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Identification of two subtypes in the rat type I angiotensin II receptor

Naoharu Iwai and Tadashi Inagami

Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN 37232, USA

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A rat adrenal cDNA library was screened by colony hybridization using a rat cDNA fragment of type I angiotensin II receptor (AT1A) previously isolated from the kidney. Two cDNA clones were identified, designated as AT1B, to have a nucleotide sequence highly homologous to and yet distinct from AT1A. The amino acid sequence of AT1B consists of 359 amino acid residues and has 96% identity with AT1A. No conspicuous difference in the ligand binding characteristics was observed between AT1A and AT1B. The mRNA for AT1B was expressed in many tissues as is the case with AT1A, and most abundantly expressed in the adrenal glands in the Sprague–Dawley rats. The existence of two subtypes in the rat type I angiotensin II receptor might explain the diverse actions of angiotensin II in various tissues.

Angiotensin II receptor

1. INTRODUCTION

Angiotensin II (AII) is a multifunctional peptide which elicits contractile, secretory, metabolic, growth, and neuronal activities in its diverse target cells [1,2,3]. The diversity in its actions has suggested the existence of several subtypes of the AII receptors. Recent introduction of new AII antagonists, such as Dup 753 and PD 123177 [4,5], have identified two major subtypes of AII receptor. While both subtypes, designated as AT1 and AT2 receptors, bind AII and its peptide analogues with the comparable avidity, AT1 receptors have high affinity for Dup 753, and AT2 receptors have high affinity for PD 123117. To date, all of the known functions of AII seem to be mediated through the AT1 receptor, and the function of the AT2 receptor remains to be determined [4,5], which consequently suggests the existence of subtypes in the type I receptors.

Recently, we and others have isolated a bovine [6] and a rat [7,8] cDNA for the AT1 receptor. The cloned AT1 receptors have the typical features of G protein coupled receptors, and are linked to phosphoinositide-specific phospholipase C. Southern blotting analysis of the rat genome using the cloned rat cDNA for AT1 receptor has suggested the existence of another closely related sequence (data not shown). Restriction enzyme mapping of the polymerase chain reaction (PCR) products from various tissues synthesized by the primers corresponding to the cloned rat cDNA for AT1, has suggested the existence of a closely related sequence expressed in the rat adrenal (data not shown). In this communication, isolation and characterization of a cDNA for a subtype of the rat AT1 receptor from the adrenal is reported.

Correspondence address: T. Inagami, Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN 37232, USA.

2. MATERIALS AND METHODS

2.1. *cDNA cloning*

A rat adrenal cDNA library was prepared using a Superscript cDNA synthesis kit (Gibco-BRL, Gaithersburg, MD). The synthesized cDNAs whose sizes were more than 2 kb were selected by agarose gel electrophoresis and were ligated to pSPORT I (Gibco-BRL), and 30,000 colonies were screened by colony hybridization using a *Kpn*I–*Sac*I fragment of the rat cDNA for the AT1 receptor [8] which covered almost all of the coding region. The hybridization was done in 35% formamide, 6×SSC (0.90 M NaCl, 0.09 M sodium citrate), 50 mM NaH_2PO_4/Na_2HPO4 (pH 7.0), and 1% sodium dodecyl sulfate (SDS) at 42°C for 12 h. The filters were washed in 2×SSC containing 1% SDS at 60°C.

Sequence analysis was done on both strands directly from double stranded plasmids using a Sequenase sequence kit (United States Biochemical Corp., Cleaveland, OH) and *Exo*III/Mung Bean nuclease deletion kit (Stratagrene, La Jolla, CA).

2.2. *RNA blot hybridization analysis*

Total RNA and poly(A)$^+$ RNA were isolated as described previously [9]. RNA hybridization analyses were done in a solution containing 50% formamide, 6×SSC, 50 mM sodium phosphate buffer (pH= 7.0), 1% SDS at 42.0°C for 12 h. The filters were washed twice at 65.0°C in a solution containing 1×SSC and 1% SDS.

2.3. *DNA transfection*

The cloned cDNA fragment (whole length) was inserted into a mammalian expression vector pcDNA I (InVitrogen, San Diego, CA). DNA transfection to Cos 7 cells was performed as described previously [6,8] using an electroporation system (Bio Rad, La Jolla, CA). Three days after transfection, the cells were subjected to ^{125}I–angiotensin II binding assay. The receptor binding assay was done as previously described [8,10].

