

Identification of Two Isoforms of Mouse Neuropeptide Y-Y1 Receptor Generated by Alternative Splicing

ISOLATION, GENOMIC STRUCTURE, AND FUNCTIONAL EXPRESSION OF THE RECEPTORS*

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Two cDNA clones homologous with human neuropeptide (NP) Y-Y1 receptor have been isolated from a mouse bone marrow cDNA library. One was thought to be the cognate of the human NPY-Y1 receptor, termed Y1 α receptor, and the other form, termed Y1 β receptor, differed from the Y1 α receptor in the seventh transmembrane domain and C-terminal tail. Analysis of the mouse genomic DNA showed that both receptors originated from a single gene. The different peptide sequences of the Y1 β receptor were encoded by separate exons, hence, these receptors were generated by differential RNA splicing. High affinity binding of [¹²⁵I]NPY to each receptor expressed in Chinese hamster ovary (CHO) cells and sequestration of [¹²⁵I]NPY after binding to each receptor were observed. In the CHO cells expressing the Y1 α receptor, intracellular Ca²⁺ increase, inhibition of forskolin-induced cAMP accumulation, and mitogen-activated protein kinase (MAPK) activation were observed by stimulation of NPY, and these responses were abolished by pretreatment with pertussis toxin. Since wortmannin completely inhibited NPY-elicited MAPK activation, we speculate that wortmannin-sensitive signaling molecule(s) such as phosphoinositide 3-kinase may lie between pertussis toxin-sensitive G-protein and MAPK. In contrast, these intracellular signals were not detected in CHO cells expressing the Y1 β receptor. Northern blots and reverse transcriptase-polymerase chain reaction analyses indicated that the Y1 α receptor was highly expressed in the brain, heart, kidney, spleen, skeletal muscle, and lung, whereas the Y1 β receptor mRNA was not detected in these tissues. However, the Y1 β receptor was expressed in mouse embryonic developmental stage (7 and 11 days), bone marrow cells and several hematopoietic cell lines. These results suggest that the Y1 β receptor is an embryonic and a bone marrow form of the NPY-Y1 receptor, which decreases in the expression during development and differentiation.

portant regulator in central and peripheral nervous systems (1). NPY is highly conserved in primary structure among species as sequences of human, rabbit, rat, and mouse are identical and differ from the porcine sequence by only a single amino acid (2). It belongs to a peptide family that also includes peptide YY (PYY) and pancreatic polypeptide (PP) (3). Mammalian NPY and PYY show 70% sequence identity, while PP is 50% homologous to NPY. NPY is widely distributed in the brain (4) and the peripheral nervous system (5), and is often co-localized with norepinephrine, *e.g.* in sympathetic perivascular nerve fibers (4, 5). Studies of various organs and cell types with peptide fragments of NPY have indicated that multiple NPY receptor subtypes exist (6). The two major receptor subtypes have been designated Y1 and Y2, and the Y1 receptor has the ability to respond to an analog of NPY modified at residues 31 and 34 ([Leu³¹,Pro³⁴]NPY) (7). The Y2 receptor subtype is defined on the basis of its affinity to the NPY peptide C-terminal fragment NPY-(13-36) (8). More recently, data from several laboratories have indicated the existence of a Y3 type receptor to which PYY shows a markedly lower affinity than NPY (9). NPY receptors have been identified in a variety of tissues, including brain, spleen, small intestine, kidney, testis, and placenta (10-12). In addition, binding sites have been noted in human cell lines such as SK-N-MC cells (neuroblastoma cell line) and HEL cells (erythroleukemia cell line) (13, 14).

NPY mRNA and NPY-like immunoreactivity has been detected in rat megakaryocytes and platelets as well as in preparations of rat mononuclear blood cells (15). In particular, high levels of NPY mRNA and NPY-like immunoreactivity were found in bone marrow of certain species of autoimmune mice that develop B-cell lymphoproliferative disorders (15, 16) and in bone marrow and peripheral lymphoblasts of children with B-cell precursor leukemia (17). These results suggest that NPY may function in hematopoietic and/or immune systems, as well as in nervous systems.

Human (18, 19) and rat (20, 21) brain NPY receptor cDNAs were isolated and showed a sequence similar to members of a G-protein coupled receptor superfamily. Ligand-binding characteristics of the expressed protein showed that the cDNA encodes the Y1-type receptor. In addition, the rat Y1-type receptor was demonstrated not only in brain but also in splenic lymphocytes, by means of polymerase chain reaction (PCR) and

Neuropeptide Y (NPY),¹ a 36-amino acid peptide, is an im-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) D63818 and D63819.

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¹ The abbreviations used are: NPY, neuropeptide Y; PYY, peptide YY; PP, pancreatic polypeptide; RT-PCR, reverse transcriptase-polymerase

chain reaction; Fura-2/AM, Fura-2 pentaacetoxymethyl ester; BAPTA/AM, 1,2-bis-(*O*-aminophenoxy)-ethane-*N,N,N',N'*-tetraacetic acid acetoxyethyl ester; CHO cells, Chinese hamster ovary cells; PTX, pertussis toxin; MAPK, mitogen-activated protein kinase; G-protein, guanine nucleotide-binding protein; kbp, kilobase pair(s); bp, base pair(s).

ligand-binding experiments (22). However, the signal transduction mechanism through the Y1-type receptor such as mitogen-activated protein kinase (MAPK) activation has not been clarified. We isolated the cognate mouse NPY-Y1 receptor cDNA from a bone marrow cell cDNA library and found a novel receptor form differing in seventh transmembrane and C-terminal tail domains. We describe here the origin of this structural diversity of the cloned mouse NPY-Y1 receptors, and provide novel information on the distribution of mRNAs encoding the two receptor isoforms, and on the cellular signaling of the cloned receptors expressed in Chinese hamster ovary (CHO) cells.

