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MOLECULAR CLONING OF THE HUMAN ANGIOTENSIN II TYPE 2 RECEPTOR cDNA

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SUMMARY: A human adult lung cDNA library was screened and one full-length human angiotensin II type 2 receptor (hAT_2R) clone was isolated and characterized. The hAT_2R cDNA clone contains a 1089-base-pair open reading frame which encodes a protein of 363 amino acid residues. The hAT_2R is approximately 92% identical in sequence to the rat and mouse AT_2R sequences. Specific binding of [^{125}I]CGP42112A was demonstrated in membranes from COS-7 cells transiently transfected with the hAT_2R cDNA. Scatchard analysis and ligand displacement profiles were typical of the AT_2R. Northern analysis demonstrated that the hAT_2R mRNA was abundantly expressed in human adult lung and in human fetal kidney. Additionally, the hAT_2R mRNA was just detectable in human adult heart and aorta. In contrast, the rat AT_2R mRNA was abundantly expressed in the rat brain and just detectable in the rat lung.

The renin-angiotensin system (RAS) plays a central role in the homeostasis of fluid volume, electrolyte balance and the maintenance of vascular tone (1-3). Recently, it was demonstrated that angiotensin II (AII), the biologically active component of the RAS, interacts with at least two pharmacologically distinct subtypes of AII receptors, AT_1R and AT_2R (4-9). The AT_1R has a high affinity for losartan, whereas AT_2R has a high affinity for PD123319 and CGP42112A and a low affinity for losartan (4-9). To date, most if not all of the known effects of AII in adult tissues are attributable to the AT_1R (10-13). Currently, the physiological function of the AT_2R or its mechanism(s) of signal transduction is poorly understood. Although it has been suggested that the AT_2R inhibits phosphotyrosine phosphatase activity (14,15). To better define the function of the AT_2R, several laboratories have cloned mouse and rat AT_2R (mAT_2R and rAT_2R) cDNAs (15-17). In this study, we report the cloning and characterization of the human AT_2R (hAT_2R) cDNA. Utilizing the hAT_2R cDNA as a probe we have examined its mRNA distribution in various adult and fetal human tissues. Furthermore, we have compared the tissue distribution of the AT_2R mRNA in human and rat.

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MATERIALS AND METHODS

Materials: AII, AI and a non-selective AII receptor antagonist, [Sar^1,Ile^8]-AII, were purchased from Sigma (St. Louis, MO). The AT_2-selective ligand CGP42112A was a gift from Ciba-Geigy (Basel, Switzerland) and Parke-Davis (Ann Arbor, MI), respectively. [^{125}I]CGP42112A was purchased from the Peptide Radioiodination Center (Washington State University, WA).

Library Screening: A human adult lung 5'-stretch cDNA library constructed in the λDR2 vector was purchased from Clontech (Palo Alto, CA). The library was screened by standard procedures utilizing a radiolabeled polymerase chain reaction (PCR) generated rat AT_2R (rAT_2R) probe. To amplify this probe an amplimer set was synthesized (i.e. sense primers 5' GGAGTCTCTGACAGTTCAATATG 3', antisense primer 5' CATTGCTAGGCTGATTACATGCATC 3') utilizing the published rAT_2R cDNA sequences (15,16). Positive clones were plaque purified and insert size was determined by PCR utilizing λDR2 insert screening amplimers (Clontech). Lambda clone $hAT_2R/7$ had an insert size of approximately 2.7 kb and was further characterized by sequencing (18).

Expression in COS-7 Cells and Ligand Binding Assays: Lambda clone $hAT_2R/7$ was converted into a plasmid, pDR2-$hAT_2R/7$, by cre-lox conversion. Both strands of the 2.7 kb hAT_2R cDNA clone were sequenced by the dideoxy sequencing method. For expression experiments, the 2.7 kb hAT_2R cDNA insert was removed by Bam HI/Xba I digestion and subcloned into the eukaryotic expression vector, pcDNA3 (Invitrogen, San Diego, CA). The new construct pcDNA3-$hAT_2R/7$ was transfected into COS-7 cells by the DEAE-dextran procedure as described previously (19). The transfected cells were harvested and membranes prepared as previously described (20). Radioreceptor binding assays were performed as previously described (15). Each experiment was performed in triplicate. Data were analyzed by the program Kinetic, EBDA, Ligand (Elsevier Biosoft, Cambridge, UK).

