Distribution of the Src-homology-2-domain-containing inositol 5-phosphatase SHIP-2 in both non-haemopoietic and haemopoietic cells and possible involvement of SHIP-2 in negative signalling of B-cells

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The termination of activation signals is a critical step in the control of the immune response; perturbation of inhibitory feedback pathways results in profound immune defects culminating in autoimmunity and overwhelming inflammation. FcγRIIB receptor is a well described inhibitory receptor. The ligation of B-cell receptor (BCR) and FcγRIIB leads to the inhibition of B-cell activation. Numerous studies have demonstrated that the SH2-domain-containing inositol 5-phosphatase SHIP (referred hereto as SHIP-1) is essential in this process. The cDNA encoding a second SH2-domain-containing inositol 5-phosphatase, SHIP-2, has been cloned [Pesesse, Deleu, De Smedt, Drayer and Erneux (1997) Biochem. Biophys. Res. Commun. **239**, 697–700]. Here we report the distribution of SHIP-2 in mouse tissues: a Western blot analysis of mouse tissues reveals that SHIP-2 is expressed in both haemopoietic and non-haemopoietic cells. In addition to T-cell and B-cell lines, spleen, thymus and lung are shown to coexpress SHIP-1 and SHIP-2. Moreover, SHIP-2 is detected in fibroblasts, heart and different brain areas. SHIP-2 shows a maximal tyrosine phosphorylation and association to Shc after ligation of BCR to FcγRIIB but not after stimulation of BCR alone. Our results therefore suggest a possible role for SHIP-2 in the negative regulation of immunocompetent cells.

Key words: immunosuppression, negative regulation, phosphatidylinositol metabolism, signal transduction.

INTRODUCTION

Immune complexes are potent activators of inflammatory cells, triggering effector responses through the cross-linking of Fc receptors such as FcϵRI or FcγRIII [1]. On B-cells, immune complexes are also negative regulators of activation triggered by stimulation of the B-cell antigen receptor (BCR). In fact, cross-linking of BCR by antigen gives rise to a sequence of intracellular events that leads to the proliferation and/or differentiation of the cells followed by antibody production. In contrast, the ligation of BCR to FcγRIIB, a low-affinity receptor for IgG, leads to a dominant-negative signal that inhibits B-cell activation [2–4]. These findings support a model in which FcγRIIB provides a powerful mechanism for B-cells to discriminate between free antigen and antigen–antibody immune complex and can thus serve to prevent the production of excess immunoglobulin.

The identification of the SH2-domain-containing inositol polyphosphate 5-phosphatase, SHIP (referred hereto as SHIP-1), as a crucial negative regulator of BCR signalling has been a major advance in the understanding of this phenomenon. Numerous studies have suggested that FcγRIIB inhibition is not mediated by a global shutdown of all enzymes implicated in BCR signalling but is instead a selective mechanism involving SHIP-1 [5–8]. SHIP-1 encodes an inositol polyphosphate 5-phosphatase that uses Ins(1,3,4,5)P_4 and PtdIns(3,4,5)P_3 as substrates. It is a 145 kDa tyrosine-phosphorylated protein with several interesting properties: an N-terminal SH2 domain, a central catalytic domain, two phosphotyrosine-binding ('PTB') consensus sequences, and three putative SH3 interacting motifs at the C-terminus [9–13] (Figure 1).

It has been proposed that SHIP-1 might regulate the proliferation and differentiation of haemopoietic cells by modulating PtdIns(3,4,5)P_3 levels and Ras activity after stimulation by antigens or cytokines [9–13]. The ligation of BCR to FcγRIIB induces the Src kinase Lyn-dependent phosphorylation of FcγRIIB on its immunoreceptor tyrosine inhibition motif (ITIM), leading to the recruitment of SHIP-1 and its tyrosine phosphorylation [5,14]. It has been demonstrated that the inositol 5-phosphatase activity of SHIP-1 can decrease the levels of PtdIns(3,4,5)P_3 and thereby counteract the pleckstrin homology domain-mediated recruitment and activation of the Bruton tyrosine kinase, leading to inactivation of phospholipase Cγ2 [7] In addition, SHIP-1 has been reported to associate through its SH2 domain to Shc during negative signalling of B-cells. This

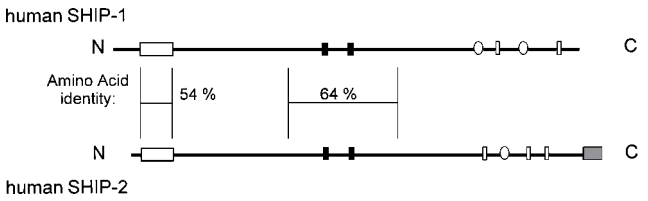

Figure 1 Alignment of SHIP-1 and SHIP-2 sequences

The sequence of SHIP-1 (U57650) is taken from [11]. The sequence of SHIP-2 (Y14385) is taken from [19]. SHIP-1 contains 1188 residues and has a predicted molecular mass of 133 kDa; SHIP-2 contains 1258 residues and has a predicted molecular mass of 142 kDa. The symbols used are as follows: N-terminal white rectangle, SH2 domain; black rectangle, 5-phosphatase motif; small white rectangle, proline-rich motif (ligand of SH3 domain); grey rectangle, SAM domain; white ellipse, NPXY site (when phosphorylated it acts as a ligand for the phosphotyrosine-binding domain).

Abbreviations used: BCR, B-cell receptor; ITIM, immunoreceptor tyrosine inhibition motif; SAM, sterile alpha motif; SH domain, Src homology domain; SHIP, SH2-domain-containing inositol 5-phosphatase; SHP, SH2-containing protein tyrosine phosphatase.

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association could prevent Grb2–Sos association to phosphorylated Shc and might thus inhibit Ras activation [15,16]. Recently, the essential role of SHIP-1 in the regulation of the immune system has been shown. It has been observed that SHIP-1 knock-out mice present a severe phenotype characterized by a shortened lifespan, splenomegaly and massive myeloid cell accumulation in the lungs [17]. Moreover, SHIP-1 $-/-$ splenic B-cells display prolonged Ca^{2+} influx, increased proliferation *in vitro*, and enhanced activation of mitogen-activated protein kinase in response to BCR–FcγRIIB ligation [18].

