Molecular Characterization of a Second Mouse Pancreatic Polypeptide Receptor and Its Inactivated Human Homologue*

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The family of mammalian neuropeptide Y (NPY)/peptide YY (PYY)/pancreatic polypeptide (PP) receptors comprises several G protein-coupled receptors, i.e. Y1, Y2, and Y4/PP1. We now report cloning of a novel member of this family named PP2. The coding region of the mouse PP2 gene reveals no introns and predicts a seven transmembrane domain (TM) receptor of 371 amino acids. Percent identities of the mouse PP2 to mouse Y1, mouse Y4/PP1 and human Y2 receptors are 53, 42, and 31, respectively. The mouse PP2 receptor expressed in COS cells binds rat 125I-PP with high affinity, i.e. IC50 = 65 pm. Pharmacological characterization of 125I-PP binding shows a rank order of potency of PP >> PYY NPY, which is similar to that of the mouse Y4/PP1 receptor. Mouse PP2 transcripts were not detectable by Northern analysis in adult tissues and in 11-, 15-, and 17-day-old embryos. However, a 9.8-kb PP2 transcript was detectable in 7-day-old mouse embryo, i.e. prior to the organogenesis of pancreas and the onset of PP production. We have also cloned the human homologue of PP2, which is a single copy gene and maps to human chromosome 5q31. Surprisingly, the human PP2 cDNAs and gene sequences display a single base deletion in the coding region. This frameshifting mutation predicts a truncated receptor of 290 amino acids without TM7. Transfection of COS-7 cells with several different human PP2 expression constructs failed to confirm any specific binding of 125I-PP, 125I-PYY, or 125I-NPY to cell membranes. These data suggest that in mouse there are at least two PP receptors, Y4/PP1 and PP2, whereas in humans, PP2 is either functionally inactive or it has acquired a PP independent function.

Pancreatic polypeptide (PP)1 is a hormone found in the general circulation, where it is released after meal ingestion (1–3). Little is known about the function of PP, which is produced and secreted primarily by certain endocrine cells of the pancreatic islets (1–3). PP-containing cells can be found also in gut and intestine (3, 4). The main known biological effects of PP are those on gastrointestinal tract and pancreatic secretion (1–5). Some activities of PP are mediated centrally via brainstem sites (6). In addition, PP may serve some unknown function in other tissues in which PP binding sites were described, including adrenal gland, brain, prostate, and liver (6–10). PP, neuropeptide Y (NPY), and peptide YY (PYY) belong to a family of structurally related 36-amino-acid peptides, which produce their effects through the interaction at multiple receptors, i.e. Y1, Y2, Y3, Y1-like, as well as a PP receptor (4, 5, 11–21). Recently, we and others have reported cloning and characterization of a PP receptor from human, rat, and mouse, termed Y4 or PP1 (15, 16, 18, 19). The Y4/PP1 receptor has subnanomolar affinity to PP and nanomolar affinity to PYY and NPY, raising the possibility that it may be shared by all three pancreatic polypeptides. Tissue distribution studies in humans and mice suggest potential roles for Y4/PP1 receptor in the gastrointestinal tract, heart, and prostate, as well as in neural and endocrine signaling.

In this report we describe cloning of a novel member of the NPY/PYY/PP receptor family termed PP2. We show that the mouse version of this gene is coding for a functional PP receptor and describe pharmacological properties of recombinant mouse PP2 receptors expressed in COS-7 cells. We also present data that suggest that the human PP2 gene is mutated, having a single base deletion, and that human PP2 receptors expressed in COS-7 cells do not interact with pancreatic polypeptides.

