MOLECULAR DETERMINANTS FOR THE COMPLEX BINDING SPECIFICITY OF THE PDZ DOMAIN IN PICK1*

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Running title: PICK1 binding specificity

PICK1 (Protein Interacting with C Kinase 1) contains a single PDZ domain known to mediate interaction with the C-termini of several receptors, transporters, ion channels and kinases. In contrast to most PDZ domains, the PICK1 PDZ domain interacts with binding sequences classifiable as type I (terminating in S/T-X-Φ; Φ, any residue) as well as type II (Φ-X-Φ*; Φ, any hydrophobic residue). To enable direct assessment of the affinity of the PICK1 PDZ domain for its binding partners we developed a purification scheme for PICK1 and a novel quantitative binding assay based on fluorescence polarization (FP). Our results showed that the PICK1 PDZ domain binds the type II sequence presented by the human dopamine transporter (-WLKV) with an almost 15-fold and >100-fold higher affinity than the type I sequences presented by protein kinase Ca (-QSAV) and the β2-adrenergic receptor (-DSLL), respectively. Mutational analysis of Lys83 in the αB1 position of the PDZ domain suggested that this residue mimics the function of hydrophobic residues present in this position in regular type II PDZ domains. The PICK1 PDZ domain was moreover found to prefer small hydrophobic residues in the C-terminal P(0) position of the ligand. Molecular modeling predicted a rank order of (Val > Ile > Leu) that was verified experimentally with up to ~16-fold difference in binding affinity between a valine and a leucine in P(0). The results define the structural basis for the unusual binding pattern of the PICK1 PDZ domain by substantiating the critical role of the αB1 position (Lys83) and of discrete side chain differences in position P(0) of the ligands.

With over 540 domains in more than 300 different proteins, PDZ (PSD-95/Discs-large/ZO-1 homology) domains are among the most common protein domains in the human genome (1-3). They mediate cellular protein-protein interactions and serve important roles in protein targeting and in the assembly of protein complexes (1; 4). PICK1 (Protein Interacting with C Kinase 1) contains a single N-terminal PDZ domain and was originally identified as an interaction partner for protein kinase Ca (PKCα) (5). In addition to its N-terminal PDZ domain, PICK1 contains a coiled-coil domain (residue 145-165 in rat PICK1) that is believed to mediate dimerization of PICK1(6), followed by a region bearing homology to Arfaptin 1 and 2 (residue 152-362), and a C-terminal acidic cluster (residue 381-389).

Although PICK1 was named for its interaction with PKCα it rapidly became clear that it had multiple interaction partners. At the current stage, the PDZ domain of PICK1 has been shown to mediate interaction with a broad range of proteins including receptor tyrosine kinases, ionotropic glutamate receptors of the AMPA and...
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kainate subtypes, metabotrophic glutamate receptors, ion channels, G-protein coupled receptors, aquaporins, transmembrane transporters and ADP-ribosylation factors (Table 1 and ref. (7)). PICK1 has been proposed to play a key role in clustering several of these protein ligands in the plasma membrane (8-11), or to target them to the perinuclear regions of the cell (10; 12-14). Moreover, given the ability of PICK1 to dimerize, it has been proposed that PICK1 is capable of recruiting PKCζ to its target proteins (6; 15; 16). Nevertheless, the physiological role of the interaction between PICK1 and several of its ligands remains unclear.

PDZ domains are ~90 residues long and consist of six β-strands (βA to βF) and two α-helices, αA and αB. In canonical PDZ interactions the PDZ domain binds the C-terminus of the interaction partner in an elongated groove as an antiparallel β-strand between the αB1 helix and the βB1 sheet, termed the PDZ binding groove (17; 18). PDZ domain interactions have been divided into three major classes; type I interactions in which the ligand terminates with (S/T)-X-Φ, type II interactions in which the ligand terminates with Φ-X-Φ (Φ is any hydrophobic residue), and type III interactions in which the ligand terminates with D/E-X-Φ (17; 18). For all three types of interactions, the side chain of the C-terminal residue (P(0)) fits tightly into a hydrophobic pocket in the domain lined by four conserved hydrophobic residues (19; 20). In type I interactions the hydroxyl group of the serine or threonine in the P(-2) position of the ligand forms a hydrogen bond with a highly conserved histidine in the αB1 position of the PDZ domain (19). In type II interactions the hydrophobic P(-2) residue interacts with a hydrophobic residue in the αB1 position in PDZ domain (17) whereas in type III interactions the negatively charged residue in P(-2) can hydrogen bond with a tyrosine in the αB1 position of the PDZ domain (18; 21).

The PDZ domain of PICK1 does not seem to conform to this classification scheme, because it binds both classical type I ligands (e.g. PKCζ and GluR5b2h) and type II ligands (e.g. GluR2 and DAT), as well as ligands without a classical PDZ interaction sequence (e.g. ARF1 and 3) (Table 1). Very few PDZ domains exhibit such mixed specificity, and the only three other PDZ domains that have been shown to bind class I and II sequences are the CIPP PDZ3 (22), the single PDZ domain of Erbin (23), and the syntenin PDZ2 (24). A high-resolution structure has not yet been obtained for the PICK1 PDZ domain, but the similarities identified in structure-assisted alignment suggest that the structure of this domain should be similar to that of other known PDZ domains. A unique feature is that the αB1 residue, which is thought to be critical for determining the specificity of the PDZ interaction, is a lysine (Lys83)(20). Interestingly, it was recently shown that a specific mutation in the carboxylate-binding loop of the PICK1 PDZ domain results in loss of interaction with the type II binding sequence of the AMPA receptor subunit GluR2, whereas binding of the type I ligand PKCζ appears unaffected (25). This suggests different binding modes of type I versus type II ligands, but does not explain their structural basis.

Here we have aimed to elucidate the determinants for the unusual binding pattern of the PICK1 PDZ domain, in the structural context of a molecular model of the protein. To this end we have developed a purification scheme for PICK1 and a quantitative binding assay based on FP that enables direct assessment of the affinity of the PICK1 PDZ domain for binding partners. Using this assay we show that the PICK1 PDZ domain has a more than 10-fold preference for the sequence presented by the human dopamine transporter (hDAT), that contains a prototypical type II binding sequence (-WLKV), over the type I sequence presented by PKCζ (-QSAV). Secondly, we provide evidence using a series of combined amino acid substitutions in the PDZ domain and in the PDZ ligands, that Lys83 in the αB1 position of the PICK1 PDZ domain is playing an important role in determining this preference. Using both the computational modeling and experimental techniques, we elucidate the nature of the interaction of hydrophobic residues in the P(0) position, and their role in the binding specificity of the PICK1 PDZ domain.

