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Functional analysis of the distal region of the third intracellular loop of PROKR2

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ABSTRACT

Mutations in the G-protein-coupled receptor PROKR2 have been identified in patients with idiopathic hypogonadotropic hypogonadism (IHH) and Kallmann syndrome (KS) manifesting with delayed puberty and infertility. Recently, the homozygous mutation V274D was identified in a man displaying KS with an apparent reversal of hypogonadism. The affected amino acid, valine 274, is located at the junction region of the third intracellular loop (IL3) and the sixth transmembrane domain (TM6). In this study, we first studied the effect of V274D and related mutations (V274A, V274T, and V274R) on the signaling activity and cell surface expression of PROKR2. Our data indicate that a charged amino acid substitution at residue 274 of PROKR2 results in low cell surface expression and loss-of-function. Furthermore, we studied the effects of two clusters of basic amino acids located at the proximal region of Val274 on the cell surface expression and function of PROKR2. The deletion of RRK (270–272) resulted in undetectable cell surface expression, whereas RKR (264–266)-deleted PROKR2 was expressed normally on the cell surface but showed loss-of-function due to a deficiency in G-protein coupling. Our data indicate that the distal region of the IL3 of PROKR2 may differentially influence receptor trafficking and G-protein coupling.

1. Introduction

Prokineticins (PK1 and PK2) are a pair of cysteine-rich peptides with a molecular weight of ≈10 kDa [1] and bind to two closely related G-protein-coupled receptors (GPCRs), i.e., PROKR1 and PROKR2, respectively [2–4]. PKs and their cognate receptors are involved in a variety of physiological functions, including gastrointestinal mobility, circadian rhythms, emotion, nociception, angiogenesis, neurogenesis, and food intake [1,5–13]. The binding of PKs to their receptors elicits various downstream signaling pathways, such as the accumulation of inositol phosphate and the mobilization of intracellular Ca^{2+} via Gαq/11 proteins, the inhibition of cAMP accumulation through Gαi/o proteins, and the stimulation of the mitogen-activated protein kinase (MAPK) via Gα/o protein-mediated signaling [2,14].

Recently, mutations in PK2 and PROKR2 have been identified in patients affected with Kallmann syndrome (KS) and/or idiopathic hypogonadotropic hypogonadism (IHH), which are disorders characterized by delayed puberty and infertility [15–19]. Hypogonadotropic hypogonadism is due to gonadotropin-releasing hormone (GnRH) deficiency. Disease-associated PROKR2 mutations may result in improper trafficking onto the plasmic membrane, an inability to bind its ligand PK2, or a disruption in its coupling to G proteins [15,20–22].

Sinisi et al. identified a homozygous V274D mutation in a man diagnosed with KS with an apparent reversal of hypogonadism [23]. The proband is an offspring of consanguineous parents. His mother, who exhibits a heterozygous V274D mutation, only showed a delayed menarche at 15 years of age, and this was followed by regular menstrual cycles [23]. The affected amino acid Val274 is located at the junction region of the third intracellular loop (IL3) and the sixth transmembrane domain (TM6) (Fig. 1A), which is a region that is involved in the activation of many GPCRs. In this study, we took advantage of this disease-associated mutation to understand the potential role of the distal region of IL3 in PROKR2 receptor signaling.

Abbreviations: ER, endoplasmic reticulum; GPCR, G-protein-coupled receptor; HEK, human embryonic kidney; IHH, idiopathic hypogonadotropic hypogonadism; IL, intracellular loop; KS, Kallmann syndrome; MAPK, mitogen-activated protein kinase; PK2, Prokineticin 2; PROKR2, Prokineticin receptor 2; TM, transmembrane domain; WT, wild-type.

