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SHP2 Associates Directly with Tyrosine Phosphorylated p90 (SNT) Protein in FGF-Stimulated Cells

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In a number of cell lines responsive to basic fibroblast growth factor (bFGF), two major tyrosine phosphorylated proteins, of molecular weights around 120kDa and 90kDa, are precipitated along with the tyrosine phosphatase SHP2 from the lysates of stimulated cells. The docker protein Gab-1 represents at least part of the 120kDa protein(s). The p90 protein was identified as the SNT protein. The two SH2 domains of SHP2 bind directly and synergistically to tyrosine phosphorylated SNT. Tyrosine phosphorylated SNT does not bind SHP1 and does not appear to be an *in vivo* substrate of SHP2 but is likely to function as an adapter protein in FGF-signalling.

Fibroblast growth factor (FGFs) are a family of at least 10 polypeptides that have mitogenic, differentiative and angiogenic activity in cells of mesenchymal, neuronal and epithelial origin (1). FGFs act by binding and activating cell surface receptor tyrosine kinases (2). The signalling mechanisms of FGFRs are poorly understood. The only characterised substrate for FGFR is PLCγ which is recruited to Tyr^{766} upon receptor autophosphorylation (3). Another site on the activated FGFR, Tyr^{653}, has been shown to be necessary for the tyrosine phosphorylation of an unidentified 89-90kDa protein (4). This protein has been shown by our group and others to associate with the SH2 domain of Grb2 adapter protein (4-7). The 89-90kDa protein was also designated as SNT (6, 7) by virtue of its binding to the cell-cycle-associated protein $p13^{suc\text{-}1}$ (8). The tyrosine phosphorylation of the SNT protein has been implicated in the differentiation of PC12 cells upon stimulation with FGF or NGF (8). The small adapter proteins Grb2 and SHC provide a link between growth factor receptors and ras, via the nucleotide exchange factor Sos (9). Sos and Grb2, but not SHC, form a complex with SNT (5). Other signalling pathways are undoubtedly activated via the formation of interactive protein complexes mediated by these and similar adapter proteins. The strategy we have employed is to identify novel proteins that complex with adapter proteins using recombinant GST-fusion proteins containing the respective SH2 domains. While investigating the specificity of SNT binding to the SH2 domains of various proteins we observed that, apart from Grb2, SNT binds to the SH2 domains of SHP2. SHP2 is tyrosine phosphorylated and activated following stimulation of cells with EGF (10,11), PDGF (12), SCF (13), Epo (14) and IL3 (15). There has been no report of FGF inducing the phosphorylation/activation of either SHP2 or SHP1 although SHP2 has been shown to play a role in FGF-stimulated mesoderm induction in both Xenopus oocytes (16) and embryonic mice (41).

SHP2 is believed to be activated by tyrosine phosphorylation (18, 19), by engagement of its SH2 domains (15,17) and by association with phospholipids in the cell membrane (20). The PDGF receptor, IRS-1 (*in vitro*), Shc in T cells, an unidentified 45kDa protein (in EGF-stimulated 293 cells) and the SIRPα transmembrane protein have been shown to be substrates of SHP2 (21-25). SHP2 binds directly to growth factor receptors, such as PDGF receptor (26,27), EGF receptor (10,11), c-KIT (14), erythropoietin receptor (28) and the IL-3R-β chain (29) in response to stimulation with their respective ligands and undergoes tyrosine phosphorylation and putative activation. Although not tyrosine phosphorylated in response to insulin, SHP2 binds to tyrosine phosphorylated IRS-1 (30) and acts as a positive mediator of insulin and prolactin signalling (31-

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Abbreviations: bFGF, basic fibroblast growth factor; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; EPO, erythropoietin; SCF, stem cell factor; NGF, nerve growth factor; IL3, interleukin 3; HRPO, Horse-radish peroxidase; SH2, src homology 2; SHP2, src homology containing phosphatase 2; SHP1, src homology containing phosphatase 1; PLC-γ, Phospholipase C-γ; ECL, enhance chemical luminiscence; FRS2, FGF receptor substrate 2; GRB2, growth factor receptor binding protein 2; SNT, suc1-associated neurotrophic factor target; Gab, Grb2 adapter binder; GST, Glutathione-S-transferase.