**EXPERIMENTAL PROCEDURES**

**Molecular biology** - The entire coding region of rat PICK1 (residues 2-416) was amplified from a
pCINEO vector by PCR using *pfu* polymerase according to the instructions by the manufacturer (Stratagene, La Jolla, CA). The primers used introduced a 5’ restriction site for MunI and 3’ restriction site for AvrII. The PCR fragment was cleaved with MunI and AvrII and cloned into the reading frame of the pET41a vector (Novagen, Madison, WI) producing an N-terminally GST fusion of PICK1. The K83H and K83V point mutations were generated by two-step PCR using *pfu* polymerase. All constructs were confirmed by restriction enzyme mapping and sequenced. The pET41 PICK1 WT, K83H and K83V vectors were transformed into the protease deficient *E.coli* strain BL21 DE3 (Novagen) carrying the pLysS plasmid.

**Purification procedure for GST fusion proteins** - Bacteria transformed with plasmids encoding the appropriate constructs were inoculated O/N in 50 mL LB media, diluted into 1L LB media, and grown to OD<sub>600</sub> 1.0 (2-3hrs). Expression of the fusion protein was induced with isopropyl-β-D-thiogalactopyranoside (500µM) for 3hrs at 30ºC. Cells were harvested and frozen at -80ºC until purification. The pellets were thawed and resuspended in buffer A (50mM Tris pH 7.4, 125mM NaCl, 20µg/mL DNAse I, 1mM DTT) and 1x bacterial protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The lysate was frozen at -80ºC and thawed and then thoroughly triturated to reduce viscosity. The lysate was clarified by centrifugation (rotor SS-34, 18000 rpm, 48000 x g, 30 min). The supernatant was incubated with glutathione-sepharose beads (Amersham Biosciences AB, Uppsala, Sweden) under slow rotation for 90 minutes at 4ºC. The beads were pelleted at 1000g for 10 minutes and washed in buffer B (50mM Tris pH 7.4, 125mM NaCl, 0.1% TX-100, 1mM DTT) by three batch washes. The protein was separated from the GST domain by cleavage with thrombin protease (Novagen) in buffer B. The protein was eluted on ice until use (usually the same day). Gels were stained with GelCode Blue Stain Reagent (Pierce Biotechnology) in order to inspect size, integrity and purity of the protein.

**Peptide synthesis and design** – Peptides were synthesized corresponding to the 13 C-terminal residues of the dopamine transporter (DAT), protein kinase C α (PKCα) and the β<sub>2</sub> adrenergic receptor (β<sub>2</sub> AR) as well as peptides carrying single point mutations in these sequences. Peptides of the wild-type (WT) sequences were labeled with Oregon Green 488 by adding N-terminal cysteine for coupling of the fluorophore. To this end the cysteine in the P-7 position of the β<sub>2</sub> AR sequence was changed to a serine. All peptides were purchased from Schafer-N, Copenhagen, Denmark as 95% pure. They were purified by reverse phase high-pressure liquid chromatography RP-HPLC and the identity was verified by mass spectrometry. Peptides were dissolved in buffer B and stored in aliquots at -20ºC until use.

**Fluorescence Polarization (FP) Plate Assay** – Saturation binding isotherms for the PDZ – peptide interactions were determined by titrating a fixed amount of Oregon green labeled peptide (40nM) with an increasing amount of PICK1 in a final volume of 100 µl. The experiments were carried out in black 96 well microtiter plates (Corning, NYC, NY) treated to reduce non-specific adsorption of peptide and protein to the plate. The system was allowed to reach equilibrium (15 min) and changes in fluorescence polarization (FP) were read in a Chameleon FP plate reader (Hidex, Turku, Finland) in the FP mode using a 488 nm excitation filter and a 535nm long pass emission filter. Each measurement is an average of 100 flashes and is carried out four times. FP was calculated according to the equation FP = (I<sub>V</sub> – g * I<sub>H</sub>)/(I<sub>V</sub> + g * I<sub>H</sub>) and equilibrium saturation binding isotherms were constructed by plotting FP versus the concentration of PICK1. To determine K<sub>d</sub>, a curve was fitted with the equation Y=FP<sub>b</sub> * X/(K<sub>d</sub>*X), where FP<sub>b</sub> is the maximal value of FP reached by complete saturation. Competition binding experiments were carried out in the same format as the saturation binding experiments using a fixed concentration of fluorescently labeled peptide (40nM) and a fixed
non-saturating concentration of purified PICK1, and an increasing concentration of unlabelled peptide. Equilibrium competition binding isotherms are constructed by plotting FP versus the concentration of unlabelled peptide. To determine $K_i$, a curve was fitted to the equation $FP = FP_f + ((FP_f − FP_b) * [R])/(K_d * (1 + X/K_i) + [R])$, with $FP_f$ and $FP_b$ being the FP value of the free and bound peptide, $[R]$ the concentration of PICK1 and $K_d$ the apparent dissociation constant determined from parallel saturation experiments. $K_i$, $FP_b$, and $FP_f$ were treated as free parameters. All equilibrium binding isotherms were repeated using at least three independent purifications ($n=3$). The replicates were fitted separately using experimentally determined values for $K_d$ and $R_t$, and average $K_i$'s ± SE are given. Extrapolated data are indicated by italics.

**RESULTS**

Dual specificity of PICK1 PDZ domain – The PDZ domain of PICK1 is one of very few PDZ domains that can bind both type I and type II sequences, and no obvious binding motifs are apparent in the reported ligands for PICK1 (Table 1). Out of 29 PICK1 PDZ interaction partners representing different binding sequences, 6 have a canonical type I PDZ binding sequence with a serine or threonine in the third to last position from the C-terminus (P(-2)), whereas 12 have canonical type II PDZ sequences with a hydrophobic residue in the P(-2) position (Table 1). Four additional interaction partners have hydrophobic P(-2) residues, but do not conform to the type II classification due to unusual residues in the P(0) position (Table 1). Indeed, the preference for the C-terminal P(0) residue also shows unusual promiscuity. PICK1 seems to prefer small and hydrophobic residues with 13 of the ligands presenting a C-terminal valine, 6 presenting an isoleucine, 3 presenting an alanine but none presenting a leucine (Table 1). However, unusual residues such as cysteine, lysine and methionine are also present in the P(0) position.

