The BAR domain protein PICK1 regulates cell recognition and morphogenesis by interacting with Neph proteins

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Abstract

Neph proteins are evolutionarily conserved membrane proteins of the immunoglobulin superfamily that control the formation of specific intercellular contacts. Cell recognition through these proteins is essential in diverse cellular contexts such as patterning of the compound eye in *Drosophila melanogaster*, neuronal connectivity in *Caenorhabditis elegans*, and the formation of the kidney filtration barrier in mammals. Here we identify the PDZ and BAR domain protein PICK1 (protein interacting with C-kinase 1) as a Neph-interacting protein. Binding required dimerization of PICK1, was dependent on PDZ domain protein interactions and mediated stabilization of Neph1 at the plasma membrane. Moreover, protein kinase C (PKCα) activity facilitated the interaction through releasing Neph proteins from their binding to the multidomain scaffolding protein Zonula Occludens 1 (ZO-1), another PDZ domain protein. In *Drosophila*, the Neph homologue Roughest is essential for sorting of interommatidial precursor cells and patterning of the compound eye. RNAi-mediated knockdown of PICK1 in the *Drosophila* eye imaginal disc caused a Roughest destabilization at the plasma membrane and a phenotype that resembled *rst* mutation. These data indicate that Neph-proteins and PICK1 synergistically regulate cell recognition and contact formation.
Organization of cells into a specific spatial configuration is a prerequisite for the development of epithelial tissues and organs and requires selective cell adhesion and cell recognition. Multiple classes of cell-surface molecules are involved in mediating cell recognition, many of which are highly conserved through evolution. Particularly important recognition molecules in humans are the Neph and Nephrin proteins (15). Mutation of members of this class of adhesion proteins results in kidney failure and severe protein loss into urine due to the disruption of the kidney filtration barrier (9, 19). Neph proteins are characterized by an extracellular domain containing five immunoglobulin repeats, a transmembrane region and an approximately 220-amino acid cytoplasmic tail that mediates protein interactions and signaling (16). Whereas mammals contain three Neph proteins (Neph1-3), two Neph-like proteins can be found in *Drosophila melanogaster* (Roughest, Kirre) and at least one in *Caenorhabditis elegans* (SYG-1)(10). Neph proteins interact with the related proteins of the Nephrin family. In the mammalian kidney it could be demonstrated that signaling through these junctional proteins directs formation, maturation and maintenance of highly specialized, interdigitating secondary processes of kidney podocytes, the visceral epithelial cells of the glomerular filtration barrier (9, 19, 46). In *Drosophila* Neph-like Roughest and Kirre mediate cell recognition and morphogenesis in the development of the fly compound eye (1, 27). The adult compound eye of *Drosophila* is composed of about 750 unit eyes, or ommatidia, that are arranged in a highly ordered crystal-like pattern. This biological crystal develops from an unpatterned single layered epithelium, the eye imaginal disc (5, 44). In the young pupa four lense secreting cone cells and two primary pigment cells are separated by a pool of undifferentiated interommatidial precursor cells. During pupal development this interommatidial lattice is shaped into a very regular honeycomb-like pattern of secondary and tertiary pigment cells. This morphogenetic process which requires cell sorting and extensive cell shaping is controlled through Neph and Nephrin proteins. Interestingly, these molecular mechanisms are evolutionarily highly conserved and have been shown to largely also apply to the development of the mammalian kidney filter making the fly eye a perfect model systems to address signaling processes at the kidney filter (6).

Although much has been learned about the role of Neph proteins in controlling morphogenetic processes the integration of adhesion and cell signaling of the cell recognition module to orchestrate cell movement, cytoskeletal reorganization and junction remodeling are poorly understood.
Here we show that Neph proteins interact with the BAR domain protein PICK1 (protein interacting with C kinase 1) and that this interaction stabilizes Neph proteins at the plasma membrane. PICK1 was first identified as an interactor of protein kinase C alpha (PKC\(\alpha\)) (36). PICK1 harbours a PDZ domain (PSD95/DlgA/ZO-1) at the N-terminus and a BAR domain (Bin/amphiphysin/Rvs) at the C-terminus. BAR domains form crescent shaped dimers and are involved in sensing and/or regulating membrane curvature, a prerequisite for vesicle formation (11, 17, 25). PICK1 has been shown to be involved in modulating the trafficking of neuronal receptors (14). We used the Drosophila eye imaginal disc to demonstrate that PICK1 and Neph proteins are involved in common pathways in vivo suggesting a critical role for BAR domain proteins in controlling important morphogenetic processes.

