Published in final edited form as:

ACS Chem Biol. 2017 September 15; 12(9): 2313–2323. doi:10.1021/acscchembio.7b00361.

Site-Specific Phosphorylation of PSD-95 PDZ Domains Reveals Fine-Tuned Regulation of Protein—Protein Interactions

Søren W. Pedersen[†], Louise Albertsen[†], Griffin E. Moran[†], Brieé Levesque[‡], Stine B. Pedersen[†], Lina Bartels[†], Hannah Wapenaar[†], Fei Ye^{§,||}, Mingjie Zhang^{§,||}, Mark E. Bowen[‡], and Kristian Strømgaard^{*,†}

[†]Center for Biopharmaceuticals, Department of Drug Design and Pharmacology, University of Copenhagen, Universitetsparken 2, DK-2100 Copenhagen, Denmark

[‡]Department of Physiology & Biophysics, Stony Brook University, Stony Brook, New York 11794, United States

[§]Division of Life Science, State Key Laboratory of Molecular Neuroscience, Hong Kong University of Science and Technology, Hong Kong, China

^{||}Center of Systems Biology and Human Health, School of Science and Institute for Advanced Study, Hong Kong University of Science and Technology, Hong Kong, China

Abstract

The postsynaptic density protein of 95 kDa (PSD-95) is a key scaffolding protein that controls signaling at synapses in the brain through interactions of its PDZ domains with the C-termini of receptors, ion channels, and enzymes. PSD-95 is highly regulated by phosphorylation. To explore the effect of phosphorylation on PSD-95, we used semisynthetic strategies to introduce phosphorylated amino acids at four positions within the PDZ domains and examined the effects on interactions with a large set of binding partners. We observed complex effects on affinity. Most notably, phosphorylation at Y397 induced a significant increase in affinity for stargazin, as confirmed by NMR and single molecule FRET. Additionally, we compared the effects of phosphorylation to phosphomimetic mutations, which revealed that phosphomimetics are ineffective substitutes for tyrosine phosphorylation. Our strategy to generate site-specifically phosphorylated PDZ domains provides a detailed understanding of the role of phosphorylation in the regulation of PSD-95 interactions.

*Corresponding Author Phone: +45 5123 6114. kristian.stromgaard@sund.ku.dk.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscchembio.7b00361.

Experimental procedures; (Figure S1) putative phosphorylation sites of PDZ domains of the disc large homologue family; (Figure S2) circular dichroism spectra of nondenatured (0 M urea) and denatured (>7.5 M urea, dashed line) PDZ1, PDZ2, and PDZ3; (Figure S3) equilibrium denaturation curves for PDZ1, PDZ2, and PDZ3 recorded at 222 nm; (Figure S4) saturation curves for PSD-95 PDZ1 and C-terminals; (Figure S5) saturation curves for PSD-95 PDZ2 and C-terminals; (Figure S6) saturation curves for PSD-95 PDZ3 and C-terminals; (Figure S7) saturation curves for noncanonical interaction between nNOS β -finger and PSD-95 PDZ1 and PDZ2; (Figure S8) Arg-to-Ala mutations of the stargazin C-terminal peptide; (Figure S9) inhibition values and curves for PSD-95 pY397-PDZ3:TARPs; (Table S1) characterization of synthetic peptides; (Table S2) characterization of recombinant PDZ domains; (Table S3) characterization of semisynthetic PDZ domains; (Table S4) stability of PDZ domains; (Table S5) K_D values (μ M) for PDZ domains; (Table S6) fold change induced by mutation relative to nonphosphorylated PDZ domains; (Table S7) kinetic rate constants for PDZ3 binding to stargazin; and references (PDF)

The authors declare no competing financial interest.

Abstract

Postsynaptic density protein of 95 kDa (PSD-95) is the most abundant scaffold protein in the postsynaptic density (PSD) of excitatory neurons. PSD-95 interacts with transmembrane proteins such as receptors and ion channels as well as cytosolic enzymes. By organizing these signaling proteins into complexes, PSD-95 plays a key role in synaptic transmission.^{1–3} PSD-95 is a member of the membrane associated guanylate kinase (MAGUK) protein family^{4,5} and contains three PSD-95/Discs-large/Zona occludens (PDZ) domains along with an SH3 and a guanylate kinase (GK) domain (Figure 1a). Many of the regulatory functions of PSD-95 are attributed to the three PDZ domains.⁶

PDZ domains are globular protein domains of approximately 90 amino acids^{7,8} and represent one of the largest classes of protein-interaction modules in the human proteome, with around 265 examples found in more than 150 proteins. They are often found in multidomain scaffolding and anchoring proteins and are linked to cellular processes such as cell polarity and migration, protein trafficking, and neuronal signal transduction.^{9,10} The canonical PDZ-binding mode involves the interaction with the C-terminus of the protein binding partners,⁷ but some PDZ domains also bind internal peptide motifs¹¹ or even phospholipids¹² through noncanonical interactions.

