Cloning of two additional catecholamine receptors from rat brain

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An approach based on the polymerase chain reaction (PCR) was used to isolate additional members of the G-linked receptor family from a rat striatal λgtII cDNA library. Priming with one degenerate probe corresponding to highly conserved consensus sequences in the third transmembrane (TM) domain of 15 G-linked receptors and sequences in the phage vector resulted in one clone (G-13) encoding a dopamine D2 receptor variant with a 29 amino acid insert in the third cytoplasmic loop. In addition, the amino acid sequence encoded by clone G-36 contained conserved sequences characteristic of the G-linked class of receptors and displayed sequence homology in TM domains with the β_2-adrenergic receptor (48%). Two conserved serine residues in TM5 postulated to be part of a ligand binding site in the adrenergic receptor, suggests that G-36 encodes a catecholaminergic receptor. Northern blot analysis confirmed the expression of G-36 in rat brain, but not in kidney, heart and lung. Several strong hybridizing bands to G-36 were obtained in both human and rat genomic DNA. The general PCR strategy employed here should prove to be extremely useful for the isolation of other members of the G-linked receptor family.

Polymerase chain reaction; Homology probing; D2 dopamine receptor; Receptor variant

1. INTRODUCTION

Dopamine D2 receptors are known to mediate the activity of adenylate cyclase, phosphatidyl inositol turnover and K^+/Ca^{2+} channels (see [1,2] for review). One D2 dopamine receptor (D2R) has been cloned [3] and shares amino acid and topographical homology, particularly in transmembrane (TM) domains, with other members of the G^1-linked receptor family [3,4]. At a functional level, the cloned D2R inhibits the activity of adenylate cyclase in a pertussis-toxin sensitive manner, and binds dopaminergic agonists and antagonists with an appropriate pharmacological specificity [3,5].

In an attempt to ascertain whether there are D2R subtypes that specifically couple to diverse effector systems we employed a variation of the homology probing approach [6–10] that involves the polymerase chain reaction [11,12] and the use of one degenerate oligonucleotide primer with cDNA from a rat striatal library as template. We report here that amplification of nucleotide sequences between the degenerate probe and sequences in the phage vector has allowed for the isolation of two additional members of the G-linked receptor family. One clone was identified as a D2R variant with a 29 amino acid insert in the third cytoplasmic loop. Another clone (G-36) contained sequence information coding for part of a novel G-linked receptor protein that shares 48% sequence homology with the β_2AR in TM domains and appears to be catecholaminergic in nature.

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Abbreviations: AR, adrenergic receptor; PCR, polymerase chain reaction; TM, transmembrane; G, guanine nucleotide binding protein

2. MATERIALS AND METHODS

2.1. *Oligonucleotides*

The oligonucleotides used in the PCR and for use as probes were synthesized at the University of Toronto facility at the Banting Institute. To design an oligonucleotide for use in the PCR, we aligned the amino acid sequence of 15 members of the G-protein-linked receptors. A degenerate oligonucleotide (TM) coding for 6 amino acids in a conserved sequence of transmembrane three was synthesized and contains a 128-fold degeneracy. One of the oligonucleotides in this mixture codes for six amino acids of the known D2R sequence (fig.1). A second oligonucleotide (VI) for use in the PCR was based on the sequence of the λgt11 vector (fig.1). BamHI linkers were included at the 5′ end of each oligonucleotide to facilitate subcloning.

2.2. *DNA amplification by PCR and subcloning*

DNA was extracted and purified from a λgt11 rat striatal cDNA library (Clontech). In a total reaction volume of 100 μl the cDNA (100 ng) was submitted to 30 cycles of PCR (Cetus-Perkin Elmer) with 1 μg each of primers TM3 and VI. The timing used was 1.5 min at 93°C, 2 min at 55°C, and 4 min at 72°C essentially as described by Sakai et al. [12], followed by a 7 min extension at 72°C. An aliquot of this reaction (10 μl) was removed and reamplified for an additional 25 cycles under the same conditions. DNA from this reaction (50 μl) was phenol extracted, ethanol precipitated, subjected to GeneClean (BIO 101), digested with *Bam*HI and *Eco*RI, and electrophoresed in soft agarose (0.8%). The DNA contained in 7 contiguous slices was subcloned into the *Bam*HI/*Eco*RI site of the plasmid sp65 (Promega), transformed with AGI cells, and transformants selected on ampicillin plates.