Materials and Methods

Reagents and plasmids

PMA (TPA, Cell Signaling Technology #9905) was used at a final concentration of 200 nM for 30 min. Full length PICK1 cDNA was cloned from a human podocyte cDNA library using standard cloning techniques. Mouse Neph1, Neph2 and Neph3 cDNAs were cloned into a modified pcDNA6 expression vector coding for the CD5 signal peptide fused to the V5-tag sequence (sV5-tag) followed by restriction sites to insert the cDNA. ZO-1 plasmids have been described before (16). A membrane-bound fusion construct of the cytoplasmic domain of Neph1 (sIg.7.Neph1\(^{cyt}\)) has been described previously (31). Antibodies were from Sigma (FLAG), Serotec (mAb V5), Millipore (pAb V5), Santa Cruz (H-300 goat pAb anti-PICK1). A polyclonal anti-Neph2 antibody has been described previously (12). For Drosophila immunofluorescence the following antibodies were used: rat anti-DE-cadherin (Developmental Studies Hybridoma Bank), rabbit anti-Pyd (kind gift from R. Cagan), mouse anti-Rst (24A5; Fischbach lab), rabbit anti-Kirre (126intra, Fischbach lab), rabbit anti-Hbs (14intra, Fischbach lab), rabbit anti-SNS (Fischbach lab).

Fly strains

All crosses were kept at 25°C. The following fly lines were used: Rst\(^{3}\) (Fischbach lab), UAS-
mCD8::GFP (Fischbach lab), GMR-GAL4 (Fischbach lab), GMR-GAL4, UAS-dicer2 and UAS-
dicer2 (gift from R. Cagan) and UAS-PICK1-RNAi (Vienna Drosophila RNAi Center, strains ID22268 and ID104486).
Cell culture and coimmunoprecipitation

HEK 293T cells were transfected with the calcium phosphate method, and incubated for 24 h. Cells were then washed with PBS, lysed in ice-cold IP-buffer (1% Triton X-100; 20 mM Tris pH 7.5; 25 mM NaCl; 50 mM NaF; 15 mM Na$_3$P$_2$O$_7$; 1 mM EDTA; 0.25 mM PMSF; 5 mM Na$_3$VO$_4$) on ice for 15 min and centrifuged (14,000 rpm, 4°C, 15 min). Supernatants containing equal amounts of total proteins were incubated for 1 h at 4°C with a polyclonal anti V5 antibody (Chemicon) that was bound to Protein G Sepharose before (1 h at 4°C) or with anti-FLAG antibody covalently coupled to agarose beads (Sigma #A2220). The precipitates were washed three times with IP-buffer and bound proteins were resolved by 10% SDS-PAGE.

For endogenous coimmunoprecipitations, tissues were freshly prepared and kidneys were perfused in situ with ice-cold PBS before lysis. Mouse brains and kidneys were homogenized using a glass potter, cleared by centrifugation, and solubilized in lysis buffer supplemented with 20 mM CHAPS and 3 mM ATP. After centrifugation at 15,000 $\times$ g (15 min, 4°C) and centrifugation at 100,000 $\times$ g (30 min, 4°C), cell lysates containing equal amounts of total protein were precleared with protein A-sepharose and then incubated for 1 hour at 4°C with the appropriate antibody, followed by incubation with 30 µl of protein A-sepharose beads for 3 hours. The beads were washed extensively with lysis buffer, and bound proteins were resolved by 10% SDS-PAGE.

RNA interference experiments

RNA interference experiments were done as described previously (29). In brief, shRNAs were designed based on the prediction of publicly available prediction programs (https://rnaidesigner.invitrogen.com/maixpress/). shRNAs were cloned into the transient microRNA expression vector pcDNA6.2-GW/emGFP/miR (Invitrogen). To monitor the efficiency of shRNA-mediated knockdown, we created a luciferase reporter construct using psicheck2 (Promega) in which the coding sequence of PICK1 was fused to the coding sequence of R. reniformis luciferase as an artificial 3’ UTR. In addition to R. reniformis luciferase, this construct expresses firefly luciferase for internal control. The reporter plasmid was cotransfected with the respective pcDNA6.2-GW/emGFP/miR shRNA construct into HEK 293T cells. R. reniformis luciferase and firefly luciferase activities were measured 24 h after transfection. Transfections and measurements were performed in triplicate. Selected hairpins (hp#1:
TGCTGTCAAATACCTGGACGATATAGGTTTTGGCCACTGACTGACCTATATCGCAGG
TATTTGA ; hp#5 TGCTGACGACAGGTACTC AAACTTCAGTTTTGGCCACTGACTGACT
GAAGTTTGTACCTGTCGT ) were cloned into one cluster (chaining) and this cluster was
GATEWAY cloned into pLenti6.2/V5-Dest (Invitrogen) for stable lentiviral expression in human
podocytes cells.

