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Predominant expression of the *src* homology 2-containing tyrosine phosphatase protein SHP2 in vascular smooth muscle cells

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Abstract *src* homology 2 (SH2)-containing protein-tyrosine phosphatase SHP2 is known to transduce positive signals from activated receptor protein-tyrosine kinases such as platelet-derived growth factor receptor (PDGFR) β and insulin receptor. Here, we demonstrate the physiological expression of SHP2 in rats. In northern and western blot analyses, SHP2 expressions were recognized in all tissues, but their expression levels varied significantly among tissues; it is lowest in the liver and kidney. Immunohistochemical staining and in situ hybridization showed SHP2 was expressed ubiquitously but predominantly in vascular smooth muscle cells (SMC). During the development of granulations, SHP2 was expressed predominantly in vascular SMC and also highly expressed in capillary cells. The functional associations of SHP2 with PDGFRβ, which transduces major growth signals in vascular SMC, identify a crucial function of SHP2 in blood vessels in consert with PDGFRβ.

Key words *src* homology 2 · Protein-tyrosine phosphatase · Immunohistochemistry · Vascular smooth muscle cells

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Introduction

Protein-tyrosine kinases (PTKs) and protein-tyrosine phosphatases (PTPs) are crucial regulatory enzymes for cellular growth, differentiation and development. Receptor PTKs initiate their signals via tyrosine phosphorylation and transduce intracellular signalling pathways [19]. These phosphotyrosines serve as binding sites for secondary signalling molecules containing *src* homology 2 (SH2) domains, which show highly specific interactions [16]. We and others have cloned a human intracellular SH2-containing protein, SHP2 (also known as SHPTP2, SHPTP3, PTP1D, PTP2C and the mouse homologue Syp) [1, 3, 4, 7, 8, 20], and its gene has been assigned to human chromosome 12q24.1 [11]. SHP2 carries two SH2 domains, which bind directly to activated growth factor receptors, such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) receptors [7, 12]. SHP2 also binds to the signalling adaptor Grb2 and insulin receptor substrate 1 upon activated tyrosine phosphorylation in response to PDGF and insulin, respectively [6, 13]. Several investigations have demonstrated that SHP2 positively transduces growth signals to activate *ras* [14, 15]. These findings are consistent with the extensive homology of SHP2 with *Drosophila corkscrew*, which functions in positive signal transduction of the *Torso*-encoded receptor PTK in concert with D-*Raf* kinase [17], and clearly show that SHP2 is a key molecule for signal transductions via tyrosine phosphorylation.

Several investigations have already shown that SHP2 transcripts are expressed ubiquitously. However, little is known about expression of its protein product. Here, we show predominant expression of SHP2 protein in vascular smooth muscle cells (SMC).

Materials and methods

For northern blot analysis, total RNA samples were extracted from various rat tissues by the guanidium/caesium chloride procedure and analysed as described [2]. Briefly, 10 μg total RNA was elec-

trophoresed (1% agarose gel), transferred to nitrocellulose filters, and hybridized with the ^{32}P-labelled SHP2 cDNA probe [1].

For preparation of anti-SHP2 polyclonal antibody, a region of the SHP2 cDNA encoding amino acids 12–441 was isolated and cloned into the bacterial expression plasmid pMAL-cRI (NEB). The anti-SHP2 sera were generated in New Zealand white male rabbits by immunizing purified bacterial recombinant pMAL-SHP2 fusion protein. The affinity-purified anti-SHP2 antibody was obtained using Affi-gel 10 coupled with the recombinant pMAL-SHP2 fusion protein. The affinity-purified anti-SHP2 antibody reacted with a 68 kDa protein of total cell lysate of rat brain tissue and recombinant 49 kDa SHP2 (amino acids 6–213)-glutathione S-transferase fusion protein (Santa Cruz) in western blot analysis, but neither preimmune serum nor the antiserum preincubated with the cognate pMAL-SHP2 fusion protein detected the SHP2 proteins (Fig. 1A), confirming SHP2 specificity.

