Direct Identification of Human Oxytocin Receptor-binding Domains Using a Photoactivatable Cyclic Peptide Antagonist

COMPARISON WITH THE HUMAN V1a VASOPRESSIN RECEPTOR*

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Christophe Breton‡‡, Hichem Chellil‡‡, Majida Kabbaj-Benmansour‡‡, Eric Carnazzi‡‡, René Seyer‡, Sylvie Phalipou‡, Denis Morin‡, Thierry Durroux‡, Hans Zingg**, Claude Barberis‡, and Bernard Mouillac‡‡ ‡‡

From the **Laboratory of Molecular Endocrinology, McGill University Health Centre, Montreal, Quebec H3A 1A1, Canada

Oxytocin (OT), a neurohypophysal nonapeptide, is among the strongest uterotonic agents known to date (1, 2). This hormone acts on the myometrium through specific OT receptors (OTRs) that show a dramatic increase in their expression pattern immediately before parturition (3). In mammals at term, OT-induced contractility of myometrial smooth muscle cells is triggered through an increase in the intracellular calcium level (4). This calcium signaling pathway is dependent on coupling the OTR to both the Gq/11 and G proteins (5). The OTR cDNA of several mammalian species has been cloned (6–9), and the deduced amino acid sequence confirmed that the receptor belongs to the large family of seven-helix transmembrane G protein-coupled receptors. OT is widely used in obstetrical practice to promote labor and delivery (10). On the other hand, blockade of the OTR may provide a unique approach for treatment of preterm labor by prolonging uterine quiescence (11–13). Therefore, much effort has been focused on the design and development of OT antagonists as potential tocolytic drugs. The utility of such OT antagonists has been demonstrated clinically by using intravenously administered 1-deamino-D-Tyr(ET)2,Thr4,Orn3[9]-vasotocin (Atosiban). This peptide antagonist has been shown to inhibit uterine contractions in women with threatened and established preterm labor (14, 15). However, the structure/function relationships of the functional domains of the OTR and the topography of the ligand-binding sites are still poorly investigated. Such knowledge should provide valuable information on the structural requirements for antagonist binding and should be helpful for the rational design of potential therapeutic agents and for a better understanding of the molecular mechanisms leading to receptor inactivation. Agonist-binding sites of the OTR have been investigated previously by site-directed mutagenesis and three-dimensional (3D) molecular modeling (16–18). In parallel, a major contribution to peptide antagonist binding affinity by the upper part of transmembrane domain (TMD) VII has been demonstrated (18).

The photoaffinity labeling technique is an essential complement to modeling and mutagenesis approaches and allows an unambiguous direct determination of the ligand/receptor contact domain of a cyclic peptide antagonist. Using a photoactivatable cyclic peptide antagonist, we were able to identify domains of the human oxytocin receptor. This approach allowed the identification of domains conserved in the V1a vasopressin receptor and provided new insights on the architecture of the human oxytocin receptor and its possible implications in the design of new therapeutic agents.
regions. So far, radiolabeled OT analogues containing a photoactivatable group at the side chain of residue 8 have been used to photolabel the OTR naturally expressed in the rat mammary gland, the guinea pig uterus, and the rabbit amnion or transfected in COS cells (recombinant porcine receptor). These studies allowed the identification of the receptor as a glycoprotein with an apparent molecular mass between 65 and 80 kDa (18–22). However, the precise localization of the covalent attachment of these ligands to the receptor has not been investigated. The present study has been performed to localize accurately peptidic antagonist-binding domains of the human OTR using a photoaffinity labeling approach. Based on the structure of the previously reported potent and high affinity cyclic peptide antagonist d(CH$_2$)$_5$[Tyr(Me)$_2$,Thr$_4$,Orn$_8$,Tyr(3$^{125}$I)-NH$_2$]vasotocin (125I-O-OTA) (23), we have designed, synthesized, and characterized a new radiolabeled photosensitive antagonist containing an azido group on the amino acid at position 9, termed 125I-ZOTA$^2$. Because this novel radioligand displays an equivalent high affinity for the human OTR and the structurally related human V$_{1a}$ arginine-vasopressin (AVP) receptor, we decided to conduct a comparative photolabeling study on both receptors. Because we already accumulated a large amount of data on linear and cyclic peptide antagonist-binding sites of the V$_{1a}$ receptor from direct receptor photoaffinity labeling experiments (24–26) as well as from site-directed mutagenesis and 3D molecular modeling studies (27–29; for review, see Ref. 30), we therefore hypothesized that this dual comparative investigation should further increase our understanding of OTR/antagonist interactions. We describe here the photolabeling of the human OTR and the human V$_{1a}$ AVP receptor with 125I-ZOTA and their proteolytic fragmentation using CNBr and Lys-C and Arg-C endopeptidases. Compilation of all the data shows that covalent attachment is restricted to three residues, Leu$^{114}$Val$^{115}$Lys$^{116}$ in the upper part of the OTR TMD III. Interestingly, the photolabeled region is equivalent in the V$_{1a}$ receptor.

