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Neuropeptide Y Receptor Gene y6: Multiple Deaths or Resurrections?

Paula Starbäck, Amanda Wraith, Henrik Eriksson, and Dan Larhammar[1]
Department of Neuroscience, Unit of Pharmacology, Box 593, Uppsala University, Uppsala SE-75124, Sweden

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The neuropeptide Y family of G-protein-coupled receptors consists of five cloned members in mammals. Four genes give rise to functional receptors in all mammals investigated. The y6 gene is a pseudogene in human and pig and is absent in rat, but generates a functional receptor in rabbit and mouse and probably in the collared peccary (*Pecari tajacu*), a distant relative of the pig family. We report here that the guinea pig y6 gene has a highly distorted nucleotide sequence with multiple frame-shift mutations. One evolutionary scenario may suggest that y6 was inactivated before the divergence of the mammalian orders and subsequently resurrected in some lineages. However, the pseudogene mutations seem to be distinct in human, pig, and guinea pig, arguing for separate inactivation events. In either case, the y6 gene has a quite unusual evolutionary history with multiple independent deaths or resurrections.

Key Words: **evolution; pseudogene; G-protein-coupled receptor; neuropeptide Y; peptide YY; pancreatic polypeptide; guinea pig; peccary.**

Neuropeptide Y (NPY) is an abundant neuropeptide that mediates a wide range of biological actions including stimulation of appetite and vasoconstriction. NPY consists of 36 amino acids and has remained highly conserved throughout vertebrate evolution (1). In mammals NPY has two related peptides called PYY (peptide YY) and PP (pancreatic polypeptide) that are expressed in the gastro-intestinal tract. The NPY receptors belong to the family of rhodopsin-like G-protein-coupled receptors. Several receptor subtypes have been classified on the basis of their pharmacology, and five distinct receptors have been cloned and characterized in mammals: Y1, Y2, Y4, Y5, and y6; for review see (2). Due to absence of a physiological correlate of the y6 receptor, the International Union of Pharmacology recommends that the y6 receptor is written by a lower-case y (2).

The y6 receptor gene was first cloned in mouse (3, 4) and rabbit (5), then in human (5, 6) along with three other primates (5). The y6 gene has also recently been cloned in pig (7) and collared peccary (*Pecari tajacu*) which belongs to the sister group of pigs (8). The subtypes Y1, Y4 and y6 form a subfamily which share approximately 50% overall amino acid identity. This subfamily shares approximately 30% identity with the Y2 and Y5 receptors (7).

The y6 receptor seems to be functional in mouse and rabbit, but it has a frame-shift mutation (a single-base deletion) in human and the other primates studied making the receptor non-functional. The pig y6 gene is also a pseudogene, but due to two 2-bp deletions in different positions than human (7). The collared peccary y6 receptor gene does not have any of these mutations, indicating that the receptor is most likely functional. In rat the y6 receptor gene seems to be absent based on both Northern and Southern blot analysis (9).

The y6 receptor shows only 79–83% amino acid sequence identity between orders of mammals and displays a widely different pharmacological profile in mouse and rabbit where the receptor is functional. In mouse the pharmacological characterization of ^{125}I-PP binding shows the following rank order of potency for y6: PP $\gg$ PYY $\geq$ NPY, which is similar to that of the mouse Y4 receptor (3). However, studies of mouse y6 in an other laboratory (4) resulted in a quite different pharmacological profile, NPY = PYY $\geq$ $NPY_{2\text{-}36}$ $\geq$ [Leu^{31},Pro^{34}]NPY = $NPY_{13\text{-}36}$ > PP, resembling an atypical Y1 receptor. The rabbit y6 receptor was reported to have a distinct rank order: PYY $\geq$ $NPY_{2\text{-}36}$ $\geq$ $NPY_{13\text{-}36}$ > NPY > [Leu^{31},Pro^{34}]NPY $\gg$ PP (5). The higher affinity of $NPY_{13\text{-}36}$ than [Leu^{31},Pro^{34}]NPY for rabbit y6 suggested a more Y2-like profile, despite the higher sequence identity with Y1 than with Y2. More recent studies (10) have shown that the mouse y6 receptor pharmacology is even more distinct from that of the other known receptor subtypes: NPY = PYY = $NPY_{2\text{-}36}$ > [Leu^{31},Pro^{34}]NPY > $NPY_{13\text{-}36}$. This suggests

[1] To whom correspondence should be addressed. Fax: 46-18-511540. E-mail: Dan.Larhammar@neuro.uu.se.

