A EUROPEAN JOURNAL OF CHEMICAL BIOLOGY

CHEMBIOCHEM

SYNTHETIC BIOLOGY & BIO-NANOTECHNOLOGY

Accepted Article

Title: Importance of a conserved Lys/Arg residue for ligand/PDZ domain interactions as examined by protein semisynthesis

Authors: Søren Wittrup Pedersen; Griffin E. Moran; Vita Sereikaite; Linda M. Haugaard-Kedström; Kristian Strømgaard

To be cited as: ChemBioChem 10.1002/cbic.201600322

Link to VoR: http://dx.doi.org/10.1002/cbic.201600322

A Journal of

ChemPubSoc Europe

www.chembiochem.org

WILEY-VCH

10.1002/cbic.201600322

Importance of a conserved Lys/Arg residue for ligand/PDZ domain interactions as examined by protein semisynthesis

Søren W. Pedersen, Griffin E. Moran, Vita Sereikaitė, Linda M. Haugaard-Kedström and Kristian Strømgaard

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

Correspondence should be addressed to S.W.P (sorenwp@gmail.com) or K.S. (kristian.stromgaard@sund.ku.dk)

Abstract

PDZ domains are ubiquitous small protein domains that are mediators of numerous protein-protein interactions, and play a pivotal role in protein trafficking, synaptic transmission and facilitating assembly of signaling-transduction complexes. In recent years, PDZ domains have emerged as novel and exciting drug targets for diseases in the brain in particular, and understanding the molecular details of PDZ domain mediated interactions is of fundamental importance. These interactions are mediated by PDZ domains binding to the protein partner through either a C-terminal peptide or internal peptide motifs. Here, we examined the importance of a conserved Lys/Arg residue in the ligand binding site of the second PDZ domain of PSD-95 by employing a semisynthetic approach. Six semisynthetic PDZ domains were successfully obtained comprising different proteogenic and non-proteogenic amino acids representing subtle changes of the conserved Lys/Arg residue, and these were tested towards four different peptide interaction partners, representing the two different binding modes. The results highlight the role of a positively charged amino acid in the β1-β2 loop of PDZ domains, and show subtle differences for canonical and non-canonical interaction partners, thus providing additional insight into the mechanism of PDZ/ligand interaction.

Keywords

semisynthesis; protein-protein interactions; PDZ domains; non-proteogenic amino acids; protein engineering

Introduction

Protein-protein interactions (PPIs) are essential for almost all cellular and biochemical processes and hence are a promising, but also challenging, class of potential drug targets.[1-4] Nature has evolved numerous protein families that govern and facilitate such PPIs,[5, 6] one example being the protein family of membrane-associated guanylate kinases (MAGUKs), which assist in forming multiprotein complexes near cellular membranes.[7, 8] The family includes the postsynaptic density protein 95 (PSD-95), which is critical for synaptic formation and function due to its ability to organize cellular components within the post synaptic density (PSD) primarily mediated via three PSD-95/discs large/zonula occludens 1 (ZO-1) (PDZ) domains.[9]

PDZ domains are one of the largest families of protein domains that facilitate PPIs, consisting of 256 human PDZ domains found in 142 different proteins.[10] PDZ domains play a pivotal role in trafficking, synaptic transmission and facilitating assembly of signaling-transduction complexes by acting as scaffolding proteins for integral membrane and cytosolic proteins.[9, 10] Notably, PDZ domains have recently emerged as novel and interesting drug targets, in particular in relation to diseases in the brain such as neurodegeneration, stroke and pain.[11-13] PDZ domains typically consist of 90-100 amino acids, which form six β-strands (β1-β6) and two α-helices (α1-α2). The ligand binding site is a shallow pocket located between the β2 strand and the α2 helix, where the ligand forms an extended antiparallel β strand (Fig. 1A).[14-16] The canonical peptide binding mode involves a peptide C-terminal carboxylate, which interacts with a conserved GLGF motif located just before the β2 strand in the PDZ domain (Fig. 1B). The GLGF motif, or carboxylate binding site, forms a cradle of backbone amides, which aligns a hydrogen bond network to the peptide C-terminal carboxylate and hence stabilizes the PDZ domain/peptide interaction. Furthermore, an extended backbone-backbone hydrogen bond network

between the ligand and the β2 strand,[16, 17] together with side chain interactions, primarily governed by the four last amino acids, are also fundamental for the canonical binding of C-terminal peptides to PDZ domains. The four C-terminal amino acids of the peptide ligand contains a motif (P_{-3}-P_0), where amino acids in position P_0 and P_{-2} are particular important for affinity and selectivity. [18, 19] So-called class I PDZ domains, such as the PDZ domains of PSD-95, recognize C-terminal peptides containing a T/S-X-V/L-COOH (X being any amino acid) motif, where a hydrophobic pocket at P_0 accommodates the V/L residue and a conserved His in the α2 helix form a hydrogen bond to the side chain hydroxyl group of the T/S residue.[15, 20] It is well established that a subset of PDZ domains recognize internal peptide motifs, exemplified by the non-canonical interaction between the β-finger in neuronal nitric oxide synthase (nNOS) and PSD-95, syntrophin and ZO-1.[20-22] The internal binding motifs form either an antiparallel β-finger or an extended conformation, which aligns the β2 strand in the PDZ domain and form interactions similar to the canonical binding mode: The P_0 site accommodates an aromatic side chain (Phe111) of the nNOS β-finger and in the P_{-2} site a hydrogen bond is formed between His residue in the α2-helix and a hydroxyl group (Thr109) in nNOS.[16, 21, 23-25] In addition, structural analysis of PDZ domains have identified interactions between the side chain of a highly conserved basic residue in the flexible β1-β2 loop, Arg/Lys (Lys165 and Arg318 in PSD-95 PDZ2 and PDZ3, respectively), which together with the GLGF loop stabilizes the carboxylate of C-terminal ligands through a water-mediated hydrogen bond (Fig. 1A).[15, 21, 26] It is known that Arg318 in PSD-95 PDZ3 can act as a switch allowing anionic strength to modulate the affinity of ligands to PDZ3[27] and interestingly, a Lys165Arg mutation in PSD-95 PDZ2, disrupted the binding to nNOS, but not the canonical binding to the C-terminal of Shaker-type K^+ channel $K_v1.4$.[28]

PSD-95 is one of the most abundant proteins in the PSD in neurons and also one of the most studied PDZ containing proteins, and thus provides an excellent model system for studies of ligand/PDZ domain interactions. PSD-95 is known to interact via its PDZ domains with the C-terminal of the GluN2B subunit of the *N*-methyl-D-aspartate (NMDA) glutamate-type receptor and nNOS. The PSD-95/nNOS/NMDA receptor complex have been demonstrated to be important in excitotoxicity, which is a process in the brain believed to be involved in diseases such as ischemic stroke,[9] and notably peptide-based inhibitors of PSD-95 PDZ domains have shown promising results in both preclinical and clinical studies as a potential treatment of ischemic stroke.[29, 30] Thus, understanding the molecular details of PDZ domain mediated interactions of PSD-95 is also of fundamental importance for development of improved inhibitors of PSD-95.

Here we address the importance of the conserved Arg/Lys in PDZ domains by employing expressed protein ligation (EPL) to introduce non-proteogenic mutations at position Lys165 in PSD-95 PDZ2 (Fig. 1C) and examine the effect on canonical and non-canonical peptide ligand interactions. Altering the length of the Lys side chain or replacing with an Arg had no effect on ligand affinity in either binding mode. Interestingly removing the positive charge of the side chain, as in citrulline and acetyl-ornithine, did not influence non-canonical interaction, but had a subtle effect upon the canonical interaction. Thus, this provides mechanistic insight into the importance of the positive charge of the conserved Lys/Arg side chain in PDZ domains, and in particular underpins putative differences between canonical and non-canonical interactions, which are fundamental for the understanding of the largest family of modulators of PPIs.

