Serine/Threonine Phosphorylation of ShcA

REGULATION OF PROTEIN-TYROSINE PHOSPHATASE-PEST BINDING AND INVOLVEMENT IN INSULIN SIGNALING

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Serine phosphorylation of the ShcA signaling molecule has been reported recently. In this work, we have identified 12-O-tetradecanoylphorbol-13-acetate (TPA)- and growth factor-induced serine/threonine phosphorylation sites in p52Shc and p66Shc. Among them, Ser\(^{29}\) in p52Shc (equivalent to Ser\(^{138}\) in p66Shc) was phosphorylated only after TPA stimulation. Phosphorylation of this site together with the intact phosphotyrosine-binding domain was essential for ShcA binding to the protein-tyrosine phosphatase PTP-PEST. TPA-induced ShcA phosphorylation at this site (and hence, its association with PTP-PEST) was inhibited by a protein kinase C-specific inhibitor and was induced by overexpression of constitutively active mutants of protein kinase C\(\alpha\), -\(\varepsilon\), and -\(\delta\) isoforms. Insulin also induced ShcA/PTP-PEST association, although to a lesser extent than TPA. Overexpression of a PTP-PEST binding-defective mutant of p52Shc (S29A) enhanced insulin-induced ERK activation in insulin receptor-overexpressing NIHc-B cells. Consistent with this, p52Shc S29A was more tyrosine-phosphorylated than wild-type p52Shc after insulin stimulation. Thus, we have identified a new mechanism whereby serine phosphorylation of ShcA controls the ability of its phosphotyrosine-binding domain to bind PTP-PEST, which is responsible for the dephosphorylation and down-regulation of ShcA after insulin stimulation.

Protein phosphorylation, a key component in the regulation of signaling pathways controlling many fundamental physiological processes, is determined not only by protein kinases, but also by protein phosphatases (1). One of the pathways controlled by protein phosphorylation and dephosphorylation is the mitogen-activated protein kinase (MAPK) pathway that converts the receptor signals into a variety of outputs (1, 2). Shc adapter/docking proteins are an important component of this pathway because they are involved in transducing the activation signals from receptor or cytoplasmic tyrosine kinases to downstream signaling cascades (3–5). At least three genes, shcA, shcB, and shcC, are known to encode Shc proteins. These share an amino-terminal phosphotyrosine-binding (PTB) domain, a central proline/glycine-rich region (CH1), and a carboxy-terminal Src homology 2 (SH2) domain (6–9). ShcA exists in three isoforms in mammalian cells, p46, p52, and p66, which differ only in the extent of their amino-terminal sequence and are produced through alternative splicing and differential use of translation initiation sites (10).

ShcA has two modules of phosphotyrosine recognition with different specificities, an amino-terminal PTB domain and a carboxy-terminal SH2 domain (11). Thus, it can associate with tyrosine-phosphorylated proteins such as receptor tyrosine kinases and cytoplasmic proteins (e.g. SHIP2) (12). ShcA has been found to be phosphatylated rapidly and efficiently by all tyrosine kinases tested to date (10). These phosphorylation sites have been mapped to Tyr\(^{239}\), Tyr\(^{240}\), and Tyr\(^{217}\) in the CH1 domain (13–15). These phosphorylated tyrosines then serve as binding sites for the SH2 domain of adapter protein Grb2 (15–17), which is constitutively associated with SOS, a ubiquitously expressed Ras guanine nucleotide exchange factor. This binding leads to recruitment of SOS to the plasma membrane, an event considered sufficient to induce Ras activation. Consistent with this model, overexpression of ShcA can transform mouse fibroblasts and induce differentiation of PC12 cells (8).

In addition to tyrosine phosphorylation, p66Shc can also be phosphorylated at serine/threonine residues in response to epidermal growth factor (EGF) (18), 12-O-tetradecanoylphorbol-13-acetate (TPA) (19), UV stress (20), Taxol (21), and endothelin-1 (22). EGF-induced serine/threonine phosphorylation of p66Shc has been implicated in the negative regulation of the MAPK pathway (18). In contrast, it has been suggested that the TPA-induced serine/threonine phosphorylation of p52Shc/p66Shc is involved in ERK activation because it leads to an increase in ShcA/Grb2 association without an increase in ShcA tyrosine phosphorylation (19). Similarly, a protein kinase C (PKC)-dependent increase in Shc/Grb2 association after TPA treatment in MCF7 cells has been attributed to ERK activation (23). Finally, Migliaccio et al. (20) reported that serine phosphorylation of p66Shc regulates stress-induced apoptotic response and life span in mammals.

