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Porcine Guanylin and Uroguanylin: cDNA Sequences, Deduced Amino Acid Sequences, and Biological Activity of the Chemically Synthesized Peptides

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Guanylin and uroguanylin are structurally related intestinal peptide hormones which were purified from a limited number of mammals and are capable of activating the particulate guanylate cyclase-C. Although the biological functions of guanylin and uroguanylin are not yet clarified in detail, they are involved in the regulation of the intestinal water and electrolyte balance. In order to verify the general importance of this hormone system in mammals, we cloned the corresponding cDNAs from pig. Here, we present the nucleotide sequences and the deduced amino acid sequences representing porcine guanylin and uroguanylin. The expression patterns of the corresponding genes, as shown by Northern hybridization and RT-PCR analysis, resemble those of the human homologues. Further, we demonstrate the bioactivity of both porcine peptide hormones by inducing the intracellular cGMP production in human T84 cells and by ion transport experiments using porcine intestinal mucosa in the Ussing chamber. © 1999 Academic Press

The membrane receptor guanylate cyclase-C (GC-C) is located in the gastrointestinal tract of mammals and is capable of binding bacterial enterotoxins, such as *E. coli* heat-stable STa. Activation of GC-C by these enterotoxins causes an increase in the intracellular level of cGMP (1) thereby activating the protein kinase cGKII (2) which subsequently phosphorylates the cystic fibrosis transmembrane conductance regulator channel (CFTR) (3). As a result, chloride and water secretion is enhanced, causing secretory diarrhoea (4). The search for endogenous ligands of GC-C led to the discovery of two novel mammalian intestinal peptide hormones that were named guanylin and uroguanylin. Guanylin was first isolated as a peptide of 15 amino acids from rat intestine (5), whereas uroguanylin was isolated originally as a 15-amino acid peptide from opossum (6) and as a 16-amino acid peptide from human urine (7).

We later succeeded in identifying circulating forms of human guanylin and uroguanylin exhibiting chain lengths of 94 and 24 amino acids, respectively (8, 9). Guanylin and uroguanylin-specific cDNA sequences have been described for a limited number of mammalian species (mouse, rat, guinea pig, and human; for EBI/GenBank database accession numbers, see Table 1). Their sizes range from 472-723 bp and they encode putative precursor proteins of 107-120 amino acids. Since these cDNAs were characterized from humans and rodents which share phylogenetically distant relations, a general importance of the guanylin/uroguanylin hormone system seems to be likely. In order to verify this thesis and to search for other possibly occurring guanylin and uroguanylin-related peptides, we amplified the corresponding cDNAs from pig using primers specific for regions conserved within the currently known amino acid sequences of the guanylin and uroguanylin precursors.

In this paper, we present the cDNA and deduced amino acid sequences of two porcine proteins representing the precursor molecules of guanylin and uroguanylin. The expression patterns of the corresponding genes were investigated by Northern hybridization and RT-PCR analysis. From the cDNA sequences obtained, we derived the amino acid sequences of the putative mature 15-amino acid guanylin and 24-amino acid uroguanylin of the pig. The peptides were chemically synthesized and their biological functionality is demonstrated by a specific cGMP assay with human T84 colon carcinoma as well as by the Ussing chamber experiments using porcine colonic mu-

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cosa. The results presented confirm an overall importance of the guanylin/uroguanylin hormone system among mammals and might facilitate the identification of related peptides and the corresponding cDNAs/genes from species other than those already mentioned.

MATERIALS AND METHODS

Oligonucleotides. The following oligonucleotides (Perkin Elmer, Weiterstadt, Germany) were used as PCR primers and hybridization probes (listed in 5′→3′ orientation): GUUP-1, TCTGAGCTCITWIRCRCAIAIYTCRCA; GUUP-2, TCTGAGTIGARWSIKTIAARMARYT; GUUP-3, RCAICCIGYRCAIGC; HUGU-3, TCTGAGCTCAAYGAYGAYTGYGARYTNTG; HUGU-4, TCTGAGCTCTGYGARYTNTGYGTNAAYGT; HUGU-5, TCTGAGCTCTGYGTNAAYGTNGCNTG; HUGU-11, TCTGAGCTCGTGCCACCACCCTGCTCTG; HUGU-15, TCTGAATTCTCATTAGAGGCAGCCGGTACACGC; OHI-1, TCTGAGCTCGAAGATCTCCTCGGCGT; OHI-2, TCAAGGACCTCCAGGAGCTCCAGAAGC; OHI-3, TCTCGAGTCTGGGCAGGTGGAGCTGC; OHI-4, TCTCGAGTCTAGATTACTAGCATCCCGCGCAGGC; SDII-1, TCTCCAGCCCATCTGCCAGTC; SDII-2, TCTCCTCAGACTGGCAGATG; SDII-3, CAGGCCTTGAGGACCATCG; SDII-4, TCTGAATTCTTATCAGCTGCAGCCGGTACAGGC; SDII-5, TCTGGATCCAGGATACAGCGGGAGCGAGATG; PIGCC-1, CACATTCTCGACCACCATGAC; PIGCC-2, ACGTTCCAGACAGGGCAGCT; UNIP-5, CCTCAGCTGCAGCTCGAG$(T)_{24}$; UNIP-6, CCTCAGCTGCAGCTCGAG; β-TUB-1, TTCCCTGGCCAGCTGAANGCNGACCTNCGCAAG; and β-TUB-2, CATGCCCTCGCCNGTGTACCAGTGNANGAAGGC.

