

SHP-2 Regulates the Phosphatidylinositol 3'-Kinase/Akt Pathway and Suppresses Caspase 3-Mediated Apoptosis

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The Src homology domain 2 (SH2)-containing tyrosine phosphatase SHP-2 has been implicated in the regulation of the phosphatidylinositol 3'-kinase (PI3K)/Akt pathway. The ability of SHP-2 to regulate the PI3K/Akt pathway is suggested to result in the positive effect of SHP-2 on cell survival. Whether SHP-2 regulates insulin-like growth factor-1 (IGF-1)-dependent activation of Akt at the level of PI3K has yet to be established. Furthermore, the identification of the down-stream apoptotic target engaged by SHP-2 in cell survival also has yet to be determined. Here, we show that overexpression of a catalytically inactive mutant of SHP-2 inhibited insulin-like growth factor-1 (IGF-1)-dependent PI3K and Akt activation. Consistent with the observation that SHP-2 participates in pro-survival signaling fibroblasts expressing a deletion within exon 3 of SHP-2, which results in a truncation of the amino-terminus SH2 domain (SHP-2^{Ex3-/-}), were hypersensitive to etoposide-induced cell death. SHP-2^{Ex3-/-} fibroblasts exhibited enhanced levels of etoposide-induced caspase 3 activity as compared to wild-type fibroblasts and the enhanced level of caspase 3 activity was suppressed by a caspase 3-specific inhibitor. Re-introduction of wild-type SHP-2 into the SHP-2^{Ex3-/-} fibroblasts rescued the hypersensitivity to etoposide-induced caspase 3 activation. The effects of abrogating SHP-2 function on cell survival were not specific to the loss of the amino-terminus SH2 domain of SHP-2 since RNAi-mediated knock-down of SHP-2 also reduced cell survival. Taken together, these data indicate that the catalytic activity of SHP-2 is required to regulate the PI3K/Akt pathway and thus likely participates in anti-apoptotic signaling by suppressing caspase 3-mediated apoptosis. *J. Cell. Physiol.* 199: 227–236, 2004. © 2003 Wiley-Liss, Inc.

Cell life and death are regulated by growth factors, which transduce their intracellular signals via a network of pathways controlled by the net level of tyrosyl phosphorylation which is regulated by the opposing actions of protein tyrosine kinases (PTKs¹) and protein tyrosine phosphatases (PTPs). PTKs activate two well characterized cell survival cascades that are regulated by tyrosyl phosphorylation. The first pathway is the Ras/Raf/extracellular-regulated kinases 1 and 2 (Erks) signaling cascade. Activation of the Erks leads to the phosphorylation and subsequent activation of Rsk which catalyzes the phosphorylation and inactivation of the Bcl-2 pro-apoptotic family member protein BAD (Bonni et al., 1999; Ballif and Blenis, 2001). The rapid post-translational modification of BAD is also accompanied by a slower transcriptional up-regulation of anti-apoptotic genes such as *Bcl-2* and *Bcl-X_L* which are induced in an Erk/Rsk/CREB-dependent pathway (Bonni et al., 1999; Jost et al., 2001). The second pathway involved in cellular survival is the phosphatidylinositol 3'-kinase (PI3K)/Akt (protein kinase B) pathway (Datta et al., 1999). It is now well established that

Abbreviations: FBS, fetal bovine serum; GFP, green fluorescent protein; IRS-1, insulin receptor substrate-1; IGF-1, insulin-like growth factor-1; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; PDGF, platelet-derived growth factor; PI3K, phosphatidylinositol 3'-kinase; Erk, extracellular-regulated kinase; SH2, Src homology domain 2; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis.

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growth factor-mediated stimulation of PI3K results in the generation of phosphatidylinositol lipids, which lead to the activation of the serine/threonine kinase, Akt. Pro-apoptotic targets such as BAD, forkhead, and caspase 9 are phosphorylated and inactivated by Akt (Datta et al., 1997; Cardone et al., 1998; Bonni et al., 1999; Shi, 2002). Caspase 9 in turn activates the effector caspase, caspase 3, which leads to apoptotic cell death (Shi, 2002). Thus, both the Ras/Raf/Erk and PI3K/Akt pathways converge to promote cell survival.

Although it is now appreciated that PTKs are critical components of cell survival, the involvement of PTPs in this process has yet to be fully defined. The ubiquitously expressed Src homology domain 2 (SH2) domain-containing protein tyrosine phosphatase, SHP-2, has been shown to positively regulate the ability of several receptor PTKs to activate the Ras/Raf/Erks signaling cascade (Noguchi et al., 1994; Yamauchi et al., 1995; Bennett et al., 1996; Saxton et al., 1997; Shi et al., 2000). In most cases, the catalytic activity of SHP-2, which is activated upon binding of its SH2 domains to their cognate phosphotyrosyl target, is required to propagate growth factor and cytokine-mediated signaling to the Erks (Neel and Tonks, 1997; Barford and Neel, 1998). Genetic evidence based on studies of the SHP-2 homolog in *Drosophila* (corkscrew) and *C. elegans* (ptp-2), substantiate the notion that SHP-2 is a positive signaling component of the Ras/Raf/Erk signaling module (Perkins et al., 1996; Allard et al., 1998; Gutch et al., 1998; Van Vactor et al., 1998). Biochemical and genetic epistasis experiments suggest that SHP-2 acts either upstream of, or parallel to, Ras (Noguchi et al., 1994; Shi et al., 2000), and this is thought to be one of the ways SHP-2 couples to the Erks. However, SHP-2 signals via multiple downstream effectors that include both Erk-dependent and independent pathways and in most cases, but not all, this involves its catalytic activity. In cases where SHP-2 signals independently of its PTP domain, it does so by acting as an adaptor by binding within its C-terminus to Grb2 (Li et al., 1993; Bennett et al., 1994; Bjorbak et al., 2001), this too could explain how SHP-2 couples to the Erk pathway.

Recent work has found that SHP-2 is involved in growth factor-mediated PI3K activation (Hakak et al., 2000; Wu et al., 2001; Zhang et al., 2002). However, whether the catalytic activity of SHP-2 is required for PI3K activation has yet to be demonstrated. Consistent with the role of SHP-2 in PI3K/Akt regulation the ability of SHP-2 to affect cell survival has been proposed based on the observation that fibroblasts containing a deletion within exon 3 of SHP-2 (SHP-2^{Ex3-/-}) exhibit reduced levels of adherence as compared to wild-type fibroblasts treated with a proteasome inhibitor (Hakak et al., 2000). Although these results suggest a role for SHP-2 in apoptosis signaling the down-stream apoptotic targets engaged by SHP-2 still remains to be identified.

In this report, we show that the catalytic activity of SHP-2 is required for insulin-like growth factor-1 (IGF-1)-mediated PI3K activity and subsequently the activation of Akt. Consistent with the role for SHP-2 in cell survival, we show that SHP-2 suppresses apoptosis via a caspase 3-dependent pathway. Taken together, these data implicate SHP-2 as playing a central role in cell survival signaling.

EXPERIMENTAL PROCEDURES

Cell culture

Fibroblasts derived from mice containing either a deletion within exon 3 of SHP-2 that removes amino acids 46–110 of the amino terminus SH2 domain (SHP-2^{Ex3-/-}) or from wild-type litter-mate controls have been described previously (Saxton et al., 1997). 293, C2C12, and NIH 3T3 cells were obtained from ATCC. Cells were cultured at 37°C, 5% CO₂ in Dulbecco's modified Eagle medium (Invitrogen Corporation, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO), 1 mM sodium pyruvate (Invitrogen Corporation), 5 U/ml penicillin, and 50 µg/ml streptomycin (Sigma).

Plasmids, transient transfections, adenoviral infection, and generation of rescued SHP-2^{Ex3-/-} stable cell lines

PIRES-green fluorescent protein (GFP) plasmids containing either wild-type or a catalytically inactive mutant of SHP-2 (SHP-2C459S) have been described previously (Kontaridis et al., 2002). Transient transfections were performed in 293 cells using standard calcium phosphate precipitation techniques. Following transient transfections, 293 cells were serum-starved for 16–24 h in DMEM containing 0.1% FBS plus 1 mM sodium pyruvate, 5 U/ml penicillin, and 50 µg/ml streptomycin. Transfected 293 cells were then re-stimulated with IGF-1 (100 ng/ml) and cell lysates were prepared. Adenovirus constructs have been described previously (Kontaridis et al., 2002) and NIH 3T3 cells were infected at $5-6 \times 10^7$ OPU per 1×10^6 cells.