The cDNA of a second SHIP closely related to SHIP-1 has been cloned [19] (Figure 1). This protein, referred to as SHIP-2, displays enzymic activity comparable to that of SHIP-1. The substrates *in vitro* are Ins(1,3,4,5)P_4 and PtdIns(3,4,5)P_3 [20,21]. Recent work by Habib et al. [21] has demonstrated that 51C/SHIP-2 associates with Shc in human non-haemopoietic cell lines stimulated with growth factors and insulin. The tissue distribution of SHIP-2 in human and mouse tissues has not yet been assessed by Western blot analysis.

The present study was undertaken to compare the distribution of SHIP-2 and SHIP-1 by Western blotting in mouse cell lines and tissues. It was observed that SHIP-2 is expressed in both haemopoietic and non-haemopoietic cells. To test the hypothesis that SHIP-2 has a similar role to that of SHIP-1 in the negative signalling of the immune system, the interactions of SHIP-2 and SHIP-1 with Shc have been compared during B-cell activation. With the use of the A20 lymphoma B-cell line as a model, the tyrosine phosphorylation of both SHIP-1 and SHIP-2 and their association with Shc have been observed in response to BCR–FcγRIIB ligation.

MATERIALS AND METHODS

Materials

Rabbit polyclonal antibodies against Shc were obtained from Affiniti (Mamhead, Exeter, Devon, U.K.). Anti-rabbit Ig and anti-mouse Ig, horseradish-peroxidase-linked whole antibodies, were obtained from Amersham Life Science (Roosendael, The Netherlands). Protein A–Sepharose CL4B was obtained from Amersham Pharmacia (Roosendael, The Netherlands). FastTrack 2.0 Kit was purchased from Invitrogen (Groningen, The Netherlands). Anti-phosphotyrosine monoclonal antibody 4G10 was purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.).

Cell lines

HeLa (human epithelial cells), NIH3T3 (mouse fibroblasts), A20 (mouse B lymphoma cell line) and NS20Y (mouse neuroblastoma cell line) were obtained through A.T.C.C. (Manassas, VA, U.S.A.). The I-E^k-restricted, pigeon cytochrome-*c*-specific T-cell hybridoma 3B4 (CD90$^+$, CD3$^+$, CD4$^+$, CD8$^-$) was derived by Dr. Fabienne Andris [22]. Murine astrocytes were purified as described in [23]. A20, 3B4 and HeLa cells were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum. Murine astrocytes, NIH3T3 and NS20Y cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum. Cells were washed with cold PBS and lysed in cold 10 mM Tris/HCl buffer, pH 7.5, supplemented with 150 mM KCl, 0.5% (v/v) Nonidet P40, 100 mM NaF, 2 mM mercaptoethanol, 1 mM Na_3VO_4 and protease and phosphatase inhibitors (1 nM okadaic acid, 5 μM leupeptin, 0.1 mM Pefabloc, 1 mM EDTA and 20 μg/ml calpain inhibitors I and II), for 30 min at 4 °C, with agitation. Protein concentration was estimated as described in [24].

Mouse tissues and spleen cells

Female Balb/c mice (6–10 weeks old) were purchased from Iffa-Credo (Bruxelles, Belgium) and maintained in our animal facility. T-cell-depleted spleen cells were obtained after complement-mediated lysis as recomended by the manufacturer (Guinea pig serum; bioMérieux, Marcy L'étoile, France) of spleen cells labelled with anti-(Thy-1.2) (clone HO-13-4A, from A.T.C.C.). The resulting population contained more than 85% Ig$^+$ cells and less than 4% CD3$^+$ cells. T-cell-enriched spleen cells were obtained after complement-mediated lysis of spleen cells labelled with anti-(I-$E^{k,d}$) (clone 14-4-4S, from A.T.C.C.), anti-CD45R (clone B220, from A.T.C.C.) and anti-CD24 (clone J11D, from A.T.C.C.). The resulting population contained more than 75% CD3$^+$ cells and less than 2% Ig$^+$ cells. Mouse tissues and spleen cells were homogenized in the following ice-cold buffer: 20 mM Tris/HCl (pH 7.5)/0.25 M sucrose/0.01% NaN_3/1 mM Na_3VO_4/protease and phosphatase inhibitors (1 nM okadaic acid/5 μM leupeptin/0.1 mM pefabloc/1 mM EDTA/20 μg/ml calpain inhibitors I and II). The homogenates were centrifuged at 13000 rev./min for 10 min. The soluble fractions were used in Western blots.

Northern blot analysis

Poly(A)-rich RNA from HeLa, A20 and 3B4 cell lines were prepared with a FastTrack 2.0 Kit, starting from 10^8 cells. The blot was probed with a 0.8 kb fragment of murine SHIP-2 cDNA corresponding to nt 538–1410 of the human counterpart (Dr. Stephane Schurmans, IRIBHN, personal communication).

Antibody production

Anti-SHIP-2 antibodies were generated in rabbits with two synthetic peptides corresponding to residues 651–670 (SEEEISFPPTYRYERGSRDT, catalytic peptide) and 1243–1258 (DPAHKRLLLDTLQLSK, C-terminal peptide) of human SHIP-2 [19,20]. Anti-SHIP-1 antibodies were generated with a peptide corresponding to residues 1222–1238 (HGKHRQEEGLLGRTAMQ, C-terminal peptide) of mouse SHIP-1 [9]. The peptides were cross-linked to keyhole limpet haemocyanin by using glutaraldehyde [25].

B-cell activation and immune precipitation

A20 B lymphoma cell line activation was performed at 37 °C with 40 μg/ml of rabbit F(ab′)$_2$ fragments against mouse IgG (Organon Teknika) or 60 μg/ml of intact rabbit IgG against mouse IgG (Organon Teknika). Stimulated or unstimulated cells (10^8) for each condition were rinsed twice in cold PBS and lysed in 1 ml of ice-cold lysis buffer. After gentle shaking for 20 min at 4 °C, the lysate was precleared for 5 min at 4 °C with 150 μl of 10% (w/v) Protein A–Sepharose. This was centrifuged at 12000 ***g*** for 30 min at 4 °C. The soluble fraction was collected and incubated with 10 μl of the antibodies and 150 μl of Protein A–Sepharose for 2 h at 4 °C. The immune complexes were recovered by centrifugation and washed four times in lysis buffer. The last wash was performed without protease and phosphatase inhibitors. One half of the immunoprecipitate was used for enzymic assay; the other was boiled in Laemmli sample buffer and subjected to SDS/PAGE [8% (w/v) gel], then transferred to nitrocellulose membranes (Protran BA 79; Schleicher and Schuell). The blots were blocked with 5% (w/v) milk powder in a buffer containing 10 mM Tris/HCl, pH 8, 150 mM NaCl and 0.05% Nonidet P40. Western blots were incubated with the relevant antisera diluted 1:500 for SHIP-1 or 1:250 for SHIP-2,

or with anti-phosphotyrosine (clone 4G10) diluted 1:1000. Immunodetection were detected with peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence system. Assays with [^{3}H]Ins(1,3,4,5)P_4 at 1 μM as substrate were performed as described [26,27].

