Polar Residues in the Transmembrane Domains of the Type 1 Angiotensin II Receptor Are Required for Binding and Coupling

RECONSTRUCTION OF THE BINDING SITE BY CO-EXPRESSION OF TWO DEFICIENT MUTANTS*

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Type 1 angiotensin receptors (AT1) are G-protein coupled receptors, mediating the physiological actions of the vasoactive peptide angiotensin II. In this study, the roles of 7 amino acids of the rat AT1A receptor in ligand binding and signaling were investigated by performing functional assays of individual receptor mutants expressed in COS and Chinese hamster ovary cells. Substitutions of polar residues in the third transmembrane domain with Ala indicate that Ser105, Ser107, and Ser109 are not essential for maintenance of the angiotensin II binding site. Replacement of Asn111 or Ser115 does not alter the binding affinity for peptidic analogs, but modifies the ability of the receptor to interact with AT1 (DuP753)- or AT2 (CGP42112A)-specific ligands. These 2 residues are probably involved in determining the binding specificity for these analogs. The absence of G-protein coupling to the Ser115 mutant suggests that this residue, in addition to previously identified residues, Asp74 and Tyr292, participates in the receptor activation mechanism.

Finally, Lys102 (third helix) and Lys199 (fifth helix) mutants do not bind angiotensin II or different analogs. Co-expression of these two deficient receptors permitted the restoration of a normal binding site. This effect was not due to homologous recombination of the CDnas but to protein trans-complementation.

The physiological actions of the vasoactive octapeptide hormone angiotensin II (AngII) in the cardiovascular, endocrine, and neuronal systems are mediated by membrane-bond receptors. Two pharmacologically distinct Ang1 receptors have been identified: AT1 and AT2 (1, 2). AT1 receptors bind biphénylimidazole antagonists such as DuP753 with high affinity and selectivity. AT2 receptors have the reverse affinities for these compounds. Cloning of both receptor types has revealed that they belong to the seven transmembrane domain receptor family (3–6). Two closely related AT1 isoforms (AT1A and AT1B) have been identified in rat and mouse species (7, 8). AT1 receptors have been shown to be coupled to G-proteins and to activate phospholipase C (PLC) (9, 10). This results in inositol trisphosphate (IP3) generation, which then causes an increase in intracellular calcium concentrations, and diacylglycerol formation, which leads to protein kinase C activation (11, 12).

The molecular location of the ligand binding domain of the G-protein-coupled receptors has been intensively investigated using genetic, biochemical, and biophysical approaches. The binding site for small molecules such as the bioamine neurotransmitters involves polar residues of the transmembrane domains (TM) (13). In contrast, the binding site for large hormones such as the pituitary glycoproteins is located in the large amino-terminal extracellular domain of the corresponding receptors (13). The ligand binding domains of peptide receptors, such as AT1, have been more recently investigated. Peptide binding by AT1 receptors is dependent on the presence of four extracellular cysteines (14) as well as on several additional residues located in the extracellular domains (15). Whereas the binding of non-peptidic ligands is unaffected by these extracellular mutations, numerous polar residues of the hydrophobic transmembrane segments are determinant in binding of non-peptidic antagonists (16–19). Two of these residues, Asp74 and Tyr292, also play an essential role in the coupling of AT1A to PLC (16, 18).

Biochemical analysis (20) and molecular modeling studies (21) indicate a major role for the third and fifth transmembrane segments (TM-III and TM-V) in AngII binding. To define the functional roles of polar residues in these transmembrane domains, substitutions of different polar residues into Ala (K102A, S105A, S107A, S109A, N111A, S115A, and K199A) were therefore created in the rat AT1A receptor (Fig. 1). The pharmacological profiles of the mutated receptors for peptidic and non-peptidic agonists and antagonists, as well as their signaling properties were analyzed.

Some of the mutants were defective for the binding of AngII and its analogs. Since the mutations were located in different domains of the AT1A receptor, we investigated whether the recently described mechanism of intermolecular complementation could be observed for this peptide hormone receptor. This phenomenon was described for different mutants of the α2C adrenergic receptors as well as the M2 and M3 muscarinic receptors when they were co-expressed, suggesting that molecular association was occurring between complementary transmembrane domains of two different defective receptors (22–24). The ligand binding and signaling properties of four binding defective AT1A mutants were therefore analyzed after their co-transfections in COS cells, in different paired combinations.