**Human podocyte culture**

Human immortalized podocytes (AB 8 cells) were cultivated as described previously (28). In
brief, cells were grown in standard RPMI 1640 medium containing 10% FCS and supplements
either at the permissive temperature of 33°C (in 5% CO2) to promote cell propagation or at the
nonpermissive temperature of 37°C (in 5% CO2) to allow the terminal differentiation.

**Pull-down assay**

MBP.Neph1\textsuperscript{57} and GST.ZO-1\textsuperscript{1-111} were expressed in E. coli and affinity purified. Purified
MBP.Neph1\textsuperscript{57} was bound to amylose resin (New England Biolabs) and incubated at 4°C for 60
min with GST.ZO-1\textsuperscript{1-111} that has been preincubated with buffer, recombinant PKCa (0.5 U;
Invitrogen), or CIAP (0.5 U; Invitrogen), respectively, for 30 min at 37°C. Bound proteins were
eluted from the resin with Laemmli buffer and subjected to western blot analysis.

**Surface labelling**

24 h after transfection HEK293T cells were starved for 3 h with serum free medium. Cells were
washed twice with PBS and surface labeled with 0.6 mg/ml EZ Link Sulfo-NHS-SS-Biotin
(Pierce) for 15 min at room temperature. Cells were incubated with quenching buffer (100 mM
glycine in PBS) for 10 min at room temperature, washed twice with PBS and lysed in IP buffer.
Precleared lysates were incubated with NeutrAvidin UltraLink Resin (Pierce) for 8 h at 4°C.
Bound proteins were eluted from the resin with Laemmli buffer and subjected to western blot
analysis.

**In vitro phosphorylation**

His.Neph1\textsuperscript{57} was expressed in *E. coli*. 5 μg of His.Neph1\textsuperscript{57} was bound to 30 μl of Ni-NTA
agarose (Qiagen). *In vitro* phosphorylation of His.Neph1\textsuperscript{57} was performed for 20 min at 30°C in
a 60 μl reaction in kinase reaction buffer containing 50 μM ATP (supplemented with 10 μCi of γ-
^{32}P-ATP (Perkin Elmer)). Phosphorylation was initiated by the addition of 0.5 U of PKCα purified from rat brain (Sigma) in enzyme dilution buffer or enzyme dilution buffer alone (control) or 0.5 U of CIAP (New England Biolabs). To monitor the incorporation of phosphate, radiolabeled His.Neph1 was visualized by SDS–PAGE and autoradiography.

Immunofluorescence

Hela cells were seeded on coverslips and transfected using Genejuice reagent (Novagen). After 24-48 h cells were fixed for 10 min in 4% paraformaldehyde. Fixed cells were incubated for 30 min in blocking solution (5% normal donkey serum, 0.1% Triton X-100) and subsequently incubated with primary and secondary antibodies. After washing in PBS, the cells were mounted in Prolong Gold antifade (Invitrogen) and subjected to immunofluorescence microscopy with an Axiovert 200M microscope / EC Plan-Neofluar 40x/1.30 Oil objective equipped with a CCD camera (all from Carl Zeiss).

For labeling of surface/endocytosed sV5.Neph1, cells were incubated at 4°C with mAb anti-V5 (Serotec) diluted in full medium for 30 min. Cells were washed with medium three times and were allowed to recover at 37°C for 30 min. Cells were then washed with PBS, fixed, but not permeabilised, with 2% paraformaldehyde for 5 min, blocked with 5% normal donkey serum in PBS and incubated with a donkey Alexa 488 coupled anti-mouse antibody (Invitrogen). After washing, cells and bound antibodies were fixed again for 5 min, before cells were incubated with permeabilizing blocking solution (5% normal donkey serum, 0.1% Triton X-100). Then a Cy3 coupled donkey anti-mouse secondary antibody (Jackson Immuno Research) was applied.

Drosophila retina immunohistology procedures were essentially as described (27). Briefly, staged pupal eye discs (18-19 h or 41-42 h after puparium formation (APF)) were dissected and fixed for 15 minutes in 4% paraformaldehyde, blocked with normal donkey serum in PBS with 0.1% Triton X-100 and incubated with primary antibody. A fluorescent dye coupled secondary antibody (Cy3, DyLight 549 or DyLight 488; Jackson ImmunoResearch) was used as the secondary antibody. Preparations were embedded in Vectashield (Vector) and image acquisition was done with a Zeiss LSM710 / AxioObserver Z.1 equipped with an EC Plan-Neofluor 40x/1.30 Oil objective (Carl Zeiss).

Adult eye fluorescence
Adult flies were frozen at -80°C. The flies were glued on a slide and autofluorescence was used to image the eye with a Zeiss LSM710 / AxioObserver Z.1 equipped with an EC Plan Neofluar 10x/0.3 objective (Carl Zeiss).

