Photolabelling the rat urotensin II/GPR14 receptor identifies a ligand-binding site in the fourth transmembrane domain

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A urotensin II (U-II) peptide analogue containing the photo-reactive *p*-benzoyl-L-phenylalanine (Bz-Phe) in the sixth position was used to identify ligand-binding sites of the rat U-II receptor, also known as GPR14. [Bz-Phe⁶]U-II bound the receptor expressed in COS-7 cells with high affinity (IC₅₀ 0.7 nM) and was as potent as U-II in the agonist-induced production of inositol phosphate. Photolabelling of the U-II receptor with ¹²⁵I-[Bz-Phe⁶]U-II resulted in the specific formation of a glycosylated ¹²⁵I-[Bz-Phe⁶]U-II–U-II receptor complex of 60 kDa. Digestion of the 60 kDa complex with endoproteinase Glu-C generated a fragment of 17 kDa circumscribing the labelled fragment to residues 148–286. Digestion of the ligand–receptor complex with endoproteinase Arg-C produced a short peptide of 4 kDa corresponding to fragments 125–148, 167–192 or 210–233. CNBr treatment of the endoproteinase-Glu-C and

-Arg-C fragments yielded 2 kDa fragments, defining the labelling site to methionine residues 184/185 of the fourth transmembrane domain. Photolabelling of two mutant receptors, M184L/M185L and M184A/M185A, led to a significant decrease in the overall yield of covalent labelling. Taken together, our results indicate that position 6 of U-II normally occupied by phenylalanine would interact with Met¹⁸⁴ and/or Met¹⁸⁵ of the fourth transmembrane domain of the U-II receptor. This information should be of significant value in the study of the interactions between U-II and its cognate receptor.

Key words: binding pocket, G-protein-coupled receptor, *p*-benzoyl-L-phenylalanine, peptide mapping, photoaffinity labelling.

INTRODUCTION

Human urotensin II (Glu-Thr-Pro-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val; U-II) is a cyclic undecapeptide shown to have potent vasoactive effects [1]. Although the peptide had originally been isolated from urophysis (a neurosecretory organ of teleost fish found on the ventral aspect of the distal end of the spinal cord) [2], the cDNA encoding its precursor has now been sequenced from many species [3–5]. *In situ* hybridization and immunohistochemistry have revealed that U-II is localized to the medulla oblongata of the brain and the spinal cord in the central nervous system, but is also present in such tissues as adrenal glands, kidney and spleen, suggesting neurohormonal or hormonal properties [3,6]. Although it is clear that U-II influences the physiological regulation of mammalian cardiovascular function, early reports led to conflicting vasoactive responses depending on species or tissues used [7,8]. Recent papers, however, have described how human U-II has potent vasodilator and cardio-stimulant responses in human tissues [8,9].

Several structural forms of the peptide have been reported in various species of fish and amphibians, with a variation in primary structure occurring in the first five to seven residues of the N-terminus [10]. However, the C-terminal cyclic hexapeptide is conserved across species, and structure–activity studies on goby (*Gillichthys mirabilis*) U-II suggested that residues 6–12 (corresponding to amino acids 4–11 in human) are sufficient for high-affinity binding to the receptor and to exert its biological activity [11]. More recently, an extensive structure–activity study of human U-II [12] has confirmed the importance of the human cyclic hexapeptide sequence for receptor activation. Moreover, similar activation levels attained by U-II peptides derived from various species (human, frog, fish and pig) have revealed that

N-terminal residues might contribute to a lesser extent to the agonistic properties of the ligand [13] than the conserved portion of U-II consisting of residues Asp⁴–Val¹¹ [11].

Recently, it has been shown that U-II is the endogenous ligand of a G-protein-coupled receptor (GPCR) [4], which was isolated from a human genomic library [6] and which possessed high sequence similarity to GPR14, an orphan receptor identified in rat [14] and currently referred to as the U-II receptor. The rat U-II receptor belongs to class A, the rhodopsin-like family, of the peptide subfamily of GPCRs and is comprised of 386 residues (389 for the human U-II receptor) [4]. The receptor possesses two potential N-glycosylation sites in the N-terminal domain (Asn²⁹ and Asn³³) and two cysteine residues in the first and second extracellular loops, which are thought to participate in disulphide bonding. Intracellular portions contain the Glu/Asp-Arg-Tyr motif, which is well conserved amongst GPCRs, and potential phosphorylation sites are found in the cytoplasmic tail. The receptor is widely expressed in human tissues, the mRNA being found in the left atrium and ventricle of the heart and in smooth-muscle cells from the coronary artery and aorta as well as endothelial cells from several vascular beds [4].

