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Involvement of tryptophan W276 and of two surrounding amino acid residues in the high constitutive activity of the ghrelin receptor GHS-R1a

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ABSTRACT

The human ghrelin receptor (GHS-R1a) is known to display a high level of signaling in the absence of ligand. The Trp276, located in the fully conserved CWXP motif of G protein-coupled receptors, is believed to function as a rotameric switch in these receptors. A comparative modelling of GHS-R1a with the motilin receptor, the most related G protein-coupled receptor to GHS-R1a known to date, but characterized by a very low ligand-independent signaling level, revealed that only two surrounding residues of Trp276, that are Val131 and Ile134, were different from these receptors. We mutated them at once in GHS-R1a to create a "motilin receptor-like" environment of Trp276 in order to study the consequences on GHS-R1a activation. We studied the pharmacological properties of the W276A, V131L-I134M GHS-R1a mutants. Basal as well as maximal ghrelin-induced signaling was assessed both by inositol-phosphate accumulation and SRE pathways. As compared to the wild type receptor, the SRE-luciferase assay displayed a markedly impaired basal activity for W276A whereas that of V131L-I134M was, strikingly, two fold increased. Nevertheless, the efficacy of ghrelin to bind or to stimulate mutant receptors remained unchanged. It is concluded that Trp276, Val131 and Ile134 have a significant impact on constitutive signaling of GHS-R1a, V131L-I134M being the first example of a GHS-R1a mutant with a higher basal activity than the wild type receptor.

1. Introduction

The physiological functions of ghrelin are mediated through the activation of the GH secretagogue receptor (GHS-R1a). GHS-R1a is mainly coupled to a Gαq protein leading to generation of inositol triphosphates (IP3) and diacylglycerol. GHS-R1a elicit also the activation of extracellular signaling-regulated kinases 1 and 2 (ERK 1/2) active on genes driven by a serum response element (SRE) (Mousseaux et al., 2006).

Referring to the «two-state» model, G protein-coupled receptors exist in equilibrium between an inactive (R) state, where the receptors are uncoupled to G-proteins, and an active (R*) state in which they can couple to and activate G-proteins (Leff, 1995). The R to R* transition of G protein-coupled receptors can occur spontaneously independently of an agonist and this is referred to constitutive activity.

Holst et al. found that the wild type GHS-R1a displays a basal signaling activity reaching 50% of the maximal activity elicited by 10^{-7} M ghrelin (Holst et al., 2003). Pantel et al. reported a natural occurring mutation in the GHS-R1a gene (Pantel et al., 2006) which deeply impaired the constitutive activity. This study provided a presumption of *in vivo* relevance of the basal activity of GHS-R1a since this mutation was harboured by two short stature suffering patients.

Schwartz et al. proposed a global toggle switch model of 7 TM receptor activation (Schwartz et al., 2006). This model pointed out the crucial role of the residue Trp in the conserved motif CWXP acting as a rotamer-switch which allows the inward movement of the sixth transmembrane segment in which it is located. In this paper, we investigate the role of this Trp residue on the basal activity of GHS-R1a.

Otherwise GHS-R1a belongs to a subclass of G protein-coupled receptors in which the most closely linked receptor to GHS-R1a is the motilin receptor which displays 86% identity in the transmembrane domains. At the opposite of GHS-R1a the motilin receptor is completely silent with respect to constitutive activity. *In vitro* mutation studies and clinical features of natural occurring mutations of GHS-R1a established that a single amino acid change can lead to an important modification of basal signaling (Holst et al., 2004; Pantel et al., 2006). Consequently, we hypothesized that few changes in the amino acid sequence of the GHS-R1a and motilin receptor might induce a great difference in their respective basal constitutive activity level, especially for residues surrounding the highly conserved Trp in the CWXP motif (Trp276 and Trp311 in GHS-R1a and motilin receptor respectively). In this aim, molecular modelling was used to build models of GHS-R1a and motilin receptor in R and R* states from the 1F88 PDB high resolution structure of rhodopsin.

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Only two differences were retrieved in the spatial environment of the CWLP Trp residue. In the TM III, Val131 and Ile134 of GHS-R1a were replaced respectively by Leu126 and Met129 in motilin receptor (Fig. 1A). Site-directed mutagenesis was employed to insert this double mutation in GHS-R1a and to create a motilin receptor-like spatial environment around Trp276.

We achieved an extensive pharmacological characterization of the W276A and V131L-I134M GHS-R1a mutants.

2. Materials and methods

2.1. Materials

Dulbecco's modified eagle's medium (DMEM), medium 199, phosphate-buffered saline (PBS), penicillin–streptomycin, and L-glutamine were all from Cambrex (Emerainville, France).

Human ghrelin was purchased from NeoMPS (Strasbourg, France). Human [^{125}I]-His (9)-ghrelin (2000 Ci/mmol) and Myo-(2-3H)inositol (15 Ci/mmol) were obtained from Amersham (Orsay, France). The AG 1-X8 anion-exchange resin was purchased from Bio-Rad (Marnes-la-Coquette, France). Bovine serum albumin (BSA) fraction V came from Euromedex (Souffelweyersheim, France). PCS Scintillation fluid was from Perkin Elmer (Courtaboeuf, France). Poly-D-lysine and probenicid were from Sigma (Lyon, France). Pluronic acid was purchased from Molecular Probes (Paris, France) and Fluo-4-AM from Interchim (Montluçon, France).

