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# Functional Diversity of $AT_2$ Receptor Orthologues in Closely Related Species

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## Abstract

**BACKGROUND—**The most striking feature of life is biodiversity. However, mechanisms of biodiversity remain poorly understood, as most protein orthologues of different species are highly homologous in sequence and identical in function. Interestingly, recent evidence has demonstrated heterogeneity for a G protein-coupled $AT_2$ receptor in both ligand binding and induction of arachidonic acid release. The present study investigated the properties of $AT_2$ receptors in closely related species.

**METHODS.—**$AT_2$ receptors cloned from human, rabbit, rat, and mouse were expressed in CHO-K1, COS-1, and HEK293 cells and characterized in ligand binding and signal transductions. Critical residues in rabbit $AT_2$ receptor attributable to heterogeneity were examined using both gain-of-function and loss-of-function approaches with mutagenesis.

**RESULTS—**The newly-cloned rabbit $AT_2$ receptor exhibits distinct biochemical and biological properties compared to its highly homologous orthologues (91% in overall amino acid sequence) of rat, mouse, and human. All these orthologues activate SH2 domain-containing phosphatase-1 (SHP-1) and show similar binding affinities for angiotensin II (Ang II) and $AT_2$-specific ligands CGP42112A and PD123319. However, reducing agent dithiothreitol (DTT) inactivates the rabbit orthologue but potentiates the others in ligand binding, a hallmark of $AT_2$ versus $AT_1$ receptor subtypes. Most interestingly, rabbit $AT_2$ receptor, but not the other orthologues, induces arachidonic acid release in various cell systems when stimulated by both Ang II and CGP42112A, the peptide antagonist. Mutagenesis studies and sequence analyses further indicate that residues $His^{106}$, $Asp^{188}$, and $Thr^{293}$ are responsible for the DTT inactivation and residues $Val^{209}$ and $Val^{249}$ are partially responsible for arachidonic acid release.

**CONCLUSION—**These results deny the co-existence of an additional $AT_2$ subtype in rabbit proximal tubule cells and demonstrate for the first time the presence of functional diversity for closely related Eutherian orthologues of a GPCR that are more than 90% homologous in the amino acid sequence.

### Keywords

Biodiversity; $AT_2$ receptor; orthologue; GPCR; angiotensin II; arachidonic acid

### Abbreviations

GPCR, G protein-coupled receptor; Ang II, angiotensin II ($Asp^1$-$Arg^2$-$Val^3$-$Tyr^4$-$Ile^5$-$His^6$-$Pro^7$-$Phe^8$); TM, transmembrane helix; AA, arachidonic acid; SHP-1; SH2 domain-containing

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phosphatase-1; rbAT$_2$, rabbit AT$_2$ receptor; rAT$_2$, rat AT$_2$ receptor; mAT$_2$, mouse AT$_2$ receptor; hAT$_2$, human AT$_2$ receptor

## Introduction

The AT$_2$ receptor belongs to the superfamily of G protein-coupled receptor (GPCR). It exerts inhibitory actions to counteract the type 1 Ang II receptor, AT$_1$, in blood pressure regulation and cell growth. In humans and mice, AT$_2$ exists as a single copy localized on the X chromosome (region Xq24-q25 and region XA2-A4, respectively) and contains no intron in its coding region. Thus the existence of multiple forms of AT$_2$, encoded by several homologous genes or derived by alternative splicing, is not possible [1-3]. However, earlier studies from several independent groups have suggested the presence of heterogeneous populations of the AT$_2$ receptor. For example, the AT$_2$ receptor in different brain regions exhibited differential sensitivity to GTPγS [4]. In rabbit proximal tubule epithelial cells, Douglas' laboratory discovered an AT$_2$ population with completely reduced binding in the presence of dithiothreitol (DTT) [5,6]. Activation of this population of AT$_2$ receptors induced arachidonic acid release in rabbit proximal tubular epithelial cells, a finding not observed in primary cells of other species [6]. In N1E-115 murine neuroblastoma cells, Reagan and Yee also observed two biochemically, pharmacologically, and immunologically different subpopulations of AT$_2$ receptors [7,8]. These investigators have therefore suggested the existence of AT$_{2A}$ and AT$_{2B}$ subtypes to explain the observed heterogeneity concerning the AT$_2$ receptors.

It is logical to assume that protein orthologues, particularly orthologues of closely related species, are identical in function because they are highly homologous (>90%) in amino acid sequence. AT$_2$ receptors share >91% amino acid identity and presumably should not be exceptional. In order to determine whether the heterogeneity of the rabbit AT$_2$ receptor results from a new subtype of AT$_2$ receptor, we have cloned an AT$_2$ receptor from rabbit proximal tubular epithelial cells to examine the structural determinants which contribute to the unique phenotypic characteristics of this species [5,6]. We now show that the rabbit AT$_2$ receptor (rbAT$_2$) is a conventional AT$_2$ receptor orthologue rather than a novel subtype of the AT$_{2B}$ receptor. However, this rabbit orthologue exhibits functional diversity in biochemical, pharmacological, and physiological properties.

