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Genomic and Expression Analysis of Canine Calcitonin Receptor-stimulating Peptides and Calcitonin/Calcitonin Gene-related Peptide*

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Calcitonin receptor-stimulating peptides (CRSPs) are new members of the calcitonin/calcitonin gene-related peptide (CT/CGRP) family identified in pigs, dogs and other domestic animals, and CRSP-1 is an active ligand for the CT receptor (CT-R). We recently sequenced porcine CRSP genes (*Crsp*s) and found similarity with the CT/CGRP gene (*Ct/Cgrp*) in sequence and genomic organization. In this study, we identified five *Crsp*s, *Crsp-1* to *Crsp-5*, in dogs. *Crsp-1* has five exons with an exon–intron organization identical to that of porcine *Crsp-1* or *Crsp-2*, while *Crsp-2* and *Crsp-3* have additional CT-2- and CT-3-coding exons like *Ct/Cgrp*. *Crsp-2* was renamed as *Ct-2/Crsp-2* because both CRSP-2 and CT-2 mRNAs were tissue-specifically expressed. *Crsp-4* and *Crsp-5* are presumably generated by retrotransposition. We postulate that *Crsp*s were generated from the gene duplication of *Ct/Cgrp*, and gained their diversity during mammalian evolution. Among the canine CTs and CRSPs, CRSP-1, CT-1 and CT-2 are active ligands for the CT-R, but CRSP-2 and others are inactive. Canine CRSP-1 and CT-2 are expressed in the central and peripheral systems, while CT-1 is localized in the thyroid gland. These findings indicate that dogs can be used for an experimental model as analysing the physiological roles of the CT/CGRP/CRSP family.

Key words: calcitonin, calcitonin gene-related peptide, calcitonin receptor, calcitonin receptor-stimulating peptide, genomic organization.

Abbreviations: CGRP, calcitonin gene-related peptide; CL-R, calcitonin-like receptor; CRSP, calcitonin receptor-stimulating peptide; *Crsp*, CRSP gene; *Crsp*-5ψ, pseudogene *Crsp*-5; CT, calcitonin; CT-coding exon, exon encoding a CT-like peptide; CT-R, CT receptor; C-terminal, carboxy terminal; *Ct/Cgrp*, CT/CGRP gene; ESE, exonic splice enhancer; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NCBI, National Center for Biotechnology Information; N-terminal, amino terminal; RACE, rapid amplification of cDNA ends; RAMP, receptor activity-modifying protein; RT–PCR, reverse transcriptase polymerase chain reaction.

We have identified calcitonin receptor-stimulating peptide-1 (CRSP-1) in domestic animals, such as pigs, dogs, cows and horses (*1–4*), which have high sequence identity with calcitonin gene-related peptide (CGRP), a potent vasodilating peptide. However, porcine CRSP-1 specifically stimulates a receptor of calcitonin (CT), a calcium-lowering peptide, with a potency about 100-fold greater than that of CT, and does not stimulate the CGRP receptor at all (*2*). In fact, CRSP-1 is shown to induce almost all the physiological effects that have been attributed to CT; the intravenous injection of porcine CRSP-1 to rats decreases the plasma calcium concentration (*2*), and its central administration alters feeding behavior, locomotor activity, body temperature and pain perception (*5*). In the pig, CRSP-1 is mainly expressed in the central nervous system and thyroid gland, and CT is exclusively present in the thyroid gland, suggesting that CRSP-1 is the sole ligand for the central CT receptors (CT-R) in this species (*2*, *4*).

We have further identified CRSP-2 and CRSP-3 in pigs and CRSP-2 in dogs (*3*, *4*). These CRSP-2 and CRSP-3 also show high sequence identity with CRSP-1 and CGRP, but stimulate neither the CT-R nor the CGRP receptor (*2–4*). Therefore, CRSPs may be divided into two groups, CRSP-1 and CRSP-2/CRSP-3, and more studies are required to elucidate the physiological roles of and differences between these highly similar but distinct peptide groups.

*The abbreviations of CT, CGRP, CL-R and related words comply with the recommendation by the Committee on Receptor Nomenclature and Drug Classification of the International Union of Basic and Clinical Pharmacology (Poyner, D.R., Sexton, P.M., Marshall, I., Smith, D.M., Quirion, R., Born, W., Muff, R., Fischer, J.A., and Foord, S.M. (2002) International Union of Pharmacology. XXXII. The mammalian calcitonin gene-related peptides, adrenomedullin, amylin, and calcitonin receptors. *Pharmacol. Rev.* **54**, 233–246).

Accession numbers of cDNA sequences determined in this study are CRSP-3, AB294187; CRSP-4, AB294188; CT-2, AB294189; CT-R, AB294191. The accession number of the cDNA sequence predicted in this study is CT-3, AB294190.

Human *Ct/αCgrp* is used instead of porcine *Ct/Cgrp* in this article, because genetic information on porcine *Ct/Cgrp* is not publicly available.

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Of note, humans and rodents have one CT and two CGRPs, αCGRP and βCGRP, but no CRSP or its gene has been identified in their genomes and tissue extracts despite intensive database searches and experimental analyses, which has seriously hampered physiological studies of CRSPs. In order to help elucidate the physiological relationship between the CT/CGRP and CRSP systems, we recently determined genomic sequences of the porcine CRSP-1, CRSP-2 and CRSP-3 genes (*Crsp-1*, *Crsp-2* and *Crsp-3*), and found their exon–intron organization to be highly similar to that of the human CT/αCGRP gene (*Ct/αCgrp*) (*6*). Human *Ct/αCgrp*, which consists of six exons, generates CT and αCGRP mRNAs by a tissue-specific alternative splicing event, *i.e*. CT mRNA in the thyroid gland and αCGRP mRNA in the neural system (*7*, *8*). Interestingly, porcine *Crsp-3* has a 6-exon structure including an exon encoding a CT-like peptide (CT-coding exon), indicating the possible generation of *Crsp-3* by gene duplication of an ancestor gene of *Ct/Cgrp*. Porcine *Crsp-1* and *Crsp-2* have 5-exon structures lacking the CT-coding exons, but *Crsp-2* shows higher sequence identity with *Crsp-3* than *Crsp-1* (*6*). These findings suggest that *Crsp*s and *Ct/Cgrp*s have increased in number and diversity during the evolution of mammals as an exception among peptide hormone families.

*Crsp*s and *Ct/Cgrp*s are hypothesized to have evolved from a common ancestor, but each mammal probably has a different set of peptide ligands for the CT-R generated from these genes, which could be separated into two categories; *i.e*. the one ligand of CT in humans and rodents, and the two ligands of CT and CRSP-1 in domestic animals. Therefore, mammalian CT-R-mediated regulatory mechanisms including the metabolism of calcium should now be recognized as more complex and specialized than those that have been simply attributed to the CT/CGRP family. We hereafter need to analyse the physiological roles of CRSPs in terms of the CT/CGRP/CRSP system, although the experimental systems available for this purpose are limited by a lack of CRSP in humans and rodents.

To fully elucidate the physiological functions of CRSPs, it is essential to establish an experimental system in which the target animal is easily used for experiments, has CRSP-2 and CRSP-3 in addition to CRSP-1 and CT/CGRP, and has genomic information that is publicly available. In dogs, we identified CRSP-1 and CRSP-2 in addition to CT/CGRP, and verified that CRSP-1 but not CRSP-2 is an active ligand for the CT-R (*3*). Information of the canine genomic DNA sequence is available from public databases, and we found that dogs have several *Crsp*s other than *Crsp-1* and *Crsp-2* in a preliminary BLAST-based search. In initial studies examining the physiological effects of CT, furthermore, dogs were frequently used in experiments (*9*). Therefore, the dog is a promising model for analysing and elucidating the physiological function of the CT/CGRP/CRSP system, which could in turn help uncover still unidentified roles of CT and CGRP in humans and rodents. In this study, we performed genomic and expressional analyses of canine *Crsp*s and *Ct/Cgrp*, and characterized the specificity and stimulatory activity of peptide ligands for the CT-R generated from these genes.

