Identification of Neuromedin U as the Cognate Ligand of the Orphan G Protein-coupled Receptor FM-3*

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Neuromedin U is a bioactive peptide first isolated from porcine spinal cord. In this paper, we demonstrate that neuromedin U is the cognate ligand for the orphan G protein-coupled receptor, FM-3, isolated originally as a homologue of neurotensin and growth hormone secretagogue receptors. Neuromedin U induced specific and evident elevation of extracellular acidification rates, arachidonic acid metabolite release, and intracellular Ca2+ mobilization in Chinese hamster ovary cells expressing human FM-3. In addition, radiolabeled neuromedin U specifically bound to membrane fractions prepared from these cells with high affinity. We subsequently analyzed the tissue distribution of neuromedin U and FM-3 mRNAs in rats using quantitative reverse transcription-polymerase chain reaction. Neuromedin U mRNA was highly expressed in the gastrointestinal tract, and the highest expression was detected in the pituitary gland. On the other hand, FM-3 mRNA was highly expressed in the small intestine and lung, suggesting that neuromedin U plays important roles in these tissues. The identification of a specific and functional receptor for neuromedin U will facilitate studies on their physiological roles and the search for receptor agonists and antagonists.

A large number of G protein-coupled receptors (GPCRs) are identified through the rapid progress of genome and cDNA analyses. However, most of their ligands are yet unidentified, so these are called "orphan" GPCRs. The identification of ligands for orphan GPCRs will promote better understanding of the regulatory mechanisms responsible for various physiological phenomena. We have recently established a strategy widely applicable to identify orphan GPCR ligands (1, 2). Our strategy is principally to search agonistic ligands by monitoring signal transductions in cells expressing orphan GPCRs. To detect cellular changes caused by the interaction of orphan GPCRs and their ligands, we have frequently measured extracellular acidification rates in Chinese hamster ovary (CHO) cells expressing orphan GPCRs (3). Almost all intracellular changes, including the activation of GPCRs, result in the acidification of the extracellular environment, and such extracellular acidification can be monitored by the microphysiometric assay using a Cytosensor (4). In the screening of various synthetic compounds, including known bioactive peptides, we found that CHO cells expressing an orphan GPCR, FM-3, were responsive to neuromedin U in the microphysiometric assay.

FM-3 was first isolated from a mouse T-cell cDNA library, and subsequently its human counterpart was isolated from a genomic library through screening with a mouse cDNA probe (5). Sequence analyses indicate that FM-3 shows moderate sequence identity with neurotensin (NT) and growth hormone secretagogue (GHS, ghrelin) (6) receptors. However, HEK-293 cells expressing FM-3 failed to bind a GHS receptor agonist (MK-0677), NT, and some known bioactive peptides; therefore, FM-3 has remained as an orphan GPCR. On the other hand, neuromedin U-8 and U-25 were isolated as bioactive peptides originally from the porcine spinal cord on the basis of their contractile activity to rat uterine smooth muscle (7, 8). Neuromedin U reportedly shows various biological activities, including hypertensive effects on rats in vivo (7, 9–12). In this report, we will demonstrate that FM-3 is a specific receptor for neuromedin U. In addition, we will show here the tissue distribution of rat neuromedin U and FM-3 mRNAs analyzed by quantitative reverse-transcription polymerase chain reaction (RT-PCR) and Southern blot analyses for human and rat FM-3 genes.

**EXPERIMENTAL PROCEDURES**

Preparation of CHO Cells Expressing GPCRs—The entire coding region of human FM-3 cDNA (5) was amplified by PCR from human brain cDNA (CLONTECH, Palo Alto, CA) and inserted into the downstream region of an SR a promoter in the expression vector pAKKO-111H (13). The resultant expression vector plasmid was transfected into dhfr− CHO cells, and then transformed dhfr+ CHO cells (CHO-hFM-3) were selected as described previously (13). CHO cells expressing human NT type 1 and rat GHS receptors were established by a similar procedure.

Peptides—Porcine neuromedin U-8 and U-25 and rat U-23 were purchased from Bachem AG (Bubendorf, Switzerland); human U-25 was synthesized by the Peptide Institute (Osaka, Japan). Other peptides were purchased from commercial suppliers.

