Integrin $\beta_3$ Phosphorylation Dictates Its Complex with the Shc Phosphotyrosine-binding (PTB) Domain

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Adaptor protein Shc plays a key role in mitogen-activated protein kinase (MAPK) signaling pathway, which can be mediated through a number of different receptors including integrins. By specifically recognizing the tyrosine-phosphorylated integrin $\beta_3$, Shc has been shown to trigger integrin outside-in signaling, although the structural basis of this interaction remains nebulous. Here we present the detailed structural analysis of Shc phosphotyrosine-binding (PTB) domain in complex with the bi-phosphorylated $\beta_3$ integrin cytoplasmic tail (CT). We show that this complex is primarily defined by the phosphorylation state of the integrin C-terminal Tyr759, which fits neatly into the classical PTB pocket of Shc. In addition, we have identified a novel binding interface which concurrently accommodates phosphorylated Tyr747 of the highly conserved NPXY motif of $\beta_3$. The structure represents the first snapshot of an integrin cytoplasmic tail bound to a target for mediating the outside-in signaling. Detailed comparison with the known Shc PTB structure bound to a target TrkA peptide revealed some significant differences, which shed new light upon the PTB domain specificity.

Integrins, a major class of non-covalent heterodimeric, glycoprotein cell surface receptors, are among the most studied and best characterized cell adhesion molecules. Integrins mediate a plethora of cell-cell, cell-extracellular matrix (ECM), and cell-pathogen interactions and hence are responsible for controlling a wide array of biological processes including homeostasis, cell migration, differentiation, adhesion, immune response etc. The unique bidirectional flow of information through integrins involves inside-out signals, which allow them to interact with extracellular ligands (such as fibrinogen, von Willebrand factor, fibronecrtin) and ligand-dependent outside-in signals which adjust the cellular response to cell-cell adhesion (1). Although our understanding of the molecular details of inside-out integrin signaling (2, 3) has grown by leaps and bounds over the past decade, the early intracellular events following the integrin-mediated ECM engagement, outside-in signal transduction, still require further clarification. With respect to the outside-in signaling, the important unanswered questions center on selective recognition of proximal effectors by integrin cytoplasmic domains at different stages of cell spreading. Phosphorylation of the integrin tails is considered to be one of the spatiotemporal mechanisms for imparting such selectivity and, indeed, phosphorylation switches are thought to be a common principle of integrin regulation. Platelet integrin $\beta_3$ cytoplasmic tail (CT) is laden with various phosphorylation sites, including two tyrosines, one serine, and multiple threonines. However, only tyrosine phosphorylation is found to be specific for the outside-in signaling (4–8) and Shc (in particular its p52 isoform) was identified as a primary signaling partner for the tyrosine-phosphorylated $\beta_3$ CT (8).

Adaptor protein Shc (Src homology 2 domain) plays a key role in mitogen-activated protein kinase (MAPK) signaling pathway (9) and can be recruited through many different types of receptors, including integrins, growth factor, antigen, cytokine, G-protein-coupled, and hormone receptors (10). In the context of the present study, it is important to mention that Shc has also been coupled to the integrin controlled cell cycle progression (11). One of the three isoforms, the p52 Shc contains three distinct domains: phosphotyrosine-binding (PTB) domain, a poorly characterized glycine/proline-rich region termed as collagen homology domain (CH1), and the SH2 domain. Previous studies have shown that two of these domains, PTB and SH2, could potentially interact with $\beta_3$ CT containing phosphorylated tyrosines (12, 13). However, based on in vitro peptide affinity chromatography assays, Higashi et al. (14) proved that p52 Shc binds to the tyrosine-phosphorylated $\beta_3$ peptide through its PTB domain.

