A New Peptidic Ligand and Its Receptor Regulating Adrenal Function in Rats*\S

Shoji Fukusumi, Hiromi Yoshida, Ryo Fujii, Minoru Maruyama, Hidetoshi Komatsu, Yugo Habata, Yasushi Shintani, Shuji Hinuma, and Masahiko Fujino

From the Discovery Research Laboratories, Pharmaceutical Research Division, Takeda Chemical Industries Ltd., Wadai 10 Tsukuba, Ibaraki, 300-4293 Japan

Received for publication, May 20, 2003, and in revised form, September 5, 2003
Published, JBC Papers in Press, September 5, 2003, DOI 10.1074/jbc.M305270200

We searched for peptidic ligands for orphan G protein-coupled receptors utilizing a human genome data base and identified a new gene encoding a preproprotein that could generate a peptide. This peptide consisted of 43 amino acid residues starting from N-terminal pyroglutamic acid and ending at C-terminal arginine-phenylalanine-amide. We therefore named it QRFP after pyroglutamylated arginine-phenylalanine-amide peptide. We subsequently searched for its receptor and found that Chinese hamster ovary cells expressing an orphan G protein-coupled receptor, AQ27, specifically responded to QRFP. We analyzed tissue distributions of QRFP and its receptor mRNAs in rats utilizing quantitative reverse transcription-polymerase chain reaction and in situ hybridization. QRFP mRNA was highly expressed in the hypothalamus, whereas its receptor mRNA was highly expressed in the adrenal gland. The intravenous administration of QRFP caused the release of aldosterone, suggesting that QRFP and its receptor have a regulatory function in the rat adrenal gland.

In the last decade, advances in cDNA and genomic DNA sequencing have revealed the existence of hundreds of G protein-coupled receptor (GPCR) genes in the human genome. Those for which the ligands have not yet been identified are referred to as orphan GPCRs. GPCRs play pivotal roles in cell-to-cell communication and in the regulation of cell functions. For this reason, the identification of endogenous ligands for orphan GPCRs will open the door for clarifying new regulatory mechanisms of the human body. Furthermore, as GPCRs are considered to be some of the most important drug target molecules, the identification of ligands of orphan GPCRs will provide opportunities for developing novel drugs (1). Orphan GPCR research is therefore important from the aspects of both basic and applied science. We have previously established a widely applicable method to identify ligands for orphan GPCRs through monitoring specific signal transductions in cells expressing orphan GPCRs (2, 3). Through this method we succeeded in identifying various orphan GPCR ligands including peptides with arginine-phenylalanine-amide (RFamide) structure at their C termini (2, 4).

The first report on a peptide with RFamide was the isolation of FMRFamide from bivalve mollusks. Since then, a number of bioactive peptides with the same structure have been found throughout the animal kingdom and are called RFamide peptides (5). It has been found that more than 20 RFamide peptide genes encode over 50 distinctive peptides in the nematode Caenorhabditis elegans (6). In mammals, four RFamide peptide genes have so far been identified, that is neuropeptide FF (7, 8), prolactin-releasing peptide (2), RFamide-related peptide (RFRP) (4), and metastin (9). All of their receptors have been identified through orphan GPCR research. Based on the identification of prolactin-releasing peptide and RFRP, we have proposed that a variety of RFamide peptide exist and have physiological functions even in mammals and that RFamide peptides are evolutionally related to members of the neuropeptide Y family (8). Another group has recently reported the identification of a new RFamide peptide and its receptor (10). However, their precise molecular and functional characterizations remain to be elucidated. In our present research, we have independently found the same new RFamide peptide gene utilizing a human genome data base and subsequently identified its receptor among orphan GPCRs by applying our previously established method. Here we report the molecular characterization of this newly identified RFamide peptide and its receptor including their binding properties and precise tissue distribution determined through in situ hybridization. In addition, we demonstrate that they function to regulate hormone secretion from the adrenal gland in rats.

EXPERIMENTAL PROCEDURES

Cloning of QRFP cDNA—To isolate a human QRFP cDNA by reverse transcription (RT)-PCR, we designed primers (5'-ATGGTAGGCCT-TACCCCTGATCTAC-3' and 5'-CAAATCCTTCCAAGGGCTCTG-GCCT-3') based on the Celera Discovery Systems and Celera Genomics-associated data base. PCR was performed in a reaction mixture (25 μl in total) containing a 0.2 μM concentration of the primers, a template cDNA synthesized from human brain poly(A)+ RNA (Clontech) using a Marathon cDNA amplification kit (Clontech), 0.1 mM dNTPs, 1.25 units of KlenTaq DNA polymerase (Clontech), and 2.5 μl of a buffer provided by the manufacturer. This was conducted at 94 °C for 2 min followed by 40 cycles at 98 °C for 10 s, at 63 °C for 20 s, and at 72 °C for 60 s. We obtained a PCR product of about 300 bp containing a full coding region and determined its nucleotide sequence with an ABI Prism 377 DNA
Amino acid sequences of human, bovine, rat, and mouse QRFP prepropeptides. The open arrowhead shows the predicted cleavage site of the N-terminal secretory signal peptide sequence. The motifs that could generate the C-terminal RFamide structure are underlined. The closed arrowhead indicates the N terminus of the fully active QRFP. Residues that are identical in at least two of the species are boxed. Amino acid numbers are shown on the right. Nucleotide and amino acid sequences for human, bovine, rat, and mouse cDNAs will appear in the DDBJ/EMBL/GenBank databases with the accession numbers AB109625, AB109626, AB109627, and AB109628, respectively.