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that y6 is not involved in appetite regulation in mouse. The receptor was found to couple by inhibiting cAMP accumulation like the other NPY receptors (5, 10).

We have recently cloned and functionally expressed the four receptors Y1, Y2, Y4, and Y5 in guinea pig (11–14) and also cloned the five known mammalian NPY receptor genes including y6 in domestic pig (7). To trace the extraordinary evolutionary history and possible functional role of the y6 gene in mammals, we present in this paper the cloning of the y6 receptor gene in the guinea pig and discuss it in relation to the previously cloned mammalian y6 genes as well as our recently cloned y6 gene in the collared peccary, a distant relative of the pig.

MATERIALS AND METHODS

Approximately 600,000 clones from a library in the vector EMBL3 (Clontech) were screened with a probe containing the coding region of the mouse Y5 receptor gene. The clone was kindly provided by Dwayne Johnson (Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN). Hybridization was done at 50° in 25% formamide, 6× SSC, 10% dextran sulfate, 5× Denhardt's solution and 0.1% SDS. Filters were washed twice at room temperature and twice for 30 min at 65° in 0.5× SSC/0.1% SDS. Four individual phage clones hybridized to the probe and the two most strongly hybridizing clones were selected for further characterization.

The two clones were enzymatically digested with HindIII (Amersham Life Science), which gave two hybridizing fragments, large enough to contain the receptor gene. The fragments were ligated into a pUC18-HindIII vector (Amersham Pharmacia Biotech) and sequenced with vector primers. Based on the sequence produced from the primers, guinea pig-specific sequencing primers were designed to sequence the rest of the fragment. Sequence determinations were done with the ABI PRISM Big Dye terminator cycle sequencing ready reaction kit (Perkin Elmer). The sequence reactions were run on an ABI PRISM 310 Genetic Analyzer (Perkin Elmer).

The phylogenetic tree was constructed with the neighbor-joining method (15) of the DNASTAR Megalign software. The full coding regions were manually aligned and the pig, human and guinea pig sequences were restored with respect to the mouse and rabbit sequences. Pairwise sequence analysis was performed using Phylogenetic Analysis by Maximum Likelihood (PAML) (Yang, Z., 2000, Version 3.0, University College London, London, England). Specifically, a pairwise analysis using the codon-based substitution model was implemented (16) where gaps are removed (thus insertions and deletions are not considered) and multiple substitutions corrected for. Parameters included the transition/transversion rate ratio, base frequencies at the three codon positions (F3 × 4) and equal distances among amino acids.

RESULTS

The guinea pig y6 receptor gene was isolated by screening a genomic library with a mouse y6 probe. Two hybridizing phage clones were identified, one of which was subcloned into a plasmid vector and sequenced. Alignment with the mouse and rabbit y6 genes showed that the coding region is intronless like in other mammals and has a length of 1120 base pairs. The guinea pig y6 nucleotide sequence displays 75–79% identity to the five known full-length receptor sequences (mouse, rabbit, human, pig, and peccary). However, the guinea pig y6 gene has numerous disruptions of the open reading frame. There are no less than eleven frame-shift mutations. In the alignment shown in Fig. 1, the frame-shift mutations have been compensated to regenerate the continuous open reading frame corresponding to mouse and rabbit y6. Also the human and pig sequences have been adjusted to allow amino acid sequence comparison. After compensation, the guinea pig y6 sequence has three premature termination codons. In addition, there is one in-frame insertion of 3 codons and two in-frame deletions of 1 and 5 codons, respectively. A few dramatic amino acid replacements can also be identified, most conspicuously two prolines in transmembrane region (TM) 2 and one arginine each in TM5 and TM6. Thus, the guinea pig y6 gene cannot encode a functional receptor protein.

At the amino acid level, the conceptual guinea pig y6 protein displays 65–73% identity to the other mammalian sequences (Table 1). These are 79–83% identical to each other, except that pig and peccary which belong to the same order of mammals (Artiodactyla) display 90% identity. In the transmembrane regions, the guinea pig sequence is 73–76% identical to the other sequences, which are 82–89% identical among each other (not shown). The amino acid alignment in Fig. 1 was used to calculate the distance tree shown in Fig. 2 with the neighbor-joining method. The tree confirms that the guinea pig sequence is the most divergent of the known mammalian sequences.

