Identification of two novel chicken GHRH receptor splice variants: implications for the roles of aspartate 56 in the receptor activation and direct ligand–receptor interaction

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Abstract

In this study, two novel GHRHR receptor splice variants, named chicken GHRHR-v1 (cGHRHR-v1) and cGHRHR-v2 respectively, were identified from chicken pituitary using RT-PCR assay. cGHRHR-v1 is characterized by an N-terminal deletion of 36 amino acid residues, including an aspartate at position 56 (Asp56) conserved in G protein-coupled receptor B-I subfamily. cGHRHR-v2 is a carboxyl-terminal truncated receptor variant with four putative transmembrane domains, which arose from alternative use of a splice acceptor site on intron 8. Using the pGL3-CRE-luciferase reporter system, the functionality of the two variants was examined in Chinese hamster ovary cells. cGHRHR-v1 was shown to be capable of transmitting signal upon agonist stimulation, but cGHRHR-v2 could not. Both GHRH and pituitary adenylate cyclase-activating peptide (PACAP) could activate cGHRHR-v1 at high dosages (GHRH ≥ 10⁻⁸ M; PACAP ≥ 10⁻⁶ M) and GHRH was much more potent than PACAP, suggesting that cGHRHR-v1 is a functional membrane-spanning receptor with an impairment in high-affinity ligand binding, rather than in receptor activation and ligand-binding specificity. This finding also points out the possibility that Asp56 is not a critical determinant for receptor activation and direct ligand–receptor interaction. To substantiate this hypothesis, using site-directed mutagenesis, two receptor mutants with replacement of Asp56 by Ala or Gly were generated. Expectedly, chicken or human GHRH could still activate both receptor mutants with reduced potencies (about 2- to 14-fold less potent). Taken together, our findings not only suggest that cGHRHR variants may play a role in controlling normal pituitary functions, but also support that Asp56 is nonessential for receptor activation and direct ligand–receptor interaction.


Introduction


In mammals, GHRH receptor splice variants with altered signaling properties and ligand-binding affinity have been reported (Mayo 1992, Hashimoto et al. 1995, Zeitler et al. 1998, Hassan 2001). In rats, a GHRHR variant with an insertion of 41 amino acids in the third intracellular loop (IL3) could bind GHRH with high affinity, but fails to increase the intracellular cAMP levels (Mayo 1992, Miller et al. 1999).
In contrast, another rat GHRHR variant with a substitution of the last five amino acids in the carboxyl terminus retains the ability to promote cAMP accumulation when tested in non-GHRHR expressing cells (Zeitler et al. 1998). In humans, the retention of the intronic sequence generates a variant encoding a C-terminally truncated GHRHR with five transmembrane domains (Hashimoto et al. 1995, McElvaine & Mayo 2006). Interestingly, though this receptor variant could not bind GHRH, it could form a complex with the full-length receptor and inhibit GHRH binding, thereby altering the signaling activity of GHRHR gene may generate multiple functional GHRHR variants and represent an additional level to control the actions of GHRH under different physiological conditions (Minneman 2001). However, the presence of GHRHR variants and their potential roles in the pituitary of nonmammalian vertebrate species have not been reported.

In our previous study, we amplified a chicken GHRHR (cGHRHR) cDNA fragment without exon 3 by RT-PCR assay (Wang et al. 2006), suggesting that GHRHR variants may be present and function in the chicken pituitary. Therefore, the present study aims at the identification and characterization of these splice variants. As a result, two novel GHRHR variants, named cGHRHR-v1 and cGHRHR-v2 respectively, have been identified. cGHRHR-v2 is a C-terminal truncated receptor variant with four putative transmembrane domains and cGHRHR-v1 is a variant with an N-terminal deletion of 36 amino acids including the aspartic acid residue at position 56 (Asp⁵⁶). Strikingly, neither deletion of 36 amino acids nor mutation of Asp⁵⁶ could abolish the ligand binding and signaling property of cGHRHR. These findings suggest a role of pituitary cGHRHR variants under normal physiological conditions, and also lead us to rethink the role of Asp⁵⁶ or residues 50–85 in the interaction between GHRH and GHRHR.

