**PROKR2** missense mutations associated with Kallmann syndrome impair receptor signalling activity

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Kallmann syndrome (KS) combines hypogonadism due to gonadotropin-releasing hormone deficiency, and anosmia or hyposmia, related to defective olfactory bulb morphogenesis. In a large series of KS patients, ten different missense mutations (p.R85C, p.R85H, p.R164Q, p.L173R, p.W178S, p.Q210R, p.R268C, p.P290S, p.M323I, p.V331M) have been identified in the gene encoding the G protein-coupled receptor prokineticin receptor-2 (PROKR2), most often in the heterozygous state. Many of these mutations were, however, also found in clinically unaffected individuals, thus raising the question of their actual implication in the KS phenotype. We reproduced each of the ten mutations in a recombinant murine Prokr2, and tested their effects on the signalling activity in transfected HEK-293 cells, by measuring intracellular calcium release upon ligand-activation of the receptor. We found that all mutated receptors except one (M323I) had decreased signalling activities. These could be explained by different defective mechanisms. Three mutations (L173R, W178S, P290S) impaired cell surface-targeting of the receptor. One mutation (Q210R) abolished ligand-binding. Finally, five mutations (R85C, R85H, R164Q, R268C, V331M) presumably impaired G protein-coupling of the receptor. In addition, when wild-type and mutant receptors were coexpressed in HEK-293 cells, none of the mutant receptors that were retained within the cells did affect cell surface-targeting of the wild-type receptor, and none of the mutant receptors properly addressed at the plasma membrane did affect wild-type receptor signalling activity. This argues against a dominant negative effect of the mutations in vivo.

**INTRODUCTION**

Kallmann syndrome (KS) is a developmental disease that combines hypogonadotropic hypogonadism, due to gonadotropin-releasing hormone (GnRH) deficiency, and anosmia or hyposmia, related to the absence or hypoplasia of the olfactory bulbs and tracts. KS is genetically heterogeneous. Five causal genes have been identified so far, namely KAL1, FGFR1, FGF8, PROKR2 and PROK2, encoding the extracellular glycoprotein anosmin-1, fibroblast growth factor receptor-1, fibroblast growth factor-8, prokineticin receptor-2 (a G protein-coupled receptor of class A, 1) and prokineticin-2, respectively (see 2 for review). Loss-of-function mutations in KAL1 and in FGFR1 or FGF8 underlie the X-linked recessive form of the disease and an autosomal dominant form with incomplete penetrance, respectively (3–6). PROKR2 and PROK2 have been identified by a candidate gene approach based on the phenotypes of the corresponding knock-out mice (7). Indeed, prokineticin receptor-2 null and prokineticin-2 null mice both display KS-like features, namely olfactory bulb morphogenetic defects and a severe atrophy of the reproductive system.

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related to markedly reduced numbers of GnRH-synthesizing cells in the hypothalamus (8–10).

The first mutations in PROKR2 were found in 14 out of 192 independent KS patients, either in heterozygous state (10 patients) or in homozygous (or compound heterozygous) state (4 patients). Notably, of the ten different mutations identified, only one was a frame-shifting mutation (p.20fsX43), while nine were missense mutations (p.R85H, p.R164Q, p.L173R, p.W178S and p.V331M; see Fig.1A). Moreover, three missense mutations present in heterozygous state in KS patients (p.R268C, p.P290S, p.V331M) and two additional missense mutations not found in the initial cohort of KS patients were detected in control individuals from the general population (7). Since then, one of the latter mutations (p.R85C) has also been found in individuals affected by KS or isolated hypogonadotropic hypogonadism (C.D., unpublished data and 11). This raises the question of the pathogenic role of the various PROKR2 missense mutations in KS. Here, we address this issue by means of two complementary approaches. Firstly, we took advantage of the crystal structures of rhodopsin and the β2-adrenergic receptor, two G protein-coupled receptors (GPCRs) belonging to the same subfamily as PROKR2 (1), to build a three-dimensional model of PROKR2 and predict how each of the ten missense mutations identified in KS patients could affect the structure and functioning of the receptor. Secondly, we tested the functional effect of each of these mutations reproduced by site-directed mutagenesis, on PROKR2 signalling in transfected HEK-293 cells.

