3-Phosphohistidine Cannot Replace Phosphotyrosine in High-Affinity Binding to Phosphotyrosine Binding or *Src* Homology 2 Domains

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ABSTRACT: Posttranslational phosphorylation of proteins is an important event in many cellular processes. Phosphorylated tyrosine residues can serve as association sites for other proteins in signal transduction cascades of tyrosine kinase receptors. Formation of phosphohistidine residues in proteins has been found in eukaryotic and prokaryotic organisms. Furthermore, it has been suggested that phosphohistidine might substitute for phosphotyrosine in conferring high-affinity binding to proteins involved in signal transduction. We have analyzed the ability of 3-phosphohistidine to associate with the known phosphotyrosine-specific phosphotyrosine binding and *src* homology 2 protein domains. From our binding studies using synthetic peptides, we conclude that 3-phosphohistidine cannot replace phosphotyrosine in conferring high-affinity binding to the phosphotyrosine binding domain of *shc* or the *src* homology 2 domain of phospholipase C-γ1.

Regulation by transient phosphorylation of proteins is a common process in a wide variety of biological processes, including signal transduction events (*1*). The phosphorylation of serine, threonine, and tyrosine residues in proteins in eukaryotic organisms has been well established in many steps of signal transduction. Phosphorylation of these residues can lead to the activation of enzymatic activities or the association of other proteins with the newly phosphorylated residue in a sequence-specific context (*2*). The association of proteins with phosphorylated tyrosine residues plays a major role in many signal transduction pathways of tyrosine kinase receptors. After receptor activation, tyrosines within the receptor itself become phosphorylated, leading to the association of other proteins with the newly formed phosphotyrosine residues in the receptor. These associations come about through phosphotyrosine-specific binding modules. As of today, two such modules have been identified, the SH2 domain and the PTB[1] domain. Specificity of the binding interaction is determined by amino acids surrounding the phosphotyrosine residue. In the case of the SH2 domain, the sequence carboxy terminal to the phosphotyrosine determines which SH2 domain can bind with high affinity. PTB domains recognize a motif, "NXX(pY)", in which amino acid residues amino terminal to the phosphotyrosine are important for binding specificity. The motif "NPX(pY)" is the most commonly identified PTB domain binding site. Whereas the SH2 domain has a requirement for a phosphorylated tyrosine for binding to take place, certain PTB domains seem to be able to also bind to unphosphorylated tyrosine in the "NPXY" sequence context (*3*).

A recent report (*4*) suggests that the PTB domain of the signaling adaptor protein *shc* can interact with a peptide sequence in which the tyrosine in the "NPXY" motif is replaced by a histidine residue. The authors of this paper show that *shc* can associate with the signaling protein PTP-PEST. PTP-PEST is not tyrosine-phosphorylated but bears a sequence motif, ^{599}NPLH^{602}, that resembles that of the PTB domain-specific motif. Peptide competition studies revealed that the *shc*/PTP-PEST interaction can be competed by a PTP-PEST-derived peptide, containing the "NPLH" sequence, albeit at higher concentrations than a peptide derived from the epidermal growth factor receptor containing the "NPX(pY)" sequence. The authors speculate that analogous to the tyrosine residue in the previously characterized PTB domain-specific sequence, "NPXY", the histidine residue 602 of the "NPLH" sequence in PTP-PEST may be phosphorylated *in vivo*. Histidine phosphorylation could thereby lead to an increased binding affinity of the *shc* PTB domain to the ^{599}NPLH^{602} sequence in PTP-PEST.

It is estimated that phosphohistidine may account for approximately 6% of total protein phosphorylation in prokaryotes as well as eukaryotes (*6*). Although not as abundant as serine- and threonine phosphorylation, histidine phosphorylation apparently is more common than phosphorylation of tyrosine residues.

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[1] Abbreviations: BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; Fmoc, fluorenylmethoxycarbonyl; FGFR1, fibroblast growth factor receptor 1; GST, glutathione *S*-transferase; HA, hemagglutinin; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PBS, phosphate-buffered saline; (pH), 3-phosphohistidine; PLC-γ1, phospholipase C-γ1; PMSF, phenylmethanesulfonyl fluoride; PTB, phosphotyrosine binding; PTP-PEST, phosphotyrosine phosphatase-PEST; (pY), phosphotyrosine; RP-HPLC, reversed phase high-performance liquid chromatography; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SH2, *Src* homology 2; *T-Ag*, *T-antigen.*

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In prokaryotes, several signal transduction events are governed by so-called two-component systems that involve the formation of phosphorylated histidine residues (*5*). These systems typically consist of a sensor protein and a response regulator protein. Sensor proteins autophosphorylate in response to specific inputs. The site of phosphorylation in most cases is a conserved histidine residue. The sensor protein transduces signals by transferring the phosphate group to an aspartic acid residue in a second component, the response regulator. Phosphorylation of the response regulator leads to an output signal such as transcriptional activation or protein−protein interaction (*5*).

