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RESEARCH ARTICLE

Evidence that prokineticin receptor 2 exists as a dimer in vivo

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Abstract Prokineticins are proteins that regulate diverse biological processes including gastrointestinal motility, angiogenesis, circadian rhythm, and innate immune response. Prokineticins bind two closed related G-protein coupled receptors (GPCRs), PKR1 and PKR2. In general, these receptors act as molecular switches to relay activation to heterotrimeric G-proteins and a growing body of evidence points to the fact that GPCRs exist as homo- or heterodimers. We show here by Western-blot analysis that PKR2 has a dimeric structure in neutrophils. By heterologous expression of PKR2 in *Saccharomyces cerevisiae*, we examined the mechanisms of intermolecular interaction of PKR2 dimerization. The potential involvement of three types of mechanisms was investigated: coiled-coil, disulfide bridges, and hydrophobic interactions between transmembrane domains. Characterization of differently deleted or site-directed PKR2 mutants suggests that dimerization proceeds through interactions between transmembrane domains. We demonstrate that co-expressing binding-deficient and signaling-deficient forms of PKR2 can re-establish receptor functionality, possibly through a domain-swapping mechanism.

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Keywords Prokineticin · Prokineticin receptor 2 · GPCR · Dimerization · Receptor fragment

Abbreviations

GPCR	G-protein coupled receptor
TMD	Transmembrane domain
PKR	Prokineticin receptor

Introduction

G-protein coupled receptors (GPCRs) are versatile biological sensors that are responsible for the majority of cellular responses to hormones and neurotransmitters as well as for the senses of sight, smell, and taste [1]. All these receptors share a central core formed by seven transmembrane helices (from TM1 to TM7) connected by intracellular and extracellular segments, and two terminal regions: an extracellular amino-terminal part and an intracellular carboxyl-terminal end. GPCRs are classified into three main families according to the structure and length of their amino-terminus and the localization of the agonist-binding site [2]. Signal transduction is associated with a set of changes in the tertiary structure of the receptor that are recognized by the associated intracellular partners, in particular the G-proteins. A growing body of evidence points to the fact that GPCRs exist as homo- or heterodimers [3–5], and the role of dimerization in receptor functioning is under extensive investigation [6, 7].

A small protein (Bv8) isolated from amphibian skin secretions was characterized in our laboratory [8]. Later on, two mammalian orthologues were identified and named prokineticin 1 and 2 (PK1 and PK2) [9, 10]. Intensive research over the past few years has shown that the

biological activities of Bv8/prokineticin proteins range from angiogenesis and involvement in reproduction and cancer to neuronal survival and neurogenesis, hypothalamic hormone secretion, circadian rhythm control, and modulation of complex behaviors, such as feeding and drinking [11–13]. The high expression level of human Bv8/prokineticins in bone marrow, lymphoid organs, and leukocytes suggested an involvement of these peptides also in hematopoiesis and inflammatory and immunomodulatory processes [14–17]. Prokineticins bind two different GPCRs with high sequence identity (about 85%) that belong to the neuropeptide Y receptor class in family 1 and are both able to bind PK1 and PK2 with similar affinity [18–20]. The absence of specificity suggests that binding to one of the two receptors is determined solely by the relative concentration and availability of receptor and ligand. The different tissue expression of prokineticins and their receptors would explain the specific role of these proteins in the various districts. PKR1 is expressed in peripheral organs such as the neuroendocrine system, the gastrointestinal tract, in macrophages and in the reproductive organs. PKR2 is expressed in peripheral organs such as the pituitary gland and thyroid, testis, and ovary, and it is suggested to have a key role in the brain. In the immune system, it is predominantly expressed in neutrophils and it is thought to be the main receptor implicated in the hematopoietic effects of Bv8 [17]. Mutations in the PKR2 gene are responsible for Kallmann syndrome, a developmental disorder combining congenital hypogonadotropic hypogonadism with anosmia or hyposmia [21].

We show here by Western–blot analysis that PKR2 has a dimeric structure in neutrophils. To investigate the mechanism of PKR dimerization, PKR2 has been heterologously expressed in *Saccharomyces cerevisiae* that was demonstrated to be an efficient system to study mammalian GPCRs [22]. This has allowed us to confirm that PKR2 is dimeric. Moreover, characterization of differently deleted or site-directed PKR mutants suggests that dimerization proceeds through a domain-swapping mechanism.

Materials and methods

Neutrophil isolation and membrane preparation

Polymorphonuclear leukocytes were isolated by established procedures. Briefly, healthy human blood (20 ml) was treated with 25 units heparin and diluted 1:1 with saline. Cells were separated on Ficoll-Hypaque density gradient (1.080, 1.122, and 1.133 g/ml). The tubes were centrifuged at 300 × *g* for 25 min at room temperature. Pellet was resuspended in lysis buffer (25 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM PMSF, and 10 μg/ml leupeptin and pepstatin) and homogenized with 20 strokes in a ground glass homogenizer. Samples were centrifuged at 40,000 × *g* for 40 min. The resulting membrane pellet was resuspended in lysis buffer with addition of 1% Triton X-100 and incubated for 1 h at RT. Samples were centrifuged at 40,000 × *g* for 30 min. Membrane aliquots were stored at −80°C.

Strains and media

The *S. cerevisiae* strain used for β-galactosidase assays and PKR2 expression was Cy12946 (*MATα FUS1p-HIS3 GPA1Gα i2(5) can1 far1Δ1442 his3 leu2 sst2Δ2 ste14::trp1::LYS2 ste3Δ1156 tbt1-1 trp1 ura3*), a generous gift of Addison D. Ault. Specifically, Cy12946 expresses a chimeric Gα subunit (GPA1$G\alpha_{i2(5)}$) in which the carboxyl-terminal five amino acids of the yeast Gα subunit, GPA1, are replaced by the carboxyl-terminal five residues of mammalian $G\alpha_{i2}$. The use of GPA1 $G\alpha_{i2(5)}$ required deletion of *SST2*, which down-regulates the pheromone response pathway by accelerating the GTPase activity of GPA1. *S. cerevisiae* was routinely grown in YPD (1% yeast extract, 2% peptone, 2% dextrose) medium. Synthetic defined (SD) medium for yeast selection after transformation consisted of 0.67% yeast nitrogen base (without amino acids) supplemented with 2% glucose (or other carbon sources) and the appropriate auxotrophic supplements.

