Role of the protein tyrosine phosphatase SHP-1 (Src homology phosphatase-1) in the regulation of interleukin-3-induced survival, proliferation and signalling

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The tyrosine phosphatase SHP-1 (Src homology phosphatase-1) has been widely implicated as a negative regulator of signalling in immune cells. We have investigated in detail the role of SHP-1 in interleukin-3 (IL-3) signal transduction by inducibly expressing wild-type (WT), C453S (substrate-trapping) and R459M (catalytically inactive) forms of SHP-1 in the IL-3-dependent cell line BaF/3. Expression of WT SHP-1 had little impact on IL-3-induced proliferation, but enhanced apoptosis following IL-3 withdrawal. Expression of R459M SHP-1 increased the proliferative response of BaF/3 cells to IL-3 and increased cell survival at low doses of IL-3 and following IL-3 withdrawal. Investigation into the biochemical consequences resulting from expression of these SHP-1 variants demonstrated that the β chain of the IL-3 receptor (Aic2A) was hypo-phosphorylated in cells expressing WT SHP-1 and hyper-phosphorylated in those expressing R459M SHP-1. Further, ectopic expression of the trapping mutant, C453S SHP-1, protected Aic2A from dephosphorylation, suggesting that Aic2A is a SHP-1 substrate in BaF/3 cells. Examination of overall levels of tyrosine phosphorylation demonstrated that they were not perturbed in these transfectants. Activation-specific phosphorylation of STAT (signal transducer and activator of transcription) 5a/b, protein kinase B and ERK (extracellular-signal-regulated kinase)-1 and -2 was also unaffected by expression of WT or R459M SHP-1. However, overall levels of IL-3-induced tyrosine phosphorylation of STAT5 were reduced upon expression of WT SHP-1 and increased when R459M SHP-1 was expressed, consistent with STAT5 being a potential SHP-1 substrate. These results demonstrate that SHP-1 acts to negatively regulate IL-3-driven survival and proliferation, potentially via regulation of tyrosine phosphorylation of Aic2A and STAT5.

Key words: Aic2A, inducible expression, signal transducer and activator of transcription 5 (STAT5), substrate-trapping.

INTRODUCTION

Interleukin-3 (IL-3) is a multipotent haemopoietic growth factor, which acts on progenitor, myeloid and mast cells to induce cell proliferation, promote cell survival and facilitate differentiation [1]. The high-affinity IL-3 receptor (IL-3R) is composed of a heterodimer consisting of a 70 kDa IL-3-specific α subunit and a 140 kDa β chain [2,3]. IL-3 treatment induces the rapid and transient phosphorylation of tyrosine residues in the cytoplasmic region of the β subunit, mediated by Jak [4] and Src [5] family tyrosine kinases. Phosphorylated tyrosine residues on the receptor form docking sites for phosphotyrosine-binding and Src homology 2 (SH2) domain-containing proteins. In combination with the tyrosine phosphorylation of other cellular proteins, these events trigger a series of intracellular signalling cascades that include the signal transducer and activator of transcription 5 (STAT5), phosphoinositide 3-kinase (PI 3-kinase) and Ras/mitogen-activated protein kinase (MAPK) pathways [6].

In order to maintain sensitivity and responsiveness in signal transduction, there is a requirement for tyrosine phosphorylation events to be reversed. This dephosphorylation is mediated by a family of protein tyrosine phosphatases (reviewed in [7]). Src homology phosphatase-1 (SHP-1) and SHP-2 are two closely related protein tyrosine phosphatases that are involved in the regulation of haemopoietic cell signalling. They share a similar overall structure, consisting of two N-terminal SH2 domains, a phosphatase domain C-terminal to these, and a C-terminal tail region believed to be involved in the regulation of phosphatase activity [8]. SHP-1 is a 64 kDa protein and is expressed in all haemopoietic cell lineages, with limited expression occurring in some epithelial lineages [9,10].

The original characterization of SHP-1 as a negative regulator of haemopoiesis was facilitated by the discovery that the mutations responsible for the *motheaten* (*me*) and *motheaten viable* (*me^v*) phenotypes in mice map to the SHP-1 gene locus [11,12]. These mice suffer from a plethora of haemopoietic abnormalities, which are primarily the result of over-proliferation and activation of myeloid and erythroid haemopoietic lineages [13]. B cells from *me* mice are hyper-responsive to B cell antigen receptor stimulation [14], and the positive selection, activation, proliferation and apoptosis of *me* T cells are enhanced following ligation of the T cell antigen receptor [15,16]. Haemopoietic cells from *me* mice hyper-proliferate in response to erythropoietin [13], macrophage colony-stimulating factor [17] and granulocyte–macrophage colony-stimulating factor [18]. γ-Irradiation-induced apoptosis is defective in B cells, T cells and macrophages derived from *me* mice [19], and *me* T cells stimulated through the Fas receptor exhibit an apoptosis defect [20]. Furthermore, expression of catalytically inactive SHP-1 reduces the level of apoptosis in the myelo-monocytic cell line U937 [21]. In

Abbreviations used: Aic2A, β chain of the murine interleukin-3 receptor; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; $DiOC_6$, 3′,3′-dihexyloxacarbocyanine iodide; ERK, extracellular-signal-regulated kinase; HBSS, Hanks buffered saline solution; IL-3, interleukin-3; IL-3R, interleukin-3 receptor; Ψm, mitochondrial membrane potential; MAPK, mitogen-activated protein kinase; *me*, *motheaten*; PI 3-kinase, phosphoinositide 3-kinase; PKB, protein kinase B; rmIL-3, recombinant murine IL-3; SH2, Src homology 2; SHP-1, Src homology phosphatase-1; STAT, signal transducer and activator of transcription; Tet, tetracycline; tTA, tetracycline-sensitive transactivator; WT, wild type; XTT, sodium 3′-{1-[(phenylamino)carbonyl]-3,4-tetrazolium]-bis-(4-methoxy-6-nitro)benzene sulphonic acid hydrate.

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MCF-7 cells, SHP-1 has been shown to be required for the induction of apoptosis by the somatostatin and Fas receptors [22]. A negative role for SHP-1 in regulating the IL-3-induced proliferation of the myeloid cell line DA-3 has been suggested following the use of antisense technology to alter levels of SHP-1 expression [23].

These findings have led to the characterization of SHP-1 as a negative regulator of proliferation and apoptosis, and have been substantiated by the discovery that SHP-1 is recruited to a wide variety of ITIM (immunoreceptor tyrosine inhibitory motif)-bearing inhibitory co-receptors in haemopoietic cell lineages. These co-receptors localize SHP-1 to its substrates in signalling complexes and thereby facilitate the modulation of receptor-mediated signalling thresholds [24]. SHP-1 also associates with cytokine receptors, including EpoR [25,26], the IL-3R β-subunit [23,27] and c-*kit* [28]. It has been suggested that SHP-1 plays a negative role in cytokine signal transduction by dephosphorylating these receptors and the tyrosine kinases associated with them [24,26].

We have previously demonstrated that SHP-1 associates with βc [27], Aic2A (the β chain of the murine IL-3R), Gab2 and the inhibitory receptor PIR-B [29], and that βc, Aic2A and Gab2 are potential *in vitro* substrates of SHP-1 [29]. Here, using regulated expression of wild-type (WT), catalytically inactive and substrate-trapping versions of SHP-1, we present a detailed investigation of (a) the effects of SHP-1 on IL-3-induced proliferation and survival, and on apoptosis following IL-3 withdrawal; (b) the role of SHP-1 in modulating IL-3-induced biochemical signalling cascades; and (c) the identification of potential *in vivo* substrates for SHP-1.

