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Cloning and characterization of a novel receptor to pancreatic polypeptide, a member of the neuropeptide Y receptor family

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Abstract **We report isolation of a murine gene, NPYR-D, which predicts an intronless novel G protein-coupled receptor of 375 amino acids. Percent identities of NPYR-D to the cloned Y1, Y2, rat Y4/PP1 and human Y4/PP1 receptors are 45, 32, 92 and 76, respectively. Southern blots indicate that NPYR-D and human Y4/PP1 receptor genes are species homologues. Rat [^{125}I]pancreatic polypeptide ([^{125}I]rPP) bound to NPYR-D-transfected COS-7 cell membranes with a high affinity, i.e. IC_{50}=90 pM. Pharmacological characterization of [^{125}I]rPP binding showed a rank order of potency of PP >> PYY ≥ NPY, such that PYY and NPY were at least 5000-fold weaker than PP. Interestingly, [^{125}I]rPYY binding produced the same rank order, but PYY and NPY were only 25-fold weaker than PP, which had an IC_{50} value of approximately 120 pM. Tissue distribution studies in mouse and humans suggest potential roles of this novel receptor in the gastrointestinal tract, heart, prostate, as well as in neural and endocrine signalling.**

Key words: G protein coupled receptor; Gene structure; Gene expression

1. Introduction

Pancreatic polypeptide (PP), neuropeptide Y (NPY) and peptide YY (PYY) belong to a family of structurally related 36-amino-acid peptides which have functions in both neural and endocrine signalling [1–4]. Among these pancreatic polypeptides, NPY is the most prominent, having potent biological effects on many targets in the brain and periphery. NPY is known to regulate central and peripheral nervous systems, cardiovascular activity, and neuroendocrine axis including reproduction and food intake [1–4].

Relatively little is known about the functional significance of PP, a hormone released from certain endocrine cells of pancreatic islets after meals [5,6]. PP is also found in endocrine cells of the gastrointestinal tract, and functional PP receptors in small intestine and colon have been described [5–7]. All three pancreatic polypeptides are present in the general circulation. The permeability of the blood–brain barrier allows circulating PP in physiological concentrations to bind to brainstem PP receptors in area postrema, interpenduncular nucleus, dorsomedial nucleus and nucleus tractus solitarius [8]. Centrally administered PP acting in brainstem nuclei can inhibit pancreatic secretion and stimulate gastric secretion and gastric motility in rats [9,10]. In addition, PP binding sites have been described in other peripheral tissues, e.g. in the adrenal gland and liver [11,12].

Pancreatic polypeptides produce their effects through interaction at multiple receptors. Pharmacological and physiological studies have described several different NPY receptor subtypes, i.e. Y1, Y2, Y3, as well as a PP receptor [1–4,7,12]. Molecular cloning and characterization of the Y1 subtype of NPY receptors demonstrated that it is a member of the superfamily of G protein coupled receptors [13–15]. Very recently, during the course of this work, cloning and characterization of Y2, Y4 and PP1 receptors has also been reported [16–20].

In this report we describe cloning of a mouse gene encoding a novel member of the neuropeptide Y receptor family, NPYR-D. We show that this receptor has very high affinity for PP and that it is highly homologous to human and rat Y4/PP1 receptors [17,19,20]. We also describe pharmacological properties of recombinant NPYR-D receptors expressed in COS-7 cells, and regional distribution of this novel receptor in mouse and human tissues.

2. Materials and methods

Library screening, cloning, sequencing and other manipulations were carried out by standard methods [21]. Mouse 129SV genomic library (gift of Dr. Ann Davis) was custom constructed in phage lambda Fix II by Stratagene. Sequence of a 53-mer oligonucleotide probe corresponding to TM7 of cloned NPY receptors, was as follows: 5′-AAG CCA TAA AAT ATG GGG TTI ACA CAI GTG GAI ATC ATI GCI RYI AGG TGG CA-3′ (I = inosine). About 800,000 recombinant phages were screened with the radiolabeled oligonucleotide probe. Hybridizations were at 42°C in 5× SSPE (1× SSPE = 150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA), 0.1% SDS, 2× Denhardt's solution, 250 μg/ml herring sperm DNA, 125 μg/ml hydrolysed yeast RNA and 2×10^7 cpm/ml of radiolabelled probe; membranes were washed 2× for 30 min at 60°C in 2× SSPE and 0.1% SDS. Filters were then exposed to Kodak XAR5 film for 2–11 days. 14 positive phages were plaque purified and two of these (MM37 and MM51) were selected for detailed analysis by restriction mapping, Southern blotting, subcloning and sequencing. A 6 kb *XbaI*/*NotI* fragment of phage MM37 was subcloned into pSK Bluescript (Stratagene) and found to contain the entire coding region of the NPYR-D gene (Genbank Data Library accession number U40189).

