Mapping Peptide-binding Domains of the Human V1a Vasopressin Receptor with a Photoactivatable Linear Peptide Antagonist*

(Received for publication, December 3, 1996, and in revised form, July 24, 1997)

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The study of antagonist-binding domains of the human V1a vasopressin receptor was performed using a radiiodinated photoreactive peptide antagonist. This ligand displayed a high affinity for the receptor expressed in Chinese hamster ovary cell membranes, and specifically labeled two protein bands with apparent molecular mass at 85–90 and 46 kDa. Our results clearly show that the V1a receptor is degraded during incubation with the ligand and that the 46-kDa species is probably the result of the 85–90-kDa species proteolytic cleavage. Truncation of the receptor was then confirmed by deglycosylation with N-glycosidase F. A monoclonal antibody directed against a c-Myc epitope added at the receptor NH2 terminus allowed immunoprecipitation of the 85–90-kDa photolabeled species. The 46-kDa photolabeled protein never immunoprecipitated, indicating that the truncated form of the receptor lacks the NH2 terminus region. To localize photolabeled domains of the receptor, the 46-kDa protein was cleaved with V8 and/or Lys-C endoproteinas. The identity of the smallest photolabeled fragment, observed at approximately 6 kDa, was then confirmed by mutation of the potential V8 cleavage sites. Our results indicate that covalent labeling of the vasopressin V1a receptor with the photoreactive antagonist occurs in a region including transmembrane domain VII (residues Asn327-Lys370).

Analysis of the primary sequence of these receptors suggests that they possess the same general architecture with seven transmembrane helices as other well characterized G protein-coupled receptors. Moreover, the comparison of their amino acid sequence reveals significant homology within the putative transmembrane regions (TM) and within the first and second extracellular loops as well. The natural ligands for the receptors of the AVP/oxytocin family are also closely structurally related. All are nonapeptides composed of a 6-amino acid disulfide-linked ring and a COOH terminus tripeptide.

Peptides of the AVP/oxytocin series were subjected to an extensive analysis of structure-activity relationships. These studies (for review, see Refs. 8–11) led to the production of a profusion of valuable pharmacological probes for assessing receptor selectivity and activity but failed to provide relevant information on the organization of the receptor-binding sites. Recently, we have tentatively localized and mapped the binding site of vasopressin on the V1a receptor subtype by combining three-dimensional molecular modeling and site-directed mutagenesis approaches (12). Our results suggest that the larger part of the vasopressin molecule is anchored within the transmembrane portion of the receptor in a position equivalent to that described for the cationic neurotransmitters. Since all amino acids in the receptor and hormone, potentially interacting in hormone binding, are conserved in the different members of the receptor family and natural neurohypophysial peptides, we proposed that the binding pocket identified in the V1a might be common to V2, V1b, and oxytocin subtypes (12). In this model, only the side chain of vasopressin Arg9 residue is projecting outside the transmembrane core of the receptor in a direction allowing interaction with Tyr115 in the first extracellular loop. The position of the Arg9 side chain and its role in determining part of the receptor selectivity have been experimentally confirmed. Indeed, when Tyr115 was replaced by an Asp or a Phe, the amino acids naturally occurring in the V2 and the oxytocin receptor subtypes, respectively, the agonist selectivity of the V1a receptor switched accordingly (13). A more direct proof of the involvement of the first extracellular loop in peptide hormone residue 8 binding has also been provided for the bovine renal V2 vasopressin receptor by a photoaffinity labeling study with a modified vasopressin agonist (14), and was then confirmed by site-directed mutagenesis (15).

So far all point mutations affecting agonist binding to V1a receptors were found to have no or far less pronounced effect on antagonist binding, suggesting that agonist and antagonist-binding sites might be structurally distinct (12). To further investigate structure-function relationships of AVP receptors, the aim of the present study was to localize the peptide antagonist-binding domains of the human V1a vasopressin receptor using a radiolabeled photoactivatable antagonist. Direct identification of the ligand-binding domains of membrane GPCR by the way of biochemical studies is still poorly investigated and is
limited by the receptor scarcity, hydrophobicity, and fragility. However, as shown above, the photoaffinity labeling technique is an essential complement to modeling and mutagenesis approaches and allows direct determination of the contact regions of a receptor and its ligands. To date, several classes of specific V1a antagonists have been characterized, such as cyclic and linear peptides, as well as nonpeptides (10, 16). Moreover, vasopressin antagonists with photoactivatable groups at positions 3, 4, 8, or 9 usually give very low covalent yields, peptide antagonists containing an a photoactivatable group at positions 3, 4, 8, or 9 usually give increasing concentrations of AVP (from 10−11 to 10−3 M). After stopping the reaction with perchloric acid, total inositol phosphates were extracted and purified by anion-exchange chromatography column (Dowex AG 1×8, formate form, 200–400 mesh; Bio-Rad). For each sample, a fraction containing total inositol phosphates was collected and counted.