Northern Blot Analysis: Human multiple tissue Northern blots, containing approximately 2 μg of pure poly A^+ RNA isolated from various adult or fetal tissues, were purchased from Clontech. Adult human adrenal, aorta and pituitary poly A^+ RNA was also purchased from Clontech. This RNA was fractionated on a denaturing formaldehyde/1.2% agarose gel and blotted onto Nytran (Schleicher and Schuell, Keene, NH). A rat multiple tissue Northern blot, containing 2 μg of pure poly A^+ RNA, isolated from various adult tissues, was also purchased from Clontech. Human Northern blots were probed with a radiolabeled 2.7 kb Bam HI/Xba I hAT_2R cDNA fragment isolated from pDR2-$hAT_2R/7$ and a human β-actin cDNA control probe. Rat Northern blots were probed with a radiolabeled rAT_2R PCR generated fragment and β-actin probe. All Northern blots probed with AT_2R probes were exposed to x-ray film with an intensifying screen for five days. Northern blots probed with β-actin control probes were exposed to x-ray film for one hour.

RESULTS

It is evident that vast species differences in the proportion and distribution of the AT_2R exist (reviewed in 21). To determine the appropriate human tissue to utilize for cDNA cloning, a rAT_2R PCR probe was generated and utilized for Northern analysis. Northern results suggested that the AT_2R mRNA was abundantly expressed in human lung tissue (data not shown). Therefore, a human lung cDNA library was screened by standard techniques with the rAT_2R specific PCR probe. Several positive clones were isolated. PCR analysis demonstrated that clone $hAT_2R/7$ contained the largest insert

(data not shown) and was selected for further characterization. The nucleotide and deduced amino acid sequence of the $hAT_2R/7$ are shown in Figure 1. This 2.7 kb clone harbored a 1089 bp open reading frame with a deduced amino acid sequence of 363 amino acid residues. Hydropathy analysis of the deduced amino acid sequence revealed the presence of seven putative transmembrane domains. There are multiple serine and

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-140                    GGATCCGCGCAGAATTCAAAGCATTCTGCAGCCTGAATTTTGAAGGAGTGTGTTTAGGCAC  -80
    TAAGCAAGCTGATTTATGATAACTGCTTTAAACTTCAACAACCAAAGGCATAAGAACTAGGAGCTGCTGACATTTCAAT   -1

                ●                                   ●
ATG AAG GGC AAC TCC ACC CTT GCC ACT ACT AGC AAA AAC ATT ACC AGC GGT CTT CAC TTC   60
met lys gly asn ser thr leu ala thr thr ser lys asn ile thr ser gly leu his phe   20
                ●                   ●               ●
GGG CTT GTG AAC ATC TCT GGC AAC AAT GAG TCT ACC TTG AAC TGT TCA CAG AAA CCA TCA  120
gly leu val asn ile ser gly asn asn glu ser thr leu asn cys ser gln lys pro ser   40

GAT AAG CAT TTA GAT GCA ATT CCT ATT CTT TAC TAC ATT ATA TTT GTA ATT GGA TTT CTG  180
asp lys his leu asp ala ile pro ile leu tyr tyr ile ile phe val ile gly phe leu   60

GTC AAT ATT GTC GTG GTT ACA CTG TTT TGT TGT CAA AAG GGT CCT AAA AAG GTT TCT AGC  240
val asn ile val val val thr leu phe cys cys gln lys gly pro lys lys val ser ser   80

ATA TAC ATC TTC AAC CTC GCT GTG GCT GAT TTA CTC CTT TTG GCT ACT CTT CCT CTA TGG  300
ile tyr ile phe asn leu ala val ala asp leu leu leu leu ala thr leu pro leu trp  100

GCA ACC TAT TAT TCT TAT AGA TAT GAC TGG CTC TTT GGA CCT GTG ATG TGC AAA GTT TTT  360
ala thr tyr tyr ser tyr arg tyr asp trp leu phe gly pro val met cys lys val phe  120

GGT TCT TTT CTT ACC CTG AAC ATG TTT GCA AGC ATT TTT TTT ATC ACC TGC ATG AGT GTT  420
gly ser phe leu thr leu asn met phe ala ser ile phe phe ile thr cys met ser val  140

GAT AGG TAC CAA TCT GTC ATC TAC CCC TTT CTG TCT CAA AGA AGA AAT CCC TGG CAA GCA  480
asp arg tyr gln ser val ile tyr pro phe leu ser gln arg arg asn pro trp gln ala  160

TCT TAT ATA GTT CCC CTT GTT TGG TGT ATG GCC TGT TTG TCC TCA TTG CCA ACA TTT TAT  540
ser tyr ile val pro leu val trp cys met ala cys leu ser ser leu pro thr phe tyr  180