Results

To explore the importance of the conserved Lys/Arg residue for peptide ligand binding to PDZ domains, we employed the prototypical PSD-95 PDZ2 domain, as well as four different 5/6-tetramethylrhodamine (TAMRA) labelled peptide ligands, representing both canonical ligands, the GluN2B C-terminal peptide (TAMRA-YEKLSSIESDV-COOH, GluN2B-COOH) and CRIPT (TAMRA-DTKNYKQTSV-COOH, CRIPT-COOH), as well as non-canonical ligands, a nNOS β-finger mimetic (TAMRA-cyclo(CTHLETTFTGDGTPKTIRVTQpG), nNOS β-finger)[16, 31-32] and a C-terminally amidated GluN2B peptide (TAMRA-YEKLSSIESDV-$CONH_2$, GluN2B-$CONH_2$). In order to evaluate the importance of the Lys/Arg residue, we replaced Lys165 in PSD-95 PDZ2 with Arg, as well as four non-proteogenic amino acids: First two variants of Lys, homolysine (hLys) and ornithine (Orn), where the number of methylene groups to the primary amine is varied, and then two variants of Arg, citrulline (Cit) and acetyl-ornithine (AcOrn), where the degree of protonation, as well as hydrogen bonding, is varied (Fig. 1C).

In order to introduce the non-proteogenic amino acids into PDZ domains, we explored a semisynthetic methodology for the generation of the PSD-95 PDZ2 domain comprising chemical mutations in position 165.[16] The strategy required the generation of two fragments: A synthetic 23-mer N-terminal peptide fragment (Δ_NPDZ2) and a recombinantly expressed 72-mer C-terminal protein fragment (Δ_CPDZ2), which would be ligated at a V178C site, a mutation which has been shown not to affect ligand binding.[16] The N-terminal fragment Δ_NPDZ2, comprising the residue of interest, Lys165 and a C-terminal hydrazide, was prepared by solid phase peptide synthesis (SPPS) (Fig. 2A), while the C-terminal fragment Δ_CPDZ2, containing a N-terminal Cys, was generated by bacterial overexpression

(Fig. 2B). The two fragments then underwent chemo-selective ligation and subsequent refolding to generate the binding competent, full-length PSD-95 PDZ2 domain (Fig. 2C).

Semisynthesis of PSD-95 PDZ2 domains. Six different N-terminal peptide fragments (Δ_NPDZ2-Lys, Δ_NPDZ2-hLys, Δ_NPDZ2-Orn, Δ_NPDZ2-Arg, Δ_NPDZ2-Cit and Δ_NPDZ2-AcOrn) containing mutations in position 165 were synthesized by Fmoc-based SPPS using a hydrazine-preloaded 2-chlorotrityl resin and an automated peptide synthesizer. The fragment Δ_NPDZ2-Lys was used to generate wild-type semisynthetic PSD-95 PDZ2 as a control. Three of the four non-proteogenic amino acids, hLys, Orn and Cit, were commercially available as appropriately protected building blocks. However, the acetylated ornithine building block, Fmoc-AcOrn-OH, used to generate Δ_NPDZ2 AcOrn, was prepared from Fmoc-Orn(Boc)-OH (Scheme S1). First, the carboxylic acid was protected as an allyl ester (**1**), then the *N*-Boc side chain protection group was removed by treatment with trifluoroacetic acid (TFA) and subsequently acetylated with acetyl chloride. Finally, the carboxylic acid was reconstituted by treating **2** with $Pd(PPh_3)_4$, generating Fmoc-AcOrn-OH (**3**) in good yield (32%) (Fig. S1). With all Lys/Arg analogues in hand, the six N-terminal peptides comprising C-terminal hydrazide were prepared by SPPS and obtained in good yields (16-34%) and excellent purities (>90%) (Table S1). The C-terminal protein fragment (Δ_CPDZ2) was obtained using a protocol similar to previously described.[16] In brief, a modified fusion protein of the full-length PSD-95 PDZ2 containing a N-terminal polyhistidine tag (MHHHHHHHRS) and a Factor Xa (FXa) cleavage site (IEGR) at position V178C was expressed in *E. coli*. After metal ion affinity chromatography, the isolated protein was subjected to FXa cleavage and the remaining Cys-terminal protein fragment (Δ_CPDZ2) was isolated in good yield and purity (13 mg/L, >95% pure) (Fig. S2, Table S2).

To generate full-length PDZ domains, ligations of the two fragments were initiated by oxidation of the C-terminal hydrazide of synthetic peptide (Δ_NPDZ2) with $NaNO_2$ for *in situ* generation of a C-terminal azide (Fig. 2A), which was converted to a C-terminal thioester peptide by addition of 2-mercaptophenyl acetic acid (MPAA).[33] Subsequently, the recombinant C-terminal protein fragment, Δ_CPDZ2, was added to the ligation mixture and ligations were complete after 2 h with ligation efficiencies between 68-81% evaluated by depletion of reactive C-terminal protein fragment (Fig. S3). The full-length semisynthetic PSD-95 PDZ2 domains were isolated by gel filtration, which removed non-ligated fragments and ligation buffer components while simultaneously refolding the protein into binding buffer. All six semisynthetic proteins were obtained in a purity of >95%, as observed by UPLC (Fig. 2D and S5, Table S3) and SDS-PAGE (Fig. 2E). The secondary structure of the semisynthetic PSD-95 PDZ2 domains, as well as the recombinantly expressed PSD-95 PDZ2 domain (PDZ2 WT), were examined and compared by circular dichroism (CD) spectroscopy (Fig. 2F). Gratifyingly, all spectra overlapped with a minimum at 212 nm, thus suggesting that all semisynthetic PDZ2 domains had a secondary structure and fold similar to that of PDZ2 WT.

Binding between semisynthetic PDZ domains and interaction partners. In order to address the effect of the chemically introduced mutations in the PSD-95 PDZ2 domain, we examined the binding of four different peptide ligands, GluN2B-COOH, CRIPT-COOH, GluN2B-$CONH_2$ and nNOS β-finger, to the semisynthetic PDZ domains using a fluorescence polarization (FP) assay.[16, 34] These ligands represent, as previously described, canonical (GluN2B-COOH and CRIPT-COOH) and non-canonical (GluN2B-$CONH_2$ and nNOS β-finger) binding modes of the peptide/PDZ domain interaction. The C-terminally amidated ligand, GluN2B-$CONH_2$, has previously been shown to have very low affinity towards the PSD-95 PDZ2 domain (K_D > 500 μM), mainly due to of the lack of charge in the C-

terminal and not being able to engage in the hydrogen bond network to the backbone of the GLGF motif.[16, 21] The nNOS β-finger ligand is a cyclic peptide analogue derived from nNOS, which has been shown to mimic the native non-canonical interaction between nNOS and PDZ domains with comparable binding affinities (K_D ~1.0-1.8 uM). [16, 31-32]