Overall, PTB domains comprise a large family of protein binding modules, which exhibit a conserved structural architecture similar to the pleckstrin homology (PH) domains (also termed as PH domain superfold) consisting of a core $\beta$-sandwich made of two anti-parallel $\beta$ sheets flanked by a C-terminal helix. In terms of the PTB domain ligand specificity, although...
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phosphorylated tyrosine is required for high affinity binding in case of proteins such as Shc PTB, IRS-1/IRS-2/IRS-3, Dok1, and SNT/FRS2, the PTB domains of Dab1/Dab2, ARH, Fe65, ICAPI/α, JIP-1/JIP-1b, Numb, Talin, and X11α exhibit similar or in some cases even higher affinity for non-phosphorylated peptides (15). For Shc PTB-integrin interaction, a bi-phosphorylated (pY747 and pY759) peptide has been shown to have greater binding affinity than a mono-phosphorylated (pY759) peptide (8). However, the exact structural basis underlying the second phosphorylusion site in a canonical PTB domain is not clearly understood.

Pathologically, Shc phosphorylation is linked to the stimulation of vascular endothelial growth factor (VEGF) production in tumors (16). Thus, deciphering the molecular details of this interaction may influence the development of new anti-cancer therapeutic strategies. Here we present the NMR-derived atomic view of how tyrosine phosphorylation affects interaction with Shc PTB, and we show for the first time a high resolution three-dimensional structure of Shc PTB domain in complex with bi-phosphorylated integrin β3CT peptide.

EXPERIMENTAL PROCEDURES

Expression and Purification—Cloning, expression, and purification of β3CT have been described previously (2). Tyrosine phosphorylation was achieved in vivo by expressing β3CT in TKB1 cell line from Stratagene. Details of this procedure and purification are described elsewhere.3 The Shc PTB domain (residues 17–207, see Fig. 1A) containing pET15b vector, generously provided by Dr. Zhou, was expressed in Rosetta (DE3) E. coli and have been modified to match changes in experimental conditions with the help of triple resonance NMR experiments, or in some cases even higher affinity for non-phosphorylated peptides (15). For Shc PTB-integrin interaction, a bi-phosphorylated (pY747 and pY759) peptide has been shown to have greater binding affinity than a mono-phosphorylated (pY759) peptide (8). However, the exact structural basis underlying the second phosphorylusion site in a canonical PTB domain is not clearly understood.

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Paramagnetic Labeling—To introduce a spin label, a new peptide, BP_3Cys, was chemically synthesized (NEO-peptides, Inc.) with an additional cysteine residue at the C terminus (Fig. 1B). A typical spin labeling reaction involved 50 mM Na_2HPO_4, 50 mM NaCl at pH 6.5, ~0.4 mM BP_3Cys, and ~5 mM cysteine-specific spin label, 3-maleimido-PROXYL (Sigma-Aldrich), hereafter referred to as mProxyl. The reaction was allowed to proceed for 1 h at room temperature. The unreacted mProxyl was then removed from mProxyl-BP_3Cys by using combination of gel-filtration chromatography on HiLoad 16/60 Superdex 75 column in 50 mM Na_2HPO_4, 50 mM NaCl at pH 6.5 and reverse phase HPLC on PROTO C4 column (The Nest Group, Inc.). 15N HSQC spectrum was collected on sample containing 0.18 mM spin-labeled peptide (mProxyl-BP_3Cys) mixed with 0.1 mM 15N Shc PTB in 50 mM Na_2HPO_4, 50 mM NaCl, 1 mM TCEP, 7% D_2O, 1 mM DSS buffer at pH 6.5 and 35 °C. An additional 15N HSQC spectrum on the 15N-labeled Shc PTB: Shc PTB domain (14) and phosphorylation of Tyr^{759} of β_3CT is essential to mediate this interaction as only the peptides containing pY^{759} have shown affinity toward GST-fused Shc (8). To further confirm and structurally characterize these findings, we have employed Nuclear Magnetic Resonance spectroscopy (NMR). To pinpoint the residues/regions involved in the Shc PTB-β_3CT interaction, we began with the chemical shift mapping experiments. Non-labeled Shc PTB domain was mixed with 15N-labeled non-phosphorylated β_3CT (hereafter referred to as β_3NP), Tyr^{747} mono-phosphorylated β_3CT (hereafter referred to as β_3MP) and Tyr^{747}Y^{759} bi-phosphorylated β_3CT (hereafter referred to as β_3BP) at the ratio 2:1 and the associated chemical shifts perturbations were monitored (expanded regions of superimposed HSQC spectra are shown in Fig. 2: (A) β_3NP; (B) β_3MP, and (C) β_3BP). As expected, Shc PTB addition had no effect on HSQC spectrum of β_3NP. In contrast, both β_3MP and β_3BP HSQC spectra show significant differential line-broadening and several peaks disappearance along with some small shifts in resonance frequencies upon addition of Shc PTB (with an exception of the very last C-terminal residue Thr^{762} of β_3BP, which demonstrates substantial chemical shift, Fig. 2C). This phenomenon is probably due to the intermediate exchange between free and bound states of β_3MP and β_3BP (28) combined with relatively large molecular weight of the complex (about 33 kDa complex versus 8 kDa for β_3CT alone). The ratio of the peak intensities along with chemical shifts perturbations for β_3NP and β_3BP residues plotted as a function of residue numbers is shown in Fig. 2, D and E, respectively. Combination of the differential line broadenings and chemical shift perturbations suggests the regions involved in interaction between phosphorylated β_3CT and Shc PTB, namely (i) residues from Asp^{740} to Ala^{750}, surrounding 744NPLpY^{747} motif, in case of β_3MP, and (ii) almost the entire C terminus, extending from Asp^{740} to Gly^{762} and encompassing both 744NPLpY^{747} and 756NITpY^{759} motifs plus the region connecting them in case of β_3BP. Because the affected region for β_3BP upon Shc PTB addition is much broader than the one for β_3MP, it can be argued that the binding site around 744NPLpY^{747} motif is a complimentary one and serves to stabilize β_3CT orientation within the complex which has been defined by the primary binding site around 756NITpY^{759} motif.