Microphysiometric Assays—Extracellular acidification rates were measured with a Cytosensor (Molecular Devices Corp., Sunnyvale, CA).
After CHO-hFM-3 or mock-transfected (i.e. only the vector without FM-3 cDNA was transfected) CHO cells were dispersed with trypsin, and they were dispensed into cell capsules (Molecular Devices) at 2.7 × 10^5 cells per capsule and cultured overnight, respectively. Then each cell capsule was attached to the device, and the cells were continuously loaded with RPMI-1640 medium (Molecular Devices) containing 0.1% bovine serum albumin (BSA) until the acidification rates became stable. The acidification rates were measured every 120 s (flow on at 100 µl/min for 80 s; flow off for 8 s; measuring acidification rates for 30 s).

Ca^{2+} Mobilization Assays—CHO-hFM-3 and mock-transfected CHO cells were seeded in black-walled, clear-base, 96-well tissue culture plates (Costar, Cambridge, MA) at 2.5 × 10^5 cells per well and cultured overnight. The cells were then incubated at 37 °C for 1 h in HEPES-buffered Hank’s balanced salt solution (pH 7.4) containing 2.5 mM probenecid and 4 µM Fluo-3AM (Dojindo, Kumamoto, Japan). The cells were washed four times with the solution without Fluo-3AM, and then changes in intracellular Ca^{2+} concentrations were measured with a fluorometric-imaging plate reader system (FLIPR; Molecular Devices) before and after the addition of agonists.

Arachidonic Acid-Metabolite Release Assays—The arachidonic acid (AA)-metabolite release assay was performed principally according to the method described previously (14).

Receptor Binding Assays—A tyrosine residue at the N terminus of porcine U-8 was radioiodinated with Na[125I] (IMS-30, Amersham Pharmacia Biotech) using a method with lactoperoxidase (Sigma) (15). After incubation, labeled peptides and unlabelled peptides were separated by reverse-phase high performance liquid chromatography. Aliquots of the labeled peptide were stored at −30 °C until use. The membrane fractions of CHO-hFM-3 cells were prepared by a method described previously (16). They were incubated with 125I-labeled porcine U-8 in 100 µl of the binding buffer containing 0.1% BSA in 96-well microplates (Sarocutherford, Corning Costar Corp., Cambridge, MA) at room temperature for 1.5 h. To determine the amounts of nonspecific binding, unlabeled porcine U-8 (final concentration at 10 −6 M) was simultaneously added to the wells. After the incubation, bound and free radioactivities were separated through rapid filtration using glass-fiber filter units (GF/C, Packard Instrument Co., Meriden, CT) equipped with a 96-well cell harvester (Packard). The filter units were completely dried, and Microcinti O (Packard) was added to each well. The radioactivity of each well was then measured with a TopCount liquid scintillation counter (Packard).

Cloning of Rat FM-3 cDNA—Rat FM-3 cDNA was isolated from poly(A)^+ RNA of rat brain. We first designed the primers, 5'-GGGCTTGCGGGTTGGCTGCTGAG-3' and 5'-CCGGTGGCTGCTGAGCCGAGATAGAA-3', and then performed PCR in a reaction mixture (25 µl) containing 0.2 mM dNTPs, 1.25 units of KlenTaq DNA polymerase (CLONTECH), and 2.5 µM concentration of each primer, a template DNA fragments thus obtained were recovered from the agarose gel and then purified using a gel and blot imaging system (Storm model 860, Amersham Pharmacia Biotech). Hybridization was performed at 59 °C overnight in a hybridization solution containing 0.5× Na2HPO4 (pH 7.2), 7% SDS, 1 mM EDTA, and 1% BSA. The filters were washed with 0.5× SSC (1× SSC = 0.18 M NaCl and 0.09 M sodium citrate) containing 0.1% SDS at 59 °C and then exposed to imaging plates (Fuji Film Co., Tokyo, Japan) for 48 h, and hybridization signals were detected using a gel and blot imaging system (Storm model 860, Amersham Pharmacia Biotech).