The nonsynonymous and synonymous rates were calculated using the codon-based maximum likelihood method of (16) using a pairwise analysis. The nonsynonymous/synonymous rate ratio is an indicator of selective pressures or constraints. Thus, where the rate ratio is very low (about 0) the gene is under purifying selection, while at values greater than 1 there is positive selection for amino acid replacements. For values around 1 there is neutral or very relaxed selection. For the y6 comparisons (Table 2), all nonsynonymous rates were lower than synonymous for all the y6 pairwise comparisons (ratios in the range 0.178–0.513), indicating that the sequences are or have been subjected to *some* purifying selection. The rabbit and mouse sequences consistently give lower ratios than the human, pig and guinea pig sequences, suggesting that the former are still under stronger purifying selection. The lowest ratio is for the rabbit-mouse comparison, the two genes that have been shown to give rise to functional receptor proteins. The two highest ratios in the table are for the comparisons between guinea pig with human and pig, all three of which are pseudogenes, in agreement with lower or lost purifying selection in these lineages.

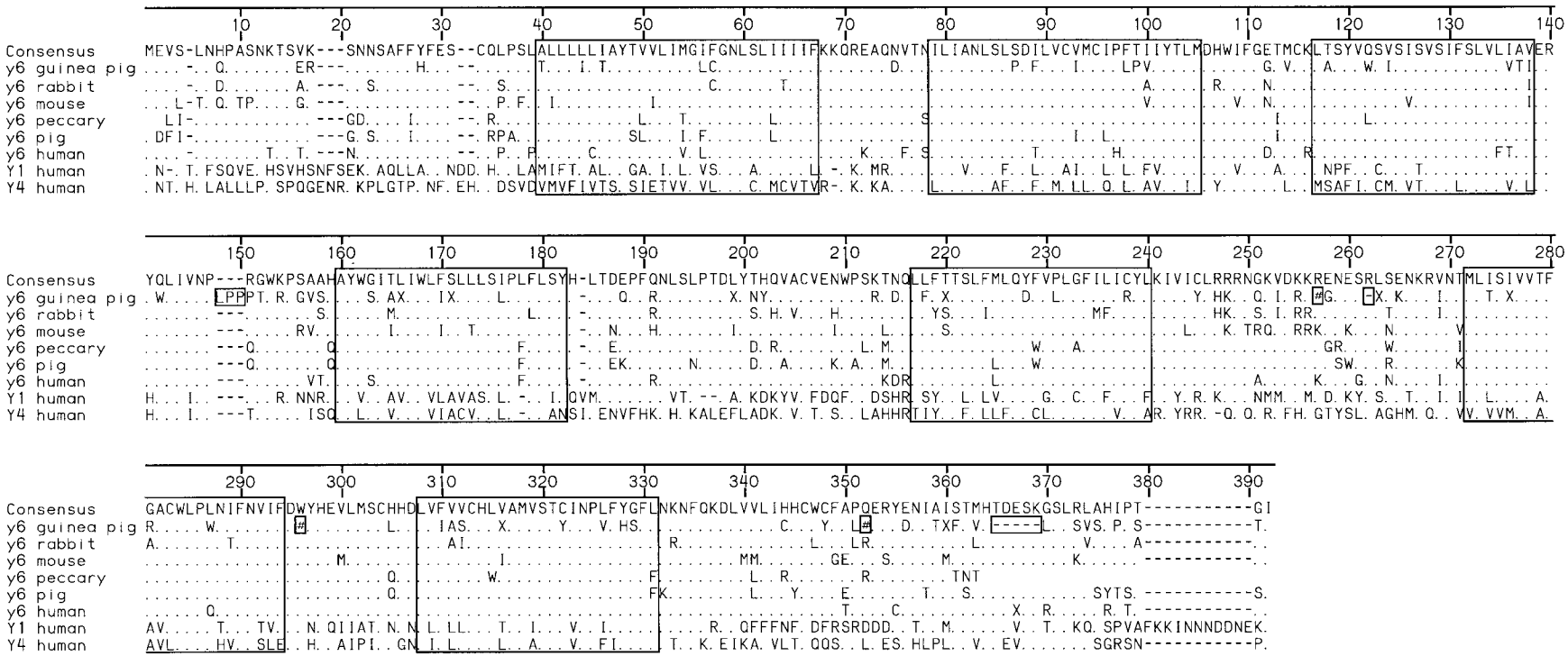

FIG. 1. Alignment of NPY receptor protein sequences for y6, human Y1, and human Y4. The y6 pseudogene in guinea pig presented here as well as the human and pig y6 pseudogene sequences have been adjusted to maintain the original open reading frame in order to facilitate alignment of amino acid sequences. In the guinea pig sequence, the three termination codons in this adjusted reading frame have been marked with boxes (alignment positions 257, 296, and 352). One in-frame insertion (148) and two in-frame deletions (262, 365) in the guinea pig sequence have also been marked with boxes. Large boxes enclose transmembrane regions.

TABLE 1

Percentage Amino Acid Sequence Identity of NPY Receptors Based upon the Alignment in Fig. 1

	y6 rabbit	y6 mouse	y6 peccary	y6 pig	y6 human	Y1 human	Y4 human
y6 guinea pig	73	70	69	65	71	43	38
y6 rabbit	—	83	81	79	82	50	44
y6 mouse		—	79	79	82	50	44
y6 peccary			—	90	83	51	41
y6 pig				—	82	49	44
y6 human					—	50	42
Y1 human						—	43
Y4 human							—

Note. The guinea pig, human and pig sequences have been adjusted to compensate for frame-shift mutations to allow alignment of the original open reading frame.

DISCUSSION