**RESULTS AND DISCUSSION**

**Bioinformatic prediction of the functional consequences of the mutations**

We used the three-dimensional structures of the bovine rhodopsin (12,13) and human β2 adrenergic receptor (14,15) to build a homology model of the murine Prokr2 (Fig. 1B). Based on this model, the non-conservative W178S, L173R and P290S mutations that affect residues within PROKR2 transmembrane domains are predicted to impair stability or correct folding of the receptor, and consequently its cell surface-targeting. Indeed, Trp178 and Pro290 are the most conserved residues of transmembrane domains 4 and 6, respectively (positions 4.50 and 6.50, according to the Balles-teros and Weinstein nomenclature, 16), and Leu173 is in a hydrophobic environment that would not easily put up with a positively charged arginine residue. The Q210R mutation, affecting a residue located in extracellular loop 2, may impair ligand-binding of the receptor. The four mutations affecting arginine residues in the intracellular loops (R85C, R85H, R164Q and R268C) could influence G protein activation. Finally, the M323I and V331M conservative mutations located in transmembrane domain 7 are predicted to have limited impact on the receptor properties. To test these predictions, we reproduced each mutation in a recombinant haemagglutinin (HA)-tagged mouse Prokr2 by site-directed mutagenesis, and measured the PROK2-evoked signalling activity, cell surface amount and agonist-binding of the mutant receptors.

**All PROKR2 missense mutations except one impair intracellular Ca²⁺ increase evoked by PROK2**

PROKR2 is a Gq-coupled receptor that promotes inositol-phosphate production, and intracellular Ca²⁺ mobilization (17–19). We analyzed the signalling activity of the HA-tagged Prokr2 mutants by measuring the intracellular release of Ca²⁺ induced by the ligand ProK2. We found that the signalling activity of all mutants except one (M323I) was impaired, as indicated by the right shift of the agonist dose–response curves, and the decreased maximal responses (Fig. 2A and Table 1). Mutations P290S, W178S, Q210R and R164Q had the most deleterious effects on Ca²⁺ release. Our results are consistent with those recently obtained by Cole et al. (11), who also studied the deleterious effect of ten PROKR2 missense mutations on intracellular Ca²⁺ mobilization, including the R85C, R164Q, L173R, W178S and V331M mutations analyzed here. Western blot analysis of the transfected HEK-293 cells showed that all mutant receptors were produced in similar amounts, and with the expected molecular weight for a SDS-resistant dimeric form (~80 kDa) (Fig. 2B).
The M323I mutation has so far been found only in compound heterozygous KS patients, together with a deleterious frame-shifting mutation (7). Based on the present results, this conservative missense mutation may not be considered pathogenic, although the possibility that it affects a Ca\textsuperscript{2+} independent PROKR2 signalling pathway, not explored in this study, should not be excluded (11).

Transmembrane domain mutations L173R, W178S and P290S impair cell surface-targeting of the receptor

The amount of receptor present in the plasma membrane was estimated for each mutated Prokr2 by means of ELISA quantification in intact (i.e. non-permeabilized) cells, using the HA epitope at the extracellular N-terminal end of the receptor (see Materials and Methods). Seven out of ten mutant receptors were found in substantial amounts (>50% of the wild-type receptor amount) at the cell surface. In contrast, the L173R, W178S and P290S receptors had low cell

