Tyrosyl phosphorylation of Shp2 is required for normal Erk activation in response to some, but not all growth factors.

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Running Title: Tyrosyl Phosphorylation of Shp2 regulates Erk activation
Summary

The protein-tyrosine phosphatase Shp2 is required for normal activation of the Erk MAP kinase (MAPK) in multiple receptor tyrosine kinase (RTK) signaling pathways. In fibroblasts, Shp2 undergoes phosphorylation at two C-terminal tyrosyl residues in response to some (FGF and PDGF), but not all (EGF and IGF) growth factors. Whereas the catalytic activity of Shp2 is required for all Shp2 actions, the effect of tyrosyl phosphorylation on Shp2 function has been controversial. To clarify the role of Shp2 tyrosyl phosphorylation, we infected Shp2-mutant fibroblasts with retroviruses expressing wild type (WT) Shp2 or mutants of either (Y542F, Y580F) or both (YDF) C-terminal tyrosines. Compared to WT cells, Erk activation was decreased in Y542F- or Y580F-infected cells in response to FGF and PDGF, but not EGF. Mutation of both phosphorylation sites resulted in a further decrease in growth factor-evoked Erk activation, although not to the level of the vector control. Immunoblot analyses confirm that Y542 and Y580 are the major sites of Shp2 tyrosyl phosphorylation, and that Y542 is the major Grb2 binding site. However, studies with antibodies specific for individual Shp2 phosphorylation sites reveal unexpected complexity in the mechanism of Shp2 tyrosyl phosphorylation by different RTKs. Moreover, because Y580F mutants retain nearly wild type Grb2-binding ability, yet exhibit defective PDGF-evoked Erk activation, our results show that association of Grb2 with Shp2 is not sufficient for promoting full Erk activation in response to these growth factors, thereby arguing strongly against the “Grb2-adapter” model of Shp2 action.
**Introduction**

Many signaling pathways regulating cell growth, proliferation, differentiation and migration involve protein-tyrosyl phosphorylation which, in turn, is controlled by protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). Most peptide growth factor receptors are transmembrane PTKs, termed receptor tyrosine kinases (RTKs). Ligand binding activates the intrinsic PTK activity of RTKs, leading to the phosphorylation of several substrates, most of which have SH2 (src homology-2) or PTB (phosphotyrosine-binding) domains. Tyrosyl phosphorylation of such signal relay molecules can alter their structure and/or function, thereby regulating enzyme activity, protein localization, and/or the assembly of multimeric signaling complexes (1). These events, in turn, control downstream signaling pathways, such as the Ras/Raf/Mek/Erk cascade (2,3).

Shp2 is a ubiquitously expressed non-transmembrane PTP that plays an essential role in most, if not all, RTK signaling pathways. In all of these pathways, Shp2 function is required for normal activation of the Erk MAP kinase and its downstream transcriptional targets (4-7). Experiments using dominant negative (catalytically inactive) Shp2, as well as studies of primary fibroblasts and cell lines from Shp2 mutant mice generated by homologous recombination (8), have shown that Shp2 acts upstream of Ras in insulin/(insulin-like growth factor) IGF-1 (9) and epidermal growth factor (EGF) signaling (10). Although likely, it has not been shown that Shp2 acts upstream of Ras in other RTK signaling pathways. Moreover, studies of mammalian cell systems, as well as genetic analyses of the *Drosophila* Shp2 ortholog Corkscrew (Csw), suggest that Shp2 also acts downstream of Ras and/or in a parallel signaling pathway (5-7).

