Discovery of a Novel Glucagon-like Peptide (GCGL) and Its Receptor (GCGLR) in Chickens: Evidence for the Existence of *GCGL* and *GCGLR* Genes in Nonmammalian Vertebrates

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Glucagon (GCG), glucagon-related peptides, and their receptors have been reported to play important roles including the regulation of glucose homeostasis, gastrointestinal activity, and food intake in vertebrates. In this study, we identified genes encoding a novel glucagon-like peptide (named *GCGL*) and its receptor (*GCGLR*) from adult chicken brain using RACE and/or RT-PCR. GCGL was predicted to encode a peptide of 29 amino acids (cGCGL$_{1-29}$), which shares high amino acid sequence identity with mammalian and chicken GCG (62–66%). GCGLR is a receptor of 430 amino acids and shares relatively high amino acid sequence identity (53–55%) with the vertebrate GCG receptor (GCGR). Using a pGL3-CRE-luciferase reporter system, we demonstrated that synthetic cGCGL$_{1-29}$, but not its structurally related peptides, *i.e.* exendin-4 and GCG, could potently activate GCGLR (EC$_{50}$: 0.10 nM) expressed in Chinese hamster ovary cells, indicating that GCGLR can function as a GCGL-specific receptor. RT-PCR assay revealed that *GCGL* expression is mainly restricted to several tissues including various brain regions, spinal cord, and testes, whereas *GCGLR* mRNA is widely expressed in adult chicken tissues with abundant expression noted in the pituitary, spinal cord, and various brain regions. Using synteny analysis, *GCGL* and *GCGLR* genes were also identified in the genomes of fugu, tetraodon, tilapia, medaka, coelacanth, and *Xenopus tropicalis*. As a whole, the discovery of *GCGL* and *GCGLR* genes in chickens and other nonmammalian vertebrates clearly indicates a previously unidentified role of GCGL-GCGLR in nonmammalian vertebrates and provides important clues to the evolutionary history of *GCG* and *GCGL* genes in vertebrates. **(*Endocrinology* 153: 5247–5260, 2012)**

It is well-documented that in mammals, glucagon (GCG) and its structurally similar peptides, glucagon-like peptide 1 (GLP1), glucagon-like peptide 2 (GLP2), glucose-dependent insulinotropic polypeptide (GIP), play critical roles in many physiological processes, such as the regulation of glucose homeostasis (1, 2). The three peptides, GCG, GLP1, and GLP2, are reported to be coencoded by a single glucagon gene. The tissue-specific posttranslational processing of the large proglucagon precursor generates distinct peptides in different tissues. GCG is predominantly released by α-cells of pancreatic islets in response to low glucose levels, whereas GLP1 and GLP2 are preferentially produced in intestinal L-cells and the central nervous system (CNS) (3–5). Unlike glucagon gene, *GIP* gene encodes a single bioactive peptide of 42 amino acids and is predominantly expressed in the intestinal K cells (6). It is reported that glucagon can enhance hepatic glucose production via glycogenolysis and gluconeogenesis and thus oppose glucose-lowering effect of insulin (5), whereas both GLP1 and GIP have been viewed as incretin hormones to stimulate glucose-dependent insulin secretion of pancreatic islet β-cells in response to

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Abbreviations: CHO, Chinese hamaster ovary; CNS, central nervous system; c, chicken; GCG, glucagon; *GCGL*, glucagon-like gene; GCGLR, *GCGL* receptor; GCGR, GCG receptor; GIP, glucose-dependent insulinotropic polypeptide; GIPR, GIP receptor; GLP1, glucagon-like peptide 1; GLP1R, GLP1 receptor; GLP2, glucagon-like peptide 2; GLP2R, GLP2 receptor; h, human; PKA, protein kinase A; RACE, rapid amplification of cDNA ends; SNF8, ESCRT-II complex subunit, homolog (*S. cerevisiae*); UBE2E, ubiquitin-conjugating enzyme E2Z.

food ingestion (7, 8). In addition to their actions in controlling glucose metabolism, GCG, GLP1, and GIP have also been reported to be involved in other physiological processes, including the regulation of gastrointestinal activity, food intake, and energy expenditure (7, 9, 10). By contrast, much less is known about the biological actions of GLP2 in mammals (11). There is increasing evidence showing that GLP2 is involved not only in controlling food intake, nutrient absorption, and gastric emptying, but also in stimulating crypt cell proliferation and bowel growth mediated by locally produced growth factors (11–13). It is becoming clear that the biological actions of the four peptides are mediated by their specific receptors: GCG receptor (GCGR), GLP1 receptor (GLP1R), GLP2 receptor (GLP2R), and GIP receptor (GIPR) (5, 10, 14–16). All these receptors belong to the same G protein-coupled receptor (GPCR) B1 subfamily, which also includes the receptors for secretin (SCT), GHRH, vasoactive intestinal polypeptide (VIP), and pituitary adenylate cyclase-activating polypeptide (PACAP) (5).

As in mammals, GIP is encoded by *GIP* gene and GCG, GLP1 and GLP2 by glucagon gene(s) in fish and birds (1, 17–21), and the actions of the four peptides are also likely mediated by the specific or predicted potential receptors for GCG, GLP1, GLP2, and GIP (19, 22–27), although the actions of some peptides differ significantly between different groups of vertebrates (28, 29), such as the opposite actions of GLP1 in controlling glucose metabolism between fish and mammals (28, 30).

Strikingly, in addition to the identification of the four peptides and their potential specific receptors in chickens noted above by us and other laboratories (19, 24, 25, 31), we have identified a novel ligand-receptor pair, named GCGL and GCGL receptor (GCGLR) in chickens in the present study. GCGL is a novel GCG-like peptide of 29 amino acids that shares high amino acid sequence similarity to mammalian and chicken GCG (62–66%) (named GCG2 in GenBank, EU718628). GCGLR is a GCGL-specific receptor (EU718627) (2008), which shares relatively high amino acid sequence identity to chicken GCGR and its structurally related receptors (GLP1R, GIPR, and GLP2R). Interestingly, *GCGL* and *GCGLR* genes were also identified in genomes of other nonmammalian vertebrate species including teleost fish. Similar to our findings, recently, *GCGL* and *GCGLR* genes were also predicted to exist in the genomes of some nonmammalian vertebrates by Irwin and Prentice (2011), and the *GCGL* was named as exendin gene and *GCGLR* as glucagon receptor-like receptor (*GRLR*); however, the two genes have not yet been functionally characterized (32). The functional characterization of *GCGL* and *GCGLR* genes in chickens, together with the identification of *GCGL* and *GCGLR* genes in nonmammalian vertebrates including teleost fish in the present study, not only raises the first clear and important concept that this novel ligand-receptor pair may exist and function in nonmammalian vertebrates (confirmed at least in chickens), but also provides critical clues to the evolutionary history of vertebrate *GCG*, *GIP*, and *GCGL* genes. The major content of this work was presented in The Plant and Animal Genomes XVIII Conference (Jan. 9–13, 2010, San Diego, CA) (33).

Materials and Methods

Chemicals and hormones

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO), and restriction enzymes were obtained from Amersham Biosciences (GE Healthcare Bio-Sciences Corp, Piscataway, NJ) unless stated otherwise. H89 was purchased from Calbiochem (Merck KGaA, Darmstadt, Germany), and human (h) GCG was purchased from Bachem (Bachem, Inc. Torrance, CA). Chicken GCGL (cGCGL), GLP1 ($cGLP1_{7-36NH2}$), GLP2 (cGLP2), GIP (cGIP), secretin (cSCT), VIP (cVIP), and Gila monster exendin-4 were synthesized using solid-phase Fmoc chemistry (GL Biochem, Shanghai, China). The purity of synthesized peptides is greater than 95% (analyzed by HPLC), and their structure was verified by mass spectrometry (GL Biochem).

Total RNA extraction

Adult chickens were killed and different tissues were collected and stored at −80 C until used. Total RNA was extracted from chicken tissues with TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions and resuspended in diethylpyrocarbonate-treated H_2O. The experiments were carried out according to the guidelines provided by the Animal Ethics Committee of Sichuan University.