EXPERIMENTAL PROCEDURES

Materials—The sources of materials used were as follows: the SMART RACE cDNA amplification kit and Advantage 2 DNA polymerase from Clontech (Palo Alto, CA, USA), rTaq DNA polymerase and KOD plus DNA polymerase from Toyobo Co. Ltd (Osaka, Japan), the QuickPrep mRNA purification kit from GE Healthcare UK Ltd (Little Chalfont, Buckinghamshire, England), the SuperScript® first strand synthesis system, the SuperScript® III first strand synthesis system, DNase I, pCR®2.1-TOPO®, Lipofectamine Plus reagent and the pcDNA3.1(+) expression vector from Invitrogen Corp. (Carlsbad, CA, USA), Symmetry 300™ C_{18} column (5 μm; 4.6 × 250 mm) from Waters Corp. (Milford, MA, USA) and 3-isobutyl-1-methylxanthine from Nacalai Tesque Inc. (Kyoto, Japan).

Synthetic porcine CT was purchased from Bachem AG (Bubendorf, Switzerland). Synthetic human adrenomedullin was purchased from Peptide Institute Inc. (Osaka, Japan). Synthetic canine CRSP-1 (SCNSATCVAHWLG GLLSRAGSVANTNLLPTSMGFKVYN with an intramolecular disulphide linkage between Cys^2 and Cys^7) and CRSP-2 (SSCKDGPCVTNRLEGWLARAERMVKNTFM PTDVDPEAFGHQHKELAA with an intramolecular disulphide linkage between Cys^3 and Cys^8) were custom-synthesized by solid phase techniques using Fmoc (*N*-(9-fluorenyl) methoxycarbonyl) chemistry at Peptide Institute Inc., as reported previously (*2*). Synthetic canine CT-1 (CSNLSTCVLGTYSKDLNNFHT FSGIGFGAETP-NH_2 with an intramolecular disulphide linkage between Cys^1 and Cys^7) and canine CT-2 (CSNLSTCVLGTYTQDLNKFHTFPQTAIGVGAP-NH_2 with an intramolecular disulphide linkage between Cys^1 and Cys^7) were custom-synthesized by the solid phase method using Fmoc chemistry at Sigma Genosys, Sigma-Aldrich Japan (Hokkaido, Japan). The synthetic porcine CGRP (SCNTATCVTHRLAGLLSRSGGMVKSNFVPTDV GSEAF-NH_2 with an intramolecular disulphide linkage between Cys^2 and Cys^7) was custom-synthesized by the solid phase method at American Peptide Company, Inc. (Sunnyvale, CA, USA). The N-Tyr derivative of canine CT-2[23-32]-NH_2 (YPQTAIGVGAP-NH_2) was kindly synthesized by Dr K. Kangawa of this institute on an 431A peptide synthesizer (Applied Biosystems, Foster City, CA, USA) using the Fmoc strategy. All these peptides were purified by RP-HPLC, and the correct synthesis was verified by mass spectrometry and an amino acid analysis.

Database Searches for Canine Crsps—We performed computer-assisted homology searches for cDNA sequences of canine CRSP-1 (686 bp) and CRSP-2 (711 bp) (*3*) in the canine genomic database (Dog Genome Resources, URL: http://www.ncbi.nlm.nih.gov/projects/genome/guide/dog/) using the BLAST search algorithm at the National Center for Biotechnology Information (NCBI) (*10*) to identify canine *Crsp-1* and *Crsp-2*. To identify CRSP-related genes, we performed BLAST-based searches for amino acid sequences of canine CRSP-1 and CRSP-2 precursor (127 residues, respectively) in the same database.

Purification of mRNA, Synthesis of cDNA, and Amplification of cDNA Fragments—The protocols for

the animal experiments were approved by the ethics committee for animal experiments of our institute. Canine tissues obtained soon after death by exsanguination under anaesthesia were dissected and their wet weights were determined. Purification of mRNAs derived from the canine thyroid gland and hypothalamus was performed using a QuickPrep mRNA purification kit. The double-stranded cDNAs were synthesized from 0.5 μg of canine thyroid gland poly(A)$^+$ RNA for CRSP-3, CRSP-4, CRSP-5, CT-2 and CT-3, and 0.5 μg of canine hypothalamus poly(A)$^+$ RNA for CT-R, respectively, using the SuperScript® first strand synthesis system for RT–PCR, according to the manufacturer's instructions. The following gene-specific primers were used: GAGCCACCGGCTGCCTAA and GCTTCAGGGTC CACATCG for CRSP-3; CCACCGGCTGCCTGCAGG and CCTTCTTCCTGGGGTCATGTCACTGCTG for CRSP-4; TCGTGAGCTGCAGCAGAG and GTCTTCAGGGTCC ACATG for CRSP-5; CCAGCCTCGACTCCTTCC and CATGCCAATGTGAGGGCG for CT-2; CCACTGGAATC TGCTACG and CATGCCAATGTGAGGGCA for CT-3; and GCCTTGGCTGCCAAAGGGTGACTCTG and GCGG ATGACTCTTGCTCG for CT-R. For each PCR, we used 1/200 of the cDNA reaction mixture, a pair of PCR-specific primers (0.4 μM each) and rTaq DNA polymerase, according to the manufacturer's instructions. PCR products were resolved on agarose gels (Agarose S, Wako, Osaka, Japan) and extracted using standard techniques. DNA fragments were then ligated into the plasmid pCR®2.1-TOPO®, according to the manufacturer's instructions. The DNA sequence was determined by the dideoxynucleotide chain termination method using an AB3130xl DNA sequencer (Applied Biosystems).

Rapid Amplification of cDNA Ends (RACE)—The first strands for 5′- and 3′-RACE cDNA were synthesized from 0.05 μg of canine thyroid gland poly(A)$^+$ RNA for CRSP-3 and CT-2 and 0.5 μg of canine hypothalamus poly(A)$^+$ RNA for CT-R using the SMART™ RACE cDNA amplification kit. The universal primer mix for 5′-RACE was used with the following gene-specific antisense primers: GGATGCATATTTCGTCCTCAGTGAGC for CRSP-3; CATGCCAATGTGAGGGCG for CT-2 and CAG AGTCACCCTTTGGCAGCCAAGGC for CT-R. The universal primer mix for 3′-RACE was used with the following gene-specific sense primers: GAGCCACCGGCTGCC TAA for CRSP-3; CCAGCCTCGACTCCTTCC for CT-2; and GCCGGGGACATCCCGGTTTACATC for CT-R. For each PCR, we used 1/100 of the cDNA reaction mixture, a pair of the above-described primers, and the Advantage 2 DNA polymerase, according to the manufacturer's directions (*11*).

Measurement of mRNA Levels by RT–PCR—The purification of poly(A)$^+$ RNA derived from canine organs was performed using a QuickPrep mRNA purification kit. DNase I treatment of the poly(A)$^+$ RNA (0.25 μg) was performed prior to reverse transcription using the SuperScript® III first strand synthesis system with oligo dT_{20} primers, according to the manufacturer's directions. For each PCR, we used 1/100 of the cDNA reaction mixture, a pair of PCR-specific primers (0.4 μM each) and KOD plus DNA polymerase, according to the manufacturer's instruction. The amplification was performed for 35 cycles (each cycle consisted of 15 s at 94°C, 30 s at 60°C and 1 min at 68°C) and the PCR products were then separated on a 2% agarose gel and stained with ethidium bromide. The following gene-specific primers were used: TCAGTGAGAAGGAAGGGC and ACCACACCCCCAGATCTG for CGRP; CGCTGTACCAG GTGGGCT and AGACCTTGAAGCCCATGC for CRSP-1; CCACGGTGCCATCGGACA and GCTTCAGGGTCCACA TCG for CRSP-2; GTGCCATCGCCTGACATT and GCT TCAGGGTCCACATCG for CRSP-3; TCAGTGAGAAGG AAGGGC and TCAATGGGAGCAAGAAGG for CT-1; CCAGCCTCGACTCCTTCC and CCGACCCCAATTGCA GTC for CT-2; GCCGGGGACATCCCGGTTTACATC and GCGGATGACTCTTGCTCG for CT-R and TCACTGC CACCCAGAAGA and TGGTCATTGAGGGCAATG for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Correct amplification was confirmed by DNA sequencing.

