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A PKD1L3 splicing variant in taste buds is not cleaved at the G protein-coupled receptor proteolytic site

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Abstract

Mutations in polycystin proteins PKD1 and TRPP2 lead to autosomal dominant polycystic kidney disease. These two proteins form a receptor-ion channel complex on primary cilia. PKD1 undergoes an autoproteolysis at the N terminal G-protein-coupled receptor proteolytic site (GPS), which is essential for the function of PKD1. Whether GPS cleavage happens in other PKD proteins and its functional consequence has remained elusive. Here we studied the GPS cleavage of PKD1L3, a protein that associates with TRPP3 in taste cells and may play a role in sour taste. Our results show that PKD1L3 also undergoes GPS cleavage. Mutation at the GPS abolishes the cleavage, and the non-cleavable mutant does not traffic to the plasma membrane when associated with TRPP3. We also found that a splicing variant of PKD1L3, which was originally identified in taste buds, is not cleaved. Amino acids L708 and S709, which are missing in this splicing variant, are crucial for the GPS cleavage of PKD1L3 and the trafficking of the PKD1L3/TRPP3 complex. Our results gain insight into the molecular mechanism of the GPS cleavage of PKD1L3. The presence of the non-cleavable variant suggests the potential *in vivo* function of uncleaved PKD proteins.

Keywords

polycystic kidney disease; PKD1L3; TRPP3; polycystin; GPS cleavage; alternative splicing

1. Introduction

Autosomal Polycystic Kidney Disease (ADPKD) is one of the most common genetic diseases which affects 1 in 400 to 1000 individuals [1]. The disease is caused by mutations in PKD1 (also known as polycystin-1) or TRPP2 (also known as polycystin-2 or PKD2) respectively [2]. PKD1 belongs to the polycystic kidney disease (PKD) protein family which consists of 5 members: PKD1, PKD1L1, PKD1L2, PKD1L3, and PKDREJ [3]. PKD

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proteins contain 11 transmembrane domains, a large extracellular N-terminus and a short intracellular C-terminus. TRPP2 belongs to the polycystin subfamily of the transient receptor potential (TRP) ion channel superfamily [4], which has three members, TRPP2, TRPP3 and TRPP5 [2]. TRPP proteins have six transmembrane domains with intracellular N- and C-termini. PKD proteins interact with TRPP proteins to form heteromeric receptor-ion channel complexes [2, 5, 6]. Previously, both PKD1/TRPP2 and PKD1L3/TRPP3 complexes were shown to contain one PKD and three TRPP proteins [7–10], and the PKD1L3 subunit is involved in forming the ion channel with TRPP3 [9].

PKD proteins were found structurally related to cell adhesion G protein-coupled receptors (aGPCR) [11]. A key feature of aGPCRs is that they undergo an autoproteolytic cleavage at the G protein-coupled receptors proteolysis site (GPS) within a conserved GPCR-Autoproteolysis INducing (GAIN) domain, which is located right before the first transmembrane segments [12]. The cleavage happens within the “HL^T(S)” motif (^: scissile bond) [13], and yields the extracellular N-terminal fragment (NTF) and the membrane-embedded C-terminal fragment (CTF) that tethered together through non-covalent interactions [11, 12]. The GAIN domain and the HL^T motif is conserved in some PKD proteins [12], and PKD1 was found to undergo GPS cleavage [14, 15], which is crucial for PKD1’s trafficking and function [16, 17], and many PKD1 pathogenic mutations alter this process [12, 15]. Non-cleavable PKD1 mutants showed significantly reduced *in vivo* function in mice [17, 18]. In spite of the crucial role of GPS cleavage, little is known on how this cleavage affects the ion channel function of the PKD1/TRPP2 complex. This is due to the lack of knowledge on the activation mechanism of this complex, which makes the functional study very challenging. The recently developed GOF mutants of TRPP2 channel provide new avenue for functional study of PKD1/TRPP2 complex [19, 20], which may help on solving this issue. The GPS cleavage in other PKD proteins and its functional consequences also remains elusive.

Besides PKD1/TRPP2, several other PKD/TRPP complexes have been identified. Among them, the PKD1L3/TRPP3 complex can be activated by low pH through a so-called “off-response” mechanism [9, 21, 22], which is named since its current only show up when the applied acid is washed off [21]. The PKD1L3/TRPP3 complex was suggested to be involved in sour taste reception since both proteins are expressed and colocalized in sour sensing taste cells [22–24], and TRPP3-knockout mice or mice with genetic ablation of TRPP3-expressing taste receptor cells showed sour taste defect [24, 25].