Flow cytometry

Cells were analysed by flow cytometry with a FACScan cytometer (Beckton–Dickinson, Mountain View, CA, U.S.A.). The cells were incubated with 2.4G2 [rat anti-(mouse FcγRII/III)], to prevent antibody binding to Fc receptor, for 10 min before being stained, then incubated with FITC-coupled 145-2C11 [anti-CD3ϵ, hamster Ig, available through A.T.C.C., kindly provided by Dr. Oberdan Leo (Université Libre de Bruxelles, Bruxelles, Belgium)]. In all cases, cells were gated according to size and scatter to eliminate dead cells and debris from the analysis.

RESULTS

Specificity of antibodies against SHIP-1 and SHIP-2

Our SHIP-1 antibody was made against a C-terminal peptide of SHIP-1 and is comparable to that made previously in Parker's laboratory and used in platelets [27]. Antibodies against SHIP-2 were prepared against two peptides chosen in the C-terminus and the catalytic part of the sequence of SHIP-2, as outlined in the Materials and methods section. The peptide sequences chosen as SHIP-2 antigens (catalytic and C-terminal peptides) had no similarity to any regions of SHIP-1. A series of controls were performed to assess the specificity of our antibodies. Lysates were prepared from A20 lymphoma B-cells and immunoprecipitated with anti-(SHIP-1) or anti-(SHIP-2 C-terminus) antibodies. As shown in Figure 2, antibodies against SHIP-1 and SHIP-2 immunoprecipitated 145–130 kDa and 160–150 kDa protein doublets when probed with our C-terminal antibodies (Figures 2C and 2A respectively). We tested whether the peptide used as SHIP-2 antigen could prevent immunoprecipitation with our anti-(SHIP-2 C-terminus) antibodies. The SHIP-2 catalytic antibodies did not work in immunoprecipitation but recognized the SHIP-2 160–150 kDa doublet in Western blots [after immunoprecipitation with our anti-(SHIP-2 C-terminus) antibodies; Figure 2B]. Thus similar results (i.e. protein bands of 160–150 kDa) were obtained in our blots when probed with antibodies against the catalytic region or against the C-terminal end of SHIP-2 (Figures 2A and 2B). The SHIP-2 C-terminal peptide used as antigen specifically prevented the immunoprecipitation of SHIP-2 but not SHIP-1 (Figures 2A–2C). SHIP-2 protein bands of 160–150 kDa were not detected after immunoprecipitation with our SHIP-1 antibody. No signal was detected with

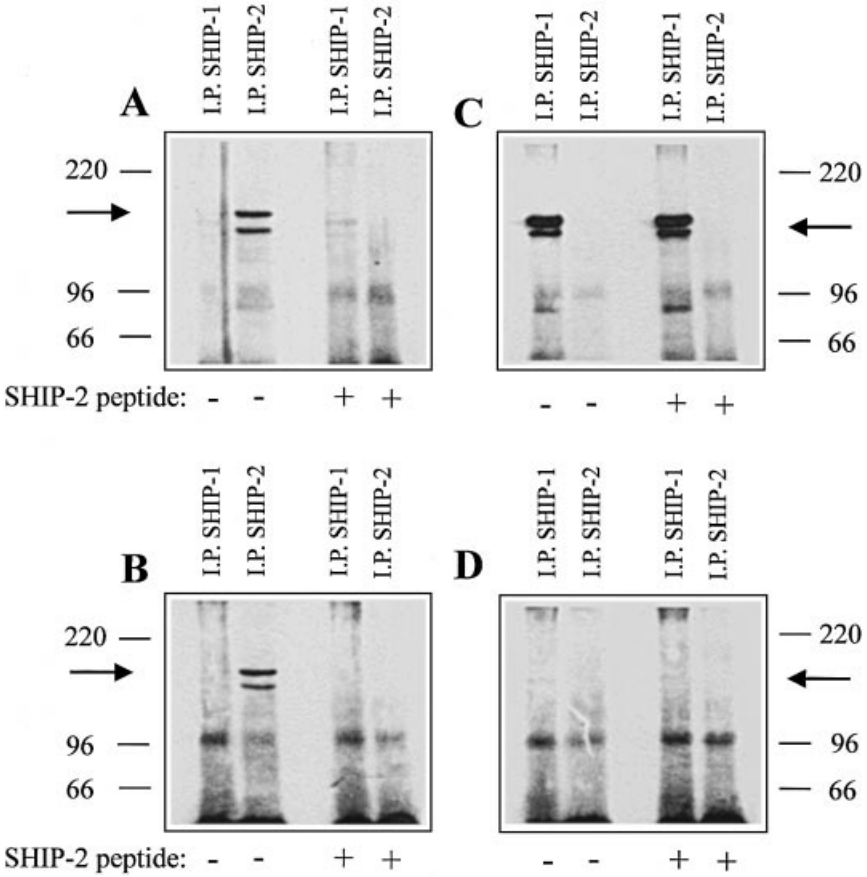

Figure 2 Immunoprecipitation of SHIP-1 and SHIP-2 protein in A20 lymphoma B-cells

A20 B-cells were lysed and immunoprecipitated (I.P.) with anti-(SHIP-1) antibodies or anti-(SHIP-2 C-terminus) antibodies in the presence or absence of 1 μg of SHIP-2 C-terminal peptide. Samples were resolved by SDS/PAGE and probed with (**A**) anti-(SHIP-2 C-terminus) antibodies, (**B**) anti-(SHIP-2 catalytic region) antibodies, (**C**) anti-(SHIP-1) antibodies or (**D**) anti-(SHIP-2) preimmune sera. The results are representative of three separate experiments. The positions of molecular mass markers are indicated (in kDa) at the left and right; SHIP-1 and SHIP-2 molecular masses (130–145 kDa and 150–160 kDa respectively) are indicated by arrows.

