

Phosphatidylinositol 4,5-bisphosphate stimulates phosphorylation of the adaptor protein Shc by c-Src

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Abstract The adaptor protein Shc was prepared as glutathione *S*-transferase fusion proteins (GST–Shc) and used as *in vitro* substrate for c-Src. Since phosphotyrosine-binding domain of Shc has been shown to bind phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) [Zhou et al. (1995) *Nature* 378, 584–592], effect of PtdIns(4,5)P₂ on the phosphorylation of GST–Shc by c-Src was examined. PtdIns(4,5)P₂ stimulated the phosphorylation of GST–Shc without any effect on the c-Src activity as judged by both its autophosphorylation and phosphorylation of exogenous substrate, Cdc2 peptide. On the other hand, phosphatidylserine, phosphatidic acid, phosphatidylinositol, and phosphatidylinositol 4-phosphate but not phosphatidylcholine stimulated the c-Src activity itself. *K_m* for GST–Shc in the presence of 1 μM PtdIns(4,5)P₂ was calculated to be 90 nM. The PtdIns(4,5)P₂-dependent phosphorylation of GST–Shc was inhibited by a GST–fusion protein containing the phosphotyrosine-binding domain of Shc. These results suggest that PtdIns(4,5)P₂ can act as a regulator of phosphorylation of Shc by c-Src through its binding to Shc.

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Key words: Shc; c-Src; Tyrosine phosphorylation; Phosphatidylinositol 4,5-bisphosphate; Phosphotyrosine-binding domain

1. Introduction

For past several years, great attention has been paid to the intracellular signaling pathways provoked by cell surface receptor tyrosine kinases, such as growth factor receptors/kinases. In these systems, protein tyrosine phosphorylation is established to play a pivotal role in the signaling events, such as Ras-dependent activation of the mitogen-activated protein kinase cascade. Recently, a number of receptors that lack intrinsic tyrosine kinase activity, such as heterotrimeric G-protein-coupled receptors and antigen receptors of T- and B-cells, have also been found to activate tyrosine kinase pathways [1]. In particular, it is well established that tyrosine phosphorylation of the adaptor protein Shc is indispensable in both kinase receptor and non-kinase receptor systems [2–5]. Shc exists in three isoforms: 46 kDa, 52 kDa, and 66 kDa.

These Shc molecules contain three structural and functional domains: an amino-terminal phosphotyrosine-binding (PTB) domain, a central collagen-homologous region containing a major tyrosine phosphorylation site, and a carboxyl-terminal Src homology 2 (SH2) domain. It has been suggested that activated receptor tyrosine kinases phosphorylate Shc directly, and phosphorylated Shc recruits another SH2-containing adaptor protein Grb2 to the plasma membrane [2,6]. Grb2 binds to a guanine nucleotide exchanging factor, Sos, that is directly responsible for the activation of Ras [2,6]. In case of non-kinase receptor systems, however, identity of the tyrosine kinases responsible for Shc phosphorylation has not yet been elucidated. Recently, it has been demonstrated that lysophosphatidic acid and angiotensin II, which act through the heterotrimeric G-protein-coupled receptors, cause functional and physical interaction between Shc and Src family tyrosine kinases, such as c-Src and Fyn [7,8].

In the present study, we prepared glutathione *S*-transferase fusion proteins containing a full length of 46- or 52-kDa isoform of Shc (GST–Shc: GST–P46 and GST–P52), and phosphorylation of GST–Shc by c-Src was examined in a purified system. Since phosphotyrosine-binding domain of Shc has been shown to bind PtdIns(4,5)P₂ [9], the effect of PtdIns(4,5)P₂ and some other phospholipids on the phosphorylation of GST–Shc was examined. The results obtained raise a possibility that tyrosine phosphorylation of Shc by c-Src is regulated by the interaction between Shc and PtdIns(4,5)P₂.

