Molecular Determinants for the Complex Binding Specificity of the PDZ Domain in PICK1*

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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 (ST)XΦ; Φ, any residue) as well as type II (ΦXΦ; 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. 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 a 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 (LysαB1) 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/Disc-large/ZO-1 homology)1 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, 2). 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 (PKCa) (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 PKCa, 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 L-α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate subtypes, metabotropic glutamate receptors, ion channels, G protein-coupled receptors, aquaporins, transmembrane transporters, and ADP-ribosylation factors (see Table I 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 PKCαs 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 αB helix and the βB 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)XΦ, type II interactions in which the ligand terminates with ΦXΦ (Φ is any hydrophobic residue), and type III interactions in which the ligand terminates with (D/E)XΦ (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 the PDZ domain (17), whereas in type III interactions the negatively

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‡ The abbreviations used are: PDZ, (PSD-95-Disc-large-ZO-1 homology); PICK1, protein interacting with C-kinase-1; DAT, dopamine transporter; PKCa, protein kinase Ca; β2AR, β2 adrenergic receptor; WT, wild type; FP, fluorescence polarization; GST, glutathione S-transferase.

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charged residue in P(2) can hydrogen bond with a tyrosine in the aB1 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. PKCa and GluR5(2)) and type II ligands (e.g. GluR2 and dopamine transporter (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 aB1 residue, which is thought to be critical for determining the specificity of the PDZ interaction, is a lysine (Lys(28)) (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 -o-amino-3-hydroxy-5-methyl-4-isoxazol propionic acid (AMPA) receptor subunit GluR2, whereas binding of the type I ligand PKCa 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 fluorescence polarization (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 DAT (hDAT), which contains a prototypical type II binding sequence (WALKV), over the type I sequence presented by PKCa (QSAV). Second, we provide evidence using a series of combined amino acid substitutions in the PDZ domain and in the PDZ ligands, that Lys(28) in the aB1 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-terminal GST fusion of PICK1. The pET41 PICK1 WT, K83H, and K83V vectors were transformed into the protease-deficient Escherichia 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 overnight in 50 ml of LB media, diluted into 1 liter of LB media, and grown to an OD of 1.0 (2–3 h). Expression of the fusion protein was induced with 0.1 mM isopropyl-β-d-thiogalactopyranoside (500 μM) for 3 h at 30 °C. Cells were harvested and frozen at −80 °C until purification. The pellets were thawed and resuspended in buffer A (50 mM Tris, pH 7.4, 125 mM NaCl, 20 μg/ml DNase I, 1 mM dithiothreitol) and 1× bacterial protease inhibitor mixture (Sigma). The lysate was frozen at −80 °C and thawed and then thoroughly trituated to reduce viscosity. The lysate was clarified by centrifugation (rotor SS-34, 18,000 rpm, 48,000 × g, 30 min). The supernatant was incubated with glutathione-Sepharose beads (Amersham Biosciences AB, Uppsala, Sweden) under slow rotation for 90 min at 4 °C. The beads were pelleted at 1,000 × g for 10 min and washed in buffer B (50 mM Tris, pH 7.4, 125 mM NaCl, 0.1% Triton X-100, 1 mM diithiothreitol) 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). Samples of 25 μl were taken from the protein solution for determination of protein concentration and SDS-PAGE.

**Protein Determination** was carried out using the BCA Protein Assay Reagent kit (Pierce, Rockford, IL) according to the manufacturer's protocol using bovine serum albumin as standard. Gels were stained with GelCode Blue Stain Reagent (Pierce) to inspect size, integrity, and purity of the protein.

**Peptide Synthesis and Design**—Peptides were synthesized correspondingly to the 13 C-terminus residues of the DAT, PKCa, and the β2 adrenergic receptor (β2AR) 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 β2AR 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 and the identity was verified by mass spectrometry. Peptides were dissolved in buffer B and aliquots at −20 °C until purification. A K83V vectors were transformed into the protease-deficient strain BL21 DE3 (Novagen, Madison, WI) producing an N-terminal GST fusion of PICK1.