## Methods and materials

### Materials

The monoclonal anti-c-Myc antibody (Roche, clone 9E10), oligonucleotides (Sigma-Genosys and MWG Biotech), Ang II and [Sar$^1$,Ile$^8$]Ang II ( Bachem), PD123319 and CGP42112A (RBI), [$^3$H]-arachidonic acid (NEN Life science), Protein Tyrosine Phosphatase Assay Kit (Upstate Biotech), DTT and other chemicals (Sigma) were purchased. $^{125}$I-[Sar$^1$,Ile$^8$]Ang II (2200 Ci/mmol) was supplied by Dr. Robert Speth, University of Washington, Pulman, WA.

### DNA cloning, mutagenesis, PCR, and expression

The cDNA of rbAT$_2$ was cloned from total RNA isolated from rabbit primary proximal tubule epithelial cells by RT-PCR with degenerate oligonucleotide primer FD1 (GTGGTT/CA/TCACTGTTTTGTTGTCAAAA) and FD2 (CAGCTGTTGGTGAATCCCAG/AGAGGATGGCAAAA), and SMART™ RACE cDNA Amplification Kit (ClonTech). The cloned cDNA was fully sequenced and modified for expression in cell lines: 1) To contain a consensus Marilyn-Kozak sequence and a unique Eco RI site at the 5′ end and Not I site at the 3′ end of the gene; and 2) To encode a decapeptide (EQKLISEEDL) epitope tag for a monoclonal antibody anti-c-Myc at the 5′ end after the start codon. The epitope tagged cDNA was subcloned into a shuttle vector pcDNA6 (Invitrogen). Identical modification was applied

to rat, mouse, and human $AT_2$ genes subcloned into the pcDNA6 vector for expression. Mutant $AT_2$ receptors were prepared by the restriction fragment-replacement method and the PCR method. DNA sequence analysis was done to confirm each mutant construct. For expression of receptor proteins, 3 μg of column (Qiagen) purified plasmid DNA per $10^7$ cells was used in transfection. CHO-K1, COS-1, and HEK293 cells (ATCC), cultured in ATCC preferred medium supplemented with 10% fetal bovine serum, were transfected by the GenePORTER™ transfection reagents (GTS Inc). For PCR detection of intronless $AT_2$ receptors, genomic DNAs of human, mouse, rat, and rabbit purchased from ClonTech were used as templates for conventional PCR with respective sense and antisense primers derived from known sequences available in the GeneBank.

### Southern blot

A DNA fragment probe of 740bp was prepared by Eco RI digestion of a TOPO TA vector plasmid DNA (Invitrogen) that contains a PCR insert of $rbAT_2$ synthesized with primer FD1 and FD2. This DNA probe (20 ng/μl) was labeled with AlkPhos Direct Kit (Amersham) following the company's protocol. Genomic DNAs of rabbit, human, rat, and mouse (ClonTech) were digested with Eco RI and visualized by chemiluminescent detection with CDP-*Star* (Amersham) after gel separation, membrane transfer, and hybridization with the labeled probe.

### Western blot of receptors expressed in CHO-K1 cells

Post-nuclear supernatant of CHO-K1 cells solubilized in lysis buffer (50 mM Tris-HCl, pH 6.8, 1% CHAPS, 5 mM EDTA, pH 8.0) containing 50 μg/ml PMSF, 10 μg/ml of Benzamidine, 10 μg/ml of Bacitracine, 10 μg/ml of Leupeptin, and 2 μg/ml of Aprotinine, was used for SDS-polyacrylamide gel electrophoresis (PAGE) and western blotting analysis. Receptor polypeptides were visualized using 1:1000 dilution of mouse monoclonal anti-c-Myc antibody and 1:3000 dilution of secondary anti-mouse antibody coupled to peroxidase and the ECL system (Santa Cruz) as described [9-11].

### DTT treatment, ligand binding assay, and AA release assay

DTT treatment of membrane preparations, saturation binding, and competitive binding assay were performed as described earlier [9-11]. AA release was assayed based on published methods [6] with minor modifications. Briefly, CHO-K1 cells and transfected CHO-K1 cells cultured in 6-well plates were allowed to grow for 24 h before they were labeled with [$^3$H]-AA (0.3 μCi/ml or 10 nM [$^3$H]-AA) overnight. The cells were washed three times with basal medium (serum-free medium plus 25 mM HEPES, pH 7.5) and then treated with 1.0 ml Basal medium containing Ang II for 15 min at 37°C. The stimulation was stopped by putting the plates on ice and the medium was transferred into pre-chilled appropriate tube for centrifugation at 15,600xg and at 4°C. Count 0.5 ml medium (half the volume) in 5 ml scintillation tubes. For total cellular radioactivity, cells in each well were solubilized with 1 ml of 1 M NaOH and counted in 5 ml scintillation tubes. [$^3$H]-AA released into the medium is expressed as percent of the total cellular radioactivity and referred to as fractional release: Fractional [$^3$H]-AA release = (count in the medium /count in the cells)*%.