2. Materials and methods

2.1. Materials

c-Src was purified from bovine brain as described previously [10]. Bacto-tryptone and Bacto-yeast extract were purchased from Difco. A synthetic tyrosine kinase-substrate peptide, termed Cdc2 peptide, was prepared as described [10]. Leupeptin was purchased from Peptide Institute (Osaka). (*p*-Amidinophenyl)methanesulfonyl fluoride hydrochloride was from Wako Pure Chemicals (Osaka). [γ -³²P]ATP (35020) was obtained from ICN. Phosphatidylserine (P-7769), phosphatidylcholine (P-5911), and phosphatidic acid (P-9511) were purchased from Sigma. Phosphatidylinositol (PtdIns) (A-38) was obtained from Serdary Research Laboratories. Phosphatidylinositol 4-phosphate (PtdIns(4)P) and PtdIns(4,5)P₂ were prepared from bovine brain as described previously [11]. All the phospholipids were lyophilized from the chloroform solution and suspended in distilled water or 20 mM Tris-HCl (pH 7.5) by sonication. Mouse NIH 3T3 cell cDNA library was purchased from Stratagene. A bacterial expression vector for glutathione *S*-transferase (GST)–fusion protein, pGEX-2T, was from Pharmacia Biotech. Other chemicals of analytical grade were from Wako or Nacalai (Kyoto).

2.2. Bacterial expression and purification of GST–fusion Shc proteins

DNAs encoding p46, p52 and PTB domain of mouse Shc were obtained by polymerase chain reaction (PCR), using a cDNA library

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Abbreviations: PTB domain, phosphotyrosine-binding domain; SH2, Src homology 2; GST, glutathione *S*-transferase; PtdIns(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; PtdIns, phosphatidylinositol; PtdIns(4)P, phosphatidylinositol 4-phosphate; PCR, polymerase chain reaction; SDS-PAGE, SDS–polyacrylamide gel electrophoresis

of mouse NIH 3T3 cell as template. The amino acid residues encoded by these Shc constructs were: P46 (46–469), P52 (1–469), and PTB (1–210) (Fig. 1A). PCR primers used were: the sense primer for P52 and PTB, 5'-GTTTGAATTCTCATGGGACCTGGGG-TTTCCTAC-3'; the antisense primer for PTB, 5'-GAAAGAATT-CACGGTGGATTCTCTGAG-3'; the antisense primer for P52 and P46, 5'-GAAAGAATTACACTTTCCGATCCACGGGTGCTG-3'. All the primers contained a restriction site for *Eco*RI as indicated by underlines. After PCR amplification, PCR products were digested with *Eco*RI and ligated into the cognate site of the bacterial expression plasmid pGEX-2T so that the products were expressed as GST-fusion proteins. All constructs were confirmed in their orientation, length, and sequence by DNA sequencing.

Cultures of *Escherichia coli* strain DH5 α containing the GST-fusion constructs were grown at 25°C in 300 ml of L-broth (10 mg/ml Bacto-tryptone, 5 mg/ml Bacto-yeast extract, 5 mg/ml NaCl) containing 50 μ g/ml ampicillin. When the A₆₀₀ reached 0.3, isopropyl-1-thio- β -D-galactopyranoside was added to a final concentration of 1.5 mM and incubation was continued for an additional 2 h at 25°C. Following manipulations were carried out at 4°C. Bacteria were collected by centrifugation at 2000 \times g for 5 min, and the pellet was washed with 250 ml of phosphate-buffered saline. The washed cells were resuspended in 10 ml of sonication buffer (20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM EGTA, 10 mM β -mercaptoethanol, 10 μ g/ml leupeptin, 500 μ M (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride) and sonicated 5 times for 20 s each with TOMY UD-201 ultrasonic disrupter (Tomy Seiko). The sample was centrifuged at 15000 \times g for 20 min. The supernatant was then loaded onto a column of 1 ml of glutathione-Sepharose 4B (Pharmacia Biotech) equilibrated with 30 ml of sonication buffer. The column was washed with 50 ml of sonication buffer containing 0.5 M NaCl. GST-fusion proteins were eluted from the glutathione-Sepharose 4B with 5 ml of 50 mM Tris-HCl (pH 8.0) containing 5 mM reduced glutathione (Boehringer Mannheim). Eluted materials were dialyzed against dialysis buffer (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 10% glycerol) and stored at -80°C until use. One liter each of recombinant bacterial cultures yielded 3.46 mg, 0.27 mg, 0.16 mg, and 0.09 mg of soluble GST, GST-P46, GST-P52 and GST-PTB, respectively. Protein was determined spectrophotometrically with the use of Bio-Rad protein assay mixture and bovine serum albumin as a standard.

