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Serine 77 in the PDZ Domain of PICK1 Is a Protein Kinase Cα Phosphorylation Site Regulated by Lipid Membrane Binding

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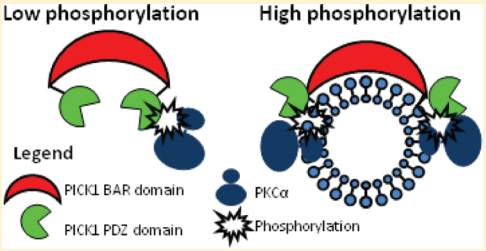
ABSTRACT: PICK1 (protein interacting with C kinase 1) contains an N-terminal protein binding PDZ domain and a C-terminal lipid binding BAR domain. PICK1 plays a key role in several physiological processes, including synaptic plasticity. However, little is known about the cellular mechanisms governing the activity of PICK1 itself. Here we show that PICK1 is a substrate in vitro both for PKCα (protein kinase Cα), as previously shown, and for CaMKIIα (Ca^{2+}-calmodulin-dependent protein kinase IIα). By mutation of predicted phosphorylation sites, we identify Ser77 in the PDZ domain as a major phosphorylation site for PKCα. Mutation of Ser77 reduced the level of PKCα-mediated phosphorylation ∼50%, whereas no reduction was observed upon mutation of seven other predicted sites. Addition of lipid vesicles increased the level of phosphorylation of Ser77 10-fold, indicating that lipid binding is critical for optimal phosphorylation. Binding of PKCα to the PICK1 PDZ domain was not required for phosphorylation, but a PDZ domain peptide ligand reduced the overall level of phosphorylation ∼30%. The phosphomimic S77D reduced the extent of cytosolic clustering of eYFP−PICK1 in COS7 cells and thereby conceivably its lipid binding and/or polymerization capacity. We propose that PICK1 is phosphorylated at Ser77 by PKCα preferentially when bound to membrane vesicles and that this phosphorylation in turn modulates its cellular distribution.



PICK1 (protein interacting with C kinase 1) is a scaffolding protein first identified as an interaction partner for PKCα (protein kinase Cα).[1] Functional studies have suggested that PICK1 plays a key role in molecular mechanisms underlying synaptic plasticity.[2] Furthermore, PICK1 has been implicated in diverse pathophysiological processes such as schizophrenia,[3,4] neuropathic pain,[5,6] cocaine addiction,[7] addiction relapse,[8] cerebral ischemia,[9,10] breast cancer,[11] and defects in spermatogenesis.[12,13]

PICK1 contains an N-terminal protein binding PDZ (PSD-95/Discs-large/ZO-1) domain and a C-terminal lipid binding BAR (Bin/amphiphysin/Rvs) domain.[14] The PDZ domain of PICK1 has been reported to interact with more than 40 proteins in addition to PKCα.[15,16] Most of the known ligands are the C-termini of different membrane proteins such as the GluA2/A3 subunits of the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid)-type ionotropic glutamate receptor,[17] the metabotropic glutamate receptor mGluR7,[18] kainate-type ionotropic glutamate receptors,[19] ASIC (acidic sensing ion channel) I and II,[20] and various neurotransmitter transporters, including the dopamine transporter (DAT)[21,22] and the GLT1b glutamate transporter.[23]

BAR domains represent dimeric α-helical modules that possess a distinctive capability to both sense and impose membrane curvature.[24−26] Thus, PICK1 is likely to exert its function in relation to its several PDZ binding partners via curvature-dependent lipid membrane interactions. Interestingly, the PDZ domain itself has been implicated in mediating lipid membrane binding.[27] There is also strong evidence that the lipid binding capacity of the PICK1 BAR domain is autoinhibited and that release of this inhibition requires binding of ligand to the PDZ domain in conjunction with membrane recruitment of PICK1.[28,29] The PICK1 BAR domain mediates moreover dimerization of PICK1 and in doing so allows the protein to bring two PDZ domain binding partners into the proximity of each other.[16]

A growing number of studies have provided improved insight into the cellular mechanisms underlying the various functions of PICK1. In synaptic plasticity, PICK1 is believed to mediate a decrease in the level of surface expression of postsynaptic GluA2-containing AMPA receptors, a key process in LTD (long-term depression).[30,31] PICK1 is also thought to mediate the presynaptic clustering of mGluR7[32] by facilitating phosphorylation and surface stabilization of mGluR7 by PKCα.[33] In relation to ASICs, PICK1 seems critical for the PKCα-mediated regulation of ASIC currents and plasma membrane expression.[34,35] ASICs are involved in the processes underlying hippocampal synaptic plasticity[36] and in patophysiological processes such as hyperalgesia.[37] PICK1 has furthermore been thought to regulate surface expression of other interaction partners such as GLT1b and DAT.[21,23]

Despite the involvement of PICK1 in a multitude of different cellular functions, little is known about the regulatory mechanisms controlling PICK1 activity. PICK1 has been shown to be a substrate for PKCα[1] and thought to become phosphorylated following activation of Ca^{2+}/calmodulin-dependent kinase IIα (CaMKIIα), although direct phosphorylation was not shown.[38]

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Here we show that indeed PICK1 is a direct substrate for PKCα as well as for CaMKIIα. By applying a systematic site-directed mutagenesis strategy, we map Ser77 to the PDZ domain of PICK1 as the major in vitro PKCα site. We show that the presence of lipid vesicles dramatically increases the level of PKCα-mediated PICK1 phosphorylation, whereas binding of the peptide ligand to the PDZ domain decreases it. Finally, we find that mimicking the phosphorylated state of Ser77 by mutation of the residue to aspartate (S77D) markedly reduces the extent of spontaneous clustering of eYFP (enhanced Yellow Fluorescent Protein)-bound PICK1 (eYFP−PICK1) in transfected COS7 cells, consistent with the possible impairment of lipid binding and/or polymerization. Taken together, the data identify a PKCα phosphorylation site in PICK1 that affects the cellular distribution of the PICK1 molecule and thus its regulation of PDZ binding partner function.

MATERIALS AND METHODS

Molecular Biology. The Quick Change method (Stratagene, La Jolla, CA) was used for single-amino acid substitutions. As a template, we used the pET41 plasmid (Stratagene) encoding GST (gluthathione *S*-transferase)-coupled rat PICK1 (GST−PICK1) as previously described[39] or the peYFP-C1 plasmid (Clontech) encoding eYFP−PICK1 as described previously.[28] GST−PICK1(136−416) was generated like GST−PICK1 by amplification of residues 136−416 of rat PICK1 from a pciNEO vector (kind gift from K. Dev). The primers introduced an N-terminal MunI and a C-terminal AvrII site that were used to clone the fragment into the pET41 vector. All primers were obtained from DNA technology A/S (Aarhus, Denmark). All mutant sequences were verified by restriction enzyme mapping and DNA sequencing and transformed into BL21 DE3 pLysS (Novagen).