Photoaffinity Labeling Experiments on CHO Membranes with [125I]3-N3-Phpa-LVA—The photoactivatable peptide was radioiodinated at positions 3, 4, 8, or 9 usually give increasing concentrations of AVP (from 10−11 to 10−3 M). After stopping the reaction with perchloric acid, total inositol phosphates were extracted and purified by anion-exchange chromatography column (Dowex AG 1×8, formate form, 200–400 mesh; Bio-Rad). For each sample, a fraction containing total inositol phosphates was collected and counted.

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Antagonist-binding Domains of the Human Vasopressin V1a Receptor

V1a antagonist-binding domains have been identified.

EXPERIMENTAL PROCEDURES

Construction of Epitope-tagged Human V1a Vasopressin Receptor—The human V1a receptor cDNA was a generous gift of Dr. M. Thibonnier (23). A stretch of nucleotides coding for a 10-amino acid epitope (EQKLISEEDL) of the human c-Myc protein (24) was inserted after the initiating Met codon of the human V1a vasopressin receptor cDNA using oligonucleotide-directed mutagenesis. The phosphorothioate technique (25) was used to select the mutated expression vector (pCMV) (25). The presence of the sequence coding for the epitope was verified by using oligonucleotide-directed mutagenesis. The phosphorothioate digestion was performed on these concentrated samples.

Affinities for [125I]HO-LVA were determined in saturation binding using concentrations ranging from 10 to 600 pM. Affinities for other ligands were determined from competition experiments using 50–70 pM [125I]HO-LVA as the radioligand. The concentrations of the unlabeled ligands varied from 1 pM to 100 μM. Nonspecific binding was determined in the presence of 200 nM HO-LVA. The data were analyzed by nonlinear least-squares regression using the computer program LIGAND (29).

Inositol Phosphate Assays—Inositol phosphate accumulation was determined as described (30). Briefly, CHO cells expressing V1a receptors were plated and grown in 6-well dishes for 48 h in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). The cells were then labeled for 24 h with [2H]inositol (10–20 Ci/mmol; NEN Life Science Products) at a final concentration of 1 μCi/ml in serum-free, inositol-free medium (Life Technologies, Inc.). Cells were washed twice with PBS, equilibrated at 37 °C in PBS for 1 h, and then incubated in PBS supplemented with 10 mM LiCl for 10 min. CHO cells were incubated for 15 min with increasing concentrations of AVP (from 10−11 to 10−3 M). After stopping the reaction with perchloric acid, total inositol phosphates were extracted and purified by anion-exchange chromatography column (Dowex AG 1×8, formate form, 200–400 mesh; Bio-Rad). For each sample, a fraction containing total inositol phosphates was collected and counted.

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Antagonist-binding Domains of the Human Vasopressin V1α Receptor

The photoactivatable peptide antagonist [125I]3-N3-Phpa-LVA was used to map peptide-binding domains of the human V1α receptor (23). The c-Myc epitope is inserted after methionine residue 1. Three potential N-glycosylation sites are shown as Asn-N*, Asn-N*, and Asn-N*. Shadowed circles indicate a consensus sequence spanning the cleavage site for a metalloproteinase (35). Potential cleavage sites for V8 and Lys-C endoproteases are also indicated as follows: shadowed triangles for Lys-C protease and shadowed squares for V8 protease. The smallest photolabeled fragment with [125I]3-N3-Phpa-LVA and resulting from double digestion is represented in black.

Site-directed Mutagenesis of the Human V1α Vasopressin Receptor—Point mutations (W304A, F307V, and F308L also named W613A, F616V, and F617L according to the modeling numbering (32)) were transiently expressed in CHO cells by electroporation as described previously (Amersham Sculptor Kit). The mutations were verified by direct dideoxynucleotide sequencing. The mutant receptors were then digested with N-glycosidase F (Boehringer Mannheim) for 2 days at 37 °C. Deglycosylated receptors were analyzed by SDS-PAGE using 12% gels.

Protease Digestion of the Partially Purified Receptors—The electroluted receptors were subjected to digestion with Lys-C or V8 proteases. Double digestions were also performed (first digestion with Lys-C followed by a second one with V8). In some cases, deglycosylated receptors were also digested with Lys-C or V8 endoprotease. Endoproteinase Lys-C (sequencing grade, Boehringer Mannheim) was used at 0.2 μg/ml (50 μl). The digestion was performed in 25 mM Tris-HCl pH 8.5, 1 mM EDTA, 0.1% SDS at 37 °C for 16–24 h. V8 protease (endoproteinase Glu-C, Boehringer Mannheim) was used at 0.2 μg/assay (50 μl) and digestion was realized in 50 mM NH4HCO3, pH 7.8, 0.2 mM EDTA, 0.1% SDS at 25 °C for 2 days. To perform double fragmentations, the Lys-C digestion was followed by sample washing and concentration using Microcon-3 (Amicon). Then, concentrated samples were diluted in V8 enzyme buffer and subjected to the second digestion. Results of protease digestions were analyzed by a Tricine discontinuous SDS-PAGE system (10–16.5% gels), which is better for the separation of small molecular mass species (30). Gels were then fixed in glacial acetic acid:methanol:dimethyl sulfoxide:water (10:50:2:38), dried and exposed to Kodak XAR-5 films at −80 °C.

