An Alternative Phosphorylation Switch in Integrinβ2 (CD18) Tail for Dok1 Binding

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Integrins are involved in cell migration and adhesion. A large number of proteins interact with the cytoplasmic tails of integrins. Dok1 is a negative regulator of integrin activation and it binds to the phosphorylated membrane proximal NxxY motif in a number of integrin β tails. The β tail of the β2 integrins contains a non-phosphorylatable NxxF motif. Hence it is unclear how Dok1 associates with the β2 integrins. We showed in this study using NMR and cell based analyses that residues Ser745 and Ser756 in the integrin β2 tail, which are adjacent to the NxxF motif, are required for Dok1 interaction. NMR analyses detected significant chemical shift changes and higher affinity interactions between Dok1 phospho-tyrosine binding (PTB) domain and integrin β2 tail peptide containing pSer756 compared to pSer745. The phosphorylated β2 peptide occupies the canonical ligand binding pocket of Dok1 based on the docked structure of the β2 tail-Dok1 PTB complex. Taken together, our data suggest an alternate phosphorylation switch in β2 integrins that regulates Dok1 binding. This could be important for cells of the immune system and their functions.

Integrins are a large family of cell surface αβ heterodimers that mediate cell-cell and cell-ECM interactions necessary for many physiological processes, including hemostasis, wound healing, immunity and developmental biology. Each subunit of an integrin has a large extracellular region that binds to ligands, a single-pass transmembrane domain that transduces activation signal across the plasma membrane and a short cytoplasmic tail (except integrin β4) that binds to an expanding list of cytoplasmic proteins. Except β4 and β8, integrin β tails contain two highly conserved NxxY/F (x: other amino acid) motifs that are docking sites for cytoplasmic proteins. The membrane proximal NxxY/F motif is a binding site for talin, a well-established cytoskeletal protein that directly activates integrins. The two isoforms of talin in vertebrates (talin 1 and 2) are 4.1-ezrin-radixin-moesin (FERM)-containing proteins. The FERM domain lies in the head region of talin, and a phosphotyrosine binding (PTB) fold in its F3 subdomain has been shown to bind the membrane proximal NPLY747 motif of the integrin β3 tail. This form of interaction is not limited to talin because it extends to other cytoplasmic proteins containing PTB folds, including negative regulator of Notch signaling (Numb), downstream target of c-Abl (Dab) and docking protein 1 (Dok1; p62Dok)5.

Dok1 is a member of the Dok family of adaptor proteins and it is expressed in lymphoid and myeloid cells. Dok1 and Dok2 are negative regulators of immune cell signaling and Dok1 has been reported to bind with p120RasGAP, a negative regulator of the Ras-ERK pathway. All seven members of the Dok family of proteins contain an N-terminal pleckstrin homology (PH) domain, a central PTB fold and multiple SH2 and SH3 binding sites. Dok1 and talin have overlapping binding sites which include the NPLY747 motif in the integrin β3 tail. Therefore it is unlikely that both molecules can simultaneously bind the integrin β tail. Indeed biophysical analyses have shown that phosphorylated Y747 enhances Dok1 binding over that of talin, suggesting that NPLY747 is a phosphorylation switch. Unlike talin,
Dok1 does not activate integrins. Dok1 is a negative regulator of integrin activation by competing with talin for binding to integrin β1A, β3 and β7 tails containing the membrane proximal NxxY motif. The leukocyte-restricted β2 integrins comprise four members that have different α subunits but a common β2 (CD18) subunit, namely αL/β2, αM/β2, αX/β2 and αD/β2. The importance of the β2 integrins is underscored by the rare autosomal disease Leukocyte Adhesion Deficiency (LAD) I in which afflicted individuals have a compromised immune system because of defective adhesive and migratory properties of their leukocytes. The molecular basis of LAD I is the reduced expression and/or expression of dysfunctional β2 integrins in leukocytes as a result of mutation(s) in the ITGβ2 gene. Dok1 has been reported to bind the integrin β2 tail. However, the corresponding Dok1 binding region in β2 contains an NPLF754 motif that does not allow phosphorylation. This begs the question if there is an alternative phosphorylation site(s) in the region that regulates Dok1 binding. Residues Ser745 and Ser756 flank the NPLF754 motif in the integrin β2 tail. The corresponding Ser residues are absent from the β3 tails of β3, β5 and β6 integrins (Table 1). However, the β tails of β1A, β1D, and β7 integrins contain a Ser residue at an equivalent position to that of Ser 756 in the β2 tail. In vitro studies have shown that integrin β2 Ser745 and Ser756 are phosphorylatable but only the former is dependent on PKCs in T cells. These observations suggest an interesting possibility that the phosphorylation state of the integrin β2 tail could promote higher affinity interaction with Dok1 PTB (Table 2). Due to limited solubility of the full-length unphosphorylated β2 tail at high concentrations, 15N-1H HSQC spectra of Dok1 PTB domain were obtained using a fragment of the integrin β2 tail. The chemical shifts of the backbone resonances including 15N and 1H of the Dok1 PTB domain have been previously assigned.