EXPERIMENTAL PROCEDURES

Materials—Materials were obtained from the following sources; [¹²⁵I]NPY (81.4 TBq/mmol) and [³²P]dCTP (111 TBq/mmol) from DuPont NEN; human [Leu³¹,Pro³⁴]NPY, human NPY-(13–36), porcine PYY, and genistein from Sigma; human NPY from Peptide Institute, Inc. (Osaka); λZAP II vector from Stratagene; Oligotex dT30-Super from Takara Shuzo (Kyoto); expression vector pcDNA1/neo from Invitrogen; forskolin and wortmannin from Seikagaku Kogyo (Tokyo); BAPTA/AM from Dojin (Kumamoto); pertussis toxin (PTX) from Funakoshi (Tokyo); U-73122 from BIOMOL Research Laboratory Inc.; anti-mouse MAPK (Erk-2) monoclonal antibody from Upstate Biotechnology Inc.; cDNAs of human brain, kidney, lung, vascular endothelial cells, smooth muscle, leukocytes, and bone marrow cells from Clontech; and various DNA-modifying enzymes from Takara Shuzo (Kyoto). All other chemicals were of analytical grade. Reagents for cell culture were from Nissui (Tokyo) and Life Technologies, Inc. WEHI-3 cells and L5178Y cells were obtained from the RIKEN Cell Bank (Tsukuba). ST2 cells were kindly provided by the JCRB Cell Bank (Tokyo).

PCR Analysis of the Human NPY-Y1 Receptor—To obtain the human NPY-Y1 receptor cDNA fragment, primers H1 (5'-ATGAATCAACAT-TATTTCCAG-3') and H2 (5'-TTGATCAAAGCACACGTATTTGTC-3') were designed based upon published sequences (18, 19) and PCR was carried out using 1 μg of human brain cDNA. PCR was performed for 30 thermal cycles under the following conditions: denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min, extension at 72 °C for 1 min. The amplified DNA fragment (605 bp long), termed hY1, was subcloned into pBluescript SK- vector and sequenced. To investigate the existence of the NPY-Y1 receptor in the human tissues, PCR was performed under the above conditions, using a template cDNA (1 μg) of human brain, kidney, lung, vascular endothelial cells, smooth muscle, leukocytes, or bone marrow cells. The amplified DNAs were separated by electrophoresis on a 0.8% agarose gel, transferred onto a nylon membrane (Hybond-N⁺, Amersham), then hybridized with the ³²P-labeled hY1 fragment. Hybridization was carried out at 65 °C for 2 h in Rapid Hybridization Buffer (Amersham) and the filter was washed at 65 °C for 30 min in 0.1 × SSC containing 0.1% SDS.

Screening of the Mouse Bone Marrow Cell cDNA Library—Poly(A)⁺ RNA was purified from adult mouse bone marrow cells according to Chomczynski and Sacchi (23), using Oligotex dT30-Super. A mouse bone marrow cell cDNA library was constructed in λZAP II, using poly(A)⁺ RNA and screened (5 × 10⁵ clones) with hY1 labeled by Random Priming Kit (Amersham). Hybridization conditions were as follows: 6 × SSC, 5 × Denhardt's solution (0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone, 0.1% Ficoll 400), 0.5% SDS, 400 μg/ml heat-denatured salmon sperm DNA, 20 μg/ml *Escherichia coli* DNA, and the radiolabeled probe at 55 °C for 17 h. Filters were washed for 20 min with 2 × SSC, 0.1% SDS at 55 °C. Positive plaques were purified, and the isolated cDNA-bearing phages were rescued to the plasmid form using a helper phage. Clones were analyzed by restriction digestion and sequenced using the dideoxynucleotide chain termination method on double-stranded template (24).

Mouse NPY-Y1 Receptor Gene Isolation and Analysis—A mouse genomic DNA library (λFIX II) was purchased from Stratagene and screened (1.5 × 10⁶ plaques) with ³²P-labeled probe. To isolate the common region of both receptors and Y1β receptor specific exon, the *EcoRI*-*PstI* fragment of the Y1α receptor and 3'-untranslated region of the Y1β receptor were used as probes, respectively. Isolated clones were subcloned into the *NotI* site of pBluescript SK- vector and analyzed by restriction digestion and partial sequencing.

Southern Blot Analysis of Mouse Genomic DNA—Mouse genomic DNA was purchased from Clontech. The mouse genomic DNA was digested with *Bam*HI, *Pst*I, *Hind*III, *Kpn*I, or *Xho*I. The fragments were separated by electrophoresis on a 0.8% agarose gel, transferred onto a

nylon membrane (Hybond-N⁺), then hybridized with a ³²P-labeled *EcoRI*-*PstI* fragment of the NPY-Y1α receptor. Hybridization was carried out at 65 °C for 2 h in Rapid Hybridization Buffer and the filter was washed at 65 °C for 30 min in 0.2 × SSC containing 0.1% SDS.

RT-PCR Analysis—RT-PCR analysis was carried out using poly(A)⁺ RNA isolated from mouse bone marrow cells. The first-strand cDNA was synthesized using First-strand Synthesis Kit (Pharmacia). The forward oligonucleotide primers were derived from the common region to the Y1α and Y1β receptor (SP1: 5'-CCCATCT GACTCTCACAGGCT-GTCT-3' and SP2: 5'-AGATATACATTCCGCTTAAAAGGAG-3') and the reverse primers were derived from the unique regions of the Y1α receptor (aAP1, 5'-TAAAAGATGGGGTTGACGCAGGTGG-3'; and aAP2, 5'-CATGGTAGACATGGCTATGGTCTCG-3') and the Y1β receptor (bAP1, 5'-AGTCAACGCAAACATGAGTACCC-3'; and bAP2, 5'-ATGGAAATACATCAGGCTCAGAGG-3'), respectively. PCR was performed using 1 μg of the synthesized cDNA with 30 cycles of 1 min of denaturation at 94 °C, 2 min of annealing at 57 °C, and 1 min of extension at 72 °C. The amplified products were separated by electrophoresis on a 0.8% agarose gel, transferred onto a nylon membrane (Hybond-N⁺), then hybridized with a ³²P-labeled probe. The probe DNA fragment (170 bp long) was prepared by PCR reaction, using the following primers P1 (5'-ATGGACAAGATCCGGGACAG-3') and P2 (5'-AGCAGATTGTGGTTGCAGGT-3') and the Y1α receptor cDNA as a template, under the thermal cycling conditions described above. Hybridization was carried out at 65 °C for 2 h in Rapid Hybridization Buffer and the filter was washed at 65 °C for 30 min in 0.2 × SSC containing 0.1% SDS.

