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CLONING, CHARACTERIZATION, AND EXPRESSION OF TWO ANGIOTENSIN RECEPTOR (AT-1) ISOFORMS FROM THE MOUSE GENOME

Hiroyuki Sasamura, Lutz Hein, Jose E. Krieger,
Richard E. Pratt, Brian K. Kobilka and Victor J. Dzau

Falk Cardiovascular Research Center
Stanford University School of Medicine
300 Pasteur Drive, Stanford, California 94305

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Summary: We report the existence of two structurally distinct forms of the angiotensin receptor AT-1 in the mouse. A Balb/c mouse genomic library was screened by homology screening with a polymerase chain reaction (PCR) amplified probe. Restriction mapping and sequencing of the isolated genes revealed the presence of two receptor isoforms, here named the mouse AT-1a and AT-1b receptors, containing 22 different amino acids. Receptor binding studies performed on COS-7 cells transfected with the two receptors revealed that they had similar binding profiles for angiotensin II, angiotensin III and AT-1 or AT-2 specific antagonists. Because many of the structural differences were in the carboxy terminal putative intracellular domain, we speculate that these isoforms may differ in their regulation, signal transduction, or desensitization mechanisms.

The renin-angiotensin system plays a role in the control of systemic blood pressure, and fluid and electrolyte metabolism. In addition to its actions in the control of vascular tone, aldosterone secretion, and sodium reabsorption in the kidney, angiotensin II also exerts local effects on tissue growth and function (1). Although some previous data from angiotensin receptor binding studies suggested the possible existence of more than one type of angiotensin receptor (2), it was only recently that a clearcut pharmacological distinction between angiotensin receptors was made possible with the development of AT-1 and AT-2 receptor specific ligands (3) (4). Further studies have shown that almost all the known systemic effects of angiotensin II are mediated by the AT-1 receptor (2). Interestingly, differences in the signal transduction mechanisms mediating AT-1 receptor actions in some tissues have been reported (5). This observation suggests the existence of subtypes within the AT-1 receptor . Further characterization of the AT-1 receptor is particularly important, since it has been implicated in the pathogenesis of disorders such as hypertension (6) and arteriosclerosis (7). The design of potent and selective antagonists based on the structure of the receptor subtypes could lead to the development of novel drugs with increased specificity and reduced side effects.

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In this study, we report the cloning and characterization of two structurally distinct forms of the AT-1 receptor which may have important and novel physiological and pharmalogical significances.

Materials and Methods

Preparation of the probe: The probe for plaque screening was produced by PCR amplification of mouse genomic DNA using primers corresponding to areas of high homology (bases 144-163 and bases 667-685) in the recently published rat and bovine AT-1 receptor cDNA sequences (8) (9). The sequences of the two PCR primers were 5'-ggtggtgattgtcatttact-3' and 5'-gaatttcataagccttctt-3'. PCR was performed at 94°C for 1 min, 55°C for 1 min, 72°C for 2 min for 30 rounds. The amplified fragment was subcloned into pGem7Zf(+) (Promega Biotech), sequenced, and used as the probe for screening.

Screening of the Mouse Genomic Library: The mouse genomic library was made from partial Sau3AI digested DNA from the liver of an individual male Balb/c mouse (Clontech). Approximately $5x10^5$ plaques from the library were screened by plaque hybridization with the prepared probe, and 7 positive single genomic clones were isolated. EcoRI digestion of PCR products obtained from these 7 clones revealed that 3 out of the 7 (clones 5, 7, and 9) contained internal EcoRI sites, whereas the remaining 4 (clones 2, 4, 6, and 12) did not. One clone from each group was selected for further analysis. In the case of clone 5, phage DNA was purified using standard techniques (10), and then an 8 kb Bam HI restriction fragment from this DNA was subcloned into pBluescript II SK(+) (Stratagene), designated pMARa1.0. From this was derived a 1.8 kb Hind III fragment which was subcloned to produce pMARa1.1. In the case of clone 4, a 1.7 kb EcoRI fragment was subcloned into pBluescript II KS(+), designated pMARb1.0. pMARa1.1 and pMARb1.0 were sequenced using a combination of the nested deletion mutant method, and the advancing primer method (10) . Sequencing was performed by the dideoxy chain termination method (11) using both Sequenase and Taq DNA polymerase (United States Biochemical).