First, the K_D values for binding of the four peptide ligands to PDZ2 WT and the semisynthetic control protein (PDZ2-Lys) were determined by saturation binding experiments (Fig. 3). The affinities for the canonical ligands, GluN2B-COOH and CRIPT-COOH, were found to be in the lower micromolar range (4.9 μM and 17 μM for PDZ2 WT and 5.4 μM and 17 μM for PDZ2-Lys, respectively) and for the non-canonical ligands, nNOS β-finger and GluN2B-$CONH_2$, the K_D values were ranging from the low to the high micromolar range (1.2 μM and 314 μM for PDZ2 WT and 1.2 μM and 240 μM for PDZ2-Lys, respectively). The K_D values for these ligands are generally the same for the expressed PDZ2 WT and the semisynthetic PDZ2-Lys, which strongly indicates that the semisynthetic strategy provides structurally intact PDZ domains. Next, affinities of the four ligands were determined for the five mutated semisynthetic PDZ domains (Fig. S6, Table S4). First, the two proteins mutated with Lys analogues, PDZ2-hLys and PDZ2-Orn, respectively were examined, and here binding was generally unaffected by either side chain elongation (hLys) or shortening (Orn). Compared to PDZ2 Lys, the mutations only gave moderate changes in affinity for both canonical and non-canonical ligands, exemplified by binding of CRIPT-COOH to PDZ2-hLys (K_D = 6.7 μM) and PDZ2-Orn (K_D = 3.4 μM), respectively (Fig. 4). Interestingly, replacing Lys with Arg, PDZ2-Arg, as also found in related PDZ domains, [15] led to a marginal increase in the affinity with K_D = 2.7 μM and 156 μM for GluN2B-COOH and GluN2B-$CONH_2$, respectively, while affinity of the nNOS β-finger decreased by 1.8 fold (K_D = 2.1 μM). PDZ domains comprising Arg analogues, PDZ2-Cit and PDZ2-AcOrn, both showed binding

properties different from those containing either Lys or Arg in the same position. Interestingly, both introduction of Cit or AcOrn led to increase in K_D values for GluN2B-COOH (18 μM and 49 μM, respectively) and CRIPT-COOH (17 μM and 52 μM). In contrast, when examining the nNOS β-finger, a slight increase in affinity to both PDZ2-Cit (K_D = 1.1 μM) and PDZ2-AcOrn (K_D = 0.89 μM) was observed. Finally, binding of GluN2B-$CONH_2$ to PDZ2-AcOrn was slightly decreased (K_D = 464 μM), while no change in affinity was observed for binding to PDZ2-Cit (K_D = 259 μM).

Discussion

Alteration of Lys in the β1-β2-loop changes binding preferences of canonical PDZ2 binders. The C-terminal of the GluN2B subunit is a canonical binder to PSD-95 PDZ2 and the importance of hydrogen-bonding[16, 17] and side chain mutations[34] have previously been studied for this interaction, but so far no X-ray crystal structure has been solved for this PDZ2 domain in complex with a peptide ligand. In contrast, the first X-ray crystal structure of a ligand/PDZ complex was solved with the CRIPT C-terminal peptide bound to PSD-95 PDZ3, which is currently the established model for the canonical binding mode.[15] The CRIPT peptide has preference for the PDZ3 vs. the PDZ2 domain of PSD-95, with K_D values of 2.3 μM and 16 μM for PSD-95 PDZ3 and PSD-95 PDZ2, respectively,[18, 34] still it is assumed that that CRIPT binds to both PDZ2 and PDZ3 in the canonical binding mode given the high sequence similarity of the proteins, including a conserved His (PDZ2 H225, PDZ3 H372) in the α2 helix and the carboxylate binding site (PDZ2 G169-F172, PDZ3 G322-F325).[35] One notable difference between the two domains is the conserved basic residue in the loop region between β1-β2 (PDZ2 K165, PDZ3 R318), which facilitates a hydrogen bond to the C-terminal carboxylate of the peptide ligand through a water molecule (Fig. 1A). Although the β1-β2 loop of PDZ2 contains the same number of amino acids as PDZ3, the X-ray crystal structure of apo PDZ2 indicate that this loop is

bending away from the GLGF site in contrast to PDZ3. This does however not seem to affect the position of the basic moiety from the conserved Lys/Arg residue as the position of the Lys amino group in PDZ2 still overlaps with the Arg guanidinium group in PDZ3.

Here, we examined the molecular details of this conserved basic residue in PSD-95 PDZ2, and by employing a semisynthetic approach we could introduce refined chemical modifications in its side chain. Three of the five semisynthetic PDZ2 domains, PDZ2-hLys, PDZ2-Orn and PDZ2-Arg, have positively charged side chains, with variation in both the number of methylene groups separating the amino group and the nature of the basic moiety (Fig. 1C). Compared to PDZ2-Lys, extending, PDZ2-hLys, or shortening, PDZ2-Orn, the side chain of Lys165 has only limited effect on binding affinity towards GluN2B-COOH and CRIPT-COOH, whereas replacing with Arg induces a small affinity increase. Despite the differences, the side chains of hLys and Arg have a similar distance from the terminal nitrogen of the amine/guanidinum to the backbone (Fig. 1C) thereby making them similarly positioned to form a hydrogen bond to the water molecule. In contrast, introduction of the Arg analogues, PDZ2-Cit and PDZ2-AcOrn, that do not have a net charge, but can still participate in hydrogen bonding, led to a moderate decrease in affinity of the canonical ligands. This suggests that the net charge of the basic side chain may play a role in the canonical peptide/PDZ domain interaction. A similar tendency has previously been observed for the peptide/syntrophin interaction, where Lys86, corresponding to Lys165 in PSD-95 PDZ2, was mutated to Met leading to a loss in binding affinity ($\Delta\Delta G_{binding}$ = 1.5 kcal mol^{-1}).[36]

Another interesting difference between the six side chain modifications is the ability to stabilize the position of the charge. The side chain of Arg has three nitrogens compared to one in Lys and its analogues, which allows Arg to form a larger number of interactions.[37] In the X-ray crystal structure

of CRIPT/PDZ3,[15] the Arg318 may form hydrogen bonds to the water molecule near the GLGF motif, as well as to the backbone carbonyls of Leu379 and Gly383 given the close proximity (3.0 Å and 2.7 Å, respectively) to the latter. These additional intramolecular interactions could stabilize the position of the positive charge in PDZ2-Arg, but not in PDZ2-Lys, which could explain the slightly lower K_D values for the canonical binders.

Non-canonical PDZ2 domain interactions are charge independent. PSD-95 PDZ2 interacts with the β-finger of nNOS through a non-canonical binding, where the ligand backbone aligns with the β2 strand forming an extended β-sheet.[16, 21] The X-ray crystal structure of the nNOS β-finger and the syntrophin PDZ domain suggests that this interaction is not mediated by a water molecule near the GLGF-site, which can be explained by the β-finger not having a free carboxylate moiety.[15] This suggests that the basic residue (Lys86 in syntrophin) might not be directly involved in non-canonical interactions. When comparing the sequences of PDZ domains that interact with nNOS (Fig. S7),[22, 38-41] it is seen that both Lys and Arg are found in the position of the conserved basic residue. Thus, the specific nature of the basic residue is apparently not important for the non-canonical interaction. In contrast, it has previously been reported that the interaction between nNOS and PSD-95 PDZ2 was completely disrupted upon an Arg to Lys mutation in this position.[28] Here, we observed that regardless of the substitution introduced into position 165 of PSD-95 PDZ2, the affinity towards the nNOS β-finger was changed less than 2 fold. The same observations were seen for the affinity of GluN2B-$CONH_2$ having a K_D value for PDZ2 WT in the high micromolar range. Thus, it seems that the removal of the charge in this position does not affect the non-canonical interaction, which is in agreement with examination of the nNOS/syntrophin interaction, where the Lys86Met mutation induced only a very modest change in binding ($\Delta\Delta G_{binding}$ = 0.5 kcal mol^{-1}).[36] In addition, our results

are in agreement, with the conclusions drawn from the X-ray crystal structure of the nNOS/syntrophin complex, that the conserved basic residue is less important for the non-canonical interaction.

Conclusion. Here, we have addressed the importance of a basic residue (Lys or Arg), which is conserved in many PDZ domains, for the binding of both canonical and non-canonical ligand/PDZ domain interactions. We took advantage of a semisynthetic strategy for PDZ domains and introduced non-proteogenic amino acids instead of Lys165 in the PSD-95 PDZ2 domain. Specifically, we incorporated two Lys analogues by introducing hLys and Orn, and similarly two Arg analogues, Cit and AcOrn. This provided succinct information on differences in the canonical and non-canonical interaction in PDZ domains, where the former prefers amino acids bearing charged side chains, whereas in the latter there was no difference in ligand affinities regardless of the mutation introduced. Thus employing a semisynthetic approach to generate PDZ domains, allowed us to decipher the molecular details about a key residue in PDZ domain interactions, and the methodology offers the opportunity to introduce a wealth of additional amino acids into PDZ domains.