Characterization of Immunoreactive CT-2 in the Thyroid Gland—Canine thyroid gland was boiled and extracted, and the extracts were desalted and condensed with a Sep-Pak C_{18} cartridge as reported (*12*). The condensate was separated by reversed phase HPLC with a Symmetry 300™ C_{18} column (5 μm, 4.6 × 250 mm) using a gradient elution of CH_3CN in the presence of 0.1% trifluoroacetic acid.

Immunoreactive CT-2 was measured by radioimmunoassay using antiserum #516-3 against canine/human CT[23-32]-NH_2 (final dilution of 1/81,000). N-Tyr-CT-2[23-32]-NH_2 was radioiodinated by the lactoperoxidase method (*13*), and its monoiodinated form was purified by reversed phase HPLC and used as a tracer.

Cell Culture—COS-7 cells (ATCC, CRL-1651) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum supplemented with 100 μg/ml penicillin and 100 units/ml streptomycin in a humidified atmosphere of 95% air, 5% CO_2 at 37°C.

Measurement of Intracellular cAMP Production—Previously established pcDNA3.1(+) expression systems for porcine CT-R, calcitonin-like receptor (CL-R) and three isoforms of receptor activity-modifying proteins (RAMPs), RAMP-1, -2 and -3, were used in this study (*2*, *14*). All combinations of the two cDNAs of one of the two receptors and one of the three RAMPs and empty vector (cDNA ratio 1:4) were co-transfected into COS-7 cells with Lipofectamine Plus reagent according to the manufacturer's protocol. Canine CT-R cDNA encoding the complete open reading frame was ligated into pcDNA3.1(+) expression vector and transfected into the COS-7 cells as described earlier except in the absence of RAMPs. The empty vector was also introduced into the COS-7 cells for a mock transfection. Following a 24-h incubation, the transfected cells were washed twice with Dulbecco's modified Eagle's medium, dissolved in 20 mM HEPES, pH 7.4, containing 0.5 mM 3-isobutyl-1-methylxanthine and 0.05% BSA, and incubated in this medium for 30 min at 37°C. The medium was then replaced with 200 μl of the same medium in which the sample of interest was dissolved, and incubation was continued at 37°C for another 30 min. Aliquots (100 μl) of the incubation medium were succinylated, evaporated and then subjected to a radioimmunoassay for cAMP as reported previously (*2*).

Statistical Analysis—The statistical analysis was performed using Student's *t*-test, with the level of significance set at $P < 0.05$.

RESULTS

Identification of Crsps *in the Canine Genomic Database*—We first screened the canine genomic database with the BLAST program to identify *Crsp-1* and *Crsp-2* using the reported canine CRSP-1 or CRSP-2 cDNA sequence as a query (*3*). *Crsp-1* and *Crsp-2* (described later as *Ct-2*/*Crsp-2*) were identified unambiguously and were deduced to consist of five and six exons, respectively (Fig. 1).

To identify CRSP-related genes in the canine genomic database, we further screened the database with the BLAST program using the amino acid sequences of canine CRSP-1 and CRSP-2 precursors as queries. We identified six candidates homologous to either the CRSP-1 or CRSP-2 precursor, including canine *Crsp-1*, *Crsp-2* and *Ct*/*Cgrp*. We sorted out three new candidates for *Crsp*s, and tentatively designated them *Crsp-3*, *Crsp-4* and *Crsp-5* (described later as *Crsp-5ψ*). *Crsp-3* was deduced to consist of six exons like *Ct*/*Cgrp* and *Crsp-2*, while *Crsp-4* and *Crsp-5* appeared to be intronless (Fig. 1).

The exon–intron organizations of canine *Crsp-1* to *Crsp-5* are schematically represented in Fig. 1, along with those of canine *Ct*/*Cgrp* (*15*), and all CRSP-related genes of pigs (porcine *Crsp-1*, *Crsp-2* and *Crsp-3*) (*6*) and humans (human *Ct*/*αCgrp*, *βCgrp* and *CALCP*) (*16–18*) except for porcine *Ct*/*Cgrp*. *Ct*/*Cgrp* is present in the pig but its DNA sequence is not available in the public database, since sequencing of the porcine genome has not yet been completed. Human *Ct*/*αCgrp* and *βCgrp* are registered in the database as *CALCA* and *CALCB*, respectively, and human *CALCP* is a pseudogene that has only two exons homologous to exons 2 and 3 of human *Ct*/*αCgrp* or *βCgrp* (*18*). All these CRSP-related genes, except for canine *Crsp-4*, *Crsp-5* and human *CALCP*, have similar exon–intron organizations and encode a 5′-noncoding sequence, a signal sequence, an amino (N-) terminal intervening peptide, CRSP/CGRP peptide and a 3′-noncoding sequence in exons 1, 2, 3 and the last 2 exons, respectively. These data clearly indicate that *Crsp-3* is a member of this gene family, and that all the genes other than canine *Crsp-4* and *Crsp-5* are classified into one family, the CT/CGRP/CRSP gene family.

Canine *Ct*/*Cgrp* has an additional exon encoding a CT-1 peptide (*15*). Based on the highly similar exon–intron organization among canine *Crsp-1*, *Crsp-2*, *Crsp-3* and *Ct*/*Cgrp*, we searched in detail for CT-coding exons in *Crsp-1*, *Crsp-2* and *Crsp-3*. In *Ct*/*Cgrp*, the CT-coding exon is located between exon 3 encoding an N-terminal intervening peptide and exon 5 encoding the CGRP peptide (*16*). However, the distance between the respective exons in canine *Crsp-1* (363 bp) is much shorter than that in canine *Ct*/*Cgrp* (2,143 bp), and no CT-coding exon was found (Fig. 1). On the other hand, *Crsp-2* and *Crsp-3* were deduced to encode CT-like peptides in exon 4 based on the criteria mentioned above and other general rules, and we tentatively designated these peptides CT-2 and CT-3.

Cloning of CRSP-3, CRSP-4, CRSP-5, CT-2 and CT-3 cDNAs—We next tried to clone cDNAs encoding the CRSP-3, CRSP-4, CRSP-5, CT-2 and CT-3 precursors, because *Crsp-3* to *Crsp-5* as well as *Ct-2* and *Ct-3* have been identified solely by searching genomic databases and there is no evidence for their expression. By the 5′- and 3′-RACE methods, the cDNA encoding the CRSP-3 precursor was cloned and found to be 688 bp in length (Supplementary Fig. 1A), whose deduced amino acid sequence is shown in Fig. 2.

