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Research paper

Identification by photoaffinity labeling of the extracellular N-terminal domain of PAC1 receptor as the major binding site for PACAP

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ABSTRACT

Pituitary adenylate cyclase-activating polypeptide (PACAP) exerts many crucial biological functions through the interaction with its specific PAC1 receptor (PAC1-R), a class B G protein-coupled receptor (GPCR). To identify the binding sites of PACAP in the PAC1-R, three peptide derivatives containing a photoreactive *p*-benzoyl-phenylalanine (Bpa) residue were developed. These photosensitive PACAP analogs were fully biologically active and competent to displace radiolabeled Ac-PACAP27 from the PAC1-R. Subsequently, the ^{125}I-labeled photoprobes were used to anchor the PAC1-R expressed in Chinese hamster ovary cells. Photolabeling led to the formation of two protein complexes of 76 and 67 kDa, representing different glycosylated forms of the receptor. Proteinase and chemical cleavages of the peptide–receptor complexes revealed that ^{125}I[Bpa^{0}, Nle^{17}]PACAP27, ^{125}I[Bpa^{6}, Nle^{17}]PACAP27 and ^{125}I[Nle^{17}, Bpa^{22}]PACAP27 covalently labeled the Ser^{98} – Met^{111} segment, the Ser^{124} – Glu^{125} dipeptide and the Ser^{141} – Met^{172} fragment, respectively. Taking into account the topology of the PAC1-R, these segments are mainly located within the extracellular N-terminal domain, indicating that this PAC1-R domain is the major binding site of PACAP27. The present study constitutes the first characterization of the binding domains of PACAP to its specific receptor and suggests heterogeneity within the binding mode of peptide ligands to class B GPCRs.

1. Introduction

G protein-coupled receptors (GPCRs) are membrane-bound proteins that transduce intracellular molecular signals initiated by the selective binding of ligands [1]. The class B family of GPCRs, also known as secretin-like or class II, is a small family of GPCRs with low sequence homology versus class A (rhodopsin-like) and class C (glutamate-like) families [2]. The class B GPCR family comprises 15 members with no apparent orphan receptor [1]. They all contain a relatively large extracellular (EC) N-terminal domain (~100–160 residues) and a juxtamembrane domain consisting of seven transmembrane (TM) α-helices [3]. Class B GPCRs are glycosylated at Asn residues within consensus sequences located in the N-terminal portion of the protein and, in some cases, in EC loops [3]. These receptors display between 21% and 67% sequence identity and most of the variations are located in the EC N-terminal region [1]. Class B GPCRs all contain several conserved cysteine residues in the first and second EC loop. In addition, most of them comprise six cysteines that form a network of disulfide bridges in the EC N-terminal domain [1]. Class B GPCRs bind structurally-related peptides including calcitonin, growth hormone-releasing hormone (GHRH), gastric inhibitory polypeptide (GIP), glucagon, secretin, vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) [1]. These relatively large (~30–40 residues) endogenous peptide ligands act as hormones or neurohormones

Abbreviations: Bip, biphenylalanine; Bpa, *p*-benzoyl-phenylalanine; CHO, Chinese hamster ovary; CNBr, cyanogen bromide; EC, extracellular; GHRH, growth hormone-releasing hormone; GIP, gastric inhibitory polypeptide; GLP-1, glucagon-like peptide 1; GPCR(s), G protein-coupled receptor(s); hPAC1-R, human PAC1 receptor; NCS, *N*-chlorosuccinimide; NOE, Nuclear Overhausser Effect; Nle, norleucine; PAC1-R, PAC1 receptor; PAC1-Rs, PAC1-R short splice variant; PACAP, pituitary adenylate cyclase-activating polypeptide; PNG-ase F, peptide *N*-glycanase F; PTH, parathyroid hormone; SAR, structure–activity relationships; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; TM, transmembrane; VIP, vasoactive intestinal peptide.

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and play important biological functions, such as blood glucose regulation, calcium homeostasis and neurotransmission [3]. Accordingly, class B GPCRs are attractive targets for treating various diseases such as type II diabetes [4], osteoporosis [5] and depression [6]. For instance, the class B GPCR ligands, glucagon, calcitonin and parathyroid hormone (PTH), are currently used in the clinic [1].

A general mechanism for ligand interaction with class B GPCRs, known as the two-domain model, has emerged [3]. This model, inferred from structure–activity relationships (SAR), site-directed mutagenesis and chimeric receptors studies, proposes that the C-terminal region of the peptide binds to the EC N-terminal domain of the receptor. Following this initial docking, the N-terminal segment of the ligand interacts with the juxtamembrane domain of the receptor and this interaction initiates receptor activation and intracellular signaling. Spatial approximation between peptide ligand and intact receptor obtained by photoaffinity labeling studies of class B GPCRs generally supports this model. For instance, substitution of carboxy-terminal residues of secretin by the photoreactive element, *p*-benzoyl-phenylalanine (Bpa) led to the labeling of the EC N-terminal domain of the secretin receptor, within the first 38 residues [7]. On the other hand, position 5 of secretin has been shown to physically interact with the third EC loop of the receptor [8]. Photoaffinity labeling studies of other class B GPCRs, such as PTH [9], calcitonin [10] and glucagon-like peptide 1 (GLP-1) [11] receptors, are also consistent with the two-domain model. However, labeling of the VPAC1 receptor, a class B GPCR, using various VIP photoprobes has revealed physical interactions within the EC N-terminal domain, including peptide ligands with photoreactive residues at positions 0 and 6 [12,13], suggesting heterogeneity within the mechanism of peptide interaction with class B GPCRs.