**FP Plate Assay**—Saturation binding isotherms for the PDZ-peptide interactions were determined by titrating a fixed amount of Oregon Green-labeled peptide (40 nm) 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, New York) treated to reduce nonspecific adsorption of peptide and protein to the plate. The system was allowed to reach equilibration (15 min) and changes in FP were read in a Chameleon FP plate reader (Hidex, Turku, Finland) in the FP mode using a 488-nm excitation filter and a 535-nm 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 = [C × (1 − g × 1P0 + g × 1P)] and equilibrium saturation binding isotherms were constructed by plotting FP versus the concentration of PICK1. To determine a curve was fitted with the equation: FP(T) = FP(0) + f([R]p × (1 + X/Kf))([R]p × (1 + X/Kf)), where FP(T) 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 (40 nm) and a fixed non-saturating concentration of purified PICK1, and an increasing concentration of unlabeled peptide. Equilibrium competition binding isotherms are constructed by plotting FP versus the concentration of unlabeled peptide. To determine a curve was fitted with the equation: FP(T) = FP(0) + f([R]p × (1 + X/Kf))([R]p × (1 + X/Kf)), where FP(T) is the maximal value of FP reached by complete saturation. The replicates were fitted separately using experimentally determined values for Kassp and Rb, and average Kc values ± S.E. are given. Extrapolated data are indicated by italics in tables.

**Computational Analysis and Molecular Modeling**—Models of the PICK1-peptide complexes were developed as follows: the PICK1 model was built using the MODELLER program (26) based on the structures of the highly similar PDZ domains of InaD and syntrophyn (28% identity) in their peptide-bound forms, which served as templates. Loop regions were optimized with MODLOOP (27), and optimal side chain rotamers were found using SCWRL (28). Peptides were docked with a simulated annealing based approach that was shown to dock 5–8-amino acid long peptides to x-ray structures with root mean square deviation <2 Å, and to homology models with root mean square deviation <3 Å compared with the corresponding crystal structures.2 After docking, the structures of the peptide, the PDZ domain, and the complex were minimized for 300 steps, and the interaction energy (ΔE) was calculated as the difference in non-bonded energy of the free peptide and the PDZ domain versus the non-bonded energy of the complex according to Equation 1.

\[
E_r = (E_{r\text{free}} + E_{\text{complex}}) - (E_{r\text{free}} + E_{\text{complex}}) = (E_{r\text{free}} + E_{\text{complex}}) - (E_{r\text{free}} + E_{\text{complex}})
\]

2 Y. Niv and H. Weinstein, manuscript in preparation.
**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 II sequences, and no obvious binding motifs are apparent in the reported ligands for PICK1 (Table I). 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 I). Four additional interaction partners have hydrophobic P(−2) residues, but do not conform to the type II classification because of unusual residues in the P(0) position (Table I). 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 16 ligands presenting a C-terminal valine, 6 presenting an isoleucine, 3 presenting an alanine, but none presenting a leucine (Table I). 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 FP. In the assay we used peptides corresponding to the 13 C-terminal residues of PKCo, which has a type I PDZ binding sequence -QSAV, and of the 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 β2AR was included as a control for the specificity of the saturation binding assay. Like the PKCo sequence, the β2AR sequence contains a type I PDZ binding sequence (-DSLL), but unlike the PKCo 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 sulhydryl-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 tracer than the unlabeled PKCo peptide was titrated with an increasing amount of non-labeled peptide (data not shown). In agreement with the saturation binding experiments, the unlabeled DAT peptide was more avid by the side chain of the P(−2) residue than the unlabeled PKCo peptide (Fig. 2A). In this competition binding experiments in which fixed concentrations of PICK1 and the fluorescently labeled peptide were titrated with an increasing amount of non-labeled peptide (data not shown).

