Individual protein tyrosine kinases and phosphatases target multiple substrates; this may generate conflicting signals, possibly within a single pathway. Protein-tyrosine phosphatase ε (PTPε) performs two potentially opposing roles: in Neu-induced mammary tumors, PTPε activates Src downstream of Neu, whereas in other systems PTPε can indirectly down-regulate MAP kinase signaling. We now show that the latter effect is mediated at least in part via the adaptor protein Shc. PTPε binds and dephosphorylates Shc in phosphotyrosine-independent manner mediated by the Shc PTB domain and aided by a sequence of 10 N-terminal residues in PTPε. Surprisingly, PTPε dephosphorylates Shc in a kinase-dependent manner; PTPε targets Shc in the presence of Src but not in the presence of Neu. Using a series of point mutants of Shc and Neu, we show that Neu protects Shc from dephosphorylation by binding the PTB domain of Shc, most likely competing against PTPε for binding the same domain. In agreement, PTPε dephosphorylates Shc in mouse embryo fibroblasts but not in Neu-induced mammary tumor cells. We conclude that in the context of Neu-induced mammary tumor cells, Neu prevents PTPε from targeting Shc and from reducing its promitogenic signal while phosphorylating PTPε and directing it to activate Src in support of mitogenesis. In so doing, Neu contributes to the coherence of the promitogenic role of PTPε in this system.

The human genome contains 90 genes that encode tyrosine kinases (1) and 107 genes that encode tyrosine phosphatases (2). Similar numbers of genes from either category generate products that act on proteins, 85 PTKs and 81 PTPs. Tyrosine kinases and phosphatases are then vastly outnumbered by their potential substrates. These enzymes target multiple substrates each and may play distinct, sometimes opposite, roles in different physiological circumstances. One such example is PTP1B, which down-regulates insulin receptor signaling and several other growth factor receptor pathways (3, 4) but up-regulates Ras signaling (5). Taken to an extreme, a single enzyme may activate a signaling pathway by targeting one substrate but simultaneously contribute toward inactivating the same pathway via a second substrate.

Nevertheless, the outcomes of signaling pathways are specific, coherent, and reproducible, implying that molecular mechanisms exist to prevent or to control situations where opposite signals can be generated by the same enzyme. Much is known about regulation of PTPs by mechanisms such as proteolysis (6, 7), inhibitory dimerization (8–13), reversible oxidation (14–16), and phosphorylation (13, 17–19). However, it remains unclear how the potential conflicts between the activities of a particular PTP on several of its substrates within a single system are prevented or resolved.

The two major protein forms of PTPε are the receptor type (RPTPε) and non-receptor type (cyt-PTPε) enzymes (20–22). Both forms can support or inhibit mitogenic signaling in a context-dependent manner. We have shown that RPTPε supports the transformed phenotype of mouse mammary tumor cells induced in vivo by an activated Neu transgene. RPTPε activates c-Src; lack of RPTPε in Neu-induced mammary tumor cells reduced c-Src activity, caused the cells to display aberrant morphology, and reduced their proliferation rate in culture and in vivo (23). Activation of c-Src by RPTPε was shown to be dependent upon phosphorylation of the PTP at its C-terminal tyrosine Tyr966 by Neu (24). A Neu-RPTPε-Src signaling pathway exists therefore in mammary tumor cells induced by Neu, in which Neu phosphorylates RPTPε, thereby driving the phosphatase to activate c-Src and to contribute to the transformed phenotype of these cells (24). On the other hand, a clear role for RPTPε and cyt-PTPε in down-regulating signaling events was shown in the context of insulin receptor signaling (25–27), ERK and MAPK signaling (28, 29), endothelial cell proliferation (30), and transformation and tumorigenicity of M1 leukemia cells (31). The molecular mechanisms that regulate and balance between these opposing roles remain unclear.