Experimental details

General information. Unless otherwise stated, starting materials were obtained from commercial suppliers and used without further purification. Fmoc protected amino acids, coupling reagents and trifluoroacetic acid (TFA) were purchased either from Peptides International (Louisville, KY, USA), Sigma Aldrich (St. Louis, MO, USA) or Iris Biotech (Marktredwitz, Germany). All reagents and solvents were used without further purification. The reactions for the Fmoc-AcOrn-OH synthesis were monitored by thin-layer chromatography (TLC) using silica gel coated aluminum plates 60F-254

(Merck). A CombiFlashRf^{TM} system (Teledyne ISCO, Lincoln, NE, USA) was used for flash column chromatography with samples being loaded onto GraceResolv^{TM} flash cartridges (Grace, Columbia, MD, USA). Silica gel chromatography was performed using Merck silica gel 60, 230-400 mech. NMR spectroscopy was performed on Bruker 400 MHz apparatus. ^{1}H NMR chemical shifts were recorded in ppm, using $CDCl_3$ and TMS as internal standard (0.00 ppm). The following abbreviations are used: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. ^{13}C NMR shifts are recorded in ppm using the residual non deuterated solvent as internal standard ($CDCl_3$ ^{13}C 77.0 ppm). NMR spectra were analyzed using MestReNova version 10.0 by Mestrec Laboratories. Mass spectra were obtained with an Agilent 6410 Triple Quadrupole Mass Spectrometer instrument using electron spray coupled to an Agilent 1200 HPLC system (ESI-LC/MS) with a C18 reverse phase column (Zorbax Eclipse XBD-C18, 4.6 × 50 mm), autosampler and diode-array detector using a linear gradient of the binary solvent system of H_2O/acetonitrile (ACN)/formic acid (A: 95/5/0.1 and B: 5/95/0.086) with a flow rate of 1 mL/min, and gradient elution from 0-100% B in 5 min. Purity was determined by ultra performance liquid chromatography (UPLC) using an Acquity UPLC BEH C18 1.7µm 2.1 × 50 mm column over a gradient of 5-95% Buffer B in 7 min.

Synthesis of Fmoc-AcOrn-OH

Allyl 2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-5-((tert-butoxycarbonyl)amino) pentanoate (1). Fmoc-Orn(Boc)-OH (1.36 g, 3 mmol) was dissolved in dimethylformamide (DMF) (10 mL) and Cs_2CO_3 (1.96 g, 6 mmol) was added to reaction mixture, followed by slow addition of allylbromide (0.39 mL, 4.5 mmol). The reaction mixture was stirred for 3 h at room temperature (rt). The mixture was diluted with H_2O (10 mL). The aqueous layer was extracted with ethyl acetate (EtOAc) (3 × 15 mL), the combined organic phases were washed with brine (20 mL), dried ($MgSO_4$) and excess solvent

removed *in vacuo*. The crude product was purified by silica gel chromatography (heptane/EtOAc 3:2) to afford **1** as colorless liquid (1.21 g, 82%). δ_H (400 MHz, $CDCl_3$) 7.82–7.71 (2 H, m), 7.65–7.55 (2 H, m), 7.45–7.36 (2 H, m), 7.36–7.28 (2 H, m), 5.90 (1 H, ddt, *J* 16.5, 11.0, 5.8), 5.38–5.20 (2 H, m), 4.65 (2 H, d, *J* 5.8), 4.41 (3 H, d, *J* 7.1), 4.22 (1 H, t, *J* 6.9), 3.14 (2 H, s), 1.79 (2 H, m) and 1.44 (9 H, s); δ_C (100 MHz $CDCl_3$) 172.1, 156.1 (2 × C), 143.9 (2 × C), 141.5 (2 × C), 131.6 (2 × C), 127.9, 127.2, 125.2 (2 × C), 120.1, 119.2, 118.0, 79.5, 67.1, 66.2, 53.8, 47.4, 40.1, 30.1, 28.6 (3 × C) and 26.2.

Allyl 2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-5-acetamidopentanoate (2). 1 (1.2 g, 2.4 mmol) was treated with TFA (5.5 mL, 72 mmol). The reaction mixture was stirred at rt and after 20 min the reaction mixture was diluted with dichloromethane (DCM) (15 mL). The solvent was subsequently removed *in vacuo*. The procedure was repeated three times to afford the primary amine. The crude primary amine was dissolved in DCM (10 mL) and cooled to 0 °C, followed by addition of triethylamine (1 mL, 7.2 mmol) and acetyl chloride (0.205 mL, 2.88 mmol). The reaction was then heated to rt and stirred for another 2 h until all starting material was consumed. The solvent was removed *in vacuo* and the residue was purified by silica gel column chromatography (DCM/methanol (MeOH) 5:1) to afford **2** as a white solid (848 mg, 81% over two steps). δ_H (400 MHz, $CDCl_3$) 7.77 (2 H, dd, *J* 7.5, 1.0), 7.60 (2 H, dd, *J* 7.5, 4.3), 7.44–7.36 (2 H, m), 7.31 (2 H, tt, *J* 7.5, 1.3 Hz), 5.90 (1 H, ddt, *J* 16.5, 11.0, 5.8), 5.68 (1 H, s), 5.49 (1 H, d, *J* 8.2), 5.38–5.22 (2 H, m), 4.65 (2 H, d, *J* 5.8), 4.40 (3 H, m), 4.22 (1 H, t, *J* 6.9), 3.34–3.21 (2 H, m), 1.96 (3 H, s), 1.94–1.67 (2 H, m) and 1.58 (2 H, q, *J* 8.1, 7.4); δ_C (100 MHz, $CDCl_3$) 172.0, 170.3, 143.8 (2 × C), 141.5 (2 × C), 131.5 (2 × C), 127.9, 127.2, 125.2 (2 × C), 120.2, 120.1, 119.3, 67.2, 66.3, 53.7, 47.3, 39.1, 30.4, 25.6 and 23.4; LCMS obs. [M+H] 437.2, calcd. [M+H] 437.2.

2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-5-acetamidopentanoic acid (3). 2 (829 mg, 1.9 mmol) and $Pd(PPh_3)_4$ (220 mg, 0.19 mmol) were dissolved in dry tetrahydrofuran (THF) (50 mL) and placed under nitrogen, morpholine (0.33 mL, 3.8 mmol) was added dropwise and the reaction mixture was stirred at rt for 20 min. The solvent was removed *in vacuo* and the resulting residue was dissolved in DCM (10 mL) and washed with 1M HCl (2 × 5 mL), dried ($MgSO_4$) and excess solvent removed *in vacuo*. The crude product was purified by silica gel chromatography (DCM/MeOH 10:1) to afford **3** as a white solid (241 mg, 32%). δ_H (400 MHz, $CDCl_3$) 7.73 (2 H, d, *J* 7.5), 7.57 (2 H, m), 7.43–7.32 (2 H, m), 7.28 (2 H, dd, *J* 7.5, 1.2), 6.30 (1 H, s), 5.83 (1 H, d, *J* 7.5), 4.36 (3 H, d, *J* 7.2), 4.18 (1 H, t, *J* 7.0), 3.26 (2 H, td, *J* 13.7, 7.5), 1.95 (3 H, s), 1.91–1.65 (2 H, m) and 1.63–1.49 (2 H, m); δ_C (100 MHz, $CDCl_3$) 174.8, 172.0, 156.5, 143.8 (2 × C), 141.4 (2 × C), 127.9 (2 × C), 127.2 (2 × C), 125.2 (2 × C), 120.1 (2 × C), 67.2, 53.6, 47.2, 39.4, 30.0, 25.3 and 23.0; LCMS obs. [M+H] 397.2, calcd. [M+H] 397.21; UPLC >95% purity.