The three-dimensional model of the human V1α vasopressin receptor was constructed using the procedure already extensively described in a previous paper (12) for the rat V1α vasopressin receptor. In a final step, the model was energy minimized with the "Amber All Atom" parameter set installed in SYBYL 6.2 (TRIPOS Associates Inc.) to relax the structure and remove bad steric contacts.

RESULTS

Binding and Coupling Properties of the Wild-type Receptor—The primary sequence of the human V1α vasopressin receptor, deduced from its cDNA cloning, and its possible transmembrane arrangement are shown on Fig. 1. The wild-type V1α vasopressin receptor was stably transfected in hamster CHO cells and its pharmacological properties determined using [125I]HO-LVA, a highly selective V1α receptor subtype agonist (26), as the radioligand. The affinity of the V1α receptor for this radiolabeled ligand was directly determined by saturation binding experiments (see Table I). A Kd value of 38 ± 7 pM (n = 3) was associated with a maximum binding capacity reaching 5–7 pmol of receptors/mg of membrane proteins (increased to 15–17 pmol when cells were treated with sodium butyrate). The affinities of 5 agonists (AVP, oxytocin, Thr3Gly7OT, dDAVP, and Phe2Orn8VT) and 3 specific antagonists for the V1α subtype (cyclic, linear, and non-peptide) were deduced from competition binding experiments. The results obtained are reported in Table I. The observed high affinities for AVP, for the V1α agonist Phe3Orn8VT, and the three V1α selective antagonists together with the observed low affinities for oxytocin, for the oxytocin selective agonist Thr3Gly7OT, and the V2 selective agonist dDAVP, clearly indicate that the receptor expressed in CHO cells has the expected pharmacological profile of the human V1α receptor (23). Furthermore, the expressed receptor is functionally coupled to phospholipase C. Stimulation of transfected CHO cells by vasopressin induced a maximum 6–8-fold increase in inositol phosphate accumulation with an apparent Kd value close to the measured Kd value for vasopressin binding (see Table I). The linear photoactivatable antagonist used in this study to map peptide-binding domains of the human V1α vasopressin receptor ([125I]l3-N3-Phpa-LVA) binds with high affinity to membranes from transfected CHO cells. A Kd value of 137 ± 37 pm (n = 3) was obtained, a value which is close to that reported for the rat liver plasma membrane receptor (54 ± 20 pm (19)).

Photoaffinity Labeling of Human V1α Vasopressin Receptor—The photoactivatable peptide antagonist [125I]3-N3-Phpa-
LVA, containing an arylazido group (Fig. 2A), was used to covalently label the human V1a vasopressin receptor. Membrane preparations from CHO cells expressing the wild-type receptor were irradiated and subjected to SDS-PAGE analysis. As shown in Fig. 2B (lane 3), the photolabeled receptor migrated as 2 broad bands centered approximately at 85–90 and 46 kDa, respectively. The receptor labeling could be completely suppressed by an excess of vasopressin (10 μM), resulting in a predominance of the 85–90 kDa band. The yield of covalent binding, measured as the fraction of specifically bound radioactivity recovered in the labeled bands, was 13.6 ± 0.5% (n = 8).

To explore the possibility that the photolabeled material in the 46-kDa band could originate from a proteolytic cleavage of that present in the 85–90-kDa band under the influence of endogenous proteases, CHO membranes were incubated under conditions likely to affect the extent of endogenous proteolysis. Incubation at 4 °C for 1 h, in the presence of protease inhibitors and ZnCl₂, resulted in a predominance of the photolabeled band of high molecular mass 85–90 kDa (Fig. 3A, lane 1) on SDS-PAGE. In this case, the 85–90-kDa band accounted for about 75% of the radioactivity associated to covalently-bound receptor, while the minor band at 46 kDa accounted for 25% (Fig. 3B). Incubation of the membranes with the photoactivatable ligand for a longer period of time (150 min) at a higher temperature, 30 °C, and in the absence of protease inhibitors and ZnCl₂, resulted in a predominant production of the low molecular weight species (Fig. 3A, lane 3).

In this condition, the band at approximately 46 kDa accounted for 70% of the radioactivity migrating on the gel, while 30% were incorporated into the 85–90-kDa band. The photoreactive azido group is in the meta position on the aromatic ring of the phenylpropionyl moiety considered as peptide position 1. The peptide was radiiodinated at position 9 on phenyl moiety of the tyrosyl residue. Autoradiogram of photolabeled CHO cell membranes, 500 μg of membranes from untransfected CHO cells (lanes 1 and 2), wild-type V1a transfected cells (lanes 3 and 4), or epitope-tagged V1a transfected cells (lanes 5 and 6) were photolabeled with [125I]3-N3-Phpa-LVA as described under “Experimental Procedures.” To define nonspecific photolabeling of the membranes, AVP (10 μM) was added (lanes 2, 4, and 6) or not (lanes 1, 3, and 5). Irradiated and washed membranes were separated onto a 12% SDS-polycrylamide gel. Equivalent amounts of proteins were loaded into the wells. The gel was dried and exposed overnight at −80 °C to film Kodak XAR-5. Molecular weight markers are indicated (kDa) on the left. In some experiments, to determine the covalent binding yield of the photolabeling reaction, gels were also cut in 5-mm slices and the radioactivity was counted. B is representative of at least three distinct experiments.