Protein Purification. GST−PICK1 wild type (WT) or mutants and GST−PICK1(136−416) were purified as described previously.[39] Briefly, 50 mL of LB medium containing 34 μg/mL chloramphenicol (MP Biomedicals) and 50 μg/mL kanamycin (Invitrogen) was inoculated with *Escherichia coli* BL21 DE3 pLysS containing the pET41 vector encoding the appropriate PICK1 sequence. The bacteria were grown overnight at 37 °C, diluted into 1 L of LB medium containing 34 μg/mL chloramphenicol and 50 μg/mL kanamycin, and grown at 30 °C. When the right OD (0.6−1.0 at 600 nm) was obtained, protein expression was induced by addition of isopropyl β-D-thiogalactopyranoside (Sigma-Aldrich) to a final concentration of 0.5 mM. After induction for 3 h at 30 °C, the bacteria were pelleted at 8000*g* for 12 min. The pellets were resuspended on ice in 25 mL of ice-cold lysis buffer [50 mM Tris base (pH 7.4) (Angus Chemicals), 125 mM NaCl (Merck), 1% Triton X-100 (Sigma-Aldrich), 20 μg/mL DNase 1 (Sigma-Aldrich), and 1 mM dithiothreitol (DTT) (Sigma-Aldrich)]. The lysates were frozen for 40 min at −80 °C and thawed again in a 4 °C water bath, to achieve proper lysis of the bacteria. The cell debris was subsequently pelleted at 47000*g* for 30 min, and the supernatant was transferred to new tubes. For each batch, 0.75 mL of glutathione Sepharose 4B beads (Amersham Bioscience) was washed three times in wash buffer (50 mM Tris, 125 mM NaCl, 0.01% Triton X-100, and 1 mM DTT) and then added to the supernatant. GST-coupled proteins were allowed to bind to the glutathione beads during incubation for 90 min at 4 °C under continuous rotation. Next, the beads were pelleted at 3000*g* for 5 min and washed three times with wash buffer. Triton X-100 (0.1%) was used in wash buffer instead of 0.01% for proteins purified for the fluorescence polarization assay. To cleave the protein from the GST domain and the coupled beads, the beads were resuspended in 0.5 mL of wash buffer containing 0.3 unit of thrombin (Novagen), transferred to Bio-Spin disposable chromatography columns (Bio-Rad), and incubated overnight at 4 °C with rotation.

In Vitro Phosphorylation with PKCα. For each reaction, approximately 3 μg of purified protein was diluted in protein wash buffer to a final volume of 10 μL and then mixed with 10 μL of reaction buffer [120 mM Tris (pH 7.5), 60 mM $MgCl_2$ (Merck), and 6 mM $CaCl_2$ (Merck)], 5 μL of PKC Lipid Activator (Upstate, Frederikssund, Denmark), and 10 ng of PKCα (Upstate). Finally, 2 μCi of [γ-^{32}P]ATP (Amersham Bioscience, Pb108) was added and the mixture incubated at 30 °C for 30 min. The reaction was stopped via placement of the mixture on ice and addition of 10 μL of sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) loading buffer [200 mM Tris-HCl (pH 6.8), 400 mM DTT, 8% SDS, 0.4% bromophenol blue, and 40% glycerol]. The samples were analyzed via 10% SDS−PAGE. The front of the gel, which contained all unbound ATP, was subsequently removed, and the gel was washed for 3 × 10 min in distilled water before being stained with GelCode Blue Stain Reagent (Thermo Scientific). The gel was finally wrapped in plastic, placed in a cassette with a storage phosphor screen (Amersham Bioscience), and left overnight. The screen was scanned with a Molecular Imager FX (Bio-Rad). The Coomassie-stained gel was scanned with a Fluor-S MultiImager (Bio-Rad). The densities of the protein bands as well as the phosphorylation bands were determined by use of Discovery Series Quantity One 1-D Analysis version 4.5 (Bio-Rad). The ratio between the phosphorylation density and the protein density was calculated and compared to the ratio determined for PICK1 WT that was defined as 100%.

In Vitro Phosphorylation with CaMKIIα. For each reaction, approximately 3 μg of purified protein was diluted in protein wash buffer to a final volume of 15 μL. The reaction buffer [100 mM Hepes (Sigma-Aldrich), 20 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM DTT, 200 μg/mL BSA (Sigma-Aldrich), 0.6 mM ATP (Sigma-Aldrich), 2 μM calmodulin (Sigma-Aldrich), and 30 nM CaMKIIα (kindly provided by R. J. Colbran)] was mixed and incubated for 10 min at 30 °C. Fifteen microliter of reaction buffer was then mixed with 15 μL of protein before the addition of 2 μCi of [γ-^{32}P]ATP and incubation at 30 °C for 10 min. The reaction was stopped via placement of the mixture on ice and addition of 10 μL of SDS−PAGE loading buffer. The SDS−PAGE procedure was the same as described for the PKCα assay. The results were calculated as explained for the PKCα assay, except that the density of the CaMKIIα autophosphorylation band was subtracted from the density of the PICK1 phosphorylation band.

Preincubation with Vesicles or Peptides Prior to Phosphorylation by PKCα. To investigate the effect of vesicle binding on PKCα-mediated phosphorylation of PICK1, the protein was preincubated in 10 μL of protein wash buffer with 3 μg of vesicles prepared from Folch fraction type I (Sigma-Aldrich B1502) and extruded, yielding a diameter of 100 nm. The mix was preincubated for 30 min on ice, and control samples without vesicles were treated the same way. To investigate the effect of peptide binding, either DAT C13 or DAT C13 V to D (see Fluorescence Polarization Assay for a description of the peptides) was added to each sample to a final concentration of 67 μM, and the mixture was incubated for 10 min at 30 °C prior to the addition of reaction buffer,

kinase activator, PKCα, and 2 μCi of [γ-^{32}P]ATP (Amersham Bioscience, Pb108).

Fluorescence Polarization Assay. All peptides were purchased from Schafer-N (Copenhagen, Denmark). The peptides corresponded to the 13 C-terminal residues of the human DAT, PKCα, and GluA2. In addition to the DAT WT peptide (DAT C13), a mutant with the C-terminal Val substituted with Asp was used (DAT C13 V to D): DAT C13, EVRQFTLRHWLKV; DAT C13 V to D, EVRQFTLRHWLKD; PKCα C13, NPQFVHPILQSAV; GluA2 C13, EGYNVYGIESVKI. Fluorescence polarization binding experiments were conducted as described in ref 39. Briefly, saturation experiments were performed with DAT C13 with an additional N-terminal cysteine residue coupled to the fluorophore Oregon Green 488 (Schafer-N), termed OrG DAT C13. The assay was conducted in 96-well microtiter plates (black, 1/2 area, nonbinding surface, Corning). Purified protein was diluted in 100 μL of TBS [50 mM Tris (pH 7.4) and 125 mM NaCl], resulting in a $^1/_3$ log dilution row with 12 points spanning four decades for each protein. OrG DAT C13 was added to each well to a final concentration of 6.7 nM, and the plate was incubated on ice for 10 min before being read in a Chameleon Fluorescence Polarization Reader (Hidex) in FP mode, using a 488 nm excitation filter and a 535 nm long pass emission filter. FP was calculated according to the equation FP = $(I_v - gI_h)/(I_v + gI_h)$, where I_v is the intensity in the vertical plane and I_h the intensity in the horizontal plane. The apparent K_d was determined by the equation

$$Y = (\mathrm{FP}_{\text{bound ligand}} - \mathrm{FP}_{\text{free ligand}})[\mathrm{PICK1}]/(K_d + [\mathrm{PICK1}]) + \mathrm{FP}_{\text{free ligand}}$$