**Experimental Procedures**

Radioiodination and Azidation of the Antagonist Probe—The detailed procedure of radioiodination and azidation leading to 125I-ZOTA has been described elsewhere.$^2$ Briefly, a strategy consisting of the solid-phase synthesis of the precursor nonpeptide d(CH$_2$)$_5$[Tyr(Me)$_2$,Thr$_4$,Orn$_8$,Phe(3$^{125}$I,4NH$_2$)-NH$_2$]vasotocin and its subsequent radioiodination and complete azidation was designed. The HPLC-purified precursor compound was done with an excess of NaNO$_2$ (10 M) was first iodinated with Na$^{125}$I (1 mCi, Amersham Pharmacia Biotech) in a phosphate buffer (pH 6.8) using Iodo-Gen (Pierce) as an oxidant. The monoiodinated peptide d(CH$_2$)$_5$[Tyr(Me)$_2$,Thr$_4$,Orn$_8$,Phe(3$^{125}$I,4NH$_2$)-NH$_2$]vasotocin was purified by HPLC (Waters C$_{18}$, µBondapack column). Then diazotization and azidation of this compound was done with an excess of NaN$_3$ (10 M) in HCl (12 m) at 0 °C in dim light for 1 h before addition of NaN$_3$ (10 M, progressively allowed to reach room temperature yielding the final product d(CH$_2$)$_5$[Tyr(Me)$_2$,Thr$_4$,Orn$_8$,Phe(3$^{125}$I,4NH$_2$)-NH$_2$]vasotocin (125I-O-OTA). The 125I-ZOTA was purified by HPLC, and its specific activity was 2200 Ci/mmol. The corresponding nonradiolabeled azidated antagonist leading to 50% inhibition, [OT] was kept at 10 nM, and [AVP] was varied from 1 nM to 1 μM in saturation and competition binding assays as described previously (25–29). Briefly, membranes were incubated in Buffer A supplemented with 1 mg/ml BSA and with radiolabeled and displacing peptides for 30 min (with [3H]AVP) or 60 min (with 125I-O-OTA and 125I-ZOTA). Affinities ($K_i$) for 125I-O-OTA and 125I-ZOTA (concentrations from 20 to 2000 pm) as well as for [3H]AVP (concentrations from 0.1 to 20 nM) were directly determined from saturation experiments. Affinities ($K_i$) for the antagonists were determined by competition experiments using [3H]AVP (= 1–2 nM) as the radioligand. The concentrations of the unlabeled ligands varied from 1 pm to 10 μM. In saturation and competition experiments, depending on the radiolabeled peptide, nonspecific binding was determined by adding unlabeled AVP (10 μM), I-OTA (400 nM), or I-ZOTA (400 nM). Bound and free radioactivity was separated by filtration over Whatman GF/C filters presoaked in a 10 mg/ml BSA solution for 3–4 h. The ligand binding data were analyzed by nonlinear least squares regression using the computer program LIGAND (34).

Inositol Phosphate Assays—The antagonist activity of I-ZOTA was determined by measuring inhibition of OT-stimulated inositol phosphate (IP) accumulation as described previously (25, 26). Briefly, CHO cells expressing the human OTR were plated and grown in six-well dishes for 48 h in Dulbecco's modified Eagle's medium supplemented and then labeled for 24 h with myo[2-3H]inositol (10–20 Ci/mmol, PerkinElmer Life Sciences) at a final concentration of 1 μCi/ml in a serum-free, inositol-free medium (Life Technologies, Inc.). Cells were washed twice with phosphate-buffered saline medium, equilibrated at 37 °C in phosphate-buffered saline for 1 h, and then incubated for 5 min in phosphate-buffered saline supplemented with 10 mM LiCl in the presence or absence of increasing concentrations of I-ZOTA (from 10$^{-12}$ to 10$^{-6}$ M). CHO cells were stimulated for 15 min with 10–5 M OT (a concentration close to the $K_{inact}$ value determined in CHO cells). The reaction was stopped by adding ice-cold perchloric acid. After neutralization of the samples, total IPs and the standard were extracted and purified by anion-exchange chromatography (Dowex AG 1-X8 column, formate elution). For each sample, the same amount of total IPs was collected and counted. $K_{inact}$ constants were calculated as $K_{inact} = IC_{50}$/1 + [OT]/$K_{a}$ in which $IC_{50}$ is the antagonist leading to 50% inhibition, [OT] was kept at 10 nM, and $K_{a}$ is the concentration of OT inducing half-maximum accumulation of IPs ($K_{a}$ = 12.9 nM in CHO cells expressing the wild-type human OTR receptor (17)).

Photoaffinity Labeling of the Receptors with 125I-ZOTA—Photoaffinity labeling experiments were carried out as described previously with the V$_{1a}$ receptor photoactivatable antagonists (25, 26). In short, membranes expressing OTR or V$_{1a}$ (500 μg) were resuspended in 4 ml of binding Buffer A containing BSA (0.5 mg/ml) in Corex glass tubes. Then 125I-ZOTA (= 2 nM) was added to the membranes, and samples were incubated at 30 °C for 1 or 3 h. The samples were then centrifuged at 44,000 g (for assays with 125I-OTA and 125I-ZOTA) or 10–15 min (for assays with [3H]AVP) topellet and 60 min (with125I-OTA and 125I-ZOTA) for the unlabeled ligand [3H]AVP (concentrations from 0.1 to 20 nM) were directly determined from saturation experiments. $K_i$ for the radiolabeled and competitive antago-$n$ist I-ZOTA were determined by competition experiments using [3H]AVP (= 1–2 nM) as the radioligand. The concentrations of the unlabeled ligands varied from 1 pm to 10 μM. In saturation and competition experiments, depending on the radiolabeled peptide, nonspecific binding was determined by adding unlabeled AVP (10 μM), I-OTA (400 nM), or I-ZOTA (400 nM). Bound and free radioactivity was separated by filtration over Whatman GF/C filters presoaked in a 10 mg/ml BSA solution for 3–4 h. The ligand binding data were analyzed by nonlinear least squares regression using the computer program LIGAND (34).
Electrophoresis of the Photoelectroeluted OT and V1a Receptors—Photola
elabeled receptors as a percentage of the total number of receptors
ternalized proteins were first subjected to SDS-PAGE using 12% acryl
amide gels as described above. After electrophoresis, the labeled bands
digested with CNBr or Lys-C/Arg-C endoproteinase. CNBr, Lys-C, and
Arg-C cleave proteins specifically at the carboxyl terminus of methion
nine, lysine, and arginine residues, respectively. Double digests were also performed (first digestion with Arg-C followed by a second
digested with CNBr). CNBr cleavage of the electroeluted OTR and V1a
was carried out in 100 μL of 70% (v/v) formic acid containing a few
crystals of CNBr. The mixture was incubated in the dark for 24 h at
room temperature under argon and then washed by adding 500 μL
of water. The sample volume was reduced under vacuum, and formic acid was removed by solvent exchange with water. Endoproteinase Lys-C (sequencing grade from *Lysobacter enzymogenes*, Roche Molecular
Biochemicals) was used at 0.2 μg/assay in a final volume of 50 μL.