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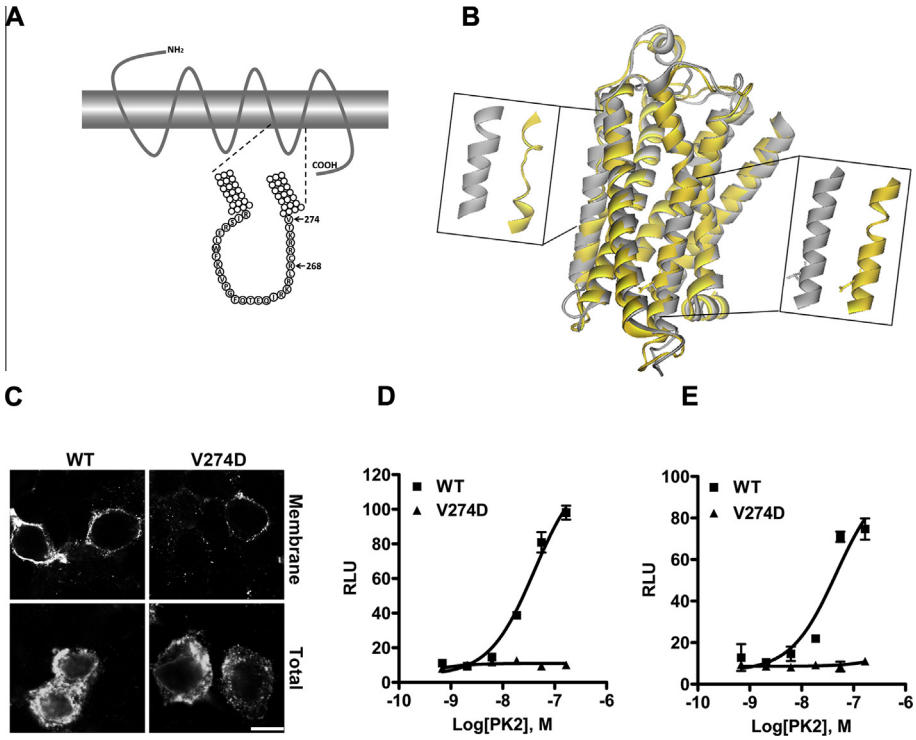

Fig. 1. The V274D mutation of PROKR2 resulted in reduced cell surface expression and loss-of-function. (A) A schematic illustrating the WT PROKR2 structure and indicating the sequences of IL3. The arrows indicate two amino acids (R268 and V274) at IL3 that have been found to be mutated in patients with Kallmann syndrome. (B) Superimposed three-dimensional image of WT (gray) and V274D (orange) PROKR2. The boxed images show the aa174–190 (left) and the aa270–288 (right) regions. In the superimposed image, we only considered the residues 52–348 because both the N- and the C-terminal residues are highly unreliable. (C) The V274D mutation resulted in lower cell surface expression of PROKR2, as demonstrated through an immunofluorescence assay. Scale bar, 7.5 μm. (D and E) V274D is a loss-of-function mutation, as demonstrated through an aequorin-based calcium mobilization assay (D) and an Egr-Luc-based assay (E).

2. Materials and methods

2.1. Antibodies

Mouse monoclonal M2 anti-FLAG antibody and the M2 anti-FLAG antibody conjugated with FITC were purchased from Sigma (Sigma, St Louis, WA, USA).

2.2. Plasmids

The full-length human PROKR2 (hPROKR2) was amplified using PCR and inserted in place of mPROKR2 in pRK5-mPROKR2 (a kind gift of Philippe Rondard, Université Montpellier I & II, France) [20]. The resultant protein possesses the mGlu5 signal peptide followed by a FLAG-tag and the complete hPROKR2 sequence without the first methionine. Plasmids encoding PROKR2 harboring various mutations were engineered using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instruction.

2.3. Immunofluorescence assay

Human embryonic kidney (HEK) 293 cells were plated on cover slips and transfected with 250 ng of wild-type (WT) PROKR2 or the mutant constructs. At 48 h after transfection, the cells were fixed with 4% paraformaldehyde for 30 min at room temperature. Some of the cells were then permeabilized for 1 h in 0.2% Triton X-100. The cells were incubated with blocking solution (PBS, 3% bovine serum albumin, and 5% goat serum) for 1 h and were then incubated overnight at 4 °C with primary anti-FLAG M2 antibody (1:200 dilution). After the cells were washed with PBS, the cells were incubated with the fluorescence-labeled secondary anti-mouse antibodies (1:300 dilution; Invitrogen, Carlsbad, CA, USA) for 2 h in a dark room. After incubation with 4,6-diamino-2-phenylindole (DAPI; Invitrogen, Carlsbad, CA, USA) for 2 min, the cells were mounted in Fluoromount medium (Sigma, St Louis, WA, USA), and the fluorescence images were examined with a laser scanning confocal system installed on a Carl Zeiss microscope (Zeiss, Gottingen, Germany) with a 5000× oil immersion objective. The images were analyzed using the Metaphor software package.