Materials and Methods

Chemical and hormones

Human pancreatic GHRH (hpGHRH1–40) and ovine pituitary adenylate cyclase-activating polypeptide (oPACAP38) were purchased from Bachem (Bachem Inc., Torrance, CA, USA). Chicken GHRH (cGHRH1–41) and (cGHRH1–47) were synthesized by GL Biochem (GL Biochem. Shanghai, China). hpGHRH1–40, oPACAP38, cGHRH1–47, and cGHRH1–31 were first dissolved in distilled water and then diluted to working concentrations with medium before use.

Animals

Chicken embryos and adult chickens were kindly provided by Kadoorie Agricultural Research Centre (Hong Kong, China). All experiments were performed under license issued from the Government of the Hong Kong Special Administrative Region and endorsed by the Animal Experimentation Ethics Committee of The University of Hong Kong.

RNA extraction and RT-PCR assay

Pituitaries from chicken embryos, sexually immature and mature chickens were isolated, and attached brain tissues were carefully removed under a dissection microscope. Then, total RNA was extracted using Tri Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instruction. RT was carried out at 42°C for 2 h in a total volume of 10 μl, consisting of 2 μg RNA, 1× single-strand buffer, 10 mM dithiothreitol, 0.5 μg oligo (dT), and 100U Superscript II (Invitrogen). One microliter of the first-strand cDNA was used as the template for each PCR. PCR was performed under the following conditions: 2 min at 94°C for denaturation, followed by 25 (or 30, 35, or 40) cycles of 30 s at 95°C, 30 s at 54°C, and 60 s at 72°C, ending with a 7-min extension at 72°C. The primers used were listed in Table 1. The PCR products were electrophoresed in 1.5%-agarose gel, stained with ethidium bromide, and visualized under u.v. illumination.

Real-time RT-PCR quantitation of GHRHR, GHRHR-v1, and GHRHR-v2 expression in chicken pituitaries at different stages

To examine the relative expression levels of GHRHR and two GHRHR variants (GHRHR-v1 and GHRHR-v2) in chicken pituitaries at different stages, pituitaries from chicken embryos (day 13, N=4), sexually immature (3 weeks, N=4), and mature chickens (25 weeks, N=4) were collected for quantitative real-time RT-PCR assay.

Before real-time RT-PCR assay, four plasmids containing cDNA fragment of β-actin, or full-length cDNAs of GHRHR or GHRHR variants, were quantitated by a UV-Visible Recording Spectrophotometer (Shimadzu Corp., Tokyo, Japan), and the copy number of DNA molecule was calculated. Then, these serially diluted plasmids with known copy number were used to set the standard curves in all real-time PCR assays.

The real-time PCR was performed on the iCycler Real-time PCR Detection System (Bio-Rad) in a volume of 20 μl containing 0.5 μl RT product, 1× PCR buffer, 0.2 mM each dNTP, 2.5 mM MgCl₂, 0.2 μM each primer, 0.5 U Taq DNA polymerase (Invitrogen), and 1 μl EvaGreen (Biotium Inc., Hayward, CA, USA). The PCR profile consisted of 40 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s, and 80°C for 7 s for signal detection. To assess the specificity of PCR amplification, melting curve analysis and agarose gel electrophoresis were performed at the end of the PCR to confirm that a specific PCR band was produced. In addition, the identities of the PCR products for all genes were confirmed by sequencing analysis (Perkin–Elmer, Foster City, CA, USA). In the quantitative real-time RT-PCR assays, PCR was carried out in duplicate for evaluation of its reproducibility.

Finally, according to the standard curve set in each quantitative real-time PCR, the copy numbers of target
transcript (including β-actin, GHRHR, GHRHR-v1, and GHRHR-v2) in RT samples were calculated.