It has been shown that p52Shc/p66Shc proteins are associated with the protein-tyrosine phosphatase PTP-PEST and that this association can be enhanced in HeLa cells by PKC activators like TPA, but not by EGF (24). The sites involved in the association of p52Shc/p66Shc and PTP-PEST were mapped to the PTB domain in ShcA and an NPLH sequence in the carboxyl
termination of PTP-PEST (25). This was the first report showing phosphotyrosine-independent binding of the PTB domain of ShcA to a target protein. Murine PTP-PEST is a ubiquitously expressed cytosolic phosphatase of 112 kDa characterized by the presence of a so-called PEST sequence that is thought to confer protein stability (26). PTP-PEST is phosphorylated by PKC and protein kinase A at Ser29 and Ser35, and this serine phosphorylation down-regulates its activity (27). Association with other proteins (28) may also control the activity of protein-tyrosine phosphatases, and ShcA has been suggested to recruit PTP-PEST to its substrates for dephosphorylation (24). PTP-PEST has been shown recently to associate with and dephosphorylate ShcA in B cells, contributing to negative regulation of lymphocyte activation via inactivation of the Ras pathway (29). Activated insulin receptor phosphorylates various cellular substrates at tyrosine residues (30). ShcA is one of these substrates and has been shown to play an important role in insulin-induced ERK activation (31–33). Tyrosine phosphorylation of ShcA at residue 317 has been shown to play an important role in signal transduction to MAPK by insulin in Rat1 fibroblasts expressing the insulin receptor (33).

In this report, we have identified TPA- and growth factor-induced serine/threonine phosphorylation sites in ShcA. One of these phosphorylation sites has been characterized and shown to be involved in binding to the protein-tyrosine phosphatase PTP-PEST. Phosphorylation of this site was induced by over-expression of some constitutively active isoforms of PKC, but not by others. Furthermore, binding of PTP-PEST to ShcA downregulated insulin-induced ERK activation. We have shown also that TPA-induced ERK activation does not involve serine phosphorylation of Shc in mouse embryo fibroblasts.

**EXPERIMENTAL PROCEDURES**

**Materials**—Insulin purified from bovine pancreas was purchased from Sigma, TPA, horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies, ECL reagents, and protein A-Sepharose were from Amersham Biosciences. EGF from purchased from R&D Systems. The MEK inhibitor PD98059 and the PKC inhibitor bisindolylmaleimide were from Calbiochem. Anti-TPP-PEST antibodies were kindly provided by Dr. Jerrold Olefsky and were maintained in DMEM containing 10% CS and transfected 20 h later by the calcium phosphate precipitation method (Amersham Biosciences). Luciferase expression was determined as described (36).

**Transient Transfections, Immunoprecipitations, and Western Blot Analysis**—NIH3T3 cells were transfected as described (19). Briefly, 1 × 106 cells were plated in 10-cm dishes and transfected 20 h later with the desired plasmids as indicated using Lipofectamine (Invitrogen) according to the manufacturer’s instructions. Stable cell lines were selected in 400 μg/ml hygromycin B. Clones were isolated and screened by immunoblotting with anti-HA antibody. The parent cell line transfected with pCDNA3 + pX343 was used as a control.

**Transient Transfection and Analysis of Reporter Gene Expression**—Shc−/− cells (0.1 × 10⁶/well) were plated in 35-mm six-well culture plates with 2 ml of DMEM containing 10% CS and transfected 20 h later with the calcium phosphate precipitation method (Amersham Biosciences). Luciferase expression was determined as described (36).

**Identification of TPA- and Growth Factor-induced Serine Phosphorylation Sites in p52 Shc and p66 Shc**—To identify phosphoamino acid residues, stable cell lines were used as a control. ShcA mutants in which Ser138 of p66 Shc or Ser29 of p52 Shc were replaced by alanine or aspartate were cotransfected with plasmid pX343 (35) expressing a hygromycin resistance gene in HIRc-B cells at a ratio of 9:1 using LipofectAMINE (Invitrogen) according to the manufacturer’s instructions. Stable cell lines were selected in 400 μg/ml hygromycin B. Whole cell extracts were immunoprecipitated with mouse anti-HA monoclonal antibodies (12CA5). The immunoprecipitates were analyzed by Western blotting using either anti-HA or anti-phospho-ERK antibodies or other antibodies as indicated. An enhanced chemiluminescence detection method (Amersham Biosciences) was employed, and the membrane was exposed to Kodak X-Omat LS or Biomax MR film. In some experiments, specific signals were quantitated by scanning the films.