**Image acquisition and processing**

Fluorescent images were acquired using Zeiss Axiovision 4.8 and Zeiss ZEN 2009 (Carl Zeiss) and assembled using Adobe Photoshop and Illustrator CS4 (Adobe Inc.) or ImageJ (26). For better visibility of membrane staining in Fig. 2, gamma was set to 0.45 in Axiovision. ImageJ software was used for Fluorescence intensity measurements in Fig. 8 and intensity profiles in Fig. 2 (RGB line profile plugin).

**Results and Discussion**

**Neph proteins bind PICK1 via PDZ domain-mediated interactions.** To identify critical regulators of the Neph/Nephrin cell recognition module we generated a cDNA library from human podocytes. The cDNA library was subjected to yeast-two-hybrid screens using the intracellular domains of Neph2 and Neph3 as baits. These screens identified PICK1 as a major interactor of Neph2 and Neph3 (58% of hits in the Neph2 screen, 18% in the Neph3 screen). PICK1 is a 55 kDa cytosolic protein with several protein interaction domains (45).

To confirm the interaction we cloned PICK1 from the human podocyte cDNA library and performed coimmunoprecipitation experiments from transiently transfected HEK 293T cells. These experiments indicated that PICK1 interacts with all three Neph proteins and confirmed the interaction with Nephrin (Fig. 1A) (42). The interaction could also be demonstrated for endogenous proteins precipitated from mouse kidney and mouse brain lysates (Fig. 1B and not shown), suggesting an *in vivo* complex formation of Neph and PICK1 proteins. PICK1 is expressed in podocytes as demonstrated in knockdown experiments using a human podocyte cell line (Fig. 1C,D).

Neph proteins contain a cytoplasmic tail that harbours a type I PDZ binding motif at the very carboxyl terminus (16). Since PICK1 contains a PDZ domain we tested whether the Neph/PICK1 interaction is mediated via a PDZ domain-mediated mechanism. Deletion of the last three amino acids representing the PDZ domain binding motif (ΔTHV) at the carboxyl terminus of Neph1, Neph2 and Neph3 strongly abrogated binding to PICK1 (Fig. 1G). Moreover, deletion of the PDZ
domain in PICK1 also abrogated the interaction (Fig. 1G) indicating that the Neph-PICK1 protein complex forms via PDZ domain mediated interactions.

In PICK1 a short stretch at the N-terminus of the BAR domain, identified as a coiled-coil motif, mediates self association (3) (Fig. 1F). To test whether dimerization of PICK1 may affect interaction with Neph proteins, we mutated valine at position 150 and tyrosine at position 163 into proline. These mutations were expected to interfere with the formation of a coiled-coil structure (Fig. 1F) and thus thought to abrogate dimerization. Co-immunoprecipitation experiments using wild-type PICK1, as well as the coiled coil mutant PICK1_{V150P/Y163P} (cc-mut) indicated that dimerization of PICK1 through the coiled-coil motif is required for the association of PICK1 with Neph proteins (Fig. 1G). Taken together, these data suggest that functional PICK1 dimers are able to bind Neph proteins and that the interaction is mediated through the PDZ domain of PICK1 and the PDZ-binding motif within the carboxyl terminus in Neph1-3.

PICK1 and Neph proteins colocalize at the plasma membrane and in intracellular vesicles derived from the plasma membrane. PICK1 has recently emerged as a central mediator in regulating endocytosis and/or recycling of neuronal receptors such as AMPA-type glutamate receptors (GluR2) and metabotropic glutamate receptors (mGluR7) (7, 24, 40, 14). In these systems PICK1 can act in either direction: GluR2 is removed from the plasma membrane in a PICK1 dependent manner, while surface localization of mGluR7 is stabilized in a PICK1 dependent manner (21, 24, 40). Given the specific interaction between Neph proteins and PICK1, we speculated that PICK1 may be involved in recycling Neph adhesion proteins. To directly test this hypothesis, we performed immunofluorescence experiments. Coexpression of PICK1 and Neph proteins in Hela cells identified a colocalization of these proteins. Besides the expected colocalization of the expressed proteins in the endoplasmic reticulum we found a strong colocalization at the plasma membrane and in intracellular vesicles (Fig. 2). Strikingly, colocalization of Neph1 and PICK1 as well as plasma membrane staining of PICK1 was lost in a deletion mutant lacking the PDZ domain (Fig. 2 and data not shown). Recruitment of PICK1 to the membrane is a known prerequisite for the activation of the BAR domain (23).