Results of cleavage were analyzed by a Tricine discontinuous SDS-

Chemical and Enzymatic Cleavage of the Photola
eluted CHO membranes (100 μg) or electroeluted OTR was resuspended in deglycosylation buffer (100 mM NaH2PO4/NaHPO4, pH 8.0; 10 mM EDTA; 1% digitonin; 1% 2-mercaptoethanol;

The affinity of 125I-ZOTA, which differs from the OT an-
tagonist 125I-OTA. The photosensitive

Antagonist Binding to Oxytocin and Vasopressin Receptors

The structure of 125I-ZOTA is compared with that of the natural hor

Photoaffinity Labeling of the Human OT and V1a Receptors—

Endoproteinase Lys-C (sequencing grade from *Clostridium his-
tolyticum*, Roche Molecular Biochemicals) was used at 0.5 μg/assay for 24 h in a final volume of 50 μL of 50 mM Tris-HCl, pH 7.8; 10 mM
CaCl2. To perform double fragmentations, the Arg-C digestion reaction
was dried under vacuum. The pellet was resuspended in 100 μL of 70% (v/v) formic acid, and CNBr cleavage was performed as indicated above.

Deglycosylation of the Photola
eluted CHO membranes (100 μg) or electroeluted OTR was resuspended in deglycosylation buffer (100 mM NaH2PO4/

The digestion was performed in 25 mM Tris-HCl, pH 8.5; 1 mM EDTA;

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### Table I

<table>
<thead>
<tr>
<th>Receptors</th>
<th>$^{125}$I-OTA $K_d$</th>
<th>$^{125}$I-ZOTA $K_d$</th>
<th>$[^{3}H]$AVP $K_d$</th>
<th>I-ZOTA $K_d$</th>
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</thead>
<tbody>
<tr>
<td>OT</td>
<td>0.18 ± 0.04 (7)</td>
<td>0.25 ± 0.04 (3)</td>
<td>1.36 ± 1.00 (3)</td>
<td>0.9 ± 0.03 (3)</td>
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<tr>
<td>$V_{1a}$</td>
<td>0.93 ± 0.06 (3)</td>
<td>0.46 ± 0.09 (3)</td>
<td>0.7 ± 0.17 (3)</td>
<td>1.38 ± 0.21 (3)</td>
</tr>
<tr>
<td>$V_{1b}$</td>
<td>ND</td>
<td>0.37 ± 0.05 (3)</td>
<td>5665 ± 1910 (3)</td>
<td></td>
</tr>
<tr>
<td>$V_{2}$</td>
<td>1.36 ± 0.45 (3)</td>
<td>204 ± 100 (3)</td>
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</table>

* Affinities ($K_d$) of $^{125}$I-OTA and $^{125}$I-ZOTA for the human OTR and $V_{1a}$ were directly determined in saturation experiments.

* Affinities ($K_d$) of $[^{3}H]$AVP for all four wild-type receptor subtypes were directly determined in saturation experiments and have already been published (25, 26).

* Affinities ($K_d$) of the nonradiolabeled I-ZOTA were determined in competition assays by displacement of $[^{3}H]$AVP.

#### Fig. 2. Photoaffinity labeling of the human OTR with $^{125}$I-ZOTA; comparison with the human $V_{1a}$ receptor.

A, photolabeling of the human OTR. Membrane proteins (500 µg) from CHO cells expressing the OTR were incubated for 1 h with $^{125}$I-ZOTA and irradiated at 254 nm for 1–4 min (lanes 2–5). To define nonspecific photolabeling of the membranes, no irradiation was performed (lane 1), or OT was added at a saturating concentration (10^{-5} M) during the incubation step (lane 6). Irradiated and washed membranes were separated on a 12% SDS-PAGE system. Equivalent amounts of proteins (25 µg) were loaded onto each well. B, deglycosylation of the photolabeled OTR. CHO cell membranes (100 µg) expressing the receptor were photolabeled and solubilized in deglycosylation buffer in the presence (lane 2) or absence (lane 1) of N-glycosidase F. Equivalent amounts of radioactivity (600,000 cpm) were loaded in each lane, and proteins were separated by SDS-PAGE using 12% gels. C, comparison of human OTR and $V_{1a}$ receptor photolabeling with $^{125}$I-ZOTA. CHO cell membranes (500 µg) expressing the OTR (lane 3) or the $V_{1a}$ receptor (lane 1) were incubated for 1 h with the photoactivatable antagonist and irradiated for 1 min. Equivalent amounts of photolabeled membranes (25 µg) were loaded in each lane before separation by SDS-PAGE. Nonspecific photolabeling of membranes was assessed by adding an excess of AVP (10^{-2} M) (lane 2) or OT (10^{-2} M) (lane 4) during incubation with $^{125}$I-ZOTA. For each experiment described in this figure, the gels were dried and exposed overnight at −80 °C to Kodak XAR-5 films, and autoradiograms are shown. Molecular mass markers are indicated (kDa) on the left. Each panel in this figure is representative of three distinct experiments.

In Fig. 2B, this enzymatic treatment converted the unique 70–75-kDa protein band (lane 1) into two bands of ~48 and 38 kDa (lane 2), confirming that the receptor contained carbohydrates. As shown in Fig. 3, the human OTR contains in its extracellular NH$_2$-terminal domain three potential N-glycosylation sites located at Asn$^5$, Asn$^{15}$, and Asn$^{36}$ (6, 38). The molecular mass of the smaller band migrating at 38 kDa (including the antagonist) is close to the theoretical mass of the receptor core deduced from the cDNA sequence (42.8 kDa). As reported for the guinea pig uterine OTR (21), the deglycosylation treatment led to a reaction product with a molecular mass ~50% of the mass of the native receptor that likely represents a final digestion product. The nature of the band obtained at 42 kDa upon photolysis remains unknown. It is interesting to note that deglycosylation of the photolabeled guinea pig receptor resulted in a protein band migrating at 38 kDa as well (21). Taking into account insensitivity of the human OTR to the plasma membrane metalloproteinase proteolysis (18), the upper band at 48 kDa likely represents a form of partial receptor deglycosylation rather than a proteolytic receptor fragment. Indeed, when the human OTR was transiently expressed in CHO cells or when the 70–75-kDa band was electrolecuted from preparative SDS-polyacrylamide gels, deglycosylation of the photolabeled materials always gave rise to both 38- and 48-kDa species (not shown). Taken together, the N-glycosidase F effect on the receptor protein and the lack of endoglycosidase H action (data not shown) suggest that the mature OT receptor is a glycoprotein that contains a high percentage of asparagine-linked oligosaccharides.