The ShcA gene yields three protein products, p46, p52, and p66. Shc proteins can associate with a variety of activated tyrosine kinase growth factor receptors and can be phosphorylated
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by them on tyrosines Tyr\textsuperscript{239}/Tyr\textsuperscript{240}/Tyr\textsuperscript{317} (numbered as in p52 Shc). The phosphorylated Shc proteins serve as adaptors, leading to recruitment of the Grb2-SOS complex and resulting in activation of downstream signaling. p66 Shc was found to be a proapoptotic factor activated in response to oxidative stress (32). In this study, we show that PTPε dephosphorylates the three protein products of the Shc\textalpha gene following phosphorylation by Src. In so doing, PTPε also inhibits the Shc-Grb2 association and phosphorylation of ERK1 and ERK2 further downstream. In the context of Neu-induced tumor cells, this activity of RPTPε should convey an antmitogenic signal, thus antagonizing the promitogenic contribution of RPTPε through its activation of Src. Surprisingly, RPTPε does not target Shc in cells in which the predominant kinase is Neu, including in Neu-transformed mammary tumor cells, due to strong physical interaction between Neu and Shc. We conclude that Neu balances between two opposing activities of RPTPε. Neu phosphorylates RPTPε and drives it to activate Src while binding Shc tightly and preventing the phosphatase from dephosphorylating the adaptor molecule. As a result, Neu prevents the potential conflict between these pro- and antimitogenic activities of PTPε in mammary tumors, enabling PTPε to support mitogenic signaling in a self-consistent and coherent manner.

EXPERIMENTAL PROCEDURES

Materials—The following cDNAs used in this study were described previously: wild-type cyt-PTPε (33), D245A cyt-PTPε (34), R283M cyt-PTPε (28), and Y683F cyt-PTPε (33); the N-terminal mutants R4M-, S8M-, T11M-, S22M-, and Δ11–26 cyt-PTPε (35); RPTPε and p67 (36); Y527F Src (23); wild type Neu (NeuN), activated Neu (V644E Neu, NeuNT), and the Tyr→Phe mutant of NeuNT (Y1024F, Y1144F, Y1201F, Y1226F, Y1227F, Y1253F Neu; Neu NYPD) (37). All of the above cDNAs were cloned into pCDNA3 (Invitrogen). In some cases, PTPε or Neu cDNAs were tagged with FLAG or HA at their C-terminal ends. Also used in this study were glutathione S-transferase (GST) fusion proteins of wild-type (WT) Shc, Y239F Shc, Y239F, Y240F Shc, and Y317F Shc, cloned in the pEG3 eukaryotic expression vector, kind gifts of Dr. Koki Ravech (University of Virginia) and Dr. Tony Tiganis (Monash University, Australia). Bacterial plasmids for expressing GST fusion proteins of full-length GST as well as its SH2 and PTB domains were a generous gift of Prof. Yechiel Zick (The Weizmann Institute of Science, Rehovot, Israel). The GST-Grb2 construct was described previously (33). S154P Shc and R401L Shc were generated from WT Shc by site-directed mutagenesis; a FLAG tag was added to their C termini, and their sequences were verified prior to cloning into pcDNA3.

Antibodies used included rabbit polyclonal anti-PTPε (21), anti-Shc (BD Transduction Laboratories, San Jose, CA), anti-phospho-ERK1/ERK2 (Sigma), anti-phospho-ERK1/ERK2 (Cell Signaling Technology), anti-Tyr(P)\textsuperscript{317} Shc, and anti-Tyr(P)\textsuperscript{239/240} Shc (Cell Signaling Technology, Danvers, MA), and anti-Neu (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Monoclonal antibodies used included anti-glutathione S-transferase (Calbiochem), anti-v-Src (Calbiochem), anti-FLAG (M2) (Sigma), and anti-Grb2 (BD Transduction Laboratories). Anti-phosphotyrosine antibodies were used for immunoprecipitation (clone PY20; Transduction Laboratories) and protein blotting (clone PY99; Santa Cruz Biotechnology).