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1 The abbreviations used are: AngII, angiotensin II; AT1 receptor, type 1 angiotensin II receptor; AT2 receptor, type 2 angiotensin II receptor; G-protein, guanine nucleotide-binding protein; PLC, phospholipase C; IP3, inositol trisphosphate; IP, inositol phosphate; TM, transmembrane domain; CHO, Chinese hamster ovary; PCR, polymerase chain reaction; RT-PCR, reverse transcription PCR.

2P. Broto, unpublished results.
EXPERIMENTAL PROCEDURES

Site-directed, insertion, and deletion mutagenesis—Expression plasmids coding for the mutated receptors were constructed following two types of strategies, using either the wild type rat AT1a cDNA fragment (2.2 kilobase pairs) inserted in M13mp19, or a described synthetic cDNA sequence (1.1 kilobase pairs) into which multiple restriction sites had been introduced (25).

K102A, S107A, K199A, and L168–188 were generated in the M13mp19 construct. Four oligonucleotides were synthesized on a PCR-Mate (Applied Biosystems): 5'-CAC CTA TGT GCC GTC GCT TCG-3' for replacement of Lys102; 5'-GTC GCT GGC GCC AGT TTC-3' for replacement of Ser107; 5'-GGC CTT ACC GCC AAT ATT CTG-3' for replacement of Lys109; 5'-G CGA CCA GTC ATC CAC CAG TGC TCG ACG CTC CCC ATA GGG CTG-3' deleting the sequence coding for the second extracellular loop. An uracil-containing M13mp19-AT1a DNA was used as template in an in vitro mutagenesis reaction using the synthetic oligonucleotides (Muta-Gene D Kit, Bio-Rad). Transformation into a strain with a functional uracil N-glycosylase allows selection against the parental unmutated strain.

The other mutations were performed by deletion of a restriction fragment and replacement with an appropriate linker. The Δ(25–35) mutant was obtained by deleting the 360-base pair HindII-BspHI fragment corresponding to the amino-terminal extracellular region of AT1a, and replacing with double-stranded linker corresponding to sense oligonucleotide 5'-AG CTG ACC ATG GCC GTC TAC ATA TTA GCT-3'.

The S105A, S109A, N111A, and S115A mutants were constructed using four double-stranded linkers corresponding to sense oligonucleotides: 5'-CCG GAT AAG GCC CT-3', 5'-GGC CTT ACC GCC AAT ATT CTG-3', 5'-GGC CTT ACC GCC AAT ATT CTG-3', and 5'-GGC CTT ACC GCC AAT ATT CTG-3'. Replacement of Ser107;5 replacement of Ser109;5 replacement of Lys199;5 replacement of Ser115. These linkers were inserted, respectively, in the Nrul-Mul (for Ser105, Ser109, and Asn111) and Mul-Pml1 (for Ser115) single restriction sites of the synthetic cDNA.

The other AT1a mutants were constructed using the same strategy. These constructions were used to control the amino (Ins[Nter]) and/or carboxy terminus (Ins[Cter]) of the AT1a coding sequence and were used here as a control to verify the absence of homologous recombination between two co-transfected AT1a cDNAs. The two inserted sequences, corresponding to sense oligonucleotides (FLAG sequence, SIS, Eastman Kodak Co.); 5'-AGG ATC CAA GAT GAC-3'). Their their 3'-terminal restriction sequence 5'-TCCTAATGTTGCCATCGCTTC-3' (for EcoRI site) were, respectively, inserted in the HindII-Eagl and BstBI-Xbal restriction sites of the synthetic cDNA. To produce the construction Ins[Nter]-Ins[Cter] containing the double insertion, the EcoRI-Xbal fragment of Ins[Cter] was inserted into the expression vector containing HindII-EcoRI fragment of Ins[Nter]. The mutated sequences were verified by dyeode sequencing using Sequenase version 2 (United States Biochemical Corp.). DNase was constructed as described previously (16). All the mutated AT1a cDNAs were subcloned into the expression vector pCE2 (26).