Myc-tagged wild-type SHP-2 was sub-cloned into the EcoRI restriction site of the pBabe retroviral vector. Infectious retroviral particles were generated using the BOSC23 retroviral packaging cells (Pear et al., 1993) that were transiently transfected with either pBabe or pBabe-SHP-2 retroviral plasmids using calcium phosphate in complete DMEM media containing 10% FBS plus 25 µM chloroquine. pBabe and pBabe-SHP-2 retroviral supernatants were used to transduce SHP2^{Ex3-/-} fibroblasts in the presence of polybrene (4 µg/ml). Puromycin-resistant (5 µg/ml) SHP2^{Ex3-/-} fibroblasts were isolated and screened for SHP-2 expression.

Immunoprecipitation, immunoblot analyses, and immune complex kinase assays

Cell lysates were prepared and immunoprecipitation and immunoblotting were performed as described previously (Kontaridis et al., 2002). Antibodies used were: polyclonal anti-phospho-Erk (Thr202/Tyr204) and anti-phospho-Akt (Ser473) antibodies (Cell Signaling Technology, Beverly, MA); polyclonal anti-SHP-2 and anti-Akt-1/2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); monoclonal anti-phosphotyrosine 4G10 and polyclonal anti-Akt-1 antibody (Upstate Biotechnology, Lake Placid, NY). Dr. John Blenis (Harvard Medical School, Boston, MA) kindly provided the anti-Erk antibody. The p85 antibodies were generously provided by Lloyd Cantley (Yale University School of Medicine, New Haven, CT). Densitometric analyses of immunoblots were performed using Lab Works 4.0 Image Analysis software (UVP Incorporated, Upland, CA).

For Akt kinase assays, cells were lysed in kinase buffer (1% NP-40 lysis buffer, 10% glycerol, and 20 mM NaF), clarified by centrifugation at 20,800g for 20 min and normalized for protein concentration using the Bradford assay. Endogenous Akt-1 was immunoprecipitated for 2 h at 4°C. Immune complexes were collected on protein A sepharose, washed twice with wash buffer (1% NP-40 lysis buffer, 10% glycerol, 1 mM NaVO₃) and twice with kinase buffer (20 mM Hepes (pH 7.4), 10 mM MnCl₂, 10 mM MgCl₂) and re-suspended in 50 µl kinase reaction buffer (supplemented with 1 mM dithiothreitol, 50 µM ATP, 15 µCi γ^{32} P-ATP and 2.5 µg of histone H2B (Roche Applied Sciences, Indianapolis, IN)). Reactions were carried out for 15 min at 30°C, stopped by the addition of sample buffer, and heated to 95°C for 5 min. Samples were separated by 10% SDS-PAGE, coomassie stained, and dried. Autoradiography was performed to visualize phosphorylated histone H2B.

Insulin receptor substrate-1 (IRS-1)-associated PI3K activity was assessed by immunoprecipitating IRS-1 overnight at 4°C, immune complexes were washed twice with NP-40 lysis buffer supplemented with 1 mM NaVO₃, twice with ST (150 mM NaCl–50 mM Tris HCl, pH 7.4) buffer, and twice with PI3K buffer (20 mM Hepes pH 7.5, 5 mM MgCl₂, 1 mM EGTA). Phosphatidylinositol and phosphatidylserine (Avanti Polar Lipids, Alabaster, AL) were dried under argon and resuspended in lipid resuspension buffer (20 mM Hepes pH 7.5, 1 mM MgCl₂, 1 mM EGTA) and sonicated for 15 min. Immunoprecipitates were resuspended in PI3K reaction buffer (10 µCi γ^{32} P-ATP, 100 µM ATP pH 7.5, 2 µg phosphatidylinositol, 1 µg phosphatidylserine, in PI3K buffer). The kinase reaction was carried out for 20 min at room temperature and stopped by the addition of 1M HCl. Lipids were extracted with 1:1 CHCl₃:MEOH, spotted onto TLC plates [pretreated with potassium oxalate solution (1 mM EDTA pH 7.9, 40% MEOH, 1% potassium oxalate) for 30 min at room temperature and air dried] and were resolved in 43:48:4:7 CHCl₃:MEOH:acetic acid:water, phospholipids were detected by autoradiography.

RNAi-mediated suppression of SHP-2

We generated 21 nucleotide dsRNA with 3' overhanging dimers of uridine as described by the Silencer siRNA Construction Kit (Ambion, Boston, MA). The following target sequences (antisense strands) were used for SHP-2 RNAi knockdown: no. 1 5' AACACTGGG-AACACTGGGGACTACTATGAC3'; no. 4 5' AAATGTG-TCAAGTACTGGCCT3'; no. 2 5' AAAAGAAGCAGAGA-AGCTGCT3'. Proliferating C2C12 myoblasts were washed with PBS and trypsinized using 1× Trypsin/EDTA (Sigma). Transfection of each double-stranded siRNA was performed using oligofectamine reagent according to the manufacturer's instruction (Invitrogen Life Technologies, Carlsbad, CA).

Apoptosis assays

Cells were serum-starved for 24 h in DMEM containing 0.1% FBS, then treated for the indicated times and concentrations with etoposide (Sigma). Non-adherent and adherent cells were collected, washed once, and re-suspended in PBS. Trypan blue (0.1% (v/v)) was added and cells were counted immediately. Caspase 3 activity

was measured according to manufacturers' instructions (ApoAlert Caspase 3 Fluorescent Assay Kit, Clontech, Palo Alto, CA). Caspase 3 activity was normalized to total protein content for each sample. Statistical significances were calculated using a two-way *t*-test analysis assuming equal variances. Flowcytometric analyses were performed on floating and attached cells that were harvested after 1–4 days, fixed in 70% ethanol for 1 h on ice and then spun for 5 min at 1,300 rpm. Cells were incubated in PBS containing 1 mg/ml RNAse (Roche) for 30 min at 37°C, propidium iodide (Sigma) was added at a final concentration of 8 mg/ml, and samples were analyzed for DNA content by flow cytometry using FACS SCAN (Becton Dickinson, Bedford, MA).

RESULTS

The catalytic activity of SHP-2 is required for IGF-1-induced activation of PI3K

Fibroblasts derived from mice that contain a deletion within exon 3 of SHP-2 express a truncated form of SHP-2 that lacks the amino terminal SH2 domain (SHP-2^{Ex3-/-}) (Saxton et al., 2000). The absence of the amino terminal SH2 domain results in the failure of SHP-2 to appropriately localize thereby resulting in the expression of a hypomorphic "loss-of-function" allele of SHP-2. It has been shown previously that IGF-1-induced Akt activation is reduced in SHP-2^{Ex3-/-} fibroblasts (Wu et al., 2001; Zhang et al., 2002). However, it has yet to be determined whether this effect occurs as a consequence of SHP-2 inhibiting IGF-1-mediated PI3K activation. To determine whether SHP-2 is required for IGF-1-induced PI3K activity we tested the ability of IGF-1 to induce PI3K activation in wild-type and SHP-2^{Ex3-/-} fibroblasts. Serum-starved wild-type and SHP-2^{Ex3-/-} fibroblasts were either left untreated or were stimulated with IGF-1 for 10 min and the amount of IRS-1-associated PI3K activity was determined by immunoprecipitating with anti-IRS-1 antibodies. As shown in Figure 1A, as compared to wild-type fibroblasts, SHP-2^{Ex3-/-} fibroblasts exhibited dramatically reduced levels of IRS-1-associated PI3K activity. Next we examined whether overexpression of a catalytically inactive mutant of SHP-2 that contains a deletion within the PTP domain of SHP-2 (SHP-2ΔP) could inhibit IGF-1 induced PI3K activity. NIH 3T3 cells were infected with adenoviruses that express either a GFP vector control, wild-type SHP-2 or SHP-2ΔP. After 24 h, the cells were serum-starved and then re-stimulated with IGF-1 (50 ng/ml) for 10 min. Figure 1B (lower part) shows that the level of overexpression of wild-type and the catalytically inactive mutant of SHP-2 are approximately four-fold above endogenous SHP-2 levels. Following stimulation with IGF-1, IRS-1-associated PI3K activity becomes induced (Fig. 1B, upper part). In fibroblasts infected with SHP-2ΔP, a dramatic reduction in the amount of PI3K-associated with IRS-1 in response to IGF-1 stimulation was observed, as compared with adenoviral GFP and SHP-2 wild-type infected NIH 3T3 cells (Fig. 1B, upper part). These data clearly demonstrate that the catalytic activity of SHP-2 is required for IGF-1-induced PI3K activity.