TTT CGA GAC GTC AGA ACC ATT GAA TAC TTA GGA GTG AAT GCT TGC ATT ATG GCT TTC CCA  600
phe arg asp val arg thr ile glu tyr leu gly val asn ala cys ile met ala phe pro  200

CCT GAG AAA TAT GCC CAA TGG TCA GCT GGG ATT GCC TTA ATG AAA AAT ATC CTT GGT TTT  660
pro glu lys tyr ala gln trp ser ala gly ile ala leu met lys asn ile leu gly phe  220

ATT ATC CCT TTA ATA TTC ATA GCA ACA TGC TAT TTT GGA ATT AGA AAA CAC TTA CTG AAG  720
ile ile pro leu ile phe ile ala thr cys tyr phe gly ile arg lys his leu leu lys  240

ACG AAT AGC TAT GGG AAG AAC AGG ATA ACC CGT GAC CAA GTC CTG AAG ATG GCA GCT GCT  780
thr asn ser tyr gly lys asn arg ile thr arg asp gln val leu lys met ala ala ala  260

GTT GTT CTG GCC TTC ATC ATT TGG TGC CTT CCC TTC CAT GTT CTG ACC TTC CTG GAT GCT  840
val val leu ala phe ile ile cys trp leu pro phe his val leu thr phe leu asp ala  280

CTG GCC TGG ATG GGT GTC ATT AAT AGC TGC GAA GTT ATA GCA GTC ATT GAC CTG GCA CTT  900
leu ala trp met gly val ile asn ser cys glu val ile ala val ile asp leu ala leu  300

CCT TTT GCC ATC CTC TTG GGA TTC ACC AAC AGC TGC GTT AAT CCG TTT CTG TAT TGT TTT  960
pro phe ala ile leu leu gly phe thr asn ser cys val asn pro phe leu tyr cys phe  320

GTT GGA AAC CGG TTC CAA CAG AAG CTC CGC AGT GTG TTT AGG GTT CCA ATT ACT TGG CTC 1020
val gly asn arg phe gln gln lys leu arg ser val phe arg val pro ile thr trp leu  340
                                                    #
CAA GGG AAA AGA GAG AGT ATG TCT TGC CGG AAA AGC AGT TCT CTT AGA GAA ATG GAG ACC 1080
gln gly lys arg glu ser met ser cys arg lys ser ser ser leu arg glu met glu thr  360

TTT GTG TCT TAAACGGAGAGCAAAATGCATGTAATCAACATGGCTACTTGCTTTGAGGCTCACCAGAATTATTTTT 1156
phe val ser END                                                                 363

AAGTGGTTTTAATAAAATAATAAAATTTCCCCTAATCTTTTCTGAATCTTCTGAAACCAAATGTAACTATGTTTATCGT 1235
CCAGTGACTTTCAGGAATGCCCATTGTTTTCTGATATGTTTGTACAAGATTTCATTGGTGAGAC               1299
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Fig. 1. Nucleotide and deduced amino acid sequence of the hAT_2R. The predicted 363 amino acid residue sequence is shown below the nucleotide sequence. Putative transmembrane domains as determined by hydrophobicity analysis are underlined. Putative N-glycosylation sites are indicated with closed circles at amino acid residues 4, 13, 24, 29 and 34. A consensus protein kinase C phosphorylation site is indicated by a (#).

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                                                                    TM-1
hAT2R    MKGNSTLATTSKNITSGLHFGLVNISGNNESTLNCSQKPSDKHLDAIPILYYII   54
rAT2R    ..D.FSF.A..R....S.P.DNL.AT.T...AF...H..A....E.......M.   54
mAT2R    ..D.FSF.A..R....SRP.DNL.AT.T...AF...H.......E.......M.   54

                                  TM-2
hAT2R    FVIGFLVNIVVVTLFCCQKGPKKVSSIYIFNLAVADLLLLATLPLWATYYSYRY   108
rAT2R    .....A......S.....................................L.....   108
mAT2R    .....A......S.....................................L.....   108

                TM-3                                        TM-4
hAT2R    DWLFGPVMCLVFGSFLTLNMFASIFFITCMSVDRYQSVIYPFLSQRRNPWQASY   162
rAT2R    ..........................................................   162
mAT2R    ..........................................................   162

                                                            TM-5
hAT2R    IVPLVWCMACLSSLPTFYFRDVRTIEYLGVNACIMAFPPEKYAQWSAGIALMKN   216
rAT2R    V.........................................................   216
mAT2R    V.........................................................   216

