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Specific Tyrosine Phosphorylations Mediate Signal-Dependent Stimulation of SHIP2 Inositol Phosphatase Activity, while the SH2 Domain Confers an Inhibitory Effect To Maintain the Basal Activity

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ABSTRACT: SH2 domain-containing 5-inositol phosphatase (SHIP2) is implicated in the development of type 2 diabetes and cancer. Tyrosine phosphorylation of SHIP2 is shown to enhance its phosphatase activity. Using IP4 as a substrate, we show here that tyrosines 986, 987, and 1135 are critical for EGF-induced stimulation of SHIP2 activity. SHIP2 with a disrupted SH2 domain (R47G mutation) displays higher constitutive activity than wild-type SHIP2. Deletion of the C-terminus region similarly activates SHIP2. Thus, the SH2 domain of SHIP2, in conjunction with the C-terminus, confers an inhibitory effect to maintain a low basal activity, and signal-induced tyrosine phosphorylations overcome this effect to activate SHIP2.

Phosphoinositide (PI) lipids are critical second messengers in cell signal transduction pathways. PI lipids regulate glucose metabolism, cell survival and proliferation, and cell adhesion−migration processes. Phosphatidylinositol 3,4,5-trisphosphate (PIP3)[1] generated by the activated phosphoinositide 3-kinase (PI 3-kinase) is central to these regulations (*1*). SHIP2 phosphoinositol phosphatase dephosphorylates PIP3 (*2*), and its function is important for glucose and energy homeostasis (*3*) (reviewed in ref (*4*)). We and others have described a role for SHIP2 in cytoskeleton remodeling and ligand-dependent receptor endocytosis (*5*−*9*) that is linked to increased cancer development and metastasis (*10*−*12*). Because of its broad biological and therapeutic significance (*4*, *13*), it is important to improve our understanding of the mechanisms regulating SHIP2 function.

Early studies of the regulation of SHIP2 function have found no evidence for extracellular signal-induced changes in the enzymatic activity, in spite of specific ligand-induced tyrosine phosphorylation of SHIP2 (*7*, *14*, *15*). These studies therefore suggested that tyrosine phosphorylation-dependent protein interactions may regulate SHIP2 function by altering its subcellular localization. However, a recent report describes significant stimulation of the 5-phosphatase activity of SHIP2 upon its tyrosine phosphorylation in 1321N1 astrocytoma cells treated with vanadinium compounds (*16*). Further, this report shows that EGF or hydrogen peroxide (H_2O_2) treatment of HeLa cells activates SHIP2 activity in correlation with its tyrosine phosphorylation levels. As this is a significant turnaround from a well-accepted conclusion about the role of tyrosine phosphorylation in the regulation of SHIP2 activity, we reinvestigated the effects of EGF and H_2O_2 on SHIP2 phosphatase activity. By making use of a simple nonradioactive in vitro assay to measure SHIP2 phosphatase activity on a 3-phosphorylated soluble inositol molecule, IP4 (inositol 1,3,4,5-tetrakisphosphate), we confirm the activation of SHIP2 by EGF and H_2O_2. Furthermore, we identify the tyrosine residues involved in the EGF-induced activation of SHIP2. Interestingly, our experiments reveal an inhibitory mechanism, mediated by the SH2 domain and the C-terminus of SHIP2, responsible for the low-level activity under unstimulated conditions.

A purified protein fragment of SHIP2 (amino acids 288−926) containing the catalytic domain was used to develop an in vitro SHIP2 phosphatase assay. A soluble inositol, inositol 1,3,4,5-tetrakisphosphate (IP4), was employed as a surrogate substrate in the malachite green-based phosphatase assay. IP4 contains the 3-phosphorylated inositol headgroup identical to that of PIP3 and is a high-affinity SHIP2 substrate in vitro (*2*, *17*). Activity of SHIP2 on IP4 reflects the PIP3 phosphatase activity of SHIP2 (*16*) (Figure 1 of the Supporting Information). This assay produces a low background level in the absence of either the enzyme or the substrate and shows a linear response with an increase in the concentration of the enzyme (Figure 1A). Using this assay, we quantified the activity of SHIP2 immunoprecipitated from the HeLa cells. EGF or H_2O_2 treatment of 15 h serum-starved, confluent cultures increased the level of SHIP2 tyrosine phosphorylation (Figure 1B) and stimulated the phosphatase activity by ∼2.5-fold (Figure 1C). These results concur with those of Batty et al. (*16*).

