Socs1 binds to multiple signalling proteins and suppresses Steel factor-dependent proliferation

Paulo De Sepulveda¹, Klaus Okkenhaug^{1,2},
Jose La Rose¹, Robert G.Hawley³,
Patrice Dubreuil⁴ and Robert Rottapel^{1,2,5,6}

¹Ontario Cancer Institute, Princess Margaret Hospital, 610 University Avenue, Toronto M5G 2M9, ²Department of Immunology, ³Department of Medicine, University of Toronto, Toronto, Ontario, Canada, ⁴Hematopoiesis Department, Holland Laboratory, American Red Cross, 15601 Crabbs Branch Way, Rockville, MD 20855, USA and ⁵Molecular Hematology Laboratory Unite 119, Institut National de la Santé et de la Recherche Médicale, 27 Boulevard Lèi Roure, Marseille, France

⁶Corresponding author
e-mail: Rottapel@oci.utoronto.ca

We have identified Socs1 as a downstream component of the Kit receptor tyrosine kinase signalling pathway. We show that the expression of Socs1 mRNA is rapidly increased in primary bone marrow-derived mast cells following exposure to Steel factor, and Socs1 inducibly binds to the Kit receptor tyrosine kinase via its Src homology 2 (SH2) domain. Previous studies have shown that Socs1 suppresses cytokine-mediated differentiation in M1 cells inhibiting Janus family kinases. In contrast, constitutive expression of Socs1 suppresses the mitogenic potential of Kit while maintaining Steel factor-dependent cell survival signals. Unlike Janus kinases, Socs1 does not inhibit the catalytic activity of the Kit tyrosine kinase. In order to define the mechanism by which Socs1-mediated suppression of Kit-dependent mitogenesis occurs, we demonstrate that Socs1 binds to the signalling proteins Grb-2 and the Rho-family guanine nucleotide exchange factors Vav. We show that Grb2 binds Socs1 via its SH3 domains to putative diproline determinants located in the N-terminus of Socs1, and Socs1 binds to the N-terminal regulatory region of Vav. These data suggest that Socs1 is an inducible switch which modulates proliferative signals in favour of cell survival signals and functions as an adaptor protein in receptor tyrosine kinase signalling pathways.

Keywords: Grb2/SH2/SH3/signal transduction/Vav

Introduction

Receptor tyrosine kinases (RTKs) and their ligands initiate signalling pathways that determine cell fate during both invertebrate and vertebrate development (Pawson and Bernstein, 1990). The important role RTKs play in mammalian development is underscored by the pleiotropic abnormalities observed in mice that carry loss-of-function mutations within the dominant white (*W*) locus. The *W* locus encodes the cellular homologue of the retrovirally transduced Kit RTK. *W* mice with mutations that diminish

Kit kinase activity lack hair pigmentation, suffer from hematopoietic defects and are infertile. A similar constellation of developmental defects is observed in *Steel* mice that carry loss-of-function mutations in the Kit ligand. In humans Kit mutations cause piebaldism, a syndrome resulting in abnormal skin and hair pigmentation and deafness (Fleischman *et al.*, 1991; Giebel and Spritz, 1991). The cellular impairment underlying the *W* phenotype reflects the inability of neural crest-derived melanoblasts, hematopoietic stem cells (HSCs) and primordial germ cells to proliferate, differentiate, migrate and/or survive during embryogenesis and adult life (Russell, 1979; Silvers, 1979).

The role of Kit and Steel factors in hematopoiesis has been particularly well studied (Broudy, 1997; Lyman and Jacobsen, 1998). Kit is expressed on early hematopoietic progenitor cells that self-renew and are capable of reconstituting all hematopoietic lineages in irradiated hosts (Okada *et al.*, 1991; Ikuta and Weissman, 1992; Orlic *et al.*, 1993). Steel factor alone can support the survival and self-renewal of HSCs *in vitro* and *in vivo* (Li and Johnson, 1994; Keller *et al.*, 1995). However, in combination with other hematopoietic growth factors, Steel factor enhances the expansion of cells committed to both the lymphoid and myeloid lineages (McNiece *et al.*, 1991a,b; Ulich *et al.*, 1991; Pietsch *et al.*, 1992; Tsuji *et al.*, 1992; Ploemacher *et al.*, 1993). Therefore, depending on the cellular context and local growth factor milieu, Steel factor manifests diverse functions during early and late hematopoiesis by virtue of its capacity to maintain and expand HSCs and participate in proliferative and differentiative events associated with lineage commitment. Little is currently known about the signal-transduction molecules that regulate Steel factor-mediated control of the cell cycle of HSCs or those which are involved in the activation of lineage-specific developmental programs.

The mechanism by which Kit initiates diverse cellular responses results in part from the specific array of signal-transducing pathways engaged by the activated Kit receptor. Kit activates canonical signal-transduction pathways including those regulated by phosphatidylinositol 3'-kinase (PI3K), Ras and phospholipase-C-gamma (PLC- γ) that are common to many growth factor and cytokine receptors (Lev *et al.*, 1991; Rottapel *et al.*, 1994). The activation of PI3K has been linked to mitogenesis (Blume-Jensen *et al.*, 1994; Serve *et al.*, 1995), differentiation (Kubota *et al.*, 1998) and cell adhesion (Serve *et al.*, 1995). PI3K has been shown to support survival and chemotactic responses in other receptor systems (Wennstrom *et al.*, 1994; Yao and Cooper, 1995). Additional signalling molecules that couple to Kit include Vav (Alai *et al.*, 1992), Jak2 (Weiler *et al.*, 1996), Dok (Carpino *et al.*, 1997), Tec (Tang *et al.*, 1994) and the tyrosine phosphatase SHP-1 (Kozlowski *et al.*, 1998). Mutations which uncouple

Kit from the SHP-1 phosphatase renders these receptors hypersensitive to Steel factor stimulation, suggesting that SHP-1 serves to downregulate the activated receptor through dephosphorylation (Kozlowski *et al.*, 1998). The function of Vav, Jak2, Dok and Tec in the context of the Kit signalling network has not been established.

In order to identify other signalling molecules that participate in Kit signal-transduction events in hematopoietic cells, we utilized the yeast two-hybrid system to isolate cDNAs encoding Kit-binding proteins. One of the cDNA clones (clone #99) isolated with this method encodes Socs1/Jab/Ssi-1/Tip3 (Endo *et al.*, 1997; Naka *et al.*, 1997; Ohya *et al.*, 1997; Starr *et al.*, 1997) (hereafter referred to as Socs1 for suppressor of cytokine signaling as this name reflects its most generic function). We show that Socs1 binds to Kit *in vivo* and is induced as an early response gene following Kit activation. Constitutive expression of Socs1 suppressed Kit-mediated proliferation of hematopoietic and fibroblast cells. While Socs1 has recently been reported to suppress cytokine signalling as a Janus kinase pseudosubstrate inhibitor, Socs1 did not inhibit Kit phosphorylation or the phosphorylation of Kit substrates suggesting that suppression of Kit signalling occurred by interference with other signal-transduction pathways. In support of this hypothesis, we provide evidence that Socs1 binds to the negative regulatory N-terminus of the hematopoietic-specific Rac exchange factor, Vav, and to the Src homology 3 (SH3) domains of the SOS adapter molecule, Grb2.

Results

Isolation of Kit-interacting proteins

A yeast two-hybrid assay (Fields and Song, 1989) was used to identify new proteins that interact with the cytoplasmic domain of the murine Kit receptor. A similar approach has been used successfully to identify protein partners for the insulin, Met and colony stimulating factor 1 (CSF-1) RTKs (O'Neill *et al.*, 1994; Weidner *et al.*, 1996; Bourette *et al.*, 1997). For the bait, the entire intracellular kinase domain of Kit was expressed as a fusion protein with the DNA-binding- and the dimerization-domain of LexA. The LexA-Kit bait was used to screen a VP-16 target library derived from the multipotent murine hematopoietic cell line EML-C1, which is capable of cytokine-induced differentiation into erythroid, myeloid and lymphoid lineages (Tsai *et al.*, 1994). EML requires Steel factor to maintain its multipotency and therefore should express the transcripts encoding signalling molecules that participate in Kit-mediated signal-transduction events important in early hematopoiesis. The screening was validated by the isolation of genes encoding proteins known to interact with Kit including p85 α , p85 β , Grb2 and PLC- γ (Lev *et al.*, 1991; Rottapel *et al.*, 1991; Blume-Jensen *et al.*, 1994). In addition to these genes, we identified clone #99, a partial cDNA encoding an SH2-containing protein (Table I). The tyrosine kinase dependence of this interaction was determined by demonstrating that the yeast clone #99 cured of the LexA-wild type Kit bait and mated with AMR70 yeast containing a LexA-kinase dead (D790N) Kit bait no longer interacted. Substitution of Asp790 with Asn within the phosphotransferase domain is derived from the severe *W*⁴² allele and abolishes the catalytic activity

Fig. 1. Schematic representation of the Socs1 protein. The SH2 domain, the diproline (PxxP) motifs and a track of eight serine residues (Poly-Ser) are shown. The partial cDNA obtained from the yeast two-hybrid screen (clone #99), which encompasses the N-terminal sequences and the SH2 domain, is indicated by a horizontal line.