EXPERIMENTAL PROCEDURES

General Procedures, Cloning, and Sequencing—Library screening, cloning, Northern and Southern blotting, PCR, and other manipulations were carried out by standard methods (22) as described previously (16). Reverse transcription coupled with PCR (RT-PCR) was carried out with human brain mRNA (Clontech Labs, Palo Alto, CA) as a template and using a RT-PCR kit (Perkin Elmer Cetus, Norwalk, CT). Degenerate oligonucleotide primers corresponding to cloned NPY receptors (11–13) were as follows: 5'-ATG GAY CAY TGG RTI TTY GGI GA-3' and 5'-ATG AAG CAA ARI GGI CCR AAR TAY TG-3' (where I is inosine, Y is C or T, R is A or G). PCR conditions were 94 °C, 1 min, 55 °C, 2 min, and 72 °C, 1 min, for 36 cycles. PCR product of 370 bp was purified on 1.5% agarose gel, digested with Bcl I to remove amplified Y1 receptor cDNAs and subcloned. Several cloned sequences, including pG8 (Table I), represented a novel sequence related to NPY receptors. Plasmid pY3.12 and additional longer cDNAs for this novel gene (Table I) were obtained by screening of a human heart cDNA library (Stratagene, La Jolla, CA) using the insert of pG8 as the probe. Inserts of plasmids pY7(a-e) (Table I) were obtained by PCR of human genomic DNA of five unrelated individuals (Bios Laboratories, New Haven, CT) with the following primers: 5’-CAA GGA CAA CAA GAG CAT CAC CAC-3’ (FNYPYB) and 5’-ACC AAG TGG CAA ACT ACA AAT ACC-3’ (RNPYRB). Mouse genomic clones were obtained by screening of 500,000 recombinant phage from a mouse 129/SV genomic library in phage A Fix II (18). Two positive phage were plaque-purified and one of these was selected for detailed analysis. A 1.6-kb HindIII fragment was subcloned into pcDNA3 (Invitrogen, San Diego, CA) and found to contain the entire coding region of the mouse PP2 gene. Manual sequencing was carried out by Lark Sequencing Technologies (Houston, TX) with Sequenase kit (U.S. Biochemical Corp.); in some sequencing reactions dTTP was used. Automated sequencing was carried out at Yale University W. M. Keck facility, using cycle sequencing with Taq polymerase, fluorescent-dideoxynucleotide terminators (Perkin-Elmer Corp.) and an Applied Biosystems 373A Stretch DNA sequencing apparatus. The nucleotide sequences of human and mouse PP2 DNAs have been depos-

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‡ The abbreviations used are: PP, pancreatic polypeptide; NPY, neuropeptide Y; PYY, peptide YY; kb, kilobase(s); bp, base pairs; TM, transmembrane domain; RT, reverse transcription; PCR, polymerase chain reaction; STS, sequence tagged site; FISH, fluorescent in situ hybridization; h, human; r, rat; m, mouse.
A sequence tagged site (STS) for human PP2 was developed with the following two primers: 5’-CAT ATG AGA CAG GCA GTC TTA GC-3’ (reverse primer RHYB) and 5’-AGA TTG GCT CGT ATA ACA ACA GG-3’ (forward primer FHYB). PCR with these two primers gave a 174-bp product, which could be confirmed by digestion with XmnI. This STS marker was used to type a panel of somatic cell hybrids for chromosome mapping (Bios Laboratories). Southern blots of genomic DNA digested with EcoRI and BamHI were purchased from Bios Laboratories. A P1 human genomic clone E2182 for PP2 was obtained by custom screening of a P1 human genomic library with cDNA insert of pY3.12 at Bios Laboratories. The identity of this P1 clone was confirmed by Southern blotting and by using our STS marker. FISH was carried out at Bios Laboratories. DNA from clone E2182 was labeled with digoxigenin-dUTP by nick translation. Labeled probe was combined with sheared human DNA and hybridized to normal metaphase chromosomes derived from PHA-stimulated peripheral blood lymphocytes in a solution containing 50% formamide, 10% dextran sulfate, and 2× SSC. Specific hybridization signals were detected by incubating the hybridized slides in fluorescein conjugated antidigoxigenin antibodies. The chromosomes were then counter stained with propidium iodide and analyzed.

Expression Analyses—Multiple tissue Northern blots were purchased from Clontech Labs. Membranes were hybridized and washed under high stringency conditions as described previously (16). A human β-actin cDNA was used to probe all filters to control for unequal loading or transfer. For RT-PCR, human poly(A)-containing RNA from various tissues (Clontech Labs) was reverse transcribed and resulting cDNAs were used for PCR with the primers FHYB and RHYB as described above. The PCR product of 174 bp was resolved on 1.5% agarose gels and confirmed by Southern blot analysis.

