Inactivation of a Novel Neuropeptide Y/Peptide YY Receptor Gene in Primate Species*

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Neuropeptide Y (NPY), peptide YY (PYY), and pancreatic polypeptide (PP) belong to a family of structurally related peptides which have numerous functions in both neural and endocrine signaling. By homology screening, we cloned a novel gene sharing the highest homology with the NPY Y1 receptor gene from humans, rabbits, and several other species. This novel gene of rabbit encodes a functional NPY/PYY receptor, designated Y2b, which prefers NPY1234 as rather than [Leu]2[Pro]3NPY despite its higher identity with the Y1 receptor. Although, at low levels, mRNA was detected in the tissues and brain regions, including hypothalamus. Further, sequence data revealed that this gene is the orthologue of the recently cloned mouse novel NPY receptor, Y5. However, our study demonstrates that the receptor function of this gene has been inactivated in primates by a frameshift mutation occurring early in primate evolution. This novel NPY receptor represents the first neurotransmitter receptor identified that has universally lost its receptor function in primate species. Interestingly, despite its inactivation in humans, the transcripts were abundantly detected in the heart and skeletal muscle, suggesting a novel function of the human gene.

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¶The abbreviations used are: NPY, neuropeptide Y; PYY, peptide YY; PP, pancreatic polypeptide; RT-PCR, reverse transcription-polymerase chain reaction; RACE, rapid amplification of cDNA ends; TMD, transmembrane domain; h, human; p, porcine; CHO, Chinese hamster ovary; G3PDH, glyceral-3-phosphate dehydrogenase.

Neuropeptide Y (NPY), peptide YY (PYY), and pancreatic polypeptide (PP) belong to a family of structurally related peptides which have numerous functions in both neural and endocrine signaling. These peptides exert their actions via receptors on the targeted neurons and peripheral cells. Several receptor subtypes have been defined by their ability to bind NPY, PYY, PP, and derivatives of these peptides. Earlier studies classified this receptor family into at least three receptor subtypes, Y1, Y2, and Y3 (1). The existence of additional receptors were also proposed, “atypical” Y1 receptor (mediating the feeding response of NPY in hypothalamus), PP-prefering receptor (exerting PP activity), and PYY-prefering receptor (2, 3). Molecular cloning studies have revealed the structure of Y1, Y2, and Y4/PP1 receptors (4–9). These are all heptahelix (seven-transmembrane regions) receptors which couple to G-proteins. The Y4/PP1 receptor has higher affinity for PP than PYY and NPY, suggesting this receptor to be a member of PP receptors.

As yet unknown members of this NPY receptor family are expected to be identified by molecular cloning. Very recently, during the course of the present work, the cloning and characterization of mouse Y5 (10) and rat Y5 (11) receptors was reported. The mouse Y5 receptor has significant homology with the Y1 receptor, but the rat Y5 receptor has little identity with the previously cloned NPY receptors, showing that these receptors are not species orthologues, despite having the same name, Y5. In the present report, we describe the cloning of a novel gene sharing the highest homology with the Y1 receptor from humans, rabbits, and several other species. Our study revealed that this novel gene is the orthologue of the mouse Y5 receptor gene and encodes functional NPY/PYY receptor in rabbits, which we have named Y2b, but has been inactivated for receptor function in primates by a frameshift mutation occurring early in primate evolution.

EXPERIMENTAL PROCEDURES

Isolation of a Human cDNA Clone—Human caudate nucleus and hypothalamus cDNA libraries were purchased from Clontech. Approximately 700,000 plaques for each library were lifted with nylon membranes, and hybridization was done with human Y1 cDNA fragments (position 42–1412 refers to HUMNEYPEPY in the GenBank™ data base) as a probe. The membranes were hybridized with 32P-labeled probe (1 × 106 cpm/ml) for 20 h at 34°C in buffer containing 5 × SSPE, 5 × Denhardt’s, 0.1% SDS, 100 μg/ml denatured salmon sperm DNA, and 30% formamide. The membranes were washed twice in 1 × SSC, 0.1% SDS at room temperature, and twice for 30 min in 1 × SSC, 0.1% SDS at 34°C. Twelve and eight individual clones were selected from the caudate nucleus and hypothalamus cDNA library, respectively. Sequences were determined with an automated fluorescent dye DNA Sequencer (ABI). The existence of a frameshift in the human Y2b gene and in the transcripts was confirmed by PCR using independent genomic DNAs or by RT-PCR using total brain, hypothalamus, heart, and skeletal muscle poly(A) RNA. Human Y2b reverse mutation was produced using PCR, and the reading frame was confirmed by sequencing.