Cell Culture—Human embryonic kidney (HEK293) cells and SYF cells (immortalized mouse fibroblasts lacking Src, Yes, and Fyn (39)) were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen), 4 mM glutamine, 50 units/ml penicillin, and 50 µg/ml streptomycin. HEK cells were transfected by the calcium phosphate technique as described (33), whereas SYF cells were transfected by the FuGENE 6 transfection reagent (Roche Applied Science). Metabolic labeling of cells was performed at 70% confluency; cells were incubated for 30 min in methionine-deficient DMEM, followed by overnight incubation in similar medium supplemented with [\textsuperscript{35}S]methionine (175 µCi (1000 Ci/mmol) per 5 ml of medium per plate). Spontaneously immortalized embryonic fibroblasts were prepared from PTPε-deficient or wild-type mice by the 3T3 method. NIH3T3 cells were transformed by expression of v-Ha-Ras. Both types of cells were grown in DMEM supplemented as above, which, for the embryonic fibroblasts, also contained 6 × 10\textsuperscript{−3} M β-mercaptoethanol. Mammary tumor cells isolated from spontaneous tumors of mice carrying the V664E Neu transgene and genetically lacking PTPε (23) were grown in DMEM supplemented with 10% fetal calf serum (Hyclone, Logan, UT) and glutamine and antibiotics as above. RPTPε was expressed in these cells by retrovirus-mediated infection as described (23).

Immunoprecipitation and Protein Blot Analysis—Cells or tissues were lysed in buffer A (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1% Nonidet P-40, supplemented with protease inhibitors (1 mM N-(α-aminooethyl) benzene-sulfon fluoride, 40 µM bestatin, 15 µM E64, 20 µM leupeptin, 15 µM pepstatin; Sigma) and 0.5 mM sodium pervanadate. Pervanadate was replaced with 5 mM sodium iodoacetate when the association between Shc and D245A cyt-PTPε was examined. Protein amounts were determined using the Bio-Rad protein assay system with bovine serum albumin as a standard. Immunoprecipitation was performed using 1–3 mg of total cell lysate and protein A and/or protein G-Sepharose beads (Amersham Biosciences or Santa Cruz Biotechnology), as described (24). Eukaryotic GST pull-down studies were performed by rocking 800 µg of cellular proteins with glutathione-agarose beads (Pierce) that had been previously blocked in 1% skim milk, followed by three washes in buffer A. Immunoprecipitated material and crude cell lysates were subject to SDS-PAGE and blotting as described (7).

Purification of Bacterial GST Fusion Proteins—Bacterial GST fusion proteins were grown in Escherichia coli DH5α bacteria and purified by binding to glutathione-agarose beads (Pierce) previously blocked in 1% milk. Following three washes in NETE buffer (0.5% Nonidet P-40, 20 mM Tris, pH 8, 100 mM NaCl, 1 mM EDTA), proteins were eluted in 20 mM glutathione, 50 mM Tris, pH 8.0. The quantity and purity of the purified proteins were examined by SDS-PAGE followed by Gel-code staining (Pierce).

In Vitro Dephosphorylation of Shc—Eukaryotic GST-Shc fusion proteins were expressed in HEK293 cells together with Neu or Y527F Src. Cells were lysed in buffer A as above, supplemented with sodium pervanadate, and rocked gently with...
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glutathione-agarose beads (Pierce) for 3 h followed by 10 washes in buffer A. FLAG-tagged cyt-PTPε was expressed in HEK293 cells and was immunoprecipitated using anti-FLAG M2 affinity beads (Sigma) followed by three washes with buffer A, two washes with buffer B (100 mM KCl, 0.5 mM EDTA, 20 mM Hepes, pH 7.6, 0.4% Nonidet P-40, 20% glycerol), and two washes with buffer 54K (150 mM NaCl, 50 mM Tris, pH 7.9, 0.5% Triton X-100), all without pervanadate supplement. FLAG-tagged cyt-PTPε was then eluted by incubation of the beads with Elution Buffer (20 mM Hepes, pH 7.3, 200 mM NaCl, 1 mM EDTA, 0.1 mM EGTA, 1 mg/ml FLAG peptide) (Sigma). The integrity, purity, and amount of GST-Shc and PTPε were analyzed by SDS-PAGE followed by silver staining (Bio-Rad) using a bovine serum albumin standard curve. Dephosphorylation reactions were conducted in 150 μl of activity buffer (50 mM MES, pH 7, 0.5 mM dithiothreitol, 0.5 mg/ml bovine serum albumin), to which 100 ng of purified, eluted cyt-PTPε and a similar amount of Shc bound to beads were added. Following gentle rocking at 32 °C for 7 h, samples were analyzed by SDS-PAGE.