Since the current of the PKD1L3/TRPP3 complex is recordable, this complex is a good model for studying the effects of the GPS cleavage on channel function of PKD/TRPP complexes. In this study we confirmed the GPS cleavage of PKD1L3, and studied the effect of GPS cleavage on trafficking and channel activity of the PKD1L3/TRPP3 complex. More interestingly, we found that the GPS cleavage is missing in a splicing variant of PKD1L3, which was identified in taste receptor cells [23], and investigated its molecular mechanism. Our results gain insight into the essential role of the GPS cleavage in trafficking and function of the PKD1L3/TPPP3 complex, and indicate a potential role of the non-cleavable forms of the PKD proteins.

2. Materials and Methods

2.1. DNA Constructs

cDNAs for mouse PKD1L3 (NCBI accession number AY164486), PKD1L3 splicing variant 1a (NCBI # DQ382345), and human TRPP3 (NCBI # NM_016112) were used. For *Xenopus* oocytes experiments, PKD1L3 cDNA with an HA tag fused on the N-terminus, or with an HA tag and FLAG tag on the N- and C-termini respectively, and TRPP3 cDNA with either no tag or a FLAG tag fused to N-terminus were cloned into a modified pGEMHE2 vector for *in vitro* transcription. For HEK 293T cell experiments, PKD1L3 constructs were cloned into pDisplay vector, and TRPP3 was cloned in pCDNA 3.1(−) vector. All PKD1L3 constructs were fused with the murine Ig k-chain leader sequence on the N-terminus.

2.2. Immunofluorescence

Immunofluorescence of HEK 293T cells was done with a protocol described previously [26]. Mouse monoclonal anti-HA (BioLegend), anti-FLAG (Sigma), and secondary antibody Alexa Fluor 488 and Alexa Fluor 594 (Life Technologies) were used. Coverslips were imaged with a Leica confocal microscope.

2.3. Surface Protein Biotinylation

Proteins on *Xenopus* oocyte plasma membrane was isolated with Pierce Cell Surface Protein Isolation kit as described previously [27], and detected with Western blot.

2.4. SDS-PAGE and Western blotting

Protein samples from cells, *Xenopus* oocytes, or mouse tissue lysates (abcam) were run on 4–12% Bolt Bis-Tris gel (Life Technologies) and transferred to PVDF membrane. Primary antibodies mouse monoclonal anti-HA (Covance), anti-Flag (Sigma), and rabbit polyclonal anti-PKD1L3 antibody (Thermo Fisher Scientific), and secondary antibodies goat anti-mouse antibody (Sigma) or Alexa Fluor 680 goat anti-mouse antibody (Thermo Fisher Scientific) were used. Blot signals were visualized with either Bio-Rad or Li-COR Odyssey imaging system.

2.5. Electrophysiology

Xenopus oocytes were injected with cRNAs obtained from *in vitro* transcription (50ng of cRNA per oocyte) and incubated at 18°C for four to five days. Channel current was then recorded with two-electrode voltage clamp (TEVC) method at room temperature and in a bath solution containing 100 mM NaCl, 0.5mM $MgCl_2$and 2 mM HEPES, pH 7.5. The same solution was adjusted to pH 2.6 with citric acid for acid application. Protocols for TEVC recording for the acid-induced off-response of TRPP3-PKD1L3 complex has been described in detail previously [9].

3. Results:

3.1. Mouse PKD1L3 is cleaved at the N-terminal GPS site.

Sequence alignment shows PKD1L3 contain the conserved HL^T sequence in the GAIN domain [12]. Therefore, we started with confirming that PKD1L3 is cleaved at the N-terminal GPS site (Fig. 1A). PKD1L3, with HA and FLAG tagged fused to N- and C-termini respectively, expressed in *Xenopus* oocytes showed two bands when blotted with either anti-HA or anti-FLAG antibody (Fig. 1B, left image). The higher band which ran above 250 kDa in both blots is the full-length protein. The smaller bands, which ran at ~190 kDa and ~140 kDa when blotted with anti-HA and anti-Flag antibody respectively (Fig. 1B), match well to the calculated sizes of the cleaved NTF (170 kDa) and CTF (132 kDa) if the cleavage happens at the GPS site. In both blots, the smaller bands have a higher intensity than the full-length bands, indicating that most proteins were cleaved (Fig. 1B), similar to what was found in PKD1 [15]. To find out whether PKD1L3 is cleaved *in vivo*, we detected the endogenously expressed PKD1L3 protein in mouse tissue lysates from various organs. The result showed that PKD1L3 was only detected from tongue sample with an anti-PKD1L3 antibody against the NTF of PKD1L3 (Fig. 1B, right image). Only cleaved PKD1L3-NTF fragment showed up in this blot while the full-length protein band may be too weak to be detected. The specificity of this antibody was confirmed by detecting PKD1L3 overexpressed in HEK 293T cells (Fig S1). Thus, our data show that most PKD1L3 undergoes GPS cleavage after expressed in *Xenopus* oocyte or HEK 293T cell, as well as *in vivo* (tongue).