**Metabolic Labeling of ShcA and Phosphoamino Acid Analysis**—NIH3T3 cells in 10-cm dishes were transfected with expression vectors for either HA-p52 Shc or HA-p66 Shc. After 10 h, the medium was changed to phosphate-free DMEM containing 0.1% dialyzed calf serum for 16 h, followed by a 4-h incubation with [32P]orthophosphate (1 mCi/ml) performed in DMEM with 0.1% CS and 50 ng/ml TPA. Treated cells were collected by centrifugation (500 × g for 5 min) and then harvested; and HA-ShcA was purified as follows. Cell extracts were incubated with anti-HA antibodies prebound to the beads for 2 h. After washing, bound proteins were eluted with 1 ml of buffer containing 50 mTris-HCl (pH 7.5), 0.1% Nonidet P-40, 4 μg leupeptin, and 1 mg/ml aprotinin. Aliquots of cell lysate were taken for protein determination, and the remainder was subjected to SDS-PAGE using 10% gels. Bands corresponding to HA-ShcA were excised. Phosphopeptide mapping and phosphoamino acid analysis were performed as described previously (37).

**Mass Spectrometry**—HA-ShcA in gel slices (2 μg of metabolically unlabeled Shc + 2 μg of metabolically labeled Shc) was reduced with dithiothreitol, alkylated with iodoacetamide, and digested with trypsin (Promega) (39). Nanoelectrospray ionization tandem mass spectrometry was performed according to Wilm and Mann (40), and phosphopeptides were detected by m/z – 79 precursor ion scanning (41). The mass spectra were acquired on an ABI 300 triple quadrupole mass spectrometer (PE Sciei, Toronto, Ontario, Canada) equipped with a nanoelectrospray ionization source (Protana, Odense, Denmark).

**Edman Degradation and Phosphate Release**—Tryptic phosphopeptides were separated by liquid chromatography-mass spectrometry, and the [32P]labeled phosphopeptides were lyophilized and subjected to solid-phase Edman degradation using an automated Model 477A sequenator (Applied Biosystems). Fractions from each cycle of the Edman degradation were lyophilized, redissolved in 50% acetonitrile, and spotted onto a thin-layer chromatography plate before exposure to a PhosphorImager screen.

**RESULTS**

**Identification of TPA- and Growth Factor-induced Serine/Threonine Phosphorylation Sites in p52 Shc and p66 Shc**—In addition to tyrosine phosphorylation, ShcA has been reported by our laboratory (20) and others (20–22) to be serine/threonine-phosphorylated in response to various stimuli, including TPA, EGF, insulin, UV stress, H₂O₂, Taxol, and endothelin-1. As we showed previously (19), both p52 Shc and p66 Shc are serine-
phosphorylated after TPA stimulation. To further characterize this phosphorylation, HA-tagged p52\textsuperscript{Shc} and p66\textsuperscript{Shc} proteins were overexpressed, radiolabeled in vivo, and immunoprecipitated from unstimulated or TPA- and FGF-2-stimulated NIH\textsuperscript{3}T3 cells. After separation by SDS-PAGE, bands corresponding to p52\textsuperscript{Shc} and p66\textsuperscript{Shc} were excised and digested overnight with trypsin. Two-dimensional gel electrophoresis analysis of the trypsin digests revealed enhanced labeling in several spots after TPA or FGF-2 stimulation. Phosphoamino acid analysis of these spots revealed that enhancement in p52\textsuperscript{Shc}-derived spots after TPA stimulation was due to serine phosphorylation, whereas after FGF-2 stimulation, it was due to...
tyrosine phosphorylation (data not shown). The enhancement in p66 Shc-derived spots was due to both tyrosine and serine phosphorylation after FGF-2 stimulation and only serine phosphorylation after TPA stimulation (data not shown).