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2.3. *Southern, Northern blotting and sequencing*

Minipreparation of plasmid cDNA from 70 randomly selected colonies, was restriction digested and the DNA fragments prepared for Southern blotting [13]. Each plasmid was Gene-Cleaned and sequenced (Sequenase, USB). Total RNA, from various rat tissues was prepared by acid guanidinium thiocyanate extraction [14], and Northern blotted. Oligonucleotides and cDNA clones were labelled with ^{32}P as described [13].

3. RESULTS AND DISCUSSION

The amino acid residues in the third TM domain of all G-linked receptors are highly conserved [4]. To probe for D2R variants a degenerate oligonucleotide was prepared (TM3; a 24-mer of 128-fold degeneracy). The sequence for this degenerate oligonucleotide (TM3) and the PCR strategy employed are depicted in fig.1A and 1B. One of the oligonucleotides contained in the mixture codes for the known D2R [3] (fig.1B). Amplification of a rat striatal cDNA library was achieved using TM3 in conjunction with an oligonucleotide prepared from sequences in the λgt11 phage vector (VI). The underlined sequences at the 5′-end of each oligonucleotide contains a restriction endonuclease site to facilitate subsequent subcloning of amplified PCR products.

Fig.1C depicts an ethidium bromide stain of PCR amplified rat striatal cDNA fragments. Following electrophoresis and Southern transfer, nitrocellulose membranes were probed with a ^{32}P end-labeled oligonucleotide (5′-CATGGTCTGGATCTCAAAGAACTT-3′) that codes for a portion of the third cytoplasmic loop of the D2 receptor (amino acids 311–318) [3]. As illustrated in an adjacent lane of fig.1C, two fairly diffuse hybridizing bands of 850 and 750 bp were identified and these bands confirmed that the PCR conditions used were suitable for the amplification of D2 receptor DNA.

PCR amplified products ranging in size from 200 to 1500 bp were subcloned into the plasmid sp65. Minipreparation of plasmids with subcloned inserts were Southern blotted and probed with the D2R specific oligonucleotide. One of these clones (G13) initially recognized by Southern blotting (fig.1D) coded for the D2 receptor, as revealed by nucleotide sequencing [3]. In addition to the previously reported sequence of D2R [3], clone G13 contained a 29 amino acid insert (encoded by an 87 bp insert) whose deduced amino acid sequence and position within the third cytoplasmic loop is shown in fig.2. Recent evidence also suggests the existence of a 87 bp insert of the D2 receptor in human pituitary and brain [15]. However, no sequence information on this insert or the position of the insert within the third cytoplasmic loop has been reported. The functional significance of the D2R variant in either rat or human is unknown, but it is tempting to speculate that these inserts may allow for D2 dopamine receptor regulation of other effector systems including PI turnover and ion channel activity. Receptor G-protein interactions have been postulated to involve N-terminal residues within the 3rd cytoplasmic loop of the receptor, connecting the 5th and 6th TM helices [16,17].