Like its close relative, Shp1, Shp2 contains two N-terminal SH2 domains, a tyrosine phosphatase catalytic (PTP) domain, and a C-terminus with two tyrosyl phosphorylation sites, Y542 and Y580, and an interposed proline-rich domain (Figure 1A). Multiple studies have established that PTP activity is required for all actions of Shp2, including its role in the Ras/Erk pathway (5,6). In contrast, the molecular details and functional consequences of tyrosyl phosphorylation of Shp2 have remained controversial. Shp2 undergoes phosphorylation at both Y542 and Y580 in response to many (but not all) stimuli, including
many (but not all) peptide growth factors (11-13). Both of these sites conform to the consensus for binding to the Grb2 SH2 domain (YXNX), and accordingly, tyrosyl phosphorylated Shp2 binds Grb2. But whereas Bennett *et al.* reported that Y542 is the major phosphorylation and Grb2 association site (14), two other studies concluded that Y580 is the major Grb2 binding site (15,16). The reason(s) for these discrepancies is (are) not clear.

The effects of tyrosyl phosphorylation on Shp2 enzymatic activity and RTK signaling are also in dispute. The finding that Grb2 binds tyrosyl phosphorylated Shp2, together with the requirement for Shp2 upstream of Ras in the Erk pathway (see above) suggested an “adapter model” of Shp2 action (14,17). In this model, tyrosyl phosphorylated Shp2 contributes to Ras (and ultimately, Erk) activation by recruiting Grb2/Sos complexes to the vicinity of the plasma membrane; hence, Shp2 functions as an adapter. Notably, it is unclear how the requirement for Shp2 catalytic activity can be reconciled with this model. Others have argued that tyrosyl phosphorylation of Shp2 stimulates its catalytic activity (12,18). Early studies found a temporal correlation between Shp2 tyrosyl phosphorylation and increased Shp2 activity (12). However, upon growth factor stimulation, Shp2 binds via its SH2 domains to RTKs and/or scaffolding adapters (e.g., Gab/Dos, IRS, FRS family members) at the same time that it becomes tyrosyl phosphorylated (11-13). Because engagement of the SH2 domains of Shp2 markedly enhances PTP activity (19), these early studies could not distinguish between the effects of tyrosyl phosphorylation and SH2 domain engagement on Shp2 catalytic activity. More recently, protein ligation techniques were used to introduce a phosphonate at either Y542 or Y580 of Shp2 (18). Phosphonate incorporation at Y542 resulted in intramolecular binding to the N-SH2, whereas phosphono-Y580 binds to the C-SH2. Moreover, both phosphonate derivatives were found to stimulate catalytic activity (to an extent similar to SH2 domain engagement) and display enhanced biological activity in a microinjection assay. These data argue for an “enzyme activation” model for Shp2 tyrosyl phosphorylation. Because Shp2 rapidly autodephosphorylates, however, a stable phosphonate might not have the same effect in cells as a transiently phosphorylated tyrosyl residue. The function of Grb2 binding in the
enzyme activation model also is unclear.

Most importantly, there has been no clear demonstration that tyrosyl phosphorylation of Shp2 is required for its signaling functions. Several studies have investigated the effects of over-expressing tyrosyl phosphorylation site mutants of Shp2 on RTK signaling in vertebrate systems. Bennett et al. found no effect of such mutants on EGF and PDGF signaling in transiently transfected 293 cells (20), nor did these mutants inhibit FGF-induced Erk activation, elongation movements or mesoderm induction in *Xenopus* embryos (21). Csw also has a tyrosyl phosphorylation site that binds Grb2, and a mutant lacking this site rescues *csw* mutant embryos when expressed under heat shock promoter control (22). These results argue that the effects (if any) of tyrosyl phosphorylation on Shp2/Csw function can be bypassed, at least at sufficiently high levels of expression.