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34). SHP2 may have an additional function as an adapter molecule because following treatment with PDGF, SCF, Epo or IL-3, Grb2 associates, via its SH2 domain, with tyrosine phosphorylated SHP2 (14,15,30,35). It has also been reported that Grb2 associates with SHP2 via its COOH-terminal SH3 domain, following EGF stimulation of cells (36).

With the implication that SHP2 is in some way involved in FGF signalling pathways we set out to identify tyrosine phosphorylated proteins that bind to the phosphatase, to ascertain the nature of the binding and to see whether such proteins were potential physiological substrates.

MATERIALS AND METHODS

Cells. Swiss 3T3 cells (ATCC CCL 92, Rockville, MD), human E5 thymic cells (a kind gift from Dr. E.F. Potworoski, Institut Armand-Frappier, Canada), rat phaeochromocytoma PC12 cells, human MRC 5 fibroblasts and human 293 embryonic kidney epithelial cells were cultured as described previously (7,39).

Antibodies and reagents. A monoclonal antibody to phosphotyrosine (PY20H), SHP1, PDGF-receptor antibodies were from Transduction Laboratories (Lexington, KY). SHP2 and GST antibodies and Grb2(SH2) and SHP2(SH2) N + C terminal GST fusion protein were from Santa Cruz Biotechnology (Santa Cruz, CA). Gab-1 antibodies and $p13^{suc-1}$ were from Upstate Biotechnology, Inc (Lake Placid, NY). The polyclonal antibody to SIRPα was a generous gift from Dr. Axel Ullrich, Max-Planck Institute fur Biochemie, Martinsried, Germany. Horseradish peroxidase-conjugated anti-rabbit or mouse IgGs were from Sigma (St. Louis, MO). EGF was from Genzyme (Cambridge, MA), PDGF-BB was from Sigma. Basic FGF was from Boehringer Mannheim (Mannheim, FRG). The ECL western blotting detection kit was from Amersham (Bucks, U.K.)

SDS–PAGE, immunoblotting and far western blotting. 7.5% SDS-PAGE was routinely carried out, followed by electroblotting onto PVDF membranes for probing with antibodies and detection by ECL. Far western blotting was used to investigate the direct binding of proteins to the SHP2(SH2) N+C/GST fusion protein. After precipitation with $p13^{suc-1}$ the eluted proteins were separated by SDS-PAGE, blotted onto PVDF membranes and incubated with the SHP2(SH2)N + C /GST fusion protein. The blots were incubated with anti-GST antibody which was subsequently detected by an anti-rabbit IgG/HRP antibody and the signals revealed by ECL. Blots were washed (4×10min) between each antibody incubation with 0.1% Tween-20 in PBS.

Single and sequential precipitations. The single and sequential precipitations were carried out as described previously (7) except in some cases lysates were first precipitated with SHP2 antibodies prior to reprecipitation with GST fusion proteins.

Production of SHP2-GSTs and transfections studies. The cDNAs of SHP2 N- and C-terminals (42) were generated by direct PCR from the template pXJ41SHP2 (gift from Dr. Catherine Pallen, IMCB), and subcloned into pGEX vectors to produce the corresponding GST fusion proteins in the E.coli DH5-α. To generate mammalian overexpression constructs, the full-length cDNA of SHP2 in pXJ41SHP2 or SHP1 in pXJ41neoSHP1 was subcloned into pcDNA3. Cys-Serine catalytic mutants (SHP2C459A) were made by site-directed mutagenesis using the QuickChange kit (Stratagene). 15μg of each plasmid was transfected into cells using Tfx-50 (Promega) according to manufacturer's instructions. 48h after transfection, cells were quiesced overnight and analysed for their expression and assessment for SNT phosphorylation.