Table I. Proteins which interact with Kit in the yeast two-hybrid system^a

VP-16 partners/bait	LexA-Kit	LexA-KitW42	LexA-lamin
Grb2	++	-	-
PLC γ	++	-	-
p85 α	++	-	-
p85 β	++	-	-
clone #99	++	-	-

^aThe signs plus and minus indicate that an interaction as detected by a β -galactosidase assay was observed (+) or not (-). KitW42 is a kinase dead allele of Kit (mutation D790N).

of Kit (Reith *et al.*, 1990). The specificity of the interaction of clone #99 with Kit was substantiated by demonstrating the absence of β -galactosidase transactivation between VP-16-#99 and LexA-lamin control bait (Table I).

Structure of clone #99 RNA and protein

Using the partial sequence of clone #99 as a probe, we isolated a full-length cDNA clone from an oligo(dT) primed cDNA library derived from the EML-C1 cell line. The largest clone was 1193 base pairs in length and contained an open reading frame of 336 nucleotides that encoded a 212 amino acid polypeptide with a predicted mol. wt of 23.7 kDa. Clone #99 is identical to Socs1 (Endo *et al.*, 1997; Naka *et al.*, 1997; Starr *et al.*, 1997). The Socs1 protein contains an unusual stretch of eight consecutive serine residues in the N-terminus. The N-terminal arm also contains a type I (residues 41–47) and a type II (residues 34–39) diproline motif, the defining determinants for SH3 domain binding (Yu *et al.*, 1994; Sparks *et al.*, 1996) (Figure 1). The C-terminal residues of Socs1 define a region of homology, designated the Socs box (Hilton *et al.*, 1998), the Cis homology (CH) domain (Masuhara *et al.*, 1997), or the SC motif (Minamoto *et al.*, 1997) present in seven other Socs family members and 12 putative open reading frames, suggesting a conserved, but presently unknown, function (Hilton *et al.*, 1998).

Socs1 mRNA is induced as an early response gene by Steel factor

In cultured cells of hematopoietic origin, Socs1 transcripts were abundant in primary bone marrow-derived mast cells and detected at lower levels in EML-C1, Ba/F3, and the

Fig. 2. Induction of Socs1 mRNA transcription by cytokines in hematopoietic cells. Bone marrow-derived mast cells [(A) and (C)] and Ba/F3-Kit cells (B) grown in the presence of media containing IL-3, were deprived of cytokines for 5 h, then stimulated for 1 h with the indicated cytokines or IgE cross-linking antibodies. In (C), factor-deprived mast cells were incubated with serum (lane 2), or Steel factor (SF) (lanes 1, 3, 4 and 5) for 1 h in the absence (lanes 1 and 2) or presence (lanes 3–5) of cycloheximide (CHX). Ten micrograms/lane of total RNA was analysed by Northern blot hybridization. The full-length *Socs1* cDNA probe detected a 1.4 kb transcript. Hybridization to the L32 probe was used to control for RNA loading.

T cell lines EL-4 and VCD28 (not shown). No Socs1 mRNA was detected in adherent rodent fibroblasts. The expression of Socs1 mRNA in mast cells and Ba/F3 cells decreased to background levels within 5 h of interleukin 3 (IL-3) withdrawal. Exposure of growth factor-starved mast cells to Steel factor, IL-3 or IL-4 for 60 min potently induced Socs1 transcripts (Figure 2A). Aggregation of FcεRI, which is expressed at high levels on mast cells, did not affect Socs1 transcript levels. Socs1 mRNA expression was similarly induced in Kit-transfected Ba/F3 cells following Steel factor or IL-3 stimulation (Figure 2B). Induction of Socs1 RNA in both mast cells and Ba/F3-Kit cells was rapid and detected as early as 15 min following Steel factor stimulation (data not shown). The induction of Socs1 mRNA was resistant to the protein synthesis inhibitor cycloheximide indicating that Socs1 is an immediate-early gene induced following Steel factor stimulation (Figure 2C). Our data demonstrate that in addition to members of the hematopoietin receptors including EpoR, granulocyte-macrophage CSF receptor (GM-CSF-R), IL-3R and IL-6R (Naka *et al.*, 1997; Starr *et al.*, 1997), activation of Kit, a member of the RTK family can induce Socs1 mRNA levels.

Kit and Socs1 form an SH2-dependent complex *in vivo*

To determine whether Kit and Socs1 interact in mammalian cells, both proteins were expressed in 293T cells and co-immunoprecipitations were performed. Socs1 was expressed as a fusion protein with the hemagglutinin (HA) epitope transcribed in-frame at its N-terminus and immunoprecipitated with anti-HA mAbs from 293T cell lysates. Protein complexes containing epitope-tagged Socs1 were detected using polyclonal rabbit sera directed against the Socs1 N-terminus including the SH2 domain (Figure 3A, lanes 5, 10 and 11). Kit bound to Socs1 in response to ligand-induced activation of Kit, suggesting that the interaction required phosphorylation of the receptor (Figure 3A, lanes 10 and 11). Similarly, we observed the inducible association of phosphorylated Kit with Socs1 in the IL-3-dependent Ba/F3 cells transfected with both Kit and Socs1 proteins (Figure 3B, lane 3). Mutation of the conserved arginine within the phosphotyrosine-binding pocket of the Socs1 SH2 domain (R105K), known to disrupt phosphotyrosine binding for the Abl and GAP SH2 domains (Marengere and Pawson, 1992; Mayer *et al.*, 1992), abolished the interaction of Socs1 with Kit demonstrating that this complex was dependent on an SH2-phosphotyrosine interaction (Figure 3C, lanes 5 and 6).

Kit kinase activity is not suppressed by Socs1

Socs1 has recently been reported to suppress the kinase activity of Janus kinase family members and the Btk/Itk kinase family member Tec in 293 cells (Endo *et al.*, 1997; Naka *et al.*, 1997; Ohya *et al.*, 1997). To establish whether Socs1 functions as an inhibitor of the Kit protein tyrosine kinase, we measured the phosphotyrosine content of Kit following Steel factor stimulation in 293T cells expressing Kit alone, or Kit with Socs1. Kit was immunoprecipitated from unstimulated or stimulated 293T cells and blotted with antiphosphotyrosine antibodies. The coexpression of Socs1 did not prevent the induction of Kit phosphorylation (Figure 3D, lanes 1 and 3). Moreover, we examined the phosphorylated state of the fraction of Kit bound to Socs1 and detected high levels of phosphorylated Kit complexed to Socs1 (Figure 3E, lanes 5 and 6). The phosphorylation of Vav, a substrate of Kit (Alai *et al.*, 1992), was similarly undiminished by Socs1 expression (Figure 3D, lanes 5 and 6). Phosphorylated Vav was observed in Socs1 immune complexes, suggesting a direct or indirect interaction between Socs1 and Vav (Figure 3D, lane 9). These results were recapitulated in Ba/F3 cells stably expressing both Kit and Socs1 (Figure 3F). These data suggest that Socs1 does not inhibit the Kit catalytic activity nor impair the phosphorylation of the Kit substrate Vav.

Socs1 binds to several members of the receptor tyrosine kinase family

We tested the capacity of Socs1 to bind to other receptor tyrosine kinase family members in a yeast two-hybrid experiment. Yeast L40 clones containing VP-16 #99 were mated with yeast AMR70 clones containing LexA alone, LexA-lamin or LexA fused with the kinase domains of Flt3, PDGF-R, CSF1-R or Tek. As a negative control each of the AMR70 clones was mated with L40 yeast containing VP-16 alone. These clones were sequentially tested for

Fig. 3. Socs1 inducibly binds to Kit *in vivo*. (A) Lysates from 293T cells transfected with Kit (lanes 1–4), Socs1 (lane 5), or both Kit and Socs1 (lanes 6–11) were used for immunoprecipitation of Kit (lanes 3, 4, 6 and 7); Socs1 via its HA tag (lanes 1, 2, 5, 10 and 11) or isotype control antibodies (lanes 8 and 9). The upper panel was blotted with anti-Kit antibodies while the lower panel was blotted with anti-Socs1 immune serum. Cells were stimulated (+) or not (–) with Steel factor for 5 min. Lanes 6, 8, 10 and 7, 9, 11 were derived from the same transfected 293T cells. (B) Socs1 and Kit interaction in Ba/F3 cells. Ba/F3–Kit cells stably expressing an HA epitope-tagged Socs1 or transfected with the control vector were serum starved for 5 h and were stimulated (+) or not (–) with Steel factor for 2 min. In the top panel, lysates were used for immunoprecipitation of HA-Socs1 (lanes 2–5). The lower panels show the expression levels of Kit and Socs1 in the lysates. (C) Socs1 SH2 domain is required for binding to Kit. Lysates from 293T cells transfected with Kit (K) (lanes 1 and 2), Kit and Socs1 (S) (lane 3 and 4), or Kit and Socs1 mutated at R105 in the phosphotyrosine-binding pocket of the SH2 domain (S*) (lanes 5 and 6) were used for immunoprecipitation of Socs1 (HA) and blotted with either anti-Kit antibodies (upper panel) or anti-Socs1 antibodies (lower panels). (D) Socs1 does not impair the phosphorylation of the Kit substrate Vav. Cells transfected with Kit (lanes 1–9), Vav (lanes 2, 3, 5, 6, 8 and 9) and Socs1 (lanes 3, 6 and 9) were stimulated with Steel factor for 5 min. Lysates were used to immunoprecipitate Kit (lanes 1–3), Vav (lanes 4–6) or Socs1 (lanes 7–9), and blotted with the antiphosphotyrosine antibody 4G10 (upper panel). Samples of the same lysates were used in lanes 1, 4, 7, lanes 2, 5, 8 and lanes 3, 6, 9, respectively. Controls for Kit, Vav and Socs1 protein expression are shown in the lower panel. (E) Socs1 does not suppress ligand-induced Kit phosphorylation. Kit and Socs1 plasmids were co-transfected into 293T cells and stimulated (+) or not (–) with Steel factor. Protein from lysates were immunoprecipitated by Kit- (lanes 1 and 2) or Socs1- (lanes 5 and 6) specific antibodies or isotype control mAbs (lanes 3 and 4) and immunoblotted with antiphosphotyrosine antibodies. (F) Socs1 does not inhibit the phosphorylation of Vav in Ba/F3–Kit cells. Ba/F3–Kit cells stably expressing an HA epitope-tagged Socs1 or transfected with the control vector were serum starved for 5 h and were stimulated (+) or not (–) with Steel factor for 2 min. The levels of Vav phosphorylation and expression are shown in the two top panels. The bottom panels show the phosphorylation and expression levels of Kit and Socs1 expression.

their capacity to transactivate the LacZ reporter gene. All four RTKs interacted with VP-16 Socs1 but not with VP-16 alone (data not shown).