Because all of the above studies used over-expression approaches, a requirement for Shp2 tyrosyl phosphorylation might have been obscured. To clarify the role of tyrosyl phosphorylation of Shp2, we retroviral gene transduction to reconstitute 3T3-immortalized fibroblasts derived from Shp2 mutant mice (8,23) with wild type (WT) Shp2 and Shp2 mutants lacking either or both tyrosyl phosphorylation sites. This approach allowed us to directly compare the signaling abilities of WT Shp2 and phosphorylation site mutants expressed at comparable levels. Our results show that tyrosyl phosphorylation is, in fact, required for normal levels and kinetics of Erk activation in some (FGF, PDGF), but not all (EGF) signaling pathways in fibroblasts. Our data also confirm our previous report that Y542 is the major Grb2 binding site in tyrosyl phosphorylated Shp2, and suggest that, in contrast to the predictions of the adapter model, Grb2 binding is not sufficient to mediate the signaling function of Shp2 in the Ras/Erk pathway.

**Experimental Procedures**

**Cell lines and culture**

3T3-immortalized fibroblasts (Ex3-/- cells) derived from a Shp2 Exon3-deficient mouse (23) were maintained in Dulbecco’s modified Eagles medium (DMEM) containing 10 % fetal bovine serum (FBS), 100 U/ml of penicillin and 100 ug/ml streptomycin.
cDNAs encoding WT human Shp2 (24) and its Y542F, Y580F and Y542,580F (14) mutants were inserted into the retroviral vector pBABE puro (25). Additional details regarding the generation of these constructs are available from T.A. upon request. The resulting retroviral vectors were co-transfected with the Ecopack packaging vector (generously provided by Dr. D. G. Gilliland, Brigham and Women’s Hospital, Boston) into 293T cells (26). Twenty-four (24) hours post-transfection, viral supernatants were collected and used to infect Shp2-mutant cells in the presence of polybrene (4 ug/ml) for 4h. Pools of infected cells were recovered following 1 week of puromycin (2 ug/ml) treatment and used for experiments.

For growth factor stimulations, cells were starved for at least 16 h in serum-free DMEM, and then exposed to EGF (20 ng/ml), IGF-I (50 ng/ml), FGF (20 ng/ml) or PDGF (20 ng/ml). In some experiments, the Src-selective inhibitor SU6656 (Calbiochem) was added to a final concentration of 1uM for 1h before stimulation.

**Immunoprecipitations and immunoblotting**

Cells were washed three times with ice-cold PBS, and lysed in a buffer containing 1% (w/v) Triton X-100, 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 1 mM Na\textsubscript{3}VO\textsubscript{4}, 1 mM phenylmethylsulfonyl fluoride, 10 ug/ml aprotinin, 10 ug/ml leupeptin, 0.5 ug/ml antipain and 0.5 ug/ml pepstatin. Lysates were centrifuged at 10,000 g at 4C for 30 min, and the protein concentration of clarified lysates was determined by BCA assay (Pierce, Rockford, IL). Immunoprecipitations and immunoblotting were performed as described previously (23,27). The monoclonal anti-phosphotyrosine antibody 4G10 was purchased from Upstate Biotechnology Inc, (Lake Placid, N.Y.); monoclonal anti-Shp2 and -Grb2 antibodies were from Transduction Laboratories (Lexington, Ky.). Polyclonal anti-Shp2 and anti-MAPK antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.). Polyclonal anti-phosphoMAPK and phospho-SHP-2 (Tyr542) and (Tyr580) antibodies were from Cell Signaling Technology (Beverly, Mass.). All immunoreagents were used at the concentrations recommended by their manufacturers.

**MAP kinase assays**
Cells were lysed in 20 mM Tris HCl [pH 7.4], 150 mM NaCl, 1% Nonidet P-40, 1 mM Na3VO4, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride, and clarified by centrifugation at 10,000 g for 30 min. Clarified lysates were incubated overnight at 4°C with 2 ug of polyclonal anti-Erk2 antibodies (Santa Cruz Biotechnology). Protein A-Sepharose (30 ul) was added, and the mixture was rotated at 4°C for 30-60 min. Immune complex kinase assays using myelin basic protein (MBP) as substrate were performed as described previously (28). Incorporated 32P was visualized by autoradiography, and quantified by using NIH image 1.62. Data were evaluated by factorial analysis of variance and Newman-Keuls multiple range test.
Results and Discussion