The phosphorylation of histidine residues has been observed in eukaryotic cells. The *Sln1* protein in yeast that is involved in osmosensing shares high amino acid homology with the bacterial two-component protein systems, including a presumed histidine autophosphorylation site (*7*). The *Arabidopsis thaliana* gene, *ETR1*, also encodes a histidine kinase, which mediates the ethylene response (*8*). Phosphohistidine was originally detected in mammalian cells in mitochondria derived from bovine liver (*9*) and nuclei from rat tissue (*10*). Recently it was found that phosphorylation of a histidine on the cytoplasmic tail of P-selectin takes place following platelet activation with thrombin or collagen (*11*). Thus, it appears that this posttranslational modification is increasingly recognized in many systems that are related to signal transduction. The difficulties in studying histidine phosphorylation events have their origin in the high energy state of the phosphate−imidazole bond, which resembles that of a phosphoramidate. Phosphohistidine is therefore not as stable as its phosphoester relatives and hydrolyzes readily under acidic conditions, which are common in experimental techniques (*12*).

The recent reports of the presence of phosphohistidine in mammalian cells combined with the importance of phosphate esters of the hydroxy amino acids tyrosine, serine, and threonine in many aspects of signal transduction prompted us to investigate if 3-phosphohistidine can replace phosphotyrosine in binding to the PTB or SH2 domains. In this report, we analyze the binding of synthetic peptides bearing the "NPX(pY)" or "NPX(pH)" sequence motifs to the PTB domain of *shc*. These studies were made possible by our recent development of methods for the synthesis and analysis of 3-phosphohistidine-containing peptides (*13*). We show that 3-phosphohistidine cannot replace phosphotyrosine in high-affinity binding to the PTB domain. Our studies also indicate that 3-phosphohistidine cannot substitute for phosphotyrosine in binding to the SH2 domain of PLC-γ1.

EXPERIMENTAL PROCEDURES

Peptide Synthesis. Peptides were synthesized using Fmoc chemistry. After cleavage and deprotection, the peptides were purified by RP-HPLC and lyophilized. Peptide identity was confirmed using an LCQ ion trap mass spectrometer (FinniganMat, San Jose, CA). For the synthesis of 3-phosphohistidine-containing peptides, we used a method we recently developed (*13*). Briefly, 0.5 mg of lyophilized peptide was dissolved in 10 mM ammonium bicarbonate, and the pH was adjusted to 8 with 0.1 M NaOH. Ten milligrams of potassium phosphoramidate was added, and the mixture was tumbled overnight at room temperature (*12, 14*). Purification of the peptides was carried out on a Vydac pH-stable reversed phase column (The Separations Group, Hesperia, CA) using 10 mM ammonium bicarbonate as solvent A and acetonitrile/10 mM ammonium bicarbonate (9:1) as solvent B. A linear gradient was applied, increasing solvent B concentration from 0% to 65% over 60 min at a flow rate of 0.5 mL/min. Peptides were detected at 215 nm. The eluted peptides were analyzed by mass spectrometry and stored in solution at 4 °C. For the synthesis of radiolabeled peptides, 10 mg of peptide resin was acetylated with 1 mCi of tritium-labeled acetic anhydride (Amersham, Arlington Heights, IL) in 500 μL of dimethylformamide/pyridine (1:1), overnight. Cleavage, purification, and phosphorylation of the radiolabeled peptides were carried out as described above for the unlabeled peptides. The erbB2- and erbB3-derived peptides were synthesized and purified by Chiron Mimotopes (Clayton, Australia) as described previously (*15*).