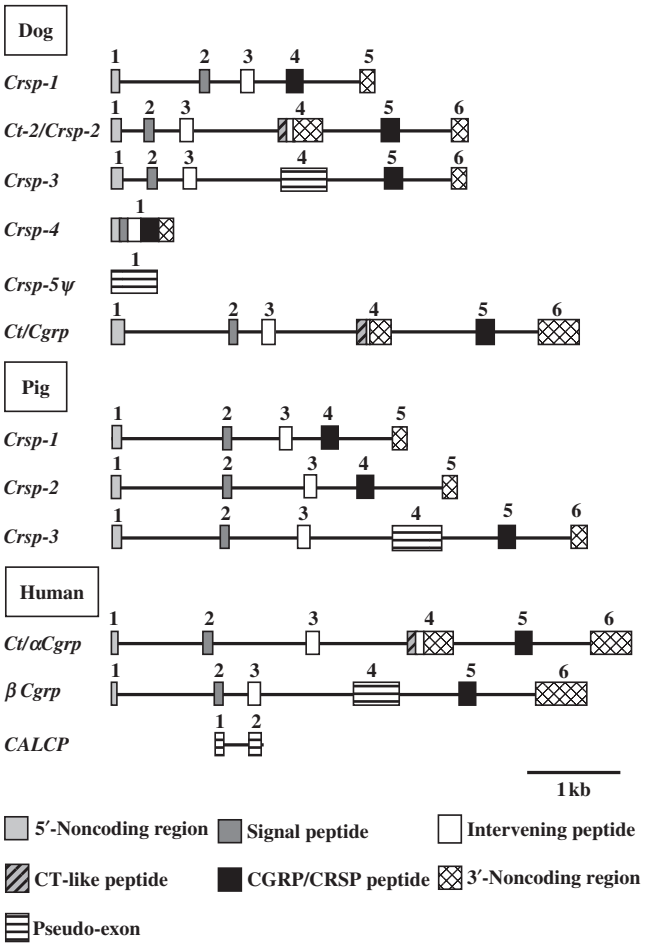

Fig. 1. **Schematic representation of exon–intron organizations of canine *Crsp*s, along with those of canine *Ct/Cgrp* and all CRSP-related genes of pigs and humans except for porcine *Ct/Cgrp*.** *Ct*/*Cgrp* is present in the pig but its DNA sequence is not available, and human *Ct*/*αCgrp* and *βCgrp* are registered as *CALCA* and *CALCB*, respectively, in the public database. Canine *Crsp-5ψ* and human *CALCP* are pseudogenes. Exons and introns are schematically indicated by boxes and lines, respectively. The exons corresponding to the 5′-noncoding region, signal peptide, intervening peptide, CT-like peptide, CGRP/CRSP peptide, 3′-noncoding region and pseudo-exon are indicated as noted at the bottom. Not only exons but also pseudo-exons are numbered to compare the exon–intron organization. Exons 1 and 2 of human *CALCP* correspond to exons 2 and 3 of human *βCgrp*, respectively. Exon 4 of canine *Crsp-3* and of human *βCgrp* are pseudo-exons, and exon 4 of porcine *Crsp-3* is presumably a pseudo-exon because of its lack of expression in porcine tissues.

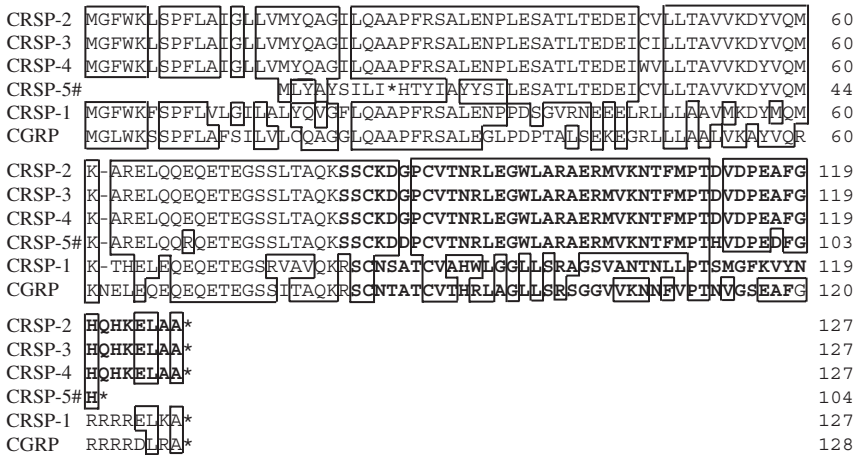

Fig. 2. **Alignment of deduced amino acid sequences of canine CRSP-1, CRSP-2, CRSP-3, CRSP-4 and CGRP precursors, and putative translation product of *Crsp-5* (CRSP-5#).** *Crsp-5* is deduced to be a pseudogene, but CRSP-5# is aligned to show its high sequence identity with CRSP-2, CRSP-3 and CRSP-4. Residues conserved in more than three precursors are boxed. Deduced amino acid sequences for mature peptides are shown in boldface. Amino acid numbers are shown on the right and the stop codon is marked with an asterisk.

Canine *Crsp-4* was located in intron 5 of another protein, LOC475245, and was assumed to be intronless (Fig. 1) in contrast with known genes of the CT/CGRP/CRSP family. We designed specific primers that span the coding region of CRSP-4, cloned the CRSP-4 cDNA of 441 bp in length by the PCR method (Supplementary Fig. 1B), and aligned its deduced amino acid sequence in Fig. 2.

The predicted canine CRSP-5 cDNA sequence has been registered in the database as hmm44701 which consists of two exons. However, the amino acid sequence coded in exon 1 of this gene was not homologous to sequences of other canine *Crsp*s and the splice donor site at the 5′ end of the exon 2 was CA, which does not satisfy the GT/AG rule (*19*), suggesting that the cDNA sequence registered as hmm44701 is incorrect. We revised the open reading frame of *Crsp-5* according to the general rule and deduced this gene to be intronless like *Crsp-4*. The revised CRSP-5 cDNA sequence had a stop codon around the N-terminal of its putative translation product (CRSP-5#), but was added to Fig. 2 to show its high sequence identity with other CRSPs. We tried to clone a cDNA encoding a CRSP-5 precursor using primers based on the highly conserved regions among *Crsp*s, but failed to amplify this cDNA even though we used reverse transcribed cDNAs that were prepared from 20 different tissues, i.e., cerebral cortex, cerebellum, hypothalamus, thalamus, midbrain, pons/medulla oblongata, pituitary, cardiac atrium, cardiac ventricle, thyroid gland, adrenal gland, stomach, small intestine, large intestine, liver, lung, spleen, pancreas and ovary (data not shown). Based on these results, *Crsp-5* is deuced to be a pseudogene.

The deduced amino acid sequences of the precursors for CRSP-1 to CRSP-4 are aligned and compared with each other in Fig. 2, along with CGRP and a putative translation product of *Crsp-5*. The amino acid sequences of the CRSP-1, CRSP-3, CRSP-4 and CGRP precursors showed 52, 99, 99 and 58% identity with the sequence of the CRSP-2 precursor, respectively. In the case of *Crsp-5*, its putative amino acid sequence in Fig. 2 shares 79% identity with that of the CRSP-2 precursor. The CRSP-3 and CRSP-4 precursors did not possess a typical prohormone convertase cleavage site at either end of the deduced biologically active sequence unit, as with the case of the CRSP-2 precursor (*20*). Thus, we presumed that the mature CRSP-3 and CRSP-4 peptides consist of 47 amino acids, starting from two residues prior to the disulphide ring structure (Fig. 2, boldface) (*3*). The amino acid sequences of mature CRSP-2, CRSP-3 and CRSP-4 peptides were identical with each other and showed 43 and 23% identity with those of CGRP and CRSP-1 peptides, respectively.

Based on the actual expression and high amino acid sequence identity with canine CRSP-1 and CRSP-2, three candidates for CRSP-related genes identified by searching the database were finally designated as *Crsp-3*, *Crsp-4* and pseudogene *Crsp-5 (Crsp-5ψ)*. CRSP-2, CRSP-3 and CRSP-4 peptides, and their precursors have identical and almost identical amino acid sequences, respectively, and show high similarity to CGRP and its precursor, suggesting that these three peptides and precursors should be categorized into one group in the canine CT/CGRP/CRSP family.

The cDNA encoding a CT-2 precursor was cloned by the 5′- and 3′-RACE methods. This cDNA was 819 bp long, and its deduced amino acid sequence showed typical features of a secretory precursor protein for a biologically active peptide, including a signal sequence, dibasic cleavage sites and a cleavage/amidation site (Fig. 3A). The deduced mature CT-2 peptide is 32 amino acids in length, and has an intramolecular disulphide linkage and C-terminal amide structure that are essential for eliciting biological activity. Thus, *Crsp-2* was renamed as *Ct-2*/*Crsp-2*.