Comparison of amino acid sequences of known RFamide peptides and QRFP. Residues with C-terminal RFamide structure are boxed. P-RP, prolactin-releasing peptide; NPFF, neuropeptide FF; NPAF, neuropeptide AF; MetEnk-amide, Met-enkephalinamide.
lyzed by means of RT-PCR using an ABI Prism 7700 sequence detector as described elsewhere (14). The following primer sets and probes were used: 5′-AGCAGCTTCCCTCTAG-3′, 5′-GGGTGCTCTTCCGAGTTG-3′, and 5′(FAM)-AGGCGAGCTTCCGAGAGC-TAMRA-3′ for rat QRFP mRNA and 5′-CGGAAGACTGGAAATTCTG-3′, 5′-ATGTTGCTTCTTGTGTCTCCTGCA-TAMRA-3′, and 5′(FAM)-AGCAAGAT-TATCCAGCAACATCCTGCA-TAMRA-3′ for rat AQ27 mRNA. PCR was performed at 50 °C for 10 min for the reaction of uracil-N-glycosylase to prevent the amplification of carried over PCR products, at 95 °C for 2 min for the activation of AmpliTaq Gold DNA polymerase, and for 40 cycles at 95 °C for 15 s and at 57 °C for 80 s for the amplification.

In Situ Hybridization—Under pentobariaxal anesthesia, male Wistar rats (8–9 weeks old) were perfused with 4% paraformaldehyde via the left cardiac ventricle. Frozen sections prepared from the brains and adrenal glands were hybridized with digoxigenin-labeled antisense RNA probes synthesized from full-length rat QRFP and AQ27 template cDNAs according to the floating method described previously (15). Visualization of QRFP and AQ27 mRNAs was conducted with alkaline phosphatase-conjugated anti-digoxigenin antibody using 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate.

Assays for Plasma Hormone Concentrations—Male Wistar rats (8–9 weeks old) were provided with food and water ad libitum and kept under controlled lighting (lights on 8:00 to 20:00) and temperature (25 °C) until used for the experiments. A cannula was inserted into the right jugular vein of each rat under sodium pentobarbital anesthesia (50 mg/kg). The cannula-implanted rats were housed in individual cages where they were kept for 3 days before the experiments. The day after the implantation, 10 μl of phosphate-buffered saline either with or without the peptide was injected into the right jugular vein via the inserted cannula. Before the intravenous injection of the peptide, 400 μl of blood was withdrawn from each rat through the cannula, and 0.01 M EDTA with aprotinin (300 kallikrein-inactivating units/ml) was immediately added. This was performed between 13:00 and 17:00. Plasma aldosterone concentrations were determined by radioimmunoassay. To analyze other adrenal gland hormones (i.e. corticosterone and testosterone), other sets of rats were treated in the same manner, and their plasma concentrations were measured as described previously (8).

RESULTS

Identification of a Novel RFamide Peptide Gene—We searched for unknown members of the RFamide peptide family in the human genome database using queries to detect repetitive patterns generating RFamide peptide (i.e. RFGR or RFGRK where RF is followed by G as an amide donor and by R or K as a proteolytic cleavage site) and a secretory signal peptide sequence upstream of the patterns as reported previously (8). Signal sequences were predicted with the SignalP Version 2.0 software program (16). By this search, we found a human genomic sequence that possibly encoded an RFamide peptide (i.e. QRFP). On the basis of the sequences detected, we isolated human, bovine, rat, and mouse cDNAs with full coding regions by RT-PCR. The isolated cDNAs encoded preproteins with amino acid lengths of 124–136 (Fig. 1). In each prepropeptide sequence, 17 or 18 amino acid residues at the N-terminal were thought to comprise the secretory signal peptide. Two RFGR motifs were found in the human prepropeptide. The motif at the C-terminal side was conserved among the different species, but that at the N-terminal was not. Based on these sequence analyses, we predicted that an RFamide peptide (QRFP) would be produced from the C-terminal motif in the human prepropeptide. In addition, we noticed that the C-terminal amino acid sequence of the predicted RFamide peptide (i.e. YGGFMRFamide) quite resembled that of Met-enkephalaminamide (i.e. YGGFMRFamide) (Fig. 2). However, QRFP did not show apparent homology to other known mammalian RFamide peptides except for its RFamide structure.