Cloning the full-length cDNAs of chicken glucagon-like gene (*GCGL*) and GCGL receptor (*GCGLR*) from adult chicken brain

Using chicken *GCG* and *GCGR* sequence as references, we searched the chicken genome database using BlastX and obtained the partial genomic sequences of chicken *GCGL* and *GCGLR*, after which gene-specific primers were then designed to amplify 5′-cDNA ends of *GCGL* and *GCGLR* genes from adult chicken brain using SMART-RACE (rapid amplification of cDNA ends) cDNA Amplification (RACE) Kit (CLONTECH Laboratories, Inc., Palo Alto, CA) (Supplemental Table 1 published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). The amplified PCR products were cloned into pBluescript SK(+/−) vector (Stratagene, La Jolla, CA) through T/A cloning and sequenced by Genetic Analyzer ABI3100 (PerkinElmer, Foster City, CA). Based on these cDNA sequences, new primers were designed to amplify the full-length cDNAs of *cGCGL* and *cGCGLR* either by 3′-RACE or by RT-PCR from chicken brain. The amplified PCR products were cloned and sequenced. Finally, the full-length cDNAs of two genes were determined by sequencing at least three independent clones.

Reverse transcription (RT) and PCR (RT-PCR)

RT was performed at 42 C for 2 h in a total volume of 10 μl consisting of 2 μg total RNA from chicken tissues, 1× Single-Strand Buffer, 0.5 mM each deoxynucleotide triphosphate, 0.5 μg oligo-deoxythymidine, and 100 U moloney murine leukemia virus reverse transcriptase (Promega Corp., Madison, WI). All negative controls were conducted under the same condition without reverse transcriptase added.

RT-PCR assays were performed to examine mRNA expression of *GCGL* and *GCGLR* in chicken tissues according to our previously established method (34). For β-actin gene, 23 cycles of 30 sec at 95 C, 30 sec at 58 C, and 60 sec at 72 C were used followed by a 5-min extension at 72 C. For *cGCGL* and *cGCGLR* genes, 30 (or 35) cycles of 30 sec at 95 C, 30 sec at 60 C, and 45 sec at 72 C were used followed by a 5-min extension at 72 C. The primers used were listed in Supplemental Table 1. The PCR products were visualized on a UV-transilluminator (Bio-Rad Laboratories, Inc. Hercules, CA) after electrophoresis on 2% agarose gel containing ethidium bromide. To confirm the specificity of PCR, the identity of PCR products was verified by sequencing.

Functional characterization of chicken *GCGL* and *GCGLR* in cultured Chinese hamster ovary (CHO) cells

Based on the cloned cDNA sequence of chicken *GCGLR*, gene-specific primers were used to amplify the open reading frame from adult chicken brain using high-fidelity *Taq* DNA polymerase (Roche Diagnostics, Basel, Switzerland) (Supplemental Table 1). The amplified PCR products were cloned into the pcDNA3.1 (+) expression vector (Invitrogen). The expression plasmids for chicken GCGR (cGCGR), GLP1R, VIP type I receptor (cVPAC$_1$), two GHRH receptors (cGHRHR1 and cGHRHR2), and secretin receptor (cSCTR) were constructed as described in our previous studies (24, 25, 35–37).

To determine whether cGCGL$_{1–29}$ could activate the mammalian GLP1R as potently as exendin 4, the expression plasmid of pig GLP1 receptor (pGLP1R) was also constructed and used in this study (Supplemental Fig. 1).

According to our previously established method, the functionality of cGCGL and cGCGLR, was examined in CHO cells by a system of cotransfected pGL3-CRE-luciferase reporter construct and receptor expression plasmid (35). In brief, CHO cells were cultured in DMEM supplemented with 10% (vol/vol) fetal bovine serum (HyClone Laboratories, Inc., Logan, UT) in a six-well plate at a density of 3×10^5 cells per well 1 d before transfection. At 70% confluency, cells were transiently cotransfected with 700 ng of pGL3-CRE-luciferase reporter construct and 200 ng of pcDNA3.1 expression plasmid (or empty vector) using Lipofectamine (Invitrogen). After 24 h of culture, transfected cells were trypsinized and cultured in a 96-well plate at a density of 2×10^4 cells per well at 37 C for 24 h before peptide treatment. After removal of medium from a 96-well plate, cells were treated with serum-free DMEM containing indicated concentrations of peptide (or peptide-free medium) for 6 h at 37 C before being harvested for luciferase assay. After removal of culture medium, CHO cells were lysed by adding 50 μl of 1×Passive Lysis Buffer (Promega) per well, and the luciferase activity of 15 μl of cellular lysates was determined using luciferase assay reagent (Promega).

Data analysis

The luciferase activities in each treatment group were expressed as relative fold increase as compared with the control group (without peptide treatment). The data were analyzed by one-way ANOVA followed by Dunnett's test using GraphPad Prism 4 (GraphPad Software, San Diego, CA). To validate our results, all experiments were repeated at least two to three times.

Results

Discovery of a novel GCG-like gene (*GCGL*) in chickens

During the process of functional characterization of glucagon receptor (GCGR) and its ligand in chickens (24), we searched the chicken genome database (http://www.ensembl.org) using chicken glucagon cDNA sequence (Accession no. S78477) as a reference and identified a genomic sequence on the chromosome E22C19W28_E50C23, which may encode a novel glucagon-like peptide (named GCGL in this study). Then, we cloned the full-length cDNA of *GCGL* gene from chicken brain using the RACE method. The cloned GCGL (or named GCG2 in GenBank) is 731 bp in length and encodes a precursor of 81 amino acids (EU718628) (Fig. 1 and Supplemental Fig. 2). The putative GCGL peptide is flanked by dibasic residues (KR) at its N and C terminals characteristic of cleavage sites for prohormone convertases (38), and thus the mature GCGL peptide is predicted to be 29 amino acids (cGCGL$_{1–29}$). Sequence alignment showed that cGCGL$_{1–29}$ shares relatively high amino acid sequence identity (62%–66%) to glucagon (GCG$_{1–29}$) and a comparatively lower identity to GLP1 (52–55%) and GLP2 (34–62%) of birds and mammals (1). Moreover, cGCGL$_{1–29}$ shares a relatively higher degree of sequence identity to the two fish GCGs (GCGa and GCGb) (55–59%) than to fish GLP1s (45–48%) and GLP2 (41%) (28). cGCGL$_{1–29}$, likewise, shares relatively higher amino acid sequence identity to lamprey GCG (66%) (AF159707) than to lamprey GLP1 (55%) (39, 40). In addition, cGCGL$_{1–29}$ also shares a certain degree of amino acid sequence identity with the first 29 amino acids of vertebrate GIP (31–45%), exendin-3 (41%) of Mexican beaded lizard (*Heloderma horridum*) (AJ580309), and exendin-4 (41%) of Gila monster (*Heloderma suspectrum*) (U77613) (Fig. 1) (41–43). In contrast, a low degree of identity (25–30%) was noted between cGCGL$_{1–29}$ and other members in glucagon/secretin superfamily including VIP, PACAP, GHRH, and secretin (37, 44). According to the relatively high amino acid sequence identity of GCGL$_{1–29}$ to GCG, this novel glucagon-like peptide is designated as GCGL and the gene encoding this peptide is named *GCGL* gene in this study.