Identification of Rat FM-3 and Its Related Gene—The EcoRI-digested rat genomic DNA was electroeluted with 0.8% agarose gel, and the DNA fragments were extracted from the gel using Suprec-01 DNA recovery cartridges (Takara Shuzo Co., Kyoto, Japan). Rat FM-3 and its related genomic gene fragments were amplified by PCR with primers (5'-AGTCGGCTGGAGGTTGGAACATCC3' and 5'-CTTGCTCCAGGTAGATCC3') designed from the second extracellular and C-terminal coding regions of the rat FM-3 cDNA, under the conditions at 94 °C for 2 min, followed by 35 cycles at 94 °C for 10 s, 62 °C for 10 s, and 72 °C for 60 s. Based on the partial rat cDNA sequence thus obtained, we synthesized various primers and isolated cDNA fragments, which covered a full coding region, by using a method consisting of 5' and 3' rapid amplification of cDNA ends with a Marathon cDNA amplification kit. Finally, we isolated a rat FM-3 cDNA with a full coding region from the rat brain cDNA by PCR with a primer set (5'-GGGCTTGGCTGCTGAGCCGAGATAGAA-3' and 5'-GGGCTCTAGGCGTGGGTCTCTGTGCGATCC-3').

Quantitative Analyses for Rat Neuromedin U and FM-3 mRNAs by RT-PCR—Poly(A)^+ RNAs were prepared from the tissues of 8- to 12-week-old rats and cDNAs were synthesized from them as described previously (14). Poly(A)^+ RNAs of the placenta and mammary glands were prepared from female rats at 17 days in pregnancy. We quantitated rat FM-3 and neuromedin U mRNAs by means of a Prism 7700 sequence detector (PE Biosystems, Foster City, CA) with primer sets (5'-CATCAGTGGTGCCAAGAAGGAGC3' and 5'-TTCTCGCTCTGTTTGTTTGCTCTGAGGC3' for neuromedin U; 5'-CAGGGAGACAGTGACGAAGGAAGGAGC3' and 5'-GGATCAAATGAGACCGAAGGAGGAGGAGC3' for FM-3) and fluorescent-labeled probes (5'FAM)-TGGGCTTGGTGCTCGAGTATTTTCCA(TAMRA)3' for neuromedin U; 5'(FAM)-TGGGCTTGGTGCTCGAGTATTTTCCA(TAMRA)3' and 5'-GGGCTTGGTGCTCGAGTATTTTCCA(TAMRA)3' for FM-3), respectively. PCR was carried out in a 25-µl reaction mixture prepared with a TaqMan universal PCR master mix (PE Biosystems) containing a cDNA synthesis reaction from 4 ng of poly(A)^+ RNA, a 0.2 µM concentration of each primer, and 0.1 µM probe. PCR was performed under the following conditions: at 50 °C for 10 min for the reaction of uracil-N-glycosylase to prevent the amplification of PCR products carried over; at 95 °C for 15 min for the activation of AmpliTaq Gold DNA polymerase; and 40 cycles of 95 °C for 15 s and 60 °C for 60 s. To obtain calibration curves, we amplified the known amounts of rat neuromedin U and FM-3 cDNA fragments in the same manner as the samples. Good linear relationships were obtained between the amount of rat neuromedin U and FM-3 cDNA input and the release of the reporter dye within the range of 10 to 10^6 copies, respectively. Rat glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA expression was also measured as an internal control using rodent G3PDH control reagents (PE Biosystems) according to the manufacturer's instructions. In most tissues, the expression levels of G3PDH mRNA ranged from 0.7 × 10^3 to 13.3 × 10^3 copies/ng of poly(A)^+ RNA, except in the skeletal muscle and costal cartilage (45.6 × 10^3 and 22 × 10^3 copies/ng of poly(A)^+ RNA, respectively).