Identification of a Receptor for QRFP—As all of the RFamide peptides previously found in mammals act as ligands for certain GPCRs, we presumed that this newly identified RFamide

TABLE 1

| Name        | Sequence            | Binding assay IC50 | cAMP assay EC50 |
|-------------|---------------------|--------------------|-----------------|
| QRFP(43)    | EDEGSAGFPAGKTEGTSGPLGLAMALTLLNGYSRKKGGSFPR-NH2 | n.d.               | 2.7             |
| QRFP(26)    | TSGPLNLAEVLNGYSRKKGGSFPR-NH2                  | 3.2                | 3.9             |
| QRFP(28OH)  | TSGPLNLAEVLNGYSRKKGGSFPR-NH2                  | 1,200              | >1,000          |
| QRFP(23)    | GLNLAEVLNLNGYSRKKGGSFPR-NH2                   | 15                 | 8.9             |
| QRFP(19)    | LGNLAEVLNGYSRKKGGSFPR-NH2                     | 49                 | 440             |
| QRFP(15)    | LGNLAEVLNGYSRKKGGSFPR-NH2                     | 37                 | 460             |
| QRFP(10)    | RKKGGSFPR-NH2                                   | 220                | >1,000          |
| QRFP(8)     | KCGGGSFPR-NH2                                   | 2,900              | >1,000          |
| QRFP(7)     | NGGGSFPR-NH2                                    | >10,000            | >1,000          |
| QRFP(28n)   | EDGSEAGFPAGKTEGTSGPLGLAMᕙ                  | >10,000            | >1,000          |

Interaction of various lengths of QRFP with AQ27

Agnostic activities of QRFPs were determined by cAMP production-inhibitory assay. Binding activities were determined by competitive binding assay using [125I-Tyr32]QRFP(43). Pyroglutamic acid is shown as <E.
peptide would also function as a ligand for a GPCR. We have previously searched for ligands of various orphan GPCRs by exposing synthetic peptides to CHO cells expressing orphan GPCRs (2, 3). We found that CHO cells expressing the orphan GPCR, AQ27, weakly but specifically responded to Met-enkephalinamide peptide (data not shown). AQ27 is a novel GPCR that we isolated from human brain poly(A)⁺ RNA based on public genome information (GenBank™ accession number AAQ270411), and has been found to be identical to GPR103 as recently reported (17). We isolated its rat and mouse counterparts from their respective brain poly(A)⁺ RNA by RT-PCR. The amino acid sequences deduced from human, rat, and mouse cDNA are aligned in Supplemental Fig. 1. These had amino acid lengths of 431 or 433 and showed 84–96% amino acid identity to each other. Among the GPCRs for which ligands are known, AQ27 showed homology with OT7T022 (the receptor for RFRP) and HLWAR77 (the receptor for neuropeptide FF), that is 30 and 32% amino acid identity, respectively, as determined by the Gapped Blast program. As a result of phylogenetic analysis, GPR103 has been predicted to be an RFamide peptide receptor (18). We therefore inferred that QRFP might act as a ligand for AQ27. To examine this, we synthesized a short peptide with an amino acid length of 7 (GGFSFRFamide). We derived this from the C-terminal RFGR motif of the human QRFP preproprotein because, in RFamide peptides, C-terminal portions are essential sites to bind receptors, and even short C-terminal peptides frequently retain receptor binding activity (19). We subjected this short peptide to an assay with HEK293 cells transiently expressing AQ27 and a reporter gene (cAMP-responsive element-luciferase). This assay was modified from the method of Chen et al. (20, 21). They demonstrated that the activation of GPCRs coupled with Gs and/or Gq causes an increase in the transcription of the cAMP-responsive element promoter. We modified their method for detecting Gq signals so that, by our technique, Gq signals were detected as the increase of luciferase activity in the presence of forskolin. As AQ27 coupled to Gq, we detected the activation of AQ27 treated with the peptide as the increase of luciferase activity (data not shown). However, the agonistic activity of this peptide appeared to be very weak with its effective dose ranging from 10⁻⁸ to 10⁻⁶ M (data not shown).

**Determination of a Fully Active Form of QRFP**—As the agonistic activity of the 7-amino acid-long synthetic peptide was considerably weak, we thought that a longer form of the peptide would show full activity. To ascertain this, we expressed the human QRFP cDNA in CHO cells and examined whether more effective peptidic ligands were secreted in the culture supernatant. As we could detect specific and strong stimulatory activity on HEK293 cells expressing AQ27 in the culture supernatant, we decided to purify the ligands for AQ27 from the supernatant, and even short C-terminal portions are essential sites to bind receptors, and even short C-terminal peptides frequently retain receptor binding activity (19). We subjected this short peptide to an assay with HEK293 cells transiently expressing AQ27 and a reporter gene (cAMP-responsive element-luciferase). This assay was modified from the method of Chen et al. (20, 21). They demonstrated that the activation of GPCRs coupled with Gs and/or Gq causes an increase in the transcription of the cAMP-responsive element promoter. We modified their method for detecting Gq signals so that, by our technique, Gq signals were detected as the increase of luciferase activity in the presence of forskolin. As AQ27 coupled to Gq, we detected the activation of AQ27 treated with the peptide as the increase of luciferase activity (data not shown). However, the agonistic activity of this peptide appeared to be very weak with its effective dose ranging from 10⁻⁸ to 10⁻⁶ M (data not shown).