When tyrosyl-phosphorylated IRS-1 recruits the SH2 domain-containing p85 regulatory subunit, resulting in the activation of PI3K (Backer et al., 1992). Thus, one

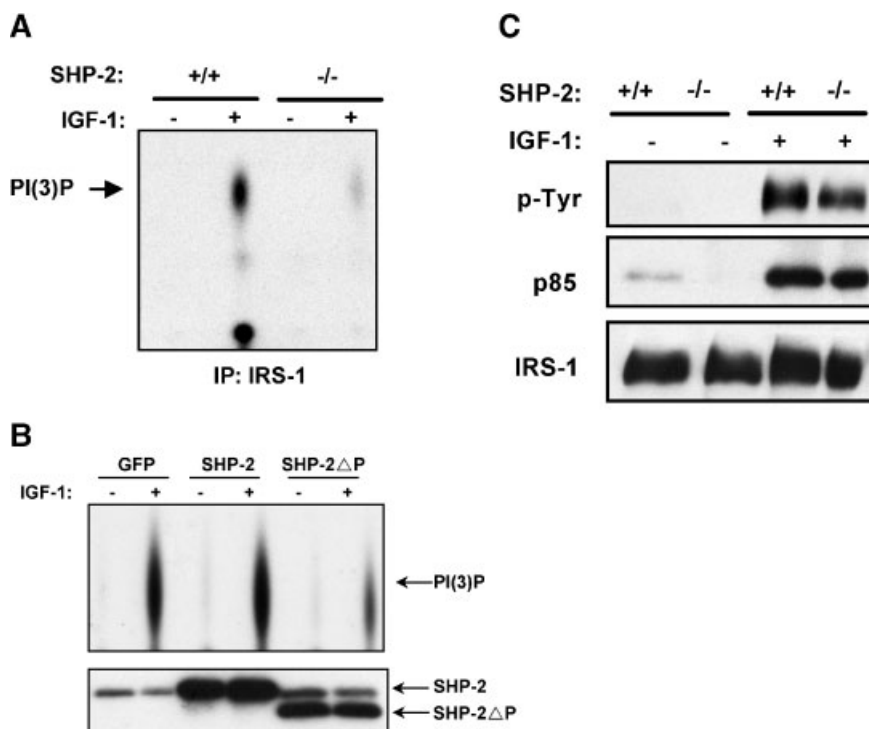


Fig. 1. The catalytic activity of SHP-2 is required for insulin-like growth factor-1 (IGF-1) induced activation of phosphatidylinositol 3'-kinase (PI3K). **A**: Wild-type and SHP-2^{Ex3-/-} fibroblasts were stimulated with IGF-1 (100 ng/ml) and lysates were subjected to immunoprecipitation for insulin receptor substrate-1 (IRS-1) using anti-IRS-1 antibodies. Immune complexes were used to determine the amount of IRS-1-associated PI3K activity as described in "Experimental Procedures." **B**: NIH 3T3 cells were infected with adenovirus

expressing green fluorescent protein (GFP) (vector control), GFP-SHP-2 or GFP-SHP-2ΔP, serum-starved then re-stimulated with IGF-1 (50 ng/ml). IRS-1-associated PI3K activity was determined. Lysates were immunoblotted with anti-SHP-2 antibodies. **C**: Wild-type and SHP-2^{Ex3-/-} fibroblasts were stimulated with IGF-1 (100 ng/ml) and lysates were subjected to immunoprecipitation for IRS-1. Immune complexes were resolved and immunoblotted with anti-phosphotyrosine (p-Tyr), anti-p85, and anti-IRS-1 antibodies as indicated.

explanation for the defect in PI3K signaling in SHP-2^{Ex3-/-} fibroblasts could be that IRS-1 tyrosyl phosphorylation and/or its association with the p85 subunit is decreased resulting in the diminished activation of PI3K. To test this possibility, we compared IRS-1 tyrosyl phosphorylation in wild-type and SHP-2^{Ex3-/-} fibroblasts following IGF-1 stimulation. These results showed that IRS-1 becomes tyrosyl phosphorylated in SHP-2^{Ex3-/-} fibroblasts following IGF-1 stimulation (Fig. 1C). Importantly, equivalent levels of p85 complexed with IRS-1 in wild-type and SHP-2^{Ex3-/-} fibroblasts (Fig. 1C). These data indicated that the IRS-1/p85 complex is appropriately recruited in SHP-2^{Ex3-/-} fibroblasts and thus the defect in PI3K activity in response to IGF-1 is unlikely to be a consequence of the failure to recruit the p85/110 complex to IRS-1.

SHP-2 is required for IGF-1-induced Akt activation

We next examined the ability of the SHP-2^{Ex3-/-} fibroblasts to activate the PI3K effector serine/threonine kinase Akt in response to IGF-1, in addition to serum and platelet-derived growth factor (PDGF). As previously demonstrated we found that Erk activation, as a function of phosphorylated Erk, was inhibited in the SHP-2^{Ex3-/-} fibroblasts as compared with wild-type fibroblasts following stimulation with serum, IGF-1, or

PDGF (Fig. 2A). Next, we tested whether growth factor-induced activation of Akt was also defective in SHP-2^{Ex3-/-} fibroblasts. When wild-type fibroblasts are rendered quiescent by serum starvation and then re-stimulated for 10 min with FBS, IGF-1, or PDGF, Akt became activated as indicated by its phosphorylation on Ser473 (Fig. 2B, upper parts). However, in response to either serum, IGF-1 or PDGF, SHP-2^{Ex3-/-} fibroblasts failed to fully activate Akt (Fig. 2B, upper parts). To confirm that in the SHP-2^{Ex3-/-} fibroblasts Akt activity is indeed defective in response to growth factor stimulation, we performed direct immune complex kinase assays on endogenous Akt from both wild-type and SHP-2^{Ex3-/-} fibroblasts. These results confirmed that SHP-2^{Ex3-/-} fibroblasts failed to activate Akt in response to serum, IGF-1, and PDGF (Fig. 2B, middle parts, lower part).

We considered the possibility that because these SHP-2^{Ex3-/-} fibroblasts express SHP-2 ((Saxton et al., 1997) and Fig. 3A), albeit mis-localized, that the apparent lack of Akt activation could be due to a delay in the endogenous mutant of SHP-2 responding to IGF-1 activation. To investigate this, time course experiments were carried out following both PDGF and IGF-1 stimulation. We found that there was no detectable increase in either Erk or Akt activation in SHP-2^{Ex3-/-} fibroblasts for up to 4 h post IGF-1 or PDGF stimulation (Fig. 2C). These