An understanding of the underlying molecular basis of how U-II binds and activates the U-II receptor is of key importance in the rational design of new pharmacophores. We have sought to probe into the putative interactions of the peptide with its cognate receptor by covalently modifying the U-II receptor with a photoreactive analogue of U-II containing the photoactivatable amino acid *p*-benzoyl-L-phenylalanine (Bz-Phe). The Bz-Phe-based photoaffinity-labelling approach [15] has been successfully employed to identify binding sites in several peptide receptors [16–18]. Here we report on the specific interaction of ¹²⁵I-labelled [Bz-Phe⁶]U-II with residues Met¹⁸⁴/Met¹⁸⁵ of the fourth trans-

Abbreviations used: AngII, angiotensin II; Bz-Phe, *p*-benzoyl-L-phenylalanine; DMEM, Dulbecco's modified Eagle's medium; ECL, extracellular loop; Endo Arg-C, endoproteinase Arg-C; ET-1, endothelin-1; GPCR, G-protein-coupled receptor; M184L (etc.), Met¹⁸⁴ → Leu mutant (etc.); PNGase-F, peptide N-glycosidase F; U-II, urotensin II.

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membrane domain of the rat U-II receptor. This is the very first study giving important insights into how U-II interacts with its receptor using a direct identification approach.

EXPERIMENTAL

Materials

BSA, bacitracin, soybean trypsin inhibitor and CNBr were from Sigma. Protein N-glycosidase F (PNGase-F; EC 3.5.1.52), staphylococcal V8 protease (EC 3.4.21.19) and endoproteinase Arg-C (Endo Arg-C; EC 3.4.22.8) were from Roche Diagnostics. The cDNA clone of the rat urotensin II receptor (GPR14) subcloned in the mammalian expression vector pcDNA₃ was kindly provided by Dr Brian O'Dowd (Department of Pharmacology, University of Toronto, Toronto, Ontario, Canada). LIPOFECTAMINE[®] and culture media were obtained from Life Technologies (Gaithersburg, MD, U.S.A.). U-II peptides were synthesized in our laboratories using Fmoc (fluoren-9-ylmethoxycarbonyl)-protected amino acids and Wang resin (a *p*-benzyloxybenzyl alcohol resin from Novabiochem, Laufelfingen, Switzerland). Cysteine residues were protected by acetamidomethyl groups, which were removed by using mercury acetate. Disulphide-bond formation was achieved by oxidation with DMSO [19]. The synthetic peptides were purified to homogeneity by reverse-phase HPLC. ¹²⁵I-U-II and ¹²⁵I-[Bz-Phe⁶]U-II (sp. radioactivity 1000 Ci/mmol) were prepared with IODO-GEN[®] (1,3,4,6-Tetrachloro-3a,6a-diphenylglycoluril; Pierce Chemical Co.) as described by Fraker and Speck [20]. Briefly, 25 µl of 1 mM peptide solution was incubated with 50 µg of IODO-GEN[®], 65 µl of 100 mM borate buffer, pH 8.5, and 1 mCi of Na¹²⁵I for 30 min at room temperature before being purified by HPLC on a C-18 column. The specific radioactivity of the labelled peptides was determined by self-displacement and saturation-binding analysis.

PCR mutagenesis

Mutant receptor cDNAs were constructed by oligonucleotide-directed mutagenesis (Expand High Fidelity PCR System; Roche Diagnostics) using the rat GPR14 as template. Two sets of forward and reverse oligonucleotides were constructed to introduce mutations simultaneously at both Met¹⁸⁴ and Met¹⁸⁵. PCR products were re-inserted in pcDNA₃ and mutagenesis was confirmed by nucleotide sequencing.

Cell culture and transfections

COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal-bovine serum, 100 i.u./ml penicillin and 100 µg/ml streptomycin at 37 °C. Semi-confluent cells (70%) in 100-mm-diameter Petri dishes were transfected using the LIPOFECTAMINE[®] method (Gibco BRL). Transfected cells were grown for 48 h before photoaffinity labelling and binding assays.

Binding experiments

COS-7 cells were washed once with PBS and subjected to one freeze-thaw cycle. Broken cells were then gently scraped into washing buffer [20 mM Tris/HCl (pH 7.4)/5 mM MgCl₂], centrifuged at 2500 *g* for 15 min at 4 °C, and resuspended in binding buffer [20 mM Tris/HCl (pH 7.4)/5 mM MgCl₂/0.1% BSA/0.01% bacitracin]. For saturation binding assays, broken cells (50–75 µg of protein) were incubated for 1 h at room temperature in binding buffer containing increasing concentrations of ¹²⁵I-U-II or ¹²⁵I-[Bz-Phe⁶]U-II (50 Ci/mmol) in a final volume

of 0.5 ml. For competition binding assays, broken cells were incubated for 1 h with 0.1 nM ¹²⁵I-U-II (1000 Ci/mmol) as tracer and increasing concentrations of U-II, [Bz-Phe⁶]U-II, [Bz-Phe⁷]U-II or [Bz-Phe¹¹]U-II. Bound radioactivity was separated from free ligand by filtration through GF/C filters pre-soaked for at least 3 h in binding buffer. Non-specific binding was measured in the presence of 1 µM unlabelled U-II. Receptor-bound radioactivity was evaluated by γ-radiation counting.