To explore the structural basis for this complex binding specificity of PICK1 we expressed the full-length protein in *E. Coli* and established a purification procedure. The purified protein was used in a binding assay based on Fluorescence Polarization (FP). In the assay we used peptides corresponding to the 13 C-terminal residues of PKCβ, which has a type I PDZ binding sequence —QSAV, and of the human dopamine transporter (hDAT), which has a type II PDZ binding sequence -WLKV, both of which are known to bind PICK1 (5; 8). A peptide corresponding to the 13 C-terminal residues of the
β2 AR was included as a control for the specificity of the saturation binding assay. Like the PKCα sequence, the β2 AR sequence contains a type I PDZ binding sequence (-DSLL), but unlike the PKCα sequence it was believed not to bind PICK1. The 13-mer peptides used for saturation binding experiments all had an N-terminal cysteine that allowed fluorescent labeling with the sulphydryl-reactive fluorophore Oregon Green maleimide. In the binding assay we took advantage of the predicted decrease in rotational diffusion of the fluorescently labeled peptides upon binding to a larger protein. Thus, we could detect the decrease in rotational diffusion upon binding of the peptides to PICK1 as an increase in FP. The increase in FP is illustrated by the saturation binding experiments shown in Fig. 1 in which a fixed concentration of fluorescently labeled peptide was titrated with an increasing amount of PICK1 (Fig. 1). The saturation binding experiments suggested that the DAT peptide bound with highest affinity to the purified preparation of PICK1 with an apparent Kd value of ~1 µM. The PKCα peptide bound with an almost 10-fold lower apparent affinity than the DAT peptide, whereas the β2 AR peptide bound with very low apparent affinity (Fig. 1). To further validate the specificity of the interaction between the peptides and PICK1 we mutated Ala87 in PICK1 to leucine. The larger side chain of the mutant residue was according to the model described below predicted to fill out the hydrophobic pocket normally occupied by the side chain of the P(-2) residue in the ligand. Consistent with this idea the mutation essentially eliminated binding of the both the DAT and PKCα peptide (data not shown).

Next, we carried out competition binding experiments in which fixed concentrations of PICK1 and of the fluorescently labeled peptide were titrated with an increasing amount of non-labeled peptide (Fig. 2A). In agreement with the saturation binding experiments, the unlabeled DAT peptide was more than one order of magnitude more potent in displacing the fluorescently tagged tracer than was the unlabeled PKCα peptide. In further agreement with the saturation binding experiments, the β2 AR peptide was much less potent than the two other peptides. From the competition binding experiments it was possible to calculate Kᵢ values for the interaction of the peptides with PICK1 (Table 2). We should note that the calculated Kᵢ values represent the most accurate estimate of the actual affinities. Thus, the absolute affinities obtained in the saturation binding assay might be affected both by the attached fluorophore and by the ratio between functional and non-functional protein in different purified preparations. As described in Experimental Procedures, the unavoidable depletion in the competition assay is accurately accounted for in the reported Kᵢ values.

Mimicking a canonical type II interaction

- Figure 3 shows a model of PICK1 binding a peptide corresponding to eight C-terminal residues of the DAT (-TLHRWLV). The peptide binds in an extended fashion on a largely hydrophobic surface, and a number of specific interactions with side chains in the PDZ domain can be identified (Fig. 3A). Of particular interest is the interaction with the αB1 position, which is occupied by a lysine (Lys83), a feature unique among all known PDZ domains. In the model, the aliphatic chain of Lys83 is part of a hydrophobic pocket that also includes Val84, Val86 and Ala87 and accommodates the leucine at the P(-2) position of the ligand (Fig. 3A). According to the model, there is, however, no predicted interaction interactions between residues in the PDZ domain and the charged nitrogen head group of Lys83; hence, it would be predicted that the aliphatic chain of Lys83 acts as a hydrophobic residue present in regular type II PDZ domains and, accordingly, that its charge would not contribute much to affinity. To test this hypothesis and thus to mimic a canonical type II interaction, the atypical lysine in the αB1 position was substituted into valine, a hydrophobic residue commonly seen at this position (e.g. in CASK (34)). The substitution (K83V) was predicted to fully preserve the hydrophobic pocket (Fig. 3B). Moreover, the presence of the additional hydrophobic residues in the P(-2) pocket (Val84, Val86, Ala87) was predicted to enable a number of favorable hydrophobic interactions of the DAT peptide in the K83V mutant as well (Fig. 3B). In agreement with these predictions, the experimental data showed that the affinity for the DAT peptide increased slightly (2-3-fold; Fig. 2B and Table 2).
The affinity of the K83V mutation for the PKCα peptide was similar to the DAT peptide increased (~6-fold). This caused the PDZ domain to be more promiscuous than the wild type (Fig. 2B and Table 2). Notably, this observation was consistent with our molecular model of PICK1 binding the PKCα peptide. As shown in Fig. 4A, the peptide is displaced away from the αB helix in WT PICK1 and thereby positioned in a manner slightly different from the typical placement occupied by the DAT peptide. In the K83V mutant, however, the PKCα peptide recovers the typical placement in the groove (Figure 4B) and conceivably with it acquires a higher affinity.

Mimicking a canonical type I interaction - Next, we sought to mimic a canonical type I interaction by substituting the αB1 lysine (Lys83) with a histidine. This mutation swapped the specificity of the PDZ domain, so that the affinity for the PKCα peptide (K_i= 0.54±0.07 µM) was much higher than that of the DAT peptide (K_i=21±2 µM) (Fig. 2C and Table 2). Note that the largest change in affinity was the increase for PKCα (~60-fold), whereas the affinity of DAT only decreased 10-fold (Table 2). Interestingly, the affinity for the β2 AR peptide which has a type I sequence at its C-terminus, like PKCα did not increase (Fig. 2C and Table 1). This suggests that despite the presence of an apparent optimal histidine in the αB1 position, the peptide fits poorly into the PDZ binding groove.