Construction of expression plasmids and transfection: For pMARa1.1, the long 5' non-coding region (-700 bp) was reduced by digestion of the linearized plasmid with exonuclease III for 150 seconds at 30°C, and treating with mung bean nuclease prior to religation, then sequencing. The insert was then removed by digestion with BssH I and Bam HI and subcloned into the expression vector pBC12BI (12) to produce the expression plasmid pMARa2.0. For pMARb1.0, the orientation of the insert was altered by digestion with EcoRI and religation in the opposite orientation in pBluescript II to produce pMARb1.1. pMARb1.1 was double-digested with BamHI and HindIII, and the insert subcloned into the same sites in pBC12BI to obtain pMARb2.0. Transfection of COS-7 cells with the expression plasmids were performed by the DEAE-dextran method, as described previously (13).

Receptor binding assays: 72 hours after transfection, the cells were lysed in buffer containing 10 mM Tris-HCl and 1 mM EDTA (pH7.4) and scraped off plates. Membrane homogenates were prepared as described(13). Binding assays were performed in 500 µl of buffer (75 mM Tris-HCl, 1mM EDTA, 12.5 mM $MgCl_2$, 0.1% bovine serum albumin, pH 7.4) for 1 h at 22°C. Saturation isotherms were performed by incubating the membranes with varying concentrations of [^{125}I]-angiotensin II (2,200 Ci/mmol; New England Nuclear). Nonspecific binding was determined by the addition of 10^{-5} M unlabelled angiotensin II. Competition experiments were performed in the presence of varying concentrations of angiotensin II or angiotensin III (Sigma) or the AT-1 and AT-2 receptor specific antagonists PD 134756 (otherwise known as DuP 753) and PD 123319 (Parke-Davis) (14). Radioactivity bound to membranes was separated from free ligand by filtration through GF/C filters (Whatman). The binding data were analyzed by nonlinear regression using GraphPAD software (GraphPAD Software Inc.).

Results and Discussion

The technique of genomic cloning has revealed the existence of closely related subtypes for several G-protein coupled receptors that contain a seven transmembrane domain structure, such as the adrenergic receptors (15), the dopamine receptor (16), and the muscarinic receptors (17). These receptor subtypes may share common pharmacological characteristics, but are structurally distinct. The different properties and physiological functions of some of these receptor subtypes are currently being investigated.

In this study, our strategy involved the cloning of the angiotensin receptor isoforms from a genomic library from an inbred strain of mouse (Balb/c). At an early stage in the study, we found evidence that the restriction pattern of PCR products from our clones were different, suggesting two distinct genomic sequences. The subsequent analyses of the clones revealed the presence of two structurally distinct receptor types which we name the AT-1a and the AT-1b receptor.

Both open reading frames of the AT-1a and the AT-1b receptors encode for a protein of 359 amino acids (Fig. 1, 2). The calculated molecular weight of the AT-1a receptor is 40,855 Da and of the AT-1b receptor is 40,949 Da. Hydrophobicity profiles reveal seven putative transmembrane domains whose positions are similar in the two receptors. No introns are found within the coding region of either receptors.