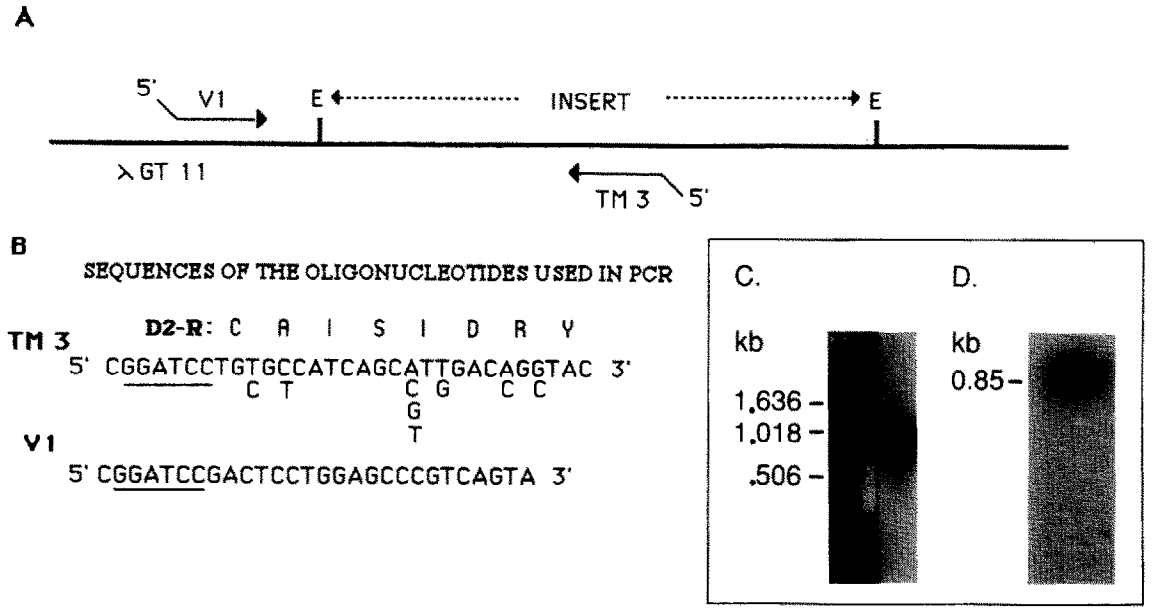

Fig.1. (A) Strategy used in the PCR amplification of rat striatal λgt11 cDNA library with a primer to TM3 and one to sequences in the phage vector (VI). Inserts in the library are flanked by *Eco*RI sites (E). (B) Sequences of oligonucleotide primers used in the PCR. D2R amino acid sequences encoded by the 24 base TM3 degenerate oligonucleotide is also shown. The underlined nucleotide sequence indicates the position of the *Bam*HI linkers. (C) Ethidium bromide stain of PCR amplified products and Southern blot depicting hybridizing bands to a [^{32}P]labelled oligonucleotide probe constructed to code for part of (amino acid sequence 311–318) the D2 receptor [3]. Size markers are indicated on the left. (D) Southern blot of clone G-13 (D2R variant).

Single strand sequencing of 36 randomly selected clones from the PCR revealed the presence of a clone (G-10) that contained 130 bases coding for deduced amino acid sequences that shared conserved consensus sites characteristic of G-linked receptors within the 3rd and 4th TM domain. Subsequent Southern blotting of the 70 randomly selected clones with G-10 revealed a larger clone (G-36) containing a 454 bp insert. The deduced amino acid sequence of G-36 and alignment with 15 other members of the G-linked family of receptors is illustrated in fig.3.

Of all the cloned G-linked receptors aligned in fig.3, G-36 contains the longest 2nd extracellular loop of 27 amino acids (fig.3). The functional significance of this loop for receptor mediated events has yet to be determined. In addition, G-36 displays 48% sequence homology in TM domains with β_2AR, 40% and 38% with α_2AR and 5-HT$_{1A}$ receptors and 32% with the D2R.

Recent site directed mutagenesis and pharmacophore mapping experiments on the β_2AR have suggested that two serine residues in the 5th TM domain are critical for agonist binding and activation of the receptor [18]. Moreover, these serine residues are conserved in G-linked receptors that bind catecholaminergic agonists, but not in those whose endogenous ligand does not contain a catechol moiety. As depicted in fig.3, G-36 has conserved serine residues (indicated by arrows) within the 5th TM domain and thus, would be predicted to belong to a catecholaminergic receptor family. At present it is difficult to ascertain whether G-36 represents an additional adrenergic receptor, D2R variant or possibly the D1 dopamine receptor. The fact that G-36 only shares limited homology (<50%) within TM regions with other G-linked receptors would argue against it being an adrenergic receptor variant, since homologies >60% are observed between the various adrenergic receptor subtypes aligned in fig.3.

Northern blots were prepared to examine the mRNA expression of G-36 in several tissues. As depicted in fig.4A, G-36 was expressed in brain but not in tissues from rat kidney, heart or lung (data not shown). Moreover, as seen in fig.4B, Southern blotting of digested human or rat genomic DNA revealed a

