Identification and Function of Disulfide Bridges in the Extracellular Domains of the Angiotensin II Type 2 Receptor†

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ABSTRACT: The angiotensin II (AngII) receptor family is comprised of two subtypes, type 1 (AT_1) and type 2 (AT_2). Although sharing low homology (only 34%), mutagenesis has identified some key residues that are conserved between both subtypes, including four extracellular cysteines. Previous AT_1 mutagenesis demonstrated that the cysteines form two disulfide bonds, one linking the first and second extracellular loops and another connecting the amino terminus to the third extracellular loop. The importance of these AT_1 disulfides in ligand binding is supported by the effect of dithiothreitol (DTT). DTT breaks disulfide bonds, thereby strongly inhibiting ligand binding in AT_1 receptors. Despite retaining the same cysteines, AT_2 receptor ligand binding is paradoxically enhanced by DTT. Thus, we constructed a series of AT_2 cysteine mutations, either individually or paired, to establish the role of the cysteines and the source of DTT's effects. The AT_2 cysteine mutants surprisingly confirmed that the cysteines form disulfide bonds in the same manner as in the AT_1 subtype. However, breaking the AT_2 disulfide bridges yielded two responses. As in AT_1 receptors, mutations disrupting the disulfide bond between the first and second extracellular loops reduced AT_2 binding by 4-fold. In contrast, mutations breaking the disulfide bridge between the amino terminus and the third extracellular loop increased AT_2 binding, mimicking DTT's effect on this subtype. Further analysis of AT_1/AT_2 chimeric exchange mutants of these domains suggested that the AT_2 amino terminus and third extracellular loop may possess latent binding epitopes that are only uncovered after DTT exposure.

Angiotensin II (AngII)[1] is an octapeptide involved in body fluid homeostasis. Its behavioral and physiological effects include increased salt appetite, increased thirst, and a pressor response. The peptide exerts its actions by binding to cell surface receptors (*1*). At least two main subtypes of AngII receptors, referred to as type 1 (AT_1) and type 2 (AT_2), have been identified. Although the two subtypes bind AngII with identical affinities (3−5 nM), development of subtype-selective ligands has aided in the pharmacological and functional analyses of these distinct receptors (*2*). The AT_1 receptor binds the AT_1-specific nonpeptide antagonist losartan with high affinity, whereas the AT_2 receptor binds with high affinity the AT_2-specific ligands PD123319 and CGP42112A. With the cloning of these subtypes (*3*−*6*), more detailed structural information on these receptors has become available. Interestingly, although the two subtypes bind AngII with identical affinities, they share a relatively low 34% amino acid homology.

Use of molecular biological techniques to introduce specific mutations into proteins has proven to be an invaluable research strategy in the analysis of protein structure and function. With respect to GPCRs, this strategy has been extensively used to map the structural elements that define ligand binding, G-protein coupling, and receptor activation of several receptor systems. In the study of AngII receptors, mutagenesis experiments have been conducted primarily on the AT_1 subtype. Thus far, these studies have identified several AT_1 binding epitopes for AngII and its related analogues (*7*−*13*), as well as for the AT_1-selective antagonist losartan (*14*). In addition, other investigators have mapped critical residues and protein regions that are important in receptor activation and G-protein coupling for this subtype (*15*−*20*). The mutational data have led to several computer models of how AngII and its related ligands bind to the AT_1 receptor (*9, 21*). In contrast, analogous information on the AT_2 subtype has been lacking. Moreover, since the two subtypes share a relatively low level of homology, the extent to which the AT_1 mutagenesis and modeling data are applicable to the AT_2 subtype remains equivocal. While it may be expected that conserved residues explain shared receptor properties, namely, the binding of AngII and its analogues, our previous work has demonstrated that the binding of AngII is much more complex. While the receptors do share some common AngII binding epitopes, other contact sites that are unique to each subtype also must exist (*22*−*25*).

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[1] Abbreviations: AngII, angiotensin II; AT_1 receptor, angiotensin II type 1 receptor; AT_2 receptor, angiotensin II type 2 receptor; DTT, dithiothreitol; SARILE, [Sar[1],Ile[8]]angiotensin II; GPCR, G-protein-coupled receptor; NT, amino terminus; ECL3, third extracellular loop; SOE, splicing by overlap extension.

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An aspect of the AT_1 receptor that has been examined by mutagenesis involves the four extracellular cysteine residues, one in the amino terminus and one in each of the three extracellular loops. When the cysteine residues were mutated, either individually or in combination, ligand binding to the AT_1 receptor diminished by 10-fold (*8*, *10*). Results obtained from the paired cysteine mutants led to the assignment of two disulfide bridges connecting these cysteine residues: one bridge between the first and second extracellular loops and a second bond between cysteines in the amino terminus and the third extracellular loop. Indeed, these mutational data seem to provide the molecular basis for the observed inhibition of ligand binding in AT_1 receptors by the reducing agent dithiothreitol (DTT). DTT disrupts disulfide bridges, therefore breaking the putative AT_1 disulfides and rendering the receptor unable to bind AngII (*10*). Surprisingly, while these four extracellular cysteine residues are conserved in the AT_2 receptor subtype, the presence of DTT paradoxically enhances the affinity of this subtype for AngII (*5*, *6*, *26*). This suggested differences in the manner that the conserved cysteine residues contribute toward binding activity in the two subtypes. We sought to more clearly identify the role of these homologous cysteine residues in the AT_2 subtype and investigate the basis of the unique AT_2 response to DTT. To address these issues, a series of AT_2 point mutations of the key cysteines, either individually or paired, as well as a series of AT_1/AT_2 receptor chimeras were generated. Their ligand binding properties and the response to DTT were then analyzed.

EXPERIMENTAL PROCEDURES

Materials. Monoiodinated ^{125}I-AngII was obtained from Amersham Corp. (Arlington Heights, IL). Unlabeled AngII and related peptides, HEPES, aprotinin, 1,10-*o*-phenanthroline, and poly(ethylenimine) (PEI) were from Sigma Chemical Co. (St. Louis, MO). All other chemicals and reagents were purchased from Fisher Scientific (Pittsburgh, PA) and were of the highest obtainable grade.

Mutagenesis Techniques. All mutations, including the substitutions of Cys^{35}, Cys^{117}, Cys^{195}, and Cys^{290} to alanine in the AT_2 receptor and all AT_1/AT_2 chimeric receptors, were achieved by a modified version of the splicing by overlap extension (SOE) technique (*27*). This procedure involved using the polymerase chain reaction (PCR) in two steps: generation of individual fragments followed by splicing of the fragments. Briefly, the two fragments were first amplified by PCR using specially designed complementary and overlapping primers that introduced the desired mutation. The two fragments were then used along with distal primers in a PCR reaction to produce the final product. As a refinement to enhance the fidelity of SOE, a small amount of Pfu DNA polymerase (1:100 Pfu:Taq) was added to the PCR reactions.