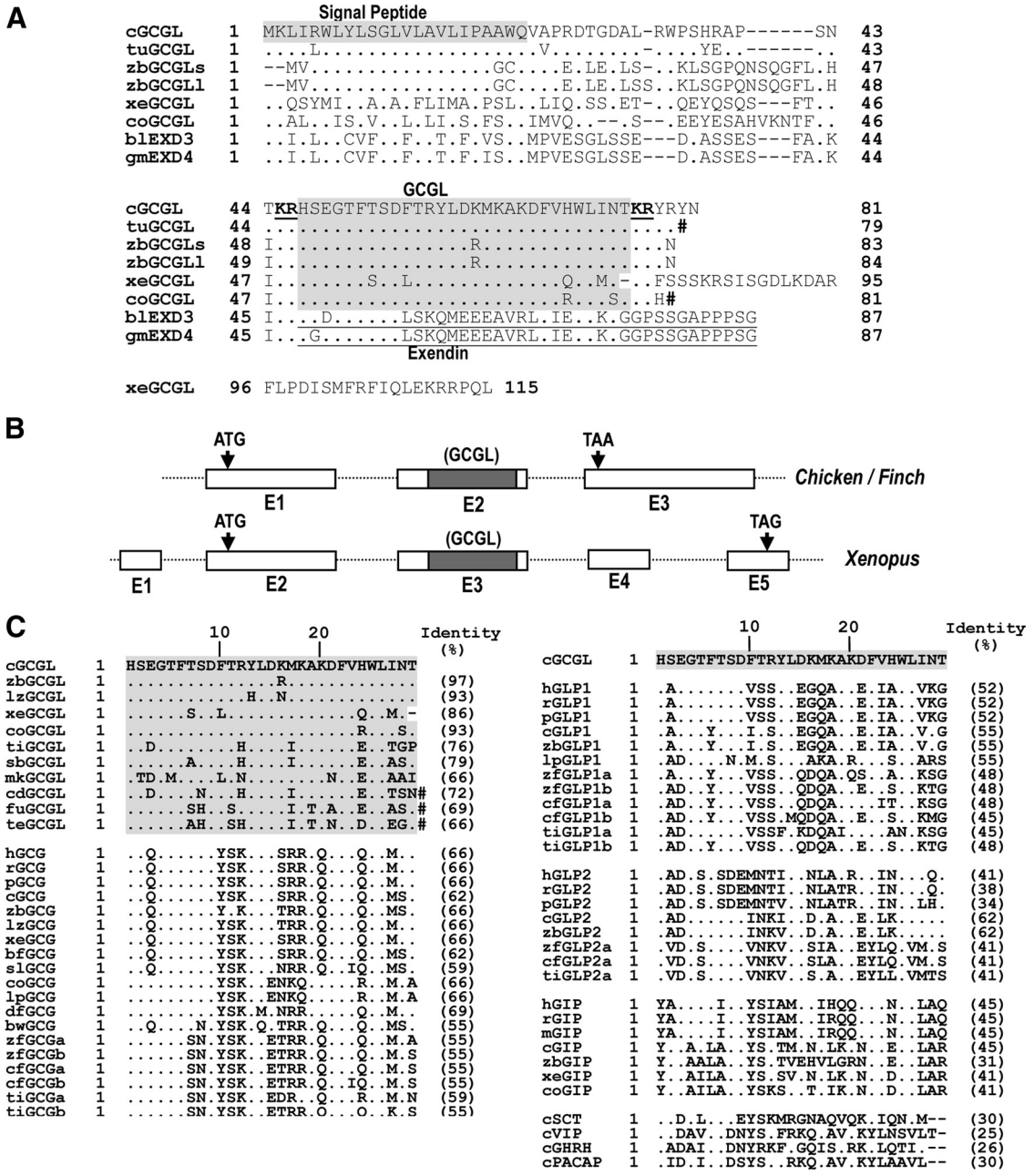

FIG. 1. A**,** Amino acid sequence alignment of chicken GCGL precursor (cGCGL: EU718628) with that of turkey (tuGCGL), zebra finch (zbGCGLs: short form, JQ689171; zbGCGLl: long form, JQ689170), *X. tropicalis* (xeGCGL: CN072883), coelacanth (coGCGL), or with that of exendin-3 from Mexican beaded lizard (blEXD3, AJ580309) and exendin-4 from Gila monster (gmEXD4, U77613). Turkey GCGL was predicted according to the genomic sequence on chromosome 4 and coelacanth GCGL according to the genomic sequence on Scaffold_JH126563; the mature GCGL peptide is *shaded*; the putative dibasic amino acid recognition sites (KR) for proteolytic cleavage are *bold* and *underlined*; *dots* indicate amino acids identical to chicken GCGL precursor, and *dashes* represent gaps in the sequence. The peptide sequences of exendin-3 and -4 are *underlined*. # indicates that C-terminal sequences of coelacanth and turkey GCGL precursor have not been determined. B, Exon (E) organization of *GCGL* gene in chicken, zebra finch, and *X. tropicalis*. The regions coding for mature GCGL peptide are *shaded*. *Arrows* indicate the putative start and stop codon. C, Amino acid sequence alignment of chicken GCGL ($cGCGL_{1-29}$) with GCGL or other structurally related peptides, including GCG, GLP1, GLP2, GIP, SCT, VIP, PACAP, and GHRH from chicken (c) and other species including zebra finch (zb), anole lizard (lz), *X. tropicalis* (xe), coelacanth (co), Nile tilapia (ti), Stickleback (sb), medaka (mk), cod (cd), *Takifugu rubripes* (fu), Tetraodon (te), human (h), rat (r), pig (p), bullfrog (bf), salamander (sl), sea lamprey (lp), dogfish (*df*), bowfin (bw), zebrafish (zf), and catfish (*cf*). The amino acid sequences of GCGL and its structurally related peptides were either retrieved from GenBank or predicted according to genomic sequences (http://www.ensembl.org) (see Supplemental Table 2) or retrieved from an review article (28). *Dots* indicate amino acids identical to chicken GCGL peptide, and *dashes* represent gaps in the sequence. # indicates that C-terminal sequences of GCGL peptide from these species have not been determined.

Identification of glucagon-like gene (*GCGL*) in other nonmammalian vertebrates

To determine whether *GCGL* gene exists in other vertebrate species, we used chicken GCGL cDNA as a reference and performed a search in the genome databases (http://www.ensembl.org) of different classes of vertebrate species including humans, zebra finch, turkeys, anole lizards, *Xenopus tropicalis*, cods, Nile tilapia, stickleback, fugu, tetraodon, medaka, and coelacanth. *GCGL* gene could be identified in all nonmammalian species examined, and the predicted GCGL peptides share high degree of amino acid sequence identities to $cGCGL_{1–29}$ (66–97%), although the peptide length of GCGL appears to vary between species (Fig. 1 and Supplemental Fig. 3 and Supplemental Table 2). In contrast, *GCGL* gene could not be identified in human and other mammalian genomes.

To confirm the expression of *GCGL* in other species, we also successfully cloned cDNAs of *GCGL* from zebra finch brain (JQ689170; JQ689171) (Fig. 1 and Supplemental Fig. 4) and identified two EST sequences of *Xenopus GCGL* deposited in GenBank (CN072882; CN072883). These findings further confirmed the existence and expression of *GCGL* gene in other nonmammalian vertebrate species.

Discovery of a novel GCGLR in chicken and nonmammalian vertebrates

The identification of novel GCGL peptide in all nonmammalian vertebrates led us to hypothesize that GCGL may have its specific receptor. To test this possibility, using chicken GCGR as a reference, we searched the chicken genome databases and identified a genomic sequence encoding a putative novel GCGR-like receptor on an unknown chromosome (Un_random:20427245:20427988:1). Then, we cloned the full-length cDNA of this receptor from adult chicken brain. The cloned receptor is 1458 bp in length and encodes a protein of 430 amino acids (EU718627). It shares high amino acid identity not only to GCGR (53–55%) of chicken and human, but also to GLP1R (48–53%) and GIPR (48–49%) of mammals. According to the pharmacological property examined in the following section, this novel receptor is designated as GCGL-specific receptor (GCGLR) in this study. Like other members in GPCR B1 family, chicken GCGLR has a large extracellular domain characterized by the presence of six conserved cysteine residues, three putative *N*-linked glycosylation sites, seven transmembrane domains, and an intracellular carboxyl terminus (Fig. 2). Moreover, the other characteristic residues and motif known to be shared by GPCR B1 family members, such as RLAK motif for G protein-coupling, were noted in GCGLR, clearly indicating that it should be classified as a novel member in this subfamily (5, 45).

To determine whether *GCGLR* gene exists in other vertebrate species, we further searched the genome databases of human, anole lizard, *X. tropicalis*, tetraodon, medaka, stickleback, Nile tilapia, fugu, and coelacanth. As expected, GCGLR, which shares high amino acid sequence identity (63–86%) to chicken GCGLR, was identified in all the nonmammalian species examined (Fig. 2 and Supplemental Fig. 5), suggesting the existence of *GCGLR* gene in nonmammalian vertebrates. In contrast, *GCGLR* gene could not be identified in human and other mammalian genomes.

Functional characterization of chicken GCGL and GCGLR

To determine whether $cGCGL_{1–29}$ is a bioactive peptide and cGCGLR is a functional receptor, cGCGLR and its structurally related receptors for chicken GCG, GLP1, VIP, GHRH, and SCT was transiently expressed in CHO cells and subjected to treatment with $cGCGL_{1–29}$ and its related peptides, including chicken GLP1, GLP2, GIP, VIP, SCT and human GCG. Receptor activation was then monitored by a pGL3-CRE-luciferase reporter system established in our previous study (35).