Construction of expression plasmids encoding cGHRHR, cGHRHR-v1, cGHRHR-v2, and cGHRHR mutants

In this experiment, the expression plasmids encoding cGHRHR (1280 bp), cGHRHR-v1 (1172 bp), and cGHRHR-v2 (1291 bp) were first constructed. In brief, the coding regions were amplified by PCR using the antisense primer containing an XbaI site at the 5′-end (5′-TCTAGAGTCTTTAGCACACAGA-3′) and the sense primer with a BamHI site at the 5′-end (5′-GGATCCTAGCATGTCATACCA-3′). PCR condition was as follows: 2 min at 94 °C for denaturation, followed by 31 cycles of amplification (94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min) and a 10-min final extension at 72 °C. The PCR products were cloned into pBluescript SK (Stratagene, La Jolla, CA, USA) and sequenced by ABI3100 Genetic Analyzer (Perkin–Elmer). The fragments with correct sequences were released by restriction enzyme digestion (BamHI and XbaI) and subcloned into pcDNA3.1 (+) expression vector (Invitrogen).

To generate the cGHRHR mutants with a replacement of aspartate 56 by alanine (Asp56→Ala56) or glycine (Asp56→Gly56), site-directed mutagenesis PCR was used in this study. The mutagenic primers were used to introduce a single amino acid substitution at position 56 (Asp56→Ala56 or Asp56→Gly56) of cGHRHR. The sequences of the mutagenic primers were listed in Table 1. Using the plasmid containing the open reading frame (ORF) region of cGHRHR as template, the ORF region was amplified and cloned into pBluescript SK (+/−) vector (Stratagene). After sequencing analysis, inserts with the mutated sites were released by restriction enzyme digestion and subcloned into pcDNA3.1 (+) expression vector (Invitrogen; Fig. 5). In this study, the two cGHRHR mutants were named cGHRHR-A (Asp56→Ala56) and cGHRHR-G (Asp56→Gly56) respectively.

Functional analysis of cGHRHR, cGHRHR-v1, cGHRHR-v2, cGHRHR-A, and cGHRHR-G

Chinese hamster ovary (CHO) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA), 100 IU/ml penicillin G, and 100 µg/ml streptomycin (Life Technologies Inc., Grand Island, NY, USA) in a 90 cm culture dish (NUNC, Rochester, NY, USA) and incubated at 37 °C with 5% CO2. CHO cells were first subcultured on six-well plates and grown for 24 h before transfection. Then, the cells were co-transfected with a mixture containing 700 ng pGL3-CRE-luciferase reporter construct (Wang et al. 2007), 300 ng of different pcDNA3.1 expression plasmids (or empty pcDNA3.1 vector), and 6 µl

### Table 1 Primers used

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aAll primers were synthesized by Invitrogen (Hong Kong).
bPrimers were used to detect the expressions of both GHRHR and GHRHR-v1.
cPrimer was designed to amplify GHRHR-v2 specifically.
dThe restriction sites are underlined and the changed nucleotide is in bold typeface.
ePrimers were designed to prepare cGHRHR mutants by site-directed PCR mutagenesis.
DOSPER (Roche Diagnostics). Transfected cells were grown in fresh DMEM for an additional 24 h. CHO cells were trypsinized and then cultured in a 96-well plate at 37 °C for an additional 24 h before hormone treatment. hpGHRH1–40, oPACAP38, cGHRH1–47, and cGHRH1–31 were diluted by serum-free DMEM to the indicated concentrations. After 6-h treatment (serum-free medium used as a control), CHO cells were harvested with 1 × passive lysis buffer for luciferase assay (Promega). The luciferase activity was measured by a Luminometer (Lumat LB9507, EG&G Berthold, Bad Wildbad, Germany) according to the manufacturer's instruction.

Data analysis
The luciferase activities in each treatment group were expressed as relative fold increase as compared with the control group (without hormone treatment). The expression level of each mRNA transcript in each sample was first calculated as the ratio to that of β-actin and finally expressed as the percentage of the expression level of GHRHR in day 13 embryonic pituitaries. The data were analyzed by one-way ANOVA followed by Dunnett's test or student's t-test using GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA). To validate our results, all experiments were repeated at least twice.