Flt3 is a RTK structurally related to Kit that is expressed in early hematopoietic multipotent progenitors and in

lymphoid progenitors. The Flt3 ligand supports the survival and differentiation of early B cell progenitors (Ray *et al.*, 1996). We verified the capacity of Flt3 and Socs1 to form an inducible and stable protein complex *in vivo* in a transfection assay. Flt3 was transiently expressed in 293T

Fig. 4. Socs1 inducibly binds to Flt3 *in vivo*. Lysates of cells expressing a Fms-Flt3 chimeric receptor (FF3) and HA-Socs1 were subjected to immunoprecipitation using either anti-Flt3 rabbit polyclonal serum, the anti-HA mAb 12CA5 or a 12CA5 isotype control (IgG2b). Protein complexes were probed with anti-Flt3 antibodies (upper panel) or Socs1 antibodies (lower panels). Cells were treated (+) or not (-) with CSF-1 (700 ng/ml) for 5 min. An equal amount of lysate derived from the same transfection was used in the '-' and the '+' lanes, respectively.

cells in the presence or absence of ectopically expressed Socs1. Socs1 was immunoprecipitated using HA mAbs from serum-starved or Flt3-activated cells and the immune complexes were blotted with anti-Flt3-specific antibodies. Flt3 co-precipitated with Socs1 following Flt3 activation (Figure 4, lane 6).

Socs1 binds to the guanine nucleotide exchange factors Vav and Vav2

In order to identify downstream signalling pathways which are coupled to Kit by Socs1, we performed a yeast two-hybrid screen using the full-length Socs1 as a bait against the EML-C1 cDNA library. One of the interacting clones was identified as the Rho-family GTPase exchange factor (GEF) Vav. The capacity of Socs1 to bind to Vav in mammalian cells was verified by co-transfection of plasmids encoding both Socs1 and Vav into 293T cells (Figure 5A). The cells were lysed and Socs1 protein immunoprecipitated. Proteins present in the Socs1 immune complex were separated by SDS-PAGE and immunoblotted for Vav. Vav formed a stable complex with both Socs1 and with Socs1 harbouring a mutation in the phosphotyrosine-binding pocket of the SH2 domain (R105K) (Figure 5A, lanes 3 and 5). The VP-16-Vav fusion protein identified in the Socs1 yeast two-hybrid screen encompasses the leucine-rich and an acidic region in the Vav N-terminus. This region of Vav is highly conserved (>80% similarity) with sequences present in the N-terminus of the recently identified second Vav family member Vav2 (Henske *et al.*, 1995; Schuebel *et al.*, 1996). We tested the capacity of Vav2 to bind to Socs1 by co-immunoprecipitation. As demonstrated in Figure 5B Socs1 and Vav2 formed a protein complex in 293T cells consistent with the notion that conserved residues present in the N-termini of both Vav and Vav2 contain the determinants necessary for Socs1 binding.

Socs1 binds to SH3-containing proteins

Socs1 protein contains a class I and a class II diproline motif in its N-terminal arm. We tested whether SH3 domains of known signalling proteins could bind to Socs1 *in vitro*. Immobilized glutathione *S*-transferase (GST)-SH3 domain fusion proteins were mixed with lysates from

Fig. 5. Socs1 constitutively binds to Vav and Vav2 *in vivo*. (A) Lysates from 293T cells transfected with Vav (lanes 1, 3, 5 and 6) and Socs1 (lanes 2, 3 and 6) or Socs1 with a mutation in the phosphotyrosine-binding pocket of the SH2 domain (lanes 4 and 5) were used to immunoprecipitate Socs1 (upper panel). Immune complexes were probed with anti-Vav antibodies. The lower panels show Vav and Socs1 protein expression, respectively. (B) Lysates from 293T cells transfected with Vav2 (lanes 1, 3, 4 and 5) and Socs1 (lanes 2, 3 and 5) or Socs1 with a mutant SH2 domain (lane 4) were used to immunoprecipitate Socs1 (upper panel). Immune complexes were probed with anti-Vav2 antibodies. Lower panels show Socs1 and Vav2 protein expression, respectively. NIS, non-immune serum.

293T cells expressing epitope-tagged Socs1. The protein complexes were washed and separated by SDS-PAGE then blotted with HA antibodies. Socs1 co-precipitated with the SH3 domains of linker-type molecules such as Grb2, p85 and Nck, but not with the proline-binding WW domains derived from Yap and dystrophin. The SH3 domains from the tyrosine kinases Itk and Fyn also interacted with Socs1 but not those derived from Abl, Src, Lck, Fgr, Hs1, PLCγ, Vav, Gap and spectrin (Figure 6A).

Molecular characterization of mutant alleles of the *Caenorhabditis elegans* Grb2 homologue *sem5* have identified single point mutations that diminish the binding capacity of the SH3 domains to their target proteins (Clark *et al.*, 1992; Rozakis-Adcock *et al.*, 1993). We used these loss-of-function mutations defined in the SH3 domains of Sem5 to verify the domain requirements for the Grb2-Socs1 interaction *in vivo*. Myc-tagged wild-type Grb2 or Grb2 variants carrying mutations in either the N-terminal (P49L) or C-terminal (G203R) SH3 domains, or an internal deletion within the SH2 domain (Grb3-3), were co-transfected with Socs1 in 293T cells. Grb2 transfected protein carrying a loss-of-function mutation in both the N- and C-terminal SH3 domains abrogated Socs1 binding, while mutating either the N-terminal or C-terminal SH3 domain severely reduced binding suggesting that both SH3 domains are required for optimal binding of Grb2 to Socs1 (Figure 6B). Grb3-3, a Grb2 isoform with a nonfunctional SH2 domain (Fath *et al.*, 1994), bound to Socs1 at levels comparable to wild-type Grb2. These

Fig. 6. Socs1 is an SH3-binding protein *in vitro*. (A) Lysates from 293T cells transfected with HA-Socs1 were incubated with the SH3 domains derived from 14 signalling proteins, the WW domains of Yap and dystrophin, the full-length Grb2 and the SH2 domain of p85 expressed as GST-fusion proteins immobilized on glutathione-Sepharose beads. Co-precipitating protein complexes were washed and probed with HA mAbs to detect Socs1. Immobilized recombinant GST was used as a negative control. The electrophoretic mobility of Socs1 is shown in the lysate lane. (B) Grb2 binds to Socs1 via its SH3 domains *in vivo*. Upper panel, plasmids encoding Grb2 constructs: wild-type Grb2, Grb2 carrying loss-of-function mutations in both SH3 domains or in either the C- or N-terminal SH3 domains, or Grb3-3 lacking a functional SH2 domain, were co-expressed with Socs1 in 293T cells. Socs1 was immunoprecipitated from cellular lysates and the immune complexes resolved by SDS-PAGE were immunoblotted for Grb2. Middle and lower panels, Socs1 and Grb2 protein expression in the lysates used in the upper panel are shown by immunoblotting with the respective antibodies.

results show that Socs1 can bind to Grb2 *in vivo* via the Grb2 SH3 domains and that this interaction is independent of the Grb2 SH2 domain.

Socs1 is a negative regulator of Kit and Flt3 mitogenic signals

To investigate the function of Socs1 in the context of Kit-dependent signalling, Socs1 was transfected into two different hematopoietic cell lines whose growth is dependent on Kit activation, EML-C1 and Ba/F3–KitΔ27. Socs1 was expressed using the bi-cistronic retroviral vector pMiev (Cheng *et al.*, 1997). The pMiev-Socs1 plasmid contains an internal ribosome entry site (IRES) inserted between cDNA encoding Socs1 and the enhanced green fluorescent protein (EGFP). As a result, cells infected with

pMiev-Socs1 express a single mRNA species encoding both the Socs1 and the EGFP proteins. Cells expressing EGFP and hence Socs1 were readily identified and purified by fluorescence-activated cell sorting (FACS).