Vector construction

PKR2 cDNA was amplified by PCR using as template human brain Marathon-Ready cDNA (Clontech) with oligonucleotides MET-*Kpn*I and Dw1-*Apa*I designed using the available PKR2 sequence (accession n. AL121755) and cloned in pBluescript. By digestion with *Kpn*I and *Eco*RI, the insert was cloned in pYES3/CT (Invitrogen) upstream of the sequence coding for His-tag, under the control of the *GAL1* promoter (PKR2-HIS). PKR2 was also cloned in pGAD424 (Clontech) under the control of the constitutive phosphoglycerate kinase (PGK1) promoter, modified by excision of the GAL4 activation domain (PGAD-PKR2).

A schematic drawing of the PKR2 mutants used in this study is reported in Fig. 1 and oligonucleotides used for construction of the various mutants are reported in Table 1.

Mutant ΔN term-R2-HIS deleted for the region 1–18 was constructed by cloning *Kpn*I–*Eco*RI the PCR fragment obtained with oligo ΔN-up and Dw2 in plasmid pYES3/CT.

Construct for the expression of Trunk-R2 was obtained by removal of a fragment *Apa*I–*Apa*I from PGAD-PKR2 and self-ligation, resulting in the creation of a stop codon after amino acid 235 of the PKR2 sequence.

To construct mutant ΔTMD7-R2 we amplified by PCR the region between aa 235–311 with oligonucleotides 235-

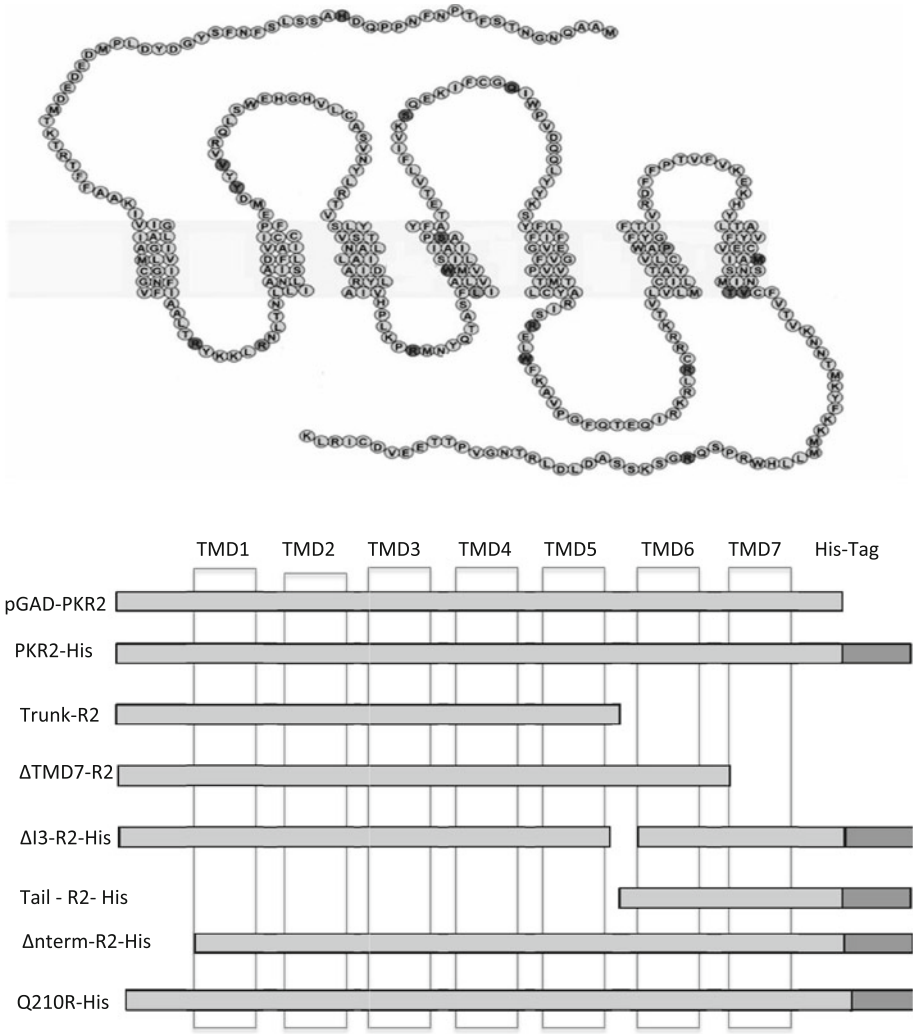

Fig. 1 Topology of PKR2 receptor (*upper panel*). Schematic of the PKR2 mutants analyzed in this study (*lower panel*). Each mutant is designated by the identities of the TMD that were retained. The protein region remaining in each deletion mutant is indicated with a *solid black line*. TMDs are indicated with *shaded boxes* and His-tag as a *black box*

Table 1 Oligonucleotides used for construction of the various mutants

Oligonucleotides	Sequence	T (°C)
MET-*Kpn*I	5′-CCG GTA CCA TGG CAG CCC AGA ATG G-3′	66.3
Dw1-*Apa*I	5′-TT GGG CCC TTC AGC CTG ATA CAG TCC-3′	63.4
ΔN-up	5′-TAG GTA CCA TGA CCA TGC CTC CTC T-3′	66.4
Dw2	5′-TTG AAT TCC TTC AGC CTG ATA CAG TCC-3′	63.4
235-up	5′-CTG GGC CCC TCT ACT ACA AGT CCT A CT-3′	63.4
DTM7-dw	5′-AAG AAT TCG TAG TGC TTT TCC TTC ACG-3′	61.9
272-up	5′-AAG GGC CCT AAG ACG GTC CTG G-3′	65.8
Tail up	5′-CTG GTA CCC TCT ACT ACA AGT CCT A CT-3′	63.6
Dw3	5′-TTG CGG CCG CCT TCA GCC TGA TAC AGT CC-3′	63.2
Q1	5′-ATC TTC TGT GGC CGG ATC TGG CCT GTC GAC CAG-3′	74.5
Q2	5′-CTG GTC GAC AGG CCA GAT CCG GCC ACA GAA GAT-3′	74.5

up and ΔTM7 and the fragment was inserted *Apa*I–*Eco*RI in Trunk-R2.