Southern blots of genomic DNA digested with *Eco*RI and *Bam*HI were purchased from Bios Labs (New Haven, CT). Mouse NPYR-D probe (which consisted of a 1.2 kb DNA corresponding to the entire coding region) was radiolabeled by random priming and hybridized at 37°C in 5× SSPE, 29% formamide, 0.5% SDS, 2× Denhardt's, 100 μg/ml herring sperm DNA, and 4×10^7 cpm/ml of radiolabelled probe. Blots were washed at 50°C in 0.1× SSPE, 0.5% SDS for 2×30 min and exposed for 40 h. Human Y4/PP1 receptor DNAs were obtained independently by PCR with degenerate oligonucleotide primers and subsequent screening of a small intestine cDNA library (data not shown). The human Y4/PP1 receptor probe consisted of a 0.6 kb *PstI* fragment containing the coding region after TMV and about 0.2 kb of 3′ untranslated region.

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Abbreviations: NPY, neuropeptide Y; PYY, peptide YY; PP, pancreatic polypeptide; kb, kilobases; bp, base pair; r, rat; TM, transmembrane domain; RT-PCR, reverse transcription-polymerase chain reaction.

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Multiple tissue Northern blots were purchased from Clontech Labs (Palo Alto, CA). Mouse and human probes were as described above for Southern blots. Following prehybridization, membranes were hybridized at 42°C in 5× SSPE, 50% formamide, 0.5% SDS, 2× Denhardt's, 100 μg/ml herring sperm DNA, and 4×10^7 cpm/ml of radiolabelled DNA probe. Membranes were washed 2× for 30 min at 54°C in 0.1× SSPE and 0.5% SDS. Filters were then exposed to Kodak XAR5 film for 2–12 days. 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 following primers: F19R, 5′-CCA TGG CAA GCA AGT GGC ACA C-3′ and F19F, 5′-CTC TGC ATG TGT TCA ACA GCC TG-3′. The PCR product of 99 bp was resolved on 1.5% agarose gels and confirmed by Southern blot analysis.