Expression in COS-7 and CHO Cells—The COS-7 cell line was cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum plus 0.5 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (all from Boehringer Mannheim). Two days after plating (3 × 10^6 cells/75 cm²), cells were transfected or co-transfected with 1 μg of plasmid DNA, unless otherwise indicated, by the DEAE-dextran-chloroquine method (27). Binding studies or inositol phosphate (IP) production in response to increasing concentrations of AngI as was described previously (32). Cells were subcultured in 12-well plates and labeled with 2 μCi/ml [3H]myo-inositol for 24 h and then incubated with AngI at 37°C for 30 min in presence of 10 μM LiCl. After purification on a Dowex anion exchange resin (AG+ 1-X8 resin, Bio-Rad), the total radioactivity IP fraction was measured.

RT-PCR Analysis for Detection of Homologous Recombination—Total RNA was prepared from COS-7 cells transfected with Ins[Nter], Ins[Cter], or Ins[Nter]-Cter plasmids using the guanidino thiocyanate method (33). Total RNA (5 μg) was treated with RNase-free DNase (Boehringer Mannheim) to avoid plasmid DNA amplification. Complementary DNAs were synthesized with Moloney murine leukemia virus reverse transcriptase (Boehringer Mannheim) using 10 pmol of a primer, corresponding to the 3’ region of the carboxy-terminal insertion sequence 5’-TTT GTC ATC GTC ATC TTT GTT-3’. This particular primer allows only reverse transcription of mRNA containing the carboxy-terminal insertion sequence to avoid any heterologous amplification. A similar argument can be made for Lys199. Since two previous studies also showed that substitutions of this residue with Gln causes a major decrease in AT1a expression in COS-7 cells transfected with Ins[Nter], Ins[Cter], or Ins[Nter]-Cter plasmids using the guanidino thiocyanate method (33). Total RNA (5 μg) was treated with RNase-free DNase (Boehringer Mannheim) and then amplified using 1 unit of Taq DNA polymerase (Boehringer Mannheim) and 10 pmol of each primer in a 25-μl volume. Thirty cycles of the PCR were performed using 94°C, 30 s; 60°C, 30 s; 72°C, 1 min. The primers used for PCR amplification correspond to the following sequences: primer 1 (5’- AAA GAC GAT GCC GAT AAG GCC CT-3’); primer 2 (5’- A AGG ATC CAA GAT GAC GCC GAT AAC GCC CCC A-3’); primer 3 (5’- A CTC CAC TAC GAA ACA AGC ACA C-3’) and primer 4 (5’- GCT TTC GGT TTG GTA TTT TGC ATC-3’). The mutagenesis sequence, SIS, Eastman Kodak Co.): 5'-AG-CTT ACC ATG GCC TAC AAA GAC GAT GCC GAT AAG GCC CCT AAT TCT TC-3’ (5’-sequence) and 5’-G CAA GTG GAC GAC GAT AAC GAC TAC AAA GAC GAT GAC GAT AAA TGA CGG ACC GT-3’ (3’-sequence) were, respectively, inserted in the HindII-Eag1 and BstBI-Xbal restriction sites of the synthetic cDNA. To produce the construction Ins[Nter]-Ins[Cter] containing the double insertion, the EcoRI-Xbal fragment of Ins[Cter] was inserted into the expression vector containing HindII-EcoRI fragment of Ins[Nter].

The mutated sequences were verified by dyeode sequencing using Sequenase version 2 (United States Biochemical Corp.). DNase was constructed as described previously (16). All the mutated AT1a cDNAs were subcloned into the expression vector pCE2 (26).

Expression in COS-7 and CHO Cells—The COS-7 cell line was cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum plus 0.5 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (all from Boehringer Mannheim). Two days after plating (3 × 10⁶ cells/75 cm²), cells were transfected or co-transfected with 1 μg of plasmid DNA, unless otherwise indicated, by the DEAE-dextran-chloroquine method (27). Binding studies or inositol phosphate measurements were done 48 h after transfection.