Stable Expression in CHO-K1 Cells—CHO-K1 cells were maintained in Ham's F-12 medium containing 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum. For all experiments, cells were grown at 37 °C in a humidified atmosphere with 5% CO₂. Each full-length cDNA fragment (Y1α or Y1β receptor) was subcloned into the mammalian expression vector pcDNA1/neo and the plasmid was transfected into CHO-K1 cells by electroporation. Stably transfected cells were selected in medium containing 1 mg/ml geneticin (G418, Sigma) and clonal cell lines were obtained by limiting dilution. G418-resistant clones were expanded and tested for the ability to bind [¹²⁵I]NPY.

Binding Assay—Cell membranes of these transfected cells, prepared as described previously (25), were resuspended in ice-cold assay buffer I (50 mM Tris-HCl, pH 7.4, 2 mM CaCl₂, 5 mM KCl, 120 mM NaCl, 1 mM MgCl₂, 0.1% bacitracin). Each type of cell membranes was incubated with various concentrations (Scatchard plot analysis) or 1.0 nM (displacement experiments) of [¹²⁵I]NPY in 0.2 ml of assay buffer II (50 mM Tris-HCl, pH 7.4, 2 mM CaCl₂, 5 mM KCl, 120 mM NaCl, 1 mM MgCl₂, 0.1% bovine serum albumin (Sigma), 0.1% bacitracin) at 25 °C for 1 h. The reaction was terminated by adding 2 ml of ice-cold assay buffer II, and the unbound radioactive component was removed by rapid filtration. The bound radioactivity was measured in a γ-counter (1470 WIZARD, Pharmacia). The protein concentration was determined by the method of Bradford (26) using bovine serum albumin as a standard.

Internalization of NPY in Transfected CHO Cells—Transfected cells (2 × 10⁵ cells) were cultured in 12-well plates. [¹²⁵I]NPY binding was measured on whole cell monolayers, as described (21). To determine internalized [¹²⁵I]NPY, monolayers were treated for 3 min with 200 mM Gly-HCl, pH 3, 200 mM NaCl (subsequent to binding incubation) to remove [¹²⁵I]NPY bound to the exterior surface of the cells, then the acid-resistant (internalized) radioactivities were measured as described (27–30).

Measurement of Intracellular Ca²⁺ Concentrations—The cells were incubated in HEPES/Tyrode buffer containing 3 μM Fura-2-pentaacetoxymethyl ester (Fura-2/AM) (Dojin) for 1.5 h at room temperature. Measurements of intracellular Ca²⁺ concentration in the Fura-2-loaded cells were performed as described previously (31).

Measurement of cAMP—Five × 10⁴ cells were plated in a 24-well plate 48 h before the assay. For experiments using PTX, PTX (50 ng/ml) was added to the medium 24 h before the assay. Thirty min prior to the addition of the ligand to the medium we added 0.5 mM 3-isobutyl-1-methylxanthine (Sigma), then switched to a serum-free medium containing the indicated concentrations of NPY, 0.5 mM 3-isobutyl-1-methylxanthine, and 20 μM forskolin. After a 30-min incubation at 37 °C, the reaction was stopped and the cells lysed by the addition of the final 6% (w/v) trichloroacetic acid, then left at -80 °C. cAMP was measured using a cAMP Assay Kit (Amersham), according to the manufacturer's protocol.

MAPK Assay—MAPK activity was measured as described (32), using myelin basic protein as a substrate.

Western Blotting—Cell lysates were run on an SDS-polyacrylamide

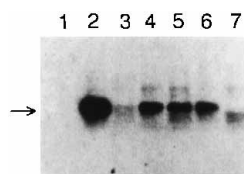


FIG. 1. PCR analysis of the human NPY-Y1 receptor mRNA. PCR reaction and hybridization were carried out as described under "Experimental Procedures." Lane 1, smooth muscle; lane 2, brain; lane 3, vascular endothelial cells; lane 4, kidney; lane 5, lung; lane 6, bone marrow cells; and lane 7, leukocytes.

gel (10% polyacrylamide) and transferred to nitrocellulose membranes. The membranes were then probed with anti-Erk-2 monoclonal antibody and a peroxidase-labeled second antibody. The antibody staining was visualized with an ECL Detection System (Amersham).

Northern Blot Analysis—Mouse Multiple Tissue Northern blot and Mouse Embryo Multiple Tissue Northern blot were purchased from Clontech that contained 2 μ g of poly(A)⁺ RNA from various sources. Hybridization was carried out at 65 °C for 2 h in Rapid Hybridization Buffer, and the filter was washed at 65 °C for 30 min in 0.2 \times SSC containing 0.1% SDS. The Y1 α receptor and the Y1 β receptor specific regions (3'-untranslated regions) were used as probes, respectively.

RESULTS

cDNA Cloning of Two Molecular Isoforms of the Mouse NPY-Y1 Receptor—A human NPY-Y1 receptor cDNA was cloned from a human brain cDNA library (18, 19). This receptor consists of 384 amino acids and has seven putative transmembrane domains, as noted in other members of the G-protein coupled receptor superfamily. Using reverse transcription polymerase chain reaction (RT-PCR), we found that mRNA encoding the human NPY-Y1 receptor was expressed not only in brain, kidney, and lung, but also in bone marrow cells, whereas it was not observed in vascular endothelial cells, smooth muscle, and leukocytes (Fig. 1).

To determine the functional role of the NPY-Y1 receptor in bone marrow cells, we isolated and sequenced mouse NPY-Y1 receptors from a mouse bone marrow cell cDNA library. Upon screening of approximately 5 \times 10⁵ plaques of the bone marrow cell cDNA library, using the human NPY-Y1 receptor fragment as a probe, we obtained 2 positive clones. One of the isolated clones, represented by Y1 α receptor, has a 2279-bp insert DNA containing a 1146-bp open reading frame. Since analysis of the predicted amino acid sequence indicates that the polypeptide encoded by this cDNA has seven transmembrane regions typical of G-protein coupled receptors and is 93% homologous to the human NPY-Y1 receptor, it is thought to be the mouse homologue of the NPY-Y1 receptor (Fig. 2A, a and b). The cytoplasmic tail of this receptor contains 6 serine residues and 4 threonine residues, as possible phosphate acceptors, and as observed in the human NPY-Y1 receptor. Furthermore, there seem to be four N-linked glycosylation sites in the N-terminal domain and the extracellular loops.