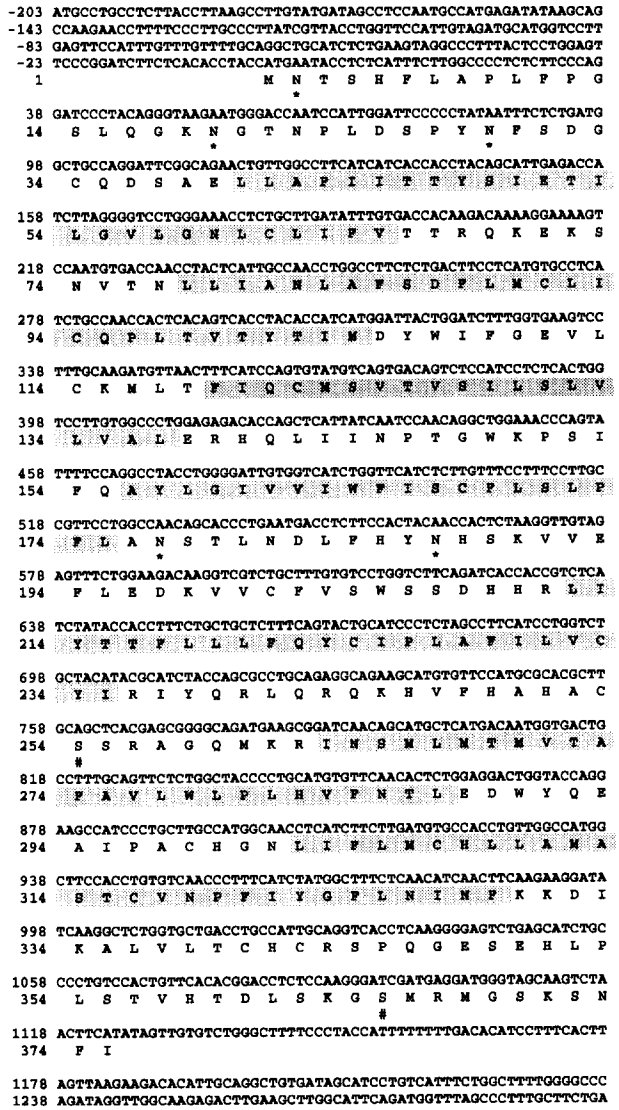


Fig. 1. The nucleotide sequence and the deduced amino acid sequence of the coding region of the mouse NPYR-D gene. The N-terminus of the protein is numbered +1 and the seven putative transmembrane domains (TM1–TM7) are indicated by shading. Potential sites for N-linked glycosylation (*) and phosphorylation by protein kinase C ( ) are indicated. The nucleotide sequence of the 1500 bp NPYR-D has been submitted to Genbank Data Library under accession number U40189.

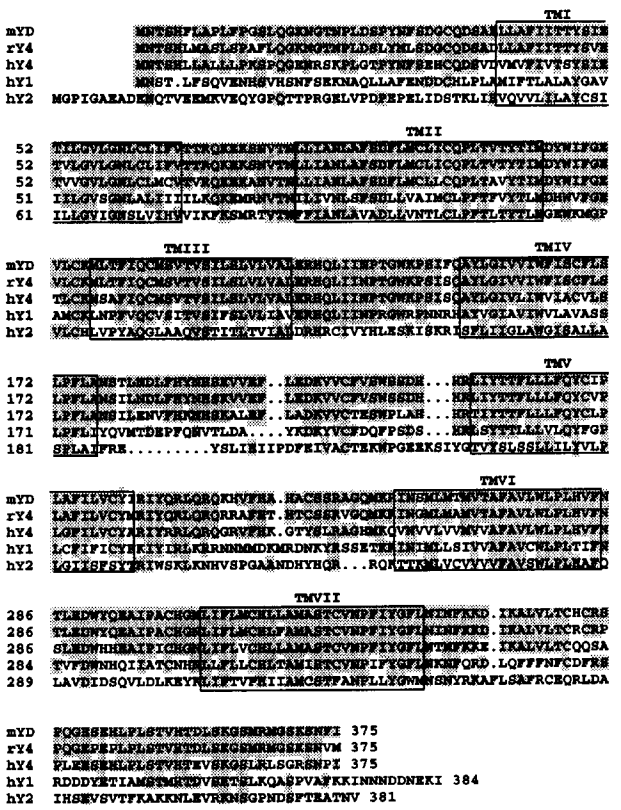


Fig. 2. Alignment of the deduced amino acid sequence of the mouse NPYR-D (mYD) with the sequences of rat Y4/PP1 receptor (rY4), human Y4/PP1 receptor (hY4), human Y1 receptor (hY1) and human Y2 receptor (hY2) [14–20]. Amino acid residues identical to the mouse NPYR-D sequence are indicated by shading and the seven putative transmembrane domains are boxed.

