Characterization of the Mouse $\alpha$1D-Adrenergic Receptor Gene

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ABSTRACT—$\alpha$1-Adrenergic receptors ($\alpha$1-ARs) play critical roles in the regulation of a variety of physiological processes. Increasing evidence suggests that multiple receptor subtypes of $\alpha$1-ARs regulate these physiological processes. Molecular cloning has identified three distinct cDNAs encoding $\alpha$1-AR subtypes ($\alpha$1A, $\alpha$1B and $\alpha$1D) that are structurally homologous. Among the $\alpha$1-AR subtypes, the function of the $\alpha$1D-AR remains unclear. In order to examine the physiological role of $\alpha$1D-AR, we cloned and characterized a gene for the mouse $\alpha$1D-AR. Using a mouse $\alpha$1D-AR cDNA as a probe, we isolated the gene for the mouse $\alpha$1D-AR from a mouse genomic library. The $\alpha$1D-AR consists of two exons and an intron that interrupts the coding region of the putative sixth transmembrane domain. The 5′-flanking region of exon 1 contains neither a TATA box nor a CAAT box but is high in GC content and contains several Sp1 binding sites (GC boxes). This pattern is similar to promoters described for other members of $\alpha$1-ARs. The untranslated region also contains putative cyclic AMP response elements. Isolation of this gene will allow further investigation, via gene knock-outs and deletion mutants, of the mechanisms of transcriptional regulation and a greater understanding of the physiological role of $\alpha$1D-AR.

Keywords: $\alpha$1D-Adrenergic receptor, Gene

Adrenergic receptors (ARs) belong to the super family of G-protein coupled, seven transmembrane domain receptors. In response to external catecholamine stimuli, these receptors mediate a variety of cellular processes. ARs are broadly divided into $\alpha$ and $\beta$ types based on their pharmacological specificity. Each class is further divided into several subtypes based on studies using both pharmacological and molecular cloning approaches. Three subtypes of the $\alpha$1-AR class ($\alpha$1A-, $\alpha$1B- and $\alpha$1D-ARs) have been identified in several species and have been shown to be distributed in various tissues, where they mediate diverse effects (1−4). Pharmacological studies using subtype-selective agents have been performed (1−4); however, the physiological role of each $\alpha$1-AR subtype is still mostly unknown. Mutant mice that lack a specific $\alpha$1-AR subtype have also been used to investigate the physiological role of each subtype. Recently, Cavalli et al. found that mice lacking the $\alpha$1B-AR gene had decreased blood pressure and aortic contractile response when exposed to $\alpha$1-AR agonists, suggesting that $\alpha$1B-AR mediates responses induced by $\alpha$1-AR agonists (5).

The $\alpha$1D-AR gene, one of the members of the $\alpha$1-AR subfamily, has not yet been cloned. The $\alpha$1D-AR gene has been shown to be widely expressed in a variety of tissues in mice (6), rats (6–8), rabbits (9) and humans (10). However, the physiological role or the regulatory mechanism of expression of the $\alpha$1D-AR gene is not clearly understood. We cloned and characterized the mouse $\alpha$1D-AR gene to gain a better understanding of its role in physiological and pathological states.

MATERIALS AND METHODS

Materials

Materials were obtained from the following manufacturers: a mouse genomic 129 SVJ mouse genomic library (Strategene, La Jolla, CA, USA); nylon membranes (Hybond N) and [32P]dCTP, Amersham (Tokyo); Random Primer DNA Labeling Kit, Takara (Kyoto); and other materials, Wako Pure Chemical Industries, Ltd. (Osaka).

Isolation and characterization of phage clones containing the mouse $\alpha$1D-AR gene

A cDNA fragment (1.5 kbp) of the mouse $\alpha$1D-AR
gene was isolated by the reverse transcription-polymerase chain reaction (RT-PCR). Primers were designed to amplify the entire coding region. The sense primer was 5'-GCTGCAGCCGTGATGACTTTCCGC-3', and the antisense primer was 5'-CCGAAGACGGAGCCGGCTTTCA-3'. Total RNA was isolated from brains of C57BL6 mice using an isogene reagent (Nippon Gene, Tokyo). The cDNA was synthesized using 10 µg of total RNA, 1 pmol of random hexamer, and 20 units of murine myeloma leukemia virus (MMLV) reverse transcriptase in a final volume of 50 µl. The PCR protocol was as follows: initial 5-min incubation at 94°C, followed by 30 PCR amplification cycles. One PCR amplification cycle consisted of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 1 min. Each 50-µl reaction mixture contained 1 µl aliquot of cDNA solution, 10 pmol of each primer, 2.5 units of Taq DNA polymerase (TakaRa Ex Taq, Takara) and the manufacturer's buffer. The amplified products were subcloned into a pGEM vector (Promega, Madison, WI, USA).