Expression of PTB and SH2 Domains. The PTB domain of human *shc* (amino acids 1−238) and the amino-terminal SH2 domain of bovine PLC-γ1 (amino acids 547−659) were expressed in *E. coli* (HB101) as GST fusion proteins using pGEX vector constructs (Pharmacia, Piscataway, NJ). The cells were harvested following centrifugation at 5000 rpm for 5 min, lysed in extraction buffer A (50 mM Tris-HCl, pH 8, 5 mM EDTA, 0.25 mg/mL lysozyme, 50 μg/M NaN_3) for 5 min, and then subjected to buffer B (1.5 M NaCl, 100 mM $CaCl_2$, 100 mM $MgCl_2$, pH 7, 20 μg/mL DNaseI, 10 mM PMSF, 50 μg/mL each of aprotinin and leupeptin) for another 5 min. The cell lysates were then centrifuged at 15 000 rpm for 1 h, the pellet was discarded, and the supernatant was subjected to affinity chromatography using a glutathione−Sepharose column (Pharmacia). The resin was then washed 3 times with PBS, and for analysis, an aliquot was subjected to SDS−PAGE. Both fusion proteins showed the expected molecular weight bands.

Radioactive Binding Assays. GST-PTB/*shc* or GST-SH2/PLC-γ1 fusion proteins (8−10 μg), absorbed to glutathione−Sepharose beads, in 200 μL of binding buffer (20 mM Tris-HCl, pH 7.4, 250 mM NaCl, 0.1% BSA, and 10 mM dithiothreitol) were incubated with 5×10^4 cpm of tritium-labeled peptides (4×10^4 cpm/μg) and in the case of the GST-PTB/*shc* fusion protein with various amounts of unlabeled peptides as competitors (Table 1). Incubation was carried out overnight, and radioactivity associated with the unwashed gluthathione−Sepharose beads was determined by scintillation counting. Data are expressed as percent binding of control in the absence of inhibitor after background was subtracted.

ELISA Competition Binding Assays. The assay was performed by a modification of a previously described method (*15*). Immulon 4 microtiter plates (Dynatech Laboratories, Inc., Chantilly, VA) were coated with 50 μL/well of 20 μg/mL anti-influenza HA antibody 12CA5 (Boehringer Mannheim, Indianapolis, IN) in PBS by overnight incubation at ambient temperature. The plates were washed 3 times with 200 μL of PBS and blocked with 3% BSA in PBS. Sf9 cell lysate containing recombinant HA-PTB/*shc* fusion protein was diluted 10-fold in binding buffer (20 mM HEPES, pH 7.8, 75 mM KCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 0.01% Triton X-100, 0.1% BSA, 100 μM PMSF, 1 μg/mL leupeptin, 200 μg/mL aprotinin, 0.7 μg/mL pepstatin). Fifty microliters μL of diluted lysate was added into each well and incubated overnight at 4 °C to capture PTB domain protein on the plates, and the plates were washed 3 times

Table 1: Peptides Used in Binding Studies to PTB and SH2 Domains

peptide sequence	protein
Ac-PLSFTNPLHSDDW-NH_2	PTP-PEST (594−606)
Ac-PLSFTNPL(pH)SDDW-NH_2	
Ac-PLSFTNPLYSDDW-NH_2	
Ac-PLSFTNPL(pY)SDDW-NH_2	
Ac-LSNPTYSV-NH_2	*T-Ag* (245−252)
Ac-LSNPT(pY)SV-NH_2	
biotin-PAFSPAFDNL(pY)(pY)WDQNSSEQG	erbB2 (1211−1231)
AFDNL(pY)(pY)WDQNS	erbB2 (1216−1227)
fluorescein-AFDNL(pY)(pY)WDQN	
AFDNPD(pY)WHSRL	erbB3 (1216−1227)
fluorescein-PLSFTNPL(pH)SDDW-NH_2	PTP-PEST (594−606)
fluorescein-PLSFTNPLHSDDW-NH_2	
Ac-LTSNQAYLDLS-NH_2	FGFR1 (760−770)
Ac-LTSNQA(pY)LDLS-NH_2	
Ac-LTSNQAHLDLS-NH_2	
Ac-LTSNQA(pH)LDLS-NH_2	

with binding buffer. Nonbiotinylated peptide inhibitors (50 μL/well, Table 1) were incubated at the indicated concentrations in 50 mM ammonium bicarbonate buffer, pH 8.0, with plate-captured PTB domain protein at ambient temperature for 1 h. After the preincubation, 50 μL of 10 nM biotinylated erbB2-derived peptide (Table 1) in ammonium bicarbonate buffer was added, and the incubation was continued for 1 h. After incubation, the plates were washed 3 times with PBS. To detect bound ligand, 100 μL of 1:5000 diluted europium-labeled streptavidin (Wallc, Turku, Finland) was added and incubated for 1 h at ambient temperature. The plates were washed 3 times, and 100 μL of enhancement solution (Wallc) was added to each well. Chemiluminescence was read using a Wallc DELFIA reader after shaking vigorously for 5 min with a plate shaker. Data are expressed as percent binding of control in the absence of inhibitor after background was subtracted.