RESULTS AND DISCUSSION

To investigate the likely tyrosine phoshorylation of SHP2 by FGF, Swiss 3T3 cells were treated with FGF (10ng/ml) or PDGF (10ng/ml) for 5 minutes, lysed and the lysates were subject to immunoprecipitation with anti-SHP2 antibodies. The bound proteins were eluted and analysed for tyrosine phosphorylation. From Fig. 1A, it is apparent in Swiss 3T3 cells that FGF and PDGF induced the tyrosine phosphorylation of several proteins that were co-precipitated with SHP2. Two bands appeared to be common to each agonist, a broad band around 115-130kDa and a narrower band around 75kDa. A broad band around 85-95kDa was seen only in lane 2 (FGF-treated) but was not present in lysates from PDGF-treated cells (lane 3). The 75kDa band was shown by western blotting to be SHP2 (data not shown). The tyrosine phosphorylation of SHP2 in the lysates from FGF-stimulated cells was always observed to be less that that seen with EGF or PDGF. A similar pattern of tyrosine phosphorylation was observed in SHP2 immunoprecipitates from FGF-stimulated E5 or MRC5 cells (data not shown). FGF therefore induced the tyrosine phosphorylation of two proteins (or groups of proteins) of approximately 90 and 120kDa that complex with SHP2 in FGF-sensitive cell lines. We therefore set out to identify these proteins.

Two tyrosine phosphorylated proteins in the 115-130 kDa molecular weight range, SIRPα and Gab-1, have recently been reported to be co-precipitated by anti-SHP2 antibodies (25,43). To verify this observation, Swiss 3T3 cells were stimulated with FGF, lysed and the lysates were subjected to immunoprecipitations with either anti-SIRP or anti-Gab-1 antibodies before separation on 7.5% SDS-PAGE and immunoblotting with antiphosphotyrosine antibodies. The result shown in Fig. 1B (left panel) shows that FGF stimulates the tyrosine phosphorylation of Gab-1 but not SIRP protein (data not shown). To demonstrate the association of SHP2 in the complex of proteins precipitated with anti-Gab-1 antibodies the anti-phosphotyrosine blots were stripped and reprobed with anti-SHP2 antibodies and the location of SHP2 was revealed. There was a significant increase in the amount of SHP2 that associates with Gab-1 in FGF-stimulated cells when compared with unstimulated cells (Fig. 1B, right panel). However, there is only a low level of tyrosine phosphorylation signal associated with SHP2 which could be seen only on longer exposure of the ECL-treated blot.

The 90kDa protein had a similar appearance and specificity to the SNT protein that is tyrosine phosphorylated following FGF stimulation and was shown previously to bind sequentially to $p13^{suc-1}$ and Grb2(SH2) using precipitation/recapture techniques (6,7). We used similar recapture techniques to identify the p90 protein that binds to SHP2 as SNT. Swiss 3T3 cells were treated with FGF at 10ng/ml for 10 minutes, lysed and