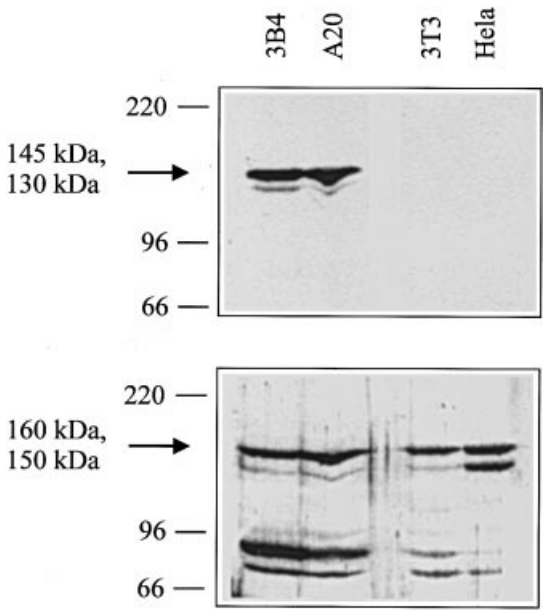

Figure 3 Comparison of the expression of SHIP-1 and SHIP-2 in cell lines

The expression levels of SHIP-1 and SHIP-2 were compared by immunoblot analysis of 90 μg of protein from whole cell lysates of 3B4 mouse T-cell hybridoma, 140 μg of A20 mouse B-cells, 70 μg of NIH-3T3 mouse fibroblasts and 120 μg of HeLa cells, as indicated. Samples were resolved by SDS/PAGE and probed with anti-(SHIP-1) antibodies (upper panel) or anti-(SHIP-2 C-terminus) antibodies (lower panel). The results are representative of three separate experiments. The positions of molecular mass markers are indicated (in kDa) at the left.

our anti-(SHIP-2 C-terminus) preimmune serum (Figure 2D). These results indicate that our antibodies against SHIP-1 and SHIP-2 did not cross-react in A20 cells. As shown below, the SHIP-2 160–150 kDa doublet was present in a very large number of cells and also in primary embryonic mouse fibroblasts, but was absent from SHIP-2-deficient immortalized fibroblasts (S. Schurmans, personal communication).

SHIP-2 is expressed in both haemopoietic and non-haemopoietic cell lines

A series of experiments were performed to compare the distribution of SHIP-2 and SHIP-1 in a variety of cell lines; we used HeLa cells as a positive control for SHIP-2 [21]. As shown by the Western blot analysis in Figure 3, only the A20 and 3B4 lymphoid cell lines expressed SHIP-1 with a known characteristic pattern of 145 and 130 kDa (as reported in [9] and [11]). In contrast, SHIP-2 is expressed in each of the cell lines (i.e. 3B4, A20, 3T3 and HeLa) with an apparent molecular mass of 160 kDa (Figure 3, lower panel). A Western blot analysis of the cell lines also showed the lower-molecular-mass band at approx. 150 kDa and several additional protein bands lower than 100 kDa. Those low-molecular-mass bands (less than 100 kDa) disappeared after immunoprecipitation with our anti-(SHIP-2 C-terminus) antibodies (see Figure 2), suggesting that those were non-specific because our antibodies were not purified. No signal was detected with our preimmune serum (results not shown). A Northern blot analysis also demonstrated the presence of a SHIP-2 transcript of approx. 5 kb present in HeLa cells, in the two murine B lymphoma cell lines (A20 and BCL1) and in mouse brain (results not shown).

Ins(1,3,4,5)P_4 phosphatase activity in cell immunoprecipitates made with antibodies against SHIP-1 and SHIP-2

Lysates were prepared from HeLa, 3B4 and A20 cells, immunoprecipitated with antibodies against SHIP-1 or SHIP-2 and tested for enzymic activity. Ins(1,3,4,5)P_4 phosphatase activity was detected in SHIP-2 immunoprecipitates of HeLa, A20 and 3B4 cells, in agreement with our immunoblot analysis (Table 1). It can be seen that (1) antibodies against SHIP-1 immunoprecipitated a significant phosphatase activity only in 3B4 and A20 cells, which is consistent with the SHIP-1 distribution (Figure 3) and (2) Ins(1,3,4,5)P_4 phosphatase activity that was immunoprecipitated with antibodies against SHIP-2 in the presence of the antigen, i.e. SHIP-2 C-terminal peptide (1 μg/ml), was no longer detectable.

SHIP-2 is expressed in various mouse tissues

As SHIP-2 protein was expressed in haemopoietic and non-haemopoietic cell lines, we compared the expression of SHIP-2 and SHIP-1 in murine tissues by Western blotting. Protein extracts from HeLa and 3B4 cells were used as controls. SHIP-1 expression was particularly high in the tissues rich in lymphoid cells such as lung, thymus and spleen (Figure 4A). In contrast, SHIP-2 was expressed in all tissues tested, particularly in heart and brain, in which low levels of SHIP-1 had previously been reported [27]. As seen previously in cell lines, the SHIP-2 C-terminal peptide prevented SHIP-2 immunoprecipitation in heart, brain and spleen tissues (Figure 4B). This was not observed in SHIP-1 immunoprecipitation in spleen. To discriminate further

Table 1 Ins(1,3,4,5)P_4 5-phosphatase activity of SHIP-1 and SHIP-2 in various cell lines after immunoprecipitation with anti-(SHIP-1) or anti-(SHIP-2) antibodies

Phosphatase activity was measured by the dephosphorylation of 1 μM Ins(1,3,4,5)P_4. Number of cells used: HeLa, 10^8; 3B4, 10^8; A20, 3×10^8. Abbreviation: n.d., not detectable. Results are means $\pm$ S.D. for four independent determinations.

		Phosphatase activity (pmol/min per ml)		
Immunoprecipitated with	SHIP-2 peptide	HeLa fibroblast	3B4 T cell hybridoma	A20 B-cell lymphoma
Anti-(SHIP-1)	−	n.d.	107.3 $\pm$ 2.2	111.6 $\pm$ 22.4
	+	n.d.	100.1 $\pm$ 8.8	122.4 $\pm$ 13.6
Anti-(SHIP-2)	−	23.1 $\pm$ 1.4	10.0 $\pm$ 1.9	12.8 $\pm$ 0.8
	+	n.d.	n.d.	n.d.