Results
Identification of two novel cGHRHR splice variants: cGHRHR-v1 and cGHRHR-v2
To identify cGHRHR variants, primers amplifying the full-length cGHRHR were used (Table 1). As shown in Fig. 4A, a minor PCR band was noted, coinciding with the finding in our previous study (Wang et al. 2006). Sequencing analysis revealed that this full-length cDNA lacks 108 bp (corresponding to exon 3) and encodes a receptor of 383 amino acids with seven intact transmembrane domains. Strikingly, loss of exon 3 results in a deletion of 36 amino acid residues (residues 50–85) in the large extracellular domain including an aspartate at position 56, a glycine at position 67, two tryptophans, and three cysteines conserved in all members of B-I GPCR subfamily (Fig. 1).

Figure 1
(A) Detection of GHRHR [bright band] and GHRHR-v1 [faint band] expressions in adult chicken pituitary by RT-PCR. Numbers indicate the PCR cycles used. (B) Alignment of the N-terminal extracellular domain of chicken GHRHR (cGHRHR: NP_001032923) with that of human GHRHR (hGHRHR: NP_000814), rat GHRHR (rGHRHR: NP_036982), mouse GHRHR (mGHRHR: NP_001003685), goldfish GHRH-like peptide receptor (gGHRH-LR: AF048819), human PACAP type I receptor [hPAC1: NP_001109], zebrafish PACAP type I receptor [zfPAC1: AY738798], mouse VIP type II receptor [mVIP2: NP_035833], and human secretin receptor [hSCTR: NP_002971]. The aspartate 56 (D; arrow), glycine (G), tryptophans (W), and cysteines (C) are boxed. The shaded regions are shown to be involved in the formation of splice variant of cGHRHR, hPAC1, and hSCTR. Dots indicate amino acid residues identical to cGHRHR and dashes represent gaps in sequence. TM1, transmembrane domain 1.
Using the same primers, we isolated another cGHRHR variant (Genbank accession no.: EF433463), which is 11 bp longer than cGHRHR. Unlike cGHRHR-v1, this variant is resulted from the alternative use of a splice acceptor site at intron 8 (Fig. 2B). The alternative mRNA splicing leads to the retention of 11 bp intronic sequence and formation of a premature stop codon located on exon 9 (Fig. 2B). Using a primer specific to 11 bp intronic sequence (Table 1), this receptor variant could be amplified from the pituitary, whereas no PCR band was found using plasmid containing cGHRHR as template, confirming the specific amplification of cGHRHR-v2 (Fig. 2C). Interestingly, this splice transcript encodes a C-terminally truncated cGHRHR variant with the first four transmembrane domains and an altered C-terminal tail (16 amino acids; Fig. 2A). Herein, this truncated GHRHR variant is designated as cGHRHR-v2.

Figure 2  (A) The deduced amino acid sequence of cGHRHR splice variant 2 (cGHRHR-v2). The four putative transmembrane domains are shaded and labeled as TM1, TM2, TM3, and TM4 respectively. The altered C-terminal tail (16 amino acids) is boxed. The inserted partial sequence (11 bp) of intron 8 is boxed and shaded. (B) Arrow indicates the alternate splice acceptor site (ag) on intron 8. The usage of this splice acceptor site results in the retention of 11 bp sequence (boxed) of intron 8 and formation of a premature stop codon in exon 9 (underlined). (C) Detection of cGHRHR (wt) and cGHRHR-v2 (v2) mRNA in adult chicken pituitary. Plasmid containing the full-length cGHRHR (plasmid (wt)) was used as template to test the specificity of PCR. RT: RT product from adult chicken pituitary. Numbers in brackets indicate the PCR cycle numbers used.
Relative expression levels of GHRHR, GHRHR-v1, and GHRHR-v2 in the chicken pituitary

To evaluate the relative mRNA levels of GHRHR, GHRHR-v1, and GHRHR-v2 in chicken pituitary at different developmental stages, pituitaries from day 13 embryos, sexually immature (3 weeks), and mature chickens were chosen for quantitative real-time RT-PCR assay in this study. Consistent with our RT-PCR results (Figs 1A and 4A), cGHRHR was shown to be abundantly expressed at all stages examined, with the highest expression level observed in 3-week pituitary. In contrast, cGHRHR-v1 was detected to be expressed at a level much lower than cGHRHR regardless of the developmental stage of pituitary used (Fig. 3). And its expression level is only about 5–20% of that of cGHRHR in most individuals examined. Compared with cGHRHR-v1, cGHRHR-v2 was shown to have even much lower abundance, and its expression level is <5% of that of cGHRHR in most individuals investigated (Fig. 3).