RESULTS

Shc and PTPε Associate in Vivo—Previous studies have shown that PTPε can down-regulate MAPK signaling but does so indirectly (28), most likely by targeting an upstream molecule(s). In order to examine this issue, we studied the effect of PTPε on the adaptor protein Shc as a possible candidate for mediating these effects. Endogenous Shc proteins formed stable associations with endogenous cyt-PTPε or RPTPε in several tissues and cell types, including in Ras-transformed NIH3T3 cells, Jurkat T-cells, and mouse brain (Fig. 1). p66 Shc and p52 Shc were precipitated with PTPε; p46 Shc was not visible in these experiments due to interference of the precipitating antibody heavy chain. Similar association was observed in HEK293 cells, which express endogenous p46 and p52 Shc, upon expression of full-length, WT cyt-PTPε or of various other forms of PTPε (Fig. 2A). The substrate-trapping mutant D245A cyt-PTPε (34, 40) bound cyt-PTPε as strongly as WT Shc; similar binding was observed also when the inactive R283M cyt-PTPε or Y683F cyt-PTPε, which lacks the key phosphorylation site analogous to Tyr639 in RPTPε, was used (Fig. 2A). These results indicate that a stable complex containing both cyt-PTPε and Shc exists and that this interaction does not require PTPε activity or C-terminal phosphorylation of the phosphatase. In contrast, the receptor RPTPε and the shorter p67 form associated with Shc less strongly (Fig. 2A). The only structural difference between cyt-PTPε and p67 is the presence of a unique sequence of 27 amino acid residues at the N terminus of cyt-PTPε (36). In order to determine whether sequence motifs present in this region affect binding to Shc, we examined the ability of a series of N-terminal mutant cyt-PTPε proteins to bind Shc. cyt-PTPε truncation mutants lacking the first 4, 8, or 11 amino acids tended to bind Shc more strongly than full-length cyt-PTPε. Mutants of cyt-PTPε lacking residues 11–27 or lacking the first 22 residues bound Shc similar to p67 (Fig. 2B). We conclude that sequences between positions 12 and 22 of cyt-PTPε are important for binding Shc, whereas residues 1–4 of cyt-PTPε may inhibit this association. No obvious sequence motifs that bind PTB domains are present within this region, possibly suggesting that these residues contribute indirectly to the Shc-cyt-PTPε association, as discussed further below.

The Shc-PTPε interaction was recapitulated also when bacterially produced GST fusion proteins of various domains of Shc were used to pull down cyt-PTPε from lysates of eukaryotic cells. A fusion protein containing the PTB domain of Shc bound cyt-PTPε strongly, whereas a protein containing the Shc SH2 domain did not bind cyt-PTPε at all (Fig. 2C). Interestingly, a GST fusion of full-length p52 Shc bound cyt-PTPε less well than a fusion protein containing only the PTB domain. These results indicate that the Shc-PTPε interaction is mediated by the PTB domain of Shc and suggest that other sequences within Shc may antagonize this interaction. Similar binding was observed when the GST-Shc fusion proteins were used to pull down hyperphosphorylated cyt-PTPε from cells pretreated

FIGURE 1. Endogenous PTPε interacts with endogenous Shc. A, Ras-transformed NIH3T3 cell lysates, which express endogenous cyt-PTPε, were precipitated with PTPε antibodies, and precipitates were blotted against Shc (top) or PTPε (bottom; the arrow marks cyt-PTPε). Lys, cell lysate; Ab, precipitating antibody; IP, immunoprecipitation; WB, Western blot. B, similar to A using Jurkat T-cells, documenting co-immunoprecipitation of endogenous cyt-PTPε with Shc in these cells as well. The arrow (bottom) marks cyt-PTPε. C, RPTPε binds Shc in brain. p66 and p52 Shc were precipitated from lysates of brains of WT and PTPε-deficient (KO) mice. Precipitates were blotted against PTPε (top) and Shc (bottom). In all panels, the asterisks mark the heavy chain of the precipitating antibody, which prevents viewing of p46 Shc; molecular mass markers in this and in subsequent figures are in kDa.
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The interaction is constitutive and is independent of the tyrosine phosphorylation status of either Shc or cyt-PTPe.