Either wild-type AT_1 or AT_2 cDNA, which have previously been isolated from the murine neuroblastoma N1E-115 cell line (*26*, *28*), served as the template in these PCRs depending on the appropriate primers. Primers used to construct the AT_2 cysteine point mutants are as follows: AT_2-C35A mutant (forward primer = 5′-CGCCTTTAATGCCTCACACAAACCATC-3′ and reverse primer = 5′-GTTTGTGTGAGGCATTAAAGGCGGACTC-3′), AT_2C117A mutant (forward primer = 5′-ACCTGTGATGGCCAAAGTGTTTGGTTC-3′ and reverse primer = 5′-CAAACACTTTGGCCATCACAGGTCCAAA-3′), AT_2C195A mutant (forward primer = 5′-TGTGAATGCTGCTATTATGGCTTTCCC-3′ and reverse primer = 5′-AGCCATAATAGCAGCATTCACACCTAA-3′), and AT_2C290A mutant (forward primer = 5′-CATTAATAGCGCTGAAGTTATAGCAGTC-3′ and reverse primer = 5′-CTATAACTTCAGCGCTATTAATGATACC-3′). The $[AT_1NT]AT_2$ chimera was constructed using the forward primer 5′-CACAGTTACATATTGGAAGCAATTCCTGTTCTC-3′ and the reverse primer 5′-TGCTTCCAATATGTAACTGTGCCTGCCAGC-3′. The $[AT_1$-ECL3$]AT_2$ chimera was made using the SOE procedure in three steps. First, the AT_1 receptor sequence from the third extracellular loop to its carboxyl tail was added to an AT_2 receptor using the primers 5′-GACCTTCTTGGATGTGCTGATTCAGCTGGG-3′ (forward primer) and 5′-CAGCACATCCAAGAAGGTCAGAAC-3′ (reverse primer). The protein region from the seventh transmembrane domain to the cytoplasmic tail was then substituted with the AT_1 receptor sequence using primers 5′-CGTGGACACTGCACTTCCTTTTGCCATCC-3′ (forward primer) and 5′-GGAAGTGCAGTGTCCACGATGTCG-3′ (reverse primer). The $[AT_2$-ECL3$]AT_1$ chimera was also made in three steps. First, the AT_2 receptor sequence from the third extracellular loop to its carboxyl tail was added to an AT_1 receptor using the primers 5′-CACATTCCTGGATGCTCTGACCTGGAT-3′ (forward primer) and 5′-CAGAGCATCCAGGAATGTGAATATTT-3′ (reverse primer). The protein region from the seventh transmembrane domain to the cytoplasmic tail was then substituted with the AT_1 receptor sequence using primers 5′-CATTGACCTGGCCATGCCCATAACCATC-3′ (forward primer) and 5′-GGGCATGGCCAGGTCAATGACTGCTAT-3′ (reverse primer). Generation of the AT_2 amino-terminal partial truncation mutant ([del 1−16]AT_2) utilized the primer 5′-GCGGGATCCAAAATGAGCCGTCCTTTTGATAATCTCAACGC-3′ in a single PCR session using wild-type AT_2 cDNA as a template. The AT_2C35A/C290A and AT_2C117A/C195A double point mutants as well as the $[AT_1NT/ECL3]AT_2$ combined chimeric receptor were created by a series of restriction enzyme digests and subsequent ligation of appropriate portions of receptor from other previously generated AT_2 cysteine mutants or AT_1/AT_2 chimeras.

Reaction conditions for all PCR were 30 cycles of 94 °C (1 min), 55 °C (1 min), and 72 °C (1 min). Following purification using Wizard PCR Preps (Promega, Madison, WI), the two fragments were combined in the overlap extension reaction using the same PCR conditions as described above. Following production of the full-length mutant receptors, the cDNA constructs were subcloned into the expression vector pCR3 (Invitrogen, Carlsbad, CA). Complete DNA sequencing for each mutant was then performed in order to verify the desired mutations as well as to ensure that PCR misincorporations did not occur within the cDNA constructs.

Cell Culture Techniques. COS-1 cells were grown on T150 plastic plates in DMEM (high glucose) supplemented with 10% fetal calf serum and 2 mM glutamine, 50 units/mL penicillin, and 50 μg/mL streptomycin in a humidified atmosphere of 5% CO_2 and 95% O_2 at 37 °C.

Cell Membrane Preparation. Wild-type and mutant AngII receptor cDNAs were transiently transfected into COS cells

using LipofectAMINE (GIBCO/BRL) following the manufacturer's protocol. Two days after transfection, cell membranes were prepared from the transfected cells as previously described (*29*). Briefly, medium was removed from culture dishes, and cells were rinsed three times in ice-cold 20 mM Tris-HCl (pH 7.4) and 150 mM NaCl. Cells were then incubated for 10−15 min at 4 °C in 20 mM Tris-HCl (pH 7.4), removed with a rubber policeman, and homogenized with a Dounce homogenizer. Following centrifugation at 48000*g* for 20 min, the membrane pellet was washed once in assay buffer, a solution of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM $MgCl_2$, 0.2% heat-inactivated BSA, 0.3 TIU/mL aprotinin, and 100 μg/mL 1,10-phenanthroline. Following a second centrifugation at 48000*g* for 20 min, the final membrane pellet was resuspended in assay buffer at a protein concentration of 1 mg/mL as determined by the BCA protein assay (Pierce, Rockford, IL).

Radioligand Binding Assays. Radioligand binding assays were performed as described previously (*29*). In brief, the binding assays were initiated by the addition of 100 μL of membrane protein (30 or 50 μg) to 150 μL of assay buffer containing various concentrations of radioligand (^{125}I-AngII) and unlabeled AT_2 receptor agonists and antagonists in the presence and absence of 10 mM DTT. Saturation isotherms used at least six concentrations of ^{125}I-AngII, ranging from 0.2 to 6.0 nM. Competition assays used 0.68 nM ^{125}I-AngII and unlabeled competitor in concentrations ranging from 10^{-11} to 10^{-6} M. The incubations continued for 60 min at 25 °C and were terminated by rapid dilution with 5 mM Tris-HCl (pH 7.4) and 150 mM NaCl and vacuum filtration on glass-fiber filters presoaked with 0.3% PEI. The glass-fiber filters were then counted in an LKB γ counter (counting efficiency of 60%). Specific binding was defined as binding in the presence of 1 μM unlabeled AngII.