2.3. Protein kinase assay

Protein kinase assay of c-Src was carried out in the standard reaction mixture (25 μ l) containing 50 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 2 μ M [γ -³²P]ATP (3.7 kBq/pmol), 1 mM dithiothreitol, 2 μ l of the purified c-Src fraction (200 ng protein) with or without exogenous substrate: 1 mM Cdc2 peptide, 1 μ M GST-P46 or 1 μ M GST-P52. Specific activity of c-Src used in this study was 90 pmol/min/mg under the standard kinase assay conditions using 1 mM Cdc2 peptide as exogenous substrate [10]. When phosphorylation was examined in the presence of various phospholipids, phospholipids and GST-PTB or GST were pre-incubated with exogenous substrates at 30°C for 10 min. The reaction was initiated by the addition of c-Src and [γ -³²P]ATP, proceeded at 30°C for 10 min and terminated by the addition of SDS-sample buffer [12] followed by boiling for 3 min. When *K_m* was determined for phosphorylation of GST-P46 and GST-P52, 20 μ M [γ -³²P]ATP (0.37 kBq/pmol) was used instead of 2 μ M ATP. Phosphorylated proteins and Cdc2 peptide were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [12] using 10% and 18% polyacrylamide slab gels, respectively. Gels were stained with Coomassie Brilliant Blue to confirm the position of proteins and the peptide, and phosphorylated bands were visualized by BAS2000 Bioimaging Analyzer (Fuji film, Tokyo). To calculate the specific activity of phosphate incorporation, the bands were excised from the gels and their radioactivity was determined with a liquid scintillation counter (Beckman LS 6000IC).

3. Results and discussion

To examine the phosphorylation of Shc by c-Src, GST-fusion proteins containing 46-kDa (GST-P46) or 52-kDa (GST-P52) isoform of Shc and PTB domain of Shc (GST-PTB) were designed to be expressed in bacteria (Fig. 1A). Preparations of GST-fusion proteins used in this study were analyzed by SDS-PAGE followed by protein staining with Coomassie Brilliant Blue (Fig. 1B). GST-P46 (lane 1), GST-P52 (lane 2), and GST-PTB (lane 3) migrated as the proteins of 73 kDa, 78 kDa, and 50 kDa, respectively. Preparation of GST-P52 occasionally displayed degradation prod-

