Epidermal Growth Factor–Enhanced Human Angiotensin II Type 1 Receptor

Deng-Fu Guo, Tadashi Inagami

Abstract  The human angiotensin II type 1 (AT₁) receptor gene was isolated and its promoter function analyzed by deletion mutant promoter/luciferase constructs in transfected Cos 7 cells. We found that epidermal growth factor enhanced the human AT₁ promoter activity twofold to threefold. The region between -227 and -366 bp from the 5' end of the cDNA was mapped for a base sequence responsive to the epidermal growth factor stimulation. By computer analysis, PEA3 transcription factor was located in this region and was shown to bind to the promoter by gel shift assay in Cos 7 and HepG2 cells. These results indicated that the human AT₁ receptor enhanced by epidermal growth factor may be due to PEA3 binding to the human AT₁ promoter. (Hypertension. 1994;23[part 2]:1032-1035.)

Key Words  • angiotensin II  • epidermal growth factor-urogastrone  • promoter regions (genetics)

Methods

Cloning of the Genomic Library

A human lymphocyte genomic library in lambda DASH II was obtained from Stratagene. A total of 10⁷ lambda phage plaques were screened on nylon membranes (Hybond-N, Amersham) by using an EcoRI–Ace I fragment of the human AT₁ cDNA as a probe. Hybridization was done in 50% formamide, 6× SSC (1× SSC consisted of 0.15 mol/L NaCl and 0.015 mol/L sodium citrate, pH 7.0), 1% sodium dodecyl sulfate (SDS), 2× Denhardt's solution (0.1% Ficoll, 0.1% bovine serum albumin, and 0.1% polyvinylpyrrolidone), and 10 mmol/L sodium phosphate buffer for 16 to 24 hours at 42°C. Membranes were washed twice in 2× SSC and 1% SDS at 60°C. A positive clone was subcloned into the Bluescript KS(+) vector for determination of base sequences.

Plasmids

The 5'-flanking region of the human AT₁ gene spanning the first exon was subjected to double restriction enzyme digestion with HindIII and Cla I and subcloned in the Bluescript vector. To construct a human AT₁ receptor promoter/luciferase system, we treated the plasmid containing HindIII and Cla I fragment with exonuclease III by using the Erase-a-Base System (Promega Corp). Nine deletion mutants were generated, inserted into the GL2 basic vector (Promega), and named pLucl through pLuc9.

Expression of Human AT₁ Promoter/Luciferase Constructs

Cos 7 cells were grown in Dulbecco's modified Eagle medium containing 10% calf serum. DNA (10 μg) of each construct was transfected into 1.8×10⁶ Cos 7 cells in 300 μL phosphate-buffered saline by electroporation in 0.2-cm gap cuvettes (150 V, 250 microfarads) using a Gene Pulser apparatus (Bio-Rad Laboratories). The cell lysates were prepared 48 hours after transfection by using the Luciferase Assay System (Promega). Luciferase activity of 40-μg protein lysates was measured with a liquid scintillation counter (LS1801, Beckman Instruments). Transfection efficiency was normalized to the levels of pSV-β-galactosidase activity (Promega), which was cotransfected into Cos 7 cells. For experiments with EGF treatment, 100 ng/mL human recombinant EGF (UBI) was added to the culture of the transfected Cos 7 cells 24 hours after electroporation, and luciferase activity was measured as above.

Angiotensin II (Ang II) is one of the major regulators of the cardiovascular system through its vasoconstrictor and aldosterone-secreting activities, adrenergic facilitation, and many other activities.1 In addition, its mitogenic and hypertrophic effects are well known. Reflecting the versatility of its biologic activities, Ang II receptors are present in a wide variety of tissues.2 Ang II interacts with the pharmacologically distinct isoforms of cell-surface receptors type 1 (AT₁) and type 2 (AT₂). The AT₁ receptor mediates many of the classic functions assigned to Ang II to date, whereas the functions of the AT₂ receptor have yet to be established.3

The cDNAs encoding AT₁ from bovine adrenal4 and rat vascular smooth muscle cells (VSMCs)5 were recently isolated by expression cloning, and their amino acid sequences showed that AT₁ is a G protein–coupled receptor with seven transmembrane helical domains. Additionally, the human AT₁ receptor cDNA and genomic DNA have been isolated by us and others.6-10

Epidermal growth factor (EGF) is a potent growth modulator of a variety of cultured cells, including VSMCs.11 EGF is also involved in cellular hypertrophy.12 However, possible interactions between the renin–Ang II system and EGF have not yet been clarified.13 To elucidate the mechanism of transcriptional regulation of the human AT₁ gene and in an effort to provide insight into the regulation of human AT₁ expression, we isolated and analyzed the 5'-flanking region of human AT₁. We constructed and analyzed human AT₁ promoter/luciferase constructs and observed that EGF is a regulator that enhances human AT₁ receptor expression through a PEA3 transcriptional factor in its 5'-flanking region.

From the Department of Biochemistry, Vanderbilt University, School of Medicine, Nashville, Tenn. Correspondence to Tadashi Inagami, Department of Biochemistry, Vanderbilt University, School of Medicine, Nashville, TN 37232.
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[56x70]at -24 to -28, -481 to -485, and -1213 to -1217 bp, putative TATA boxes were found at -46 to -52, -967 to -973, and -1078 to -1085 bp upstream of the 5' end of the human AT1 receptor gene, approximately 1.2 kb was completely sequenced. Recognized consensus sequences are shown in boldface and labeled: TATA box, CAAT box, Sp1 (Sp1 on figure) recognition sequence, cyclic AMP-induced responsive element (CRE) sequence, HNF5 sequence, AP2 recognition sequence, and GC box recognition sequence.