Inositol Trisphosphate Assay. Transfected COS cells were loaded with [^{3}H]inositol (4.5 μCi/mL D-MEM) for 18 h prior to assay. Transfected cells were then stimulated with agonist for 30 s, rinsed once with ice-cold phosphate-buffered saline, and then rapidly lysed in 1 mL of 10% trichloroacetic acid. Insoluble materials were pelleted at 16000*g*. The pellets were solubilized in 500 μL of 1% sodium dodecyl sulfate in 0.1 M NaOH for protein quantification. The supernatant from each lysate was extracted five times with 2 volumes of water-saturated ether. Following the final extraction, the aqueous layers were neutralized by addition of sodium bicarbonate and EDTA to final concentrations of 6 and 15 mM, respectively. The aqueous supernatants were added to 1 mL AG1-X8 anion-exchange resin columns (Bio-Rad Labs, Hercules, CA), and inositol phosphates were separated by stepwise elution with increasing concentrations (0−1 M) of ammonium formate in 0.1 M formic acid (*30*). The amount of IP_3 eluted from each column was quantitated by liquid scintillation counting in Tru-Count scintillation cocktail (IN/US Systems, Inc., Tampa, FL).

Data Analysis. All data were analyzed using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA). The results are presented as means ± standard error. Statistical analysis was performed with the aid of SuperANOVA software (Abacus Concepts, Berkeley, CA). ANOVA was performed on the binding data, and the Student−Newman−Keuls test was used as a post hoc test. Unless specifically noted, the significance level was set at $P < 0.01$.

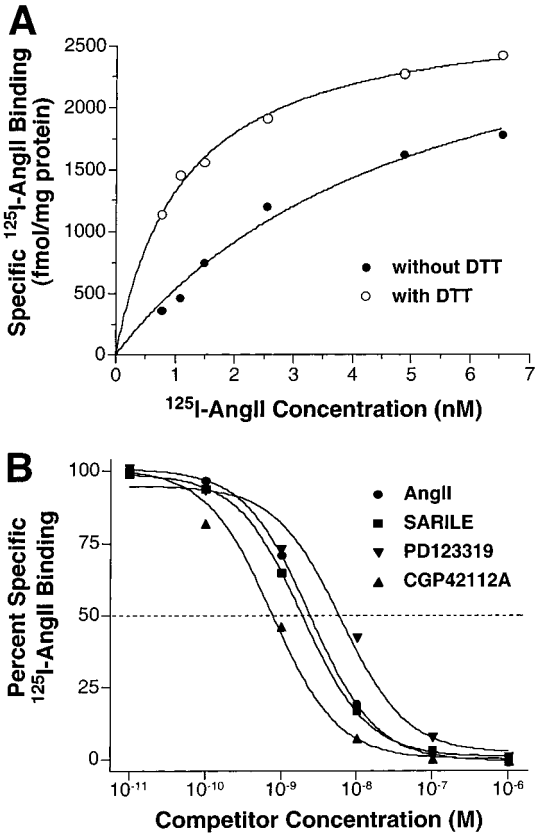

FIGURE 1: Representative binding curves for the angiotensin II type 2 (AT_2) receptor. Using radioligand binding assays with ^{125}I-AngII, extensive pharmacological characterizations were conducted for the AT_2 receptor and for each mutant receptor. (A) A representative saturation isotherm is shown for the wild-type AT_2 receptor. The binding data were subjected to the curve fitting using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA) in order to determine affinity (K_d) and expression level (B_{max}) values. Similar procedures were used to generate the pharmacological data for all receptors shown in Tables 1 and 3. (B) A representative competition analysis is shown for the wild-type AT_2 receptor. Unlabeled competitors AngII, SARILE, CGP42112A, and PD123319 were used to displace ^{125}I-AngII binding. Using GraphPad Prism software, the binding data were analyzed to determine the respective K_i values for each competing ligand. Similar procedures were used to generate the pharmacological data for all receptors shown in Table 2.

RESULTS

To investigate the binding properties of the AT_1 and the AT_2 receptor subtypes, saturation binding analyses were conducted for the two receptors using ^{125}I-AngII in the presence and absence of 10 mM DTT. Representative saturation isotherms for the AT_2 receptor, without and with the DTT treatment, are shown in Figure 1A. The AT_1 and AT_2 receptors exhibited dramatic differences in AngII binding in the presence of DTT (Table 1); specific binding was completely abolished in the AT_1 receptor by the addition of DTT, whereas the AT_2 receptor displayed a significant ($P < 0.01$), nearly 4-fold increase in its affinity for AngII in the presence of this reducing agent. Within the AT_1 receptor, four extracellular cysteines have been shown to form two sets of disulfide bridges. DTT's disruption of these disulfide bonds has been hypothesized to be the basis of AT_1's decreased ligand binding by this reducing agent. Interestingly, these same four extracellular cysteines are also conserved in the AT_2 subtype. To evaluate the role of these

Table 1: Binding Affinity (K_d) of Angiotensin II for Wild-Type and Mutated Angiotensin II Type 2 Receptors[a]

receptor	K_d (AngII)	K_d (AngII + 10 mM DTT)
AT_1 wild type	5.3 ± 1.4 ($n = 3$)	no specific binding ($n = 3$)
AT_2 wild type	3.4 ± 0.4 ($n = 8$)	0.9 ± 0.7 ($n = 7$)[b]
AT_2C35A	1.8 ± 0.3 ($n = 6$)	0.6 ± 0.1 ($n = 5$)[b]
AT_2C117A	13.7 ± 0.7 ($n = 3$)[c]	1.0 ± 0.3 ($n = 3$)[b]
AT_2C195A	12.0 ± 2.1 ($n = 3$)[c]	3.4 ± 0.4 ($n = 3$)[b,d]
AT_2C290A	2.0 ± 0.4 ($n = 7$)	0.8 ± 0.2 ($n = 4$)

[a] Radioligand binding was measured as described in Experimental Procedures with 30 μg of AT_2C35A or AT_2C290A or 50 μg of AT_2C117A or AT_2C195A mutant or 20 μg of wild-type membrane protein and ^{125}I-AngII (ranging from 0.2 to 6 nM) in the presence and absence of 10 mM dithiothreitol (DTT). Nonspecific binding was determined in the presence of 1 μM unlabeled AngII. Data are presented in nanomolar as the means ± standard error. The number of independent experiments is shown in parentheses. [b] DTT had a significant effect on binding affinities (K_d), $P < 0.01$. [c] Cysteine mutation had a significant effect on binding compared to wild-type AT_2, $P < 0.01$.[d] A significant difference in binding affinity after comparing all K_d's in the presence of DTT ($P < 0.01$).