EML-C1 cells express Kit endogenously, require Steel factor for the maintenance of their multipotency and die within 24 h following Steel factor privation. A variant Ba/F3 cell line harbouring an oncogenic form of the Kit receptor (Ba/F3–KitΔ27) rendering them growth factor-independent (N.Casteran, R.Rottapel and P.Dubreuil, unpublished). Both cell lines were infected either with pMiev or with the pMiev-Socs1 retrovirus. Low, intermediate and high GFP expressing cells were isolated by flow-cytometry cell sorting (Figure 7A) and Socs1 protein levels in each of the populations were determined by Western blot analysis of cellular lysates (Figure 7B). Ba/F3–KitΔ27 cells infected with either the empty vector or cells expressing intermediate or high levels of Socs1 were plated at an initial density of 40 000 cells/ml and grown under normal conditions for 7 days. Over the course of the 7-day assay, the number of control Ba/F3–KitΔ27 cells increased three times faster than the cells expressing intermediate levels of Socs1. Ba/F3–KitΔ27 cells that expressed high levels of Socs1 failed to proliferate during the 7-day assay (Figure 7C), although these cells remained viable as measured by the exclusion of the vital-stain Trypan blue. The capacity of Socs1 to suppress Steel factor-dependent proliferation in EML-C1 cells was similar to the results observed with Ba/F3–KitΔ27 (Figure 7C). The fraction of viable EML-C1 cells expressing Socs1 was quantified by staining with Annexin V and 7-amino-actinomycin D (7AAD) (Figure 8). EML-C1 cells grown in Steel factor contained ~20% of apoptotic and dead cells 48 h after sorting, whereas EML-C1 deprived of Steel factor during the same period had uniformly died. Although EML-C1 cells expressing Socs1 showed no increase in their numbers, 60% were viable at 48 h and ~50% were viable at 64 h (Table II).

Both Kit and Flt3 have been shown to support anchorage-independent growth in rodent fibroblasts (Lev *et al.*, 1990; Maroc *et al.*, 1993; Caruana *et al.*, 1998). We wished to determine whether the mitogenic potential of Kit and Flt3 in fibroblasts was suppressed by the expression of Socs1 as measured by colony formation in soft agar. A population of NIH 3T3 cells that stably express Kit (PK9) and a Rat2 cell population expressing a chimeric Fms/Flt3 receptor (Raff8) both demonstrated ligand-dependent colony formation in the presence of their cognate ligands after 14 days of culture (Figure 9, left-hand panels). PK9 and Raff8 cells were secondarily infected with the pMiev-Socs1 retrovirus to achieve high Socs1 expression. The expression of Socs1 did not affect the morphology of the cells and did not affect monolayer proliferation under growth conditions of 10, 5, 1 or 0.5% fetal calf serum (FCS) compared with pMiev infected controls and was not therefore generally toxic to the cells. However, the constitutive expression of Socs1 severely reduced both the number and the size of the colonies in response to either Kit or Flt3 activation (Figure 9, right-hand panels). Therefore, Socs1 suppresses the transformed phenotype induced by the activation of the Kit and Flt3 receptors in fibroblasts. These data, together with those

Fig. 7. Constitutive expression of Socs1 in hematopoietic cells suppresses Kit-mediated mitogenesis. **(A)** EML-C1 (left panel) and Ba/F3Δ27 (right panel) cells were infected with the pMiev-Socs-GFP retrovirus expressing both Socs1 and the GFP protein from a single bicistronic mRNA species. The infected cells were then sorted for low (5), intermediate (3) or high (4) expression of GFP. Cells infected with the pMiev vector alone (2) were sorted for high GFP expression as controls. **(B)** Socs1 protein expression in the sorted populations of EML-C1 (left panels) and Ba/F3Δ27 (right panels). Lysates obtained from the sorted populations shown in (A) were immunoblotted with either Socs1 (upper panel) or β -actin (lower panel) specific antibodies. Lane 1, non-transfected cells; lane 2, cells infected with the pMiev vector alone and sorted for high GFP expression; lanes 3, 4 and 5 represent cells transfected with pMiev-Socs1 and sorted for intermediate, high or low GFP expression respectively. **(C)** EML-C1 (left panel) or Ba/F3Δ27 (right panel) cells which express intermediate (population 3), or high (population 4) levels of Socs1 shown in (A) were plated in triplicate at a density of 40×10^3 cells/ml. Cells infected with the empty vector (vector high) were used as controls. Cells excluding the vital-stain Trypan blue were counted on days 3, 5, 6 and 7.

Fig. 8. Detection of apoptotic and dead cells in EML-C1 cells. EML-C1 cells grown with Steel factor (top left), EML-C1 grown without Steel factor (top right), EML-C1 cells grown with Steel factor and infected with the Miev vector (bottom left) or EML-C1 cells grown with Steel factor and infected with Miev-Socs1 (bottom right) were stained 48 h after they had been sorted for GFP expression. Annexin V and 7-amino-actinomycin D were used to detect apoptotic and dead cells.

described above, suggest that Socs1 may function as an inducible 'switch' which attenuates Kit-dependent mitogenic signals while maintaining cell survival signals.

Table II. Percentage of viable cells (Annexin⁻, 7-AAD⁻) in EML-C1 cultures

EML-C1 culture	Time post sort ^a (h)			
	0	24	48	64
+ Steel factor	87	81	77	75
- Steel factor	85	19	1	1
Miev + Steel factor ^a	77	75	77	79
Miev-Socs1 + Steel factor ^a	86	58	60	47

^aEML-C1 infected with the Miev or Miev-Socs1 retroviruses were sorted by flow cytometry for high GFP expression as outlined in Figure 7. AnnexinV and 7-amino-actinomycin D (7AAD) were used to determine the percentage of viable cells.

Discussion

In this report we describe the molecular interactions and the function of a new Kit-binding protein, Socs1, cloned from the Steel factor-dependent multipotent hematopoietic cell line EML-C1. We have shown that: (i) Socs1 is expressed in cell lineages which express Kit (i.e. cells of hematopoietic origin); (ii) the expression of Socs1 message is regulated as an early response gene by Steel factor in primary bone marrow-derived mast cells and Kit-transfected Ba/F3 cells; (iii) Socs1 binds to Kit via its SH2 domain in a phosphotyrosine-dependent manner; (iv) the forced expression of Socs1 in hematopoietic cell lines whose growth depend on Kit activation suppresses mitogenesis responses while maintaining cell survival signals; and (iv) Socs1 is an adaptor-type molecule that binds to Grb2 and Vav.

Socs1 binds to signalling proteins

The Socs1 N-terminus contains both class I and II SH3-binding determinants and is a functional SH3-binding protein. Specifically, Grb2 forms a constitutive complex

Fig. 9. Socs1 suppresses anchorage-independent growth of fibroblasts. Ligand-induced colony formation of fibroblasts that express Kit (PK9) (upper panel) or a Fms-Flt3 chimeric receptor (RAFF8) (lower panel) infected either with pMiev or Miev-Socs1 retrovirus. Cells sorted for high GFP expression were plated at a density of 10^6 cells/ml in the presence or absence of growth factors (250 ng/ml Steel factor or 100 ng/ml CSF-1) in 0.36% agar. Colonies were photographed after 3 weeks for PK9 and after 2 weeks for RAFF8 following the initiation of cultures.

with Socs1 via its SH3 domains. In order to identify other signalling pathways with which Socs1 interacts, we subjected Socs1 to a yeast two-hybrid screen and showed that Socs1 binds to the hematopoietic-specific GEF Vav. Vav is rapidly phosphorylated on tyrosine in response to ligation of a wide variety of antigen and cytokine receptors (Bustelo and Barbacid, 1992; Bustelo *et al.*, 1992; Margolis *et al.*, 1992; August *et al.*, 1994; Weng *et al.*, 1994) including Kit (Alai *et al.*, 1992). The exchange activity of Vav is positively regulated by tyrosine phosphorylation (Crespo *et al.*, 1996) suggesting that Vav may be a positive effector of Kit function in hematopoietic cells. Mice deficient in Vav demonstrate abnormal thymocyte development and attenuated T cell receptor (TCR) activation in peripheral lymphocytes (Fischer *et al.*, 1995; Tarakhovsky *et al.*, 1995; Zhang *et al.*, 1995). Vav has recently been shown to coordinate lymphocyte cytoskeletal reorganization with cell-cycle progression (Fischer *et al.*, 1998; Holsinger *et al.*, 1998) which is consistent with its role as a Rac1 GEF. Deletion of N-terminal sequences within the leucine rich calponin-like domain (Castresana and Saraste, 1995) renders Vav-transforming in NIH 3T3 cells (Katzav *et al.*, 1989), suggesting that this region may negatively regulate the exchange activity of the molecule or direct Vav to specific subcellular locations. We have cloned Vav as a Socs1-binding protein using the yeast two-hybrid technique and have demonstrated that Socs1

binds to Vav in mammalian cells. The binding site of Socs1 is contained within the N-terminal region of Vav encompassed by amino acids 1–199, a region of the protein that is highly conserved with the second Vav family member Vav2. The N-termini of Vav and Vav2 may be functionally conserved since Socs1 bound to both molecules. The interaction between Socs1 and Vav did not require a functional phosphotyrosine-binding pocket in Socs1 SH2 domain or the Vav SH3 domains. We are currently searching for the minimal Socs1-binding determinant sequence of Vav by deletion mutagenesis and are determining whether Socs1 modulates the exchange activity of Vav.

The Rac and Rho small GTP-binding proteins regulate the formation of specific cytoskeletal structures. Rac induces lamellipodia formation and membrane ruffling (Ridley *et al.*, 1992), while Rho stimulates the formation of actin stress fibres and focal adhesion plaques (Ridley and Hall, 1992). Steel factor induces mast cell chemotaxis (Meininger *et al.*, 1992) and adhesion to fibronectin matrix (Serve *et al.*, 1995), and enhances degranulation (Bischoff and Dahinden, 1992; Vosseller *et al.*, 1997), all of which requires reorganization of cytoskeletal structures. Ligand-induced activation of Kit stimulates cytoskeletal responses including membrane ruffling and stress-fibre formation (Blume-Jensen *et al.*, 1991; Vosseller *et al.*, 1997) suggesting a role for Rac and Rho in Kit signalling pathways. We are currently examining the capacity of Socs1 to regulate Kit-induced cytoskeletal changes in cells of hematopoietic origin.