### Immunoprecipitation and SHP-1 activity assay

Twenty-four hours after co-transfection with $AT_2$ and SHP-1 (kindly provided by M. Thomas, Washington University, St. Louis, USA), CHO-K1 cells were starved overnight and then stimulated with Ang II for 3 minutes. After treatment, cells were rinsed with ice-cold PBS, then solubilized for 30 min at 4°C in lysis Buffer (50 mM Tris-HCl, pH 7.5, 1.5% CHAPS, 150 mM NaCl, 5 mM EDTA), containing 0.5 mM Sodium orthovanadate, 10 μg/ml of Benzamidine, 10 μg/ml of Bacitracine, 10 μg/ml of Leupeptin, and 2 μg/ml of Aprotinine, and

50 μg/ml PMSF. After centrifugation at 15,000g for 15 min, the resulting supernatant (1-1.5 mg of proteins) was incubated for 2 h at 4°C with 10 μl of monoclonal anti-SHP-1 antibody prebound to Sepharose Protein A (Santa Cruz). The immunocomplexes were washed five times with washing buffer (identical to the lysis buffer but without 1.5% CHAPS and 0.5 mM Sodium orthovanadate). At the end of washing, the immunocomplexes were resuspended in PTPase buffer (50 mM Tris-HCl, pH 7.0, 1 mg/ml Bovine serum albumin, 5 mM DTT). The PTP activity was assayed by measuring the release of inorganic phosphate from phosphopeptides based on a malachite green detection system [12] using the PTPase assay Kit (Upstate) following the company's protocol.

### Statistical Analysis

The results are expressed as the mean ± S.E.M. of two to five independent determinations. The significance of measured values was evaluated with an unpaired Student's *t*-test.

## Results

### Cloning and sequence analysis of a rabbit $AT_2$ receptor

To determine whether an $AT_{2B}$ subtype exists in the rabbit kidney cortex and to elucidate the mechanism of functional diversity of the renal $AT_2$, we cloned the renal $AT_2$ from rabbit kidney cortex (GeneBank accession number AF451328). The polypeptide encoded by the open reading frame consists of 363 amino acids and shares a high identity of 91% in overall amino acid sequence with its human, rat, and mouse $AT_2$ orthologues (Fig. 1). This high degree of identity falls within the range for many other protein orthologues including $AT_1$. The $rbAT_2$ bears all the structural features of the other $AT_2$ orthologues, including seven transmembrane helices (TM) and four cysteine residues located on the extracellular domains. Sequence alignment identified 19 non-conserved unique residues for $rbAT_2$ when compared to the human, rat and mouse orthologues as shown in Figure 1. Of these 19 residues, 63.2% located in the N-terminal and TM I, 10.5% in the TM II-VII, 10.5% in intracellular domains, and 15.8% in extracellular domains. In addition, the $rbAT_2$ is short of one potential *N*-glycosylation site at $Asn^{24}$ due to the $Phe^{26}$ substitution in comparison to the other three orthologues.

### The $rbAT_2$ is a conventional $AT_2$ orthologue

Polymerase chain reaction (PCR) using rabbit genomic DNA as templates for amplification produced a single band of 1.1kb (data not shown). This PCR product is identical to the complete coding region of the $rbAT_2$ gene as confirmed by DNA sequencing, indicating its intronless property of the coding region similar to the other three orthologues. Southern blot using an 800bp fragment of $rbAT_2$ DNA as probe detected a single band, suggesting little possibility of existence of other $AT_2$ subtypes (Fig. 2). Thus, we conclude that this $rbAT_2$ is an orthologue of the human, rat, and mouse $AT_2$, rather than a new $AT_{2B}$ subtype.

### Identical ligand binding affinity but distinct DTT sensitivity of $rbAT_2$

The $rbAT_2$ must reproduce the similar function observed in rabbit kidney cortex in a surrogate cell system since the data above has ruled out the presence of $AT_2$ heterogeneity [5,6]. To first examine the ligand binding property, expression of rabbit, human, rat, and mouse $AT_2$ orthologues in COS-1 and CHO-K1 cell lines was verified by Western blot [9-11] with a specific anti-c-Myc antibody which recognizes the c-Myc tag at the N-terminal of the receptors (Fig. 3). Saturation binding of $^{125}$I-[$Sar^1$,$Ile^8$]Ang II and competitive binding of $^{125}$I-[$Sar^1$,$Ile^8$]Ang II against [$Sar^1$,$Ile^8$]Ang II, and $AT_2$-specific ligand CGP42112A and PD123319 were applied to the four $AT_2$ orthologues, respectively [9-11]. Table I summarizes the data and shows little difference in binding affinity among four orthologues in the presence or absence of DTT treatment. Analogous to the human, rat, and mouse $AT_2$, the rabbit