Cyt-PTPe Reduces Shc Phosphorylation in a Kinase-specific Manner

In order to examine the physiological consequences of the Shc-cyt-PTPe interaction, we expressed the phosphatase in cells and examined its effect on phosphorylation of endogenous Shc in 293 cells. Basal phosphorylation of Shc in this system is low, hence Shc was activated by co-expression of PTKs. When the endogenous p46 and p52 forms of Shc were phosphorylated in the presence of activated (Y527F) Src, cyt-PTPe reduced their phosphorylation levels significantly (Fig. 3A). Catalytically inactive R283M cyt-PTPe did not reduce Shc phosphorylation, indicating that this effect requires PTPe activity. Similar results were obtained also when wild-type Src was used (not shown) and when experiments were repeated in fibroblasts lacking endogenous Src, Fyn, and Yes (Fig. 3B). The latter cells also express p66 Shc, whose phosphorylation was reduced in the presence of cyt-PTPe. Interestingly, the presence of cyt-PTPe did not reduce phosphorylation of Shc when the latter was phosphorylated by Neu (Fig. 3, C and D). The distinction between Neu and Src also affected downstream signaling events; the presence of cyt-PTPe reduced the association of Shc with Grb2 and reduced activation of ERK1 and ERK2 in the presence of Src but not in the presence of Neu (Fig. 3D). Similar effects and a similar distinction between Neu and Src were observed when cyt-PTPe was replaced with RPTPe (Fig. 3, E and F). In separate experiments, cyt-PTPe could not reduce phosphorylation of Shc in the presence of the epidermal growth factor receptor (not shown), suggesting that the “protective” effect of Neu may be shared also by related kinases.

Importantly, PTPe affected Shc phosphorylation in a kinase-dependent manner also when endogenous proteins were examined. Phosphorylation of Shc and of ERK was increased in spontaneously immortalized embryonic fibroblasts from mice genetically lacking PTPe (28, 34) in comparison with WT cells that express the phosphatase (Fig. 4A). In contrast, phosphorylation of Shc was consistently similar in mammary tumors induced in vivo by activated Neu in mice lacking PTPe (23) and in the same cells reconstituted with RPTPe (Fig. 4B), in line with observations presented in Fig. 3, D and E. Since cyt-PTPe and

with sodium pervanadate, indicating that the Shc-cyt-PTPe interaction is independent of tyrosine phosphorylation of the phosphatase (Fig. 2C). The Shc PTB domain binds the nonreceptor type phosphatase PTP-PEST by targeting a tyrosineless NPLH sequence motif located at the C terminus of the PTP (41). cyt-PTPe contains a similar sequence, NPSH, at positions 266–269 within its D1 PTP domain. However, mutating the above four residues to alanines did not affect binding of cyt-PTPe to Shc, indicating that the NPSH motif is not a target of the Shc PTB domain (not shown). Replacement of tyrosines 239, 240, or 317 in Grb2, which play important roles in binding Grb2 and in Shc signaling (42), with nonphosphorylatable phenylalanine residues also had no effect on cyt-PTPe binding (not shown). Together with the strong ability of bacterially produced GST-Shc proteins to bind cyt-PTPe (Fig. 2C), this result indicates that tyrosine phosphorylation of Shc is also not required for the interaction with cyt-PTPe. In all, we conclude that the Shc-cyt-PTPe interaction is mediated by the PTB domain of Shc and is supported by residues 12–22 of cyt-PTPe;
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FIGURE 3. cyt-PTPe reduces phosphorylation of Shc in a kinase-specific manner. A, cyt-PTPe dephosphorylates Src-phosphorylated Shc. HEK293 cells were transfected with combinations of cyt-PTPe or its inactive mutant R283M cyt-PTPe (RM), with or without activated (YS27F) Src (YP). Tyrosine-phosphorylated proteins were immunoprecipitated, and phospho-Shc was detected among them using anti-Shc antibodies. B, similar to A, in fibroblasts lacking endogenous Src, Yes, and Fyn (SYF cells), cyt-PTPe dephosphorylates endogenous Shc, including p66 Shc, in the presence of Src. The top two panels show different exposures of the same blot. C, PTPe cannot dephosphorylate Neu-phosphorylated Shc. HEK293 cells were transfected with wild-type Neu and cyt-PTPe as indicated. Phospho-Shc was examined as in A. D, dephosphorylation of Shc by PTPe down-regulates downstream signaling events. HEK293 cells expressing cyt-PTPe, activated Src, and activated Neu as indicated were analyzed for Shc-Grb2 association, activation of ERK, and phosphorylation of Shc. Except where immunoprecipitation (IP) is indicated, crude cell lysates were used in blotting studies. E, similar to D, using RPTPe. F, bar diagram depicting relative amount of phospho-Shc (normalized to precipitated Shc) in the presence of Src, Neu, and the phosphatase as indicated. Shown are the mean ± S.E.; n = 2–5 repeats per bar.*, significant (p = 0.048) by Student's t test. IP, immunoprecipitation; WB, Western blot.