Abbreviations: I = Inosinenucleotide, W = A or T, S = G or C, R = A or G, Y = T or C, M = A or C, K = G or T, N = A, C, G, or T. Underlined regions do not correspond to the cDNA sequences but contain restriction endonuclease recognition sites to facilitate easy cloning of the PCR fragments obtained.

Molecular biological standard procedures. RNA extraction, Northern hybridization, and Southern hybridization were performed according to Sambrook et al. (10). cDNA first strand synthesis, analytical reverse transcriptase polymerase chain reaction (RT-PCR), and DNA fluorescence sequencing were performed as described previously (11, 12). PCR fragments were cloned using either the pGEM 5Zf T-vector system (Promega, Madison, USA) (13), or the plasmid pBSK+ (Stratagene, Heidelberg, Germany) by means of restriction endonuclease recognition sequences included in the 5′-*add on* regions of some of the primers which were compatible with those occurring within the multi-purpose cloning site of the vector. For analytical RT-PCR, the following primer pairs were used: porcine guanylin: OHI-2/OHI-3, porcine uroguanylin: SDII-4/SDII-5, porcine GC-C: PIGCC-1/PIGCC-2, β-tubulin: β-TUB-1/β-TUB-2. Southern blot analysis of the RT-PCR products was performed using the oligonucleotides OHI-1 and SDII-2 as hybridization probes for cDNA fragments specific for porcine guanylin and uroguanylin, respectively. Nucleotide sequences were compared by means of the MacMolly program package (SoftGene, Berlin, Germany) using a window size of 12 nucleotides allowing two mismatches.

Preparative PCR amplification of cDNA. Two degenerate antisense primers (GUUP-1, GUUP-3) were deduced from the amino acid sequence (C E (L/I) C (V/A) (N/Y) (V/A) A C (T/A) G C) conserved at the C-termini of the human guanylin and uroguanylin precursor sequences (14-16). A degenerate sense primer (GUUP-2) was deduced from an amino acid sequence motif (L E (S/M) V (K/Q) K) conserved within the N-termini of the known guanylin and uroguanylin precursor sequences from different species (16).

5 μg porcine jejunum total RNA was reverse transcribed using 50 pmol GUUP-3 primer and 200 units SuperscriptII reverse transcriptase (Gibco BRL, Eggenstein, Germany) according to the manufac-

TABLE 1

Comparison of the Nucleotide Sequences of Guanylin and Uroguanylin-Specific cDNAs from Different Species

	SDGCAP1	CPGUAMRN2	CPGUAMRN1	HSGNLNA	RNGUANYL	MMGUANYLA	SDGCAPII	CPUGUMRNA	HS342791	RNU75186	MMU90727
CPGUAMRN2	65.7										
CPGUAMRN1	47.1	44.1									
HSGNLNA	57.2	67.0	54.2								
RNGUANYL	54.7	50.4	41.6	64.3							
MMGUANYLA	47.7	63.1	44.2	65.7	86.4						
SDGCAPII	29.1	35.0	25.3	35.5	40.3	38.2					
CPUGUMRNA	23.4	28.5	25.1	29.4	41.6	36.5	68.8				
HS342791	36.1	39.2	41.2	29.0	34.0	16.2	58.6	65.5			
RNU75186	30.5	25.4	31.2	33.2	30.7	24.3	46.9	54.2	45.5		
MMU90727	45.5	30.3	37.8	33.5	30.8	34.3	55.1	61.8	59.5	72.8	
DV49353	23.3	24.9	37.4	24.9	27.7	27.2	18.7	31.1	39.9	20.1	22.4