For western blot analysis, normal Wistar male rat tissues were homogenized with RIPA buffer [100 mM sodium chloride (NaCl), 2 mM EDTA, 10 mM sodium orthovanadate, 1 mM PMSF, 1% NP40 and 50 mM TRIS (pH 7.2)] and their protein concentrations were calculated with Protein Assay (BioRad). Each lysate (75 µg) was subjected to 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and transferred to immobilon (Millipore). The blots were incubated with blocking solution containing 3% bovine serum albumin, 10 mM TRIS [pH 8.2] and 140 mM NaCl, incubated with the affinity-purified anti-SHP2 antibody (2 µg/ml) for 2 h, followed by an additional 1 h of incubation with peroxidase-conjugated anti-rabbit IgG antibody (Amersham, Buckinghamshire, UK). The reactivity was visualized with an enhanced chemiluminescence detection system (Amersham).

For immunohistochemistry, 5 µm thick cryostat sections from fetal rats (obtained from 16-day-old fetal Wistar rats) or adult Wi-

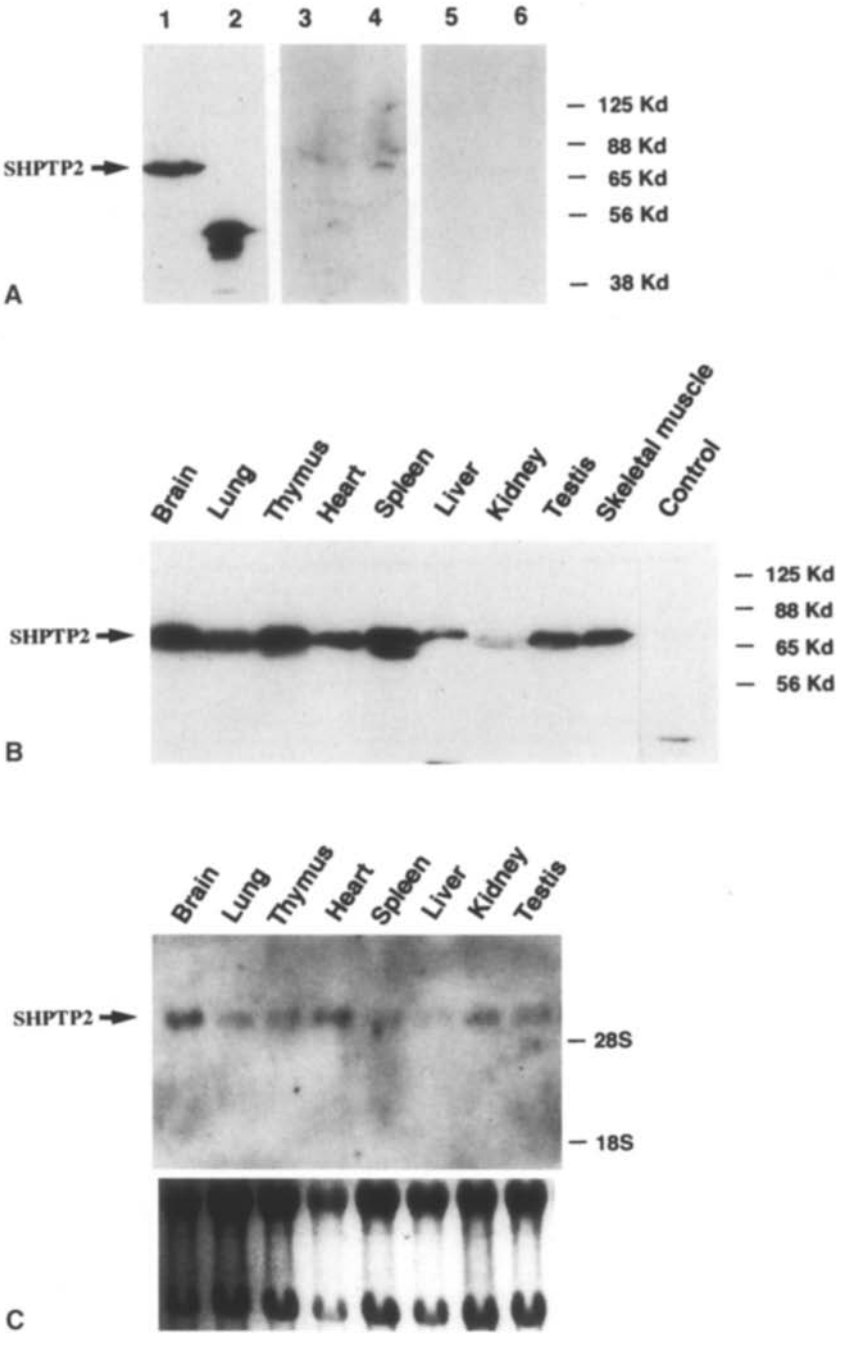

Fig. 1A–C *src* homology 2-containing tyrosin phosphatase protein (SHP2) expression in various rat tissues. **A** Total lysate (75 µg) of rat brain (lanes 1, 3 and 5) and 2 µg of the recombinant 49-kDa SHP2(amino acids 6–213)-glutathione S-transferase fusion protein (lanes 2, 4 and 6) were loaded onto 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The 68-kDa SHP2 protein in rat brain lysate and the recombinant 49-kDa SHP2 protein were detected by an antiserum (2 µg/ml) to SHP2 (lane 1 and 2, respectively), but greatly inhibited by 10 µg/ml of the cognate pMAL-SHP2 fusion protein (lanes 3 and 4). Preimmunized rabbit IgG (2 µg/ml) did not show the signals (lanes 5 and 6). **B** Each lane was loaded with the lysate (150 µg) of adult rat tissues homogenized in RIPA buffer. A major band of 68-kDa SHP2 protein is shown in all tissues we examined, and its expression level varies greatly. These findings were consistent with two other independent experiments. The reactivity was visualized with an enhanced chemiluminescence detection system. **C** Total RNA samples (10 µg/lane) extracted from various adult rat tissues were analysed by northern blots using a human SHP2 probe [1]. The positions of ribosomal RNA markers are indicated to the right of the blot. Ribosomal RNAs stained with ethidium bromide are shown for relative amounts of total RNA loaded