We then characterized the other clone represented by Y1 β receptor. The sequence was identical to the Y1 α receptor from the 5'-untranslated region to the third extracellular region, but was completely different in the seventh transmembrane, the cytoplasmic tail and 3'-untranslated region (Fig. 2A, a and c). Addition of different DNA fragments at position 908 (numbering from the first base of the coding sequence) of the Y1 α receptor created another reading frame downstream from this junction, which extends the coding region to a new stop codon located 14 bp downstream. As a consequence, a 79-amino acid C-terminal fragment of the Y1 α receptor was replaced with a new 4-amino acid fragment in the C-terminal end of the Y1 β receptor. Thus, the Y1 β receptor does not carry part of the seventh transmembrane and C-terminal tail (Fig. 2B). To confirm the existence of two forms of mouse NPY-Y1 receptor in

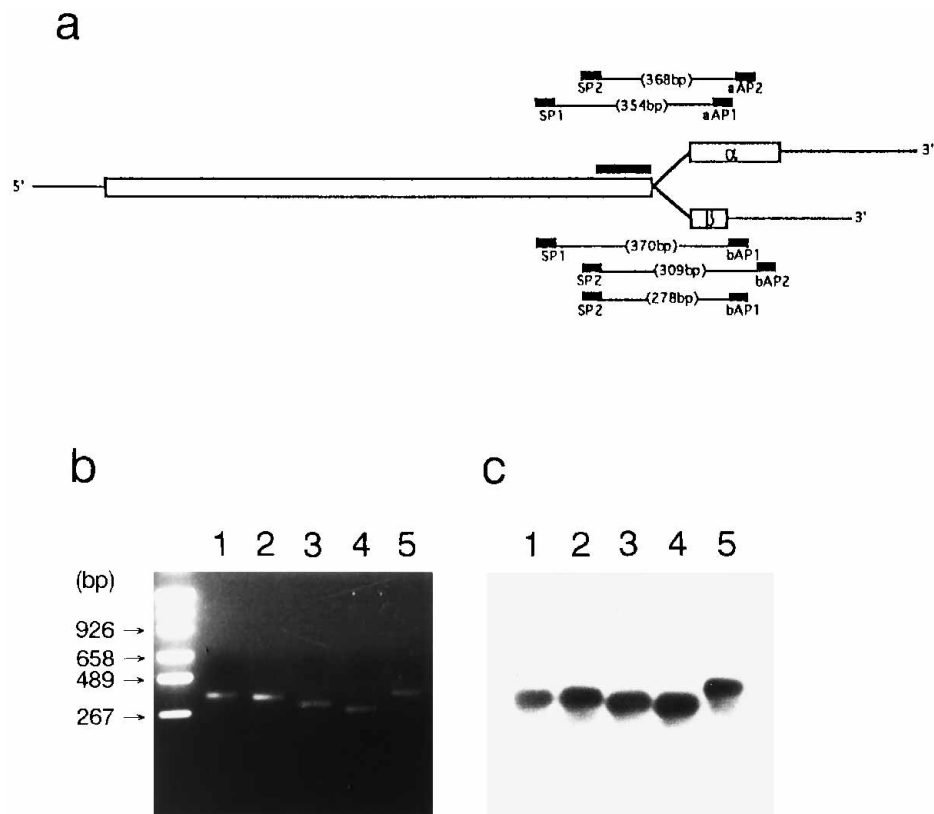
mouse bone marrow cells, RT-PCR analysis was carried out with template cDNAs constructed with mRNAs of the bone marrow cells. As shown in Fig. 3, the amplified products corresponding to each clone were detected. Thus, mRNAs encoding two isoforms of mouse NPY-Y1 receptor exist in bone marrow cells.

Isolation and Characterization of Mouse NPY-Y1 Receptor Genomic Clones—To determine whether or not Y1 α and Y1 β receptors are produced from a single gene, genomic structure of the mouse NPY-Y1 receptor was characterized. First, Southern blot analysis of the mouse genomic DNA indicated the presence of single *Bam*HI, *Pst*I, *Hind*III, *Kpn*I, and *Xho*I restriction fragments, when probed with the *Eco*RI-*Pst*I fragment which corresponds to a part of the common region to both Y1 α and Y1 β receptor cDNAs (Fig. 4a). This means that the Y1 α and Y1 β receptors originated from a single gene.

Upon screening of approximately 1.5 \times 10⁶ plaques of a mouse genomic library with the above probe (the *Eco*RI-*Pst*I fragment), we obtained 5 positive clones. One of the isolated clones (λ MY1 α 2), approximately 23 kbp, was analyzed in detail. Restriction mapping of this clone is shown in Fig. 4b. Partial sequence and Southern blot analysis revealed that this clone contained the entire Y1 α receptor open reading frame, including the 5'- and 3'-untranslated regions (Fig. 2A, a and b, and Fig. 4b), but not the Y1 β receptor-specific region (Fig. 2c). The mouse genomic library was thus rescreened using a DNA probe corresponding to the Y1 β receptor-specific cDNA sequences: at positions 980 to 1300 (320 bases) of the Y1 β receptor (Fig. 2A, c). Screening of approximately 1.5 \times 10⁶ plaques yielded 3 positive clones. One of the isolated clones (λ MY1 β 11), approximately 13 kbp, was extensively characterized. Restriction mapping, sequencing of the partial fragments that hybridized to the probes, and comparison with the both receptor cDNAs allowed for determination of the intron/exon structure of the NPY-Y1 receptor gene. As shown in Fig. 4b, the Y1 α receptor has at least two introns. The first intron, approximately 6.4 kbp long, was located at -147/-148 in the 5'-untranslated region of the Y1 α receptor. Since the major transcriptional start points of the mouse NPY-Y1 receptor were determined at positions -167, -182, -238, -247, and -263 in the 5'-untranslated region (33), the first exon consists of about 20~120 bp. The second intron (108 bp long) is located just after the proposed fifth transmembrane domain at position 697. A similar organization (locations of first and second introns) was observed in the human NPY-Y1 receptor gene (34). The Y1 β receptor is produced by RNA splicing of the third intron, more than 15 kbp long, located downstream from position 908 of the Y1 α receptor. Sequences around the putative junctions are shown in Fig. 4c. These sequences fit well with the proposed consensus sequences for RNA splicing. These results taken together indicate that the variation in C-terminal peptides could be produced by alternative splicing of mRNA from the single gene encoding the mouse NPY-Y1 receptor. The (TG)₂₉ and (GC)₁₀ sequences, alternating purine/pyrimidine repeat, and the potential left-handed Z-DNA-forming sequences (35-39), were found in tracts of up to 50 bp long in the Y1 α receptor 3'-untranslated region (Fig. 2b). Although the function of these sequences is unknown, the potential Z-DNA-forming sequences in the third intron might play a substantial role for splicing of the Y1 β receptor mRNA.