To construct mutant ΔI3-R2-HIS that presents deletion of the ic3 loop from 235 to 272, PCR was performed with oligonucleotides 272-up and Dw2 and the product was inserted *Apa*I–*Eco*RI in Trunk-R2.

To construct mutant Tail-R2-HIS, the sequence corresponding to region 236–383 was amplified with oligonucleotides tail up and Dw3 and inserted in pYES3/CT *Kpn*I–*Not*I.

Mutation Q210R was introduced in PKR2-HIS by using QuickChange site-directed mutagenesis kit (Stratagene)

with oligonucleotides Q1 and Q2, according to the manufacturer's instructions.

The fusion of the α-factor signal sequence cloned at the 5′-end of the Bv8 sequence to allow secretion of the mature polypeptide was recovered from PS-BV8#13 [23] and cloned *Bam*HI–*Eco*RI in p413ADH under the control of the constitutive alcohol dehydrogenase (ADH) promoter (p413-Bv8).

Plasmid Cp1021 possesses the *FUS1-lacZ* construct that allows production of *β*-galactosidase when the pheromone pathway is stimulated.

Reporter gene assays

Transformation of Cy12946 yeast cells was performed by the lithium acetate protocol [24]. Yeast transformants were grown at 30°C to mid-logarithmic phase in appropriate selection media containing 2% raffinose as the sole carbon source. Protein expression was induced by the addition of galactose to a final concentration of 20 g/l. After 16 h (culture OD_{600} 0.8–1), *β*-Gal activity was determined using 2.5 mM *o*-nitrophenyl-*β*-D-galactopyranoside (ONPG). Assays performed on different days were normalized to the average wild-type activity obtained. Three independent yeast clones from each transformation were used.

Total yeast membranes

Yeast cells from a 200–ml overnight culture (OD_{600} 0.8–1) were collected by centrifugation. All subsequent manipulations were conducted on ice. Cells were washed in water and re-centrifuged. The cell pellet was then weighed and resuspended in ice-cold lysis buffer (25 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM PMSF and 10 μg/ml leupeptin and pepstatin). Glass beads were added to the suspension, and the cells were broken by vigorous vortexing. To minimize protein denaturation, the sample was put on ice for 1 min after vortexing. This procedure was repeated four times. Samples were centrifuged at 13,200 rpm for 1 min. Subsequently, membranes were pelleted by centrifugation at 40,000 × *g* for 40 min. The resulting membrane pellet was resuspended in lysis buffer with addition of 1% Triton X-100 and incubated for 1 h at RT. Samples were centrifuged at 40,000 × *g* for 30 min and the supernatant containing the membrane extracts, were stored at −80°C.

Western blotting of membrane extracts

Samples were fractionated by SDS–PAGE on a 10% gel and transferred to a polyvinylidene difluoride membrane. The membrane was probed with anti-His monoclonal antibody horseradish peroxidase conjugate (Roche) 1:5,000, or with anti-PKR2 goat polyclonal antibody (sc-54316, Santa Cruz Biotechnology) 1:3,000 followed by rabbit anti-goat IgG horseradish peroxidase conjugate. The immunoreactive bands were visualized using an ECL detection system (BM Chemiluminescence Blotting Substrate, Roche).

Disulfide trapping

Cross-linking reagents, final concentration 7.5 μM 1,10-phenantroline and 2.5 μM $CuSO_4$ was added to membrane preparations for 30 min at RT. The reaction was terminated with 10 mM *N*-ethylmaleimide for 20 min at RT. The samples were resolved by SDS–PAGE in the absence of reducing agents. The effects of cross-linking were examined by immunoblotting.

Results

Detection of PKR2 dimers in neutrophils

Neutrophils play a central role in innate immunity providing the first line of protection against pathogens by secreting a variety of cytokines and chemokines that promote tumor growth and angiogenesis. Bv8 is highly expressed in tumor-infiltrating neutrophils and induces neutrophil migration at very low concentration by activation of PKR2 expressed in neutrophils. Bv8 produced by neutrophils within the tumor might attract additional neutrophils in the tumor mass and at the same time promote angiogenesis locally [17].

Western-blot analysis of endogenous PKR2 was performed on membrane extracts from human polymorphonuclear leukocytes, with a commercial antibody that was raised against a peptide corresponding to the amino-terminal 18 aa of human PKR2. As shown in Fig. 2a (lane 1), a band at 45 kDa corresponding to a PKR2 monomer was clearly visible, together with a prominent band at an apparent molecular mass approximately twice that of the monomer (72–95 kDa), suggesting the existence of an SDS-resistant dimeric species of the receptor. The results of three independent experiments are shown in Fig. 2a. Membrane extracts from human glioma U373 cells which do not express PKR2 [25] were used as negative control (Fig. 2b, lane 3).

Cupric orthophenanthroline (CuP) is a redox catalyst widely employed to study contact interfaces involving cysteine residues [26]. Therefore, if PKR2 oligomerizes, CuP treatment may produce a disulfide-linked dimer or oligomer if the interface has cysteine residues in close proximity. However, treatment with CuP did not increase the amount of the higher-molecular-weight species (Fig. 2a, lane 2).