To clarify the physiological function of tyrosyl phosphorylation of Shp2, we reconstituted Ex3-/- fibroblasts with WT Shp2, mutants of either (Y542F, Y580F) or both (YDF) tyrosyl phosphorylation sites, or the parental retrovirus as a control (Figure 1A). All experiments were performed with pools of retrovirally infected cells to average out possible inter-clonal variation. Immunoblot analysis using antibodies directed against the Shp2 C-terminus revealed that the levels of expression of the reconstituted WT and mutant Shp2 proteins were comparable in each pool (Figure 1B), thus enabling direct comparison of their signaling properties. An immunoreactive species smaller than WT Shp2 also was detected in each pool, including cells expressing the parental retrovirus alone. As described previously (8), this truncated Shp2 species arises as a consequence of splicing around the neomycin resistance cassette that was used to replace Exon 3. Although the N-terminally truncated protein has markedly increased PTP activity, because of its missing N-SH2 domain, it fails to target appropriately, and in all settings tested thus far, behaves as an Shp2 hypomorph (8,10,23,29-32).

Because of the key role of Shp2 in RTK-evoked Erk activation, we compared the response of WT- and mutant Shp2-reconstituted cells to several growth factors. In WT cells, FGF and PDGF stimulation resulted in strong tyrosyl phosphorylation of Shp2. In contrast, IGF-1 and EGF failed to evoke detectable Shp2 tyrosyl phosphorylation (Figure 1C). Why stimulation with some, but not all RTKs leads to tyrosyl phosphorylation of Shp2 is not known, although this difference may be more apparent than real. When over-expressed in heterologous cells, the insulin receptor can evoke detectable Shp2 tyrosyl phosphorylation (33), and EGF-stimulated tyrosyl phosphorylation of Shp2 also has been reported in some cell systems (13,15). Conceivably, RTK-evoked tyrosyl phosphorylation of Shp2 occurs only when surface expression of the receptor exceeds a certain minimal level. We have not quantified EGFR, PDGFR, IGF-1R or FGFR surface expression in WT or Shp2 mutant-reconstituted cells. However, we did observe higher and/or more sustained growth factor-evoked Erk activation in cells exposed to saturating levels of FGF or PDGF (which stimulate Shp2 tyrosyl phosphorylation), compared to those treated with saturating
levels of EGF or IGF (Figure 1D). Previous studies have shown that increasing RTK expression levels can prolong the kinetics of Erk activation (34,35). Although several other explanations remain possible, these data, along with reports of EGF- or insulin/IGF1-evoked Shp2 tyrosyl phosphorylation in other cell systems, are consistent with the possibility that Shp2 phosphorylation depends on the strength of RTK signal delivered. Alternatively, the ability of a particular growth factor to evoke Shp2 tyrosyl phosphorylation may depend on the proximity of Shp2 to the activated RTK. Notably, the PDGFR has a direct binding site for Shp2 (36,37), whereas Shp2 is recruited to the FGFR signaling complex via FRS-2, which binds constitutively to the FGFR (38,39). In contrast, Shp2 is recruited to the EGFR via Gab1 (40), and the insulin/IGF-1Rs primarily via members of the IRS family (41), which associate less tightly with the respective activated RTKs.