Deglycosylation of the Labelled Vasopressin Receptor—As shown on Fig. 4, treatment of photolabeled membranes with N-glycosidase F before SDS-PAGE shifted apparent molecular mass of the 85–90- and 46-kDa protein bands to approximately 53 and 36 kDa, respectively, indicating that they both were glycosylated and contained at least one potential N-glycosylation site. The NH₂-terminal extracellular region of the human vasopressin receptor contains two potential N-linked glycosylation sites (N-X-(S/T)) at Asn14 and Asn27. An additional N-linked glycosylation site is present at the level of the second extracellular loop (Asn150). A molecular mass of 53 kDa is close to the theoretical mass deduced from the receptor cDNA sequence (48.3 kDa including molecular mass of the agonist).
Antagonist-binding Domains of the Human Vasopressin V1a Receptor

SDS-PAGE also revealed two broad bands with molecular mass at approximately 85–90 and 46 kDa, respectively (see Fig. 2, lane 5). These two bands are homologous to the radiolabeled bands derived from the wild-type receptor although the expected increase in size due to the presence of an additional 10-residue epitope on the tagged receptor could not be evidenced. The epitope-tagged receptor labeling was also specific because an excess of vasopressin totally suppressed the signal (lane 6). Finally, the apparent molecular weight of the deglycosylated epitope-tagged receptor was equivalent to that of the wild-type receptor (data not shown).

CHO cell membranes expressing either the wild-type or the epitope-tagged receptor were incubated with the photoactivatable antagonist, photolyzed, and solubilized. The solubilized material was then subjected to immunoprecipitation using an anti-c-Myc monoclonal antibody directed against the 9E10 epitope. Two different incubation conditions with the photoaffinity probe were selected: a condition in which preferential photolabeling of the 85–90-kDa protein band occurred (1 h at 4°C with protease inhibitors and ZnCl2), the other in which the major photolabeled protein was the 46-kDa species (150 min at 30°C without ZnCl2 and protease inhibitors). As shown in Fig. 5, SDS-PAGE electrophoresis of the immunoprecipitates derived from epitope-tagged receptors revealed the 85–90-kDa but not the 46-kDa band even under conditions favoring its production. In this experiment (Fig. 5, lane 4), the immunoprecipitated receptor appeared as a sharp radiolabeled band and its apparent molecular weight could be more precisely determined on interpolation (88.9 ± 0.7 kDa (n = 5)). Control experiments with immunoprecipitates derived from the wild-type receptor failed to reveal any labeled band. These results indicate that the 46-kDa species do not contain the NH2-terminal part of the receptor at a site proximal to the Asn27–Asn196 glycosylation site.

Protease Digestions of the Labeled Receptors—To identify

FIG. 3. Degradation of photolabeled V1a receptor during incubation with [125I]3-N3-Phep-LVA. CHO cell membranes (500 μg) expressing the wild-type V1a receptor were incubated with the photoactivatable antagonist in different conditions and for different times. ZnCl2 (0.1 mM) and protease inhibitors (leupeptin, benzamidine, and soybean trypsin inhibitor) were added (lanes 1 and 2) or not (lane 3) in the incubation medium. Different incubation times were compared: 60 min (lanes 1 and 2) and 150 min (lane 3). Finally, different temperatures were tested: 4°C (lane 1) and 30°C (lanes 2 and 3). Equivalent amounts of photolabeled membranes were loaded in each lane before separation on SDS-PAGE. A, autoradiogram of photolabeled membranes. The gel was dried and exposed 3 h at −80°C to Kodak XAR-5 film. B, the gel was cut in 5-mm slices and radioactivity was determined.  ■ corresponds to lane 1, ○ to lane 2, ▲ to lane 3. Migration of molecular weight markers is indicated on the left (A) and on the upper scale (B). The figure is representative of three distinct experiments.

Even if N-glycosidase F treatment appears not to be complete, this would suggest that the 85–90-kDa band could correspond to the native V1a receptor glycoprotein. On the other hand, the protein band at 36 kDa possessed an apparent molecular weight significantly smaller than the molecular weight calculated from the protein core. This result thus confirmed that the lower photolabeled protein band at 46 kDa (see Fig. 2) clearly proceeded from proteolytic degradation but was still glycosylated. Taking into account that molecular weight of the smaller deglycosylated protein is 36 kDa, proteolytic cleavage of the receptor must occur between Asn27 and Asn196.