We next went on to test whether PICK1 may influence vesicular trafficking, targeting or stability of Neph1 at the plasma membrane. Endocytosis assays using a pulse chase approach did not show gross differences of overall internalization of Neph1 in the presence or absence of PICK1 (not shown). However, surface biotinylation experiments revealed that expression of PICK1
significantly increased the amount of plasma membrane Neph1 whereas plasma membrane targeting of a control plasma membrane protein (transferrin receptor) was not affected (Fig. 3). These data indicated that PICK1 acts to stabilize Neph1 at the plasma membrane. BAR domains act as sensors and/or regulators of membrane curvature (17). Since BAR domain dimers bind preferentially to liposomes of specific size (25), it is not unlikely that different BAR domains have slightly different geometries, which in turn determines the type of vesicle or invaginated membrane that is bound (14). PICK1 may therefore associate with a certain subset of vesicles. To test whether PICK1 specifically associates with Neph proteins in endocytic vesicles we performed a fluorescence based endocytosis assay (Fig. 4A). Hela cells were cotransfected with sV5.Neph1 and with FLAG.PICK1. Living cells were pulse labeled with a mouse anti-V5 antibody, washed and transferred to 37°C for 30 min to allow internalization of protein antibody complexes. After fixation the plasma membrane pool of Neph1 and the internalized Neph1 proteins were stained in different colors. PICK1 co-staining revealed a perfect colocalization with endocytosed Neph1 originating from the plasma membrane (Fig. 4A). Interestingly, a subpopulation of the Neph1-PICK1 positive vesicles stained positive for the late endosome marker Rab7, which supported the concept that PICK1 may act to recycle Neph proteins from the late endosome and related compartments (Fig. 4B).

PKC activity controls preferential binding of PICK1 and release from the plasma membrane associated multidomain protein Zonula occludens 1 (ZO-1). PICK1 has been shown to be important for neuronal biology such as hippocampal and cerebellar longterm depression, mainly by targeting neuronal receptors to endocytosis or retrieval through a PDZ domain interaction which is dependent on PKCα activity (18, 20, 37). Moreover, recent studies suggested that PICK1 and PKCα act together to stabilize mGluR7 at the plasma membrane. This stabilization required phosphorylation of mGluR7 by PKCα which in turn enhanced the binding of mGluR7 to PICK1 (40). Since phosphorylation of neuronal receptors disrupted their binding to the PDZ domain-containing scaffolding proteins such as ABP/GRIP but did not affect binding to PICK1 (22) we tested whether PKCα activity also influenced binding of Neph1 to the scaffolding protein Zonula occludens 1 (ZO-1). We had previously demonstrated that Neph1 binds ZO-1, a large multidomain PDZ protein similarly to the GluR2-ABP/GRIP interaction (16). ZO-1 interacts with Neph1 at the plasma membrane providing a scaffold for Neph1 signaling activity (16, 32) and it has been suggested that Neph-dependent cell recognition requires constant
remodeling of the cell junction (2, 15). Activation of PKCα with the phorbol ester PMA resulted in an inhibition of ZO-1 binding to Neph1 (Fig. 5A) without affecting the PICK1-Neph1 interaction (Fig. 5B). Of note, overexpressed PICK1 also co-precipitated with FLAG-tagged ZO-1 as shown in Fig. 5A. Repeated experiments revealed that this interaction was quite weak and only occurred with overexpressed proteins. To test whether ZO-1 is part of the endogenous PICK1 complex we used a tandem affinity purification approach and characterized the interacting proteins (not shown). Although this study identified a series of specific partner proteins including the published BAR domain protein ICA-69, ZO-1 was not among these candidates (not shown).

Inhibition of ZO-1 binding to Neph1 was strongly enhanced in the presence of PICK1 (Fig. 5A). To test whether PKCα was able to phosphorylate the cytoplasmic tail of Neph1 to control ZO-1 binding, Neph1 and ZO-1 were recombinantly expressed and purified and subjected to PKCα phosphorylation followed by pull-down experiments. PKCα treatment resulted in direct phosphorylation of the Neph1 cytoplasmic tail (Fig. 5D) and disrupted the interaction of ZO-1 with Neph1 (Fig. 5C). Consistent with these findings coexpression of PKCα with ZO-1 and Neph1 inhibited the interaction in HEK 293T cells (Fig. 5E). Taken together, these data show that the Neph1/ZO-1 interaction is dynamically regulated by PKCα and PICK1 and suggest that PICK1 may act in concert with PKC activity to control Neph1 recycling to the plasma membrane.

dPICK1 and Roughest, a Drosophila homolog of Neph1, cooperatively control patterning of the Drosophila compound eye. Drosophila eye development involves cell sorting, cell death, and remodeling of cell contacts and shapes – processes that have been shown to be controlled by Neph and Nephrin mediated adhesion and signaling (1, 10, 10, 13, 27). The primary pigment cells express Nephrin proteins, Hbris (Hbs) and Sticks-and-stones (Sns), that interact with the Nephrin-like proteins, Roughest (Rst) and Kin-of-irre (kirre), present on neighbouring cells. Heterophilic adhesion between Nephrin- and Nephrin-like proteins results in remodeling contacts between cells to favor their contact with the pigment cells (1, 10). This sorting and culling process results in a pattern with a precise number and position of interommatidial cells (44).