Although *Crsp-3* also encoded a CT-like peptide, CT-3, in exon 4, a stop codon was located just preceding the CT-3 peptide in the putative open reading frame of the CT-3 precursor. We tried to clone the cDNA encoding

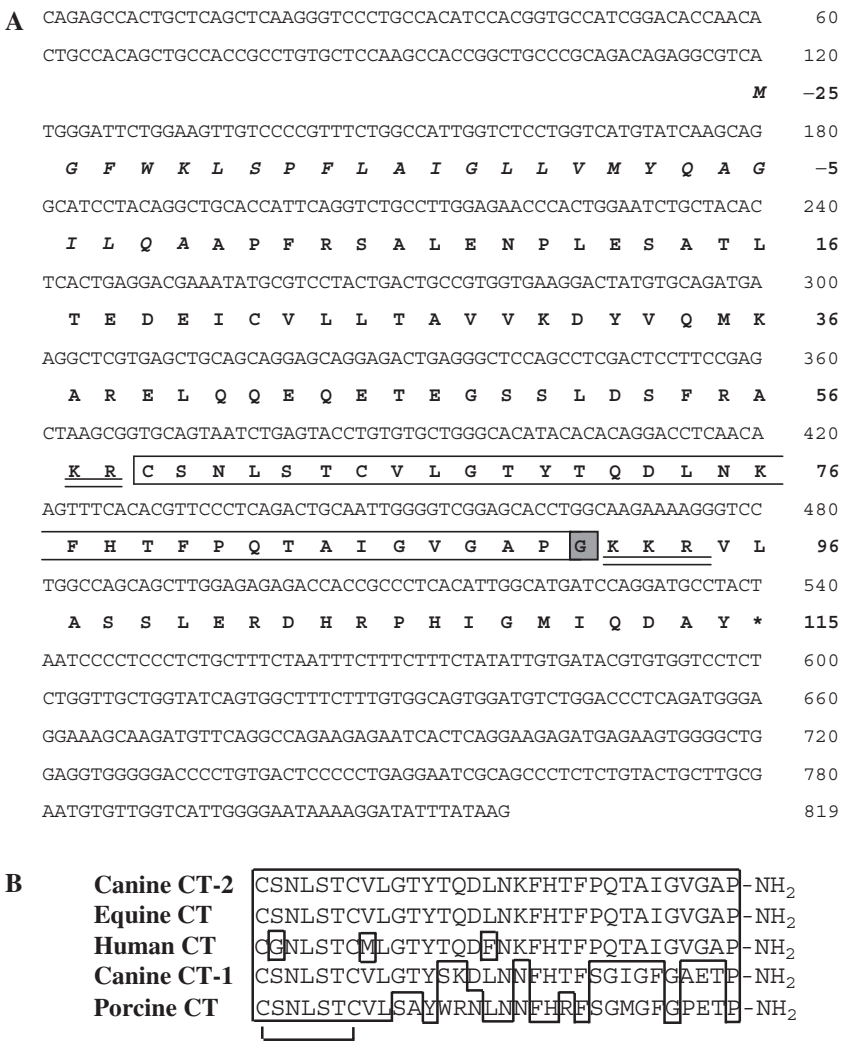

Fig. 3. **Nucleotide and deduced amino acid sequence of canine CT-2 precursor (A), and alignment of deduced mature amino acid sequences of canine CT-2, equine CT, human CT, canine CT-1 and porcine CT peptides (B).** (A) Nucleotide and amino acid numbers are shown on the right, and the stop codon is marked with an asterisk. The deduced amino acid sequence of the canine CT-2 precursor is shown in boldface, a putative signal peptide is in boldface/italics, and the mature canine CT-2 peptide is boxed. A donor glycine of the C-terminal amide is shaded, and cleavage sites of either end of CT-2 peptide are doubly underlined. (B) The bracket shows an intramolecular disulphide linkage (between the 1st and 7th residues), and residues conserved in more than three peptides are boxed.

the CT-3 precursor, but failed to amplify this cDNA in the 20 different tissues listed earlier in contrast with CRSP-3 mRNA (data not shown). Thus, exon 4 of *Crsp-3* is recognized as a pseudo-exon and CT-3 mRNA is probably unexpressed.

Mammalian CTs have well-conserved amino acid sequences, but are generally classified as human-type and porcine-type peptides based on their amino acid sequence similarity to human CT or porcine CT (*21*). Each mammalian species has been recognized to have one CT of either the human-type or porcine-type. Canine CT-1 shows higher similarity to porcine CT in the amino acid sequence, while canine CT-2 is more similar to human CT, indicating that dogs have two CTs, a porcine-type and a human-type (Fig. 3B).

Tissue Expression of Canine CRSP and CT mRNAs—The mRNA levels of CRSP-1, CRSP-2, CRSP-3, CGRP, CT-1 and CT-2 were estimated in the 20 canine tissues by the RT–PCR method (Fig. 4). Although the cDNA sequences of CRSP-1, CRSP-2 and CRSP-3 are highly similar to each other, a PCR system specific for each cDNA was designed and used in this study. However, we were not able to prepare specific primers for CRSP-4 mRNA and to measure its expression level, because the cDNA sequence of CRSP-4 is nearly identical with that of CRSP-2 (99%) or CRSP-3 (98%).

As shown in Fig. 4, CRSP-1, CRSP-2 and CRSP-3 mRNAs were primarily expressed in the central nervous system, particularly in the hypothalamus. In the peripheral tissues, CRSP-1 mRNA was significantly observed in the thyroid gland, and CRSP-2 mRNA was detected in the thyroid gland, adrenal gland, spleen and ovary. The expression profile of CRSP-3 mRNA was similar to that of CRSP-2 mRNA, and CRSP-3 mRNA was further

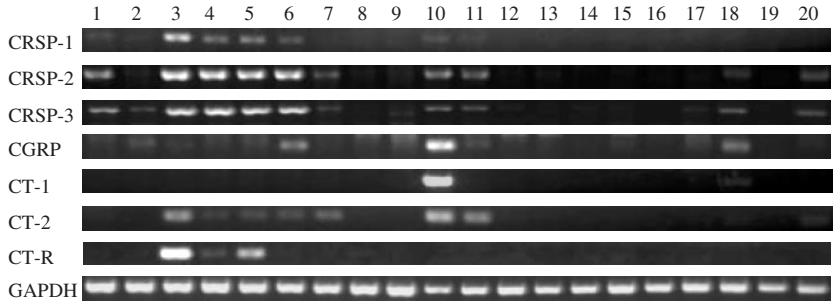

Fig. 4. **Tissue expression levels of canine CRSP-1, CRSP-2, CRSP-3, CGRP, CT-1, CT-2, CT-R and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs analysed by RT–PCR.** RT–PCR was performed as described in 'EXPERIMENTAL PROCEDURES' section using poly(A)$^+$ RNA independently purified from 20 tissues. PCR products were electrophoresed in agarose gels, and visualized with ethidium bromide staining. RT–PCR amplification of GAPDH mRNA is proof of the integrity of respective RNA preparations and reverse transcription. *Lane 1*, cerebral cortex; *lane 2*, cerebellum; *lane 3*, hypothalamus; *lane 4*, thalamus; *lane 5*, midbrain; *lane 6*, pons/medulla oblongata; *lane 7*, pituitary; *lane 8*, cardiac atrium; *lane 9*, cardiac ventricle; *lane 10*, thyroid gland; *lane 11*, adrenal gland; *lane 12*, kidney; *lane 13*, stomach; *lane 14*, small intestine; *lane 15*, large intestine; *lane 16*, liver; *lane 17*, lung; *lane 18*, spleen; *lane 19*, pancreas; *lane 20*, ovary.

detected in the lung. On the other hand, high levels of CGRP, CT-1 and CT-2 mRNAs were detected in the thyroid gland. The expression of CGRP mRNA was also observed in the central nervous system, particularly in the pons/medulla oblongata, and further in the spleen. CT-1 mRNA was expressed weakly in the spleen but not at all in the central nervous system, whereas CT-2 mRNA was expressed in the central nervous system, particularly in the hypothalamus, and in the adrenal gland. Taken together, the expression of CT-1 mRNA is strictly limited to the thyroid gland, while CRSP-1, CRSP-2 and CRSP-3 mRNAs as well as CT-2 mRNA are widely distributed both to the central nervous system and to the peripheral tissues such as the thyroid and adrenal glands.

