 signal transduction.

EXPERIMENTAL

cDNA constructs

WT SHP-2 was a gift from Dr U. Dechert (BRAIN, Darmstadt, Germany). C459S and D425A substitutions were created by PCR-based site-directed mutagenesis as described previously [39]. All mutations were verified by DNA sequencing. PCR products were cloned into the *Bam*HI and *Eco*RI sites of the Myc epitope tagging vector, pBluescript-N-Myc2 [40]. Myc-tagged SHP-2s were subcloned into the tetracycline-response vector pUHD10-3neo [41,42].

Cell culture

Murine IL-3-dependent BaF/3 cells expressing a tetracycline transactivator (tTA) were a gift from Dr A. Mui (DNAX, Palo Alto, CA, U.S.A.) [43]. They were cultured and electroporated (10 μ g of DNA at 960 μ F, 450 V for 1×10^7 cells) as described previously [40]. After a 48 h recovery, cells were placed under selection with 1 mg/ml G418, 1 μ g/ml puromycin and 2 μ g/ml tetracycline. Clones were picked after 10–14 days, expanded and screened for inducible expression of the introduced cDNAs.

Screening for tetracycline-regulated expression

Antibiotic-resistant clones were expanded in the presence of 2 μ g/ml tetracycline. Cells were washed three times in $1 \times$ Hanks buffered saline solution, containing 20 mM Hepes and plated at 1×10^5 cells/ml in the absence or presence of 2 μ g/ml tetracycline. After 24 h, cells were washed once in PBS, lysed, and 20 μ g of protein was used per sample for immunoblotting.

Induction of expression in bulk cultures and cell stimulations

SHP-2 expression was induced for 24 h as described above. IL-3 stimulations and cell lysis were performed as described previously [9]. Protein concentrations were determined using the Bradford assay, according to the manufacturer's recommendations (Bio-Rad), and equivalent amounts of protein were used for immunoblotting or precipitation.

Subcellular fractionation and IP (immunoprecipitation)

Subcellular fractionation and IPs were performed as described previously [9,44,45]. The following rabbit polyclonal antibodies were used: 1 μ g anti-Gab2 (06-967; Upstate Biotechnology, Lake Placid, NY, U.S.A.), 1 μ g anti-SHP-2 (sc-280; Santa Cruz Biotechnologies, Santa Cruz, CA, U.S.A.), 1 μ g anti-STAT5 (sc-835; Santa Cruz), 2 μ g of anti-Aic2A (sc-677; Santa Cruz; where Aic2A stands for the β -chain of the murine IL-3R), 5 μ g of anti-myc epitope tag mouse monoclonal antibody (9E10) or 3 μ g of anti-CD31 (cluster determinant 31)/PECAM-1 (platelet endothelial cell adhesion molecule-1) monoclonal antibody (558 736; BD Biosciences, Cowley, Oxfordshire, U.K.).

SDS/PAGE and immunoblotting

SDS/PAGE and immunoblotting were performed as described previously [44,45]. The following primary antibodies were used: anti-phosphotyrosine antibody 4G10 at 0.1 μ g/ml (05-321, Upstate Biotechnologies); 1:1000 for polyclonal antibodies against phosphorylated forms of ERK1 and ERK2 (9101; Cell Signalling Technology, Beverly, MA, U.S.A.), JNKs (Jun N-terminal kinases, 9251; Cell Signalling Technology) and phosphorylated STAT5a/b (05-495; Upstate Biotechnologies); 0.5 μ g/ml for 9E10 and anti-Gab2 antibody and 0.2 μ g/ml for anti-Aic2A, anti-SHP-2, anti-STAT5, anti-ERK1 (sc-93; Santa Cruz) and anti-JNK antibodies (9252; Cell Signalling Technology). Secondary antibodies conjugated to horseradish peroxidase were used at 0.05 μ g/ml (Dako, Denmark). Immunoblots were developed using ECL (Amersham Biosciences, Chalfont, Bucks., U.K.) and Kodak X-AR 5 film and stripped and reprobed using standard conditions [10]. In some cases, densitometry was performed on blots from at least three independent experiments.

Preparation of nuclear extracts

Nuclear extracts were prepared as described previously [46] using 5×10^6 cells per extraction. Clarified supernatants were retained and protein concentrations determined. For immunoblotting, 20 μ g of protein per sample was used.

Flow cytometry and apoptosis assays

For cell-surface staining of PECAM-1, BaF/3 transfectants were cultured at low (1×10^4 /ml) or high (1×10^5 /ml) density, washed and stained in PBS containing 2 % (v/v) foetal bovine serum with anti-PECAM-1 or isotype matched control (IgG2a, κ) and FITC-conjugated anti-rat secondary antibody. For analysis of apoptosis, transfectants were cultured in the presence or absence of 2 μ g/ml tetracycline for 24 h, washed and cultured with no IL-3 or 1 pg/ml IL-3 for 24 h $\pm$ tetracycline at 5×10^4 cells/ml. cells (1×10^6) were stained in the dark for 15 min with 10 nM DiOC₆ (3,3'-dihexyloxycarbocyanine iodide; Sigma) in PBS [47–49]. Samples were washed once with PBS and 10 000 events were analysed per sample using a FACS Vantage system (BD Biosciences, Cowley, Oxfordshire, U.K.).

Statistical analyses

Two-tailed paired Student's *t* tests were used to analyse the results.

RESULTS

Expression of SHP-2 variants in IL-3-dependent BaF/3 cells

Previous investigations support a role for SHP-2 in IL-3 signalling. However, little information exists on the identity of the

Figure 1 Inducible expression of catalytically inactive and WT SHP-2 in BaF/3 cells

Cells were incubated for 24 h in the presence (+) or in the absence (–) of 2 μ g/ml tetracycline (Tet) and then treated for 10 min with IL-3 (3) or left untreated as controls (C). Samples were immunoblotted using an anti-SHP-2 polyclonal antibody. The results from C459S clone 12, D425A clone 18 and WT clone 12 are shown. These clones were representative of three independent clones expressing each variant. Molecular-mass standards are shown in kDa, and positions of the SHP-2 variants (*myc*-SHP-2) and endogenous SHP-2 are indicated.

key protein substrates targeted by SHP-2 or on the functional responses regulated by SHP-2 in IL-3-dependent cells. To address these issues, we used the tetracycline off-regulated gene-expression system to express variants of SHP2 in IL-3-dependent BaF/3 cells [41,42]. This system has been a valuable tool in our laboratory for analysing proteins that have detrimental effects on cell growth [40,46,50]. Most importantly, it enables the effects of expressing a mutant SHP-2 protein to be directly compared with the same cell clone not expressing the SHP-2 variant, allowing a more reliable study of any resulting effects observed. An inducible expression also bypasses many of the problems of clonal variation, which are difficult to control adequately and are often encountered when clones constitutively expressing mutant proteins are used for delineating protein function. We generated two mutated forms of SHP-2, based on substrate trapping versions of PTP1B [38]. C459S SHP-2 has Cys-459 replaced by a Ser residue and has been used widely as a dominant-negative, catalytically inactive form of SHP-2 [21–23,51]. The second mutant we generated has an Ala replacing Asp at position 425 (D425A SHP-2). This SHP-2 mutant has not been previously used for cellular expression studies. We also expressed WT SHP-2, mainly for control purposes.

BaF/3 cells stably expressing tTA were transfected with response plasmids encoding N-terminal *myc* epitope-tagged versions of WT, C459S and D425A forms of SHP-2. The inducible expression was assessed, and three independent clones for each SHP-2 variant, showing low basal and consistent inducible expression in the absence of tetracycline were selected for further analyses; examples are shown in Figure 1. We screened more than 50 clones for each SHP-2 variant and routinely observed 1–2-fold overexpression, consistent with levels achieved using other cell and expression systems [21,22]. Maximal expression was observed 24 h after tetracycline removal and was maintained for 72 h (results not shown).

SHP-2 variants interact with a similar profile of tyrosine-phosphorylated proteins in response to IL-3

We determined whether the expressed SHP-2 variants interacted with a profile of proteins similar to those previously demonstrated [8–10]. Specific IP of *myc*-tagged SHP-2 variants revealed that each variant complexed with tyrosine-phosphorylated proteins of 135, 97 and 80 kDa after stimulation with IL-3 (Figure 2A, lanes 4, 8 and 12). This complex was similar to the profile observed in SHP-2 IPs from parental BaF/3 cells (Figure 2A, lane 14).