To verify the role of tyrosine phosphorylation in SHIP2 activation, we made use of a series of mutant SHIP2 genes. Structural domains of SHIP2 and the mutants we employed in the study are shown in a schematic of SHIP2 amino acid sequence (Figure 2A). We used two catalytically inactive SHIP2 mutants, ΔRV (inositol phosphatase domain deleted) and D690A (phosphatase signature motif), as negative controls.

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[1]Abbreviations: SHIP2, SH2 domain-containing inositol 5-phosphatase; PIP3, phosphatidylinositol 3,4,5-trisphosphate; H_2O_2, hydrogen peroxide; IP4, inositol 1,3,4,5-tetrakisphosphate.

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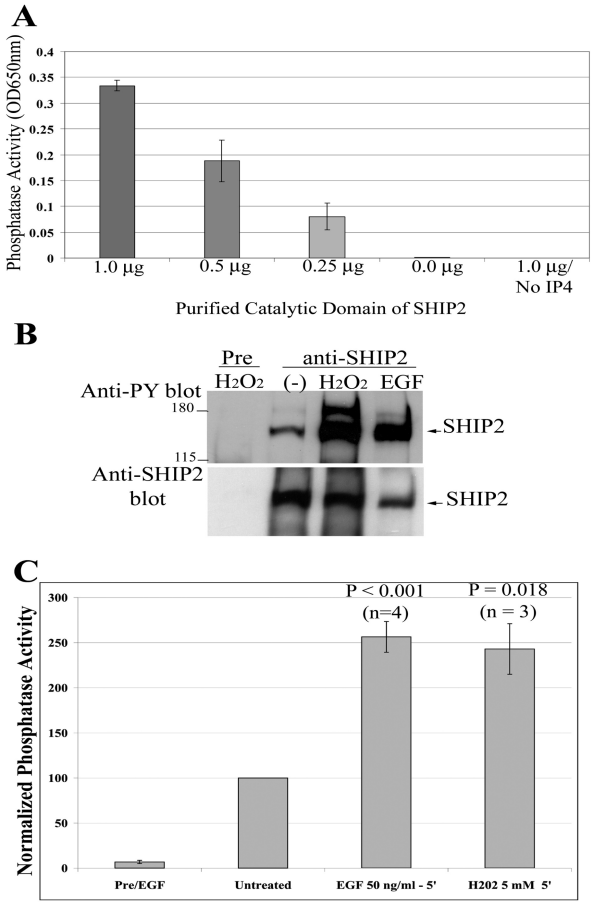

FIGURE 1: Tyrosine-phosphorylated SHIP2 is more active. (A) Malachite green assay for measuring the phosphatase activity of the bacterially expressed SHIP2 catalytic domain using IP4 (100 μM) as a substrate. (B) Anti-SHIP2 immunoprecipitations (IP) from untreated [serum-starved for 15 h (−)], H_2O_2-treated (5 mM for 5 min), or EGF-treated (50 ng/mL for 5 min) HeLa cells were analyzed in anti-phosphotyrosine (PY) and anti-SHIP2 Western blots. Pre is the control antibody used for the IP. (C) Phosphatase activity of immunopurified SHIP2 from EGF- or H_2O_2-treated HeLa cells after normalization to the untreated sample. *P* values (Student's *t* test) are for comparison with the untreated sample.