EXPERIMENTAL

Cell lines and tissue culture

A stable BaF/3 transfectant cell line expressing tTA (tetracycline-sensitive transactivator) (BaF/3 tTA) was generously donated by Dr A. Mui (DNAX, Palo Alto, CA, U.S.A.) [30]. BaF/3 tTA and derivatives were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% (v/v) fetal calf serum (Sigma), 20 μM 2-mercaptoethanol, 100 units of penicillin/streptomycin, 2 mM glutamine, 2 μg/ml tetracycline (Tet) and 5% (v/v) conditioned medium from JWW3/WEHI 3B cells as a source of IL-3.

Generation of WT, R459M (catalytically inactive) and C453S (substrate-trapping) SHP-1 pUHD10-3 constructs

Plasmids encoding WT and R459M SHP-1 were generously provided by Professor B. Neel (Beth Israel Deaconess Medical Center, Boston, MA, U.S.A.). The coding sequences were amplified with Vent DNA polymerase (New England Biolabs) according to the manufacturer's recommendations using the following oligonucleotide primers: sense primer (incorporating an *Eco*RI site for in-frame cloning into the Myc epitope tagging vector pBluescript-N-Myc2), 5′-TGCGGAATTCTTATGGTG-AGGTGGTTTCAC-3′; antisense primer (including an *Eco*RV site for cloning purposes), 5′-CCTGATATCCACCGCTCAC-TTCCTCTT-3′. PCR products were digested with *Eco*RI and *Eco*RV and subcloned into pBluescript-N-Myc2, as described previously [31]. Epitope-tagged WT and R459M SHP-1 cDNAs were subcloned into pUHD10-3neo [32,33]. To generate C453S SHP-1 pUHD10-3, site-directed mutagenesis was performed on WT SHP-1 pUHD10-3 using the Stratagene QuikChange™ site-directed mutagenesis protocol. Each construct was verified by sequencing.

Preparation and characterization of stable transfectants

BaF/3 tTA cells were electroporated as described previously [31]. An aliquot of 10 μg of linearized DNA was used per 1×10^7 cells at 960 μF and 450V. Cells were plated at 5×10^5/ml for 48 h in the presence of 2 μg/ml Tet, and then plated on to 96-well trays in 1 mg/ml G418, 1 μg/ml puromycin and 2 μg/ml Tet at 10000 cells per well. After 7–14 days, clones were expanded and screened for inducible expression, as described previously [31]. Clones showing low basal and consistent inducible expression of the protein of interest were selected for further study. Three independent clones from each transfection were selected for further analyses.

Cytokine-dependent proliferation assay

XTT (sodium 3′-{1-[(phenylamino)carbonyl]-3,4-tetrazolium}-bis-(4-methoxy-6-nitro)benzene sulphonic acid hydrate) dye-reduction assays were performed in 96-well trays, with quadruplicate samples for each treatment as described previously [31,34]. Following a 72 h incubation, XTT was added to each well and trays were incubated for 3–5 h to allow for development. The amount of the soluble formazan product generated in each well was measured on a Dynatech MR5000 plate reader at 450 nm before the maximum absorbance for the assay was reached (maximum 1.8–2.0 absorbance units). Mean and S.D. values were calculated for each treatment point.

Trypan Blue exclusion assay

Transfectants were plated at 1×10^5 cells/ml in the presence or absence of 2 μg/ml Tet for 24 h to induce expression. Cells were washed three times in Hanks buffered saline solution (HBSS) and plated at 1×10^4 cells/ml in the presence or absence of 2 μg/ml Tet in RPMI 1640 medium containing 0, 1 or 20 pg/ml recombinant murine IL-3 (rmIL-3; R&D Systems). Cells were incubated at 37 °C. After 18, 24 and 48 h, aliquots of cells were removed, pelleted and resuspended in 100 μl of HBSS. Then 100 μl of Trypan Blue solution (Sigma) was mixed with the cells, and viable cells (not stained) and dead cells (stained blue) were counted. Each sample was counted in duplicate.

Measurement of mitochondrial membrane potential (Ψm)

Transfectants were induced for 24 h, washed three times in HBSS and resuspended at 1×10^4 cells/ml in the presence or absence of Tet in RPMI 1640 medium containing either 0 or 1 pg/ml rmIL-3. Cells were incubated at 37 °C for 24 h, washed once in PBS and incubated for 30 min in 10 nM 3′,3′-dihexyloxacarbocyanine iodide ($DiOC_6$; Sigma) [35,36]. After one PBS wash, 10000 events were analysed per sample by flow cytometry using a FACS Vantage System (Beckton Dickinson) on parameter FL1 (fluorescent channel 1). As a control, 1×10^6 IL-3-grown parental BaF/3 cells were treated with $DiOC_6$ in the same way. Half were then treated with 50 μM of the mitochondria-uncoupling agent carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP; Sigma) for 15 min.

Induction of cells in bulk culture, cell stimulation and immunoprecipitation

Transfectants were washed three times with HBSS, resuspended at 1×10^5 cells/ml in the presence or absence of 2 μg/ml Tet and cultured for 24 h. Cells were stimulated using rmIL-3 as previously described [37]. Cell pellets were lysed in ice-cold solubilization buffer [50 mM Tris/HCl, pH 7.5, 10% (v/v) glycerol, 1% (v/v) Nonidet P-40, 150 mM NaCl, 5 mM EDTA,

1 mM NaF, 40 μg/ml PMSF, 10 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin and 0.7 μg/ml pepstatin]. Insoluble cell debris was removed by pelleting at full speed for 1 min, and the supernatant was transferred to a clean tube. Immunoprecipitations were performed as described previously [37]. Antibodies against IL-3Rβ (sc-677) and STAT5b (sc-835) were used at 1 μg/ml (Santa Cruz Biotechnology).

SDS/PAGE and immunoblotting

SDS/PAGE and immunoblotting were performed as described previously [37]. Primary antibodies were used at the following concentrations: anti-phosphotyrosine antibody 4G10 at 0.1 μg/ml (05-321; Upstate Biotechnology, Lake Placid, NY, U.S.A.); anti-Myc epitope tag antibody 9E10 at 0.5 μg/ml; anti-SHP-1 at 0.1 μg/ml (sc-287; Santa Cruz); anti-IL-3Rβ at 0.2 μg/ml (sc-677; Santa Cruz); anti-STAT5a/b at 0.04 μg/ml (sc-855, Santa Cruz); anti-phospho-STAT5a/b (Y694/699) at 0.1 μg/ml (05-495; UBI); anti-ERK (extracellular-signal-regulated kinase)-1/2 at 1:1000 dilution (sc-93; Santa Cruz); anti-phospho-ERK-1/2 at a 1:1000 dilution (9101; Cell Signaling Technology); anti-protein kinase B (PKB) at a 1:1000 dilution (9272; Cell Signaling Technology); anti-phospho-PKB (S473) at a 1:1000 dilution (9271; Cell Signaling Technology).

Secondary antibodies conjugated to horseradish peroxidase were used at a concentration of 0.05 μg/ml (Dako). Immunoblots were developed using the ECL® system (Amersham Pharmacia) and Kodak X-AR5 film. Immunoblots were stripped and re-probed using standard conditions, as previously described [38].

Statistical analyses

Paired Student's t tests were used to analyse data as indicated.