Using the same experimental conditions (incubation for 1 h at 30 °C followed by 1 min of UV irradiation), CHO cell membranes expressing the human AVP $V_{1a}$ receptor were photolabeled with $^{125}$I-ZOTA. Two broad bands at 85–90 and 46 kDa were covalently labeled (Fig. 2C, lane 1). Equivalent results were obtained in earlier photolabeling studies using two $V_{1a}$-selective photoactivatable peptide antagonists (25, 26). The photolabeling of both 85–90- and 46-kDa protein bands was receptor-specific because labeling could be completely suppressed by adding 10 µM AVP (Fig. 2C, lane 2). The covalent binding efficiency of the probe to the $V_{1a}$ receptor, calculated as the specifically bound radioactivity recovered in the two labeled bands, was estimated at 3% (multiple determinations). As demonstrated in two previous studies (25, 26), the 46-kDa photolabeled band originated from a proteolytic cleavage of the entire receptor migrating at an apparent molecular mass of 85–90 kDa. Indeed, the relative abundance of the 46-kDa species
hydrophobicity analysis and primary sequence comparison and alignment. The three potential well as the predicted arrangement of the protein in the cell membrane. The position of the TMD was predicted from G protein-coupled receptor

Fig. 3. Schematic representation of the human OTR. The primary sequence of the receptor deduced from the cloned cDNA (6) is shown as well as the predicted arrangement of the protein in the cell membrane. The position of the TMD was predicted from G protein-coupled receptor hydrophobicity analysis and primary sequence comparison and alignment. The three potential N-glycosylation sites are shown at Asn6, Asn15, and Asn36. Shaded residues (Phe91–Gln96) indicate a consensus sequence of the cleavage site for a metalloprotease (41, 42). Predicted cleavage sites for CNBr (black diamonds), Lys-C (black triangles), and Arg-C (black squares) are also indicated. The tripeptide fragment covalently labeled with the 125I-ZOTA antagonist is highlighted (residues Leu124–Lys136) at the top of TMD III.

(∼50% in Fig. 2C, lane 1) could be significantly reduced when incubation with 125I-ZOTA was done in the presence of ZnCl2 and protease inhibitors for 1 h at 4 °C (data not shown). As illustrated in Fig. 4, the sequence Phe103–Gln108 in the V1a receptor corresponds to a potential cleavage site for a metalloprotease present in the membrane preparation. This enzyme has been demonstrated to digest the bovine renal V2 receptor between Gln92 and Val93 upon ligand binding (18). As demonstrated previously with two radioiodinated photoactivatable linear peptide antagonists (25, 26), we found here that the human V1a is sensitive to metalloproteinase proteolysis as well during incubation with the cyclic peptide antagonist 125I-ZOTA. Interestingly, although the proteolytic cleavage site of the metalloproteinase is conserved in the human OTR (sequence Phe91–Gln96), this receptor is resistant to the proteolysis (even with a longer 3-h incubation time, data not shown), indicating that other residues, domains, or specific conformations are necessary for an efficient enzymatic action.

Fragmentation of the Human OT and V1a Photolabeled Receptors and Identification of the Covalently Labeled Regions—To identify antagonist-binding domains covalently bound to the photoactivatable cyclic peptide 125I-ZOTA, photolabeled bands were excised from preparative SDS-polyacrylamide gels, electroeluted, and subjected to chemical cleavage with CNBr or enzymatic digestion with Lys-C and Arg-C endoproteinases. Based on our present knowledge of the localization of ligand-binding sites on G protein-coupled receptors (for review, see Ref. 39), we have systematically excluded fragments corresponding to putative intracellular portions of the receptors as potential domains covalently bound to the ligand. For the human OTR, the unique photolabeled 70–75-kDa band was used to perform the different fragmentation reactions (see Fig. 3 for the localization of CNBr, Lys-C, and Arg-C cleavage sites in the OTR sequence). The photoactivatable peptide itself is protected from the different degradations because it does not possess Met, Lys, or Arg residues.

The experimental OTR fragmentation patterns derived from CNBr, Lys-C, Arg-C, or Arg-C + CNBr digestions are presented in Fig. 5. For a better understanding of the results, a theoretical fragmentation map of the OTR is shown in Fig. 6. CNBr cleavage of the photolabeled 70–75-kDa OTR yielded three small radiolabeled bands migrating at molecular masses of ∼4.5, 5.5, and 6.5 kDa (Fig. 5A, lane 2). This result restricted the site of covalent attachment to only one receptor domain, Lys125–Met133. Digestion of the photolabeled OTR with Lys-C endoproteinase yielded four fragments at molecular masses of ∼5, 8, 11, and 16 kDa (Fig. 5B, lane 2). Because the smallest fragment migrated at ∼5 kDa, only two peptide sequences, His130–Lys136 and Met127–Lys206, could be covalently bound to 125I-ZOTA. Fragment Ala192–Lys238 (3.1 kDa) encompassing TMD V was eliminated as a potential candidate because of the absence of CNBr cleavage sites in this region. Digestion of the photolabeled 70–75-kDa OTR with Arg-C endoproteinase produced four labeled bands at molecular masses of ∼3.4, 4, 7.8, and 10 kDa (Fig. 5C, lane 2). Several fragments could account for this result, such as Met1–Arg27, Val81–Arg65, Leu114– Arg137, or Leu155–Arg178. Considering the cleavage results with CNBr or Lys-C endoproteinase, there was only one reasonable candidate of ∼3.4 kDa, spanning from Lys124 to Arg137 and including the entire TMD III. Successive treatment of the 70–75-kDa photolabeled receptor with Arg-C and CNBr (Fig. 5D, lane 3) generated a new fragment with a molecular mass of ∼3 kDa, which is slightly smaller than the 3.4 kDa obtained with Arg-C alone (Fig. 5D, lane 2) or the 4.5 kDa produced with CNBr alone (Fig. 5A, lane 2). Among the predicted fragments resulting from a digest with Arg-C, there is only one that
fulfills the criteria of having a molecular mass of \(\sim 3 \text{ kDa}\) and possessing an internal CNBr cleavage site, namely the fragment Leu\(^{114}\)–Met\(^{123}\). Compilation of the data highlighted an overlap of the different fragmentations in the upper part of TMD III (see Fig. 6) and indicated that covalent binding of \(^{125}\text{I}-\text{ZOTA}\) is restricted to the three amino acid residues Leu\(^{114}\)–Val\(^{115}\)–Lys\(^{116}\) (see Fig. 3).