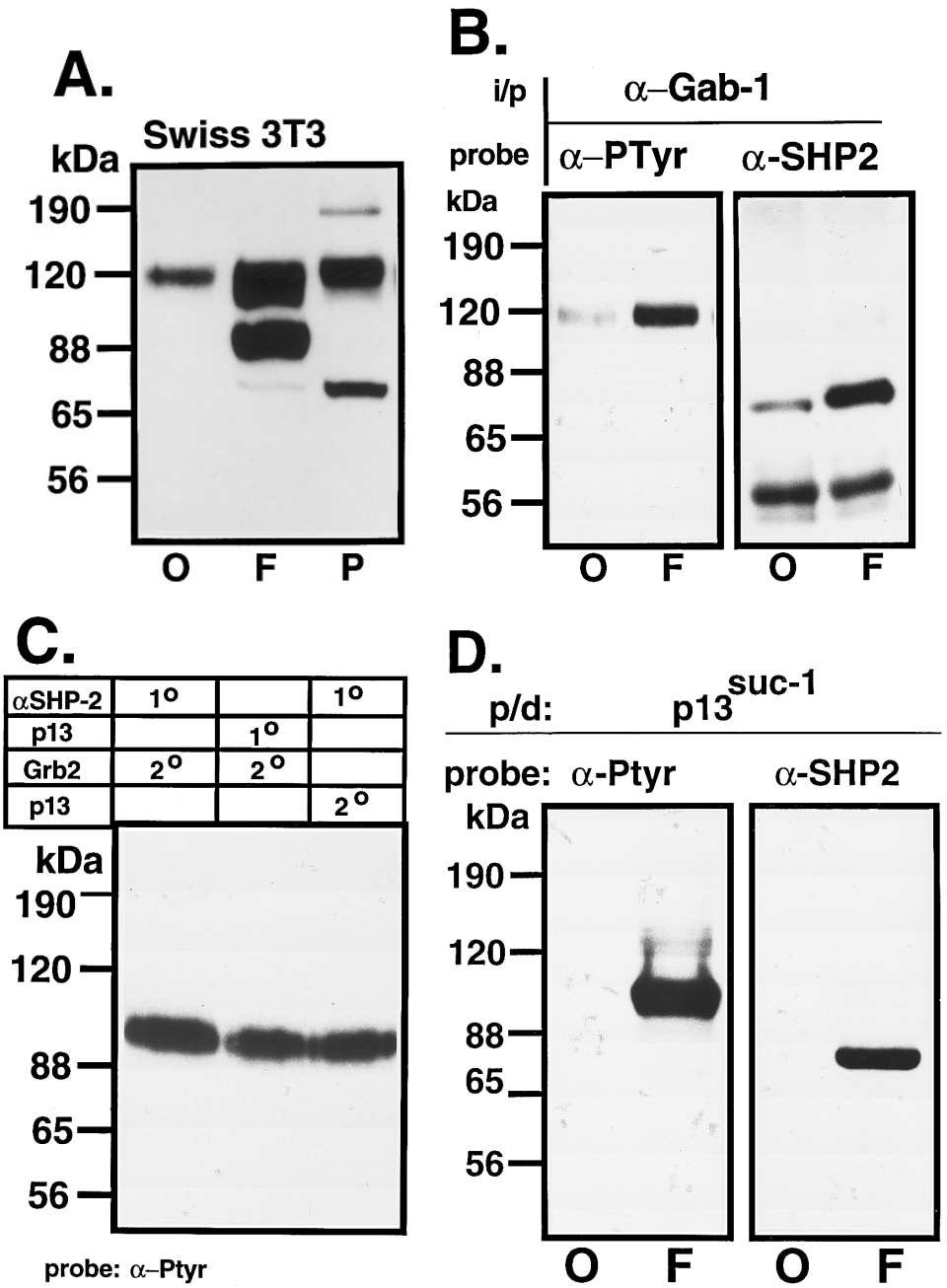

FIG. 1. (**A**) SHP2 immunopreciptation of the lysates from FGF-stimulated cells. Swiss 3T3, cells were stimulated with bFGF or PDGF for 10min before lysis. Lysates were incubated with anti-SHP2 antibodies, the proteins eluted and separated by SDS–PAGE and analysed for tyrosine phosphorylation using anti-phosphotyrosine antibodies and ECL. O - unstimulated cells, F - bFGF(10ng/ml) and P - PDGF (10ng/ml). (**B**). Immunoprecipitation of Gab-1. Swiss 3T3 cells were left unstimulated (O) or stimulated with bFGF (10ng/ml) (F) for 10min. Lysates were immunoprecipitated with anti-Gab-1 antibodies, the bound proteins eluted, separated by SDS-PAGE and immunoblotted initially with anti-phosphotyrosine antibodies and revealed by ECL (left panel). The blot was subsequently stripped and reprobed with anti-SHP2 antibody. (**C**). Precipitation/recapture of p90 protein. Swiss 3T3 cells were activated with bFGF and prepared for precipitation, as above. Lysates were incubated primarily (1°) with anti-SHP2 antibodies or $p13^{suc-1}$ protein. The bound proteins were eluted off and recaptured (2°) by Grb2(SH2)/GST fusion proteins or again by $p13^{suc-1}$ protein. The proteins were again eluted off, separated on SDS–PAGE, immunoblotted with anti-phosphotyrosine antibodies and revealed by ECL. (**D**). Association of SNT and SHP2. Swiss 3T3 cells were left unstimulated (O) or activated with bFGF (F) and prepared for precipitation, as above. The lysates were incubated with $p13^{suc-1}$ protein and the eluted proteins were separated by SDS–PAGE and immunoblotted initially with anti-phosphotyrosine antibodies and treated with ECL (left panel). The blot was subsequently stripped and reprobed with anti-SHP2 antibodies (right panel).