Experiment 1: $cGCGL_{1–29}$ can potently activate cGCGLR expressed in CHO cells

As shown in Fig. 3A, synthetic $cGCGL_{1–29}$ could stimulate luciferase activity of CHO cells via activation of cGCGLR in a dose-dependent manner, and the EC_{50} value is 0.10 nM. Although $cGCGL_{1–29}$ could activate cGLP1R expressed in CHO cells, its potency (EC_{50}, 32.0 nM) is nearly 320-fold lower in activating cGLP1R than cGCGLR. In contrast, $cGCGL_{1–29}$ failed to activate chicken glucagon receptor (cGCGR) (24), VIP type I receptor ($cVPAC_1$) (36), two GHRH receptors (cGHRHR1 and cGHRHR2) (36), and secretin receptor (cSCTR) at any concentration tested (10^{-12} to 10^{-6} M) (37). These findings clearly indicated that $cGCGL_{1–29}$ can act as a potent ligand for cGCGLR.

Experiment 2: GCGLR is a receptor specific to $cGCGL_{1–29}$

To determine whether cGCGLR is a receptor specific to $cGCGL_{1–29}$, cGCGLR expressed in CHO cells was subjected to treatment with cGCGL and its structurally related peptides, including GCG, GLP1, GLP2, GIP, VIP, and SCT. As shown in Fig. 4A, cGCGLR could be activated not only by cGCGL, but also by human GCG (hGCG), cGLP1, cGLP2, and cGIP. The order of the potency is $cGCGL_{1–29}$ (EC_{50}, 0.10 nM) $\gg$ hGCG (EC_{50},

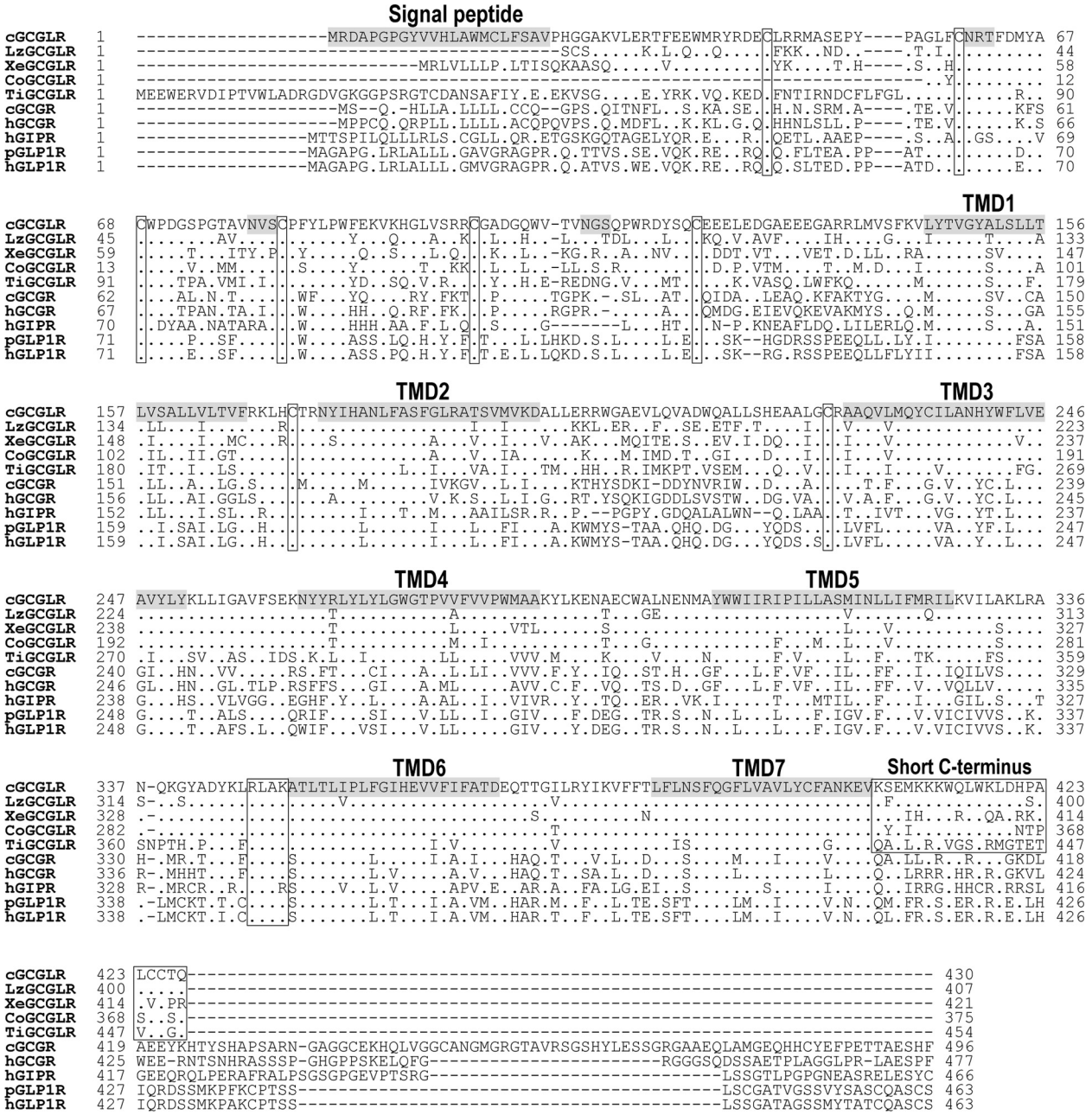

FIG. 2. Amino acid sequence alignment of chicken GCGLR (cGCGLR: EU718627) with that of Anole lizard (lzGCGLR: XP_003227767), *X. tropicalis* (XeGCGLR: XP_002932473), coelacanth (coGCGLR: ENSLACP00000003585), Nile tilapia (TiGCGLR: ENSGACP00000012006), or with human (hGCGR: NP_002053) and chicken GCGR (cGCGR: EF624352), or with human GIPR (hGIPR: NP_0001555), or with pig (pGLP1R: JQ085965) and human GLP1R (hGLP1R: NP_000151). The seven transmembrane domains (TMD) are *shaded* and labeled accordingly; the conserved cysteine residues are *boxed*; the putative *N*-linked glycosylation sites (NXT/S, where X represents any amino acid residue except proline) are *shaded*; the RLAK (or RLAR) motif for G protein coupling in the third intracellular loop is *boxed*; the short intracellular carboxyl terminus of GCGLR is *boxed*; and *dots* indicate amino acids identical to cGCGLR, and *dashes* represent gaps in the sequence.

32.39 nM) ≈ cGLP1 (EC_{50}, 35.28 nM) ≫ cGLP2 (EC_{50}, >100 nM) > cGIP. Because $cGCGL_{1–29}$ is 300-fold more potent in activating cGCGLR than any other peptide tested, it strongly suggests that cGCGLR is a receptor specific to cGCGL.

Despite the high degree of structural similarity noted between cGCGLR and cGCGR, cGCGR, unlike cGCGLR, could be activated only by hGCG (Fig. 4B), indicating that cGCGR is a receptor highly selective to glucagon.

Experiment 3: cGCGLR is functionally coupled to intracellular protein kinase A (PKA) signaling pathway

To determine whether cGCGLR is functionally coupled to intracellular protein kinase A (PKA) signaling pathway, as other members in the GPCR B1 subfamily (5),