The concentration of the different PICK1 variants was calculated on the basis of the apparent K_d obtained from the saturation binding experiments, so equal amounts of peptide binding proteins were used in each experiment. The competition assay was conducted using 96-well microtiter plates in which 12 wells were used for each binding curve; 45 μL of protein at an appropriate dilution was added to a $^1/_3$ log dilution row of peptide diluted in 45 μL of TBS. Finally, 10 μL of 0.067 μM DAT OrG was added, and the plate was incubated on ice for 10 min before being read. The obtained fluorescence polarization values were analyzed by nonlinear regression analysis in Prism 5.0 (GraphPad Software Inc., San Diego, CA), and the affinities for the peptides were calculated as described previously.[39]

Vesicle Pull-Down Assay. The lipid film was prepared as described previously[40] from Folch fraction type I and rehydrated in 300 mM D-sorbitol to a final concentration of 50 mg/mL. The samples were sonicated, vortexed, and extruded 15 times through a filter with a pore size of 100 nm. For the pull-down assay, 10 μM protein was incubated with 10 mg/mL vesicles in 150 μL of TBS [Tris-buffered saline (pH 7.4)] containing 1 mM DTT for 30 min on ice and spun down at 140000*g* for 15 min at 4 °C. The supernatant was transferred to new tubes, and the pellet was resuspended in 150 μL of TBS with 1 mM DTT; the supernatant and pellet were analyzed via 10% SDS−PAGE and stained with GelCode Blue Stain Reagent (Thermo Scientific), and relative protein levels were quantified with AlphaEaseFC (Alpha Innotech Corp.).

Cell Culture and Transfection. COS7 cells were grown in DMEM (Dulbecco's modified Eagle's medium) 1965 with 10% fetal calf serum (HyClone, Logan, UT), 2 mmol/L glutamine, and 0.01 mg/mL gentamicine (Invitrogen) at 37 °C in a humidified 5% CO_2 atmosphere. The transfections were performed as reverse transfections; i.e., a mixture of 0.1 μg of C1-eYFP-PICK1 plasmid, 0.3 μL of lipo2000 (Invitrogen), and 47 μL of OptiMEM (Invitrogen) was spotted in the well of an eight-well Lab-Tek chambered coverglass (155411, Nunc), and the coverglass was incubated for 20 min before addition of 40000 cells in 50 μL of OptiMEM. After 5 h, the medium was changed to normal growth medium. The cellular distribution of eYFP−PICK1 was analyzed 24−48 h after transfection in live cells by epifluoresence microscopy (Carl Zeiss TM210). Images were acquired with a 40× oil objective and a YFP dichroic filter (excitation at 514 nm). Image acquisition was performed with MetaMorph (Meta Imaging, Universal Imaging, version 4.6.); settings were fixed throughout an experiment. Images were analyzed with ImageJ. The counting of cells containing a cluster was performed manually. A cluster was defined as an area with an intensity 1.5 times higher than the average intensity in the cytosol. At least 10 pictures were taken, and approximately 50 cells were counted of each sample.

Western Blotting. COS7 cells (2×10^6) transfected with C1-eYFP-PICK1 plasmid were lysed in 1 mL of Tris-HCl (pH 7.4) containing 1% (v/v) Triton X-100, 5 mM *N*-ethylmaleimide, and protease inhibitor cocktail (Roche Diagnostics). After solubilization for 30 min at 4 °C, the cell debris was removed by centrifugation at 16000*g* for 20 min at 4 °C. The protein concentration was determined in the supernatant, and equal protein amounts of each sample were analyzed via 10% SDS−PAGE as described above. After being blotted to the PVDF membrane, the membrane was split in two around 50 kDa, and eYFP−PICK1 was detected in the top part by 1:1000 rabbit anti-GFP (Abcam ab 290) and actin in the bottom part by 1:2000 rabbit anti-actin (Sigma A2066) and then 1:5000 secondary HRP-conjugated antibodies (Pierce). The signal was quantified using the ECL kit (Amersham) and FluorChem HD2 (Alpha Innotech Corp.) and AlphaEaseFC (Alpha Innotech Corp.). The level of eYFP−PICK1 was normalized to the corresponding actin level, and then the level of eYFP−PICK1 S77D was calculated relative to that of eYFP−PICK1 WT.

RESULTS

PICK1 Is an in Vitro Substrate for PKCα and CaMKIIα. In vitro phosphorylation experiments with purified PICK1 confirmed that PICK1 is a substrate for PKCα (Figure 1A) as previously described.[1] SDS−PAGE of the phosphorylation sample revealed a ^{32}P-labeled band corresponding to the elution of purified PICK1 (molecular mass of ∼50 kDa) visualized by a BlueGel stain (Figure 1A). In vitro phosphorylation experiments also indicated that PICK1 is a substrate for CaMKIIα at least in vitro (Figure 1B,C). CaMKIIα is autophosphorylated, and because of the similar molecular sizes of CaMKIIα and PICK1, it was not possible to separate the two proteins via SDS−PAGE. Therefore, CaMKIIα was preincubated with unlabeled ATP prior to phosphorylation of PICK1 to minimize the background signal from CaMKIIα autophosphorylation (Figure 1B). To conclusively separate the signal from PICK1 and CaMKIIα phosphorylation, the experiment was also performed with N-terminally truncated PICK1(136−416), which would be separated from CaMKIIα by SDS−PAGE (Figure 1C). Panels B and C of Figure 1 demonstrate together that PICK1 is a substrate for CaMKIIα in vitro and that phosphorylation, at least to some extent, takes place beyond residue 135.

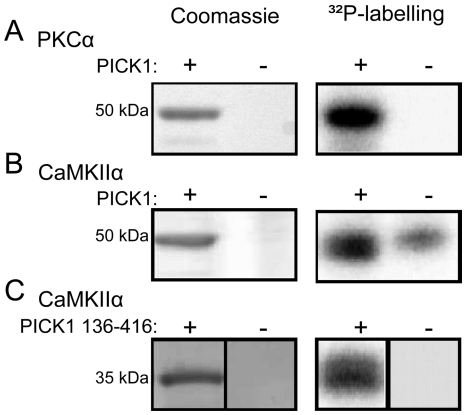
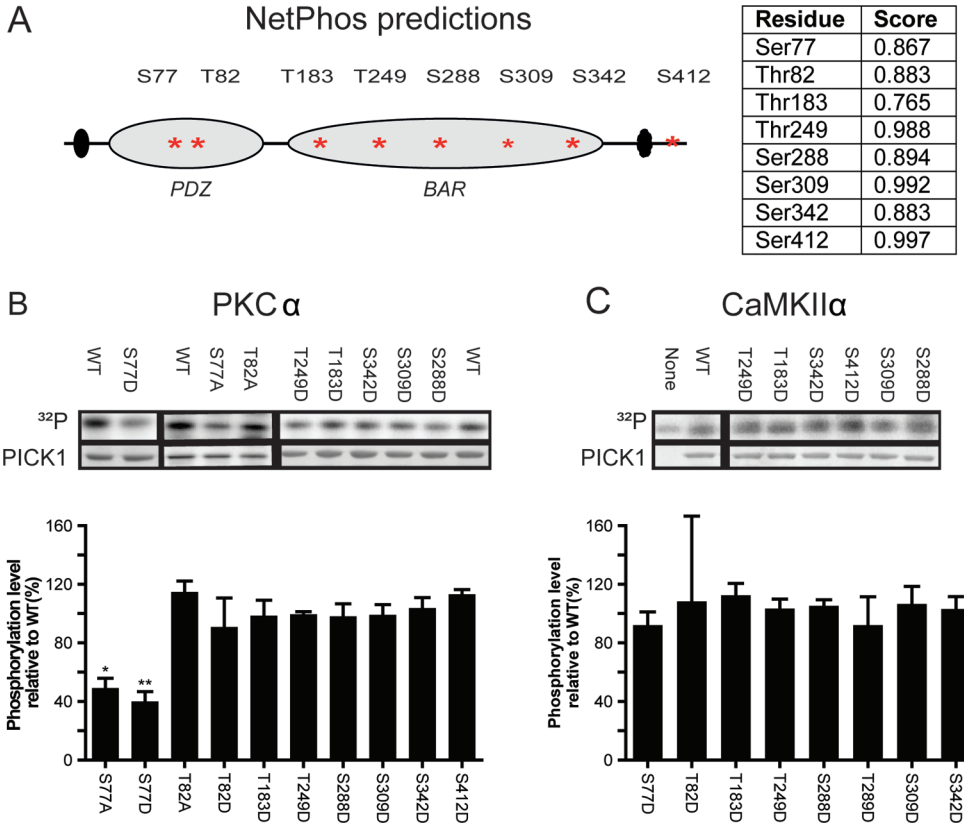