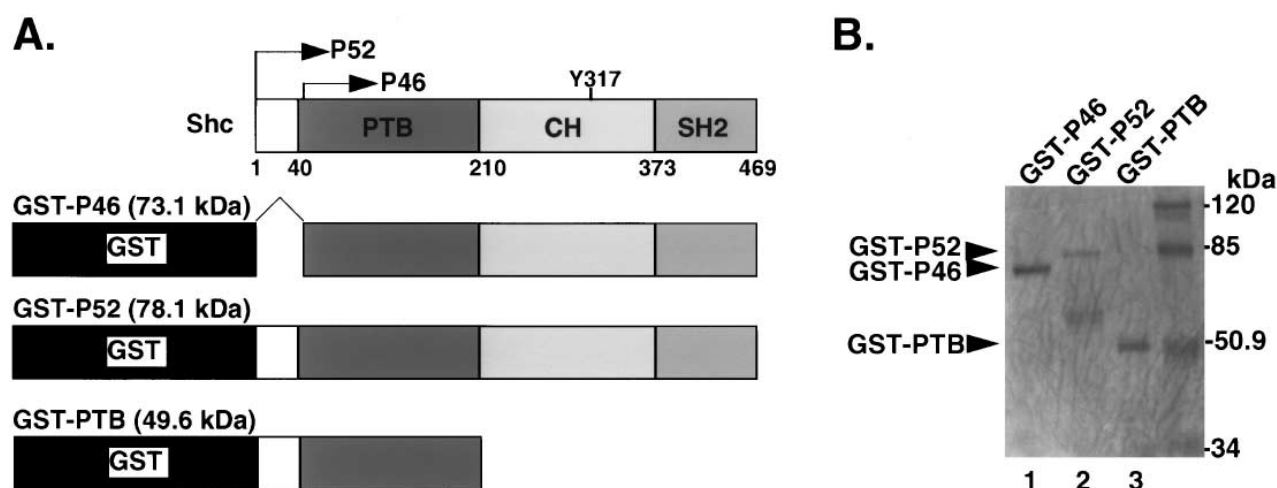


Fig. 1. Preparation of mouse p52^{shc}, p46^{shc}, and Shc PTB domain as GST-fusion proteins. Schematic representation (A) and Coomassie Brilliant Blue-stained SDS-PAGE (B) of the preparations of GST-fusion proteins used in this study are shown. Preparation of cDNAs for each construct and its bacterial expression and purification were carried out as described in Section 2. A: Calculated molecular mass of each GST-fusion protein is shown in parentheses. p52^{shc} (P52) and p46^{shc} (P46) result from differential translation initiation at two proximal ATG sites which correspond to methionine 1 and methionine 46 of P52, respectively. Three domains of Shc are shown: amino-terminal phospho-tyrosine-binding domain (PTB), collagen-homologous domain (CH), and the carboxyl-terminal Src homology 2 domain (SH2). The major phosphorylation site in human Shc [17], Tyr³¹⁷ (Y317), is also indicated. Mouse Shc has a deletion of four amino acids before Tyr³¹⁷ [18], thus correct residue number for the tyrosine phosphorylation site is 313. B: 1 μ g of each preparation of GST-fusion protein was analyzed by SDS-PAGE. Pre-stained molecular size markers (Bio-Rad); β -galactosidase (120 kDa), bovine serum albumin (85 kDa), ovalbumin (50.9 kDa), and carbonic anhydrase (34 kDa).

ucts at 52 kDa (lane 2) and 26 kDa (not shown here), suggesting a proteolytic cleavage around the junction of GST (26 kDa) and P52.

First, we examined the phosphorylation of GST-P46 and GST-P52 by c-Src under the standard assay conditions. However, phosphorylation of the GST-Shc proteins could not be detected in spite of the fact that Cdc2 peptide, a synthetic peptide substrate, was effectively phosphorylated (data not shown). Zhou et al. have demonstrated that PtdIns(4,5)P2 and PtdIns(4)P specifically bind to the PTB domain of Shc while other phospholipids such as phosphatidylserine, phosphatidic acid, phosphatidylcholine, phosphoethanolamine and phosphatidylinositol (PtdIns) do not [9]. In addition, De Corte et al. have recently demonstrated that gelsolin, a known PtdIns(4,5)P2-binding protein, can be phosphorylated by c-Src in a PtdIns(4,5)P2-dependent manner [13]. These observations led us to think that PtdIns(4,5)P2 binding to the PTB domain may alter the structure of Shc as a substrate of c-Src. In Fig. 2, effect of various phospholipids on the phosphoryl-

ation of 1 μ M of GST-P46 or GST-P52 by c-Src was examined. As control experiments, autophosphorylation and phosphorylation of Cdc2 peptide by c-Src were also examined in the presence of phospholipids. Phospholipids used were phosphatidylserine, phosphatidylcholine, phosphatidic acid, PtdIns, PtdIns(4)P, and PtdIns(4,5)P2. All the phospholipids, except phosphatidylcholine and PtdIns(4,5)P2, were found to stimulate the c-Src activity as judged by the autophosphorylation and Cdc2 peptide phosphorylation (lanes 1–3 and 4–6). Correspondingly, in the presence of these phospholipids, phosphorylation of GST-Shc was also stimulated (lanes 7–9 and 10–12). Therefore, it is concluded that these abundant phospholipids act on c-Src rather than substrate proteins or peptide. It has been demonstrated that Shc is constitutively tyrosine-phosphorylated in Rous sarcoma virus-transformed cells [14]. It means that deregulated and fully active v-Src can phosphorylate Shc efficiently. Thus, it is possible to think that some phospholipids, which exist abundantly in the plasma membrane, can activate c-Src and enable it to phospho-