Fluorescence Polarization Binding Assays. Fluorescence polarization as a direct measure of binding to *shc* PTB domain was adapted from a previously described method (*12*). Recombinant *shc* PTB domain protein was purified as described (*13*), and diluted in binding buffer without BSA. Diluted PTB domain protein was incubated at the indicated concentrations in a 96 well black microtiter plate (Dynatech Laboratories, Inc.) with 5 nM fluoresceinated peptide ligand (Table 1) in a final volume of 100 μL, and the plate was shaken for 10 min. Fluorescence polarization was read using a FPM2 fluorescence polarization plate reader (Dynatech Labs, Inc.). Results are expressed as fluorescence polarization $\times 10^{-3}$ (mP) as described (*16*).

RESULTS

To study the differences in binding between phosphotyrosine- and 3-phosphohistidine-containing peptides to the PTB domain of *shc*, we used PTP-PEST-derived peptides (Table 1). 3-Phosphohistidine-containing peptides were synthesized as described under Experimental Procedures. Histidine-phosphorylated peptides were separated from unphosphorylated peptides by RP-HPLC using an ammonium bicarbonate solvent system (*13*). Phosphopeptide identity was confirmed by mass spectrometry (Figure 1). All peptides that were used in the studies were modified on the amino terminus by either an acetyl group or a fluorescein group. Therefore, no phosphorylation of the amino-terminal amino group could occur during the reaction with potassium phosphoramidate. Previous studies have shown that long incubation of histidine with potassium phosphoramidate leads to the 3-phosphorylated histidine isomer (*12*). ^{1}H-NMR analysis of a synthetic peptide that underwent histidine phosphorylation using the same reaction conditions showed that prolonged incubation with potassium phosphoramidate also leads to the 3-phospohistidine isomer in peptides (*13*). We therefore assume that all the peptides that were used for the binding studies carry a phosphate group on the 3-position of the imidazole ring. Since phosphohistidine residues are labile, we subjected all 3-phosphohistidine-containing peptides to RP-HPLC analysis after the completion of the binding studies that are described below. In all three cases, we found that the peptides retained the phosphate group during the various binding assays.

We substituted unphosphorylated and phosphorylated tyrosine and histidine residues within the "NPLX" sequence and performed binding competition experiments with the known high-affinity interaction between the *shc* PTB domain and the *T-Ag*-derived peptide Ac-LSNPT(pY)SV-NH_2 (*18*). Binding competition data are presented in Figure 2. Both phosphotyrosine-containing peptides derived from *T-Ag* or PTP-PEST led to a significantly better competition of labeled *T-Ag* peptide (5×10^4 cpm/1.25 μg) binding to the PTB domain of *shc* than all the other peptides, including the 3-phosphohistidine-containing peptide. As shown in Figure 2, the phosphotyrosine-containing peptides derived from *T-Ag* and PTP-PEST competed for *shc* PTB domain binding with an IC_{50} of 2.0×10^{-5} M and 6.31×10^{-7} M, respectively. The apparent higher affinity of the PTP-PEST-derived peptide over the *T-Ag*-derived peptide is probably due to the greater peptide sequence length. Binding affinities for the PTP-PEST-derived peptides with an unphosphorylated or 3-phosphorylated histidine residues were at least 10 times lower than the ones for the tyrosine-phosphorylated PTP-PEST or *T-Ag*-derived peptides.

Because variations in experimental conditions can affect results from *in vitro* competition binding assays, we also assessed the ability of "NPX(pH)"-containing peptides to bind the *shc* PTB domain in two other independent assay systems. Tyrosine-phosphorylated, erbB2-derived peptides have been previously shown to bind the *shc* PTB domain with high affinity in an assay based on an ELISA format (*15*). As described under Experimental Procedures, PTB domain protein tagged with the HA epitope was immobilized on microtiter plates by capture with an anti-HA monoclonal antibody and incubated with a biotinylated, tyrosine-phosphorylated erbB2-derived peptide. After being washed, the bound peptide was detected with europium-coupled streptavidin and quantified by chemiluminescence. Competition binding studies were performed by preincubation of the PTB domain-coated microtiter wells with increasing concentrations of nonbiotinylated, PTP-PEST-derived peptides containing nonphosphorylated or 3-phosphorylated histidine within the "NPXH" motif (Table 1). Control experiments were performed in which the nonbiotinylated, competing peptides contained tyrosine-phosphorylated "NXX(pY)" sequences derived from either erbB2 or erbB3 (Table 1). As shown in Figure 3, the erbB2 and erbB3 peptides competed for *shc* PTB domain binding under these conditions with an IC_{50} of approximately 100−300 nM, consistent with previously reported results (*15*). Both the nonphosphorylated