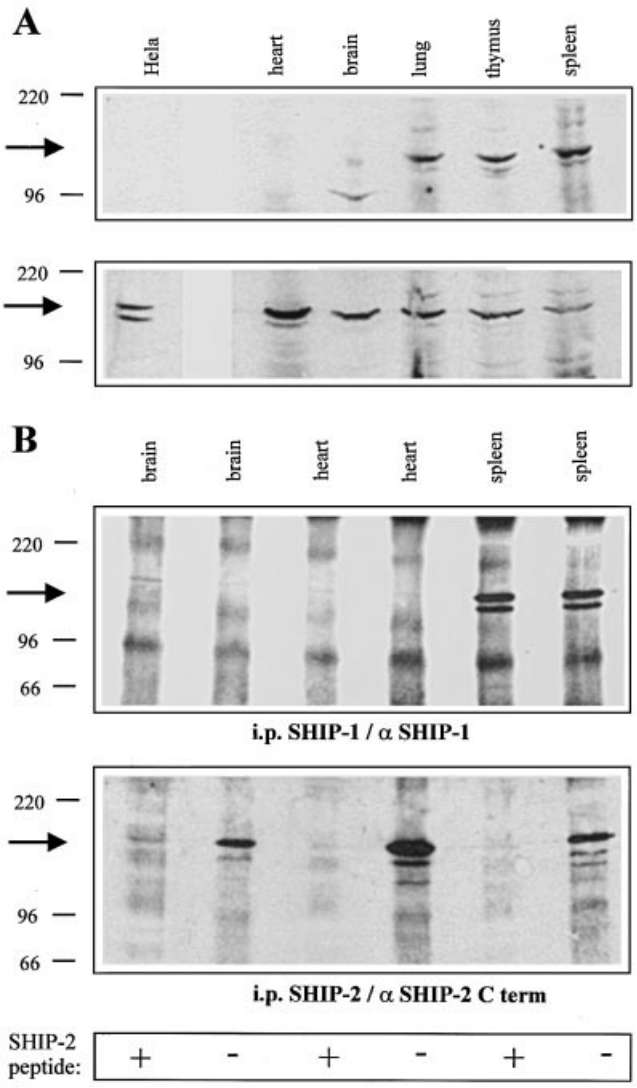

Figure 4 Western blot analysis of SHIP-1 and SHIP-2 expression in mouse tissues

(**A**) The expression levels of SHIP-1 (upper panel) and SHIP-2 (lower panel) were compared in protein from HeLa cells (120 μg of protein), heart (150 μg), brain (140 μg), lung (140 μg), thymus (35 μg) and spleen (120 μg), as indicated. (**B**) Mouse heart, brain and spleen were lysed and immunoprecipitated (i.p.) with anti-(SHIP-1) antibodies or anti-(SHIP-2 C-terminus) antibodies, in the presence or absence of 1 μg of SHIP-2 C-terminal peptide. The blots were probed with anti-(SHIP-1) antibodies (α SHIP-1) or anti-(SHIP-2 C-terminus) antibodies (α SHIP-2 C term). The positions of molecular mass markers are indicated (in kDa) at the left; SHIP-1 and SHIP-2 molecular masses (130–145 kDa and 150–160 kDa respectively) are indicated by arrows.

the expression of SHIP-2 in various brain areas, we checked by Western blotting the cortex, the cerebellum, freshly cultured murine astrocytes and NS20Y murine neuroblastoma cells. SHIP-2 was expressed in all these cells (Figure 5).

The two major populations of cells obtained after the mechanical dissociation of spleen cells are B-cells and T-cells. We compared spleen cells, T-cell-enriched spleen cells and T-cell-depleted spleen cells (B-cell-enriched population of cells) for expression of SHIP-1 and SHIP-2. Figure 6 shows that SHIP-2 expression was not dependent on the presence of T-cells in spleen, arguing in favour of the expression of SHIP-2 in B-cells.

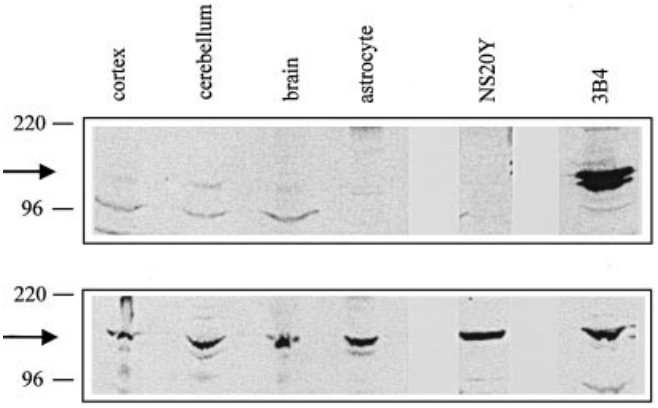

Figure 5 Expression of SHIP-1 and SHIP-2 in the nervous system

The expression levels of SHIP-1 and SHIP-2 were compared by immunoblot analysis in 3B4 T-cells (90 μg of protein), cortex (130 μg), cerebellum (70 μg), brain (140 μg), mouse astrocytes (35 μg) and NS20Y cells (150 μg), as indicated. The blots were probed with anti-(SHIP-1) (upper panel) antibodies or anti-(SHIP-2 C-terminus) antibodies (lower panel). The results are representative of two separate experiments. The positions of molecular mass markers are indicated (in kDa) at the left; SHIP-1 and SHIP-2 molecular masses (130–145 kDa and 150–160 kDa respectively) are indicated by arrows.

Moreover, when the same amounts of protein were loaded on the gel, SHIP-1 was expressed in spleen cells and T-cell-depleted spleen cells but at much lower levels in T-cell-enriched spleen cells.