Activation of cGHRHR-v1 by chicken GHRH or human GHRH

To examine the signaling property of cGHRHR-v1, cGHRHR-v1 recombinant construct was transiently expressed in CHO cells and challenged with human GHRH (hpGHRH1–40) or chicken GHRH (cGHRH1–47 or cGHRH1–31). To monitor the changes of intracellular cAMP levels, a pGL3-CRE-luciferase reporter system was used as reported in our previous study (Wang et al. 2007). Surprisingly, both chicken and human GHRH could activate cGHRHR-v1 at \( \geq 10^{-8} \) M dosages (Fig. 4), suggesting that cGHRHR-v1 is still likely to be a functional membrane-spanning receptor. Though GHRH could activate both cGHRHR and cGHRHR-v1, it should be noted that GHRH is \( \sim 1000 \)-fold less potent in activating cGHRHR-v1 than in activating cGHRHR (Fig. 4).

Asp\(^{56}\) is not a crucial determinant for receptor activation and direct ligand–receptor interaction

Deletion of N-terminal 36 amino acid residues including Asp\(^{56}\) fails to abolish the receptor-activated cAMP–PKA signaling pathway (Fig. 4), leading us to speculate that Asp\(^{56}\) may not be crucial for receptor activation and direct ligand–receptor interaction. To test this possibility, two receptor mutants with a replacement of Asp\(^{56}\) by Ala or Gly (Asp\(\rightarrow\)Ala or Asp\(\rightarrow\)Gly) were generated and tested in the pGL3-CRE-luciferase reporter system (Fig. 5). Strikingly, both chicken and human GHRH could activate cGHRHR-G (Asp\(\rightarrow\)Ala) or cGHRHR-A (Asp\(\rightarrow\)Gly) with slightly reduced potencies (\( \sim 2-\) to 14-fold less potent), suggesting that Asp\(^{56}\) is not critical for receptor activation and direct interaction between cGHRHR and cGHRHR (Fig. 5).

Activation of cGHRHR-v1 and cGHRHR mutants by oPACAP38

To determine whether cGHRHR-v1 and cGHRHR mutants could abolish the ligand-binding selectivity, PACAP\(_{38}\), a structurally and functionally related peptide, was used in this experiment. Interestingly, ovine PACAP\(_{38}\) (oPACAP\(_{38}\)) activated both cGHRHR-v1 and cGHRHR mutants (cGHRHR-A or cGHRHR–G; Fig. 6). However, its potency is comparatively lower than human or chicken GHRH (about 100-fold less potent), clearly suggesting that mutation of Asp\(^{56}\), or deletion of residues 50–85, could not abolish the ligand-binding selectivity of cGHRHR (Fig. 6).
Effects of co-expressing cGHRHR and cGHRHR-v1 on cGHRHR signaling in CHO cells

The co-expression of cGHRHR and cGHRHR-v1 in the pituitary suggests that cGHRHR-v1 may modify cGHRHR signaling in normal pituitary. To test this possibility, we co-expressed cGHRHR and cGHRHR-v1 in CHO cells and monitored the changes in ligand-stimulated luciferase activity. Interestingly, when treated with GHRH, particularly at low concentrations (10^{-12} to 10^{-7} M), the fold increase in luciferase activity appeared to be significantly reduced in the presence of equal amount of cGHRHR-v1 and cGHRHR expression plasmids when compared with cells expressing cGHRHR alone (Fig. 7). However, when the two expression plasmids were co-transfected at a ratio of 5:1 (cGHRHR:cGHRHR-v1), only a minor difference was noted between cells expressing cGHRHR alone and cells expressing both cGHRHR and cGHRHR-v1 (data not shown).