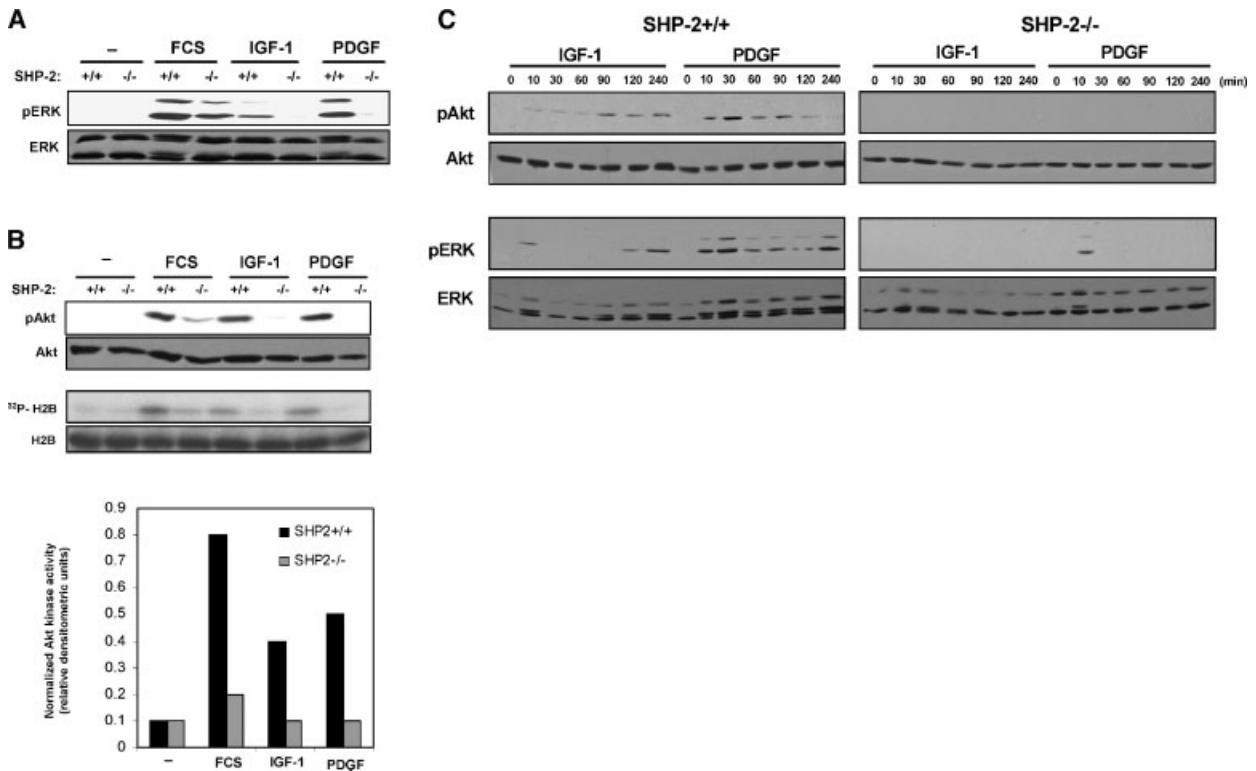


Fig. 2. Growth factor-induced activation of Akt is defective in SHP-2^{Ex3-/-} fibroblasts. Cells were serum-starved for 24 h and then either left unstimulated or were re-stimulated for 10 min with 10% FBS, IGF-1 (100 ng/ml), or platelet-derived growth factor (PDGF, 100 ng/ml). Cells were lysed and immunoblotted for (A) phospho-extracellular-regulated kinase (pErk) and Erk (B) phospho-Akt (pAkt) and Akt (upper parts) and in vitro Akt kinase assays (lower parts). The

graph below shows the mean densitometric units of the autoradiograph of ³²P-histone H2B normalized to the mean densitometric units of the coomassie-stained histone H2B protein. C: Wild-type and SHP-2^{Ex3-/-} fibroblasts were stimulated with either IGF-1 (100 ng/ml) or PDGF (100 ng/ml) for the indicated times, pErk, Erk, pAkt, and Akt immunoblots were then performed.

data argue that the failure of either PDGF or IGF-1 to activate Akt in SHP-2^{Ex3-/-} fibroblasts is not simply due to a delay in the kinetics of their activation, but rather an intrinsic inability of these fibroblasts to stimulate the PI3K/Akt pathway.

Rescue of the Akt kinase activity defect in SHP-2^{Ex3-/-} fibroblasts

In order to prove definitively that the loss of Akt activity in SHP-2^{Ex3-/-} fibroblasts was dependent upon SHP-2 we generated SHP-2^{Ex3-/-} fibroblasts that repressed SHP-2. SHP-2^{Ex3-/-} fibroblasts were infected with either pBabe as a control or pBabe-SHP-2 retrovirus. Clones that exhibited resistance to puromycin were selected for analyses of SHP-2 expression levels. Two independent clones were selected: one that was transduced with the retroviral vector alone (Ex3-/-V) and one that expressed SHP-2 at levels comparable to that of wild-type fibroblasts (Ex3-/-WT) (Fig. 3A). Examination of phospho-Erk levels in Ex3-/-WT fibroblasts in response to IGF-1 resulted in a level of Erk activation that was comparable to that of wild-type fibroblasts (Fig. 3B). These results are consistent with the notion that functional restoration of SHP-2 rescues the Erk pathway. We then assessed both Akt kinase activity and phospho-Akt levels following IGF-1 stimulation in the Ex3-/-WT rescued fibroblasts. Stimula-

tion of SHP-2^{Ex3-/-} and Ex3-/-V fibroblasts with IGF-1 failed to induce either Akt kinase activity or phospho-Akt immunoreactivity (Fig. 3C). In contrast, Ex3-/-WT fibroblasts rescued the Akt signaling defect in response to IGF-1 (Fig. 3C). Densitometric analysis of the Akt kinase activity indicated that the Ex3-/-WT fibroblasts achieved levels of Akt activation following IGF-1 stimulation that were approximately 70% of that produced in wild-type fibroblasts following IGF-1 stimulation (Fig. 3C, lower part).

The catalytic activity of SHP-2 is required for IGF-1 induced Akt activity

We next examined if the catalytic activity of SHP-2 is required for IGF-1-induced PI3K activation. 293 cells were transiently transfected with vector, wild-type, or a catalytically inactive (SHP-2-CS) mutant of SHP-2. Following transfection, 293 cells were serum-starved and then re-stimulated with IGF-1 for 10 min. Under these conditions greater than 75% of the cells are transfected (data not shown). First, as a control, phospho-Erk levels were examined, since the catalytic activity of SHP-2 has been shown to be required for IGF-1-induced Erk activation (Shi et al., 1998). As expected, IGF-1 induced phospho-Erk was blocked when the catalytically inactive mutant of SHP-2 was overexpressed (Fig. 4A, upper parts). Analysis of these same cell lysates

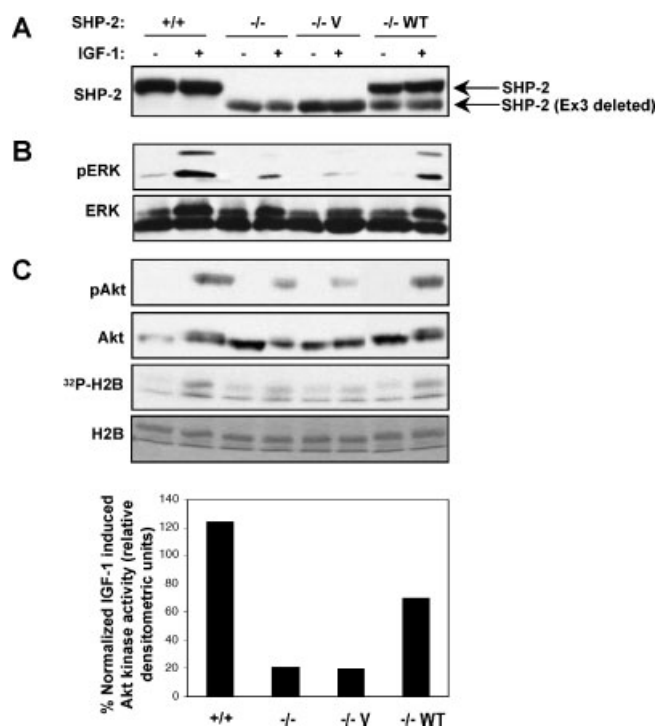


Fig. 3. Rescue of Akt defect in SHP-2^{Ex3-/-} fibroblasts. **A:** Shown are immunoblot analyses for SHP-2 expression using anti-SHP-2 antibodies on cell lysates prepared from puromycin-resistant SHP-2^{Ex3-/-} fibroblasts containing vector control (-/-V) or wild-type SHP-2 (-/-WT). **B:** Wild-type, SHP-2^{Ex3-/-}, -/-V, and -/-WT fibroblasts were either left unstimulated or re-stimulated with IGF-1 (100 ng/ml). Lysates were immunoblotted for pErk (upper part) and Erk (lower part). **C:** Cell lysates from B were subjected to either immunoblotting with anti-pAkt or anti-Akt antibodies (upper parts) or in vitro Akt kinase assays (lower part). The graph shown below represents densitometry of the Akt kinase assay performed above. IGF-1-induced Akt kinase activities were calculated by normalizing the mean densitometric units of ³²P-histone H2B to that of the corresponding coomassie-stained histone H2B protein. The normalized IGF-1-induced Akt kinase activities are represented as a percentage of the IGF-1-stimulated and unstimulated values.

revealed that the catalytic activity of SHP-2 was also required for activation of Akt, since 293 cells expressing the catalytically inactive mutant of SHP-2 were inhibited in their ability to activate Akt following IGF-1 stimulation (Fig. 4B, upper parts). Overexpression of SHP-2-CS to these levels resulted in an approximate inhibition of IGF-1-induced Akt activation by 50% as determined by quantitative densitometric analysis (Fig. 4B, lower part). IGF-1-induced Akt activation was also inhibited in HA-Akt-1 immunoprecipitates derived from transiently transfected 293 cells co-expressing the catalytically inactive mutant of SHP-2 (data not shown).