Immunoprecipitation of the Photolabeled Receptors—The conclusions derived from the deglycosylation experiments could be confirmed by using the human V1a vasopressin receptor tagged at its amino terminus with the 9E10 epitope (EQKLISEEDL, amino acid residues 411–420 of human c-Myc protein, see Fig. 1). The epitope-tagged receptor was well expressed in CHO cells: 5 pmol/mg of membrane proteins (increased to 15–17 when cells were treated with sodium butyrate). Its pharmacological and functional properties were undistinguishable from those of the wild-type receptor (Table I). Photolabeling of the epitope-tagged receptor followed by
peptide-binding domains covalently-bound to the photoactivatable linear antagonist, photolabeled receptors were electroeluted from a preparative SDS-PAGE and then subjected to enzymatic cleavage by endoproteases. Lys-C protease and V8 protease (Glu-C) were used either alone or in combination. Lys-C and V8 proteases cleave proteins specifically at the COOH-terminal side of lysine and the COOH-terminal side of glutamate residues, respectively. They are thus expected to preserve the photoactivatable ligand which do not possess these cleavage sites. In a first series of experiments, both the 85–90- and 46-kDa labeled bands were excised from SDS-PAGE and subjected to protease digestions separately. Fragmentation patterns were identical (data not shown), indicating that the covalently-bound radioactivity is exclusively associated with the peptide sequence present in the 46-kDa fragment spontaneously generated during membrane incubation. Detailed analysis of the proteolytic fragmentation of the photolabeled receptors was thus conducted using material recovered from the 46-kDa band. Moreover, the potential proteolytic radiolabeled fragments that could be generated from Lys-C and V8 digestions of the 46-kDa species are listed in Table II.

**Fig. 5. Immunoprecipitation of epitope-tagged V1a vasopressin receptor.** Equivalent quantities of CHO cell membranes (500 μg) expressing either the wild-type (lanes 1 and 3) or the epitope-tagged (lanes 2 and 4) V1a receptor were incubated with [125I]3-N3-Phpa-LVA for 1 h at 4 °C (lanes 1 and 2) in the presence of ZnCl₂ and protease inhibitors or 150 min at 30 °C (lanes 3 and 4) without ZnCl₂ and protease inhibitors. Membranes were then photolabeled and solubilized as described under “Experimental Procedures.” Solubilized receptors were washed and concentrated on Centricon 30. Wild-type and epitope-tagged V1a receptors were immunoprecipitated with mouse monoclonal antibody 9E10 (1/20) and anti-mouse IgG second antibody coupled to agarose beads. Immunoprecipitated receptors were subjected to SDS-PAGE analysis using a 12% gel. The gel was dried and exposed overnight to Kodak XAR-5 film at -80 °C. The autoradiogram shown is representative of at least three distinct experiments. Molecular weight markers are indicated on the left.

**Fig. 6. Fragmentation of the human V1a vasopressin receptor with V8 and Lys-C proteases.** CHO cell membranes (500 μg) expressing the wild-type human V1a vasopressin receptor were incubated with the [125I]3-N3-Phpa-LVA for 150 min at 30 °C without ZnCl₂ and protease inhibitors, a condition which favors the preferential photolabeling of 46-kDa receptor species. The membranes were separated on a preparative 12% SDS-PAGE gel and the photolabeled receptor was electroeluted, washed, and concentrated as described under “Experimental Procedures.” Equivalent amounts of photolabeled receptors were used in each protease digestion assay (30,000 cpm). The samples were then loaded on discontinuous 10–16.5% Tricine gels. A, Lys-C protease digestion. The “partially purified” receptor was treated (lanes 2–4) or not (lane 1) with Lys-C endoproteinase for 24 h (excepted for lane 2, 16 h). In lane 4, the receptor was first deglycosylated with N-glycosidase F. B, V8 and V8 + Lys-C protease digestions. The electroeluted receptor was treated (lanes 2 and 4) or not (lane 1) with V8 protease for 48 h. In lane 3, the receptor was subjected to a double fragmentation with Lys-C and V8 proteases. In lane 4, the Lys-C inhibitor was removed after the digestions. The figure shows autoradiograms of dried gels exposed to Kodak XAR-5 films at -80 °C for 24 h. Molecular weight markers are indicated on the left. Each assay is representative of at least three distinct experiments.

