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Binding specificity of the ectodomain of the parathyroid hormone receptor ☆

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ABSTRACT

The parathyroid hormone (PTH)1 receptor is a member of the class B G protein-coupled receptor (GPCR) family and regulates bone and mineral metabolism of vertebrates. A truncated highly active parathyroid hormone fragment PTH (1–34) exerts stimulatory effects on the receptor and is used for treatment of osteoporosis. To study the interacting amino acids of the natural peptide ligand PTH (1–84) with the ectodomain of its receptor we used peptide micro arrays on solid cellulose membranes. The amino acids Arg20 and Trp23 within the identified core binding stretch PTH (20–26) were found to be most important for affinity to the ectodomain of PTH1R. Isothermal titration calorimetry and NMR spectroscopy allowed peptide binding studies in solution and verified peptide positions required for high affinity. With this combination of biochemical and biophysical methods we extend former findings on this essential interaction and can now provide a strategy to screen for optimized therapeutic peptides.

1. Introduction

The human parathyroid hormone receptor 1 (hPTH1R) is characterized by a large extracellular N-terminal domain and seven transmembrane spanning helices and belongs to the class B or secretin family of G protein-coupled receptors (GPCRs). According to the nomenclature, receptors of this superfamily control basic cellular processes and are specifically activated by peptide hormones including glucagon, glucagon-like peptide 1 (GLP-1R) [1–3], glucose-dependent insulinotropic polypeptide (GIPR) [4] and parathyroid hormone (PTH1R). Their N-terminal domains are stabilized by three highly conserved disulfide bonds. Ligand binding induces a conformational change in the receptor either resulting in the activation of adenylate cyclase or phospholipase-C [5] via G protein binding and signaling. Parathyroid hormone (PTH) specifically binds to PTH1R and thereby regulates the blood calcium and phosphate levels [6]. PTH is also a potent agonist of PTH2R, a related receptor which shares about 50% sequence homology with PTH1R [7].

The peptide hormone is secreted by parathyroid glands whenever the calcium levels in the blood are reduced or blood phosphate concentrations are increased [8]. Due to its involvement in diseases like osteoporosis and malignancy associated hypercalcemia [9], PTH represents a valuable therapeutic tool. A synthetic PTH (1–34) variant is used to treat osteoporosis by decelerating the rate of bone loss [10,11]. To use PTH as a drug, it is important to achieve comprehensive knowledge about its interaction with the cognate G protein-coupled receptor.

In addition to the molecular structure of the ligand bound complex it is of great pharmaceutical interest to understand the role of individual residues involved in the ligand–receptor interaction [12,13]. This facilitates the development of improved agonists and antagonists for treatment of osteoporosis. Moreover, these findings could help to elucidate the mechanism of receptor activation also for other members of this class of GPCRs and to develop respective therapies.

In the present study, we overexpressed the N-terminal domain of PTH1R (nPTH1R) in *E. coli* as inclusion bodies (Ibs). The nPTH1R aggregate was dissolved in guanidine hydrochloride and successfully refolded in vitro [14,15]. Full binding activity to the peptide fragment PTH (1–37) could be recovered as demonstrated by isothermal titration calorimetry (ITC). Further truncated variants of PTH and peptides with specific amino acid exchanges were designed to identify the functionally important amino acid residues. Critical positions were obtained from peptide library screens. In addition we recorded NMR data to analyze

Abbreviations: Ibs, inclusion bodies; IMAC, immobilized metal ion affinity chromatography; PTH1R, parathyroid hormone receptor; GPCR, G protein-coupled receptor.

☆ Dedicated to Rainer Rudolph.

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PTH (1–34) binding to its receptor ectodomain at a residue-by-residue basis. We put our data into the context of the recently published crystal structure of the N-terminal domain of nPTH1R in complex with PTH (15–34) [12]. The NMR measurements show additional interaction sites at the N-terminus of PTH, which were not accessible in the crystal structure.