Characterization of the Cloned NPY-Y1 Receptors in CHO Cells—To examine ligand binding properties and signal transduction through each receptor, the coding region of each clone was subcloned in a mammalian expression vector and transfected into CHO-K1 cells (termed CHO-NPY α for the Y1 α receptor and CHO-NPY β for the Y1 β receptor). [¹²⁵I]NPY specif-

FIG. 3. RT-PCR analysis of the two isoforms of NPY-Y1 receptor mRNAs in bone marrow cells. *a*, schematic presentation of PCR primers, corresponding positions in each cDNA and expected sizes of PCR products. *b*, gel electrophoresis of amplified products. *Lanes 1–5* correspond to PCR analysis with a primer pair of SP2 and aAP2 in *lane 1*; SP1 and aAP1 in *lane 2*; SP2 and bAP2 in *lane 3*; SP2 and bAP1 in *lane 4*; SP1 and bAP1 in *lane 5*. *c*, Southern blot analysis of amplified products. After gel electrophoresis (*panel b*), DNA products were transferred onto nylon membrane and Southern blots made using an internal DNA fragment (prepared by PCR as described under "Experimental Procedures") as a probe (represented by *solid bar* in *a*).



whereas internalization, defined as bound [125 I]NPY to each cell not dissociable from the cells by acid (pH 3) treatment, was maximal (78.4% of total binding for the CHO-NPY α cells and 67.2% of total binding for the CHO-NPY β cells) after 30 min, and negligible internalization occurred at 4 °C (data not shown). These results suggest that NPY is internalized similarly after binding to each receptor.

To further determine the functional properties of each receptor, intracellular second messengers were investigated by measuring NPY-evoked intracellular Ca $^{2+}$ mobility, cAMP accumulation, and MAPK activation in the transfected cells. Upon addition of NPY (10 nM) to the CHO-NPY α cells, intracellular Ca $^{2+}$ was elevated (190 ± 28 nM, mean \pm S.D., $n = 5$, Fig. 6A, *a*) and forskolin (20 μ M)-stimulated cAMP accumulation was inhibited ($49.5 \pm 3.4\%$ inhibition, mean \pm S.D., $n = 4$). The intracellular Ca $^{2+}$ response was receptor-dependent, being elicited by NPY, PYY, and [Leu 31 ,Pro 34]NPY, but not by NPY-(13–36) (data not shown). Elevation in intracellular Ca $^{2+}$ by NPY, PYY, and [Leu 31 ,Pro 34]NPY was comparable to that observed with the human NPY-Y1 receptor (18). A transient generation of inositol trisphosphate evoked by NPY application, and NPY-induced intracellular Ca $^{2+}$ increase was markedly suppressed by pretreatment with U-73122 (5 μ M), a phospholipase C inhibitor (40, 41) (data not shown). When the CHO-NPY α cells were treated with PTX (50 ng/ml, 24 h), intracellular Ca $^{2+}$ increase and inhibition of cAMP production were abolished (the former shown in Fig. 6A, *b*, and the latter not shown). This suggests that the Y1 α receptor couples to PTX-sensitive G-protein(s), probably G $_i$ /G $_o$. Several G-protein coupled receptors are reported to activate the signaling pathway involving MAPK activation (42). NPY at 10 nM activated MAPK in the CHO-NPY α cells (peaked at 3–5 min) (Fig. 6B). Since the NPY-elicited MAPK activation was completely abolished by PTX treatment (50 ng/ml, 24 h), the NPY receptor-mediated MAPK activation via PTX-sensitive G-protein(s) (Fig. 6C). Recently, we found that the platelet-activating factor-

stimulated MAPK activation in guinea pig leukocytes is mediated by Ca $^{2+}$ -dependent and Ca $^{2+}$ -independent/wortmannin-sensitive pathways (43). To determine the pathways of MAPK activation by NPY in the CHO-NPY α cells, the effect of wortmannin was examined. Pretreatment of the cells with wortmannin inhibited MAPK activation in response to NPY, with a half-maximal inhibition observed at an inhibitor dose of 50–100 nM (data not shown). As shown in Fig. 6C, 500 nM wortmannin completely inhibited NPY (10 nM)-elicited MAPK activation. Treatment with wortmannin, at the concentration used in this experiments, did not affect intracellular Ca $^{2+}$ mobilization (Fig. 6A, *c*). As shown in Fig. 6, *A* (*panel d*) and *C*, MAPK activity was not inhibited by the treatment of 20 μ M BAPTA/AM (intracellular Ca $^{2+}$ chelator), while it completely abolished the NPY-induced intracellular Ca $^{2+}$ increase. These observations suggest that the Y1 α receptor mediates MAPK activation via a Ca $^{2+}$ -independent but a wortmannin-sensitive pathway. Wortmannin-sensitive signaling molecules such as phosphoinositide 3-kinase may lie between MAPK activation and PTX-sensitive G-protein(s).

In contrast, NPY-evoked cell responses such as intracellular Ca $^{2+}$ increase, inhibition of cAMP production, or MAPK activation was not detected in the CHO-NPY β cells, although specific binding of [125 I]NPY to the Y1 β receptor was observed (Fig. 7). These results suggest that the cytoplasmic tail of the Y1 α receptor contributes to G-protein(s) activation.