star rats were air-dried and fixed in cold acetone for 10 min. The sections were blocked with 10% goat serum in phosphate-buffered saline [PBS; 137 mM NaCl, 2.7 mM potassium chloride, 8 mM disodium hydrogen phosphate (Na_2HPO) and 1.5 mM potassium dihydrogen phosphate (KH_2PO_4)] for 2 h at room temperature. The sections were then incubated with the affinity-purified anti-SHP2 antibody (10–20 μg/ml), a monoclonal antibody 1A4 to α-smooth muscle (α-SM) actin (DAKO; 0.9 μg/ml), an affinity-purified factor VIII related antigen antiserum (DAKO; 10 μg/ml), or pre-immunized rabbit antibody (20 μg/ml) in PBS containing 2% BSA overnight at 4° C. After the reaction, the detection and colour development were performed using HISTOFINE kits (Nichirei, Japan). To confirm the signals, the affinity-purified SHP2 antibody (20 μg/ml) was pre-incubated with SHP2(amino acids 12–441)-fusion protein and used for immunostaining. For generation of granulation tissue, 12-week-old male Wistar rats were anaesthetized and 1 ml of complete Freund's adjuvant (DIFCO, Mich.) with 1 mg ovalbumin was injected subcutaneously twice at 2-week intervals after immunization of ovalbumin.

SHP2 in situ hybridization probes were prepared from a pBluescript plasmid (Stratagene, La Jolla, Calif.) containing a 0.3 kb fragment of human SHP2 cDNA insert. The riboprobe was generated by incorporation of digoxigenin-labelled UTP following Genius RA labelling kit instructions (Boehringer, Mannheim, Mannheim, Germany). Six micrometre-thick paraffin-embedded specimens, from CB-17 mouse, which were fixed with 4% paraformaldehyde were processed as described before [18], and hybridization was carried out at 50° C for 36 h, followed by RNase digestion, and washing with 0.5–2× standard saline citrate. The slides were incubated with immunogold-conjugated anti-digoxigenin polyclonal antibody and visualized by silver enhancement with counterstaining with methyl-green. Using the sense-riboprobe of SHP2, there were no distinct signals (data not shown).