Measurement of agonist-induced production of inositol phosphates

Accumulation of inositol phosphates was determined essentially as described [21], with the following modification. Production of inositol phosphates was induced with 100 nM U-II and [Bz-Phe⁶]U-II or 10 µM [Bz-Phe⁷]U-II and [Bz-Phe¹¹]U-II for 20 min at 37 °C in buffer containing 10 mM LiCl. Inositol phosphates were sequentially eluted from the anion-exchange column by the addition of an ammonium formate/formic acid solution of increasing ionic strength. Fractions containing inositol phosphates were collected and counted for radioactivity in a liquid-scintillation counter.

Photoaffinity labelling

Photoaffinity labelling was performed as previously described [16]. Transfected COS-7 cells were incubated for 1 h at room temperature in 0.5 ml of binding buffer containing the photo-reactive radioligand (5 nM), in the presence or absence of U-II, angiotensin II (AngII) or endothelin-1 (ET-1). Cells were then washed, irradiated for 1 h at 0 °C under filtered UV light in washing buffer and centrifuged. The pellet was solubilized in modified radioimmunoprecipitation buffer ['m-RIPA'; 100 mM Na₂HPO₄ (pH 8.5)/25 mM EDTA/0.1 mg/ml soybean trypsin inhibitor/1% (v/v) Nonidet P40] containing Complete Protease Inhibitor Cocktail[®] (Roche Diagnostics). After centrifugation (13000 *g* for 30 min at 4 °C), the supernatants were kept at –80 °C until further analysis.

Partial purification of the labelled complex

Photolabelled receptors were partially purified as previously described [16,22]. Briefly, photolabelled receptors were separated by SDS/PAGE before being dried and exposed to BioMax[®]MS films (Kodak). Bands corresponding to receptor-ligand complexes were cut from the gels and submitted to passive elution. Eluted proteins were then concentrated, divided into portions and kept at –20 °C.

Endoglycosidase, proteolytic and chemical digestions

For endoglycosidase digestions, partially purified photolabelled receptors were resuspended in digestion buffer containing 0.5% Nonidet P40. PNGase-F (500 units/ml) was added, and samples were incubated at room temperature for 16 h. For proteolytic digestions, partially purified photolabelled receptors (5000–10000 c.p.m.) were resuspended in 25 µl of digestion buffer [100 mM NH₄HCO₃ (pH 8.0)/0.1% SDS] and were incubated for 5–7 days at room temperature. Under these conditions V8 protease cleaves at the C-terminal side of glutamate residues. For Endo Arg-C digestion, partially purified photolabelled receptors (5000–10000 c.p.m.) were resuspended in 75 µl of digestion buffer (containing 90 mM Tris/HCl, pH 7.6, 8.5 mM CaCl₂, 5 mM dithiothreitol, 0.5 mM EDTA and 0.01% SDS) and samples were incubated for 16 h at 37 °C. When subsequent digestions were needed, products of the first digestion were run on a 16.5% (w/v) acrylamide/Tris/Tricine gel and the corresponding band was eluted in water containing 0.1% SDS. Eluted proteins were

A **B****Extracellular**

Figure 1 (A) Amino acid sequence of hU-II and photoreactive hU-II analogues and (B) two-dimensional representation of the primary structure of the rat U-II receptor and its potential cleavage sites by specific proteases and CNBr

(A) Residues represented in **bold** characters correspond to amino acid modifications. (B) Black closed circles indicate sites of hydrolysis for CNBr. **Bold** circles indicate recognition sites for V8 protease. Grey closed circles indicate recognition sites for Endo Arg-C. Putative sites of N-glycosylation on asparagine-29 and -33 are also shown.

freeze-dried and submitted to subsequent digestion. For CNBr hydrolysis, partially purified photolabelled receptors (5000–10000 c.p.m.) were resuspended in a 1:1 mixture of 50% trifluoroacetic acid and CNBr (200 mg/ml) and incubated at room temperature, in the dark for 18 h. Reactions were terminated by adding 1 ml of water and the material was freeze-dried.

Analysis of the products of proteolysis and chemical cleavage

The products of proteolysis and chemical cleavage were analysed by SDS/PAGE using 16.5% polyacrylamide/Tris/Tricine gels, followed by autoradiography on X-ray films (Kodak BioMax[®] MS) with intensifying screens. ¹⁴C-Labelled low-molecular-mass

protein standards (Amersham Biosciences) were used to determine apparent molecular masses.

RESULTS

Binding specificity and functional properties of photoreactive analogues

Figure 1(A) shows the primary structure of human U-II and the photoreactive U-II analogues used in the present study. Phe⁶, Trp⁷ and Val¹¹ were successively replaced by the photolabile Bz-Phe to give [Bz-Phe⁶]U-II, [Bz-Phe⁷]U-II and [Bz-Phe¹¹]U-II respectively. The peptides also contain a tyrosine residue, present in the native peptide at position 9, for radioiodination. In

Figure 2 Specific photoaffinity labelling of rat U-II receptor from transiently transfected COS-7 cells