RPTPe affect Shc phosphorylation and downstream signaling in similar manners (Fig. 3, D and E) (results not shown), we believe that this difference in Shc phosphorylation is due to the presence of Neu as the dominant kinase in these mammary tumor cells (Fig. 4B) versus the absence of a single dominant kinase in immortalized fibroblasts (Fig. 4A). We note that in some experiments phosphorylation of ERK1 and ERK2 was unchanged in PTPe-deficient mammary tumor cells (e.g. see Fig. 4B), whereas in other studies of these cells, ERK phosphorylation was increased relative to cells expressing RPTPe. This suggests that RPTPe affects phosphorylation of ERK in this system not only through Shc.

Further studies focused on cyt-PTPe. A possible explanation for the kinase-dependent ability of cyt-PTPe to dephosphorylate Shc is that Src and Neu phosphorylate Shc at distinct sites, some of which may not be dephosphorylated by cyt-PTPe. In order to study this possibility, we examined phosphorylation of Shc at tyrosine residues 239, 240, and 317 using phospho-specific antibodies. Neu and Src phosphorylated Shc at these sites equally well (Fig. 5A); in agreement with previous results, cyt-PTPe reduced phosphorylation at Tyr239/240 and Tyr317 in the presence of Src but not in the presence of Neu (Fig. 5A). This result indicates that although both kinases can phosphorylate Shc at these critical sites, cyt-PTPe is nevertheless prevented from dephosphorylating them in the presence of Neu.

In separate experiments, analysis by two-dimensional gel electrophoresis revealed different migration patterns along the p1 axis for Shc isolated from cells expressing Src versus Neu (not shown), strongly suggesting that these kinases do modify Shc covalently, directly or indirectly, in manners that are at least partially distinct. Nevertheless, these distinct modification patterns do not account for kinase-dependent dephosphorylation of Shc, since kinase specificity was not detected when purified Shc was dephosphorylated by cyt-PTPe in vitro. In these experi-
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Figure 5. Mechanistic dissection of the kinase dichotomy. A, PTPε dephosphorylates Shc at sites Tyr239/240 and Tyr317. HEK293 cells expressing cyt-PTPε, Y527F Src, and Neu, as indicated, were analyzed for phosphorylation of Shc at Tyr239/240 and Tyr317 using phospho-specific antibodies. B, purified PTPε can dephosphorylate Shc in vitro irrespective of the phosphorylating kinase. GST-Shc was purified from HEK293 cells expressing Y527F Src or Neu. Following incubation with cyt-PTPε that had been purified from separate cells, phosphorylation of GST-Shc was analyzed by protein blotting (top). Also shown are amounts of purified GST-Shc and PTPε present (bottom). C, Neu, but not Src, adheres tightly to Shc. HEK293 cells expressing cyt-PTPε, FLAG-tagged Shc, Neu (N), or activated Src (S), as indicated, were labeled with [35S]methionine. Shc was precipitated via its FLAG tag, and the precipitates were analyzed by SDS-PAGE on a 6–15% gradient gel. Proteins were transferred to membrane, which was then exposed to film. Proteins noted were identified by probing the membrane with specific antibodies (not shown). IP, immunoprecipitation; WB, Western blot.
phosphorylation of Shc was detected; phosphorylation was reduced significantly upon co-expression of cyt-PTPε (Fig. 6, lanes 3 and 4). However, upon added expression of Neu, Shc phosphorylation was not reduced and remained at levels observed in cells expressing Src alone (Fig. 6, lanes 3, 4, and 6). This level of phosphorylation was similar to that observed in cells expressing Neu with or without added cyt-PTPε (Fig. 6, lanes 1 and 7). This result indicates that the “protective” effect of Neu dominates over the “permissiveness” of Src.