The findings are consistent with direct observations from our molecular model of PICK1. Regarding the DAT peptide, substitution of Lys83 with histidine was predicted to preserve the hydrophobic pocket although with some distortion (Fig. 3B), which would explain the moderate decrease in affinity of the DAT peptide for the K83H mutant (Table 2). With respect to the PKCα peptide, the model includes a hydrogen bond between the histidine in position 83 and P(-2) that corresponds to a conventional type I docking of the peptide in the binding groove (Fig. 4C). Thus, the models indicate that the observed increases in the affinity of the PKC peptide for the mutant constructs have different origins. For the K83V mutant, it is due to a significant reorientation of the peptide backbone (compare Figure 4A and 4B), while for the K83H mutant the increased affinity is due to the addition of a direct interaction of the P-2 and αB1 side chains.

The dependence of the specificity mediated by the αB1 residue in the PICK1 PDZ domain, on the interaction with the P(-2) residue in the ligand was directly supported by the observed changes in affinity that resulted from the interchange of the P(-2) residues in the DAT and PKCα peptides. For the DAT peptide, substitution of the P(-2) leucine into a type I serine caused the expected interchange in specificity i.e., the affinity for the PICK1 WT decreased 18-fold compared to the WT peptide, whereas the affinity for the PICK1 K83H increased 19-fold compared to the WT peptide (Fig. 5 and Table 3). For the PKCα peptide, we observed the expected opposite effect upon substitution of the P(-2) serine into a leucine, i.e. the affinity for the PICK1 WT was increased 19-fold compared to the WT peptide, whereas the affinity for PICK1 K83H was decreased 19-fold compared to the WT peptide (Fig. 5 and Table 3).

Assessing the role of the P(0) residue of the ligand – There are several examples of PDZ domains that display a notable selectivity for specific hydrophobic residues at the P(0) position. For example, it has long been known that the PDZ domains of the MAGUK proteins PSD-95, PSD-93, SAP102, and SAP97 bind almost exclusively to peptides with Val at P(0) (35; 36). Conversely, the NHERF PDZ domains are prototypical examples of PDZ domains that select for peptides with a Leu at P(0). Additional examples of P(0) selective domains are AF-6 (Val) and Shank (Leu) (see http://icb.med.cornell.edu/services/pdz/start).

For other PDZ domains for which many ligands are known (e.g. those found in GIPC, CASK and Veli) no apparent selectivity have been discerned. To gain insight into the role the P(0) position for the binding specificity of PDZ domains we probed computationally the affinity of PICK1 for peptides terminating in various hydrophobic residues. As a control, we first attempted to reproduce the preference of MAGUK PDZ domains (in this case the third PDZ domain of PSD-95) for peptides terminating in a valine and the preference of NHERF for peptides terminating in a leucine. Importantly, a simple minimization–based approach using the Charmm Par22 force field (30) and a recently developed
The present study provides the first direct quantitative assessment of binding affinities between the PDZ domain of PICK1 and its binding partners. Using this new development, we provide novel insight into the structural context of the unusual binding properties of the PICK1 PDZ domain from molecular models of the constructs. The measured binding affinities revealed an affinity of the C-terminal DAT peptide of 2.3±0.1µM, which is comparable to the affinity of other PDZ interactions measured with in-solution methods (36-40). Studies using solid-phase assays have reported significantly higher affinities for PDZ domain interactions (17; 41); but increasing evidence suggests that these solid-based methods - such as assays based on the Biacore platform or ELISA assays - tend to overestimate intrinsic thermodynamic affinities due to dense coating of wells or chips (36; 42; 43). In contrast to the DAT peptide, the affinity of the PKCa peptide, which is a typical type I ligand, was almost 15-fold lower (33±2µM). This suggests that despite the apparent promiscuity, the PDZ domain of PICK1 may have a preference for type II ligands. In fact, this is
indirectly supported as well by the markedly higher number of known type II ligands for PICK1 as compared to type I ligands (Table 1). It should also be noted that an affinity of 30-35 µM might be considered only borderline for physiological interactions (36; 44) and is thus surprising given that PICK1 originally was identified as a protein interacting with PKCα. This raises the question whether other structural elements in PKCα could contribute to the binding in addition to the 13 C-terminal residues contained in the peptide analyzed in this study. Previous to this study there were, however, no data supporting this possibility for the interaction between PKCα and PICK1; hence, the only available data are based on a yeast two-hybrid assay that supports a canonical PDZ interaction in which the extreme C-terminus, including the four last residues of PKCα, plays a key role (45). For other well-characterized canonical PDZ domain interactions in general there is also no evidence for involvement of residues beyond the last ~10 C-terminal residues. Nonetheless, this does not exclude the putative existence of additional interactions between the two proteins that remain to be identified.

The PICK1 PDZ domain is unique in that it contains a lysine in the αB1 position (Fig. 3). Domains that preferentially bind type I ligands usually have a histidine at this position, which forms a hydrogen bond with the P(-2) Ser/Thr of the ligand. In contrast, domains that preferentially bind type II ligands usually have a hydrophobic residue in this position, e.g. a valine as in CASK (34). We found that mutation of Lys83 to valine (K83V) causes a moderate increase in affinity for the DAT peptide (Table 2 and Fig. 2B). This suggested, in agreement with our molecular model, that the aliphatic chain of the αB1 lysine is likely to serve a function corresponding to hydrophobic residues present in this position in regular type II PDZ domains, and that the residue charge is not directly responsible for peptide binding at least for the peptides tested. Mutation of Lys83 to valine also increased the affinity for the PKCα peptide. Thus, K83V appears more promiscuous than the wild type suggesting that the PICK1 promiscuity cannot be attributed to the atypical lysine in the αB1 position. The gain of affinity of the PKCα peptide for the K83V mutant is furthermore consistent with our model indicating that in WT the PKCα peptide is displaced away from the αB helix compared to the typical peptide binding mode displayed by the DAT peptide, but recovers the typical placement in K83V (Fig. 4). Of interest, the data are in overall agreement also with a previously proposed model for the dual binding of the type I sequence in PKCα and the type II sequence in GluR2 by PICK1 (6).

