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In vitro modification of substituted cysteines as tool to study receptor functionality and structure–activity relationships

Daniel Rathmann, Xavier Pedragosa-Badia, Annette G. Beck-Sickinger *

Institute of Biochemistry, Faculty of Biosciences, Pharmacy and Psychology, Leipzig University, D-04103 Leipzig, Germany

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Dedicated to Gunter Fischer on the occasion of his 70th birthday

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ABSTRACT

Mutagenic investigations of expressed membrane proteins are routine, but the variety of modifications is limited by the twenty canonical amino acids. We describe an easy and effective cysteine substitution mutagenesis method to modify and investigate distinct amino acids in vitro. The approach combines the substituted cysteine accessibility method (SCAM) with a functional signal transduction readout system using different thiol-specific reagents. We applied this approach to the prolactin-releasing peptide receptor (PrRPR) to facilitate biochemical structure–activity relationship studies of eight crucial positions. Especially for $D^{6.59}C$, the treatment with the positively charged methanethiosulfonate (MTS) ethylammonium led to an induced basal activity, whereas the coupling of the negatively charged MTS ethylsulfonate nearly reconstituted full activity, obviously by mimicking the wild-type charged side chain. At $E^{5.26}C$, $W^{5.28}C$, $Y^{5.38}C$, and $Q^{7.35}C$, accessibility was observed but hindered transfer into the active receptor conformation. Accordingly, the combination of SCAM and signaling assay is feasible and can be adapted to other G-protein-coupled receptors (GPCRs). This method circumvents the laborious way of inserting non-proteinogenic amino acids to investigate activity and ligand binding, with rising numbers of MTS reagents allowing selective side chain modification. This method pinpoints to residues being accessible but also presents potential molecular positions to investigate the global conformation.

Prolactin-releasing peptide (PrRP)[1] is a member of the RFamide peptide family and is found in the hypothalamus, brainstem, and medulla oblongata [1–3]. It exerts a wide variety of physiological effects, including anorexia, nociception, stress response, and arterial blood pressure [4]. PrRP features two equipotent isoforms: PrRP31 (31 residues) and an N-terminally truncated PrRP20 (20 residues) [5,6]. Virtually all of the known physiological effects of PrRP are produced by the activation of the PrRP receptor (PrRPR), which belongs to the G-protein-coupled receptor (GPCR) superfamily [6]. This huge receptor family of surface proteins represents a prominent target, with approximately 30% of currently available drugs manipulating their activity [7,8]. GPCRs consist of a seven-transmembrane helix (TMH) structure, an intracellular C-terminal domain, an extracellular N-terminal tail, and three intracellular and three extracellular loops (ELs) (see Fig. 1). The seven TMHs of GPCRs constitute structural support for signal transduction.

Site-directed mutagenesis has been performed extensively in GPCR systems to investigate a plethora of fields, including constitutively active receptor mutants (CAMs), specific regions/motifs, ligand binding, receptor conformations in the presence of agonists, antagonists, and inverse agonists, and G-protein binding. Besides the frequently used scanning alanine mutagenesis (SAM), the scanning cysteine accessibility method (SCAM) [9–11] is another ingenious approach for the systematic identification of residues in TMHs that contribute to the binding pocket of a GPCR. This technique has been used to map numerous GPCR systems, transporters, and ion channels, as reviewed by Javitch and coworkers [9]. Especially, the use of CAMs and their respective wild-type (wt) background provides interesting insights into conformational differences between active and inactive GPCR conformations [12,13].

SCAM is based on the reaction of sulfhydryl-specific reagents with a single cysteine residue, which is introduced by mutagenesis one at a time (see Fig. 2A). These reagents are usually derivatives of methanethiosulfonate (MTS) such as the positively charged MTS ethylammonium (MTSEA) and MTS ethyltrimethylammonium (MTSET) and the negatively charged MTS ethylsulfonate (MTSES) (see Fig. 2). SCAM reaction relies on the fact that MTS reagents

* Corresponding author. Fax: +49 341 9736909.
E-mail address: beck-sickinger@uni-leipzig.de (A.G. Beck-Sickinger).