The three PDZ domains of PSD-95 bind to the C-termini of a broad repertoire of partners including the *N*-methyl-D-aspartate (NMDA) sensitive glutamate receptor,¹³ stargazin¹⁴ and other transmembrane α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor regulating proteins (TARPs),¹⁵ G protein-coupled frizzled receptors,¹⁶ and protein kinase C (PKC).¹⁷ In addition, PSD-95 binds neuronal nitric oxide synthase (nNOS) through a noncanonical interaction.^{18,19} Importantly, modulation of the PDZ-mediated interactions of PSD-95 has recently attracted great interest as a novel drug target in the treatment of ischemic stroke, where inhibitors of the PDZ domains are currently in both preclinical and clinical development.^{20–22}

Synaptic activity is highly regulated by the phosphorylation of PSD-95. Multiple phosphorylation sites within PSD-95 were identified in mass spectrometry studies²³ and additional sites are predicted by computational tools (Figure 1b).^{24,25} Several of these phosphorylation sites are found within the PDZ domains and may be of particular importance for regulation of PSD-95 interactions. In addition, sequence alignment of PDZ domains within the disc large (Dlg) family members most closely related to PSD-95 (Figure S1) indicates that several Ser, Thr, and Tyr residues are conserved, which could suggest evolutionary conserved phosphorylation sites.²⁶

Phosphorylation of PSD-95 has been shown to affect PDZ interactions. Phosphorylation of Y397 in PDZ3 by the Src tyrosine kinase has been shown to decrease the interaction with the C-terminus of cysteine-rich PDZ-binding protein (CRIPT).^{27,28} Similarly, phosphorylation of S73 in PDZ1 by Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) was reported to selectively abolish binding to the GluN2A subunit of the NMDA receptor, without affecting the interaction with the GluN2B subunit.²⁹ Finally, phosphorylation of Y236 and Y240 in PDZ2 has been found in proteomic studies,²³ but the kinase(s) responsible is unknown. Immunoblotting has shown that these sites are phosphorylated in mice under ischemic conditions, but their physiological effects remain enigmatic. Generally, little quantitative information is known regarding how phosphorylation of the PDZ domains in PSD-95 modulates interactions with its binding partners. Thus, it is unclear how phosphorylation may affect the functionality of PSD-95 in neuronal signal transduction.

Studying the effects of phosphorylation is most commonly achieved using recombinant kinases or by introducing phosphomimetic mutations, such as Asp or Glu, through sitedirected mutagenesis.³⁰ The negatively charged Asp or Glu residues are chosen to mimic the negative charge of the phosphate. However, from a chemical perspective there are substantial differences between a carboxylic acid and a phosphate group, raising questions as to how well such mutations mimic phosphorylation. Moreover, in the case of a phosphotyrosine (pTyr), Asp and Glu residues lack the aromatic moiety, calling into question their ability to act as mimetics of pTyr. Because phosphomimetics are often the only option, concerns about their general effectiveness are often left unaddressed.

To circumvent the problems associated with producing phosphorylated proteins, we employed expressed protein ligation (EPL).³¹ Here, we used the technology to generate site-specifically phosphorylated PDZ domains of PSD-95, which are necessary to explore the effects of phosphorylation on ligand specificity and selectivity. We selected four physiologically important phosphorylation sites (S73, Y236, Y240, and Y397), as outlined above, and introduced the phosphorylated amino acids site-specifically using two different semisynthetic strategies. This involved synthesis of either the C-terminal (pY236, pY240, and pY397) or N-terminal (pS73) phosphorylated fragments followed by EPL. In parallel, we expressed wild type PDZ domains and their corresponding phosphomimetic mutants (S73D, Y236E, Y240E, and Y397E), which we compared to the phosphorylated proteins. We found that phosphorylation induced modulatory effects, which fine-tuned the binding of PSD-95 to C-terminal peptide ligands. Interestingly, the interaction between PDZ3 and stargazin showed a large affinity increase upon phosphorylation, so we explored this interaction in more detail by NMR titration studies and single molecule binding studies.

Importantly, this effect was not observed with the Y397E phosphomimetic mutation. In general, we found some correlation between the effect of phosphorylated residues and phosphomimetics, but there was substantial variation with a number of important outliers.

RESULTS AND DISCUSSION

Generation of Site-Specifically Phosphorylated PDZ Domains of PSD-95.

Generation of site-specifically phosphorylated proteins can be achieved by treatment with a specific kinase. This requires that the kinase is known and that the kinase-mediated phosphorylation is selective and efficient. Phosphorylated PDZ domains have previously been generated using recombinant kinases, as exemplified by generation of PSD-95 pY397-PDZ3 using Src kinase.²⁷ The reaction was neither efficient nor selective, but pure phosphorylated protein was obtained. To avoid these problems, we therefore generated site-specifically phosphorylated PDZ domains of PSD-95 by expressed protein ligation, which is an excellent tool for elucidating the effect of post-translational modifications (PTMs). We generated the three PDZ domains from PSD-95 carrying either one or two phosphorylated residues. The phosphorylation sites S73 and Y397 are targets of CaMKII³² and Src kinases,^{23,27} respectively. The putative kinase(s) responsible for phosphorylating Y236 and Y240 have not been identified; however, the sites are predicted to be targets of Src.