We also mutated Lys83 to a histidine causing a switch in preference from the DAT peptide to the PKCα peptide (Fig. 2). To the best of our knowledge, this is the first example of a switch in type I/type II preference for a PDZ domain based on a single amino acid substitution. The switch was mostly due to a substantial increase in affinity for the PKCα peptide rather than a decrease in affinity for the DAT peptide. The increase in affinity of the PKCα peptide is most likely due to a hydrogen bond between the inserted histidine and P(-2) Ser in this peptide, as seen in typical type I interactions (17; 19). Strong evidence for such an interaction was supported by interchanging the P(-2) residue of the DAT and PKCα peptides and testing them against WT PICK1 and the K83H mutation (Fig. 5). As expected, substitution of leucine with serine in P(-2) of the DAT peptide increased the affinity for K83H, but decreased the affinity for WT PICK1. In contrast, substitution of serine with leucine in P-2 of the PKCα peptide resulted in decreased affinity of K83H and increased affinity for the WT (Fig. 4).

In addition to the C-termini of the DAT and PKCα, we tested a peptide corresponding to the C-terminus of the β2 adrenergic receptor (β2 AR). The β2 AR peptide displayed low affinity for the PICK1 PDZ domain; nonetheless, the affinity was still higher than that of the DAT peptide with the C-terminal aspartate substitution (Fig. 2). This is not surprising because the peptide presents a canonical class I sequence. Interestingly, a simple substitution in the β2 AR peptide of the P(0) leucine with the predicted optimal valine (β2 AR SAV) increased the affinity around 4-fold, i.e. to an affinity similar to that of the PKCα peptide (PKCα SAV) (Table 5). This indicates a role for the P(0) position in determining the preference of PICK1 among different type I ligands. A
surprising finding was that neither the $\beta_2$ AR WT peptide ($\beta_2$ AR SLL), nor the $\beta_2$ AR SLV mutant displayed the major affinity increase for K83H that we observed for PKC$\alpha$ SAV (Table 5). Thus, although PKC$\alpha$ SAV and $\beta_2$ AR SLV have almost the same affinity for PICK1 WT, their mode of interaction with K83H must be substantially different. The difference does not involve the leucine in P(-1) of the $\beta_2$ AR peptide, since substitution of the P(-1) residues of the two peptides does not change the affinities (data not shown). Accordingly, it is likely that structural elements upstream the canonical PDZ binding sequence are responsible for this difference.

To elucidate the role of the P(0) position in the interacting ligand for the binding affinity to a given PDZ domain we used a computational approach to predict the preferred hydrophobic side chains in this position. The calculations were validated by correct prediction of the P(0) preferences for two well-characterized PDZ domains, PSD-95 PDZ3 (Val-selective) and NHERF PDZ1 (Leu-selective). The calculations for PICK1 predicted a Val $>$ Ile $>$ Leu order of preference, which corresponds to the frequency of occurrence of these residues in known PICK1 interaction partners listed in Table 1 (these show a clear preference for valine (13 times) and isoleucine (6 times) as compared to leucine (0)). In our binding assay we were able to confirm this order of preference predictions quantitatively (Fig. 6, 7 and Table 1). For example, changing the P(0) valine to a leucine caused an almost 5-fold decrease in affinity of the PKC$\alpha$ peptide and a 16-fold decrease in affinity of the DAT peptide (Fig. 6, 7 and Table 1). This indicates that the hydrophobic side chain required in position P(0) for optimal affinities of both type I and type II peptides, must also be of a well-defined size to fit in the hydrophobic pocket. Thus, the residue in the P(0) position of the ligand is a critical determinant for PICK1 binding selectivity of both type I and type II ligands. Nonetheless, the role of P(0) for the binding specificity of PICK1 is still not as remarkable as that demonstrated previously in canonical type I selective PDZ domains such as in PDZ 1 and 2 of PSD-95 and SAP102 (36). Hence, PICK1 is also relatively promiscuous with regard to the P(0) position.

The results show that the energetic cost of substituting P(0) is somewhat context specific. For example, the substitution of P(0) with the smaller alanine was much less energetically costly in the PKC$\alpha$ peptide than in the DAT peptide (Fig. 7). This agrees well with the fact that the three known PICK1 ligands with an alanine in the P(0) position are all type I ligands (Table 1). The context specificity of the P(0) substitutions also supports the inferences from our molecular model that the type I binding mode of PICK1 might be different from the type II binding mode (Fig. 4). This agrees as well with the recent findings by Dev et al. (25) showing that substitution of Lys27 in the carboxylate binding loop of the PICK1 PDZ domain was tolerated much better by the PKC$\alpha$ peptide than by the type II ligand from GluR2 (-SVKI). Notably, however, our current data strongly suggest that the divergence is due to a different mode of insertion into the PDZ binding groove, rather than to separate binding sites for the two peptides proposed in (25). Thus the PKC$\alpha$ peptide can compete for the fluorescently tagged DAT peptide, both peptides are affected by substitution of the $\alpha$B1 residue in the PDZ domain, and both peptides are affected similarly by substitution of the C-terminal valine.

The primary conundrum that motivated this paper was the apparent lack of consensus among the ligands reported to interact with PICK1 and, in particular, the ability of PICK1 to bind both type I and type II sequences. Such promiscuity has been reported for only a few other PDZ domains; the CIPP PDZ3 domain(22), the PDZ domain of Erbin (23), and the syntenin PDZ2 domain (24). At this stage, substantial information is available regarding the structural basis for the promiscuity of Erbin and syntenin(24; 46). In contrast to PICK1, Erbin contains a histidine in the $\alpha$B1 position and an NMR study has suggested that type I peptides bind in the traditional way with the P(-2) serine of the ligand forming a hydrogen bond with the histidine in the $\alpha$B1 position (46). Our data indicate that such a hydrogen bond is not formed by P(-2) in PICK1 unless the WT $\alpha$B1 position is substituted with a histidine. Thus, the WT PDZ domain of PICK1 must recognize type I ligands through a binding mode distinct from that of Erbin. The difference between Erbin and PICK1 is further underlined by...
the fact that Erbin binds primarily type I sequences whereas PICK1 predominantly binds type II sequences (Table 1).