Results

Using an affinity-purified polyclonal antibody showing specific binding to SHP2 (Fig. 1A), we examined SHP2 protein expression in various tissues from adult rats, including brain, lung, thymus, heart, spleen, liver, kidney, testis and skeletal muscle. Western blot analysis showed that SHP2 protein was expressed in all tissues examined; it was most abundant in the spleen, thymus, brain and heart, and was found at low levels in the liver and kidney (Fig. 1B).

We also examined SHP2 mRNA expression in normal adult rat tissues. Northern blot analysis of adult rat tissues, including the brain, lung, thymus, heart, spleen, liver, kidney, and testis, indicated ubiquitous expression of SHP2 mRNA (approximately 6.8 kb) with the highest levels in the brain and heart (Fig. 1C). These findings are consistent with the results of northern blot analysis in human [4] and mouse tissues [7], and therefore, SHP2 mRNA expression pattern is conserved among these mammals. Taken together, SHP2 protein expression is overall consistent with its mRNA expression but, as seen in the kidney, it varied by some unknown mechanism.

To investigate SHP2 expression more precisely, we used immunohistochemical analysis with the affinity-purified anti-SHP2 antibody. The analysis revealed distinctly stained cells in blood vessels (Fig. 2A), and faint staining was also seen in adult rats throughout many tissues (data not shown) consistent with results showing ubiquitous expression of SHP2 protein. The staining with the antibody to SHP2 was specific, as control experiments using the pre-incubated affinity-purified anti-SHP2 antibody did not reveal these stained cells (Fig. 2D). To determine whether SHP2 is expressed in endothelial cells or vascular SMC, we examined the expressions of factor VIII related antigen and α-SM actin in the serial tissue sections. The cells expressing SHP2 protein

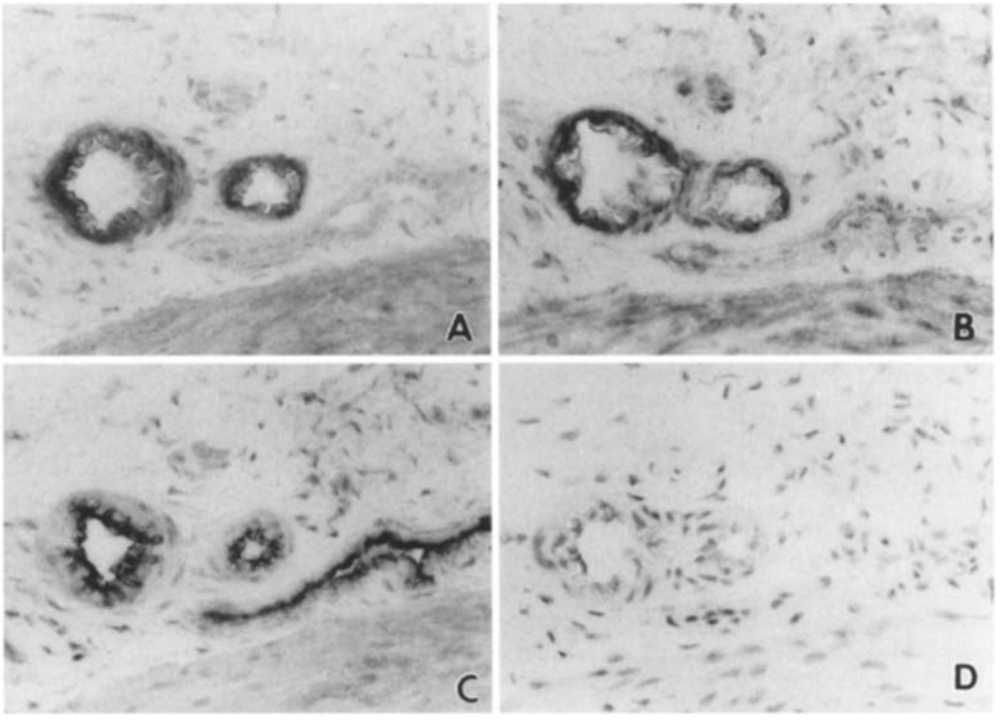

Fig. 2A–D Immunohistochemical staining of SHP2. Serial frozen sections from small intestine in adult Wistar rats were immunostained with anti-SHP2 (**A**), anti-α-smooth muscle (SM) actin (**B**) and anti-factor VIII related antigen antibodies (**C**). Affinity-purified anti-SHP2 antibody produced distinct staining of blood vessels and the stained cells also expressed α-SM actin, but did not express factor VIII related antigen. The immunoreactions were not detected when pre-incubated anti-SHP2 antibody was used (**D**). ×400