orthologue also exhibits an 8-fold increase in affinity in the presence of 5 mM DTT [9]. However, distinct from the human, rat, and mouse $AT_2$, the rabbit orthologue shows an 80% (4.8 fold) decrease in $B_{max}$ in the presence of 5 mM DTT as compared to no DTT treatment. As a result, the binding curve of $^{125}$I-[Sar$^1$,Ile$^8$]Ang II for $rbAT_2$ is in sharp contrast to the curves for the human, rat, and mouse orthologues (Fig. 4). Of 19 non-conserved unique residues, His$^{106}$, Asp$^{188}$, and Thr$^{293}$ are the amino acids that locate in the extracellular domains of the $rbAT_2$. Deletion of the N-terminal (the first 33 amino acids) of the $rbAT_2$ (mutant $rbAT_{2N}$) does not alter the property of DTT inactivation (Table I).

### All four $AT_2$ orthologues activate SHP-1 but only the $rbAT_2$ mediates AA release

Activation of an $AT_2$ by Ang II in rabbit kidney epithelial cells has been shown to induce arachidonic acid (AA) release [5,6]. Consistently, Ang II stimulation induced marked [$^3$H]-AA release in CHO-K1 cells transiently transfected with $rbAT_2$. The addition of an $AT_2$-specific antagonist PD123319 inhibited the [$^3$H]-AA release. However, [$^3$H]-AA release was not observed in CHO-K1 cells transiently transfected with human, rat, and mouse $AT_2$ orthologues (Fig. 5). These results were reproduced using COS-1 and HEK293 cell lines (data not shown).

Ang II stimulation of human $AT_2$ in CHO-K1 and PC12 cells has been shown to activate SHP-1, a SH2 domain-containing phosphatase [13,14]. Contrary to the [$^3$H]-AA release, all four $AT_2$ orthologues activated SHP-1 with no appreciable differences in CHO-K1 cells as detected by tyrosine phosphatase assay of SHP-1 specific immunoprecipitates (Fig. 6).

### Key residues determine AA release

The most critical regions directly specifying G protein selectivity are the cytoloop 2 and 3 domains [15]. To understand the molecular mechanism of $rbAT_2$-mediated [$^3$H]-AA release, $rbAT_2$ mutant, V209A-V249I, and its rat reciprocal counterpart, A209V-I249V, were constructed simply by switching a DNA fragment between rabbit and rat wild-type $AT_2$ receptors. The two mutants maintained all the binding properties of their own wild type (data not shown). The mutant V209A-V249I of $rbAT_2$ lost 64% activity in [$^3$H]-AA release whereas the mutant A209V-I249V of rat $AT_2$ gained 30% activity (Fig. 5), indicating that Val$^{209}$ and Val$^{249}$ of the $rbAT_2$ contribute to [$^3$H]-AA release. Substitution of amino acid in the N-terminus of $AT_2$ receptor shows no effect on AA release since $rbAT_{2N}$ maintains full activity (Fig. 5).

### Variations in potential phosphorylation sites

Phosphorylation is a fundamental mechanism in regulation of GPCR functions. We analyzed potential sites of Ser, Thr, and Tyr phosphorylation for these four orthologues by NetPhos 2.0 and detected several unique sites for each orthologue as summarized in Table II [16]. This suggests another potential mechanism to understand how substitution of a seemingly non-critical amino acid in the microenvironment could impose profound impact on receptor function through alteration of phosphorylation.

## Discussion

Almost all receptors including the $AT_2$ may act differently in different locations of the same species. This exciting and complex phenomenon is beyond the sphere of this study because this type of difference displayed by an identical receptor is unlikely caused by a receptor itself. In contrast, this study deals with functional differences caused by a receptor itself due to structural differences. In the $AT_2$ receptor, most variations (~50%) of amino acids are located within the short N-terminal region of the receptor orthologues (Fig. 1). However, these variations reveal little functional diversity in the study as demonstrated by the $rbAT_{2N}$ with

comparison to the wild-type rbAT$_2$. Thus, we speculate that the amino acid sequence in the N-terminus may specify the species signal or origin for the AT$_2$ receptors.

Substitution of His$^{106}$, Asp$^{188}$, and Thr$^{293}$ in the rbAT$_2$ may increase the DTT accessibility to the highly conserved disulfide bond of Cys$^{117}$-Cys$^{195}$ of the receptor [9]. Breakage of this disulfide bond in a G protein-coupled receptor results in complete loss of function due to mis-folding [9].

Residue Ala$^{63}$ located in the cytoloop 1 also may directly attribute to AA release [12] since the gain-of-function and loss-of-function were not equivalent and not close to 100%. In addition, residues located in the TM domains (Tyr$^{37}$, Gln$^{43}$, Arg$^{48}$, Phe$^{53}$, Ser$^{59}$, and Val$^{94}$) may indirectly and collectively affect G protein coupling selectivity and efficiency through alteration of critical GPCR conformations [10,11,15].