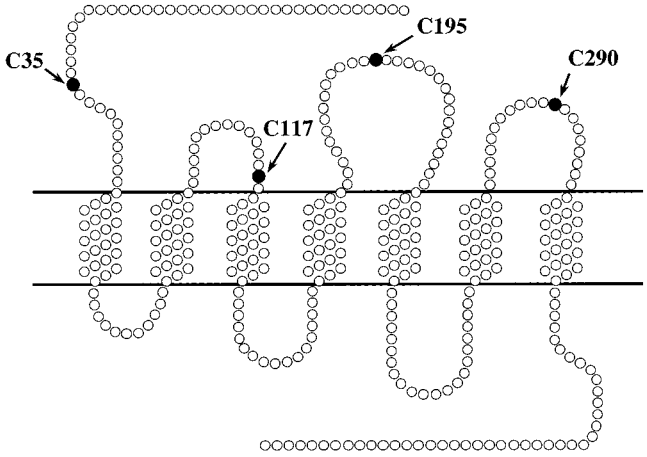

FIGURE 2: Location of the four conserved extracellular cysteines within the AT_2 receptor. Four extracellular cysteine residues (Cys^{35}, Cys^{117}, Cys^{195}, and Cys^{290}) are conserved in the AngII receptor family and their locations within the AT_2 receptor are shown highlighted in black. Alanine substitutions of these cysteines were created as described in Experimental Procedures.

cysteine residues (Cys^{35}, Cys^{117}, Cys^{195}, and Cys^{290}) with respect to AT_2 receptor binding and this subtype's unique DTT sensitivity, point mutations were constructed by substituting each of the four cysteines with alanines (Figure 2). Binding affinities for ^{125}I-AngII in the presence and absence of 10 mM DTT were then determined by saturation binding analysis for each of the four AT_2 cysteine mutants and compared to the wild-type receptors. The alanine substitutions resulted in two distinct responses: mutations of either Cys^{117} or Cys^{195} resulted in significant, 4-fold decreases in AngII affinity, whereas substitutions of either Cys^{35} or Cys^{290} produced receptors that exhibited small, albeit nonsignificant, 2-fold increases in AngII binding affinities (Table 1). For all four cysteine point mutants, the presence of DTT further enhanced AngII binding, but the extent of this increase varied. The addition of DTT increased AngII binding in mutants of Cys^{35}, Cys^{117}, and Cys^{290} to K_d values near that of DTT-treated wild-type receptor. Although the Cys^{195} mutant did exhibit increased AngII binding when subjected to DTT, this DTT-treated mutant still bound AngII nearly 4-fold less efficiently than a similarly treated wild-type AT_2 receptor ($P < 0.01$).

Competition binding analysis was also performed on each single point mutant to determine whether the effects of the cysteine mutations extended to other ligands. Ligands tested include AngII, the peptidic antagonist [Sar^1,Ile^8]angiotensin II (SARILE), the AT_2-selective peptidic agonist CGP42112A, and the AT_2-selective nonpeptide PD123319. A representative competition curve for the wild-type AT_2 receptor is shown in Figure 1B. Overall, the rank order of potency of the four ligands tested remained unchanged by each of the cysteine mutations, indicating that these mutations did not create any large global conformational changes in the ligand binding pocket of the AT_2 receptor (Table 2). However, there were some subtle differences in the effects of the Cys^{35} and Cys^{290} mutations on the binding of the peptidic ligands—AngII, SARILE, and CGP42112A—compared to the binding of the nonpeptide antagonist PD123319. Although the changes were not statistically significant, the observed differences in the mutants compared to the wild-type receptor may provide clues to overall structural differences in the manner that peptides and nonpeptides interact with the AT_2 receptor. Both of the Cys^{35} and Cys^{290} mutants displayed some enhanced, DTT-like affinity for the peptidic ligands compared the wild-type receptor. In contrast, the affinity for the nonpeptide PD123319 was unaffected by these cysteine mutations. Since Cys^{35} and Cys^{290} reside in the amino terminus and the third extracellular loop, respectively, these results further suggested that these extracellular domains may contribute to the unique AT_2 property of enhanced ligand binding upon DTT treatment. Collectively, the saturation binding analyses combined with the competition binding analyses suggest that mutating the extracellular cysteines affects overall ligand binding in the AT_2 receptor and that these residues may be categorized on the basis of similarity of effects into two distinct pairs, with one set comprised of Cys^{35} and Cys^{290} and another consisting of Cys^{117} and Cys^{195}.

One possibility for the two sets of cysteines is that the residues within each set form a disulfide bridge, with Cys^{35} linked to Cys^{290} and Cys^{117} linked to Cys^{195}. Thus, to test this possibility, double cysteine mutants (AT_2C35A/C290A and AT_2C117A/C195A) were formed by splicing the single point mutants into appropriate combinations. Saturation binding analysis with ^{125}I-AngII was then performed to compare the binding properties of the double cysteine mutants to their respective single point mutants as well as to the wild-type AT_2 receptor. If a pair of cysteine residues are involved in a disulfide bond, it is expected that the effect on ligand binding of a double mutation will not be greater than individually mutating each residue of the putative disulfide bond. Alternatively, if the decrease in affinity of such double mutations is cumulative and multiplicative, then it is unlikely that a disulfide bond links the two cysteines. The double mutant AT_2C117A/C195A displayed a similar level of decreased AngII affinity (10.3 ± 1.7 nM, $n = 3$, $P < 0.01$), in agreement with the affinities found for the single point mutants AT_2C117A and AT_2C195A. Similarly, the double mutant AT_2C35A/C290A also showed a comparable, not statistically different, degree of enhanced binding of AngII (2.1 ± 0.8 nM, $n = 5$) in comparison to the single mutant counterparts AT_2C35A and AT_2C290A. These results further support the fact that the AT_2 receptor possesses two

Table 2: Competition Binding Analysis for the Binding of AngII, CGP42112A, PD123319, and SARILE to AT_2 Wild-Type and Cysteine Mutants[a]

competitor	AT_2 wild type	AT_2C35A	AT_2C117A	AT_2C195A	AT_2C290A
AngII	3.7 ± 1.7 ($n = 5$)	1.7 ± 0.8 ($n = 4$)	3.3 ± 1.1 ($n = 4$)	3.7 ± 1.1 ($n = 3$)	1.9 ± 0.5 ($n = 3$)
CGP42112A	0.7 ± 0.1 ($n = 5$)	0.2 ± 0.04 ($n = 3$)	0.7 ± 0.2 ($n = 4$)	1.3 ± 1.1 ($n = 3$)	0.2 ± 0.03 ($n = 3$)
PD123319	7.6 ± 1.0 ($n = 5$)	10.7 ± 1.6 ($n = 3$)	5.0 ± 1.0 ($n = 3$)	7.5 ± 6.2 ($n = 3$)	15.2 ± 1.2 ($n = 3$)
SARILE	1.5 ± 0.6 ($n = 3$)	0.7 ± 0.2 ($n = 5$)	0.9 ± 0.3 ($n = 5$)	3.9 ± 1.1[b] ($n = 3$)	0.7 ± 0.01 ($n = 3$)

[a] Binding of ^{125}I-AngII to cell membranes was determined in the presence of increasing concentrations of unlabeled peptide (AngII, CGP42112A, and SARILE) and nonpeptide (PD123319) ligands as described in Experimental Procedures. The data are expressed as K_i in nanomolar as the means ± standard error. The number of independent experiments is shown in parentheses. [b] K_i for SARILE in AT_2C195A was significantly different compared to the other mutants ($P < 0.01$).

disulfide bonds, one pairing Cys^{35} and Cys^{290} forming a bridge between the amino terminus and the third extracellular loop and another linking Cys^{117} and Cys^{195} connecting the first and second extracellular loops.