To confirm the hypothesis that 756NITpY^{759} motif occupies the canonical PTB site and to map the residues involved from Shc PTB side, we performed similar chemical shift mapping experiments. Non-labeled β_3NP, β_3MP, β_3BP solutions were mixed with 15N-labeled Shc PTB at the ratio 2:1. The superimposition of expanded regions of HSQC spectra for 15N-labeled Shc PTB mixed with β_3NP, MP, BP is depicted in Fig. 3A (Shc PTB alone is shown in black; with β_3NP, in blue; β_3MP, in red; and β_3BP, in lime; the superimposition of entire spectra is presented in supplemental Fig. S1A). As predicted, there were no changes in the 15N-labeled Shc PTB HSQC spectrum upon addition of the full-length non-labeled β_3NP. Addition of β_3MP to 15N-labeled Shc PTB leads to several small shifts in resonance frequencies as well as appearance of few additional (doublet) weak peaks probably representing a small population of protein in bound conformation. In contrast, there are signifi-
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FIGURE 2. Summary of 15N-labeled β3NP, MP, BP, and non-labeled Shc PTB interactions. The superimposition of 15N-1H HSQC spectra of 15N-labeled (A) β3NP (black) in the presence of Shc PTB (blue); (B) β3MP (red) in the presence of Shc PTB (blue); (C) β3BP (lime) in presence of Shc PTB (blue); important residues are labeled and marked with arrows; (D) normalized intensity ratios (I/I0) of the 15N-labeled β3MP, β3BP with and without non-labeled Shc PTB plotted as a function of residue number; (E) chemical shift changes of 15N-labeled β3MP, β3BP due to the addition of non-labeled Shc PTB. The column height for residue Thr762 in case of 15N-labeled β3BP + non-labeled Shc PTB (Delta [ppm] of 0.199 ppm) has been truncated for the sake of clarity. Bars are colored as follows: β3MP (dark gray) and β3BP (light gray). Delta [ppm] refers to the combined HN and N chemical shift changes, according to the equation: Δδ(HN,N) = [(ΔδHN)2 + (ΔδN)2]1/2, where Δδ = δbound − δfree. All the experiments were performed at 35 °C and at pH 6.1, in the buffer containing 50 mM NaH2PO4, 5 mM NaCl, 5 mM DTT, 7% D2O, and 1 mM DSS.