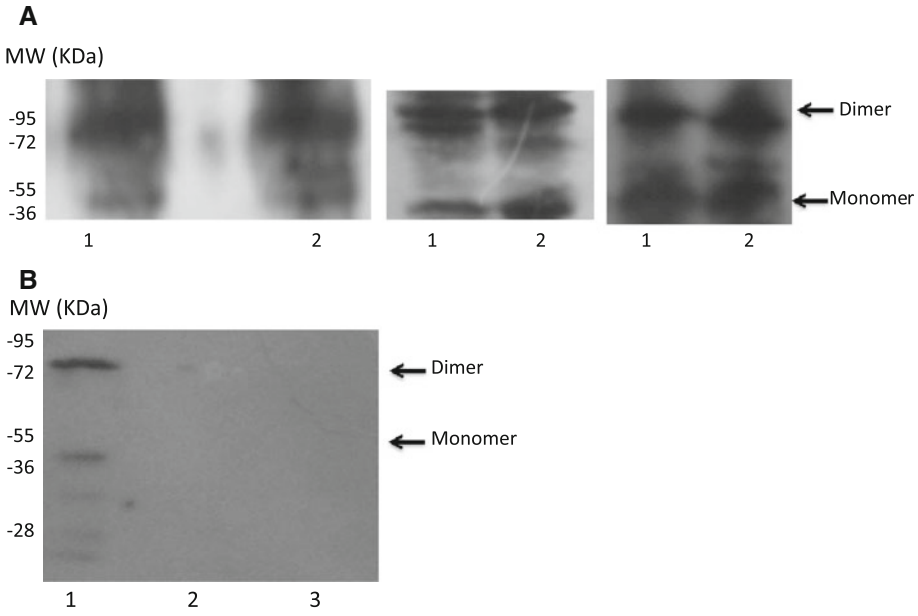

Fig. 2 Western-blot analysis of endogenous PKR2 in human neutrophils using anti-PKR2 antibody. **a** *Lane 1*, neutrophil membrane extracts; *lane 2*, neutrophil membrane extracts cross-linked with CuP. Three independent experiments are shown. **b** *Lane 1*, membrane extracts from yeast expressing PKR2; *lane 2*, membrane extracts from yeast CyCp; *lane 3*, membrane extracts from human glioma U373 cells

Functional expression of PKR2 in yeast

To confirm the oligomeric structure of PKR2 and to investigate the mechanism of dimerization, we expressed the protein in yeast. The striking resemblance between the components of the yeast pheromone pathway and mammalian GPCR signaling system make yeast an attractive host for the in vivo reconstitution of mammalian GPCRs [22].

Our initial goal was to design a strategy that allowed the functional expression of the human prokineticin receptors in yeast. We first cloned the human PKR2 coding sequence containing a His-tag at the C terminus into the yeast expression plasmid pYES3/CT, thus placing PKR2 expression under the control of the strong yeast *GAL* promoter (PKR2-HIS).

We next introduced the plasmid PKR2-HIS in yeast strain Cy12946 expressing a mutant version of Gpa1p in which the last five amino acids of Gpa1p were replaced with the corresponding residues present in mammalian G_{aq} [27]. Because for large peptides, it is difficult to penetrate the yeast cell wall, we decided to express Bv8 with autocrine system in which the yeast cells produce the ligand as well as the receptors. Therefore, to provide a signal to activate the mutant and native receptors, we co-expressed Bv8 fused to a pre-pro-α factor secretory sequence in vector p413 (p413-Bv8).

To address the functional integrity of the PKRs expressed in yeast, we employed a β-galactosidase reporter bioassay. Initially, the bioassay was configured using a control yeast strain transformed to coexpress β-Gal under control of FUS1 with the PKR2 receptor and Gpa1p (strain CyCp). When this strain was transformed with p413-Bv8 and incubated with galactose to induce PKR2 expression, activation of PKR2 was evident, as β-galactosidase activity was observed (Fig. 3b). Thus, the bioassay provides an effective readout of GPCR–ligand interaction and we therefore applied this assay to monitor PKR2 activation. Western-blot analysis with a polyclonal antibody raised against the His-tag evidenced two immunoreactive bands at approximately 45 and 95 kDa (Fig. 3a). In general, the pattern of immunoreactive PKR2 bands that we obtained in yeast with the His-tag or the PKR2 antibody closely resembled that observed in neutrophil cells. No signal was present in membranes of untransformed yeast CyCp with either antibody (Fig. 2b, lane 2 and Fig. 3a, lane 2). A ΔN term-R2-HIS mutant lacking the amino-terminal region 1–18 (that contains the four potential N-glycosylation sites of PKR2) was identical to WT for activity and capacity of dimerization (Fig. 3a, b), demonstrating that glycosylation is not required for dimerization of PKR2.

In order to directly determine if the higher-molecular-weight species corresponded to a specific PKR2 homodimer, we devised a co-immunoprecipitation strategy that exploits coexpression in yeast of the mutant ΔN term-R2-HIS with untagged full-length PKR2. The receptors were immunoprecipitated with the anti-His antibody, subjected to SDS–PAGE, and immunodetected using a commercial polyclonal antibody raised against a peptide corresponding to the amino-terminal 18-amino acids of PKR2, which does not recognize the ΔN term-R2-HIS mutant. As seen in Fig. 4, anti-His (a), anti-PKR2 (b)), detection of the anti-His immunoprecipitate with the PKR2 antibody revealed both the 45 and the 90-kDa forms of the receptor. This indicates that the two molecular species (His-tagged ΔN term-R2 and full-length untagged PKR2) were co-immunoprecipitated