We next analyzed the proteolytic fragmentation patterns of the human V\(_{1a}\) receptor (see Fig. 7), and the theoretical digestion map of this receptor is shown again in Fig. 6. In two previous studies (25, 26), we demonstrated that the 46-kDa photolabeled band corresponding to a truncated glycosylated V\(_{1a}\) receptor from Val\(^{105}\) to the carboxyl-terminal end is derived from the entire 85–90-kDa photolabeled receptor and contains all the covalently bound radioactivity. Therefore, the proteolytic fragmentation of the V\(_{1a}\) receptor was only conducted using the material recovered in the 46-kDa protein (see Fig. 4 for the localization of CNBr, Lys-C, and Arg-C cleavage sites in the V\(_{1a}\) sequence). CNBr cleavage of the photoaffinity-labeled 46-kDa species yielded three labeled fragments migrating at molecular masses of \(\sim 4, 5.5,\) and \(7.5 \text{ kDa}\) (Fig. 7A, lane 2). Apart from the additional 4-kDa band, the CNBr cleavage pattern was similar to the one described previously using the photoactivatable linear peptide antagonist \(^{125}\text{I}-\text{[Lys(3N\text{Phpa})\text{8}]HO-LVA}\) (26). Considering that CNBr cleavage and Lys-C protease digestion of the V\(_{1a}\) receptor are quite similar when the photolabeling is performed either with \(^{125}\text{I}-\text{ZOTA}\) or \(^{125}\text{I}-\text{[Lys(3N\text{Phpa})\text{8}]HO-LVA}\), the Val\(^{105}\)–Lys\(^{128}\) sequence likely corresponds to the 5-kDa labeled band produced upon Lys-C fragmentation (the NH\(_2\) terminus of this fragment is defined by the metalloproteinase cleavage site, Val\(^{105}\)). Arg-C endoproteinase digestion of the photolabeled 46-kDa receptor yielded three labeled fragments at molecular masses of \(\sim 4, 6.5,\) and 10 kDa (Fig. 7C, lane 2). Only two fragments could account for a labeled band with an electrophoretic migration at 4 kDa: Val\(^{105}\)–Arg\(^{116}\) and Val\(^{126}\)–Arg\(^{149}\). Although the Val\(^{126}\)–Arg\(^{149}\) fragment is the best candidate, labeling of both regions could be in agreement with results obtained from the CNBr cleavage and the Lys-C digestion. To confirm this localization, successive treatment of the 46-kDa photolabeled receptor with Arg-C and CNBr was done, generating a new fragment at a molecular mass of \(\sim 3 \text{ kDa}\) (Fig. 7D, lane 2), a result equivalent to that determined with \(^{125}\text{I}-\text{[Lys(3N\text{Phpa})\text{8}]HO-LVA}\) (26). Considering that CNBr cleavage and Lys-C protease digestion of the V\(_{1a}\) receptor are quite similar when the photolabeling is performed either with \(^{125}\text{I}-\text{ZOTA}\) or \(^{125}\text{I}-\text{[Lys(3N\text{Phpa})\text{8}]HO-LVA}\), the Val\(^{105}\)–Lys\(^{128}\) sequence likely corresponds to the 5-kDa labeled band produced upon Lys-C fragmentation (the NH\(_2\) terminus of this fragment is defined by the metalloproteinase cleavage site, Val\(^{105}\)). Arg-C endoproteinase digestion of the photolabeled 46-kDa receptor yielded three labeled fragments at molecular masses of \(\sim 4, 5.5,\) and 10 kDa (Fig. 7C, lane 2). Only two fragments could account for a labeled band with an electrophoretic migration at 4 kDa: Val\(^{105}\)–Arg\(^{116}\) and Val\(^{126}\)–Arg\(^{149}\). Although the Val\(^{126}\)–Arg\(^{149}\) fragment is the best candidate, labeling of both regions could be in agreement with results obtained from the CNBr cleavage and the Lys-C digestion. To confirm this localization, successive treatment of the 46-kDa photolabeled receptor with Arg-C and CNBr was done, generating a new fragment at a molecular mass of \(\sim 3 \text{ kDa}\) (Fig. 7D, lane 2), which is slightly smaller than the 4-kDa bands obtained with CNBr or Arg-C alone. The labeled receptor fragment at \(\sim 3 \text{ kDa}\) corresponds most likely to the Val\(^{126}\)–Met\(^{135}\) sequence. The identification of this fragment as the site of covalent binding for \(^{125}\text{I}-\text{ZOTA}\) would be entirely consistent with the results of the CNBr, Lys-C, and Arg-C cleavages described above. Compilation of all the fragmentation patterns (see Fig. 6) highlighted that the covalent attachment of \(^{125}\text{I}-\text{ZOTA}\) to the human V\(_{1a}\) receptor might also be restricted to the three amino acids Val\(^{126}\), Val\(^{127}\), and Lys\(^{128}\) in the upper part of the TMD III (see Fig. 4) that are homologous to Leu\(^{114}\)–Val\(^{115}\)–Lys\(^{116}\) in the OTR.
To confirm further the localization of the covalent binding of $^{125}$I-ZOTA in the TMD III of the human OTR, we next carried out site-directed mutagenesis of the potential CNBr cleavage sites in this region. Thus, M78A and M123A mutant receptors were first constructed (see Fig. 3 for localization of the CNBr and Lys-C/Arg-C endoproteinases). CHO cell membranes (500 μg) expressing the OTR were incubated for 1 h with $^{125}$I-ZOTA and irradiated for 1 min. Sample proteins were then separated on a preparative 12% gel, and the photolabeled 70–75-kDa species was electroeluted, washed, and concentrated as described under "Experimental Procedures." Equivalent amounts of electroeluted photolabeled OTR were used in each digestion or chemical cleavage assay (15,000–25,000 cpm). The samples were then loaded on discontinuous 10–16.5% Tricine gels. A, CNBr chemical cleavage. The electroeluted receptor was treated (lane 2) or not (lane 1) with CNBr for 24 h in the dark at room temperature. B, Lys-C protease digestion. The 70–75-kDa species was treated (lane 2) or not (lane 1) with the protease for 24 h at 37 °C. C, Arg-C protease digestion. The electroeluted receptor was treated (lane 2) or not (lane 1) with the protease for 24 h at 37 °C. D, Arg-C protease/CNBr double fragmentation. The radiolabeled OTR was treated with Arg-C protease alone (lane 2) or in two steps with Arg-C protease followed by CNBr cleavage (lane 3). The figure shows autoradiograms of dried gels exposed to Kodak XAR-5 films at −80 °C for 48–72 h. Molecular mass markers are indicated on the left in each panel. Each assay is representative of at least three distinct experiments.