CHO K1 cells were maintained in Ham’s F-12 medium (Boehringer Mannheim) supplemented with 10% fetal calf serum plus 0.5 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The CHO AT1a clones have been previously described (16, 28). To establish the CHO K11A and CHO S115A clones, CHO cells were transfected with 10 μg of the corresponding plasmid and 2 μg of the selection marker pSV2neo using the calcium phosphate precipitation method (29). Transfected cells were selected by their resistance to 750 μg/ml G418 (Life Technologies, Inc.) and cloned by limiting dilution.

Binding Experiments—[3H]-AngI was labeled by the chloramine-T method (30) ([3H]-[Sar1]-AngI (Table I) and [3H]-[AngI], or the non-peptide antagonist [3H]-DuP753 (data not shown). These data suggest that these mutations result in a loss of the structural integrity necessary for peptide and non-peptide binding or that they are not expressed at the membrane. Latter experiments (see below) show that co-expression of these two receptor mutants results in normal ligand binding. It is therefore concluded that these mutants are expressed at the cell surface and that Lys102 and Lys199 are essential for the AT1a binding site. Two previous studies also describe the important role of Lys102 and Lys199, respectively (14, 15). In the case of Lys102, it was proposed that its substitution would provoke an overall alteration in receptor structure in view of its position at the neighboring disulfide bridge. Our co-expression study indicates that substitution of this residue does not cause a global alteration in receptor structure as protein complementation can occur to produce chimeric receptors, functional in binding AngI. Thus, it seems likely that Lys102 represents an overlapping point in binding site for peptide (AngI and [Sar1]-AngI) and non-peptide ligands (DuP753). A similar argument can be made for Lys199. Since substitution of this residue with Glu causes a major decrease in affinity for AngI (14) and substitution with Ala provokes the
complete abolition of AngII and DuP753 binding, we propose that Lys199 is required for ionic interaction with carboxy-terminal COOH of AngII as well as with the acidic group of the tetrazole of DuP753.

Five single point mutants in the TM-III, S105A, S107A, S109A, N111A, and S115A, recognized ¹²⁵I-[Sar¹]AngII with $K_d$ values similar to those of the wild-type receptor (Table I). The pharmacology of these five mutants for different peptidic or non-peptidic ligands was then analyzed (Table II). All of these mutants exhibited AngII and [Sar¹,Ala⁸]AngII binding affinities that did not markedly differ from those of the wild-type receptor. However, some differences are observed in the binding affinity of the pseudopeptidic CGP42112A or non-peptidic DuP753 compounds, specific for AT₂ and AT₁ receptors, respectively, for the N111A and S115A mutants. N111A showed a significant increase (8-fold) in affinity for CGP42112A and a significant decrease (45-fold) in affinity for DuP753. S115A had an affinity for the DuP753 similar to the wild-type but a significantly increased affinity for CGP42112A (7-fold).

These results indicate that the polar residues Ser105, Ser107, and Ser109 are not essential for the binding of peptidic or non-peptidic ligand to the AT₁A receptor. The absence of a major role for Ser¹⁰⁷ in the binding of [Sar¹,Ala⁸]AngII and DuP753 has been demonstrated previously (17). This Ser residue is conserved in mammalian AT₁ receptors, but it is replaced by an alanine in the amphibian AngII receptor. Our data suggest that it is not responsible for the specific pharmacological profile observed for the amphibian receptor, which recognizes peptidic but not non-peptidic antagonists. Furthermore, our results demonstrate that this residue is not a contributor to the binding of either peptidic agonists, or of the specific AT₁/AT₂ non-peptidic ligands. In contrast, these studies stress the importance of the residues Asn¹¹¹ and Ser¹¹⁵ in binding the specific AT₁/AT₂ ligands. These results could be related to two previous reports; the substitution of Asp⁷⁴, present in TM-II, by an asparagine (16) and the substitution of the Tyr²⁹², present in TM-VII, by a phenylalanine (18) provoke a lower affinity for DuP753 and a higher affinity for CGP42112A. Despite their locations on different transmembrane domains, these 4 residues are probably positioned within a small distance of each other in the plasma membrane, suggesting that Asp⁷⁴, Asn¹¹¹, Ser¹¹⁵, and Tyr²⁹² could belong to a specific AT₁/AT₂ ligand binding site. Molecular modeling studies suggest that these residues lie in a plane that is three or