Figure 1. In vitro phosphorylation of PICK1 by PKCα and CaMKIIα. The illustrated gels are from representative experiments demonstrating specific phosphorylation of PICK1 by the three kinases: (left) Commassie-stained gels and (right) incorporation of ^{32}P assessed by autoradiography. (A) PKCα in vitro phosphorylation of PICK1 WT. A control without PICK1 WT is included to show background phosphorylation (n = 3). (B) CaMKIIα in vitro phosphorylation of PICK1 WT. The background from CaMKIIα autophosphorylation is minimized by preincubation with unlabeled ATP before addition of PICK1 and ^{32}P-labeled ATP; the right lane of the right gel shows the remaining background (n = 3). (C) CaMKIIα in vitro phosphorylation of PICK1(136–416) (n = 3).

Identification of a PKCα Phosphorylation Site in PICK1. To map the PKCα phosphorylation site(s) in PICK1, we first identified putative sites by use of the NetPhos prediction program.[41] We chose to analyze the eight serine or threonine residues with the highest predicted phosphorylation probability score for being a phosphorylation site (>0.85). The probability scores are presented in Figure 2A together with the position of the potential phosphorylation sites in a schematic figure of PICK1. All eight residues were substituted with aspartate one at a time, and the resulting mutants were purified upon expression in *E. coli*. The purified mutants were analyzed by in vitro phosphorylation and the phosphorylation levels quantified by phosphoimaging analysis. Figure 2B shows the quantifications of the relative phosphorylation level of PICK1 mutants by PKCα for three to five experiments. Substitution of Ser77 with aspartate significantly reduced the level of phosphorylation compared to that of PICK1 WT (39 ± 7.7%; p < 0.01; n = 4). A similar reduction was seen via mutation of Ser77 to alanine (47 ± 7.7%; p < 0.05; n = 3) (Figure 2B). None of the other substitutions resulted in phosphorylation significantly different from that of WT. This strongly suggests that Ser77 in the PICK1 PDZ domain is a PKCα phosphorylation site. Moreover, the extent of the decrease suggests that Ser77 is the most prominent PKCα site at least in vitro.



Residue	Score
Ser77	0.867
Thr82	0.883
Thr183	0.765
Thr249	0.988
Ser288	0.894
Ser309	0.992
Ser342	0.883
Ser412	0.997

Figure 2. Screening for phosphorylation sites by site-directed mutagenesis. (A) Schematic drawing of PICK1 showing the approximate localization of the potential phosphorylation sites together with a table showing the phosphorylation potential score predicted by NetPhos 2.0. (B and C) Tests of the mutants in the PKCα in vitro phosphorylation assay (B) and the CaMKIIα in vitro phosphorylation assay (C). Top panels are representative SDS–PAGE gels showing ^{32}P incorporation and Coomassie stains. Bottom panels show quantitative phosphorylation levels relative to that of WT. Data are means ± the SE of three to five experiments. Mutation of S77 results in a significant decrease in the PKCα-mediated phosphorylation level [S77A, 48 ± 7.7% (n = 3); S77D, 39 ± 7.7% (n = 4) (means ± SE)]. One asterisk indicates p < 0.05, and two asterisks indicate p < 0.01 (one-way ANOVA followed by Dunnett's multiple-comparison test). Each n represents a unique protein batch.

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We attempted to validate that Ser77 represents a PKCα phosphorylation site also in a cellular context by use of mass spectrometry analysis after isolation of PICK1 tagged at the N-terminus with enhanced yellow fluorescent protein (eYFP) from transfected HEK293 cells. However, because of an unusually high lysine/arginine content in the area around Ser77 in the primary structure of PICK1, we were unable to isolate a peptide containing Ser77 and thus unable to determine its cellular phosphorylation state (data not shown).

The Ser/Thr mutants described above, as well as that with Thr289 substituted with aspartate, were also tested in the CaMKIIα in vitro phosphorylation assay. T289 was not predicted by NetPhos but is positioned in a CaMKIIα consensus site.[42] However, none of the substitutions reduced the level of CaMKIIα in vitro phosphorylation (Figure 2C).

Vesicle Binding Increases the Level of Phosphorylation of PICK1 by PKCα. PICK1 can bind to lipid membranes both via its BAR domain[14] and via a putative lipid binding patch of the PDZ domain.[27] Because PKCα also is capable of binding to lipid membranes,[43] we envisioned that the presence of a lipid membrane could act as a scaffold to facilitate phosphorylation by bringing both PKCα and PICK1 to the membrane. Furthermore, it is known that the affinity of some PKC substrates can be enhanced by lipid-induced α-helix formation.[44] Conversely, Ser77 is located close to the reported lipid binding patch (the CPC motif) and could be shielded by lipid membrane binding (Figure 3A). We decided, therefore, to test whether the presence of lipid vesicles would affect phosphorylation of PICK1 by PKCα. Addition of vesicles (prepared from total brain lipids and extruded to obtain a maximal diameter size of 100 nm) dramatically increased the level of phosphorylation of PICK1 by PKCα (971 $\pm$ 197%; $p < 0.01$; $n = 3$) (Figure 3B). Moreover, this effect was almost abolished in the phosphomimic PICK1 S77D. This reduction could result from either weakened vesicle binding by PICK1 because of the introduction of a negative charge close to the positively charged lipid binding patch or direct removal of the phosphorylation site sensitive to vesicle binding. To discriminate between these possibilities, we also tested the S77A substitution to mimic the unphosphorylated state of Ser77. Introduction of the S77A mutation likewise reduced the level of phosphorylation, making it unlikely that the observed difference between PICK WT and S77 substitution mutants is caused by weakened vesicle binding of the mutants. Therefore, we conclude that incubation with vesicles increases the level of in vitro phosphorylation of PICK1 by PKCα and that this effect is predominantly due to an increased level of phosphorylation of Ser77.