Expression of the Y1 α and Y1 β Receptor in Mouse Tissues—The relative abundance of mRNAs for the two receptors in various tissues was investigated by Northern blot analysis using differential DNA regions of the Y1 α and Y1 β receptor as probes. Fig. 8*a* indicates that the NPY-Y1 α receptor mRNA, approximately 4.0 kb, was highly expressed in the brain, heart, kidney, spleen, skeletal muscle, and lung, whereas the mRNA of Y1 β receptors was not detected in these tissues. Surprisingly, the Y1 β receptor mRNA, approximately 4.5 kb, was highly expressed in the 7- and 11-day embryo (Fig. 8*b*). Since

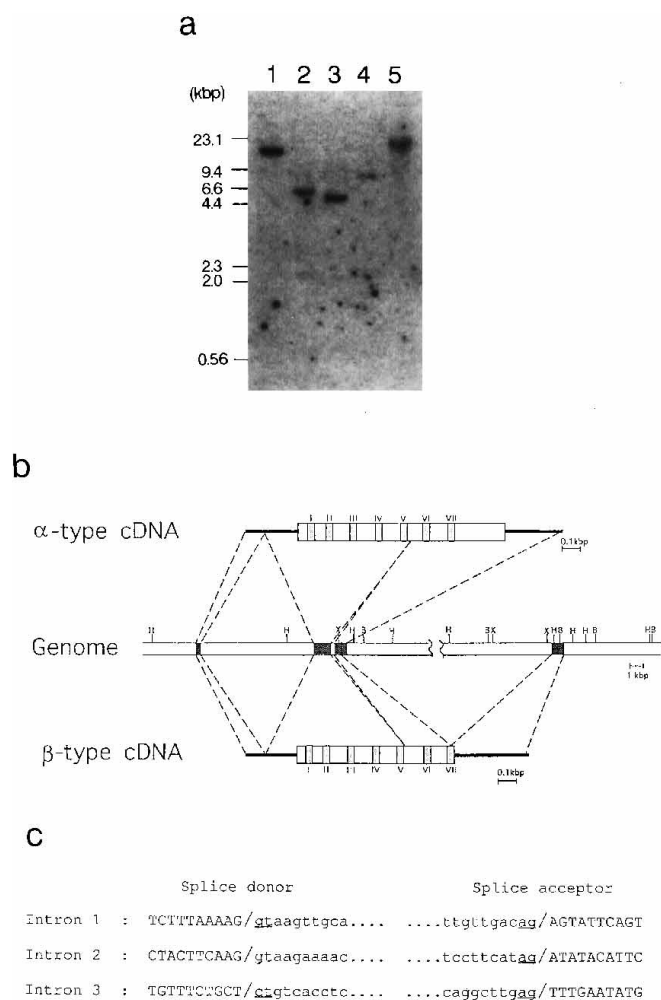


FIG. 4. Genomic analysis of the mouse NPY-Y1 receptor gene. *a*, Southern blot analysis of mouse genomic DNA. Mouse genomic DNA was digested with *Bam*HI (lane 1), *Pst*I (lane 2), *Hind*III (lane 3), *Kpn*I (lane 4), or *Xho*I (lane 5). Hybridization analysis was carried out as described under "Experimental Procedures" with the *Eco*RI-*Pst*I fragment of NPY-Y1 α receptor as a probe. *b*, organization of the mouse NPY-Y1 receptor gene and its relationship to both mRNAs. *c*, boundary sequences of the exon/intron. Exon and intron are represented by *capital* and *lower case* letters, respectively.

each isolated clone is approximately 2 kbp long, the full-length transcript probably contains a longer 3'-untranslated sequence. The expression of the Y1 β receptor was further investigated by RT-PCR in various hematopoietic cell lines. The expression of the Y1 β receptor mRNA was detected in ST2 (bone marrow stroma cells), M1 (myeloblasts), L5178Y (lymphoma cells), WEHI-3 (myelomonocytes), and embryonic stem cells (E14.1), but not in P815 cells (mastocytoma cells) (data not shown). These results are interpreted to mean that mRNA of the Y1 β receptor is generated by tissue- and development-specific alternative splicing. The Y1 β receptor is specifically expressed in bone marrow cells and at the early stage of embryonic development and might play a substantial role in these tissues and cells.

DISCUSSION

We report here the isolation of two isoforms of the NPY-Y1 receptor cDNA designated Y1 α , a mouse homologue of the human NPY-Y1 receptor, and Y1 β , a truncated form of Y1 α (Fig. 2). RT-PCR, Southern blots, and genomic DNA analyses show that these two isoforms are generated from a single gene by alternative RNA splicing.

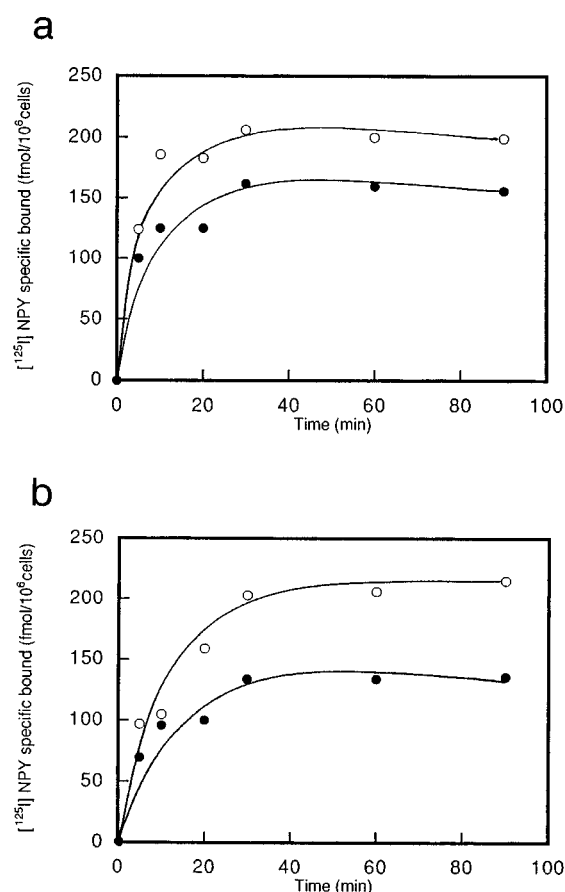


FIG. 5. Time course of [¹²⁵I]NPY binding to the CHO-NPY α cells (a) or the CHO-NPY β cells (b). The cell monolayers were washed and incubated with 1 nM [¹²⁵I]NPY in the absence or presence of 1 μ M NPY (nonspecific binding) for various times at 37 °C. Duplicate cultures were then analyzed for total binding (*open circles*) or internalized ligand (*closed circles*) as described under "Experimental Procedures." In the latter case, cells were treated for an additional 3 min at 4 °C with 200 mM Gly-HCl, pH 3, 200 mM NaCl, and then cell associated ligand was determined.