The trypsin digests from the same experiment were also subjected to fractionation by HPLC. Two equivalent peptides derived from p52Shc and p66Shc isoforms with similar elution profiles showed enhanced phosphorylation after TPA treatment, but almost no phosphorylation under basal conditions or after FGF-2 treatment (Fig. 1A). Further analysis of these two peptides by nanoelectrospray ionization tandem mass spectrometry showed them to be identical to the sequence HGS-FVNKPTR (Fig. 1B). This peptide corresponds to the sequence of amino acids 136–145 in the p66 isoform and amino acids 27–36 in the p52 isoform. As this peptide contains only one serine residue, we conclude that the TPA-specific phosphorylation sites are Ser29 in p52Shc and Ser138 in p66Shc. Another peptide derived from p66Shc eluted later than the first peptide (50 min) and showed enhanced incorporation of 32P after TPA or FGF-2 stimulation (Fig. 1A). Nanoelectrospray ionization mass spectrometry analysis of this peptide in positive ion full-scan mode showed two masses of 5205 and 5287 Da. These masses could be accounted for by an ShcA tryptic peptide with a mass of 5126 Da containing one or two phosphorylation sites. To determine the phosphorylation sites in such a long peptide, we isolated the 32Pi-labeled peptide by liquid chromatography fractionation and subjected the peptides to solid-phase Edman degradation. The peptide released 32P in cycles 15 and 22 of the Edman degradation, which correspond to Thr29 and Ser36 (data not shown).

Phosphorylation of ShcA at Ser29 and Ser138 in the p52 and p66 Isoforms, Respectively, Regulates Its Binding to PTP-PEST—ShcA associates with PTP-PEST, and this association has been shown to increase after PKC activation (24). We confirmed in NIH3T3 cells that TPA treatment enhanced the association of PTP-PEST specifically with isoforms p52Shc and p66Shc, but not p46Shc (Fig. 2A). Because the p46Shc isoform lacks the 45-amino acid N terminus to the PTB domain encompassing the serine residue whose phosphorylation is induced upon TPA treatment, we considered the possibility that this serine phosphorylation is required for ShcA binding to PTP-PEST. Serine-to-alanine mutations in p52Shc and p66Shc prevented ShcA binding to PTP-PEST (Fig. 2B). A serine-to-aspartate mutation of the same residues in both isoforms of ShcA caused constitutive association with PTP-PEST and insensitivity to TPA stimulation for further enhanced association (Fig. 2C). Using in vitro binding assays, Charest et al. (25) have mapped the interaction of ShcA with PTP-PEST to the PTB domain of ShcA showing Ser29 (in red) in the amino-terminal fragment extending away from the binding pocket. The stretch of amino acids 107–116 is indicated in purple.
PKC isoforms can induce Shc serine phosphorylation. A, TPA (but not EGF or FGF-2) induces the association of ShcA with PTP-PEST. NIH3T3 cells were transiently transfected with HA-p52Shc and either unstimulated or stimulated for 10 min with 100 ng/ml TPA, 100 ng/ml EGF, or 10 ng/ml FGF-2. Cell extracts were immunoprecipitated (IP) using anti-HA antibody and analyzed with anti-PEST or anti-HA antibodies. IB, immunoblot. B, TPA-induced Shc/PTP-PEST association is PKC-dependent. NIH3T3 cells were transfected with HA-p66Shc and incubated overnight. Cells were treated with 500 nM bisindolylmaleimide (PKC Inhibitor) for 45 min or left untreated and were then stimulated with 100 ng/ml TPA for 10 min. The association of ShcA with PTP-PEST was analyzed as described above. C, overexpression of constitutively active PKC isoforms can induce ShcA serine phosphorylation and hence association with PTP-PEST. Expression vectors for PKC isoforms or an empty vector (EV) were cotransfected with 5 µg of HA-p52Shc. Cells were serum-starved for 16 h and lysed. Cell lysates were immunoprecipitated with anti-HA antibody and analyzed for PTP-PEST association as described for A. D, serine phosphorylation of ShcA at the PTP-PEST-binding site is MEK-independent. NIH3T3 cells were transfected with 100 ng/ml TPA for 45 min with PD98059 or left untreated, stimulated with 100 ng/ml TPA for 10 min, and lysed. Cell lysates were immunoprecipitated with anti-HA antibodies and analyzed for PTP-PEST association as described for A (upper two panels). Total cell lysates were also analyzed for ERK activation using anti-phospho-ERK (P-Erk) and anti-ERK antibodies (lower two panels).