Substitution of Ser77 Does Not Affect the Peptide Binding Capacity of the PICK1 PDZ Domain. Ser77 is localized to the PICK1 PDZ domain and thus to the domain that binds the extreme C-termini of PICK1's many interaction partners (Figure 4A). Although Ser77 is localized away from the binding groove, we wanted to test whether phosphorylation of this residue could affect the binding capacity of the PDZ domain. To test this, we employed our previously described fluorescence polarization binding assay that is based on detection of binding of a fluorescently tagged peptide ligand to purified PICK1 in solution.[39] As a fluorescently tagged ligand, we used a peptide equivalent to the 13 C-terminal residues of the dopamine transporter (OrG DAT C13), which displays the highest affinity reported for the PICK1 PDZ domain.[39] We performed the experiments as competition binding assays using three unlabeled peptides, which are known to bind the PICK1

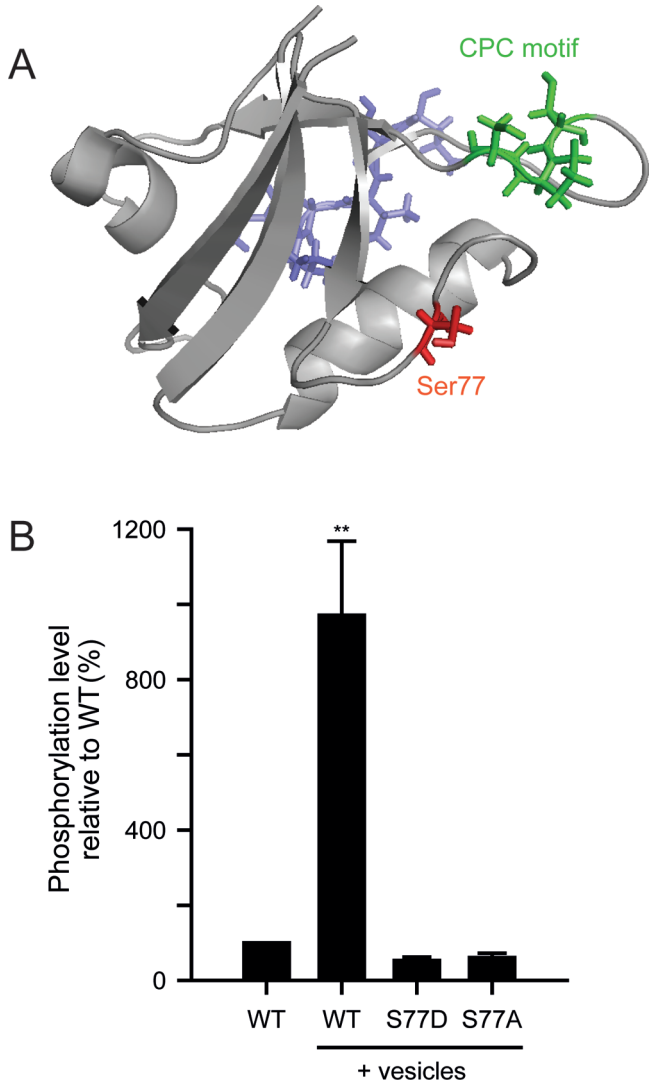

Figure 3. Effect of lipid vesicles on PKCα-mediated phosphorylation of PICK1. (A) Structural model of the PICK1 PDZ domain highligting the position of Ser77 (red). The CPC motif in the reported lipid binding patch is colored green.[27] The GluA2 ligand in the binding pocket is colored light blue.[7] The presented model is based on the coordinates of Protein Data Bank entry 2PKU.[27] (B) PKCα-mediated phosphorylation of purified PICK1 preincubated with vesicles. The level of phosphorylation of PICK1 WT without vesicles (control) is defined as 100%, and the levels of phosphorylation of PICK1 WT, PICK1 S77D, and PICK1 S77A with vesicles are presented relative to the level of phosphorylation of PICK1 WT without vesicles: 971 $\pm$ 197% for PICK1 WT, 53.1 $\pm$ 9.1% for PICK1 S77D, and 60.8 $\pm$ 11.6% for PICK1 S77A (means $\pm$ SE). Two asterisks indicate $p < 0.01$ [one way-ANOVA followed by Dunnett's multiple-comparison test ($n = 3$)]. Error bars represent the SE.

PDZ domain, i.e., peptides equivalent to the C-termini of DAT (DAT C13), GluA2 (GluA2 C13), and PKCα (PKCα C13). As a negative control, we used a DAT peptide in which the last residue was changed to aspartate (DAT C13 V to D), which disrupts the interaction with the PICK1 PDZ domain.[39] In Figure 4B, the y-axis shows fluorescence polarization (FP) (arbitrary units, millipolarization), which reflects the rotational diffusion of the fluorescently tagged peptide when it is bound to PICK1 in solution. Increasing the concentration of the unlabeled peptide reduces the FP value as the fluorescently labeled tracer is displaced from the larger protein, thereby

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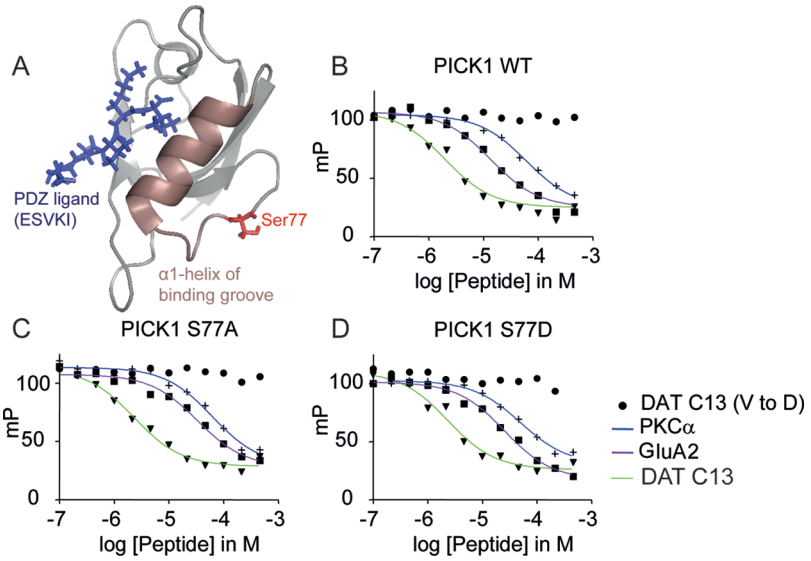

Figure 4. Mutating Ser77 does not affect the ligand binding affinity of the PICK1 PDZ domain. (A) Structural model of the PICK1 PDZ domain highligting the position of Ser77 relative to the PDZ binding pocket. The C-terminus of GluA2 in the PDZ binding groove is colored blue. Ser77 is colored red. The α-helix that is part of the binding pocket and connected to S77 is colored light purple. The presented model is based on the coordinates of Protein Data Bank entry 2PKU.[27] (B–D) Fluorescence polarization competition binding curves for binding of the peptide to PICK1 WT, PICK1 S77A, and PICK1 S77D, respectively. A fixed concentration of Oregon Green-labeled DAT peptide (OG DAT C13, ~6.7 nM) was incubated together with purified PICK1 and the indicated concentrations of unlabeled peptides corresponding to the 13 C-terminal residues of PKCα, GluA2, or DAT C13. DAT C13 V to D represents a negative control peptide in which the C-terminal residue was changed from Val to Asp. Data are shown as bound relative to maximal bound OG DAT C13 (means of triplicate measurements ± SE) and representative of three measurements.

increasing its rotational diffusion. The three peptides (DAT C13, GluA2 C13, and PKCα C13) all inhibited binding of OrG DAT C13 with the same potency in S77D and S77A as in the WT (Figure 4B and Table 1). Hence, neither removal of the side chain nor mimicking phosphorylation of Ser77 affected binding of the ligand to the PDZ domain.