A mouse genomic library from the 129 SVJ mouse was screened for clones carrying the mouse α1D-AR gene using the plaque hybridization method. Approximately 2 × 10⁶ plaques from the library were screened using the mouse α1D-AR cDNA as a probe. Nylon membranes were lifted in duplicate from each plate, and the DNA on the membranes was treated with a denaturing solution (0.5 N NaOH, 1.5 N NaCl) and a neutralizing solution (0.5 N Tris-HCl pH 7.4, 1.5 N NaCl), followed by baking at 80°C for 2 h in an oven. The probe was labeled with [α-32P]dCTP by the random priming method. The filters were hybridized in 0.5 M sodium phosphate (pH 7.2), 7% sodium dodecyl sulfate (SDS), 1% BSA, 1 mM EDTA, 0.05 N sodium chloride, and 1 × 10⁶ cpm/ml of 32P-labeled probe at 55°C for 8–10 h. The filters were washed in 40 mM sodium phosphate at 55°C, 1% SDS and 1 mM EDTA. The positive clones were isolated by three rounds of purification and were subjected to restriction map analysis and partial sequencing. Fragments that hybridized to the cDNA probe by Southern blot analysis were subcloned into a pBluescript KS vector and sequenced on both strands using the Dye Terminator Cycle Sequencing method, according to the manufacturer’s protocol.

Southern blot analysis

Mouse genomic DNA was prepared from E14 cells. Ten micrograms of DNA was digested with restriction enzymes, fractionated by gel electrophoresis on a 0.8% agarose gel and then transferred to a Nylon membrane. The filter was hybridized in a buffer containing 6 × standard saline citrate (SSC) (1× SSC denotes 0.15 M NaCl and 0.015 M trisodium citrate), 5 × Denhardt’s solution, 50% deionized formamide, 0.25% SDS, 10 µg/ml de-natured salmon testis DNA and a 1 × 10⁶ cpm/ml of 32P-labeled probe. After hybridization for 16–20 h at 42°C, the filter was washed in 40 mM sodium phosphate, 1% SDS and 1 mM EDTA.

Primer extension analysis

Poly (A+)-RNA was prepared from mouse tissues using Isogen (Nippon Gene) followed by oligo (dT)-cellulose chromatography. Primer extension analysis was performed as described previously (11). A synthetic oligonucleotide (5'-ACTGCTCGCCGGGCTCTTCGAAA GTGAC-3'), complementary to nucleotides +54 to +25 relative to the translation initiation site of the mouse α1D-AR gene, was end-labeled using [γ-32P]ATP and T4 polynucleotide kinase. The labeled primers were hybridized to RNA by incubating for 20 min at 90°C. After precipitation of the annealed primer and template, the hybridized primers were extended by incubation with 20 units of avian myeloblastosis virus (AMV) reverse transcriptase in a reverse transcriptase buffer containing 0.5 mM deoxynucleotide triphosphate. The sample was precipitated by the addition of ethanol, denatured and analyzed on a 6% sequencing gel.

RESULTS

Southern blot analysis of the mouse α1D-AR gene

Mouse genomic DNA was digested individually with two different restriction enzymes (EcoRV and HindIII), blotted and then probed with mouse α1D-AR cDNA. Specific bands, 7.5 kb and 5.0 kb, and 4.8 kb, 4.7 kb and 3.8 kb, were detected after digestion with EcoRV and HindIII, respectively (Fig. 1). The sizes of the hybridized fragments are consistent with restriction maps derived from phage clones. The results indicate that the mouse α1D-AR gene is encoded by a single gene and that there is no pseudogene.

Cloning the mouse α1D-AR gene

To find the gene encoding the mouse α1D-AR, a genomic library from a 129 SVJ mouse was screened using the mouse α1D-AR cDNA as a probe. Two independent clones were isolated from the library and were analyzed by digestion with restriction enzymes and by partial sequencing. We found that these two clones do not overlap. One clone contained the nucleotide sequence corresponding to the first exon encoding amino acids 1–364 of the mouse α1D-AR and the other contained the second exon of the mouse α1D-AR encoding amino acids 365–562. Exon 1 consists of the 5'-noncoding region and the coding region up to the distal part of the putative sixth transmembrane domain. Exon 2 contains the rest of the coding region, as well as the 5'-noncoding region. These
results, combined with that of the Southern blot analysis, indicate that the mouse α1D-AR gene spans over 10 kb (Fig. 2). To define the position and boundaries of the exon blocks, the restriction fragments identified by Southern hybridization were subcloned, and their sequences were determined. The 5.0-kb SacI, 4.8-kb HindIII and 6.5-kb BamHI fragments from the first clone (Fig. 1) were subcloned into pBluescript KS and were sequenced in both directions. These fragments contained exon 1 and part of intron 1. The 3.0-kb XbaI, 4.7 kb HindIII and 3.8 kb HindIII fragments from the second clone were subcloned into pBluescript KS and were sequenced in both directions. These fragments contained exon 2 and part of intron 1. The intron is more than 5 kb long and interrupts the putative sixth transmembrane domain (Figs. 2 and 3). The GT-AG sequence is conserved for splice sites, while traditional intron sequences adjacent to the consensus splice site are conserved. The consensus sequence for a potential polyadenylation signal (AATAAA) was present in the 3'-noncoding region, starting 1056 bases downstream from the stop codon (Fig. 3).