In fibroblasts, Shp2 is required for sustained Erk activation in response to EGF, IGF-1, FGF and PDGF (8,10,23,32). To assess the effects of Shp2 tyrosyl phosphorylation, we monitored Erk activation by phospho-specific antibody immunoblotting in cells expressing WT Shp2 or the phosphorylation site mutants (Figure 2). As expected, restoring WT Shp2 expression increased Erk activation in response to all growth factors, particularly at later time points, compared to vector control-infected cells (Figures 2B-F). Consistent with the absence of tyrosyl phosphorylation of Shp2 in response to EGF stimulation, we found no difference in EGF-evoked Erk activation between WT, Y542F-, or Y580F-expressing cells (Figure 2A). Mutation of both Y542 and Y580 also had no effect on EGF-evoked Erk activation (data not shown). In contrast, Erk activity induced by FGF (Figure 2B) or PDGF (Figure 2C) was decreased in Y542F and Y580F mutant cells compared to WT cells, although it remained higher than that in control (vector alone) cells. Erk activation was decreased even further in cells expressing the double phosphorylation site mutant (YDF) than in single mutant (Y542F, Y580F)-expressing cells, although the YDF mutant retained some ability to restore Erk activation compared to vector control cells (Figures 2D,E). Direct assessment of Erk activity by immune complex kinase assays confirmed that, compared to WT-expressing cells, Erk activation in response to FGF or PDGF was
impaired in cells expressing phosphorylation site mutants of Shp2 (Figure 2F). Taken together, these data indicate that tyrosyl phosphorylation of Shp2 enhances its ability to restore normal Erk activation to Shp2-mutant cells, although mutants of Shp2 that are unable to become tyrosyl phosphorylated retain some function.

Next, we examined the effect of each Y>F mutation on total tyrosyl phosphorylation of Shp2 and its association with Grb2 in growth factor-stimulated cells. Interestingly, the truncated mutant Shp2 expressed in Ex3-/− fibroblasts (and all of its derivative lines) retained the ability to undergo tyrosyl phosphorylation in response to PDGF, although to a significantly lower extent than WT Shp2-expressing cells (Figure 3A). These data suggest that, in contrast to a previous report on EGFR signaling (10), truncated Shp2 can be recruited to the vicinity of the PDGFR. Thus, the Ex3-/− protein may have some residual signaling potential and, consistent with this notion, we have found that unlike Ex3-/− embryos, which die at E8.5-10, totally null Shp2 embryos succumb before E6.5 (W. Yang and B.G.N., unpublished data). Surprisingly, however, total tyrosyl phosphorylation of Shp2 in cells expressing either Y542F or Y580F, as measured by immunoblotting with monoclonal anti-phosphotyrosine antibody 4G10 (Figure 3A) or several other anti-phosphotyrosine antibodies (data not shown) appeared to be reduced to levels seen in cells expressing only the parental retrovirus (Figure 3A). Similar results were obtained following stimulation with FGF (Figure 3B). Even more surprising, although the Y542F mutant lost virtually all Grb2-binding ability, PDGF-evoked Grb2 binding to Shp2 in Y580F-expressing cells was restored to WT levels, despite the apparent lack of any Shp2 tyrosyl phosphorylation of this mutant (Figure 3A). Likewise, the Y580F mutant retained about half the Grb2 binding ability of WT Shp2 in FGF-stimulated cells, despite lacking any obvious tyrosyl phosphorylation as detected by anti-phosphotyrosine immunoblotting (Figure 3B). These results appeared to suggest dissociation between Shp2 tyrosyl phosphorylation (as measured by total anti-phosphotyrosine immunoblotting) and Grb2 binding. On the other hand, they also suggested that Y542 was required for most of the ability of Grb2 to bind to Shp2 following PDGF or FGF stimulation.

These are at least two potential explanations for this apparent paradox. Both tyrosines
might be necessary for efficient phosphorylation of Shp2. Alternatively, the anti-phosphotyrosine antibodies used for immunoblotting might not recognize monophosphorylated Y542 or Y580 effectively. To distinguish between these possibilities, we used recently developed phosphospecific antibodies that recognize Y542 or Y580 (Figures 3C, D). Immunoblotting with pY542-specific antibodies revealed that, following PDGF stimulation, Y542 was phosphorylated to comparable extents in WT and Y580F cells. In contrast, no phosphorylation was detected in Y542F-expressing cells, confirming the specificity of these antibodies for pY542 (Figure 3C, left panel). Immunoblotting with anti-pY580 revealed no phosphorylation in Y580F cells, as expected. Surprisingly, however, Y542F-expressing cells exhibited an ~50% decrease in Y580 phosphorylation (Figure 3C, right panel). These data suggest that Y542 must be phosphorylated to promote efficient Y580 phosphorylation in response to PDGF stimulation. Following PDGF stimulation, the Y542F and Y580F mutants also exhibited increased mobility on SDS-PAGE compared to WT Shp2. Whether this reflects the direct effects of tyrosyl phosphorylation on the mobility of Shp2 in SDS-PAGE or a more indirect consequence of tyrosyl phosphorylation, e.g., on seryl/threonyl phosphorylation of Shp2, remains to be determined.