2.4. *Polymerase chain reaction*

To assess the ratio of the expression levels of AT1A mRNA to that

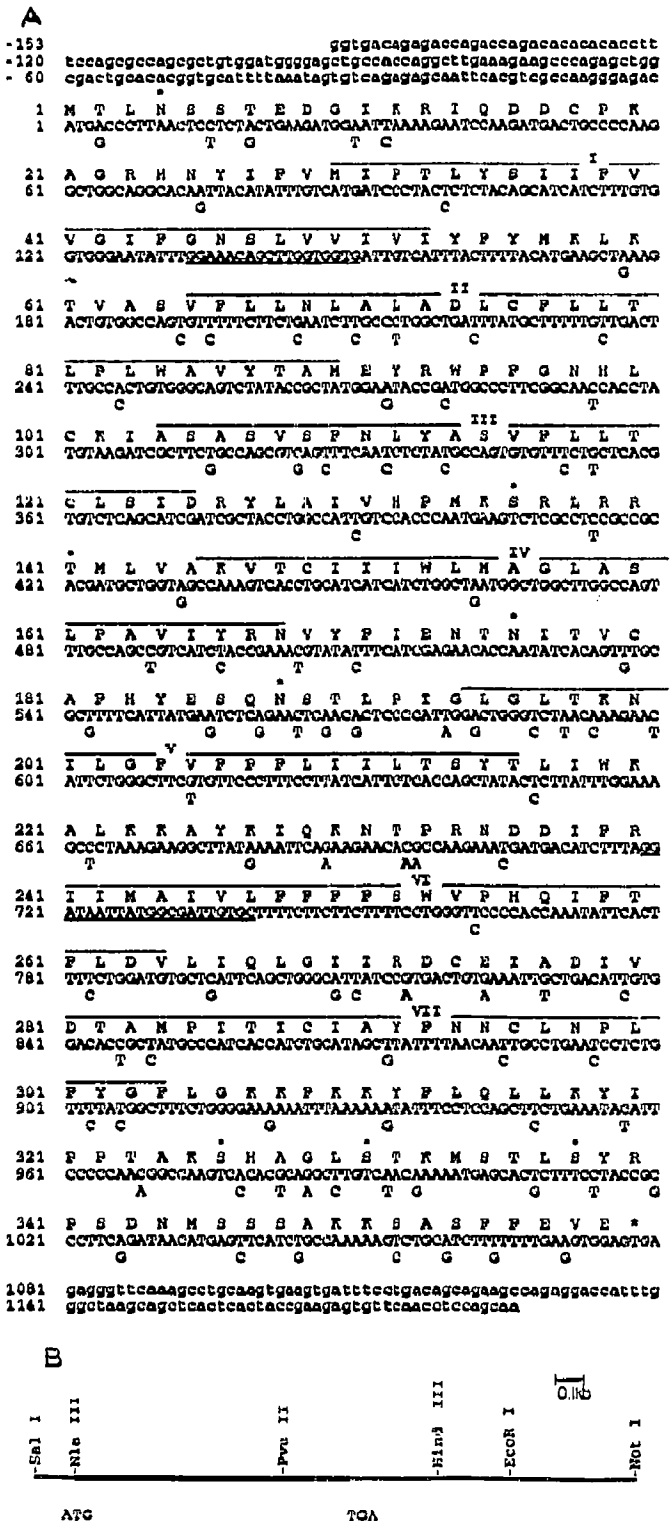

Fig. 1. Nucleotide sequence of AT1B. (A) Nucleotide and deduced amino acid sequence of AT1B. The deduced amino acid sequence is shown above the nucleotide sequence, and the putative transmembrane domains I-VII are indicated above the amino acid sequence. Three possible *N*-glycosylation sites are indicated by asterisks; potential phosphorylation sites are indicated by dots. The nucleotide sequence of the coding region of AT1A is shown below that of AT1B. Only the sequences of the different nucleotides are shown. The underlined regions correspond to the primers' sequences used in the PCR analysis (Fig. 3). (B) Restriction map of the 2.3 kb clone. The 5' *Sal*I site and 3' *Not*I site are the insertion sites of the cDNA into pSPORT I. The thick line indicates the coding region.

of AT1B mRNA, the polymerase chain reaction (PCR) method was employed. The primers used were as follows:

5'-sense primer = 5'-GGAAACAGCTTGGTGGTG-3' (133–150)
3'-antisense primer = 5'-GCACAATCGCCATAATTATCC-3' (739–719)