Serine Phosphorylation-induced ShcA/PTP-PEST Association

The three-dimensional structure of the PTB domain, including the amino-terminal end of p52Shc, has been reported to be constitutively associated with Grb2 (38). However, some other mutants of conventional PKCβI and PKCβII isoforms were unable to induce this association. Phosphorylation of the p66Shc isoform at Ser50 has been reported to be MEK-dependent (18, 44). We asked whether phosphorylation of the site regulating PTP-PEST binding in ShcA is also MEK-dependent. Pretreatment of cells with the MEK inhibitor PD98059 for 45 min had no effect on TPA-induced ShcA phosphorylation or its association with PTP-PEST (Fig. 3D). As expected, TPA-induced ERK activation was inhibited by PD98059 (Fig. 3D, lower panel).

TPA- and PKCa-induced ERK Activation Is Upstream of SOS, but Independent of Serine Phosphorylation of ShcA at the Site Regulating PTP-PEST Binding in NIH3T3 Cells and in ShcA−/− Mouse Embryonic Fibroblasts—Previously, we showed that TPA induces activation of Ras/ERK signaling pathway upstream of SOS, involving serine phosphorylation of ShcA (19). Because TPA induces Shc/Grb2 association without inducing tyrosine phosphorylation of Shc, we suggested that TPA induces ERK activation through serine phosphorylation-dependent Shc/Grb2 association (19). PTP-PEST has been reported to be constitutively associated with Grb2 (45). We also found that PTP-PEST associated with Grb2 and that the association was unaffected by TPA treatment (data not shown). This prompted us to consider the possibility that TPA-
induced ShcA association with Grb2 is indirect and through PTP-PEST. We then postulated that an ShcA mutant unable to bind PTP-PEST would act as a dominant-negative molecule in TPA-induced ERK activation. However, overexpression of such an ShcA mutant had no effect on activation of coexpressed ERK after TPA stimulation in NIH3T3 cells (data not shown). As the absence of an effect may have been due to insufficient competition with endogenous wild-type Shc, we next examined TPA-induced ERK activation in Shc−/− mouse embryo fibroblasts (46). Again, TPA strongly activated ERK in these cells irrespective of overexpression of wild-type or PTP-PEST binding-defective ShcA (Fig. 4A). Nevertheless, PKC-mediated ERK activation was suppressed by coexpression of dominant-negative SOS (Fig. 4B). These results suggest that PKC activates the Ras/ERK signaling pathway upstream of SOS, involving molecule(s) other than Shc.

PTP-PEST Binding to Shc Down-regulates Insulin-induced ERK Activation in Human Insulin Receptor-overexpressing HIRc-B Cells—As PTP-PEST association with Shc has been shown to negatively regulate B cell activation (29), we tested whether PTP-PEST association with Shc affects its tyrosine phosphorylation. Because TPA did not induce tyrosine phosphorylation of ShcA in NIH3T3 cells or in ShcA−/− fibroblasts transiently expressing ectopic ShcA (19), we addressed this question using insulin-induced ShcA tyrosine phosphorylation in HIRc-B cells overexpressing the human insulin receptor. ShcA is one of the key mediators of insulin-induced ERK activation. Upon ligand binding, the insulin receptor phosphorylates Tyr317 of ShcA, which serves as a binding site for Grb2 (33). Insulin has been shown also to induce serine phosphorylation of p66Shc, as well as p52Shc, although the latter did not show a shift upon SDS gel electrophoresis (44). We found that p52Shc (but not p66Shc) was associated with PTP-PEST in HIRc-B cells and that this association could be slightly increased by insulin stimulation (Fig. 5A). To find out the importance of this association in insulin signaling, we derived HIRc-B cell lines by stably transfecting expression vectors coding for HA-tagged wild-type p52Shc or the HA-tagged p52Shc S29A mutant (PTP-PEST binding-defective). Clones expressing comparable amount of proteins were selected and used for analysis. Co-immunoprecipitation analysis using anti-HA antibodies showed that only wild-type p52Shc (but not p52Shc S29A) associated with PTP-PEST after TPA stimulation (Fig. 5B). The HIRc-B cells overexpressing the p52Shc S29A mutant were more responsive to insulin in terms of ERK activation (3- and 1.8-fold enhancement after 5 and 10 min, respectively) than the cells overexpressing wild-type p52Shc (Fig. 5C). This result was confirmed in two different sets of stable cell lines. In parallel, basal as well as insulin-induced tyrosine phosphorylation was much higher in the p52Shc S29A mutant than in wild-type p52Shc (Fig. 5D). Furthermore, overexpression of PTP-PEST in HIRc-B cells had a negative effect (50% decrease) on insulin-induced ERK activation (Fig. 5E). This suggests that PTP-PEST is a negative regulator of insulin-induced ERK activation in these cells.