Despite the lack of seventh transmembrane domain and cytoplasmic tail, the Y1 β receptor shows ligand binding specificities identical to those of the Y1 α receptor. Walker *et al.* (44) reported that ionic interactions between the positively charged amino acids of NPY and negatively charged residues of the human NPY-Y1 receptor are involved in ligand-receptor interaction. By means of site-directed mutagenesis, substitution of acidic residues (aspartic acids and glutamic acids) present in the three extracellular loops of the human NPY-Y1 receptor yielded proteins unable to bind [¹²⁵I]NPY. In contrast, deletion of the 51 residues in the C-terminal tail of the receptor resulted in a loss of 9 negatively charged residues but had no significant effect on affinity of the receptor for NPY. They suggested that extracellular loops of the NPY-Y1 receptor are involved in NPY binding, through ionic interaction. Since the Y1 β receptor has a similar affinity to NPY, the extracellular loops rather than the seventh transmembrane domain plays a critical role for the ligand binding. Hunyady *et al.* (30) showed that PTX-sensitive G_i-proteins did not appear to play a role in endocytosis of angiotensin II (AT_{1a}) receptor, since the receptor showed normal internalization kinetics in PTX-treated cells. They demonstrated that endocytosis of the AT_{1a} receptor was independent of agonist-induced signal transduction, and receptor internalization and activation of phospholipase C led to different structural requirements of the receptor. Such independence of sequestration and the signal transduction was observed with the

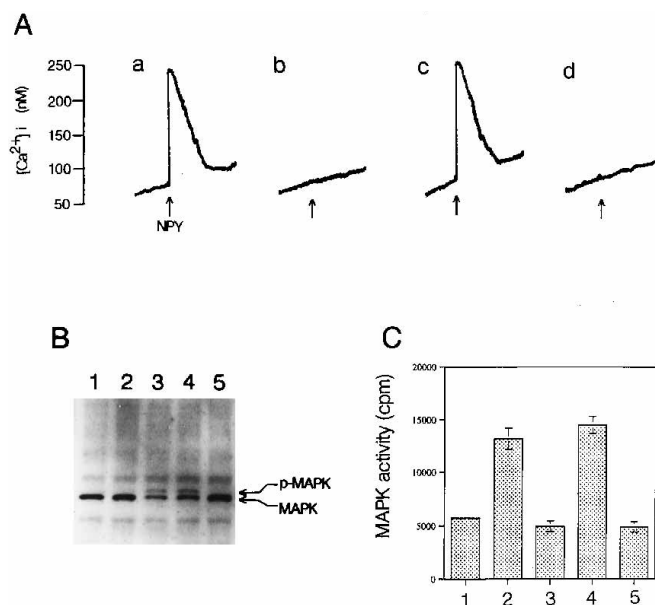


FIG. 6. Effect of PTX, wortmannin, and BAPTA/AM on intracellular Ca^{2+} increase and MAPK activation in the CHO-NPY α cells. For experiments using PTX, PTX (50 ng/ml) was added to the medium 24 h before the assay. Wortmannin treatment and BAPTA/AM loading were carried out as described (43). **A**, representative tracing of intracellular Ca^{2+} mobilization by NPY (10 nM) in the Fura-2/AM loaded-CHO-NPY α cells. *Trace a*, control cells; *trace b*, PTX-treated cells; *trace c*, wortmannin-treated cells; *trace d*, BAPTA/AM-loaded cells. **B**, activation of MAPK by NPY. Electrophoretic mobility shift of p42 MAPK detected by immunoblot analysis. The cells were serum-starved for 20 h before stimulation. After prewarming for 10 min at 37 °C, the cells were challenged with 10 nM NPY, and cell lysates were prepared at the indicated times. *Lane 1*, 0 min; *lane 2*, 0.5 min; *lane 3*, 3 min; *lane 4*, 5 min; and *lane 5*, 30 min. Detection of MAPK was carried out as described under "Experimental Procedures." The positions of MAPK and its phosphorylated form (p-MAPK) are shown by arrows. **C**, effect of PTX, wortmannin, and BAPTA/AM on NPY-evoked MAPK activation. *Lane 1*, non-stimulated cells; *lane 2*, cells stimulated with 10 nM NPY for 3 min; *lanes 3–5*, NPY (10 nM)-stimulated cells pretreated with PTX (*lane 3*), or with 20 μM BAPTA/AM at 25 °C for 30 min (*lane 4*), or with 500 nM wortmannin at 37 °C for 10 min (*lane 5*). Preincubation conditions are the same as in **A**. The columns and vertical bars show the mean and S.D., respectively.

neurotensin receptor (45). We propose that the β -type receptor has a functional role for NPY internalization, since sequestration of [^{125}I]NPY was observed in the β -type receptor expressed CHO cells after ligand-receptor binding. Intracellular sequestration of the NPY-receptor complex might provide a specific message to the cells.

Although the two isoforms show identical ligand affinities, there are differences in cell signaling properties. The Y1 α receptor elicits a PTX-sensitive intracellular Ca^{2+} increase, inhibition of cAMP accumulation, and MAPK activation, whereas the Y1 β receptor evokes no such responses. Irie *et al.* (46) reported that the C-terminal tail of prostaglandin-E EP $_3$ subtype receptor was essential for activation of G-protein. They showed that the C-terminal tail-truncated prostaglandin-E EP $_3$ subtype receptor retained the potential to form the agonist/receptor/G $_i$ -protein ternary complex but failed to activate G $_i$ -protein. Hence, we speculate that the C-terminal tail of NPY-Y1 receptor contributes to the activation of G-protein(s). Production of multiple isoforms by alternative splicing has been noted in the rhodopsin-type receptor family, such as D $_2$ dopaminergic receptor (47–49), prostaglandin-E EP $_3$ subtype receptor (50, 51), thromboxane A $_2$ receptor (52), metabotropic glutamate receptor (53), neurokinin-1 receptor (54), MCP-1 receptor (55), and somatostatin receptor (56). All these receptor