Examination of the amino acid sequence of the two receptors reveals that they differ in 22 amino acids, giving an overall sequence identity of 93.9%. These different amino acids are dispersed throughout the whole receptor, but are particularly prevalent in the carboxy terminal intracellular domain. The putative transmembrane domains are well conserved except for 3 amino acid differences in the 5th and 6th transmembrane domains. Some of the amino acid changes outside the transmembrane domains, (for example the substitution of lysine for glutamate at amino acid 227, and the substitution of glutamate for lysine at amino acid 275) could result in possible conformational differences. Of particular interest is the existence of differences involving serine residues in the carboxy terminal intracellular region which may affect phosphorylation. Since phosphorylation of receptors is considered to be involved in the regulation of G-protein coupled receptors (18), this could result in differences in the regulation of these two receptors. The overall amino acid identity of the AT-1a receptor with the published bovine cDNA sequence is 92.2%, and 98.6% with the rat cDNA sequence, whereas the corresponding figures for the AT-1b receptor are 89.7% and 94.4% (Fig. 3).

Binding studies performed on membranes from COS-7 cells transfected with the AT-1a or AT-1b receptors revealed no major differences in ligand binding properties (Fig. 4). Membranes from the specifically transfected cells bound [^{125}I]-angiotensin II with a dissociation constant of 0.35±0.06 nM (AT-1a) and 0.27±0.05 nM (AT-1b) respectively. The number of receptors expressed in COS-7 cells ranged between 400 - 1200 fmol/mg protein. No specific binding was detectable in untransfected COS-7 cells. In competition experiments, both receptors displayed a binding profile characteristic for AT-1 receptors, i.e. high affinity for the AT-1 specific compound PD134756 (DuP 753) and low affinity

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  -72  TGGATTTTTATTTTTTCCAGATCAAGTGCATTTTGAACAGTGTCTGAGACCAACTCAACCCAGAAAAGCAAA     -1

ATG GCC CTT AAC TCT TCT ACT GAA GAT GGC ATC AAA AGA ATT CAA GAT GAC TGC CCC AGG     60
Met ALA Leu Asn Ser Ser THR Glu Asp Gly Ile Lys Arg Ile Gln Asp Asp Cys Pro ARG     20
                 *

GCT GGC AGG CAC AGT TAC ATA TTT GTC ATG ATC CCT ACT CTC TAC AGC ATC ATC TTT GTG    120
Ala Gly Arg His SER Tyr Ile Phe Val Met Ile Pro Thr Leu Tyr Ser Ile Ile Phe Val     40

GTG GGA ATA TTT GGA AAC AGC TTG GTG GTG ATC GTC ATC TAC TTT TAC ATG AAG CTG AAG    180
Val Gly Ile Phe Gly Asn Ser Leu Val Val Ile Val Ile Tyr Phe Tyr Met Lys Leu Lys     60

ACT GTG GCC AGT GTC TTT CTT CTA AAT CTC GCC CTG GCT GAC TTA TGC TTT TTG CTG ACT    240
Thr Val Ala Ser Val Phe Leu Leu Asn Leu Ala Leu Ala Asp Leu Cys Phe Leu Leu Thr     80

TTG CCC CTG TGG GCT GTC TAT ACC GCT ATG GAA TAC CGC TGG CCC TTC GGC AAT CAC CTA    300
Leu Pro Leu Trp Ala Val Tyr Thr Ala Met Glu Tyr ARG Trp Pro Phe Gly Asn His Leu    100

TGT AAG ATC GCT TCG GCC AGC GTC AGT TTC AAC CTC TAC GCC AGC GTG TTC CTG CTC ACG    360
Cys Lys Ile Ala Ser Ala Ser Val Ser Phe Asn Leu Tyr Ala Ser Val Phe Leu Leu Thr    120

TGT CTC AGC ATC GAT CGC TAC CTG GCC ATT GTC CAC CCG ATG AAG TCT CGC CTC CGC CGC    420
Cys Leu Ser Ile Asp Arg Tyr Leu Ala Ile Val His Pro Met Lys Ser Arg Leu Arg Arg    140

ACG ATG CTG GTG GCC AAA GTC ACC TGC ATC ATC ATC TGG CTG ATG GCT GGC TTG GCC AGT    480
Thr Met Leu Val Ala Lys Val Thr Cys Ile Ile Ile Trp Leu Met Ala Gly Leu Ala Ser    160