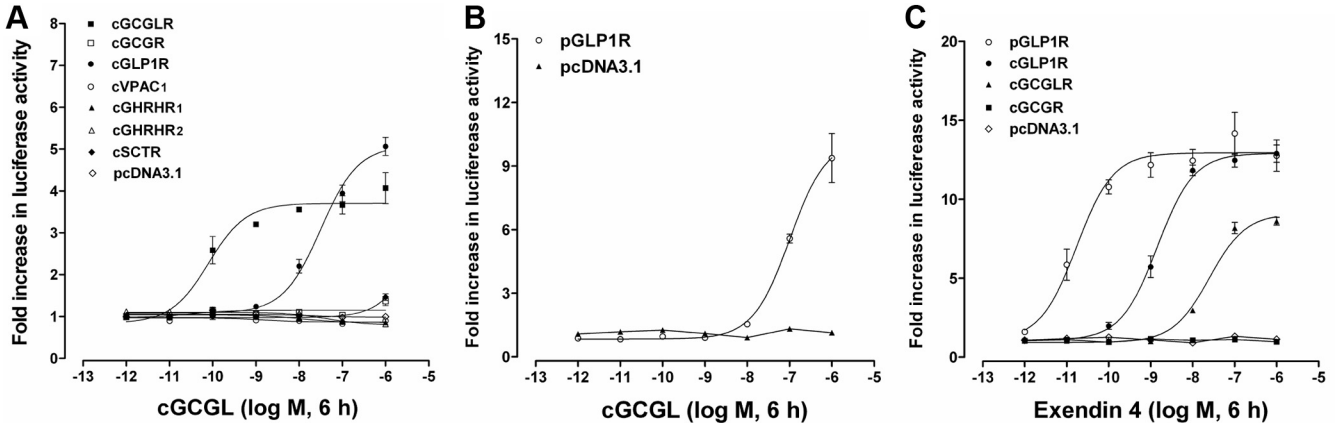

FIG. 3. A, Activation of cGCGLR, cGCGR, cGLP1R, cVPAC$_1$, cGHRHR$_1$, cGHRHR$_2$, and cSCTR upon chicken GCGL (cGCGL$_{1-29}$, 10^{-12} to 10^{-6} M, 6 h) treatment, monitored by a system of cotransfection of pGL3-CRE-luciferase reporter construct and receptor expression plasmid in cultured CHO cells. The functionality of cGCGR, cGLP1R, cVPAC$_1$, cGHRHR$_1$, cGHRHR$_2$, and cSCTR used in this experiment was reported in our previous studies (24, 25, 35–37). B, Activation of pig GLP1R (pGLP1R) upon cGCGL treatment (cGCGL$_{1-29}$, 10^{-12} to 10^{-6}M, 6 h), monitored by a system of cotransfection of pGL3-CRE-luciferase reporter construct and receptor expression plasmid in cultured CHO cells. C, Activation of pig GLP1R (pGLP1R), chicken GLP1R (cGLP1R), chicken GCGLR (cGCGLR), and chicken GCGR (cGCGR) upon exendin-4 treatment (exendin-4, 10^{-12} to 10^{-6} M, 6 h), monitored by a system of cotransfection of pGL3-CRE-luciferase reporter construct and receptor expression plasmid in cultured CHO cells. Cotransfection of the empty pcDNA3.1 vector and pGL3-CRE-luciferase reporter construct was used as an internal control in the three experiments, and peptide treatment did not increase the luciferase activity of CHO cells at any concentration tested. Each data point represents mean ± SEM of three replicates.

cGCGLR activation was examined in the presence or absence of H89, a specific PKA inhibitor. As shown in Fig. 4C, H89 (10 μM) treatment could completely abolish cGCGL (10 nM)-induced luciferase activity of CHO cells, confirming that cGCGLR expressed in CHO cells is functionally coupled to the intracellular PKA signaling pathway.

Experiment 4: exendin-4 is potent in activating pig GLP1R, but not cGCGLR

The structural similarity shared between chicken GCGL and exendin-4 of Gila monster (or exendin-3 from beaded lizard) points to a possibility that exendin(s) identified in reptiles may function as a potential ligand for GCGLR (Fig. 1). To test this, cGCGLR expressed in CHO

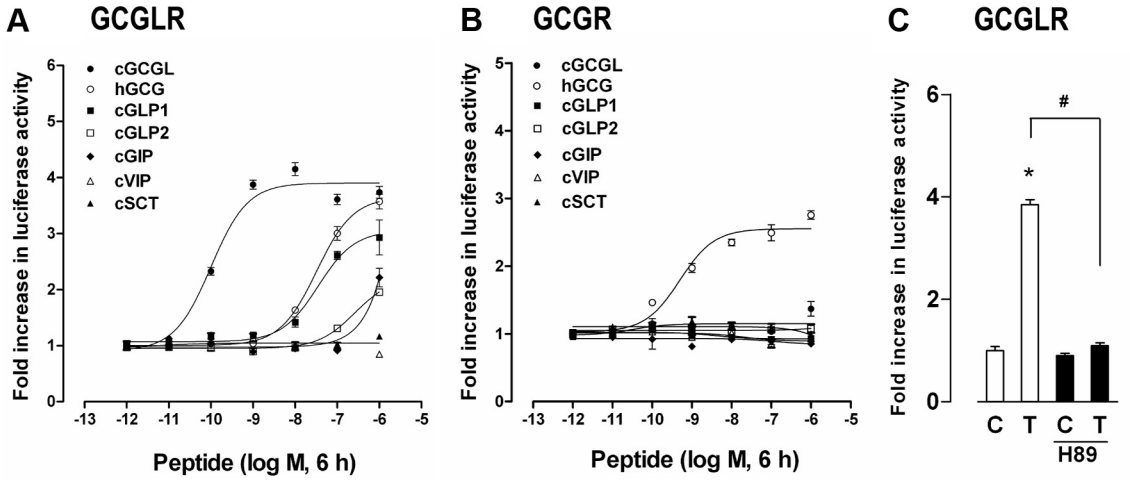

FIG. 4. A, Activation of chicken GCGLR or B**)** activation of chicken GCGR by GCGL and its structurally related peptides including human GCG (hGCG), chicken GLP1 (cGLP1), chicken GLP2 (cGLP2), chicken GIP (cGIP), chicken VIP (cVIP), and chicken secretin (cSCT) (10^{-12} to 10^{-6}M, 6 h), monitored by a system of cotransfection of pGL3-CRE-luciferase reporter construct and receptor expression plasmid in cultured CHO cells; cotransfection of the empty pcDNA3.1 vector and pGL3-CRE-luciferase reporter construct was used as an internal control, and each peptide treatment did not increase the luciferase activity of CHO cells at any concentration tested (data not shown). Each data point represents mean ± SEM of three replicas. C, Effects of H89 (10 μM) on cGCGL$_{1-29}$ (10 nM, 6 h)-induced luciferase activities of CHO cells cotransfected with pGL3-CRE-luciferase reporter construct and chicken GCGL receptor (cGCGLR) expression plasmid. H89 was added 1 h before peptide treatment. In this graph, T represents peptide treatment and C represents control without peptide treatment. Each data point represents mean ± SEM of three replicates. *, $P \leq 0.01$ *vs.* control (in the absence of H89); #, $P \leq 0.01$ *vs.* peptide treatment (in the presence of H89).

cells was treated with exendin-4, and pig and chicken GLP1R was used as controls. As shown in Fig. 3, exendin-4 could potently activate pig GLP1R (pGLP1R) with an EC_{50} value of 0.017 nM, and it also activated chicken GLP1R with an EC_{50} value of 1.44 nM. In contrast, exendin-4 could only activate cGCGLR with a much lower potency (EC_{50}, 24.97 nM).

To determine whether $cGCGL_{1–29}$ could activate pig GLP1R as potently as exendin-4, we examined the potency of $cGCGL_{1–29}$ in activating pGLP1R expressed in CHO cells. As shown in Fig. 3, $cGCGL_{1–29}$ could activate the pGLP1R only at higher concentrations (≥100 nM).

These findings clearly indicate that exendin-4 of Gila monster is functionally distinct from $cGCGL_{1–29}$, and cGCGLR also cannot act as a receptor specific to exendin-4.

Tissue distribution of chicken *GCGL* and *GCGLR* mRNA

To elucidate the physiological roles of GCGL and GCGLR in chickens, using RT-PCR, we examined the mRNA expression of *GCGL* and *GCGLR* in adult chicken tissues including heart, duodenum, kidney, liver, lung, muscle, ovary, pituitary, spleen, testes, pancreas, spinal cord, and various regions of brain including telencephalon, midbrain, cerebellum, hindbrain, and hypothalamus. As shown in Fig. 5, strong PCR signal could be detected only in the testes, hypothalamus, and hindbrain of different chicken individuals when low PCR cycle was used, suggesting a comparatively high mRNA expression level of *GCGL* in these chicken tissues. When PCR cycles were increased to 35 cycles, moderate PCR signal was detected in spinal cord and other parts of brain, and very weak PCR signal in duodenum and ovary, whereas no PCR signal was detected in other tissues.

Unlike the restricted expression of *cGCGL* in chicken tissues, *GCGLR* mRNA was detected to be widely expressed in most chicken tissues examined except heart, liver, and muscle. Interestingly, strong PCR signal was consistently noted in the pituitary and spinal cord, as well as in nearly all brain regions. In contrast, only very faint PCR signal was detected in duodenum and spleen (Fig. 5).

In this study, we also examined the mRNA expression of *GCGL* and *GCGLR* genes in other parts of the gastrointestinal tract, including proventriculus, gizzard, jejum, ileum, cecum, and colon. No PCR signal was detected in other parts of the gastrointestinal tract (data not shown).