Table 1. Comparison of amino acid sequences of integrin cytoplasmic tails highlighting the NxxY/F motif (blue) and potential phosphorylation of Ser residues (red).

| Name | Residue numbering | Sequence |
|------|------------------|----------|
| β1A  | 752-798          | KLLMIHHDRREFAKFEKMNAKWDTGEPNPYYKSATTTTVNPKYEYGK |
| β1D  | 752-801          | KLLMIHHDRREFAKFEKMNAKWDTQENPYKSPIINNFKNPYNVKGRAKGL |
| β2   | 724-769          | KALIHLSDLREYRRFEKKLKSQWNNDNPLFKSATTVVMPKAFEAS |
| β3   | 742-788          | KLLITIHDRKEFRAFEEKERARAKWDTANPNLYKEATSTFTNITYRGRT |
| β5   | 743-799          | KLLVTIHDRFREAFKQSLRSRYEMASNPLYRPKSTHTVDFTNKFNKSYNGTVD |
| β6   | 731-788          | KLLVSFHDREKEVFAERSKAXWQTGTNPNLYRGSTSTFKNVTYKHREKQKVDLSTD |
| β7   | 747-798          | RLSVEIYDREYRSFEKQQQQLNWKKQDSNPNLYKSATTTTNPRFQEDAPTL |

Results

Binding of the PTB domain of Dok1 with phosphorylated full-length integrin β2 tail peptides. Interactions between Dok1 PTB domain with full-length integrin β2 tail phosphorylated either at Ser745 (pSer745-β2 or Ser756 (pSer756-β2) were investigated by analyzing the changes in 15N-1H HSQC spectra of the PTB domain. In these experiments, a series of 15N-1H HSQC spectra of 15N-labelled PTB domain were acquired at various concentrations of integrin β2 tail peptides (Fig. 1A,B). 15N-1H HSQC spectra of the PTB domain showed discernable chemical shift changes upon addition of phosphorylated integrin β2 tail peptides. The chemical shifts of the backbone resonances including 15N and 1H of the Dok1 PTB domain have been previously assigned.