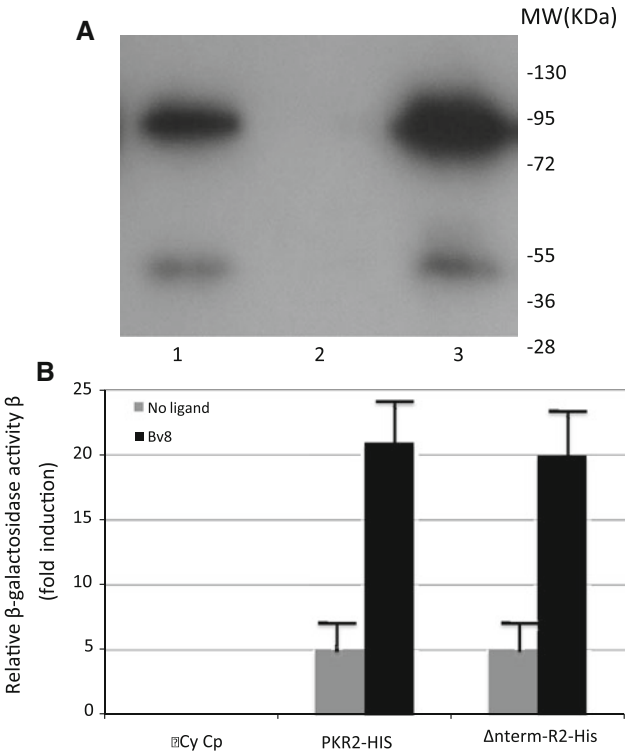

Fig. 3 Functional expression of PKR2 in yeast. **a** Western-blot analysis of membrane extracts using anti-HIS antibody: *lane 1*, PKR2; *lane 2*, CyCp; *lane 3*, ΔN term-R2-HIS. **b** *β*-galactosidase activity of strains expressing the indicated PKR2 variants. WT and mutant PKR2 were expressed episomally in yeast containing a FUS1-lacZ gene reporter with or without co-expression of ligand Bv8. Data show the *β*-Gal activity obtained with each construct relative to plasmid Cp1021 alone. Data are the average with standard error of the mean of three or more replicate experiments ($n \geq 3$) done in duplicate

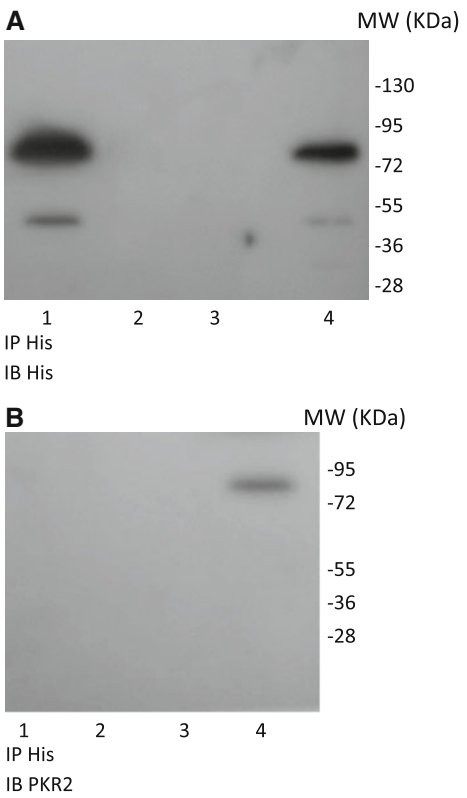

Fig. 4 Membranes from yeast cells co-transformed with ΔN term-R2-HIS and PKR2 alone or together were prepared and subjected to immunoprecipitation (*IP*) with anti-HIS antibodies. Thereafter, the immunoprecipitates were resolved by SDS–PAGE under reducing conditions and immunoblots (*IB*) were probed with anti-HIS (**a**) and anti-PKR2 (**b**). *Lane 1*, ΔN term-R2-HIS; *lane 2*, PKR2; *lane 3*, CyCp; *lane 4*, ΔN term-R2-HIS/PKR2

as part of a complex, consistent with the higher-molecular-weight form being a PKR2 homodimer.

Dimerization of some GPCRs was shown to be increased or decreased following agonist stimulation [5]. To examine whether PKR2 dimerization was influenced by agonist treatment, membrane extracts of yeast cells endogenously expressing Bv8 were subjected to SDS–PAGE and Western-blot analysis. Figure 5 shows that the presence of Bv8 does not alter the ratio of dimeric to monomeric forms of the receptor. These results are consistent with the concept that the formation of PKR2 oligomers was not affected by receptor activation, showing that PKR2 oligomer formation was ligand-independent.

It was reported that deletion of the third intracellular loop increased the level of recombinant muscarinic receptor in yeast crude membrane fraction [28]. Possibly the ic3 loop may be detrimental to the efficient expression of receptor in yeast, perhaps by serving as target for yeast proteases leading to rapid receptor degradation. We created a mutant ΔI3-R2-HIS deleted for PKR2 intracellular loop 3 from residue 235 to 272. However, mutant ΔI3 was expressed at the same level of WT, it was dimeric (Fig. 5a) and it showed basal activity that was not increased in the presence of Bv8 (Fig. 5b). Lack of response to the ligand might be expected because studies in a mammalian expression system have shown that the membrane ic3 loop proximal region (10–15 aa) is required for productive receptor/G-protein coupling.

Reconstitution of functional PKR2 receptor by co-expression of amino- and carboxyl-terminal receptor fragments

In 1988, Kobilka et al. split the *β*2-adrenoreceptor into two fragments, one containing TMD 1 to 5 and the other containing TMD 6 and 7. The transfection of these two fragments together resulted in recovery of the function of the *β*2-adrenoreceptor [29]. Since then, this phenomenon has been demonstrated for other GPCRs [30–32]. These finding are consistent with the idea that these integral

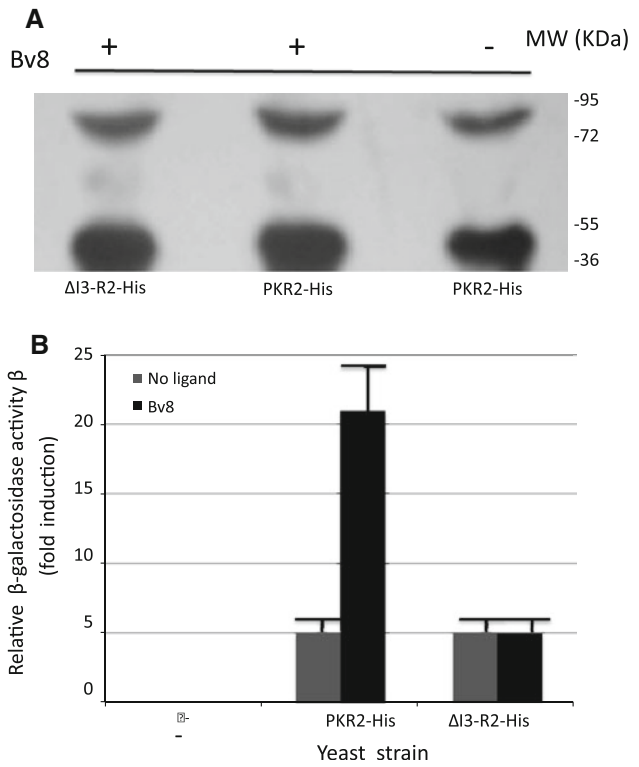

Fig. 5 Analysis of full-length and ΔI3 mutant PKR2 expressed in yeast. **a** Immunoblotting. Membrane extracts were prepared from yeast transformed with ΔI3-R2-HIS and PKR2-HIS with and without Bv8. Equal amounts of membrane protein (10 µg) were separated by SDS–PAGE and receptors were visualized via Western blotting using anti-HIS antibody. **b** *β*-galactosidase activity. *β*-Gal activity was assayed in yeast strains expressing the indicated proteins, as described in Materials and methods. Data are the average with standard error of the mean of three or more replicate experiments ($n \geq 3$) done in duplicate