**Determination of a Fully Active Form of QRFP**—As the agonistic activity of the 7-amino acid-long synthetic peptide was considerably weak, we thought that a longer form of the peptide would show full activity. To ascertain this, we expressed the human QRFP cDNA in CHO cells and examined whether more effective peptidic ligands were secreted in the culture supernatant. As we could detect specific and strong stimulatory activity on HEK293 cells expressing AQ27 in the culture supernatant, we decided to purify the ligands for AQ27 from the culture supernatant. To do so, we used affinity column chromatography using a monoclonal antibody (1F3) for RFRP-1 (22)

was added to the cells after 10 s. The concentrations of peptide used were 10⁻⁵ M (○), 10⁻⁶ M (●), 10⁻⁷ M (□), 10⁻⁸ M (■), 10⁻⁹ M (▲), and 10⁻¹⁰ M (△). Data are shown as representative traces. B, inhibition of cAMP production by various forms of QRFP in CHO cells expressing AQ27. The inhibition of forskolin-stimulated cAMP increase in CHO-hAQ27 was measured in the presence of the indicated concentration of QRFP(43) (○), QRFP(26) (●), QRFP(260EF) (□), QRFP(23) (■), QRFP(19) (▲), QRFP(15) (△), QRFP(10) (+), and QRFP(7) (□). Scatchard plot for binding of [¹²⁵I-Tyr³²]QRFP to AQ27. Membrane fractions prepared from CHO-hAQ27 cells were incubated with increasing concentrations of [¹²⁵I-Tyr³²]QRFP(43), and the bound and free ligands were separated when the binding reached equilibrium. Data are plotted as bound (B, pmol/mg⁻¹ of protein) versus bound/free (B/F, pmol mg⁻¹ nM⁻¹). Each symbol represents a mean value with a S.E. in triplicate determinations.

**Fig. 4. Analysis of interaction between QRFPs and AQ27.** A, QRFP-induced changes in Ca²⁺ concentrations in CHO cells expressing AQ27. CHO-hAQ27 cells were loaded with Fluo-3/AM, and their changes in fluorescence were measured by FLIPR. Human QRFP(43)
because 1F3 could cross-react with the short form of QRFP (GGFSFRFamide). The culture supernatant (2.4 L) was boiled and centrifuged, and the resulting supernatant was applied to the affinity chromatography. Elution was conducted with 0.2M glycine-HCl (pH 2.2) containing 0.5 M NaCl. The eluate was subsequently fractionated through a Vydac C18 218TP5415 column with a 20–35% linear gradient of CH3CN, and each fraction was examined in assays with HEK293 cells transiently expressing human AQ27 and a reporter gene. Finally active fractions in the assays were applied to a RPC C2/C18 SC2.1/10 column chromatography. AQ27-stimulatory activities were detected in eluted fractions, which corresponded to at least two peaks (i.e. peaks 1 and 2 in Fig. 3A). Although peaks 1 and 2 matched absorbance peaks at 215 nm, the main absorbance peak at 280 nm matched only with peak 1. In the C-terminal region of QRFP there is a Tyr residue that should give absorbance at 280 nm. Since the absorbance of peak 2 was faint at 280 nm, we believe that it contained impurities. In the reporter gene assay, both peaks appeared to reach plateau levels of luciferase activity. Considering these results, we concluded that peak 1 was a major product derived from the QRFP cDNA-transfected CHO cells and that peak 2 was not a major peak, although it showed activity.