RESULTS

Generation, selection and characterization of BaF/3 transfectants expressing SHP-1 variants

SHP-1 has been implicated as a negative regulator of signalling pathways in numerous haemopoietic cells [24]. However, only a single study, which relied on an antisense-based approach, has suggested that SHP-1 acts as a negative regulator of IL-3-induced proliferation [23]. Furthermore, to date, the key biochemical targets of SHP-1 involved in this functional response have not been explored. In support of a role for SHP-1 in IL-3 signalling, we have demonstrated that SHP-1 associates with the β chain of the IL-3R and *in vitro* can dephosphorylate phosphopeptides based on tyrosine residues 612 and 750 of human βc [27]. Recently we have identified other potential SHP-1 substrates using *in vitro* analyses in IL-3-dependent BaF/3 cells [29]. Hence, prior to our initiating the study reported herein, it was not known whether SHP-1 was involved in the regulation of other functional responses to IL-3, which IL-3-induced biochemical signalling pathways were regulated by SHP-1 and whether the potential substrates we have identified *in vitro* are *bone fide* substrates within IL-3-dependent cells. Given the reported actions of SHP-1 as a negative regulator of proliferation, we used the Tet-off regulated gene expression system [32] to investigate the role of SHP-1 in IL-3-dependent cells. This system has been a valuable tool in our laboratory for analysing proteins that have detrimental effects on cell growth [31,39]. It allows for the effects of expression of a mutant protein to be examined within the same cell, ensuring that the effects of a given mutant are examined more reliably. Inducible expression also bypasses many of the problems encountered when clones constitutively expressing mutant proteins are used for delineating protein function, as such strategies are subject to the vagaries of clonal variation, which are often difficult to control for adequately.

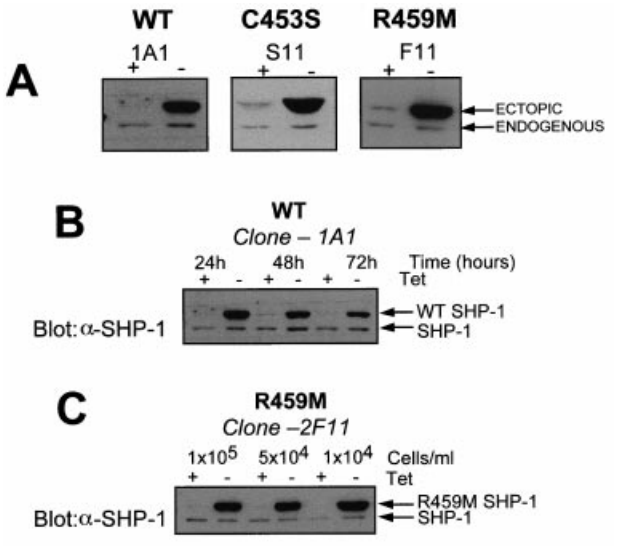

Figure 1 Inducible expression of SHP-1 constructs in BaF/3 cells

Clones expressing WT, R459M and C453S SHP-1 were incubated in the presence (+) or absence (−) of 2 μg/ml Tet for 24 h. Clones showing high levels of expression and high inducibility were selected. (**A**) Expression levels and inducibility of expression in clones selected for further analyses. (**B**) WT transfectants were incubated in the presence (+) or absence (−) of 2 μg/ml Tet and aliquots of cells were taken at the indicated times after the initiation of induction. (**C**) Cells were cultured as in (**A**), but cultured at densities of 1×10^5, 5×10^4 or 1×10^4 cells/ml. Immunoblotting was performed using rabbit polyclonal anti-SHP-1 antibodies (α-SHP-1). The positions of the expressed protein and of endogenous SHP-1 are shown.

In order to examine the functional role of SHP-1 in IL-3 signalling in greater detail, we chose to express a version of SHP-1 in which arginine-459 was replaced by methionine (R459M SHP-1), which generates a catalytically inactive, dominant-negative form of SHP-1 [40]. WT SHP-1 was also expressed. As an additional tool, we also expressed a version of SHP-1 in which cysteine-453 had been replaced by serine (C453S SHP-1). This form of SHP-1 has been shown to act as a trapping mutant in previous studies, in addition to being catalytically inactive [40]. All three versions of SHP-1 were tagged at the N-terminus with a Myc epitope and transfected into BaF/3 tTA cells. Antibiotic-resistant clones were screened for inducible expression of the SHP-1 variants. Immunoblotting with anti-SHP-1 antibodies allowed levels of induced expression to be compared with levels of endogenous SHP-1. Figure 1(A) shows expression of the exogenous protein in the representative clones for each SHP-1 variant that were selected for use in the present study. Similar levels of expression were observed in additional independent clones that were also used for the investigations presented herein (results not shown). Figure 1(B) shows, using WT SHP-1 clone 1A1 as an example, that expression was maintained for 72 h. Figure 1(C) shows, using R459M clone 2F11 as an example, the levels of expression achieved in cells plated at the different densities subsequently used in this study.

Expression of R459M SHP-1 increases the IL-3-induced proliferation of BaF/3 cells

To investigate whether the expression of WT or R459M SHP-1 influences IL-3-induced proliferation, BaF/3 cells expressing

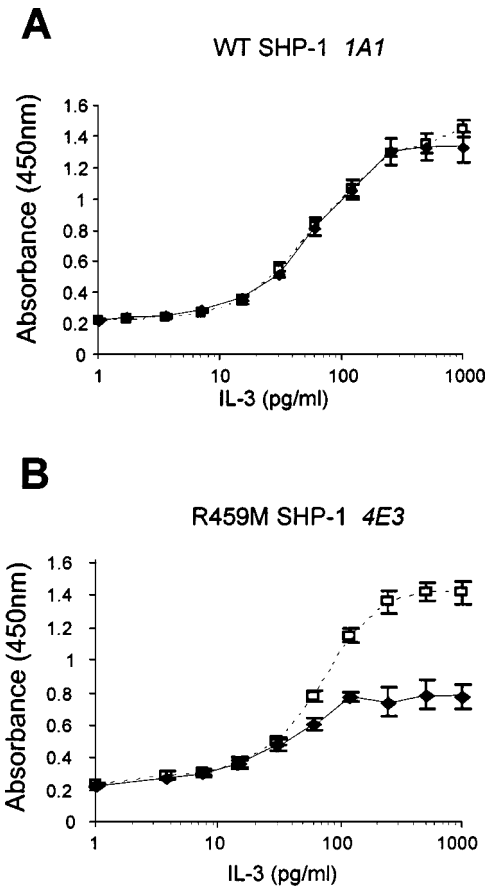

Figure 2 Effects of expression of WT and R459M SHP-1 on IL-3-induced proliferation

(**A**) XTT bioreduction assays were performed as described in the Experimental section. Experiments shown are those in which the cells were cultured in the presence (◆; solid lines) or absence (□; broken lines) of Tet for a period of 24 h prior to the assay being set up. (**A**) Effect of expressing WT SHP-1 (clone 1A1) upon the IL-3-responsiveness of BaF/3 cells. (**B**) Effect of expressing R459M SHP-1 (clone 4E3) upon the IL-3-responsiveness of BaF/3 cells. Results are representative of three independent experiments and for two different clones in each case. Means ± S.D. are plotted for each point from quadruplicate samples. Cells incubated in the absence of IL-3 gave an average reading of 0.2 absorbance unit.