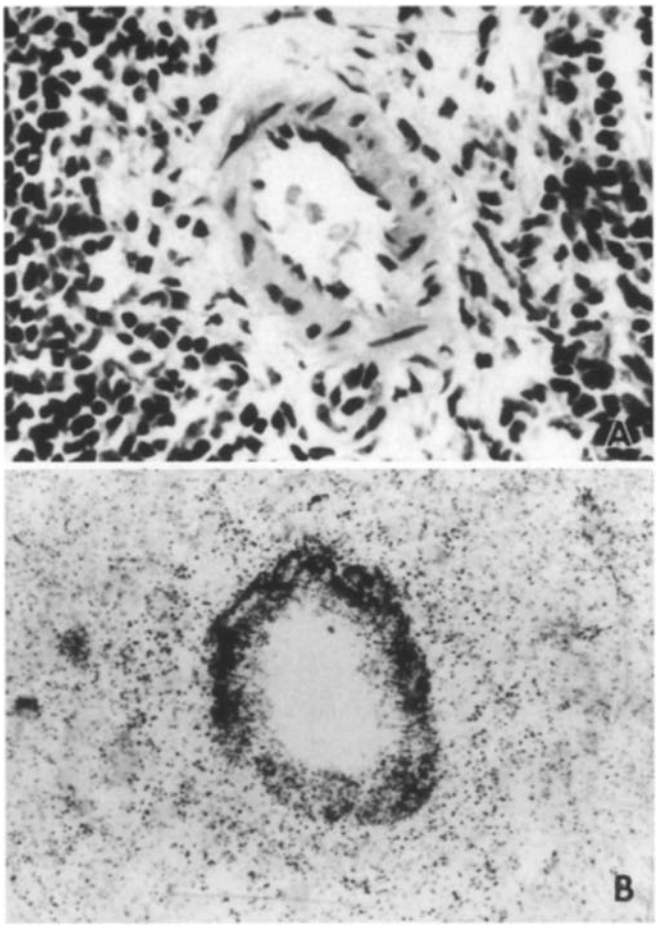

Fig. 3A, B In situ localization of SHP2 mRNA. **A** The serial tissue section was stained with haematoxylin and eosin. ×400. **B** The in situ hybridization study with probe to SHP2 showing preferential expression in vascular SM cells