We further confirmed CT-2 mRNA expression by detecting and characterizing its translated product in the thyroid gland. In tissue extracts of the thyroid gland, CT-2-like immunoreactivity was detected by the antibody against synthetic CT-2. In the reversed phase HPLC, a majority of the immunoreactivity emerged at the elution time of CT-2 but not at that of CT-1 abundantly present in the same tissue (Supplementary Fig. 2).

Sequence Elements Regulating Alternative Processing in Canine Ct-2/Crsp-2—Alternative processing of human CT/CGRP mRNA has been well examined and is reported to be regulated at the levels of polyadenylation and splicing, with several of the RNA sequence elements and protein factors involved having been identified to balance the inclusion of the CT-coding exon (*22–25*). In the polyadenylation, intronic enhancer elements present downstream of exon 4 are known to regulate its inclusion, but these elements are highly conserved in both canine *Ct-2/Crsp-2* and human *Ct/Cgrp* (data not shown). In the case of the splicing, there are three dominant mechanisms regulating this event, and we examined them in the canine *Ct-2/Cgrp-2*. First, the non-canonical branch point for the CT-specific acceptor upstream of exon 4 and the canonical branch point for the CGRP-specific acceptor upstream of exon 5 are reported to lead to the inclusion of exon 5 (*26–28*). The branch points upstream of exon 4 in canine *Ct/Cgrp* and *Ct-2/Crsp-2* were non-canonical uracil and cytosine, respectively, while their branch points upstream of exon 5 were identical to a canonical adenine (Fig. 5). Thus, the branch points upstream of exons 4 and 5 in human *Ct/αCgrp* are conserved in these canine genes. Secondly, two exonic splice enhancers (ESEs), ESE A and ESE B, located in exon 4 were reported to be required for inclusion of exon 4 in human CT mRNA (*22*, *29*, *30*). The exon 4 sequences in canine *Ct/Cgrp*, *Ct-2/Crsp-2* and human *Ct/αCgrp* were highly conserved in these three genes (Fig. 5).

Thirdly, Fox-1 and Fox-2 proteins are known to function as neuron-specific splicing regulators in the CT/CGRP system, and are expressed in the muscle, heart and brain tissues (*31*). In humans, Fox-1 and Fox-2 proteins tightly suppress the inclusion of exon 4 in neurons by binding two GCAUG silencer elements located upstream and downstream of the 5′ end of exon 4 that are beside the non-canonical branch point and in the vicinity of ESE A, respectively (*25*). Thus, we searched for GCAUG silencer elements around the exon 4 starting point in *Ct/Cgrp* and *Ct-2/Crsp-2*. Canine *Ct/Cgrp* has no GCAUG silencer element close to ESE A, but two GCAUG silencer elements are present beside the non-canonical branch point (Fig. 5). In the case of canine *Ct-2/Crsp-2*, one GCAUG silencer element exists beside the non-canonical branch point, but no additional silencer element is present in exon 4 (Fig. 5). These results suggest that the binding of Fox-1 and Fox-2 proteins to the GCAUG silencer element is critical to the tissue-specific alternative processing in the maturation of CT/CGRP mRNA and CT-2/CRSP-2 mRNA, and a reduced binding site of Fox-1 and Fox-2 in canine *Ct-2/Crsp-2* probably results in the expression of CT-2 mRNA in the brain via a cancellation of suppression.

Specificity of Canine CTs and CRSPs in the Porcine Receptor Systems—Adrenomedullin and CGRP receptors are composed of CL-R and RAMPs. The CGRP receptor consists of CL-R and RAMP-1, while the adrenomedullin receptor consists of CL-R and RAMP-2 or RAMP-3 (*32*). In the case of the CT-R, RAMPs are also reported to partially alter its specificity and activity (*33*). We have

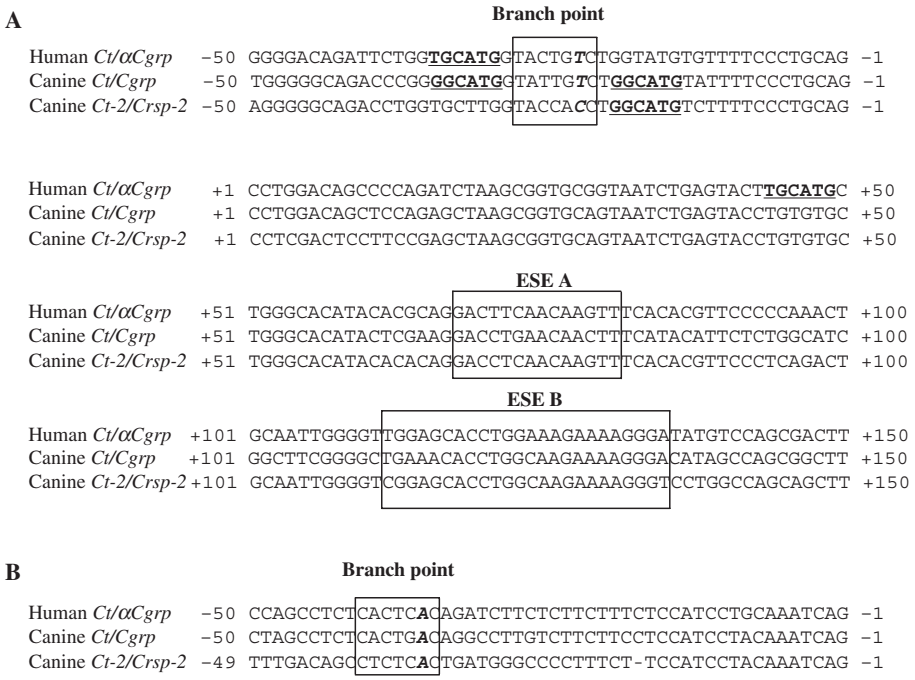

Fig. 5. **Nucleotide sequences regulating alternative processing in human *Ct/Cgrp*, canine *Ct/Cgrp* and canine *Ct-2/Crsp-2***. Putative branch point sequences for the CT-specific acceptor (A) and CGRP-specific acceptor (B) are boxed, and the branch points are indicated in boldface/italics (A, B). Exonic enhancer elements (ESEs) A and B are boxed (A). The GCAUG silencer elements that interact with Fox-1 and Fox-2 proteins are indicated in underlined boldface (A). Numbers represent bp numbers upstream (−) or downstream (+) of the 3′ splice site of exon 4 (A) or of the 3′ splice site of exon 5 (B).

previously established expression systems for all receptor complexes in COS-7 cells by the cloning of these five cDNAs in the pig, and reported the specificity and cAMP-producing activity of canine CRSP-1 and CRSP-2 (*3*). In this study, therefore, we first examined effects of canine CT-1 and CT-2 on these receptor complexes. Canine CT-1 or CT-2 did not stimulate cAMP production via the CL-R, and co-expression of RAMPs did not enhance affinity for the CL-R similar to canine CRSP-1 and CRSP-2 (Fig. 6). Canine CT-1 and CT-2 stimulated the cAMP production via the CT-R, but their stimulatory effect was weaker than that of canine CRSP-1 at 100 nM. Co-expression of RAMPs, especially in the case of RAMP3, rather reduced the cAMP-producing activity of CT-1 and CT-2 as well as that of CRSP-1 (Figs. 6A–C). On the other hand, canine CRSP-3 and CRSP-4, which is identical to CRSP-2, did not at all stimulate the cAMP production via either the CT-R or CL-R even in the presence of RAMPs (Fig. 6D).

Cloning of Canine CT-R and Biological Activity of Canine CTs and CRSPs in the Canine CT-R System— As described previously, each mammalian species has a porcine-type CT or human-type CT, and the specificity and potency of each CT differ in the CT-R of each species (*34*). Since dogs have three active ligands for the CT-R, i.e., porcine-type CT, human-type CT and CRSP-1, these ligands may have an affinity for the canine CT-R different from that for the porcine CT-R. Thus, it is necessary to construct an expression system for the canine CT-R to correctly evaluate their biological activity.