Socs1 is a inducible switch suppressing Steel factor-mediated proliferation while maintaining Steel factor-dependent cell survival

We have found that the forced expression of Socs1 in either the EML-C1 or BaF/3–Kit Δ 27 cell lines attenuated the proliferative response of these cells to Kit activation while maintaining factor-dependent cell survival. Survival in the absence of proliferation may favour differentiation events to occur (Fairbairn *et al.*, 1993). We are currently investigating whether expression of Socs1 in EML-C1 cells induces differentiation followed by cell death as an explanation of the elevated incidence of apoptosis observed in these cells. These results should be compared with those reported by Starr and Naka (Starr *et al.*, 1997; Naka *et al.*, 1997). The myelomonocytic leukemic cell line M1 differentiates into mature macrophages, ceases to proliferate and apoptoses in response to IL-6 or LIF. Expression of Socs1 antagonized the effects of IL-6 or LIF by blocking differentiation and promoting DNA synthesis in response to these cytokines. Therefore Socs1 appears to function as a signalling switch, which, depending on the cell line and the cytokine, shifts the response to cytokines either towards proliferation or towards cell survival.

The growth-suppressive action of Socs1 on Kit-mediated signalling might result from the capacity of Socs1 to inhibit the kinase activity of Jak2, a substrate of Kit. In support of this idea, Kit and Jak2 form a constitutive complex and Steel factor stimulates the rapid phosphorylation of Jak2 (Weiler *et al.*, 1996). Treatment of FDCP-1 cells with Jak2 antisense oligonucleotides results in a partial impairment in Steel factor-dependent proliferation

(Weiler *et al.*, 1996) and the capacity of Steel factor to support CFU-mix colony formation in fetal liver hematopoietic progenitors derived from Jak2-deficient mice is reduced by ~60% compared with normal controls (Parganas *et al.*, 1998). These data suggest that inhibition of Jak2 would not fully explain the complete block in Kit-mediated proliferation of EML-C1 and Ba/F3–Kit Δ 27 cells expressing Socs1 that we have observed. We tested for the possibility that Socs1 may function as an inhibitor of Kit kinase activity and found that coexpression of Socs1 with Kit did not impair ligand-induced phosphorylation of the receptor or the capacity of Kit substrates such as Vav for becoming phosphorylated. We propose that Socs1 may interrupt other signal-transduction pathways in addition to those lying downstream of the Janus kinase family and conjecture that the interaction of Socs1 with Grb2 and Vav may mediate some of its growth-suppressive effects.

Socs1 may perform an important function for Steel factor-mediated signalling in hematopoietic progenitor cells. Kit is expressed on hematopoietic precursor cells which at high frequency are capable of long-term reconstitution of irradiated recipient mice at high frequency (Okada *et al.*, 1991; Ikuta and Weissman, 1992; Orlic *et al.*, 1993). Although Steel factor alone provides a weak proliferative stimulus for hematopoietic progenitors, it promotes the survival and self-renewal of hematopoietic stem cells with long-term repopulating ability *in vitro* in the absence of cell division (Li and Johnson, 1994; Keller *et al.*, 1995). The combination of Steel factor plus IL-11 retains long-term repopulating activity that can be serially transplanted up to quaternary recipients (Holyoake *et al.*, 1996). Sustained stem cell self-renewal *in vivo* is also dependent on Steel factor (Miller *et al.*, 1997). In combination with other growth factors such as G-CSF, GM-CSF, IL-3, IL-7, IL-11 and IL-12, however, Steel factor is a potent mitogen (McNiece *et al.*, 1991a,b; Ulich *et al.*, 1991; Pietsch *et al.*, 1992; Tsuji *et al.*, 1992; Ploemacher *et al.*, 1993) and in combination with other hemopoietins increases the frequency of erythroid, myeloid and lymphoid progenitor cells. The signalling pathways that define Kit-dependent survival responses versus those that support proliferation are presently unknown. Steel factor-dependent induction of Socs1, however, may function to modulate Kit signals that mediate cell survival and thus co-ordinate the processes of self-renewal and lineage commitment in hematopoiesis.

Materials and methods

Cells and culture conditions

Bone marrow-derived mast cells were cultured as outlined (Reith *et al.*, 1990) and were grown in OPTI-modified Eagle's medium (MEM), 10% fetal bovine serum (FBS) and 0.5% of conditioned media from X63-IL-3 cells containing IL-3 (Karasuyama and Melchers, 1988). EML-C1 were a gift of S.Tsai and were grown in Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% horse serum and 15% conditioned media from BHK/mKL containing Kit-ligand (gift from S.Tsai). Ba/F3 cells were grown in Roswell Park Memorial Institute (RPMI) media 10% FBS and 2% of X63-IL-3 conditioned media containing IL-3. Ba/F3–Kit Δ 27 are Ba/F3 cells rendered IL-3-growth-independent and are transfected with a mutant form of Kit which is constitutively active as a result of a deletion in the juxtamembrane domain of Kit (N.Casteran, R.Rottapel and P.Dubreuil, unpublished). Ba/F3–Kit Δ 27 cells were grown in RPMI and 5% FBS. 293T and GP+E-86 cells were grown in DMEM and 5% FBS. All media and sera were purchased from Gibco-BRL.

Plasmids

pBTM116-Kit: the mouse cDNA encoding the Kit intracellular domain (nucleotides 1646–2988) was amplified by PCR and cloned at the *Eco*RI site in the vector pBTM116 in-frame with LexA. The same strategy was used to clone the W⁴² Kit allele. pBTM116-Socs1: the Socs1 cDNA from pSK-Socs1 was cloned at the *Sal*I site of pBTM116Src in-frame with LexA. Similarly, cDNA encoding full-length Socs1, the N-terminal domain corresponding to amino acids 1–79 and the C-terminal domain corresponding to amino acids 168–212 of Socs1 were cloned in the *Sal*I site of pBTM116. Mammalian expression vectors were as follows. pMT3-Socs1: the coding sequence of Socs1 from pSK-Socs1 was cloned at the *Eco*RI site of pMT3 in-frame with the HA epitope tag. pMT3-Socs1-SH2* carries the Socs1 cDNA with a mutation in codon 105 (CGC changed to AAG) generated by overlapping PCR. pMiev-Socs1: the HA-Socs1 DNA fragment was amplified from pMT3-Socs1 and subcloned in the *Xho*I and *Sal*I sites of pMiev vector. pMT3-Vav2: human Vav2 cDNA (a gift from D.J.Kwiatkowski) was cloned in pMT3 in-frame with the HA coding sequence. pCMV-Vav: murine Vav cDNA (a gift from J.Penniger) was cloned in the pFLAG-CMV-5 vector (Kodak). Grb2 expression vectors (Kavanaugh *et al.*, 1996), pECE-Kit (Rottapel *et al.*, 1991) and pECE-Flt3 (Maroc *et al.*, 1993) are described elsewhere. The pLexa-RTKs plasmids were a gift from L.Rohrschneider.

Yeast two-hybrid screen

Plasmids, *Saccharomyces cerevisiae* strains, selective media and the transformation protocol have been described previously (Vojtek and Hollenberg, 1995). The murine Kit intracellular domain nucleotides 1657–2988, corresponding to amino acids 544–975 was inserted into the pBTM116 vector, resulting in LexA–Kit fusion protein. The *S.cerevisiae* L40 strain was transformed and selected in medium lacking uracil and tryptophan. A colony containing the LexA–Kit bait was then transformed with the VP-16 cDNA library derived from the multipotential hematopoietic cell line EML (Tsai *et al.*, 1994; Lioubin *et al.*, 1996). Five million transfected clones were screened for colonies containing a VP-16 fusion protein interacting with the bait on the basis of transactivation of the *His3* reporter gene after 3 days growth on medium lacking uracil, tryptophan, leucine, lysine and histidine. Candidate clones were then screened for β -galactosidase activity using a colorimetric assay. The positive yeast clones were then cured of the bait plasmid and mated with AMR70 yeast expressing either LexA–Kit (the bait), a kinase-inactive mutant of Kit (LexA–Kit⁴²), or LexA–lamin. Clones which interacted with Kit, but not Kit⁴² or lamin were further analysed. Purified DNA isolated from positive clones was sequenced and compared with DNA databases using the BLAST program at the National Center for Biotechnology Information (NCBI).

The same procedure was used for the yeast two-hybrid screen of Socs1. The full-length Socs1 cDNA was inserted into the pBTM116 vector which contained a constitutively active form of the Src tyrosine kinase cloned into the *Pvu*II site of pBTM116. Colonies expressing a VP-16 fusion protein that interact with the Socs1 bait in a phosphotyrosine-independent manner were cured of the pBTM116-Src-Socs1 plasmid and mated with AMR70 containing pBTM116-Socs1 plasmid.

Isolation of full-length Socs1 cDNA

A λ phage library (λ ZAPII vector, Stratagene) containing cDNAs obtained by oligo(dT) priming of mRNAs expressed in EML-C1 cells was screened using clone #99 as a probe. After the second round of screening, phagemids (pBluescript SK plasmids) were obtained from the positive phages and sequenced. Two independent cDNA clones (pSK-Socs1) were sequenced. Both clones started at the same nucleotide but had a different poly(A) tail. The sequence of pSK-Socs1 has been deposited in the DDBJ/EMBL/GenBank database (accession No. AF120490).