These primers corresponded to the regions where no sequence divergence was noted between AT1A and AT1B. Ten-to-twenty micrograms of total RNA from various tissues were reverse-transcribed using random primers as a primer, and the resultant cDNA mixtures were amplified by PCR [8] using the two primers described above. The reaction profile included 30–35 cycles of denatureration at 95°C for 30 s, annealing at 54°C for 60 s, and polymerization at 72°C for 90 s. Contamination of genomic DNA in the sample RNAs was neglected by subjecting the RNAs directly to PCR amplification in which no significant product was synthesized. The DNA fragment synthesized from AT1A mRNA should have seven *Hae*III sites, and that from AT1B should have four *Hea*III sites. The digested PCR products were electrophoresed on a 6% polyacrylamide gel. A trace amount of [α^{32}P]dCTP was included in the PCR reaction mixture for autoradiography. The whole cDNA fragment of AT1A and AT1B were inserted into pBluescript KS(+) (Stratagene). Synthetic RNA copies of AT1A and AT1B mRNA were synthesized using T7 RNA polymerase according to the manufacturer's recommended method after linearization of the template plasmids.

2.5. *Animals*

Male Sprague–Dawley rats, spontaneously hypertensive rats, and Wistar–Kyoto rats were obtained from Taconic Farms (Germantown, NY).

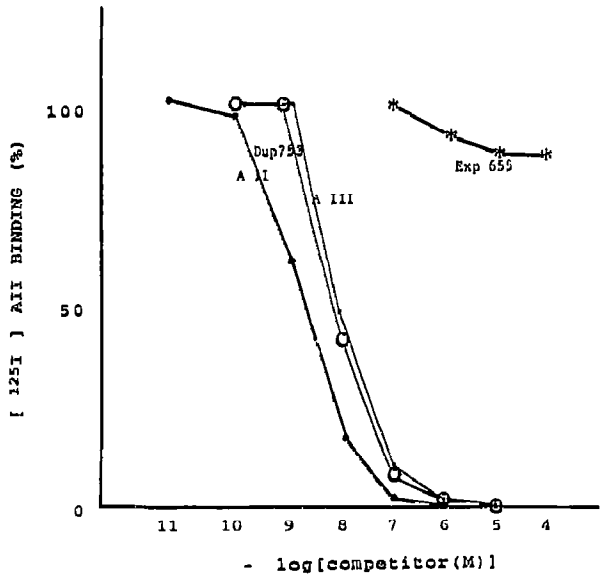

Fig. 2. Binding characteristics of AT1B. Displacement of ^{125}I-labelled angiotensin II (0.2 nM) by unlabelled angiotensin II (•), Dup 753 (○), angiotensin III (■), and Exp 655 (*). Each point represents a mean value of triplicate experiments.

3. RESULTS AND DISCUSSION

From the 30,000 clones screened, two clones were isolated which hybridized to the rat AT1 cDNA fragment. The insert size of one clone was 2.3 kb and that of the other was 2.2 kb. These two clones were identified to correspond to the same mRNA based on the nucleotide sequence analyses, and were revealed to be distinct from the cDNA for the rat AT1 receptor previously cloned. Figure 1 shows the nucleotide sequence of the 2.3 kb cDNA, and the deduced amino acid sequence. The AT1 receptor which was isolated previously from the rat kidney is designated AT1A, and the cDNA which was isolated in the present study is designated as AT1B in this communication. The amino acid sequence of AT1B also consists of 359 amino acid residues, has 96% sequence identity with AT1A, and shares features commonly found in other G protein coupled receptors. No significant sequence homology was noted in the untranslated region shown in the Fig. 1 between AT1A and AT1B.

The positions of the putative transmembrane domains I–VII are tentatively assigned on the basis of a hydropathy profile and a sequence comparison with other G protein coupled receptors. There are several potential sites for post-translational modification in AT1B which include three cosensus sites for *N*-glycosylation in extracellular loops, one of which is in the N-terminus preceeding the first transmembrane domain and the other two are in the third extracellular loop. One cysteine residue is present in each of the four extracellular loops which would form two disulfide bridges essential for ligand binding [11]. Several serine