Figure 2 Association of SHP-2 variants with tyrosine-phosphorylated proteins

C459S, D425A and WT SHP-2 clones were incubated in the presence (+) or absence (–) of 2 μ g/ml Tet for 24 h. Transfectants and parental BaF/3 cells were stimulated for 10 min with 10 ng/ml rIL-3 (IL-3) or left untreated as controls (CON). (A) Cell extracts from 5×10^6 cells were used to prepare anti-*myc* epitope tag or anti-SHP-2 IPs. (B) Only C459S SHP-2 transfectants were used. Whole cell lysate (WCL, 20 μ g) was removed before IP with either anti-*myc* epitope tag or anti-SHP-2 antibodies. Immunoblotting with 4G10 was performed to detect tyrosine-phosphorylated proteins (α -PY). The immunoblot for the C459S samples in (A) was stripped and reprobed with a polyclonal antibody raised against Gab2 (lower panel) and in (B) with anti-SHP-2 polyclonal antibody (lower panel). Molecular-mass standards are shown in kDa, and the positions of p135, p97, SHP-2 and p48 are indicated.

For a direct comparison, anti-*myc* TAG and anti SHP-2 IPs were prepared from cell extracts of C459S SHP-2 transfectants and are shown in Figure 2(B). A 135 kDa protein co-precipitated with all the expressed SHP-2 variants after IL-3 stimulation. A 135 kDa protein was also distinctly observed in samples prepared from C459S SHP-2-expressing cells that were not stimulated (Figure 2A, lane 2; 2B, lane 5), suggesting that this SHP-2 variant may have a higher affinity for this protein (see below for further details). Reprobing the C459S blot shown in Figure 2(A) with an anti-Gab2 antibody indicated that at least a portion of the 97 kDa phosphotyrosyl protein that co-precipitated was Gab2 (Figure 2A lower panel), consistent with previous observations [29,32,39,52]. It is also noteworthy that a protein of approx. 48 kDa was clearly present at higher levels in precipitates prepared from transfectants expressing C459S SHP-2 and was present in both untreated and IL-3-treated cells (Figure 2A). The 80 kDa protein in the anti-TAG IPs and the 68 kDa protein in the anti-SHP-2 IPs co-migrated with proteins immunoreactive with anti-SHP-2 antibodies on reprobing (Figure 2B, lower panel), suggesting that they represent, at least in part, epitope-tagged SHP-2 and endogenous SHP-2. The fact that we did not detect any endogenous SHP-2 in C459S SHP-2 precipitates suggests that SHP-2 is not a substrate for itself.

Expression of C459S SHP-2 prolonged tyrosine phosphorylation of cellular proteins

We were interested in the biochemical signals regulated by SHP-2 in IL-3 signalling. Identification of the key substrates of SHP-2 is critical to furthering our understanding. Therefore, we investigated whether expression of any of the SHP-2 variants affected

Figure 3 Prolongation of tyrosine phosphorylation of cellular proteins by C459S SHP-2

C459S SHP-2 transfectants were incubated in the presence (+) or absence (–) of 2 μ g/ml Tet for 24 h. (A, C) Cells were treated for the indicated times (in min) with 10 ng/ml IL-3 or left untreated (0). (B, D) Cells were stimulated for 10 min with 10 ng/ml of IL-3 (3). (B) Cells were washed and samples taken at the indicated times (in min) or left untreated (0 or C). (A, B) 20 μ g of protein/sample was immunoblotted in duplicate. (C) Anti-myc TAG immunoprecipitates were prepared from 500 μ g of protein/sample. (D) Anti-TAG precipitates were prepared from cytosolic and membrane fractions equivalent to 2×10^7 cells. Samples were separated by either 10% (upper) or 7% (lower) acrylamide gels to examine the location of the 135 and 48 kDa proteins respectively. Blots were probed to detect tyrosine-phosphorylated proteins (α -PY), SHP-2 or Gab2. The positions of prolonged tyrosine-phosphorylated proteins are indicated, as are the positions of endogenous and C459S-SHP-2.

tyrosine phosphorylation of cellular proteins. Cells were either stimulated continuously with IL-3 (Figure 3A) or stimulated for 10 min with IL-3, washed free of IL-3 and samples taken over the next 6 h (Figure 3B). In the presence of C459S SHP-2, an increase in tyrosine phosphorylation of proteins of 97 and 135 kDa was observed, most noticeable at 120, 240 and 360 min after IL-3 treatment. Similar time courses in cells expressing equivalent

levels of D425A or WT SHP-2 revealed no alterations in tyrosine phosphorylation (results not shown) and identical results were observed in independent clones. To examine whether C459S SHP-2 was trapping these proteins, we prepared anti-TAG IPs from extracts treated for prolonged periods of time with IL-3. As shown in Figure 3(C), phosphotyrosyl proteins of 135, 97, 80 and 48 kDa were precipitated after 10, 120 and 240 min IL-3 treatment. Reprobing with anti-Gab2 antibodies (Figure 3C, lower panel) suggested that, as shown in Figure 2, at least a portion of the 97 kDa protein was Gab2. Reprobing with anti-SHP-2 antibodies suggested that at least a fraction of the 80 kDa was C459S SHP-2, precipitated by the anti-TAG antibody (results not shown). These results suggested that expression of C459S SHP-2 leads to alterations in tyrosine phosphorylation of discrete proteins, probably, by trapping key residues on these substrates.

We also examined the subcellular localization of the proteins trapped by C459S SHP-2. Cytosolic and membrane fractions were prepared and C459S SHP-2 was precipitated. As shown in Figure 3(D), the 135 kDa phosphotyrosyl protein was present in the membrane fraction, albeit faintly, probably due to dephosphorylation during the fractionation process and the fact that only a small fraction of SHP-2 was membrane-localized in BaF/3 cells [9]. The 48 kDa protein was located almost exclusively in the cytosol. We attempted to identify this protein and examined whether it corresponded to SHP-2 substrates of a similar molecular weight identified in other cell systems. Investigations revealed that p48 did not correspond to the membrane localized protein PZR (protein zero related) [53,54]. We also investigated whether p48 corresponded to the Shc adaptor protein, which was heavily tyrosine-phosphorylated in response to IL-3 [55], but found no evidence that Shc tyrosine phosphorylation was altered upon expression of C459S SHP-2 (results not shown). Further investigations are required to identify this putative substrate.

The 135 kDa protein trapped by C459S SHP-2 is PECAM-1/CD31

Trapping of a 135 kDa membrane-localized protein by SHP-2 in BaF/3 cells has been reported previously [52], and the present studies confirm this. However, the identity of this protein has not been reported. Hence we undertook studies to characterize this protein further. We found that the 135 kDa protein did not correspond to signal-regulatory protein α , CD19 or paired immunoreceptor-B (results not shown). All of them have been shown to be SHP-2 substrates in other systems. Next, we assessed whether p135 was the inhibitory immunoreceptor PECAM-1/CD31 because, previously, this has been shown to be a substrate for SHP-2 [56]. Cell-surface expression of PECAM-1 was high on C459S SHP-2 transfectants grown at both low density (1×10^4 /ml) and higher density (1×10^5 /ml), as shown in Figure 4(A). Expression of C459S SHP-2 led to increased tyrosine phosphorylation of PECAM-1 in the absence of IL-3 treatment and this was maintained for 240 min after IL-3 treatment (Figure 4B). Expression of the D425A SHP-2 had no consistent effect on levels of PECAM-1 tyrosine phosphorylation (results not shown). In a more detailed time-course analysis (see Figure 4C), it can be seen that, in the precipitates prepared from cells maintained in tetracycline, IL-3 treatment increases PECAM-1 phosphorylation (seen at 10 and 60 min time points). This has not been reported previously. At all times, PECAM-1 phosphorylation was enhanced in samples expressing C459S SHP-2, consistent with it being protected from dephosphorylation by C459S SHP-2 and thus being a substrate for SHP-2. Immunoblotting with anti-SHP-2 antibodies demonstrated that both endogenous and ectopically expressed C459S SHP-2 associated with PECAM-1, consistent with earlier reports. These results are consistent with a portion of