Introduction of the four phosphorylated residues, pS73, pY236, pY240, and pY397, into the three PDZ domains of PSD-95 required two different semisynthetic routes, as pS73 is located in the N-terminal portion of PDZ1, while pY236 and pY240 as well as pY397 are in the C-terminal portion of PDZ2 and PDZ3, respectively (Figure 2). In all cases, the phosphorylated PDZ domains were obtained by a ligation of a synthetic peptide fragment containing the phosphorylated residue and a recombinantly expressed protein fragment, which were subsequently refolded to generate the functional PDZ domain. For pS73-PDZ1, a synthetic N-terminal fragment was generated by solid-phase peptide synthesis (SPPS) containing pS73 and a C-terminal hydrazide (Figure 2a and Table S1). The remaining C-terminal part of PDZ1 was recombinantly expressed in *E. coli* as a polyhistidine fusion protein containing a factor Xa (FXa) recognition site (IEGR), which after treatment with FXa generated the protein fragment with an N-terminal Cys residue (Table S2). With the two fragments in hand, the ligation reaction was initiated by oxidation of the peptide C-terminal hydrazide, converting it into a peptide thioester derivative by *in situ* thiolysis. Subsequent reaction with the N-terminal Cys residue of the recombinant protein fragment generating the desired pS73-PDZ1 domain (Table S3). The generation of phosphorylated PDZ2 (pY236-PDZ2, pY240-PDZ2, and pY236-pY240-PDZ2) and PDZ3 (pY397-PDZ3) required generation of a synthetic C-terminal phosphopeptide fragment and recombinant N-terminal protein fragment (Figure 2b). The synthetic peptide fragments containing the pTyr residues were prepared by SPPS with all peptides containing an N-terminal Cys residue (Table S1). The corresponding protein fragment was expressed as a fusion protein containing an N-terminal *Mycobacterium xenopi* Gyrase A (*Mxe GyrA*) intein and a polyhistidine tag, which provided the N-terminal protein fragment with a C-terminal thioester after thiolysis (Table S2). The synthetic and recombinant fragments for the four phosphorylated PDZ domains were subsequently combined in a ligation reaction, which was followed by desulfurization in

a radical mediated reduction. This converted the generated Cys residue into the native Ala residue (Table S3). Finally, all five phosphorylated PDZ domains were exchanged into binding buffer by diafiltration (Figure 2c and d).

Protein Folding and Stability of Phosphorylated PDZ Domains.

To confirm that the phosphorylated PDZ domains were correctly folded, we assessed their secondary structures with circular dichroism (CD). We recorded the far-UV spectrum (190–250 nm) for each of the phosphorylated PDZ domains along with the recombinantly expressed wildtype (WT) PDZ1, PDZ2, and PDZ3 (Figure S2). Gratifyingly, the CD spectra of the semisynthetic phosphorylated PDZ domains demonstrated an overall resemblance to the spectra for the WT PDZ domains with an ellipticity minimum between 208 and 212 nm.

We also compared the thermodynamic stability of the five phosphorylated PDZ domains to their corresponding WT PDZ domains by measuring their CD spectra as a function of increasing urea concentration (Figure S3). This revealed that phosphorylation caused different degrees of destabilization to the PDZ domain fold (Table S4). For the pS73-PDZ1 domain, the stability decreased with a $\Delta\Delta G_{D-N}^{H_2O}$ of 0.31 kcal mol⁻¹ compared to nonphosphorylated PDZ1. Similarly, singly phosphorylated pY236-PDZ2 and pY240-PDZ2 were destabilized by 0.30 and 0.22 kcal mol⁻¹, respectively. The doubly phosphorylated pY236-pY240-PDZ2 showed an additive reduction of stability by 0.56 kcal mol⁻¹. Interestingly, the pY397 PDZ3 domain showed a much more dramatic effect with a 1.71 kcal mol⁻¹ reduction in stability compared to WT PDZ3.

Overall, we found that phosphorylation destabilized the PDZ domains. This is in contrast to a previous report of a phosphorylated Erbin PDZ domain.³³ However, the Erbin PDZ domain contained an artificially designed phosphorylation site, which might not be representative of endogenously phosphorylated PDZ domains. It is worth noting that both reported and predicted phosphorylation sites in the PDZ domains of the Dlg family are primarily found in secondary structural elements rather than in loop regions (Figure S1). This includes Y236 and Y240 in the $\beta 5$ region of PDZ2 and Y397 found in the $\alpha 3$ helix of PDZ3 (Figure 2c). A reduction in stability supports previous studies demonstrating the importance of the $\alpha 3$ helix for the PDZ3 domain fold.^{28,34} Additionally, a previous NMR study of pY397-PDZ3 showed that phosphorylation perturbed PDZ3 domain compactness by repositioning the $\alpha 3$ -helix.²⁷

Site-Specific Phosphorylations Perturb the Canonical PDZ/Ligand Interaction Profile.

All three PDZ domains of PSD-95 bind so-called class I C-terminal peptide ligands, which are characterized by the C-terminal consensus sequence S/T-X- Φ -OH (X is any amino acid; Φ is a hydrophobic amino acid). Large-scale *in vitro* screenings of PDZ/ligand interactions have identified numerous endogenous binding partners for the three PDZ domains in PSD-95.^{35,36} However, the effect of PDZ domain phosphorylation on these interactions has not been thoroughly examined.