Chemical shift perturbations of a number of residues including Leu52, Arg54, Tyr56, Gly57, Val61, Phe65, Arg69 at the central region of the integrin β2 tail was determined from the combined chemical shift changes in the presence of increasing ligand concentrations with Kd values of 98 μM and 215 μM for pSer756-β2 and pSer745-β2 tail peptides, respectively (Fig. 1E,F). These data suggest that phosphorylation at Ser756 as compared to Ser745 of the integrin β2 tail promotes higher affinity interaction with Dok1 PTB (Table 2). Due to limited solubility of the full-length unphosphorylated β2 tail at high concentrations, 15N-1H HSQC spectra of Dok1 PTB domain were obtained using a fragment of the integrin β2 tail. The leukocyte-restricted β2 integrins comprise four members that have different α subunits but a common β2 (CD18) subunit, namely αL/β2, αM/β2, αX/β2 and αD/β2.
Figure 1. Dok1 binds to the integrin β2 tail upon phosphorylation of Ser745 and Ser756. (A and B) represent the 15N-1H HSQC spectra of the Dok1 PTB domain, showing chemical shift changes at 0 mM (red contour), 0.4 mM (cyan contour), 1 mM (violet contour) and 2 mM (orange contour) concentrations of pSer745 (A) and pSer756 β2 tail (B). (in inset) Chemical shift changes of residue Arg69 and Val61 of the PTB domain of Dok1 are shown. Bar diagrams showing combined chemical shift perturbation for 15N and HN resonances of each residue of the Dok1 PTB domain upon binding to pSer745-β2 (C) and pSer756-β2 (D). Note in (D) residues showing resonance broadening upon additions of phosphorylated β2 tail are marked as asterisks. The dotted lines in (C and D) marked average chemical shift perturbation. The secondary structural elements are shown at the top of each plot. Normalized chemical shift differences are plotted against the concentrations of pSer745-β2 (E) and pSer756-β2 (F) for Dok1 PTB domain to determine equilibrium dissociation constants (Kd) values.
Table 2. Dissociation constant ($K_d$) values determined for integrin $\beta_2$ tail and Dok1 interactions.

| $\beta_2$ tail peptides | $K_d$ (mM) |
|-------------------------|-----------|
| pSer745-β2              | 0.215 ± 0.02 |
| pSer756-β2              | 0.098 ± 0.02 |
| KT15                    | 16 ± 0.3 |
| pSer756-KT15            | 0.189 ± 0.03 |
| KT18EE                  | 8.49 ± 0.2 |

Conformations and interactions of phosphorylated integrin $\beta_2$ tail fragment with PTB domain. To gain further insight into phosphorylated integrin $\beta_2$ tail-Dok1 interactions, we made use of a pSer756-KT15 (KSQWNNDNPLFKSAT) peptide. $^{15}$N-$^1$H HSQC spectra of the Dok1 PTB domain were acquired at various concentrations of pSer756-KT15 peptide. Chemical shift perturbations were detected for residues of Dok1 PTB domain located at the canonical ligand binding site akin to that detected using the full-length pSer756-32 tail (Fig. 4A). The extent of chemical shift changes of Dok1 in the presence of unphosphorylated KT15 was also limited (Fig. 2B). An apparent $K_d$ value of 16 mM was determined for the interaction between Dok1 PTB domain and unphosphorylated KT15 (Fig. 2C, Table 2). Compared with pSer756-β2 and pSer745-β2 tails, the unphosphorylated integrin $\beta_2$ tail interacts weakly with the Dok1 PTB domain. Similar observations were made in interactions studies using unphosphorylated integrin $\beta_3$ tail with the Dok1 PTB domain. Chemical shift perturbations caused by pSer756-β2 tail were mapped onto the 3-D structure of Dok1 PTB domain (Fig. 3). Most of the binding residues are located in the C-terminal $\alpha$-helix and the $\beta$ strand-loop-$\beta$ strand-loop of the seven stranded $\beta$-sheet of the PTB domain (Fig. 3). These structural elements of Dok1 PTB domain are involved in binding pTyr peptide ligands.12,19.
discernible STD effect in free solution (Supplementary Fig. 2). STD experiments were also performed with unphosphorylated KT15 peptide with Dok1 PTB domain but there was apparently no or low STD effect, indicating a lack of binding with the Dok1 PTB domain (Supplementary Fig. 3).