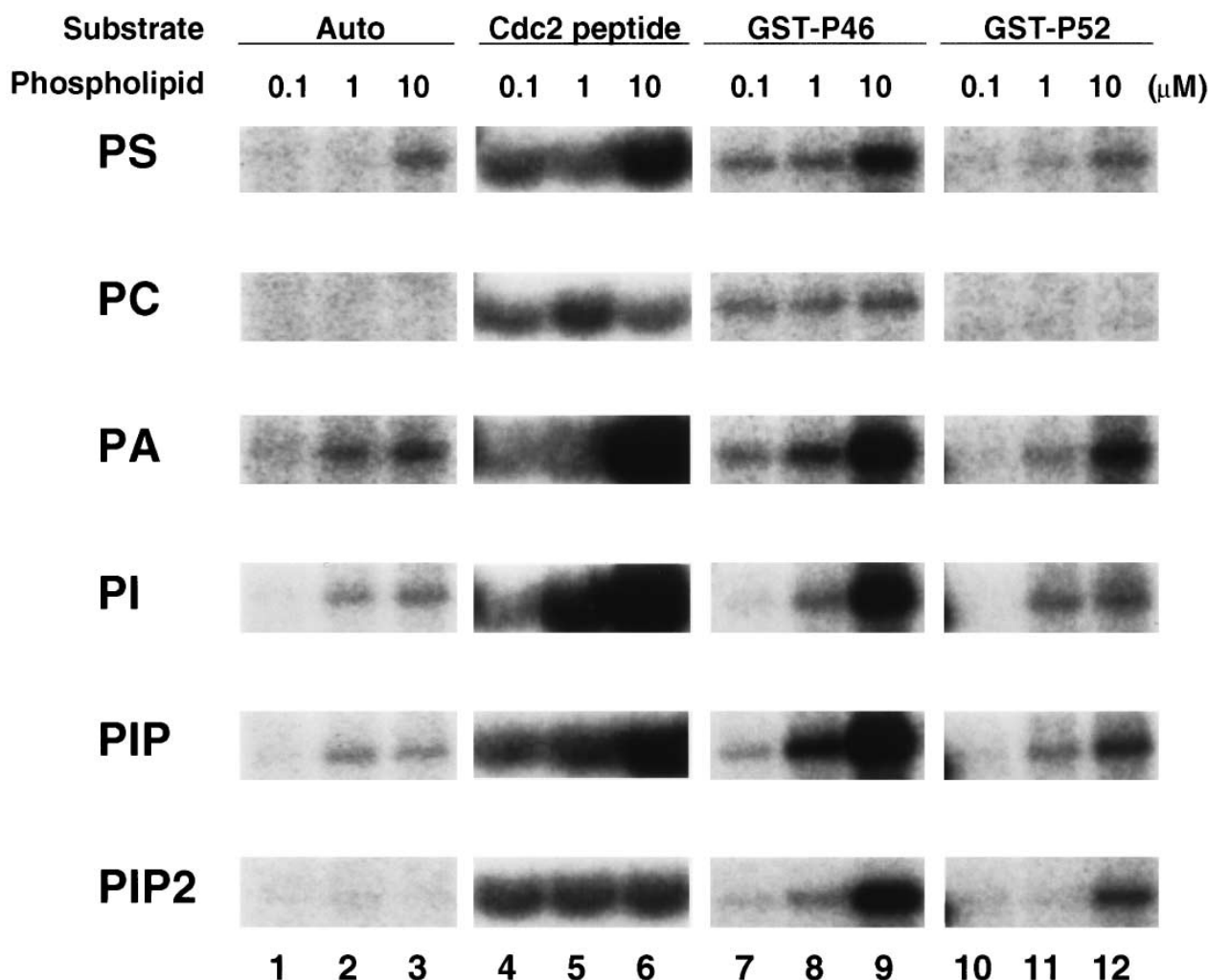


Fig. 2. Effect of various phospholipids on autophosphorylation of c-Src and phosphorylation of Cdc2 peptide and GST-Shc by c-Src. Auto-phosphorylation of c-Src (lanes 1–3), and phosphorylation of Cdc2 peptide (lanes 4–6), GST-P46 (lanes 7–9) and GST-P52 (lanes 10–12) by c-Src were examined in the presence of 0.1 μ M (lanes 1, 4, 7, and 10), 1 μ M (lanes 2, 5, 8, and 11) or 10 μ M (lanes 3, 6, 9, and 12) of various phospholipids in the standard reaction mixture as described in Section 2. Phosphorylated proteins were separated by SDS-PAGE using 10% polyacrylamide gels and analyzed by BAS2000 Bioimaging Analyzer. Phosphorylation of Cdc2 peptide was analyzed by SDS-PAGE on 18% gels. Results are representative of three independent experiments. PS, phosphatidylserine; PC, phosphatidylcholine; PA, phosphatidic acid; PI, PtdIns; PIP, PtdIns(4)P; PIP2, PtdIns(4,5)P2.

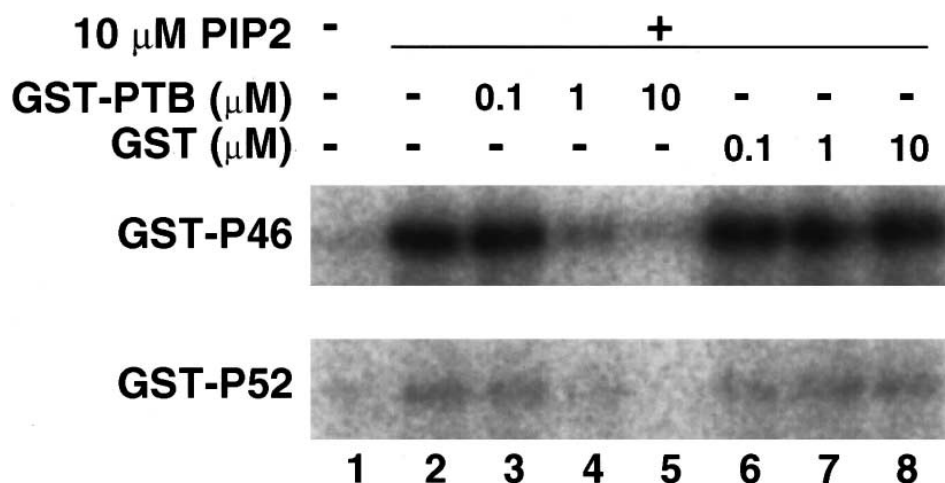


Fig. 3. Shc PTB domain inhibits the PtdIns(4,5)P₂-dependent phosphorylation of GST–Shc by c-Src. Phosphorylation of GST–P46 (upper panel) and GST–P52 (lower panel) by c-Src was carried out in the absence (lane 1) or the presence (lanes 2–8) of 10 μ M PtdIns(4,5)P₂ and various concentrations (0.1 μ M, 1 μ M, 10 μ M) of GST–PTB (lanes 3–5) or GST (lanes 6–8). Results are representative of two independent experiments. Phosphoimage of GST–P46 and GST–P52 is shown.