Table 1. Binding Affinities for PICK1 WT, PICK1 S77A, and PICK1 S77D[a]

	DAT C13	GluA2 C13	PKCα C13	DAT C13 V to D
WT	1.05 [0.987–1.14]	7.6 [6.4–9.1]	49 [38–63]	ND
S77A	1.5 [1.4–1.6]	9.8 [8.0–12]	28 [27–29]	ND
S77D	1.1 [1.0–1.3]	8.8 [8.1–9.6]	29 [25–33]	ND

[a]Data are K_i values in micromolar (means [SE interval]) calculated from fluorescence polarization competition binding curves. A fixed concentration of Oregon Green-labeled DAT peptide (OG DAT C13, ~6.7 nM) was incubated together with purified PICK1 WT, PICK1 S77A, or PICK1 S77D and increasing concentrations of unlabeled peptides corresponding to the 13 C-terminal residues of PKCα, GluA2, or DAT C13. DAT C13 V to D represents a negative control peptide in which the C-terminal residue was changed from Val to Asp. Data were analyzed by nonlinear regression analysis as described in Materials and Methods. ND, not determined.

Phosphorylation of PICK1 by PKCα Is Not Affected by Binding of PKCα to the PICK1 PDZ Domain. The C-terminus of PKCα is a ligand for the PICK1 PDZ binding pocket.[15] Therefore, we investigated whether the possible binding of PKCα to the PICK1 PDZ binding pocket was required for its phosphorylation of PICK1. In this experiment, we used a PICK1 mutant (A87L) that cannot accommodate a peptide in the PDZ binding pocket.[39] PICK1 A87L was phosphorylated to the same level as PICK1 WT (92 ± 14%; not significant; n = 4) (Figure 5A), indicating that binding of PKCα to the PICK1 PDZ domain is not critical for phosphorylation of PICK1 by the kinase.

Binding of the Peptide to the PDZ Domain Decreases the Level of Phosphorylation of PICK1 by PKCα. The experiment described above suggests that binding of PKCα to PICK1 is not required for PICK1 phosphorylation; however, the experiment does not address whether occupancy of the PDZ binding crevice per se affects the phosphorylation of PICK1 by PKCα. To address this question, we preincubated purified PICK1 with either the DAT C13 peptide that binds the PDZ domain with high affinity or the nonbinding DAT C13 V to D peptide. Preincubation with DAT C13 resulted in a significant decrease in the level of phosphorylation of PICK1 compared to preincubation with DAT C13 V to D (70.5 ± 9.6%; $p < 0.05$; n = 3) (Figure 5B). The decrease was not due to the simple presence of DAT C13 in the mixture; i.e., no significant difference in phosphorylation level was observed when the nonbinding A87L mutation was preincubated with DAT C13 compared to DAT C13 V to D (95 ± 6.9%; not significant; n = 5) (Figure 5B). We also analyzed the S77D mutation to assess whether the reduced level of phosphorylation in the presence of DAT C13 was due to the reduced level of phosphorylation of Ser77. Surprisingly, preincubation with DAT C13 also resulted in a decrease in the level of phosphorylation of PICK1 S77D compared to preincubation with DAT C13 V to D (75.9 ± 6.4%; $p < 0.05$; n = 3) similar to what was observed for the WT. Taken together, the data suggest that the overall level of phosphorylation of PICK1 by PKCα is decreased when a peptide is bound to the PICK1 PDZ domain; however, this effect is not caused by blocking the phosphorylation of Ser77.

The Phosphomimic eYFP–PICK1 S77D Displays a Decreased Clustering Tendency When Overexpressed in COS7 Cells. Ser77 is located in the proximity of the lipid

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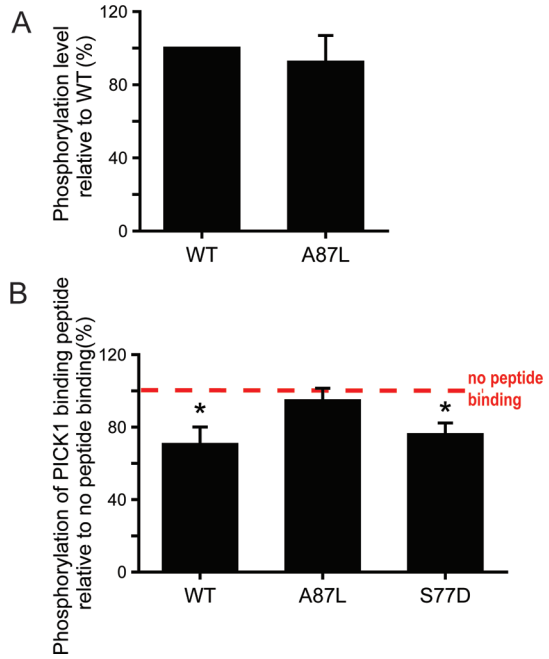

Figure 5. Phosphorylation of PICK1 by PKCα does not require binding of PKCα to the PICK1 PDZ domain, and binding of the peptide to the PDZ domain decreases the level of phosphorylation. (A) PKCα mediated in vitro phosphorylation of PICK1 A87L relative to PICK1 WT. Purified PICK1 WT or PICK1 A87L was phosphorylated in vitro by PKCα and analyzed by SDS−PAGE, and phosphorylation was quantified as described in Materials and Methods. Data are means ± SE; PICK1 A87L, 92.4 ± 14.5% (n = 3). Error bars represent the SE. (B) PKCα mediated in vitro phosphorylation of PICK1 WT, PICK1 S77D, and PICK1 A87L preincubated with the DAT-LKV peptide. Data are shown as phosphorylation level normalized to the phosphorylation level of PICK1 WT, PICK1 S77D, or PICK1 A87L preincubated with the nonbinding DAT-LKD control peptide (red dashed line): 70.5 ± 9.6% for PICK1 WT, 75.9 ± 6.4% for PICK1 S77D, 94.6 ± 6.9% for PICK1 A87L (means ± SE; n = 3). An asterisk indicates $p < 0.05$ in one sample t test.

binding patch of the PDZ domain (Figure 3A), and consequently, we wanted to test if mimicking its phosphorylation affected the cellular distribution of PICK. In the experiments, we used PICK1 with eYFP fused to the N-terminus (eYFP−PICK1). Figure 6A shows representative confocal microscopy pictures of the distribution in transiently transfected COS7 cells of eYFP−PICK1 WT and eYFP−PICK1 S77D. In agreement with previous reports, eYFP−PICK1 formed discrete cytosolic clusters, which have been reported to reflect the membrane binding capacity of PICK1.[14] In the eYFP−PICK1-expressing cells, at least one discrete cytosolic cluster was observed in 73 ± 4% of the cells, whereas for eYFP−PICK1 S77D-expressing cells, discrete clusters were observed in 49 ± 3% of the cells. Thus, the number of cells containing at least on cluster is significantly reduced for eYFP−PICK1 S77D compared to eYFP−PICK1 (Figure 6B). The difference in the number of cluster-containing cells could be related to different expression levels of the two construct; however, no apparent difference was observed in transfection efficiency, and Western blots of cell lysates showed similar expression of the two constructs (Figure 6C). This immediately suggests that the lipid binding capacity of the phosphomimicking mutant eYFP−PICK1 S77D was reduced. We attempted to test this more directly using in vitro lipid vesicle pull-down experiments; however, we were not