[^{125}I]Rat pancreatic polypeptide ([^{125}I]rPP) and [^{125}I]rat peptide YY ([^{125}I]PYY) were purchased from NEN-DuPont (Boston, MA). PYY, NPY and (2–36)NPY were synthesized by Bayer Corp. Rat pancreatic polypeptide (rPP), human pancreatic polypeptide (hPP), and (Leu^{31}-Pro^{34})NPY were purchased from Penisula Laboratories Inc. (Belmont, CA). COS-7 cells (ATCC) were grown in DMEM media supplemented with 2 mM glutamine, 10% fetal bovine serum, 1 mM sodium pyruvate and antibiotic/antimycotic. Cells at 70% confluency were transfected with plasmids using the lipofectamine method (GIBCO-BRL). The coding region of NPYR-D was cloned into pcDNA3 (Invitrogen, San Diego, CA) to generate expression vector pG25. 15 μg DNA and 90 μl of lipofectamine were added to each flask. Media was completely replaced 24 h post transfection, and membranes were harvested 24 h later. The binding assays were performed on GF/C Millipore 96-well plates pretreated with 0.02% polyethylenimine (PEI) for at least 2 h prior to use. The PEI was aspirated from the plates on a vacuum manifold immediately before the samples were added to the wells. All peptides, tissue and radioligands were diluted with binding buffer (137 mM NaCl, 5.4 mM KCl, 0.44 mM KH_2PO_4, 1.26 mM $CaCl_2$, 0.81 mM $MgSO_4$, 20 mM HEPES, 1 mM DTT, 0.1% bacitracin, 100 mg/ml streptomycin sulfate, 1 mg/ml aprotinin and 10 mg/ml soybean trypsin inhibitor, pH 7.4, with 0.3% BSA). Samples for binding consisted of 1–50 μg of protein, 25 pM [^{125}I]rPP or 50 pM [^{125}I]PYY, and peptide dilution in a final volume of 200 μl. Nonspecific binding was defined by 1 μM rPP or 1 μM PYY, respectively. After a 2 h incubation at room temperature with constant mixing, the samples were aspirated on a vacuum manifold, washed with three 200 μl aliquots of ice-cold binding buffer. Binding data were analyzed using the non-linear regression curve-fitting program RS/1 (BBN Software Products Corp., Cambridge, MA).

Rabbit antisera were generated against the human Y4/PP1 receptor carboxy-terminal 17-mer peptide coupled to keyhole limpet hemocyanin. Total homogenates (40 μg protein) of human tissues were fractionated on SDS-polyacrylamide gels (4–20%), transferred onto nitro-

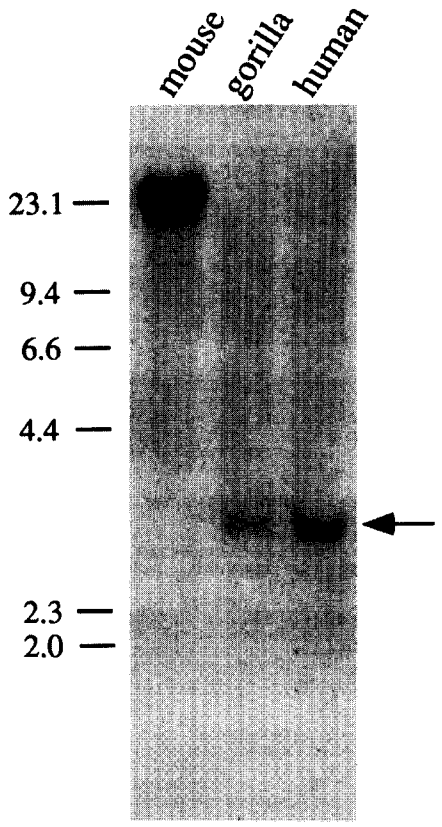


Fig. 3. Southern blot analysis of *Eco*RI-digested genomic DNA, using a mouse NPYR-D probe under moderate stringency. Each of the species examined revealed a single hybridizing band likely representing the NPYR-D gene species homologues. This blot was stripped and re-probed with a human Y4/PP1 receptor probe. The arrow indicates the position of the human Y4/PP1 receptor gene *Eco*RI fragment, which was detected by hybridization under high stringency conditions. Similar data were also obtained with *Bam*HI-digested genomic DNA.

cellulose membranes and probed with antisera diluted in non-fat milk (1:100). Alkaline phosphatase conjugated goat anti-rabbit antibody and color development reagent were from Immun-Blot Assay Kit (Bio-Rad Labs).

3. Results and discussion

To find genes that encode novel members of the NPY receptor family, a mouse genomic library was screened with a radiolabeled 53-mer oligonucleotide corresponding to the seventh transmembrane region (TM7) of the cloned NPY receptors. Two genomic clones (λMM-37 and λMM-51) were found to contain a novel gene named NPYR-D. A 6 kb fragment of λMM-37 was subcloned, and sequencing identified the entire open reading frame of NPYR-D. No evidence for introns was found in the coding region of NPYR-D; this is not surprising, since many G protein coupled receptors are known to be intronless.