the lysates were incubated with $p13^{suc-1}$ fusion protein or precipitated with SHP2 antibodies prior the recapturing the precipitated proteins as described in *Materials and Methods.* Figure 1C demonstrates that different combinations of precipitation/recapture show a similar outcome. All three lanes show only one 90kDa protein. As it has been previously demonstrated that the $p13^{suc-1}$-binding p90 protein is the same as the protein that binds to the SH2 domain of Grb-2 it follows that this is also the same protein that is precipitated by SHP2 antibodies. This protein was originally designated as SNT by virtue of its binding to the $p13^{suc-1}$ protein. When $p13^{suc-1}$ was used to precipitate proteins from the lysates of FGF-stimulated and unstimulated cells there was a 90kDa tyrosine phosphorylated protein in the lysates from FGF-treated cells and not from untreated cells, as shown previously (Fig. 1D, left panel). When the membrane was stripped and probed with anti-SHP2 antibodies, SHP2 was seen only in the lysates of FGF-treated cells (Fig.1D, right panel). It is again noteworthy that although a significant amount of SHP2 protein is detected in the lane from FGF-stimulated cells there is no apparent tyrosine phosphorylation of this protein. The above data indicates that p90 SNT and Gab-1 associate in complexes with SHP2.

Since most interactions of SHP2 with other proteins occur via its SH2 domains, experiments were carried out to determine if SNT and Gab-1 from FGF-stimulated cells could bind to the SH2 domains of SHP2. Swiss 3T3 cells were left untreated, as controls, or treated with FGF(10ng/ml) for 10 minutes, lysed and the lysates were incubated with either GST-fusion proteins containing the SH2(N + C-terminal) domains from SHP2 or fusion proteins that contained either the N or C SH2 domain. The bound proteins were eluted, separated by SDS PAGE, blotted onto a PVDF membrane and probed with anti-phosphotyrosine antibodies. The result from such an experiment is shown in Fig. 2A. There was no binding of any 115kDa protein which suggest that Gab-1 from FGF-stimulated cells does not bind to any of the SHP2 SH2 domains. p90(SNT) was present in the lanes from FGF-stimulated cells. Only very low binding of SNT is observed with either the N or C terminal SH2 from SHP2 but synergistic binding is seen when both domains are present in the fusion protein. This is not without precedence as it has been observed that SHP2 shows similar binding synergy to a 100kDa tyrosine phosphorylated protein in myeloid cells (38).