Figure 4 Prolongation of tyrosine phosphorylation of PECAM-1/CD31 by C459S SHP-2 expression

(A) C459S SHP-2 transfectants were cultured at either 1×10^4 or 1×10^5 cells/ml for 24 h prior to staining with an anti-PECAM-1 antibody to detect surface expression. Ten thousand events were analysed per sample by flow cytometry, and fluorescence detected on FL-1. The shaded peaks represents staining with isotype matched control antibody and the unshaded peaks represents staining with the PECAM-1 antibody. (B, C) C459S SHP-2 transfectants were incubated in the presence (+) or absence (–) of $2 \mu\text{g/ml}$ Tet for 24 h. Cells were stimulated for 10 min with 10 ng/ml of IL-3, washed, and samples taken at the indicated times (in min) or left untreated (0). PECAM-1 immunoprecipitates (IPs) were prepared from 500 μg of each cell extract, except for the 10 min time points in (B), where only 250 $\mu\text{g/IP}$ was used (hence the comparatively decreased signal with these two samples, which are included for completeness). Immunoblotting with 4G10 was performed to detect tyrosine-phosphorylated proteins ($\alpha\text{-PY}$). The same immunoblots were stripped and reprobed with polyclonal antibodies against SHP-2 (lower panels). The positions of PECAM-1/CD31 and SHP-2 are indicated.

the 135 kDa protein identified in time-course analyses (shown in Figures 3A and 3B) as being PECAM-1, and demonstrate, for the first time, that PECAM-1 is a substrate for SHP-2 in IL-3 signalling responses.

Gab2 and Aic2A are SHP-2 substrates

Previous studies by us and others have indicated that Gab2 (97 kDa) is a potential substrate for SHP-2 [9,29,32,39,52], and we have *in vitro* data suggesting Aic2A (140 kDa) is also a potential SHP-2 substrate [39]. As shown in Figure 5(A), Gab2 tyrosine phosphorylation was increased at later times after IL-3 stimulation in the presence of C459S SHP-2, most clearly noticeable at 240 min. Densitometry of independent experiments gives average increases of 26% at 120 min and 117% at 240 min in samples expressing C459S SHP-2. At later time points, little tyrosine phosphorylation of Gab2 could be detected. We also detected C459S SHP-2 in Gab2 precipitates for prolonged periods (Figure 5A), consistent with the results shown in Figure 3(C), and it is present in greater proportions, compared with endogenous SHP-2. Expression of D425A (Figure 5B) or WT SHP-2 (results not shown) at similar levels did not have a consistent protective effect on Gab2. In addition, neither of these variants was present

Figure 5 Prolongation of Gab2 tyrosine phosphorylation by C459S SHP-2 expression

C459S and D425A SHP-2 clones were incubated in the presence (+) or absence (–) of $2 \mu\text{g/ml}$ Tet for 24 h. (A, B) Cells were stimulated for 10 min with 10 ng/ml IL-3, washed and samples taken at the indicated times (in min) or left untreated (0). (C) Cells were pretreated for 15 min with 100 μM pervanadate before treatment with 10 ng/ml IL-3 for 10 min (3). To prepare Gab2 IPs, 500 μg of each cell extract was used. Immunoblotting with 4G10 was performed to detect tyrosine-phosphorylated proteins ($\alpha\text{-PY}$). The same immunoblots were stripped and reprobed with polyclonal antibodies against Gab2 (middle panels) and SHP-2 (lower panels). Molecular-mass standards are shown in kDa, and the positions of Gab2 and SHP-2 are indicated.

in Gab2 precipitates for sustained times. In the case of D425A SHP-2, there was clearly a lesser amount of the variants present in Gab2 precipitates at later times, compared with endogenous levels, a situation opposite to what we observed for C459S SHP-2. Pretreatment with pervanadate potentiated the effect of C459S SHP-2 on Gab2 tyrosine phosphorylation (Figure 5C, lanes 3 and 4). This was not observed in D425A or WT SHP-2 transfectants (results not shown). This evidence, taken with the results above, is consistent with previous work, suggesting that Gab2 is an important substrate of SHP-2 downstream of the IL-3R.

Effects of expression of the SHP-2 mutants on Aic2A tyrosine phosphorylation were also assessed. C459S SHP-2 expression afforded a moderate, but nonetheless consistently observed, increase in IL-3-induced Aic2A tyrosine phosphorylation at early times (10 min) after IL-3 treatment (Figure 6A). On average, a 50% increase ($\pm$ S.E.M. 14.5%, $P < 0.05$) was apparent compared with the level of Aic2A phosphorylation observed in cells

Figure 6 Increase in Aic2A tyrosine phosphorylation by C459S SHP-2 expression

C459S and D425A SHP-2 clones were treated as described in the legend to Figure 5, with (C) being pretreated with pervanadate. Anti-Aic2A immunoprecipitates were prepared from 500 μ g of cell lysate. Tyrosine-phosphorylated Aic2A was detected by immunoblotting with 4G10 (α -PY). Blots were stripped and (A–C) reprobed with an anti-Aic2A antibody (lower panels), whereas (D) was reprobed with anti-TAG antibody, 9E10. Molecular-mass standards are shown in kDa, and the positions of Aic2A and C459S SHP-2 are indicated. (E) The mean level of IL-3-induced Aic2A tyrosine phosphorylation in the absence of Tet is indicated as a percentage of the tyrosine phosphorylation measured in + Tet + IL-3 samples (latter set at 100% for comparative purposes). Densitometry was performed on four independent experiments for each SHP-2 variant. The mean, S.E.M. and significance (*P*) values are shown.

maintained in tetracycline and treated with IL-3 (Figure 6E). Pre-treatment with a low dose of pervanadate enhances detectable levels of tyrosine phosphorylated Aic2A [39]. Correspondingly, Aic2A tyrosine phosphorylation was also enhanced in cells expressing C459S SHP-2 that had been pretreated with pervanadate (see Figure 6C). We also demonstrate the presence of C459S SHP-2 in Aic2A precipitates (Figure 6D) and levels were increased after IL-3 stimulation. Expression of neither D425A SHP-2 (Figure 6B) nor WT (Figure 6E) affected Aic2A tyrosine phosphorylation in the absence or presence of pre-treatment with pervanadate (results not shown). Collectively, our results indicate that Aic2A is an SHP-2 substrate, although the moderate effects observed are consistent with only one or two sites on Aic2A being targeted by SHP-2. Owing to the very distinct kinetic differences observed, we consider it unlikely that Aic2A contributes to the 135 kDa protein protected by C459S SHP-2 expression in long-term IL-3 stimulations (Figure 3).

STAT5 is not an SHP-2 substrate in BaF/3 cells

STAT5a and STAT5b become tyrosine-phosphorylated after IL-3 stimulation of BaF/3 cells [3], with phosphorylation of Y694 (STAT5a) and Y699 (STAT5b) being necessary for their dimerization and translocation to the nucleus. *In vitro* analyses have suggested that STAT5 is a potential SHP-2 substrate [57], and work published during the preparation of this paper, largely performed in non-haemopoietic cells, supported previous findings [58]. Therefore it was important to investigate whether expression of SHP-2 mutants in BaF/3 cells perturbed IL-3-induced STAT5 phosphorylation. Expression of none of the SHP-2 variants affected overall levels of STAT5a/STAT5b tyrosine phosphorylation induced by IL-3 (see Figure 7A). Site-specific STAT5a/STAT5b phosphorylation at Y694/699 and translocation to the nucleus

Figure 7 Insignificant effect of expression of SHP-2 variants on STAT5a/b phosphorylation or translocation

Transfectants were incubated in the presence (+) or absence (–) of 2 μ g/ml Tet for 24 h. Cells were treated for the indicated times (in min) with 10 ng/ml IL-3 or left untreated (0). (A) To prepare STAT5 immunoprecipitates, 250 μ g of each cell extract was used. 4G10 was used to detect tyrosine-phosphorylated STAT5 (upper panels) and reprobed with a polyclonal antibody against STAT5 (lower panel). (B) Cytosolic and nuclear extracts were prepared and 20 μ g of protein was immunoblotted with a phospho-specific STAT5 antibody (upper panels) and reprobed with α -STAT5 (lower panels). Positions of phosphorylated and unphosphorylated STAT5 are indicated.