Figure 2. Interactions of Dok1 with unphosphorylated β2 tail KT15 peptide. (A) Overlay of $^{15}$N-$^{1}$H HSQC spectra of the Dok1 PTB domain, showing chemical shift changes at protein:peptide concentration ratios 1:0 (red contour), 1:1 (cyan contour), 1:3 (violet contour) and 1:6 (orange contour). Two HSQC peaks corresponding to residue Arg54 at ~9.00 ppm and residue Tyr56 at ~7.9 ppm showed KT15 binding induced perturbation (arrows). (B) Normalized chemical shift differences for Dok1 PTB domain are plotted against the concentrations of KT15 peptide to determine the $K_d$ value. (C) Bar diagram showing combined chemical shift perturbations for $^{15}$N and HN resonances of each residue of the Dok1 PTB domain upon binding to KT15 peptide (protein:peptide concentration ratio 1:6).
Docking of pSer756-KT15 peptide with Dok1 PTB domain. We utilized the structural information of Nak peptide in complex with the PTB domain of Numb protein for modeling the backbone conformation of the pSer756-KT15 peptide (See Materials and Methods). The 11-residue Nak peptide adopts largely extended conformation when in complex with Numb PTB domain. The sequence of Nak peptide, GFxxxxxxFP, shows some similarity with pSer756-KT15 peptide in terms of the composition of residues and the residues that are involved in binding to the target protein. Two Phe in the Nak peptide, GFxxxxxFP, define a sequence pattern similar to WxxxxxF in the pSer756-KT15 peptide. Overall topologies of Dok1 PTB domain in complex with pSer756-KT15 (left panel), RET peptide (middle panel) and Numb PTB domain with the Nak peptide (right panel) are shown (Fig. 5). In these structures, PTB domain-peptide complexes are defined by extended conformations of the peptide ligands occupying the binding region comprising the C-terminal long helix and β5 and β6 strands. The peptides are also in close association with the surface of the PTB domains. In the docked structure of pSer756-KT15/Dok1 PTB domain, there are a number of potential molecular interactions that may stabilize the complex. The phosphate group of pSer756 is in close proximity to the side-chain guanidinium groups of residues Arg54, Arg69 and Arg70 of the PTB domain, suggesting salt-bridge and/or hydrogen bond interactions (Fig. 6A). These Arg residues are also involved in the recognition of the pTyr in RET and integrin β2 tail peptide12,19. Apart from these interactions, aromatic-aromatic stacking and cation-π contacts may be involved between Dok1 PTB domain Phe89 and Arg58 with Trp747 of the pSer756-KT15 peptide (Fig. 6B). Hydrophobic packing interactions may occur between residues Phe754 and Leu753 in the NPLF motif of pSer756-KT15 peptide with residue Ile96 of the Dok1 PTB domain. In addition, polar and ionic interactions appear to be sustained between residues Asn749/Glu93, Asp750/Arg55 of pSer756-KT15 peptide and Dok1 PTB domain (Fig. 6B).

Cell-based analyses of Dok1 binding to integrin β2 cytoplasmic tail. We examined the binding of Dok1 to integrin β2 cytoplasmic tail in cells using FRET assay. The myeloid leukemia K562 cells that do not express endogenous β2 integrins were transfected with expression plasmids of integrin αL and Dok1-CFP (CFP fused to the C-terminus of Dok-1) and integrin β2-YFP wild-type or S745G/S756G or S745E/S756E. Since the integrin β2 tail assumes extended conformation in complex with Dok1, we have chosen Ser to Gly mutations in the β2 tail. The technical control groups were cells transfected with the indicated plasmids but the Dok1-CFP was substituted with empty CFP plasmid. The expression levels of integrin β2-YFP constructs and Dok1-CFP were examined by immunoblotting (Fig. 7A). Transfected cells were subjected to YFP-photobleach FRET analyses. Significant FRET signal was detected in cells transfected with αL, Dok1-CFP and integrin β2-YFP wild-type compared with control group cells transfected with αL, CFP and integrin β2-YFP. Cells transfected with either integrin β2 S745G/S756G or S745E/S756E had significantly reduced FRET signal. These data suggest that Dok1 binds to the integrin β2 cytoplasmic tail and the association requires β2 Ser745 and/or Ser756. However, poor FRET signal was detected when integrin β2 Ser745 and Ser756 were both substituted with the acidic residue Glu to mimick phosphorylation. This is line with our NMR experiments that showed weak interactions.
(Kd ~ 8.49 mM) between β2 peptide fragment containing Ser to Glu substitutions with the Dok1 PTB domain (Table 2, Supplementary Fig. 4). Ser to Glu or Asp substitution has been commonly used to interrogate the properties of phosphorylated Ser but the inability of Asp/Glu to mimic phosphorylated Ser has also been reported 25. Hence, it is possible that the single carboxylate group on the side chain of Glu is insufficient to reconstitute the double negative charge and bulky phosphate moiety on phosphorylated Ser. We have attempted to detect the phosphorylation of integrin β2 cytoplasmic tail on Ser745 and Ser756 by immunoprecipitation of wild-type integrin αLβ2 from transfected K562 cells and the T cell line Jurkat followed by immunoblotting with either an anti-phospho Ser antibody or anti-integrin β2 phospho Ser745 obtained from commercial sources. However, we were unable to obtain definitive results possibly due to poor specificity and reactivity of these antibodies (data not shown).