Sequence analysis of NPYR-D revealed a 1125-bp open reading frame encoding 375 amino acid residues with a calculated molecular mass of 42,634 Da (Fig. 1). 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. Analysis of NPYR-D protein for regional hydrophobicity revealed seven putative transmembrane domains (TMs), the typical hallmark of G protein coupled receptors. There are 5 potential N-linked glycosylation sites, 3 in the N-terminus and 2 in the second extracellular loop connecting TMIV and TMV. The NPYR-D protein contains residues conserved in many G protein receptors, including an acidic residue in TMII and cysteine residues in the first and second extracellular loop which may form a disulfide bridge [22]. The carboxyl terminal region contains 7 serines and 3 threonines which may be substrates for protein kinases; phosphorylation has been implicated in receptor desensitization [23]. The C-terminus also contains cysteine residues which may be sites for fatty acid modification [24].

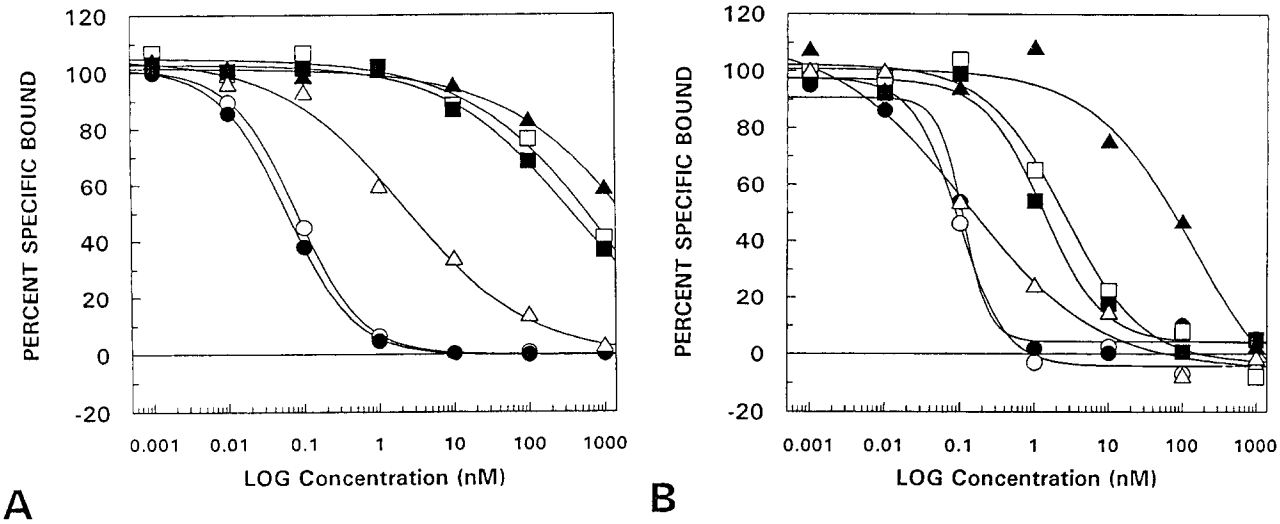


Fig. 4. Comparison of competition curves for various peptides in transiently expressed NPYR-D membranes, using (A) [^{125}I]rat PP and (B) [^{125}I]PYY. Competing peptides were rPP (empty circles), hPP (filled circles), NPY (empty squares), PYY (filled squares), (Leu^{31}Pro^{34})NPY (empty triangles) and (2–36)NPY (filled triangles). Binding conditions are described in section 2.

Table 1
Comparison of IC_{50} values for various peptides from [^{125}I]rPP and [^{125}I]PYY binding to COS-7 cell membranes transiently expressing NPYR-D receptors. Values are the average of at least 2 independent experiments.

PEPTIDE	IC_{50} (nM)	
	[^{125}I]rPP	[^{125}I]PYY
human PP (hPP)	0.054	0.11
rat PP (rPP)	0.097	0.11
(Leu^{31}Pro^{34})NPY	4	0.35
PYY	500	3
NPY	790	3
(2-36)NPY	2350	83

During the course of this work we became aware that the cloning and characterization of human and rat Y4/PP1 receptors have been presented in a preliminary form [17,19,20]. Comparison of the NPYR-D sequence (Fig. 2) with these sequences showed that NPYR-D has 92% identity to rat Y4/PP1 receptor and 76% identity to human Y4/PP1 receptor. Additional comparisons revealed 45% identity with the cloned Y1 receptor [13–15] and lower, 32% identity, with the cloned Y2 receptor [16,18]. A drosophila NPY receptor [25] has only 28% identity with NPYR-D, and unrelated G protein receptors have approximately 25–31% identity with NPYR-D.