SHIP-2 associates with Shc during ligation of the BCR and FcγRIIB receptor in B-cells

SHIP-1 has been reported to have a role in negative signalling for receptors that regulate the immune response [5–7,16,18]. SHIP-2 growth-factor-induced tyrosine phosphorylation [21], its association with Shc [13,21], and its ability to hydrolyse PtdIns(3,4,5)P_3 [20,21] suggest that SHIP-2 could have a similar role. No results have in fact been reported about a possible role of SHIP-2 in immune effector cells. In view of the tissue and cell distribution of SHIP-2 (see above, particularly the expression of SHIP-2 in a B-cell-enriched population of cells), we studied the tyrosine phosphorylation of SHIP-2 and its possible interaction with Shc in the A20 B-cell line. This model [6,7] offers the possibility of comparing the effect of positive or negative signalling in the same cells that we have shown to co-express SHIP-1 and SHIP-2.

The effects of the stimulation of BCR [by the F(ab′)$_2$ fragment of antibody against mouse IgG: positive signal] and the ligation of BCR to FcγRIIB (by intact antibody against mouse IgG: negative signal) on the tyrosine phosphorylation of SHIP-2 were compared. Tyrosine-phosphorylated bands of approx. 160, 150 and 145 kDa were immunoprecipitated with antibodies against SHIP-2 and detected with anti-phosphotyrosine monoclonal antibody in response to either positive or negative signalling (I. P. SHIP-2, Figure 7). The identity of the 145 kDa additional band is unknown; it could be a protein phosphorylated on tyrosine that associates with SHIP-2. Maximal phosphorylation was observed after 2 min with intact Ig (Figure 7). In agreement with previously published results (see [5,28]), SHIP-1 phosphorylation tested in parallel presented kinetics similar to those of a major phosphorylated band of 145 kDa. The same

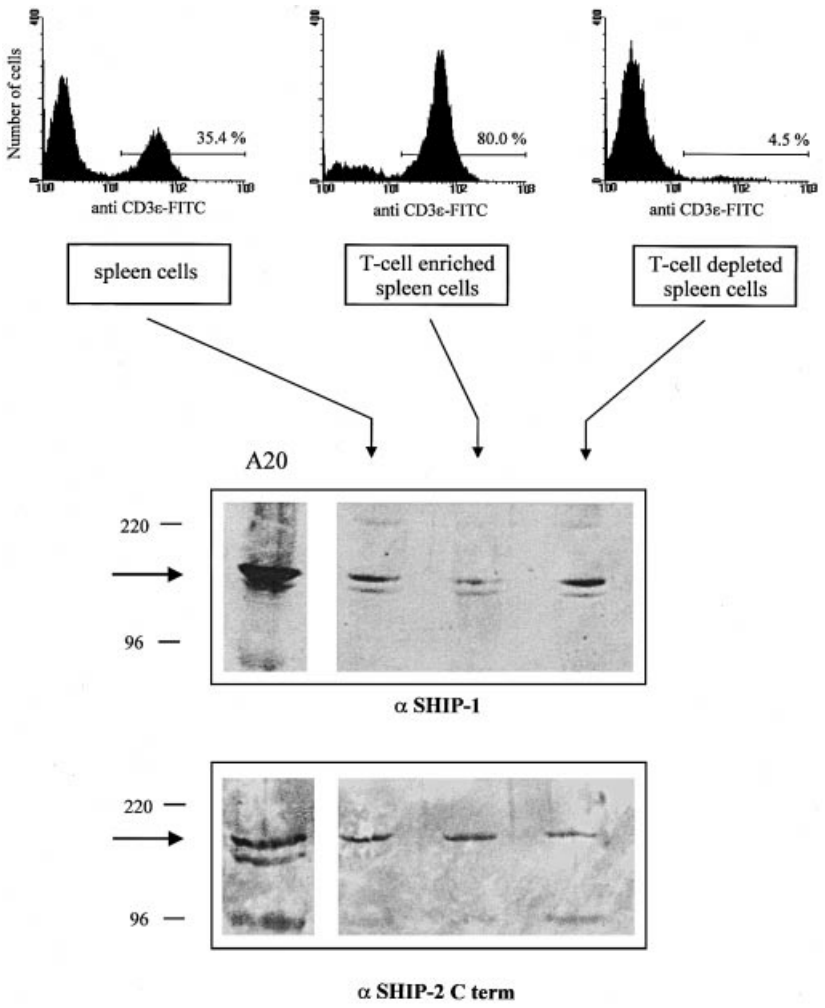

Figure 6 Expression of SHIP-1 and SHIP-2 in various spleen cell populations

The expression levels of SHIP-1 and SHIP-2 were compared by immunoblot analysis of A20 B-cells (140 μg of protein), spleen cells (36 μg), T-cell-enriched spleen cells (35 μg) and T-cell-depleted spleen cells (38 μg), as indicated. The blots were probed with anti-(SHIP-1) (α SHIP-1) antibodies or anti-(SHIP-2 C-terminus) antibodies (α SHIP-2 C term). The percentage of CD3ϵ-positive cells in each populations was analysed by one-colour immunofluorescence after staining with FITC-labelled anti-CD3ϵ antibody. The positions of molecular mass markers are indicated (in kDa) at the left; SHIP-1 and SHIP-2 molecular masses (130–145 kDa and 150–160 kDa respectively) are indicated by arrows.

amounts of SHIP-2 (or SHIP-1) were present in each condition tested, as shown when our blots were probed with antibodies against SHIP-2 (or SHIP-1) respectively (Figure 7). SHIP-2 tyrosine phosphorylation had recently been reported in non-haemopoietic cell lines stimulated by epidermal growth factor, platelet-derived growth factor and insulin [21]. B-cells were not tested, because Habib et al. did not detect SHIP-2 in A20 cells with their antibodies. The rapid kinetics of phosphorylation that we observed in response to ligation of BCR to FcγRIIB was comparable to the kinetics described for stimulation with epidermal growth factor and platelet-derived growth factor but was different from the long-lasting (2 h) SHIP-2 phosphorylation in response to insulin-like growth factor 1, nerve growth factor or insulin.

We tested the association of SHIP-2 to Shc at 2 min of stimulation. Although SHIP-1 and SHIP-2 were clearly detected in anti-Shc immunoprecipitates of intact IgG-stimulated cells, SHIP-1 was detected at lower levels and SHIP-2 was barely detectable in cells stimulated with $F(ab')_2$ fragments of mouse IgG. SHIP-1 and SHIP-2 were not detected in Shc immunoprecipitates of unstimulated cells (Figure 8).