Expression Studies in COS-7 Cells—COS-7 cells were grown and transfected using the LipofectAMINE method (Life Technologies, Inc.) as described previously (16). 125I-Labeled rat pancreatic polypeptide (125I-rPP), 125I-labeled rat peptide YY (125I-PYY), and rat 125I-NPY were purchased from DuPont NEN. PYY, NPY, and (2–36)PYY were synthesized by Bayer Corp. Rat pancreatic polypeptide (rPP), human pancreatic polypeptide (hPP), and (Leu31Pro34)NPY were purchased from Peninsula Laboratories Inc. (Belmont, CA). Expression vectors were constructed in pcDNA3 (Invitrogen). Samples for binding consisted of 2–150 μg of membrane protein, 100 pM 125I-rPP, and peptide concentration in a final volume of 200 μl. Nonspecific binding was defined by 1 μM rPP. The binding assays were performed on GF/C Millipore 96-well plates as described previously (16). Data were analyzed using the nonlinear regression curve-fitting program RS/1 (BBN Software Products Corp., Cambridge, MA).

Plasmid Constructs—To remove the 5′-untranslated region of clone Y3.12, we utilized a unique HindIII site within the 5′ leader. Plasmid pY3.12 was cut with HindIII and religated. The resultant plasmid pY3.12dhada 5′-untranslated leader of 100 bp. To insert a single T base following the CCCC sequence in TM6, we used PCR mutagenesis. PCR primer TFIYNPYB, 5′-TCC ATC GTC GTC ACC TTT GGA GCC TGC TGG CTG CCC ATG AAT TCT TAC G-3′, was designed to take advantage of a unique Bst XI site upstream of the mutated position. PCR was carried out using pY3.12dhA as a template and primers

The sequences of the mouse PP2 receptor and its human homologue. In both cases the sequence around the predicted initiator methionine codon agrees with the consensus sequence and this methionine codon is preceded by an in-frame stop codon. The NH2 terminus of the proteins are numbered +1, and the putative transmembrane domains (TM1–TM7) are underlined. Nucleotide sequences are shown from a unique HindIII site. The human PP2 sequence compared to mouse sequence has a single base deletion in the coding region, corresponding to TM6 (indicated by a # sign). Gaps (periods) were introduced to optimize the alignment.
The PCR product was digested with BstEII and EcoRV and purified. pY3.12dH was digested with BstEII and EcoRV, purified, and ligated to PCR fragment BstEII-EcoRV. The resultant plasmid pG22 was sequenced from BstEII site until 39 end to confirm the sequence and the single base insertion.

RESULTS

The nucleotide and deduced amino acid sequences of the mouse PP2 receptor and its human homologue are shown in Fig. 1. The mouse sequence was obtained from a genomic clone, showed no evidence of introns, and revealed a 1113-bp open reading frame encoding 371 amino acid residues with a calculated molecular mass of 42,713 Da (Fig. 1). The nucleotide sequence identity between mouse and human PP2 is 83% within the coding region.

Sequence of the human PP2 was determined with clones pY3.12 and pG18, which represent cDNAs isolated from a human heart cDNA library. The resultant plasmid pG22 was sequenced from BstEII site until 3’ end to confirm the sequence and the single base insertion.

Analysis of mouse PP2 protein for regional hydrophobicity revealed seven putative transmembrane domains (TMs), the typical hallmark of G protein coupled receptors. There are three potential N-linked glycosylation sites, two in the NH2

terminus and one in the second extracellular loop connecting TMIV and TMV. The mouse PP2 protein contains residues conserved in many G protein receptors, including an acidic residue (Asp) in TMII and cysteine residues in the first and second extracellular loop that may form a disulfide bridge (23). Within human PP2 cDNA, a single base insertion following to sequence CCCC in TM6 would shift the reading frame and extend it throughout TM7 and the COOH terminus, similar to

Fig. 2. Amino acid sequence alignment of the mouse PP2 (pMP2) with the sequences of the mouse Y4/PP1 receptor (mY4), mouse Y1 receptor (mY1), and human Y2 receptor (hY2) (11, 14, 16). Amino acid residues identical to the mouse PP2 sequence are indicated by shading and the seven putative transmembrane domains are boxed. Gaps (periods) were introduced to optimize the alignment.
Comparison of the mouse PP2 protein with human PP2 (up to TM6) reveals 78% identity. Additional comparisons of the mouse PP2 sequence reveal 42% identity with the cloned Y4/PP1, 53% with mouse Y1 receptor and lower, 31%, identity with the cloned Y2 receptor (Table II). A Drosophila NPY receptor (26) has only 28% identity with mouse PP2, and unrelated G protein receptors have approximately 25–29% identity. An alignment of the mouse PP2 with other cloned mammalian NPY/PYY/PP receptors is shown in Fig. 2.