An additional prediction of the above model is that Neu that cannot physically bind Shc would be unable to protect it from dephosphorylation. In order to examine this possibility, we turned to catalytically active Neu in which all known auto-phosphorylation sites had been mutated (Y1024F,Y1144F,Y1201F, Y1226F,Y1227F,Y1253F Neu; NYPD Neu (37)) and which cannot bind Shc. Indeed, NYPD Neu did not protect Shc from dephosphorylation (Fig. 7C). These results and those obtained with the S154P Neu mutant lead us to conclude that disruption of the association between Shc and Neu prevents Neu from protecting Shc from dephosphorylation by PTPε.

Finally, the model by which Shc is protected from dephosphorylation by physical binding to Neu suggests that virtually all phosphorylated Shc found in cells expressing Neu should be associated with and protected by the kinase (and possibly also with other molecules that protect it). In order to verify this, we expressed Neu with or without added cyt-PTPε in cells, exhaustively precipitated over 90% of cellular Neu, and examined the distribution of Shc and phospho-Shc between the precipitated material and remaining supernatant. In agreement with the model, all detectable Tyr(P)239/240 Shc was found in the precipitated fraction, whereas significant amounts of nonphosphorylated Shc were found in both the precipitated and nonprecipitated fractions. Similar results were obtained in the presence or absence of cyt-PTPε (Fig. 8). We conclude that although not all Shc molecules associate with Neu, phospho-Shc molecules are found only in association with the Neu signaling complex. In all, these results are consistent with tight association between Neu and Shc preventing cyt-PTPε from dephosphorylating Shc and down-regulating signaling events further downstream.

**DISCUSSION**

Results presented here indicate that cyt-PTPε and RPTPε can down-regulate phosphorylation of Shc at its major phosphorylation sites Tyr239/240 and Tyr317, thus reducing association of Shc with Grb2 and activation of ERK1 and ERK2 further downstream. The ability of purified cyt-PTPε to dephosphorylate purified phospho-Shc in vitro together with presence of both molecules in a stable complex suggests that Shc is a substrate of PTPε. PTPε down-regulates activity of ERK1 and ERK2 and other mitogen-activated protein kinases in an indirect manner (28); results presented here suggest therefore that Shc is one of the upstream molecules that mediate the effect of PTPε on ERK.

Shc and cyt-PTPε interact constitutively via the PTB domain of Shc and in a manner supported by the presence of residues R401L.) were phosphorylated by Src, were dephosphorylated by cyt-PTPε in the presence of Src, and bound cyt-PTPε equally well (Fig. 7B). These results agree with the central role of the Shc PTB domain in mediating the phosphotyrosine-based interactions between Shc and Neu and with the phosphotyrosine-independent nature of the association between cyt-PTPε and Shc. These results also demonstrate that in this system, the Shc SH2 domain does not play a significant role in mediating associations of Shc with Neu, Src, or cyt-PTPε.

In order to examine whether S154P Shc, which cannot bind Neu, is protected from dephosphorylation by cyt-PTPε, we expressed wild type or S154P Shc in HEK293 cells together with Neu, Src, and cyt-PTPε (Fig. 7C). Both WT and S154P Shc were phosphorylated by Src (Fig. 7C, lanes 3 and 4) and were dephosphorylated by cyt-PTPε (Fig. 7C, lanes 5 and 6). Additional expression of Neu protected wild-type Shc from dephosphorylation, in agreement with previous results in this study but did not protect S154P Shc from dephosphorylation (Fig. 7C, lanes 7 and 8). In these studies, S154P Shc retained 20.1 ± 8.6% of the phosphorylation of WT Shc (mean ± S.E., n = 3, p = 0.011 by Student’s t test). These results and those obtained with the NYPD Neu mutant lead us to conclude that disruption of the association between Shc and Neu prevents Neu from protecting Shc from dephosphorylation by PTPε.