Southern Blot Analyses for FM-3 Gene—Human and rat genomic DNAs (5 µg, CLONTECH) were digested with EcoRI, subjected to electrophoresis on 0.8% agarose gel, and transferred to nylon membrane Biodyne B (Pall BioSupport Corp., Glen Cove, NY). These filters were hybridized with human and rat FM-3 cDNA probes (0.3 kb), which were amplified by PCR with primers corresponding to the coding region of the second and forth transmembrane domain, after labeling with [32P]dCTP (NEN Life Science Products) using a Multiprime DNA-labeling system (Amersham Pharmacia Biotech). Hybridization was performed at 59 °C overnight in a hybridization solution containing 0.5 M Na2HPO4 (pH 7.2), 7% SDS, 1 mM EDTA, and 1% BSA. The filters were washed with 0.5× SSC (1× SSC = 0.18 M NaCl and 0.09 M sodium citrate) containing 0.1% SDS at 59 °C and then exposed to imaging plates (Fuji Film Co., Tokyo, Japan) for 48 h, and hybridization signals were detected using a gel and blot imaging system (Storm model 860, Amersham Pharmacia Biotech).

Identification of the Neuromedin U Receptor—CHO-hFM-3 cells were transfected with the following synthetic compounds on CHO-hFM-3 cells in the microphysiometric assay and found that specific and dose-dependent promotion of extracellular acidification was induced in these cells by porcine U-8 (Fig. 1). At the dose of 10^−7 mol/l...
The significant elevation of the acidification rates was observed and this elevation reached a maximum (220% of the basal level). AA release from CHO cells expressing GHS or NT receptor, respectively. As shown in Fig. 4, only porcine U-8 could induce AA release in CHO-hFM-3 cells. In contrast, GHRP-6 and NT specifically induced AA release from CHO cells expressing GHS or NT receptor, respectively, but porcine U-8 did not induce AA release from these cells as well as mock-transfected CHO cells.

Specific Binding of Neuromedin U to FM-3—We examined

\[ \text{Specific AA release from CHO-hFM-3 cells after stimulation with neuromedin U derived from different species.} \]

CHO-hFM-3 cells were incubated overnight with \(^3\)H-labeled arachidonic acid. After being washed, the cells were incubated for 30 min with human U-25 (○), rat U-23 (△), porcine U-25 (□), and porcine U-8 (●). Radioactivities released into supernatants were then measured. Each symbol represents a mean value with a standard error in triplicate determinations.

Specific Changes of Ca\(^{2+}\) Mobilization and AA Release Induced in CHO-hFM-3 Cells by Neuromedin U—We analyzed cellular changes induced in CHO-hFM-3 cells by neuromedin U. In the FLIPR assay, we found that porcine U-8 induced the rapid mobilization of intracellular Ca\(^{2+}\) at the doses from 10\(^{-7}\) to 10\(^{-9}\) M (Fig. 2). However, porcine U-8 at 10\(^{-7}\) M did not induce the Ca\(^{2+}\) mobilization in mock-transfected CHO cells. Because extracellular AA release is caused by the activation of phospholipase A\(_2\), which is closely linked to Ca\(^{2+}\) influx, we next compared the potency of human U-25, porcine U-25 and U-8, and rat U-23 in CHO-hFM-3 cells in the AA release assay (Fig. 3). The potency of rat U-23 seemed to be about two times higher than that of others with a median effective concentration (EC\(_{50}\)) of 1.3 \(\times\) 10\(^{-9}\) M.

FM-3 shares significant amino acid sequence homology with NT type 1 and GHS receptors. To confirm the specific interaction of FM-3 and neuromedin U, we applied porcine U-8, a synthetic GHS of GHRP-6 (17), and human NT at 10\(^{-7}\) M on CHO-hFM-3 cells, mock-transfected CHO cells, and CHO cells expressing NT type 1 or GHS receptor, respectively. As shown in Fig. 4, only porcine U-8 could induce AA release in CHO-hFM-3 cells. In contrast, GHRP-6 and NT specifically induced AA release from CHO cells expressing GHS or NT receptor.
the binding of neuromedin U to FM-3. $^{125}$I-Labeled porcine U-8 efficiently bound to the membrane fractions prepared from CHO-hFM-3 cells. Unlabeled neuromedin U inhibited this binding in a dose-dependent manner (Fig. 5A). Rat U-23 was the most potent in this competitive binding assay with median inhibitory concentration (IC$_{50}$) of $3 \times 10^{-11}$ M. On the other hand, IC$_{50}$ values of human and porcine U-25 were $1.4 \pm 10^{-10}$ M and $1.7 \pm 10^{-10}$ M, respectively. Porcine U-8 was the least potent with IC$_{50}$ of $5.1 \pm 10^{-11}$ M. However, GHRP-6, human NT, and pancreatic peptide (PP) had no detectable inhibitory activity at $10^{-7}$ M in this assay. Scatchard analysis showed that CHO-hFM-3 cells expressed a single class of high affinity binding sites for $^{125}$I-labeled porcine U-8 (Fig. 5B). The dissociation constant ($K_d$) was $6.6 \times 10^{-11}$ M, and the number of maximal binding sites ($B_{max}$) was 2.4 pmol mg$^{-1}$ protein.