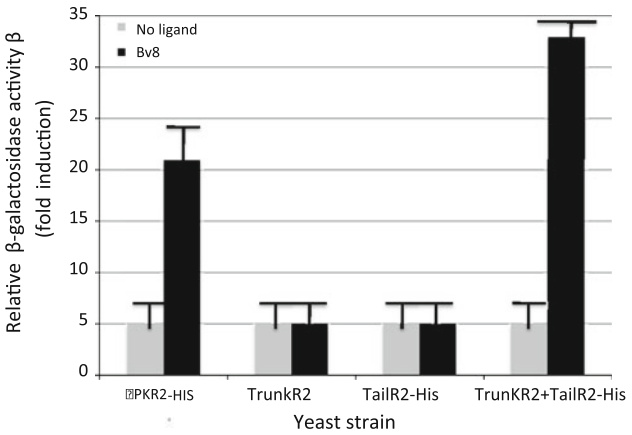

Fig. 6 Functional reconstitution of split PKR2 in yeast. *β*-Gal activity was assayed in the strains expressing the indicated proteins, as described in Materials and methods section. Data are the average with standard error of the mean of three or more replicate experiments ($n \geq 3$) done in duplicate

membrane proteins are composed of two or more independent folding domains that are inserted into the lipid bilayer as independent units and then assembled to form a functional transmembrane protein [33].

We expressed in yeast the two domains of PKR2, one containing TMD 1 to 5 (Trunk-R2) and the other containing TMD 6 and 7 (Tail-R2-HIS), separately or together and tested the induction of *β*-gal expression. Neither of the PKR2 fragments alone are capable of inducing the transduction cascade in yeast. However, co-expression of the two fragments determines reconstitution of a functional receptor complex, as shown in Fig. 6.

Mechanism of dimerization: identification of domains of the PKR2 receptor involved in dimerization

Residues or domains involved in GPCR dimerization differ depending on the receptor. Thus, we next attempted to determine the PKR2 domains responsible for its dimerization/oligomerization. The structural mechanisms of GPCR dimerization are often incompletely understood, but several models have been proposed: (1) coiled-coil interactions mediated by the carboxyl-terminal domain, (2) covalent inter-subunit disulfide bonding, and (3) interaction between transmembrane domains. However, as more data emerges, it seems probable that dimerization occurs using a combination of these mechanisms. We have analyzed the contribution of these three types of interactions to PKR dimerization. To this end, several receptor mutants were generated. Figure 1 schematically describes the deletion and site-specific mutant constructs that were used in this study.

1. Coiled-coil interactions between the carboxyl-terminal domains: In order to study the occurrence of carboxyl-terminal coiled-coil contacts, we prepared an expression construct encoding a truncated protein that lacks the seventh TM fragment and the cytosolic carboxyl-terminal extension (designated ΔTMD7-R2). ΔTMD7-R2 should not be able to establish interactions involving the C terminus of PKR2. Western-blot analysis of extracts from yeast cells expressing ΔTMD7-R2 or WT His-tagged PKR2 revealed a very similar pattern, with SDS-resistant dimers (Fig. 7a). Therefore, the truncated mutant exhibited normal oligomerization, thus excluding carboxyl-terminal coiled-coil interactions as the mechanism of dimerization. The functional status of ΔTMD7-R2 was analyzed: as expected (see below), ΔTMD7-R2 expressed in yeast cells in the presence of endogenously produced Bv8 did not generate an increase of *β*-galactosidase activity (Fig. 7b).
2. Covalent inter-subunit disulfide bonding: Compelling studies have demonstrated that the amino-terminal

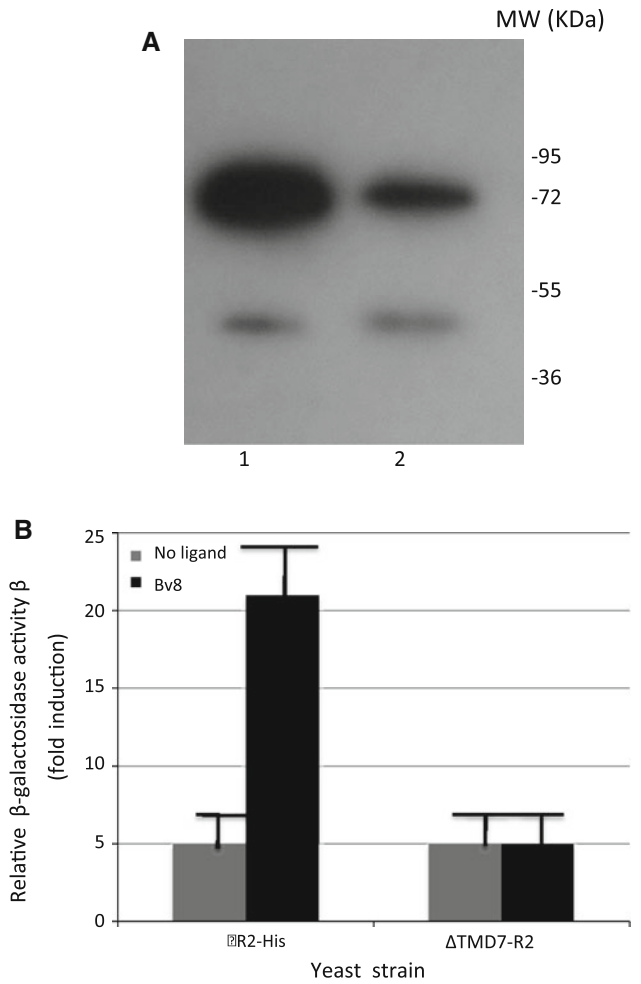

Fig. 7 Analysis of full-length and ΔTMD7-R2 mutant PKR2 expressed in yeast. **a** Immunoblotting using anti-PKR2 antibody. *Lane 1*, ΔTMD7-R2, *lane 2*, PKR2-HIS. **b** *β*-galactosidase activity. *β*-Gal activity was assayed in yeast strains expressing the indicated proteins, as described in Materials and methods. Data are the average with standard error of the mean of three or more replicate experiments ($n \geq 3$) done in duplicate