Dok-1 binds integrin β cytoplasmic tail and it is a negative regulator of integrin activation 13,14. To date, however, there is a lack of cell-based evidence demonstrating Dok-1 dissociating from integrin β cytoplasmic tail in the presence of an activation signal. Hence we examined if chemokine-induced cell activation leads to the dissociation of Dok-1 from the integrin β2 cytoplasmic tail. The chemokine RANTES and its receptor CCR5 were used in our study because RANTES has been reported to stimulate leukocyte adhesion and migration via activation of integrin αLβ226,27. We generated stable K562 expressing Dok1-CFP by antibiotic selection. These cells were then transfected with expression plasmids of integrin αL and β2-YFP and CCR5. The expression levels of these proteins were verified by flow cytometry analyses (Fig. 7C). Cells were then treated without or with RANTES and FRET analyses

Figure 4. Analyses of interactions of pSer756 KT15 peptide with Dok1 PTB domain. (A) Bar diagram showing combined chemical shift perturbations for 15N and HN resonances of residues of the Dok1 PTB domain upon binding to pSer756 KT15 peptide. Residues showing resonance broadening are marked (asterisks). (B) Normalized chemical shift differences for Dok1 PTB domain are plotted against the concentrations of pSer756 KT15 peptide to determine the Kd value. (C) 31P NMR spectra of pSer756 KT15 peptide in free solution and in the presence of Dok1 PTB domain at different peptide:protein molar ratios (1:0 (red), 1:0.5 (blue), 1:1 (cyan) and 1:3 (green)). (D) Saturation transfer difference (STD) NMR spectrum of pSer756-KT15 peptide in the presence of Dok1 PTB domain. The off-resonance or the reference spectrum is shown (top).
performed on these cells (Fig. 7D). FRET signal was significantly lower in cells treated with RANTES compared with untreated cells. These data suggest that Dok-1 dissociates from integrin β2 cytoplasmic tail in the presence of RANTES/CCR5 activation signal.

**Discussion**

A large number of cytosolic proteins, including talins, kindlins, filamins, Dok1, 14-3-3, are known to interact with the β tail of integrins and they regulate the ligand-binding activity of these integrins. Interactions with cognate proteins can be modulated by phosphorylation of the integrin β tail at specific sequence motifs. The membrane proximal NxxY motif found in the β tail of a number of integrins interacts with the talin head domain. IgFLN domains of filamin and PTB domain of Dok1.

Phosphorylation of Tyr in the NPxY motif enables binding of the Dok1 PTB domain with Ile96 of the PTB domain are observed. In addition, there can be polar and ionic interactions: Asp749 (K15) with Glu93 (Dok1) and Asp750 (K15) with Arg55 (Dok1).
This led us to hypothesize that an alternate phosphorylation switch involving Ser745 and Ser756 regulates the interaction of Dok1 PTB with the integrin β2 tail. Previous studies have demonstrated the functional consequences of Ser745 and Ser756 phosphorylation in integrin β2 tail. However, there has been no in depth study to our knowledge demonstrating an integrin β2 tail binding protein that recognizes these phosphorylated serines. This study demonstrates that the PTB domain of Dok1 binds to integrin β2 tail, which is dependent on the phosphorylation state of Ser745 and Ser756. NMR experiments using phosphorylated full-length integrin β2 tail peptides showed that the affinity of Dok1 PTB was higher with integrin β2 tail having phosphorylated Ser756 compared with phosphorylated Ser745. It is noteworthy that the integrin β1A, β1D and β7 tails also contain a potential