SHP-2^{Ex3-/-} fibroblasts show increased sensitivity to etoposide-induced cell death and caspase 3 activation

We hypothesized that if SHP-2^{Ex3-/-} fibroblasts were intrinsically defective in their pro-survival ability then these fibroblasts should exhibit an increased sensitivity to etoposide-induced apoptosis. To determine if there is a survival defect in SHP-2^{Ex3-/-} fibroblasts we utilized the pharmacological apoptosis-inducing agent etoposide

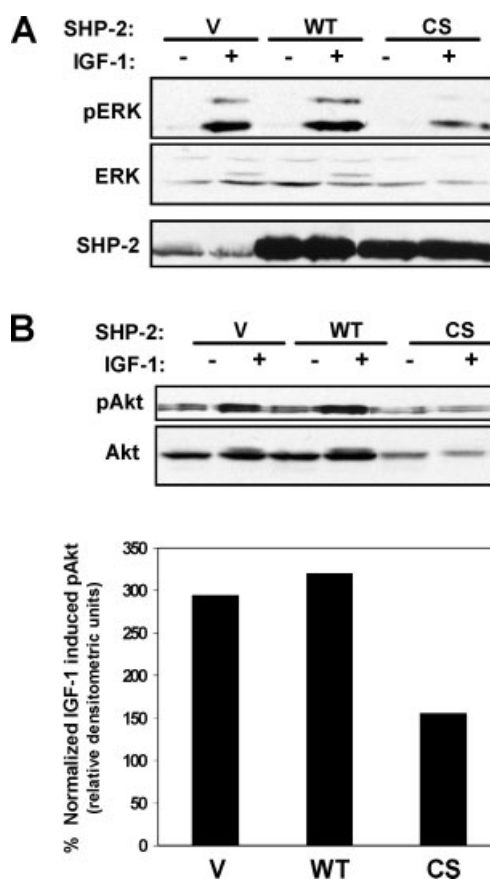


Fig. 4. Catalytically inactive SHP-2 blocks Akt activity. Serum-starved 293 cells were transfected with pIRES-GFP vector control (V), pIRES-GFP-SHP-2 (WT), or pIRES-GFP-SHP-2-CS, a catalytically inactive mutant of SHP-2 (CS). Under these conditions at least 75% of the cells are transfected as determined by visualizing for GFP-positive cells (data not shown). Twenty four hours post-transfection, cells were stimulated for 10 min with IGF-1, lysed and immunoblotted for (A) pErk, Erk (upper parts) and SHP-2 (lower part), (B) pAkt and Akt. The graph below in B represents the percent IGF-1-induced phospho-Akt levels (derived from the mean densitometric units from normalized immunoblots).

which induces apoptosis via a pathway resulting in the release of cytochrome c (Yang et al., 1997). Wild-type and SHP-2^{Ex3-/-} fibroblasts were serum-starved for 24 h and then treated with different concentrations of etoposide for 5 h, after which cell death was quantitated by trypan blue exclusion. When SHP-2^{Ex3-/-} fibroblasts were exposed to etoposide, these fibroblasts exhibited an enhanced level of cell death as compared to wild-type fibroblasts (Fig. 5A). At saturating etoposide concentrations, SHP-2^{Ex3-/-} fibroblasts were up to two-fold more sensitive to etoposide-induced cell death than wild-type fibroblasts (Fig. 5A).

To confirm that these fibroblasts were undergoing apoptotic cell death, we treated the SHP-2^{Ex3-/-} and wild-type fibroblasts with 25 μ m etoposide for up to 5 h and measured etoposide-induced caspase 3 activity (Fig. 5B). As a control, wild-type and SHP-2^{Ex3-/-} fibroblasts grown in the presence of 10% FBS, exhibited minimal caspase 3 activity (Fig. 5B). The addition of etoposide to SHP-2^{Ex3-/-} fibroblasts induced an

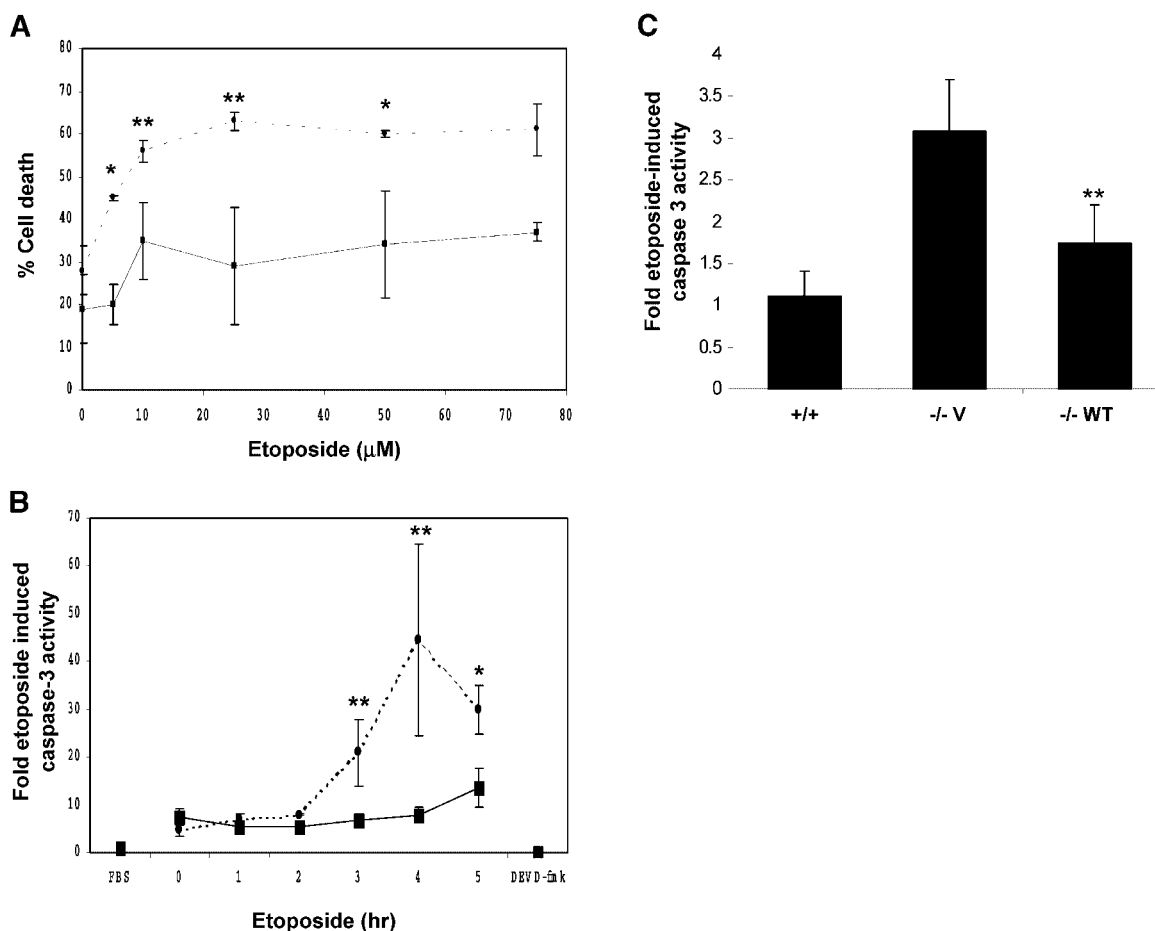


Fig. 5. Re-expression of functional SHP-2 in SHP-2^{Ex3}-/- fibroblasts suppresses the sensitivity to etoposide-induced cell death. **A:** Cell death was performed as described in "Experimental Procedures." Percent etoposide-induced cell death was calculated by dividing the percent cell death in etoposide-treated cells by the percent cell death in untreated cells. Data are representative of the mean \pm the standard error of the mean of three independent experiments. **B:** Wild-type (solid line) or SHP-2^{Ex3}-/- (dashed line) fibroblasts were serum-starved for 24 h, 25 μ M etoposide was added to the cells for the indicated time periods. Control cells were maintained in 10% FBS. Non-adherent and adherent cells were pelleted and caspase 3 activity was assessed. Samples pre-treated with DEVD-fmk caspase 3