### Table 1. Functional characterization of Prokr2 mutants in transfected HEK-293 cells

<table>
<thead>
<tr>
<th>Prokr2</th>
<th>Cell surface amount(^a) (% of WT)</th>
<th>Ligand binding(^b) IC\textsubscript{50} ± SEM (nM)</th>
<th>Signalling activity(^c) EC\textsubscript{50} ± SEM (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>100</td>
<td>17.7 ± 3.6</td>
<td>15.9 ± 3.9</td>
</tr>
<tr>
<td>R85C</td>
<td>59.3 ± 16.4</td>
<td>13.0 ± 2.3</td>
<td>81.8 ± 22.7</td>
</tr>
<tr>
<td>R85H</td>
<td>64.0 ± 9.5</td>
<td>18.9 ± 8.0</td>
<td>95.1 ± 34.9</td>
</tr>
<tr>
<td>R164Q</td>
<td>88.5 ± 6.5</td>
<td>13.4 ± 2.2</td>
<td>ND</td>
</tr>
<tr>
<td>L173R</td>
<td>21.6 ± 7.5</td>
<td>ND</td>
<td>63.5 ± 20.7</td>
</tr>
<tr>
<td>W178S</td>
<td>14.2 ± 2.1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Q210R</td>
<td>97.6 ± 9.8</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>R268C</td>
<td>82.2 ± 12.0</td>
<td>12.5 ± 2.3</td>
<td>40.9 ± 11.1</td>
</tr>
<tr>
<td>P290S</td>
<td>9.9 ± 3.3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>M323I</td>
<td>56.1 ± 8.1</td>
<td>22.3 ± 4.1</td>
<td>10.1 ± 4.9</td>
</tr>
<tr>
<td>I331M</td>
<td>71.9 ± 3.8</td>
<td>13.1 ± 4.0</td>
<td>60.2 ± 20.7</td>
</tr>
</tbody>
</table>

ND, not determined.

\(^a\)ELISA.

\(^b\)Displacement of \(^{125}\text{I}-\text{MIT-1}\) by PROK2.

\(^c\)Intracellular calcium release upon stimulation by PROK2.
surface levels (Fig. 2C and Table 1) despite quite large total cell amounts (see Fig. 2B), thus suggesting that these mutants had difficulties passing the quality control system. This was confirmed by the quantification of total protein levels by ELISA in permeabilized cells. Concomitant ELISA quantifications in permeabilized and non-permeabilized cells also showed that the other mutant receptors were correctly addressed to the cell surface (Fig. 2C). Notably, although similar low levels of the L173R, W178S and P290S receptors were detected at the cell surface, the L173R receptor was still able to evoke intracellular Ca\textsuperscript{2+} release upon ligand-activation, whereas the W178S and P290S receptors were not (see Fig. 2A and C). This indicates that the W178S and P290S mutations not only impair cell surface-targeting of the receptor, but also its ability to activate the Gq protein.

Intracellular loop mutations R85C, R85H, R164Q, R268C and transmembrane domain mutation V331M presumably impair G protein-coupling

The four mutations located in PROKR2 intracellular loops (R85C, R85H, R164Q and R268C) and the murine I331M mutation (standing in for the V331M human mutation) located in the seventh transmembrane domain impaired receptor signaling upon agonist-stimulation, with R164Q (second intracellular loop) having the most severe effect (see Fig. 2A). The deleterious effect of these mutations could not be explained by impaired cell surface-targeting of the mutant receptors (see Fig. 2C) or impaired agonist affinity (see Fig. 3B). These mutations are thus likely to impair G protein-coupling of the receptor.

Mutant receptors do not exert a dominant negative effect on the wild-type receptor in co-transfected HEK-293 cells

Prokr2 knock-out mice display an abnormal, KS-like phenotype only in the homozygous state (9). Most KS patients carrying mutations in PROKR2 are, however, heterozygous. We reasoned that the pathogenic effect of some of the PROKR2 missense mutations found in heterozygous state could result from intracellular retention of the wild-type receptor by the mutant one, that is a dominant negative effect similar to that reported for other GPCRs (21–23). To determine if the L173R, W178S and P290S receptors that failed to reach the plasma membrane could exert such a dominant negative effect, we measured the amounts of wild-type receptor at the cell surface and in the whole cell, by means of ELISA without and with cell permeabilization, respectively, in co-transfected HEK-293 cells producing both Flag-tagged wild-type and HA-tagged mutant receptors. We found that cell surface-targeting of the wild-type receptor was not affected by any of these three mutants (Fig. 4A).
mutant receptors upon coexpression with the Flag-tagged detected between the HA-tagged wild-type and any of the Fig. S1), a significant difference in the response could not be saturation of the calcium signal (see Supplementary Material, usually lower than the theoretical values, presumably due to each other. Even though measured calcium responses were coexpressed receptors do not affect the signalling activity of Prokr2 and HA-tagged mutant Prokr2 in HEK-293 cells, and measured upon coexpression of Flag-tagged wild-type receptors that are properly targeted to the plasma membrane and for the wild-type receptor. For each pair of coexpressed receptors, the measured intracellular Ca2+ responses mediated by the coexpressed Flag-tagged wild-type Prokr2 and HA-tagged wild-type or mutant Prokr2, upon 300 nM PROK2 stimu-lation, are shown for each of the seven mutant receptors that are properly targeted to the plasma membrane and for the wild-type receptor. For each pair of coexpressed receptors, the measured intracellular Ca2+ response (black histograms) is represented as the proportion of the expected calcium response if the two coexpressed receptors do not affect the signalling activity of each other (grey histograms). This theoretical value was calculated for every pair of coexpressed receptors, by adding the expected contributions of each receptor to the overall signalling activity according to its amount at the cell surface (see Materials and methods). Data are means ± SEM of four independent experiments. A significant difference is not detected for any of the mutants studied, i.e. those that are prop-erly addressed to the cell surface (R85H, R268C) and those that are not (L173R, W178S, P290S) (Student’s t-test, P > 0.05). (B) Functional analysis of Prokr2 signalling activity when wild-type and mutant receptors are coexpressed. The intracellular Ca2+ responses according to its amount at the cell surface (see Materials and methods). Data are means ± SEM of four independent experiments. A significant difference is not detected between the HA-tagged wild-type and any of the mutant receptors (Student’s t-test, P > 0.05).