**FIG. 1. Bidimensional representation of the rat AT₁A receptor amino acid sequence.** Gray circles indicate the residues replaced by alanine using site-directed mutagenesis. Black circles indicate the deleted residues. The amino-terminal sequence Met¹-Ala²-Leu³ ...Tyr²⁶-Ile²⁷ ...Phe²⁸ was replaced by Met-Ala-Cys-Tyr-Ile-Phe. The sequence of the second extracellular loop, Ile¹⁶⁵-His¹⁶⁶-Arg¹⁶⁷ ...Ser¹⁸⁹-Thr¹⁹⁰-Leu¹⁹¹ was replaced by Ile-His-Arg-Cys-Seth-Leu. Numbering is according to Ref. 3.

**TABLE I**

| Receptor | Cell type | $K_d$ (nM) | $B_{max}$ sites/cell |
|----------|-----------|-----------|----------------------|
| Wild type | COS       | 1.33 ± 0.30 | 433,827 ± 91,586     |
|           | CHO       | 1.17 ± 0.12 | 155,349 ± 25,023     |
| K102A     | COS       | No detectable specific binding |
| S105A     | COS       | 1.74 ± 0.35 | 604,602 ± 93,918     |
| S107A     | COS       | 0.75 ± 0.19 | 128,843 ± 24,450     |
| S109A     | CHO       | 0.58 ± 0.08 | 39,405 ± 1,118       |
| S110A     | COS       | 1.17 ± 0.23 | 493,908 ± 127,951    |
| N111A     | COS       | 0.73 ± 0.20 | 117,028 ± 28,565     |
|           | CHO       | 2.51 ± 0.98 | 29,307 ± 1,914       |
| S115A     | COS       | 1.35 ± 0.57 | 112,546 ± 39,930     |
| K199A     | COS       | No detectable specific binding |

Angiotensin AT₁A Receptor Mutants
Angiotensin AT₁A Receptor Mutants

Table I
Affinity constants (Kᵢ) of wild-type and mutant receptors for AngII agonists and antagonists
Data represent the means ± S.E. obtained from three to five separate experiments in which each point is performed in duplicate.

|                  | Wild type | S105A | S107A | S109A | N111A | S115A |
|------------------|-----------|-------|-------|-------|-------|-------|
| Kᵢ (nM)          |           |       |       |       |       |       |
| AngII            | 4.59 ± 0.74 | 4.25 ± 1.32 | 2.40 ± 0.43 | 3.48 ± 0.50 | 1.29 ± 0.71 | 6.70 ± 3.03 |
| [Sar¹,Ala⁸]AngII | 1.98 ± 0.36 | 3.37 ± 1.22 | 1.26 ± 0.27 | 1.90 ± 0.53 | 1.27 ± 0.51 | 1.29 ± 0.33 |
| DuP753           | 2.36 ± 0.39 | 4.00 ± 1.46 | 2.46 ± 1.04 | 1.08 ± 0.24 | 107 ± 37*  | 6.08 ± 1.85 |
| CGP42112A        | 3727 ± 912  | 4010 ± 662  | 2686 ± 793  | 4018 ± 858  | 471 ± 184* | 522 ± 164* |

* p < 0.05 versus wild-type.

Table II
Affinity constants (Ki) of wild-type and mutant receptors for AngII agonists and antagonists
Data represent the means ± S.E. obtained from three to five separate experiments in which each point is performed in duplicate.

|                  | Wild type | S105A | S107A | S109A | N111A | S115A |
|------------------|-----------|-------|-------|-------|-------|-------|
| Ki (nM)          |           |       |       |       |       |       |
| AngII            | 4.59 ± 0.74 | 4.25 ± 1.32 | 2.40 ± 0.43 | 3.48 ± 0.50 | 1.29 ± 0.71 | 6.70 ± 3.03 |
| [Sar¹,Ala⁸]AngII | 1.98 ± 0.36 | 3.37 ± 1.22 | 1.26 ± 0.27 | 1.90 ± 0.53 | 1.27 ± 0.51 | 1.29 ± 0.33 |
| DuP753           | 2.36 ± 0.39 | 4.00 ± 1.46 | 2.46 ± 1.04 | 1.08 ± 0.24 | 107 ± 37*  | 6.08 ± 1.85 |
| CGP42112A        | 3727 ± 912  | 4010 ± 662  | 2686 ± 793  | 4018 ± 858  | 471 ± 184* | 522 ± 164* |

* p < 0.05 versus wild-type.