### Tissue Distribution of Neuromedin U and FM-3 mRNA in Rats

We analyzed the tissue distribution of rat neuromedin U and FM-3 mRNAs. Because the sequence of rat FM-3 cDNA has not been reported, we designed some primers based on the known human and mouse FM-3 cDNA sequences (5) and isolated a rat FM-3 cDNA by RT-PCR (Fig. 6). The isolated cDNA encoded an open reading frame with 412-amino acid length. The N-terminal portion of rat FM-3 was slightly longer than those of human and mouse FM-3. Rat FM-3 shared 71% and 80% amino acid sequence identity with human and mouse FM-3, respectively. When analyzing rat FM-3 cDNAs amplified...
from jejunum poly(A)⁺ RNA by RT-PCR, we found that some splicing variants existed in rat FM-3 cDNAs. One of them, which was a major PCR product, contained an intron in the sixth transmembrane domain and did not seem to encode a functional receptor (data not shown). Therefore, to avoid the amplification of this variant, we designed primers and a probe to quantify FM-3 mRNA by RT-PCR using an ABI Prism 7700 sequence detector.

As shown in Fig. 7 (upper panel), the highest level of neuromedin U mRNA was detected in the pituitary gland (9.6 × 10⁳ copies/ng of poly(A)⁺ RNA). In the gastrointestinal tract, high levels of expression were detected in the duodenum and jejunum (7.6 × 10³ and 5.6 × 10³ copies/ng of poly(A)⁺ RNA, respectively), whereas the expression levels were low in the caecum (0.3 × 10³ copies/ng of poly(A)⁺ RNA) but high levels in the colon and rectum (2.4 × 10³ and 3.0 × 10³ copies/ng of poly(A)⁺ RNA, respectively). In the central nervous system, moderate levels of the expression were detected in the striatum, hypothalamus, medulla oblongata, and spinal cord (0.4 × 10³ to 1.0 × 10³ copies/ng of poly(A)⁺ RNA). In the other peripheral tissues, moderate levels of expression were observed in the thyroid gland, trachea, testis, and ovary (0.3 × 10³ to 1.2 × 10⁴ copies/ng of poly(A)⁺ RNA).

FM-3 mRNA was mainly detected in the small intestine and lung (2.1 × 10³ to 3.1 × 10⁴ copies/ng of poly(A)⁺ RNA) (Fig. 7, lower panel). There was a clear difference in the expression levels between the small and large intestine: expression levels in the small intestine were greater than those in the large intestine. FM-3 mRNA levels in the central nervous system were rather low (0.02 × 10³ to 0.04 × 10³ copies/ng of poly(A)⁺ RNA). A very low level of FM-3 mRNA expression was detected in the pituitary gland where the highest level of neuromedin U mRNA was detected.

Southern Blot Analyses for FM-3 Gene—We analyzed human and rat FM-3 genes by Southern blot analyses after the electrophoresis of human and rat genomic DNAs digested with EcoRI. As shown in Fig. 8A, by using a radiolabeled human FM-3 cDNA fragment as a probe, we detected a clear hybridized band (indicated with a in lane 1) of 10.5 kb in the human genomic DNA. This band was expected to be derived from the human FM-3 gene. In addition, we detected a fainter band (6 in lane 1) around 5.3 kb in the human genomic DNA, suggesting that a gene with a similar DNA sequence to the FM-3 gene exists in the human genome. Using the same probe, we detected two hybridized bands of 19 and 8 kb (c and d, respectively, in lane 2) in the rat genomic DNA, suggesting that there are two genes corresponding to the human FM-3 gene in rats. On the other hand, by using a radiolabeled rat FM-3 cDNA probe, we detected a single band (a in lane 3) in the human genomic DNA at the same position when hybridizing with the human cDNA probe. These results indicated that the isolated rat FM-3 gene actually corresponded to the human FM-3 gene. We could not detect a hybridization signal at the position corresponding to the band b in lane 1, suggesting that its sequence was not as similar to the rat FM-3 as it was to the human FM-3. Using the rat probe, we detected clearly two hybridized bands (c and d, respectively, in lane 4) in the rat genomic DNA at the same position when hybridizing with the human cDNA probe, suggesting that there are two FM-3-like genes in the rat genomic DNA.