We then endeavoured to determine if some of the mutant receptors that are properly targeted to the plasma membrane could have a dominant negative effect on the receptor signalling activity. The calcium response to 300 nM PROK2 was measured upon coexpression of Flag-tagged wild-type Prokr2 and HA-tagged mutant Prokr2 in HEK-293 cells, and compared with the value that would be expected if the two coexpressed receptors do not affect the signalling activity of each other. Even though measured calcium responses were usually lower than the theoretical values, presumably due to saturation of the calcium signal (see Supplementary Material, Fig. S1), a significant difference in the response could not be detected between the HA-tagged wild-type and any of the mutant receptors upon coexpression with the Flag-tagged wild-type receptor (Fig. 4B).

Taken together, our results argue against a dominant negative effect of these mutations in vivo, and therefore support the current hypothesis of a digenic or oligogenic inheritance of KS in patients heterozygous for PROKR2 mutations (24). Indeed, many of the mutations have also been found in clinically unaffected first-degree relatives (7,11,25 and C.D., unpublished data), which strongly suggests that the presence of monoallelic PROKR2 mutations is not sufficient to produce the disease phenotype.

MATERIALS AND METHODS

Materials

Purified human recombinant PROK2 and the [125I]-MIT-1 were obtained from Fitzgerald Industries International, Inc. (Concord, MA, USA) and Perkin-Elmer (Courtaboeuf, France), respectively.

Plasmids

A cDNA containing the entire coding region of the murine Prokr2 (GenBank NM_144944) was obtained from embryonic day 10 brain RNAs, by RT–PCR using the following set of primers: mProkr2F 5'-CGACCGCTGGACCCCAGAAG AACAC-3' and mProkr2R 5'-CTAGTCTAGACTATTTA GTCGATACAATCC-3' (underlined sequences denote MluI and XbaI restriction sites for subsequent cloning of the PCR fragment). This PCR fragment was inserted, in place of the mGlu5-encoding MluI-XbaI fragment, into recombinant pRK5 plasmid vectors coding for the HA-tagged or Flag-tagged metabotropic glutamate receptor mGlu5. The final recombinant vectors code for a protein possessing the mGlu5 signal peptide followed by an N-terminal HA-tag or Flag-tag and the Prokr2 entire coding sequence. Plasmids encoding the various mutant Prokr2 harbouring each of the ten PROKR2 missense mutations identified in KS patients were then engineered by using the QuikChange mutagenesis protocol (Stratagene, La Jolla, CA, USA).

Transfection experiments

HEK-293 cells were cultivated in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, and transfected by electroporation as described previously (26). In simple transfection experiments, 10⁷ cells were transfected with 2 µg of recombinant plasmid DNA encoding either wild-type or mutant Prokr2, and completed to a total amount of 10 µg plasmid DNA with empty pRK5 vector. In co-transfection experiments, 10⁷ cells were transfected with 0.5 and 2 µg of the plasmids encoding the Flag-tagged wild-type Prokr2 and HA-tagged wild-type or mutant Prokr2, respectively, completed to a total amount of 10 µg plasmid DNA with empty pRK5 vector.