![Image of autoradiograms showing digestion and cleavage of OTR and V1a receptor](http://www.jbc.org/)

### Fig. 6. Theoretical fragmentation maps of the OTR and V1a receptor

The theoretical fragmentation maps of the OTR (A) and V1a receptor (B) using CNBr, Lys-C endoproteinase, or Arg-C endoproteinase are shown. TMDs are represented as solid black lines. Cleavage sites for CNBr and enzymes are marked with black dots, and the sizes of the theoretical fragments are indicated. Only fragments possessing a molecular mass larger than 1 kDa are shown. In each digestion, the best candidate fragment for covalent attachment of $^{125}$I-ZOTA, based on experimental digestions, is shown as a solid black line. For the V1a receptor, cleavage of the receptor with a metalloproteinase during incubation with $^{125}$I-ZOTA is indicated by an arrow. The resulting radiolabeled 46-kDa protein used in the different fragmentation reactions starts at Val$^{105}$. For a direct comparison between these theoretical fragmentation maps and the experimental fragmentation results, the molecular mass of the photoactivatable antagonist $^{125}$I-ZOTA covalently attached to the receptors has to be added (1.3 kDa).

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**Site-directed Mutagenesis of the Human OT and V1a Receptors**—To confirm further the localization of the covalent binding of $^{125}$I-ZOTA in the TMD III of the human OTR, we next carried out site-directed mutagenesis of the potential CNBr cleavage sites in this region. Thus, M78A and M123A mutant receptors were first constructed (see Fig. 3 for localization of...
these Met residues). Moreover, to eliminate the possibility that photolabeling might have occurred elsewhere in the receptor, other potential CNBr cleavage sites in TMD VI and TMD VII were also mutated (M296A, M315A, M330A; see Fig. 3). All mutant receptors were transiently expressed in CHO cells and photolabeled with $^{125}$I-ZOTA. CNBr cleavage fragments obtained using radiolabeled mutant receptors were then compared with the fragments obtained with the wild-type OTR. The fragmentation patterns of M296A, M315A, and M330A mutant receptors were equivalent to that of the wild-type OTR (the three labeled fragments at 4.5, 5.5, and 6.5 kDa were recovered, data not shown), confirming that regions including TMD VI and TMD VII were not photolabeled. By contrast, as illustrated in Fig. 8, the CNBr cleavage of both M78A and M123A mutant receptors only produced a unique major 6.5-kDa radiolabeled band (lanes 4 and 6). The two smaller bands at 4.5 and 5.5 kDa visualized in the wild-type OTR fragmentation (lane 2) were not present anymore, confirming that the covalently bound domain of the OTR with $^{125}$I-ZOTA is delimited by Met78 and Met123 residues.

To confirm as well our hypothesis regarding the localization of the covalent attachment site of $^{125}$I-ZOTA to TMD III in the human V1a receptor, we used in this study a K128A mutant V1a receptor (Lys128 constitutes a potential cleavage site for Lys-C protease, see Fig. 4), which was constructed and described previously (26, 29). Following expression in CHO cells and photolabeling of the mutated receptor, the corresponding 46-kDa radiolabeled fragment was digested with Lys-C, and the resulting fragmentation was compared with that obtained with the wild-type receptor. As shown in Fig. 9, the 5-kDa band produced upon cleavage of the wild-type receptor with Lys-C endoproteinase (lane 2) was no more visible when the mutant receptor was treated with K128A (lane 4). This fragmentation pattern is representative of three distinct experiments.

**Fig. 7.** Single and double fragmentations of the human V1a vasopressin receptor with CNBr and Lys-C/Arg-C endoproteinases. CHO cells membranes (500 μg) expressing the wild-type human V1a vasopressin receptor were incubated for 3 h (a condition that favors the preferential accumulation of the 46-kDa species) with $^{125}$I-ZOTA and irradiated for 1 min. Membrane proteins were then separated on a preparative 12% gel, and the photolabeled 46-kDa species was electroeluted, washed, and concentrated as described under "Experimental Procedures." Equivalent amounts of electroeluted photolabeled V1a receptor were used in each digestion or chemical cleavage assay (15,000–25,000 cpm). A, CNBr chemical cleavage. The electroeluted receptor was treated (lane 2) or not (lane 1) with CNBr for 24 h in the dark at room temperature. B, Lys-C protease digestion. The electroeluted receptor was treated (lane 2) or not (lane 1) with Lys-C protease for 24 h at 37 °C. C, Arg-C protease digestion. The electroeluted receptor was treated (lane 2) or not (lane 1) with Arg-C protease for 24 h at 37 °C. D, Arg-C protease/CNBr fragmentation. The electroeluted V1a receptor was treated (lane 3) or not (lane 1) with Arg-C protease alone or subjected to a double fragmentation with Arg-C protease and CNBr (lane 2). The figure shows autoradiograms of dried gels exposed to Kodak XAR-5 films at −80 °C for 48–72 h. Molecular mass markers are indicated on the left in each panel. Each assay is representative of at least three distinct experiments.