Solid-phase peptide synthesis (SPPS)

Resin loading with hydrazine. 2-Chlorotrityl chloride resin (4.26 mmol, 1.42 mmol g^{-1} loading, Iris Biotech) was washed alternatingly with DMF (3 × 5 mL) and DCM (3 × 5 mL) twice. Resin was swollen in DMF/DCM (1:1) for 30 min and subsequently washed with DMF (3 × 5 mL). 16 mmol NH_2NH_2 and 5 mmol triethylamine (TEA) in DMF was added to the resin and agitated for 30 min. The resin was washed with DMF and the procedure was repeated. After the second coupling, the resin was washed by alternating DMF and DCM and capped using 5 mL DMF/MeOH/N,N-diisopropylethlyamine (DIPEA) (8:1.5:0.5) for 10 min, washed with DMF and repeated once.

Manual coupling of Fmoc-Gly-OH. The resin was washed twice alternating with DMF (3 × 5 mL) and DCM (3 × 5 mL) before flow wash with DCM. 2 eq. of Fmoc-Gly-OH, 4 eq. 2-(1H-7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate (HATU) and 4 eq. DIPEA in DMF were added directly to the resin. After 1 h of coupling, the resin was flow washed with DMF. Subsequently, the resin was capped using 5 mL DMF/MeOH/DIPEA (8:1.5:0.5) for 10 min, washed with DMF and repeated once. Finally, the resin was washed with DCM and dried overnight under vacuum.

Microwave assisted Fmoc-SPPS. All peptides were synthesized in 0.1 mmol scale using the prepared hydrazine linked resin. Automated microwave-assisted SPPS was carried out using a Liberty Blue microwave peptide synthesizer (CEM, Matthews, NC, USA) equipped with a 30 mL Teflon reaction vessel. Fmoc-protected amino acids were made up as 0.2 M solutions in DMF. An activator solution was made up as 0.5 M *N,N'*-diisopropylcarbodiimide (DIC) in DMF with 0.01% DIPEA and an activator base solution was made up as 1 M ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma) in DMF with 0.01% DIPEA. Peptide synthesis was performed using the following settings: deprotection: 150 W, 15 s, 75 °C followed by 30 W, 60 s, 90 °C, coupling: 190 W, 180 s, 75 °C followed by 36 W, 120 s, 90 °C. The introduction of Fmoc-hLys(Boc)-OH and Fmoc-Orn(Ac)-OH in Δ_NPDZ2 hLys and Δ_NPDZ2 AcOrn, respectively, was performed manually using 2 eq. Fmoc-protected amino acid, 2 eq. HATU and 4 eq. DIPEA and agitated for 1 h at rt. The coupling efficiency was monitored by Kaiser Test (Sigma-Aldrich, St. Louis, MO, USA). After the coupling was completed, the resin was capped by treating with 5 mL DMF/MeOH/DIPEA (8:1.5:0.5) for 10 min, washed with DMF and repeated once. The synthesis continued on the peptide synthesizer.

Cleavage of peptides from resin. Peptides were cleaved from resin using a cleavage cocktail containing TFA/triisopropylsilane (TIPS)/H_2O (90:5:5) for 2.5 h at rt. After cleavage, the peptides were precipitated using ice cold diethyl ether and subsequently spun down. The isolated pellet was dissolved in a mixture of buffer A (95% H_2O, 5% ACN, 0.1% TFA) and buffer B (95% ACN, 5% H_2O, 0.1% TFA) and lyophilized.

Purification of peptides. Lyophilized peptides were purified by preparative reverse phase high performance liquid chromatography (RP-HPLC) (Waters, Milford, MA, USA) using a ZORBAX 300SB-C18 PrepHT 21.2 × 250 mm 7 µm column (Agilent, Santa Clara, CA, USA) over a gradient of 20-50% Buffer B over 25 min. 10 mL fractions were collected using an automated fraction collector and pure fractions were pooled and lyophilized. Peptides were characterized by LC-MS and purity determined by UPLC.

Fluorescently labeled peptides were synthesized and purified as previously described.[16]

Recombinant protein expression

DNA subcloning. Codon optimized DNA gene cassettes were purchased from Invitrogen (Carlsbad, CA, USA) containing HindIII and KpnI surrounding the PDZ2 domain comprising residues 155-249 of PSD-95. This insert was cloned into a pRSET expression vector from Invitrogen (Carlsbad, CA, USA) and verified by DNA sequencing (Eurofins MWG Operon, Germany). Two mutations (V178C and Y190W) were introduced by standard site-directed mutagenesis protocol (Stratagene) by primers (forward primer V178C 5'-CTA TCG CGG GTG GTT GTG GTA ATC AGC ATA TTC -3´; reverse primer V178C 5´-GAA TAT GCT GAT TAC CAC AA CCA CCC GCG ATA-3´; forward primer Y190W 5'- GCG ATA ACA GCA TTT GGG TGA CCAA AAT TAT-3´ reverse primer Y190W; 5´-

CAA TAA TTT TGG TCA CCC AAA TGC TGT TAT CGC-3´) . The factor Xa (FXa) sites were introduced by PCR amplification with elongated (+ 16 bp) primers sFXA (5´-NNN ATC GAG GGA AGG TGT NNN-3´) and asFXA thereby inserting four extra amino acids (Ile-Glu-Gly-Arg) next to the V178C mutation.

Protein overexpression. Expressions of PDZ2 domains were generally conducted as previously described.[16] In brief, *E. coli* BL21-DE3 pLys cells were transformed with PDZ2 containing pRSET cDNA and grown over night (ON) in 6 L ZYM5052 medium. The cells were harvested, resuspended and lysed into solid guanidinium hydrochloride (GnHCl) and sodium dihydrogen phosphate giving a final concentration of 6 M and 0.05 M, respectively. pH was adjusted to 7.5 and the protein was purified using 2 × 5 mL HisTraps (GE Healthcare Life Sciences) according to the protocol. To generate the C-terminal fragment of PDZ2, the PDZ2 IEGR construct was dialyzed into 50 mM TRIS, 150 mM NaCl, 1 mM $CaCl_2$, pH 7.5 ON at 4 °C. 1 unit of Factor Xa (HTI technologies, Essex Junction, VT, USA) were added per mg of protein generating the desired Cys-terminal fragment after ON incubation at 4 °C. A reverse HisTrap and RP-HPLC purification was performed afterwards according to previous described protocols.[16]

Ligation and refolding. The lyophilized synthetic peptide (2.0 mmol, 1.5 eq.) was dissolved in 300 µL acidic buffer (6 M GnHCl, 0.2 M sodium phosphate, pH 3.0) and cooled to -10 °C. 60 uL of a 0.2 M $NaNO_3$ solution in acidic buffer (6 M GnHCl, 0.2 M sodium phosphate, pH 3.0) was added dropwise to the peptide solution. After incubation for 20 min, the C-terminal hydrazide was fully converted and the peptide was mixed with 10 mg (1.3 mmol) of the C-terminal protein fragment dissolved in 300 µL ligation buffer (6 M GuHCl, 0.2 NaPi, 60 mM MPAA, 40 mM TCEP, pH 7.1). The ligation was incubated at rt for 2 h and 1 M DTT was added to give a final DTT concentration of 10 mM. ÄKTA

Explorer 100 Air (GE Healthcare) equipped with a Superdex HiLoad 16/600 75 pg (GE Healthcare) gel filtration column equilibrated with 50 mM sodium phosphate, pH 7.4. The ligation mixture was injected and the full-length PDZ domain collected after approximately 84 mL. The purified protein was concentrated in Amicon Ultra-15 cutoff 10 kDa (EMD Millipore, Billerica, MA, USA) and concentration was determined using absorbance at $\lambda_{280\ nm}$ measured on a NanoDrop 1000 spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Purity was determined by UPLC and SDS-PAGE gel.