2. Materials and methods

2.1. Expression, purification and refolding of nPTH1R

The nPTH1R was produced recombinantly using the expression vector pET15b nPTH1R [15] and *E. coli* strain Rosetta pLys as a host. Fed batch fermentation was conducted in a 10 L Biostat ED bioreactor (B. Braun, Melsungen, Germany) with an initial volume of 6 L at 37 °C. Protein expression was induced at an optical density ($OD_{600\ nm}$) of 55 by 1 mM IPTG. For purification and refolding, a protocol according to Grauschopf et al. was used with minor modifications [15,16]. Briefly, cells were lysed and isolated inclusion bodies were stored at −20 °C. The receptor domain was enriched from solubilized Ibs by IMAC. For renaturation of nPTH1R a folding reactor (FairMenTech, Göttingen, Germany) at a constant temperature (4 °C) was used. The denatured protein solution was added into a stirred refolding buffer. Four pulses with a maximum of 50 μg/mL protein were injected in intervals of 6 h into the vessel containing 2 L of 50 mM Tris–HCl, pH 8 at 4 °C, 1 M L-arginine, 1 mM EDTA, 5 mM GSH and 1 mM GSSG. The incubation was continued for four days after the last pulse. Further purification was achieved via hydrophobic interaction chromatography using a phenyl sepharose 6 fast flow column (Pharmacia, Uppsala, Sweden) and decreasing ammonium sulphate concentrations in a linear gradient from 1 M to 0 M over 120 mL. Size exclusion chromatography with a Superdex 75 prep grade column (Pharmacia, Uppsala, Sweden) was used as final purification step. The protein yield was determined spectrophotometrically. Protein solutions were concentrated via an Amicon® Ultra-15 device with 10.000 MWCO (Millipore, Billerica, USA).

2.2. Peptide synthesis on continuous cellulose membrane support

The peptide chains were synthesised on Whatman 50 paper (140×110 mm) semi automatically using an Auto-Spot Robot APS 222 (Abimed GmbH, Langenfeld, Germany). Array design was performed using the software from Jerini Bio Tools GmbH (Berlin, Germany). For peptide synthesis, conventional Fmoc/tBu chemistry was applied [17]. Prior to peptide chain synthesis, the cellulose membranes were modified with fluorenylmethoxycarbonyl (Fmoc) beta-alanine as first anchor residue [18]. Subsequently, the Fmoc protection groups were cleaved and 0.2 μL of a 0.3 M Fmoc-beta-alanine-OPfp solution was used to configurate 17×25 spots with a beta-alanine-beta-alanine anchor and a diameter of 4 mm each. Excessive reactants were removed with dimethylformamide (DMF) and residual free amino functions on the membrane were acetylated with 5% acetanhydride and 2% diisopropylethylamine (DIPEA) in DMF. Again the Fmoc protection groups were cleaved and the membrane was stained with 0.01% bromophenol blue (BPB) in DMF to ensure the correct coupling reaction between the beta-alanines by indicating the unprotected N-terminal amino group. The peptides were synthesized from the C-terminal to the N-terminal position in a cyclic threefold coupling procedure with specific amino acids for the desired sequence. For that purpose 0.3 μL of a 0.3 M solution of the amino acids with Fmoc protection groups dissolved in either N, N-dimethylformamide (DMF) or N-methyl-2-pyrrolidinone (NMP) were added stepwise to the membrane. After coupling the last amino acid, all Fmoc protection groups were cleaved with 20% piperidine in DMF and the membranes were acetylated with 5% acetanhydride in DMF and DIPEA. In a final step, the peptide side chain protection groups were removed by washing the membrane in 50% trifluoroacetic acid (TFA) in dichloromethane (DCM) and triisopropylsilane (TIS). Finally, the membranes were washed, dried and stored in sealed bags at −20 °C.