PCR Amplification of Species Orthologue—Using primers 5′-CTCTGTGCGTACAGTAGGAC-3′ and 5′-CTGCGTGCAGAATTACGACG-3′ produced from human Y2b cDNA, the orthologous gene fragments of the primates and rabbit were amplified by PCR at 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min for 34 cycles (12). Primers 5′-TCTCTGTGTGCGTCCATCC-3′ and 5′-AACATGAGAACCAGTGGAC-3′ produced from the rabbit sequence also amplified the mouse and hamster orthologue as well. The PCR products were sequenced directly to avoid the influence of PCR error. To obtain ORF of the rabbit cDNA, the 5′- and 3′-RACE method was employed using a Marathon cDNA Amplification Kit (Clontech) according to the manufacturer’s protocol. Briefly, the cDNA of rabbit brain and skeletal muscle was ligated to the cDNA adaptor. For RACE, the adaptor-ligated cDNA was amplified with nested primers made from the partial rabbit DNA in combination with...
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Marathon adaptor primers. Using primers 5'-AAAATCTAGATCAGGATTTGCTAGAC-3' and 5'-AACAGATATCTCATTGCTAGCC-3' (underlined sequences represent artificial XhoI site) made from the sequence of the RACE products, the ORF was amplified from rabbit brain and skeletal muscle cDNA and genomic DNA as described previously (13).

Expression and Functional Assay—Rabbit Y2b, human Y2b, and the reverted-mutant cDNA were ligated into mammalian expression vector pEF-BOS (dhfr) containing dhfr gene as a selective marker (14, 15). For transient expression study, the plasmid constructs and no insert vector were separately transfected into COS-1 cells as described previously (13). To establish a rabbit Y2b expressed cell line, the plasmid construct was transfected into CHO (dhfr-) cells using LipofectAMINE (Life Technologies, Inc.) as described previously (15). The transfected cells were divided into single cells, and a monoclonal cell line showing Y2b-transfected cells as described previously (15).

Study of mRNA Distribution—Total RNA from various rabbit tissues and brain regions was treated with DNase (RNase-free, Nippongene) to eliminate the contamination of genomic DNA. Then the RNA was reverse-transcribed as described previously (13). Using primers 5'-TTTCCTGTTGCTTCTTACC-3' and 5'-GACCCAGCTATCTTTCCTG-3' (Boxed nucleotide sequence and that of H-Y2bRM is shown above and below, respectively), Y2b cDNA was amplified from the cDNA pool or the corresponding RNA by PCR at 94°C for 30 s, 60°C for 30 s, and 72°C for 1 ms for 25 cycles. The amplified products were transferred to nylon membrane and hybridized with 32P-labeled probe made from the full-length Y2b cDNA. The G3PDH cDNA was amplified using human G3PDH primers (Clontech) by PCR at 94°C for 30 s, 60°C for 30 s, 50°C for 30 s, 30 s, and 72°C for 1.5 min for 30 cycles. Northern membranes containing 5 μg of poly(A)+ RNA from different human tissues and brain areas were purchased from Clontech. Northern analysis of human Y2b transcripts. Additional 3'-RACE experiments using human skeletal muscle poly(A)+ RNA indicated the main size of human Y2b transcripts to be 3 kilobases, the same size as shown in the figure (data not shown).

RESULTS AND DISCUSSION

To identify novel members of the NPY/PYY/PP receptor family, human caudate nucleus and hypothalamus cDNA libraries were screened using human Y1 cDNA (4, 5) as a probe. Two overlapping clones from the caudate nucleus library were found to have the highest nucleotide sequence identity to the Y1 receptor (54%), indicating that this cDNA encoded a novel heptahelix receptor for the NPY/PYY/PP family. Although these clones covered the entire ORF, alignment with human Y1 indicated the existence of a frameshift between the sixth and seventh putative transmembrane domains (TMD). Subsequent RT-PCR and 3'-RACE experiments revealed no intron in the ORF and no RNA editing or alternative splicing in the tissues determined, confirming that this gene has a premature stop codon downstream from the sixth TMD, giving a 290-amino acid protein (Fig. 1). PCR experiments of human/rodent somatic and hybridized Southern blot analysis indicated that the human genome contains a single copy of this gene on chromosome 5 (data not shown).