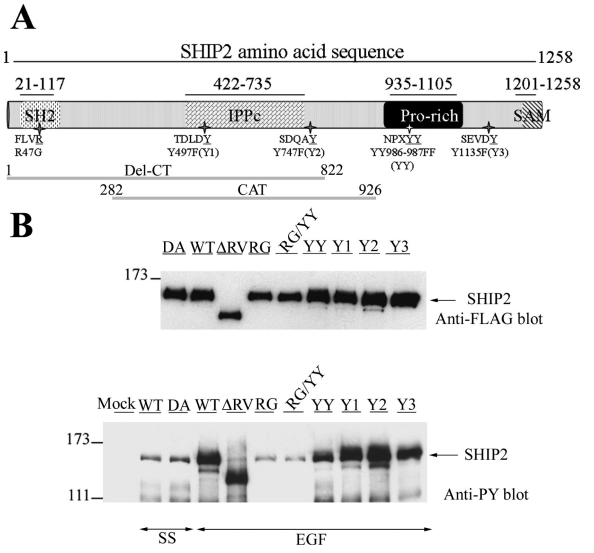

FIGURE 2: Domain structure of SHIP2 showing the critical tyrosine residues and their contribution to the EGF-induced phosphorylation. (A) Schematic of SHIP2 protein domain structure showing the critical amino acid residues targeted to study the effect of tyrosine phosphorylation on SHIP2 activity. Regions encoded by the truncated SHIP2 mutants, Del-CT and CAT, are also shown. (B) Anti-FLAG Western blot of anti-FLAG immunoprecipitates showing the expression of FLAG-tagged SHIP2 proteins in HeLa cells transfected with the indicated construct. An anti-phosphotyrosine (PY) blot shows the tyrosine phosphorylation of SHIP2 in response to EGF (50 ng/mL for 5 min) following serum starvation for 15 h. The following SHIP2 constructs were used: DA (D690A), WT (wild type), ΔRV (phosphatase domain deleted with EcoRV digestion), RG (R47G), RG/YY (R47G and YY-FF), YY (YY986−987FF), Y1 (Y497F), Y2 (Y747F), and Y3 (Y1135F).

FLAG-tagged wild-type and mutant SHIP2 genes were transiently expressed in HeLa cells and immunoprecipitated using the anti-FLAG (M2) antibody. Figure 2B shows the expression levels and the EGF-induced tyrosine phosphorylation of these proteins. The R47G mutation abolished the EGF-induced SHIP2 tyrosine phosphorylation, as previously reported for PDGF in primary astrocytes (*15*). We reasoned that this mutation will consequently abolish the EGF stimulation of SHIP2 activity. On the other hand, the activity of R47G-SHIP2 is elevated to a level similar to that of EGF-stimulated, tyrosine-phosphorylated, wild-type SHIP2 (Figure 3A).

Next, we tested four possible tyrosine phosphorylation sites (NetPhos2.0 score of ~0.6 and above) by using phenylalanine substitution mutations at Y497 (Y1), Y747 (Y2), Y1135 (Y3), and Y986 and Y987 (YY). Among the four putative sites, YY (positions 986 and 987 forming the NPXY motif) and Y3 (Y1135) residues are more prominent sites of EGF-induced phosphorylation (Figure 2B). The phosphatase assay of anti-FLAG immunoprecipitates revealed the EGF-induced activation of wild-type SHIP2 and varying degrees of suppression of this effect in all four tyrosine mutants (Figure 3B). As observed with the R47G mutant, a double mutant containing both R47G and YY substitutions showed an elevated level of activity similar to that of the EGF-treated wild-type protein. The YY and Y3 mutants showed no significant EGF-dependent activation when compared to either the untreated wild type or the untreated respective mutant. Levels of EGF-induced activation of Y1 and Y2 were significantly higher than that of the untreated wild type but lower than that of the EGF-treated wild type (Figure 3B). The activities of YY, Y1, Y2, and Y3 were not significantly different from that of wild-type SHIP2 under untreated serum-starved condition (Figure 3C and Figure 2 of the Supporting Information). Thus, YY986−987 and Y1135 constitute the primary target sites for EGF-induced activation, and Y747 and Y497 may play a supplemental role.

We then tested whether the elevated activity of R47G is independent of EGF treatment. Indeed, R47G exhibited an ~2-fold higher activity compared to that of the wild type under unstimulated serum-starved conditions (Figure 3C). Similar results were also obtained in HEK293 cells (data not shown). These results indicate that an inhibitory effect is mediated by the SH2 domain of SHIP2. Interestingly, SHIP2 enzymatic activity is thought to be important for suppressing signal-induced PIP3 levels, whereas PTEN 3-phosphatase regulates the turnover of this lipid in the unstimulated state. It is possible that a SH2 domain-interacting protein may interfere with the conformational flexibility of SHIP2 to restrict its activity. Alternatively, there could be an inter- or intramolecular interaction between the SH2 domain and the C-terminus of SHIP2. As none of the tyrosine mutants we examined show an elevated activity similar to that of R47G, they are unlikely to be the sites for this