Next, we carried out competition binding experiments in which fixed concentrations of PICK1 and 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 avid by the side chain of the P(−2) residue than the unlabeled PKCo peptide (data not shown).
three similar experiments. For the experiments, tracer (40 nM) was incubated with a fixed subsaturating amount of WT PICK1, K83H, or K83V and titrated with increasing amounts of unlabeled peptides. After 15 min of incubation, fluorescence polarization (FP) values were determined. $K_i$ values were determined from non-linear regression analysis of binding data as described under “Experimental Procedures.” Data are mean ± S.E. of the indicated number of experiments.

Further agreement with the saturation binding experiments, the $\beta_2$ AR peptide was much less potent than the two other peptides. From the competition binding experiments it was possible to calculate $K_i$ values for the interaction of the peptides with PICK1 (Table II). We should note that the calculated $K_i$ 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 proteins in different purified preparations. As described under “Experimental Procedures,” the unavoidable depletion in the competition assay is accurately accounted for in the reported $K_i$ values.

Mimicking a Canonical Type II Interaction—Fig. 3 shows a model of PICK1 binding a peptide corresponding to eight C-terminal residues of the DAT (-TLHRWLKV). 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 $\alpha 1$ position, which is occupied by a lysine (Lys86), 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 $\Phi(2)$ position of the ligand (Fig. 3A). According to the model, there is, however, no predicted 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 $\alpha 1$ 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 $\Phi(2)$ pocket (Val84, Val86, and Ala87) was predicted to enable a number of favorable hydrophobic interactions of the DAT peptide in the K83V mutants 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 II).

The affinity of the K83V mutation for the PKC$\alpha$ 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 II). Notably, this observation was consistent with our molecular model of PICK1 binding the PKC$\alpha$ peptide. As shown in Fig. 4A, the peptide is displaced away from the $\alpha B$ helix in WT PICK1 and thereby positioned in a manner slightly different from the typical placement occupied by the DAT pep-

**FIG. 1. PICK1 saturation binding.** Fluorescently labeled peptides (40 nM) corresponding to the C-terminal 13 residues of DAT, PKC$\alpha$, and $\beta_2$ AR were titrated with increasing amounts of purified WT PICK1 protein. After 15 min of incubation, 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, PKC$\alpha$, and $\beta_2$ 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, PKC$\alpha$, and $\beta_2$ AR. C, competition binding to K83H of Oregon Green-labeled DAT peptide (PKC$\alpha$) with unlabeled peptides corresponding to the 13 C-terminal residues of DAT, PKC$\alpha$, and $\beta_2$ AR. Data are representative of at least three similar experiments. For the experiments, tracer (40 nM) was incubated with a fixed subsaturating amount of WT, K83H, or K83V and titrated with increasing amounts of the indicated unlabeled peptides. After 15 min of incubation, FP values were determined. The $K_i$ of the DAT peptide for PICK1 WT was 2.3 ± 0.1 μM (n = 12), and the $K_i$ of the PKC$\alpha$ peptide was 33 ± 2 μM (n = 9).

**TABLE II**

<table>
<thead>
<tr>
<th></th>
<th>PICK1 WT</th>
<th>PICK1 K83V</th>
<th>PICK1 K83H</th>
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<tbody>
<tr>
<td></td>
<td>$\mu M$</td>
<td>S.E. $n$</td>
<td>$\mu M$</td>
</tr>
<tr>
<td>DAT WT</td>
<td>2.3 0.1 12</td>
<td>1.92 0.02 3</td>
<td>2.10 0.02 12</td>
</tr>
<tr>
<td>PKC$\alpha$ WT</td>
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<td>5.5 0.4 3</td>
<td>0.54 0.07 9</td>
</tr>
<tr>
<td>$\beta_2$ AR WT</td>
<td>245 6 3</td>
<td>235 40 3</td>
<td>210 20 3</td>
</tr>
</tbody>
</table>
In the K83V mutant, however, the PKCβ/H9251 peptide recovers the typical placement in the groove (Fig. 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 PKCβ/H9251 lysine (Lys83) with a histidine. This mutation swapped the specificity of the PDZ domain, so that the affinity for the PKCβ/H9251 peptide ($K_i = 0.54 \pm 0.07 \mu M$) was much higher than that of the DAT peptide ($K_i = 21 \pm 2 \mu M$) (Fig. 2C and Table II). Note that the largest change in affinity was the increase for PKCβ (~60-fold), whereas the affinity of DAT only decreased 10-fold (Table II). Interestingly, the affinity for the β2AR peptide, which has a type I sequence at its C terminus, like PKCβ/H9251 did not increase (Fig. 2C and Table I). 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, sub-
PICK1 Binding Specificity