Figure 8 Effect of C459S and WT SHP-2 expression on MAPK activation

C459S (A, C) or WT SHP-2 (B, D) clones were incubated in the presence (+) or absence (–) of 2 μ g/ml Tet for 24 h prior to being stimulated for the indicated periods of time (in min) with 10 ng/ml rIL-3, or left untreated as controls (0). (A, C, D) Cytosolic (cyt) and (B) nuclear (nuc) extracts were prepared and 20 μ g of protein was immunoblotted with antibodies specific for activated forms of ERK1 and 2 (A and B, α -pERK, upper panels) or activated forms of JNKs (C and D, α -pJNK). The membranes were reprobed with an anti-pan ERK antibody (A and B, α -ERK, lower panel) or with an anti pan-JNK antibody (C and D, α -JNK, lower panel).

were also unaffected by expression of the SHP-2 variants (Figure 7B), implying that STAT5a and STAT5b are not substrates of SHP-2 *in vivo*.

SHP-2 involved in the regulation of MAPK activation in response to IL-3

Many reports support a role for SHP-2 in the activation of ERKs [10,21–27,29,33]. Expression of C459S SHP-2 decreased IL-3-induced phosphorylation of ERK1 and ERK2, most apparent at later times (120–360 min), as shown in Figure 8(A). After 120 min IL-3 stimulation, cells expressing C459S SHP-2 showed a decrease in ERK phosphorylation of, on average, 42% ($\pm 12.6\%$, $P < 0.05$) compared with the level observed in cells maintained

in tetracycline. Expression of WT SHP-2, on the other hand, enhanced IL-3-induced ERK1 and ERK2 activation, particularly at later times (60–240 min), in both cytosolic and nuclear extracts (Figure 8B). At 60 min, the average increase in WT SHP-2 expressing cells compared with + tetracycline cells was 102% ($\pm 18\%$, $P < 0.05$), and at 240 min the average increase compared with + tetracycline sample was 38% ($\pm 2.7\%$, $P < 0.01$). Expression of D425A SHP-2 had no effect on ERK activation (results not shown). The pan-ERK antibody demonstrates equality of loading, and consistent with our previous observations [46], under basal conditions, ERK1 and ERK2 are present in the nuclear fractions of BaF/3 cells.

We also examined effects of expression of the different SHP-2 variants on IL-3-induced activation of the JNK family of MAPKs. Interestingly, expression of C459S SHP-2 augmented IL-3-induced JNK activation and this was clearly observed after 30, 60 and 120 min of IL-3 treatment (Figure 8C). In cells treated for 30 min with IL-3, expression of C459S SHP-2 led to, on average, a 73% increase ($\pm 15.5\%$, $P < 0.02$) in IL-3-induced JNK phosphorylation when compared with the level in the same cells which had been maintained in tetracycline. After 60 min stimulation with IL-3, a 356% increase ($\pm 48.2\%$, $P < 0.02$) in JNK phosphorylation was observed, and after 120 min stimulation with IL-3, a 165% increase ($\pm 1.54\%$, $P < 0.0001$) in JNK phosphorylation was observed in those cells expressing C459S SHP-2 when compared with the levels of JNK phosphorylation measured for the same cells maintained in tetracycline. WT SHP-2 had no effect on IL-3-induced activation of JNKs (Figure 7D). These results suggest that SHP-2 plays opposing roles in the regulation of MAPKs, positively regulating ERK activation and negatively regulating JNK activation in response to IL-3.

Expression of C459S SHP-2 decreases cell survival at suboptimal doses of IL-3

SHP-2 has been reported to be involved in the regulation of proliferation in a number of systems [22,24,59]. IL-3 dose-response assays were performed using reduction of XTT (sodium 3'-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulphonic acid hydrate) as an indicator of metabolic activity relating to cell growth. We found that expression of none of the variants significantly affected IL-3-induced proliferation. We also examined effects on IL-3-induced survival. A decrease in mitochondrial membrane potential precedes apoptosis and can be used to measure early apoptotic events [47,49]. DiOC₆ stained cells that had an intact mitochondrial potential and weakly stained cells whose mitochondrial membrane potential was depolarized [49]. DiOC₆ staining of IL-3-grown BaF/3 yielded a single population of strongly stained cells. This was decreased after treatment with the mitochondrial uncoupling agent CCCP (see Figure 9A), the left-shifted cells representing those undergoing apoptosis. Transfectants were incubated with or without tetracycline in the presence of 20 pg/ml IL-3, which supports some proliferation in a 72 h growth assay (results not shown), 1 pg/ml IL-3, which we have found to support survival of BaF/3 for 24–48 h but has a limited ability to induce proliferation, or no IL-3. In the presence of 20 pg/ml IL-3, more than 95% of cells remained viable and no effect of SHP-2 variant expression on cell survival was observed (results not shown). Representative flow cytometry profiles shown in Figure 9(B) demonstrate the effects observed after 24 h when C459S SHP2 was expressed and are summarized in Table 1. C459S SHP-2 expression in clone 12 led to average decreases of 13.4% ($\pm 0.8\%$, $P < 0.01$) and 11.3% ($\pm 2.4\%$, $P < 0.05$) in cells surviving after 24 h culture in 1 pg/ml IL-3 or no IL-3 respectively. Similar effects were observed in a

Figure 9 Reduction in cell survival due to C459S SHP-2 expression

Cells were incubated in the presence or absence of 2 μ g/ml Tet for 24 h, washed and incubated for a further 24 h (+ or – Tet) with 1 pg/ml IL-3 or no IL-3. Cells were stained with 10 nM DiOC₆ and 10 000 events were analysed per sample by flow cytometry. (A) Parental BaF/3 cells in the presence or absence of CCCP. (B) C459S SHP-2 transfectants.

Table 1 Effect of expression of SHP-2 variants on cell survival in suboptimal doses of IL-3 and after IL-3 withdrawal

Percentage decrease in cells with intact mitochondrial membrane potentials in transfectants induced to express the indicated SHP-2 variant, and the results were compared with those maintained in a non-induced state. Data used were from three independent experiments. The mean, S.E.M. and significance (*P*) values are shown and were calculated using a two-tailed paired Student's *t* test.

SHP-2 variant	Decrease in cells with intact mitochondrial membrane potentials (%)	
	1 pg/ml IL-3	No IL-3
C459S		
Clone 12	13.4 $\pm$ 0.8, <i>P</i> < 0.01	11.3 $\pm$ 2.4, <i>P</i> < 0.05
Clone 47	9.6 $\pm$ 0.9, <i>P</i> < 0.01	4 $\pm$ 0.7, <i>P</i> < 0.05
Wild-type	2 $\pm$ 1.4	0.5 $\pm$ 1.0

second, independent clone (clone 47), although at a decreased level, quite probably because this clone consistently expressed less C459S SHP-2. Expression of WT SHP-2 had no significant effect on survival in the absence or presence of 1 pg/ml IL-3 (see Table 1). Very similar results were obtained when cell viability was measured by Trypan Blue exclusion. C459S SHP-2 expression decreased viability by 17% ($\pm$ 0.5%, *P* < 0.001) after 24 h in 1 pg/ml IL-3 and by 20% ($\pm$ 1.25%, *P* < 0.001) after 48 h

in culture. No differences in cell viability were observed when either D425A or WT SHP-2 was expressed. In the light of these findings, we specifically compared the XTT results obtained at 1 pg/ml, and found that, in the presence of C459S SHP-2 expression, the average absorbance readings were decreased by 10.5% ($\pm$ 1.75%, *P* < 0.01) compared with a reduction of 2% ($\pm$ 0.61%) when WT SHP-2 was expressed. These changes in XTT assays were consistent with the apoptosis and viability results. Despite these effects on cell survival, expression of C459S SHP-2 had no detectable effect on IL-3-induced activation of protein kinase B (results not shown).