The guinea pig y6 receptor gene is in a clear state of molecular decay with multiple inactivating mutations. It displays numerous frame-shift mutations and three premature stop codons as well as several drastic amino acid replacements in some of the transmembrane regions. Thus, the gene cannot give rise to a functional full-length receptor protein. The high nucleotide sequence identity of 75–79% to y6 in other mammals confirms that this gene is an orthologue of y6. This is also supported by Southern hybridization to guinea pig genomic DNA with a probe from mouse (not shown). After adjustment of the reading frame, the hypothetical guinea pig y6 protein displays 65–73% overall identity to the other mammals which are 79–83% identical to each other (Table 1).

The y6 receptor gene displays a quite odd behaviour during mammalian evolution. It is a pseudogene in species from at least three mammalian orders, namely human, pig and guinea pig, all of which have frame-shift mutations. In rabbit and mouse, the gene has a continuous coding sequence giving rise to a functional gene, although the receptor protein displays quite different pharmacological properties in these two species and the physiological role of the receptor has yet to be established. The gene appears to maintain an open reading frame in the collared peccary despite its close distance of only approximately 30 Myr to the pig (8). The rat gene has gone undetected despite the availability of a mouse probe, suggesting that y6 has been lost from the rat genome (9). In this context it is important to note that the guinea pig is no longer regarded a rodent as it displays quite divergent sequences both for mitochondrial (17, 18) and nuclear genes (19). The guinea pig should at least be considered a very early branch in the rodent tree, possibly even a distinct order.

The most obvious inactivating mutations, the frame-shifts, are distinct in human, pig and guinea pig. In the primates studied, the y6 gene is inactivated by a frame-shift mutation in TM6 leading to a stop codon seven amino acids after TM6 (5, 6). Pig y6 has the first frame-shift somewhat earlier in the region encoding the second extracellular loop (7). The guinea pig y6 sequence presented here has its first frame-shift mutation already in the second intracellular loop (between TM3 and TM4). The fact that none of the frame-shifts is shared between primates, pig and guinea pig, together with the loss of y6 in rat, suggest that the y6 gene has been inactivated independently in these four lineages of mammals.