[1] *Abbreviations used:* PrRP, prolactin-releasing peptide; PrRPR, PrRP receptor; GPCR, G-protein-coupled receptor; TMH, transmembrane helix; EL, extracellular loop; CAM, constitutively active receptor mutants; SAM, scanning alanine mutagenesis; SCAM, scanning cysteine accessibility method; wt, wild-type; MTS, methanethiosulfonate; MTSEA, MTS ethylammonium; MTSET, MTS ethyltrimethylammonium; MTSES, MTS ethylsulfonate; IP, inositol phosphate; HPLC, high-performance liquid chromatography; cDNA, complementary DNA; EYFP, enhanced yellow fluorescent protein; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; TMD, transmembrane domain.

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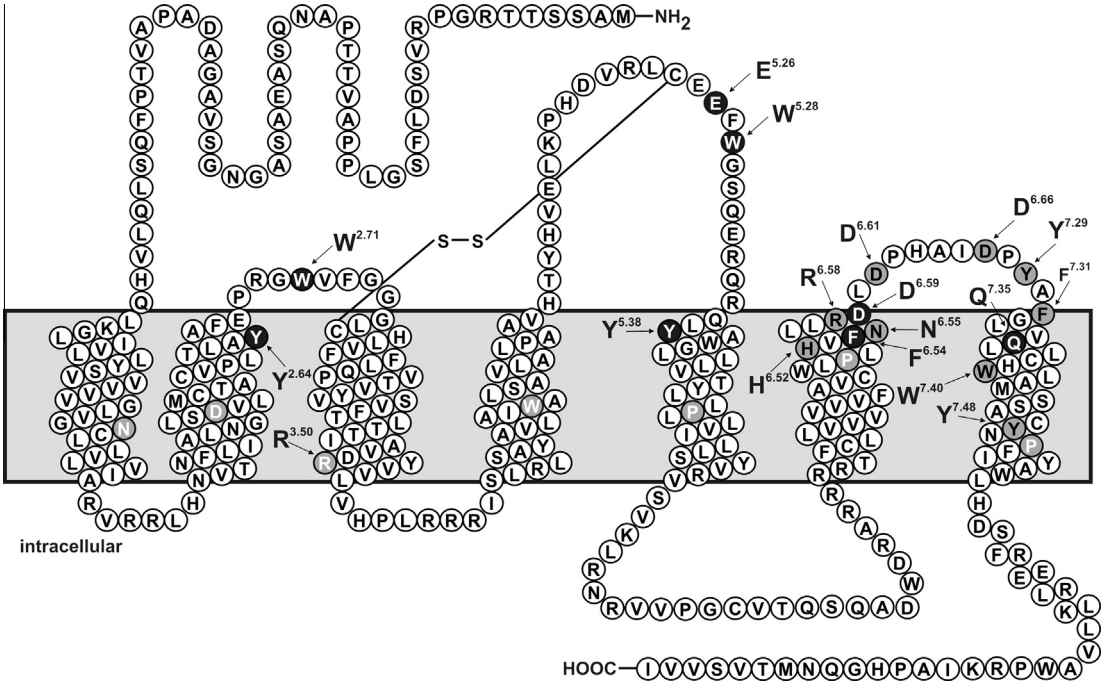

Fig.1. Snake plot representing the sequence of the human prolactin-releasing peptide receptor and investigated residues. Selective alanine substitutions revealed residues of different impact for ligand recognition. The residues shown in black letters with gray circles were unaffected by substitution to alanine, whereas the residues in black circles with white letters revealed a dramatic loss in ligand recognition. It was previously found that $E^{5.26}$ and $D^{6.59}$ were direct ligand binding partners, representing parts of the binding crevice together with the potential residues $Y^{2.64}$, $W^{2.71}$, $W^{5.28}$, $Y^{5.38}$, and $F^{6.54}$ [17,18]. The numbering of the residues was performed in accordance with Ballesteros and Weinstein [21], and the most conserved residue in each TMH is highlighted by white letters with gray background.