Table III
Parameters of AngII-induced stimulation of inositol phosphate production mediated by wild-type and mutant receptors expressed in COS-7 or CHO cells
Data represent the means ± S.E. obtained from four to six separate experiments.

|                  | Wild type | S105A | S107A | S109A | N111A | S115A |
|------------------|-----------|-------|-------|-------|-------|-------|
| E₅₀ (nM)         |           |       |       |       |       |       |
| COS              | 0.26 ± 0.09 | 0.44 ± 0.18 | 0.30 ± 0.14 | 0.35 ± 0.20 | 0.40 ± 0.23 | ND    |
| CHO              | 152 ± 55  | 225 ± 75  | 353 ± 132  | 196 ± 68  | 122 ± 18  | 18 ± 8* |

* p < 0.05 versus wild-type. ND, not detectable.

Angiotensin AT₁A Receptor Mutants

Fig. 2. AngII-induced stimulation of total inositol phosphate production in COS cells expressing wild-type or mutant AT₁A receptors. Dose-response curves were performed for the wild-type (□), S105A (●), S107A (△), S109A (▲), N111A (☐), and S115A (○) AT₁A receptors. The results are expressed as the ratio of the [³H]IP fraction (counts/min) derived from cells after exposure to agonist versus those obtained from cells exposed to buffer alone. Data points represent the mean ± S.E. of three independent experiments carried out in duplicate.

four α-helical turns below the membrane surface and therefore buried deep in the lipid bilayer.

Residues Involved in G-protein Coupling and Mechanisms of Receptor Activation—Agonist binding to the AT₁ receptor leads to the activation of PLC, which hydrolyzes a membrane phospholipid (phosphoinositide diphosphate) and produces two second messengers, IP₃ and diacylglycerol. Therefore, the efficiency of AT₁ coupling and signaling can be estimated by measuring the increased production of either specific IP₃ or total IP in response to increasing concentrations of agonist. It is generally accepted that the intensity of the maximal IP response to agonist (Eₘₐₓ) is dependent on the number of binding sites at the surface of the cells and that the half-maximal response is obtained with an agonist concentration (EC₅₀) similar to the Kᵢ of the agonist (34). Consequently, the coupling efficiency of different AT₁A mutants can be estimated and compared using the EC₅₀ and the ratio Eₘₐₓ/Bₘₐₓ. Using these parameters, coupling of S105A, S107A, S109A, N111A, and S115A AT₁A receptors was compared with that of the wild-type AT₁A, after transient expression in COS-7 cells. No detectable IP response was observed in non-transfected COS-7 cells (data not shown), whereas a dose-dependent stimulation of IP production was measured in cells expressing the wild-type, the S105A, S107A, S109A, and N111A mutants. The half-maximal response (EC₅₀) of these different AT₁A receptors was obtained with AngII concentrations varying between 0.26 and 0.44 nM (Table III), which are in a similar range to the corresponding Kᵢ values of AngII for these receptors (Table I).
The maximal stimulation of IP production (Fig. 2) is similar to the wild-type receptor for S105A and S109A and corresponds to a 6-fold increase above the basal production of total IP. These results show a functional coupling for these two mutants. In contrast, the maximal stimulation of IP production by the S107A and N111A mutants (Fig. 2) is lower (2-3-fold increase above the basal production), but these receptors were expressed at lower levels in COS-7 cells (1.1–1.3 x 10^5 sites/cell) than the wild-type or other mutants (4.3 to 6.0 x 10^5 sites/cell). Similarly, after stable expression in CHO cells, the expression level was also reduced by 5- or 6-fold for the S107A and N111A mutants as compared to the wild-type receptor (Table I). However, the ratio Emax/Bmax x 10^6 sites and the E50 value were similar for the two mutants as compared to the wild-type receptor (Table III). These results indicate that the S107A and N111A mutations do not alter the ability of the receptor to couple to G-protein, but probably interfere with the biosynthesis and/or cell surface expression of AT1A.