Figure 7. Analyses of the interaction between integrin β2 tail and Dok1 in cells. (A) K562 cells transfected with the indicated expression plasmids were lysed. Proteins were resolved on 10% SDS-PAGE under reducing conditions and immunoblotted (IB) with either anti-GFP or anti-Dok1 antibodies. (B) Transfected K562 cells were subjected to YFP-photobleach FRET analyses. Each data point represents the mean ± S.D. of ≥30 cells analyzed. (C) Flow cytometry analyses of K562 stable line expressing Dok1-CFP that were transfected with integrin αLβ2-YFP and CCR5. Wild-type K562 cells were used as the control group. (D) FRET analyses of cells in (C) that were treated without or with chemokine RANTES (50 ng/ml) for 10 min at 37°C. 60 and 39 cells were analyzed for conditions without and with RANTES treatment, respectively. Data point represent mean ± S.D. *p < 0.05, Student’s t test.
phosphorylatable Ser residue at the equivalent position of Ser756 in the β2 tail (Table 1). pSer756-β2 tail occupies the canonical binding pocket of Tyr phosphorylated peptides at the C-terminal long helix and β5-β6 strands of the PTB domain. The docked structure of Dok1 PTB with the integrin β2 pSer756-KT15 peptide provides information on their relative orientation and the molecular interactions involved. In this model, the phosphate group of pSer756-KT15 is found in a close proximity to the side chains of residues Arg54 and Arg69 of the PTB domain that allows the formation of ionic interactions and/or hydrogen bonds. Indeed 31P NMR spectra of pSer756-KT15 peptide showed significant chemical shift changes when in complex with the Dok1 PTB domain (Fig. 4C) and 2H NMR HSQC spectra of the PTB domain showed high chemical shift perturbations of these corresponding Arg residues upon addition of the phosphorylated integrin β2 tail peptide (Fig. 1D). The docked structure of the phosphorylated integrin β2 peptide/PTB domain suggests packing interactions between residues Leu731 and Phe732 in the NPLF motif and Trp725 of the β2 tail with residue Ile96 and residues Arg58 and Phe89 of the PTB domain, respectively (Fig. 6). STD-NMR studies demonstrated that the aromatic ring of residues Trp725 and Phe732, and the aliphatic side chains of residues Leu731 and Thr758 are in close proximity to the PTB domain of Dok1 (Fig. 4D). Alongside, Ile96, Arg58 and Phe89 of the PTB domain demarcated chemical shift perturbations when in complex with the phosphorylated β2 tail (Fig. 1D). The binding affinity of the phosphorylated integrin β2 tail with the PTB domain of Dok1 is rather low. We hypothesize that in a physiological system, this binding affinity can be enhanced by their membrane localization. Indeed, the PH domain of Dok1 protein has been shown to be important for Dok1 targeting to the membrane35. Interactions between integrin tails and their binding partners are found to be of higher affinity when the integrin tails are anchored in lipids36,37. It has also been reported that membrane interactions stabilize the binding of the head domain of talin to integrin tails38,39. Although we have not examined the interaction of di-phosphorylated (pSer745 and pSer756) integrin β2 tail peptide with Dok1 PTB domain in this study, we conjecture that the binding affinity may be further enhanced when both Ser are phosphorylated. This will be investigated in future work. In a cellular system, the simultaneous phosphorylation of both Ser745 and Ser756 could have a synergistic effect on the recruitment of Dok1 to the integrin β2 tail. In this regard, phosphorylation of Ser745 or Ser756 or both under different conditions may allow phosphorylation switch in the integrin β2 tail that regulates its interaction with Dok1. These findings suggest that minor sequence variations amongst the integrin β2 sequence may influence many aspects of integrin activation and its downstream signaling. In this regard, the phosphorylation of integrin β2 tail in cells because antibodies of high specificity and reactivity are unavailable to us. However, others have shown that these residues can be phosphorylated37,38.