The PAC1 receptor (PAC1-R) (Fig. 1), a prototypical class B GPCR, has been recently recognized as an attractive pharmacological target for the treatment of neurological insults [14] such as fetal alcoholism syndrome [15], cerebral ischemia [16] and drug-induced toxicity [17]. The activity of PAC1-R is modulated by PACAP, a C-terminally amidated neuropeptide that exists in two isoforms, a 38-amino acid peptide (PACAP38) and its 27-residue N-terminal fragment (PACAP27) [18]. SAR studies have shown that the N-terminal domain of PACAP is essential for the activation of PAC1-R whereas central and C-terminal regions of the peptide are important for receptor specificity and high-affinity binding [19]. Indeed, truncation of the first two residues of PACAP38 suppresses the PAC1-R agonistic activity [20], while C-terminally shortened PACAP27 fragments behave as complete PAC1-R agonists showing a decrease of their binding affinity [21]. These SAR data are consistent with the two-domain model of interaction of peptide ligands with class B GPCRs. The solution structure of the complex formed by the isolated EC N-terminal domain of PAC1-R short splice variant (PAC1-Rs) and the antagonist PACAP(6-38) has been determined by NMR. This study revealed that the C-terminal region of PACAP binds as an α-helix to the EC N-terminal domain by means of specific electrostatic and hydrophobic interactions [22]. Moreover, direct Nuclear Overhausser Effect (NOE) contacts between segment 29–34 of the ligand and residues 117–119 of the isolated EC domain were reported [22]. On the other hand, no NOE connectivities between residues 6–9 of PACAP(6-38) and the N-terminal domain of PAC1-Rs were observed, suggesting that these residues interact with other part of the receptor [22]. These results correlate with the two-domain model. Nonetheless, physical interactions between full-length functional PAC1-R and its endogenous ligand are still not characterized. Such information would help in understanding the mode of action of PACAP at the molecular level. Particularly, elucidation of the mechanisms by which PACAP binds to the PAC1-R could facilitate the rational design of pharmacologically attractive compounds acting selectively at the PAC1-R and could lead to innovative therapeutic strategies. In this context, the present study was undertaken to determine the physical sites of interaction between the human PAC1 receptor (hPAC1-R) and its endogenous ligand, PACAP, by means of photoaffinity labeling.

2. Materials and methods

2.1. Photoligand synthesis

Using the solid phase synthesis strategy combined with the Boc chemistry methodology, photosensitive PACAP27 analogs were obtained by substituting Phe^{6} and Tyr^{22} with Bpa and also by incorporating Bpa at the N-terminal amine function (position 0).

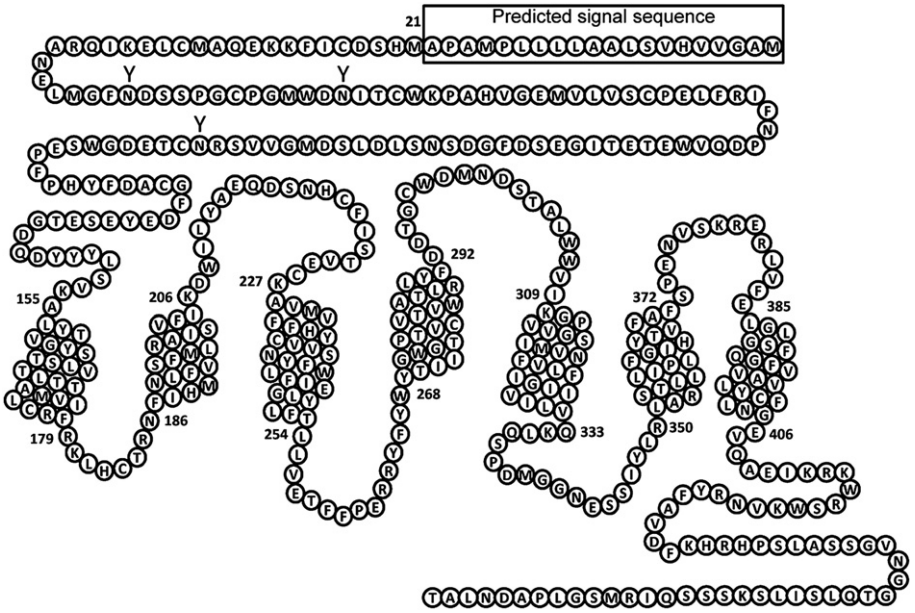

Fig. 1. Topography of the human PAC1 receptor. The location of the extracellular loops and the transmembrane domains of the human PAC1 receptor (Swissprot #P41586) were predicted using the Kyte and Doolittle hydropathy analysis plot [33] and the shareware HMMTOP [34].