Since mouse NPYR-D and human Y4/PP1 receptor proteins are 76% identitical and species homologs are typically highly similar (>90% amino acid identity), we have investigated whether these two genes are species homologues. Southern blot analysis of mouse and human genomic DNA with NPYR-D and Y4/PP1 probes suggested the existence of a single gene in both species. Cross-hybridization with mouse NPYR-D probe suggested that its human species homologue is indeed the human Y4/PP1 receptor gene (Fig. 3). The somewhat unexpected lower homology may indicate substantial structural and functional divergence of these receptors.

The coding region of NPYR-D was cloned into the expression vector pcDNA3 and the resultant plasmid pG25 was transfected into COS-7 cells. Binding of [^{125}I]rPP to membranes transiently transfected with NPYR-D was very high, indicating that PP may be the endogenous ligand for this mouse clone. Typically, greater than 95% specific binding was observed with [^{125}I]rPP, while [^{125}I]PYY had only 50% specific binding at best. Of the peptides tested, both rat and human PP had the highest affinity for NPYR-D. Surprisingly, there were pharmacological differences observed when comparing the pharmacological profiles for both radioligands (Fig. 4A,B). Of particular note were the affinities for NPY and PYY, which were only 25-fold weaker than rPP when [^{125}I]PYY was the radioligand, as compared to more than a 5,000-fold difference when [^{125}I]rPP was used (Table 1). A possible reason for this discrepancy between radioligands could be due to differences in affinity states recognized by [^{125}I]rPP and [^{125}I]PYY.

Northern analysis of mouse and human tissues was performed to examine transcript sizes and regional differences in mRNA abundance. In the mouse, NPYR-D appears to