Mutations at the HL^T motif, including mutating the L (−1) to histidine (H), have been shown to abolish the GPS cleavage in PKD1 [15]. To further confirm that the cleavage of PKD1L3 happens at the HL^T site, we introduced the L to H (L1044H) mutation in PKD1L3, and tested the cleavage after expressing it in *Xenopus* oocytes. As shown in Fig. 1C, the mutant only gave the full-length band while the cleaved NTF band is missing, indicating that GPS cleavage is abolished. These data strongly supports the cleavage occurrence at the GPS in PKD1L3.

We further tested how the GPS cleavage affects the function of PKD1L3/TRPP3 complex by measuring the acid-induced off-response after expressed the two proteins in oocytes. The result shows that Introducing the L1044H mutation abolished the off-response current (Fig. 1D and 1E). Since GPS cleavage has been shown to be crucial for the trafficking of PKD1 [16, 17], we then tested how the GPS mutation affects the plasma membrane trafficking of the PKD1L3/TRPP3 complex. In the presence of TRPP3, the WT PKD1L3 protein can traffic to plasma membrane when expressed in HEK 293T cells (Fig. 1F, top left two panels), as shown previously [22]. However, when the non-cleavable mutant PKD1L3_L1044H was expressed with TRPP3, its plasma membrane expression is greatly reduced (Fig. 1F, top right panel). This result shows that without GPS cleavage of PKD1L3, the plasma membrane trafficking of PKD1L3/TRPP3 complex is majorly abolished, which is consistent with our function data in oocytes. Thus, GPS cleavage is crucial for the trafficking and function of the PKD1L3/TRPP3 complex.

3.2. A splicing variant of PKD1L3 does not undergo GPS cleavage.

Alternative splicing yields mRNA with different inclusion of exons. Proteins translated from these mRNA can be structurally and biochemically different from each other and carry out variable functions [28]. PKD1L3 mRNA also undergoes alternative splicing [29]. A previous study have found several PKD1L3 splicing alternatives in taste buds of mouse circumvallate papillae [23]. One of the alternatively spliced variants, PKD1L3 variant 1a (PKD1L3–1a), has three differences compared to the originally reported PKD1L3 [29], the version used in our above experiments. The PKD1L3–1a RNA is missing a 60 bp region at the beginning of exon 7, has an extra 90 bp exon 7A right after exon 7, and an extra 120 bp exon 33 at 3' of the RNA (Fig. S2) [23]. The two variable regions around exon 7 lead to missing of 20 amino acids (Q698-F717), and an addition of 30 amino acids in a downstream region in PKD1L3–1a (Fig. S2). Both changes are about 300 amino acids away from the HL^T cleavage site.

In our experiment, we found that PKD1L3–1a gave no acid induced off-response when co-expressed with TRPP3 in *Xenopus* oocytes (Fig. 2A). Since exon 7 and 7A is located within the GAIN domain, we then checked whether the GPS cleavage is affected in PKD1L3–1a. The result showed that indeed PKD1L3–1a expressed in *Xenopus* oocytes was not cleaved (Fig. 2B). We further checked its surface expression and found that PKD1L3–1a did not traffic to the plasma membrane when co-expressed with TRPP3 in HEK 293T cells, (Fig. 2C), consistent with the lack of channel function in *Xenopus* oocytes.

3.3. L708 and S709 are crucial for GPS cleavage in mouse PKD1L3.

Since partial deletion of exon 7 in PKD1L3–1a lead to 20 amino acids missing at the beginning of the GAIN domain (Fig. 3A), we next focused on this region to gain insight into the molecular mechanism. We paid our attention to the sequence "DLSE" in the center of these 20 amino acids, since D707 and E710 are the only two charged amino acids. We first deleted "DLSE" in the original PKD1L3 protein and found that this deletion mutant (PKD1L3_ΔDLSE) lost channel function after co-expression with TRPP3 in oocytes (Fig. 3B). We then further neutralized D707 and E710 to generate the PKD1L3_NLSQ mutant and found it has full channel function in complex with TRPP3 (Fig. 3B). Thus, the charged D707 and E710 are not important for channel function. We then mutated L708 and S709 to negatively-charged amino acids and generated the mutant PKD1L3_DEDE. Interestingly, this mutant lost all channel function (Fig. 3B). Actually, the function of PKD1L3 is very sensitive to these two amino acids since mutating either one of them (PKD1L3_L708E or PKD1L3_S709D) is sufficient to abolish the current (Fig. 3B).