Inability of cGHRHR-v2 in activating cAMP–PKA signaling pathway upon ligand stimulation

To demonstrate the functionality of cGHRHR-v2, cGHRHR-v2 was also transiently expressed in CHO cells. As shown in Fig. 4, neither chicken GHRH (cGHRH_{1-31}) nor human GHRH (hGHRH_{1-40}) activated the cGHRHR-v2 signaling pathway in CHO cells.
and cGHRH1–47 nor human GHRH (hpGHRH1–40) could activate cGHRHR-v2 and signal through cAMP–PKA pathway at any dosage tested. When cGHRHR-v2 and cGHRHR were co-transfected into CHO cells at different ratio (1:1 or 1:5), no difference was found in agonist-induced fold increase in luciferase activity between cGHRHR transfected and co-transfected cells (data not shown).

**Discussion**

In this study, two GHRHR splice variants, named cGHRHR-v1 and cGHRHR-v2, have been identified from chicken pituitary. cGHRHR-v1 is a receptor variant with an N-terminal deletion of 36 amino acid residues, including an aspartate at position 56, two tryptophans and three cysteines, which are fully conserved in GPCR B-I subfamily (Harmar 2001, Mayo et al. 2003). cGHRHR-v2 is a C-terminal truncated receptor with four putative transmembrane domains. To our knowledge, our study is the first to report two novel GHRHR variants expressed in the pituitary of vertebrate species.

Although alternative mRNA splicing of GHRHR gene has been found in the normal pituitaries of both mammals and chickens, the alternative splicing site of chicken ghrhr gene is shown to be different from its mammalian counterparts. In mammals, mRNA alternative splicing occurs at the third IL (Mayo 1992, Hashimoto et al. 1995, McElvaine & Mayo 2006) or the C-terminal tail of GHRHR (Hashimoto et al. 1995, Zeitler et al. 1998). In chickens, alternative mRNA splicing of
cGHRHR was noted at two novel sites. One is located on exon 3 and the other is located downstream of the fourth transmembrane domain (Figs 1 and 2). Despite the lack of information on exon 3 deletion of GHRHR from other species, a similar N-terminal spliced variant (hPAC1vs) of PACAP type I receptor, a GPCR having close evolutionary relationship to GHRHR, has been identified in human cerebellum tissues (Dautzenberg et al. 1999). In addition, the similar deletion of 36 amino acids (encoded by exon 3) had also been reported in secretin receptor identified from human gastrinomas (Ding et al. 2002b). These evidence strongly suggest that the N-terminal deletion (exon 3 deletion) may be a potential mechanism for generating splice variants of secretin/
PACAP/GHRH receptors. Although no splicing occurs in intron 8 of mammalian GHRHR, retention of intronic sequence appears to be common in generating functional splice variants of GPCRs (Kilpatrick et al. 1999). In humans, retention of intron 10 (561 bp) leads to the formation of a truncated GHRHR splice variant with five transmembrane domains, which is expressed in normal pituitary and pituitary adenomas (Hashimoto et al. 1995, McElvaine & Mayo 2006).

cGHRHR-v1 involves a deletion of 36 amino acid residues, a region which is generally believed to be crucial for direct ligand–receptor interaction of subfamily B-I GPCRs (Carruthers et al. 1994, Couvineau et al. 1995, Gaylinn 2002), raising an issue regarding the functionality of

Figure 6 Activation of GHRHR-v1 (A) and two GHRHR mutants (GHRHR-A and GHRHR-G) (B) by ovine PACAP38 (oPACAP; 10^{-12}-10^{-10} M, 6 h). The pGL3-CRE-luciferase reporter construct was co-transfected with different expression plasmids to monitor the changes in intracellular cAMP levels of CHO cells upon PACAP treatment. Each data point represents mean ± S.E.M. of three replicates.