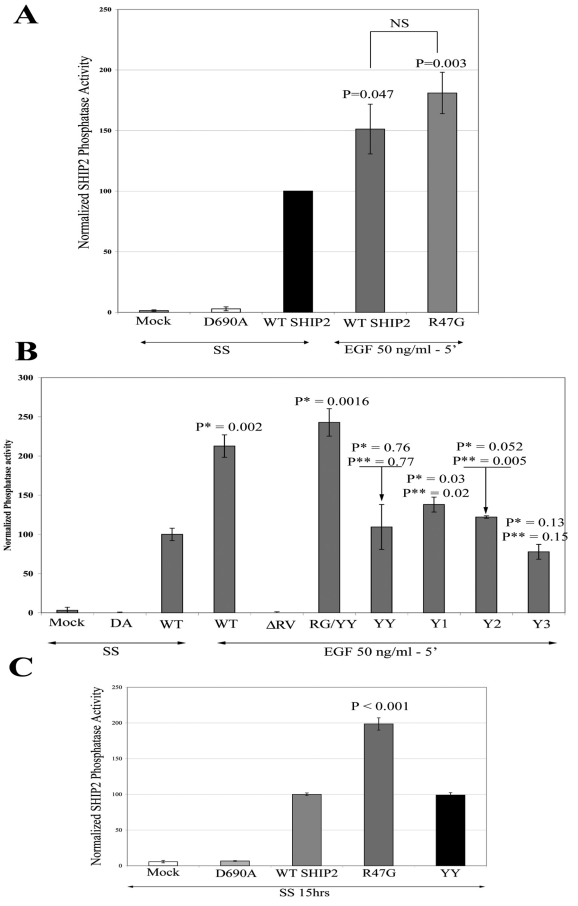

FIGURE 3: Inhibitory and stimulatory mechanisms of SHIP2 phosphatase activity. Phosphatase activity of immunopurified SHIP2 from HeLa cells normalized to the untreated sample. Amounts of SHIP2 proteins were equalized on the basis of anti-FLAG blots. HeLa cells, transfected with the indicated SHIP2 construct, were serum-starved for 15 h (SS) before EGF treatment (50 ng/mL for 5 min). A Student's t test was used to determine the statistical significance: (A) $n = 4$ (in triplicate) and (B and C) representative experiment (in triplicate), reproduced four times with similar results. (A) P values are for comparison with values of the untreated wild type, and NS denotes not significant. (B) P^* values are for comparison with values of the untreated wild type (SS), and P^{**} values are for comparison with the activity of the respective mutant under untreated, serum-starved conditions (from Figure 2 of the Supporting Information) ($P = 0.01$ for EGF-treated WT vs EGF-treated Y1 and 0.003 for EGF-treated WT vs EGF-treated Y2). (C) P value for comparison with the value of the wild type.

SH2 domain interaction. As with the EGF-induced effect, redundancy is a distinct possibility. One or more of the other tyrosines located at the C-terminus (e.g., positions 886, 1162, and 1213) may be involved in this regulation. We tested this notion using two truncated constructs of SHIP2: one with a deletion in the C-terminal region (Del-CT) and the other truncated at both the N- and C-termini (contains the central catalytic domain; termed CAT). Both truncated SHIP2 genes are more active than the wild type (Figure 3 of the Supporting Information) (the difference between the two mutants, although intriguing, is not statistically significant). Therefore, the C-terminal region clearly plays an important role in the negative regulation of SHIP2.

Identification of a mechanism for suppressing SHIP2 activity could be harnessed for targeted anti-diabetes and anti-cancer therapies. Although PIP3 is thought to be the physiological substrate for SHIP2, studies on the effects of SHIP2 on soluble inositol levels have not yet been reported. IP4 binds to many PH domain-containing proteins and possibly competes with PIP3. IP4 also competes with inositol 3,4,5-trisphosphate (IP3), a calcium signaling trigger. A negative role for IP4 has been illustrated in B cell survival and selection (*18*). Our results showing the regulation of IP4 5-phosphatase activity of SHIP2 by the extracellular signals raise an intriguing possibility of a biological role for SHIP2 in IP4 turnover.

SUPPORTING INFORMATION AVAILABLE

Comparison of SHIP2 activity on IP4 and PIP3, basal activity of tyrosine mutants of SHIP2, activity data from two truncated SHIP2 genes, and experimental methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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