Significant changes in HSQC spectrum of Shc PTB upon addition of β3BP indicating substantial conformational rearrangement of Shc PTB, a hallmark of Shc PTB domain (17). However, low mutual solubility of Shc PTB and full-length β3CT constructs prevented us from conducting a full scale, thorough NMR structural investigation of Shc PTB-β3BP interaction.

To circumvent the solubility issue and to understand this interaction in atomic details, we have synthesized three phosphorylated tyrosines containing peptides representing different regions of the full-length β3CT, which are involved in interaction with Shc PTB (Fig. 1B). Similar chemical shift changes in HSQC spectrum of Shc PTB upon addition of β3BP, according to the equation:

\[ \Delta \delta (HN,N) = (\Delta \delta_{HN})^2 + (\Delta \delta_N)^2 \]

Even the greater conformational rearrangement is achieved via addition of BPβ3Peptide. Fig. 3C depicts these crucial differences in chemical shifts perturbations between MPCβ3 versus BPβ3Peptide plotted as a function of Shc PTB residue number, which provides us with the essential information about the possible binding site for the 744NPLpY747 motif. Explicitly, three Shc PTB regions are particularly different: (a) residues 78T–E80 of the helix α2, (b) residues Arg104 and Leu106 of the long loop connecting helix α2 and strand β2 and (c) residues 159D–V164 of the loop connecting strand β5–β6. Mapping these regions on the available Shc PTB structure (PDB ID: 1SHC) (12) suggests...
that accommodation of 744NPpY747 motif requires some movement of the helix α2 and the flexible loop connecting α2 to β3 strand.

To conclude, our titrations results are in good correlation with Cowan et al. (8) demonstrating that the phosphorylation of Tyr759 β3CT is essential to mediate direct integrin interaction with Shc PTB except that the NMR methods have allowed us to observe a weak interaction between Shc PTB and β3 peptides containing only pY747, which the biochemical assays could not detect.

Structural and Dynamic Characterization of the Complex—

Based upon our titration experiments, we could predict the possible mode of interaction of Shc PTB with BPβ3Peptide (27 residues, Fig. 1B): positively charged side-chain of Arg104 forming a salt-bridge with negatively charged phosphate group of pY747 whereas pY759 occupying the canonical PTB site. To fully characterize this interaction at atomic level, we have determined the three-dimensional solution structure of Shc PTB-BPβ3Peptide complex using modern triple resonance NMR methods (described in details under “Experimental Procedures”). Inter-molecular NOEs were paramount in defining the orientation of BPβ3Peptide within the complex, which was later independently confirmed by paramagnetic relaxation enhancement (PRE) experiments (see below). Table 1 summarizes the structural statistics for the final 15 water refined structures with lowest energies and Fig. 4, A and B depict the backbone superimposition and ribbon representation of these structures respectively (the secondary structural features are presented in supplemental Fig. S2).

As seen in case of Shc PTB-TrkA complex, the N-terminal region of Shc PTB (residues 17–35) are dynamically unstructured (12), whereas the core-structured region of Shc PTB, encompassing residues 38–201, adopts a well known PH domain superfold in complex with BPβ3Peptide: a seven-stranded β-sandwich composed of two anti-parallel β-sheets capped by a C-terminal α-helix (α3). Moreover, it contains two additional α-helices, N-terminal α1 and α2 connected to strands β1 and β2 respectively. Examination of this complex reveals the details of the binding sites for both pYs (Fig. 4C). As expected, residues 754FYNITpY759 (representing the classical consensus ψxNPxpY Shc PTB recognition motif with an exception of an isoleucine replacing proline residue) sits in the canonical PTB groove, an elongated cleft located between helix α3 and stand β3, with negatively charged phosphate group forming salt-bridges with positively charged side chains/amides of the Arg757, Arg177, Lys169, and Gln148. The residues 756STF754 of BPβ3Peptide adopt an anti-parallel β-strand conformation aligned to β5 strand of Shc PTB with hydrophobic amino acid, Phe754, maintaining the majority of α3-β5-α2 sheet.