We determined the structure of the purified peptide in peak 1 as follows. Since the quantity of the peptide in peak 1 was calculated to be less than 1 pmol from its absorbance peak height, we analyzed it by MALDI-TOF mass spectrometry (Fig. 3B) and found that it produced a protonated molecular ion (m/z 4505.7). Considering this together with other evidence, we concluded the structure of the peptide to be <EDEGSEATGFL-PAAGEKTSGPLGNLAELNGYSRKGFSFRFamide (<E indicates pyroglutamic acid). Although we could not determine from the mass spectrometric data whether or not the C-terminal residue was amidated, we determined the C-terminal RFamide structure of the peptide by the following reasoning. (i) The cDNA sequence of QRFP had motifs to generate RFamide peptides, and we knew that the synthetic 7-amino acid-long peptide with C-terminal RFamide showed weak but significant agonistic activity in the reporter assay (data not shown). (ii) It has been well established that RFamide structure is essential for RFamide peptides to interact with their receptors (23). (iii) We confirmed with synthetic peptides that the nonamidated form of this peptide showed drastically reduced agonistic activity (Table I). (iv) The M + H+ calculated average masses of the amidated and nonamidated form were 4504.9 and 4505.9, respectively. Although the M + H+ observed average mass (m/z 4505.7) seemed to be closer to the M + H+ calculated average mass of the nonamidated peptide than that of the amidated form, both M + H+ calculated average masses were within the allowable error limit (0.05%) of delayed extraction MALDI-TOF mass spectrometry in linear mode using external calibration. Therefore, the mass spectrometric data did not contradict the predicted RFamide structure. We determined the N-terminal structure as follows. (i) It is well known that an N-terminal glutamine or glutamic acid residue is easily circularized and converted to pyroglutamic acid (24). (ii) The calcu-
lated molecular weight of the nonpyroglutamylated form was 4521.9, which differed from the mass spectrometric data (i.e. 4505.7) beyond the error limited. We therefore concluded that the purified peptide of peak 1 possessed the predicted structure. Our results indicated that this peptide was generated from the preproprotein encoded by the cDNA through processing. We therefore named it QRFP for pyroglutamylated RFamide peptide. In the reporter gene assays, the purified peptide significantly suppressed cAMP production in CHO-hAQ27 after the stimulation. We also detected the suppression of cAMP production in CHO-hAQ27—We next determined intracellular Ca$^{2+}$ and cAMP changes in CHO-hAQ27 after stimulation with QRFP. As shown in Fig. 4A, we detected a rapid rise of intracellular Ca$^{2+}$ concentrations in CHO-hAQ27 after the stimulation. We also detected the suppression of cAMP production in these cells (Fig. 4B and Table I). These results suggest that AQ27 couples to G proteins, that is G$i/0$ and G$q$, in CHO cells. Because the purified QRFP had a long N-terminal region, we chemically synthesized various forms of QRFP and determined their cAMP production-inhibitory activities. QRFP inhibited the cAMP production of CHO-hAQ27 in a dose-dependent manner. The 43-amino acid-long QRFP, that is QRFP(43), was the most potent with a 50% inhibitory concentration (EC$_{50}$) of 2.7 nM (Table I). Serial deletion of the N-terminal region gradually attenuated the inhibitory activity of QRFP. However, the efficacy of the inhibitory activity of the peptides did not change (Fig. 4B). A nonamidated form of the peptide, that is QRFP(26OH), and an N-terminal portion of QRFP, that is QRFP(28n), did not show the evident inhibitory activities, indicating that the C-terminal structure of QRFP is crucial in its interaction with the receptor.

### Table II

| Abbreviation | Name                                | AQ27 | QRFP |
|--------------|-------------------------------------|------|------|
| Pir          | Piriform cortex                     | +++  | –    |
| CxA          | Cortex-amygdala transition zone     | +++  | –    |
| VP           | Ventral pallidum                    | ++   | –    |
| BSTMA        | Bed nucleus stria terminalis, medial division, anterior part | +   | –    |
| BSTLP        | Bed nucleus stria terminalis, lateral division, posterior part | +   | –    |
| BSTLV        | Bed nucleus stria terminalis, lateral division, ventral part | +   | –    |
| LPO          | Lateral preoptic area               | +++  | –    |
| ADP          | Anterodorsal preoptic nucleus       | +++  | –    |
| VMPO         | Ventrolateral preoptic nucleus      | +    | –    |
| MPOM         | Medial preoptic nucleus, medial     | +    | –    |
| AHA          | Anterior hypothalamic area, anterior part | +  | –    |
| AHC          | Anterior hypothalamic area, central part | +  | –    |
| AHP          | Anterior hypothalamic area, post part | +  | –    |
| MCPO         | Magnocellular preoptic nucleus      | ++   | –    |
| PaAP         | Paraventricular hypothalamic nucleus, parvicellular part | +   | –    |
| PaDC         | Paraventricular hypothalamic nucleus, dorsal cap | +   | –    |
| PVA          | Paraventricular thalamic nucleus, anterior | + | –    |
| PVP          | Paraventricular thalamic nucleus, posterior | + | –    |
| VMHC         | Ventromedial hypothalamic nucleus, central part | + | –    |
| VMHDM        | Ventromedial hypothalamic nucleus, dorsomedial part | + | –    |
| VMHVL        | Ventromedial hypothalamic nucleus, ventrolateral part | ++ | –    |
| ZI           | Zona incerta                        | +++++| –    |
| RCh          | Retrochiasmatic area                | +    | –    |
| ArcD         | Arcuate nucleus, dorsal part        | +    | –    |
| ArcM         | Arcuate nucleus, medial part        | +    | –    |
| ArcL         | Arcuate nucleus, lateral part       | +    | –    |
| ArcMP        | Arcuate nucleus, medial posterior part | + | –    |
| PH           | Posterior hypothalamic area         | +    | –    |
| LH           | Lateral hypothalamic area           | +    | –    |
| MPT          | Medial pretectal nucleus            | +    | –    |
| IGL          | Intergeniculate leaf                | +    | –    |
| M1           | Primary motor cortex                | +    | –    |
| M2           | Secondary motor cortex             | +    | –    |
| Cg1          | Cingulate cortex, area 1            | +    | –    |
| Cg2          | Cingulate cortex, area 2            | +    | –    |
| MeAD         | Medial amygdaloid nucleus, anterodorsal part | + | –    |
| MZMG         | Marginal zone medial geniculate     | +    | –    |
| BIC          | Nucleus of brachium inferior colliculus | ++ | –    |
| DRD          | Dorsal raphe nucleus, dorsal part   | ++   | –    |
| DRV          | Dorsal raphe nucleus, ventral part  | ++   | –    |
| DRC          | Dorsal raphe nucleus, caudal part   | ++   | –    |
| MnR          | Median raphe nucleus                | +    | –    |
| LDTg         | Laterodorsal tegmental nucleus      | +    | –    |
| Ppy          | Parapyramidal nucleus               | +    | –    |
| LC           | Locus caeruleus                     | ++   | –    |
| CGA          | Central gray, α part                | +++  | –    |
| CGB          | Central gray, β part                | +++  | –    |
| Sol          | Nucleus of solitary tract           | +    | –    |
A competition with an IC50 of 0.52 nM. The deletion of the N-terminal sequence in QRFP gradually diminished its binding affinity to AQ27 and was almost parallel to the decrease of cAMP production-inhibitory activities.