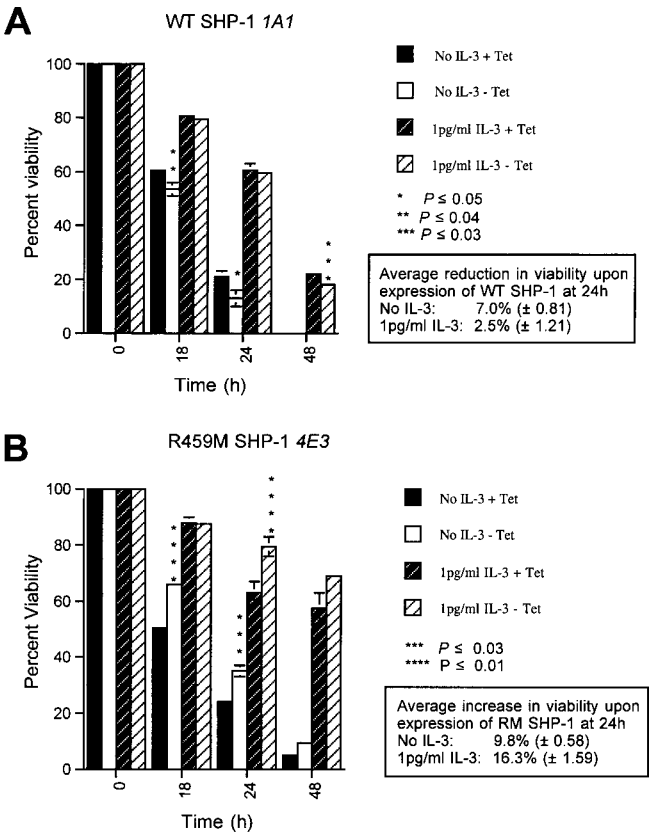

Figure 3 Effects of expression of WT and R459M SHP-1 on cell survival

Trypan Blue exclusion assays were performed as described (see the Experimental section) and the percentage of viable cells was determined at the times shown. The results are representative of the effects of expression of WT or R459M SHP-1 seen in two independent clones for each SHP-1 variant. Means ± S.D. are plotted for each point from quadruplicate samples. Paired Student's *t* tests were applied to the data: $^{*}P \leqslant 0.05$, $^{**}P \leqslant 0.04$, $^{***}P \leqslant 0.03$ and $^{****}P \leqslant 0.01$ denote significant differences compared with + Tet samples at the same time and under the same treatment conditions. The boxes beside each panel give the average values for the effects of expression of each SHP-1 variant on cell viability at 24 h, which were calculated from three independent experiments. Values in parentheses are S.E.M.

either WT or R459M SHP-1 were subjected to IL-3 dose–response assays. Reduction of XTT by NAD^+/NADPH oxidoreductases to generate a coloured formazan was used an indicator of metabolic activity and growth of the cells [34]. Transfectants were grown in the presence or absence of Tet for 24 h prior to being set up in the assay. Expression of WT SHP-1 had no significant effect on the IL-3-dependent proliferation of BaF/3 cells (Figure 2A). In contrast, expression of R459M SHP-1 consistently and significantly increased proliferation in response to IL-3 at both optimal (> 200 pg/ml rmIL-3) and suboptimal (< 200 pg/ml rmIL-3) doses (Figure 2B). The increased growth in the − Tet R459M SHP-1 samples necessitated that the assays were read prior to the maximum absorbance being reached. Due to the correspondingly decreased incubation time, the absorbance values for the + Tet R459M SHP-1 samples appear lower than those for the WT SHP-1 clones. The effects of expression of R459M SHP-1 on growth were observed with two independent clones (results not shown), and are consistent with a previous report implicating SHP-1 as a regulator of IL-3-driven proliferation [23].

Expression of R459M SHP-1 increases the survival of BaF/3 cells

The increase in proliferation observed upon expression of R459M SHP-1 could arise through two potential mechanisms: enhanced cell survival or an increase in the cell cycling rate. SHP-1 has been reported to both facilitate [19,21] and prevent [41] apoptosis in different haemopoietic cells, but this has not been examined directly in IL-3-dependent cells. Therefore we examined whether expression of R459M or WT SHP-1 had any effects on cell survival in the presence of suboptimal doses of IL-3 and following IL-3 withdrawal. Expression was induced for 24 h and the cells were cultured for a further 24 h in 0, 1 or 20 pg/ml IL-3. Both 20 and 1 pg/ml are suboptimal IL-3 concentrations, and while 20 pg/ml can still support some growth of BaF/3 cells (see Figure 2), 1 pg/ml cannot. After 24 h, the proportion of cells remaining viable was determined by Trypan Blue exclusion. A typical example of the effects observed is shown in Figure 3, with averaged effects shown in the boxes. When incubated with 20 pg/ml IL-3, each of the transfectants, in the presence or absence of Tet, remained 99–100% viable over the 48 h time period of the assay (results not shown). Cells expressing WT SHP-1 showed little change in their viability relative to + Tet

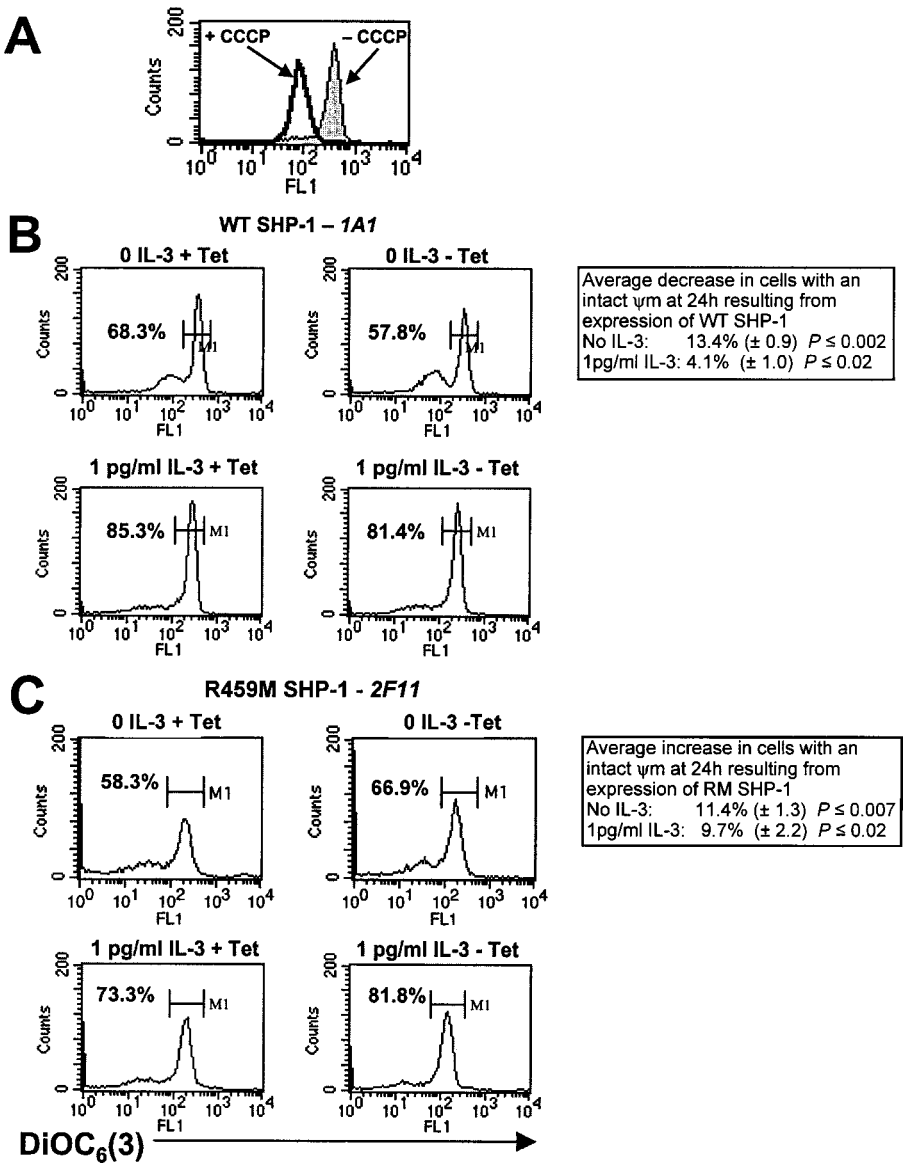

Figure 4 Effects of expression of WT and R459M SHP-1 on Ψm

$DiOC_6$ staining was performed as described (see the Experimental section). (**A**) Abrogation of mitochondrial membrane potential by the mitochondrial uncoupling agent CCCP. (**B**) Effect of expressing WT SHP-1 upon the mitochondrial membrane potential of BaF/3 cells in either the absence or the presence of 1 pg/ml rmIL-3 after a 24 h period. (**C**) Effect of expressing R459M SHP-1 upon the mitochondrial membrane potential of BaF/3 cells in either the absence or the presence of 1 pg/ml rmIL-3 after a 24 h period. Marker M1 shows the proportion of 10000 cells counted that retained their mitochondrial membrane potential 24 h after the start of the assay. The boxes beside (**B**) and (**C**) give the mean percentage difference in the number of cells with an intact Ψm at 24 h. Values in parentheses are S.E.M. The paired Student's *t* test was used to calculate significance in each case.

controls when cultured with 1 pg/ml IL-3, but did show a consistent decrease in viability when cultured in the absence of IL-3 ($P \leqslant 0.04$ at 18 h and $P \leqslant 0.05$ at 24 h). In contrast, although no effect was seen on cells cultured with 20 pg/ml IL-3 (results not shown), expression of R459M SHP-1 increased the viability of BaF/3 cells in the absence of IL-3 ($P \leqslant 0.01$ at 18 h and $P \leqslant 0.03$ at 24 h) or in the presence of 1 pg/ml IL-3 ($P \leqslant 0.01$ at 24 h; see Figure 3B).