Mutation of Ser115 results in a dramatic reduction of the ability of the receptor to mediate AngII-induced IP formation, despite the fact that this receptor is expressed at similar levels to those of the wild-type receptor in CHO cells (Table III). Therefore, this polar residue in TM-III plays a crucial role in agonist-induced activation of the AT1A receptor, responsible for G-protein coupling and signal transduction. Two other polar residues (Asp74 and Tyr292) deeply located in the TM-II and -VII, respectively, have also been shown to be essential for receptor activation and coupling (16, 18). According to a computer model for AT1A receptor activation, Marie et al. have proposed that the carboxylate group of Asp74 and the hydroxyl group of Tyr292 are linked by an hydrogen bond. Further analysis of the polar residues interaction within the seven transmembrane segments by computer modeling permits the development of an hypothetical model for the role of these 3 residues (Asp74, Ser115, and Tyr292) in receptor activation. In the absence of ligand, Tyr292 and Asp74 could be linked by an hydrogen bond. This interaction could be displaced by binding of AngII or other agonist, resulting in the formation of a new hydrogen bond between Tyr292 and Ser115, required for the receptor activation and therefore G-protein coupling. This hypothetical model needs to be confirmed experimentally.

Co-expression of Different Mutants—Using different AT1A mutants, we investigated the intermolecular interactions of AT1A receptors by co-expression experiments. It has been proposed that the transmembrane domains of multiple helix proteins such as G-protein-coupled receptors, are independently folded and stable in the lipid bilayer and assembe between each other in a second stage to form their classical tertiary structure (22). Several lines of experimental evidence indicate that formation of this functional tertiary structure does not necessarily involve the transmembrane domains of a single molecule, but can be achieved using either complementary fragments of truncated receptors, or two complementary domains from two independent but intact molecules of protein (22–24). One example of such functional complementation be-

### Table IV

| Receptor          | Kd (nM) | Bmax (pmol sites/cell) |
|-------------------|---------|-----------------------|
| Wild type         | 2.44 ± 0.75 | 632,537 ± 156,218     |
| D74N              | 0.78 ± 0.12 | 166,590 ± 21,172      |
| D74N/S115A        | 0.95 ± 0.28 | 85,623 ± 7,641        |
| K102A/K199A       | 3.62 ± 1.39 | 29,039 ± 5,783        |

### Table V

| Receptor          | K102A/K199A |
|-------------------|-------------|
| AngII             | 3.21 ± 0.60 |
| [Sar1,Ala8]AngII  | 0.92 ± 0.23 |
| DuP753            | 1.88 ± 0.86 |
| CGP42112A         | 1968 ± 752  |

Fig. 3. Effect of co-transfection of varying K102A/K199A DNA ratios on receptor density. Data represent the means ± S.E. obtained from at least two separate experiments in which each point is performed in duplicate. *, p < 0.05 versus 10/40 and 40/10 ratios of K102A/K199A DNA (µg).
formed by protein complementation within the cell membrane.

As the K102A and K199A mutants, two deletion mutants Δ(3–25) and Δ(168–188) are unable to bind the peptidic agonists \( ^{125}\text{I}-[\text{Sar}^1]\text{AngII} \) and \( ^{3}\text{H}\text{AngII} \), or the non-peptidic antagonist \( ^{3}\text{H}\text{DuP753} \) (data not shown). Co-expression of all four \( \text{AT}_{1A} \) mutants in different paired combinations was performed to determine whether intermolecular complementation could be observed for this peptide hormone receptor. COS-7 cells co-transfected with the two deletion mutants Δ(3–25)/Δ(168–188) did not display any specific binding for \( ^{125}\text{I}-[\text{Sar}^1]\text{AngII} \) (data not shown). In contrast, co-transfection of COS-7 cells with K102A/K199A resulted in the appearance of specific binding sites for the \( ^{125}\text{I}-[\text{Sar}^1]\text{AngII} \) (Table IV). These sites displayed ligand binding properties similar to those of the wild-type receptor in terms of \( K_i \) and specificities for different ligands (Table V). Furthermore, the maximum number of binding sites detected with \( ^{125}\text{I}-[\text{Sar}^1]\text{AngII} \) depends on the K102A/K199A ratios (Fig. 3).