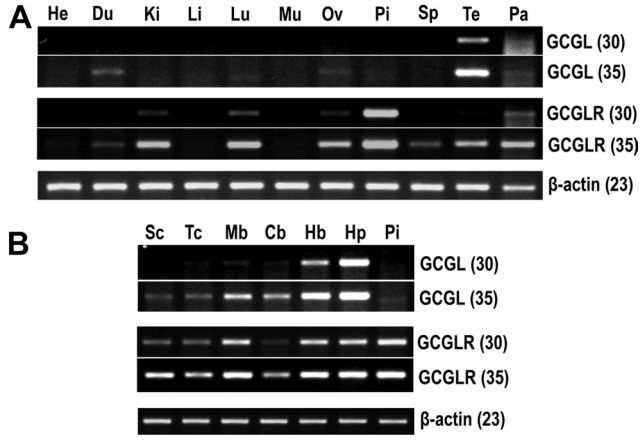

FIG. 5. A, RT-PCR detection of the mRNA expression of *GCGL* and *GCGLR* in adult chicken tissues including heart (He), duodenum (Du), kidneys (Ki), liver (Li), lung (Lu), muscle (Mu), ovary (Ov), pituitary (Pi), spleen (Sp), testes (Te) and pancreas (Pa). B, RT-PCR detection of *GCGL* and *GCGLR* mRNA expression in adult chicken pituitary (Pi), spinal cord (Sc) and various brain regions including telencephalon (Tc), midbrain (Mb), cerebellum (Cb), hindbrain (Hb), and hypothalamus (Hp). *Number in bracket* indicates the number of PCR cycles used.

GCGL and *GCGLR* are located in distinct syntenies conserved between chicken and other nonmammalian vertebrate species

To trace evolutionary history of *GCGL* and *GCGLR* genes in vertebrates, we performed synteny analyses by searching the gene information flanking *GCGL* and *GCGLR* genes in genomes of chicken (or turkey), human, *X. tropicalis*, coelacanth, and Nile tilapia. *GCGL* gene was found to be located in a synteny conserved in nonmammalian vertebrates (Fig. 6). In contrast, *GCGL* gene could not be identified in human genome, although its neighboring genes could be identified on human chromosome 12, suggesting that it may have been lost in mammals. Similarly, *GCGLR* gene was also found to be located in a synteny conserved in all nonmammalian species examined, but it seemed to be lost in mammals as indicated by synteny analysis (Fig. 7).

Discussion

In this study, a novel GCG-like peptide, GCGL, and its specific functional receptor (GCGLR) have been identified from chicken brain. RT-PCR assay revealed that *GCGL* mRNA expression is mainly restricted to several tissues including the CNS, whereas *GCGLR* is abundantly expressed in the CNS and pituitary. Moreover, we also provided solid evidence that *GCGL* and *GCGLR* genes exist in nonmammalian vertebrates including teleost fish. To our knowledge, our study represents the first to identify and functionally characterize this novel ligand-receptor pair (GCGL-GCGLR) in vertebrates (33).

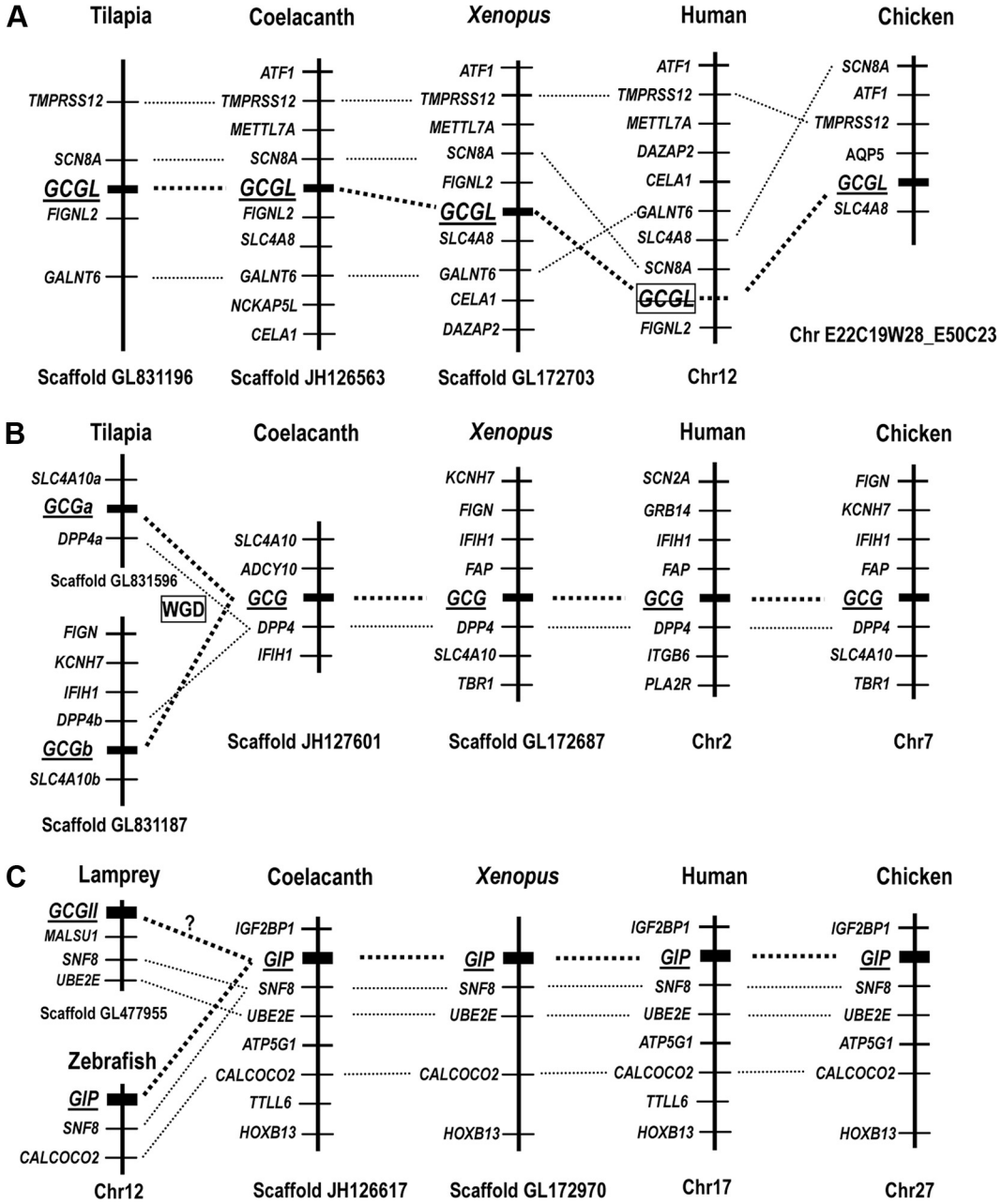

FIG. 6. A, *GCGL* is located in a synteny conserved between chicken, *X. tropicalis*, coelacanth, and tilapia. *Dotted lines* indicate the syntenic genes identified in these species. *GCGL* gene could not be identified in humans; however, the neighboring genes flanking chicken and *Xenopus GCGL* gene could be found in human chromosome 12, implying that *GCGL* gene may have been lost in the mammalian lineage during evolution. B and C, *GCG* and *GIP* genes are located in the other two distinct syntenies conserved between chickens, humans, *X. tropicalis*, coelacanth, and tilapia (or zebrafish). The two glucagon genes (*GCGa* and *GCGb*) identified in tilapia are likely generated by a whole genome duplication event (WGD) occurred in the teleost lineage. *Dotted lines* indicate the syntenic genes identified in these species. Interestingly, the *SNF8* and *UBE2E* genes adjacent to the lamprey *GCGII gene* (AF159708) were also identified in a conserved synteny containing *GIP* gene of human, chicken, *Xenopus*, coelacanth, and zebrafish, suggesting that the lamprey *GCGII* gene may have a much closer evolutionary relationship to vertebrate *GIP* gene than to glucagon/*GCGL* gene. However, whether vertebrate *GIP* gene is orthologous, or paralogous, to lamprey glucagon II gene requires further investigation. Genes were named based on their annotations in the human genome. Chr, Chromosome.