As an additional measure of cell survival, we assessed effects on Ψm, the loss of which has been demonstrated to be an early, irreversible event that facilitates the induction of apoptosis [36]. $DiOC_6$ stains mitochondria in a manner that is dependent upon the integrity of Ψm [35]. Thus $DiOC_6$ can be used as a tool to measure the loss of Ψm as cells initiate apoptosis, and hence can be employed to assess the level of apoptosis in a cell population by flow cytometry. CCCP is a mitochondrial uncoupling agent that abolishes Ψm, and its effect on the $DiOC_6$ staining of BaF/3 cells can be seen in Figure 4(A). WT and R459M SHP-1 transfectants were cultured in the presence or absence of Tet for 24 h, and then cultured for a further 24 h in the presence or absence of 1 pg/ml IL-3 (plus or minus Tet). The Ψm profiles of the populations were then determined using $DiOC_6$. Representative examples of the data collected are shown for WT (Figure 4B) and R459M (Figure 4C) SHP-1 transfectants, with the boxes in each case showing the average changes in the numbers of cells with an intact Ψm. Expression of WT SHP-1 reduced the

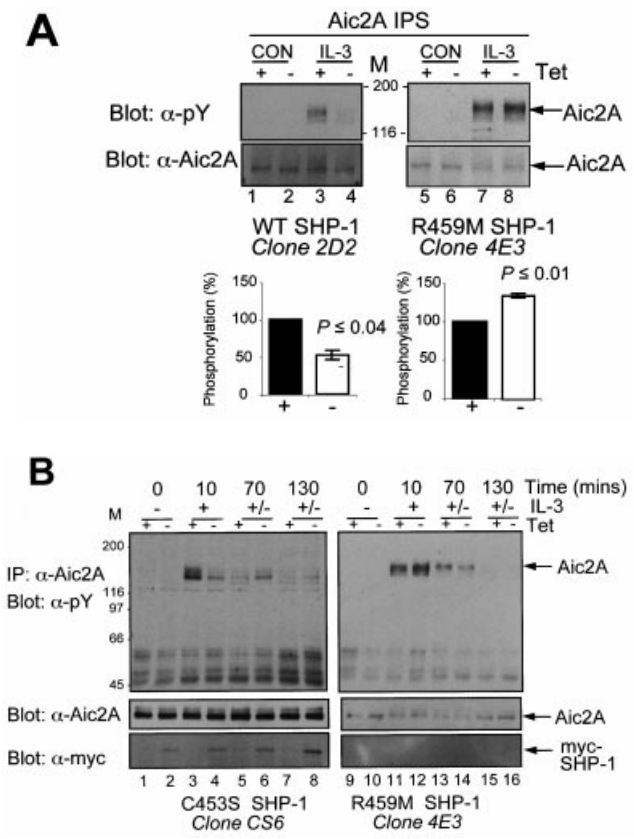

Figure 5 Effects of expression of WT, R459M and C453S SHP-1 on IL-3-induced tyrosine phosphorylation of Aic2A

(**A**) WT (left panels) and R459M (right panels) transfectants were incubated in the presence (+) or absence (−) of 2 μg/ml Tet for 24 h prior to being stimulated with 4 ng/ml IL-3 for 10 min or left unstimulated as a control (CON). (**B**) C453S SHP-1 transfectants were cultured and stimulated as in (**A**), but the cells were then washed to remove the IL-3 and resuspended in serum-free medium. These cells were again incubated at 37 °C and samples were taken at the times shown. Immunoprecipitations (IPs) were prepared using anti-Aic2A antibodies and 500 μg of each cell lysate. Immunoblotting with 4G10 was used to detect tyrosine-phosphorylated proteins (α-pY). Immunoblots were then stripped and re-probed with anti-Aic2A antibodies to allow parity of loading to be examined (α-Aic2A) and in (**B**) with antibody 9E10 to see whether the expressed protein was precipitated with Aic2A (α-myc). Molecular mass standards are shown in kDa (M). The position of Aic2A is indicated in each case. The lower panels of (**A**) show the average changes in Aic2A tyrosine phosphorylation. Results from three independent experiments were densitometrically scanned and the mean changes in tyrosine phosphorylation of Aic2A resulting from expression of each SHP-1 variant were determined. Values for +Tet +IL-3 conditions were set at 100%, as this was the control for each clone, and the average changes are shown for the −Tet +IL-3 samples. Bars indicate S.E.M. Student's paired *t* tests were applied to the results, and *P* values are depicted in each case for the −Tet compared with the +Tet samples.

proportion of cells with an intact Ψm in the population cultured in the absence of IL-3 by an average of 13.4% ($P \leqslant 0.002$; Figure 4B). This suggested that apoptosis was being facilitated by WT SHP-1. In contrast, Figure 4(C) shows that expression of R459M SHP-1 increased the proportion of cells with an intact Ψm both in the absence of IL-3 (average increase 11.4%; $P \leqslant 0.007$) and in the presence of 1 pg/ml IL-3 (average increase 9.7%; $P \leqslant 0.02$), indicating that expression of R459M SHP-1 was acting to enhance viability. These results demonstrate a role for SHP-1 in regulating cell survival at lower doses of IL-3. However, these results also suggest that increased cell survival is not likely to underpin the enhanced proliferation observed upon expression of R459M, because at doses of IL-3 of $\geqslant 20$ pg/ml there was no detectable increase in cell survival.

SHP-1 modulates the IL-3-induced tyrosine phosphorylation of Aic2A

We hypothesized that perturbations in the mechanisms of IL-3 signalling would be demonstrable that would account, individually or in combination, for the functional effects of expression of WT and R459M SHP-1. In addition, a rigorous and detailed examination of the biochemical signalling pathways regulated by SHP-1 in IL-3-dependent cells has not been carried out previously. Initially, we examined whether expression of the SHP-1 variants affected overall levels of IL-3-induced tyrosine phosphorylation. We performed both short and long IL-3 time-course analyses [see Figures 6A and 6B (panels i); and results not shown], but expression of neither WT or R459M SHP-1 had any consistent effects on overall levels of tyrosine phosphorylation. We have reported previously that βc [27] and Aic2A [29] are potential SHP-1 substrates *in vitro*. In order to examine whether tyrosine phosphorylation of Aic2A is regulated by SHP-1 *in vivo*, Aic2A immunoprecipitates were prepared from WT and R459M SHP-1 transfectants that had been cultured in the presence or absence of 2 μg/ml Tet and then stimulated with 4 ng/ml IL-3 or left unstimulated as a control. The IL-3-induced tyrosine phosphorylation of Aic2A (Figure 5A, lanes 1 and 3) was dramatically decreased by the expression of WT SHP-1 (Figure 5A, lane 4) and slightly increased by the expression of R459M SHP-1 (Figure 5A, lane 8). These results were repeated in independent clones, and were observed at both the submaximal dose of 4 ng/ml and the maximal stimulatory dose of 10 ng/ml IL-3 (results not shown). Densitometric scanning of data from three independent experiments showed there to be an average decrease in IL-3-induced Aic2A tyrosine phosphorylation upon expression of WT SHP-1 of $\sim 50\%$ ($P \leqslant 0.04$), compared with an average increase of 35% ($P \leqslant 0.01$) upon expression of R459M SHP-1.