DISCUSSION

In both mast cells and B-cells, SHIP-1 has been shown to inhibit immune receptor activation by binding to the tyrosine-phosphorylated ITIM of the co-receptor FcγRIIB [5,16,29]. The inactivation of SHIP-1 in B-cells results in enhanced proliferation in response to BCR–FcγRIIB ligation, indicating that FcγRIIB-mediated inhibition of BCR signalling is dependent on SHIP-1 [18]. The inhibition of B-cell activation by SHIP-1 might involve its 5-phosphatase activity's acting on PtdIns(3,4,5)P_3, one of its substrates *in vitro* [9] as determined in intact cells [7]. Alternatively, SHIP-1 might compete with Grb2 for Shc binding [15,16] as it has been shown that SHIP-1$-/-$ splenic cells display an enhanced mitogen-activated protein kinase activation in response to BCR–FcγRIIB ligation [18].

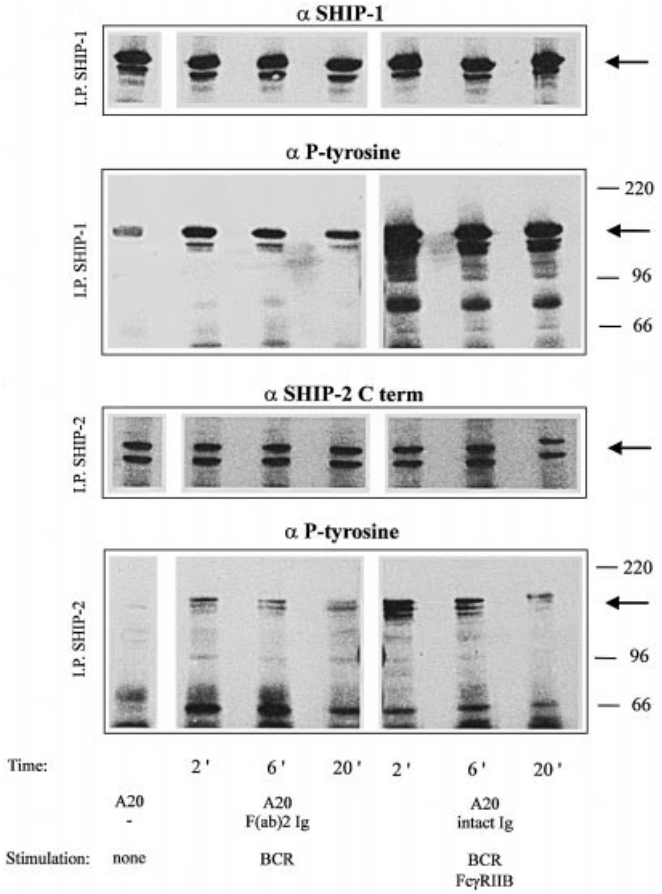

Figure 7 Kinetics of tyrosine phosphorylation of SHIP-1 and SHIP-2 proteins in response to ligation of BCR to FcγRIIB

A20 cells (10^8 cells) were incubated for 2, 6 or 20 min with medium, rabbit F(ab′)2 fragments against mouse IgG or intact rabbit IgG against mouse IgG. The cells were lysed and immunoprecipitated (I.P.) with anti-(SHIP-1) antibodies or anti-(SHIP-2 C-terminus) antibodies. From half of the immunoprecipitate, blots were probed with anti-phosphotyrosine (α P-tyrosine). From the other half, blots were probed with anti-(SHIP-1) (α SHIP-1) antibodies or anti-(SHIP-2 C-terminus) (α SHIP-2 C term) antibodies as control. The results are representative of two separate experiments. The positions of molecular mass markers are indicated (in kDa) at the right; the arrows indicate the position of SHIP-1 and SHIP-2 respectively.

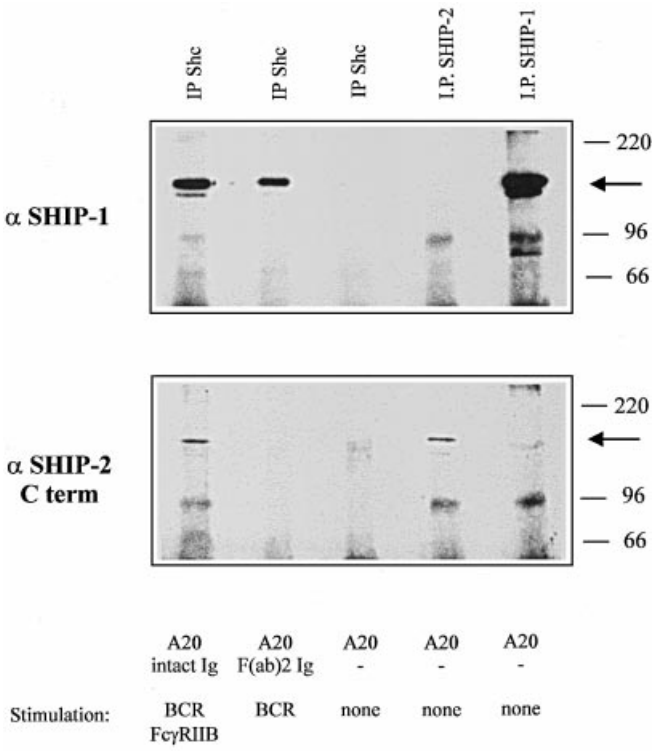

Figure 8 Ligation of BCR to FcγRIIB induces association of Shc with SHIP-1 or SHIP-2

A20 cells (10^8 cells) were incubated for 2 min with medium, rabbit $F(ab')_2$ fragments against mouse IgG or intact rabbit IgG against mouse IgG. Immunoprecipitation (I.P.) was performed with anti-Shc, anti-(SHIP-1) or anti-(SHIP-2 C-terminus) antibodies. The samples were analysed by Western blotting probed with anti-(SHIP-1) antibodies (upper panel) or anti-(SHIP-2 C-terminus) antibodies (lower panel). The results are representative of two separate experiments. The positions of molecular mass markers are indicated (in kDa) at the right; the arrows indicate the position of SHIP-1 and SHIP-2 respectively.

From a sequence comparison, SHIP-2 is related to SHIP-1 (Figure 1): its central part shows 64% amino acid identity with SHIP-1. SHIP-2 has an SH2 domain, a single NPXY site and a C-terminal proline-rich region. Recombinant SHIP-2 in bacteria demonstrates Ins(1,3,4,5)P_4 and PtdIns(3,4,5)P_3 phosphatase activities [20]. Therefore, on the basis of its sequence and phosphatase activity, SHIP-2 seems to be an alternative signalling protein that could have a similar function to that of SHIP-1 in signalling. Therefore the cell and tissue distributions of SHIP-1 and SHIP-2 could help in understanding the functional role of the two phosphatases.