**DISCUSSION**

**TPA Induces Serine Phosphorylation of ShcA—**ShcA is an adapter/docking protein whose tyrosine phosphorylation in the CH1 domain plays a pivotal role in relaying growth factor signals from receptors to downstream signaling molecules. Recently, several groups, including ours, have reported serine phosphorylation of the p66Shc isoform induced by various extracellular stimuli such as growth factors and TPA (19), hydrogen peroxide and UV irradiation (20), Taxol (21), and endothelin-1 (22). In this work, we identified three serine/threonine phosphorylation sites in p66Shc and one serine phosphorylation site in p52Shc. Although phosphorylation at Ser36 of p66Shc and Ser29 of p52Shc was specifically induced by TPA, phosphorylation at Ser36 and Thr39 in p66Shc was induced by both TPA and FGF-2. Phosphorylation at Ser36 in p66Shc has been reported already (20) and appears to be the main phosphorylation site in this isoform. Therefore, although all three ShcA isoforms contain three tyrosine residues whose phosphorylation is induced by growth factors (10), they differ with respect to the mode of serine/threonine phosphorylation; p66Shc has one phosphothreonine and two phosphoserine residues, p52Shc has one phosphoserine, and p46Shc has none. The presence of the three isoforms in many mammalian cell lines strongly suggests a specific role for each isoform and each serine phosphorylation site. Phosphorylation at Ser29 has been shown to have a role in oxidative stress-induced apoptosis (20). As the kinases responsible for this phosphorylation vary depending on the stimulus (20, 21, 47), the biological role of this phosphorylation may also vary. Very little is known about its role when it is induced by signals other than oxidative stress. In this study, however, we concentrated on only one of these phosphorylation sites, i.e. Ser138 in p66Shc and Ser29 in p52Shc.

3 A. Faisal and Y. Nagamine, unpublished data.
We have shown here that phosphorylation at Ser 29 of p52Shc and an intact PTB domain are both required for TPA-induced PTP-PEST binding to ShcA. Because in vitro binding assays showed that a peptide fragment from the PTB domain of ShcA lacking the amino-terminal region including Ser 29 could still bind to PTP-PEST (25), it is likely that Ser 29 is acting as a regulatory site for protein/protein interaction at a different site. In the previous experiment (25), the fragment containing Ser 29 but not amino acids 107–116 did not bind PTP-PEST.

There are numerous examples showing that the catalytic activity of an enzyme is modulated by its phosphorylation at a site away from the catalytic center through conformational change (48, 49). The activities of a vast number of protein kinases in signal transduction are regulated this way. Also well documented are various cases of phosphorylation-induced protein/protein interaction involving this very phosphorylation site, namely recognition of a phosphotyrosine residue in a signaling molecule by the SH2 or PTB domain of another signaling molecule (50). However, the phosphorylation-induced ShcA/PTP-PEST interaction is unique in that it involves phosphotyrosine-independent binding of the PTB domain of ShcA to PTP-PEST and is induced by the serine phosphorylation of Shc, although the phosphoamino acid is not directly involved in the protein/protein interaction. This adds a novel mode of regulation by protein phosphorylation at serine residues. This is not, however, a general mode of regulation of Shc PTB domain binding to its targets because, although Shc requires serine phosphorylation for binding to PTP-PEST after TPA stimulation, its binding to the EGF receptor after EGF stimulation can occur in the absence of serine phosphorylation. An S29A mutant of p52Shc can bind to the EGF receptor after EGF stimulation as efficiently as wild-type p52 Shc in NIH3T3 and HIRc-B cells (data not shown). Because ShcA binding to PTP-PEST is via a histidine residue as opposed to a consensus phosphotyrosine residue in the NPXY motif in the ShcA PTB domain targets, this could imply that phosphorylation at Ser 29 perhaps has a role in augmenting ShcA binding to targets in which the preferred Shc PTB domain-binding sequence is not available. Three-dimensional structural modeling of the PTB domain, including the amino-terminal end of the p52 Shc isoform, located Ser 29 in a fragment protruding away from the PTB domain (Fig. 2E). This suggests that regulation by Ser 29 phosphorylation of PTP-PEST binding to the PTB domain is not simply through steric hindrance by the phosphate group. It may involve more dynamic changes in protein structure. As the mod-