| Proteases Fragments Molecular massa (kDa) |
|-----------------|-----------------|-----------------|
| Lys-C           | His₁²⁹⁹-Lys₁⁵₅₈ | 4.8             |
|                  | Thr¹⁵⁹⁹-Lys₁⁹₀₆ | 6.2             |
|                  | Ala₂⁰⁰₄-Lys₂⁵₀  | 7.2             |
|                  | Met₂⁹²₄-Lys₃₇₀ | 10.6            |
|                  | Glu₃⁷₅₄-Lys₄₀₂ | 4.6             |
| V8              | Val₁⁹⁴₄-Glu₂₆₀₉ | 8.9             |
|                  | Glu₃⁷₁₄-Glu₃₂₄ | 8.7             |
|                  | Asn₂²⁷₄-Glu₃₇₁ | 6.5             |
|                  | Asp₇⁷₇₄-Thr₈₄₈ | 6.2             |
|                  | His₁²⁹⁹-Lys₁⁵₅₈ | 4.8             |
|                  | Thr¹⁵⁹⁹-Glu₁⁹₀₃ | 5.5             |
|                  | Ala₂⁰⁰₄-Lys₂⁵₀  | 7.2             |
|                  | Met₂⁹²₄-Glu₂₆₀₉ | 5.4             |
|                  | Asn₂²⁷₄-Lys₃₇₁ | 6.4             |
|                  | Asp₇⁷₇₄-Lys₄₀₂ | 4.5             |

*In all cases, the molecular mass of the photolabile antagonist (1.5 kDa) is included in the value (e.g. fragment Met₂⁹²₄-Lys₃₇₀, noted at 10.6 kDa, corresponds to the amount of the peptidic fragment mass of 9.1 kDa with that of the ligand 1.5 kDa).*

*Fragments containing potential N-glycosylation sites.*
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...and ~6 kDa. In some experiments, another fragment at ~18 kDa was also present. When deglycosylated receptor was digested with V8 protease, the 6-kDa band was still observed, indicating that this photolabeled fragment does not contain an N-linked glycosylation site (Fig. 6B, lane 4). Thus, V8 proteolytic fragment Val194-Glu260, including a potential glycosylation site (see Table II), cannot account for the photolabeled binding domain of the receptor. Only three fragments Glu261, Glu324, Asn327-Glu371, and Asp377-Thr418, with molecular masses at 8.7, 6.5 and 6.2 kDa, respectively (see Table II), could correspond to the 6-kDa protein band. Double fragmentation of photolabeled V1a receptor with Lys-C and V8 proteases revealed 2 major digests (Fig. 6B, lane 3) at ~10.5 and ~6 kDa. In some cases, a third photolabeled fragment was also obtained at ~15 kDa, probably when double digestion was not complete.

When photolabeled receptor was subjected to V8 digestion only or double fragmentation, the smallest fragment always possessed an apparent molecular mass at ~6 kDa. The Lys-C/V8 digestion map of the human V1a vasopressin receptor shows only one proteolytic peptide that kept a molecular mass (6.4 kDa, see Table II) equivalent to that obtained with V8 digestion only (6.5 kDa, Table II). This smallest peptide includes transmembrane region VII and part of the COOH-terminal region (see Fig. 1) and spans from Asn327 to Lys370. The isolated radiolabeled 6-kDa fragment probably corresponds to this sequence.

To verify this hypothesis, site-directed mutagenesis of the potential V8 protease cleavage sites in this receptor domain was done at Glu324–Glu326 and Glu371–Glu376 of the human V1a receptor, respectively (see Fig. 1). Mutated receptors have been expressed in CHO cells and their binding properties toward [125I]3-N3-Phpa-LVA verified. The photosensitive ligand binds to mutant receptors Asp324–Asp326V1a and Asp371–Asp376V1a with an equivalent affinity to that for the wild-type V1a vasopressin receptor ($K_d$ values of 62.7 ± 12.7 and 55.0 ± 18.5 pM ($n$ = 3), respectively). Photolabeling of the mutated receptors was also performed and V8 fragmentation of corresponding 46-kDa radiolabeled species was studied in details and compared with that of the wild-type receptor. As seen in Fig. 7, the radiolabeled 6-kDa species, resulting from the wild-type receptor V8 fragmentation (lane 2), was not present any more when both mutant receptors were digested by the protease (lanes 4 and 6). As V8 potential cleavage sites Glu324–Glu326 and Glu371–Glu376 have been suppressed, the expected molecular mass of the smallest photolabeled species should be 13.8- and 11.9-kDa, respectively. For each mutant, a ~12–13-kDa photolabeled fragment was obtained (Fig. 7, lanes 4 and 6), a size consistent with that of the expected fragments. Moreover, V8 fragmentation of the mutant Asp371–Asp376V1a also produced an unexpected fragment at ~9 kDa that could result from a nonspecific cleavage. In conclusion, the V8 fragmentation of both mutants allowed us to define NH2- and COOH-terminal side of the photolabeled fragment and to confirm its identity.

**DISCUSSION**

A linear photoactivatable antagonist has been used to irreversibly label the human V1a vasopressin receptor expressed in CHO cells. Properties of this peptide have already been described (19). Briefly, this compound combines several advantages over those previously characterized: high affinity and selectivity, possibility of radioiodination, and high covalent binding yield. In this study, we have also shown that fragmentation of the photolabeled receptor only generates a small number of radiolabeled fragments. Moreover, the smaller fragment derives from other higher molecular weight fragments produced by partial proteolysis. This would indicate that the [125I]3-N3-Phpa-LVA covalently binds upon irradiation to a limited number of sites closely located on the receptor peptide sequence. Finally, the extent of nonspecific binding is very low. Indeed, when AVP was present during incubation with the photoactivatable antagonist, covalently-bound radioactivity in the solubilized samples was almost undetectable (see Fig. 2). It therefore appears that [125I]3-N3-Phpa-LVA is indeed a valuable tool to localize antagonist-binding sites on the human V1a receptor.