inhibitor were serum-starved and treated with etoposide for 3 h. Caspase 3 activity (μ M AFC/ μ g protein) was normalized to uninduced (10% FBS) samples. Assays were performed in duplicate and data represent the mean \pm the standard error of the mean from three separate experiments. Statistical significances were performed using a one-tailed two sample student's *t*-test **P* < 0.05 and ***P* < 0.1. **C:** Wild-type (+/+), -/-V, and -/-WT fibroblasts were serum-starved for 24 h before stimulating for 5 h with etoposide. Caspase 3 activity was measured as described under "Experimental Procedures." These data are representative of the mean \pm the standard error of the mean of three independent experiments. Statistical significances were performed using a one-tailed two sample student's *t*-test ***P* < 0.1.

increase in caspase 3 activity, which peaked at 4 h. In contrast, etoposide-treated wild-type fibroblasts exhibited relatively little change in caspase 3 activity throughout the 5 h time course (Fig. 5B). The enhanced level of caspase 3 activity observed in the SHP-2^{Ex3}-/- fibroblasts were suppressed by pre-incubation with the caspase 3-specific inhibitor DEVD-fmk peptide (Fig. 5B).

To establish that the rescue of Akt activity correlated with a rescue of cell death sensitivity, we examined whether re-expression of SHP-2 in SHP-2^{Ex3}-/- fibroblasts rescued the sensitivity to etoposide-induced caspase 3 activity. Wild-type fibroblasts, Ex3-/-V and Ex3-/-WT fibroblasts were exposed to etoposide (25 μ M) for 5 h and caspase 3 activity was measured. These experiments revealed that Ex3-/-WT fibroblasts exhibited reduced sensitivity to etoposide as compared to Ex3-/-V control as shown by a reduced level of

caspase 3 activity following etoposide treatment (Fig. 5C). These data demonstrate that re-expressing wild-type SHP-2 in SHP-2^{Ex3}-/- fibroblasts rescues the survival defect and that SHP-2 positively regulates cell survival and inhibits apoptosis via the caspase 3 effector pathway in fibroblasts.

To extend the observation that SHP-2 positively regulates cell survival, we asked whether down-regulation of SHP-2 in a different experimental system also resulted in decreased cell survival. To test this, the expression of SHP-2 in the murine myoblast C2C12 cell line was suppressed using RNAi oligonucleotides. We identified three RNAi oligonucleotides in which SHP-2 expression levels could be effectively ablated with varying degrees of efficiency (Fig. 6A). When C2C12 myoblasts were treated with a combination of RNAi-1 and 4 (10 nM) an increase in the percentage of apoptotic

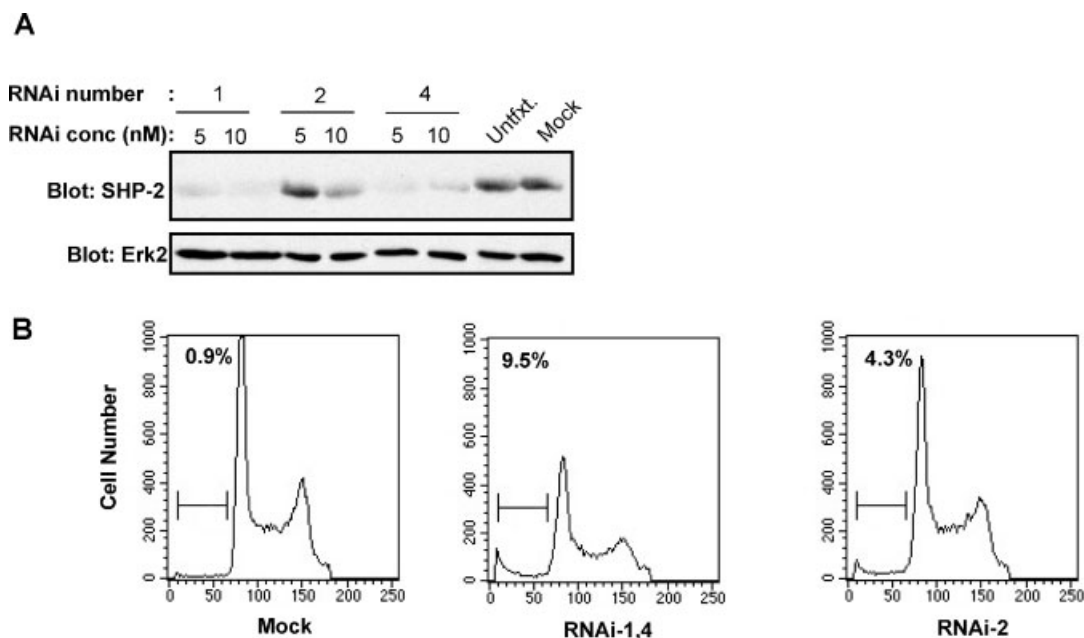


Fig. 6. RNAi-mediated suppression of SHP-2 induces apoptosis. **A:** C2C12 myoblasts were either left untreated (mock) or transfected without RNAi (untfxt.) or were transfected with RNAi-1, 2, and 4 at either 5 or 10 nM as described in "Experimental Procedures." SHP-2 expression was determined using anti-SHP-2 antibodies (upper part)

and as a loading control anti-Erk2 immunoblots (lower part) were performed. **B:** C2C12 myoblasts were either mock transfected or were transfected with a combination of RNAi-1 and 4 (10 nM) or RNAi-2 (10 nM). Hypodiploid/sub-G1 (apoptotic) DNA content was determined by propidium iodide staining and flowcytometry.

myoblasts were induced as compared to mock-transfected cultures (Fig. 6B). An intermediate level of apoptosis was induced when a concentration of RNAi-2 (10 nM) was used which partially suppresses SHP-2 expression as compared to RNAi-1 and -4 at equivalent concentrations (Fig. 6B). Taken together, these results demonstrate that SHP-2 also serves to maintain survival in myoblasts (Fig. 6B) as well as in fibroblasts (Fig. 5), suggesting that SHP-2 likely plays a fundamental role in the regulation of cell survival.

DISCUSSION

In this study, we demonstrate that the non-membrane protein tyrosine phosphatase, SHP-2, positively regulates cell survival by suppressing caspase 3 activity. The molecular basis for the ability of SHP-2 to promote cell survival in this manner is presumably due to the observation that SHP-2 is required for PI3K/Akt activation (Figs. 2–4). Others have demonstrated that SHP-2 is involved in IGF-1-induced Akt activation (Hakak et al., 2000; Wu et al., 2001; Zhang et al., 2002). However, it had not yet been established whether this is a consequence of SHP-2 regulating IGF-1-mediated PI3K activity. Our data clearly show that SHP-2 indeed regulates Akt by functioning to stimulate IGF-1-mediated PI3K activity (Fig. 1A), thus assigning SHP-2 upstream of PI3K. SHP-2 has the capacity to signal both in a catalytic-dependent and independent manner. In most cases, but not all, the catalytic activity of SHP-2 is required for downstream signaling (Tonks and Neel, 2001). Given the complexity of how SHP-2 signals it remained formerly possible that the ability of SHP-2 to regulate PI3K could occur by a mechanism that is independent of its catalytic activity. For example, activation

of Ras can be mediated via the ability of SHP-2 to bind Grb2 within its carboxyl terminus (Li et al., 1993; Bennett et al., 1994; Bjorbak et al., 2001). The activation of c-Src has also been suggested to be positively effected by SHP-2 in a non-catalytic dependent manner (Walter et al., 1999) and c-Src itself can participate in direct phosphorylation and activation of Akt (Chen et al., 2001; Conus et al., 2002). Here, we show that the catalytic activity of SHP-2 is required for PI3K and subsequently Akt activation following IGF-1 stimulation (Fig. 1B). Thus, activation of SHP-2's catalytic domain serves to couple the IGF-1 receptor to the PI3K/Akt pathway.