As an additional means of determining whether Aic2A is a *bone fide* substrate of SHP-1, we investigated the effect of expression of the C453S substrate-trapping mutant version of SHP-1 on Aic2A tyrosine phosphorylation. IL-3 induces rapid tyrosine phosphorylation of Aic2A, which is quickly reversed following the subsequent removal of IL-3 (Figure 5B, lanes 3, 5 and 7). The effects of expressing C453S and R459M SHP-1 on this reversal of Aic2A tyrosine phosphorylation revealed that it was partially prevented by the expression of C453S SHP-1 (Figure 5B, lanes 6 and 8), but not of R459M SHP-1 (Figure 5B, lanes 14 and 16). This indicates that specific phosphotyrosine residues on Aic2A are being trapped by C453S SHP-1, protecting them from dephosphorylation. Immunoblotting of the SHP-1 variants demonstrated that, following IL-3 stimulation, increasing levels of C453S SHP-1, but not R459M SHP-1, were co-precipitated with Aic2A (Figure 5B, lower panels), further indicating that C453S SHP-1 was incrementally 'trapping' Aic2A. These findings strongly suggest that Aic2A is an *in vivo* substrate of SHP-1. Of further interest is the observation that C453S SHP-1 initially lowered IL-3-induced tyrosine phosphorylation of Aic2A, before then protecting it following the removal of IL-3.

Role of SHP-1 in the regulation of downstream pathways

In light of the dramatic attenuation of the tyrosine phosphorylation of Aic2A as a result of WT SHP-1 expression, and the slight but consistent potentiation of Aic2A tyrosine phosphorylation afforded by expression of R459M SHP-1, it was

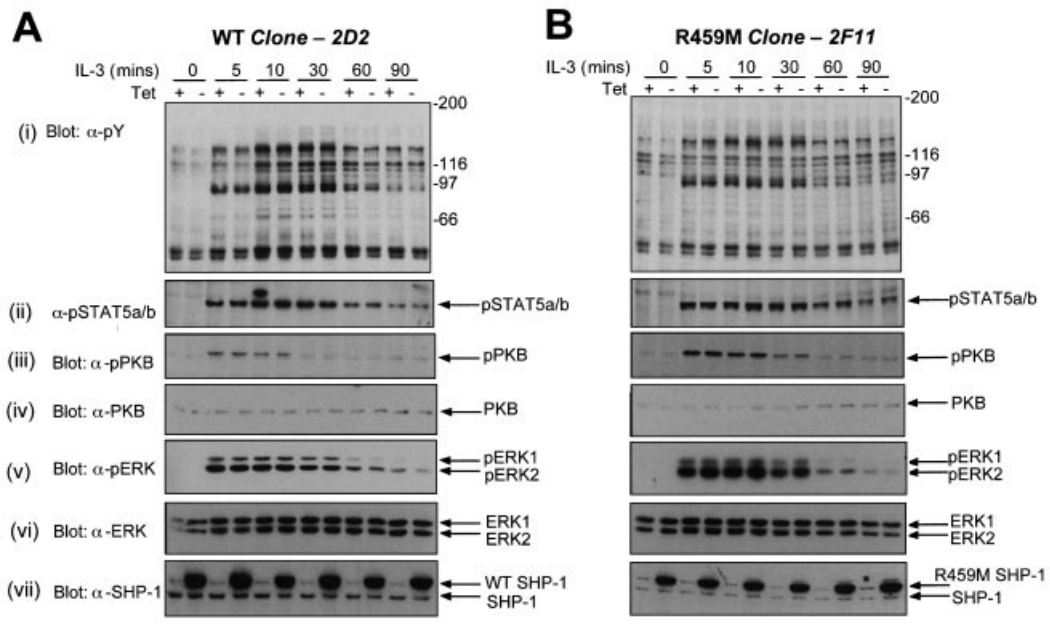

Figure 6 Effects of expression of WT and R459M SHP-1 on IL-3-induced activation of downstream pathways

WT (**A**) and R459M (**B**) SHP-1 transfectants were incubated in the presence (+) or absence (−) of 2 μg/ml Tet for 24 h prior to being stimulated with 4 ng/ml IL-3 for 90 min or left unstimulated as a control (0 min). Samples of the cells were taken after the times shown. Portions of 25 μg of each sample were separated by SDS/PAGE on either a 7.5% or a 10% (w/v) acrylamide gel. Immunoblots of the 7.5% acrylamide gels were probed with the anti-phosphotyrosine antibody 4G10 antibody [**A**(i) and **B**(i); α-pY], then stripped and reprobed with an antibody specific for tyrosine-phosphorylated STAT5a/b [**A**(ii) and **B**(ii); α-pSTAT5a/b]. Identical samples separated through 10% acrylamide gels were probed and re-probed sequentially with antibodies specifically recognizing serine-phosphorylated PKB [**A**(iii) and **B**(iii); α-phos-PKB] and ERK-1/2 [**A**(v) and **B**(v); α-phos-ERK]. Immunoblots were also stripped and re-probed with anti-PKB [**A**(iv) and **B**(iv); α-PKB], anti-ERK [**A**(vi) and B(vi); α-ERK] and anti-SHP-1 [**A**(vii) and **B**(vii); α-SHP-1] antibodies to show parity of loading. Results are representative of three experiments in each case.

speculated that effects might be seen on the subsequent activation of downstream biochemical signalling pathways. The principle pathways believed to mediate the functional outcomes of IL-3 stimulation are the STAT5a/b, PI 3-kinase and MAPK pathways [6]. The effects of expressing WT and R459M SHP-1 on these pathways were investigated by performing IL-3 time courses and by immunoblotting equivalent quantities of cytosolic cell lysates with antibodies that specifically detect phosphorylated (and hence activated) components of these cascades.

Phosphorylation of tyrosine-694/699 of STAT5a/b is required for it to dimerize and to activate DNA binding activity [42]. A phospho-specific antibody was used to examine the effects of expression of WT and R459M SHP-1 on the phosphorylation of STAT5a/b at Tyr-694/699. As shown in Figures 6(A) and 6(B) (panels ii), expression of neither WT SHP-1 nor R459M SHP-1 affected the tyrosine phosphorylation of Tyr-694/699 of STAT5a/b following IL-3 stimulation.

Next, we assessed activation of the PI 3-kinase cascade. Phosphorylation of PKB at both serine-473 and threonine-308 occurs in a PI 3-kinase-dependent manner, and is required for the full activation of PKB [43]. We used a phosphospecific antibody that recognizes phosphorylated Ser-473 to assess the activation of PKB by IL-3. Expression of neither WT nor R459M SHP-1 had any effect on the phosphorylation of PKB at Ser-473 (Figure 6, panels iii). These findings were supported by *in vitro* kinase assays performed with PKB (results not shown).

The activation of ERK-1 and -2 by IL-3 was investigated using phospho-specific antibodies raised against threonine-202 and tyrosine-204 of ERK-1 and -2 which, when phosphorylated by MEK (MAPK/ERK kinase), activate the serine/threonine kinase activity of ERK and hence facilitate the induction of immediate-early gene expression [44]. Expression of neither WT nor R459M SHP-1 had any clear effects upon ERK-1 and -2 phosphorylation (Figure 6, panels v). We also examined the activation of JNK (c-Jun N-terminal kinase) and p38 MAPKs, but again no consistent alterations were observed (results not shown).