The tyrosyl phosphorylation site mutations had a somewhat different effect on FGF signaling. Whereas mutation of Y580 had no effect on Y542 phosphorylation in PDGF signaling, FGF-evoked phosphorylation of Y542 was reduced by ~50% in Y580F-expressing cells; note that Y580F and WT Shp2 are expressed at comparable levels in these cells (Figures 1B, 3D). As in PDGF signaling, however, Y542 phosphorylation also was required for efficient phosphorylation of Y580 (Figure 3D).

Taken together, these data suggest a model in which, in the absence of growth factor stimulation, the C terminus of Shp2 is in a “closed” conformation in which the tyrosyl phosphorylation sites, Y580 in particular, are relatively inaccessible. Following PDGF stimulation, Y542 probably becomes phosphorylated first, and then promotes “opening” of the closed form, permitting efficient phosphorylation of Y580. Consistent with our previous work, but in contrast to the conclusions of others (15,16), Y542 must also
be the major Grb2 binding site in response to PDGF, because Grb2 binding is essentially normal in Y580F-expressing cells, whereas it is absent in cells expressing Y542F. Y542 phosphorylation probably occurs first in response to FGF stimulation as well, but here, Y580 must also undergo phosphorylation for WT levels of Y542 phosphorylation to be attained. Further studies will be required to determine whether Y580 phosphorylation is required for effective phosphorylation of Y542 by its respective kinase, or to stabilize it from dephosphorylation, most likely autodephosphorylation (33). It also is not clear whether tyrosyl phosphorylation, per se, evokes the conformational changes proposed above, or whether it acts more indirectly, e.g., by promoting seryl/threonyl phosphorylation and/or binding of other proteins. Notably, Shp2 has a proline-rich region between Y542 and Y580 (Figure 1A); conceivably, phosphorylation of either (or both) of these residues could regulate binding of an SH3 domain-containing protein. Regardless, Y542 is probably the major Grb2 binding site in response to FGF signaling as well, because in Y580F-expressing cells, Grb2 binding to Shp2 is reduced in parallel to the reduction in Y542 phosphorylation (Figures 3B, D).

One potential explanation for the apparently ordered phosphorylation of Y542 and Y580 would be if these sites were the targets of different PTKs. For example, Y542 might be phosphorylated by the RTK itself, while Y580 would be phosphorylated by a downstream PTK. Src family kinases (SFKs) are attractive candidates for such downstream PTKs, because they are activated in a variety of RTK signaling pathways (42,43). Moreover, Shp2 is tyrosyl phosphorylated in v-Src-transformed cells and also is a good Src substrate in vitro (11,44).

To test whether SFKs phosphorylate Shp2 downstream of growth factor receptors, we treated WT Shp2-expressing cells with the Src selective inhibitor, SU6656 (45). However, there was no effect of this inhibitor on either Y542 or Y580 phosphorylation in FGF or PDGF-stimulated cells (Figure 4A). As an independent test of the role of SFKs, we compared growth factor-evoked Shp2 phosphorylation in cells lacking Src, Fyn and Yes (SYF cells) and SYF cells reconstituted with Src (46). Consistent with the inhibitor studies, there was no difference in phosphorylation of either Y542 or Y580 in SYF and SYF Src
cells (Figure 4B). These data indicate that SFK are not required for phosphorylation of either Y542 or Y580 in response to FGF or PDGF, and suggest that each is a direct target of the FGFR and PDGFR, respectively. We cannot exclude the possible involvement of another downstream PTK in phosphorylation of either Y542 or Y580. Notably, however, two other types of downstream PTK, Abl and Tec family PTKs, typically require SFKs to become activated (47-49).