Characterization of 5'-noncoding region

Sequence analysis revealed that the 5'-untranslated region contains neither a TATA box nor a CAAT box sequence. However, there are several potential Sp1 binding sites (CCGCCC) and putative cyclic AMP (cAMP) response elements (CRE) ([T]GACG[T][C]) in this region (Fig. 4). The transcription initiation site was determined by primer extension analysis using mouse poly (A+)-mRNA from the brain. As shown in Fig. 5, several bands were observed upstream from the translation initiation site, suggesting that there are several transcription initiation sites.
DISCUSSION

The gene that encodes the mouse α1D-AR was isolated and its structure was determined. The mouse α1D-AR gene consists of two exons separated by a single intron of at least 5 kb at the end of the putative sixth transmembrane domain. However, the exact size of the intron could not be determined because we did not obtain overlapping genomic clones. The 5'-flanking region of exon 1 contains neither a TATA box nor a CAAT box but is high in GC content and contains several Sp1 binding sites (GC boxes). This pattern is similar to promoters described for other members of α1-ARs. Also, the 5'-untranslated region contains a putative CRE.

The structure of the mouse α1D-AR gene is very similar to other α1A-AR genes (12, 13) and α1B-AR (14, 15), which consist of two exons with one intron, a TATA-less promoter and a CRE. The similarities between these genes suggest that all α1-AR subtype genes may originate from the same ancestor. They may also be distinctly related to the rest of the AR family, including β1- (16), β2- (17), β3- (18) ARs and α2-AR subtypes (19-21) which lack introns. A comparison of the organization of these genes (Fig. 6.) was used to trace the evolutionary
relationship of these genes. Based on the genomic organization, one can predict that α1-AR subtypes originate from the same ancestor. Using a hypothetical cationic amine receptor dendrogram (22), we further predict that the α1-AR subtypes are more distantly related to α2- and β-ARs. Furthermore, the presence of a single large intron at the end of the putative sixth transmembrane domain makes the α1-AR receptors different from other AR subtypes, forming a distinct family of receptors.

The promoter region in the mouse α1D-AR gene revealed similarities in structure to human α1A-AR (13) and rat/human α1B-AR genes (14, 15). These genes lack characteristic elements in the promoter region such as a TATA box, a high GC content and a putative CRE. In the adrenergic receptor family, only human α2A-AR (23), rat α2A/D-AR (24) and turkey β-ARs were shown to contain a TATA box in the promoter region. Although the TATA-less promoter was thought to be associated with housekeeping genes, it has recently become apparent that many membrane receptor genes possess TATA-less promoters (25). Also, we found that the α1D-AR gene lacks a CAAT box in the promoter region; however, the consensus sequence of the CAAT box (GG(C/T)CGAAT CT; 7/9 matches required with CAATC intact) has been found only in human/rat α1B and mouse α2B-AR genes among the members of the AR family.

The promoter region of the mouse α1D-AR contains several GC boxes ((GT)/(G/A)GGCCG(G/T)(G/A)(G/A) (C/T)) (24) located upstream from the transcription initiation sites. Binding of Sp1 in the region may play a role in basal and/or activated transcription in the promoter without a TATA box, and transcription is often associated with Sp1 binding to GC rich regions further upstream of the promoter (25–30). Primer extension analysis suggests that transcription initiation sites are located 70–310 bp upstream from the ATG. Since multiple transcription sites have been found in numerous catecholamine receptor genes, including the human α1A-AR (13), the human α1B-AR (15), rat α1B-AR (which contains three distinct promoter regions) (14, 31, 32), rat β1-AR (33) and the human dopamine 1A receptor (34), the mouse α1D-AR gene may also contain multiple transcription initiation sites.

CREs in the α1A-AR gene have been found to be involved in regulating the expression of α1A-ARs (13). cAMP increases transcription of the α1A-AR and α1B-AR gene via activation of the cAMP response element-binding protein (CREB) family of transcription factors which interact with the CRE sequence present in the 5'-non coding region. In addition to its indirect action through CREs, cAMP can also increase transcription by interacting with other regulatory sequences such as AP-1 (TGACTCTT), AP-2 (C CGCGGGCC), Sp1 (CCGCCC), the inverted CCAAT motif (35) and the estrogen response element (36). The presence of a CRE in the promoter region of the α1D-AR gene would suggest that cAMP exerts its effects through this pathway; however, evidence of other mechanisms has introduced the need for further
Fig. 5. Analysis of the transcription initiation site using primer extension assays. $^{32}$P-labeled primer complementary to the region +25 to +54 from the ATG of the α1D-AR gene was annealed to 5 μg of mouse brain poly(A)$^+$ RNA or 5 μg of tRNA, and primer extension was performed as described in Materials and Methods. A 1-kb DNA marker (Gibco BRL, Rockville, MD, USA) was labeled with [γ-$^{32}$P]dATP and the Klenow Fragment. Extension products as well as the DNA marker were separated on a 6% sequencing gel. Arrows on the right indicate the products. Numbers on the left indicate the size of the DNA markers.

In summary, we have characterized the gene that encodes the mouse α1D-AR. Characterization of this gene will allow further investigation into the function and role of the α1D-AR.

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