FIG. 5. Relative affinities of DAT and PKCα peptides after interchanging the P(−2) residues. A, relative changes in affinity for WT PICK1 of the 13 residues C-terminal DAT and PKCα peptides upon substituting Leu and Ser in P(−2) positions. Data are mean ± S.E. of three independent experiments. B, relative changes in affinity for PICK1 K83H of the 13 residues C-terminal DAT and PKCα peptides upon substituting Leu and Ser in P(−2) positions. Data were obtained from competition binding experiments using DAT13Org or PKCαOrg (40 nM) as tracer and a fixed subsaturating amount of WT PICK1 or K83H. The affinities (Ki values) were determined from non-linear regression analysis of binding data as described under “Experimental Procedures.” Data are mean ± S.E. of three independent experiments.

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 II). 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 because of a significant reorientation of the peptide backbone (compare Fig. 4, A and B), whereas for the K83H mutant the increased affinity is because of 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 with the WT peptide, whereas the affinity for the PICK1 K83H increased 19-fold compared with the WT peptide (Fig. 5 and Table III). 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 with the WT peptide, whereas the affinity for PICK1 K83H was decreased 19-fold compared with the WT peptide (Fig. 5 and Table III).

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 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 has been discerned.

To gain insight into the role the P(0) position for the binding specificity of the 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 implicit solvent model (Screened Coulomb Potential-Implicit Solvent Model; SCP-ISM) (31) was able to reproduce the experimentally observed preferences (Table IV). Indeed, the calculated order of preference of the third PDZ domain of PSD-95 (19) for peptides corresponding to the C terminus of CRIPt was Val > Ile > Leu, whereas the order of preference of the first PDZ domain of NHERF-1 (35) for peptides corresponding to the C terminus of CFTR appeared to be Leu > Ile > Val. Application of this computational method to evaluate the binding characteristics of peptide constructs that correspond to the DAT C terminus with P(0) position mutants, interacting with a model of PICK1, indicated a preference for the P(0) position in the order Val > Ile > Leu (Table IV).

This specific computational prediction was tested experimentally by generating DAT and PKCα peptides (13-mers) with substitutions of the P(0) residue (Val, Ile, and Leu). The binding affinities of the peptides were determined from competition binding experiments using a fixed concentration of PICK1 and fluorescently tagged DAT peptide. In agreement with the computational predictions, the preference for P(0) position was Val > Ile > Leu (Figs. 6 and 7A and Table V). To interpret the predicted and the observed affinity differences in a structural context, the binding modes were analyzed in models of PICK1 with three different P(0) residues (Fig. 8). The difference in affinity was found to correlate well with the difference in the number of predicted contacts between atoms of the P(0) residues and residues in the hydrophobic pocket of PICK1 (Fig. 8).