Cells expressing the U-II receptor were photolabelled with ^{125}I -[Bz-Phe⁶]U-II, solubilized, denatured, and submitted to SDS/PAGE on a 10% or 8% acrylamide (**A** and **B** respectively) separating gels (40 μg of protein/lane), followed by autoradiography. (**A**) Photolabelling was performed in the presence of 10^{-11} M U-II (lane 1), 10^{-8} M U-II (lane 2), 10^{-6} M U-II (lane 3) and 10^{-4} M U-II (lane 4). (**B**) Total labelling (lane 1), labelling in the presence of 10^{-4} M U-II (lane 2), labelling in the presence of 10^{-6} M AngII (lane 3) and labelling in the presence of 10^{-5} M ET-1 (lane 4). Molecular-mass markers are indicated (kDa). These results are representative for at least three separate experiments.

competitive binding assays, U-II and [Bz-Phe⁶]U-II exhibited high affinities for the U-II receptor expressed in COS-7 cells with IC_{50} values of 3.2 ± 0.5 nM ($n = 4$) and 0.7 ± 0.2 nM ($n = 5$), whereas [Bz-Phe⁷]U-II and [Bz-Phe¹¹]U-II had much lower affinities [266 ± 58 nM ($n = 3$) and 466 ± 150 nM ($n = 4$) respectively]. The functional properties of these analogues were then assessed by measuring the ligand-induced $\text{Ins}(1,4,5)\text{P}_3$ production in COS-7 cells expressing the U-II receptor. Incubation of the cells with 100 nM [Bz-Phe⁶]U-II caused an increase in $\text{Ins}(1,4,5)\text{P}_3$ comparable with the level produced by native U-II, suggesting the agonist nature of the analogue (basal, 1500 c.p.m.; [Bz-Phe⁶]U-II, 23000 c.p.m.; and U-II, 30000 c.p.m.). Incubation of the cells with 10 μM of either [Bz-Phe⁷]U-II or [Bz-Phe¹¹]U-II also led to increased production of inositol phosphates (results not shown). Given the higher affinity of [Bz-Phe⁶]U-II for the U-II receptor, we conducted the photolabelling studies with this analogue.

Photoaffinity labelling of rat U-II receptor

In photoaffinity-labelling experiments using ^{125}I -[Bz-Phe⁶]U-II, two proteins migrating as 60 and 31 kDa bands on SDS/PAGE were specifically and covalently labelled (Figure 2A). The labelling of these forms of the U-II receptor was decreased in a dose-dependent manner (Figure 2A) with complete abolition at 100 μM U-II (Figures 2A and 2B, lanes 2). No decrease in labelling of either form was observed when AngII (100 μM) (Figure 2B, lane 3) or ET-1 (10 μM) (Figure 2B, lane 4) was added to the photolabelling-experiment reaction mixture, confirming the

Figure 3 PNGase-F digestion of the two ^{125}I -[Bz-Phe⁶]U-II-labelled U-II receptor species

(**A**) The partially purified photolabelled 60 kDa form of U-II receptor (10000 c.p.m.) was incubated in the absence (lane 1) or the presence (lane 2) of PNGase-F (2 units) for 16 h at room temperature. (**B**) The partially purified photolabelled 31 kDa form of U-II receptor (10000 c.p.m.) was incubated in the absence (lane 1) or the presence (lane 2) of PNGase-F (2 units) for 16 h at room temperature. Samples were run on a 10% acrylamide separating gel followed by autoradiography. Molecular-mass markers are indicated (kDa). These results are representative for at least three separate experiments.

specificity and the selectivity of the labelling. Determination of covalent incorporation yields, calculated from the ratio of total radioactivity found in isolated bands to total specific binding observed before photolysis, revealed that ^{125}I -[Bz-Phe⁶]U-II had a 60% yield of covalent incorporation on to the U-II receptor.

Deglycosylation of the ligand–receptor complexes

To verify whether the two receptor forms labelled by ^{125}I -[Bz-Phe⁶]U-II were glycosylated, both the 60 and the 31 kDa complexes were partially purified and submitted to PNGase-F digestion (Figure 3). Deglycosylation of the 60 kDa ligand–receptor complex led to the production of a 42 kDa band (Figure 3A, lane 2), corresponding to the calculated molecular mass of the U-II receptor. However, deglycosylation of the 31 kDa complex did not lower its molecular mass, suggesting its unglycosylated nature (Figure 3B, lane 2).

V8-protease digestion of the 60 kDa ^{125}I -[Bz-Phe⁶]U-II–U-II receptor complex

In an initial attempt to map the ligand–receptor contact sites, the 60 kDa photoligand–receptor complex was partially purified

Figure 4 V8-protease digestion of the partially purified 60 kDa form of photolabelled U-II receptor

(A) The partially purified photolabelled 60 kDa form of the U-II receptor (5000 c.p.m.) was incubated in the absence (lane 1) or the presence (lane 2) of V8 protease (20 μ g) for 7 days at room temperature. Samples were run on a 16.5% acrylamide/Tris/Tricine separating gel followed by autoradiography. Molecular-mass markers are indicated (kDa). These results are representative for at least three separate experiments. (B) Serpentine representation of the theoretical V8-protease fragmentation footprint for U-II receptor with cleavage at all potential sites. The numbers in square brackets indicate the molecular mass of the corresponding photoaffinity cross-linked fragment and ligand. The putative contact domain, corresponding to the non-glycosylated receptor fragment 148–286, is highlighted as dark-grey shading.