We first obtained information on the sequence of CT-R from the genomic database, but the registered amino acid sequence of canine CT-R (553 residues) was longer than that of porcine CT-R (482 residues) and human CT-R-2 (474 residues), the dominant CT-R in humans (*35*, *36*), and was not homologous in the N-terminal 1–94 sequence. We then cloned canine CT-R by the 5′- and 3′-RACE method using primers hybridizing with a region other than the N-terminal region. The cDNA encoding canine CT-R was 3518 bp in length and its open reading frame encoded 476 residues (Supplementary Fig. 3), indicating that the registered amino acid sequence was assigned to wrong predictions of first and second exons probably due to a long second intron (91 kb). The amino acid sequence of canine CT-R determined in this study showed 74 and 90% identity with that of porcine CT-R and human CT-R-2, respectively (Supplementary Fig. 4).

Expression levels of canine CT-R mRNA were estimated in the 20 tissues by the RT–PCR method (Fig. 4). Canine CT-R was highly expressed in the central nervous system, particularly in the hypothalamus and midbrain, as expected from a previous report on the porcine CT-R (*34*).

We evaluated the biological activity of canine CTs and CRSP-1 in an assay of the canine CT-R-mediated cAMP production (Fig. 7). Both CT-1 and CT-2 dose-dependently enhanced cAMP production in COS-7 cells expressing the CT-R, and showed comparable activity with a half-maximal concentration of 1.0 nM. In contrast with the data obtained in the porcine CT-R system, canine CRSP-1 weakly stimulated cAMP production and its potency was approximately 100-fold less than that of CT-1 or CT-2. CRSP-2 did not at all enhance the

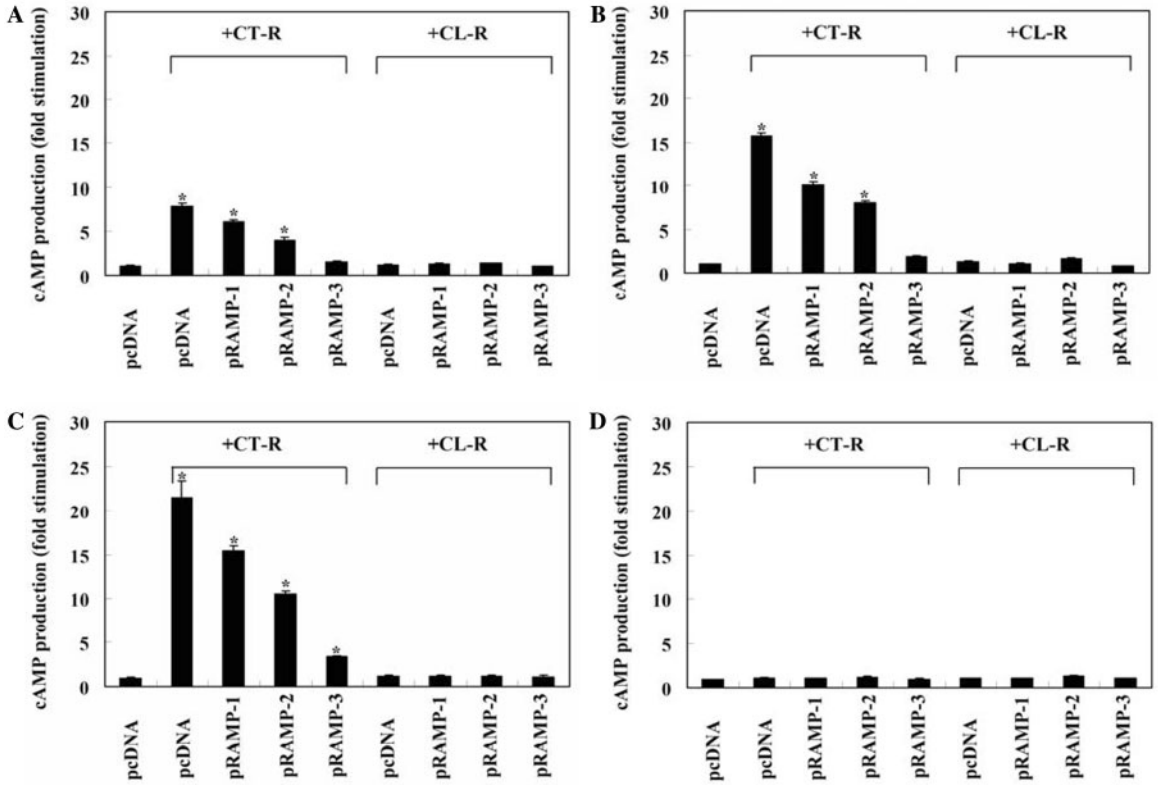

Fig. 6. **Specificity of canine CTs and CRSPs in the porcine receptor systems.** COS-7 cells were co-transfected with porcine CT-R or CL-R cDNA and RAMP-1, -2 or -3 cDNA or pcDNA, and then stimulated with 100 nM of canine CT-1 (A), CT-2 (B), CRSP-1 (C) or CRSP-2 (D). Each point represents the mean ± SE of three separate determinations. Asterisk stands for $P < 0.05$ compared with the mock-transfected control.

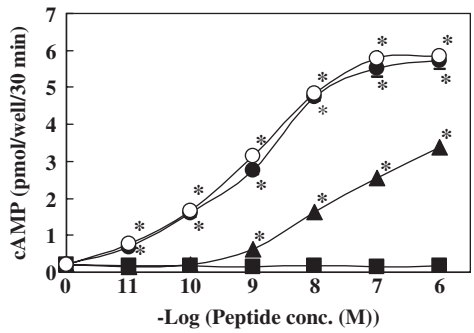

Fig. 7. **Biological activity of canine CTs and CRSPs in the canine CT-R system.** COS-7 cells were transfected with canine CT-R cDNA. The cells were then stimulated with the indicated concentrations of canine CT-1 (closed circles), CT-2 (open circles), CRSP-1 (closed triangles) and CRSP-2 (closed squares) peptides. We confirmed that each peptide did not increase the cAMP production in mock-transfected COS-7 cells. Each point represents the mean ± SE of three separate determinations. Asterisk stands for $P < 0.05$ compared with the peptide-free control.

cAMP production via canine CT-R, as in the case of porcine CT-R.

DISCUSSION

We recently sequenced porcine *Crsp-1, Crsp-2* and *Crsp-3*, and reported that the genomic organization of each gene is similar to that of human *Ct/αCgrp* (*6*). In dogs, we have previously cloned two CRSP cDNAs encoding CRSP-1 and CRSP-2 (*3*). In the present study, we identified *Crsp-1* and *Ct-2/Crsp-2* in the canine genomic database using these two cDNA sequences. We further identified three new genes, *Crsp-3*, *Crsp-4* and *Crsp-5ψ*, by searching the canine genomic database using the amino sequences of the CRSP-1 and CRSP-2 precursors.

Porcine *Crsp-1* and *Crsp-2* consist of five exons, while porcine *Crsp-3* consists of six exons including an additional CT-coding exon similar to mammalian *Ct/Cgrp* (*6*). Canine *Crsp-1* is composed of 5 exons and its exon–intron organization is compatible with that of porcine *Crsp-1* and *Crsp-2*, while canine *Ct-2/Crsp-2* consists of six exons and includes the CT-coding exon (Fig. 1). Taking pseudo-exons into account, *Crsp*s could be divided into a 6-exon type and a 5-exon type based on the number of exons. The 6-exon *Crsp* is hypothesized to be generated by gene duplication of *Ct/Cgrp* followed by conversion of the CGRP sequence into the CRSP sequence, because avians are deduced to have one *Ct/Cgrp* and no *Crsp* (*37*). On the other hand, the 5-exon *Crsp* might have been generated from the 6-exon *Crsp* by deletions of the CT-coding exon. During the evolution of mammals, the 6-exon *Crsp* and 5-exon *Crsp* are hypothesized to have been independently duplicated, along with gene duplication of *Ct/Cgrp*, and some of them may have disappeared thereafter.