DISCUSSION

The tyrosine phosphatase, SHP-2, plays important roles in the control of embryogenesis, differentiation and proliferation [18,20,22,60]. In cytokine-mediated signal transduction, it has been proposed that SHP-2 may act as both a tyrosine phosphatase and also as an adaptor molecule [25,27,33,34]. In the case of the IL-3R system, we and others have demonstrated previously that SHP-2 associates with a Gab2 [29,32,39], namely the β -chain of the IL-3R [8], and becomes phosphorylated itself, enabling interactions with Grb2 [10]. SHP-2 activity also increased after IL-3 treatment [10]. However, two key issues have not been directly addressed. First, which of the proteins inducibly tyrosine-phosphorylated in response to IL-3 are key substrates of SHP-2? Secondly, what functional role SHP-2 plays in IL-3-dependent haemopoietic cells? We have addressed both of these questions in this study using a regulated expression of WT and two variants of SHP-2 (C459S and D425A) in IL-3-dependent BaF/3 cells. The engagement of the SH2 domains of SHP-2 is critical not only for SHP-2 activation, but also for functional specificity [14,15]. Hence we felt it imperative to express full-length versions of our SHP-2 variants. We demonstrate, for the first time, that PECAM-1/CD31 is a substrate for SHP-2 in IL-3-signalling. In addition, Aic2A, Gab2 and a cytosolic protein of 48 kDa are protected from tyrosine dephosphorylation on expression of catalytically inactive, substrate-trapping C459S SHP2, consistent with the fact that they are also *in vivo* substrates for SHP-2 in IL-3 signalling. Surprisingly, none of the SHP-2 variants influenced IL-3-induced proliferation, but instead, we define a role for SHP-2 in mediating survival signalling in suboptimal doses of IL-3 and after IL-3 withdrawal.

This is the first report that identifies a role for SHP-2 in mediating survival of IL-3-dependent cells. Using two measurements to assess cell survival, one based on mitochondrial integrity and the other on Trypan Blue exclusion, expression of C459S SHP-2 in two independent clones led to decreased cell survival. These effects were apparent after IL-3 withdrawal and at a dose of 1 pg/ml IL-3, which provided a survival signal for up to 48 h but was not sufficient to induce significant levels of proliferation. These effects on survival were mirrored by small changes apparent in XTT assays at 1 pg/ml IL-3, suggesting that at low doses of IL-3, SHP-2 is an important mediator of cell survival. However, at higher doses of IL-3, expression of C459S SHP-2 had no measurable effect on cell proliferation. Another study using C459S SHP-2 has also recently reported that SHP-2 mediates the protective effects of IL-6 against dexamethasone-induced cell death [62]. Thus, in addition to regulating proliferation and differentiation, in certain cellular environments, SHP-2 also appears to play a role in transducing a survival signal.

The observation that expression of SHP-2 negatively regulates IL-3-induced activation of JNKs enhances the possibility that increased JNK activation may contribute to the observed decrease in cell survival. Interestingly, Shi et al. [24] have demonstrated that

heat-shock-induced activation of JNK is negatively regulated by SHP-2 and, hence, this may be an important target of SHP-2 action in IL-3-dependent signalling. JNKs have been implicated in the regulation of both apoptosis and cell survival. We have evidence that combinations of stimuli that generate sustained activation of JNKs in BaF/3 cells promote apoptosis through a JNK-dependent pathway [50], whereas a transient activation of JNKs appears to be required for IL-3-induced proliferation of BaF/3 cells [63]. The augmented and sustained activation of JNKs observed in cells expressing C459S SHP-2 could well contribute to the increased apoptosis observed in these cells.

The differences we observed in ERK activation in the C459S and WT SHP-2 transfectants, although between only 40 and 100%, were consistent with many previous reports [21–25,27]. Our SHP-2 variants only expressed at levels 1–2-fold above endogenous SHP2. In comparison, 5-fold overexpression of C459S SHP-2 in CHO cells resulted in only a 25% decrease in maximal insulin-induced MAPK activation [23], and overexpression by 10–15-fold in 293 cells led to only a 40% decrease in platelet-derived growth factor-induced ERK activation [64]. C459S SHP-2 expression decreased IL-3-induced ERK1 and ERK2 phosphorylation most consistently at later times (2–6 h), whereas expression of WT SHP-2 potentiated ERK1 and ERK2 phosphorylation, particularly at later times (1–4 h). The sustained phase of ERK activation has been linked to induction of cell proliferation [65] but, given our observations, this sustained phase may also be involved in maintaining cell viability.

Another important aspect of our study has been the identification of substrates of SHP-2 that are IL-3-specific. In this regard, we identify PECAM-1/CD31 as a substrate for SHP-2 in IL-3 signalling. Although it is important to note that PECAM-1 has been shown previously to be a substrate for SHP-2 in a number of mouse tissues [56], this is the first time it has been reported in relation to IL-3. It is quite probable that the 135 kDa membrane glycoprotein reported previously to be a substrate for SHP-2 in BaF/3 cells by Gu et al. [52] also corresponds to PECAM-1. PECAM-1 is a member of the immunoglobulin–immunoreceptor tyrosine-based inhibitory motif superfamily and has a number of functions that include regulation of leukocyte adhesion and transendothelial cell migration, as well as regulation of antigen-receptor signalling [66]. Mice deficient in PECAM-1 have a hyper-responsive B cell phenotype and develop autoimmunity [67]. PECAM-1 is tyrosine-phosphorylated in many cell types, which facilitates binding and recruitment of SHP-2 [66,68]. The binding of SHP-2 to immuno-receptor tyrosine inhibitory motifs within the cytoplasmic tail of PECAM-1 is required for its inhibitory actions [69]. To our surprise, we found that IL-3 induces tyrosine phosphorylation of PECAM-1. PECAM-1 can be phosphorylated by several tyrosine kinases, including those of the Src family [66], and it is known that Lyn is activated by IL-3 [2]. Hence this represents a potential mechanism whereby IL-3 may phosphorylate PECAM-1. Both positive and negative regulatory roles could be envisaged for PECAM-1 in IL-3 signalling. We observed some constitutive phosphorylation of PECAM-1 and association with SHP-2 that could serve to locate SHP-2 in the proximity of the plasma membrane and make a portion of SHP-2 available for recruitment to the IL-3 receptor. Alternatively, IL-3-induced tyrosine phosphorylation of PECAM-1 could serve to divert SHP-2 away from the IL-3 receptor, preventing dephosphorylation of Aic2A and thus allowing signalling to proceed. Further studies need to be undertaken to investigate whether the inhibitory immunoreceptor PECAM-1 plays a role in modulating IL-3 signalling.

The identification of Aic2A as a substrate for SHP-2 has not been observed previously within cells, although we had

demonstrated that it can be trapped by the C459S SHP-2 mutant *in vitro* [39]. This observation could have important implications for the regulation of IL-3 signalling. Although the effects we observed were moderate, we know from previous studies using phosphopeptides, based on tyrosines present in the cytoplasmic domain of the β -chain of the IL-3R, that only two residues, Tyr-612 and to some extent Tyr-750, can be dephosphorylated by SHP-2 *in vitro* [8]. Thus, with only two potential sites for SHP-2 dephosphorylation out of eight in the cytoplasmic tail of Aic2A, it is not surprising that we see only a 50% (on average) increase in Aic2A tyrosine phosphorylation when C459S SHP-2 is expressed. We do not observe any change in Aic2A tyrosine phosphorylation on expression of WT SHP-2, which may have been expected. However, BaF/3 cells express quite high levels of SHP-2 and these may already be sufficient to saturate Aic2A sites, the result being that expression of more SHP-2 had no added effect. SHP-2 has been implicated as a positive regulator of signalling through both its adaptor functions and catalytic activity. So, what functional role might there be for dephosphorylation of Aic2A by SHP-2? One simple explanation is that SHP-2 has an auto-regulatory function. We have shown that SHP-2 is recruited to the β -chain of the IL-3R via Tyr-612 [8]. Thus, if SHP-2 dephosphorylates this site, it would regulate its own actions. Alternatively, by dephosphorylating sites on Aic2A, SHP-2 could regulate recruitment of negative regulators of IL-3 signalling, e.g. SHP-1 [70,71] and SH2-containing inositol phosphatase. To distinguish these possibilities and determine how they relate to the observed effects on cell survival, a detailed analysis of the temporal regulation of tyrosine phosphorylation of sites on Aic2A, combined with the use of Aic2A and SHP-2 mutants, is required.