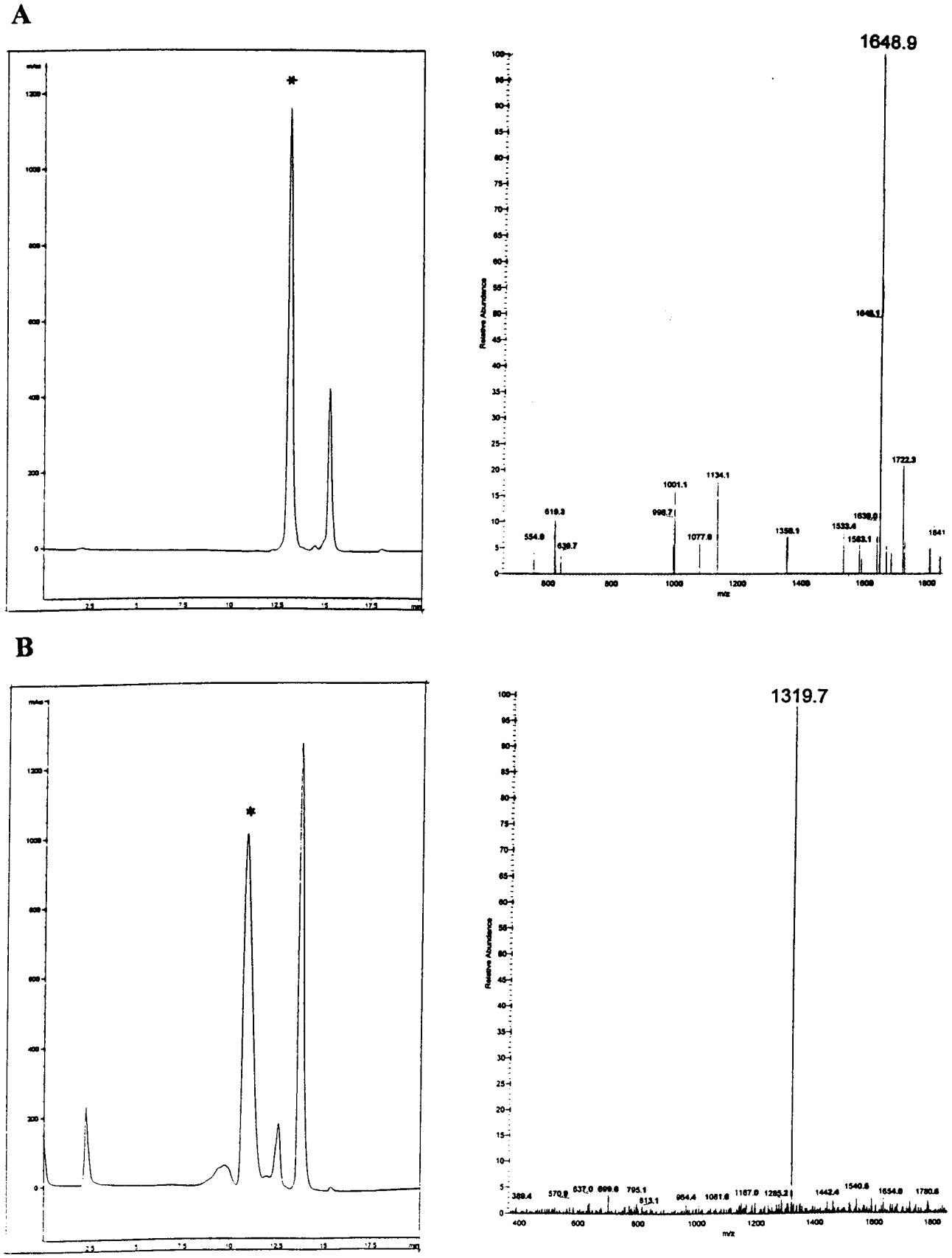

FIGURE 1: RP-HPLC traces and mass spectra of 3-phosphohistidine-containing peptides Ac-PLSFTNPL(pH)SDDW-NH_2 (A) and Ac-LTSNQA(pH)LDLS-NH_2 (B) used in binding studies to the GST−*shc* and GST−PLC-γ1 fusion proteins, respectively. The RP-HPLC peaks labeled with asterisks represent the histidine-phosphorylated peptides that were used for mass spectrometry (inset). The later eluting peaks represent the unphosphorylated peptides.

and 3-histidine-phosphorylated PTP-PEST-derived peptides competed with an apparent affinity at least 100-fold lower than that of the erbB2- or erbB3-derived peptides in the same experiment. Further, there was minimal difference in apparent affinity between peptides containing 3-phosphorylated or nonphosphorylated histidines.