An increasing number of cytosolic proteins have been identified to bind integrin tails. These interactions are regulated by many factors including the phosphorylation of integrin β tails. Given that there are sequence variations amongst the integrin β tails, there can be slight but important differences in which phosphorylation regulates these interactions. In this study, we have identified and characterized an alternate phosphorylation switch in the integrin β2 tail that regulates its interaction with Dok1. These findings will be important for future studies investigating the underlying mechanisms of β2 integrin-mediated immune responses.

Materials and Methods

Plasmids. Full-length Dok1 cDNA from a human leukocyte cDNA library (Clontech Laboratories, Mountain View, CA) was PCR amplified using relevant primers and cloned into the plasmid pcDNA3.1(+) (Invitrogen, Carlsbad, CA). Dok1 was PCR sub-cloned into the pECFP-N1 expression plasmid (Clontech) for FRET experiments. The pcDNA3 expression plasmids containing human integrin αL or integrin β2-YFP (YFP fused to the C-terminal of β2 cytoplasmic tail) have been reported previously40–42. The integrin β2-YFP S745G/S756G expression construct was generated by mutagenesis using the Quikchange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) and relevant primers. The human CCR5 pcDNA3 expression plasmid was a kind gift from Dr. R.W. Doms (MD) (University of Pennsylvania, PA).

Cell culture, transfection and fluorescence resonance energy transfer (FRET) experiments. K562 cells (ATCC, Manassas, VA) were cultured in RPMI1640 full-medium containing 10% (v/v) FBS and antibiotics. Cells (2 × 10⁶) were transfected with integrin αL plasmid (9μg), integrin β2-YFP plasmid (WT or mutant) (9μg) and Dok1-CFP or CFP plasmid (12μg) by electroporation using the Neon electroporation kit (Invitrogen) on a MP-100 pipette-type microporator (Invitrogen). YFP-photobleach FRET assay was performed essentially as described40. Immunoblottings of transfected K562 lysates were performed using rabbit anti-GFP antibody (Life Technologies, Grand Island, NY), rabbit anti-Dok1 antibody (Abcam, Cambridge, MA) and HRP-conjugated goat anti-rabbit (Avastan, Menlo Park, CA) followed by ECL detection. To generate K562 cells stably expressing Dok1-CFP, cells were transfected with Dok1-CFP plasmid by electroporation followed by serial dilutions into 96-well cell culture microtiter plates to select for stable clones in the presence of antibiotic G418 (0.6 mg/ml). Clones expressing Dok1-CFP were screened by flow cytometry analyses and verified by immunoblotting with anti-GFP antibody (Life Technologies, Grand Island, NY). Stable K562 cells expressing Dok1-CFP
transfected with integrin α2L, integrin β2-YFP and CCR5 plasmids (12 μg each) were stained with either 1 μg each of mAb M254 (anti-α2L) or mAb 2D7 (anti-CCR5, BD Biosciences, San Jose, CA) followed by APC-conjugated secondary antibody (goat anti-mouse IgG, BD Biosciences, 1:500 dilution). Samples were acquired and examined on a FACSCalibur flow cytometer and data analyzed using the Flowjo software (Tree Star Inc. Ashland, OR). For RANTES activation, transfected K562 cells were incubated in culture medium containing RANTES (50 ng/ml) (Calbiochem, Merk Millipore) for 10 min at 37°C before performing FRET analyses.