We wondered whether L708E and S709D abolish channel function by interfering with PKD1L3/TRPP3 trafficking. Surface biotinylation results from oocytes show that, when TRPP3 is co-expressed, WT PKD1L3 and the PKD1L3_NLSQ were present on the membrane, while L708E and S709D mutants were absent on the plasma membrane, indicating the trafficking of the last two mutants is eliminated. Furthermore, we noticed that in the oocyte lysate samples (Fig. 3C, bottom left image), it can be clearly seen that both single mutants almost completely abolished the GPS cleavage. Accordingly, the trafficking of TRPP3 to the plasma membrane, which heavily relies on its assembly with PKD1L3 [9, 22], was also abolished when PKD1L3_L708E or S709D were co-expressed (Fig. 3C, two

blot pictures on the right). Thus, our results show that the 20 amino acids missing in variant 1a, more specifically, amino acids L708 and S709, are crucial for the GPS cleavage of PKD1L3. Changes in this region can interfere with GPS cleavage and abolish trafficking of the PKD1L3/TRPP3 complex as well as its channel function.

3.4. Molecular mechanism based on a structural model.

With the online structure prediction server Phyre2 [30], we generated a structural model of the GAIN domain of PKD1L3 based on the GAIN domain structure of the aGPCR protein CIRL/Latrophilin 1 (CL1) [12]. The GAIN domain in CL1 is formed by subdomain A which has 5 α-helices bundled together, and the subdomain B which is full of β-sheets (Fig. 4A). The GPS domain, which is formed by five short β-sheets, is located in the last part of the GAIN domain (Fig. 4A, in purple). The model of the PKD1L3 GAIN domain, which includes the majority part of the GAIN domain, shows an overall similar structural arrangement to that of the CL1 GAIN domain. The missing 20 amino acids in PKD1L3_1a forms the second α-helix in the subdomain A (Fig. 4B, left image, in red). This helix should be structurally essential for maintaining the conformation of subdomain A. Missing this helix, or deleting “DLSE” in the center of the helix, will lead to dramatic conformational changes in GAIN doamin, and consequently interfere with GPS cleavage.

Future analysis reveals the possible reason why amino acids L708 and S709, but not D707 and E710, are essential for GPS cleavage. As shown in Fig. 4B, the two charged amino acids D707 and E710 are pointing to the outside of the helix and have no interaction with other parts of the structure. On the other hand, L708 and S709 are both pointing to the inside of the helix bundle and should play a crucial role in maintaining the structure.

4. Discussion:

In the PKD family proteins, the GPS cleavage motif “HL^T” is conserved in PKD1, PKD1L2, and PKD1L3, but not in PKD1L1 and PKDREJ [12]. Here we showed that GPS cleavage happens in PKD1L3 as well and is essential for the trafficking and ion channel activity of the PKD1L3/TRPP3 complex. Our results indicate that GPS cleavage is conserved among a subgroup of PKD proteins and further confirm its critical role in their function.

Although GPS cleavage is essential for the trafficking and function of PKD1 [18] and PKD1L3 (shown in this study), the non-cleaved PKD proteins exist *in vivo* and may play particular roles. First, since “HL^T” motif is not conserved in PKD1L1 and PKDREJ [12], these two proteins may not undergo GPS cleavage. Second, the GPS cleavage of PKD1 is not complete, and a significant amount of PKD1 remains as non-cleaved full-length protein in the embryonic kidney, indicating a special function of this form in embryonic development [18]. More interestingly, the non-cleavable mutant of PKD1, T3041V, was found to partially preserve the *in vivo* function of PKD1 in early development [18], further confirming that the full-length non-cleaved PKD1 is functional. Here, in addition to the finding that the GPS cleavage is also not complete in PKD1L3 (Fig. 1B, 2C, and 3C), we found that the GPS cleavage is missing in the splicing variant PKD1L3–1a (Fig. 2). This is significant since PKD1L3–1a was identified in tongue, the place where PKD1L3 plays its

role in sour taste. Since our results show that PKD1L3–1a does not traffic to plasma membrane when associate with TRPP3, the non-cleavable PKD1L3–1a may have a potential TRPP3-independent function *in vivo*, which reveals the roles of non-cleaved full-length PKD proteins, including PKD1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights:

- Polycystin protein PKD1L3 undergoes autoproteolysis at N-terminal GPS domain.
- GPS cleavage is essential for trafficking and function of PKD1L3/TRPP3 complex.
- Splicing variant PKD1L3–1a, found in taste buds, does not undergo GPS cleavage.
- Two amino acids, missing in PKD1L3–1a, is essential for GPS cleavage of PKD1L3.