Photolabeling of the human V1a receptor with [125I]3-N3-Phpa-LVA generated two molecular species with apparent molecular masses of ~85–90 and ~46 kDa (see Fig. 2). Previously, two molecular forms of the rat V1a receptor could also be detected following labeling with vasopressin analogues containing a photoreactive azido group into the side chain of Lys8 or into Lys3 (17, 34). More recently, the photolabeling of bovine kidney membranes with a tritiated photoactivatable agonist (arylazido group at the side chain of Lys8) also revealed two V2 receptor species with molecular masses at 58 and 30 kDa (14). We show that the 85–90-kDa molecular species, derived from the human V1a receptor is glycosylated and contains the amino-terminal part of the receptor molecule. The observed size of the deglycosylated counterpart of the 85–90 kDa roughly corresponds to the expected size of the peptidic core of the entire receptor, suggesting that the 85–90-kDa protein might correspond to the native state of the receptor. As far as the 46-kDa species is concerned, we show that its relative abundance can be reduced under particular experimental conditions (low temperature and presence of protease inhibitors). These conditions are likely to reduce the influence of endogenous proteases usually present in crude tissue or cell line membrane preparations. Further proteolytic fragmentation of the 46-kDa species generated the same radiolabeled fragments as fragmentation of the 85–90-kDa species. All these data are consistent with the conclusion that the 46-kDa species results from a proteolytic cleavage of the 85–90-kDa species. Since the 46-kDa species...
was found to lack the amino-terminal portion of the receptor and to be glycosylated, it is concluded that the site of proteolytic cleavage is located on the NH$_2$-terminal portion before the last putative glycosylation site in the receptor sequence (Asn$^{196}$). In another study, it has been clearly demonstrated that the bovine renal V2 vasopressin receptor is cleaved by a metalloproteinase during the incubation with a high-affinity ligand (35). The proteolytic cleavage occurs between Gln$^{92}$ and Val$^{93}$ and produces a NH$_2$-terminal truncated V2 receptor with a molecular mass at 30 kDa. The region spanning the cleavage site (Ph$_{103}$ to Gln$^{96}$) is highly conserved in other receptors for vasopressin and oxytocin. In the human V1a subtype, it corresponds to the sequence Phe$^{103}$ to Gln$^{108}$ (see Fig. 1). Deglycosylation of the 46-kDa photolabeled V1a receptor converted its apparent molecular mass to $\sim$36 kDa. This size is consistent with that of a proteolytic fragment which could be generated from a cleavage in this region.

As documented under “Results,” fragmentation of the wild-type as well as mutant human V1a receptors by a combination of Lys-C and V8 proteases, allowed the identification of a radiolabeled 6-kDa fragment of the receptor containing the site(s) of covalent attachment of the photoactivatable antagonist. This fragment including transmembrane region VII and part of the intracellular COOH-terminal region of the receptor very likely spans from Asn$^{327}$ to Lys$^{370}$. Based on our present knowledge on the localization of ligand-binding sites on GPCR (36), it seems reasonable to exclude part of the 6-kDa fragment corresponding to a putative intracellular portion of the receptor as a domain for the covalent attachment of the ligand. We conclude that upon UV irradiation of CHO cell membranes, a covalent linkage between the bound photoactivatable antagonist [125I]3-N$_3$-Phpa-LVA and the putative seventh transmembrane region of the human V1a receptor has occurred. This localization is consistent with the hydrophobic nature of the photoactivatable compound which could interact with the transmembrane pocket of the V1a vasopressin receptor. However, although peptide agonists and antagonists for vasopressin receptors have similar amino acid composition, they probably adopt very different conformations and probably fit into different states of the V1a receptor protein. Indeed, our previous mutagenesis study on the V1a receptor-binding sites have shown that mutations affecting agonist binding have little or no effect on antagonist binding (12).