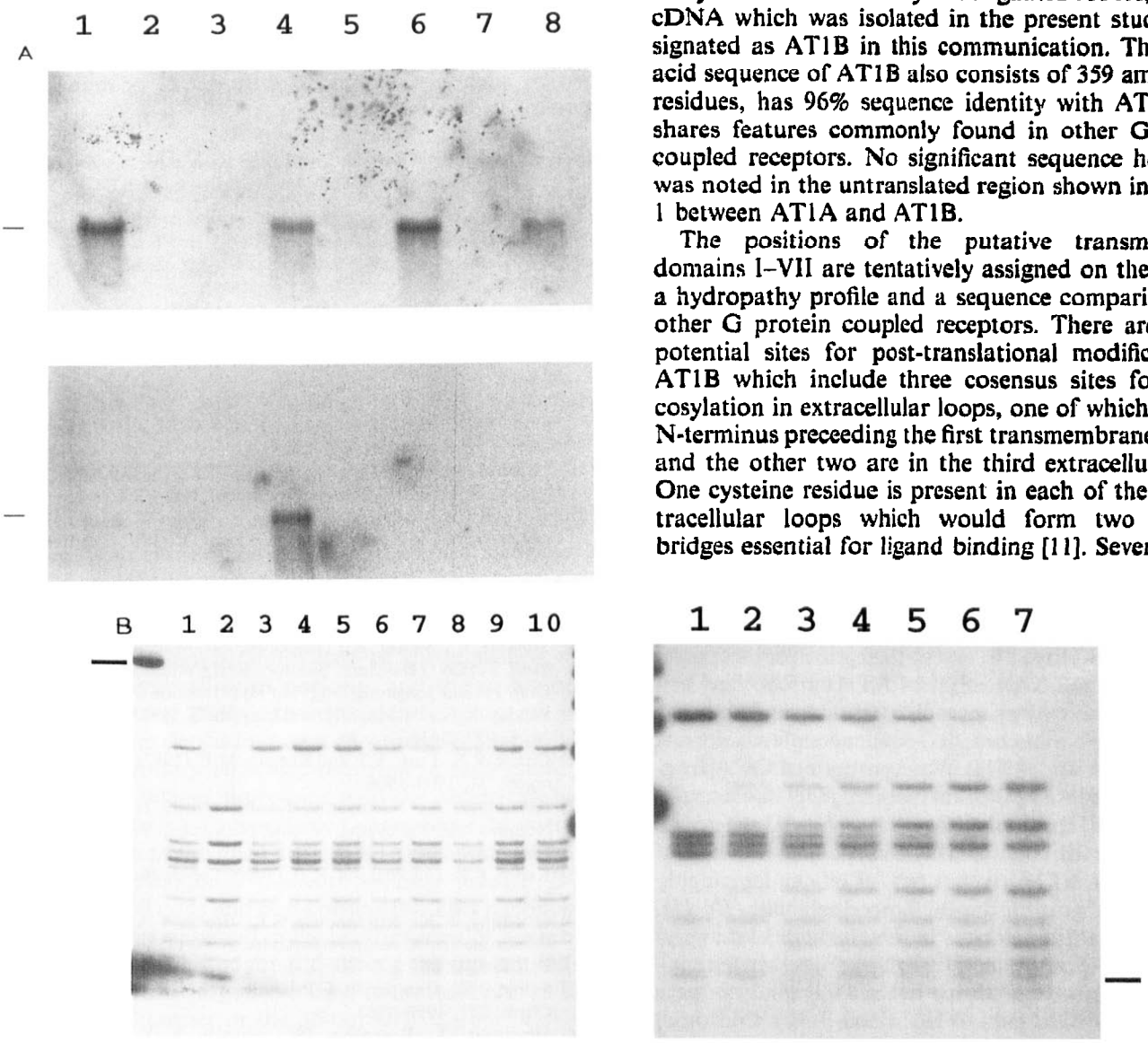

Fig. 3. Expression of the mRNA for AT1B. (A) Nothern blot analysis of AT1B. Two micrograms of the poly(A)$^+$ RNA from kidney (lane 1), brain (2), ventricle of the heart (3), adrenal (4), spleen (5), liver (6), ileum (7), and fetal skin (8) were used in the analysis. All the sample RNAs in this experiment were obtained from Sprague–Dawley rats. The upper panel was obtained by using the whole cDNA fragment of the AT1B as a probe, and the lower panel was obtained by using the *Sal*I-*Nla*III fragment (see Fig. 1B) as a probe. The position of the 18S ribosomal RNA is marked in the left side. (B) Polymerase chain reaction (PCR) analysis of the expression of AT1B mRNA. *Right panel*: the RNAs containing 10 pg of synthetic AT1B RNA (B-RNA) (lane 1), 8 pg of B-RNA and 2 pg of synthetic AT1A RNA (A-RNA) (lane 2), 6.67 pg of B-RNA and 3.33 pg of A-RNA (lane3), 5 pg of B-RNA and A-RNA (lane 4), 3.33 pg of B-RNA and 6.67 pg of A-RNA (lane 5), 2 pg of B-RNA and 8 pg of A-RNA (lane 6), and 10 pg of A-RNA (lane 7) were reverse-transcribed, and the resultant cDNA mixtures were amplified. The PCR products were ethanol precipitated and then digested with *Hae*III. The DNA fragment originating from B-RNA should give 263 bp, 103 bp, 95 bp, 90 bp, and 54 bp fragments, while that from AT1A should give 151 bp, 112 bp, 95 bp, 69 bp, 54 bp, 48 bp, 42 bp, and 34 bp. Please note that the 39 bp fragment is an artifact of primers' dimer which is indicated on the right side. *Left panel*: ten micrograms of the total RNA from the adrenals of SD rats (lane 1), livers of SD rats (2), livers of WKY (W) rats (3), livers of SHR (S) rats (4), brains of W rats (5), brains of S rats (6), kidneys of W rats (7), kidneys of S rats (8), adrenals of W rats (9), and adrenals of S rats (10) were reverse-transcribed, and the resultant cDNA mixtures were amplified by PCR. The PCR products were digested with *Hae*III restriction enzyme. Three 20-week-old SHR and WKY rats were used to isolate the sample RNAs.

and threonine residues exist in the second and C-terminal cytoplasmic domain for possible regulatory phosphorylation [12,13]. Other notable features include the highly hydrophilic N-terminal sequence, and the short third cytoplasmic loop. All these features are shared in common with AT1A.

