Cloning of the Parathyroid Hormone Receptor in Japanese Quail

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Abstract: Parathyroid hormone/parathyroid hormone-related protein receptor (PTH/PTHR type 1 receptor; commonly known as PTH1R) is a family BG protein-coupled receptor that is mainly expressed in the bone and kidney of humans where it regulates skeletal development, bone turnover, and mineral ion homeostasis. The medullary bone of female birds is formed and resorbed in the presence of estrogen and androgen, while the administration of human estrogen to male Japanese quail results in artificial formation of the medullary bone in the bone marrow cavity. Therefore, male Japanese quail medullary bone is a good model for the study of bone homeostasis, and Japanese quail PTHR may play an important role in bone metabolism. However, the expression and function of PTHR in Japanese quail are unknown, and PTHR cloning is incomplete. Therefore, in this study, we attempted to clone the PTHR cDNA of Japanese quail.

Key words: PTHR, Cloning, Japanese quail

Introduction

Parathyroid hormone/parathyroid hormone-related protein receptor (PTH/PTHR type 1 receptor; commonly known as PTH1R) is a family BG protein-coupled receptor that is expressed primarily in the bone, kidney, and cartilage, but also in tissues such as the vasculature and certain developing organs in humans and other species like mice1. PTH1R transmits stimuli provided by two different ligands: PTH, which is secreted from the parathyroid glands, and PTHrP, which is secreted from a diverse range of tissues2. The activation of PTH1R in osteoblastic cells and chondrocytes modulates the rates of proliferation and apoptosis, and leads to the production of a variety of signaling factors involved in bone and cartilage metabolism3,4. In female birds, the medullary bone is formed and resorbed in the presence of circulating estrogen and androgen5,6. Medullary bone functions as a calcium reservoir for eggshell formation7,8, and undergoes resorption and formation in synchrony with the egg position in the oviduct during egg-laying cycles9,10. Male Japanese quail can also form medullary bone in the bone marrow cavity, albeit artificially, following the administration of human estrogen11,12. This represents a good model for the study of bone homeostasis such as bone formation and resorption11,12. Moreover, Japanese quail medullary bone could be a useful source of bone metabolism-related PTHR. However, the expression and function of PTHR in Japanese quail are unknown, and PTHR cloning is unfinished. Therefore, the present study cloned the Japanese quail cDNA of PTHR.

Materials and Methods

Animals

Three 6-week-old female Japanese quails were sacrificed with an overdose of pentobarbital sodium. The kidneys and medullary bones were dissected out and immersed in liquid nitrogen. All animal procedures were performed in accordance with Okayama University guidelines.

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Figure 1. The design of nested PCR primers. We analyzed homologies between human PTH1R, human PTH2R, mouse PTH1R, mouse PTH2R, zebrafish PTH1R, zebrafish PTH2R, zebrafish PTH3R, and pig PTH1R to design primers for nested PCR in the cloning of PTHR from Japanese quail. The underlines were targeted for the design of PCR primers.

\[ \text{CAG GAA GCT GAA ATC TAC-3'}; \text{ and 3'}-\text{RACE-3, 5'}-\text{TCA TGG CTA TGC CAT ACA CAG ATG-3'} \]

After size-fractionation, PCR products were purified using agarose gel electrophoresis, and sub-cloned into the pGEM-T Easy vector (Promega, Madison, WI) for DNA sequencing.

**Sequence analyses**

All sequences were processed using the DNA Star program. The NCBI open reading frame (ORF) finder was used to identify ORFs, and putative conserved domains were searched for in the NCBI Conserved Domain Database. Amino acid alignment against other species was performed using Basic Local Alignment Search Tool (BLAST) analysis in NCBI. A phylogenetic tree was constructed using MEGA 7.0.16 software using the Neighbor Joining (NJ) method, and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test was decided from 500 replications.

**Evolutionary distances** were computed using the Poisson correction method, and are given as the number of amino acid substitutions per site.

**Results**

Partial fragments of Japanese quail PTHR

Each product of nested PCR from kidney- or medullary bone-derived
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Figure 2. The design of primers for 5'/3' RACE. We designed primers for 5'/3' RACE for the cloning of Japanese quail PTHR using the partial alignment derived from nested PCR products.