SHP-1 modulates the overall IL-3-induced tyrosine phosphorylation of STAT5a/b

In order to investigate whether the modulation of Aic2A tyrosine phosphorylation by the expression of WT or R459M SHP-1 affects the overall tyrosine phosphorylation of STAT5, STAT5 was immunoprecipitated and its level of tyrosine phosphorylation determined by immunoblotting with the anti-phosphotyrosine antibody 4G10. Expression of WT SHP-1 reduced (Figure 7A, lanes 3 and 4) and expression of R459M SHP-1 increased (Figure 7A, lanes 7 and 8) the overall tyrosine phosphorylation of STAT5 induced by IL-3. Densitometric scanning of three independent experiments for each SHP-1 variant demonstrated that expression of WT SHP-1 led to an average decrease in STAT5 tyrosine phosphorylation of $\sim 40\,\%$ ($P \leqslant 0.02$), whereas expression of R459M SHP-1 led to an increase of $\sim 35\,\%$ ($P \leqslant 0.03$).

As another means to test if STAT5 is a substrate of SHP-1, we examined the ability of C453S SHP-1 to protect STAT5 from tyrosine dephosphorylation after an initial pulse of IL-3 stimulation. C453S SHP-1 did protect STAT5 from dephosphorylation at later time points (Figure 7B, lanes 6 and 8) while R459M SHP-1 did not (lanes 14 and 16). Re-probing these blots with the anti-Myc epitope-tag antibody 9E10 showed that C453S SHP-1 co-

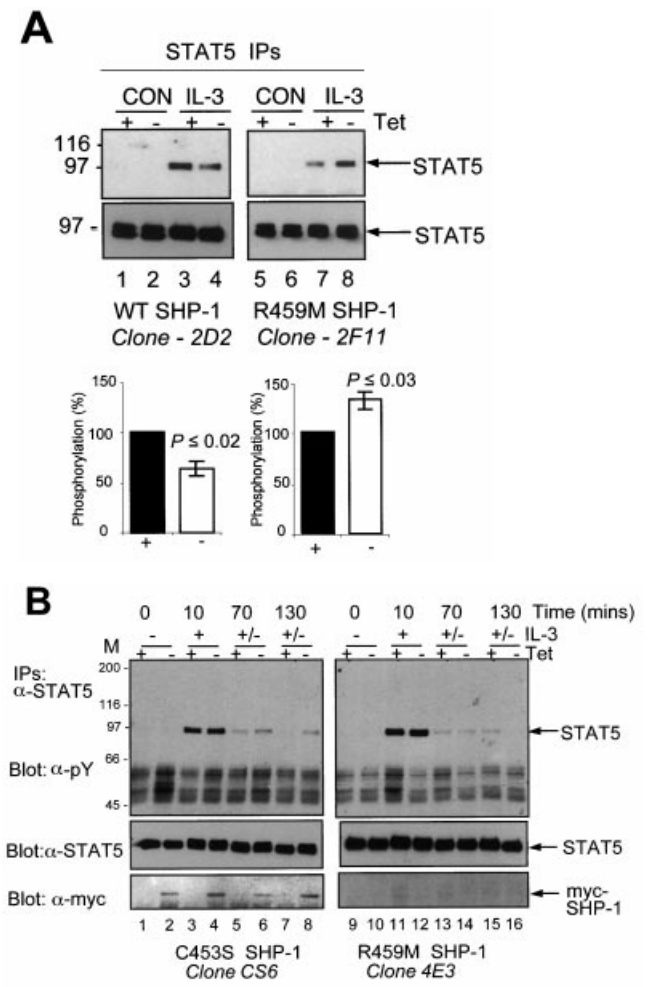

Figure 7 SHP-1 negatively regulates tyrosine phosphorylation of STAT5

(**A**) WT (left panels) and R459M (right panels) transfectants were incubated in the presence (+) or absence (−) of 2 μg/ml Tet for 24 h prior to being stimulated with 4 ng/ml IL-3 for 10 min or left unstimulated as a control (CON). Anti-STAT5a/b immunoprecipitates were prepared from 500 μg of each cell lysate per sample. (**B**) C453S (left panel) and R459M (right panel) transfectants were cultured and stimulated as in (**A**), but the cells were then washed to remove the IL-3 and resuspended in serum-free medium. These cells were incubated at 37 °C again and samples were taken after the times shown (+/−). Anti-STAT5 immunoprecipitates (IPs) were prepared as in (**A**). Immunoblotting with antibody 4G10 (α-pY) was used to detect tyrosine-phosphorylated proteins (upper panel). Immunoblots were stripped and re-probed with anti-STAT5a/b antibodies (α-STAT5a/b) to allow parity of loading to be examined (middle panel), and in (**B**) with antibody 9E10 (α-myc) to see whether the expressed protein was precipitated along with STAT5. Molecular mass standards are shown in kDa (M). The position of STAT5a/b is indicated in each case. The lower panels of (**A**) show average changes in STAT5 tyrosine phosphorylation. The results from three independent experiments were densitometrically scanned and the mean changes in the tyrosine phosphorylation of STAT5 resulting from expression of each SHP-1 variant were determined. Values for +Tet +IL-3 conditions were set at 100%, as this was the control for each clone, and the average changes are shown for the −Tet +IL-3 samples. Values are means ± S.E.M. Student's paired t test was applied to the results, and P values are depicted in each case for the −Tet compared with the +Tet samples.

precipitated with STAT5, whereas, importantly, R459M SHP-1 did not (Figure 7B, bottom panels). The protection of STAT5 tyrosine phosphorylation and the presence of C453S SHP-1, but not R459M SHP-1, in STAT5 precipitates are consistent with C453S SHP-1 trapping STAT5. However, C453S SHP-1 could be detected in STAT5 precipitates prepared from samples taken at $t = 0$, which was unexpected, although levels were increased at $t = 130$. One possibility is that this mutant binds to STAT5 that has very low levels of phosphotyrosine. Alternatively, the mutant could be bound to an additional protein, also complexed with STAT5. When the results from all three SHP-1 variants are taken together, they collectively indicate that STAT5 is a potential *in vivo* substrate of SHP-1.

DISCUSSION

SHP-1 has been widely reported to be a negative regulator of cellular signalling in immune cells [24]. However, only one previous study has investigated the functional role of SHP-1 in IL-3-dependent cells, and this relied on the use of antisense technology [23] and did not investigate effects on cell survival or downstream biochemical signalling pathways. To examine the functional role of SHP-1 in IL-3 signalling in greater depth and to investigate the key substrates and downstream pathways involved in its actions, we used an alternative approach and expressed a number of SHP-1 variants using the Tet-regulated gene expression system in IL-3-dependent BaF/3 cells. In the present study, we demonstrate that expression of the catalytically inactive R459M mutant of SHP-1 enhances the IL-3-induced proliferation of BaF/3 cells, whereas expression of WT SHP-1 does not. Importantly, based on the opposite effects of R459M and WT SHP-1 on cell survival, we also define a role for SHP-1 in regulating the apoptosis of IL-3-dependent cells. Examination of effects on potential substrate proteins demonstrated that STAT5 and Aic2A are *in vivo* substrates of SHP-1.