FIGURE 3. Summary of 15N-labeled Shc PTB and non-labeled β3CT interactions. A, superimposition of 15N-1H HSQC spectra of 15N-labeled Shc PTB (black) in presence of non-labeled β3NP (blue); β3MP (red); β3BP (green); pH 6.1; (B) superimposition of HSQC spectra of 15N-labeled Shc-PTB (black) in presence of non-labeled MPNβ3 (red); MPCβ3 (light blue) and BPβ3Peptide (lime); pH 6.5; Some of the important residues undergoing conformational change are labeled and marked with arrows; (C) chemical shift differences between 15N-la- beled Shc-PTB + MPCβ3 versus 15N-labeled Shc-PTB + BPβ3Peptide. Delta [ppm] refers to the combined HN and N chemical shift changes, according to the equation: Δδ(HN,N) = ((ΔδHN2 + 0.2(ΔδN2))1/2, where Δδ = δexp - δcalc. All the experiments were performed at 35°C, in the buffer containing 50 mM Na2HPO4, 50 mM NaCl, 5 mM DTT, 7% D2O, and 1 mM DSS.
The BPβPeptide orientation was further confirmed and validated by introduction of the cysteine specific paramagnetic spin label, mProxyl, attached to the C terminus of modified peptide, BPβCys, through the formation of thioether bond and measuring the distance-dependent reduction in peaks intensities in 15N-labeled Shc PTB HSQC spectrum (29). These PRE studies independently confirmed the orientation of the phosphorylated integrin tail. Briefly, paramagnetic spin label facilitates nuclear relaxation in a distance-dependent manner (1/T1), causing significant line-broadening for nuclei in proximity (<15–20 Å) to the free radical. Analysis of the reduction in NMR peaks intensities allows mapping the direct location of the spin label with respect to the protein binding surface (30). This pattern of reductions in peaks intensities of Shc PTB HSQC spectra upon addition of mProxyl-BPβCys is depicted in Fig. 5D and the intensities ratios are mapped on the surface of the complex in Fig. 4D. The affected residues are shown in color gradient from orange to yellow (most affected: orange, least affected: yellow), and residues for which we have no data are shown in gray. The expanded region of superimposed HSQC spectra is shown in supplemental Fig. S4. It should be noted that the chemical shift perturbations in Shc PTB resonances are very similar/ almost identical upon addition of BPβPeptide or modified BPβCys at 1:2 ratio indicating that the inclusion of an additional cysteine residue at the C terminus did not alter the bound conformation and/or the affinity of binding. The specific pattern of the altered cross-peaks intensities, including the significant reduction in peak intensities (Ipara/Idia <0.3) of the 15N-H-150, 168-N-V172, 201-R-R2027 regions indicates that the C-terminal mProxyl tag is, indeed, positioned near the canonical binding site. This confirms the occupancy of the binding pocket by pY759 and eliminates any possibility for the distant pY747 to be found at the same place.

Further, to better understand the backbone conformational flexibility of Shc PTB in complex with β3 integrin, we have measured the relaxation parameters T1, T2, and heteronuclear T1, T2, and heteronuclear NOE parameters (T2, Ipara/Idia ) (24). The expanded region of superimposed HSQC spectra is shown in Fig. 5, A–C. The average value of T1 is about 710 ms; the average value of T2 is about 50 ms; and the average NOE value is about 0.66. For most of the residues, T1 and T2 values do not deviate significantly beyond the experimental error from the average numbers. However, several regions, including dynamically unstructured N- (residues 17–38) and C- (residues 202–207) termini plus a stretch of residues (91–100) within loop connecting helix α2 with strand β2 (89–111), demonstrate fast internal motion with increased T2 and reduced NOE values. The flexible nature of this loop with the most profound motion associated with the residues Arg97 and Arg99 allows crucial structural changes to ensure the formation of the second phosphotyrosine-binding pocket. The motion of 100KPCSRPLS107 region within this loop is restricted because of the interaction with β3. 744NPLpY747 motif.
one of the two NXXY motif containing regions recently reviewed in details (31). Overall, these interactions are tightly controlled with phosphorylation as one of the possible regulatory mechanisms. The phosphorylation state of the tyrosine residues within NXXY motifs of β3,CT can differentially regulate β3 interactions with PTB domain-containing proteins. Talin, for example, serves as a major activator for non-phosphorylated β3 (2, 32, 33), while Dok1 binds to pY747-β3 with higher affinity and, thus, replaces talin favoring the latent state of the receptor (34). Furthermore, Cowan et al. (8) have demonstrated that phosphorylation of the Tyr 759 is essential for the direct Shc binding, which mediates outside-in signaling events (35). Until now, however, the detailed structural basis of this interaction has remained elusive. The present study exposes the exact molecular mechanism underlying the Shc PTB-β3,CT complex formation and takes our understanding of the nature of this interaction to a new level.