Figure 7 Effect of the co-expression of GHRHR and GHRHR-v1 on GHRHR signaling in CHO cells. In this experiment, CHO cells were transfected with either GHRHR or GHRHR-v1 expression plasmid, or co-transfected with both constructs at a ratio of 1:1 (GHRHR + GHRHR-v1), and then treated with chicken GHRH1–31 (A) or GHRH1–47 (B) (10^{-12}–10^{-7} M) for 6 h. The empty pcDNA3.1(+) vector was used to keep the net amount of plasmids constant among different groups. The pGL3-CRE-luciferase reporter construct was co-transfected into CHO cell to monitor the changes in intracellular cAMP levels after GHRH treatment. Each data point represents mean ± S.E.M. of three replicates. *P<0.01 versus GHRHR.
cGHRHR-v1. Interestingly, cGHRHR-v1 could be activated by human and chicken GHRH at high dosages (≥10⁻⁸ M), suggesting that cGHRHR-v1 is still likely to be a membrane-spanning receptor with a reduced ligand-binding affinity. Consistent with our finding, the human PAC1 vs over-expressed in HEK293 cells could bind PACAP with reduced affinity and signal cAMP accumulation (Dautzenberg et al. 1999). Our findings in cGHRHR-v1, together with the study on hPAC1 vs, provide two exceptional, nonetheless important, cases among all members in the GPCR B-I subfamily, in which the N-terminal deletions neither abolish receptor activation nor prevent the normal traffic of receptor variants to the cell surface, though the deleted regions may contain structural motif or functional domain critical for high-affinity ligand binding (Fig. 4; Dautzenberg et al. 1999).

The loss of an aspartic acid residue at position 56 (Asp⁵⁶), which is fully conserved among subfamily B-I GPCRs (Harmar 2001), did not impair the cGHRHR-v1 activation in the present study, strongly arguing against its suggested critical role in receptor activation and direct ligand–receptor interaction. Several studies demonstrated that point mutation of aspartate (Asp⁶⁰) in little mouse GHRHR (Godfrey et al. 1993, Lin et al. 1993) renders receptor totally defective in ligand binding and ligand-stimulated cAMP production despite its expression level similar to wild-type receptor in vitro (Gaylinn et al. 1999), claiming that this aspartic acid residue is critical for receptor activation and direct ligand–receptor interaction. This notion is also strongly supported by studies that mutation of this equivalent residue in receptors of VIP, glucagon, and GHRH-like peptide abolishes the ability of receptors to bind their respective ligands (Carruthers et al. 1994, Couvineau et al. 1995, Kee et al. 2005). However, in sharp contrast to this concept, substitution of Asp⁵⁶ with Gly (D→G, cGHRHR-G) or Ala (D→A, cGHRHR-A) has little effect on cGHRHR signaling and only causes slightly reduced potencies of GHRH on activating two receptor mutants (Fig. 5). To our knowledge, our finding provides the unique and important piece of direct evidence that the conserved Asp⁵⁶ is not a crucial determinant for receptor activation and direct ligand–receptor interaction between GHRH and GHRHR. In view of the remarkable conservation of Asp⁵⁶ in all GPCRs belonging to secretin/VIP/GHRHR subfamily (Fig. 1; Sherwood et al. 2000, Harmar 2001, Mayo et al. 2003), it leads us to hypothesize that the presence of aspartic acid residue confers these receptors a right conformational structure required for high-affinity ligand binding, and mutation of this residue may result in the formation of a steric obstacle in their binding pockets, which would interfere with the receptor–ligand interaction.

Since cGHRHR-v1 and two receptor mutants could be activated by chicken and human GHRH, it led us to further examine whether the N-terminal 36 amino acid residues or Asp⁵⁶ is involved in determining the ligand-binding specificity or selectivity of cGHRHR. In this study, PACAP₃₈, a peptide structurally and functionally related to GHRH, was used (Sherwood et al. 2000). Consistent with our previous study, oPACAP₃₈ activates cGHRHR with a potency much lower than that of human or chicken GHRH (Fig. 6; Wang et al. 2007). Similarly, oPACAP₃₈ also activates two cGHRHR mutants and cGHRHR-v1 with potencies much lower than human or chicken GHRH (Figs 4–6), clearly indicating that mutation of aspartate 56 (Asp⁵⁶) or deletion of residues 50–85 could not abolish the selectivity of cGHRHR or cGHRHR-v1. In agreement with our findings, DeAlmeida & Mayo (1998), using a chimeric receptor approach, have also demonstrated transmembrane domains and/or extracellular loops, instead of the N-terminal extracellular domain, of human GHRHR to be critical in determining the specificity of ligand binding.