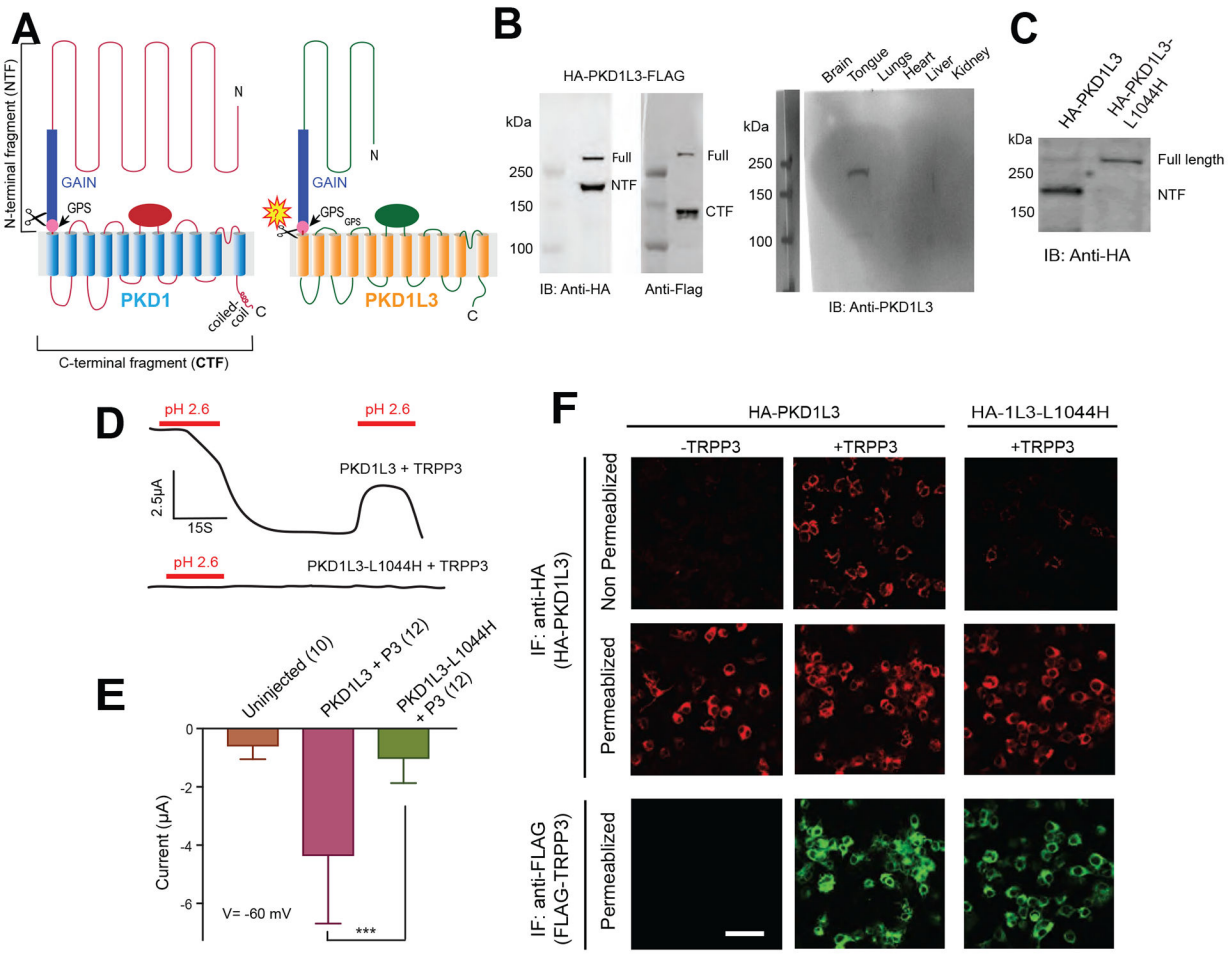

Fig. 1.