The CT-coding exon 4 of canine *Crsp-3* is probably a pseudo-exon, since CT-3 mRNA includes a stop codon and is not expressed in the tissues shown in Fig. 4. Porcine CT-2 mRNA has a complete open reading frame but its expression has not been detected (*6*), suggesting that its exon 4 is actually a pseudo-exon. Human *βCgrp* also has a CT-coding pseudo-exon that retains only a vestige of the exon. In contrast, canine CT-2 mRNA was expressed in the central nervous system as well as in the thyroid gland (Fig. 4). The existence of CT-2 peptide was confirmed in the thyroid gland by chromatographic characterization (Supplementary Fig. 2). These results finally allowed us to designate this gene as *Ct-2/Crsp-2*. On the other hand, CRSP mRNAs are expressed from all *Crsp*s shown in Fig. 1, except for canine CRSP-5 that has an unusual genomic organization. Taken together, there is a possibility that evolutional pressure was applied differently to the CT- and CRSP-coding exons.

Since CRSPs and their cDNAs were numbered according to the order of their discovery and identification in each species, it should be noted that the *Crsp-2* or *Crsp-3* in one species does not necessarily coincide with that in another as easily noticed in Fig. 1. Canine CRSP-3 and CRSP-4, which are identical to CRSP-2, are inactive ligands for CT-R and CL-R, as are porcine CRSP-2, CRSP-3, and the recently identified caprine CRSP-2 (*38*). To explore the physiological roles of these inactive CRSPs, one may approach to measure a wide range of effects after their systemic administration to dogs. In contrast, CRSP-1s retain definite features in their genes and biological activity even in different mammals.

Crsp-4 and *Crsp-5ψ* were first identified in this study, and lack introns and CT-coding exons, suggesting that these genes were generated by retrotransposition after the ancestral animals of the dog and pig evolutionarily separated from their common ancestor. Retrotransposition is a significant source of novel coding sequences in mammalian genetic evolution, and genes generated by retrotransposition generally lack introns and contain stop codons, frequently resulting in pseudogenes (*39*). It is highly probable that canine *Crsp-4* and *Crsp-5ψ* were generated by retrotransposition from *Ct-2/Crsp-2* or *Crsp-3*, because of their extremely high sequence identity. Although *Crsp-5ψ* is concluded to be a pseudogene, *Crsp-4* is unexpectedly transcribed at least in the thyroid gland and may have some biological significance (Fig. 2 and Supplementary Fig. 1B).

The expression of CT mRNA in mammals is restricted to the thyroid gland, while canine CT-2 mRNA is widely expressed not only in the thyroid gland but also in the central nervous system (Fig. 4). The difference in the tissue expression profiles of these two genes is deduced to be caused by the different numbers of binding sites of Fox-1 and Fox-2 proteins, neuron-specific splicing regulators that bind the GCAUG silencer element. In humans, Fox-1 and Fox-2 are reported to suppress in neurons the splicing from a *Ct/Cgrp* primary transcript to CT mRNA by binding to two GCAUG elements, and one mutation in either of these elements decreases the suppression of splicing to CT mRNA (*25*). Canine *Ct-2/Crsp-2* has one GCAUG element, while *Ct/Cgrp* has two. The suppressive activity of Fox-1 and Fox-2 is deduced to be weaker for the alternative splicing of a *Ct-2/Crsp-2* primary transcript to CT-2 mRNA than of a *Ct/Cgrp* primary transcript to CT-1 mRNA. As a result, CT-2 mRNA is considered to be expressed in the central nervous system and adrenal gland in addition to the thyroid gland.

Dogs have two CT-R ligands (CT-2 and CRSP-1) in the central nervous system and three CT-R ligands (CT-1, CT-2 and CRSP-1) in the peripheral tissues. CT-1 and CT-2 equi-potently stimulate canine CT-R, and are about 100-fold more potent than CRSP-1 (Fig. 7), indicating that canine CT-1 and CT-2 function as principal CT-R ligands. In pigs, CRSP-1 is the sole CT-R ligand in the central nervous system and two CT-R ligands (CT and CRSP-1) are present in the peripheral tissues, with CRSP-1 more potently stimulating CT-R. In humans, the existence of neither a central CT-R ligand nor *Crsp* in the genome has been proven, and CT exerts its activity only in the peripheral tissues. Considering these findings, it is essential to re-examine the regulatory system composed of CT, CRSP and CT-R in each mammal, since the number and localization of CT-R ligands as well as their receptor specificity differ in each species. These results further indicate that the dog is an experimental model suitable for analysing the physiological functions of all these CT-R ligands.

Canine *Crsp-1*, *Ct-2/Crsp-2* and *Crsp-3* are located in the vicinity of *Ct/Cgrp* on chromosome 21 similar to porcine *Crsp*s and *Ct/Cgrp* on chromosome 2 (*6*), or human *Cgrp*s and *CALCP* on chromosome 11 (*18*) (Fig. 8). *Crsp-4* and *Crsp-5ψ*, which are presumably generated by retrotransposition, are present on chromosomes 14 and 22, respectively. Previously, we assumed that porcine *Crsp-1*, *Crsp-2* and *Ct/Cgrp* correspond to human *βCgrp*, *CALCP* and *Ct/αCgrp*, respectively, based on their positional order on the chromosomes (*6*). The present results lead us to hypothesize that canine *Crsp-1*, *Ct-2/Crsp-2*, *Crsp-3* and *Ct/Cgrp* correspond to porcine *Crsp-2*, *Ct/Cgrp*, *Crsp-3* and *Crsp-1*, respectively. From a comparison of these genes on the canine and human chromosomes, canine *Crsp-1*, *Ct-2/Crsp-2* and *Ct/Cgrp* are assumed to match human *CALCP*, *Ct/αCgrp* and

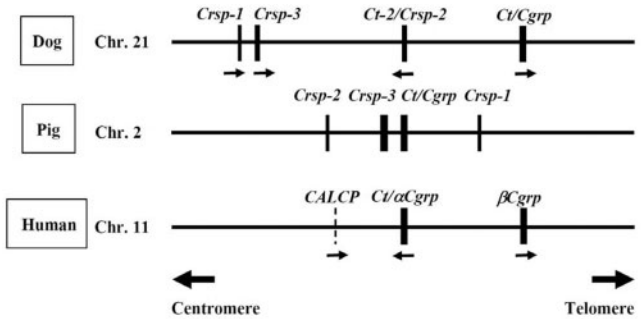

Fig. 8. **Relative chromosomal positions of *Crsp*s and *Cgrp*s in dogs, humans and pigs.** A vertical line and a dashed line indicate a gene and a pseudo-gene, respectively, and the line width is correlated with the length of each gene. Small arrows indicate the direction of each gene, and large arrows indicate the direction of the chromosome. The directions of porcine *Crsp-1*, *-2* and *-3* and *Ct/Cgrp* have not been determined yet. Canine *Crsp-4* and *Crsp-5* are present on chromosomes 14 and 22, respectively.

βCgrp, respectively. In any matching of these genes, however, there are discrepancies which are difficult to explain with a simple evolutional relationship. The difficulty in elucidating the relation between *Ct/Cgrp* and *Crsp*s in mammalian species, in contrast with other mammalian peptide hormone genes, such as natriuretic peptide genes (*40*), leads us to speculate that the *Crsp*s in each animal have been generated by repeated gene duplication and deletion after the divergence of dogs, pigs and humans from their common ancestor depending on their necessity in each species.

In conclusion, we identified five canine *Crsp*s, compared them with porcine and human genes of the CT/CGRP/CRSP family, and analysed the biological activity of peptides generated from these genes. Based on the results, we postulate that *Crsp*s were generated from the gene duplication of *Ct/Cgrp*, and increased in number and diversity with further gene duplication during mammalian evolution. The differences in the number, expression profile and receptor-specificity of CTs/CRSPs suggest us a need to re-investigate the physiological significance of both the classical peptide hormone family, CT/CGRP, and the newly identified family, CT/CRSP, from the point of view of the CT-R ligands.

Supplementary data are available at *JB* online.

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