**In situ** hybridization of AQ27 mRNA in rat adrenal gland. A and C, hybridization with an antisense riboprobe. B and D, hybridization with a sense riboprobe. Positive signals by the antisense probe were mainly detected in the zona glomerulosa (A) and zona reticularis (C). No hybridization signal was detected with the sense probe in the same areas including the adrenal medulla (B and D). ZG, zona glomerulosa; ZF, zona fasciculate; ZR, zona reticularis; M, adrenal medulla. Scale bar, 100 μm.

(data not shown). These results indicate that QRFP functions specifically as a ligand for AQ27.

To examine the binding of QRFPs with AQ27, we labeled human QRFP(43) with 125I. As shown in Fig. 4C, Scatchard plot analysis indicated that the membrane fractions of CHO-hAQ27 had a single class of high affinity binding sites for [125I-Tyr32]QRFP at the dissociation constant (Kd) of 5.3 × 10−11 M and maximal binding sites (Bmax) of 0.51 pmol mg−1 of protein, indicating that QRFP binds with high affinity to AQ27 as a specific ligand. In competitive binding experiments, various lengths of QRFP inhibited the binding of [125I-Tyr32]QRFP with AQ27 (Table I). QRFP(43) proved the most potent in the competition with an IC50 of 0.52 nM. The deletion of the N-terminal sequence in QRFP gradually diminished its binding affinity to AQ27 and was almost parallel to the decrease of cAMP production-inhibitory activities.

**Tissue Distribution of AQ27 and QRFP mRNAs**—We analyzed the tissue distribution of AQ27 and QRFP mRNAs in rats by quantitative RT-PCR. The highest levels of QRFP mRNA were detected in the hypothalamus and optic nerve, while signals were detected in neurons within the piriform cortex, intergeniculate leaf, locus caeruleus, and central gray α/β part. (Table II and Supplemental Fig. 3). The sense probe for AQ27 mRNA did not give any evident signals. In the adrenal gland, AQ27 mRNA was detected in the cortex. The cortex consists of three parts, that is the zona glomerulosa, zona fasciculate, and zona reticularis. A high level of AQ27 mRNA expression was detected in the zona glomerulosa with moderate levels found in the zona fasciculata and zona reticularis (Fig. 6).

**Promotion of Aldosterone Secretion in Rats by QRFP**—The zona glomerulosa in the adrenal gland is known to secrete mineral corticoids including aldosterone. We examined the effects of QRFP on aldosterone secretion by intravenous administration into rats. As shown in Fig. 7, human QRFP(43) at doses of 40–400 nmol/kg of weight increased plasma aldosterone levels 5–15 min after administration. Five minutes after administration, plasma aldosterone concentrations in the treated rats reached about 5 times those in the untreated rats and then gradually declined reaching the basal level at 60 min. Although we examined other hormones (i.e. testosterone and corticosterone) secreted from the adrenal gland, we could not detect significant changes in their plasma levels (data not shown). Considered together with the expression of AQ27 mRNA in the adrenal gland, these results indicate that QRFP acts directly on the zona glomerulosa to induce aldosterone secretion in rats.

**DISCUSSION**

In this study, we have demonstrated that the novel RFamide peptide QRFP and its receptor exist and function in mammals. Although Jiang Z. et al. (10) have very recently reported the identification of the same peptide and its receptor independently of our research, they did not determine the form of this peptide possessing a full agonistic activity. We have shown here that a 43-amino acid-long sequence was necessary to exhibit full activity in QRFP on the basis of experiments ex-
pressing the QRFP gene in CHO cells and analyzing the interaction of the receptor with various lengths of synthetic peptides. Furthermore we unequivocally demonstrated that QRFP specifically bound with high affinity to the receptor, AQ27, on the basis of Scatchard plot analysis. The affinity of QRFP to AQ27 (i.e. \( K_d \) of \( 5.3 \times 10^{-11} \) M) was comparable in level to those of other known RFamide peptides to their receptors (2, 8, 9, 19). Multiple binding sites have been reported in the case of recombinant GPCRs, and these are affected by guanine nucleotides (25). However, under our experimental conditions, we did not detect any low affinity binding site in the absence of additional guanine nucleotide.