2.4. Calcium mobilization assay

An aequorin-based luminescent assay was used to measure the mobilization of intracellular Ca^{2+} as described previously [21].

2.5. Gene transcription reporter assay

HEK293 cells were transiently transfected with 5 ng of either WT or mutant PROKR2 plasmid and 5 ng of Egr-1-luciferase reporter (a kind gift from Nelly Pitteloud, Harvard Medical School, USA) [15]. At 24 h after transfection, the cells were stimulated with PK2 in serum-free medium containing 0.1% BSA. After 16-h incubation, the cells were lysed in luciferase assay buffer (Promega Corp., Madison, WI, USA), and the luciferase induction was analyzed using a luciferase assay system (Promega Corp., Madison, WI, USA).

2.6. Quantification of receptor expression on membrane by fluorescence flow cytometry

The expression levels of the receptors on the cell membrane were measured using a flow cytometric assay. Briefly, monolayers of transfected cells expressing the indicated FLAG-tagged receptor were dissociated from dishes using 0.04% EDTA in PBS lacking Ca^{2+}

or Mg^{2+}. The cell suspensions were washed twice in 1 ml of PBS and incubated at 4 °C for 60 min in PBS containing 1% bovine serum albumin and 2.5 μg/ml M2 anti-FLAG antibody conjugated to FITC. After three washes, the surface receptor immunoreactivity was measured through fluorescence flow cytometry (FACScan; BD Biosciences, Palo Alto, CA, USA). The fluorescence intensity of 20,000 cells was collected for each sample, and each condition was tested in triplicate samples. The Cellquest software was used to calculate the mean fluorescence intensity of the cell populations. All of the experiments were conducted at least three times with similar results.

2.7. Molecular modeling

Because there was no homology structure with sequence identity of at least 40% in the PDB database [24], the intensive mode of the Phyre2 server [25] was utilized to predict the three-dimensional structures of PROKR2. The Phyre2 server uses the alignment of hidden Markov models via HHsearch [26] to significantly improve the accuracy of alignment and the detection rate. The sequences of WT and V274D PROKR2 were submitted to the Phyre2 server, and six templates (SCOP codes [27]: c2ksaA [human Substance-P receptor], c2rh1A [human β2-adrenergic receptor], c3emlA [Human Adenosine A2A receptor], c3uonA [human M2 muscarinic acetylcholine receptor], c4djhA [human κ-type opioid receptor], and c3pdsA [human β2-adrenergic receptor]) were selected to model the protein based on heuristics to maximize the confidence, percentage identity, and alignment coverage.

3. Results

3.1. V274D mutation results in reduced cell surface expression of PROKR2

To understand the effects of the V274D mutation, we first conducted molecular modeling of the WT and V274D PROKR2 using the Phyre2 server. As expected, one amino acid change did not significantly alter the overall structure of PROKR2 (Fig. 1B). However, a more detailed analysis of the secondary structure (Fig. 1B) indicated that the V274D mutation may lead to the disruption of both TM6 and TM4 α-helices. We thus speculated that the V274D mutation may result in inefficient targeting of PROKR2 to the cell surface.

We used an immunofluorescence assay to probe PROKR2 on the cell surface. As shown in Fig. 1C, the WT PROKR2 is correctly expressed on the cell surface, whereas the V274D mutation resulted in a significant reduction in the level of PROKR2 on the cell surface. The total receptor expression was not significantly altered, as demonstrated through immunofluorescence in permeabilized cells (Fig. 1C). We also quantified the receptor expression through flow cytometry and found that the cell surface amount of V274D PROKR2 was approximately 45.7 ± 0.24% ($P < 0.01$, unpaired t-test) of that found for the WT receptor.

3.2. V274D is a loss-of-function mutation

We studied the effect of V274D mutation on the PROKR2 signal transduction using an aequorin-based assay to measure the intracellular calcium mobilization. As shown in Fig. 1D, PK2 stimulation induced a dose-dependent increase in the intracellular calcium mobilization in WT PROKR2-expressing cells. In stark contrast, the V274D PROKR2 mutant exhibited no increase in calcium mobilization. PROKR2 signaling also activates the MAPK pathway, which in turn elevates the gene expression of *Egr-1* [2,15]. We thus used a construct in which the Egr-1 promoter was fused upstream to the luciferase reporter gene (Egr-Luc) to measure the activation of the MAPK pathway by PK2 stimulation. As expected, PK2 increased the luciferase activity in a dose-dependent manner when Egr-Luc was co-transfected with WT PROKR2 (Fig. 1E). Similar to the calcium mobilization study, PK2 failed to increase the luciferase activity when Egr-Luc was co-transfected with the V274D PROKR2 mutant (Fig. 1E).