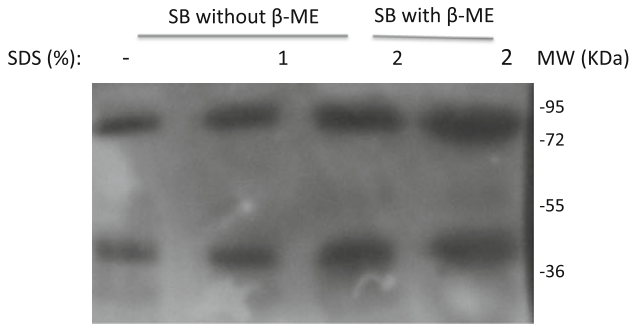

Fig. 8 Detergent-solubilized extracts were treated for 10 min with sample buffer (*SB*) with 1 and 2% SDS with or without 7.5% *β*-mercaptoethanol. Samples were analyzed by Western blotting using His-Tag antibody. The mobility of marker proteins is shown on the *left*

extracellular domain, for the presence of Cys residues involved in disulfide bonds, contributes to dimerization of the family 3 receptors, CaR, mGluR and GABAR, and among family 1 receptors, bradikinin B2 and CCR5 [7, 34].

The amino-terminal extracellular domain of PKR2 does not contain cysteines, only two Cys residues are present, located on the first and second extracellular loop, respectively. To test the possible involvement of disulfide bonds in PKR2 dimerization, we examined the effect of the reducing agent *β*-mercaptoethanol. Under reducing conditions, the relative abundance of apparent receptor monomers and dimers was not affected by as much as 2% SDS (Fig. 8). This result suggests that PKR2 dimerization does not require covalent disulfide bonding.

3. Interaction between transmembrane domains: Studies with several GPCRs have shown that the co-expression of two mutant receptors, which individually do not bind or transduce signals, results in receptors that bind and transduce signals [35]. These data imply that a functional complementation is achieved by intermolecular interactions between receptor molecules. Two basic types of GPCR dimers have been considered to date. In 'contact' dimers, the two monomers are independently folded as individual units, and establish a limited number of contacts such as disulfide bonds or hydrophobic interactions [34]. Conversely, in 'domain-swapped' dimers the monomers exchange domains connected by a hinge loop, so that the functional unit comprises a domain from each monomer [34, 36]

   a. *Co-expression of ΔTMD7-R2 and Q210R* We hypothesized that co-expression of binding- and signaling-deficient PKR mutants in yeast could restore PKR function by functional complementation. We selected two PKR mutant receptors for their inability to bind the ligand or to transduce signaling after ligand binding. The first mutant receptor was the Q210R missense mutation present in Kallmann syndrome patients harboring an inactivating Q to R mutation in the second extracellular loop [37]. The second mutant contained deletions of TM helix 7 and carboxyl-terminal region: it was chosen because of involvement of the deleted regions in G-protein coupling and second messenger generation. As expected, Q210R-HIS and ΔTMD7-R2 mutants expressed alone in yeast showed no capacity to induce *β*-gal activity, but when the

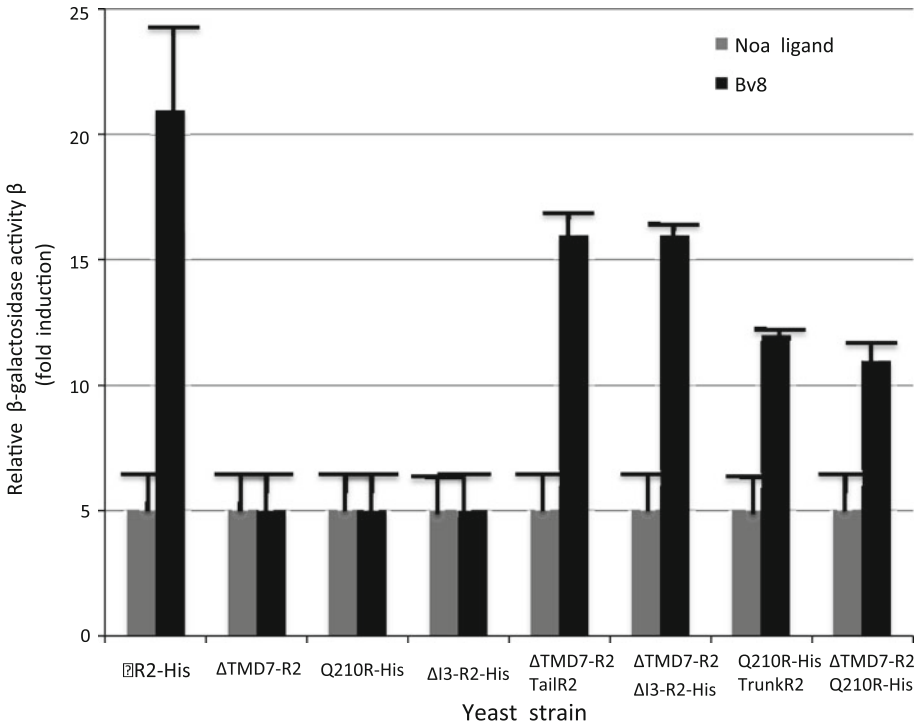

Fig. 9 Domain swapping allows dimerization of PKR2. *β*-Gal activity was assayed in yeast strains expressing the indicated proteins, as described in Materials and methods. Data are the average with standard error of the mean of three or more replicate experiments ($n \geq 3$) done in duplicate

two receptor mutants were co-expressed, the *β*-gal activity was partially restored (Fig. 9).

b. *Co-expression of PKR2 Q210R with PKR2 Trunk amino-terminal folding domain (TMDs 1–5) or ΔTMD7-R2 with PKR2 tail carboxyl-terminal folding domain (TMDs 6–7)* Based on the domain-swapped dimer model, we hypothesized that mutant receptors carrying inactivating mutations within TMDs 1–5 can be functionally rescued by supplying a receptor fragment spanning the mutated receptor portion. Thus, if domain-swapping of TMD 1–5 with TMD 6 and 7 occurred as postulated for the muscarinic/adrenergic receptor chimera [30, 36], co-expression of Trunk-R2 with Q210R-HIS would result in the rescue of PKR2 function. As a matter of fact, this is exactly what happened, as clearly shown in Fig. 9.