TTG CCA GCC GTC ATC CAC CGA AAT GTG TAT TTC ATT GAG AAC ACC AAT ATC ACT GTT TGC    540
Leu Pro Ala Val Ile His Arg Asn Val Tyr Phe Ile Glu Asn Thr Asn Ile Thr Val Cys    180
                                                    *

GCT TTT CAT TAC GAG TCC CGG AAT TCA ACG CTC CCC ATA GGA CTG GGC CTA ACC AAG AAC    600
Ala Phe His Tyr Glu Ser ARG Asn Ser Thr Leu Pro Ile Gly Leu Gly Leu Thr Lys Asn    200
                        *

ATC CTG GGC TTC CTG TTC CCT TTC CTA ATC ATT CTT ACC AGC TAT ACT CTT ATA TGG AAA    660
Ile Leu Gly Phe LEU Phe Pro Phe LEU Ile Ile Leu Thr Ser Tyr Thr Leu Ile Trp Lys    220

GCT CTA AAG AAG GCT TAT GAA ATT CAG AAG AAC AAG CCA AGA AAT GAT GAC ATC TTT AGG    720
Ala Leu Lys Lys Ala Tyr GLU Ile Gln Lys Asn LYS Pro Arg Asn Asp Asp Ile Phe Arg    240

ATA ATT ATG GCG ATT GTG CTT TTC TTC TTC TTC TCC TGG GTC CCC CAC CAA ATA TTC ACA    780
Ile Ile Met Ala Ile Val Leu Phe Phe Phe Phe Ser Trp Val Pro His Gln Ile Phe THR    260

TTC CTG GAT GTG CTG ATT CAG CTG GGC GTC ATC CAT GAC TGT AAA ATT GCC GAC ATC GTG    840
Phe Leu Asp Val Leu Ile Gln Leu Gly Val Ile His Asp Cys LYS Ile Ala Asp VLB Val    280

GAC ACT GCC ATG CCC ATA ACC ATC TGC ATA GCG TAT TTT AAC AAC TGC CTG AAC CCT CTG    900
Asp Thr Ala Met Pro Ile Thr Ile Cys Ile Ala Tyr Phe Asn Asn Cys Leu Asn Pro Leu    300

TTT TAC GGC TTT CTG GGG AAA AAA TTT AAA AAG TAT TTC CTC CAG CTC CTG AAA TAT ATT    960
Phe Tyr Gly Phe Leu Gly Lys Lys Phe Lys LYS Tyr Phe Leu Gln Leu Leu Lys Tyr Ile    320

CCC CCA AAG GCC AAG TCG CAC TCA AGC CTG TCT ACG AAA ATG AGC ACG CTC TCC TAC CGC   1020
Pro Pro Lys Ala LYS Ser His SER SER Leu Ser Thr Lys Met Ser Thr Leu Ser Tyr Arg    340
                                    +                           +

CCC TCA GAT AAC ATG AGC TCA GCC GCC AAA AAG CCT GCG TCT TGT TCT GAG GTG GAG TGA   1080
Pro Ser Asp Asn Met Ser Ser ALA Ala LYS Lys PRO Ala SER Cys SER Glu Val Glu ---    359

CAGGTTCA  1088
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Fig.1 Nucleotide and deduced amino acid sequence of the mouse angiotensin receptor AT-1a. The predicted 359-residue amino acid sequence is shown below the nucleotide sequence. Amino acids which are different from the AT-1b receptor are shown in upper case and boxed. The positions of the seven putative transmembrane regions as determined by hydrophobicity analysis are dotted underlined. The position of the nucleotides corresponding to the PCR primers selected for amplification of probe are underlined. Postulated N-glycosylation sites are indicated by asterisks; potential phosphorylation sites are indicated by a cross.

for the AT-2 specific compound PD 123319. The similar ligand binding profiles between the two receptors are consistent with the sequence analysis that the transmembrane regions are highly conserved between the two receptors.