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>5' primer 1</td>
<td>CCATCTGGCACTTGCTGTGAATAATTTATTTTATCAATAATTACAG</td>
</tr>
<tr>
<td>3' primer 1</td>
<td>AGTCTGAAACCAAGAGCTACGAGTAGAGAGTAGGTGAGCTCA</td>
</tr>
<tr>
<td>3' primer 2</td>
<td>CGACACAGTAGAACAGGAGCTGCTGAAATTCAGCCTCCTATGGCCTT</td>
</tr>
<tr>
<td>3' primer 3</td>
<td>GTTGGGGCTCTATATTGTATTTTATGCTATACAGATGTC</td>
</tr>
<tr>
<td>A 5' RACE</td>
<td>ACAGGATCTTTGGCAAGTCCATATACGTATATGGAATGGCTGTCAATCGTC</td>
</tr>
<tr>
<td>B 3' RACE</td>
<td>CCAGGAGTTTTTTGTCGACCATATACGTATATTGCCAGGGGTAAT</td>
</tr>
</tbody>
</table>

Figure 3. Nested PCR analysis. We detected a single band of around 500 bp as the nested PCR product from both the kidney and the medullary bone.

Figure 4. 5'/3' RACE amplification product. We detected two bands around 1000 bp from 5' primers (A arrows), and a single band smaller than 1000 bp from 3' primers (B).

5' RACE and 3' RACE analyses

Using the 5/3 primer pair, cDNA fragments of around 1000 bp were amplified (Fig. 4). Amplification fragments obtained from 5'-RACE had two clear bands (Fig. 4A arrows), while fragments obtained from 3'-RACE had one clear band less than 1000 bp in size (Fig. 4B). These three PCR bands were sub-cloned and sequenced.

ORF analysis of Japanese quail PTHR

The size of the cloned PTHR was 1890 bp. The ATG initiation codon was located at position 103, and the terminal codon at position 1717 (Fig. 5, asterisk). The ORF was predicted to encode a protein of 538 amino acids (Fig. 5, capital letters) with a conserved extracellular hormone receptor domain of 49 amino acids (Fig. 5, wavy underlines) containing six conserved cysteine residues (Fig. 5, circles) and an N-glycosylation consensus motif conserved with human PTH1R (Fig. 5, boxes). The signal peptide is underlined. The protein was also predicted to contain seven transmembrane receptors of the secretin family (Fig. 5, double underlines).

BLAST analysis

Amino acid alignment revealed high homology with PTHR sequences of other birds. The 10 highest BLAST scores were obtained in alignments with the chicken, falcon, little egret, mallard, euckoo, grey crowned crane, snipe, blue-crowned manakin, hoatzin, and medium ground finch (Table 1). The rates of query cover were 100% in these species.

Phylogenetic tree analysis

A phylogenetic tree of the Japanese quail PTHR ORF (Fig. 6) was generated using the NJ method. The tree contains two major clades, one containing vertebrate PTH2R and the other clusters PTH1R and PTH3R. The PTHR of Japanese quail was classified in the PTH1R branch very close to PTH1R of chicken, turkey, and zebra finch.

Discussion

We successfully cloned the PTHR of Japanese quail using 5' and 3' RACE. The nucleotide sequence was predicted to be 1890 bp, encoding a putative PTHR protein of 538 amino acids. The putative Japanese quail PTHR was identified as PTH1R not PTH2R or PTH3R. PTH1R is also known as PTH/PTHrP receptor because of its equal binding affinities for both PTH and PTHrP. PTH2R is activated by PTH and tuberoinfundibular peptide of 39 residues in humans and zebrafish, but not by PTHrP. However, PTH2R is not found in birds. PTH3R is activated by PTHrP. However, PTH3R is only found in non-mammalian vertebrates such as zebrafish, sea bream, and chicken. PTH3R was previously detected at high expression levels in all stages of chicken embryos, but at low levels in adult chicken. We did not identify PTH3R in Japanese quail in this study, which could be explained by the fact that we used adult tissues for cloning.