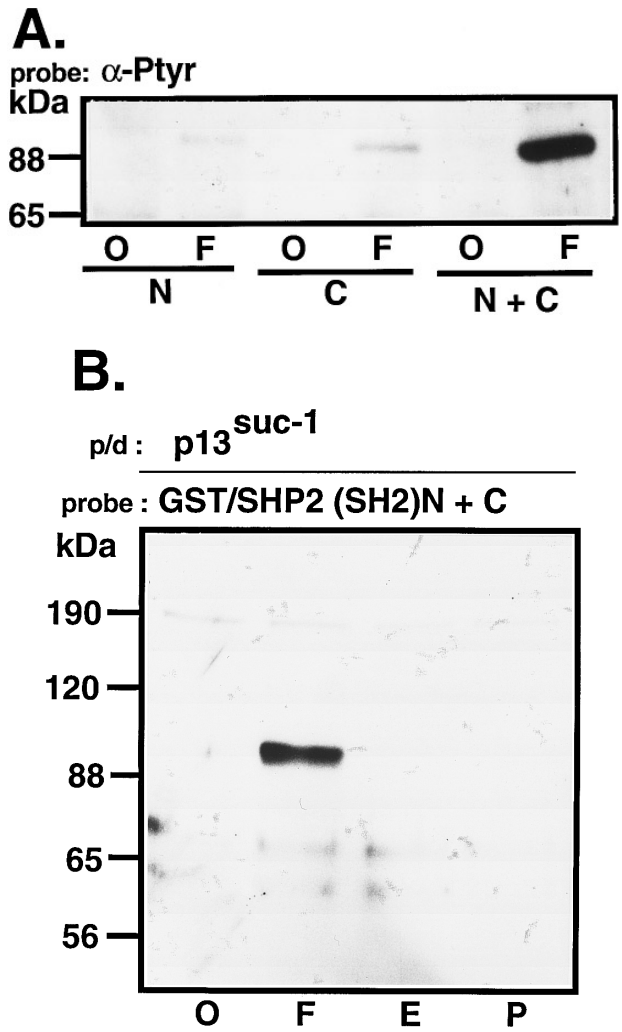

FIG. 2. (**A**) Swiss 3T3 cells were left unstimulated (O) or activated with bFGF (F) and prepared for precipitation, as above. The lysates were incubated with 15μg of GST-fusion proteins containing the NH2-terminal SH2 domain from SHP2 (N), the COOH-terminal SH2 from SHP2 (C) or the two SH2 domains (N + C). The eluted proteins were separated by SDS–PAGE and immunoblotted with anti-phosphotyrosine antibodies and treated with ECL. (**B**). Far western blot of SHP2(SH2)N + C terminal binding to $p13^{suc-1}$-binding proteins. Swiss 3T3 cells were left unstimulated (O) or activated with bFGF (F) for 10min, PDGF (P) for 10 min, EGF(E) 5min and prepared for pull-down (p/d), as above. The lysates were incubated with $p13^{suc-1}$ protein and the eluted proteins were separated by SDS–PAGE. The blots were incubated sequentially with SHP2(SH2)N + C/GST fusion protein, anti-GST antibodies and anti-rabbit IgG antibodies conjugated to horseradish peroxidase before being treated with ECL.

We next utilised a far western blotting technique to investigate whether the association between SNT and the SHP2 (SH2) N + C domains was direct. For this experiment, we utilised a GST fusion protein containing both SH2 domains of SHP2. Swiss 3T3 cells were either untreated or treated with FGF, EGF or PDGF for 5 or 10 minutes. The cells were lysed and the lysates were incubated with $p13^{suc-1}$conjugated to beads. The bound proteins were eluted, separated by SDS-PAGE, blotted onto PVDF membranes and the far western protocol as described in *Materials and Methods* was carried out. In Fig. 2B, only one band at 90kDa in the FGF lane is apparent. It has been shown previously that FGF and not PDGF or EGF induces the tyrosine phosphorylation of SNT (unpublished data). Therefore only tyrosine phosphorylated SNT binds to the SH2(N

+ C) domain of SHP2 and this binding is direct. In separate experiments using Gab-1 immunoprecipitation in the initial purification step followed by similar far western techniques, it was again apparent that tyrosine phosphorylated Gab-1 does not bind to the SH2 domains of SHP2 (data not shown).

The SIRPα docker protein was recently shown to bind directly to either SHP1 or SHP2 phosphatase. These two phosphatases shows a high percentage of homology to each other. As SNT also binds directly to SHP2, it was of interest to see whether SHP1 directly associates with this protein as well. Although SHP2 is ubiquitously expressed the expression of SHP1 is confined to haemopoietic and epithelial cells. Parallel immunoprecipitations utilising anti-SHP1 and anti-SHP2 antibodies were carried out on human 293 and rat PC-12 cells. These cell lines express both phosphatases and are responsive to FGF. The results of such an experiment, which was carried out using a similar immunoprecipitation protocols to that described for Fig. lA, are shown in Fig. 3A. In both 293 and PC-12 cells, tyrosine-phosphorylated p90(SNT) were precipitated *in vivo* by anti-SHP2 antibodies from cells stimulated with FGF. In contrast, SHP1 does not bind SNT *in vivo,* which is in agreement with the result from a previous *in vitro* SH2-binding experiment (data not shown). It appears therefore that SNT, unlike SIRPα, binds specifically to SHP2.

The identification of SNT as a SHP2 binding protein raised the possibility that SNT was a substrate for the phosphatase. To investigate this, 293 cells were transfected with vector alone (mock), the wildtype SHP2 cDNA or its mutant cDNA, where the catalytically essential cysteine 459 was mutated to alanine (SHP2C459A). The transfected cells were activated with FGF, lysed and the lysates precipitated with $p13^{suc-1}$ or Grb2(SH2) before being separated on SDS-PAGE and subsequently immunoblotted with antiphosphotyrosine antibodies. Immunoblotting whole cell lysates or SHP2 immunoprecipitations with anti-SHP2 antibodies demonstrated that the transfected cells expressed significantly increased amounts of wildtype or mutant SHP2 protein compared with the mock-transfected cells (Fig. 3B, left panel). When the tyrosine phosphorylated SNT was precipitated with either $p13^{suc-1}$ or Grb2(SH2) (Fig. 3B, right panel) there was no apparent difference in the p90/SNT signal between cells transfected with wildtype or mutant SHP2 which indicates that SNT is unlikely to be a substrate for the phosphatase. This is in contrast to recent data which demonstrate that SIRPα binds to the SH2 domains of SHP1 and SH2 and is a substrate for these phosphatases (25).