PKD1L3 undergoes N-terminal GPS cleavage. (A) Schematic diagram showing the structure of PKD1 and PKD1L3. The scissor shows the GPS cleavage site in PKD1 and the question mark denotes the GPS cleavage in PKD1L3 that is being explored in this study. (B) Western blot on the left shows the cleavage happens after PKD1L3 was expressed in *Xenopus Laevis* oocytes. Western blot on the right shows the cleaved band is present in mouse tongue tissue. Samples were blotted with an anti-PKD1L3 antibody recognizing the N-terminus of the protein. (C) Western blot shows the cleavage is missing in PKD1L3_L1044H when expressed in *Xenopus* oocytes. (D) Whole-cell off-response current induced by acid (pH 2.6, red bar) in oocytes expressing the indicated constructs. (E) Bar graph shows the average acid-induced currents at −60 mV from the PKD1L3/TRPP3 and PKD1L3_L1044H/TRPP3 in *Xenopus* oocytes. Data are shown as mean and standard deviation. ***P < 0.001. (F) Immunofluorescence shows PKD1L3_L1044H was not expressed on plasma membrane in the presence of the TRPP3. Both non-permeablized (top row) and permeablized (bottom two rows) conditions were used to show surface expression and overall expression, respectively.

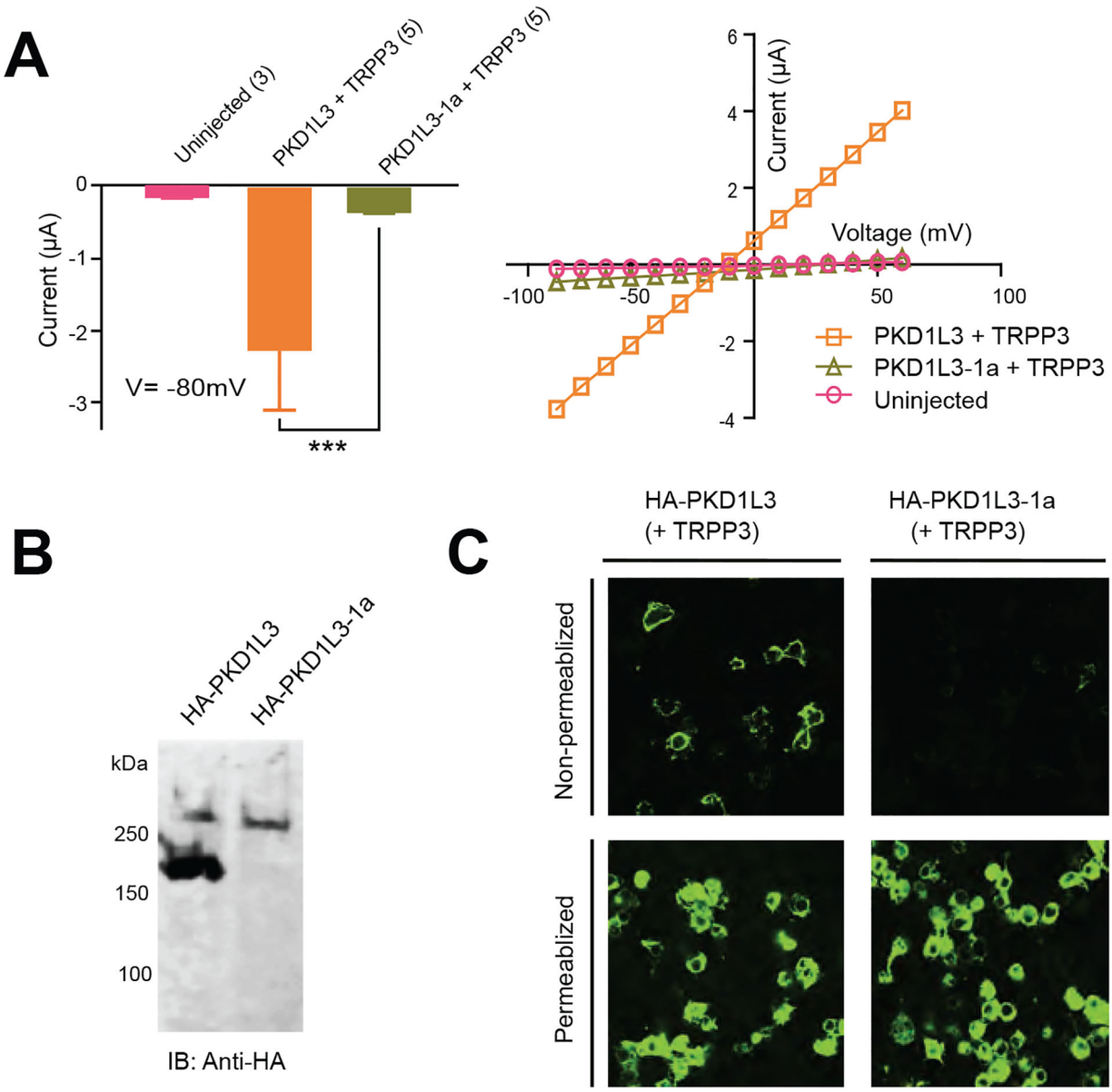

Fig. 2.