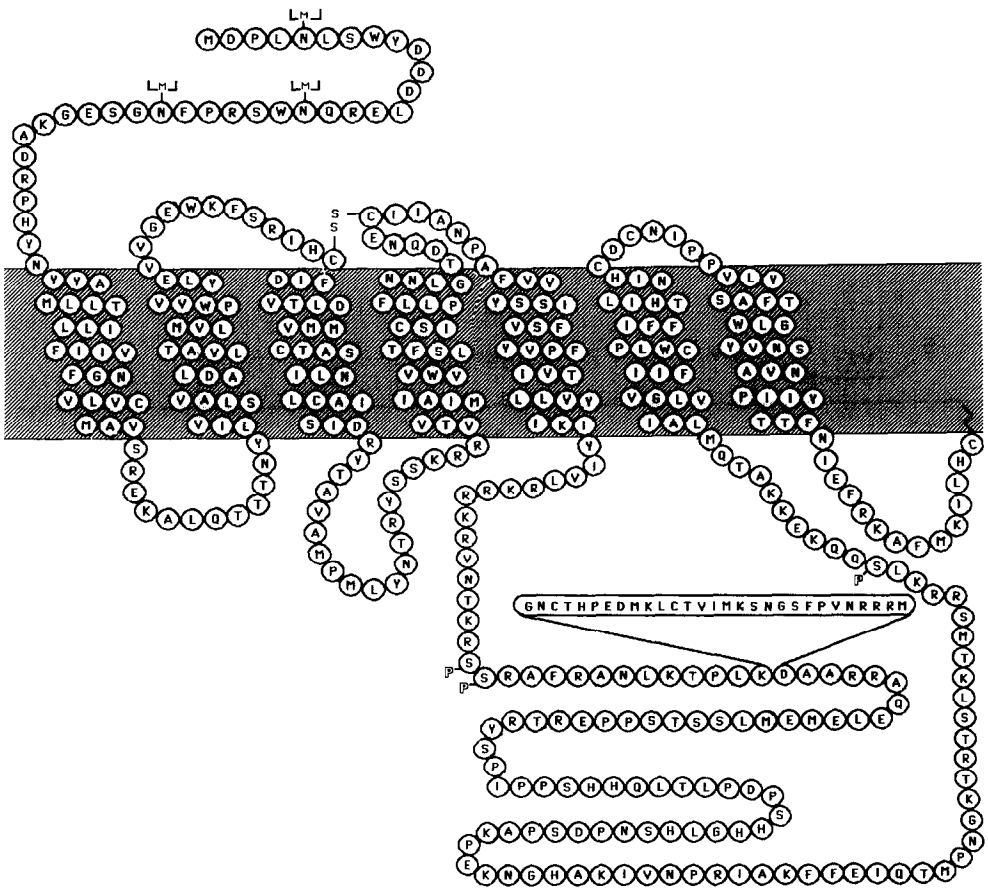

Fig.2. A model illustrating the topographical structure of the rat D2R variant in the membrane. The position of a 29 amino acid insertion in the third cytoplasmic loop is shown. Also shown are potential N-linked glycosylation sites, two possible PKA phosphorylation sites in the third cytoplasmic loop and extracellular cysteine residues suspected to be involved in disulphide bonding in the equivalent position in the β_2AR [19]. The carboxyl tail is shown anchored to the plasma membrane via palmitic acid thioesterification to Cys^{415} (a cysteine residue in the equivalent position of β_2AR is palmitoylated [20]).

Fig.3. Alignment of the amino acid sequence encoded by clone G-36 with the amino acid sequences of 15 G protein coupled receptors. This alignment is based upon a visual alignment of the sequences. The approximate position of the putative transmembrane regions are indicated by the horizontal lines above the sequences and conserved amino acids are boxed in black. HAM = hamster; α_2AR-C4 and α_2-AR-C10 = α_2AR subtypes whose genes reside on chromosomes 4 and 10, respectively.

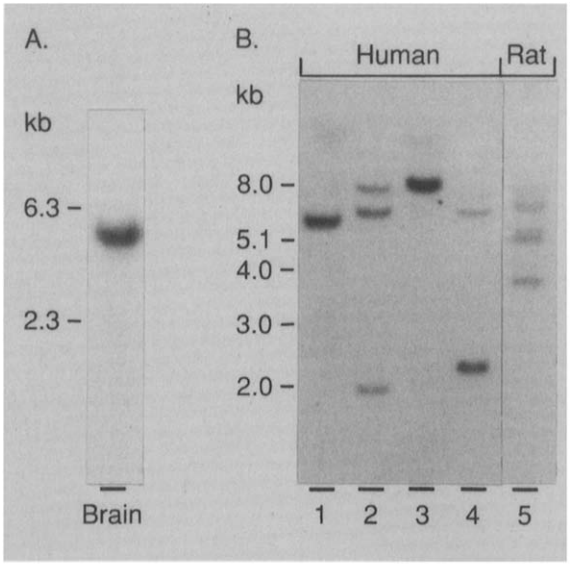

Fig.4. (A) Total brain RNA (30 μg) was subjected to blot hybridization [13] using [^{32}P]labelled clone G-36 as a probe. Molecular weight markers are shown on the left. (B) Southern blot analysis of human and rat genomic sequences homologous to clone G-36. Human DNA was digested with *Pst*I (lane 1), *Hin*dIII (lane 2), *Bam*HI (lane 3) and *Bgl*II (lane 4) and rat DNA digested with *Pst*I (lane 5) were subjected to electrophoresis and Southern hybridization [13]. Size markers are shown on the left.

number of strongly hybridizing bands. These experiments confirm that transcripts corresponding to G-36 sequences exist in the rat and that sequences homologous to G-36 are present in the human genome. In any event, it is clear that the PCR based homology probing approach described here should be extremely useful in the isolation of additional members of the G-linked receptor family.

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