Double stranded RNAs corresponding to PICK1-mRNA were expressed under the control of the eye specific driver GMR-GAL4, together with the RNAi enhancer dicer2 (8). Interestingly, knockdown of dPICK1 resulted in a rough eye phenotype closely resembling the rst phenotype (Fig. 6). The phenotype was confirmed using two independent RNAi lines (Fig. 6E,F). To
demonstrate that the PICK1 phenotype was dependent on the Neph function we tested for genetic interaction and made use of the weak allele rst\(^3\) (41). The presence of one rst\(^3\) allele in females, which have one intact copy of the rst gene on the X-chromosome, and one copy of the GMR-GAL4 transgene on the second chromosome, leads to a very mild phenotype. These flies demonstrated almost wild type patterned eyes (Fig. 7, first column). In males, however, the weak rst\(^3\) phenotype was displayed (Fig. 7, second column). The addition of RNAi-mediated knockdown of dPICK1 using GMR-GAL4, without the RNAi enhancer dicer2, did not show any dramatic effect in females carrying one rst\(^3\) allele (Fig. 7, third column). However, adding the PICK1 knockdown to the rst\(^3\) allele in males (still in the absence of dicer2) resulted in a dramatic enhancement of the phenotype clearly confirming the genetic interaction (Fig. 7, last column).

Taken together these data indicated that PICK1, very similarly to the mammalian situation, acts to support Neph/Roughest function in the fly.

To examine the PICK1 function in the fly eye in greater detail we analyzed Neph and Nephrin like proteins, collectively known as IRM proteins (10), during eye development and stained pupal eye discs at a morphogenetically highly active stage (18-19 hr APF). Interestingly, dPICK1 knockdown did not result in an obvious subcellular redistribution of IRM proteins in cells of the eye discs (Fig. 8). However, we realized that the fluorescence intensity was generally weaker in the dPICK1 knockdown eye discs as compared to controls. Quantification of the fluorescence intensity confirmed this effect (Fig. 8B,C). Knockdown of dPICK1 in the fly eye resulted in a significant decrease of Rst and Kirre expression at the plasma membrane but did not affect the expression of other proteins (E-cadherin, Pyd, SNS) (Fig. 8 B,C). Since the IRM proteins are required for cell sorting at this stage of development (27) this reduction could well explain the rough eye phenotype.

Taken together, this work highlights the important role of PICK1 in regulating the Neph-dependent cell recognition module and Neph-mediated steps in morphogenesis. Members of the Neph/Nephrin cell recognition module are conserved across species (10). They are involved in the recognition of different cell types and are mediating cell contacts of opposing cell membranes. Thus, it is highly conceivable that similar mechanisms may regulate the dynamic function of the Neph/Nephrin cell recognition module in many species. We observed an increased Neph level at the plasma membrane in the presence of PICK1 in a cell culture biotinylation assay and a decreased Neph level in dPICK1 knockdown Drosophila eye discs.
Thus, PICK1 seems to affect the rate of turnover and recycling of Neph adaptor proteins to affect their signaling abilities. In light of this hypothesis, it is important to consider that Neph proteins do not only function as adhesion molecules. They are involved in signaling for the regulation of cellular processes such as remodeling of cell contacts, organizing cell shape changes and cell sorting during Drosophila eye development (1, 10, 27), cell fusion in Drosophila muscle development (4, 38), correct wiring of the Drosophila brain (30, 39), localization and stabilisation of presynaptic sites in C. elegans (34, 35), the development and maintenance of the kidney podocyte (9, 19), or the wiring of olfactory sensory neurons in the olfactory bulb of the mouse (33). It is obvious that these processes require the tightly regulated and timed expression of the Neph/Nephrin proteins at their surface. The PDZ/BAR domain protein PICK1 has been shown to regulate the timed expression and/or recycling of associated proteins at the plasma membrane (22, 43). In conclusion, PICK1 was identified as an essential novel regulator of Neph/Nephrin proteins most likely by influencing their availability to mediate signal transduction at the plasma membrane.