rylate Shc proteins. On the other hand, PtdIns(4,5)P₂ gave an increased phosphorylation of GST–Shc without any effect on the c-Src activity itself (Fig. 2, bottom panels). Since GST could not be phosphorylated by c-Src at any concentrations of phospholipids tested (data not shown), the result suggests that PtdIns(4,5)P₂ specifically acts on Shc to interact with c-Src as a substrate and/or stabilizes a c-Src–Shc complex. Extent of phosphorylation of GST–P52 was apparently lower than that of GST–P46 at about two-times difference (Fig. 2, bottom panels). However, it was found that phosphorylation of 52-kDa protein, a possible Shc fragment derived from GST–P52 (Fig. 1B), is also taking place at an extent comparable to GST–P52 (data not shown). Thus, it can be said that PtdIns(4,5)P₂ stimulates phosphorylation of both p46 and p52 isoforms of Shc by c-Src almost equally. Calculated K_m values for GST–P46 and GST–P52 were 90 nM and 85 nM, respectively, in the presence of 1 μ M PtdIns(4,5)P₂. Similar values were obtained with 10 μ M PtdIns(4,5)P₂; however, higher concentration of PtdIns(4,5)P₂ (100 μ M) greatly inhibited the c-Src activity judged by the phosphorylation of Cdc2 peptide (data not shown). These results are quite different from the observation by De Corte et al. [13] that the phosphorylation of gelsolin at 2.5 μ M by c-Src required the PtdIns(4,5)P₂ concentration at around 100 μ M.