Northern blot analysis

Total RNAs from mouse tissues or cell lines were isolated by the Trizol procedure (Gibco-BRL). Fifteen micrograms of total RNA from tissues or 10 μ g of total RNA from cell lines were electrophoresed on formaldehyde-agarose gels and transferred to Hybond N+ (Amersham-Pharmacia Biotech) membranes. The full-length Socs1 cDNA and the L32 ribosomal gene were used as probes. The probes were labelled by random priming and the hybridizations were performed at 65°C. Membranes were washed in 0.2 $\times$ SSC, 0.1% SDS at 65°C. In some experiments, cells were stimulated with Steel factor (supernatant at 1:50), IL-3 (supernatant at 1:200) or IL-4 (supernatant at 1:1000) (Karasuyama and Melchers, 1988) for 1 h before RNA extraction. For IgE-receptor stimulation, a monoclonal IgE anti-di-nitrophenol (DNP)

(Sigma) was incubated with the cells at 10 µg/ml at 4°C for 30 min and crosslinking was achieved by adding DNP coupled to human serum albumin (Sigma) at 10 ng/ml. Cycloheximide (Sigma) at indicated concentrations was added 30 min before and during Steel factor stimulation.

Antibodies

Rabbit polyclonal serum against Socs1 was generated by using the first 173 amino acids of Socs1 in fusion with GST as an immunogen. Kit and Flt3 rabbit polyclonal sera were raised against a GST fusion protein containing the kinase inserts of each protein, respectively. Polyclonal serum against Vav was generously supplied by T.Mak and the Vav2 rabbit polyclonal serum was provided by J.Downward. The anti-HA mAb12CA5 was purified from hybridoma supernatant. Purified IgG2b was used as a control for the 12CA5 mAb. Monoclonal antiphosphotyrosine antibodies (4G10) was purchased from Upstate Biotechnology. Protein A-horse-radish peroxidase (HRP) (ICN) and goat anti-mouse IgG-HRP (Jackson Immunoresearch Laboratories) were used as secondary antibodies for Western blots.

Transfection of 293T cells

293T cells were transfected by the calcium-phosphate method as described in Maniatis *et al.* (1989). Two million cells seeded in a 100 mm tissue culture dish (Sarstedt Inc.) were transfected with 20 µg of plasmid DNA (either 10 µg of each of two expression vectors or 10 µg of one expression vector plus 10 µg of carrier DNA). Cells were lysed 36–48 h after transfection.

Immunoprecipitation and immunoblotting

Cells were starved for 18 h and then stimulated in the presence or absence of Steel factor (supernatant from CHO-KLS-C used at 1:50; gift from D.Donaldson, Genetic Institute) or CSF-1 (700 ng/ml). Stimulated cells were washed in phosphate-buffered saline (PBS) prior to lysis in ice-cold lysis buffer containing 50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM PMSF and 2 mM Na₂VO₄. Equalized whole-cell lysates were mixed for 18 h with various antibodies as specified in the text and protein A coupled to agarose beads (Amersham-Pharmacia Biotech) for immunoprecipitation. Proteins from whole-cell lysates and immunoprecipitates were separated by SDS-PAGE, then transferred to Immobilon membranes (Millipore) and blotted with various antibodies as specified in the text. An HRP-conjugated secondary antibody was detected using the enhanced chemiluminescence (ECL) (Amersham-Pharmacia Biotech).

Binding assays using GST-SH3 domain fusion proteins

GST-SH3 fusion proteins derived from Crk, Nck, p85, Abl, Src, Lck, Fyn, Itk, Fgr, Hs1, PLCγ, Vav, Gap and spectrin α were expressed in *Escherichia coli* BL21(DE3) (Stratagene) from pGEX vectors (Amersham-Pharmacia Biotech). GST fusion proteins of Grb2, p85 SH2 and the WW domains from Yap (a gift from M.Sudol) and dystrophin (a gift from D.Rotin) were similarly expressed. Bacterial cultures were grown to log phase, induced by 0.5 mM isopropyl-β-D-galactopyranoside (IPTG) (Gibco-BRL) and incubated for a further 4 h at 28°C. Bacteria were then lysed in lysis buffer and the GST fusion proteins were purified on glutathione-Sepharose beads (Amersham-Pharmacia Biotech). The amount of proteins was estimated on Coomassie-stained SDS-PAGE gels. Ten micrograms of each immobilized GST fusion proteins were mixed with 293T cell lysates expressing Socs1 and incubated for 2 h at 4°C. The beads were washed three times with 1 ml lysis buffer, and bound proteins were separated on a 10% SDS-polyacrylamide gel, transferred to Immobilon and blotted with 12CA5 anti-Ha mAbs.

Infection of cells with retroviral vectors

Packaging GP+E-86 cells were transfected by the calcium phosphate method with pMiev-Socs1 or pMiev and were sorted for expression of GFP 1 week post-transfection. These two populations of cells were used thereafter as a source of retroviruses. Infections were done by co-culture in 60 mm plates using 2×10⁶ mitomycin-treated GP+E cells and either 2×10⁶ of Ba/F3-Kit, 2×10⁶ of Ba/F3D27 or 7.5×10⁵ EML-C1 cells in 3 ml of culture media (RPMI and 5% FCS or IMDM, 20% horse serum and 15% conditioned media containing Kit-ligand) containing 4 ng/ml of polybrene. Non adherent cells were collected 48 h later and incubated in media for 24 h. Cells were then sorted for high or low expression of GFP using a cell sorter. Cells at the same concentration were plated in triplicates and aliquots counted every day or every other day for growth

curves. AnnexinV-PE and 7-amino-actinomycin D (PharMingen) staining were done according to the manufacturer's instructions.

Soft agar colony assays

Thirty-thousand Rat2 or NIH 3T3 cells, or their derivatives, were seeded into 1 ml of an upper layer containing Dulbecco's modified Eagle's medium (DMEM) 0.36% bacto-agar (Gibco-BRL) over a bottom layer containing DMEM and 0.6% bacto-agar in 6-well culture plates (Costar) for the semi-solid culture assay. Both layers were supplemented with 10% FCS, 100 UI/ml penicillin, 100 mg/ml streptomycin, MEM vitamins (Life-Technologies) with or without growth factors (either 250 ng/ml recombinant of murine Steel factor (Immunex) or 100 ng/ml of recombinant human CSF-1 (CetusChiron). The number of colonies was scored after either 14 or 21 days and representative areas were photographed.

Acknowledgements

We thank Larry Rohrschneider, Daniel Dumont, David Kwiatkowski, Marius Sudol, Daniella Rotin and Tak Mak for sharing plasmid DNA. We thank Schickwan Tsai for the EML-C1 cell line and the EML-C1-derived lambda phage library, Julian Downward for the Vav2 antibodies and Luc Merengere for the Vav antibodies. We thank Claude Quentin for his assistance with cell sorting, Jane McGlade for her help with the yeast two-hybrid work, David Hogg for the use of his sequencing facility and Philippe Poussier and Dwayne Barber for reviewing the manuscript. This work was supported by grants from the National Cancer Institute of Canada. P.D.S. was supported by fellowships from the Leukemia research Fund of Canada and the Medical Research Council of Canada. P.D. is an INSERM scientist and acknowledges support from an INSERM/MRC fellowship. R.R. is a senior research scholar of the Arthritis Society of Canada.

References

- Alai, M., Mui, A.L., Cutler, R.L., Bustelo, X.R., Barbacid, M. and Krystal, G. (1992) Steel factor stimulates the tyrosine phosphorylation of the proto-oncogene product, p95vav, in human hemopoietic cells. *J. Biol. Chem.*, **267**, 18021–18025.
- August, A., Gibson, S., Kawakami, Y., Kawakami, T., Mills, G.B. and Dupont, B. (1994) CD28 is associated with and induces the immediate tyrosine phosphorylation and activation of the Tec family kinase ITK/EMT in the human Jurkat leukemic T-cell line. *Proc. Natl Acad. Sci. USA*, **91**, 9347–9351.
- Bischoff, S.C. and Dahinden, C.A. (1992) c-kit ligand: a unique potentiator of mediator release by human lung mast cells. *J. Exp. Med.*, **175**, 237–244.
- Blume-Jensen, P., Claesson, W.L., Siegbahn, A., Zsebo, K.M., Westermarck, B. and Heldin, C.H. (1991) Activation of the human c-kit product by ligand-induced dimerization mediates circular actin reorganization and chemotaxis. *EMBO J.*, **10**, 4121–4128.
- Blume-Jensen, P., Ronnstrand, L., Gout, I., Waterfield, M.D. and Heldin, C.H. (1994) Modulation of Kit/stem cell factor receptor-induced signaling by protein kinase C. *J. Biol. Chem.*, **269**, 21793–21802.
- Bourette, R.P., Myles, G.M., Choi, J.L. and Rohrschneider, L.R. (1997) Sequential activation of phosphatidylinositol 3-kinase and phospholipase C-γ2 by the M-CSF receptor is necessary for differentiation signaling. *EMBO J.*, **16**, 5880–5893.
- Broudy, V.C. (1997) Stem cell factor and hematopoiesis. *Blood*, **90**, 1345–1364.
- Bustelo, X.R. and Barbacid, M. (1992) Tyrosine phosphorylation of the vav proto-oncogene product in activated B cells. *Science*, **256**, 1196–1199.
- Bustelo, X.R., Ledbetter, J.A. and Barbacid, M. (1992) Product of vav proto-oncogene defines a new class of tyrosine protein kinase substrates. *Nature*, **356**, 68–71.
- Carpino, N., Wisniewski, D., Strife, A., Marshak, D., Kobayashi, R., Stillman, B. and Clarkson, B. (1997) p62 (dok): a constitutively tyrosine-phosphorylated, GAP-associated protein in chronic myelogenous leukemia progenitor cells. *Cell*, **88**, 197–204.
- Caruana, G., Cambareri, A.C., Gonda, T.J. and Ashman, L.K. (1998) Transformation of NIH3T3 fibroblasts by the c-Kit receptor tyrosine kinase: effect of receptor density and ligand-requirement. *Oncogene*, **16**, 179–190.
- Castresana, J. and Saraste, M. (1995) Does Vav bind to F-actin through a CH domain? *FEBS Lett.*, **374**, 149–151.