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References


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**Figure legends**

**Figure 1**

FIG. 1. PICK1 interacts with Neph/Nephrin proteins via its PDZ domain. (A) Neph1-3 and Nephrin co-precipitate with PICK1. FLAG-tagged PICK1 and sV5-tagged Neph/Nephrin or control protein were expressed in HEK 293T cells and precipitated with anti-FLAG antibody. Western blot analysis was performed with a V5 specific antibody. Expression levels in the lysates are shown below. (B) Endogenous PICK1 interacts with Neph2 in the brain. Protein lysates from mouse brains were subjected to immunoprecipitation with a control antibody (anti-myc) or PICK1-specific antibody, washed extensively and immunoblotted with specific anti-Neph2 antibody. The last lane depicts Neph2 expression in the brain, shown here after Neph2 precipitation due to low expression levels in the lysate. (C) To create an artificial 3' UTR for luciferase, the coding sequence of human PICK1 was cloned into a bicistronic vector, fusing this sequence with the coding sequence of Renilla luciferase. Renilla luciferase activity, normalized to firefly luciferase activity that served as an expression control, was used to assay the efficiency of the shRNA-mediated knockdown of PICK1. The knock-down efficiency of chained hairpins #1&5 are shown. Scrambled shRNA (miR-neg) was used as a control. Experiments were performed in triplicate. Error bars represent SD. (D) PICK1 is expressed in human podocytes. Human podocytes were stably transduced with PICK1 hairpin#1&5 or miReg-neg, respectively. PICK1 was precipitated from cell lysates with a rabbit anti-PICK1 antibody and immunoblotted with a goat anti-PICK1 antibody. A HEK293T cell lysate with overexpressed untagged human PICK1
served as a control. (E) PICK1 co-precipitation with Neph proteins is dependent on the PDZ
domain binding motif. FLAG-tagged PICK1 and V5-tagged Neph1-3 full length proteins or V5-
tagged Neph proteins missing the PDZ-binding motif (ΔTHV) were expressed in HEK 293T cells
and precipitated with anti-V5 antibody. Western blot analysis was performed with a FLAG
specific antibody. Expression levels in the lysates are shown below. (F) Schematic representation
of PICK1 showing residues V150 and Y163 within the predicted coiled-coil domain. Predictions
using the program COILS suggests that mutation of these residues to proline impairs formation of
a functional coiled-coil domain (http://www.ch.embnet.org/software/COILS_form.html). (G)
Neph proteins co-precipitate with PICK1 in a PDZ-domain and coiled-coil domain dependent
manner. FLAG-tagged PICK1, PICK1 missing the PDZ domain (ΔPDZ), or PICK1V150P,Y163P (cc-
mut) were coexpressed with V5-tagged Neph full length proteins and precipitated with anti-
FLAG antibody. Western blot analysis was performed with a V5 specific antibody. Expression
levels in the lysates are shown below.

Figure 2
FIG. 2. PICK1 colocalizes with Neph proteins. HeLa cells were transiently transfected with the
constructs indicated (Neph constructs with sV5-tag; PICK1 constructs with FLAG-tag) and
stained with antibodies against the tags. The full length Neph proteins colocalize with full length
PICK1 in cytoplasmic patches and at the plasma membrane. PICK1ΔPDZ does not colocalize
with Neph proteins. The merged image shows the PICK1 channel in green and the Neph channel
in magenta. Nuclei are stained with DAPI (blue). Boxed regions showing cell borders are shown
in an enlarged view below each image. Intensity profiles for PICK1 (green) and Neph (magenta)
along the white line in the merged image are shown to the right to assess the colocalization of
PICK1 and Neph proteins and the random colocalization of PICK1ΔPDZ and Neph proteins.

Figure 3
FIG. 3. (A) sV5.Neph1 was expressed in HEK293T cells either in the presence or in the absence
of PICK1. Biotinylated surface-expressed proteins were precipitated with NeutrAvidin resin and
V5-tagged Neph1 was visualized by Western blot using V5 antibody. Staining for the Transferrin
receptor served as a loading control. (B) Quantitation of the blots was performed by measuring
the band intensity of Neph1 compared with the intensity of the band representing Transferrin receptor. Graphs represent means ± SEM; ****p < 0.0001 (unpaired Student’s t test). (n = 7).

**Figure 4**

FIG. 4. (A) PICK1 colocalizes with endocyosed Neph1. Hela cells were cotransfected with sV5.Neph1 and with FLAG.PICK1. Living cells were labeled with mouse anti-V5 antibody for 30 min at 4°C. Cells were then washed and transferred to 37°C for additional 30 min. Next, the cells were fixed and labeled with a secondary anti-mouse-Alexa488 antibody (green fluorescent). After washing, the bound antibodies were briefly fixed. The cells were then permeabilized and labeled with an anti-mouse-Cy3 (red fluorescent) secondary antibody to mark internalized intracellular Neph1. PICK1 was stained with a rabbit anti-FLAG antibody. PICK1 clearly colocalizes with endocytosed Neph1 in intracellular vesicles (examples are marked with circles).