We subsequently analyzed the relation of two bands hybridized in the rat genomic DNA using the FM-3 cDNA probes. After gel electrophoresis, we extracted DNA fragments at the position corresponding to each band and then performed PCR...
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With primers designed from the rat FM-3 cDNA. As shown in Fig. 8B, a DNA fragment of 1.2 kb was amplified from the extracted genomic DNA corresponding to the band c. As a result of sequence analysis for the amplified DNA fragment, we found that it completely matched with rat FM-3 cDNA sequence except for the additional sequence of an intron (334 bp), which was inserted at the position of Ile-287 in the coding region of the rat FM-3 cDNA. These results demonstrated that this fragment was derived from the rat FM-3 gene. On the other hand, a DNA fragment with 877 bp was amplified from the band d. The DNA sequence of the amplified fragment had 91% identity with that of the rat FM-3 cDNA. However, this fragment (FM-3-related gene) did not appear to encode a functional open reading frame, because one base insertion caused a frameshift and stop codons were detected in the sequence. It was thus impossible that the DNA corresponding to the band d could encode a functional receptor, although its sequence was highly similar to the rat FM-3 gene. In addition, an intron-like sequence did not exist in this FM-3-related gene. We speculated that this might be a pseudogene of FM-3. Taken together, our Southern hybridization analyses indicated that the rat FM-3 gene, which we isolated, was the orthologue of the human FM-3 gene.

DISCUSSION

So far FM-3 has remained as an orphan GPCR, because some peptides, including a synthetic GHS, NT, endothelin, vasoactive intestinal peptide (VIP), growth hormone-releasing hormone, somatostatin, thyrotropin-releasing hormone, calcitonin, and galanin were examined as to whether they could act as FM-3 ligands, but they failed to stimulate HEK293 cells expressing FM-3 (5). In this paper, we demonstrated that neuromedin U showed a specific and very potent agonistic activity to CHO-hFM-3 cells and specifically bound to their membrane fractions with high affinity at a Kd of 66 pm. Our data unequivocally demonstrate that neuromedin U is the cognate ligand of FM-3. Because neuromedin U caused Ca2+ influx and AA release in CHO-hFM-3 cells, FM-3 was hypothesized to couple to Gq in the signal transduction pathway. We could not detect apparent changes in cAMP production in CHO-hFM-3 cells treated with neuromedin U (data not shown).

Since neuromedin U was first purified from porcine spinal cord (7, 8), it has been isolated from a number of species (18). In a comparison of neuromedin U peptides derived from different species, the C-terminal five amino acid residues were totally conserved, suggesting that this region is of major importance. It has been reported that amidation of the C-terminal asparagine is indispensable for neuromedin U to exhibit biological activities (7). As shown in Fig. 8, neuromedin U shares a consensus sequence, LXXPRX-amide, with VIP and the same asparaginylamide C terminus with VIP, respectively (7). Although both PP and VIP did not stimulate CHO-hFM-3 cells, we found that Aplysia small cardiac peptide B (SCPB) (19), having a consensus motif, LXXPRX-amide, with neuromedin U, showed a very low but significant agonistic activity to CHO-hFM-3 cells (data not shown). These results suggest that the LXXPRX-amide motif is needed for neuromedin U to interact with FM-3, but in the case of PP, its N-terminal portion might hinder the interaction with FM-3. Neuromedin U shows slight sequence homology with NT and ghrelin. However, neither NT nor ghrelin stimulated CHO-hFM-3 cells (data not shown). This might be due to the fact that NT and ghrelin do not share the consensus motif LXXPRX-amide (Fig. 9).