Although properties of ligand binding to AT$_2$ receptors are well established, the full impact on biological function(s) and many other properties of the AT$_2$ receptor remain elusive. This report on one hand explains why two types of AT$_2$ receptors were detected previously in the presence and absence of DTT and indicates that no AT$_2$ subtype receptors exist in rabbit. The previously observed heterogeneity likely resulted from functional diversity. However, it is unclear to us at this stage as to what the physiological significance of the functional diversity, predominantly, the DTT sensitivity and induction of arachidonic acid release can be. Given the observation that redox potential can also serve as an important regulation mechanism for protein activation [17,18], the distinct DTT property of rabbit AT$_2$ may provide an alternative mechanism for receptor activation under pathological states in which redox potential is altered. However, it is unclear whether the cell surface receptor AT$_2$ could be influenced intracellularly by the redox potential during the receptor folding, maturation, and intracellular trafficking since alteration of redox potential may occur only inside the cell under physiological conditions. Under pathological conditions such as myocardial infarction, injury, and inflammation, interstitial redox potential may change significantly. Interestingly, the expressions of AT$_2$ receptors were increased significantly under these pathological conditions [1-3].

For protein orthologues with significantly less amino acid identity, functional diversity has been observed. For example, the human P2 nucleotide receptor P2X$_7$ binds to ligand KN-62 and KN-04 whereas rat P2X$_7$ (80% identity) does not [19]. Mammalian AT$_1$ orthologues (human, rat, mouse, rabbit, etc., with 95% identity) bind to AT$_1$-specific ligand losartan whereas amphibian AT$_1$ receptor (Xenopus, 60% identity) does not [20]. Functional diversity for protein orthologues with amino acid identity greater than 90% is rare. The collective or synergistic effect of limited substitutions of seemingly unimportant amino acids may play a more important role in functional diversity than previously appreciated. Variation of phosphorylation sites (as shown in Table II), conformation of interaction motifs, and accessibility of interaction motif or domains are potential factors that also determine functional diversity of receptor orthologues.

For G protein-coupled receptors, Granneman *et al* reported different pharmacological profiles between human and rat $\beta_3$-adrenergic receptors [21,22]. However, the human and rat $\beta_3$-adrenergic receptors share only 79% identity in amino acid sequence. Mallee et al. also documented different species selectivity for non-peptide antagonists between human and rat CGRP receptors [23], an atypical GPCR in that it requires heterodimerization of CRLR, a classical seven transmembrane receptor, with the accessory protein RAMP1. However, this difference resulted from variations of a single residue at position 74 in the accessory protein RAMP1 that bears 71% homology. Replacement of this single residue in CGRP receptor produced little difference in binding to the native peptide ligand CGRP. The mechanism of an accessory protein-mediated modulation in receptor pharmacology could be very different and

complex. One may find different pharmacological properties for receptor orthologues of closely related species. However, differences in intrinsic properties such as signal transduction are not documented.

The mechanism of biodiversity is largely unknown. Functional diversity of many individual genes may serve as one of the potential mechanisms. Most recently, identification of the human-specific gene family provided another exciting potential mechanism to explain what makes chimpanzees and humans different [24].

Biodiversity is an intriguing concept for biomedical research. Mouse, rat, and rabbit are the most widely-used animal species for man's study in biomedicine. For example, more and more genetically modified animals with diseases or disorders are used to study gene functions, produce gene products, conduct gene therapies and test new drugs. Animal genes and cells are widely used as objects or tools of molecular and cellular studies on fundamental mechanisms such as protein-protein interaction and signal transduction. In many studies, species compatibility is not considered for heterologous expression of a gene in cells or even animals. These have posed an unprecedented challenge to biomedical scientists because in many cases results from one study are not completely reproducible in another application. In the field of GPCR research, the presence of multiple subtypes of many receptors with yet no detectable difference in function represents just another example of the current challenge [25]. Elucidation of functional diversity for these GPCR subtypes would improve drug efficacy and reduce side effects. In the field of evolution and comparative biology, phylogenetic methods based on 1D sequence analysis may not be accurate in predicting the evolutionary positions of branching orders of organisms [26] if functional diversity as reported here is not considered.