Another RFamide motif was found in the N-terminal portion of the human preproprotein, that is \( ^{87} \)RFGRG\( ^{90} \). However, synthetic RFamide peptides deduced from this motif did not show high agonistic activity on HEK293 cells expressing human AQ27 (data not shown). In addition, alignment of the human, bovine, rat, and mouse preproprotein sequences revealed that this motif was not conserved in the other species (Fig. 1). Furthermore, in our experiments expressing the human QRFP cDNA in CHO cells, we could not detect agonistic peptides generated from this motif in the culture supernatant. In view of these results, we concluded that only one RFamide peptide (i.e. QRFP) would be produced from the C-terminal portion of the human preproprotein. QRFP is the fifth member of the RFamide peptide family to be found in mammals. Although we examined whether the fully active form of QRFP could cross-react with the receptors for other RFamide peptides, such cross-reaction was not detected. However, when we tested the short 7-amino acid peptide, it showed very weak cross-reactions to OT77T022 (data not shown), suggesting that QRFP is closely related to RFRPs and neuropeptide FF not only in structure but also in functions. We concluded that the long N-terminal portion of QRFP is important in establishing its specificity and affinity for AQ27.

In our analyses for the tissue distribution of QRFP and AQ27 mRNAs in rats, we found that QRFP mRNA was highly expressed in the hypothalamus, optic nerve, trachea, and mammary gland. On the other hand, high levels of AQ27 mRNA were detected in the central nervous system, adrenal gland, and testis. These results suggest that QRFP and its receptor possess multiple functions in various tissues. The very high expression of AQ27 mRNA in the adrenal gland, especially in the zona glomerulosa of the cortex, suggested that QRFP affected the function of the adrenal glands in rats. Although the major hormones secreted from the adrenal cortex are corticosterone, testosterone, and aldosterone, the zona glomerulosa is the main site producing aldosterone, which controls electrolyte metabolism. We have shown here that the intravenous injection of QRFP resulted in the increase of plasma aldosterone in rats. Our results suggest that QRFP and its receptor play a regulatory role in aldosterone secretion from the adrenal cortex in rats. The release of aldosterone is mainly controlled by angiotensin II (26). Although the stimulatory activity of QRFP appeared to be weaker than that of angiotensin II (i.e. the efficacy of QRFP was one-tenth less than that of angiotensin II), QRFP might have regulatory functions similar to angiotensin II in aldosterone secretion in rats. As we noted, QRFP has a structure quite similar to Met-enkephalinamide. Enkephalins reportedly have regulatory functions in the release of catecholamine from the medulla of the adrenal gland. In addition, neuropeptide FF exists in the adrenal gland and inhibits aldosterone release (27). Considering these results, RFamide peptides might play roles in the regulation of adrenal functions. However, in comparison with rats, AQ27 mRNA expression levels in the adrenal gland are not so high in humans and mice (10). Therefore, we cannot rule out the possibility that in each species QRFP and its receptor show different expression and functions in respect to the adrenal gland.

A high level of AQ27 mRNA expression was detected in the hypothalamus in rats. It is noteworthy that high levels of AQ27 mRNA have been detected in the hypothalamus even in humans and mice (10, 16). It is known that peptides found abundantly in frequently utilized brain structures have a role in the feeding behavior of animals. Since QRFP mRNA was detected in the retrochiasmatic nucleus and arcuate nucleus, we tested the effects of the intracerebroventricular injection of QRFP on food intake in rats. We could not, however, detect any evident effects of QRFP under our experimental conditions (data not shown). Using in situ hybridization, AQ27 mRNA was detected in the dorsal raphe nucleus, locus caeruleus, laterodorsal tegmental nucleus, and ventrolateral preoptic nucleus, which are known as regions related to sleep modulation (28), suggesting that QRFP has a regulating activity on sleep.

Based on sequence homology, AQ27 was found to correspond to NPY-1 in the nematode. Disruption of the NPY-1 gene reportedly affects the social behavior of the nematode (29). As AQ27 mRNA was detected widely in the brain including the cortex zones in rats (Table II), the suppression of AQ27 expression might influence behavior even in mammals. Future studies will be necessary to clarify the physiological significance of QRFP and its receptor.

Acknowledgments—We thank Dr. Y. Fujisawa for helpful discussion. We thank Drs. C. Kitada, T. Watanabe, K. Kikuchi, M. Harada, T. Shinohara, H. Tanaka, Y. Noguchi, M. Hosoya, and Y. Kawamata for assistance with the experiments. We also thank Celera Genomics for the use of Celera’s data bases.