**Protein expression and purification.** Human Dok1 PTB domain (Q154 – G256; Swiss-Prot Q99704, number as Q1-G103) was sub-cloned into the pET14b vector with an N-terminal six His-tag. *Escherichia coli* Rosetta cells were transformed with the expression plasmid and grown at 37°C either in LB or M9 medium containing [15N] ammonium chloride (Cambridge Isotope Laboratories). Expression of recombinant protein was induced by adding 1 mM IPTG to cells (OD600 0.6–0.7) followed by incubation for 6–12 h at 16°C. Cells were centrifuged and re-suspended in Buffer A (20 mM Tris-HCl buffer, pH 8.0) followed by affinity purification on a Nickel-NTA column (Qiagen). His-tagged Dok1 PTB domain was eluted in Buffer A containing 500 mM imidazole and dialyzed against Buffer B (50 mM sodium phosphate buffer (pH 6.0) containing 100 mM NaCl and 2 mM DTT). Protein samples were further purified by gel filtration in Buffer B on a HiLoad Superdex 75 16/26 GL preparative column that was connected to an AKTA FPLC UPC-900 system (GE Healthcare UK Ltd., England). Proteins were eluted at a flow rate of 0.5 ml/min and monitored by absorbance at λ = 276 nm.

**Synthetic peptides.** Synthetic peptides (purity >95%) of full-length β2 tail, phosphorylated full-length β2 (pSer756−β2 and pSer756/32), phosphorylated peptide fragment of β2 (pSer756−KT15), unphosphorylated KT15, and KT18EE (Table 2) were purchased from GLBioChem®, Shanghai, China.

**NMR spectroscopy.** All NMR experiments were performed on a Bruker DRX 600 MHz spectrometer equipped with an actively shielded cryoprobe. NMR data were processed using the NMRPipe and NMRRDraw suite and analysed by Sparky (T.D. Goddard and D.G. Kneller, University of California, San Francisco). The chemical shifts were directly or indirectly referenced to DSS. A series of [15N]-1H HSQC spectra of 15N-labelled Dok1 PTB domain, at 0.7 mM concentration, were acquired in the presence of different concentrations of unphosphorylated KT15, pSer745-32, pSer756-32 and pS756-KT15. The stock solutions of the above mentioned peptides were prepared in the same buffer (100 mM NaCl, 2 mM DTT). Protein samples were further purified by gel filtration in Buffer B on a HiloLoad Superdex 75 16/26 GL preparative column that was connected to an AKTA FPLC UPC-900 system (GE Healthcare UK Ltd., England). Proteins were eluted at a flow rate of 0.5 ml/min and monitored by absorbance at λ = 276 nm.

**31P NMR experiments.** 31P NMR spectra of pS756-KT15 were recorded on a Bruker DRX 400 spectrometer at 298 K. Data acquisition and processing were performed with the Topspin software (BRUKER) suite. 1-D 31P NMR spectra of pS756-KT15 at 0.2 mM in water (pH 6.2) were recorded in the presence of 100 μM DAG (dihydroxyacetone phosphate) and 50 μM DAD (dihydroxyacetone).
of different concentrations of Dok1 PTB domain. The Dok1 PTB stock solution was used in unbuffered water (pH 6.2). The pH of each sample was adjusted to 6.2 after addition of protein solutions.

**Docking of Dok1 PTB domain with pS756-KT15 peptide.** Based on the conformation of NAK peptide (pdb accession code, 1DMF.pdb), a structural model of pS756-KT15 peptide was built using INSIGHTII (Accelrys Inc.) software. The model structure of pS756KT15 was energy minimized by conjugate gradient energy minimization protocol. The model of pS756/KT15/Dok1 complex was derived using HADDOCK program (http://haddock.science.uu.nl/) based on chemical shift changes of the PTB domain. The model structure was validated using ADIT Validation Server from the Protein Data Bank (PDB) and coordinates of the complex are provided in supporting materials.

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**Author Contributions**
S.G., J.C.-Y.C., C.F., S.-M.T. and S.B. conceived and designed the experiments. S.G., J.C.-Y.C., C.F. and A.B. carried out experiments. S.G., J.C.-Y.C., C.F., A.B., S.-M.T. and S.B. analyzed the data. S.G., A.B., S.-M.T. and S.B. wrote the paper.

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