Our results also argue strongly against the Grb2 adapter model for positive signaling by Shp2. Although mutation of Y580 results in marked diminution of the ability of Shp2 to restore normal Erk activation in response to PDGFR stimulation (Figures 2C, D), Y580F Shp2 retains essentially WT Grb2 binding in PDGF-stimulated cells (Figure 3A). Likewise, Y580F retains ~50% Grb2 binding in FGF-stimulated cells. Because heterozygotic Shp2-deficient fibroblasts express about half the level of Shp2 as WT cells, yet activate Erk normally in response to FGF (8);(W. Yang and B.G.N., unpublished data), the level of Grb2 binding in Y580F cells should have been sufficient to restore Shp2 function were the adapter model correct.

Instead, our results support alternative explanations for the role of tyrosyl phosphorylation of Shp2. Cole and colleagues have provided compelling evidence that tyrosyl phosphorylation of Shp2 can result in increased Shp2 activity (18), and increased Shp2 activity might well explain our results. But in addition to becoming tyrosyl phosphorylated, Shp2 binds via its SH2 domains to the PDGFR and Gab1 following PDGF stimulation, and to Gab1 and FRS2 in response to FGF. Engagement of the N-SH2 domain of Shp2 fully activates the enzyme, so it is unclear how/why tyrosyl phosphorylation would be required to promote additional Shp2 activation. Conceivably, a significant amount of tyrosyl phosphorylated Shp2 transmits signals while unbound to the above phosphotyrosyl proteins.

However, a second alternative is suggested by the role of tyrosyl phosphorylation of the receptor tyrosine phosphatase RPTPα. Approximately 20% of RPTPα is phosphorylated constitutively on Y789 and, like Shp2, tyrosyl phosphorylated RPTPα binds Grb2 (50,51). Recent studies show that tyrosyl phosphorylation of RPTPα is...
essential for its major function, the dephosphorylation of inhibitory C-terminal phosphotyrosines of Src family PTKs (SFKs) (52,53). Inactive SFKs assume a closed state with their C-terminal phosphotyrosines bound intramolecularly to their respective SH2 domains. Phosphorylated Y789 of RPTPα competes with SFK C-terminal tyrosyl residues for binding to SFK SH2 domains, thereby “prying open” the closed form and facilitating dephosphorylation of SFK C-terminal tyrosyl residues by the RPTPα catalytic domain. Grb2 competes with SFK C-terminal phosphotyrosines for binding to RPTPα pY789; thus, Grb2 functions as an inhibitor of the biological activity of RPTPα. By analogy, tyrosyl phosphorylated Shp2 might engage the SH2 domain of a potential substrate(s), thereby increasing its accessibility to the PTP domain. If so, then key substrates for Shp2 might be identified by virtue of their ability to bind to phosphotyrosyl peptides derived from the sequences surrounding Y542 and/or Y580.

In summary, our studies reveal new complexity in Shp2 regulation and function. We have shown that phosphorylation of the two Shp2 C-terminal tyrosines most likely occurs in an ordered fashion, with Y542 phosphorylation preceeding phosphorylation of Y580. Moreover, our results show unambiguously that Y542 is the major Grb2 binding site in Shp2, at least in fibroblasts stimulated with PDGF or FGF. Most importantly, although it is well established that the PTP activity of Shp2 is essential for its positive signaling functions, we have demonstrated for the first time that tyrosyl phosphorylation has an important, but more auxiliary role, potentiating the actions of Shp2 in some, but not all, RTK signaling pathways. Further studies will be required to clarify just how tyrosyl phosphorylation regulates Shp2 function.