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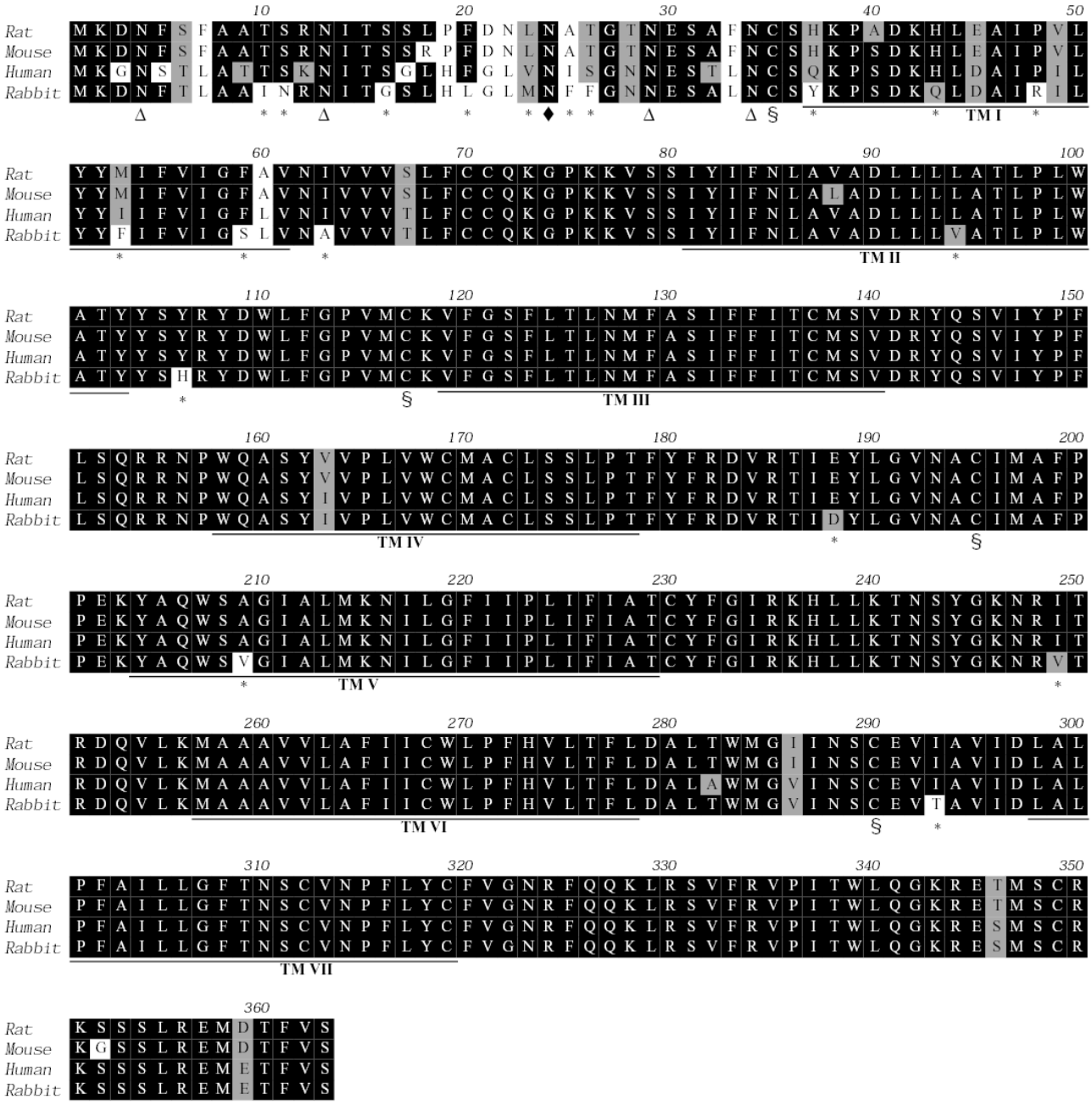


**Fig. 1.**

ClustalW alignment of $AT_2$ receptor orthologues of rat, mouse, human, and rabbit (GeneBank accession number AF451328). Putative transmembrane domains (TM I-VII) are underlined. The * indicates 19 non-conserved unique residues for rabbit $AT_2$ in comparison to rat, mouse, and human orthologues. Of the 19 residues, 12 residues (63.2%) are located in the N-terminal and TM I, 3 residues (15.8%) in extracellular domains, 2 residues (10.5%) in TM II-VII, and 2 residues (10.5%) in intracellular domains. Mark Δ indicates four potential glycosylation sites (NxS/T) present in all four orthologues. Mark ♦ at $Asn^{24}$ shows the potential glycosylation site present in all three orthologues but not in the rabbit $AT_2$ (NxF). § indicates four highly

conserved Cys residues that form two disulfide bonds of $Cys^{35}$-$Cys^{290}$ and $Cys^{117}$-$Cys^{195}$ in $AT_2$ receptor. Computer software: MacVector (Oxford Molecular, Inc.).

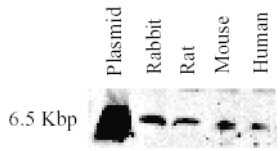


**Fig. 2.**

Southern blot detection of $AT_2$ receptor gene subtypes or isoforms. Plasmid, rat $AT_2$ receptor cDNA subcloned in pcDNA3 vector.

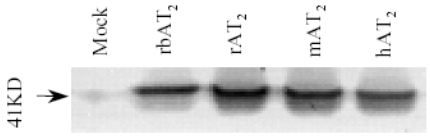


**Fig. 3.**

Western blot detection of $AT_2$ receptor orthologues expressed in CHO-K1 cells with specific anti-c-Myc antibody.