We also tested a β2AR peptide in which the leucine in the
Interaction energy differences of peptides for PSD-95, NHERF, and PICK1 with substituted hydrophobic P(0) residues

<table>
<thead>
<tr>
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<th>£E</th>
<th>ΔE_c</th>
<th>ΔE_e</th>
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<tbody>
<tr>
<td>PSD-95-3</td>
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<td></td>
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<td>+1.83</td>
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<td></td>
<td>QDTRTV</td>
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<tr>
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<td>QDTRL</td>
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<td>0.00</td>
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<td>NHERF-1-1</td>
<td>HWKLKV</td>
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<tr>
<td></td>
<td>HWLKI</td>
<td>-108.06</td>
<td>+3.96</td>
</tr>
<tr>
<td></td>
<td>HWLKL</td>
<td>-105.98</td>
<td>+6.04</td>
</tr>
<tr>
<td>PICK1</td>
<td></td>
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</table>

**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 the indicated substitutions in the P(0) position. B, competition binding to WT PICK1 of unlabeled C-terminal PKCa peptides (13 residues) containing the indicated substitutions in the P(0) position. C, competition binding to WT PICK1 of unlabeled C-terminal β2AR peptides (13 residues) containing the 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, 40 nM) was incubated with a fixed subsaturating amount of WT PICK1 and titrated with increasing amounts of unlabeled peptides. After 15 min of incubation, FP values were determined.

P(0) position was substituted with valine. This resulted in a 4-fold increase in affinity corresponding well to the loss of affinity for the other type I sequence, PKCa, upon valine to leucine substitution (Fig. 6 and Table V). Moreover, as a negative control in the P(0) substitution experiments we substituted P(0) in the DAT peptide with aspartate and this eliminated binding completely consistent with the prediction that a negatively charged residue in this position is incompatible with binding in the PDZ domain groove (Fig. 6A and Table V).

Next we explored the effect of decreasing the size of the

**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 (gray columns), or β2 AR (white columns) peptides containing the indicated substitutions in the P(0) position. B, relative decreases in affinity at K83H of C-terminal DAT (black columns), PKCa (gray columns), or β2AR (white columns) peptides containing the indicated substitutions in the P(0) position. Data were obtained from competition binding experiments using DAT13 or PKCa13 Oregon Green (40 nM) as tracer and a fixed subsaturating amount of WT PICK2 or K83H. The affinities (K_i values) were determined from non-linear regression analysis of binding data as described under "Experimental Procedures." Data are mean ± S.E. of three independent experiments.

**Table V**

<table>
<thead>
<tr>
<th></th>
<th>PICK1 WT</th>
<th>PICK1 K83V</th>
<th>PICK1 K83H</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Mean K_i</td>
<td>S.E. n</td>
<td>Mean K_i</td>
</tr>
<tr>
<td>DAT LKV</td>
<td>2.3 ± 0.1</td>
<td>12 ± 2.1</td>
<td>1.02 ± 0.02</td>
</tr>
<tr>
<td>DAT LKI</td>
<td>9.5 ± 0.9</td>
<td>3 ND³</td>
<td>64 ± 11</td>
</tr>
<tr>
<td>DAT LKL</td>
<td>37 ± 5</td>
<td>3 ND⁵</td>
<td>90 ± 17</td>
</tr>
<tr>
<td>DAT LKA</td>
<td>49 ± 3</td>
<td>3 ND</td>
<td>55 ± 7</td>
</tr>
<tr>
<td>DAT LKD</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>PKCa SAV</td>
<td>33 ± 2</td>
<td>9 ± 0.5</td>
<td>0.4 ± 0.03</td>
</tr>
<tr>
<td>PKCa SAI</td>
<td>77 ± 7</td>
<td>3 ND</td>
<td>1.46 ± 0.04</td>
</tr>
<tr>
<td>PKCa SAL</td>
<td>166 ± 15</td>
<td>3 ND</td>
<td>4.6 ± 0.3</td>
</tr>
<tr>
<td>PKCa SAA</td>
<td>40 ± 3</td>
<td>3 ND</td>
<td>0.34 ± 0.01</td>
</tr>
<tr>
<td>β2 SLL</td>
<td>245 ± 6</td>
<td>323 ± 40</td>
<td>210 ± 20</td>
</tr>
<tr>
<td>β2 SLV</td>
<td>63 ± 8</td>
<td>170 ± 20</td>
<td>31 ± 4</td>
</tr>
</tbody>
</table>

³ND, not determined.
hydrophobic side chain by mutation of P(0) to alanine in the DAT and PKCa peptides. This caused a dramatic 20-fold loss of affinity for the DAT peptide; surprisingly, however, there was hardly any loss of affinity for the PKCa peptide (Figs. 6 and 7 and Table V). This suggests a more substantial role of the P(0) position for the binding affinity of a type II ligand such as DAT, as compared with that for a type I ligand such as PKCa.