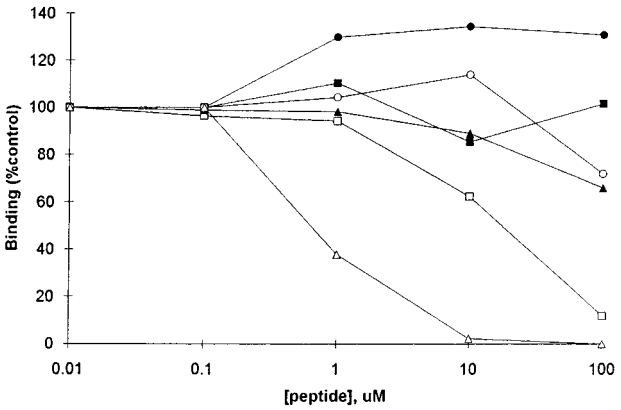

FIGURE 2: Binding competition assays with the PTB domain of *shc*. A GST−*shc* fusion protein was incubated with 5×10^4 cpm of the tritium-labeled *T-Ag*-derived peptide Ac-LSNPT(pY)SV-NH_2 and varying concentrations of unlabeled peptides as described under Experimental Procedures. Results are expressed as percent of control binding in the absence of competitor after background was subtracted. (●) Ac-PLSFTNPLHSDDW-NH_2, (○) Ac-PLSFTNPL-(pH)SDDW-NH_2, (▲) Ac-PLSFTNPLYSDDW-NH_2, (△) Ac-PLS-FTNPL(pY)SDDW-NH_2, (■) Ac-LSNPTYSV-NH_2, (□) Ac-LSNPT-(pY)SV-NH_2.

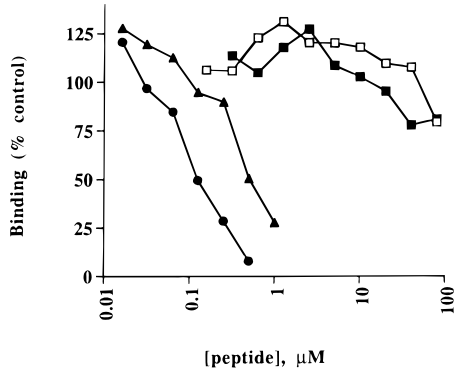

FIGURE 3: Competition binding of phosphorylated and nonphosphorylated PTP-PEST peptides to the *shc* PTB domain in an ELISA format. HA epitope-tagged *shc* PTB domain protein was captured on 96 well plates with monoclonal antibody 12CA5, and binding of the erbB2-derived peptide biotin-PAFSPAFDNL(pY)(pY)-WDQNSSEQG was quantified as described under Experimental Procedures. Nonbiotinylated PTP-PEST-derived peptides Ac-PLS-FTNPL(pH)SDDW-NH_2 (■) and Ac-PLSFTNPLHSDDW-NH_2 (□) were used as competitors. Competition binding by the erbB2-derived peptide AFDNL(pY)(pY)WDQNS (●) and the erbB3-derived peptide AFDNPD(pY)WHSRL (▲) was performed as controls. Results are expressed as percent of control binding in the absence of competitor after background was subtracted.

The data in Figures 2 and 3 were generated using competition binding assays, which are indirect assessments of peptide affinity. We wished to also directly determine whether 3-phosphohistidine can confer on NPXH motifs high-affinity binding to the *shc* PTB domain. For these experiments, we made use of a fluorescence polarization assay which directly measures solution binding in a homogeneous system which does not require washing steps or the addition of competitors (*16*). In this assay, binding of fluorescein-labeled peptides to the PTB domain induces a molecular volume change and a subsequent change in fluorescence polarization which can be quantified with a fluorescence polarization spectrophotometer. Fluorescein-labeled peptides derived from PTP-PEST and containing histidine or 3-phosphohistidine (Table 1) were incubated with increasing concentrations of purified *shc* PTB domain