We prepared a panel of 19 fluorescently labeled C-terminal peptides representing endogenous interaction partners including ionotropic glutamate receptors (GluN2A-D),

potassium channels ($K_v1.3$, $K_v1.4$, $K_v1.7$, $K_v3.3$, Kir2.1), sodium channels ($Na_v1.4$), plasma membrane calcium-transporting ATPase (PMCA), transient receptor potential cation channel subfamily V member 3 (TRPV3), G protein-coupled receptors (Frizzled), adaptor proteins (CRIPT, KIF1B, stargazin), and kinases (Cnkr2, PKC, Sapk3). We then examined the panel of peptides for their binding to the three WT PSD-95 PDZ domains, as well as the five phosphorylated PSD-95 PDZ domains, and determined the K_D values for all interactions (Figures S4–S6, Tables S5 and S6).

First, we observed that the K_D values for the interactions between phosphorylated PDZ domains and C-terminal peptides were in the lower micromolar range, which is similar to the K_D for interactions with WT PDZ domains. The highest affinity was found for the interaction between pY236-pY240-PDZ2 and $Na_v1.4$ (VRPGVKESLV) with a K_D of 2.9 μ M. The K_D values for interactions with pS73-PDZ1 were between 2 and 25 fold lower than the corresponding K_D values for phosphorylated PDZ2 and PDZ3, which agrees with observations of affinity for WT PSD-95 PDZ domains.^{35,37} An interesting exemption from this trend was KIF1B (NLKAGRETTV), which showed similar affinity for all five phosphorylated PDZ domains.

Comparing the change in affinity induced by PDZ domain phosphorylation revealed that the effect is highly dependent on both the phosphorylation site and the specific interaction partner (Figure 3, Table S6). For PDZ1, pS73 phosphorylation caused modest changes or no change in the affinity for the panel of ligand peptides ranging from a 1.2-fold increase for CRIPT (DTKNYKQTSV) to a 2.4-fold decrease for $K_v1.7$ (PAGKHMVYEV). Surprisingly, we did not observe differences between the two glutamate receptor subunits, GluN2A (KKMPSESVDV) and GluN2B (EKLSSIESDV), in their response to phosphorylation of PDZ domains as suggested in a previous study.³²

In contrast, PDZ2 phosphorylation generally had a larger impact, where the majority of the ligand affinities decreased up to 5-fold. In one case, a small increase in affinity relative to WT PDZ2 was observed: PMCA (SPLHSLETSTV). The effects from doubly phosphorylated PDZ2 (pY236-pY240-PDZ2) were comparable to those observed with singly phosphorylated domains. Thus, increasing the number of phosphorylated residues did not have additive effects on ligand affinity.

Finally, pY397 phosphorylation of PDZ3 perturbed ligand affinity to a much larger extent than did phosphorylation of PDZ1 or PDZ2. In general, most peptides showed a loss of affinity to pY397-PDZ3 relative to WT. The $Na_v1.4$ derived peptide (VRPGVKESLV) was most affected with an 11-fold reduction in affinity. Surprisingly, stargazin (NTANRRRTTPV) showed a remarkable 4.5-fold increase in affinity toward pY397-PDZ3 relative to WT PDZ3.

We also examined the effect of phosphorylation on the noncanonical interactions between PSD-95 and nNOS. We used a cyclic peptide known to mimic the interaction with the β -finger motif of nNOS (cyclo(CTHLETTFTGDGTPKTIRVTQpG)).^{38,39} As with the canonical interactions, phosphorylation of either PDZ1 or PDZ2 reduced the affinity for the β -finger mimic relative to the WT PDZ domains (Figure S7, Table S6). Affinity for pY236-

pY240-PDZ2 was similar to the singly phosphorylated PDZ domains. As observed previously, we could not detect binding of the β -finger mimic to PDZ3,¹⁹ and this was not influenced by phosphorylation.

Generation of the five site-specifically phosphorylated PDZ domains allowed us to address the effects of binding between PSD-95 and a panel of 19 peptides, representing the C-termini of endogenous interaction partners. We found that phosphorylation fine-tunes the affinity through relatively modest changes for isolated PDZ domains and monovalent peptide ligands. However, these effects on affinity could be substantially magnified in a physiological setting where ligands are often multivalent, which could result in additive effects.

Our studies provide a clear indication that these phosphorylation sites in the PDZ domains of PSD-95 do not function as an on/off switch for binding but rather seem to refine and fine-tune affinities to the binding partners. Interestingly, such a refinement in terms of selectivity toward binding partners has previously been suggested for phosphorylation of S73 in PDZ1, which was reported to abolish binding to the GluN2A subunit of NMDA receptors without affecting the interaction with the GluN2B subunit.³² However, we could not verify this change of selectivity by pS73-PDZ1 as we observed no difference in selectivity between the GluN2A and GluN2B C-terminal peptides. Thus, if there is such a differential effect on binding to the two different subunits, it seems to originate from residues upstream of the C-terminus of the NMDA receptor or other domains in PSD-95, which both have predicted CaMKII phosphorylation sites.