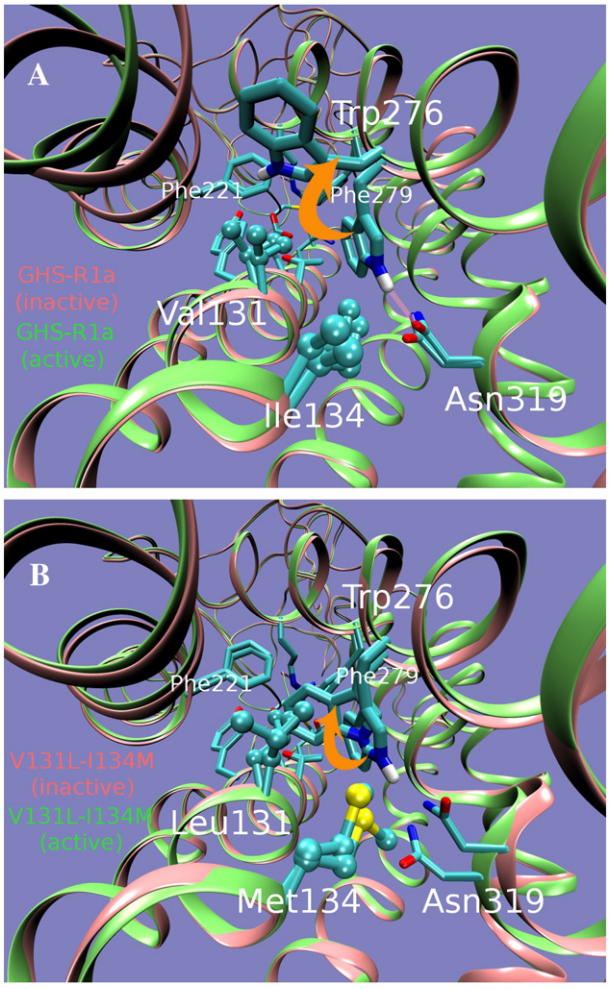

Fig. 1. Molecular model of the wild type ghrelin receptor (A) and V131L-I134M mutant receptor (B). A: Local conformational differences observed after the transition from the inactivated (pink) towards the activated (green) states of the wild type GHS-R1a receptor after rotation of the Trp276 indole chain. In each case the rotation of the Trp residue was possible without affecting the local structure of the protein. B: In the V131L-I134M GHS-R1a double mutant, rotation of the Trp276 was constricted and the local structure of the protein much more affected than in the wild type receptor (A). In the activated state of the double mutant, the Trp276 indole chain was closer to the Phe221 and Phe279 residues forming an aromatic cluster which role in GHS-R1a activation has already been mentioned. From our models, these residues are thought to stabilize the double mutant receptor in its activated state leading to an increased constitutive activity.

2.2. Molecular modelling

The molecular model of the human ghrelin receptor GHS-R1a was constructed by homology modelling taking the high resolution X-ray structure of rhodopsin as a template (Protein Data Bank accession number 1F88). We verified that using the recently published X-ray structure of the beta-2 adrenergic receptor (PDB number 2RH1) as a template instead of rhodopsin would have not changed the choice of the mutated residues (Cherezov et al., 2007; Rasmussen et al., 2007; Rosenbaum et al., 2007). After refinement of the initial GHS-R1a model through energy minimization (EM) and molecular dynamic simulations (MD) performed *in vacuo*, the putative inactive state structure of the ghrelin receptor (R) was obtained (Fig. 1A, pink). In this model, the side chain of Trp276 (TMVI) was in the $g+$ rotameric conformation (McAllister et al., 2004).

By conformational search (CS), the *t* rotameric conformation of Trp276 (TMVI), putative active state structure of the ghrelin receptor (R*), was also achieved (Fig. 1A, green). The method consisted to progressively rotate the χ 1 angle of Trp276, by steps of 5°, minimizing the energy of the whole protein at each step.

Starting from these models and by applying homology modelling we further built the corresponding putative inactive (R/$g+$) and active (R*/t) states of the motilin receptor which were also refined by the same protocol (EM, MD, and CS).

The molecular models of the GHS-R1a double mutant Val131Leu and Ile134Met (R and R*) were deducted from the wild type ghrelin receptor (R and R*) and refined by the same approach (Fig. 1B).

All calculations were performed on a Silicon Graphics Octane workstation using Biosym software (Accelrys, San Diego, CA).

2.3. Molecular biology: mutagenesis

Wild type human growth hormone secretagogue receptor 1a cloned in eukaryotic expression vector pcDNA3 was purchased from the Guthrie Research Institute (Sayre, PA, U.S.A.). Mutations were constructed by PCR using the overlap expression method (Ho et al., 1989).

Two kinds of oligonucleotide primers were used.

First, oligonucleotide cloning primers, the same for all mutants: sense primer 5′ GTGTGCTGGAATTCACCATGTGGAAC 3′ and antisense 5′ TGCATGCTCGAGCGGCCGCTCATGTATTAATAC 3′ containing the *EcoRI* and *XhoI* restriction site sequences respectively.

Second, oligonucleotide mutagenesis sense and antisense primers designed to introduce the required mutation.

For each mutant two PCR were performed using the wild type GHS-R1a cloned in pcDNA3 as a template. The first one was carried out with the sense cloning primer and the specific antisense mutagenesis primer since the second one used the specific sense mutagenesis primer and the antisense cloning primer.

The two overlapping resulting PCR products were amplified in a third PCR with the sense and antisense cloning primers. The final PCR product was gel-purified, digested with *EcoRI* and *XhoI* and then inserted into the *EcoRI* and *XhoI* sites of the plasmid pcDNA3.

All PCR experiments were performed using Pfu Ultra High Fidelity Hot Start polymerase (Stratagene, La Jolla, CA). All mutations were verified by DNA sequence analysis (Millegen, Labège France).