Although the results from the AT_2 double point mutants indicated disulfide bond pairings that are identical to those that have been proposed for the AT_1 receptor, the two subtypes exhibit striking differences in their sensitivity to DTT. The basis of the AT_1 and AT_2 differing responses to this reducing agent likely arises from other structural differences. On the basis of the enhanced AngII binding affinity of point mutants AT_2C35A and AT_2C290A, we postulated that elements in either the amino terminus (NT) or the third extracellular loop (ECL3) may be involved in enhancing AngII binding for the AT_2 receptor when disulfide bonds are broken. To test this hypothesis, additional receptor mutants were constructed and their binding affinities for AngII were determined (Figure 3).

Since the AT_2 receptor amino terminus is 16 amino acids longer than that of the AT_1 subtype, a truncation mutation ([del 1−16]AT_2) that deleted the first 16 amino acids of the AT_2 receptor was generated. This yielded an AT_2 receptor with an amino terminus of the same length as the AT_1 subtype. Saturation binding analysis demonstrated that the truncated receptor had an affinity for AngII that was not significantly different than that of wild type. Moreover, binding to the truncated receptor in the presence of DTT was unchanged, indicating that removal of the first 16 amino acids weakened the AT_2 receptor's response to DTT. To further evaluate the possible contribution of the amino terminus to the differential effects of DTT on binding for the two receptor subtypes, a chimeric receptor ([AT_1NT]-AT_2) was constructed that replaced the AT_2 amino terminus with that of the AT_1. As shown in Table 3, this chimeric exchange weakened the binding of AngII to this chimera in the absence of DTT—[AT_1NT]AT_2 exhibited a small, significant reduction in affinity compared to the wild-type AT_2 receptor. Similarly, in the presence of DTT, AngII binding affinity did increase slightly, though the effect was not statistically significant.

On the basis of the binding properties of the AT_2C290A point mutant, the third extracellular loop was another region that may be involved in the increased AngII binding affinity of the AT_2 subtype when subjected to DTT treatment. Consequently, chimeric receptors were constructed that exchanged the third extracellular domains between the two subtypes, generating [AT_1ECL3]AT_2, an AT_2 receptor with an AT_1 third extracellular loop, and its reciprocal chimera [AT_2ECL3]AT_1 (Figure 3). The [AT_1ECL3]AT_2 chimera bound AngII with wild-type affinity in the absence of DTT,

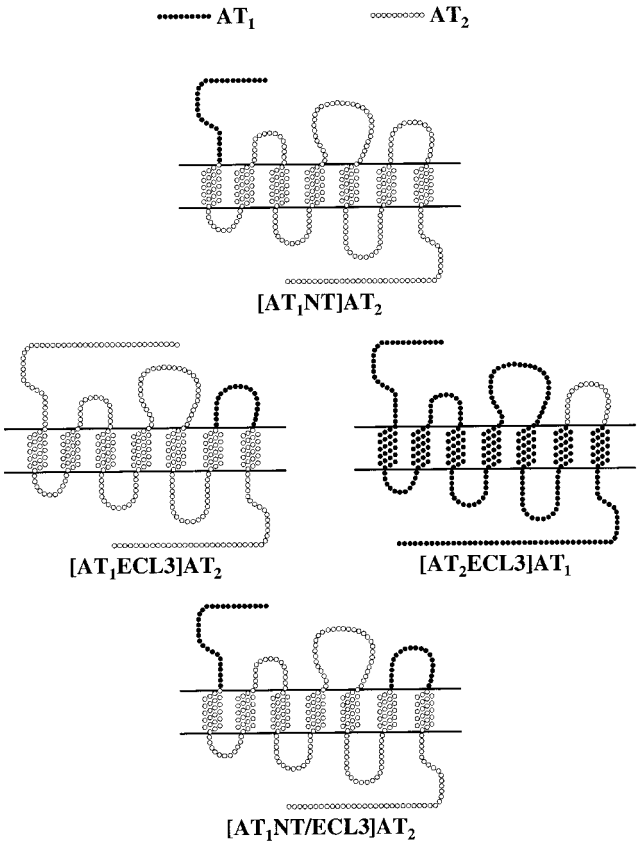

FIGURE 3: Schematic diagram of chimeric AT_1/AT_2 mutants. AT_1/AT_2 chimeric receptors were constructed as described in Experimental Procedures and shown above. Black indicates AT_1 receptor segments, while white represents AT_2 sequences. [AT_1NT]AT_2 is comprised of the AT_1 amino terminus attached to the AT_2 receptor. [AT_1ECL3]AT_2 is an AT_2 receptor containing an AT_1 third extracellular loop. [AT_2ECL3]AT_1 is an AT_1 receptor with an AT_2 third extracellular loop. [AT_1NT/ECL3]AT_2 attaches the AT_1 amino terminus and third extracellular loop onto an AT_2 receptor.

but upon addition of 10 mM DTT this chimera bound AngII with a small, significant decreased in affinity (Table 3). The mirror chimera [AT_2ECL3]AT_1 did show measurable specific binding of ^{125}I-AngII. However, the binding was nonsaturable ($K_d > 100$ nM) and thereby prohibited precise determination of its expression level and affinity for AngII. Because this chimeric receptor consists of predominately AT_1 receptor sequences, especially in the transmembrane and cytoplasmic domains, the [AT_2ECL3]AT_1 chimera retains key AT_1 structural determinants for proper G-protein coupling and receptor activation. Thus, if the chimera was successfully expressed, it would respond appropriately with an agonist to induce inositol trisphosphate (IP_3) production, a signal transduction pathway that is characteristic of the wild-type

Table 3: Binding Affinity of Angiotensin II for Wild-Type and Chimeric Angiotensin II Type 2 Receptors[a]

receptor	K_d (AngII)	K_d (AngII + 10 mM DTT)
AT_2 wild type	3.4 ± 0.4 ($n = 8$)	0.9 ± 0.7 ($n = 7$)
[del 1−16]AT_2	4.6 ± 0.8 ($n = 4$)	3.4 ± 0.8 ($n = 3$)
[AT_1NT]AT_2	8.6 ± 1.9 ($n = 4$)[b]	5.1 ± 1.7 ($n = 4$)
[AT_1ECL3]AT_2	6.2 ± 0.3 ($n = 3$)	8.8 ± 0.1 ($n = 3$)[c]
[AT_1NT/ECL3]AT_2	2.2 ± 1.1 ($n = 3$)	4.8 ± 1.1 ($n = 3$)

[a] Radioligand binding was measured as described in Experimental Procedures with 100 μg of [AT_1NT]AT_2 or [AT_1ECL3]AT_2 or 20 μg of wild-type membrane protein and ^{125}I-AngII (ranging from 0.2 to 6 nM) in the presence and absence of 10 mM dithiothreitol (DTT). Nonspecific binding was determined in the presence of 1 μM unlabeled AngII. Data are presented in nanomolar as the means ± standard error. The number of independent experiments is shown in parentheses. [b] Statistically significant difference compared to wild-type AT_2 receptor ($P < 0.01$). [c] DTT produced a significant decrease in K_d ($P < 0.01$).