We have also provided evidence that Gab2 and a 48 kDa protein are also potential SHP-2 substrates in IL-3 signalling. Gab2 has previously been identified as an SHP-2 substrate [9,29,32], and our findings were consistent with these earlier reports. The tyrosine residues on Gab2, which were dephosphorylated by SHP-2, have not been determined, but potentially include Tyr-604 (YLAL) and Tyr-633 (YVQV), which are consensus binding sites for the SH2 domains of SHP-2. If only two sites are targeted by SHP-2, then it is not unsurprising that the protection observed (Figure 5) is only moderate. The 48 kDa protein, which precipitated preferentially with C459S SHP-2, remains to be identified. We showed that p48 is present mainly in the cytosol. Therefore the finding that it is not PZR, which has been shown to be an SHP-2 substrate in other cells [53,54,72], is not surprising, given that PZR is a transmembrane protein [54]. Another potential candidate for p48 was the SHP-2-interacting transmembrane protein [73], but, due to the difference in location, we feel that it is unlikely that p48 is an SHP-2-interacting transmembrane protein. Neither does p48 correspond to isoforms of the Shc protein, which we know are inducibly tyrosine-phosphorylated after IL-3 stimulation [55]. Further purification and scale-up strategies are required to enable direct identification of this protein.

The C459S SHP-2 mutant appeared to be acting in the manner predicted by trapping substrate proteins. The fact that expression of WT SHP-2 at similar levels as C459S SHP-2 showed no effect on tyrosine phosphorylation controlled for SH2-mediated effects. However, C459S SHP-2 not only traps substrates, but is also inactive. Thus, we cannot distinguish whether the effects we have observed on cell survival are due to competition of C459S SHP-2 with endogenous SHP-2, such that it acts in a classical dominant-negative manner, or due to trapping of substrates, effectively leading to a 'blocking' of potentially key signalling molecules. To distinguish these effects, we generated an additional SHP-2 mutant, harbouring a mutation at position 465, substituting methionine for arginine, which we predicted would

be catalytically inactive, but not substrate-trapping, based on previous reports [74]. When introduced into BaF/3 tTA cells, despite repeated attempts, we failed to generate clones expressing full-length versions of this SHP-2 variant, but rather, only a cleaved product could be detected. We also generated a D425A version of SHP-2, which has not been reported previously. However, despite interacting with a similar profile of phosphotyrosyl proteins as endogenous SHP-2, it did not act as a trapping mutant. Although D425A SHP-2 lacks the critical proton-donating aspartic acid [13], it may not be completely catalytically inactive. Thus, even though it may trap substrates, it can still dephosphorylate them, resulting in a net null effect. Alternatively, this SHP-2 variant could still be subjected to inactivation via redox regulation [75], recently reported to proceed via a sulphenyl amide intermediate in the case of PTP1B [76], which could prevent substrate binding.

In summary, this study has made two important findings. First, expression of the C459S trapping form of SHP-2 has enabled the identification of key substrates, including PECAM-1/CD31, involved in IL-3 signalling. Second, we have defined a role for SHP-2 in cell survival, via a potential mechanism involving differential regulation of activation of ERKs and JNKs, suggesting that in addition to its role in cellular proliferation, SHP-2 may also be required to provide an optimal anti-apoptotic signal in cytokine-dependent cells.

The authors thank Joe Zhao for providing the anti-PZR antibodies and Nick Paling for helpful discussions and comments. This work was supported by a grant from the Wellcome Trust.