A typical spot synthesis yields 5–10 nM equalling 6–12 μg for an average 10 mer peptide. Generally a purity of more than 70% for average 6–15 mers is achieved.

2.3. Binding studies of cellulose bound peptides

The peptide sequences were derived from the sequence of PTH. Membranes with 12 mer peptides covering PTH (1–84) overlapping by 11 amino acids were covalently attached at their C-terminal end to the beta-alanine anchor and used for first screen [19,20]. Libraries with shorter peptides and amino acid substitutions were produced as indicated in S1.

The spotted membranes were rinsed 3×10 min in water, 3×10 min in DMF and again 3×10 min in water. Afterwards they were incubated in an 8 M urea-buffer with 1% SDS (w/v) and 0.15 mM beta-mercaptoethanol for 20 min to denature the peptide chains. For renaturation of the peptides, the urea concentration was reduced to zero by steps of 1 M with 20 mM Tris–HCl, pH7.5. Membranes were equilibrated at 20 °C for 10 min with protein storage buffer (50 mM Tris–HCl, pH 8 at 20 °C, 300 mM $(NH_4)_2SO_4$) and incubated with 50 μM nPTH1R in the same buffer supplemented with 0.05% Tween 20 overnight at 4 °C under gentle shaking. Subsequently, unspecifically bound nPTH1R was removed by washing the membranes with TBS buffer (4 °C), and spot bound protein was electrotransferred with a semi dry blotter (SEMI-PHOR, Hoefer, Germany) onto pre-soaked nitrocellulose membranes (VWR international, Darmstadt, Germany). In this indirect method the nitrocellulose membranes were sandwiched between blotting paper, soaked with transfer buffer (25 mM Tris–HCl, pH 7.4, 0.15 mM glycine, 0.05% Tween 20 (v/v) at 4 °C). Electrotransfer was performed at a constant voltage of 40 V, 1 mA cm^{-2}, 100 W for 90 min. To reduce unspecific interactions, the membranes were blocked in TBT buffer with 5% milk powder. The transferred protein was detected with mouse anti PTH/PTHrP-receptor antibody (Santa Cruz, Heidelberg, Germany) and peroxidase conjugated goat anti mouse antibody (Santa Cruz, Heidelberg, Germany). The final visualization was performed by using an enhanced chemiluminescence (ECL) system.

2.4. Synthesis of crystalline PTH peptides

The PTH peptides were synthesized by solid-phase peptide synthesis with the robot Syro II (MultiSynTech, Witten, Germany) using 0.15 mmol pre-loaded Fmoc-amino acid-Wang resins (NovaBiochem, Läufelfingen, Switzerland). Synthesis was done by Fmoc strategy and standard protocol with Fmoc amino acids as building blocks, PyBOP (NovaBiochem) and N-methyl-morpholine as coupling reagents in dimethylformamide. Piperidine (20%)/DMF was the standard cleavage cocktail used for Fmoc detachment. The resin was treated twice for 10 min. All couplings were performed using a four-fold excess of the Fmoc amino acid derivative PyBOP and N-methyl-morpholine in DMF. A double coupling protocol was applied. After detachment of the peptides from the resins and site-chain deprotection with TFA/DCM/TIS/thioanisol/methylmercaptoacetamide/water (80/7.5/5/2.5/2.5/2.5) at room temperature for 2 h, the crude peptides were precipitated by diethylether and purified by RP-HPLC on a Gilson 306 equipment with a SP 250/10 Nucleosil 100-7C8 column (Macherey-Nagel, Düren, Germany) utilizing a water/acetonitrile (0.1% TFA) gradient. The purified peptides were lyophilized. The purity of the peptides was evaluated by analytical HPLC with a LiChroCART®(LiChrospher® 100, RP8, 5 μm) 125×4 mm column (Merck, Darmstadt, Germany). The purification was performed using a water/acetonitrile (0.1% TFA) gradient of 5–100% in 30 min and a flow rate of 1 mL/min and the peptides were detected at 220 nm.