The organization of PTH1R is highly homologous in the rat, human, and mouse. The gene extends over 22 kb and contains at least 5 exons and 14 introns. Human PTH1R was mapped to chromosome 3p21.1-p22. In the present study, Japanese quail PTH1R showed high homology with human PTH1R. Moreover, we also identified a conserved N-glycosylation consensus motif that was homologous to the human PTH binding site, seven transmembranous loops, and six cysteine residues. This suggests that the function of Japanese quail PTH1R is conserved across species.
Figure 5. Sequence alignment of Japanese quail PTH1R. The nucleotide alignment of Japanese quail PTH1R is represented in capital letters from 1 to 1890. The start and stop codons are shown in bold and underlined, and the stop codon is asterisked. The putative PTH1R amino acid alignment is represented by single capital letters in bold from 1 to 538. Signal peptides are underlined, the conserved hormone receptor domains have wavy underlines, the seven transmembranous loops have double underlines, the conserved N-glycosylation consensus motif is underlined within a box, and the cysteine residues are circled.

quail PTH1R is similar to that of human PTH1R. PTH1R is highly expressed in bone, kidney, and growth plates, and in other tissues throughout development. Its expression in the bone and kidney of adult animals is critically associated with the homeostatic maintenance of blood calcium levels via the actions of PTH released from the parathyroid gland. The activation of PTHR1 in osteoblastic cells and chondrocytes modulates the rates of proliferation and apoptosis, and the production of a variety of signaling factors involved in bone and cartilage metabolism.

In the future, we plan to investigate the expression and function of Japanese quail PTH1R in bone metabolism using the medullary bone and our cloned PTH1R.

Competing interests
The authors have declared that no competing interest exists.
Figure 6. Phylogenetic tree of Japanese quail PTH1R. The evolutionary history was inferred using the neighbor-joining method. The optimal tree with the sum of the branch length = 2.64876491 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method, and are in the units of the number of amino acid substitutions per site. The analysis involved 26 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 298 positions in the final dataset. Evolutionary analyses were conducted using MEGA7 software. The accession numbers of the Ensembl genome browser were followed: Turkey pth1r ENSMGAP00000002429, Turkey pth3r ENSMGAP00000001447, Chicken pth1r ENSGALP00000008782, Chicken pth3r ENSGALP000000044720, zebra finch PTH1R ENSTGUP0000000207, zebra finch PTH3R ENSTGUP00000001986, opossum pth1r ENSMODP000000017460, opossum pth2r ENSMODP000000019807, human PTH1R AAA10389, human PTH2R AAH36811.2, mouse pth1r AAH3681, mouse pth2r NP644676, tropical clawed frog pth1r XP_002939411.2, Anolis lizard pth1r ENSACAP00000017570, Anolis lizard pth2r ENSACAP00000010946, Anolis lizard pth3r ENSACAP00000017570, Xenopus Pth1r ENSXETP00000007949, Xenopus Pth2r ENSXETP000000017570, Takifugu Pth1r ENSTRUP00000035460, Takifugu Pth2r ENSTRUP00000035460, Takifugu Pth3r ENSTRUP0000005220, and human secretin receptor AAA64949.

Table 1. BLAST analysis of the putative Japanese quail PTHR

<table>
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<tr>
<th>Description</th>
<th>Max Score</th>
<th>Total Score</th>
<th>Identify</th>
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<td>1119</td>
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<td>Falcon predicted parathyroid hormone/parathyroid hormone-related peptide receptor</td>
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<td>1119</td>
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<td>Little egret predicted parathyroid hormone/parathyroid hormone-related peptide receptor</td>
<td>1118</td>
<td>1118</td>
<td>99%</td>
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<td>Mallard parathyroid hormone/parathyroid hormone-related peptide receptor isoform X1</td>
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<td>1118</td>
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<td>1118</td>
<td>99%</td>
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<tr>
<td>Grey crowned crane parathyroid hormone/parathyroid hormone-related peptide receptor</td>
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<td>1117</td>
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<td>1115</td>
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The highest BLAST scores of the putative Japanese quail PTHR are shown, and all derive from birds. The rates of query cover are 100%.
References


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