**Fig. 8.** Chemical CNBr fragmentation of photolabeled M78A and M123A mutant OTR. Photolabeling of CHO cell membranes (1 mg) expressing wild-type, mutant M78A, or mutant M123A OTR as well as preparative 12% SDS-PAGE were performed as described in the legends to Figs. 2 and 5. Radioactive material at 70–75 kDa, corresponding to each receptor, was electroeluted, washed, and concentrated before being subjected to CNBr cleavage. Equivalent amounts of photolabeled receptors were used in each chemical cleavage assay (20,000 cpm). The radiolabeled samples, i.e. wild-type (lanes 1 and 2), mutant M78A (lanes 3 and 4), and mutant M123A (lanes 5 and 6) were treated (lanes 2, 4, and 6) or not (lanes 1, 3, and 5) with CNBr for 24 h in the dark at room temperature. An autoradiogram of a discontinuous 10–16.5% dried gel exposed to Kodak XAR-5 film at −80 °C for 72 h is shown. Molecular mass markers are indicated on the left. This fragmentation pattern is representative of three distinct experiments.
beled K128A mutant V1a receptor. We have first pharma-
study and demonstrated that this OT antagonist combined
three distinct experiments. This fragmentation pattern is representative of
three distinct experiments.

K128A receptor was used instead (lane 4). The Lys-C fragmen-
tation pattern yielded only the 8- and 14-kDa photolabeled
fragments, confirming that covalent binding of 125I-ZOTA to
the human V1a receptor is located in TMD III. Taken together,
these results demonstrate that upon UV irradiation the cyclic
peptide antagonist 125I-ZOTA attaches covalently to both OTR
and V1a at an equivalent position in the upper part of TMD III.

**DISCUSSION**

It is well established that OTR is a major therapeutic target
in the control of labor, thus analysis of the structure/function
relationships of its functional domains, particularly the ligand
binding pocket, is very important. However, relatively little
information on the characterization of OTR antagonist-binding
sites is currently available. In one report, the upper part of
TMD VII has been demonstrated to participate in the binding
of an 125I-OTA-derived antagonist (18). To provide further
information on structural requirements for antagonist binding
to the human OTR, we have undertaken a photoaffinity labeling
study with a novel photoactivatable cyclic peptide ligand. The
structure of I-ZOTA or that of its radiolabeled counterpart
125I-ZOTA is based on that of I-OTA for which properties were
described more than 10 years ago (23). We have first pharma-
ologically and functionally characterized 125I-ZOTA (present
study) and demonstrated that this OT antagonist combined
high affinity, low nonspecific binding, easiness and efficiency of
radioiodination, and appreciable covalent binding yield. Like
125I-OTA (40), 125I-ZOTA behaved as a nonselective compound
displaying equivalent high affinity for both the human OTR
and the human AVP V1a receptor. Taking advantage of this
lack of selectivity, we decided to conduct a comparative pho-
toaffinity labeling study on both receptors.

The photolabeled OTR migrated on SDS-polyacrylamide gels
as a unique glycosylated broad band with an apparent mole-
cular mass of 70–75 kDa. The size of the human OTR is con-
sistent with the molecular mass reported for OTRs isolated
from rat mammary gland, guinea pig uterus, or rabbit amnion
(19–22). Deglycosylation of the photolabeled OTR converted
the 70–75-kDa protein into two bands of molecular masses of
~48 and 38 kDa, the latter corresponding roughly to the ex-
pected size of the peptidic core of the native receptor as de-
scribed for the guinea pig OTR (21). By contrast, using the
same photolabeling conditions (incubation for 1 h at 30 °C
followed by 1 min of irradiation), the human V1a receptor
was degraded during incubation with the ligand, leading to two
bands of molecular masses of ~85–90 and 46 kDa. As described
previously, the 85–90-kDa molecular species corresponds to the
glycosylated native state of the receptor, whereas the 46-kDa
species represented an NH2-terminal-truncated receptor. The
latter results from a proteolytic cleavage of the 85–90-kDa
band by an endogenous metalloproteinase present in the CHO
membrane preparation (25, 26). Equivalently, photolabeling of
bovine kidney membranes with a tritiated photoactivatable
agonist containing an arylazido group at the side chain of Lys8
gave rise to two AVP V2 receptor bands, a glycosylated native
receptor at ~58 kDa and an NH2-terminal-truncated form at
30 kDa (41, 42). In that case, the proteolytic cleavage occurred
given by Gln92 and Val93 in the six-amino acid sequence FQV-
LQ, located in TMD II and conserved in all neurohypophyseal
hormone receptors, including the OTR. According to the
authors, the proteolytic cleavage of the V2 receptor would require
a receptor conformational change dependent on the agonistic
properties of the ligand (42). We have presently shown that the
human OTR is fully resistant to the proteolysis after binding of
the cyclic photoactivatable antagonist, a result previously
shown by others with a different photoactivatable antagonist
(18, 42). By contrast, the metalloproteinase cleavage occurred
in the human V1a receptor after incubation with 125I-ZOTA and
under the same experimental conditions. This finding is equiv-
alent to that obtained when incubating V1a-expressing mem-
branes with two different photoactivatable linear peptide an-
tagonists (25, 26). Taken together, the present data suggest
that cyclic as well as linear peptide antagonists are able to
induce a conformational change leading to proteolytic cleavage
in the V1a receptor, whereas the OTR remains resistant.