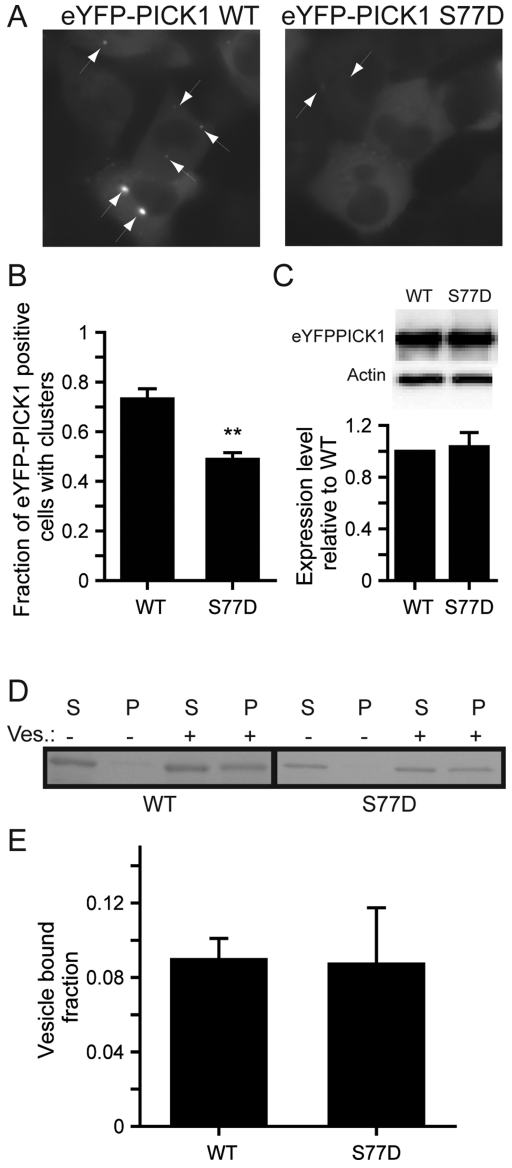

Figure 6. Clustering of eYFP−PICK1 in COS7 cells and vesicle binding. (A) Representative pictures showing weakened clustering of eYFP−PICK1 S77D compared to eYFP−PICK1 in transiently transfected COS7 cells. The arrows point to the clusters. (B) Quantification of clustering of WT compared to that of S77D. Clustering was quantified by counting the fraction of cells with at least one cytosolic cluster of eYFP−PICK1 or eYFP−PICK1 S77D. A cluster was defined as an area with an intensity of 1.5 times the average cytosolic intensity of eYFP. Data are means ± SE obtained from three independent transfections. At least 10 pictures were taken, and approximately 50 cells were counted in each sample. An asterisk indicates $p < 0.01$ in a two-sided Student's t test (n = 3); error bars represent the SE. (C) Quantification of the expression level measured by Western blot. The level of eYFP−PICK1 was normalized to actin, and the level of S77D was calculated with respect to a WT transfected in parallel (n = 5, where each n represents an independent transfection). (D) Representative gel of a lipid vesicle pull-down assay of PICK1 WT (left) and PICK1 S77D (right). Purified PICK1 WT or S77D was incubated with lipid vesicles before ultracentrifugation and analysis of pellet (P) and supernatant (S) fractions by SDS−PAGE and Western blotting. All pellets were loaded, and one-fifth of the supernatant was loaded. Ves. denotes vesicles. (E) Quantification of lipid binding in the in vitro vesicle pull-down assay. Data are given as the vesicle-bound fraction (means ± SE; n = 3). The vesicle-bound fraction was calculated as [(pellet + vesicles)/5]/(total protein + vesicles) − [(pellet + vesicles)/5]/(total protein + vesicles).

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able to reproduce the previously reported vesicle binding capacity of the PDZ domain itself (data not shown).[27] Instead, we measured the lipid binding capacity of full-length PICK1 in the same assay. In agreement with previous observations,[14] we saw robust pull-down of PICK1 by the lipid vesicles but were unable to detect a difference for full-length PICK1 WT and S77D (Figure 6D,E). This suggests that phosphorylation of Ser77 is unlikely to affect the overall lipid binding capactity of PICK1 at least in vitro. Moreover, the data suggest that the cytosolic clusters might not be a simple readout of the lipid binding capacity of PICK1 alone.

DISCUSSION

PICK1 is known to serve multiple functions in relation to its many interaction partners; however, little is known about cellular mechanisms regulating the activity of PICK1. In this study, we show that PICK1 is an in vitro substrate for two important kinases: PKCα and CaMKIIα. We identify a serine at position 77 of the PICK1 PDZ domain as the major PKCα phosphorylation site, at least in vitro. In addition, we show evidence that this phosphorylation is crucially regulated by lipid membrane binding and affects the cellular distribution of PICK1, which together are likely to have a functional impact on the interaction partners.

PICK1 was previously shown to be an in vitro substrate for PKCα;[1] however, it should be noted that Staudinger et al. conducted the in vitro phosphorylation assay with PICK1 coupled to GST, resulting in a protein with a molecular weight similar to that of PKCα. Because PKCα autophosphorylates, the possibility that the reported phosphorylation was due to autophosphorylation of PKCα cannot be completely excluded. In our study, the in vitro phosphorylation assay was performed with an untagged PICK1, resulting in separation of the two proteins by SDS−PAGE. Hence, it can now be concluded that PICK1 is indeed an in vitro substrate for PKCα.

Autophosphorylation was also an issue in the CaMKIIα in vitro phosphorylation assay because CaMKIIα autophosphorylates,[45] and PICK1 and CaMKIIα have similar molecular weights. The magnitude of the background signal from CaMKIIα autophosphorylation was reduced by preincubating CaMKIIα with cold ATP, and the observed increase in the level of phosphorylation upon addition of PICK1 can most likely be ascribed to phosphorylation of PICK1. However, to rule out the possibility that the observed effect was due to an effect of PICK1 on CaMKIIα autophosphorylation, we also performed the assay with a truncated version of PICK1, PICK1(136−416), because this can be separated from CaMKIIα by SDS−PAGE. PICK1(136−416) was also phosphorylated by CaMKIIα, and thus, we can conclude for the first time that PICK1 is an in vitro substrate for CaMKIIα and that the phosphorylation at least to some extent takes place beyond residue 135 of PICK1.