**FIGURE 6.** Neu protects Shc from dephosphorylation also in the presence of Neu; disrupting the Shc-Neu association by mutating Neu prevents Neu from protecting Shc from dephosphorylation. WT or Y1024F,Y1144F,Y1201F,Y1226F,Y1227F,Y1253F Neu (YF), which lacks all known autophosphorylation sites, were expressed in HEK293 cells together with Src and cyt-PTPε as indicated. The numbers above the top panel represent relative amounts of pShc. Phosphorylation of endogenous Shc and ERK is shown. IP, immunoprecipitation; WB, Western blot.
12–22 in cyt-PTPε. Of note, most cyt-PTPε molecules are cytosolic, whereas ~10% are nuclear and another 20% are membrane-associated. The absence of the first 27 residues of cyt-PTPε abolishes membrane localization (36), whereas residues 1–10 are required for nuclear localization (35). Since Shc is often phosphorylated by or associated with membrane-associated molecules, residues 12–22 of cyt-PTPε may contribute indirectly to the PTPε-Shc interaction by promoting membranal localization of the phosphatase. We note, however, that residues 12–22 are present also in RPTPε, which is an integral membrane protein and which binds Shc effectively but apparently more weakly. RPTPε contains also unique membrane-spanning and extracellular domains that are absent from cyt-PTPε and that may mask the contribution of residues 12–22 to binding Shc in RPTPε. Our results also suggest that residues 1–4 of cyt-PTPε, which are unique to this form of PTPε, decrease association with Shc. Further studies are required to fully resolve this issue.

An unexpected finding in this study was the inability of PTPε to dephosphorylate Shc in the presence of Neu, despite being able to do so in the presence of Src or when using purified Shc and PTPε. The “protective” effect of Neu in cells is dominant, since the presence of Neu protects Shc from dephosphorylation even in the presence of Src. Further studies strongly suggested that the molecular basis for protection by Neu is its constitutive interaction with Shc, which is mediated by the Shc PTB domain-binding phosphotyrosine residues located in the cyto-
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FIGURE 9. Model describing the effect of PTPe on Shc in the presence of Src or Neu. A, PTPe binds Shc via the latter’s PTB domain. PTPe can dephosphorylate Shc, thus countering its phosphorylation by Src. Both phosphorylation and dephosphorylation are schematically targeted at the Shc CH1 domain, where Tyr239/240 and Tyr317 are located, although this does not exclude other phosphorylation sites within Shc. B, Shc binds Neu via its PTB domain, thereby preventing PTPe from binding the same Shc molecule and protecting it from dephosphorylation. C, Neu plays a dual role versus PTPe in Neu-induced mammary tumors. Neu phosphorylates RPTP, thus driving it to activate Src (24), while binding Shc and preventing PTPe from dephosphorylating the adapter molecule (this study). In so doing, Neu supports a coherent, promitogenic role for PTPe, which the PTP has been shown to play in these cells (23).

Dephosphorylation of Shc by PTPe in the mammary tumor system would then contradict the mitogenic signal initiated by Neu and conveyed in part by PTPe-mediated dephosphorylation of Src. However, this potential for conflicting signals is avoided by the activities of Neu, the predominant kinase in the system. On one hand, Neu phosphorylates RPTP and channels its activity toward Src (24), whereas on the other hand, Neu binds Shc tightly and prevents it from being dephosphorylated by PTP. Furthermore, the “protective” effect of Neu toward Shc is dominant, ensuring that PTPe is not driven to dephosphorylate Shc as Src activity increases (Fig. 9C). In all, the present study describes a molecular mechanism by which PTP activity is modulated by directing it toward one substrate while avoiding another. In so doing, the potential for the PTP to initiate signals that are potentially conflicting is reduced, and its contribution to cell signaling is made more coherent.

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