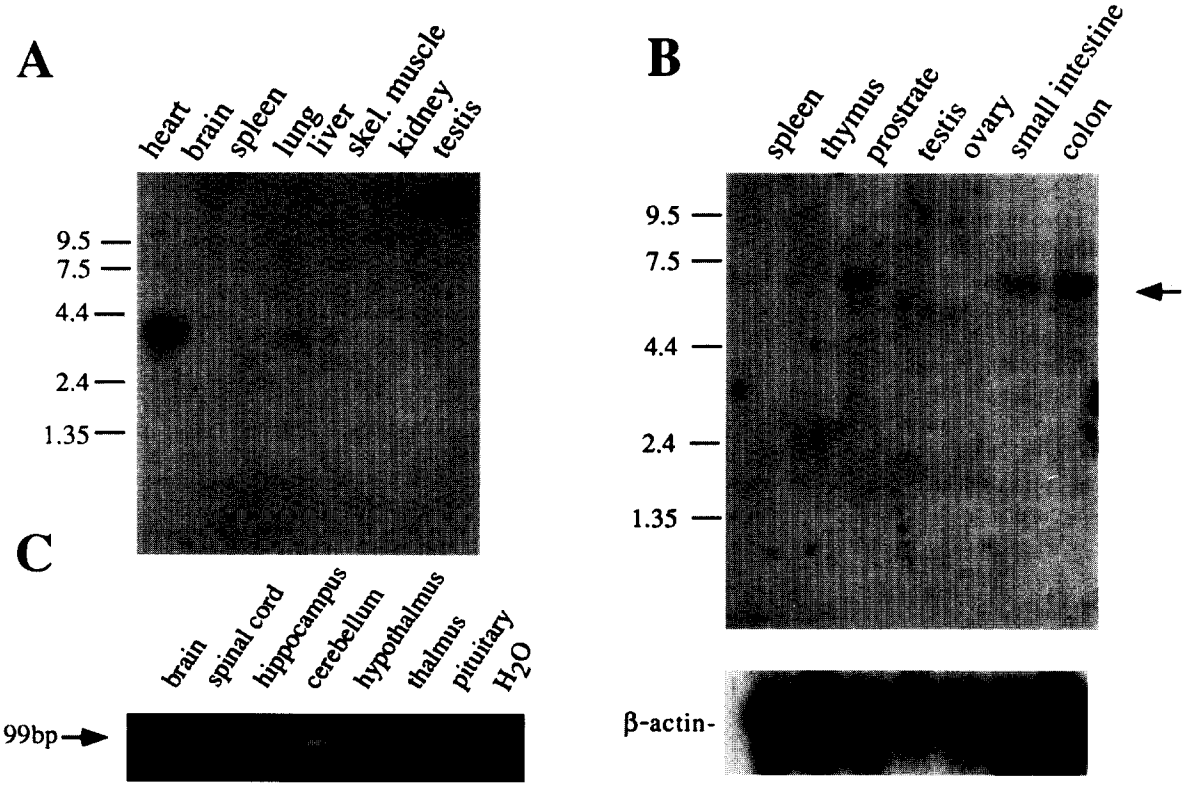


Fig. 5. Tissue distribution of NPYR-D and its human homologue. (A) Northern analysis of NPYR-D message in various mouse tissues. Blot contains about 2 μg of poly(A)$^+$ RNA from various mouse tissues. (B) Northern analysis of human Y4/PP1 receptor message in various human tissues. Blot (B) contains 2 μg of poly(A)$^+$ RNA from human tissues. (C) Expression of human Y4/PP1 receptors assesed by RT-PCR. Lane 1, brain; 2, spinal cord; 3, hippocampus; 4, cerebellum; 5, hypothalamus; 6, thalamus, 7, pituitary; 8, water.

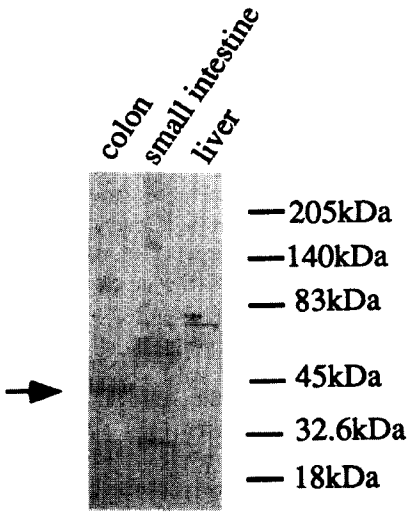


Fig. 6. Western blot analysis of human colon, small intestine and liver proteins with an antisera against C-terminus of human Y4/PP1 receptors. Arrow points to the 43 kDa band which is likely to represent the human Y4/PP1 receptor.

be synthesized as a 4.2 kb message in the heart (Fig. 5A). This message could also be detected in the small intestine (data not shown) but not in brain and other tissues examined (Fig. 5A). The human homologue of NPYR-D was detected as a 5.9 kb transcript in the small intestine, colon, prostate (Fig. 5B) and stomach (not shown). Consistent with these observations, Western blot analysis with an antiserum against the carboxy-terminus of the human Y4/PP1 receptor detected a 43 kDa protein in human colon and small intestine (Fig. 6). At lower levels, using RT-PCR, the NPYR-D message was also detectable in various parts of the human brain (Fig. 5C). This tissue distribution is consistent with previously reported effects of PP on gastrointestinal tract, such as ion transport in small intestine and colon, and gastrointestinal motility [5–7]. Expression in the CNS is consistent with brainstem-mediated effects of PP on gastrointestinal motility and gastric acid and pancreatic secretion [8–10]. The potential role of PP and its receptor in the brain may deserve further examination.

The results presented here demonstrate that the NPYR-D gene is coding for a novel PP receptor. The receptor also has an affinity for PYY and NPY, raising the possibility that this receptor may be shared by all three pancreatic polypeptides. The availability of cloned receptors should aid in the search for specific antagonists, which are required for functional characterization of pancreatic polypeptides as well as for development of potential therapeutics.

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Note added in proof

Three abstracts referenced in this manuscript, [16], [17] and [20], have now been published as full papers. These references are as follows: 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; Bard, J.A., Walker, M.W., Branchek, T.A. and Weinshank, R.L. (1995) J. Biol. Chem. 270, 26762–26765; and Lundell, I., Blomqvist, A.G., Berglund, M.M., Schober, D.A., Johnson, D., Statnick, M.A., Gadski, R.A., Gehlert, D.R. and Larhammar, D. (1995) J. Biol. Chem. 270, 29123–29128.