The NMR data presented unambiguously proves that both phosphorylated tyrosine residues are involved in interaction with Shc PTB domain, although the vital role is performed by pY759. As depicted in Fig. 4, the BPβ3,Peptide wraps itself around Shc PTB with the major focal points presented by electrostatic interactions between negatively charged phosphate groups of pY759/pY747 and positively charged side chains of Shc PTB (residues Arg67, Arg175, Lys169, Gln148 in case of pY759 and Arg104 in case of pY747). As predicted, 756NIPy759 motif occupies the canonical PTB pocket with residues 525STF724 forming an anti-parallel β strand against the β5 strand (150ISFA153) of Shc PTB. This complex also illuminates an additional novel binding site for pY747 with the characteristic, perpetual type-I β turn of 744NPLpY747 motif fitting nicely into the groove formed between helix α2 and long flexible loop connecting the strand β2 and the helix α2.

A direct comparison between Shc PTB-BPβ3,Peptide complex and Shc PTB-TrkA complex (pdb id: 1SHC), depicted on Fig. 6A, reveals major similarities with some crucial differences depicted in Fig. 6A. Overall, the Ca atoms of the structured regions (residues 41–198) superimpose to the mean structure reasonably well with an R.M.S.D. of 2.26 Å ± 0.98Å. As presented in Fig. 6B, R.M.S.D. graph most of the β strands superimpose very well with an R.M.S.D. below 1 Å. The pY759 of β3, occupying a canonical PTB site, overlaps neatly with pY190 of TrkA with small shifts in placement accompanied by corresponding movement of the loops connecting strands β4/β5 and β5/β6 (Fig. 6C). However, the regions involved in the formation of the second, pY747, binding site show remarkable differences. The position of the loop connecting the helix α2 with the strand β2 is significantly different, reflecting the biggest fluctuation in the graph with maximum R.M.S.D. over 6 Å. Based upon our titration data and the previous biochemical assays (8), this novel phosphotyrosine-binding groove formed between the long loop and helix α2, is responsible for defining the precise arrangement of this large 27 residues bi-phosphorylated integrin constituent on Shc PTB surface, thereby increasing the binding affinity as compared with the small 11 residues, mono-phosphorylated (pY759) MPCβ3. So far the exact structural role of this unusually long loop (~24 residues), exclusively found in Shc PTB domain, has not been established except for residue Arg112, situated at the beginning of β2 strand, which has been implicated in phospholipid interaction along with residues Lys116 and Lys139 (36). As per our knowledge, this is the first time when residues 100KPCSRPLS107
of this elongated loop, commonly referred as Shc loop (37), are shown to be involved in direct interaction with a phosphorylated tyrosine residue. The actual biological significance of the proximity of these two binding sites, the phospholipid and the, pY747, phosphotyrosine-binding site, is yet to be understood. However, this comparison between Shc PTB-TrkA and Shc PTB-BPβ3-Peptide, along with the NMR relaxation data, proves the flexible nature of Shc PTB loop(s) manifesting the versatility found in PTB fold.