It is likely that SNT plays a similar role in FGF-signalling as the IRS-1 protein does in the insulin signalling pathway. These proteins act as docker proteins providing tyrosine phosphorylated sites, in addition to

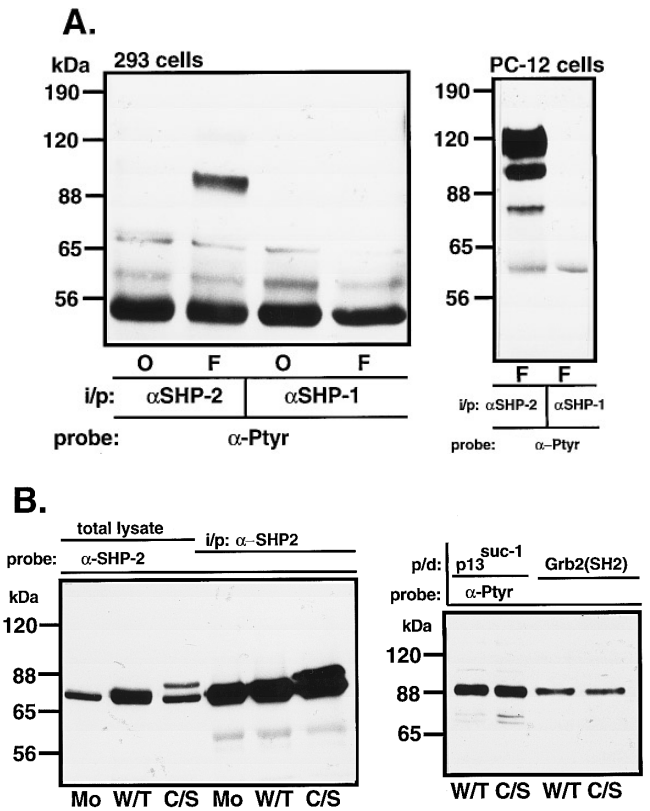

FIG. 3. (**A**). Comparative immunoprecipitations with anti-SHP1 and anti-SHP2 antibodies. 293 and PC12 cells were left unstimulated (O) or stimulated with bFGF (10ng/ml) (F) for 10min before lysis. Lysates were incubated with anti-SHP2 or anti-SHP1 antibodies, the proteins eluted and separated by SDS–PAGE and analysed for tyrosine phosphorylation using anti-phosphotyrosine antibodies and ECL. (**B**) Overexpression of wildtype and catalytically inactive SHP2 in 293 cells. 293 cells were transiently transfected with wild type (W/T) or mutated (C/S) cDNA for SHP2. Control cells were transfected with vector alone (Mo). Cells were lysed and precipitated with anti-SHP2 antibodies and these elutes or whole cell lysates were separated by SDS–PAGE and probed with anti-SHP2 antibodies and ECL (left panel). Other plates of transfected cells were treated with bFGF (10ng/ml) for 10min, lysed and the lysates were incubated with $p13^{suc-1}$ protein or Grb2(SH2)/GST fusion proteins and the eluted proteins were separated by SDS–PAGE and immunoblotted with anti-phosphotyrosine antibodies and treated with ECL (right panel).

those on the receptor, for the interaction of various adapter proteins. It is likely that SHP2 plays a role as an adapter in these two pathways. We have shown also by far western techniques that Grb2 can bind directly via its SH2 domain, to tyrosine phosphorylated sites on SHP2 which provides another means of activating the Ras/Raf/MAP kinase pathway. Whilst this manuscript was in preparation the likely protein sequence for SNT was published (37). This 56kDa protein, termed FRS2, has a membrane-anchoring myristylation site, a PTB domain and four Grb2-binding sites. The sequence of the protein and mutational analysis indicates that this protein has a role as a docker protein

in close proximity to the FGF receptor and upstream of the MAP kinase pathway. There are several sites on the protein containing the consensus binding motif for SHP2, pY-V/I/T-X-V/L/I, that was derived from binding studies using a degenerate peptide library (40). Mutations of these sites will indicate which are actual binding sites and the effects on downstream signalling pathways or neurite outgrowth.

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