Finally, we tested the effect of P(0) substitutions on binding to PICK1 K83H (Fig. 7B and Table V). Overall, we observed the same pattern as in the WT with a Val > Ile > Leu preference with a trend toward less effect of the P(0) substitutions in the K83H construct (Table V). Interestingly, in the context of K83H, the PKCa peptide remained unaffected by the P(0) alanine substitution, in contrast to the DAT peptide. It is also noteworthy that although the affinity of the β2AR peptide with a valine in P(0) was only 2-fold lower than that of the PKCa WT peptide, the affinity of this mutated peptide did not display the major increase for K83H that we observed for the PKCa WT peptide (Table V).

**DISCUSSION**

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 $2.3 \pm 0.1 \mu M$, which is comparable with 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 (1, 17); but increasing evidence suggests that these solid-based methods, such as assays based on the Biacore platform or enzyme-linked immunosorbent assays, tend to overestimate intrinsic thermodynamic affinities because of 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 ($33 \pm 2 \mu M$). 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 with type I ligands (Table I). It should also be noted that an affinity of 30–35 μM might be considered only borderline for physiological interactions (1, 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 Lys83α to valine (K83V) causes a moderate increase in affinity for the DAT peptide (Table II 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 Lys83α to valine also increased the affinity for the PKCa 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 with 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 because of 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 because of 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 βAR. The βAR 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 βAR peptide of the P(0) leucine with the predicted optimal valine (βAR SLV) increased the affinity around 4-fold, i.e. to an affinity similar to that of the PKCα peptide (PKCα SAV) (Table V). 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 βAR WT peptide (βAR SLL), nor the βAR SLV mutant displayed the major affinity increase for K83H that we observed for PKCα SAV (Table V). Thus, although PKCα SAV and βAR 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 βAR peptide, because 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 of 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 I (these show a clear preference for valine (13 times) and isoleucine (6 times) as compared with leucine (0)). In our binding assay we were able to confirm this order of preference predictions quantitatively (Figs. 6 and 7 and Table I). For example, changing the P(0) valine to a leucine caused an almost 5-fold decrease in affinity of the PKCα peptide and a 16-fold decrease in affinity of the DAT peptide (Figs. 6 and 7 and Table I). 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, substitution of P(0) with the smaller alanine was much less energetically costly in the PKCα 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 I). 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 PKCα peptide than by the type II ligand from GluR2 (-SVKI). Notably, however, our current data strongly suggest that the divergence is because of a different mode of insertion into the PDZ binding groove, rather than to separate binding sites for the two peptides proposed in Ref. 25. Thus the PKCα peptide can compete for the fluorescently tagged DAT peptide, both peptides are affected by substitution of the βB1 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 αB1 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 αB1 position (46). Our data indicate that such a hydrogen bond is not formed by P(-2) in PICK1 unless the WT αB1 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 I).

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 interleukin-5R α ligand (-DSVF). 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 S-1 pocket formed partly by residues in the β8-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, because of its small size, can hardly provide
much binding energy in a hypothetical S\(^{-1}\) pocket. Furthermore, the P(0) valine can be substituted to an alanine with only a slight decrease of the affinity (less than 2-fold) (Figs. 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 interleukin-5Ro 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 (TNFYA), 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 S\(^{0}\) pocket. This is compensated for by the P(−1) tyrosine fitting into a novel hydrophobic S\(^{-1}\) 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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REFERENCES