Recently, Abreu et al. showed that a KS-associated PROKR2 mutation (R80C) exerts a dominant negative effect on the WT receptor, presumably by interfering with the expression of the WT receptor [22]. However, the V274D PROKR2 did not show any dominant negative effect on the WT receptor, even when its DNA level was fivefold higher than that of the WT (data not shown), coincident with the fact that only a homozygous carrier is affected with KS [23].

3.3. Residue 274 is intolerant to a charged amino acid

To further understand how the precise physiochemical characteristics of residue 274 might dictate the strength of the function of PROKR2, valine 274 was converted to arginine (V274R), alanine (V274A), or threonine (V274T) through site-directed mutagenesis. The PK2-stimulated Ca^{2+} mobilization was measured in cells transiently transfected with these individual PROKR2 variants. As shown in Fig. 2A, the replacement of Val274 with alanine (V274A) and threonine (V274T) residues did not significantly alter the signal transduction of PROKR2; however, a basic amino acid (V274R) mutation resulted in a disruption of the signal transduction of PROKR2. We also characterized the signal transduction of individual PROKR2 mutants using the Egr-Luc reporter system. Consistent with the results of the calcium mobilization study, the activation of the V274A and V274T PROKR2 mutants by PK2

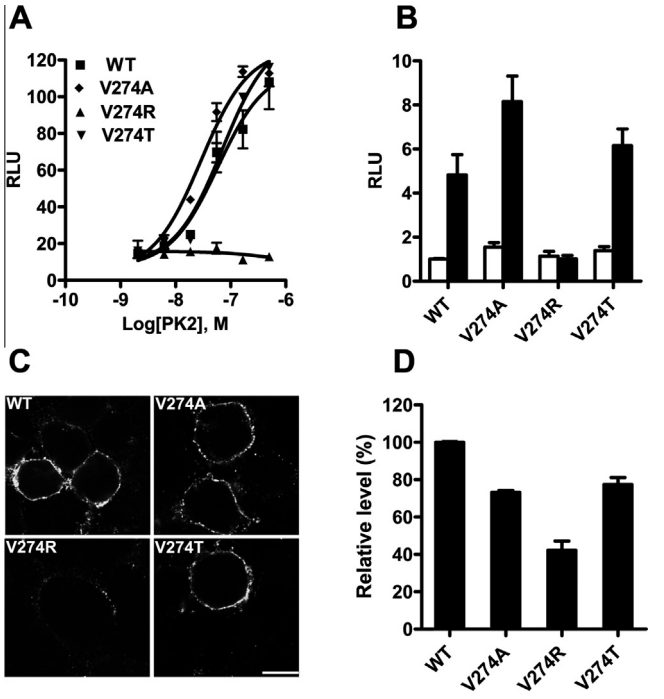

Fig. 2. Mutation analysis of Val274 of PROKR2. (A-B) The signaling activity of V274A, V274T, and V274R PROKR2 was measured using an aequorin-based calcium mobilization assay (A) and an Egr-Luc-based assay (B). In panel B, PK2 was added to a final concentration of 50 nM and incubated for 16 h. (C and D) The cell surface expression of PROKR2 with various mutations at Val274 was demonstrated using an immunofluorescence assay (C) and through flow cytometry (D). Scale bar, 7.5 μm.

increased the luciferase activity to the same degree as the WT receptor, whereas the stimulation of V274R PROKR2 failed to increase the luciferase activity (Fig. 2B).

We therefore used an immunofluorescence assay to measure the cell surface expression of the various Val274 mutants. As shown in Fig. 2C, the V274A and V274T mutations did not significantly affect the cell surface expression of this receptor. However, the cell surface expression of the V274R PROKR2 was markedly reduced. The cell surface expression of these receptors was further quantified through flow cytometry. As shown in Fig. 2D, the cell surface expression of V274R PROKR2 was only 42% of the surface expression of WT, whereas the surface expression levels of V274A and V274T PROKR2 were more than 70% of the surface expression of WT. The total protein expression was not significantly different between the WT and mutant receptors, as demonstrated through the immunofluorescence of permeabilized cells (data not shown).