                                                    TM-6
hAT2R    ILGFIIPLIFIATCYFGIRKHLLKTNSYGKNRITRDQVLKMAAAVVLAFIICWL   270
rAT2R    ..........................................................   270
mAT2R    ..........................................................   270

                                  TM-7
hAT2R    PFHVLTFLDALAWMGVINSCEVIAVIDLALPFAILLGFTNSCVNPFLYCFVGNR   324
rAT2R    ..........T...I.....................................T.....   324
mAT2R    ..........T...I.....................................T.....   324

hAT2R    FQQKLRSVFRVPITWLQGKRESMSCRKSSSLREMETFVS                   363
rAT2R    ....................T............D....                     363
mAT2R    ....................T.....G......D....                     363
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Fig. 2. Comparison of the amino acid sequences between the human, rat and mouse AT_2R. The amino acid sequences of the hAT_2R, rAT_2R and mAT_2R are shown. Amino acids are represented by their single letter code. Putative transmembrane domains are indicated with a line and are identified as TM-1 through TM-7. Amino acids in the rAT_2R and mAT_2R sequences which are different from the corresponding hAT_2R sequences are shown.

threonine amino acid residues present in the third intracellular loop and carboxyl tail domains of the hAT_2R that may be putative phosphorylation sites (22). There are also five consensus sites for N-glycosylation (23) in the amino-terminal extracellular domain of the hAT_2R. Recently, Servant et al. (24) demonstrated by photoaffinity labeling experiments, that the hAT_2R is N-glycosylated in at least three sites in vivo.

A comparison of deduced amino acid sequences of the human, rat and mouse AT_2R is shown in Figure 2. The amino acid sequence of the hAT_2R was 92% identical to the rat and mouse AT_2R cDNA sequence (15-17). Only the amino terminal extracellular domain of the hAT_2R shows divergence from the rat and mouse AT_2R (Figure 2).

Membranes from COS-7 cells transfected with the pcDNA3-hAT_2R/7 plasmid showed specific binding to [^{125}I]CGP42112A. The Scatchard plot of the binding showed K_d and B_{max} of .33 nM and .54 pmol/mg protein, respectively (Figure 3A). No specific binding was detectable in untransfected COS-7 cells (data not shown). Displacement of this binding is shown in Figure 3B. The binding of [^{125}I]CGP42112A was competed by unlabeled angiotensin analogs and AT_2R specific ligands in the following rank order:

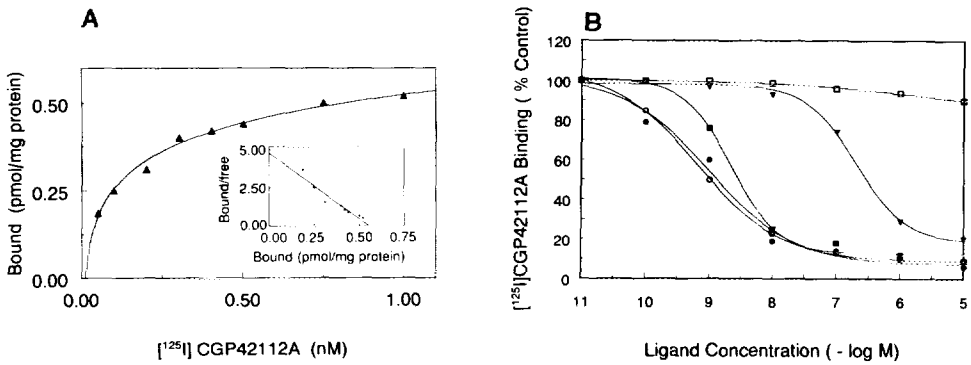

Fig. 3. Binding characteristics of [^{125}I]CGP42112A to COS-7 cells transfected with the pcDNA3-hAT$_2$R construct. A. Saturation isotherm of the specific binding of [^{125}I]CGP42112A to membranes from COS-7 cells expressing the hAT$_2$R. Inset shows a Scatchard plot of the same data. **B.** Displacement of specific [^{125}I]CGP42112A binding in the hAT$_2$R cDNA transfected COS-7 cell membranes by unlabeled Sar^1,Ile^8-AII (○), CGP42112A (●), AII (■), AI (▼) and Losartan (□). The plotted data are from a typical experiment.

Sar^1,Ile^8-AII=CGP42112A>AII>AI. Losartan (10 μM), an AT$_1$R selective antagonist (4-9), did not inhibit [^{125}I]CGP42112A binding (Figure 3B). These binding characteristics are in good agreement with those observed in the membranes from that reported for the mouse and rat AT$_2$R (15-17).