Identification of the novel *GCGL* gene and its implications for the evolutionary history of *GCG*, *GCGL*, and *GIP* genes in vertebrates

In this study, we identified a novel *GCGL* gene from chicken brain. It encodes a GCG-like peptide (named GCGL), which shares high amino acid sequence identity (62–66%) with chicken and mammalian GCGs and certain degree of amino acid sequence identity with other structurally related peptides, including vertebrate GLP1 (45–55%), GLP2 (34–62%), and GIP (31–45%), and reptilian exendin-3/4 (41%). Despite the high degree of sequence identity shared between cGCGL and cGCG, the

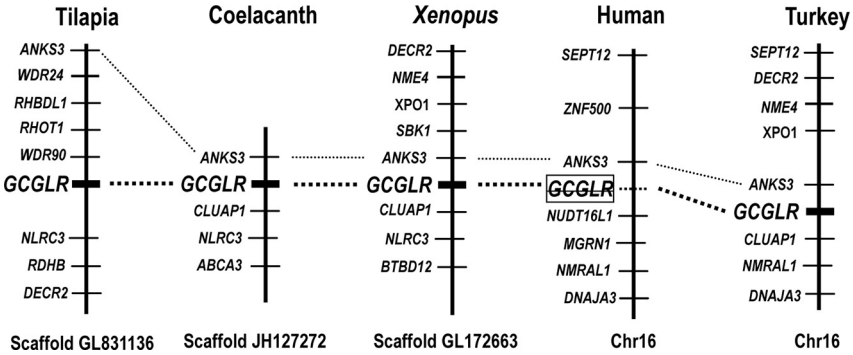

FIG. 7. *GCGLR* is located in a synteny conserved between turkeys, *X. tropicalis*, coelacanth, and tilapia. *Dotted lines* indicate the syntenic genes identified in these species. *GCGLR* gene could not be identified in humans; however, the neighboring genes of turkey, coelacanth, tilapia, and *Xenopus GCGLR* gene could be found in human chromosome 16, implying that *GCGLR* gene may have been lost in the mammalian lineage during evolution. Genes were named based on their annotations in the human genome. Chr, Chromosome.

exon composition of chicken *GCGL* and *GCG* genes is different. The coding region of *GCG* gene consists of six exons and encodes three biologically active peptides: GCG, GLP1, and GLP2 (17, 19), whereas *GCGL* gene consists of three exons in chickens (or four exons in *X. tropicalis*) and encodes a single bioactive peptide (GCGL) (Fig. 1B). The exon composition of GCGL-coding region is also different from that of *GIP* gene, which consists of six exons in chickens and mammals (1). Although the exon numbers of chicken *GCG*, *GCGL*, and *GIP* genes seem to be different, the first two exons of three genes within the coding region are organized in a highly similar fashion. The first exon encodes a signal peptide, and the second exon encodes the bioactive peptide (GCG, GCGL or GIP), and both exons are separated by a phase 2 intron (Supplemental Figs. 2–4).

The relatively high structural similarity between GCGL and GCG (/GIP) in chickens also led us to determine whether *GCGL* gene is generated by a duplication of *GCG* or *GIP* gene occurred in the avian lineage, or originated in the early history of vertebrate evolution. We searched the genome database from different groups of vertebrate species and identified the *GCGL* gene in all nonmammalian species examined, clearly indicating that *GCGL* gene already appeared before tetrapod/teleost split. This conclusion was further supported by synteny analysis. *GCGL* gene was located in a synteny conserved in tilapia, coelacanth, *Xenopus*, and chicken, whereas *GCG* and *GIP* genes are located in their two respective conserved syntenies (Fig. 6).

It has been hypothesized that *GCG* and *GIP* genes are originated by a gene duplication event (1, 2, 46). The identification of an extra *GCGL* gene in vertebrates substantiates the need to modify this hypothesis. Because three genes could be identified in different vertebrate classes including teleost fish, coelacanth, and tetrapods, it suggests that the three genes must have already appeared in the last common ancestor of teleost and tetrapods. The ancient origin of the three genes, together with the high degree of amino acid sequence identity they shared, as well as their distinct chromosome localization (Fig. 6), also led us to speculate that *GCG*, *GCGL* and *GIP* were likely originated from an ancestral GCG-like gene, which experienced two successive rounds of genome duplication events (2R hypothesis) occurred in the early history of vertebrate evolution (Fig. 8) (33, 47), although the existence of an extra GCG-like gene in vertebrates remains to be confirmed. Our hypothesis (33) is also consistent with the idea proposed by Irwin and Prentice (32).

Using synteny analysis, we also noted that *FIGNL2* and *SLC4A8* flanking *Xenopus GCGL* gene are highly homologous to *FIGN* and *SLCA10* genes which flank *Xenpous GCG* gene. This finding further supports that *GCGL*, *GCG*, and their neighboring genes are likely originated by a large-scale chromosomal duplication event or even a genome duplication event; presumably the second round of genome duplication event occurred in the early history of vertebrate evolution (Fig. 8).

It was reported that two GCG-like genes, namely glucagon I (AF159707) and glucagon II (AF159708), were identified in sea lampreys, the most ancient extant vertebrate species (39). Interestingly, using synteny analysis, we also noticed that ESCRT-II complex subunit, homolog (*S. cerevisiae*) (*SNF8*) and ubiquitin-conjugating enzyme E2Z (*UBE2E*) genes adjacent to lamprey glucagon II gene located on lamprey scaffold GL477955 are highly homologous (81–89% amino acid identity) to vertebrate *SNF8* and *UBE2E* genes adjacent to *GIP* gene, respectively (Fig. 6), suggesting that lamprey glucagon II gene may have a much closer evolutionary relationship to vertebrate *GIP* than to glucagon or *GCGL* gene (Fig. 6). However, whether vertebrate *GIP* gene is orthologous, or paralogous, to lamprey glucagon II gene requires further investigation.

Identification of a novel *GCGLR* gene in nonmammalian vertebrates

Using chicken GCGR as a reference, we searched the chicken genome and identified a novel GCGL-specific receptor. Chicken GCGLR shares high structural similarity (48–55% identity) with the receptors for GCG, GIP, GLP1, and GLP2, clearly indicating their close evolution-