(Figure 4A, lane 1) and submitted to V8-protease digestion, which cleaves on the C-terminal side of glutamate residues (Figure 1B). This digestion produced a band of apparent molecular mass 17 kDa (Figure 4A, lane 2). Two potential receptor domains appear as potential candidates: one comprising receptor domain Leu²⁴–Glu¹⁴⁷, with a calculated molecular mass of 14.8 kDa, which is glycosylated on Asn²⁹ and/or Asn³³ residues (Figure 1B), and another fragment corresponding to Arg¹⁴⁸–Glu²⁸⁶, with a calculated molecular mass of 17.5 kDa (un-

glycosylated). Since PNGase-F digestion did not change the migration pattern of the latter fragment (results not shown), this suggested the absence of N-glycosylation on this fragment. The only candidate fragment is the 139-residue polypeptide encompassing Arg¹⁴⁸ to Glu²⁸⁶ (Figure 4B).

Endo Arg-C digestion of the 60 kDa ¹²⁵I-[Bz-Phe⁶]U-II–U-II receptor complex

To further identify the binding domain, the 60 kDa photoligand–receptor complex was submitted to Endo Arg-C digestion, which specifically cleaves at the C-terminal end of arginine residues (Figure 1B). Endo Arg-C digestion (Figure 5A, lane 1) produced two bands migrating at a molecular mass of 6 kDa and 4 kDa (Figure 5A, lane 2). Since the 6 kDa band is progressively cleaved to give the 4 kDa band after prolonged digestion times (results not shown), the former is believed to originate from a partial enzyme cleavage. Thus it is assumed that the labelling occurs only on one receptor site, the smallest region corresponding to the 4 kDa Arg-C-generated fragment. Taking this into consideration with those results obtained with the V8 protease circumscribing the labelling to the 148–286 polypeptide, three fragments appear as potential candidates: Val¹²⁵–Arg¹⁴⁸ (4.2 kDa) (if labelling occurred exclusively on Arg¹⁴⁸); Lys¹⁶⁷–Arg¹⁹² (4.4 kDa); Thr²¹⁰–Arg²³³ (4.0 kDa) (Figure 5C).

Labelling occurs exclusively in the fourth transmembrane domain of the U-II receptor

In order to discriminate between the three possible domains of interaction identified by Endo Arg-C digestion as being the third, fourth or the fifth transmembrane domain, we took advantage of the exclusive presence of methionine residues (positions 184–185) within the Lys¹⁶⁷–Arg¹⁹² receptor domain. Of the three potential Endo-Arg-C-generated fragments, only the 167–192 polypeptide would be CNBr-sensitive, enabling us to identify it from the other two. We then proceeded to cleave the 4 kDa fragment previously generated by Endo Arg-C with CNBr. An adduct of 2.5 kDa migrating with the dye front was obtained (Figure 5B). Given the low resolution of this part of the gel, it could only be determined that the fragment was sensitive to CNBr cleavage. Thus, on the basis of CNBr-sensitivity, we can attribute the labelling as occurring exclusively in the fourth transmembrane domain of the receptor (Figure 5C).

¹²⁵I-[Bz-Phe⁶]U-II interacts with Met¹⁸⁴ and/or Met¹⁸⁵ of the U-II receptor

To further confirm the identity of the receptor domain interacting with the photoligand, the ¹²⁵I-[Bz-Phe⁶]U-II–U-II receptor complex was digested with V8 protease and the 17 kDa fragment was partially purified. Cleavage of this fragment with CNBr (Figure 6A, lane 1) led to the production of three distinct labelled fragments of 12.5, 6.5 and of 2 kDa (Figure 6A, lane 2), the latter co-migrating with the free ligand of calculated molecular mass 1.5 kDa (Figure 6A, lane 3). The labelled fragments able to produce this pattern are indicated in Figure 6(B). The 12.5 and 6.5 kDa entities would be the result of partial CNBr cleavage at Met¹⁸⁴ or Met¹⁸⁵, yielding fragments corresponding to Met¹⁸⁵–Glu²⁸⁶ (13 kDa) and Arg¹⁴⁸–[Met^{184/185}] (5.8 kDa). The 2 kDa fragment could either correspond to Met¹⁸⁵ covalently attached to the ligand or the ligand itself released from one or both methionine residues following CNBr treatment [23]. This led us

Figure 5 Endo Arg-C digestion of the partially purified 60 kDa form photolabelled U-II receptor

(A) The partially purified photolabelled 60 kDa form of the U-II receptor (5000 c.p.m.) was incubated in the absence (lane 1) or the presence (lane 2) of Endo Arg-C (0.5 μ g) for 16 h at 37 °C. (B) The 4 kDa Endo Arg-C fragment was incubated in the absence (lane 1) or presence (lane 2) of CNBr (100 mg/ml) for 18 h at room temperature in the dark. Samples were run on a 16.5% acrylamide/Tris/Tricine separating gel followed by autoradiography. Molecular-mass markers are indicated (kDa). These results are representative of at least three separate

experiments. (C) Serpentine representation of the theoretical Endo Arg-C fragmentation footprint for the U-II receptor with cleavage at all potential sites. Numbers in square brackets indicate the molecular mass of the corresponding photoaffinity cross-linked fragment, including radioactive ligand. Closed circles represent methionine residues present in the receptor's fourth transmembrane domain. The highlighted (grey shading) receptor fragments indicate potential contact domains with the most probable domain, fragment 167–192, highlighted in dark grey.