The phosphorylation sites we examined in PSD-95 PDZ2, pY236, and pY240 have both been identified in the rat brain by proteomic studies²³ and observed in extracts isolated from ischemic rat brain. Although phosphospecific antibodies targeting these sites have been developed, the kinase(s) mediating these phosphorylations are not known. PDZ1 and PDZ2 of PSD-95 are known to be involved in key pathways leading to increased nitric oxide levels during ischemic stroke by excessive activation of nNOS. This is facilitated by the ternary complex of the nNOS/PSD-95/NMDA receptor.^{20,40} When we examined the interaction of the nNOS β -finger toward the phosphorylated PDZ domains, we observed a decrease in affinity, which could suggest a protective feedback mechanism for regulating NO levels in the postsynapse during ischemia. In ischemia, elevated NO levels stimulate tyrosine kinases such as Src⁴¹ that could phosphorylate PDZ2, which would facilitate nNOS dissociation from the PSD-95/NMDA receptor complex. This mechanism may have important implications for regenerative repair after stroke.⁴²

Molecular Insight into the pY397-PDZ3/Stargazin Interaction.

Intrigued by the increased affinity of stargazin for pY397-PDZ3 relative to WT PDZ3, we examined this interaction in greater detail using mutational studies, biological NMR, and single molecule fluorescence resonance energy transfer (smFRET). The crystal structure of PSD-95 PDZ3 bound to a pentameric CRIPT peptide shows that Y397 is in close proximity to the P₋₅ position of the peptide.⁷ Stargazin contains two Arg residues (R318 and R319) in positions P₋₆ and P₋₅, respectively, which could interact with Y397 and pY397. To test whether this interaction could explain the increased affinity for phosphorylated PDZ3, we

prepared three stargazin C-terminal peptides containing either single (R318A or R319A) or double (R318A-R319A) Arg-to-Ala mutations and tested these peptides for binding to pY397-PDZ3 and WT PDZ3 (Figure S8). Single mutations in stargazin reduced the affinity for WT PDZ3 by 5.6-fold for R318A and 7.9-fold for R319A. The double mutation, R318A-R319A, caused a 41-fold decrease in affinity. A similar trend was observed for the interaction with pY397-PDZ3, but with even larger effects on affinity. The single mutations reduced the affinity for pY397-PDZ3 by 15-fold for R318A and 11-fold for R319A. The double mutant, R318A-R319A, showed a remarkable 119-fold loss of affinity to pY397-PDZ3. The reductions in affinity reveal that the two arginine residues are strictly required for stargazin binding to both WT and phosphorylated PDZ3 with pY397-PDZ3 being entirely dependent on these arginines for binding. Thus, it is tempting to speculate that the increased affinity upon phosphorylation could be a result of salt bridge interactions between the two Arg residues and the pY397 phosphate group (Figure 4).

As additional confirmation that phosphorylation of PDZ3 increased the affinity for stargazin, we used NMR (Figure 5). The NMR spectra of both WT PDZ3 and pY397-PDZ3 showed well-dispersed peaks as are typical for well-folded proteins. For clarity, the aromatic and amide protons in 6.5 ppm to 10.6 ppm regions are shown (Figure 5a). Next, the NMR samples were titrated with unlabeled wildtype stargazin peptide. We observed specific chemical shift changes with increasing ligand concentration for a peak at 0.1 ppm, which corresponds to a methyl group. This indicates that both WT PDZ3 and pY397-PDZ3 interact with the stargazin peptide under these experimental conditions (Figure 5b). This peak at 0.1 ppm showed fast exchange when WT PDZ3 was titrated with stargazin peptide, but intermediate exchange for the corresponding titration of pY397-PDZ3. The slower exchange confirms that stargazin binds pY397-PDZ3 with higher affinity than WT PDZ3 (Figure 5b).

To provide insight into the mechanism underlying the increased affinity, we measured the binding rate constants for the interaction of stargazin with WT PDZ3 and pY397-PDZ3 using smFRET. The full cytoplasmic domain from stargazin was expressed recombinantly and labeled with the smFRET donor at an engineered cysteine residue (S290C) that is more than 20 residues away from the PDZ ligand to prevent interference with PDZ3 binding to the C-terminus. Stargazin was appended with an N-terminal biotinylation site that allowed for directional attachment to a passivated microscope slide followed by the addition of fluorescently labeled PDZ3 in solution at 100 nM (Figure 6a). Individual binding events between PDZ3 and stargazin were clearly visible and appeared as bursts of emission from the acceptor while under donor illumination (Figure 6b). Molecules showing binding were analyzed using hidden Markov modeling and fit to a two state model (*i.e.*, bound and unbound). Histograms of dwell times in the bound and unbound states were then analyzed to extract the off rate (k_{off}) and on rate (k_{on}), respectively (Figure 6c). The k_{off} for stargazin binding to WT PDZ3 was at the temporal limit of our detection, which means that many binding events are shorter in duration than a single video frame. Our previous analysis showed that missing short events prevents accurate recovery of the full dwell time distribution.⁴³ By only analyzing binding events longer than a single frame, we can put an upper limit on k_{off} for WT PDZ3 at 11.4 s^{-1} . The k_{off} 's were similar to within measurement uncertainty for all PDZ3 variants (Table S7). For the unbound time, we analyzed the frequency of all events with fluorescence higher than the baseline. We measured a k_{on} for