Western blot analysis

Twenty hours after transfection, HEK-293 cells were washed with PBS (Ca²⁺- and Mg²⁺-free) and harvested. The membranes were prepared as described (26). For each sample,
30 µg of total proteins were subjected to SDS–PAGE in a 10% polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane (Hybond-C; Amersham Biosciences). HA-tagged proteins were probed with a rabbit anti-HA polyclonal antibody (dilution 1/400; Zymed, San Francisco, CA, USA), and then with an Alexa-Fluor® 700 goat anti-rabbit IgG antibody (dilution 1/3000; Molecular Probes Invitrogen Corporation, Carlsbad, CA, USA). Proteins were visualized by the Odyssey® Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE, USA).

Intracellular calcium measurements

After transfection, cells were seeded in polyornithine-coated, black-walled clear bottom 96-well plates, and cultivated for 20 h, as described (27). Cells were washed with freshly prepared buffer (20 mM Hepes, 1 mM MgSO₄, 3.3 mM Na₂CO₃, 1.3 mM CaCl₂, 2.5 mM probenecid, in 1 × Hank’s balanced salt solution, pH 7.4) supplemented with 0.1% bovine serum albumin, and loaded with 1 µM Ca²⁺-sensitive fluorescent dye Fluo-4 AM (Molecular Probes) for 1 h at 37°C. Cells were washed again and incubated with 50 µL of the buffer. Different concentrations of purified human PROK2 were added in the wells to reach a final volume of 100 µL, and fluorescence signals (excitation 485 nm, emission 525 nm) were measured by using the fluorescence microplate reader Flexstation (Molecular Devices) at sampling intervals of 1.5 s for 60 s. In co-transfection experiments, the calculated signalling contribution of each coexpressed receptor is the Ca²⁺ response measured when this receptor was expressed alone, divided by its cell surface amount in this condition (determined by ELISA, in arbitrary units), times its cell surface amount when it was coexpressed (ELISA, arbitrary units).

Quantification of Prokr2 at the plasma membrane by ELISA

Twenty hours after transfection, HEK-293 cells were washed with PBS and fixed with 4% paraformaldehyde in PBS for 5 min, as described (26). The cells were then either permeabilized, using 0.05% Triton X-100 for 5 min, or not permeabilized. Cells were incubated in PBS, 1% foetal calf serum (blocking solution) for 30 min. HA-tagged proteins were detected by using rat anti-HA monoclonal antibody (blocking solution) for 30 min. HA-tagged proteins were probed with a rabbit anti-HA polyclonal antibody (dilution 1/400; Zymed, San Francisco, CA, USA), and then with Alexa Fluir-Fluor® 700 goat anti-rabbit IgG antibody (dilution 1/400; Zymed, San Francisco, CA, USA) at 0.25 g/ml. Flag-tagged proteins were detected with the mouse anti-Flag monoclonal antibody M2 (Sigma, St Louis, MO, USA) at 0.8 µg/ml and goat anti-mouse IgG antibody coupled to horseradish peroxidase (Amersham Biosciences, Uppsala, Sweden) at 0.25 µg/ml.

Ligand-binding assay

The ligand-binding assay was performed on intact HEK-293 cells as described (26) using 0.06 nM [³²P]-MIT-1. The radioligand was displaced by increasing concentrations of PROK2. The curves were fitted according to the equation \( y = (y_{max} - y_{min})/(1 + [x/IC_{50}]^{nH}) + y_{min} \) using GraphPad Prism software (San Diego), where IC₅₀ is the concentration of the compound that inhibits 50% of binding radioligand and nH is the Hill coefficient.

Molecular modelling

Sequences of prokineticin receptors, ß2 adrenergic receptor and rhodopsin, were aligned with the help of the available crystal structures (Protein Data Bank accession numbers 1gzm and 2hr1) using the program VIOTO (http://bioserv.cbs. cern.fr/)(28). Molecular models of the murine Prokr2 were built using the program MODELLER 7.7 (http://salib.org/) (29) with two combined templates (PDB 1gzm and PDB 2hr1) and a small set of restraints (for transmembrane helix extensions or conservation of several hydrogen bonds). Models were evaluated using VIOTO and ERRAT (30).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

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