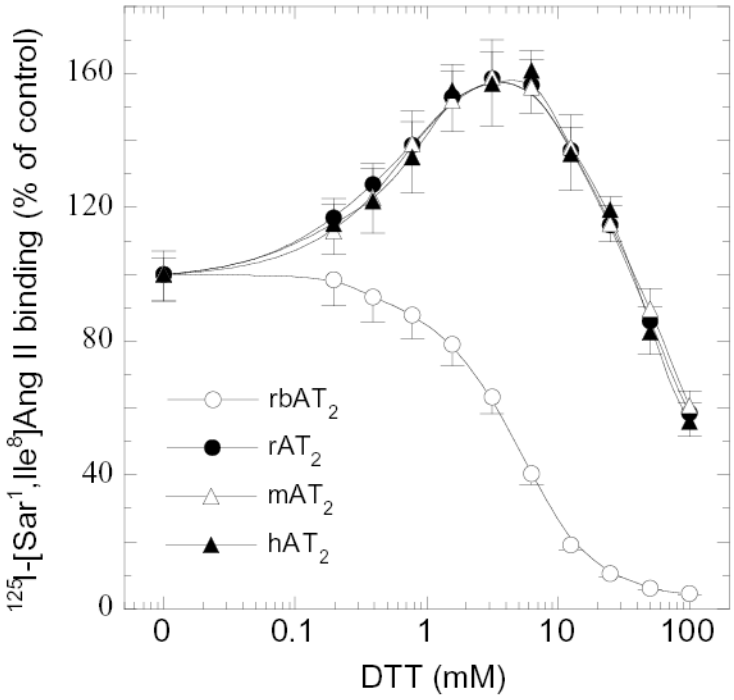


**Fig. 4.**

Effect of varying concentrations of DTT on $^{125}$I-[Sar$^{1}$,Ile$^{8}$]Ang II binding to AT$_2$ receptor orthologues. The specific binding of $^{125}$I-[Sar$^{1}$,Ile$^{8}$]Ang II in each sample was adjusted to be < 10 ± 1% (≈ 20,000) of total input cpm without any treatment. This value is represented as 100%. Samples were first exposed to DTT at 22°C for 20 min and then the ligand was added.

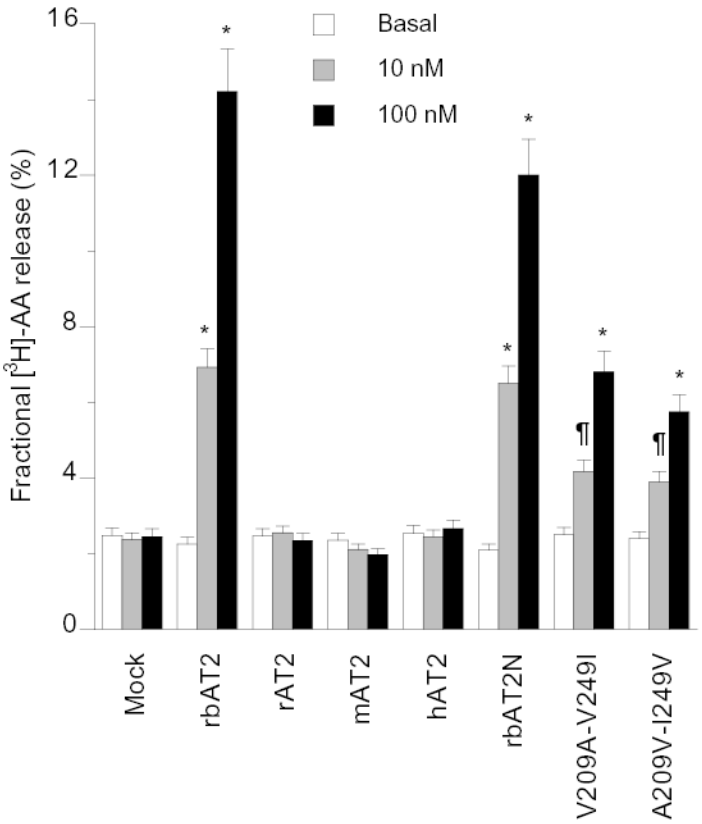


**Fig. 5.**

[$^3$H]-AA release induced by Ang II stimulation of mock-transfected and $AT_2$-transfected CHO-K1 cells. The mutant V209A-V249I of $rbAT_2$ and the mutant A209V-I249V of rat $AT_2$ maintained all binding properties of their own wild type as shown in Table 1 (data not shown). ¶, $p<0.05$, and *, $p<0.01$ by t-test in comparison to the basal of any group. No significant elevation of basal [$^3$H]-AA release was detected in $rbAT_2$ group in comparison to the basal levels of other groups. Results shown are mean ± s.e.m. of at least three independent experiments.