It is of particular interest to point out that in our study, cGHRHR mutants (cGHRHR-A or cGHRHR-G) could still be activated by human GHRH (Fig. 5). This finding appears to contrast the studies in mammals. In mice, GHRHR mutant with a substitution of aspartate (Asp⁶⁰) is unable to bind mouse GHRH and signal intracellular cAMP accumulation (Godfrey et al. 1993, Lin et al. 1993, Gaylinn et al. 1999). This remarkable difference implies that a coevolutionary event may have occurred in both mammalian GHRH and GHRHR genes (Wang et al. 2007).

Unlike cGHRHR-v1, cGHRHR-v2 could not transduce signal in response to GHRH or PACAP. The inability of signaling through cAMP pathway is not surprising since the predicted cGHRHR-v2 only has four transmembrane domains and lacks the third IL3 and C-terminal tail, both of which have been demonstrated to be critical for G-protein (Gs or Gi) coupling and activation of GPCRs (O’Dowd et al. 1988, Minneman 2001).

The co-expression of cGHRHR and its variants in normal pituitary raises an interesting issue regarding their physiological relevance in vivo. In this study, both cGHRHR and cGHRHR-v1 alone could signal cAMP–PKA pathway upon agonist stimulation; however, cGHRHR-v1 could only be activated by GHRH at high concentrations (≥10⁻⁸ M; Fig. 4), suggesting that cGHRHR-v1 is less likely to be directly involved in triggering cAMP accumulation in vivo. Interestingly, co-expression of cGHRHR-v1 and cGHRHR leads to the reduction in fold increase of ligand-stimulated luciferase activity of CHO cells and a right-shifted dose–response curve, suggesting that cGHRHR-v1 may inhibit the signaling of full-length cGHRHR when co-expressed in CHO cells. The underlying mechanism is unclear. One possible explanation is that cGHRHR-v1 may form hetero-complexes with the full-length cGHRHR on the cell surface, thereby inhibiting the function of full-length cGHRHR, as have previously been demonstrated for secretin receptor variant without exon 3 (Ding et al. 2002a,b). The expression level of cGHRHR-v1 is much lower than that of cGHRHR in pituitaries of most individuals examined (Fig. 3) and the inhibitory effect of cGHRHR-v1 is shown to be concentration–dependent in vitro (Fig. 7); thus, whether cGHRHR-v1 could modulate GHRH signaling in the

pituitary under normal physiological conditions remains to be further clarified.

There is also evidence showing that GPCR with four transmembrane domains, such as truncated gonadotropin-releasing hormone receptor and V2 vasopressin receptor (No2), could act as an inhibitor to control the signaling of GPCR by directing degradation of full-length receptor and diminishing its cell surface expression (Grosse et al., 1997, Zhu & Wess 1998). However, co-expression of cGHRHR-v2 with cGHRHR does not display any inhibitory effect on cGHRHR function despite the high concentration of cGHRHR-v2 expression plasmid used (cGHRHR-v2: cGHRHR = 1:1). Thus, the physiological relevance of cGHRHR-v2 expressed in pituitary remains to be elucidated in our future study.

In summary, two GHRHR splice variants, cGHRHR-v1 and cGHRHR-v2, were identified and shown to be expressed in normal chicken pituitaries. cGHRHR-v2 is a receptor variant with four transmembrane domains and incapable of transmitting signal upon ligand stimulation. cGHRHR-v1 is a splice variant with an N-terminal deletion of 36 amino acid residues including Asp56 conserved in GPCR B-I subfamily. Functional studies further demonstrated that both cGHRHR-v1 and cGHRHR variants with a substitution of Asp56 could be activated by GHRH and PACAP. Our study, for the first time, provides direct evidence that the conserved Asp56, though required for full function of cGHRHR, is nonessential for receptor activation and direct receptor–ligand interaction (Fig. 5).

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