AT_1 receptor. As shown in Figure 4, both the wild-type AT_1 receptor and the [AT_2ECL3]AT_1 chimera clearly demonstrated AngII-induced IP_3 turnover. The wild-type receptor exhibited an EC_{50} value of 2.36 ± 0.40 nM and a maximum stimulation of [^{3}H]IP_3 of 2344 ± 337 cpm/mg of protein (means ± SE; $n = 3$), whereas the chimeric receptor showed an EC_{50} value of 396 ± 130 nM and a maximum stimulation of [^{3}H]IP_3 of 767 ± 104 cpm/mg of protein (means ± SE; $n = 4$). This clear demonstration of AngII-induced IP_3 turnover combined with the detection of specific ^{125}I-AngII binding verified the successful expression and proper insertion into the cell membrane of the [AT_2ECL3]AT_1 chimera. Although determination of its K_d was not possible, determining the effect of DTT on binding was still possible. Single point binding assays were performed in the presence and absence of 10 mM DTT on isolated membranes containing the [AT_2ECL3]AT_1 chimeric receptor. Because the number of binding sites remains unchanged in such an experiment, any increase or decrease in specific binding would respectively indicate enhancement or reduction in affinity for AngII. For the wild-type AT_1 receptor, 10 mM DTT abolished essentially all specific binding (Figure 5A). However, when compared to the [AT_2ECL3]AT_1 chimera, AngII binding only decreased slightly in the presence of DTT (12.9 ± 0.7 fmol/mg of protein without DTT, 10.1 ± 0.1 fmol/mg of protein with 10 mM DTT; $n = 3$), indicating only a very small, significant decrease ($P < 0.05$) in binding affinity (Figure 5B).

Finally, to assess whether the amino terminus and the third extracellular loop of the AT_2 receptor may work in concert in retaining high-affinity binding of AngII when subjected to DTT, a combination chimera [AT_1NT/ECL3]AT_2 was created by splicing individual chimeric exchange mutants. The resultant chimera added the AT_1 amino terminus and its third extracellular loop onto an AT_2 receptor, and its AngII binding response to DTT was tested. As shown in Table 3, this mutant showed a small, though nonsignificant decrease in affinity for AngII with the addition of 10 mM DTT.

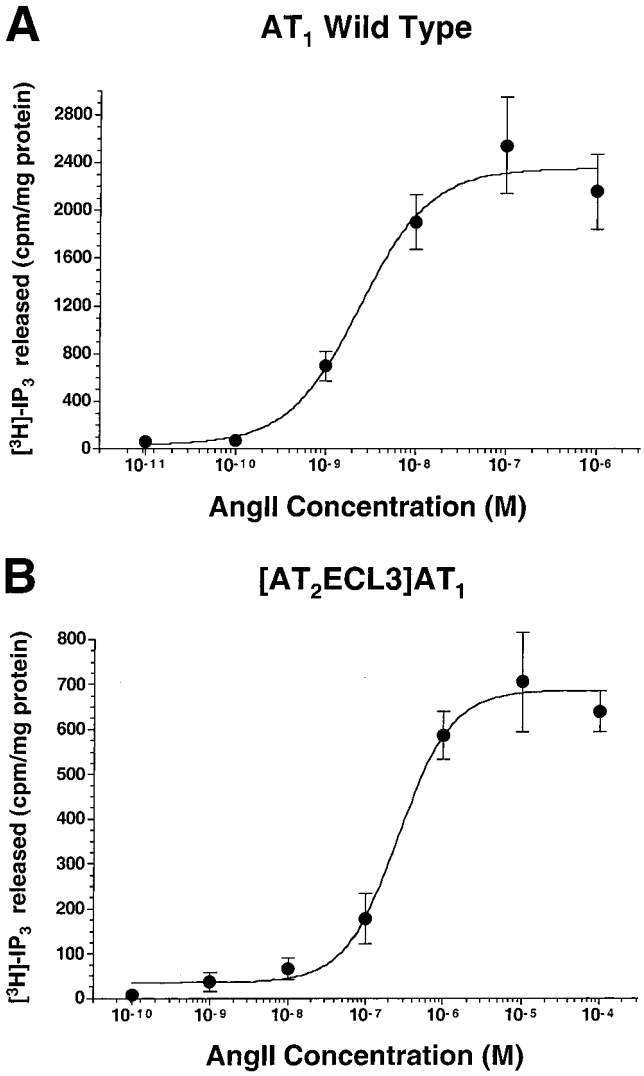

FIGURE 4: Dose−response curves of wild-type AT_1 and chimeric [AT_2ECL3]AT_1 receptors to activate intracellular signaling in response to AngII. COS cells were transfected with either (A) wild-type AT_1 receptor or (B) [AT_2ECL3]AT_1 receptor and were then later metabolically labeled with [^{3}H]inositol. After being preloaded with [^{3}H]inositol, the transfected cells were treated with increasing concentrations of AngII for 30 s. The values reported the means ± standard error ($n = 3$ for wild-type AT_1 receptors while $n = 4$ for [AT_2ECL3]AT_1 chimera).