The ligand binding characteristics for the AT1B was determined by expressing the cloned cDNA in Cos 7 cells. The binding of ^{125}I-AII to the transfected Cos 7 cells was saturable with a dissociation constant (K_D) of 2.3 nM, which is comparable to that of AT1A (1.6 nM–1.9 nM) [7,8]. No significant binding was observed in the mock-transfected Cos 7 cells. A displacement experiment (Fig. 2) of ^{125}I-AII binding indicated that DUP 753 (type I specific antagonist) is by far a more potent inhibitor than EXP 655 (type II antagonist), which indicated that AT1B has also type I character.

Expression of AT1B mRNA in various tissues was investigated by Nothern blotting (Fig. 3A). Because the untranslated region of the AT1B has no significant sequence similarity to AT1A, the fragment (*Sal* I–*Nla* III) containing the 5′ untranslated region was used as a probe. As shown in Fig. 3A (lower panel) AT1B mRNA was detected in the adrenal, and slightly in the kidney. The same pattern was obtained when using a fragment from the 3′ untranslated region (data not shown). In contrast to the lower panel, the upper panel shows a Nothern blot analysis probed with the whole cDNA of AT1B in which prominent signals were obtained in the kidney and the liver. The polymerase chain reaction method was employed to assess the ratios of the expression of AT1A mRNA to that of AT1B mRNA in various tissues. The two primers used corresponded to the regions where no nucleotide sequence difference exist between AT1A and AT1B. The synthesized DNA fragment from AT1A should have seven *Hae*III sites, and that from AT1B should have four *Hae*III sites. As shown in Fig. 3B (right panel) the molecular ratios of the mRNA for AT1A to that for AT1B can be roughly estimated by digesting the PCR products with *Hae*III. Please note that the mRNAs in the Nothern blot analysis (Fig. 3A) were obtained from Sprague–Dawley rats, and most of the mRNAs used in the PCR analysis were obtained from SHR and WKY (lane 3–10). Although the molecular ratios of AT1A mRNA to AT1B mRNA vary between tissues and strains (SD versus SHR or WKY), the mRNA for AT1B is also expressed in as many tissues as that for AT1A.

The presence of heterogeneity among the AII receptor population has been suggested from the observation that AII elicited diverse responses in various tissues, although most AII receptors in various tissues seem to possess similar binding affinities and specificities for AII and its peptide analogs. For instance, the AII receptors have been reported to be linked via G proteins to phospholipase C [14], adenylate cyclase [15] and calcium channels [16]. Whether the two subtypes of the type I AII receptors have different functional properties, such as coupling to different G proteins [17], remains to be determined.

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