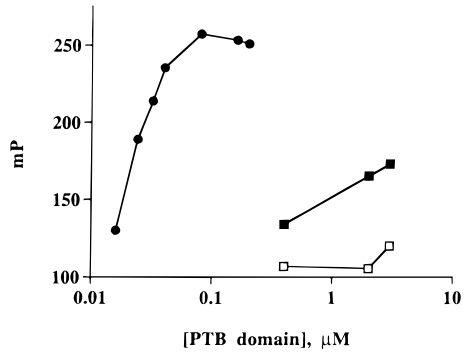

FIGURE 4: Direct assessment of binding of phosphorylated and nonphosphorylated PTP-PEST peptides to the *shc* PTB domain by fluorescence polarization. Increasing concentrations of purified *shc* PTB domain were incubated with 5 nM fluorescein-labeled peptides derived from erbB2, fluorescein-AFDNL(pY)(pY)WDQN (●), or PTP-PEST, fluorescein-PLSFTNPL(pH)SDDW-NH_2 (■), and fluorescein-PLSFTNPLHSDDW-NH_2 (□), and fluorescence polarization was determined using a FPM2 plate reader. Results are expressed as fluorescence polarization $\times 10^{-3}$.

protein, and fluorescent polarization was determined using a FPM2 plate reader. As shown in Figure 4, a phosphorylated, fluorescein-labeled control peptide derived from erbB2 and containing the "NXX(pY)" sequence bound well to PTB domain under these conditions, with an apparent affinity of approximately 50 nM. The PTP-PEST-derived peptides bound poorly, with affinities significantly greater than 1 μM. Accurate estimates of affinity for these peptides were not possible because higher concentrations of PTB domain protein interfered with the fluorescence detection (data not shown). The PTP-PEST peptide containing 3-phosphohistidine bound minimally better than the peptide containing nonphosphorylated histidine. These data demonstrate directly that phosphorylated histidine does not confer upon the PTP-PEST peptide high-affinity binding to the *shc* PTB domain, such as is observed with the "NXX(pY)"-containing erbB2 peptide. We conclude that phosphorylation of histidine in the 3-position of the imidazole ring of residue 602 in the PTP-PEST-derived peptide does not increase the relatively weak binding of this sequence to the PTB domain of *shc*.

To study the binding of 3-phosphohistidine to the other phosphotyrosine-specific module, the SH2 domain, we analyzed the association of FGFR1-derived peptides with the SH2 domain of PLC-γ1 (*19*). The association of the phosphorylated FGFR1 with the SH2 domain of PLC-γ1 is mediated by phosphotyrosine residue 766 of the receptor. We synthesized peptides based on the FGFR1 binding site for PLC-γ1 with phosphotyrosine, tyrosine, 3-phosphohistidine or histidine in place of residue 766 of the receptor-derived peptide sequence (Table 1). As expected, the radiolabeled peptide with the phosphotyrosine residue shows good binding to the SH2 domain of PLC-γ1 at a concentration of 4 μM. All other peptides, where phosphotyrosine is replaced with either tyrosine, histidine, or 3-phosphohistidine, showed no detectable binding over background at the same concentrations (Figure 5). Due to the low solubility of the 3-phosphohistidine-containing FGFR1-derived peptide, we were unable to carry out detailed binding competition studies as in the case of the PTB domain. As for the PTB domain of *shc*, a 3-phosphohistidine in place of a phosphotyrosine does not seem to confer high-affinity binding of a consensus sequence-containing peptide to the SH2 domain of PLC-γ1.

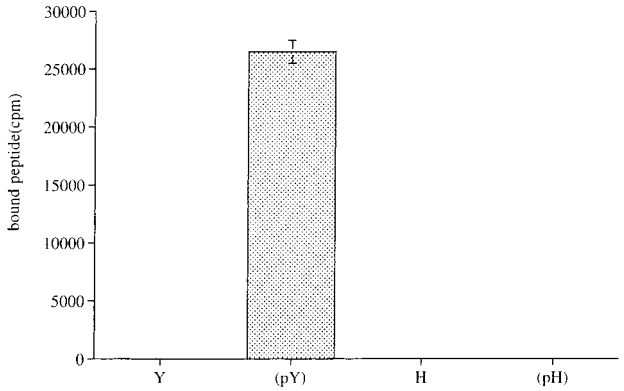

FIGURE 5: Binding assay with the N-terminal SH2 domain of PLC-γ1. A GST−PLC-γ1 fusion protein was incubated with 5×10^4 cpm of tritium-labeled peptides as described under Experimental Procedures. Background binding to GST−Sepharose was subtracted. Y: Ac-LTSNQAYLDLS-NH_2. (pY): Ac-LTSNQA(pY)LDLS-NH_2. H: Ac-LTSNQAHLDLS-NH_2. (pH): Ac-LTSNQA(pH)-LDLS-NH_2.