In the present paper we have shown that SHIP-2 is expressed in both haemopoietic and non-haemopoietic cell lines; it migrates with a molecular mass of 160–150 kDa in SDS/PAGE gels. Our results disagree with those of Habib et al. [21], in which SHIP-2 was described as a 145 kDa protein. Those authors found that A20 and HL60 haemopoietic cell lines failed to express SHIP-2. We detected SHIP-2 in three lymphoid cell lines: A20, BCL1 and 3B4. In addition, SHIP-2 was seen in various populations of spleen cells enriched in B-cells or T-cells. The reason for the non-expression of SHIP-2 in the two lymphoid cells tested by Habib et al. [21] is not understood but could result from different affinities of the antibodies used in the two studies. In our hands, the observed molecular mass of SHIP-2 (160–150 kDa) determined by Western blotting is higher than that of SHIP-1 (145–130 kDa). It is higher than the theoretical molecular mass calculated from the full-length sequence of SHIP-2 (142 kDa) [20]. It is, however, identical with the observed molecular mass of SHIP-2 on Western blots when a full-length construct is expressed in COS-7 cells after transfection (X. Pesesse, unpublished work).

The complete distribution of SHIP-2 in mouse tissues has not yet been reported. We therefore compared the tissue distributions of SHIP-1 and SHIP-2 by Western blot analysis. Our results show clearly that SHIP-2 is more broadly expressed than SHIP-1. SHIP-2 is detected in all haemopoietic tissues tested. In contrast with SHIP-1, SHIP-2 is also present in heart and brain tissues. The presence of SHIP-2 in the central nervous system could perhaps be correlated to the presence of a sterile alpha motif (SAM) in the SHIP-2 C-terminal sequence (residues 1200–1258 [19] but not present in SHIP-1; Figure 1). This SAM domain is an evolutionary conserved protein-binding domain of 60–70 residues. It has been found in proteins from such diverse organisms as fungi, protozoans and animals [29]. The possible functions of SAM-containing proteins reveal a surprisingly

common theme: the regulation of the developmental process. SAM domains are identified in the C-terminus of all known ephrin-related receptor protein tyrosine kinases, the largest group of receptor protein tyrosine kinases specifically expressed in the central nervous system [29].

The existence of two SHIPs in the inositol 5-phosphatase family is reminiscent of the existence of two SH2-containing protein tyrosine phosphatases (SHPs). SHP-1 is expressed primarily in haemopoietic cells, whereas SHP-2 is expressed ubiquitously (reviewed in [30]). In most systems SHP-1 seems to be a negative regulator of protein tyrosine kinase signalling, whereas SHP-2 has a positive (i.e. signal-enhancing) role [30]. SHP-1, SHP-2 [31] and SHIP-1 [14] bind to the same ITIM sequence of FcγRIIB. However, FcγRIIB-mediated inhibition in B-cells occurs in the absence of SHP-1 but requires SHIP-1 [32]. The role of SHP-2 is unclear [33].

We have shown here that in B-cells, SHIP-2, like SHIP-1, is tyrosine-phosphorylated and associates with Shc under negative conditions of signalling (i.e. ligation of BCR to FcγRIIB). We did not observe any change in Ins(1,3,4,5)P_4 phosphatase activity associated with SHIP-1 or SHIP-2 (i.e. determined after immunoprecipitation) when A20 cells had been stimulated (results not shown). The catalytic activity of SHIP-1 has been reported (e.g. in platelets) not to be regulated after receptor stimulation, suggesting that its subcellular localization after stimulation is important [7,27,32]. The same concept could apply for SHIP-2 molecules that might interact with SHIP-2 (and distinct from Shc) need to be identified in haemopoietic cells.

The similar behaviours of SHIP-1 and SHIP-2 in B-cells suggest a possible role for SHIP-2 in the negative regulation of immunocompetent cells mediated by FcγRIIB. A recent report [8] on Akt kinase regulation in B-cells supports this hypothesis. The activation of Akt kinase by BCR stimulation has been shown to be inhibited by wortmannin, indicating that BCR cross-linking activates Akt in a manner dependent on phosphoinositide 3-kinase. This activation is inhibited by the ligation of BCR to FcγRIIB, an effect that is not observed in SHIP-1-deficient B-cells. Thus the presence of SHIP-2 in B-cells, its phosphorylation and association to Shc during ligation of BCR to FcγRIIB (the present study) and a slight inhibition of Akt during the condition observed in SHIP-1-deficient cells [8] suggest the participation of SHIP-2 in FcγRIIB negative signalling. However, other results argue against behaviour of SHIP-1 similar to that of SHIP-2. The severity of the phenotype observed in SHIP-1-deficient mice [17] suggests that SHIP-2 does not have a compensatory effect in the absence of SHIP-1. Moreover, SHIP-2 tyrosine phosphorylation and association with Shc has been reported in non-haemopoietic cell lines stimulated by growth factors and insulin [21], indicating clearly that SHIP-2 could be involved in a signal transduction cascade with no participation of SHIP-1.

The two main conclusions from the study presented here are that SHIP-2 is expressed in both haemopoietic and non-haemopoietic cells and that, in B-cells, SHIP-2, like SHIP-1, is tyrosine-phosphorylated and associates with Shc under negative conditions of signalling. Various models of SHIP-1 mechanism of action have been presented in B-cells [5,7] and mast cells [5,34,35]; these could therefore be re-evaluated in the light of possible competitive interactions between Shc and SHIP-1 or SHIP-2.

During the preparation of this paper, Wisniewski et al. [36] reported the presence of SHIP-2 in chronic myelogenic leukaemia progenitor cells. In their cells, SHIP-2 was constitutively tyrosine-phosphorylated and associated with Shc. The authors reported a molecular mass of 155 kDa for SHIP-2, which is comparable to the 160–150 kDa that we observed in our experiments in mouse cells and tissues with our antibodies. Both SHIP-1 and SHIP-2 are expressed simultaneously in haemopoietic progenitor cells as observed in the different cell lines that we have tested.

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