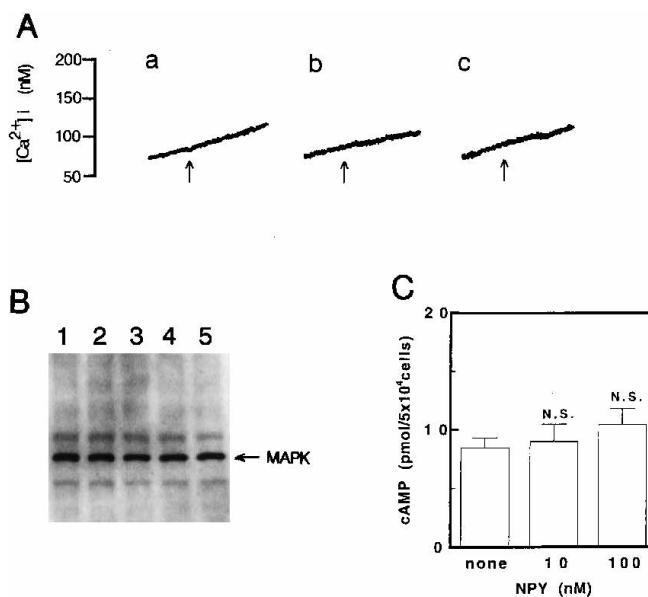


FIG. 7. Failure of Ca^{2+} mobilization, inhibition of cAMP production and electrophoretic mobility shift of p42 MAPK by NPY application to the CHO-NPY β cells. **A**, representative tracing of intracellular Ca^{2+} mobilization by NPY (10 nM) (*Trace a*), PYY (10 nM) (*Trace b*), or [Leu^{31} , Pro^{34}]NPY (10 nM) (*Trace c*) in the Fura-2/AM loaded-CHO-NPY β cells. **B**, activation of MAPK by NPY. Electrophoretic mobility shift of p42 MAPK detected by immunoblot analysis. Experimental conditions were the same as for Fig. 6B. *Lane 1*, 0 min; *lane 2*, 0.5 min; *lane 3*, 3 min; *lane 4*, 5 min; and *lane 5*, 30 min. **C**, analysis of adenylate cyclase inhibition. The CHO-NPY β cells were incubated at 37 °C for 10 min with 20 μM forskolin in the presence of the indicated concentrations of NPY, and then the intracellular cAMP concentration was determined as described under "Experimental Procedures." N.S., not significant versus control ($p > 0.05$, Student's *t* test).

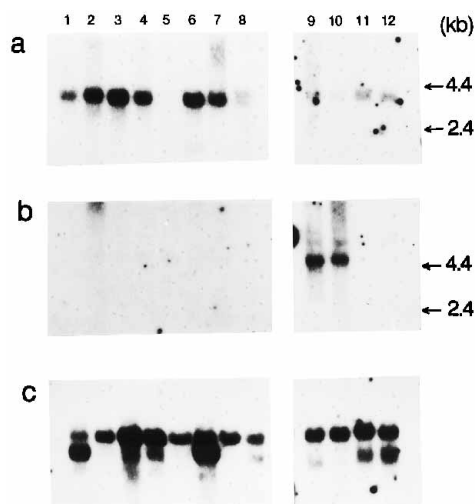


FIG. 8. Northern blot analysis of the NPY-Y1 α and NPY-Y1 β receptor mRNAs. Expression of the NPY-Y1 α (**a**) and NPY-Y1 β receptor mRNA (**b**) in various mouse tissues and embryo. Each lane contains 2 μg of poly(A) $^+$ RNA. In **c**, β -actin probe was used as an internal control. *Lane 1*, heart; *lane 2*, brain; *lane 3*, spleen; *lane 4*, lung; *lane 5*, liver; *lane 6*, skeletal muscle; *lane 7*, kidney; *lane 8*, testis; *lane 9*, embryo (7 days); *lane 10*, embryo (11 days); *lane 11*, embryo (15 days); and *lane 12*, embryo (17 days).

isoforms differ only in the third cytoplasmic loop or C-terminal tail, and these isoforms show no properties of the Y1 β receptor: truncated structure and defect of cell signaling.

In CHO-NPY α cells, NPY-induced MAPK activation via PTX-sensitive G-protein was seen to be mediated by Ca^{2+} -independent/wortmannin-sensitive pathway. Several G-pro-

tein coupled receptors, including the α_2 -adrenergic receptor (57), lysophosphatidic acid receptor (58–60), M2 muscarinic acetylcholine receptor (61), C5a receptor (62), somatostatin receptor (32, 63), and platelet-activating factor receptor (64), have been shown to stimulate MAPK activation in various cell types. The signaling pathways by which these receptors activate MAPK are poorly understood, but there are several pieces of evidence for both Ras-dependent (57–62) and Ras-independent (64–66) activation of MAPK. Koch *et al.* (67) reported that MAPK activation via PTX-sensitive pathway is mediated by the $\beta\gamma$ subunit of G_i-protein and occurs as a result of ras activation (67). Activated Ras can then act as a molecular switch causing Raf-1 activation, and subsequently leading to stimulation of MAPK cascade. Here, we obtained evidence that wortmannin-sensitive signaling molecules such as phosphoinositide 3-kinase may lie between PTX-sensitive G-protein and MAPK activation. Further experiments are on going to elucidate the target molecules of wortmannin.

Northern blots and RT-PCR analyses showed tissue- and development-specific expression of two isoforms of receptor mRNAs. The Y1 α receptor was specifically expressed in the brain, heart, kidney, spleen, skeletal muscle, lung, bone marrow cells, and several hematopoietic cell lines. In contrast, the Y1 β receptor mRNA was detected in embryo (7 and 11 days), bone marrow cells, and several hematopoietic cell lines. The functional role of the Y1 β receptor in embryonic development and hematopoietic system can be further examined by establishing knock-out mice.

NPY mRNA and NPY-like immunoreactivity is found not only in autonomic nervous systems and in the adrenal medulla but also in megakaryocytes/platelets and possibly mononuclear blood cells in rats. In humans, NPY is present in lymphocytes and monocytes (68). A high level of NPY was found in bone marrow of autoimmune mice with B-cell lymphoproliferative disorders and in children with B-cell precursor leukemia. These findings suggest a role for NPY during normal B-cell development and/or pathologic disorders of B-line cells. The existence of the NPY-Y1 type receptor in rat splenic lymphocytes was deduced from PCR evidence and from ligand-binding analyses (22). The presence of the Y1 α and Y1 β receptors in bone marrow cells suggests a role for these receptors in the hematopoietic system.

We have described here the cloning and the elucidation of genomic structure, signal transduction, and tissue distribution of two isoforms of the mouse NPY-Y1 receptor with alternatively spliced C-terminal regions. NPY-Y1 receptors form a novel repertoire of G-protein-coupled receptors that are diversified not only by mediating distinct cell signaling but also by differential expression patterns of individual receptors in mouse tissues. Additional studies on functions and regulation of diverse members of NPY receptors are underway to examine complex physiological responses of NPY in the nervous and hematopoietic systems.

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