The coding region of the mouse PP2 was cloned into the expression vector pcDNA3 and the resultant plasmid pG29 was transfected into COS-7 cells. Binding of 125I-rPP to COS-7 cell membranes was linear up to at least 50 μg of protein, and typically greater than 75% specific binding was observed with 125I-rPP. Of the peptides tested, both rat and human PP had the highest affinity for PP2 (Fig. 3). There were only small differences observed when comparing the pharmacological profiles of mouse PP2 and Y4/PP1 receptors (Table III). Specific binding of 125I-PYY to membranes was lower, with relatively high background of nonspecific binding, and was not further investigated.

In contrast to mouse PP2, transient expression of human PP2 in COS-7 cells did not yield any specific binding to 125I-rPP, 125I-PYY, or 125I-NPY. A number of different plasmids were tested with identical results (Table I). There were only small differences observed when comparing the pharmacological profiles of mouse PP2 and Y4/PP1 receptors (Table III). Specific binding of 125I-PYY to membranes was lower, with relatively high background of nonspecific binding, and was not further investigated.

The human PP2 gene coding region could be amplified by PCR from our P1 genomic clone (plasmid pG27, Table I) and showed no evidence for introns, similar to the mouse gene. Southern blot analysis of mouse and human genomic DNA with mouse and human PP2 probes suggested the existence of a single gene in both species. Cross-hybridization with mouse PP2 probe suggested that its human species homologue is...
Indeed the human PP2 gene (Fig. 4).

Typing of a somatic cell hybrid panel with our STS marker revealed that PP2 gene maps to human chromosome 5 (data not shown). To map the human PP2 gene more precisely, a human genomic P1 clone was isolated and used for FISH. This experiment resulted in the specific labeling of the long arm of a group B chromosome. A second experiment was conducted in which a genomic probe containing the nucleolar protein gene NPM, which is known to localize to 5q35 (27), was cohybridized with our P1 plasmid E2182 in order to confirm the identity of the specifically labeled chromosome as chromosome 5. Measurements of 10 specifically labeled chromosomes 5 determined that E2182 hybridization signal is located at a position that is 70% of the distance from the centromere to the telomere of the chromosome arm 5q, an area that corresponds to 5q31 (Fig. 5).

A total of 80 metaphase cells were analyzed with 74 exhibiting specific labeling.

Northern analysis of mouse and human tissues was performed to examine transcript sizes and regional differences in mRNA abundance. The human PP2 message was detected as an abundant 3.4-kb transcript in the heart. At lower levels, the same transcript was detectable also in skeletal muscle, gastrointestinal tissues, adrenal glands and some other tissues (Fig. 6). Using RT-PCR, the message was also detectable in various parts of the human brain (data not shown). In the adult mouse tissues, PP2 message was not detectable by Northern blot analysis (data not shown). However, in the developing mouse, a 9.8-kb PP2 message was detected in 7-day-old embryo but not in 11-, 15-, and 17-day-old embryo (Fig. 7).

DISCUSSION

The results presented here show that the PP2 gene is a novel member of the NPY/PYY/PP receptor family. The mouse PP2 receptor has very high affinity to PP, indicating that PP may be one of its endogenous ligands. Both Y4/PP1 and PP2 receptors may also be able to interact with PYY and NPY in vivo, although we have not investigated 125I-PYY binding to mouse PP2 receptors in detail. The affinities of PYY and NPY were low in the 125I-rPP assay, as is the case for mouse Y4/PP1 receptors (16). The percent identity between mouse Y4/PP1 and mouse PP2 proteins is relatively low (42%) considering that both receptors have very similar affinities to rat and human PP and similar rank orders of potencies for other peptides tested (Table III). While we have not investigated coupling to second messenger systems, similarity of mouse PP2 receptors to Y4/PP1, Y1 and Y2 receptors within second and third intracellular loops (Fig. 2) may indicate potential coupling to adenylyl cyclase and calcium signaling systems (13, 15, 17).