Fluorescence polarization. Dilution series of the PDZ2 domains were prepared in 50 mM sodium phosphate and ranged from 1-500 μM. TAMRA labeled peptides (400 nM) were mixed and diluted 1:1 with the different concentrations of PDZ2 in a black flat-bottom 396 well-plate (Corning Life Science, Corning, NY, USA). 11 measurements were made for each binding curve using a Safire plate-reader (Tecan) at excitation/emission wavelengths of $\lambda_{545\ nm}/\lambda_{575\ nm}$. Each experiment was performed in triplicate. The concentration of the fluorescent probe was 200 nM in all the FP experiments. The instrumental Z-factor was adjusted to obtain maximum signal and the G-factor calibrated from the blank and background wells to give an initial milli-polarization (mP) of 20. The background signal was subtracted from the obtained fluorescent polarization signal and fitted to one-sited binding model.

Circular dichroism. Circular dichroism (CD) experiments were performed using an Olis DSM 100 CD Spectrometer (Olis Inc., Bogart, GA, USA) in 1 mm quartz cuvettes (Starna Scientific Ltd., Essex, England). CD spectra were obtained using protein samples at a concentration of 200 μg/mL and data was collected in millidegrees of ellipticity (θ), which was converted into mean residue ellipticity (Θ_{MRE}) by the equation:

$$\theta_{MRE}[deg \times cm^2 \times dmol^{-1}] = \frac{\theta[\mathrm{mdeg}] \times MRW}{l[mm] \times c[mg\ mL^{-1}]} \qquad MRW = \frac{\text{Molar weight } [g\ mol^{-1}]}{\text{Number of backbone amides}}$$

where *l* is the pathway of the cuvette in mm and the *MRW* is the mean residue weight. The obtained spectrum is an average of 3 measurements at each wavelength.

Acknowledgements

This research was financed by the Danish Council for Independent Research – Medical Sciences.

10.1002/cbic.201600322

Figures

Fig. 1. Structures and sequences of PDZ domains and introduced amino acids. (A) X-ray crystal structures of PSD-95 PDZ2 (orange), PSD-95 PDZ3 (light blue) and syntrophin-PDZ (grey) domains display a similar fold. The conserved basic residues coordinate a water molecule and form hydrogen bonds to the C-terminal carboxylate of the ligand. (B) The three PDZ domains all contain a conserved Lys/Arg residue (▼, bold) positioned in the β1-β2 loop. (C) Proteogenic and non-proteogenic amino acids introduced instead of Lys165.

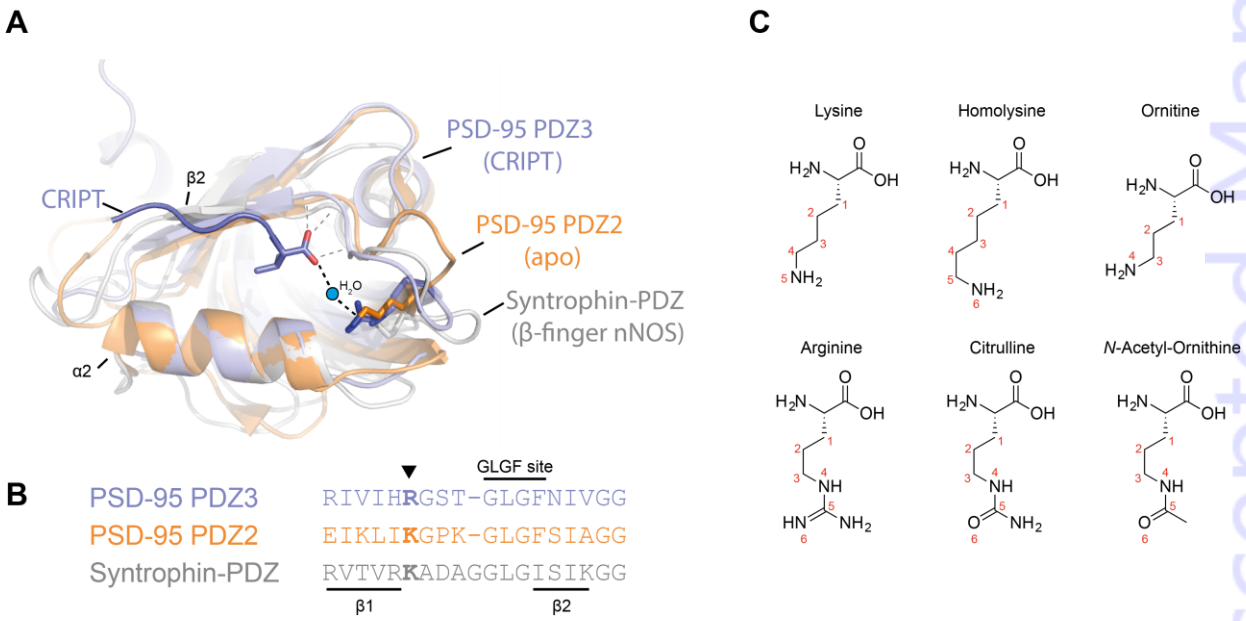
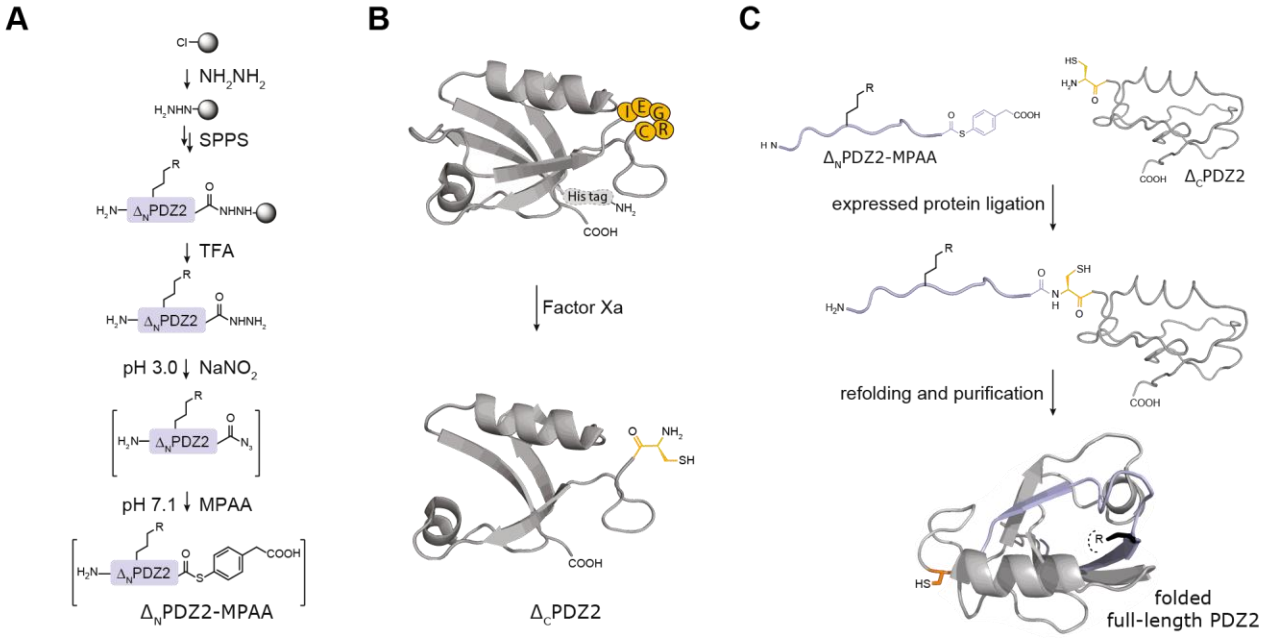
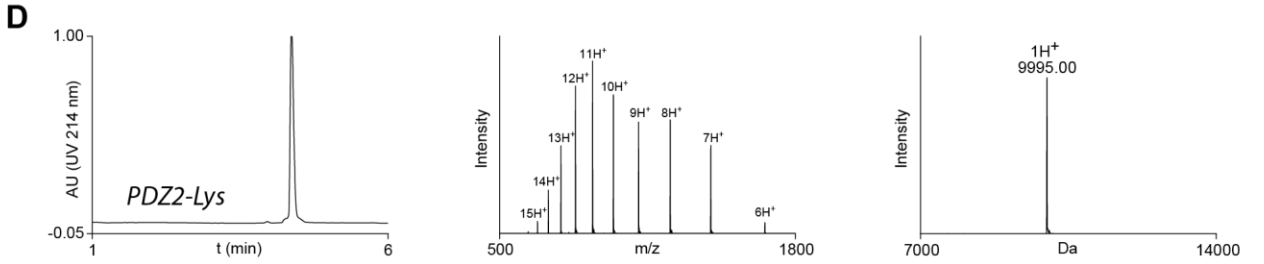
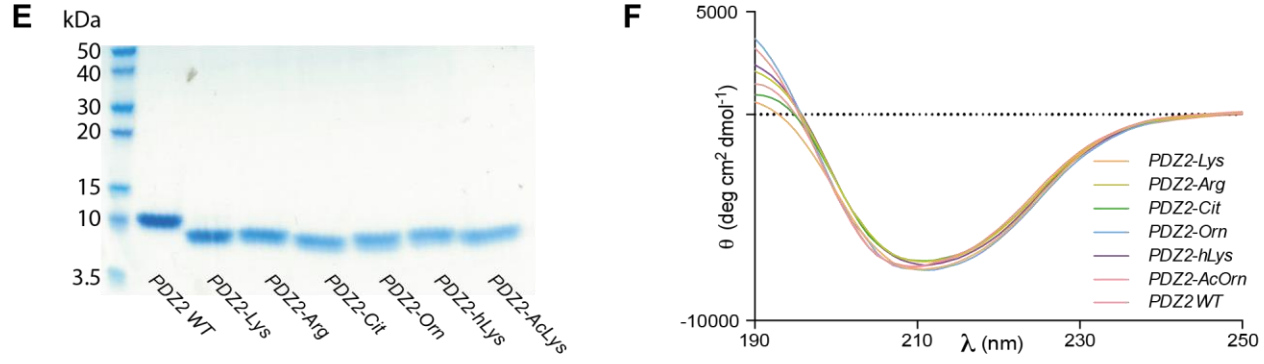