- Cheng,L., Du,C., Murray,D., Tong,X., Zhang,Y.A., Chen,B.P. and Hawley,R.G. (1997) A GFP reporter system to assess gene transfer and expression in human hematopoietic progenitor cells. *Gene Ther.*, **4**, 1013–1022.
- Clark,S.G., Stern,M.J. and Horvitz,H.R. (1992) *C. elegans* cell-signalling gene sem-5 encodes a protein with SH2 and SH3 domains. *Nature*, **356**, 340–344.
- Crespo,P., Bustelo,X.R., Aaronson,D.S., Coso,O.A., Lopez,B.M., Barbacid,M. and Gutkind,J.S. (1996) Rac-1 dependent stimulation of the JNK/SAPK signaling pathway by Vav. *Oncogene*, **13**, 455–460.
- Endo,T.A. et al. (1997) A new protein containing an SH2 domain that inhibits JAK kinases. *Nature*, **387**, 921–924.
- Fairbairn,L.J., Cowling,G.J., Reipert,B.M. and Dexter,T.M. (1993) Suppression of apoptosis allows differentiation and development of a multipotent hemopoietic cell line in the absence of added growth factors. *Cell*, **74**, 823–832.
- Fath,I., Schweighoffer,F., Rey,I., Multon,M.C., Boiziau,J., Duchesne,M. and Tocque,B. (1994) Cloning of a Grb2 isoform with apoptotic properties. *Science*, **264**, 971–974.
- Fields,S. and Song,O. (1989) A novel genetic system to detect protein–protein interactions. *Nature*, **340**, 245–246.
- Fischer,K.D., Zmudzins,A., Gardner,S., Barbacid,M., Bernstein,A. and Guidos,C. (1995) Defective T-cell receptor signalling and positive selection of Vav-deficient CD4⁺CD8⁺ thymocytes. *Nature*, **374**, 474–477.
- Fischer,K.D. et al. (1998) Vav is a regulator of cytoskeletal reorganization mediated by the T-cell receptor. *Curr. Biol.*, **8**, 554–562.
- Fleischman,R.A., Saltman,D.L., Stastny,V. and Zneimer,S. (1991) Deletion of the *c-kit* protooncogene in the human developmental defect piebald trait. *Proc. Natl Acad. Sci. USA*, **88**, 10885–10889.
- Giebel,L.B. and Spritz,R.A. (1991) Mutation of the KIT (mast/stem cell growth factor receptor) protooncogene in human piebaldism. *Proc. Natl Acad. Sci. USA*, **88**, 8696–8699.
- Henske,E.P., Short,M.P., Jozwiak,S., Bovey,C.M., Ramlakhan,S., Haines,J.L. and Kwiatkowski,D.J. (1995) Identification of VAV2 on 9q34 and its exclusion as the tuberous sclerosis gene TSC1. *Ann. Hum. Genet.*, **59**, 25–37.
- Hilton,D.J. et al. (1998) Twenty proteins containing a C-terminal SOCS box form five structural classes. *Proc. Natl Acad. Sci. USA*, **95**, 114–119.
- Holsinger,L.J., Graef,I.A., Swat,W., Chi,T., Bautista,D.M., Davidson,L., Lewis,R.S., Alt,F.W. and Crabtree,G.R. (1998) Defects in actin-cap formation in Vav-deficient mice implicate an actin requirement for lymphocyte signal transduction. *Curr. Biol.*, **8**, 563–572.
- Holyoake,T.L., Freshney,M.G., McNair,L., Parker,A.N., McKay,P.J., Steward,W.P., Fitzsimons,E., Graham,G.J. and Pragnell,I.B. (1996) *Ex vivo* expansion with stem cell factor and interleukin-11 augments both short-term recovery posttransplant and the ability to serially transplant marrow. *Blood*, **87**, 4589–4595.
- Ikuta,K. and Weissman,I.L. (1992) Evidence that hematopoietic stem cells express mouse c-kit but do not depend on steel factor for their generation. *Proc. Natl Acad. Sci. USA*, **89**, 1502–1506.
- Karasuyama,H. and Melchers,F. (1988) Establishment of mouse cell lines which constitutively secrete large quantities of interleukin 2, 3, 4 or 5, using modified cDNA expression vectors. *Eur. J. Immunol.*, **18**, 97–104.
- Katzav,S., Martin-Zanca,D. and Barbacid,M. (1989) *vav*, a novel human oncogene derived from a locus ubiquitously expressed in hematopoietic cells. *EMBO J.*, **8**, 2283–2290.
- Kavanaugh,W.M. et al. (1996) Multiple forms of an inositol polyphosphate 5-phosphatase form signaling complexes with Shc and Grb2. *Curr. Biol.*, **6**, 438–445.
- Keller,J.R., Ortiz,M. and Ruscetti,F.W. (1995) Steel factor (c-kit ligand) promotes the survival of hematopoietic stem/progenitor cells in the absence of cell division. *Blood*, **86**, 1757–1764.
- Kozlowski,M., Larose,L., Lee,F., Le,D.M., Rottapel,R. and Siminovich,K.A. (1998) SHP-1 binds and negatively modulates the c-Kit receptor by interaction with tyrosine 569 in the c-Kit juxtamembrane domain. *Mol. Cell. Biol.*, **18**, 2089–2099.
- Kubota,Y., Angelotti,T., Niederfellner,G., Herbst,R. and Ullrich,A. (1998) Activation of phosphatidylinositol 3-kinase is necessary for differentiation of FDC-P1 cells following stimulation of type III receptor tyrosine kinases. *Cell Growth Differ.*, **9**, 247–256.
- Lev,S., Yarden,Y. and Givol,D. (1990) Receptor functions and ligand-dependent transforming potential of a chimeric *kit* proto-oncogene. *Mol. Cell. Biol.*, **10**, 6064–6068.
- Lev,S., Givol,D. and Yarden,Y. (1991) A specific combination of substrates is involved in signal transduction by the kit-encoded receptor. *EMBO J.*, **10**, 647–654.
- Li,C.L. and Johnson,G.R. (1994) Stem cell factor enhances the survival but not the self-renewal of murine hematopoietic long-term repopulating cells. *Blood*, **84**, 408–414.
- Lioubin,M.N., Algate,P.A., Tsai,S., Carlberg,K., Aebersold,R. and Rohrschneider,L.R. (1996) p150^{Shp}, a signal transduction molecule with inositol polyphosphate-5-phosphatase activity. *Genes Dev.*, **10**, 1084–1095.
- Lyman,S.D. and Jacobsen,S.E. (1998) c-kit ligand and Flt3 ligand: stem/progenitor cell factors with overlapping yet distinct activities. *Blood*, **91**, 1101–1134.
- Marengere,L.E. and Pawson,T. (1992) Identification of residues in GTPase-activating protein Src homology 2 domains that control binding to tyrosine phosphorylated growth factor receptors and p62. *J. Biol. Chem.*, **267**, 22779–22786.
- Margolis,B., Hu,P., Katzav,S., Li,W., Oliver,J.M., Ullrich,A., Weiss,A. and Schlessinger,J. (1992) Tyrosine phosphorylation of *vav* proto-oncogene product containing SH2 domain and transcription factor motifs. *Nature*, **356**, 71–74.
- Maroc,N., Rottapel,R., Rosnet,O., Marchetto,S., Lavezzi,C., Mannoni,P., Birnbaum,D. and Dubreuil,P. (1993) Biochemical characterization and analysis of the transforming potential of the FLT3/FLK2 receptor tyrosine kinase. *Oncogene*, **8**, 909–918.
- Masuhara,M. et al. (1997) Cloning and characterization of novel CIS family genes. *Biochem. Biophys. Res. Commun.*, **239**, 439–446.
- Mayer,B.J., Jackson,P.K., Van,E.R. and Baltimore,D. (1992) Point mutations in the abl SH2 domain coordinately impair phosphotyrosine binding *in vitro* and transforming activity *in vivo*. *Mol. Cell. Biol.*, **12**, 609–618.
- McNiece,I.K., Langley,K.E. and Zsebo,K.M. (1991a) Recombinant human stem cell factor synergises with GM-CSF, G-CSF, IL-3 and epo to stimulate human progenitor cells of the myeloid and erythroid lineages. *Exp. Hematol.*, **19**, 226–231.
- McNiece,I.K., Langley,K.E. and Zsebo,K.M. (1991b) The role of recombinant stem cell factor in early B cell development. Synergistic interaction with IL-7. *J. Immunol.*, **146**, 3785–3790.
- Meininger,C.J., Yano,H., Rottapel,R., Bernstein,A., Zsebo,K.M. and Zetter,B.R. (1992) The c-kit receptor ligand functions as a mast cell chemoattractant. *Blood*, **79**, 958–963.
- Miller,C.L., Rebel,V.I., Helgason,C.D., Lansdorp,P.M. and Eaves,C.J. (1997) Impaired steel factor responsiveness differentially affects the detection and long-term maintenance of fetal liver hematopoietic stem cells *in vivo*. *Blood*, **89**, 1214–1223.
- Minamoto,S., Ikegame,K., Ueno,K., Narazaki,M., Naka,T., Yamamoto,H., Matsumoto,T., Saito,H., Hosoe,S. and Kishimoto,T. (1997) Cloning and functional analysis of new members of STAT induced STAT inhibitor (SSI) family: SSI-2 and SSI-3. *Biochem. Biophys. Res. Commun.*, **237**, 79–83.
- Naka,T. et al. (1997) Structure and function of a new STAT-induced STAT inhibitor. *Nature*, **387**, 924–929.
- O'Neill,T.J., Craparo,A. and Gustafson,T.A. (1994) Characterization of an interaction between insulin receptor substrate 1 and the insulin receptor by using the two-hybrid system. *Mol. Cell. Biol.*, **14**, 6433–6442.
- Ohya,K.I. et al. (1997) SOCS-1/JAB/SSI-1 can bind to and suppress Tec protein-tyrosine kinase. *J. Biol. Chem.*, **272**, 27178–27182.
- Okada,S., Nakauchi,H., Nagayoshi,K., Nishikawa,S., Nishikawa,S.-i., Miura,Y. and Suda,T. (1991) Enrichment and characterization of murine hematopoietic stem cells that express *c-kit* molecule. *Blood*, **78**, 1706–1712.
- Orlic,D., Fischer,R., Nishikawa,S.-I., Nienhuis,A.W. and Bodine,D.M. (1993) Purification and characterization of heterogeneous pluripotent hematopoietic stem cell populations expressing high levels of *c-kit* receptor. *Blood*, **82**, 762–770.
- Parganas,E. et al. (1998) Jak2 is essential for signaling through a variety of cytokine receptors. *Cell*, **93**, 385–395.
- Pawson,T. and Bernstein,A. (1990) Receptor tyrosine kinases: genetic evidence for their role in *Drosophila* and mouse development. *Trends Genet.*, **6**, 350–356.
- Pietsch,T., Kyas,U., Steffens,U., Yakisan,E., Hadam,M.R., Ludwig,W.D., Zsebo,K. and Welte,K. (1992) Effects of human stem cell factor (c-kit ligand) on proliferation of myeloid leukemia cells: heterogeneity in response and synergy with other hematopoietic growth factors. *Blood*, **80**, 1199–1206.