Effect of photolabelling on methionine double mutants at positions 184 and 185 of the U-II receptor

Since the photolabelling results on the wild-type receptor suggested that Met¹⁸⁴ and/or Met¹⁸⁵ interacted with U-II at position 6, we examined the effect of replacing these two residues with leucine or alanine by constructing the M184L/M185L and M184A/M185A U-II receptor mutants. The integrity and functionality of these receptors were assessed pharmacologically. Both mutant receptors bound [Bz-Phe⁶]U-II similarly to the wild-type receptor (0.7 nM), with K_d values of 0.8 ± 0.1 nM ($n = 3$) and $0.8 \text{ nM} \pm 0.1$ nM ($n = 3$) for M184L/M185L and M184A/M185A respectively (Figure 7A). The binding capacity ($B_{\max}$) of these two mutant receptors [4.3 and 5.2 pmol/mg of protein respectively ($n = 3$)] was also found to be similar to that of the wild-type receptor [4.8 pmol/mg of protein ($n = 3$)]. Both receptors possessed patterns of ligand-induced $\text{Ins}(1,4,5)P_3$ production comparable with those of the wild-type receptor (results not shown). Photolabelling of M184L/M185L and M184A/M185A was then performed to verify the effect of side-chain modification on the yield of covalent incorporation of [¹²⁵I]-[Bz-Phe⁶]U-II. Determination of covalent incorporation yields (calculated from the ratio of total radioactivity found in isolated bands to total specific binding observed before photolysis) revealed yields evaluated at 70% (M184L/M185L) and 40% (M184A/M185A) when compared with the wild-type receptor (Figure 7A). Indeed, the intensity of labelling of both mutant receptors is clearly weaker than that of wild-type receptor. This further demonstrates that Met¹⁸⁴ and Met¹⁸⁵ are critical for the covalent insertion of the photoligand.

We then proceeded to verify whether modifying the mutant receptors still enabled binding of the photoligand in the same region as that in the wild-type receptor. V8-protease digestion of the ligand–mutant receptor complexes produced a 17 kDa fragment similar to that of the wild-type receptor, which showed that the labelling on both mutant receptors still occurred in the Arg¹⁴⁸–Glu²⁸⁶ domain of the mutated U-II receptors (Figure 7B). Furthermore, CNBr cleavage of the whole ligand–mutant receptor complexes failed to show any ligand release, in contrast with the wild-type receptor, since only a 17 kDa fragment is produced along with a partial digestion product of 25 kDa (Figure 7C). Since CNBr-cleavage-sensitive methionine residues at the site of photoattachment are absent in both leucine and alanine double mutants of the receptor, the smaller fragment of 17 kDa would correspond to Ser¹⁴⁵–Met²⁸⁸.

DISCUSSION

Identifying the molecular determinants involved in the bimolecular recognition/interaction of ligand and receptor is of utmost

experiments. (C) Serpentine representation of the theoretical Endo Arg-C fragmentation footprint for the U-II receptor with cleavage at all potential sites. Numbers in square brackets indicate the molecular mass of the corresponding photoaffinity cross-linked fragment, including radioactive ligand. Closed circles represent methionine residues present in the receptor's fourth transmembrane domain. The highlighted (grey shading) receptor fragments indicate potential contact domains with the most probable domain, fragment 167–192, highlighted in dark grey.

Figure 6 CNBr cleavage of the partially purified V8-protease-generated 17 kDa fragment of photolabelled U-II receptor

(A) The partially purified photolabelled 60 kDa form of the U-II receptor (30 000 c.p.m.) was incubated in the presence of V8 protease (20 μ g) for 7 days at room temperature. The resulting 17 kDa labelled receptor fragment was then cut from the gel, eluted passively and concentrated using Microcon-3. The partially purified 17 kDa fragment was split in two aliquots and incubated in the absence (lane 1) or the presence (lane 2) of CNBr (100 mg/ml) for 18 h at room temperature in the dark. In lane 3, the 125 I-[Bz-Phe⁶]U-II supplemented with 10 μ g of BSA was run in parallel. Samples were run on a 16.5% acrylamide/Tris/Tricine separating gel followed by autoradiography. Molecular-mass markers are indicated (kDa). These results are representative of at least three separate experiments. (B) Serpentine representation of the theoretical CNBr fragmentation of the 17 kDa V8-protease digestion product generating the smallest entity identified as the 1.6 kDa 125 I-[Bz-Phe⁶]U-II analogue released from either Met¹⁸⁴ and/or Met¹⁸⁵ (black closed circles).