**Fig. 5. Role of PTP-PEST binding to Shc in insulin-induced ERK activation.** A, Shc/PTP-PEST association after insulin stimulation. HIRc-B cells were transiently transfected with HA-tagged PTP-PEST, starved for 16 h, and stimulated with 10 ng/ml insulin and 100 ng/ml TPA; whole cell extracts were immunoprecipitated (IP) with anti-HA (upper panels) or anti-Shc (lower panels) antibodies. Immunoprecipitates were resolved by SDS-PAGE and analyzed by immunoblotting (IB) using anti-ShcA and anti-HA antibodies. B, PTP-PEST association with wild-type (WT) p52Shc and p52Shc S29A in HIRc-B stable cell lines. HIRc-B cell lines stably expressing either wild-type HA-p52Shc or the S29A mutant were serum-starved for 16 h, stimulated with 100 ng/ml TPA, and lysed. The cell lysates were immunoprecipitated using anti-HA antibodies and analyzed with anti-PTP-PEST and anti-ShcA antibodies. C, effect of the PTP-PEST binding-defective mutant of p52Shc on insulin-induced ERK activation. HIRc-B cells stably expressing pcDNA, wild-type HA-p52Shc, or HA-p52Shc S29A were stimulated with 10 ng/ml insulin for the times indicated. Equal amounts of total cellular proteins were resolved by SDS-PAGE and immunoblotted with anti-phospho-ERK (P-Erk) and anti-ERK antibodies. Expression of Shc was analyzed by anti-HA antibodies. D, tyrosine phosphorylation of wild-type p52Shc and the PTP-PEST binding-defective mutant of p52Shc after insulin stimulation. Cells were stimulated with 10 ng/ml insulin for 10 min. Wild-type p52Shc and the S29A mutant were immunoprecipitated with anti-HA antibodies and analyzed using anti-phosphotyrosine (P-Tyr) and anti-Shc antibodies. E, PTP-PEST down-regulates insulin signaling. HIRc-B cells overexpressing HA-tagged PTP-PEST were stimulated with insulin after 16 h of starvation. Equal amounts of total cellular proteins were resolved by SDS-PAGE and immunoblotted with anti-HA, anti-phospho-ERK, and anti-ERK antibodies. All experiments were performed more than twice with similar results.
Serine Phosphorylation-induced ShcA/PTP-PEST Association

PKC phosphorylation in Chinese hamster ovary cells expressing the human insulin receptor (59). Because we have shown here that overexpression of constitutively active PKCa strongly induces Shc-PTP-PEST association, the inhibition of insulin-induced Shc tyrosine phosphorylation by activation of PKCa may be due to increased association of Shc with PTP-PEST, which may dephosphorylate it after insulin stimulation. Interestingly, a significant level of tyrosine phosphorylation was observed in p52Shc S29A before insulin induction without ERK activation, suggesting that mere tyrosine phosphorylation of ShcA is not enough for the downstream signaling to be activated. It may be that membrane localization of ShcA is also required. In this context, PTP-PEST recruitment would operate as a safety valve to suppress the runaway activation of ligand-induced signaling. Overexpression of PTP-PEST in HiR-B cells had a negative effect on insulin-induced ERK activation, possibly through dephosphorylation of ShcA. This finding was further corroborated by the observation that cells overexpressing the PTP-PEST binding-defective ShcA mutant exhibited stronger ERK activation after insulin stimulation than cells overexpressing wild-type ShcA. As PTP-PEST is a scaffold protein interacting with various other proteins, including paxillin, Cak, and Cas (29), there may be another role for ShcA/PTP-PEST interaction. Through this interaction, ShcA may mediate the ligand-induced signals not only to the Ras/ERK signaling pathway, but also to other pathways coordinating cellular activities. Future experiments will determine the range and specificity of PTP-PEST-mediated signaling.

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