There has been little information on the tissue distribution of FM-3 mRNA. FM-3 transcripts of 2 and 5 kb have been reportedly detected in some tissues, in particular, they are found by Northern blot analyses to be abundant in the testis of mice (5). To know more precise distribution of its mRNA in rats, we isolated rat FM-3 cDNA and analyzed the tissue distribution of FM-3 mRNA by the quantitative RT-PCR. However, we could not detect a high level of FM-3 mRNA expression in the testis in rats by RT-PCR. This may be due to species difference or selective amplification of FM-3 cDNAs in our analyses.

Moreover, we also analyzed the distribution of neuromedin U mRNA to compare it with that of FM-3 mRNA. Both neuromedin U and FM-3 mRNAs were highly expressed in the small intestine, whereas in the large intestine the expression levels of neuromedin U mRNA were relatively high but FM-3 mRNA expression levels were low. It is reported that neuromedin U-like immunoreactivity is abundantly detected in the gastrointestinal tract (20–26) and that neuromedin U promotes motor responses (9, 10), gut blood flows (11), and intestinal ion transport (12). Taken together, it is suggested that neuromedin U and FM-3 play important roles in the gastrointestinal tract.

It is reported that both neuromedin U mRNA and neuromedin U-like immunoreactivity are abundantly detected in human and rat pituitary gland (20, 27, 28). We also detected the highest level of neuromedin U mRNA expression in rat pituitary gland. In immunocytochemical analyses, neuromedin U-positive cells are reportedly observed in the anterior lobe of the pituitary and they are colocalized in the corticotrophs (27). In the rat brain, neuromedin U-positive nerve fibers are observed in the hypothalamic paraventricular and supraoptic nuclei (22, 27). In addition, the subcutaneous administration of neuromedin U into rats results in increasing plasma adrenocorticotropin and corticosterone concentrations (29). These facts suggest that neuromedin U is involved in the control of the hypothalamo-pituitary-adrenocortical axis. However, we detected only low levels of FM-3 mRNA expression in the hypothalamus, pituitary, and adrenal gland. In addition, it is reported that neuromedin U potently contracts isolated rat uterus (7, 8) and human bladder (9) and that abundant neuromedin U binding sites are detected in rat uterus (18, 30). However, we detected very low levels of FM-3 mRNA expression both in the uterus and bladder in rats. It has been proposed that neuromedin U acts as a neuropeptide or neuromodulator rather than a circulating hormone, because plasma neuromedin U levels seem to be fairly low (20). One possible explanation for the discrepancy of the FM-3 and neuromedin U distributions is that other unknown receptors responsible for neuromedin U may exist in these tissues. Although the results of our Southern blot analyses clearly indicated that the rat cDNA, which we isolated, was the orthologue of the human FM-3 cDNA, we detected an additional fainter band in the human genomic DNA using the human FM-3 cDNA probe.
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band suggested that a gene with homologous sequence with the FM-3 gene might exist in the human genome. It may be one of the candidate genes for a neuromedin U receptor subtype, although it might be a pseudogene as found in the rat genome. In the rat genome, we found that there was a FM-3-related gene in addition to the FM-3 gene. However, this gene did not encode a functional receptor, though it had a highly conserved sequence with the rat FM-3 gene. Under the conditions of lower stringency, in the rat genomic DNA, we could not detect additional bands hybridized clearly with the rat FM-3 probe (data not shown), suggesting that other genes with at least a highly similar sequence to FM-3 do not exist in the rat genome. Future studies are necessary to confirm whether there are functional receptor subtypes for neuromedin U.

In the lung, a high level of FM-3 mRNA expression was detected, whereas neuromedin U mRNA expression was low. The similar pattern was observed in the femur. Although there is the possibility that neuromedin U is delivered to these tissues by a certain mechanism such as neuronal processes, further studies are required to clarify pathways for neuromedin U to act on FM-3. Neuromedin U and FM-3 mRNAs were found to be localized in a variety of tissues, suggesting that they have multiple unknown functions. The discovery of a specific receptor for neuromedin U will give novel insights into the physiological significance of neuromedin U and should enhance the search for its agonists and antagonists.

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Identification of Neuromedin U as the Cognate Ligand of the Orphan G Protein-coupled Receptor FM-3
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