- Ploemacher, R.E., van Soest, P.L., Voorwinden, H. and Boudewijn, A. (1993) Interleukin-12 synergizes with interleukin-3 and steel factor to enhance recovery of murine hemopoietic stem cells in liquid culture. *Leukemia*, **7**, 1381–1388.
- Ray, R.J., Paige, C.J., Furlonger, C., Lyman, S.D. and Rottapel, R. (1996) Flt3 ligand supports the differentiation of early B cell progenitors in the presence of interleukin-11 and interleukin-7. *Eur. J. Immunol.*, **26**, 1504–1510.
- Reith, A., Rottapel, R., Giddens, E., Brady, C., Forrester, L. and Bernstein, A. (1990) W mutant mice with mild or severe developmental defects contain distinct point mutations in the kinase domain of the c-kit transmembrane receptor. *Genes Dev.*, **4**, 390–400.
- Ridley, A.J. and Hall, A. (1992) The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell*, **70**, 389–399.
- Ridley, A.J., Paterson, H.F., Johnston, C.L., Diekmann, D. and Hall, A. (1992) The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell*, **70**, 401–410.
- Rottapel, R., Reedijk, M., Williams, D.E., Lyman, S.D., Anderson, D.M., Pawson, T. and Bernstein, A. (1991) The Steel/W transduction pathway: kit autophosphorylation and its association with a unique subset of cytoplasmic signaling proteins is induced by the Steel factor. *Mol. Cell. Biol.*, **11**, 3043–3051.
- Rottapel, R., Turck, C.W., Casteran, N., Liu, X., Birnbaum, D., Pawson, T. and Dubreuil, P. (1994) Substrate specificities and identification of a putative binding site for PI3K in the carboxy tail of the murine Flt3 receptor tyrosine kinase. *Oncogene*, **9**, 1755–1765.
- Rozakis-Adcock, M., Fernley, R., Wade, J., Pawson, T. and Bowtell, D. (1993) The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the Ras activator mSos1. *Nature*, **363**, 83–85.
- Russell, E.S. (1979) Hereditary anemias of the mouse: a review for geneticists. *Adv. Genet.*, **20**, 357–459.
- Schubel, K.E., Bustelo, X.R., Nielson, D.A., Song, B., Barbacid, M., Goldman, D. and Lee, I.L. (1996) Isolation and characterization of murine *vav2*, a member of the *vav* family of proto-oncogenes. *Oncogene*, **13**, 363–371.
- Serve, H., Yee, N.S., Stella, G., Sepp-Lorenzino, L., Tan, J.C. and Besmer, P. (1995) Differential roles of PI3-kinase and kit tyrosine 821 in kit receptor-mediated proliferation, survival and cell adhesion in mast cells. *EMBO J.*, **14**, 473–483.
- Silvers, W.K. (1979) *The Coat Colors of Mice*. Springer-Verlag, New York, NY.
- Sparks, A.B., Rider, J.E., Hoffman, N.G., Fowlkes, D.M., Quillam, L.A. and Kay, B.K. (1996) Distinct ligand preferences of Src homology 3 domains from Src, Yes, Abl, Cortactin, p53bp2, PLC γ , Crk and Grb2. *Proc. Natl Acad. Sci. USA*, **93**, 1540–1544.
- Starr, R. et al. (1997) A family of cytokine-inducible inhibitors of signalling. *Nature*, **387**, 917–921.
- Tang, B., Mano, H., Yi, T. and Ihle, J.N. (1994) Tec kinase associates with c-kit and is tyrosine phosphorylated and activated following stem cell factor binding. *Mol. Cell. Biol.*, **14**, 8432–8437.
- Tarakhovskiy, A., Turner, M., Schaal, S., Mee, P.J., Duddy, L.P., Rajewsky, K. and Tybulewicz, V.L. (1995) Defective antigen receptor-mediated proliferation of B and T cells in the absence of Vav. *Nature*, **374**, 467–470.
- Tsai, S., Bartelmez, S., Sitnicka, E. and Collins, S. (1994) Lymphohematopoietic progenitors immortalized by a retroviral vector harboring a dominant-negative retinoic acid receptor can recapitulate lymphoid, myeloid and erythroid development. *Genes Dev.*, **8**, 2831–2841.
- Tsuji, K., Lyman, S.D., Sudo, T., Clark, S.C. and Ogawa, M. (1992) Enhancement of murine hematopoiesis by synergistic interactions between steel factor (ligand for c-kit), interleukin-11 and other early acting factors in culture. *Blood*, **79**, 2855–2860.
- Ulich, T.R., del Castillo, J., McNiece, I.K., Yi, E.S., Alzona, C.P., Yin, S.M. and Zsebo, K.M. (1991) Stem cell factor in combination with granulocyte colony-stimulating factor (CSF) or granulocyte-macrophage CSF synergistically increases granulopoiesis *in vivo*. *Blood*, **78**, 1954–1962.
- Vojtek, A.B. and Hollenberg, S.M. (1995) Ras–Raf interaction: two-hybrid analysis. *Methods Enzymol.*, **255**, 331–342.
- Vosseller, K., Stella, G., Yee, N.S. and Besmer, P. (1997) c-kit receptor signaling through its phosphatidylinositol-3'-kinase-binding site and protein kinase C: role in mast cell enhancement of degranulation, adhesion and membrane ruffling. *Mol. Biol. Cell*, **8**, 909–922.
- Weidner, K.M., Di Cesare, S., Sachs, M., Brinkmann, V., Behrens, J. and Birchmeier, W. (1996) Interaction between Gab1 and the c-Met receptor tyrosine kinase is responsible for epithelial morphogenesis. *Nature*, **384**, 173–176.
- Weiler, S.R., Mou, S., DeBerry, C.S., Keller, J.R., Ruscetti, F.W., Ferris, D.K., Longo, D.L. and Linnekin, D. (1996) JAK2 is associated with the c-kit proto-oncogene product and is phosphorylated in response to stem cell factor. *Blood*, **87**, 3688–3693.
- Weng, W.K., Jarvis, L. and LeBien, T.W. (1994) Signaling through CD19 activates Vav/mitogen-activated protein kinase pathway and induces formation of a CD19/Vav/phosphatidylinositol 3-kinase complex in human B cell precursors. *J. Biol. Chem.*, **269**, 32514–32521.
- Wennstrom, S., Siegbahn, A., Yokote, K., Arvidsson, A.K., Heldin, C.H., Mori, S. and Claesson, W.L. (1994) Membrane ruffling and chemotaxis transduced by the PDGF β -receptor require the binding site for phosphatidylinositol 3' kinase. *Oncogene*, **9**, 651–660.
- Yao, R. and Cooper, G.M. (1995) Requirement of phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. *Science*, **267**, 2003–2006.
- Yu, H., Chen, J.K., Feng, S., Dalgarno, D.C., Brauer, A.W. and Schreiber, S.L. (1994) Structural basis for the binding of proline-rich peptides to SH3 domains. *Cell*, **76**, 933–945.
- Zhang, R., Alt, F.W., Davidson, L., Orkin, S.H. and Swat, W. (1995) Defective signalling through the T- and B-cell antigen receptors in lymphoid cells lacking the *vav* proto-oncogene. *Nature*, **374**, 470–473.

Received July 27, 1998; revised November 23, 1998;
accepted December 22, 1998