WT PDZ3 of $3.9 \mu\text{M}^{-1} \text{s}^{-1}$, which gives a K_D of $2.9 \mu\text{M}$. The k_{on} for Y397E-PDZ3 was similar ($3.0 \mu\text{M}^{-1} \text{s}^{-1}$), which gave a K_D of $3.2 \mu\text{M}$. However, pY397-PDZ3 showed a nearly 3-fold increase in k_{on} . Thus, smFRET observed a 3-fold increase in the k_{on} upon phosphorylation, giving a 4-fold increase in K_D , in agreement with peptide binding studies (Table S7). An increase in k_{on} is consistent with an electrostatic effect arising from the interaction of arginine residues with the phosphate group.

Stargazin is member of the TARP family, which contains eight evolutionary-related members (γ -1 to γ -8; stargazin is known as γ -2). Thus, we wanted to explore whether these other TARPs interact with PSD-95 PDZ3 and subsequently how they are affected by pY397 phosphorylation. We prepared C-terminal peptides for the seven remaining TARPs and tested their affinity to WT PDZ3 and pY397-PDZ3 (Figure S9). We found that one subgroup of TARPs containing γ -1, γ -5, γ -6, and γ -7 did not bind WT PDZ3 or pY397-PDZ3, whereas the remaining TARPs (γ -2, γ -3, γ -4, and γ -8) all showed similar affinities for WT PDZ3 and pY397-PDZ3. Affinities of these TARPs for pY397-PDZ3 ranged from 3.1 to $3.8 \mu\text{M}$ and showed similarly increased affinities relative to WT as observed for stargazin (γ -2). None of the nonbinding TARP subgroups contain typical C-terminal PDZ binding motifs, whereas the binding TARP subgroups have class I PDZ ligands at their C-termini (Figure S9).⁴⁴ The four C-terminal TARP peptides that bound to PDZ3 are identical in their seven C-terminal residues, NRRTTPV, but diverge in their N-termini. This supports the notion that the affinity increase observed for the pY397-PDZ3/stargazin interaction originates close to the C-terminus.

We observed that phosphorylation of Y397-PDZ3 potentiates the interaction with stargazin. Only one other example is known where phosphorylation increased the affinity of a peptide/PDZ interaction. Phosphorylation of syndecan increased the affinity for PDZ1 of T cell lymphoma invasion and metastasis 1 (TIAM1). In this instance, phosphorylation of Tyr in the P₋₁ position of the ligand increased the affinity for the PDZ domain by 1.6 fold and was mediated by a pTyr-Arg salt bridge.⁴⁵ In PSD-95, the mechanism is similar, but the pTyr is in the PDZ3 domain and the arginine is in stargazin. NMR studies verified that the affinity of stargazin is indeed higher to pY397-PDZ3 relative to wild type PDZ3. Additionally, smFRET measurements showed that this increase in affinity was a result of an increased k_{on} , which is consistent with an ionic interaction with Arg318 and/or Arg319. Previously, stargazin had been suggested to primarily interact with PSD-95 through PDZ1 and PDZ2.^{46,47} However, we observed that the affinity was in fact higher for PDZ3. Interestingly, a recent study demonstrated that phosphorylation of stargazin resulted in elongation of the cytoplasmic domain and switched the interaction from PDZ1/2 to PDZ2/3.⁴⁸ This supports our finding that PDZ3 in PSD-95 probably plays a more important role in recognition of stargazin, particularly after phosphorylation of both proteins.

Phosphomimetic Mutations vs Phosphorylated Residues for Studying Protein–Protein Interactions.

It is an open question as to how well phosphomimetic mutations, such as Asp or Glu, mimic the effect of phosphorylated Ser or Tyr residues (Figure 7a). Such phosphomimetic mutations are frequently used in biological studies to address potential effects of

phosphorylation. However, there is little information to critically assess how well these mutations mimic the phosphorylated residue of interest.^{49,50} Having characterized the five site-specifically phosphorylated PDZ domains, we next expressed and purified the five corresponding PDZ domains containing phosphomimetic mutations: S73D-PDZ1, Y236EPDZ2, Y240E-PDZ2, Y236E-Y240E-PDZ2, and Y397E-PDZ3 (Tables S5 and S6). We then tested the phosphomimetic PDZ domains for binding using the panel of the 19 C-terminal peptides representing endogenous PSD-95 binding partners.

We found a reasonable correlation between the ligand affinities for phosphorylated and phosphomimetic residues for PDZ1 (Figure 7b). However, for the PDZ1/ β -finger non-canonical interaction, there was notable deviation with losses of affinity of 2.4- and 7.1-fold for pS73-PDZ1 and S73D-PDZ1, respectively. Thus, the phosphomimetic produced a larger reduction of affinity relative to the phosphorylated residue.