Surprisingly, the human PP2 homologue displays properties of an inactivated gene, i.e. pseudogene. The single base deletion in the human PP2 gene is not due to a cloning or sequencing artifact, since DNAs were cloned by three independent methods and sequenced by three different methods. Furthermore, the deletion was found in several different cDNAs and in genomic DNAs from five unrelated individuals of three ethnic backgrounds (Table I). The deletion predicts a truncated protein without the seventh transmembrane region and the cytoplasmic carboxyl terminus. Such deletion likely results in a nonfunctional receptor, since G protein-coupled receptors generally require TM7 for activity (e.g. see Unson et al. (28)). A number of different constructs containing the coding region of human PP2 were transfected in COS-7 cells, but none of these constructs bound to 125I-rPP, suggesting a loss of function. It is possible, however, that functional human PP2 polypeptide is not expressible in COS-7 cells. Although we have not formally shown protein production from our various human PP2 expression constructs, control experiments with mouse PP2 and rat Y1 DNAs (constructed in the same pcDNA3 expression vector) tested under identical conditions, validate the common COS-7 cell transient expression system that we employed. Insertion of a single base following to CCCC sequence in TM6 would correct the reading frame of human PP2, so that its COOH terminus would be similar to and co-linear with mouse PP2 (Fig. 1, reading frame in italics). We have constructed plasmid pG22 with such an insertion (insertion of a single T base predicts a Leu-277 following Pro-276) and determined that this construct also failed to direct production of functional PP/PYY/NPY receptors in COS-7 cells. This may indicate that the coding region of human PP2 has accumulated additional mutations that have inactivated the active site for pancreatic polypeptides.
the heart and several other tissues is of unknown significance; it may represent an attempt at compensatory up-regulation of the PP2 gene expression. In conclusion, the human PP2 gene is mutated, does not seem to interact with pancreatic polypeptides and may represent a pseudogene. Less likely, the truncated human PP2 protein may serve some other function, which does not require interaction with pancreatic polypeptides.

In some instances, truncated GPCRs have been re-activated by coexpression with other truncated GPCRs (29), and cytoplasmic domains of GPCRs might inhibit GPCR signaling (30).

Mouse PP2 message is detectable in 7-day-old mouse embryo, which is prior to the formation of pancreas by invagination of duodenal endoderm. It would be interesting to determine if PP2 message is co-localizing to the pancreatic anlage, which is already noticeable before 20-somite stage (about day 8.5–9) (31). The ligand for embryonic PP2 receptors at this early stage of development is probably not PP, since PP mesenchyme is already noticeable before 20-somite stage (about day 8.5–9) (31). The ligand for embryonic PP2 receptors at this early stage only later, at 30-somite stage (about day 10) (31); immunohistochemical data place onset of PP expression to a much later stage (32). These issues are of interest since they may shed light on a central question in organogenesis of the pancreas, i.e., identification of a common, multipotential progenitor cell from which endocrine pancreatic islet cells arise (31–34). Available data indicate that the earliest expressed islet hormone gene may be PYY (34), which is a possible endogenous ligand for mouse PP2 receptor. Future studies will also be required to elucidate the reasons for dramatic evolutionary change in structure and function of mouse versus human PP2 receptors.

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Note Added in Proof—Several novel neuropeptide Y/peptide YY/pancreatic polypeptide receptors were recently cloned. Weinberg et al. describe a mouse neuropeptide Y receptor (Weinberg, D. H., Sirinathsinghji, D. J. S., Tan, C. P., Shiao, D. J. S., Tan, C. P., Shiao, L.-L., Morin, N., Rigby, M. R., and Hu (1993) J. Biol. Chem. 268, 27720–27727) and Hu et al. (Gerald, C., Walker, M. W., Vaysse, P. J.-J., He, C., Branchek, T. A., and Weinshank, R. L. (1995) J. Biol. Chem. 270, 26758–26761)

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