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**Figure Legends**

**Figure 1.** A. Schematic representation of WT human Shp2 and mutant constructs B. Shp2 expression levels in mutant cell pools. Retroviral vectors directing expression of WT Shp2 and the indicated mutants or the parental retrovector (pBabepuro) alone (Vector) were packaged and used to infect Ex3/-/- fibroblasts as described in *Experimental Procedures*. The level of Shp2 expression in each pool was determined by immunoblotting with anti-N-terminal and anti-C-terminal Shp2 antibodies, as indicated. Note the presence in each pool of the N-terminally truncated Shp2 protein generated by the Ex3 mutation (Ex 3/-/- Shp2), which is detectable only with antibodies against the Shp2 C-terminus, as well as the reconstituted WT and mutant Shp2 proteins (recon. Shp2), as indicated. C. Level of Shp2 tyrosyl phosphorylation evoked by growth factor stimulation of WT cells. Shp2 immunoprecipitates prepared from cells stimulated with the indicated growth factors were analyzed by SDS-PAGE, followed by anti-phosphotyrosine immunoblotting. D. Differential growth factor-evoked Erk activation in WT cells. Cells were stimulated with the indicated growth factors for the indicated times as described in *Experimental Procedures* and Erk activation was monitored by immunoblotting using anti-phospho-MAPK antibodies, followed by reprobing with anti-total MAPK antibodies to control for equal loading.

**Figure 2.** Erk activation in fibroblasts expressing WT and tyrosyl phosphorylation site mutants of Shp2. Pools of cells expressing WT, mutant Shp2 or control vector (-/-), as indicated, were starved and then stimulated with EGF (A), FGF (B, D) or PDGF (C, E) for the indicated times, as described in *Experimental Procedures*. Erk activation was assessed by immunoblotting as in Figure 1 (A-E). To quantify Erk activation more precisely, Erk activity was measured by immune complex kinase assays (FGF, n=4; PDGF, n=3), as described in *Experimental Procedures* (F). All values are the mean ± SD. *, P<0.05 compared with either WT or control parental -/- cells.

**Figure 3.** Effect of tyrosyl phosphorylation site mutants on Shp2 tyrosyl phosphorylation
and Grb2 association. Cells expressing WT Shp2 or the indicated mutants were stimulated with PDGF (A, C) or FGF (B, D) for 5 min. Cell lysates were prepared and immunoprecipitated with anti-Shp2 antibodies. Immunoprecipitates were analyzed by SDS-PAGE followed by immunoblotting with anti-phosphotyrosine (clone 4G10; αpY), anti-phospho-SHP-2 (Tyr542); (αpY542), anti-phospho-SHP-2 (Tyr580); (αpY580) or anti-Grb2 antibodies, as indicated. The same membrane was stripped and reprobed with anti-Shp2 antibodies to control for loading.

**Figure 4.** SFK are dispensable for Shp2 tyrosyl phosphorylation. **A.** Cells reconstituted with WT Shp2 were exposed to the indicated growth factors with or without the Src selective inhibitor SU6656, for 5 min, and Shp2 immunoprecipitates were subjected to immunoblotting with anti-phospho-SHP-2 (Tyr542); (αpY542) or anti-phospho-SHP-2 (Tyr580); (αpY580) antibodies, as indicated. **B.** SYF cells and SYF cells reconstituted with Src were treated with PDGF or FGF for 5 min. Shp2 immunoprecipitates were subjected to immunoblotting with anti-phospho-SHP-2 (Tyr542); (αpY542) or anti-phospho-SHP-2 (Tyr580); (αpY580) antibodies, as indicated. The membrane was stripped and reprobed with anti-Shp2 antibodies to control for loading.
Figure 1
Figure 2
Figure 3
Figure 4
Tyrosyl phosphorylation of Shp2 is required for normal Erk activation in response to some, but not all growth factors
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