In contrast, for both PDZ2 and PDZ3 we observed several outliers as well as skewed correlations. The difference between pY236-PDZ2 and Y236E-PDZ2 was more pronounced (Figure 7c). Peptide ligands with K_D values above 10 μ M showed a similar affinity for phosphorylated and phosphomimetic proteins, whereas peptide ligands with K_D values below 10 μ M were less affected by the phosphomimetic than by phosphorylation. Similarly, for singly modified Y240 (Figure 7d) and the double modified Y236-Y240-PDZ2, the phosphomimetic mutants had less effect on the affinity than the corresponding phosphorylation (Figure 7e). Specifically, phosphorylation generally produced a 2–4-fold larger reduction in peptide binding affinity. Surprisingly, the differences in affinity for ligands interacting with Y397E-PDZ3 and pY397-PDZ3 domains were generally less than 2-fold. Thus, the Y397E mutation was more effective at mimicking phosphorylation than similar mutations in PDZ2 (Figure 7f). However, one striking difference was the interaction of stargazin, which showed a 4.5-fold increase in affinity to pY397-PDZ3 but no change in affinity for the corresponding phosphomimetic mutant Y397E-PDZ3. This was also confirmed by smFRET (Figure 6c). Thus, the interesting observation about the increased affinity for the phosphorylated PDZ3 domain would not have been revealed using a phosphomimetic.

In conclusion, we performed a thorough comparison of the effect of a phosphorylated residue versus a phosphomimetic residue in binding of peptide ligands to the PSD-95 PDZ domains. We found that for PDZ1 and PDZ3 a phosphomimetic residue faithfully mimics a phosphorylated residue, although important outliers were observed. In contrast, for PDZ2, phosphomimetic residues were generally less effective relative to the phosphorylated residue.

There are structural and chemical differences between phosphate and carboxylate groups, such as the degree of ionization and number of coordinating oxygen atoms. Moreover, Asp and Glu are poor mimics for the aromatic character of Tyr. Still, phosphomimetics are often the only option and are then used uncritically in part due to a lack of systematic comparisons between phosphorylated residues and phosphomimetics. Here, we have provided such a comparison by measuring almost 100 different peptide/PDZ domain interactions with either phosphorylations or phosphomimetics. We observed that in several cases Asp or Glu are

indeed faithful mimics of phosphorylation. In particular, using Asp as a surrogate for pSer in pS73-PDZ1 was generally effective. In contrast, when comparing Glu as a surrogate for pTyr the correlation is much weaker, and in most cases there are significant differences. The most interesting example was the interaction of PDZ3 with stargazin, where the phosphomimetic showed no difference relative to WT, while phosphorylation of Y397 increased the affinity. The presence of outliers and skewed correlations clearly highlights the need for caution when considering results from studies using phosphomimetics.

CONCLUSION

We have generated five site-specifically phosphorylated PDZ domains from PSD-95 and have explored how phosphorylation subtly modulated binding to a suite of interaction partners that included canonical C-terminal peptides and also the non-canonical nNOS β -finger. We discovered that a specific phosphorylation site, pY397 in PDZ3, caused an increase in affinity to the AMPA receptor auxiliary subunit stargazin, and characterized this interaction in greater detail using NMR and smFRET. Finally, we made a comprehensive comparison of how well phosphomimetic residues, Asp or Glu, mimic the corresponding phosphorylated residues. This showed that great care should be taken when drawing conclusions from studies using phosphomimetic residues. This work also underlines the importance of employing protein semisynthesis, or other technologies, that allow site-specific and efficient incorporation of phosphorylated residues.

METHODS

Details of experimental procedures are provided in the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This research was financed by the Danish Council for Independent Research, Medical Sciences to S.W.P. (4092–00402B), the Lundbeck Foundation to L.A. (R165-2013-15822), and a research grant from the Research Grant Council of Hong Kong to M.Z. (AoE-M09–12).

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Figure 1.

Phosphorylation sites of PSD-95. (a) Organization of the five domains in PSD-95: PDZ1–3, an SH3 domain, and a GK domain. (b) Primary sequence of the human PSD-95 with phosphorylation sites identified through MS-MS studies (orange) or prediction tools (yellow).

Figure 2.

Semisynthesis of phosphorylated PDZ1, PDZ2, and PDZ3 of PSD-95. (a) Semisynthetic strategy for obtaining N-terminal phosphorylation of PDZ1. The N-terminal fragment (NPDZ1 , 23 amino acids) was synthesized on 2-chlorotrityl (2CL) resin treated with hydrazine and subsequent chain elongation by SPPS generating a peptide containing a benzylated serine phosphate at position 73, which after trifluoroacetic acid (TFA) treatment cleaved the deprotected hydrazide phospho-peptide from the solid support. The C-terminal fragment (CPDZ1 , 69 AA) was recombinantly expressed in *E. coli* as a polyhistidine fusion protein containing a factor Xa (FXa) recognition site (IEGR) generating a N-terminal Cys after treatment with FXa enzyme. Ligation was initiated by oxidizing hydrazine of NPDZ1 with NaNO_2 for in situ generation of a C-terminal azide, which subsequently underwent thiolysis forming a thioester by the addition of 4-mercaptophenylacetic acid (MPAA). Ligation was performed by adding CPDZ1 generating the full-length PDZ1 domain (Table S3). (b) Semisynthetic strategy for obtaining C-terminal phosphorylated PDZ2 and PDZ3.