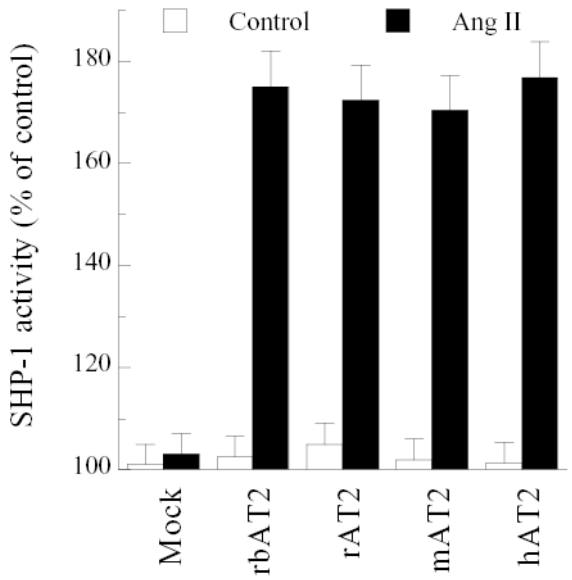


**Fig. 6.**

SHP-1 activity induced by Ang II (100 nM) stimulation of CHO-K1 cells co-transfected with SHP-1 and $AT_2$ receptor orthologues.

**Table I**

Binding affinity and $B_{max}$ of $AT_2$ receptor orthologues

| | [Sar$^1$,Ile$^8$]Ang II, ($K_i$, nM) | | | CGP42112A, ($K_i$, nM) | | | PD123319, ($K_i$, nM) | | | $B_{max}$, (pmol/mg) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Control** | **DTT (5mM)** | **-fold** | **Control** | **DTT (5mM)** | **-fold** | **Control** | **DTT (5mM)** | **-fold** | **Control** | **DTT (5mM)** | **-fold** |
| $rbAT_2$ | 0.89 ±0.15 | 0.09±0.02 | 9.89 | 0.61 ±0.13 | 0.07±0.01 | 8.71 | 17.7±2.2 | 2.4±0.43 | 7.38 | 4.05 ±0.35 | 0.85±0.38 | 4.765 |
| $rAT_2$ | 0.82 ±0.14 | 0.08±0.01 | 10.3 | 0.65 ±0.11 | 0.07±0.01 | 9.29 | 17.2±2.5 | 2.1±0.37 | 8.19 | 4.11 ±0.32 | 3.95±0.34 | 1.041 |
| $mAT_2$ | 0.83 ±0.12 | 0.08±0.01 | 10.4 | 0.63 ±0.10 | 0.08±0.01 | 7.88 | 18.4±2.8 | 2.5±0.31 | 7.36 | 4.08 ±0.36 | 4.09±0.41 | 0.998 |
| $hAT_2$ | 0.87 ±0.11 | 0.09±0.01 | 9.67 | 0.57 ±0.09 | 0.07±0.01 | 8.14 | 16.7±2.6 | 2.3±0.36 | 7.26 | 3.93 ±0.33 | 3.91±0.37 | 0.995 |
| $rbAT_{2N}$ | 2.98 ±0.76 | 0.11±0.02 | 27.1 | 0.69 ±0.12 | 0.08±0.01 | 8.63 | 17.6±2.3 | 2.3±0.38 | 7.69 | 3.78 ±0.32 | 0.62±0.35 | 6.096 |

Cell membranes were pre-treated with and without 5mM DTT at 22°C for 20 minutes. Here $rbAT_2$, $rAT_2$, $mAT_2$, and $hAT_2$ represent rabbit, rat, mouse, and human $AT_2$ receptor orthologues, respectively. The $rbAT_{2N}$ is a $rbAT_2$ mutant with deletion of the first 33 amino acids. This mutant is very similar to the wild type $rbAT_2$ except for a reduced binding affinity (3.3 fold) for [Sar$^1$,Ile$^8$]Ang II in the absence of DTT treatment. Data represent results of three or more experiments of binding isotherms followed by Scatchard analysis. Results are presented as mean ± s.e.m.

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## Table II

Phosphorylation sites of $AT_2$ orthologues

| | rbAT2 | rAT2 | mAT2 | hAT2 |
|---|---|---|---|---|
| Ser6* | NA | x | x | NA |
| Ser16* | NA | --- | x | --- |
| Ser36* | x | x | x | x |
| Ser40* | x | NA | x | x |
| Ser145 | x | x | x | x |
| Ser152 | x | x | x | x |
| Ser346 | x | NA | NA | x |
| Ser348 | x | x | x | x |
| Ser352 | x | x | NA | x |
| Ser353 | x | x | x | x |
| Ser354 | x | x | x | x |
| Thr10* | NA | x | x | --- |
| Tyr143 | x | x | x | x |
| Tyr189* | x | --- | --- | --- |
| Tyr204* | x | x | x | x |
| **Total** | **12**(61) | **11**(63) | **12**(63) | **11**(64) |

Potential sites of Ser, Thr and Tyr phosphorylation are predicted by NetPhos 2.0 Internet program(16). Here **x** means the potential phosphorylation site is predicted;

---, no potential phosphorylation is predicted;

NA, residue not available.

* Located in the extracellular domains and extracellular half region of TM domains. The superscript numbers in the brackets represent the total numbers of Ser, Thr, and Tyr residues available in the receptor orthologues, respectively.