DISCUSSION

Protein domains that have high affinities for phosphotyrosine residues play important roles in signal transduction cascades of tyrosine kinase receptors. In the case of the SH2 domain, the specificity of binding to phosphotyrosine is furthermore determined by the amino acid sequence carboxy terminal to the phosphotyrosine. In particular, the residues in the +2 or +3 positions relative to the phosphotyrosine are critical for the affinity and specificity. Different SH2 domains have different preferences for carboxy-terminal amino acid sequences. The phosphorylation of the tyrosine residue is essential for high-affinity binding to take place.

The other protein domain that can confer high-affinity binding to phosphotyrosine residues is the PTB domain. In this case, the amino acid sequence amino terminal to the phosphotyrosine residue is important for high-affinity binding to take place. PTB and SH2 domains differ in their structure, specificity, and mechanism of action. The PTB domain forms a hydrophobic interaction with the amino-terminal amino acid residues. Furthermore, the orientation of the phosphopeptide relative to the antiparallel β-sheet structure of the SH2 or PTB protein domains is different (*17*). Finally, PTB domain ligands contain a β turn, while SH2 ligands bind in an extended conformation.

A recent report on two PTB domains in the neuronal proteins, X11 and FE65, suggests that their binding to a "NPXY" sequence in the amyloid precursor protein is independent of phosphotyrosine (*3*). Another study also suggests that the association of the PTB domain of *shc* with the tyrosine phosphatase PTP-PEST is independent of phosphotyrosine (*4*). Instead, the authors report that a sequence within PTP-PEST that is similar to the originally described "NPX(pY)" consensus sequence confers low-affinity binding to the PTB domain of *shc*. In this sequence, the tyrosine residue is replaced with histidine (^{599}NPLH^{602}). In order for high-affinity binding to take place, the authors of this report speculate that histidine residue 602 in PTP-PEST is phosphorylated. Peptide competition studies showed that the nonphosphorylated, histidine-containing peptide has an intermediate affinity for the PTB domain (between that of tyrosine- and phosphotyrosine-containing peptides). This finding and the fact that other PTB domains also may not require phosphotyrosine for high-affinity binding prompted us to investigate the effects of 3-phosphohistidine in place of phosphotyrosine in the "NPX(pY)" sequence motif on the binding to the PTB domain of *shc*.

Our results using peptide competition studies as well as direct binding studies clearly show that 3-phosphorylation of the histidine residue in the ^{599}NPLH^{602} sequence of PTP-PEST does not increase the binding affinity to a significant extent. Both the unphosphorylated and the 3-histidine-phosphorylated peptides show a greatly reduced binding affinity to the PTB domain of *shc* when compared to the tyrosine-phosphorylated peptide. Previous studies with "NXX(pY)"-containing erbB2-derived peptides had revealed that their binding to the PTB domain of *shc* is greatly reduced when phosphotyrosine is replaced with phosphoserine (*20*). This result also indicates that phosphorylation alone is not sufficient to confer high-affinity binding to the PTB domain of *shc*.

In other studies, we examined the effects of a 3-phosphohistidine in place of a phosphotyrosine in a consensus sequence motif for binding to an SH2 domain. The FGFR1-derived phosphotyrosine-containing peptide binds to the SH2 domain of PLC-γ1 while the same peptide containing 3-phosphohistidine showed no binding. This indicates that 3-phosphohistidine also cannot replace phosphotyrosine in this binding interaction.

Although we cannot exclude that other 3-phosphohistidine-containing sequences bind to PTB or SH2 domains with higher affinities than those in this study, we consider this unlikely. The overall dimensions and aromatic character of the imidazole ring of histidine are quite different from those of the phenol ring of tyrosine. We therefore propose that a 3-phosphohistidine does not interact as well as phosphotyrosine with the positively charged site on the PTB or SH2 domains that is created by adjacent arginine and lysine residues. The data in this study support that hypothesis.

Recent reports on the discovery of phosphohistidine in mammalian cells have led to speculations on their possible functions. It is tempting to assume that they can provide sites of interaction with other proteins through conserved structural motifs as do phosphotyrosines. A distinctive property of phosphohistidine that one has to consider is the lability of this modification as compared to phosphotyrosine or -serine and -threonine. Slight changes in the pH or other environmental factors can lead to a rapid hydrolysis and/or transfer of the phosphate to other amino acids. This intrinsic lability of phosphohistidine could be beneficial for very rapid alterations in cells in response to environmental changes.

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