Independent inactivation is further supported in the artiodactyl lineage as the peccary lacks the frame-shift mutations seen in the pig. The reasonably low

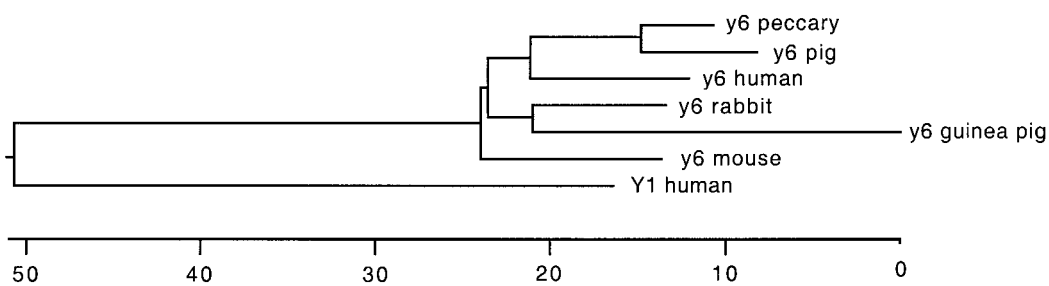

FIG. 2. Distance tree of NPY receptor y6 sequences and the human Y1 sequence. The tree was constructed with the neighbor-joining method of the DNASTAR Megalign software. The human Y4 sequence was used as outgroup. The scale shows percentage divergence.

TABLE 2

Pairwise Estimates of the Nonsynonymous/Synonmymous Ratio Using the Maximum Likelihood Method of PAML (Phylogenetic Analysis by Maximum Likelihood)

	Pig	Rabbit	Guinea pig	Mouse
Human	0.360 ± 0.056	0.316 ± 0.050	0.441 ± 0.031	0.220 ± 0.032
Pig		0.298 ± 0.057	0.513 ± 0.031	0.219 ± 0.029
Rabbit			0.347 ± 0.062	0.178 ± 0.000
Guinea pig				0.358 ± 0.016
Mouse				

Note. Values are ratios ± standard errors.

nonsynonymous/synonymous rates suggest that the y6 genes in human and pig have only recently undergone a loss of function and thereby a loss of purifying selection. Possibly these genes may have found an alternate function to the "typical NPY receptor," for example as a truncated receptor that may form a dimer with a full length receptor subtype enabling second messenger signaling in pig and human.

Alternatively, the y6 gene may have been inactivated before the mammalian orders diverged from each other 80–100 Myr ago. This may have resulted from a mutation in the promoter region or other regulatory regions. Subsequently, secondary frame-shift mutations may have followed independently in primates, pig and guinea pig. Other lineages could have regained expression, i.e., rabbit, mouse, and collared peccary (albeit functional studies have not been performed in the peccary). This scenario would presumably involve a fairly long period of altered expression or non-expression, particularly in the artiodactyl lineage, which would seem unlikely.

We favour the alternative hypothesis with four independent inactivation events in primates, guinea pig, pig, and rat. This is supported by the alignment in Fig. 1 and the distance tree in Fig. 2 which show that the guinea pig y6 gene has accumulated many more inactivating mutations than either human or pig, suggesting that the guinea pig gene was inactivated much earlier than the other two. The high ratio of nonsynonymous/synonymous substitutions (Table 2) also suggests that the guinea pig sequence has a longer history as a pseudogene. The time point for inactivation of primate y6 is difficult to estimate, but the pig gene was presumably inactivated less than 30 Myr ago after the pig and peccary lineages diverged from each other. The rat gene was lost after it diverged from mouse probably less than 12–15 Myr ago.

To our knowledge, multiple independent inactivations of the same gene in different lineages is uncommon. The best known case is probably the deficiency of L-gulono-gamma-lactone oxidase, the enzyme catalyzing the terminal step in L-ascorbic acid (vitamin C) biosynthesis, which was inactivated independently in hominoids and the lineage leading to the guinea pig (20, 21). Another possible example is the enzyme urate oxidase which may have been independently silenced in gibbons and the lineage leading to the other hominoids (22). However, the latter case could also be due to a still unidentified inactivating mutation in the promoter or in regulatory elements.

Even if the y6 gene was silenced independently in four lineages of mammals, this would only seem to be possible if the gene was already of rather modest importance in the mammalian ancestor. Its present physiological role in mouse and rabbit remains to be determined. Nevertheless, y6 most likely arose at a very early stage in vertebrate evolution prior to the origin of gnathostomes (jawed vertebrates), presumably as a result of chromosome duplications (or genome doublings) as indicated by its presence in a large chromosomal segment with extensive similarities to the regions harbouring the Y1 and Y4 genes (7). Thus, the y6 gene has been present in the lineage leading to mammals since before 400 Myr ago. We are presently attempting to clone the y6 gene in non-placentals including a marsupial and a bird (chicken) as well as even more distantly related species to further investigate the role y6 plays in these lineages.

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