REFERENCES

1. Hinuma, S., Onda, H., and Fujino, M. (1999) J. Mol. Med. 77, 495–504
2. Hinuma, S., Habata, Y., Fujii, R., Kawamata, Y., Hosoya, M., Fukuosumi, S., Kitada, O., Masuo, Y., Asano, T., Matsumoto, H., Sekiguchi, M., Kurakawa, T., Nishimura, O., Onda, H., and Fujino, M. (1998) Nature 393, 273–276
3. Tatemoto, K., Hosoya, M., Habata, Y., Fujii, R., Kakegawa, T., Zou, M. X., Kawamata, Y., Fukuosumi, S., Hinuma, S., Kitada, C., Kurakawa, T., Onda, H., and Fujino, M. (1998) Biochem. Biophys. Res. Commun. 251, 471–476
4. Hinuma, S., Shintani, Y., Fukuosumi, S., Iijima, N., Matsumoto, Y., Hosoya, M., Fuji, R., Watanabe, T., Kikuchi, K., Terao, Y., Yano, T., Yamamoto, T., Kawamata, Y., Habata, Y., Asada, M., Kitada, C., Kurakawa, T., Onda, H., Nishimura, O., Tanaka, M., Ibata, Y., and Fujino, M. (2000) Nat. Cell Biol. 10, 703–708
5. Price, D. A., and Greenberg, M. J. (1977) Science 197, 670–671
6. Li, C., Kim, K., and Nelson, L. S. (1999) Brain Res. 848, 26–34
7. Perry, S. J., Yi-Kung, H. E., Cronk, D., Bagust, J., Sharma, R., Walker, R. J., Wilson, S., and Burke, D. P. (1997) FEBS Lett. 400, 420–430
8. Elshourbagy, N. A., Ames, R. S., Fitzgerald, L. R., Foley, J. D., Chambers, J. K., Szekeres, P. G., Evans, N. A., Schmidt, D. B., Buckley, P. T., Dytko, G. M., Murdock, R. P., Milligan, G., Groaske, D. A., Tan, K. B., Shabon, U., Nuthalapati, P. W., Wang, D. Y., Wilson, S., Bergman, B. D., and Baranu, H. M. (2000) J. Biol. Chem. 275, 25965–25971
9. Ohtaki, T., Shintani, Y., Honda, S., Matsumoto, H., Hori, A., Kaneko, K., Terao, Y., Kuman, S., Takata, Y., Masuda, Y., Isihashi, Y., Watanabe, T., Asada, M., Yamada, T., Suegana, M., Kitada, C., Usuki, S., Kurakawa, T., Onda, H., Nishimura, O., and Fujino, M. (2001) Nature 411, 613–617
10. Jiang, Y., Luo, L., Gustafson, E. L., Yadav, D., Laverty, M., Murgolo, N., Vissileva, G., Zeng, M., Laiz, T. M., Behan, J., Qiu, P., Wang, L., Wang, S., Bayne, M., Greene, J., (2003) J. Biol. Chem. 278, 27652–27657
11. Hinuma, S., Hosoya, M., Og, K., Tanaka, H., Nagai, Y., and Onda, H. (1994) Biochim. Biophys. Acta 1199, 251–259
12. Fukuosumi, S., Kitada, C., Takekawa, S., Kizawa, H., Sakamoto, J., Miyamoto, M., Hinuma, S., Kitano, K., and Fujino, M. (1997) Biochem. Biophys. Res. Commun. 232, 157–163
13. Hosoya, M., Moriya, T., Kawamata, Y., Ohkubo, S., Fuji, R., Matsui, H., Shintani, Y., Fukuosumi, S., Habata, Y., Hinuma, S., Onda, H., Nishimura, O., and Fujino, M. (2000) J. Biol. Chem. 275, 29528–29532
14. Fujisawa, K., Hosoya, M., Kawamata, Y., Usuki, K., Hinuma, S., Onda, H., Nishimura, O., and Fujino, M. (2000) J. Biol. Chem. 275, 21068–21074
15. Jost, P., and Methner, A. (2002) Genome Biol. http://genomebiology.com/2002/
19. Yoshida, H., Habata, Y., Hosoya, M., Kawamata, Y., Kitada, C., and Hinuma, S. (2003) Biochim. Biophys. Acta 1593, 151–157
20. Durocher, Y., Perret, S., Thibaudneau, B., Gaumand, M. H., Kamen, A., Stocco, R., and Abramovitz, M. (2000) Anal. Biochem. 284, 316–326
21. Chen, W., Shields, T. S., Stork, P. J., and Cone, R. D. (1995) Anal. Biochem. 226, 349–354
22. Fukusumi, S., Habata, Y., Yoshida, H., Iijima, N., Kawamata, Y., Hosoya, M., Fujii, R., Hinuma, S., Kitada, C., Shintani, Y., Suenaga, M., Onda, H., Nishimura, O., Tanaka, M., Ibata, Y., and Fujino, M. (2001) Biochim. Biophys. Acta 1540, 221–232
23. Mekler, D. J. (1994) Enzyme Microb. Technol. 16, 450–456
24. Bushy, W. H., Jr., Quackenbush, G. E., Humm, J., Youngblood, W. W., and Kizer, J. S. (1987) J. Biol. Chem. 262, 8532–8536
25. Jopez-Gimenez, J. F., Villanueva, M., Brea, J., Loza, M. I., Palacios, J. M., Mengod, G., and Vilars, M. T. (2001) Mol. Pharmacol. 60, 690–699
26. Campbell, W. B., Brooks, S. N., and Pettinger, W. A. (1974) Science 184, 984–996
27. Labrouche, S., Laulin, J. P., Le Moal, M., Tramu, G., and Simonnet, G. (1998) J. Neuroendoocrinol. 7, 559–565
28. Monti, J. M., and Monti, D. (2000) Life Sci. 66, 1999–2012
29. de Bono, M., and Bargmann, C. I. (1998) Cell 94, 679–689