DISCUSSION

Much of the research on elucidating structure−function relationships within the AngII receptor family has focused on the AT_1 receptor. Domains within this subtype that are responsible for ligand binding (*7*−*14*), receptor activation (*15*−*17*, *20*), and G-protein coupling (*18*, *19*) have been identified. The growing set of AT_1 mutational data has led investigators to propose computer models that illustrate possible molecular mechanisms underlying ligand−receptor interactions (*9*, *21*). In contrast, analogous efforts on the AT_2 subtype have lagged behind. Furthermore, the extent that current AT_1 models are applicable to the AT_2 receptor is unclear due to the surprisingly low level of homology (34%) shared between the two subtypes. Some progress has been made in recent years that has begun to identify AT_2 receptor domains involved in ligand binding and receptor activation, thereby beginning to highlight structural and functional similarities and dissimilarities in the AngII receptor family. Not surprisingly, the two subtypes do share some common AngII binding epitopes. For example, some known AT_1 binding epitopes for AngII have AT_2 homologues , including Lys^{215} in the fifth transmembrane-spanning domain (*22*), Asp^{279} and His^{273} in the sixth transmembrane-spanning domain (*24*, *31*), and Arg^{182} and Asp^{297} in the extracellular

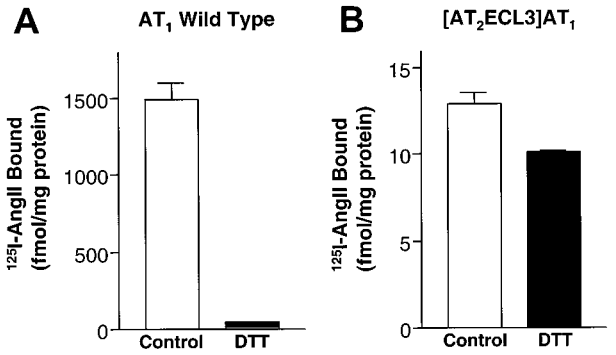

FIGURE 5: Effect of DTT on ^{125}I-AngII binding in the $[AT_2ECL3]AT_1$ chimera. The $[AT_2ECL3]AT_1$ chimera is an AT_1 receptor with an AT_2 third extracellular loop. Single point binding assays were performed on membranes from cells transfected with either $[AT_2ECL3]AT_1$ or wild-type AT_1 receptor in the presence or absence of 10 mM DTT. Binding conditions were as follows: (A) for wild-type AT_1 receptor, 1.4 nM ^{125}I-AngII with 5 μg of protein of transfected membranes, and (B) for chimeric receptor, 2.7 nM ^{125}I-AngII with 200 μg of protein of transfected membranes. Specific ^{125}I-AngII binding in the absence (control) or presence of 10 mM DTT in the two receptors is expressed as means ± standard error from three independent experiments. Each independent experiment paired the control and 10 mM DTT conditions for both the $[AT_2ECL3]AT_1$ chimera and the wild-type AT_1 receptor.

loops (*23*). Correspondingly, residues unique to each subtype have been shown to be involved in properties specific to each subtype, such as the binding of subtype-selective ligands (*14*, *32*) and G-protein coupling (*18*, *33*). However, the expected roles of conserved and nonconserved residues within the AngII receptor family are not always straightforward. For example, despite the fact that AT_1 and AT_2 receptors exhibit identical affinities for AngII, not all of the identified AT_1 binding epitopes for this peptide are preserved in the AT_2 receptor. Moreover, chimeric receptor exchanges of AT_1 and AT_2 regions with vastly dissimilar amino acid sequences can still produce mutant receptors that preserve common properties such as high-affinity AngII binding (*25*). Thus, the role of any AT_2 homologue of key AT_1 residues, identified either by mutagenesis or computer modeling, remains to be established.

The AT_1 and AT_2 receptors share four extracellular cysteine residues, one in the amino terminus and one in each of the three extracellular loops. Yamano and colleagues have demonstrated the importance of these cysteines by substituting each residue with a glycine, which resulted in mutants with 10-fold lower affinity for AngII (*8*). To identify possible disulfide linkages, further experiments paired specific cysteine mutants and suggested that two distinct disulfide bonds are formed between the cysteines (*10*). One disulfide bridge, which connects the cysteines in the first and second extracellular loops, is known to be conserved in other GPCRs (*34*, *35*). An additional, uniquely AT_1-specific disulfide bridge was also identified that linked the cysteines in the amino terminus and in the third extracellular loop. Collectively, the mutational data suggest that both disulfide bonds are integral in maintaining the overall protein conformation required for high-affinity ligand binding for the AT_1 receptor. Moreover, the well-known inhibition of AT_1 binding activity by the reducing agent DTT further supports the postulated role of the AT_1 disulfide bonds; DTT would break the disulfide bridges and thereby disrupt AT_1 binding (*10*). Paradoxically, despite conservation of the same four extracellular cysteine residues in the AT_2 receptor (Figure 2), DTT enhanced AngII binding for this subtype by approximately 4-fold (Table 1). Although the increase in binding affinity was modest, it was especially striking when compared to DTT's profound effects on the AT_1 receptor, where DTT completely abolished any specific binding of AngII. Thus, the extent that the AT_1 cysteine mutation data are applicable to the AT_2 subtype remained unclear.

We also chose to use a mutational approach to investigate the role of the conserved extracellular cysteines within the AT_2 receptor with respect to this subtype's unique DTT sensitivity and potential disulfide bond pairings. This mutational strategy has been extensively employed for many G-protein-coupled receptors (*34*−*37*) in addition to the AT_1 receptor (*10*). It is important to note that this approach, as is for all mutational strategies, is an indirect method for assigning disulfide pairings. Thus, while it is commonly expected that the effect on ligand binding of a double mutation of cysteines paired in a disulfide bond would not be greater than mutating each individual residue, the indirect nature of mutagenesis does not discount the possibility that regional conformational changes due to the mutational substitution itself could alter ligand binding affinities in a manner unrelated to disruption of a putative disulfide bond.

In characterizing AT_2 cysteine mutants, two distinct responses were observed. One response involved mutations of either Cys^{117} or Cys^{195}, which respectively reside in the first and second extracellular loops (Figure 2). Alanine substitutions of either of these residues yielded receptors with an approximately 4-fold lower affinity for AngII compared to the wild-type receptor. A similar 4-fold decrease in AngII binding was exhibited by the double point mutant $AT_2C117A/C195A$, indicating that these two cysteines are likely paired in a disulfide bond. As discussed above, this particular disulfide bond is preserved not only in the AT_1 receptor but also in other GPCRs where this linkage is critical for high-affinity ligand binding (*34*, *35*). Although the effects of AT_2's Cys^{117} or Cys^{195} mutations were not as large as observed for equivalent AT_1 mutants, this particular AT_2 disulfide bond appears to play an analogous role in maintaining proper conformation of the ligand binding pocket. When DTT was added to the binding experiments, thereby disrupting any disulfide bonds, both cysteine mutants still retained the AT_2-like response of enhanced ligand binding, although the extent of this increased affinity varied. AT_2C117A response to DTT treatment was comparable to similarly treated wild-type receptor. The effect of DTT on AT_2C195A, however, was somewhat less. One possibility is that subtle, local conformational changes may have been directly caused by the amino acid substitution itself of Cys^{195}, thus preventing the mutant from displaying a full AT_2-like response to DTT. Still, since both cysteine mutants continued to exhibit significant DTT-induced increases in ligand binding to levels comparable to DTT-treated AT_2, Cys^{117} and Cys^{195} or other elements contained in their respective extracellular domains are unlikely to be the source of the distinct AT_2 response to DTT. In contrast, alanine substitutions of Cys^{35} or Cys^{290} slightly elevated receptor affinity about 2-fold for AngII (Table 1). DTT further enhanced AngII binding for these single point mutants to levels comparable to those of the similarly treated wild-type AT_2 receptor. The double mutant

AT_2C35A/C290A also showed an identical level of increased AngII binding and thereby indicated that these two cysteines are likely joined in a disulfide bond. Collectively, our mutational experiments indicated that these four conserved residues form identical disulfide bridges in both the AT_1 and the AT_2 receptor subtypes.