The presence of only one putative frameshift and the conserved homology with the Y1 receptor prompted us to investigate the orthologous gene of other species. One primer pair made from the human cDNA sequence amplified the orthologue of the primate species chimpanzee, gorilla, and tamarin (a New World monkey), and of a nonprimate species, the rabbit. Using the rabbit sequence, the mouse and hamster orthologues were amplified. Southern blot analysis supported an orthologous gene in the chimpanzee, gorilla, and tamarin (a New World monkey), and of a nonprimate species, the rabbit. Using the rabbit sequence, the mouse and hamster orthologues were amplified. Southern blot analysis supported an orthologous gene as described previously (13).
restored by a single-base insertion, Figs. 1 and 2B) produced any specific binding sites for 125I-porcine PYY, 125I-human NPY, PYY, or PP at concentrations up to 2 nM.

Using the 5'- and 3'-RACE methods, the rabbit ORF was amplified from the brain and skeletal muscle poly(A) RNA (Fig. 2B). The gene was revealed to be intronless and to encode a novel heptahelix receptor of 371 amino acids, sharing highest identity with human Y1 (49%) followed by Y4/PP1 (41%) and Y2 (24%) receptors. The deduced amino acid sequence of the rabbit is shown above and that of mouse/hamster (identical in this region) is shown below. Consensus nucleotides are indicated by shading. ▼ and ▲ represent the position of a frameshift deletion in the primates. B, alignment of the deduced amino acid sequence encoded in the Y2b gene of rabbit (Rab-Y2b), mouse (M-Y2b/M-Y5), and human (H-Y2b), and the human reverted mutant (H-Y2bRM). Potential sites for N-linked glycosylation are indicated by •. The putative transmembrane domains (I–VII) are shown. ▼ represents the position of a frameshift in the human Y2b. Identical amino acid residues in Rab-Y2b and M-Y2b/M-Y5 and identical residues to rabbit or mouse in H-Y2b and H-Y2bRM are shaded.

FIG. 3. Pharmacological profile of rabbit Y2b receptor. A, inhibition of 125I-porcine PYY binding to membranes from CHO cells stably transfected with rabbit Y2b. Competition data are expressed as a percentage of binding in the absence of competitor peptide. Data represent the mean ± S.E. for three experiments performed in triplicate. The rank of order is as follows: human PYY (hPYY, K_i = 2.5 nM) > porcine NPY13–36 (pNPY13–36, K_i = 5.0 nM) > human hPP (hPP, K_i = 340 nM).

B, inhibition of forskolin-stimulated cAMP formation by human PYY in the rabbit Y2b-transfected CHO cells. cAMP levels obtained in the presence of forskolin (10 μM) alone were arbitrarily set at 100%. Data represent the mean ± S.E. for four experiments performed in triplicate.
determine the difference in binding specificity between Y1 and Y2 (1–3) have no functional significance in these species or that directional selection has occurred.

In rabbit, Y2b mRNA was detected in several tissues and brain regions, including hypothalamus, by RT-PCR (Fig. 4A), but was not detectable by Northern blot analysis (data not shown). Surprisingly, despite the inactivation for receptor function in humans, Northern blot analysis revealed abundant transcripts (3 kilobases) in the heart and skeletal muscle (Fig. 4B). It is presently unknown whether the frameshift mutation of the Y2b gene in the primate ancestor was neutral, i.e. Y2b was dispensable in the ancestor, or had evolutionary advances. However, considering the high levels of Y2b transcripts in human tissues, unlike the absent or low levels of transcription of inactivated genes observed to date (17–20), it is interesting to speculate that the mutation was the first step toward not only the inactivation for receptor function but also the creation of another function in the Y2b gene, and that the human Y2b gene possesses this novel function in place of a receptor for NPY/PYY.

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FIG. 4. Distribution of Y2b transcripts in rabbit and human tissues. A, RT-PCR analysis of Y2b transcripts in various rabbit tissues and brain regions. PCR products from the cDNA are indicated by +RT, those from total RNA without reverse transcription are indicated by −RT. The PCR products of G3PDH are shown as a quantitative control for each cDNA. B, Northern blot analysis of Y2b transcripts in various human tissues. Molecular size markers in kilobases are shown in the figure. The transcripts were also detected at low levels in the brain regions: subthalamic nucleus and thalamus (data not shown).