Splicing variant PKD1L3–1a does not undergo GPS cleavage. (A) Bar graph (left) and representative currents (right) show the absence of the acid-induced off-response from *Xenopus* oocytes co-expressing PKD1L3–1a and TRPP3. Statistical significance was calculated with t-test. ***P < 0.001. (B) Western blot shows the lack of GPS cleavage of PKD1L3–1a expressed in oocytes. (C) Immunofluorescence images show that HA-tagged PKD1L3–1a was not expressed on the plasma membrane when co-expressed with TRPP3 in HEK 293T cells, while PKD1L3 was. Anti-HA antibody was used to detect HA-tagged PKD1L3.

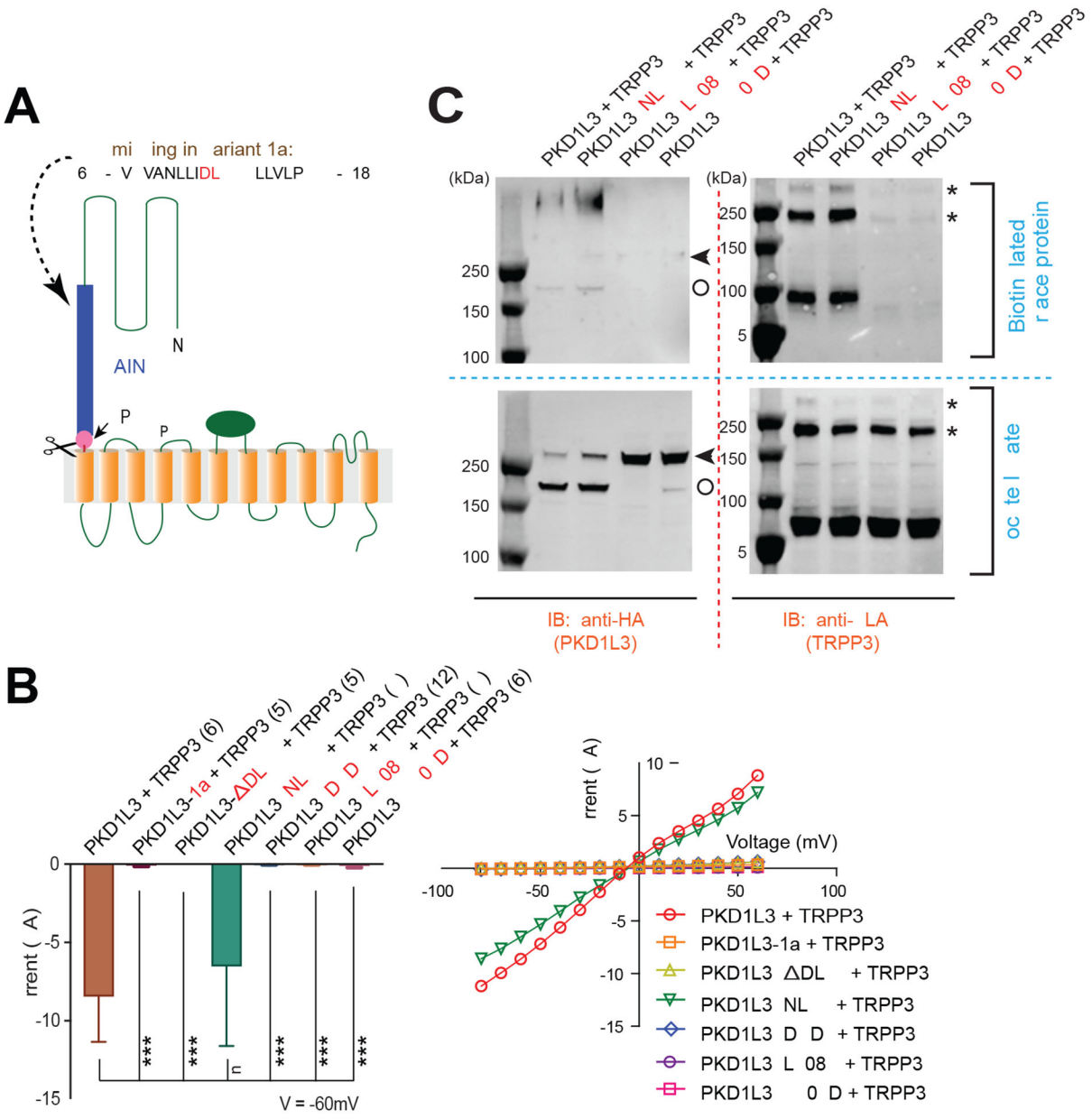

Fig. 3.

L708 and S709 are essential for GPS cleavage in PKD1L3. (A) Schematic diagram shows the relative position of the 20 amino acids, which are missing in PKD1L3–1a, in the GAIN domain. The four amino acids “DLSE” (in red) were chosen for further analysis in our following experiments. (B) Bar graph (left) and representative acid-induced off-response currents (right) from *Xenopus* oocytes injected with indicated RNA combinations. The means and standard deviations are shown in the bar graph. Statistical significances were compared between PKD1L3/TRPP3 (first bar) and the other samples with t-test. ***P < 0.001; n.s.: not significant. (C) Surface biotinylation experiment showing the lack of the GPS cleavage of the L708E and S709D mutants expressed in *Xenopus* oocytes (lower left