Our data also suggest that the mechanism of type I/type II duality is likely to be different between PICK1 and syntenin. According to the crystal structure of syntenin, the so-called type I interaction does not involve the P(-2) serine in the bound IL5Rα ligand (-D-S-V-F). Instead, binding affinity is obtained through the P(0) phenylalanine that perfectly fills the corresponding hydrophobic pocket in the PDZ domain (termed S0 in the paper using the terminology for site specificity in proteases) and through the P(-1) valine that occupies a novel S1 pocket formed partly by residues in the βB-sheet (24). A similar scenario is unlikely for type I ligands (e.g. PKCα) binding to PICK1 because the P(-1) position of PKCα is an alanine, which, due to its small size, can hardly provide much binding energy in a hypothetical S1 pocket. Furthermore, the P(0) valine can be substituted to an alanine with only a slight decrease of the affinity (less than 2 fold) (Fig. 6 and 7). This means that PICK1 can bind with relatively high affinity a peptide with alanines in the two positions (P(0) and P(-1)) that are the most important for syntenin binding of the IL5Rα ligand. Taken together, this binding mode is very unlikely for PICK1 although additional experiments are needed to fully address this question. For syntenin type II ligands, such as syndecan (T-N-F-Y-A), the crystal structure showed that they dock very similarly to the canonical description for type II interactions; nonetheless, the structure also showed that the C-terminal alanine (P(0)) is too small to fill the S0 pocket. This is compensated for by the P(-1) tyrosine fitting into a novel hydrophobic S1 pocket and by the canonical class II P(-2) interaction. However, this mechanism is incompatible with our PICK1 data that demonstrated a key role of P(0) in the binding affinity of type II ligands, with a major loss of affinity from substituting the P(0) valine with alanine (24).

In summary, we have obtained new insight into the structural basis for the molecular recognition between PICK1 and its binding partners by establishing a convenient and reliable binding assay based on FP, and interpreting the results in the structural context of molecular models. The assay allowed for a direct testing of specific predictions from a structural context. Most importantly, the use of this approach in conjunction with a series of modified peptides and mutations in the PICK1 PDZ domain provided evidence that the mechanisms underlying the ability of the PICK1 PDZ domain to recognize both type I and type II ligands conceivably is unique and distinct from the mechanism described for the few other PDZ domains showing type-I/type II duality. Given the reliability of the assay established here, it is to be expected that polarization–based assays should prove highly useful not only for further structural elucidation of PDZ interactions but in a variety of other drug discovery processes aimed at identifying small-molecule inhibitors of this type of protein-protein interactions.
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FOOTNOTES

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The abbreviations used are: PICK1, protein interacting with C-kinase-1; DAT, Dopamine transporter; PKCα, protein kinase Ca; β2 AR, β2 adrenergic receptor; WT, wild type; FP, fluorescence polarization.

FIGURE LEGENDS

Fig. 1 PICK1 saturation binding. Fluorescently labeled peptides corresponding to the C-terminal 13 residues of DAT, PKCα13 and β2 AR (DAT13 OrG, PKCα13 OrG and β2 AR13 OrG) 40 nM were titrated with increasing amounts of purified WT PICK1 protein. After 15 minutes of incubation, fluorescence polarization (FP) values were determined as a direct read-out of peptide binding to PICK1. Data are representative of at least five similar experiments.

Fig. 2 PICK1 competition binding. A, Competition binding to WT PICK1 of Oregon Green labeled DAT peptide (DAT13 OrG) with unlabeled peptides corresponding to the 13 C-terminal residues of DAT, PKCα and β2 AR. B, Competition binding to K83V of Oregon Green labeled DAT peptide (DAT13 OrG) with unlabeled peptides corresponding to the 13 C-terminal residues of DAT, PKCα and β2 AR. C, Competition binding to K83H of Oregon Green labeled DAT peptide (DAT13 OrG) with unlabeled peptides corresponding to the 13 C-terminal residues of DAT, PKCα and β2 AR. Data are representative of at least three similar experiments. For the experiments DAT13OrG (40nM) was incubated with a fixed sub-saturating amount of either WT, K83H or K83V and titrated with increasing amounts of indicated unlabeled peptides. After 15 minutes of incubation, fluorescence polarization (FP) values were determined. The K_i of the DAT peptide was 2.3±0.1µM (n=12), and the K_i of the PKCα peptide was 33±2µM (n=9).

Fig. 3. Model of PICK1 with the C-terminal DAT peptide. The PICK1 model was obtained using MOEDELLER (26), with the peptide-bound PDZ domains of InaD (1IHJ.pdb, 28% identity) and syntrophin (1QAV.pdb, 28% identity) as templates. Loop structures were refined with MODLOOP server (27), and side-chain orientations were optimized by SCWRL program (28). The peptide (TLRHWLKV) was docked using a simulated-annealing method implemented in CHARMM[4] (Niv and Weinstein, manuscript in preparation). A, Ribbon representation of wild type PICK1: Residues in the peptide binding-site are colored cyan. The residues within the black circle form the P(-2) pocket. Peptide residues are colored orange. B and C, The P(-2) pocket in PICK1 mutants K83H (B) and K83V (C). D, Surface representation of PICK1. Basic, acidic, polar and non-polar residues are colored blue, red, pink and white, respectively. Peptide residues are colored orange. Figure prepared with VMD (http://www.ks.uiuc.edu/Research/vmd/).

Fig. 4. Model of PICK1 mutants with the C-terminal PKCα peptide. The models were obtained as described in Fig. 3. A, The PKCα peptide (shown in purple) binding to WT PICK1 (in pink, with K83 side chain shown in purple). B, PKCα peptide (in red) binding to K83V mutant (in orange, V83 side chain shown in red). C, PKCα peptide (brown) complexed with K83H mutant (tan, H83 side chain shown in brown). All models are superimposed on WT PICK1 (cyan, K83 side chain blue) binding the DAT peptide (shown in blue).
Fig. 5. Relative affinities of DAT and PKCa peptides after interchanging the P(-2) residues. **Upper panel,** Relative changes in affinity at WT PICK1 and at K83H of the 13 residues C-terminal DAT peptide upon substituting Leu in P(-2) position with a Ser as found in PKCa in this position. Data are means ± S.E. of 3 independent experiments. **Lower panel,** Relative changes in affinity at WT PICK1 and at K83H of the 13 residues C-terminal PKCa peptide upon substituting Ser in P(-2) position with a Leu as found in DAT in this position. Data were obtained from competition binding experiments using DAT13OrG (40nM) as tracer and a fixed sub-saturating amount of WT PICK1 or K83H. The affinities (K_i values) were determined from non-linear regression analysis of binding data as described in Experimental Procedures. Data are means ± S.E. of 3 independent experiments.