Northern blot experiments utilizing the hAT$_2$R cDNA as a probe demonstrated that this gene was abundantly expressed in human adult lung (Figure 4A, lane 4) and just detectable in adult heart (Figure 4A, lane 1) and aorta (Figure 4B, lane 2). The hAT$_2$R mRNA was also highly expressed in human fetal kidney (Figure 4D, lane 4) and to a lesser amount in human fetal lung (Figure 4D, lane 2). In contrast, the rAT$_2$R mRNA was abundantly expressed in rat brain (Figure 4C, lane 2) and just detectable in rat lung (Figure 4C, lane 4). All Northern blots were hybridized with a β-actin control probe to assess the relative quantity of the poly A$^+$ mRNA present in each lane.

DISCUSSION

In the present study, we have demonstrated that we have isolated the hAT$_2$R cDNA. The hAT$_2$R is approximately 92% identical with the mouse and rat AT$_2$R. The hAT$_2$R cDNA has a K_d of .33 nM for the AT$_2$R antagonist [^{125}I]CGP42112A. The transiently expressed hAT$_2$R cDNA clearly has a ligand displacement profile typical of the AT$_2$R. Northern blot analyses, for the first time, demonstrate that the hAT$_2$R mRNA is abundantly expressed in the human adult lung and just detectable in heart and aorta. The function of the hAT$_2$R in the lung is not known.

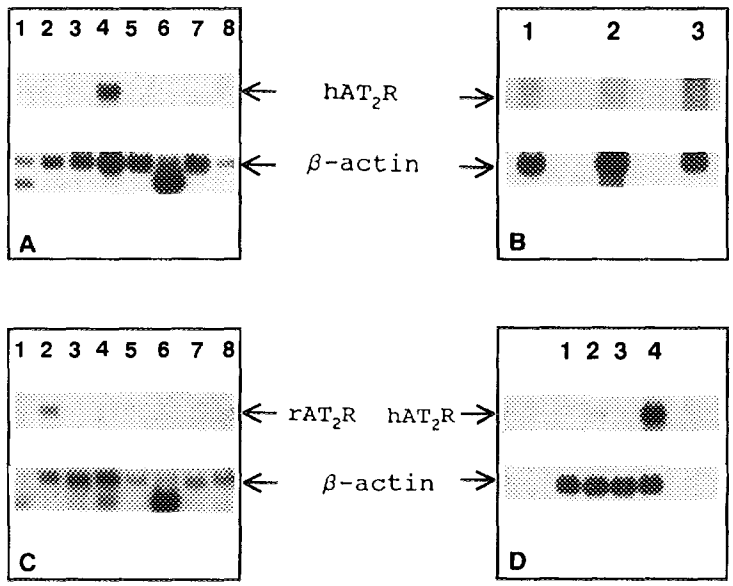

Fig. 4. Northern blot analysis. A. The human adult multiple tissue Northern was sequentially probed with a radiolabeled hAT_2R cDNA probe and a β-actin control probe. Lanes 1-8 contained, in order, two μg of poly A^+ mRNA isolated from human adult heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas. **B.** Northern blot was probed as described in A. Lanes 1-3 contained 5 μg of poly A^+ mRNA isolated from human adult adrenal, aorta and pituitary. **C.** The adult rat multiple tissue Northern blot was sequentially probed with a radiolabeled rAT_2R PCR probe and the control probe. Lanes 1-8 contained, in order, two μg of poly A^+ mRNA isolated from adult rat heart, brain, spleen, lung, liver, skeletal muscle, kidney and testis. **D.** Human fetal multiple tissue Northern blot was probed as described in A. Lanes 1-4 contain, in order, two μg of poly A^+ mRNA isolated from human fetal brain, lung, liver and kidney.

We also demonstrate that the hAT_2R mRNA is abundantly expressed in human fetal kidney and is just detectable in human fetal lung. Grone et al. (25) characterized AII receptor subtypes by in vitro autoradiography in fetal and adult human renal tissue utilizing [^{125}I]Sar^1,Ile^8-AII. They demonstrated that in human adult kidney, the hAT_2R was only present in the large preglomerular vessels. In contrast, they demonstrated that in human fetal kidney, the hAT_2R was present throughout the medulla and cortex. Therefore, these autoradiographic results support our Northern blot data. The presence of the hAT_2R in human fetal kidney strengthens the hypothesis that AII could be important for renal development.

The cloning of this cDNA will allow us to begin to investigate the physiological significance of the hAT_2R in the cardiovascular system and fetal development. Furthermore, the cloning of the hAT_2R cDNA will allow us to determine the organization of the hAT_2R gene.

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