Analogously, mutant ΔTMD7-R2, lacking the TM7 and carboxyl-terminal regions, was functionally rescued by co-expression with mutants Tail-R2, spanning the region TMD 6–7, or ΔI3-R2-HIS, lacking ic3, as evidenced by induction of *β*-gal activity in the presence of agonist, Bv8 (Fig. 9).

The functional reconstitution of missense mutations within TMDs 1–5 and ΔTMD7-R2 upon co-expression with amino- and carboxyl-terminal PKR2 domains, respectively, strongly supports formation of an oligomeric structure by a domain-swapping mechanism.

Discussion

We present evidence that recombinant PKR2 can be functionally expressed in yeast and that endogenous PKR2 oligomerizes in neutrophils. The latter result is particularly relevant, because demonstration that GPCRs dimerize in vivo is difficult to achieve, due to the low expression levels of these proteins and the lack of high-quality antibodies; as a matter of fact, most studies have been performed with transfected cells that overexpress tagged GPCRs [38]. It is important to note that we were able to investigate the oligomeric state of the native receptor, because neutrophils express high levels of PKR2 [14]. Dimerization of GPCRs is emerging as an important mechanism for the regulation of these receptors and the ability to express prokineticin receptors in yeast should greatly facilitate future structure–function studies.

Yeasts have become an established system for studying mammalian GPCR structure and function because it is possible to couple heterologous GPCRs to the pheromone signal transduction pathway. Yeast strains that lack endogenous mating factor receptors can be easily engineered, thus providing a receptor-null environment in which to study mammalian receptors. Many mammalian GPCRs (including the C5a, somatostatin, adenosine, and muscarinic receptors) can functionally couple to the yeast mating response pathway through the yeast Gα protein, Gpa1, or engineered G-proteins that contain portions of both Gpa1 and human G-proteins. This has been exploited for several purposes, including the identification of functional

important residues or of agonists capable of activating orphan GPCRs, and in establishing a functional significance of the RGS family (regulator of G-protein signaling proteins) [39]. Also, dimerization of C5a was studied in yeast by using fluorescence resonance energy transfer [40]. However, in some cases, GPCRs have been reported to be poorly expressed because they remain in the membrane of the endoplasmic reticulum or are directed to the vacuole to undergo proteolytic degradation [39]. In our hands, recombinant PKR2 was fully functional in yeast, confirming the widespread usefulness of this host system and setting the conditions for future detailed studies of this receptor.

Despite intense investigation, the domains critical for GPCR dimerization remain poorly understood. Early studies suggested key roles for both amino- and carboxyl-terminal elements in homodimeric interactions. However, increasing evidence largely based on mutational analysis, individual transmembrane (TM) domain expression and cross-linking experiments now suggests that the TM α-helical domains represent the major dimerization interface for both GPCR homodimers and heterodimers [33, 34].

Functional complementation of two identical full-length receptors harboring different missense mutations in the amino- and carboxyl-terminal domains would be the most convincing evidence demonstrating the existence of homodimers. However, it is interesting to note that our efforts to rescue the function of receptors containing mutations in the amino-terminal domain (Q210R) by co-expression with a PKR2 mutant harboring a deletion in the carboxyl-terminal domain (ΔTM7) were successful. These data imply that a functional complementation is achieved by intermolecular interaction between receptor molecules. The results of our study favor a model in which PKR2 specifically associates by a domain-swapped mechanism rather than by lateral interaction. In a domain-swapped structure, it has been suggested that GPCRs may "open up" their hydrophobic core and by domain-swapping interconvert from monomers to dimers. This mechanism has been shown to be the basis of the molecular interaction between muscarinic m2 and m3 receptors [36].

We have shown that the PKR2 receptor, as an example of class 1 GPCRs, forms dimeric complexes with both full-length and truncated constructs and provide evidence that receptor assembly occurs independently of receptor activation.

In several systems, heterodimerization alters receptor signaling. Moreover, many studies have shown that GPCRs that are fully functional when expressed alone alter their ligand binding specificity when co-expressed with other receptors [3, 33]. Understanding possible differences in the affinity and efficacy of various agonists and antagonists for the monomeric and dimeric forms of the receptors and their differential ability to transmit signals can help explain the differences in the physiological responses they induce. Understanding dimerization will provide key insights into diseases involving both functional and defective GPCRs.

Our preliminary results indicate that PKR1 and PKR2 receptor subtypes can form heterodimers. This result must be analyzed in the future because it can help us to understand the phenomena in districts where two different subtypes of the receptors are co-expressed.

Several loss-of-function mutations in the human PK2 or PKR2 genes have been described in patients with Kallmann syndrome [21]. PK2 and PKR2 are also important in other functions attributed to the central nervous system, such as maintaining circadian rhythmicity [41]. Of the two receptors for prokineticin, only PKR2 is expressed in neutrophils, suggesting that this is the main receptor implicated in regulation by prokineticin of neutrophil migration in human tumors and inflammatory disorders [17, 42]. Given the importance of PK/PKR2-mediated signaling for many physiologically relevant processes, it is critical to understand the basis of PKR2 dimerization. Our results provide a first indication that domain-swapping is effective for formation of PKR2 dimers. This finding can contribute to provide a framework for interpretation of results concerning the defects of PKR2 mutants associated with Kallmann syndrome or other pathologies.

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