The two AngII receptor subtypes exhibit dramatic differences in ligand binding when exposed to DTT, which breaks all disulfide bonds. Since the extracellular cysteines form identical disulfide bridges for both AngII receptor subtypes, other elements unique to the AT_2 receptor protein must exist and permit this subtype to maintain high-affinity binding when exposed to DTT. Although the magnitude of the observed increases in AngII binding induced by any mutations of Cys^{35} and Cys^{290} did not completely approach the levels induced by DTT treatment, the enhancing effects of these mutations were reflective of the customary AT_2 response to DTT (whereas mutations of Cys^{117} and Cys^{195} were not). This qualitative difference suggests that components within the extracellular domains where these cysteines reside may be partly responsible for AT_2's response to DTT. In addition, competition analysis of Cys^{35} and Cys^{290} mutants demonstrated that these small increases in affinity apply only to peptidic ligands, i.e., AngII, SARILE, and the AT_2-specific CGP42112A, and not to the nonpeptide AT_2 antagonist PD123319 (Table 2). These observations appear to be consistent with the hypothesis that larger peptidic ligands are more likely to interact with the extracellular domains of the receptor, whereas the binding of smaller nonpeptides such as PD1233219 are situated more deeply within the transmembrane-spanning domains and would thereby remain immune to mutations of extracellular residues. Indeed, binding epitopes within the AT_2 receptor have been mapped to extracellular domains with respect to AngII (*23*, *25*) and to CGP42112A (*32*). Since Cys^{35} and Cys^{290} reside within the amino terminus and the third extracellular loop of the AT_2 receptor, respectively, we began to explore the possible contributions of these extracellular domains in the AT_2 DTT effect.

To examine the possible role of the AT_2 amino terminus, two mutational changes were introduced. First, because the AT_2 amino terminus is 16 amino acids longer than that of the AT_1 receptor, the AT_2 amino terminus was truncated to the same length as that of the AT_1 receptor. Binding analysis revealed that the truncated receptor retained near wild-type affinity for AngII as well as some, though not statistically significant, AT_2-like DTT responsiveness. Second, a chimeric receptor [AT_1NT]AT_2, consisting of replacing the AT_2 amino terminus with that of the AT_1, was constructed to evaluate possible DTT effects on the amino-terminal cysteine in the context of native AT_1 receptor residues. This chimeric receptor bound AngII with high affinity and continued to exhibit a small, though not statistically significant, DTT enhanced binding. Overall, both amino-terminal mutants showed some blunting in the DTT enhancement of binding. Thus, this extracellular domain may only contribute slightly toward AT_2's response to DTT. To investigate the role of the third extracellular loop, chimeric receptors were created that exchanged this domain between the two subtypes. Although the [AT_1ECL3]AT_2 chimera, which replaced an AT_2 receptor's third extracellular loop with an analogous part of the AT_1 protein, possessed wild-type affinity for AngII, the presence of 10 mM DTT resulted in a slight, statistically significant decrease in AngII binding affinity (Table 3). Qualitatively, this DTT response was more AT_1-like than AT_2-like. The reciprocal chimera [AT_2ECL3]AT_1 also demonstrated a very small, statistically significant decrease in binding affinity when subjected to DTT. However, because 10 mM DTT completely abolishes all specific binding for the wild-type AT_1 receptor (Table 1), it is especially noteworthy that any specific binding was measured in DTT-treated [AT_2ECL3]AT_1, a chimera that is primarily composed of AT_1 receptor sequences. Taken together, the chimeric exchanges of the third extracellular loops suggest that, for the AT_2 receptor, this domain may contribute to this subtype's unique response to DTT. Finally, to evaluate whether the amino terminus and the third extracellular loop of the AT_2 receptor may work synergistically in retaining high-affinity AngII binding when subjected to DTT, a combination chimera [AT_1NT/ECL3]AT_2 was evaluated. The resultant chimera, with an AT_1 amino terminus and an AT_1 third extracellular loop attached to an AT_2 receptor, showed a slightly decreased affinity for AngII with the addition of DTT; i.e., this chimera was also beginning to lose the AT_2-like property of enhanced ligand binding when subjected to DTT.

In summary, our mutagenesis data indicate that for both AngII subtypes the conserved extracellular cysteine residues form two identical disulfide bonds: one that connects the first and second extracellular loops and another that links the amino terminus to the third extracellular loop. The mutational data and the resulting assignments of disulfide bridges, however, can benefit from future biochemical mapping experiments, which can more directly determine free and paired cysteine residues within the AngII receptor subtypes. Still, despite possessing identical pairs of disulfide bonds, the two subtypes exhibit strikingly different binding properties when these bonds are broken by DTT treatment. Because DTT greatly inhibits AT_1 receptor binding, the disulfide bonds maintain the ligand binding pocket and preserve high-affinity binding of peptidic ligands by forming necessary constraints on the extracellular domains of this subtype. In contrast, the AT_2 receptor displays a modest increase in AngII binding when exposed to DTT. For this subtype, the same disulfide bonds also restrict the extracellular domains. However, when the disulfide bonds are disrupted by DTT, other compensatory elements within the AT_2 receptor protein are uncovered and help to maintain, or even enhance, this receptor's binding activity. Our experiments suggest that the AT_2 disulfide bridges are of two distinct types: the link between Cys^{117} and Cys^{195} (respectively in the first and second extracellular loops) is AT_1-like in that its disruption results in decreased ligand binding, whereas the bond between Cys^{35} and Cys^{290} (respectively in the amino terminus and third extracellular loop) may mask latent peptide binding epitopes that are uncovered upon disruption of this disulfide bridge. These latent binding epitopes permit the AT_2 receptor to continue to bind peptidic ligands with a slightly higher affinity when all disulfide bonds are broken through DTT treatment. Further experiments indicate that some of these latent ligand binding epitopes may reside within the third extracellular loop of the AT_2 subtype. Other possible elements within the AT_2 amino terminus may also offer only small contributions when

the disulfide bonds are broken. These experiments represent our continuing efforts to identify the structural determinants that define AT_2 receptor binding and function and determine the extent that current AT_1 models are applicable to the AT_2 subtype. Comparing and contrasting AT_1 and future AT_2 models may establish a greater understanding of the molecular properties of the entire AngII receptor family.

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