importance in order to understand the initial steps leading to receptor activation. In the present study, photoaffinity labelling was used as a strategy to probe into the interactions of the

newly described ligand–receptor pair of U-II–U-II receptor. This approach depends on the spatial proximity between a radioactive photoligand and receptor domains to form a covalent conjugate that is subsequently mapped to identify the interaction sites. As a preliminary step, three analogues of U-II, containing Bz-Phe substituted at positions 6, 7 and 11, were prepared and characterized for their capacity to recognize and activate the U-II receptor. The physico-chemical properties of the Bz-Phe moiety, hydrophobic and aromatic, are also shared by residues 6 (Phe) and 7 (Trp), while residue 11 (Val) retains a hydrophobic nature. Although all three photoreactive analogues were able to stimulate phospholipase C-dependent production of the second messenger $\text{Ins}(1,4,5)\text{P}_3$, suggesting their agonistic nature, [Bz-Phe⁷]U-II and [Bz-Phe¹¹]U-II had poor binding affinities to the receptor. However, [Bz-Phe⁶]U-II recognized the receptor with a 5-fold higher affinity than U-II itself. Report have shown that modifications of human U-II at positions 6 and 11 are well tolerated, while a change at position 7 greatly impairs the peptide's binding properties [12]. It has been suggested that the presence of a hydrophobic amino acid at the C-terminus, as well as at positions occupied by phenylalanine and tryptophan, of U-II is crucial to the peptide's ability to interact with its receptor and to its capacity at producing biological effects. In NMR and dynamic simulated annealing studies of the solution conformation of U-II, the Phe⁶, Trp⁷ and Val¹¹ side chains were oriented on the same side of the molecule so as to form a hydrophobic pocket optimized for binding with its receptor [24]. Obviously, Bz-Phe substitution at Trp⁷ and Val¹¹ greatly altered the binding properties of the peptide, possibly by the enhanced steric hindrance effect caused by the Bz-Phe moiety with the receptor's binding pocket. Thus, based on its affinity and its capacity at stimulating the U-II receptor, it is reasonable to suppose that [Bz-Phe⁶]U-II adopts a binding mode similar to that taken by U-II itself.

In photolabelling experiments performed in transfected COS-7 cells, [Bz-Phe⁶]U-II specifically labelled the U-II receptor, which migrated as two entities, one of molecular mass $\approx$ 60 kDa and another of molecular mass $\approx$ 31 kDa. The susceptibility of the 60 kDa protein to PNGase-F treatment suggests the glycosylated nature of the receptor, a feature that would cause its molecular mass to be overestimated. This behaviour on SDS/PAGE has been observed for other glycosylated GPCRs, such as the Ang II receptors AT₁ and AT₂ [22,25]. The result further implies that the 60 kDa form corresponds to the complete receptor. Because of its insensitivity to PNGase treatment, the 31 kDa protein may originate from the intact complex lacking an N-terminal portion. It is still not clear whether this fragment is produced during the experimental procedure after binding has occurred or before binding at the cell surface, but we have observed similar photolabelling patterns using various transfected cell lines (results not shown).

Results from enzymic and chemical degradation of the photoligand–receptor complex clearly identify Met¹⁸⁴ and Met¹⁸⁵ as contact points for 125 I-[Bz-Phe⁶]U-II. Our results first showed that the labelling was restricted to the fourth transmembrane domain of the U-II receptor. A 2 kDa fragment was also produced following CNBr cleavage of the 17 kDa V8-protease-generated fragment, thus suggesting photoligand release from methionine residues [23]. Also, mutation of the photoattachment site gives further support to ligand interaction with the methionine residues. Indeed, the significant decrease in labelling yields of the M184L/M185L and M184A/M185A receptor mutants further reinforces the idea of the methionine residues as important ligand contact points. Furthermore, the absence of the 2 kDa adduct following CNBr cleavage of the two receptor mutants

clearly demonstrate that the original photoattachment site has been abolished.

Thus photolabelling coupled with mutational studies strongly suggest that transmembrane 4 participates in the bimolecular ligand–receptor complex interface and contributes to hormone binding. Here, we report a close proximity between an aromatic benzophenone amino acid of the ligand and methionine residues of the receptor to describe a ligand–receptor interaction. Identification of methionine residues as contact points in receptors have been frequently reported in labelling studies employing the benzophenone moiety [18,26]. This type of interaction appears to be favoured, since the photogenerated benzophenone radical forms a transient charge-transfer complex with the sulphur atom in the methionine residue, followed by a covalent C–H insertion into the sulphur-adjacent γ -methylene or ϵ -methyl group of methionine [15]. The increased rate of reaction of benzophenone radicals with thioether moieties would cause a pronounced selectivity towards methionine residues in a protein environment. The reactivity distance between the benzophenone radical and a methionine residue could increase the normal reactivity radius of Bz-Phe [27].