These results demonstrate that the two single-point mutants K102A and K199A are expressed at the cell surface and are able to complement each other, whereas their individual expression in COS-7 cells does not result in functional receptors.

The increased number of binding sites is correlated to the increase of the quantity of the two plasmid DNA, indicating that the appearance of these binding sites requires the presence of the two deficient mutants in equimolar amount. This protein complementation results in the restoration of a binding site similar to that of the wild-type, since it interacts with peptidic agonists and antagonists as well as with non-peptidic compounds. In contrast, there was no restoration of a ligand binding site when the two deletion mutants Δ(3–25) and Δ(168–188) were co-expressed together, or with either the K102A or K199A mutants (data not shown).

Co-expression of the K102A/K199A mutants did not result in activation of IP production (data not shown). Similarly, no IP response was detected when co-expression experiments were performed with mutant receptors (D74N and S115A) that are unable to mediate stimulation of IP production, although both have been shown to bind AngII peptides with wild-type affinities (Ref. 16 and the present report, respectively).

Because of the unexpected and somewhat provocative nature of these results, it was necessary to verify that no homologous recombination had occurred between the transfected cDNAs. This specific point was analyzed using three \( \text{AT}_{1A} \) cDNA constructs containing additional heterologous sequences inserted at either the 5’ or 3’ end or at both ends of the coding sequence (Fig. 4A). Individually expressed, these constructs produce functionally normal \( \text{AT}_{1A} \) receptors (data not shown). Expression and co-expression of these three constructions in COS cells allowed the analysis of the RNA populations by RT-PCR using pairs of oligonucleotides located either in the 5’ (primer 1) or 3’ (primer 4) insertions or in the 5’ or 3’ part of the \( \text{AT}_{1A} \) coding sequence (primers 2 and 3). RT-PCR products with primers 1 and 4 are obtained from the DNA template derived from COS cells transfected with the construction Ins[Nter-Cter] but not obtained with RNA isolated from COS cells co-transfected with the constructions Ins[Nter] and Ins[Cter] (Fig. 4B)). Thus, no homologous recombination events were detected during co-transfection of two similar constructions. The fact that no functional receptor was observed when the two deletion mutants Δ(3–25) and Δ(168–188)) were co-expressed is further evidence that homologous recombination does not occur between co-transfected cDNAs.

These results show that \( \text{AT}_{1A} \) receptors may be able to physically interact with each other to create binding sites similar to those of the wild-type. These binding sites may account for up to 5% of the total receptor molecules, assuming that the number of mutant receptors that reach the cell surface is the same as the wild-type \( \text{AT}_{1A} \) density in the same experiments (Table IV). The inability of these “reconstituted” receptors to transduce a signal suggests either that the number of functional molecules is insufficient for the response to be detected or that the conformation of these “chimeric” molecules is not optimal for G-protein coupling. Taken together, these experiments strongly suggest that de novo production of an AngII binding receptor from two functionally defective mutants is secondary to protein-protein interactions amongst transmembrane domains at the cell surface.

In conclusion, the present report identified a role for charged residues located in the external part of TM-III and TM-V (Lys\(^{102}\) and Lys\(^{199}\)) in AngII binding. Moreover, these residues and others buried deep in TM-III (Asn\(^{111}\) and Ser\(^{115}\)) are involved in the binding site of non-peptidic analogs. In addition to the Tyr\(^{292}\) and Asp\(^{34}\) residues, Ser\(^{115}\) is required for receptor activation. Finally, co-expression of these different \( \text{AT}_{1A} \) mutants has permitted the first demonstration of intermolecular complementation amongst peptide hormone receptors.
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