To further analyze the capability of PTB domains to accommodate different fragments of integrin tails we compared the known structure of integrin β3 (chimera, (34)) bound to talin (PDB ID: 1MK7) with Shc-β3 complex. Talin PTB (also known as an F3 variant of the canonical PTB) domain differs significantly from Shc PTB, it is only about half in size (~100 residues long versus ~200) with missing analogs for helices 1 and 2 and the long Shc loop and with absolutely no sequence homology even within the canonical binding pocket. However, in terms of structural architecture, the core seven-stranded β-sandwich together with C-terminal α-helix of talin PTB-β3 complex superimposes surprisingly well with Shc PTB-BPβ3-Peptide complex (see Fig. 6D), even though the residues defining the interaction from the β3 side are completely different. In both cases, two different NXXY motifs of β3 integrin form reverse turns which are further stabilized by contacts between N756–F198 for Shc and N744–T354/I356 for talin. An aromatic residue Trp 739 (at Y-8 position), as compared with the hydrophobic Ile485 of TrkA or Phe754 of β3 integrin in the complex with Shc (both found in canonical pY-5 positions), defines the antiparallel orientation of the ligand strand. Both these antiparallel strands, formed by residues 752STF754 in case of Shc and residues 739WDTA742 in case of talin, superimpose surprisingly well. However, the non-phosphorylated
PTB domains of 17 different proteins. In addition to $\beta_\alpha$CT, the cytoplasmic domains of integrin $\beta_{1\alpha}$, $\beta_3$, and $\beta_\gamma$ also demonstrated some affinities to several of these PTB domains, reflecting the intrinsic flexibility of both the PTB fold and $\beta$ integrins. Among the possible reasons for such indiscrimination is the exceptional conservation of NPXY and NXXY motifs within $\beta$ integrin tails (Fig. 6E), which along with other critical residues, coordinate integrin-PTB domains interactions. However, considering the specific nature of Shc PTB: $\beta_\alpha$CT interaction, we speculate that among all the integrin tails depicted in Fig. 6E, similar interaction with Shc PTB can be expected only in case of the tyrosine-phosphorylated $\beta_6$ (740NVTpY744) due to the presence of large hydrophobic residue, Pheme, at pY-5 position. This bulky, hydrophobic residue (corresponding to Phe754 in $\beta_\alpha$CT and Ile485 in TrkA, Fig. 6C) occupies the non-polar pocket formed between $\alpha_3$ helix and $\beta_5$ strand of Shc PTB. Furthermore, according to our Shc PTB-BP$\beta_3$Peptide complex, the placement of a large negative group (pT753) next to this pY-5 residue should cause the charge repulsion with the nearby Thr75 (from $\alpha_2$ helix of Shc PTB) along with steric hindrance with the above mentioned non-polar pocket. This is probably the most likely cause for the decreased affinity (40) of $\beta_\alpha$CT to Shc PTB observed upon Thr753 phosphorylation.

Overall, this presented comparison establishes two salient features: (i) proteins containing PTB fold can fine-tune their affinity toward their targets by an introduction of additional target-specific binding sites as the second phosphotyrosine-binding site defined in Shc; and (ii) integrin cytoplasmic tails are capable of accommodating different structural features depending upon the binding partner. This remarkable dexterity may be the underlying foundation for the crucial bidirectional flow of information through integrins. Although $\beta_3$CT interaction with Shc PTB is unique as compared with its interaction with talin or Dok1 PTB domains, a low sequence homology among PTB domains makes it very difficult to predict whether the other PTB domains will interact...
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with β integrin tails in a manner similar to Shc, talin, or Dok1. Indeed, such low sequence homology within the PTB domains simultaneously presents a challenge for the computational modeling and an opportunity for the comprehensive structural investigation.

To conclude, we have (i) confirmed the direct Shc PTB interaction with βi integrin cytoplasmic tail; (ii) demonstrated that this interaction depends strongly on the tyrosine(s) phosphorylation state of the receptor; (iii) structurally characterized Shc PTB in complex with bi-phosphorylated βs CT; and (iv) defined molecular details of the secondary non-canonical phosphotyrosine-binding site within the Shc PTB. Because Shc is involved in regulating the stimulation of VEGF production in tumor cells, our data help to understand how tyrosine phosphorylation of β integrin is linked to MAPK pathway and how it may play multiple roles in the regulation of integrin signal transduction.

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