Since PTB domain of Shc is shown to bind PtdIns(4,5)P₂ [9], we next examined whether the PtdIns(4,5)P₂-dependent Shc phosphorylation by c-Src is affected by exogenously added PTB domain. As shown in Fig. 3, GST–PTB inhibited the phosphorylation of GST–Shc in a dose-dependent manner, while GST did not. GST–PTB could not be a substrate of c-Src at any concentrations used in this study (data not shown), indicating that the inhibition was not a result of substrate competition. The result seems to be consistent with the idea that Shc PTB domain competitively binds to PtdIns(4,5)P₂ and abolishes GST–Shc phosphorylation by c-Src. However, it may be an oversimplified interpretation. Interestingly, 1 μ M GST–PTB showed significant inhibitory effect toward 10 μ M PtdIns(4,5)P₂ (Fig. 3, lane 4) and 10 μ M GST–PTB was enough for the complete inhibition (lane 5). Such effect at low molar ratio cannot be explained by a simple

competitive interaction. Therefore, it might be possible that GST–PTB also binds to c-Src and interferes with the interaction between c-Src and GST–Shc.

In this study, PtdIns(4,5)P₂ was shown to stimulate the phosphorylation of GST–Shc by c-Src. Relative content of PtdIns(4,5)P₂ among phospholipids in the plasma membrane is quite low and strictly regulated by extracellular stimuli elicited by hormones and environmental conditions. So, it is suggested that PtdIns(4,5)P₂ can act as a regulator for the phosphorylation of Shc by c-Src in an extracellular signal-dependent manner. It is well established that tyr³¹⁷ of Shc is the major site of phosphorylation and the phosphorylation is indispensable for Shc-dependent signaling pathways [2–5]. However, it is not clear whether the same tyrosine residue is phosphorylated in the presence of PtdIns(4,5)P₂. It should be clarified whether tyr³¹⁷ is the only phosphorylation site in the PtdIns(4,5)P₂-treated Shc. It is also of interest whether the products of a lipid kinase PI 3-kinase such as PtdIns(3,4,5)P₃ affects the Shc phosphorylation by c-Src because involvement of PI 3-kinase in the kinase/non-kinase receptor-mediated tyrosine phosphorylation of Shc has been documented [15,16]. As it can be concluded from the results shown in Fig. 3, the interaction of PtdIns(4,5)P₂ with PTB domain is required for the PtdIns(4,5)P₂-dependent phosphorylation of Shc by c-Src. It seems natural to surmise that the interaction between PtdIns(4,5)P₂ and Shc PTB domain serves not only for localizing Shc to the membrane as anticipated [9] but also for modulating the tyrosine phosphorylation of Shc. However, it is also possible that PtdIns(4)P, which has been shown to bind to PTB domain of Shc [9], can act as a modulator for tyrosine phosphorylation of Shc although PtdIns(4)P stimulated the c-Src activity itself in our experimental system (Fig. 2). It has been demonstrated that binding of PtdIns(4,5)P₂ to PTB domain can be eliminated partially by a phosphotyrosine-containing peptide [9]. Thus, it would be interesting to test whether such phosphotyrosine-binding to the PTB domain also affects the phosphorylation of Shc by c-Src. In the presence of PtdIns(4,5)P₂, phosphorylation of both GST–P46 and GST–P52 was equally stimulated. In many cell systems, however, the extent of expression and phospho-

rylation level of these two isoforms are quite different. Thus, the elucidation of the function of each Shc isoform may contribute to the understanding the signal transduction pathways involving Shc.

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