reacts 10^9 times faster with thiolates (S^-) than with thiols (SH) [14] and the deprotonated form of cysteine occurs to a significant extent only in the aqueous medium [15]. Thus, the sulfhydryl group of an introduced cysteine, facing toward a solvent accessible area, should react much faster with the sulfhydryl-specific reagent than sulfhydryls facing toward the interior of the protein or the lipid bilayer [16]. It is expected that after the MTS reaction with a cysteine exposed to the binding crevice, binding will decrease. If binding is unaffected, then interpretation must be done with caution. Either the cysteine is not accessible to the solvent or the mutation site is distant from the binding site.

We recently identified the double binding mode of R^{19} on the peptide ligand, which has two putative interaction partners within the PrRPR: $E^{5.26}$ and $D^{6.59}$. In addition, we were able to identify $Y^{5.38}$, $W^{5.28}$, and $F^{6.54}$ to be involved in receptor activation and ligand binding [17]. In a second study, we explored putative binding partners of EL 1 and its associated upper TMHs. With respect to receptor activation and ligand recognition, we identified $Y^{2.64}$ and $W^{2.71}$ as crucial residues when substituted to alanine (see Fig. 1) [18]. Nonetheless, the distinct role of the aromatic residues remains not fully understood and needed to be interpreted very carefully because effects of alanine substitution could be attributed to either a local interaction of the mutated residue or a global structural effect [19]. In this context, it was rarely possible to determine whether a residue is exposed to the binding crevice.

To address these limitations, we performed SCAM but for the first time coupled to a signal transduction readout assay (see Fig. 2B). This combined method allows more predictions of the particular function of residues and opens up a wide field of diverse assay setups. Most important, the exposure of the binding crevice can be determined by reaction with MTS derivatives while the receptor activity is studied in parallel. This enables an in vitro modification of side chains and contributes detailed information on functional residues. Performing an initial selective SAM of the so far not investigated EL 3 and the associated upper TMHs of the PrRPR revealed $Q^{7.35}$ as a novel interacting residue. Here, we report the application of SCAM coupled to an inositol phosphate (IP) accumulation assay at $Q^{7.35}$ and further recently described residues of influence regarding binding and/or signaling at the PrRPR (see Fig. 3) [17,18].

Materials and methods

Peptide synthesis

PrRP20 was synthesized by automated multiple solid-phase peptide synthesis on the multiple peptide synthesizer Syro II (MultiSynTech, Witten, Germany) using an orthogonal Fmoc/tBu strategy following the recently described protocol [20]. Rink amide resin and N_α-Fmoc [*N*-(9-flurenyl)methoxycarbonyl] protected amino acids were purchased from Iris Biotech (Marktredwitz, Germany).

Peptide purification and characterization was performed as described previously using preparative reverse-phase high-performance liquid chromatography (HPLC) (Vydac RP18 column, 22 × 250 mm, 10 μm, 300 Å, Grace, Deerfield, IL, USA, or Phenomenex Jupiter 10 U Proteo column, 250 × 21.20 mm, 90 Å, Aschaffenburg, Germany), analytical reversed-phase HPLC (Vydac RP18 column, 4.6 × 250 mm, 5 μm, 300 Å, Grace), and matrix-assisted laser desorption/ionization mass spectrometry (Ultraflex III MALDI–TOF/TOF mass spectrometer, Bruker Daltonics, Bremen, Germany) to yield homogeneous PrRP20 (>99% purity) [18].

Generation of PrRPR mutants

The complementary DNA (cDNA) of the PrRPR was C-terminally fused to the cDNA of the enhanced yellow fluorescent protein (EYFP) and cloned into the eukaryotic expression vector pEYFP-N1 (Clontech, Heidelberg, Germany). Mutations were introduced