The C-terminal phosphorylations were introduced by synthesis of a N-terminal Cys peptide fragment (19 AA for $_{\text{C}}\text{PDZ2}$ and 26 AA for $_{\text{C}}\text{PDZ3}$) containing one or two phosphorodiamidate-protected tyrosines yielding pTyr after TFA treatment. The larger N-terminal fragment ($_{\text{N}}\text{PDZ2}$ and $_{\text{N}}\text{PDZ3}$ of both 75 AA) was produced through recombinant expression of a fusion protein containing the desired PDZ fragment, a *Mycobacterium xenopi* Gyrase A (Mxe GyrA) intein and a polyhistidine tag. Adding 2-mercaptoethanesulfonate (MESNa) induced a thiolysis reaction, leaving the extein fragment with a C-terminal thioester. The C- and N-terminal fragment were ligated followed by a desulfurization step converting Cys to Ala. The full-length semisynthetic PDZ domains were refolded by diafiltration. (c) The three PDZ domains with the four phosphorylation sites are displayed. Structures are adapted from X-ray crystal structures of PDZ12 (PDB-ID 3ZRT) and PDZ3 (PDB-ID 1BE9). (d) LC-MS traces for the five purified semisynthetic PDZ domains. Inlet shows the deconvoluted mass (expected masses: pS73-PDZ1, 9910.5 Da; PDZ2 pY236 and pY240/pY236-pY240, 10635/10715 Da; and PDZ3 pY397, 10958 Da).

Figure 3.

Change in canonical ligand binding properties of phosphorylated PDZ domains. The fold change affinity toward C-terminal peptide ligands induced by single and double phosphorylation. Affinity increase is displayed as a left shifted fold change, whereas affinity decrease is displayed as a right shifted fold change. Fold changes for ligands having $K_D > 100 \mu\text{M}$ for nonphosphorylated proteins are not displayed.

Figure 4. Molecular details of the PDZ3–stargazin interaction. Model displaying the predicted interactions with side chains of two arginines of the stargazin C-terminal peptide and the Y397 phosphorylation of PDZ3. The R318 makes a salt bridge with the negative charge of pY397 (*top left inset*). The positive charged R319 is stabilized by salt bridges originating from two positive charges of E331 and E364 of PDZ3 (*bottom left inset*). 3D model designed from the PDZ3 X-ray crystal structure of SAP-97 cocrystallized with the pentameric peptide (RTTPV) resembling C-terminal stargazin (PDB-ID: RJXT).

Figure 5.

Structural homogeneity and stargazin binding investigated by the 1D ^1H NMR spectrum of PDZ3 WT and PDZ3 pY397. (a) The well-dispersed spectra and overall similarity of the spectra indicate that PDZ3 WT and PDZ3 pY397 are both well folded and the phosphorylation does not alter the overall conformation of the PDZ domain. (b) A selected methyl peak of the ^1H NMR spectra showing that the free and the stargazin peptide-bound forms of PDZ3 WT undergo fast to intermediate exchange, whereas the free and the stargazin peptide-bound forms of PDZ3 pY397 undergo slow exchange.

Figure 6.

Kinetics of PDZ3 and stargazin interaction studied by single molecule FRET. (a) Cartoon representation of the single molecule FRET assay used to directly observe binding interactions between PDZ3 and stargazin. The full cytoplasmic domain of stargazin was labeled with the FRET acceptor (blue star) and attached to a passivated streptavidin surface *via* N-terminal biotinylation. PDZ3 was labeled with the FRET donor (magenta star) and added in solution at 100 nM. TIRF microscopy allows selective excitation of molecules at the surface. PDZ3 binding to stargazin brings the donor and acceptor close enough together to produce FRET. (b) Representative single molecule time trace of acceptor intensity. The bar above the panels illustrates the pattern of alternating laser excitation. First, the acceptor is directly excited to identify stargazin molecules on the surface. Next, the donor is excited, and individual protein interactions are visible as bursts of acceptor intensity produced by FRET. Finally, the acceptor is directly excited to probe for acceptor photobleaching, which has not occurred in these traces. (c) Histograms of the dwell times in the bound state (top) and unbound state (bottom) for the interaction between PDZ3 and stargazin. Data are shown as circles for PDZ3 WT (black), Y397E (gray), and pY397 (orange). Data from replicate measurements were pooled and fit as a single histogram (solid lines).

Figure 7. Comparing phosphorylations and phosphomimetic approaches for PDZ domains. (a) Structural differences of phosphorylated amino acids (orange background) and phosphomimetics (gray background). The K_D values obtained from phosphorylated PDZ domains (*x-axis, orange*) and PDZ domains containing phosphomimetic mutations (*y-axis, gray*) compared for (b) PDZ1 S73, (c) PDZ2 Y236, (d) PDZ2 Y240, (e) PDZ2 Y236 Y240, and (f) PDZ3 Y397. Dashed lines: phosphomimetics induce the same effect on ligand binding as the corresponding phosphor amino acids ($K_D(\text{pS or pY}) = K_D(\text{D or E})$).