Fig. 2. Semisynthesis of PDZ2 domains introducing Lys/Arg analogues. (A). Synthesis of the N-terminal peptide fragment containing Lys/Arg analogues of PSD-95 PDZ2. The peptide thioester was prepared using Fmoc-based SPPS. Hydrazine was loaded on a 2-chlorotrityl chloride resin, followed by chain elongation and cleaved using TFA. The C-terminal hydrazide was converted into a thioester *in situ* by oxidation with $NaNO_2$ followed by thiolysis with 4-mercaptophenylacetic acid (MPAA). (B) The recombinant C-terminal fragment was obtained by expression of a fusion protein containing a histidine tag (His tag) and a FXa site (IEGRC). After expression in *E. Coli*, the protein was enzymatically digested with FXa, yielding the Δ_CPDZ2 fragment with a N-terminal Cys. (C) Ligation of the synthetic peptide and recombinant protein fragments, Δ_NPDZ2 and Δ_CPDZ2 respectively, was performed under denaturing conditions in 6 M GnHCl. After ligation, the semisynthetic PDZ2 was refolded and purified by gel filtration (>95% pure). (D) LC-MS characterization of semisynthetic PDZ2-Lys; chromatogram, mass trace and deconvoluted mass spectra. (E) 16% SDS-PAGE gel displaying the purified expressed and semisynthetic PDZ2 domains. Left lane is the protein ladder (Novex Sharp Pre-stained Protein Standard, Thermo). (F) CD spectra of the six Lys165 PDZ2 mutants and the expressed PDZ2 WT, demonstrating highly similar secondary structures. The spectra were recorded three times between 250–190 nm at 20 °C with 1 nm increments. Each curve is an average of three measurements of the same sample.

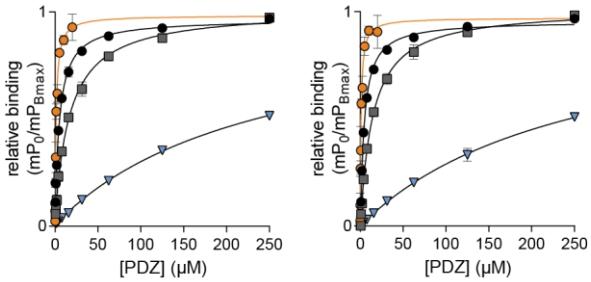






10.1002/cbic.201600322

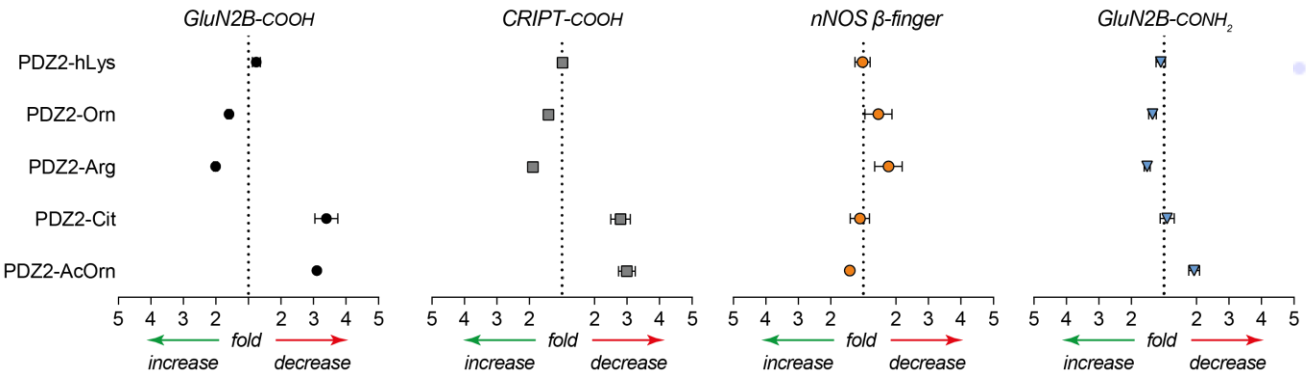
Fig. 3. Ligand binding to recombinantly expressed and semisynthetic PDZ2 domains. Saturation curves from FP assays for the expressed PDZ2 domain (upper left) and semisynthetic PDZ2 domain (upper right). Increasing amounts of PDZ2 domains were titrated against TAMRA-labeled peptides; up to 250 μM for the C-terminal peptide ligands and 25 μM for the cyclic nNOS β-finger. Assays were performed in triplicates and K_D values were determined from 50% of mP_{Bmax}.



Ligand	PDZ2 WT	PDZ2-Lys
GluN2B-COOH	4.9 ± 0.23	5.4 ± 0.25
CRIPT-COOH	17 ± 0.84	17 ± 1.0
nNOS β-finger	1.2 ± 0.18	1.2 ± 0.14
GluN2B-$CONH_2$	314 ± 33	240 ± 16

10.1002/cbic.201600322

Fig. 4. Effect on peptide binding of introducing Arg/Lys analogues into PSD-95 PDZ2. The fold change was found by dividing the K_D values for the four peptide ligands and the semisynthetic PDZ domains by the K_D value obtained from PDZ2-Lys. Affinity increase is displayed as left shifted fold change whereas affinity decrease as right shifted fold change.