The increase in IL-3-induced proliferation observed upon expression of R459M SHP-1 is consistent with this catalytically inactive mutant acting in a classical dominant-negative manner and competing with endogenous SHP-1. Our results independently support the earlier report that suggested a negative regulatory role for SHP-1 in IL-3-induced growth [23]. Interestingly, it has recently been shown that an inhibitor of SHP-1 and SHP-2 augments the proliferation of BaF/3 cells at sub-optimal doses of IL-3 [45]. Given that we observe a minimal role for SHP-2 in regulating IL-3-driven proliferation (H. Wheadon and M. J. Welham, unpublished work), this finding is consistent with the observations we report here. We have examined the basis of the functional role of SHP-1, and demonstrate that the increase in proliferation at IL-3 doses greater than 40 pg/ml seen when R459M SHP-1 was expressed is not likely to be due to decreased apoptosis, as no effects on cell survival were observed at 20 pg/ml IL-3. Rather, a reduction in SHP-1 activity resulting from expression of R459M SHP-1 may lower the threshold for IL-3 signalling, potentiating the proliferative response. Investigation of the underlying mechanism suggests that expression of R459M SHP-1 increases the growth rate of the cells, which may be due to a shortening of the cell cycle time (N. R. D. Paling and M. J. Welham, unpublished work).

The involvement of SHP-1 in the regulation of apoptosis has not been reported previously in IL-3-dependent cells, but is consistent with previous reports using other cell systems [21,22]. Using two independent assessments of cell survival, we demonstrate that expression of R459M SHP-1 results in an increase in cell survival in the presence of 1 pg/ml IL-3, and that expression of WT SHP-1 decreases cell survival upon IL-3-withdrawal. Thus SHP-1 appears to play a role in regulating apoptosis in BaF/3 cells.

Having demonstrated that SHP-1 plays a role in regulating two functional responses of BaF/3 cells, we sought to identify key SHP-1 substrates that are potentially involved in regulating these effects. Yi et al. [23] reported that an increase in SHP-1

levels reduced the overall level of IL-3-induced tyrosine phosphorylation. Our results are in contrast with this. We could not detect any consistent alterations in the overall level of tyrosine phosphorylation of cellular proteins induced by IL-3 when cells were induced to express either WT or R459M SHP-1. Yi et al. [23] also reported that decreasing SHP-1 levels (using antisense RNA) modestly increased phosphorylation of a 140 kDa protein, which was suggested to be Aic2A, although this was not demonstrated directly. We investigated effects on Aic2A tyrosine phosphorylation directly, and show definitively that overall levels of IL-3-induced Aic2A tyrosine phosphorylation were dramatically reduced upon expression of WT SHP-1, increased slightly but significantly when R459M SHP-1 was expressed, and protected by expression of C453S SHP-1. These effects are unlikely to be the result of blocking by the SH2 domains of SHP-1, as the WT, R459M and C453S forms all control for each other. We have shown previously that SHP-1 can dephosphorylate tyrosine residues 612 and 750 of the βc-subunit of the human IL-3R *in vitro* [27], and that Aic2A is a potential SHP-1 substrate *in vitro* [29]. However, the magnitude of the effect of expressing WT SHP-1 is greater than would be expected simply in terms of SHP-1 acting only on two residues of Aic2A [27]. One possible explanation is that SHP-1 is acting upstream of Aic2A tyrosine phosphorylation. SHP-1 could be acting directly on a kinase responsible for tyrosine phosphorylation of Aic2A, and we are currently investigating this possibility. Alternatively, Tyr-745 of Aic2A, which is analogous to Tyr-750 of βc, is thought to be involved in facilitating further tyrosine phosphorylation of the receptor [46]. Thus, if SHP-1 dephosphorylated this site, it would prevent further phosphorylation of the receptor. This latter model is supported by the finding that expression of C453S SHP-1 reduced the maximal IL-3-induced tyrosine phosphorylation of Aic2A, normally observed 10 min after receptor engagement. This can be explained by C453S SHP-1 trapping either Tyr-745 of Aic2A, or a tyrosine kinase that acts upon Aic2A, thus blocking its ability to mediate the tyrosine phosphorylation of Aic2A. These findings highlight the need for a more detailed examination of the phosphorylation dynamics of individual tyrosine residues of IL-3Rβ and the degree of degeneracy in their functions.

We have also identified STAT5 as a potential substrate of SHP-1, with overall levels of STAT5 tyrosine phosphorylation being reduced upon expression of WT SHP-1 and increased by R459M SHP-1 expression. Expression of C453S SHP-1 also appeared to protect STAT5 from dephosphorylation. Other groups have reported a role for SHP-1 in the regulation of STAT signalling downstream of other receptors [47,48]. However, Yu et al. [49] reported that STAT5 immunoprecipitated from IL-2-stimulated CTLL-20 cells was not dephosphorylated *in vitro* by an SHP-1–glutathione S-transferase fusion protein. Interestingly, in our studies, SHP-1 does not target Tyr-694/Tyr-699 of STAT5a/b; hence the differences observed may be because IL-2 induces phosphorylation of different, additional tyrosine residues on STAT5 compared with IL-3, which are not targeted by SHP-1. The effect of tyrosine phosphorylation of STAT5 on residues other than Tyr-694/Tyr-699 has not been determined. Further analyses of the effects of WT and R459M SHP-1 on the transcriptional activity of STAT5 would be required to assess the functional consequences of the effects we observe.

When we investigated the effects of the SHP-1 variants on specific downstream signalling cascades known to regulate proliferation and cell survival [6], we rather surprisingly failed to reveal any consistent effects on IL-3-induced activation of MAPKs or PKB. How then do we correlate the functional effects observed with the underlying biochemistry? There are a number of possibilities to explain these findings. Potentially, expression of R459M SHP-1 could be subtly modulating levels of the MAPK and PKB pathways, such that they are difficult to quantify. Effects on intracellular signalling cascades were measured over a period of 2 h, whereas proliferation was measured over 3 days. Therefore small but sustained changes in signalling would be amplified over this 3 day period and could provide sufficient signals to increase proliferation. Alternatively, distinct pathways, potentially regulated via Aic2A and/or STAT5 tyrosine phosphorylation, that couple to control of the cell cycle could be affected by expression of R459M SHP-1, resulting in increased proliferation. Tyrosine phosphorylation of Aic2A and the subsequent activation of the STAT5 pathway are critical events in the initiation of IL-3-induced cell function [6], and thus their regulation by SHP-1 could represent some of the primary mechanisms via which SHP-1 regulates IL-3-induced proliferation and survival. It is thus somewhat paradoxical that increasing the expression of WT SHP-1 does not affect IL-3-induced proliferation. One explanation may be that a proportion of Aic2A tyrosine phosphorylation is not required for proliferative signalling; significantly, there is precedent for this in the literature. Cells expressing βc that has had all its tyrosine residues mutated to phenylalanine still support proliferation in optimal IL-3, but not survival [46], suggesting that different tyrosine residues couple to distinct functional responses. Thus we propose that, while WT SHP-1 may decrease the overall tyrosine phosphorylation of Aic2A, it does not target those receptor tyrosine residues that facilitate IL-3-induced proliferation. Survival signalling, which is more dependent on the tyrosine phosphorylation of those residues being regulated by SHP-1, is affected by the expression of WT SHP-1. When SHP-1 activity is decreased by expression of the dominant-negative R459M SHP-1 mutant, the increase in Aic2A tyrosine phosphorylation may be sufficient to overcome signalling thresholds for both proliferation and survival, and so induce the increases in proliferation and survival observed. Rigorous analysis of the temporal regulation of phosphorylation and dephosphorylation of specific tyrosine residues on Aic2A, combined with analyses of Aic2A mutants and their functional consequences, are required to test this model.

In summary, we have demonstrated that SHP-1 inhibits the IL-3-induced proliferation and survival of BaF/3 cells. In addition, we have shown that SHP-1 negatively regulates tyrosine phosphorylation of the β subunit of the murine IL-3R, Aic2A, consistent with it being a primary substrate of SHP-1. We have also demonstrated that STAT5 is a potential substrate of SHP-1 in IL-3 signalling. The modulation of the tyrosine phosphorylation of Aic2A and STAT5 mediated by SHP-1 may contribute to the regulation of biological responses induced by IL-3.

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