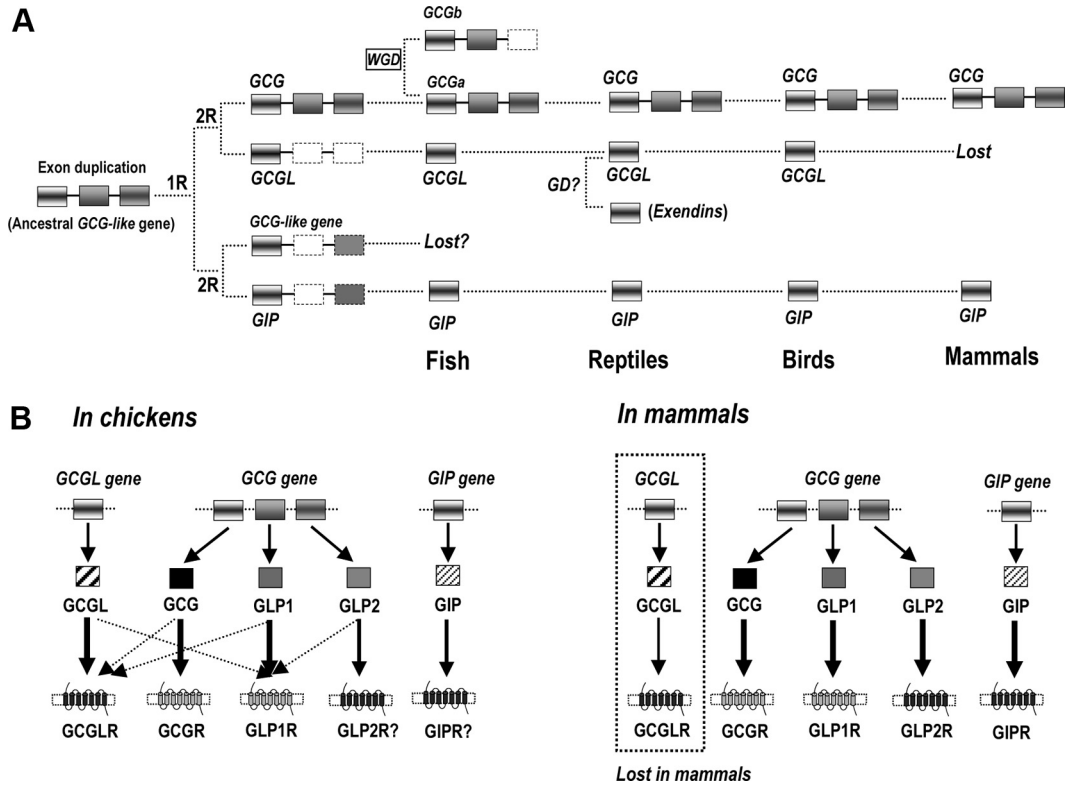

FIG. 8. A, Proposed evolutionary history of *GCGL*, *GCG*, and *GIP* genes in vertebrates. In this hypothesis, an ancestral GCG-like gene containing duplicated exons experienced two successive rounds of genome duplication event (1R/2R hypothesis) occurred in the early history of vertebrate evolution, leading to the emergence of four members: *GCGL* gene, *GCG* gene, *GIP* gene, and an extra GCG-like gene, which may have been lost during vertebrate evolution. *GCG* gene of vertebrate still retains the duplicated exons, which code for GCG, GLP1 and/or GLP2. *GIP* and *GCGL* genes only retain single exon coding for GIP or GCGL peptide, whereas the other duplicated exon(s) may have been lost during evolution (2, 60). Interestingly, multiple copies of exendin gene have been identified in particular reptilian species (*e.g.* Mexican beaded lizard and Gila monster), and these exendin precursors share high structural similarity to chicken GCGL (Fig. 1), implying that they may originate from gene duplication (GD) of *GCGL* gene occurred in a group of lizards. The two types of GCG (named GCGa and GCGb, or GCG1 and GCG2) identified in a number of teleost fish, are generated by the third round of whole genome duplication event (WGD) occurred in the teleost lineage as indicated by synteny analysis shown in Fig. 6 (61). *GCGL* gene could not be identified in any mammalian species, implying that it may have been lost during evolution. 1R and 2R represent the first round and second round of genome duplication, respectively, which occurred in the early history of vertebrate evolution (47). B, The ligand-receptor pairs identified in chickens and mammals. In chickens, GCGL, GCG, GLP1, GLP2 and GIP, which are encoded by three genes, have their specific (or potential) receptors: GCGLR, GCGR, GLP1R, GLP2R and GIPR (JQ689169), although GLP2R and GIPR have not yet been functionally characterized in chickens. Interestingly, GLP1R could also be activated by GLP2 and GCGL, and GCGLR by GCG and GLP1 at higher concentrations (≥10 nM) (*dotted arrows*); however, their physiological relevance remains to be clarified. In mammals, only four ligand-receptor pairs (GCG-GCGR, GLP1-GLP1R, GLP2-GLP2R, GIP-GIPR) have been identified. *GCGL* and *GCGLR* (*boxed*) genes identified in chicken and other nonmammalian vertebrates have been lost in mammalian lineage during evolution.

ary relationship (Supplemental Fig. 6). Compared with GCGR, GLP1R, GLP2R and GIPR identified in vertebrates, GCGLR has a shorter intracellular C-terminal tail. Because a number of serine residues in the long C-terminal tail of mammalian GLP1R or GCGR have been shown to be critical for receptor internalization and desensitization (48, 49), it is unclear whether the absence of these residues in the short C terminus of cGCGLR could affect receptor internalization and desensitization.

Using synteny analysis, we further proved that *GCGLR* gene exists in all non-mammalian vertebrate species examined, indicating that like *GCGL* gene, *GCGLR* gene also has an ancient origin. The existence of this novel receptor and its ligand not only depicts the fundamental difference in member composition of GCG, GCG-like peptides, and their receptor systems between different groups of vertebrates (Fig. 8), but also adds a further level of complexity to decipher the structural and functional changes of these ligand-receptor pairs during vertebrate evolution.

GCGL is a potential endogenous ligand for GCGLR

Despite the higher structural similarity and closer evolutionary relationship between GCG and GCGL, GCGL could not activate cGCGR at any concentration tested, ruling out the possibility that GCGL is a ligand for GCGR. Interestingly, based on the high amino acid sequence identity between the predicted GCGL precursor and exendin precursors (Fig. 1), Irwin (50) proposed that this novel *GCGL* gene is likely an exendin gene. However, unlike

exendins identified in reptiles, which can activate either mammalian VIP receptor(s) or GLP1R (51–54), chicken $GCGL_{1–29}$ could activate neither chicken VIP type I receptor ($cVPAC_1$), nor pig GLP1R potently (Fig. 3). In contrast, exendin-4, an agonist of mammalian GLP1R, could potently activate pig GLP1R. These findings strongly suggest that cGCGL is functionally distinct from exendin(s) identified in some reptile species, although exendin genes, particularly exendin-3 or exendin-4, may originate from the duplicated *GCGL* gene (Fig. 8), which has undergone convergent evolution during speciation (50).

In contrast to the little or low potency of cGCGL in activating cGCGR, cGLP1R, and pGLP1R, $cGCGL_{1–29}$ was shown to activate the newly identified GCGLR potently (EC_{50}, 0.10 nM), strongly suggesting that $cGCGL_{1–29}$ is an endogenous ligand for GCGLR.

The potential roles of GCGL and GCGLR in chickens

GCG, GLP1, GLP2, and GIP and their receptors have been reported to be involved in many physiological processes including the regulation of gastrointestinal activity, glucose metabolism, and food intake in vertebrates (5, 7, 10). However, unlike the wide tissue expression of *GCG* and *GIP* genes (31), *cGCGL* mRNA was mainly restricted to the spinal cord and various regions of brain. The tissue expression pattern of *cGCGL* is also in sharp contrast with that of reptilian exendin genes, which have been shown to be expressed exclusively in the salivary gland, but not brain, of Mexican beaded lizard and Gila monster (41, 42). Like *cGCGL*, *cGCGLR* mRNA were also found to be widely expressed in various brain regions and spinal cord. Our finding also partially coincides with a recent report by Irwin and Prentice (32), in which *GCGL* and *GCGLR* were detected in the brain of chicken and *X. tropicalis* by RT-PCR. These findings imply that GCGL and its receptor may play important roles in the CNS of chicken and other nonmammalian vertebrates, such as regulation of feeding behavior and neuronal activity, as its structurally related peptides (GLPs) played in vertebrates (7, 55).

Of particular interest to note is the abundant mRNA expression of *GCGLR* in adult chicken pituitaries (Fig. 5). This finding, together with the abundant *cGCGL* mRNA expression in the chicken hypothalamus, points out a possibility that cGCGL is likely a potential novel hypophysiotropic factor to regulate pituitary functions. However, more studies are required to substantiate this hypothesis. Similar to GCGLR expression in chicken pituitary, GLP1 receptor, a receptor homologous to GCGLR (Fig. 2), is reported to be expressed in rodent pituitary (56–58). And iv or intracerebroventricular administration of GLP1 can regulate hypothalamic-pituitary axis activity including elevating plasma ACTH levels in mammals (59). Conceivably, the abundant expression of cGCGLR in both pituitary and hypothalamus implies that like GLP1, GCGL may play active roles in the chicken hypothalamic-pituitary axis through activating GCGLR expressed at both sites. In this study, we also noted the mRNA expression of both *GCGL* and *GCGLR* in chicken gonads including testes and ovaries, implying that cGCGL is likely an autocrine/paracrine factor involved in controlling gonadal functions. Interestingly, mRNA expression of *cGCGLR*, but not *cGCGL*, could be detected in kidneys, lungs, spleen, and pancreas. Nevertheless, the question whether cGCGL plays a role in these tissues remains open to discussion.

It is well documented that mRNA expression of both *GCG* and *GIP* genes could be detected in mammalian intestine, and the gastrointestinal tract is also one of the target sites of these bioactive peptides. However, an almost indiscernible PCR signal of both *GCGL* and *GCGLR* was only noted in the duodenum, suggesting that the gastrointestinal tract is neither a major source of GCGL production nor a target site of GCGL action. Meanwhile, the little or no expression of *GCGL* in gastrointestinal tract also hints that GCGL is unlikely a key player in the enteroinsular axis, although *GCGLR* mRNA expression could be detected in the pancreas.

In summary, a novel GCGL and its receptor GCGLR have been identified from chicken brain. Functional study demonstrated that GCGLR is a GCGL-specific receptor. RT-PCR assay showed that unlike vertebrate *GCG* and *GIP* genes, *GCGL* mRNA expression is mainly confined in the CNS and testis, whereas *GCGLR* is widely expressed in chicken tissues. As in chickens, both *GCGL* and *GCGLR* genes were also identified in other nonmammalian vertebrate species including teleost fish. Our findings not only suggested that this novel ligand-receptor pair plays yet-to-be identified roles in nonmammalian vertebrates, but also provide critical clues to the evolutionary history of *GCG*, *GIP*, and *GCGL* genes in vertebrates (Fig. 8).

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The full-length cDNA sequences of chicken GCGL and GCGLR, and pig GLP1R have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers: EU718628, EU718627, and JQ085965.

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