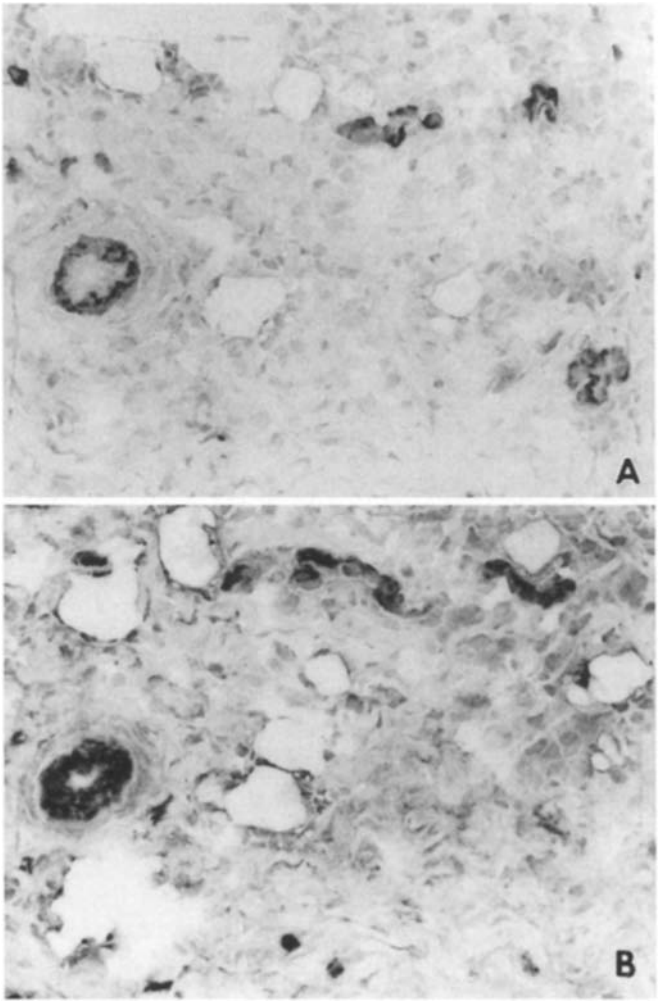

Fig. 4A, B SHP2 expression during development and granulomation tissue. Serial frozen sections from subcutaneous granulomation tissue were immunostained with the affinity-purified SHP2 (**A**) and anti-factor VIII related antigen antibodies (**B**). Affinity-purified SHP2 antibody produced distinct staining of blood vessels outside of the endothelium (which was positive for factor VIII related antigen). There are also sprouting capillary cells expressing SHP2 and the SHP2 expression was very similar to the von Willebrand factor expression. The immunoreactions were not detected when the pre-incubated anti-SHP2 antibody was used (data not shown). In the sections, there is non-absorbed oil derived from Freund's adjuvant. ×400

also showed α-SM actin (Fig. 2B), but factor VIII related antigen expression was negative (Fig. 2C), indicating SHP2 is predominantly expressed in vascular SMC.

To confirm this observation, we investigated SHP2 mRNA localization by in situ hybridization. The SHP2 mRNA was preferentially expressed in blood vessels and by comparison with haematoxylin and eosin staining, the strongly positive signals were confined to vascular SMC (Fig. 3). Thus, immunohistochemical localization of SHP2 protein is consistent with in situ localization of SHP2 mRNA.

To probe whether SHP2 expression is associated with early development of blood vessels, artificial granulation tissue was generated by several injections of complete or incomplete Freund's adjuvant with ovalbumin, and SHP2 expression there was analysed by immunohistochemical staining. In granulation tissue, the smaller vascular SMC also showed predominant expression of SHP2 as shown in Fig. 2. It is noteworthy that predominant expression of SHP2 was also seen in the several capillary cells in the granulation tissue, and the SHP2 expression was very similar to the expression of CD34 (Fig. 4).

Discussion

SHP2 is believed to be a positive regulator of PDGF, EGF and insulin growth signalling, all of which are known to bind their receptors carrying PTKs, and the ligand binding rapidly induces PTK activation. Thus, investigation of SHP2 expression is crucial for understanding how these growth signals are regulated. Here, we show predominant expression of SHP2 in vascular SMC. The predominant expression of SHP2 in vascular SMC may explain the low expression in liver and kidney which contain fewer vascular SMC. Considering the expression of PDGF receptor β (PDGFRβ) by the vascular

SMC [10] and potential functions of SHP2 as a positive regulator of PDGF-mediated growth signalling [18], SHP2 may work in concert with PDGFRβ to promote PDGF-mediated signalling in the vascular SMC.

We have also shown that the predominant expression of SHP2 is not limited to vascular SMC but is also seen in the small capillary cells in granulation tissue. Since capillary cells are endothelial cells and vascular SMC (pericytes), capillary endothelial cells may also express SHP2. Though it remains to be seen if SHP2 is predominantly expressed in capillary endothelial cells, this observation may suggest that SHP2 expression in transiently augmented in these cells. Interestingly, PDGFR has been demonstrated on capillary endothelial cells, but is absent on macrovascular endothelial cells [5], accounting for the unireponsiveness of the cells to PDGF. More recently, it has been demonstrated that SHP2 is physically associated with an autophosphorylated receptor-PTK TEK, which is expressed predominantly in the endothelial cells of actively growing blood vessels and functions in vascular development [9]. These accumulating data and our observations here strongly suggest that SHP2 may function at an early stage of angiogenesis and also operate in the control of vascular SMC.

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