REFERENCES

- Silvennoinen, O., Witthuhn, B. A., Quelle, F. W., Cleveland, J. L., Yi, T. and Ihle, J. N. (1993) Structure of the murine Jak2 protein-tyrosine kinase and its role in interleukin 3 signal transduction. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 8429–8433
- Anderson, S. M. and Jorgensen, B. (1995) Activation of src-related tyrosine kinases by IL-3. *J. Immunol.* **155**, 1660–1670
- Mui, A. L. F., Wakao, H., O'Farrell, A. M., Harada, N. and Miyajima, A. (1995) Interleukin-3, granulocyte-macrophage colony-stimulating factor and interleukin-5 transduce signals through 2 Stat5 homologs. *EMBO J.* **14**, 1166–1175
- Welham, M. J., Duronio, V., Sanghera, J. S., Pelech, S. L. and Schrader, J. W. (1992) Multiple hemopoietic growth factors stimulate activation of mitogen-activated protein kinase family members. *J. Immunol.* **149**, 1683–1693
- Gold, M. R., Duronio, V., Saxena, S. P., Schrader, J. W. and Aebersold, R. (1994) Multiple cytokines activate phosphatidylinositol 3-kinase in hemopoietic cells. Association of the enzyme with various tyrosine-phosphorylated proteins. *J. Biol. Chem.* **269**, 5403–5412
- Vanhaesebroeck, B., Welham, M. J., Kotani, K., Stein, R., Warne, P. H., Zvelebil, M. J., Higashi, K., Volinia, S., Downward, J. and Waterfield, M. D. (1997) p110 δ , a novel phosphoinositide 3-kinase in leukocytes. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 4330–4335
- deGroot, R. P., Coffey, P. J. and Koenderman, L. (1998) Regulation of proliferation, differentiation and survival by the IL-3/IL-5/GM-CSF receptor family. *Cell. Signal.* **10**, 619–628
- Bone, H., Dechert, U., Jirik, F., Schrader, J. W. and Welham, M. J. (1997) SHP1 and SHP2 protein-tyrosine phosphatases associate with βc after interleukin-3-induced receptor tyrosine phosphorylation. *J. Biol. Chem.* **272**, 14470–14476
- Craddock, B. L. and Welham, M. J. (1997) Interleukin-3 induces association of the protein-tyrosine phosphatase SHP2 and phosphatidylinositol 3-kinase with a 100-kDa tyrosine-phosphorylated protein in hemopoietic cells. *J. Biol. Chem.* **272**, 29281–29289
- Welham, M. J., Dechert, U., Leslie, K. B., Jirik, F. and Schrader, J. W. (1994) Interleukin (IL)-3 and granulocyte/macrophage colony-stimulating factor, but not IL-4, induce tyrosine phosphorylation, activation, and association of SHPTP2 with Grb2 and phosphatidylinositol 3'-kinase. *J. Biol. Chem.* **269**, 23764–23768
- Freeman, R. M., Plutzky, J. and Neel, B. G. (1992) Identification of a human src homology 2-containing protein-tyrosine phosphatase – a putative homolog of drosophila corkscrew. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 11239–11243
- Tonks, N. K. and Neel, B. G. (1996) From form to function: signaling by protein tyrosine phosphatases. *Cell (Cambridge, Mass.)* **87**, 365–368
- Hof, P., Pluskey, S., DhePaganon, S., Eck, M. J. and Shoelson, S. E. (1998) Crystal structure of the tyrosine phosphatase SHP-2. *Cell (Cambridge, Mass.)* **92**, 441–450
- O'Reilly, A. M. and Neel, B. G. (1998) Structural determinants of SHP-2 function and specificity in *Xenopus* mesoderm induction. *Mol. Cell. Biol.* **18**, 161–177
- Tenev, T., Keilhack, H., Tomic, S., Stoyanov, B., SteinGerlach, M., Lammers, R., Krivtsov, A. V., Ullrich, A. and Bohmer, F. D. (1997) Both SH2 domains are involved in interaction of SHP-1 with the epidermal growth factor receptor but cannot confer receptor-directed activity to SHP-1/SHP-2 chimera. *J. Biol. Chem.* **272**, 5966–5973
- Qu, C. K. and Feng, G. S. (1998) Shp-2 has a positive regulatory role in ES cell differentiation and proliferation. *Oncogene* **17**, 433–439
- Qu, C. K., Nguyen, S., Chen, J. Z. and Feng, G. S. (2001) Requirement of Shp-2 tyrosine phosphatase in lymphoid and hematopoietic cell development. *Blood* **97**, 911–914
- Qu, C. K., Shi, Z. Q., Shen, R., Tsai, F. Y., Orkin, S. H. and Feng, G. S. (1997) A deletion mutation in the SH2-N domain of Shp-2 severely suppresses hematopoietic cell development. *Mol. Cell. Biol.* **17**, 5499–5507
- Qu, C. K., Yu, W. M., Azzarelli, B., Cooper, S., Broxmeyer, H. E. and Feng, G. S. (1998) Biased suppression of hematopoiesis and multiple developmental defects in chimeric mice containing Shp-2 mutant cells. *Mol. Cell. Biol.* **18**, 6075–6082
- Saxton, T. M., Henkemeyer, M., Gasca, S., Shen, R., Rossi, D. J., Shalaby, F., Feng, G. S. and Pawson, T. (1997) Abnormal mesoderm patterning in mouse embryos mutant for the SH2 tyrosine phosphatase Shp-2. *EMBO J.* **16**, 2352–2364
- Frearson, J. A. and Alexander, D. R. (1998) The phosphotyrosine phosphatase SHP-2 participates in a multimeric signaling complex and regulates T cell receptor (TCR) coupling to the Ras/mitogen-activated protein kinase (MAPK) pathway in Jurkat T cells. *J. Exp. Med.* **187**, 1417–1426
- Milarski, K. L. and Saltiel, A. R. (1994) Expression of catalytically inactive Syp phosphatase in 3T3 cells blocks stimulation of mitogen-activated protein kinase by insulin. *J. Biol. Chem.* **269**, 21239–21243
- Noguchi, T., Matozaki, T., Horita, K., Fujioka, Y. and Kasuga, M. (1994) Role of SH-PTP2, a protein-tyrosine phosphatase with Src homology 2 domains, in insulin-stimulated Ras activation. *Mol. Cell. Biol.* **14**, 6674–6682
- Shi, Z. Q., Lu, W. and Feng, G. S. (1998) The Shp-2 tyrosine phosphatase has opposite effects in mediating the activation of extracellular signal-regulated and c-Jun NH₂-terminal mitogen-activated protein kinases. *J. Biol. Chem.* **273**, 4904–4908
- Gadina, M., Stancato, L. M., Bacon, C. M., Lerner, A. C. and O'Shea, J. J. (1998) Involvement of SHP-2 in multiple aspects of IL-2 signaling: evidence for a positive regulatory role. *J. Immunol.* **160**, 4657–4661
- Li, W., Nishimura, R., Kashishian, A., Batzer, A. G., Kim, W. J. H., Cooper, J. A. and Schlessinger, J. (1994) A new function for a phosphotyrosine phosphatase: linking GRB2-Sos to a receptor tyrosine kinase. *Mol. Cell. Biol.* **14**, 509–517
- Pazdrak, K., Adachi, T. and Alam, R. (1997) Src homology 2 phosphatase 2 (SHP2) tyrosine phosphatase is a positive regulator of the interleukin 5 receptor signal transduction pathways leading to the prolongation of eosinophil survival. *J. Exp. Med.* **186**, 561–568
- Cunnick, J., Dorsey, J. F., Munoz-Antonia, T., Mei, L. and Wu, J. (2000) Requirement of SHP2 binding to Grb2-associated Binder-1 for mitogen-activated protein kinase activation in response to lysophosphatidic acid and epidermal growth factor. *J. Biol. Chem.* **275**, 13842–13848
- Gu, H. H., Pratt, J. C., Burakoff, S. J. and Neel, B. G. (1998) Cloning of p97/Gab2, the major SHP2-binding protein in hematopoietic cells, reveals a novel pathway for cytokine-induced gene activation. *Mol. Cell* **2**, 729–740
- Shi, Z. Q., Yu, D. H., Park, M., Marshall, M. and Feng, G. S. (2000) Molecular mechanism for the Shp-2 tyrosine phosphatase function in promoting growth factor stimulation of Erk activity. *Mol. Cell. Biol.* **20**, 1526–1536
- Zhao, C. M., Yu, D. H., Shen, R. and Feng, G. S. (1999) Gab2, a new pleckstrin homology domain-containing adapter protein, acts to uncouple signaling from ERK kinase to Elk-1. *J. Biol. Chem.* **274**, 19649–19654
- Nishida, K., Yoshida, Y., Itoh, M., Fukada, T., Ohtani, T., Shirogane, T., Atsumi, T., Takahashi-Tezuka, M., Ishihara, K., Hibi, M. et al. (1999) Gab-family adaptor proteins act downstream of cytokine and growth factor receptors and T- and B-cell antigen receptors. *Blood* **93**, 1809–1816
- Kim, S.-O., Jiang, J., Woelsung, Y., Feng, G.-S. and Frank, S. J. (1998) Involvement of the Src homology 2-containing tyrosine phosphatase SHP-2 in growth hormone signalling. *J. Biol. Chem.* **273**, 2344–2354
- Tauchi, T., Damen, J. E., Toyama, K., Feng, G. S., Broxmeyer, H. E. and Krystal, G. (1996) Tyrosine 425 within the activated erythropoietin receptor binds Syp, reduces the erythropoietin required for Syp tyrosine phosphorylation, and promotes mitogenesis. *Blood* **87**, 4495–4501
- Kharitonov, A., Chen, Z., Sures, I., Wang, H., Schilling, J. and Ullrich, A. (1997) A family of proteins that inhibit signalling through tyrosine kinase receptors. *Nature (London)* **386**, 181–186