3.4. Truncation of RRK (270–272) disrupts the cell surface expression of PROKR2

The basic amino acids in the IL3 have been reported to be critical for the signaling through some GPCRs. There is a stretch of basic amino acids (aa264–272) located at the proximal region of Val274. We first constructed a PROKR2 receptor with a deletion in RRK (270–272). As shown in Fig. 3A, the RRK (270–272)-truncated PROKR2 receptor showed undetectable expression on the cell surface, as demonstrated through an immunofluorescence assay. Instead, the mutant receptor was expressed intracellularly. As a result, no activity was detected with this mutant receptor (data not shown).

3.5. The BBXXB motif at IL3 is critical for PROKR2 signaling

The BBXXB motif (B, basic amino acid, X, any amino acids) at IL3 has been implicated in G-protein coupling with several GPCRs. The RKRLR (264–268) sequence appears to be a putative BBXXB motif in PROKR2. Interestingly, R268C, which is a KS-associated PROKR2 mutation located in this region, has been reported to mildly affect PROKR2 signaling [20]. To further investigate the role of the BBXXB domain in PROKR2 signaling, we constructed a truncated PROKR2 receptor through the deletion of RKR (264–266). The cell surface expression of Δ(264–266) PROKR2 was similar to that obtained with the WT receptor (Fig. 3B); however, Δ(264–266) PROKR2 exhibited disrupted signaling activity, determined through the assessment of intracellular calcium mobilization (Fig. 3C).

3.6. Overexpression of Gαq rescues the activity of Δ(264–266) PROKR2

To directly test whether the Δ(264–266) and V274D PROKR2 mutants exhibit reduced G-protein coupling, we coexpressed the α subunit of Gq with PROKR2. We predicted that the decreased G protein coupling efficiencies, if there are any, would be overcome by the increased number of G proteins. Fig. 4 illustrates the effects of the overexpression of Gq α subunits on the activity of the receptors in response to stimulation by PK2. The maximal responses of Δ(264–266) PROKR2 were increased markedly by the overexpression of the Gq α subunits.

4. Discussion

The structure of GPCRs consists of seven transmembrane helices, three intracellular loops (IL1–3), and a cytoplasmic C-terminal tail. The correct structure is critical for the transport of GPCRs from the endoplasmic reticulum (ER)–Golgi pathway to the plasma membrane. Many mutations in the transmembrane domain result in immature proteins that are retained in the ER. Valine 274 of PROKR2 is located at the junction of IL3 and TM6. The KS-associated mutation V274D results in significantly lower cell surface expression and loss-of-function. Interestingly, the Val274 amino acid residue can be substituted with alanine or a polar amino acid, e.g., threonine. However, the substitution of Val274 by a positively charged amino acid, e.g., arginine, led to a phenotype similar to that obtained with the V274D mutation: low cell surface expression and loss-of-function. These results indicate that this amino acid (Val274) is intolerant to charged amino acids.

Although the V274D mutation resulted in a ≈60% reduction in cell surface expression, the mutated receptor exhibited little activity upon PK2 stimulation. One possible interpretation is that the V274D mutation further disrupted the G-protein coupling. However, the overexpression of the Gαq protein failed to significantly rescue the signaling activity of V274D PROKR2 (Fig. 4C). We therefore speculated that the resultant structure of V274D PROKR2 may not favor receptor activation. It has been indicated that the TM6 mobility plays an important role in GPCR activation. Electron paramagnetic resonance spectroscopy studies have indicated that the photoactivation of rhodopsin involves a rotation and tilting of TM6 relative to TM3 [28]. Furthermore, chemical reactivity measurements, fluorescence spectroscopy, and ultraviolent absorbance spectroscopy through the zinc crosslinking of histidines have also indicated the motion of TM6 during rhodopsin activation [29]. A similar motion of TM3 and TM6 was also observed in the β2-adrenergic receptor through fluorescence spectroscopy [29]. Studies of