References

[1] M. R. Arkin, Y. Tang, J. A. Wells, *Chem. Biol.* **2014**, *21*, 1102-1114.
[2] S. Gul, K. Hadian, *Expert Opin. Drug Discovery* **2014**, *9*, 1393-1404.
[3] L. N. Makley, J. E. Gestwicki, *Chem. Biol. Drug Design* **2013**, *81*, 22-32.
[4] H. Jubb, A. P. Higueruelo, A. Winter, T. L. Blundell, *Trends Pharmacol. Sci.* **2012**, *33*, 241-248.
[5] B. T. Seet, I. Dikic, M. M. Zhou, T. Pawson, *Nat. Rev. Mol. Cell Biol.* **2006**, *7*, 473-483.
[6] S. Yellaboina, A. Tasneem, D. V. Zaykin, B. Raghavachari, R. Jothi, *Nucleic Acids Res.* **2011**, *39*, D730-735.
[7] C. Y. Zheng, G. K. Seabold, M. Horak, R. S. Petralia, *Neuroscientist* **2011**, *17*, 493-512.
[8] C. Oliva, P. Escobedo, C. Astorga, C. Molina, J. Sierralta, *Develop. Neurobiol.* **2012**, *72*, 57-72.
[9] E. Kim, M. Sheng, *Nat. Rev. Neurosci.* **2004**, *5*, 771-781.
[10] W. Feng, M. Zhang, *Nat. Rev. Neurosci.* **2009**, *10*, 87-99.
[11] K. K. Dev, *Nat. Rev. Drug Discov.* **2004**, *3*, 1047-1056.
[12] C. N. Chi, A. Bach, K. Strømgaard, S. Gianni, P. Jemth, *BioFactors* **2012**, *38*, 338-348.
[13] Z. Khan, M. Lafon, *Curr. Med. Chem.* **2014**, *21*, 2632-2641.
[14] H. J. Lee, J. J. Zheng, *Cell Commun. Signal.* **2010**, *8*, 8.
[15] D. A. Doyle, A. Lee, J. Lewis, E. Kim, M. Sheng, R. MacKinnon, *Cell* **1996**, *85*, 1067-1076.
[16] S. W. Pedersen, S. B. Pedersen, L. Anker, G. Hultqvist, A. S. Kristensen, P. Jemth, K. Strømgaard, *Nat. Commun.* **2014**, *5*, 3215.
[17] J. N. Eildal, G. Hultqvist, T. Balle, N. Stuhr-Hansen, S. Padrah, S. Gianni, K. Strømgaard, P. Jemth, *J. Am. Chem. Soc.* **2013**, *135*, 12998-13007.
[18] M. A. Stiffler, J. R. Chen, V. P. Grantcharova, Y. Lei, D. Fuchs, J. E. Allen, L. A. Zaslavskaia, G. MacBeath, *Science* **2007**, *317*, 364-369.
[19] R. Tonikian, Y. Zhang, S. L. Sazinsky, B. Currell, J. H. Yeh, B. Reva, H. A. Held, B. A. Appleton, M. Evangelista, Y. Wu, X. Xin, A. C. Chan, S. Seshagiri, L. A. Lasky, C. Sander, C. Boone, G. D. Bader, S. S. Sidhu, *PLoS Biol.* **2008**, *6*, e239.
[20] B. Z. Harris, B. J. Hillier, W. A. Lim, *Biochemistry* **2001**, *40*, 5921-5930.
[21] B. J. Hillier, K. S. Christopherson, K. E. Prehoda, D. S. Bredt, W. A. Lim, *Science* **1999**, *284*, 812-815.
[22] B. H. Chang, T. S. Gujral, E. S. Karp, R. BuKhalid, V. P. Grantcharova, G. MacBeath, *Chem. Biol.* **2011**, *18*, 1143-1152.
[23] R. R. Penkert, H. M. DiVittorio, K. E. Prehoda, *Nat. Struct. Mol. Biol.* **2004**, *11*, 1122-1127.
[24] H. Tochio, Q. Zhang, P. Mandal, M. Li, M. Zhang, *Nat. Struct. Mol. Biol.* **1999**, *6*, 417-421.
[25] H. Tochio, Y. K. Mok, Q. Zhang, H. M. Kan, D. S. Bredt, M. Zhang, *J. Mol. Biol.* **2000**, *303*, 359-370.
[26] O. Kocher, G. Birrane, K. Tsukamoto, S. Fenske, A. Yesilaltay, R. Pal, K. Daniels, J. A. Ladias, M. Krieger, *J. Biol. Chem.* **2010**, *285*, 34999-35010.
[27] C. N. Chi, A. Engström, S. Gianni, M. Larsson, P. Jemth, *J. Biol. Chem.* **2006**, *281*, 36811-36818.
[28] S. H. Gee, S. Quenneville, C. R. Lombardo, J. Chabot, *Biochemistry* **2000**, *39*, 14638-14646.

[29] A. Bach, B. H. Clausen, M. Møller, B. Vestergaard, C. N. Chi, A. Round, P. L. Sørensen, K. B. Nissen, J. S. Kastrup, M. Gajhede, P. Jemth, A. S. Kristensen, P. Lundström, K. L. Lambertsen, K. Strømgaard, *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, 3317-3322.
[30] M. D. Hill, R. H. Martin, D. Mikulis, J. H. Wong, F. L. Silver, K. G. Terbrugge, G. Milot, W. M. Clark, R. L. Macdonald, M. E. Kelly, M. Boulton, I. Fleetwood, C. McDougall, T. Gunnarsson, M. Chow, C. Lum, R. Dodd, J. Poublanc, T. Krings, A. M. Demchuk, M. Goyal, R. Anderson, J. Bishop, D. Garman, M. Tymianski, ENACT trial investigators, *Lancet. Neurol.* **2012**, *11*, 942-950.
[31] S. Seedorff, C. Appelt, M. Beyermann, P. Schmieder, *Biochem. Biophys. Res. Commun.* **2010**, *395*, 535-539.
[32] C. Hoppmann, S. Seedorff, A. Richter, H. Fabian, P. Schmieder, K. Rück-Braun, M. Beyermann, *Angew. Chem. Int. Ed*. **2009**, *48*, 6636-6639.
[33] G. M. Fang, Y. M. Li, F. Shen, Y. C. Huang, J. B. Li, Y. Lin, H. K. Cui, L. Liu, *Angew. Chem. Int. Ed.* **2011**, *50*, 7645-7649.
[34] A. Bach, C. N. Chi, T. B. Olsen, S. W. Pedersen, M. U. Roder, G. F. Pang, R. P. Clausen, P. Jemth, K. Strømgaard, *J. Med. Chem.* **2008**. *51*, 6450-6459.
[35] Y. Ivarsson, *FEBS Lett.* **2012**, *586*, 2638-2647.
[36] B. Z. Harris, F. W. Lau, N. Fujii, R. K. Guy, W. A. Lim, *Biochemistry* **2003**, *42*, 2797-2805.
[37] S. Sokalingam, G. Raghunathan, N. Soundrarajan, S. G. Lee, *PLoS One* **2012**, *7*, e40410.
[38] H. Cui, A. Hayashi, H. S. Sun, M. P. Belmares, C. Cobey, T. Phan, J. Schweizer, M. W. Salter, Y. T. Wang, R. A. Tasker, D. Garman, J. Rabinowitz, P. S. Lu, M. Tymianski, *J. Neurosci.* **2007**, *27*, 9901-9915.
[39] K. Schuh, S. Uldrijan, M. Telkamp, N. Rothlein, L. Neyses, *J. Cell Biol.* **2001**, *155*, 201-205.
[40] G. M. Riefler, B. L. Firestein, *J. Biol. Chem.* **2001**, *276*, 48262-48268.
[41] B. L. Firestein, D. S. Bredt, *J. Biol. Chem.* **1999**, *274*, 10545-10550.

10.1002/cbic.201600322

Table of Contents

PDZ domains are mediators of numerous protein-protein interactions, and understanding the molecular details these interactions are of fundamental importance. Here, we applied expressed protein ligation to generate six semisynthetic PDZ domains containing proteogenic and non-proteogenic amino acids. This allowed us to highlight the importance of a conserved Lys/Arg residue in PSD-95 PDZ2.