Fig. 6. Changes in affinity upon substitution of the P(0) position of DAT, PKCa and the β2 AR. **A,** Competition binding to WT PICK1 of unlabeled C-terminal DAT peptides (13 residues) containing indicated substitutions in the P(0) position. **B,** Competition binding to WT PICK1 of unlabeled C-terminal PKCa peptides (13 residues) containing indicated substitutions in the P(0) position. **C,** Competition binding to WT PICK1 of unlabeled C-terminal β2 AR peptides (13 residues) containing indicated substitutions in the P(0) position. Data are representative of at least three similar experiments. For the experiments Oregon Green labeled DAT peptide (DAT13OrG, 40nM) was incubated with a fixed sub-saturating amount of WT PICK1 and titrated with increasing amounts of indicated unlabeled peptides. After 15 minutes of incubation, fluorescence polarization (FP) values were determined.

Fig. 7. PICK1 preference for small hydrophobic residues in the P(0) position. **A,** Relative decreases in affinity at WT PICK1 of C-terminal DAT (black columns), PKCa (grey columns) or β2 AR (white columns) peptides containing indicated substitutions in the P(0) position. **B,** Relative decreases in affinity at K83H of C-terminal DAT (black columns), PKCa (grey columns) or β2 AR (white columns) peptides containing indicated substitutions in the P(0) position. Data were obtained from competition binding experiments using DAT13OrG (40nM) as tracer and a fixed sub-saturating amount of WT PICK2 or K83H. The affinities (K_i values) were determined from non-linear regression analysis of binding data as described in Experimental Procedures. Data are means ± S.E. of 3 independent experiments.

Fig 8. Models of the interaction of the residue at the P(0) position, with the hydrophobic pocket between secondary structure elements αB and βB of the PICK1 PDZ domain. **Top,** valine, middle; isoleucine, bottom; leucine. For clarity, only the P(0) residue is shown for the peptide binding in the pocket, in the energetically optimal positions for each peptide. White lines represent interactions (distance < 4.0Å) between PICK1 and P(0) atoms. All atoms involved in interactions are indicated with white-rimmed circles. The higher number of contacts with PICK1 atoms for valine (11) than for isoleucine (8) or leucine (8) is in agreement with the preference of PICK1 for peptides with valine at the P(0) position. It is noteworthy that all side-chain atoms of valine and isoleucine are involved in interactions, but the Cβ and Cγ atoms of leucine are not. This explains the calculated and measured reduction in affinity of PICK1 for peptides with leucine at the P(0) position, compared to either isoleucine or valine.
### Table 1.
Proteins shown to interact with the PDZ domain of PICK1

| Ligand name                      | Sequence         | Type | Methods                  | Ref.   |
|----------------------------------|------------------|------|--------------------------|--------|
| Protein Kinase Cα                | NPQFVPILQSAV     | I    | YTH, co-loc, co-IP       | (5)    |
| Ephrin receptor A7               | AQMLHLHGTGQV     | II   | YTH, GST-PD, Co-IP       | (9)    |
| Ephrin B1                        | MPPQSPANIYKV     | II   | YTH, GST-PD, Co-IP       | (9)    |
| Ephrin receptor B2               | MRAMQNQSQVEV     | II   | YTH, GST-PD, Co-IP       | (9)    |
| Muscle specific kinase           | ERMCERAGTVSV     | II   | YTH                      | (9)    |
| GluR2 (AMPA)                     | EGVNYGIESVKI     | II   | YTH, GST-PD              | (10; 47) |
| GluR3/4 (AMPA)                   | EGVNYGTESVKI     | II   | YTH, GST-PD              | (47)   |
| GluR4C (AMPA)                    | EGVNYGTESIKI     | II   | YTH                      | (9)    |
| GluR5b (KAR)                     | CHQRTQHKETVA     | I    | YTH, GST-PD              | (48)   |
| GluR6 (KAR)                      | FNDRRLPGKEMTA    | I    | YTH Weak, GST-PD         | (48)   |
| mGluR7A                          | AKKKYVSYNLLVI    | II   | YTH, GST-PD weak         | (49; 50) |
| mGluR7B                          | QKSVTWYTIPTV     | ?    | YTH, GST-PD weak         | (49; 50) |
| Anionic Exchanger 1              | EGRDDEVAMPV      | ?    | YTH                      | (51)   |
| Anionic Exchanger 2              | EGVDEYNEMPV      | ?    | YTH                      | (51)   |
| Aquaporin 1                      | ADDINSRVEKPK     | ?    | YTH weak, GST-PD         | (51)   |
| Aquaporin 2                      | LHSPQSLPRGSA     | I    | YTH                      | (51)   |
| Aquaporin 9                      | ENNLEKHELSEM    | ?    | YTH                      | (51)   |
| ARF1 GTPase                      | GLDWLSNQRNQK     | ?    | YTH                      | (52)   |
| ARF3 GTPase                      | GLDWLANQNLNEK    | ?    | YTH                      | (52)   |
| Dopamine transporter             | EVRQFTLRHVKTV    | II   | YTH, GST-PD, FP, Co-IP   | (8)    |
| Norepinephrine transp.           | DIRQFQLQHLA1     | II   | YTH strong, Co-IP        | (8)    |
| Serotonin transporter            | TEIPCGDIRNAV     | ?    | YTH weak                 | (8)    |
| ERBB2/HER2 RTK                   | AENPEYLGLDPVF    | II   | YTH, GST-PD, Co-IP       | (53)   |
| ERBB4/HER4 RTK                   | LPPPPYHRHTNV     | I    | YTH                      | (53)   |
| PrPR GPCR                        | APHGQNMVSVWI     | II   | Co-IP, Co-loc            | (54)   |
| BNaC1/ASIC2a                     | LQTALGFLTEIA     | ?    | YTH, GST-PD, Co-IP       | (12)   |
| BNaC2/ASIC1a                     | HHPAPRTSSDDIC    | ?    | YTH, Co-IP               | (12)   |
| Netrin receptor UNC5H1           | PDAGLFTVSEAC     | ?    | YTH, GST-PD, Co-IP       | (55)   |
| CAR cell adhesion                | VMIPQAQSKGSI     | I    | Co-IP, Co-loc            | (56)   |

YTH, yeast two-hybrid; co-IP, co-immunoprecipitation; co-loc, co-localization; GST-PD, gluthathione-S-transferase fusion protein pull down, FP; fluorescence polarization. Note that some of these sequences are evolutionary closely related meaning that information provided is redundant to some extent.
Table 2.
Binding affinities of C-terminal peptides for PICK1 WT and mutants.