Growth factors, such as IGF-1 and PDGF, promote cell survival by initiating the Ras/Raf/Erk and PI3K/Akt signaling cascades. SHP-2^{Ex3-/-} fibroblasts fail to stimulate both IGF-1 and PDGF-induced Erk and Akt activation, indicating that both of these pro-survival pathways are disrupted in these cells. Since the activation of Erk, as well as Akt, in response to growth factors is defective in fibroblasts that lack functional SHP-2, it is likely that the requirement for SHP-2 in maintaining cell survival stems from its ability to regulate both the Ras/Raf/Erk and PI3K/Akt pathways (Parrizas et al., 1997). Consistent with this, our results show that SHP-2^{Ex3-/-} fibroblasts exhibit enhanced sensitivity to etoposide-induced apoptosis as compared to wild-type fibroblasts (Fig. 5). The loss of SHP-2 function was causal to the enhanced sensitivity to cell death in response to etoposide since re-introduction of functional SHP-2 in the SHP-2^{Ex3-/-} fibroblasts ameliorated etoposide-induced sensitivity to cell death (Fig. 5C). This conclusion is further supported by the observation that apoptosis was induced in proliferating C2C12 myoblasts in which SHP-2 expression was reduced

using RNAi (Fig. 6). These observations strongly support the general requirement for SHP-2 in the maintenance of cell survival. Previous work from Hakak et al. (2000) demonstrated that SHP-2 was required for anti-apoptotic signaling. Our data indicates that as compared to wild-type fibroblasts the activity of caspase 3 is enhanced in SHP-2^{Ex3-/-} fibroblasts (Fig. 5) suggesting that SHP-2 suppresses apoptosis by inhibiting the release of cytochrome c from mitochondria. Together, these data therefore define a link between SHP-2 and the apoptotic signaling machinery. In contrast to the results presented here and those of others (Hakak et al., 2000), Yuan et al. (2003) have recently shown that SHP-2 is required to promote DNA damage-induced cell death in response to cisplatin. Loss-of-function SHP-2^{Ex3-/-} fibroblasts used in these studies were instead found to be resistant to cisplatin-induced DNA damage. The precise mechanism for the actions of SHP-2's response to cisplatin-induced DNA damage, as well as etoposide, likely resides in identifying the direct substrates of SHP-2 that are dephosphorylated in response to these various agents.

The ability of PI3K to be recruited to tyrosyl phosphorylated IRS-1 following stimulation with IGF-1 appears to be unaffected in SHP-2^{Ex3-/-} fibroblasts (Fig. 1C) suggesting that SHP-2 does not regulate PI3K activation by controlling the ability of the p85 regulatory subunit to interact with IRS-1. These experiments also indicate that SHP-2 does not dephosphorylate either IRS-1 and/or p85 following IGF-1 stimulation. Interestingly, our data differ from those reported previously in which enhanced tyrosyl phosphorylation of IRS-1 in response to insulin was observed on a mutant form of IRS-1 that lacks the binding site for SHP-2 (Myers et al., 1998). It is certainly conceivable that SHP-2 dephosphorylates a single tyrosyl residue on IRS-1, as it is unlikely that we would be able to detect a single hyperphosphorylated tyrosyl residue in the background of the total mass of tyrosyl phosphorylated IRS-1. This is in fact the case with Gab-1 dephosphorylation by SHP-2, in which SHP-2 serves to dephosphorylate the PI3K binding site on Gab-1 in response to EGF (Zhang et al., 2002). Consistent with the data presented here, Zhang et al. (2002) also demonstrated a requirement for SHP-2 to positively regulate IGF-1-induced Akt activity. Given that both EGF- and IGF-1-dependent regulation of PI3K requires the catalytic activity of SHP-2 suggests that the PTP domain of SHP-2 exhibits a high degree of substrate selectivity. This substrate selectivity is likely to be dictated by the precise composition of the protein-protein interactions exhibited by SHP-2 downstream of different signaling pathways. When bound to IRS-1, SHP-2 may be presented to a sub-set of substrates that are not present and/or accessible as compared to when it is bound to Gab-1. These issues are again, only likely to be resolved completely when the substrates for SHP-2 in these various signaling pathways have been identified.

The precise mechanism for how SHP-2 regulates PI3K activation is unknown. Biochemical approaches in mammalian systems have demonstrated that SHP-2 can function upstream of Ras (see Introduction). Over-expression of a catalytically inactive mutant of SHP-2 inhibits insulin- and EGF-induction of Ras-GTP-loading when either measured directly (Noguchi et al., 1994;

Shi et al., 2000) or through a Ras-GTP-binding assay (Yart et al., 2001). Here, we show that the catalytic activity of SHP-2 is also required for IGF-1-induced PI3K activity (Fig. 5C). The assignment of SHP-2 as an upstream regulator of Ras is consistent with the observation that SHP-2 is required for both IGF-1-induced Erk and Akt pathways. Activated Ras has multiple effectors, one of them is Raf which couples to the Erk cascade and the other is PI3K which couples to Akt (Rodriguez-Viciano et al., 1994). SHP-2 may therefore be required to activate PI3K via its ability to stimulate Ras-GTP exchange following growth factor stimulation. This working hypothesis is supported by the observation that activated Ras is sufficient to stimulate the PI3K/Akt pathway (Datta et al., 1996). Exactly how SHP-2 interfaces with the PI3K pathway remains to be defined.

In summary, our data provide direct evidence that PI3K activation in response to IGF-1 requires the catalytic activity of SHP-2. The requirement for SHP-2 in the regulation of the PI3K/Akt survival pathway correlated with the enhanced sensitivity to caspase 3-mediated apoptosis when SHP-2 function was abrogated. Further studies are now underway in order to define the precise mechanistic basis for how SHP-2 regulates PI3K, which should further our understanding of how SHP-2 couples this pathway to the regulation of cell survival. Since production of phosphoinositide lipids has been implicated in diseases such as cancer, our data suggest that pharmacological inhibition of SHP-2's phosphatase activity could serve as a potential cancer chemotherapeutic target.

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LITERATURE CITED

- Allard JD, Herbst R, Carroll PM, Simon MA. 1998. Mutational analysis of the SRC homology 2 domain protein-tyrosine phosphatase corkscrew. *J Biol Chem* 273(21):13129-13135.
- Backer JM, Myers MG, Shoelson SE, Chin DJ, Sun X-J, Miralpeix M, Hu P, Margolis B, Skolnik EY, Schlessinger J, White M. 1992. Phosphatidylinositol 3'-kinase is activated by association with IRS-1 during insulin stimulation. *EMBO J* 11(9):3469-3479.
- Ballif BA, Blenis J. 2001. Molecular mechanisms mediating mammalian mitogen-activated protein kinase (MAPK) kinase (MEK)-MAPK cell survival signals. *Cell Growth Differ* 12(8):397-408.
- Barford D, Neel BG. 1998. Revealing mechanisms for SH2 domain mediated regulation of the protein tyrosine phosphatase SHP-2. *Structure* 6(3):249-254.
- Bennett AM, Tang TL, Sugimoto S, Walsh CT, Neel BG. 1994. Protein-tyrosine-phosphatase SHPTP2 couples platelet-derived growth factor receptor β to Ras. *Proc Natl Acad Sci USA* 91:7335-7339.
- Bennett AM, Hausdorff SF, O'Reilly AM, Freeman RM, Neel BG. 1996. Multiple requirements for SHPTP2 in epidermal growth factor mediated cell cycle progression. *Mol Cell Biol* 16:1189-1202.
- Ejorbak C, Buchholz RM, Davis SM, Bates SH, Pierroz DD, Gu H, Neel BG, Myers MG, Jr., Flier JS. 2001. Divergent roles of SHP-2 in ERK activation by leptin receptors. *J Biol Chem* 276(7):4747-4755.
- Bonni A, Brunet A, West AE, Datta SR, Takasu MA, Greenberg ME. 1999. Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. *Science* 286(5443):1358-1362.

- Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, Frisch S, Reed JC. 1998. Regulation of cell death protease caspase-9 by phosphorylation. *Science* 282(5392):1318–1321.
- Chen R, Kim O, Yang J, Sato K, Eisenmann KM, McCarthy J, Chen H, Qiu Y. 2001. Regulation of Akt/PKB activation by tyrosine phosphorylation. *J Biol Chem* 276(34):31858–31862.
- Conus NM, Hannan KM, Cristiano BE, Hemmings BA, Pearson RB. 2002. Direct identification of tyrosine 474 as a regulatory phosphorylation site for the Akt protein kinase. *J Biol Chem* 277(41):38021–38028.
- Datta K, Bellacosa A, Chan TO, Tsichlis PN. 1996. Akt is a direct target of the phosphatidylinositol 3-kinase. Activation by growth factors, v-src and v-Ha-ras, in SF9 and mammalian cells. *J Biol Chem* 271(48):30835–30839.
- Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME. 1997. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* 91(2):231–241.
- Datta SR, Brunet A, Greenberg ME. 1999. Cellular survival: A play in three Acts. *Genes Dev* 13(22):2905–2927.
- Gutch MJ, Flint AJ, Keller J, Tonks NK, Hengartner MO. 1998. The *Caenorhabditis elegans* SH2 domain-containing protein tyrosine phosphatase PTP-2 participates in signal transduction during oogenesis and vulval development. *Genes Dev* 12(4):571–585.
- Hakak Y, Hsu YS, Martin GS. 2000. Shp-2 mediates v-Src-induced morphological changes and activation of the anti-apoptotic protein kinase Akt. *Oncogene* 19(28):3164–3171.
- Jost M, Huggett TM, Kari C, Boise LH, Rodeck U. 2001. Epidermal growth factor receptor-dependent control of keratinocyte survival and Bcl-xL expression through a MEK-dependent pathway. *J Biol Chem* 276(9):6320–6326.
- Kontaridis MI, Liu X, Zhang L, Bennett AM. 2002. Role of SHP-2 in fibroblast growth factor receptor-mediated suppression of myogenesis in C2C12 myoblasts. *Mol Cell Biol* 22(11):3875–3891.
- Li W, Nishimura R, Kashishan A, Batzer AG, Kim WJH, Cooper JA, Schlessinger J. 1993. A new function for a phosphotyrosine phosphatase: Linking GRB2-Sos to a receptor tyrosine kinase. *Mol Cell Biol* 14:509–517.
- Myers MGJ, Mendez R, Shi P, Pierce JH, Rhoads R, White MF. 1998. The COOH-terminal tyrosine phosphorylation sites on IRS-1 bind SHP-2 and negatively regulate insulin signaling. *J Biol Chem* 273:26908–26914.
- Neel BG, Tonks NK. 1997. Protein tyrosine phosphatases in signal transduction. *Curr Opin Cell Biol* 9(2):193–204.
- Noguchi T, Matozaki T, Horita K, Fujioka Y, Kasuga M. 1994. Role of SH-PTP2, a protein-tyrosine phosphatase with src homology 2 domains, in insulin-stimulated ras activation. *Mol Cell Biol* 14:6674–6682.
- Parrizas M, Saltiel AR, LeRoith D. 1997. Insulin-like growth factor 1 inhibits apoptosis using the phosphatidylinositol 3'-kinase and mitogen-activated protein kinase pathways. *J Biol Chem* 272(1):154–161.
- Pear WS, Nolan GP, Scott ML. 1993. Production of high-titer helper-free retroviruses by transient transfection. *Proc Natl Acad Sci USA* 90:8392–8396.
- Perkins LA, Johnson MR, Melnick MB, Perrimon N. 1996. The nonreceptor protein tyrosine phosphatase corkscrew functions in multiple receptor tyrosine kinase pathways in *Drosophila*. *Dev Biol* 180(1):63–81.
- Rodriguez-Viciana P, Warne PH, Dhand R, Vanhaesebroeck B, Gout I, Fry MJ, Waterfield MD, Downward J. 1994. Phosphatidylinositol-3-OH kinase as a direct target of Ras. *Nature* 370:527–532.
- Saxton TM, Henkemeyer M, Gasca S, Shen R, Rossi DJ, Shalaby F, Feng GS, Pawson T. 1997. Abnormal mesoderm patterning in mouse embryos mutant for the SH2 tyrosine phosphatase Shp-2. *EMBO J* 16(9):2352–2364.
- Saxton TM, Ciruna BG, Holmyard D, Kulkarni S, Harpal K, Rossant J, Pawson T. 2000. The SH2 tyrosine phosphatase shp-2 is required for mammalian limb development. *Nat Genet* 24(4):420–423.
- Shi Y. 2002. Mechanisms of caspase activation and inhibition during apoptosis. *Mol Cell* 9:459–470.
- Shi ZQ, Lu W, Feng GS. 1998. The Shp-2 tyrosine phosphatase has opposite effects in mediating the activation of extracellular signal-regulated and c-Jun NH2-terminal mitogen-activated protein kinases. *J Biol Chem* 273(9):4904–4908.
- Shi ZQ, Yu DH, Park M, Marshall M, Feng GS. 2000. Molecular mechanism for the Shp-2 tyrosine phosphatase function in promoting growth factor stimulation of Erk activity. *Mol Cell Biol* 20(5):1526–1536.
- Tonks NK, Neel BG. 2001. Combinatorial control of the specificity of protein tyrosine phosphatases. *Curr Opin Cell Biol* 13(2):182–195.
- Van Vactor D, O'Reilly AM, Neel BG. 1998. Genetic analysis of protein tyrosine phosphatases. *Curr Opin Genet Dev* 8(1):112–126.
- Walter AO, Peng ZY, Cartwright CA. 1999. The Shp-2 tyrosine phosphatase activates the Src tyrosine kinase by a non-enzymatic mechanism. *Oncogene* 18(11):1911–1920.
- Wu CJ, O'Rourke DM, Feng GS, Johnson GR, Wang Q, Greene MI. 2001. The tyrosine phosphatase SHP-2 is required for mediating phosphatidylinositol 3-kinase/Akt activation by growth factors. *Oncogene* 20(42):6018–6025.
- Yamauchi K, Milarski KL, Saltiel AR, Pessin JE. 1995. Protein-tyrosine-phosphatase SHPTP2 is a required positive effector for insulin downstream signaling. *Proc Natl Acad Sci USA* 92:664–668.
- Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, Peng TI, Jones DP, Wang X. 1997. Prevention of apoptosis by Bcl-2: Release of cytochrome c from mitochondria blocked. *Science* 275(5303):1129–1132.
- Yart A, Laffargue M, Mayeux P, Chretien S, Peres C, Tonks N, Roche S, Payrastra B, Chap H, Raynal P. 2001. A critical role for phosphoinositide 3-kinase upstream of Gab1 and SHP2 in the activation of ras and mitogen-activated protein kinases by epidermal growth factor. *J Biol Chem* 276(12):8856–8864.
- Yuan L, Yu W-M, Yuan Z, Haudenschield CC, Qu C-K. 2003. Role of SHP-2 tyrosine phosphatase in the DNA damage-induced cell death response. *J Biol Chem* 278(17):15208–15216.
- Zhang SQ, Tsiaras WG, Araki T, Wen G, Minichiello L, Klein R, Neel BG. 2002. Receptor-specific regulation of phosphatidylinositol 3'-kinase activation by the protein tyrosine phosphatase Shp2. *Mol Cell Biol* 22(12):4062–4072.