- 36 Kuhne, M. R., Pawson, T., Lienhard, G. E. and Feng, G. S. (1993) The insulin receptor substrate 1 associates with the SH2-containing phosphotyrosine phosphatase Syp. *J. Biol. Chem.* **268**, 11479–11481
- 37 Liu, L., Damen, J. E., Ware, M. D. and Krystal, G. (1997) Interleukin-3 induces the association of the inositol 5-phosphatase SHIP with SHP2. *J. Biol. Chem.* **272**, 10998–11001
- 38 Flint, A. J., Tiganis, T., Barford, D. and Tonks, N. K. (1997) Development of 'substrate-trapping' mutants to identify physiological substrates of protein tyrosine phosphatases. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 1680–1685
- 39 Wheadon, H., Paling, N. and Welham, M. J. (2002) Molecular interactions of SHP1 and SHP2 in IL-3 signalling. *Cell. Signal.* **14**, 219–229
- 40 Craddock, B. L., Orchiston, E. A., Hinton, H. J. and Welham, M. J. (1999) Dissociation of apoptosis from proliferation, protein kinase B activation, and BAD phosphorylation in interleukin-3-mediated phosphoinositide 3-kinase signaling. *J. Biol. Chem.* **274**, 10633–10640
- 41 Gossen, M., Bonin, A. L. and Bujard, H. (1994) Control of gene activity in higher eukaryotic cells by prokaryotic regulatory elements. *Trends Biotechnol.* **12**, 58–62
- 42 Gossen, M. and Bujard, H. (1992) Tight control of gene-expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5547–5551
- 43 Mui, A. L. F., Wakao, H., Kinoshita, T. and Miyajima, A. (1996) Suppression of interleukin-3-induced gene expression by a C-terminal truncated stat5: role of stat5 in proliferation. *EMBO J.* **15**, 2425–2433
- 44 Welham, M. J., Duronio, V. and Schrader, J. W. (1994) Interleukin-4-dependent proliferation dissociates p44^{erk-1}, p42^{erk-2}, and p21^{ras} activation from cell growth. *J. Biol. Chem.* **269**, 5865–5873
- 45 Welham, M. J. and Schrader, J. W. (1992) Steel factor-induced tyrosine phosphorylation in murine mast cells: common elements with IL-3-induced signal transduction pathways. *J. Immunol.* **149**, 2772–2783
- 46 Craddock, B. L., Hobbs, J., Edmead, C. and Welham, M. J. (2001) Phosphoinositide 3-kinase-dependent regulation of IL-3-induced proliferation: involvement of mitogen-activated protein kinases, SHP2 and Gab2. *J. Biol. Chem.* **276**, 24274–24283
- 47 Marchetti, P., Castedo, M., Susin, S. A., Zamzami, N., Hirsch, T., Macho, A., Haeflner, A., Hirsch, F., Geuskens, M. and Kroemer, G. (1996) Mitochondrial permeability transition is a central coordinating event of apoptosis. *J. Exp. Med.* **184**, 1155–1160
- 48 Rottenberg, H. and Wu, S. L. (1998) Quantitative assay by flow cytometry of the mitochondrial membrane potential in intact cells. *Biochim. Biophys. Acta* **1404**, 393–404
- 49 Zamzami, N., Marchetti, P., Castedo, M., Zanin, C., Vayssiere, J. L., Petit, P. X. and Kroemer, G. (1995) Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death *in vivo*. *J. Exp. Med.* **181**, 1661–1672
- 50 Wheadon, H. and Welham, M. J. (2003) The coupling of TEL/PDGR β R to distinct functional responses is modulated by the presence of cytokine: involvement of mitogen-activated protein kinases. *Blood* **102**, 1480–1489
- 51 Yamauchi, K., Milarski, K. L., Saltiel, A. R. and Pessin, J. E. (1995) Protein-tyrosine-phosphatase SHPTP2 is a required positive effector for insulin downstream signalling. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 664–668
- 52 Gu, H. H., Griffin, J. D. and Neel, B. G. (1997) Characterization of two SHP-2-associated binding proteins and potential substrates in hematopoietic cells. *J. Biol. Chem.* **272**, 16421–16430
- 53 Zhao, R. X. and Zhao, Z. J. (2000) Dissecting the interaction of SHP-2 with PZR, an immunoglobulin family protein containing immunoreceptor tyrosine-based inhibitory motifs. *J. Biol. Chem.* **275**, 5453–5459
- 54 Zhao, Z. J. and Zhao, R. (1998) Purification and cloning of PZR, a binding protein and putative physiological substrate of tyrosine phosphatase SHP-2. *J. Biol. Chem.* **273**, 29367–29372
- 55 Welham, M. J., Duronio, V., Leslie, K. B., Bowtell, D. and Schrader, J. W. (1994) Multiple hemopoietins, with the exception of interleukin-4, induce modification of Shc and mSos1, but not their translocation. *J. Biol. Chem.* **269**, 21165–21176
- 56 Xu, F. P., Xu, M. J., Zhao, R. X., Guerra, A., Zeng, F. H. and Zhao, Z. J. (2002) Tyrosine phosphatases SHP-1 and SHP-2 are associated with distinct tyrosine-phosphorylated proteins. *Exp. Cell Res.* **272**, 75–83
- 57 Yu, C. L., Jin, Y. J. and Burakoff, S. J. (2000) Cytosolic tyrosine dephosphorylation of STAT5 – potential role of SHP-2 in STAT5 regulation. *J. Biol. Chem.* **275**, 599–604
- 58 Chen, Y., Wen, R. R., Yang, S., Shuman, J., Zhang, E. E., Yi, T. L., Feng, G. S. and Wang, D. M. (2003) Identification of Shp-2 as a Stat5A phosphatase. *J. Biol. Chem.* **278**, 16520–16527
- 59 Ugi, S., Maegawa, H., Kashiwagi, A., Adachi, M., Olefsky, J. M. and Kikkawa, R. (1996) Expression of dominant negative mutant SHPTP2 attenuates phosphatidylinositol 3'-kinase activity via modulation of phosphorylation of insulin receptor substrate-1. *J. Biol. Chem.* **271**, 12595–12602
- 60 Saxton, T. M. and Pawson, T. (1999) Morphogenetic movements at gastrulation require the SH2 tyrosine phosphatase Shp2. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 3790–3795
- 61 Reference deleted
- 62 Chauhan, D., Pandey, P., Hideshima, T., Treon, S., Raje, N., Dacies, F. E., Shima, Y., Rosen, S., Avraham, S., Kharbanda, S. et al. (2000) SHP2 mediates the protective effect of interleukin-6 against dexamethasone-induced apoptosis in multiple myeloma cells. *J. Biol. Chem.* **275**, 27845–27850
- 63 Smith, A., RamosMorales, F., Ashworth, A. and Collins, M. (1997) A role for JNK/SAPK in proliferation, but not apoptosis, of IL-3-dependent cells. *Curr. Biol.* **7**, 893–896
- 64 Zhao, R. and Zhao, Z. J. (1999) Tyrosine phosphatase SHP-2 dephosphorylates the platelet-derived growth factor receptor by enhancing its downstream signalling. *Biochem. J.* **338**, 35–39
- 65 Meloche, S., Seuwen, K., Pages, G. and Pouyssegur, J. (1992) Biphasic and synergistic activation of p44(Mapk) (Erk1) by growth-factors – correlation between late phase activation and mitogenicity. *Mol. Endocrinol.* **6**, 845–854
- 66 Jackson, D. E. (2003) The unfolding tale of PECAM-1. *FEBS Lett.* **540**, 7–14
- 67 Wilkinson, R., Lyons, A. B., Roberts, D., Wong, M. X., Bartley, P. A. and Jackson, D. E. (2002) Platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) acts as a regulator of B-cell development, B-cell antigen receptor (BCR)-mediated activation, and autoimmune disease. *Blood* **100**, 184–193
- 68 Maas, M., Wang, R., Paddock, C., Kotamraju, S., Kalyanaramen, B., Newman, P. J. and Newman, D. K. (2003) Reactive oxygen species in duce reversible PECAM-1 tyrosine phosphorylation and SHP-2 binding. *Am. J. Physiol.-Heart Circ. Physiol.*, DOI 10.1152/ajpheart.00509.2003
- 69 Newman, D. K., Hamilton, C. and Newman, P. J. (2001) Inhibition of antigen-receptor signaling by platelet endothelial cell adhesion molecule-1 (CD31) requires functional ITIMs, SHP-2, and p56(lck). *Blood* **97**, 2351–2357
- 70 Paling, N. R. D. and Welham, M. J. (2002) The role of the protein tyrosine phosphatase SHP-1 in regulation of interleukin-3-induced survival, proliferation and signalling. *Biochem. J.* **368**, 885–894
- 71 Yi, T., Mui, A. L. F., Krystal, G. and Ihle, J. N. (1993) Hematopoietic cell phosphatase associates with the interleukin-3 (IL-3) receptor β chain and down-regulates IL-3-induced tyrosine phosphorylation and mitogenesis. *Mol. Cell. Biol.* **13**, 7577–7586
- 72 Zannettino, A. C. W., Roubelakis, M., Welldon, K. J., Jackson, D. E., Simmons, P. J., Bendall, L. J., Henniker, A., Harrison, K. L., Niutta, S., Bradstock, K. F. et al. (2003) Novel mesenchymal and haematopoietic cell isoforms of the SHP-2 docking receptor, PZR: identification, molecular cloning and effects on cell migration. *Biochem. J.* **370**, 537–549
- 73 Pfeiffer, K. I., Marie-Cardine, A., Simeoni, L., Kuramitsu, Y., Leo, A., Spicka, J., Hilgert, I., Scherer, J. and Schraven, B. (2001) Structural and functional dissection of the cytoplasmic domain of the transmembrane adaptor protein SIT (SHP2-interacting transmembrane adaptor protein). *Eur. J. Immunol.* **31**, 1825–1836
- 74 Timms, J. F., Carlberg, K., Gu, H. H., Chen, H. Y., Kamatkar, S., Nadler, M. J. S., Rohrschneider, L. R. and Neel, B. G. (1998) Identification of major binding proteins and substrates for the SH2-containing protein tyrosine phosphatase SHP-1 in macrophages. *Mol. Cell. Biol.* **18**, 3838–3850
- 75 Meng, T. C., Fukuda, T. and Tonks, N. K. (2002) Reversible oxidation and inactivation of protein tyrosine phosphatases *in vivo*. *Mol. Cell.* **9**, 387–399
- 76 Salmeen, A., Andersen, J. N., Myers, M. P., Meng, T. C., Hinks, J. A., Tonks, N. K. and Barford, D. (2003) Redox regulation of protein tyrosine phosphatase 1B involves a sulphenyl-amide intermediate. *Nature* **423**, 769–773

Received 31 July 2003/20 August 2003; accepted 22 August 2003

Published as BJ Immediate Publication 22 August 2003, DOI 10.1042/BJ20031160