(B, C) Coexpression of PICK1 stabilizes Neph1 at the plasma membrane. (B) A subpopulation of PICK1 / Neph1 endocytosed vesicles is positive for the late endosome marker Rab7. Hela cells were cotransfected and treated as in (A), except that extracellular V5 epitopes were not labeled but blocked with a anti-mouse-antibody (non fluorescent). In addition, Rab7 was stained with a rabbit anti-Rab7 antibody. A subpopulation of the vesicles is positive for endocytosed Neph1, PICK1 and Rab7.

**Figure 5**

FIG. 5. PKCα and PICK1 cooperatively regulate the Neph1/ZO-1 plasma membrane complex.

(A) FLAG-tagged ZO-1\textsuperscript{1-503} and V5-tagged Neph1\textsuperscript{59} were coexpressed with PICK1 or empty vector. ZO-1 was precipitated with anti-FLAG antibody. Western blot analysis was performed with a V5 specific antibody to detect coprecipitated Neph protein. Activation of protein kinase C (PKCα) with 200 nM PMA inhibits the interaction (lane 5 and 6). Coexpression of PICK1 further augments the PKC effect on the Neph1/ZO-1 interaction (lane 1 and 2) without affecting protein levels (middle and lower panels). (B) PMA does not influence the PICK1/NEHP1 interaction. Experimental setting as in (A) with the constructs indicated. (C, D) The interaction Neph1/ZO-1 is regulated through PKCα activity. (C) Pull down assay of bacterially expressed GST.ZO-1\textsuperscript{1-111} and MBP.Neph1\textsuperscript{59} in the presence of buffer, PKCα or calf intestinal alkaline phosphatase.
Addition of PKCα abrogates the Neph1/ZO-1 interaction whereas CIAP did not affect the interaction. (D) Kinase assay of bacterially expressed His.Neph1α in the presence of radioactively labeled ATP and buffer, PKCα or CIAP. Addition of PKCα led to phosphorylation of Neph1α. Autoradiograph after in vitro phosphorylation of His.Neph1α (E) Membrane bound Neph1α (sIg7.Neph1α) or a control plasmid (sIg.7.cdm12), and FLAG-tagged ZO-11.503 were expressed in HEK 293T cells and precipitated with protein G. Western blot analysis was performed with a FLAG specific antibody. Overexpression of PKCα abrogated the Neph1/ZO-1 interaction.

**Figure 6**

FIG. 6. Knockdown of dPICK1 causes a rst mutant-like phenotype. (A-C) Scanning electron micrographs of adult eyes. (A-B) Controls have a wild-type patterned eye. (C) GMR-GAL4 driven knockdown of dPICK1 (RNAi transgene on the third chromosome) leads to a rough eye phenotype. The RNAi enhancer dicer2 was coexpressed. (D-E) Midpupal eye imaginal discs stained for DE-cadherin. GMR-GAL4 driven dPICK1 knockdown with two independent RNAi transgenes lead to the same Rst-like phenotype. Many ommatidia are not separated by a single, but by a double row of interommatidial cells (arrows). The RNAi enhancer dicer2 was coexpressed. (D) Driver line control. (E) dPICK1 RNAi transgene on the second chromosome. (F) dPICK1 RNAi transgene on the third chromosome.

**Figure 7**

FIG. 7. Genetic interaction between Rst and dPICK1. The genotype for each column is indicated on top. First row: Images of adult eyes. The weak phenotypes of the Rst3 allele (2nd column) and dPICK1 RNAi knockdown (3rd column) enhance each other to a severe phenotype if both lesions are present in males (rightmost column). Pupal eye imaginal discs (3 individual discs per genotype, 42 h APF) stained with anti-Pyd illustrate the same effect. In the eye imaginal discs of these animals clear sorting defects are visible. Many ommatidia are not separated by a single, but by a double row of interommatidial cells (arrows).
FIG. 8. Knockdown of dPICK1 does not change the subcellular distribution but the total protein level of IRM proteins. (A) Pupal eye imaginal discs (18-19 h APF) stained with the antibodies indicated. The subcellular localization of the stained proteins is indistinguishable between dPICK1 knockdown (GMR-GAL4; UAS-dPICK1RNAi > UAS-dicer2) and control (GMR-GAL4 > UAS-dicer2). Brightness and contrast were adjusted for optimal visibility of the localization the proteins (B,C) Quantitation of the fluorescence intensity. Genotypes as in (A). Quantitation of the eye discs was performed by measuring the mean fluorescence intensity in dPICK1 knockdown eye discs compared with control eye discs. Graphs represent mean fluorescence intensities of individual eye discs (each data point one eye disc) ± SEM; ****P < 0.0001, ***P <=0.0003 (unpaired Student’s t test).