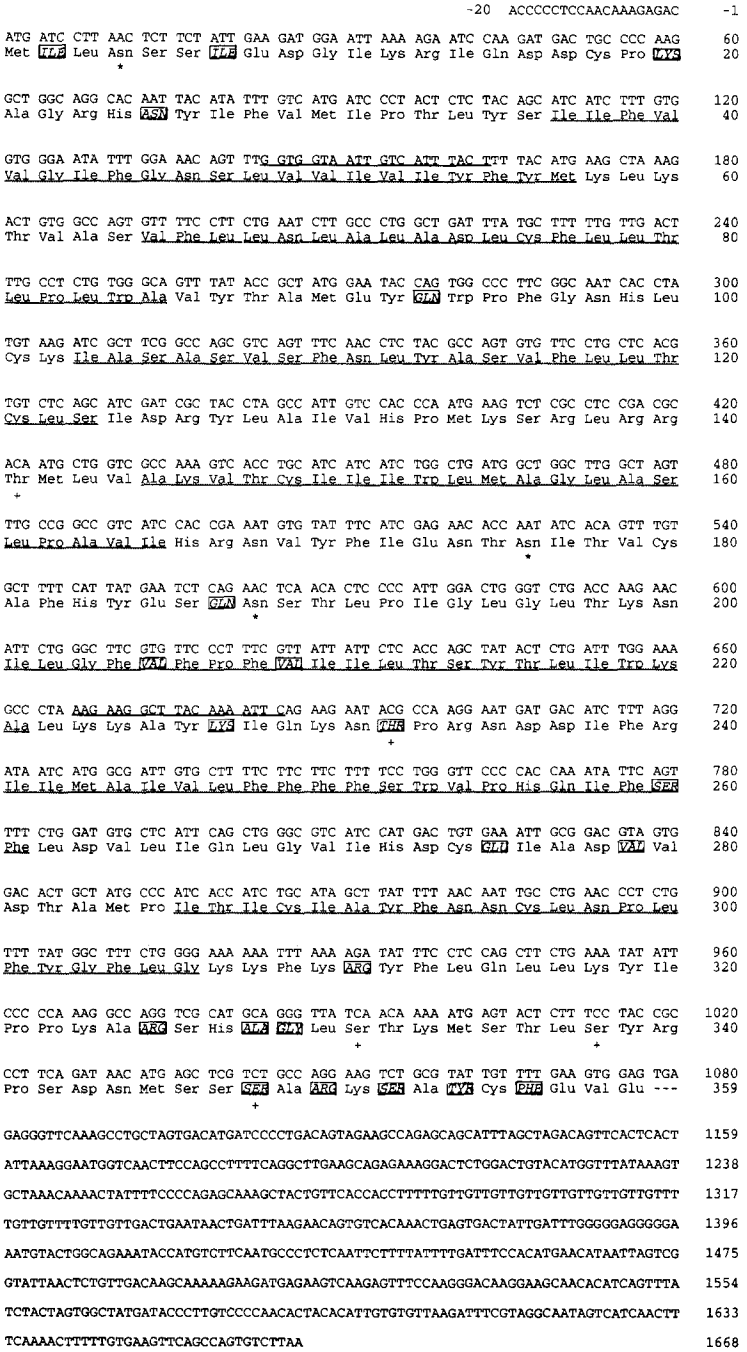

Fig.2 Nucleotide and deduced amino acid sequence of the mouse angiotensin receptor AT-1b. Symbols are as described in Fig.1.

In conclusion, our data suggest that structurally distinct forms of the AT-1 receptor exist in the mouse. Since the AT-1 receptor mediates most of the known actions of angiotensin II, the discovery of AT-1 receptor isoforms could have important implications in the

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                                                            I                                 II
MOUSE A    MALNSSTEDGIKRIQDDCPRAGRHSYIFVMIPTLYSIIFVVGIFGNSLVVIVIYFYMKLKTVASVFLLNLALADLCFLLTLPLWAVYTAMEYRWPFGNHL  10
MOUSE B    .I....I............K....N..................................................................Q.......
RAT        ......A............K................................................................................
BOVINE     .I.................K....N...I.....................................................................Y.