|                | PICK1 WT   |                      | PICK1 K83V |                      | PICK1 K83H |                      |
|----------------|------------|----------------------|------------|----------------------|------------|----------------------|
|                | Mean $K_i$ | SE  | n  | Mean $K_i$ | SE  | n  | Mean $K_i$ | SE  | n  |
| DAT WT         | 2.3        | 0.1 | 12 | 1.02        | 0.02 | 3  | 21          | 2   | 12 |
| PKCα WT        | 33.0       | 2   | 9  | 5.5         | 0.4  | 3  | 0.54        | 0.07 | 9  |
| $\beta_2$ WT   | 245        | 6   | 3  | 230         | 40   | 3  | 210         | 20   | 3  |

Binding affinities were obtained from competition binding experiments in which Oregon Green labeled DAT peptide (DAT13 OrG) was incubated with a fixed sub-saturating amount of either PICK1, K83H or K83V and titrated with increasing amounts of unlabeled peptides. After 15 minutes of incubation, fluorescence polarization (FP) values were determined. $K_i$ values were determined from non-linear regression analysis of binding data as described in Experimental Procedures. Data are means ± S.E. of indicated number experiments.
Table 3.
Binding affinities of C-terminal peptides for PICK1 WT and mutants.

|                | PICK1 WT |                | PICK1 K83H |                |
|----------------|----------|----------------|------------|----------------|
|                | Mean $K_i$ (µM) | SE | n | Mean $K_i$ (µM) | SE | n |
| DAT LKV        | 2.3      | 0.1            | 12         | 21             | 2  | 12 |
| DAT SKV        | 42       | 7              | 3          | 1.10           | 0.06 | 3 |
| PKCα SAV       | 33.0     | 2              | 9          | 0.54           | 0.07 | 9 |
| PKCα LAV       | 1.7      | 0.1            | 3          | 10.4           | 0.6  | 3 |

Binding affinities were obtained from competition binding experiments in which Oregon Green labeled DAT peptide (DAT13 OrG) was incubated with a fixed sub-saturating amount of either PICK1, K83H or K83V and titrated with increasing amounts of unlabeled peptides. After 15 minutes of incubation, fluorescence polarization (FP) values were determined. $K_i$ values were determined from non-linear regression analysis of binding data as described in Experimental Procedures. Data are means ± S.E. of indicated number experiments.
Table 4

*Predicted Binding energies of PSD-95, NHERF and PICK1 with substituted hydrophobic P(0) residues.*

| PDZ    | Peptide | $E_i$     | $\Delta E_i$ |
|--------|---------|-----------|--------------|
| PSD-95-3 | KQTSV   | -144.52   | 0.00         |
|        | KQTSI   | -142.36   | +2.16        |
|        | KQTSL   | -137.39   | +7.13        |
| NHERF-1- | QDTRV   | -173.51   | +1.83        |
|        | QDTRI   | -175.28   | +0.06        |
|        | QDTRL   | -175.34   | 0.00         |
| PICK1  | HWLKV   | -112.02   | 0.00         |
|        | HWLKI   | -108.06   | +3.96        |
|        | HWLKL   | -105.98   | +6.04        |

Interaction energy differences of peptides for PSD-95-3, NHERF-1 or PICK1. Peptides interacting with PSD-95-3 correspond to the C-terminus of CRIPT, peptides interacting with NHERF-1 correspond to the C-terminus of CFTR, while peptides interacting with PICK1 correspond to the DAT C-terminus. Energy differences are reported in kcal/mol.
Table 5.
Binding affinities of C-terminal peptides substituted in P(0) for PICK1 WT and mutants.

|        | PICK1 WT | PICK1 K83V | PICK1 K83H |
|--------|----------|------------|------------|
|        | Mean $K_i$ (µM) | SE | n | Mean $K_i$ (µM) | SE | n | Mean $K_i$ (µM) | SE | n |
| DAT LKV | 2.3 | 0.1 | 12 | 1.02 | 0.02 | 3 | 21 | 2 | 12 |
| DAT LKI | 9.5 | 0.9 | 3 | ND | 24 | 5 | 3 |
| DAT LKL | 37 | 5 | 3 | ND | 64 | 11 | 3 |
| DAT LKA | 49 | 3 | 3 | ND | 90 | 17 | 3 |
| DAT LKD | - | - | - | - | - | - | - |
| PKCα SAV | 33 | 2 | 9 | 5.5 | 0.4 | 3 | 0.54 | 0.07 | 9 |
| PKCα SAI | 77 | 7 | 3 | ND | 1.46 | 0.04 | 3 |
| PKCα SAL | 166 | 15 | 3 | ND | 4.6 | 0.3 | 3 |
| PKCα SAA | 40 | 3 | 3 | ND | 0.34 | 0.01 | 3 |
| $\beta_2$ SLL | 245 | 6 | 3 | 230 | 40 | 3 | 210 | 20 | 3 |
| $\beta_2$ SLV | 63 | 8 | 6 | 170 | 20 | 3 | 31 | 4 | 6 |

Binding affinities were obtained from competition binding experiments in which Oregon Green labeled DAT peptide (DAT13 OrG) was incubated with a fixed sub-saturating amount of either WT PICK1, K83H or K83V and titrated with increasing amounts of unlabeled peptides. After 15 minutes of incubation, fluorescence polarization (FP) values were determined. $K_i$ values were determined from non-linear regression analysis of binding data as described in Experimental Procedures. Data are means ± S.E. of indicated number experiments. ND = not determined.
Fig. 1

![Graph showing the effect of PICK1 concentration on mP for different receptors: DAT OrG, PKCα OrG, and β2 AR OrG.](http://www.jbc.org/)
Fig. 5

A

PICK1 WT

mP

DAT LKV
DAT SKV
PKC SAV
PKC LAV

B

PICK1 K83H

mP

DAT LKV
DAT SKV
PKC SAV
PKC LAV

Log [Peptide] in M
Fig. 6
Fig. 7

A

Fold decrease in affinity relative to Val

WT

DAT
PKC
β2 AR

Ala
Val
Ile
Leu

B

Fold decrease in affinity relative to Val

K83H

DAT
PKC
β2 AR

Ala
Val
Ile
Leu

Residue in P(0)
Molecular determinants for the complex binding specificity of the PDZ domain in pick

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