**Results**

The 5' Flanking Region of the Human AT1 Receptor Gene

For characterization of the nucleotide sequence of the region upstream of exon 1 in the human AT1 receptor gene, approximately 1.2 kb DNA sequence upstream of exon 1 was completely sequenced. For identification of the sequence of elements that might be involved in the control of human AT1 receptor gene expression, the 5'-flanking region was analyzed with a computer program that identified known regulatory sequences. A result suggesting that they have a regulatory role was obtained. Three overlapping Sp1 recognition sequences (consensus sequence, GGCGG) were located at -116 to -121 and -121 to -126 bp, respectively. One GC box was present at -245 to -255 bp. A cyclic AMP-induced responsive element (CRE; consensus sequence, GGTCA) was present at -177 to -181 bp. Two transcription factor AP2 binding sites (consensus sequence, CCCA/CNG/CG/CG/Q were present at -255 to -261 bp. One GC box was present at -291 to -296 bp. These results are shown in Fig 1.

**Detection Analysis of the Human AT1 Promoter by Transient Transfection**

All the human AT1 promoter/luciferase chimeric constructs were introduced into Cos 7 cells by electroporation. The construct pLuc4 gave the highest luciferase activity; therefore, its luciferase level was used as a reference (set as 100%) in each series of experiments. The promoter/luciferase constructs and relative activity of luciferase of each construct are shown in Fig 2. Compared with the highest luciferase expression in pLuc4, pLuc5 gave only 65% of pLuc4. A CAAT box sequence was found in the region of pLuc4 beyond that of pLuc5. The transfectant with pLuc8 that contains overlapping double Sp1 binding sites gave only 7% luciferase activity. The transfectant with pLuc9 gave a background level of luciferase activity comparable to that with the basic plasmid (pGL2) alone. For mapping the EGFR-responsive region of the promoter, Cos 7 cells transfected with each of the nine human AT1 promoter/luciferase chimeric constructs were treated with 100 ng/mL EGF for 24 hours, and the luciferase activity was measured. The luciferase activity increased 2.5- to 2.9-fold in pLuc1 through pLuc5 but not in pLuc6 through pLuc9. Therefore, the EGF-responsive region should be located between the 5' ends of pLuc5 and pLuc6, that is, between -227 and -366 bp.
computer search, a PEA3 transcription factor (−291 to −296 bp), an AP2 active protein (−255 to −263 bp), and a GC box (−245 to −255 bp) were found in this region. Because PEA3 can be activated by EGF, we investigated by gel shift assay whether PEA3 can bind to the human AT₁ receptor.

Gel Shift Assay Analysis

To demonstrate the presence of PEA3 activity in Cos 7 and HepG2 cell nuclear extracts, we used a PEA3-specific oligonucleotide. Protein binding to the 32P-labeled PEA3 oligonucleotide effectively competed with the specific unlabeled oligonucleotide but not with the AP2 oligonucleotide as shown in Fig 3. This demonstrates that PEA3 activity is present in the cells and presumably interacts with the appropriate sequence in the human promoter.

Discussion

In the present study we sequenced and analyzed the human AT₁ receptor promoter region and also demonstrated that EGF upregulated the human AT₁ receptor. The sequence of the human AT₁ receptor promoter is unusual because it contains the characteristics of promoters of both a housekeeping gene and a regulated gene. Most eukaryotic promoters have a TATA box, an upstream regulatory sequence such as the CAAT box, and tissue-specific or hormonal-responsive elements. Conversely, a typical housekeeping gene promoter does not contain a TATA box but instead has GC-rich regions. The human AT₁ receptor promoter contains not only three TATA boxes and three CAAT boxes but also two Sp1 binding sites, a GC box, and a PEA3 site. These are features of a class of promoters that has complex regulatory processes. The present study of functional features of the promoter region using deletion-mutated promoter/luciferase constructs demonstrates that the maximal expression of the human AT₁ gene requires the sequence from −642 bp. Comparison of the promoter activity of pLuc4 with that of pLuc5 suggests that the region between pLuc4 and pLuc5 was the most critical for the promoter function.

We were not able to detect human AT₁ receptor expression in long passaged human cell lines such as HepG2 and HeLa cells by Northern hybridization.
Thus, EGF may exert its hypertrophic effects through a double mechanism, its direct effect mediated by the urokinase plasminogen promoter. The present observation that the EGF-increased promoter function in human cells may be positively regulated by EGF.

However, we observed that rat AT₁A receptor is actively expressed in rat aortic vascular smooth muscle cells isolated from Sprague-Dawley rats. Rat AT₁A, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and ethidium bromide staining are shown. Minus sign indicates without epidermal growth factor (EGF) treatment; plus sign, with 100 ng/mL EGF treatment for 24 hours.

Figure 4. Northern analysis of rat angiotensin type 1A (AT₁A) receptor expression in rat aortic vascular smooth muscle cells isolated from Sprague-Dawley rats. Rat AT₁A, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and ethidium bromide staining are shown. Minus sign indicates without epidermal growth factor (EGF) treatment; plus sign, with 100 ng/mL EGF treatment for 24 hours.

In conclusion, EGF may exert its hypertrophic effects through a double mechanism, its direct effect mediated by the EGF receptor and its indirect positive effect on AT₁ receptor expression.

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