image), and the lack of surface expression of their complexes with TRPP3 (pictures on the top row).

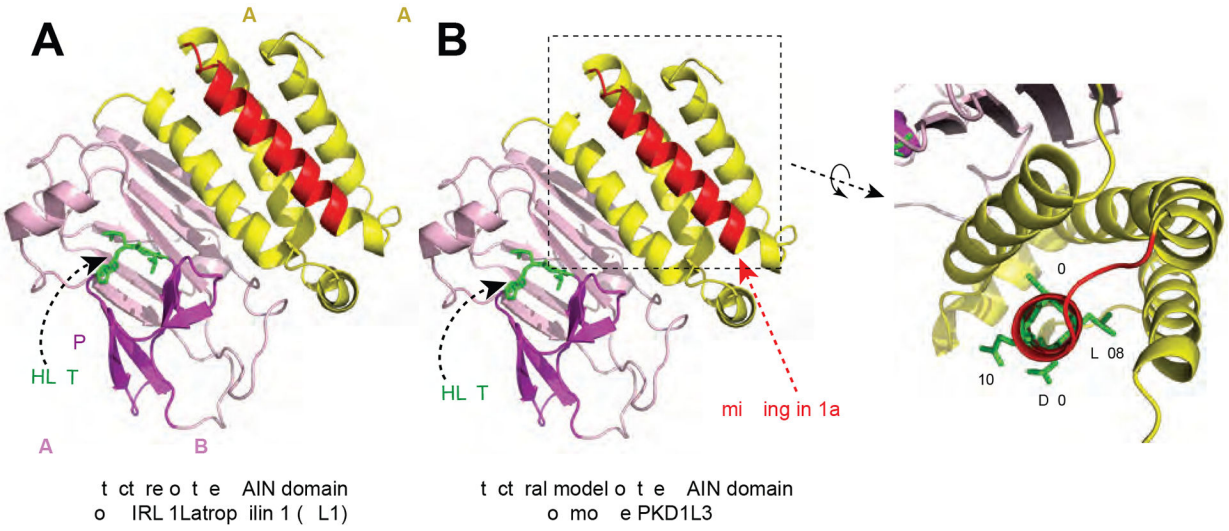

Fig. 4.

The molecular mechanism of the roles of L708 and S709 in maintaining GPS cleavage revealed by a structural model of the PKD1L3 GAIN domain. (A) The crystal structure of the GAIN domain of cell-adhesion GPCR CL1 (PDB access code 4DLQ) [12], showing the two subdomains A and B, the GPS domain, and the HL^T cleavage site. Helix in red indicates the corresponding position of the missing structure in PKD1L3_1a shown further in B. (B) Structural model of the GAIN domain of the PKD1L3 built with Phyre2 [30]. Left: the structure model of PKD1L3 GAIN domain shows great overall similarity to the structure of the GAIN domain of CL1. Splicing in PKD1L3–1a leads to the helix missing in subdomain A, which is indicated in red. Right: A zoom-in and top view of the missing helix in 1a, showing that L708 and S709 are pointing to the center of helix bundle and may play a role in stabilizing subdomain A of the GAIN domain.