The activities of PKCα and CaMKIIα are important for synaptic plasticity in which PICK1 also plays a major role. Thus, we speculated that phosphorylation of PICK1 by these kinases can modulate the function of PICK1. In this study, we focused on the phosphorylation of PICK1 by PKCα, and by site-directed mutagenesis, we identified Ser77 as a major phosphorylation site at least in vitro. Unfortunately, our attempt to identify Ser77 as a phosphorylation site in a cellular context failed because of the high lysine and arginine content in the area around Ser77, which prevented isolation of peptides containing Ser77 and their subsequent identification by mass spectrometry. Therefore, we can still only speculate that Ser77 is a phosphorylation site in vivo, although we find it likely given the high degree of phosphorylation seen in vitro and the high score obtained for the site according to the NetPhos prediction program.[41]

To improve our understanding of how PKCα phosphorylation of PICK1 might be regulated in a cellular system, we tested the effect of lipid binding on PKCα-mediated phosphorylation of PICK1. Performing the phosphorylation assay in the presence of lipid vesicles resulted in an approximately 10-fold increase in the level of phosphorylation of PICK1. Moreover, the vesicle-induced increase in the level of phosphorylation was abolished by mutation of Ser77, strongly indicating that the observed effect was due to an increased level of phosphorylation of Ser77. Thus, recruitment of PICK1 to the membrane might be a key element in promoting the phosphorylation of Ser77. Interestingly, because the lipid vesicles selectively affect phosphorylation of Ser77, the observed increase more likely reflects a conformational change in PICK1 upon lipid binding than a scaffolding of PICK1 and PKCα on the lipid vesicles.

It is also interesting to note that phosphorylation of PICK1 by PKCα was independent of the interaction of the PICK1 PDZ domain and the C-terminus of PKCα; i.e., the PDZ binding deficient mutant, PICK A87L, was phosphorylated to the same extent as WT. This might reflect that PKCα does not bind in the PDZ domain binding groove when phosphorylating PICK1. We observed, however, that binding of a peptide ligand, and thus another putative cellular interaction partner of PICK1, reduced the level of phosphorylation of PICK1. Remarkably, the peptide ligand also reduced the level of phosphorylation of PICK1 when Ser77 was mutated, indicating shielding of a phosphorylation site other than Ser77 by the peptide or by conformational changes in PICK1 that disfavor phosphorylation of a site other than Ser77.

Ser77 is located in the PICK1 PDZ domain close to the reported lipid binding patch in the PDZ domain[27] as shown in Figure 3A. This patch was suggested to add to the lipid binding capacity of the BAR domain and thereby to be important for the role of PICK1 in synaptic plasticity.[27] The vesicle-induced increase in the level of phosphorylation strongly suggests that Ser77 is not docked directly in the membrane; nonetheless, the phosphorylation of Ser77 could be envisioned to decrease the capacity of the PICK1 PDZ domain to bind to negatively charged lipid membranes. In agreement with this prediction, we observed that the phosphomimic S77D compromised the clustering tendency of eYFP−PICK1 in COS7 cells. Previous studies have suggested that this clustering tendency represents a measure of PICK1's lipid binding capacity.[14] However, we could not reproduce the reported lipid binding of the PICK1 PDZ domain[27] in a vesicle pull-down assay, and the phosphomimic S77D did not reduce the lipid binding capacity of full-length PICK1. Nevertheless, it is important to stress that indeed the lipid binding capacity of the PDZ domain could be involved in the cellular localization of PICK1 even though we cannot reproduce it in a simplified in vitro assay, especially because the lipid binding of the BAR domain has been shown to be autoinhibited in full-length PICK1.[28] Furthermore, the clustering of PICK1 could also reflect oligomerization and/or polymerization of the protein as seen for other BAR domain proteins,[46] which might be important for subcellular localization of PICK1 and its interaction partners. Thus, we speculate that phosphorylation decreases the level of

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polymerization of PICK1 in addition to potentially decreasing the lipid binding capacity.

In summary, this study identifies PICK1 as a phosphoprotein subject to phosphorylation by major kinases such as PKCα and CaMKIIα. Moreover, our data suggest that PKCα-mediated phosphorylation is dynamically governed by the interplay between the cellular localization of PICK1 and the occupancy of the PDZ binding pocket (summarized in Figure 7). Among several possible sites, we identify Ser77 as a main site for PKCα-mediated phosphorylation and show how lipid binding enhances phosphorylation of this site and how this phosphorylation in turn is likely to affect its cellular redistribution. Future studies should further clarify the complex role of phosphorylation in regulating the multiple cellular functions of PICK1.

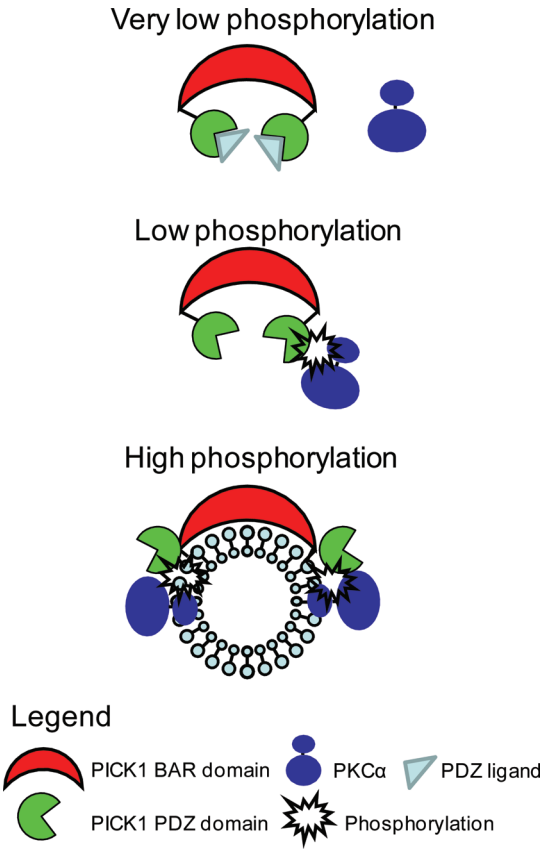

Figure 7. Proposed model of regulation of PKCα phosphorylation of PICK1. Our data suggest that PKCα-mediated phosphorylation is dynamically governed by the interplay between the cellular localization of PICK1 and the occupancy of the PDZ binding pocket. A very low level of phosphorylation of PICK1 is expected when it is not bound to membranes and when a peptide ligand is bound in the PDZ domain. A low level of phosphorylation of PICK1 would be seen when PICK1 neither binds to a membrane nor has a PDZ ligand bound to the PDZ binding pocket. The highest level of phosphorylation of PICK1 would be expected when the protein is bound to membranes with no peptide ligand bound to the PDZ binding pocket. The phosphorylation would be predicted to cause dissociation of PICK1 from the membrane and likely facilitates its cellular redistribution.

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Author Contributions

I.A.-J. performed the molecular biology, the protein purifications, the in vitro phosphorylation assays, and the cellular distribution assay. T.S.T. performed the vesicle pull-down assay. K.L.M. and I.A.-J. performed the fluorescence polarization assay. I.A.-J., U.G., and K.L.M. designed the research and wrote the manuscript.

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ABBREVIATIONS

AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ASIC, acidic sensing ion channel; BAR, Bin/Amphiphysin/Rvs; CaMKIIα, Ca^{2+}-calmodulin-dependent protein kinase IIα; DAT, dopamine transporter; eYFP, enhanced Yellow Fluorescent Protein; LTD, long-term depression; PDZ, PSD95/Disc large/ZO1; PICK1, protein interacting with C kinase 1; PKCα, protein kinase Cα; SE, standard error.

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