                  III                                   IV
MOUSE A    CKIASASVSFNLYASVFLLTCLSIDRYLAIVHPMKSRLRRTMLVAKVTCIIIWLMAGLASLPAVIHRNVYFIENTNITVCAFHYESRNSTLPIGLGLTKN  20
MOUSE B    ......................................................................................Q.............
RAT        ....................................................................................................
BOVINE     ......................................................L.......TI.....F................Q.....V.......

                 V                                      VI                                           VII
MOUSE A    ILGFLFPFLIILTSYTLIWKALKKAYEIQKNKPRNDDIFRIIMAIVLFFFFSWVPHQIFTFLDVLIQLGVIHDCKIADIVDTAMPITICIAYFNNCLNPL  30
MOUSE B    ....V...V.................K....T...........................S..............E...V.....................
RAT        ............................................................................S.......................
BOVINE     ........................T.........K....K..L..................M.......L.R....E............L..........

MOUSE A    FYGFLGKKFKKYFLQLLKYIPPKAKSHSSLSTKMSTLSYRPSDNMSSAAKKPASCSEVE   359
MOUSE B    ..........R.............R..AG..................S.R.S.Y.F...
RAT        ...............................................S.......F...
BOVINE     ...........................N...............E.GN.ST...P.I...
```

Fig.3. Homology comparison of the deduced amino acid sequences of the mouse AT-1a and AT-1b sequences with those reported for bovine and rat receptors based on published cDNA sequences. Amino acids are shown by their single letter code. The 7 putative transmembrane domains are indicated with a line. Amino acids in the mouse AT-1b, rat, and bovine sequences which are different from the corresponding mouse AT-1a sequences are indicated.

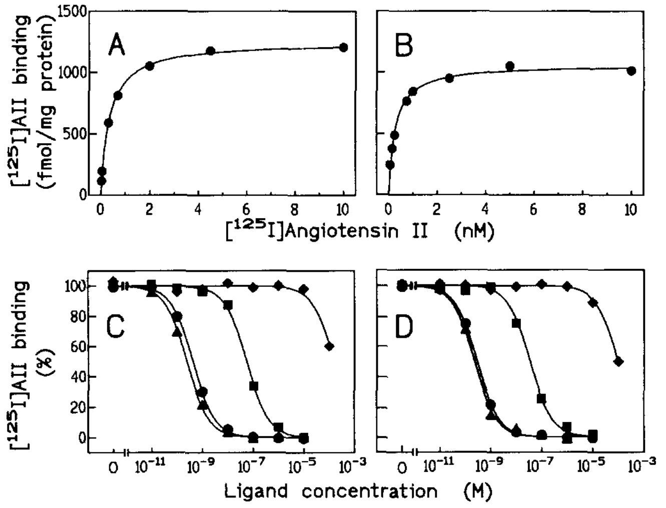

Fig.4 Binding of $[^{125}I]$-angiotensin II to membranes prepared from COS-7 cell transfected with the mouse AT-1a receptor (A,C) and the AT-1b receptor (B,D).
(A,B) Saturation isotherms of specific $[^{125}I]$-angiotensin II binding.
(C,D) Displacement of $[^{125}I]$-angiotensin II (0.1 nM) binding by unlabelled angiotensin II (circles), angiotensin III (triangles), AT-1 specific ligand PD 134756 (squares) and AT-2 specific ligand PD 123319 (diamonds). Data shown are means of a representative experiment.

understanding of the control of body homeostasis by the renin-angiotensin system. Further studies are required to elucidate the physiological consequences of the existence of structurally distinct types of the AT-1 receptor. Specifically, we hope to clarify the contribution of the sequence differences to the conformation of the receptor or to the mode of phosphorylation of the receptor, which may influence the regulation, signal transduction, or desensitization of the AT-1 receptor isoforms.

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