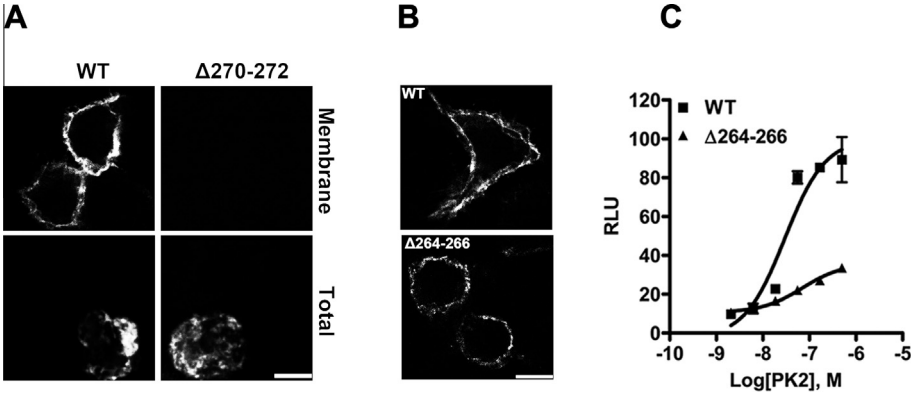

Fig. 3. Effect of the deletion of residues 264–266 and 270–272 on the cell surface expression and function of PROKR2. (A and B) Cell surface expression of Δ(270–272) (A) and Δ(264–266) (B) PROKR2, as demonstrated through an immunofluorescence assay. Scale bar, 7.5 μm. (C) The function of Δ(264–266) PROKR2 was analyzed through an aequorin-based calcium mobilization assay.

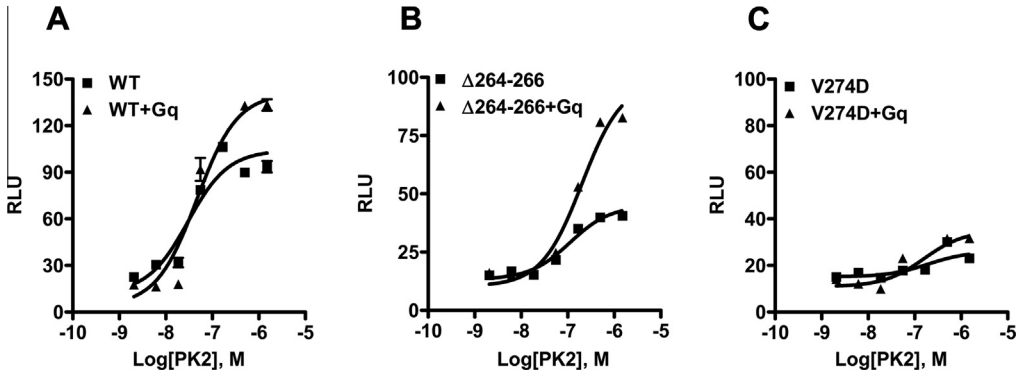

Fig. 4. Effect of Gαq overexpression on the signaling activity of WT (A), Δ(264–266) (B), and V274D (C) PROKR2. WT, V274D, and Δ(264–266) PROKR2 were transfected with or without a Gαq-expressing plasmid, and the signaling activity of these receptors was measured through an aequorin-based calcium mobilization assay.

the β2-adrenergic receptor labeled with fluorescent probes at the cytoplasmic end of TM6 have shown that agonists induce a rotation or tilting movement of the cytoplasmic end of TM6 similar to that observed in rhodopsin [29].

The basic-amino-acid-rich domain in IL3 plays important roles in receptor signaling. In particular, a BBXXB motif in several receptors has been reported to couple G proteins [30,31]. The RKRLR (264–268) sequence of PROKR2 appears to be a consensus sequence of BBXXB. The deletion of RKR (264–266) did not affect the cell surface expression of PROKR2; however, this mutant receptor exhibited very weak activity upon PK2 stimulation. We suggest that positively charged residues are needed in this region (264–266) of PROKR2 to allow for efficient interaction with Gαq. This interpretation derives from our findings that the overexpression of the α subunits of Gq reverses the loss of the apparent potency of PK2 in cells expressing Δ(264–266) PROKR2.

In summary, through a combination of molecular modeling and functional analysis of a disease-associated PROKR2 mutation (V274D), we demonstrated that a charged amino acid at this location (Val274) disrupts the transmembrane structure of the receptor and lead to loss-of-function. The deletion of RRK (270–272) resulted in undetectable cell surface expression, whereas RKR (264–266)-deleted PROKR2 was expressed normally on the cell surface but showed loss-of-function due to deficient G-protein coupling. Our data indicate that the distal region of the IL3 of PROKR2 may differentially influence receptor trafficking and G-protein coupling.

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