Recent data report significant reductions in photolabelling efficacy when replacing a native interacting methionine residue within the receptor with a related amino acid such as leucine or when shortening side-chain lengths by substitution with alanine [28,29]. Our results strongly suggest that Met¹⁸⁴ and Met¹⁸⁵ constitute important determinants of the U-II receptor binding pocket, but we cannot rule out the possibility that other receptor residues might also assist in accommodating Phe⁶ of U-II in the binding pocket. Given the hydrophobic nature of the benzophenone side chain, the interactions depicted are likely to occur with hydrophobic receptor residues. However, this type of interaction does not always accurately reflect the true binding dynamics of the native ligand, but ensures identification of residues in close proximity to the targeted amino acid. A good example of this is provided in a recent study where Lys²⁷ of parathyroid hormone was substituted by Bz-Phe, which linked to a leucine residue within the parathyroid-hormone receptor, while numerous glutamate residues were adjacent to the interaction site [18]. It was pointed out that the glutamate residues probably contributed extensively to the recognition process of the native ligand containing lysine [30]. Therefore, careful interpretation of the results should then be considered when assessing direct contact points.

Our results provide information as to the identification of those residues within the U-II receptor that participate in the

Figure 7 Photolabelling and digestions of wild-type and mutant U-II receptors

(A) Cells expressing equal amounts of wild-type, M184A/M185A and M184L/M185L receptors (lanes 1, 2 and 3 respectively) were photolabelled with ¹²⁵I-[Bz-Phe⁶]U-II, solubilized, denatured, and submitted to SDS/PAGE on a 10% acrylamide separating gel (40 μ g of protein/lane), followed by autoradiography. Molecular-mass markers are indicated (kDa). Receptor amount was monitored with binding assays. Photolabelling efficiencies are indicated as mean labelling percentages compared with the wild-type receptor. K_d values evaluated using ¹²⁵I-[Bz-Phe⁶]U-II saturation binding assays are also indicated. (B) The partially purified photolabelled 60 kDa form of the U-II receptor, M184A/M185A and M184L/M185L (5000 c.p.m.) were incubated in the absence (lane 1, 3 and 5 respectively) or the presence (lane 2, 4 and 6 respectively) of V8 protease (20 μ g) for 7 days at room temperature. (C) The partially purified photolabelled 60 kDa form of the U-II receptor, M184A/M185A and M184L/M185L (10000 c.p.m.) were incubated in the absence (lane 1, 3 and 5 respectively) or the presence (lane 2, 4 and 6 respectively) of CNBr (100 mg/ml) for 18 h at room temperature in the dark. Samples were run on a 16.5% acrylamide/Tris/Tricine separating gel followed by autoradiography. Molecular-mass markers are indicated (kDa). These results are representative for at least three separate experiments. U-II-SCN, U-II thiocyanate.

interaction with the ligand, urotensin II. The superfamily of GPCRs is extensive and diverse, with ligands as varied as photons, odorants, biogenic amines, peptides and large glycoproteins. Our understanding of the bimolecular recognition process for small ligands such as biogenic amines is that they will interact with residues found within transmembrane domains [31]. Interaction of peptide ligands would appear to be more complex, with determinants being located within transmembrane domains as well as within extracellular loops. For example, Ang II adopts an extended structure when bound to its receptors (AT₁ and AT₂) due to interactions with residues of transmembrane domains and extracellular loops [16,25,27]. Studies on ligand–receptor interactions directed toward the vasopressin receptor V1a suggest a similar interaction mode, with the hydrophobic part of the peptide embedded in transmembrane domains while the more hydrophilic region positions itself in close contact with extracellular domains of the receptor [32]. However, despite different binding modes, GPCRs seem to follow a standard pathway leading to G-protein activation, a direct consequence of ligand-induced transmembrane movement. Indeed, various studies on rhodopsin and adrenoceptors have demonstrated that significant transmembrane rotations occur following agonist binding [33,34].

The high-resolution structure of rhodopsin has provided very useful insights into the structure of GPCRs which, despite low sequence identity, possess similar topologies [35]. The receptor inactive state depicted by this high-resolution structure shows the second extracellular loop (ECL2) ‘capping’ the 11-*cis*-retinylidene-chromophore-binding pocket. It also has been reported that residues from ECL2 would contribute to the formation of the active form of rhodopsin, suggesting that this flexible domain might play an important role in the activation process [36]. Indeed, many GPCRs have been shown to bind their ligand through interactions mediated by the ECL2 or adjacent domains [37,38]. Activation determinants located in ECL2 and leading to constitutive receptor signalling have also been reported among a subfamily of GPCRs [39]. Our results point to the important contribution of transmembrane domain 4 adjacent to ECL2 of the U-II receptor, in docking with residue 6 found within the highly conserved cyclic portion of U-II. Further studies combining both mutagenesis, photoaffinity-labelling approaches and molecular modelling will provide information crucial to the development of models defining the binding pocket of this receptor. Because of U-II’s putative role in heart failure and other cardiovascular pathologies, the design of novel ligands could be facilitated by the knowledge brought on by these models.

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