It is now commonly accepted that peptide ligands, agonists, or antagonists partly bind to transmembrane domains of their receptors (for review, see Refs. 36 and 37). This has been demonstrated with different approaches. Using a photofinity labeling technique, Maggio and co-workers (38) have shown that the COOH-terminal hydrophobic sequence of substance P (SP) inserts into a hydrophobic ligand-binding pocket localized into the upper part of the murine neurokinin NK-1 receptor transmembrane domains and the remainder of the SP molecule interacts with residues on the extracellular face of the receptor. These conclusions have then been confirmed on rat (39) and human (40) NK-1 receptors. The idea that peptide ligands partly bind to transmembrane regions of their receptors has also been nicely demonstrated on neurokinin NK-2 receptors using a novel approach with fluorescent ligands (41). Turcatti et al. (41) have shown that the binding site on the NK-2 receptor for the amino-terminal end of peptide antagonists is buried in the hydrophobic pocket of the receptor protein and clearly distinct from the binding site for the amino-terminal end of peptide agonists which is extracellular. Targeting the seventh helix of the V1a vasopressin receptor as the site of [125I]3-N$_3$-Phpa-LVA covalent binding is also consistent with mutational studies on other GPCR for peptide ligands, showing that aromatic residues localized in transmembrane VI and VII play a key role in antagonist binding. Indeed, Tyr$^{397}$ at the top of helix VII in NK-1 receptor appears to participate in the binding of all neuropeptide receptor and in an aromatic interaction with at least one nonpeptide antagonist (42). A role in the binding of antagonists for aromatic amino acids belonging to the upper part of helix VI and highly conserved in most GPCR, has also been demonstrated. In the terminology used in receptor modeling studies (for review, see Refs. 43 and 44), these amino acids constituting an aromatic cluster have been numbered as residues 613, 616, and 617 by reference to their position in the transmembrane sequence (for instance, residue 613 is the thirteenth residue in the helix VI). As examples, distances in Å (displayed in yellow) occurring between residues Phe-616 and face to face residue Ile-707 or Ala-711 are shown (e.g. 3.68 Å between Phe-616 and Ile-707).

FIG. 8. Three-dimensional model of the human V1a vasopressin receptor. A side view of the upper regions of the receptor is shown from a direction parallel to the cell membrane surface. The positioning of transmembrane domains I to VII is anticlockwise and their COOH chain is shown in magenta. Transmembrane regions VI and VII are labeled in green. The side chains of residues Trp-613, Phe-616, and Phe-617 in TM VI and neighborhood residues Ile-707 and Ala-711 in TM VII are highlighted in white. Receptor residues are labeled according to the modeling numbering: the first digit indicates the transmembrane α-helix, the next two digits indicate the rank of the residue in this transmembrane domain (e.g. Phe-616 is the sixteenth residue in helix 6). As examples, distances in Å (displayed in yellow) occurring between residue Phe-616 and face to face residue Ile-707 or Ala-711 are shown (e.g. 3.68 Å between Phe-616 and Ile-707).
Table III

| Binding of AVP and 3-N3-Phpa-LVA to the wild-type and mutant human V1a receptors | \( K_i \) (nM) | \( [\mathrm{3H}]\text{AVP} \) | 3-N3-Phpa-LVA |
|---------------------------------------------|----------------|-----------------|----------------|
| WT human V1A                                 | 0.67 ± 0.17 (3) | 0.24 ± 0.07 (3) | 0.35 (3)       |
| W613A V1a                                   | 0.87 ± 0.33 (3) | 0.47 ± 0.11 (3) | 0.78 (3)       |
| F616V V1a                                   | 2.24 ± 0.29 (4) | 430 ± 119 (3)   | 0.29 (4)       |
| F617L V1a                                   | 0.98 ± 0.33 (5) | 0.78 ± 0.15 (3) | 0.17 (3)       |

* Affinity \((K_i)\) of wild-type and mutant V1a receptors for \([\mathrm{3H}]\text{AVP}\) was directly determined in saturation experiments. Affinity \((K_i)\) for 3-N3-Phpa-LVA was obtained in competition binding assays by displacement of \([\mathrm{3H}]\text{AVP}\) used at \( \sim 1-2\ \text{nM}\). Data were analyzed as described under “Experimental Procedures.” All values in this table are expressed as the mean \(\pm\) S.E. calculated from at least three independent determinations.

Tyr(Me)\(^2\)-Phe\(^3\) of the ligand and the receptor, which has been generated by UV irradiation, is probably around 3 Å (48). As illustrated in Fig. 8, the proposed three-dimensional model of the human V1a vasopressin receptor predicts an equivalent distance between the aromatic residue cluster in TM VI and neighborhood residues of TM VII (for example, the distance between Phe-616 in helix VI and lle-707 in helix VII facing each other, is approximately 3.6 Å). To verify a potential role of residues constituting the aromatic cluster in the V1a vasopressin receptor, Trp-613, Phe-616, and Phe-617 have been replaced by residues loosing their aromatic character. The binding properties of mutant V1a receptors W613A, F616V, and F617L to the photoactivatable ligand have been studied (see Table III). Interestingly, substitution of phenylalanine 616 with a valine resulted in a \( >1700\)-fold reduction in antibody affinity and only a 4-fold reduction in AVP binding. Combining photolabeling and site-directed mutagenesis of the human V1a vasopressin receptor, we propose a potential interaction between the hydrophobic NH\(_2\) terminus of the peptide antagonist and the aromatic cluster of transmembrane helix VI.

Acknowledgments—We are grateful to Dr. M. Thibonnier (Cleveland, OH) for providing the human V1a receptor cDNA. Many thanks to A. Cohen-Solal for production of ascites fluid containing 9E10 monoclonal antibody and to M. Passama and L. Charvet for help with the illustrations.

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