Using CNBr chemical cleavage and Lys-C/Arg-C protease
digestions of the photolabeled human OTR and V1a, we dem-
strated that 125I-ZOTA covalently bound to the upper part of
TMD III. This result has been confirmed using double fragmen-
tation (CNBr followed by Arg-C protease) experiments and
site-directed mutagenesis of potential CNBr or Lys-C cleavage
sites in this OTR or V1a receptor region. The covalently at-
ached region of both receptors has been restricted to three
amino acid residues only, Leu114-Val115-Lys116 in the OTR,
which corresponds to Val126-Val127-Lys128 in the human V1a
receptor (Figs. 3 and 4). Based on high resolution x-ray
structure of bovine rhodopsin (43), these residues are predicted
to be located at the top of TMD III. Interestingly, the Leu114-
Val115-Lys116 motif in the OTR and the corresponding Val126-
Val127-Lys128 motif in the V1a are located in a position homol-
gous to that of Glu113 in rhodopsin known for interacting with

**FIG. 9.** Endoproteinase Lys-C fragmentation of the photola-
beled K128A mutant V1a receptor. Photolabeling of CHO cell mem-
branes (1 mg) expressing wild-type or mutant K128A V1a receptors was
performed as described in the legend to Fig. 7. The corresponding
46-kDa photolabeled species were then electroeluted from a preparative
12% SDS-polyacrylamide gel and subjected to Lys-C endoproteinase
digestion. Equivalent amounts of photolabeled receptors were used in
digestion assay (10,000 cpm). The radiolabeled samples, i.e. wild-
type (lanes 1 and 2) or mutant K128A (lanes 3 and 4) were treated (lanes
2 and 4) or not (lanes 1 and 3) with Lys-C proteinase for 24 h at 37 °C.
An autoradiogram of a discontinuous 10–16.5% dried gel exposed to
Kodak XAR-5 film at ~80 °C for 72 h is shown. Molecular mass markers
are indicated on the left. This fragmentation pattern is representative of
three distinct experiments.
selective photoactivatable linear peptide antagonists, 125I-OTA-related cyclic peptide antagonist (18).

between the receptors and 125I-ZOTA. It is very likely that the linear and cyclic peptide antagonists as well as for SR 49059, peptide antagonists. In the human OTR, the top of TMD VII has been identified so far to be covalently attached to photolabile peptide antagonists are indicated with black triangles. In the human V1a receptor, the top of TMD VII, the first extracellular loop, and the top of TMD III have been photolabeled with 125I-3N3Phpa-LVA (25), 125I-[Lys(3N3Phpa)8]HO-LVA (26), and 125I-ZOTA (this study), respectively. In the human OTR, only the top of TMD III has been demonstrated so far to be covalently attached to 125I-ZOTA (this study). Residues or regions likely to be involved in peptide antagonist binding affinity, on the basis of site-directed mutagenesis studies, are indicated with black spheres. In the human V1a receptor, Glu128 (TMD II), Lys128 (TMD III), Gln185 (TMD IV), and Phe307 (TMD VI) play a role in the binding of V1a-selective cyclic (29) and linear (25, 26) peptide antagonists. In the human OTR, the top of TMD VII has been proposed to be a major contributor to high affinity binding of an 125I-OTA-related cyclic peptide antagonist (18).

the retinal Schiff base. As illustrated in Fig. 10, this photoaffinity labeling study not only constituted the first direct identification of the human OTR antagonist-binding sites determined so far but also allowed us to localize a third photolabeled region in the V1a receptor (25, 26). Indeed, the first extracellular loop and the upper part of TMD VII of the V1a receptor were identified as contact regions for two structurally related V1a-selective photoactivatable linear peptide antagonists, 125I-[Lys3N3Phpa]3HO-LVA and 125I-3N3Phpa-LVA. The Lys128 residue in the human V1a receptor, corresponding to Lys116 in the human OTR, is well conserved throughout the AVP/OT receptor family (30). Moreover, this residue has been shown to play a pivotal role in the binding of agonists (27, 29), two different classes of peptide antagonists such as linear peptides (25, 26) or the cyclic peptide d(CH2)5[Tyr(Me)2]AVP (29) (see also Fig. 10), and also the nonpeptide compound SR 49059 (29). In the present study, the presence of Lys128 residue in the tripeptide sequence covalently attached to the ligand is very interesting. As for V1a-selective linear and cyclic peptide antagonists as well as for SR 49059, this Lys residue might be responsible for a direct interaction between the receptors and 125I-ZOTA. It is very likely that the protonated amine group of Lys128 or Lys116, depending on the presence of Lys128 residue in the tripeptide sequence covalently attached to 125I-ZOTA or Atosiban (14, 15) and to generate meaningful information on receptor/antagonist interactions, it will be necessary to perform other photoaffinity labeling studies.

In conclusion, covalent attachment of 125I-ZOTA to the upper part of TMD III of both the OTR and V1a receptor is fully consistent with our previous 3D antagonist/V1a receptor models. Taken together, these findings suggest once again as demonstrated previously that agonist and peptide antagonist binding pockets might be common to all receptor subtypes of the OT/AVP family and that specific residues can differentiate agonist versus antagonist binding in these receptors (29). The delineation of a three-amino acid “contact domain” is a major
step “en route” to a more detailed mapping of the molecular interactions between OT-related ligands and the OTR. The use of other photoactivatable antagonist ligands as well as molecular modeling of the OTR based on the recent 3D crystal structure of bovine rhodopsin (43) should be very helpful in the future for rationalizing the design of OT antagonists based on the receptor structure.

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Direct Identification of Human Oxytocin Receptor-binding Domains Using a Photoactivatable Cyclic Peptide Antagonist: COMPARISON WITH THE HUMAN V1a VASOPRESSIN RECEPTOR

Christophe Breton, Hichem Chellil, Majida Kabbaj-Benmansour, Eric Carnazzi, René Seyer, Sylvie Phalipou, Denis Morin, Thierry Durroux, Hans Zingg, Claude Barberis and Bernard Mouillac

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