Note. The sequence identities between guanylin and uroguanylin-specific cDNAs from different species are shown as percentages. Porcine guanylin cDNA (SDGCAP1) shares the highest sequence identity with guanylin cDNAs of other species, whereas the porcine uroguanylin cDNA (SDGCAPII) shares the highest sequence identity with the corresponding uroguanylin-specific cDNAs. In both cases, the guinea pig sequences exhibit the highest similarity with the porcine sequences (65.7% and 68.8%, respectively). The cDNA sequences are indicated by their EBI/Genbank identifiers as follows: SDGCAP1: porcine (*Sus domestica*) guanylin cDNA, accession no. Z73607; SDGCAPII; porcine (*Sus domestica*) uroguanylin cDNA, accession no. Z83746; CPGUAMRN1: guinea pig (*Cavia porcellus*) cDNA for guanylin-like protein (splice variant), accession no. Z74735; CPGUAMRN2: guinea pig (*Cavia porcellus*) guanylin cDNA, accession no. Z74736; HSGNLNA: human (*Homo sapiens*) guanylin cDNA, accession no. M97496; RNGUANYL: rat (*Rattus norvegicus*) guanylin cDNA, accession no. M95493; MMGUANYLA: mouse (*Mus musculus*) guanylin cDNA, accession no. M95175; CPGUMRNA: guinea pig (*Cavia porcellus*) uroguanylin cDNA, accession no. Z74738; HS342791: human (*Homo sapiens*) uroguanylin cDNA, accession no. U34279; RNU75186: rat (*Rattus norvegicus*) uroguanylin cDNA, accession no. U75186; MMU90727: mouse (*Mus musculus*) uroguanylin cDNA, accession no. U90727; DV49353: opossum (*Didelphis virginiana*) uroguanylin cDNA, accession no. U49353.

turer's instructions. Subsequently, the enzyme was inactivated by incubation for 10 min at 70°C, and the RNA was hydrolysed for 10 min at 50°C after addition of 5 units of RNAseH (Gibco BRL). cDNA first strands were purified by means of Glassmax spin columns (Gibco BRL) and eluted in a volume of 50 μl water.

Several PCR protocols using different combinations of the primers GUUP-1, GUUP-2, and GUUP-3 and the human uroguanylin cDNA (16) -derived primer HUGU-11 were tried. Finally, the following reaction conditions led to the amplification of porcine partial guanylin and uroguanylin cDNA: Amplification of porcine guanylin cDNA: Primers GUUP-1/GUUP-2, 5× (95°C, 20 sec; 46°C, 20 sec; 70°C, 90 sec); 5× (95°C, 20 sec; 42°C, 20 sec; 70°C, 90 sec); 28× (95°C, 20 sec; 37°C, 1 sec; 70°C, 90 sec). Amplification of porcine uroguanylin cDNA: Primers GUUP-2/GUUP-3/HUGU-11 (nested PCR), 5× (95°C, 20 sec; 46°C, 20 sec; 70°C, 90 sec); 5× (95°C, 20 sec; 40°C, 20 sec; 70°C, 90 sec); and 28× (95°C, 20 sec; 37°C, 1 sec; 70°C, 90 sec).

Reactions were performed in a total volume of 100 μl in a model 9600 thermal cycler (Perkin Elmer) under the following general conditions: 10 mM Tris-HCl pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 1/10 of each cDNA first strand, 200 μM of each dNTP, 0.4 μM of each PCR primer, 2 units *Taq* DNA polymerase (Biomol, Hamburg, Germany), hot start at 78°C.

From the partial cDNA sequences obtained, highly specific primers (OHI-1, OHI-3, OHI-4, and SDII-2) were constructed and used together with a human uroguanylin-derived primer (HUGU-15) for amplification of the 3′- and 5′-termini of both the porcine guanylin and uroguanylin cDNAs, as described earlier (16).

For 3′-RACE experiments, the cDNA first strand synthesis was primed using the oligo(dT) primer UNIP-5 whose 5′-add on region corresponds to the primer UNIP-6. In the case of porcine guanylin cDNA, the first amplification step was performed as follows (general conditions as described above): 25× (95°C, 20 sec; 58°C, 20 sec; 70°C, 1 min), primers OHI-2/UNIP-6. 5 μl of the reaction mixture was then subjected as a template to a second amplification step under the same conditions but with 40 cycles. The 3′-terminus of porcine uroguanylin cDNA was amplified using the primer combination SDII-1/UNIP-6 for the first step as described above. Here, the second amplification step was performed with 35 cycles and the primer combination SDII-3/UNIP-6.

For 5′-RACE experiments, cDNA first strand synthesis was primed using the oligonucleotide OHI-4 for porcine guanylin, and GUUP-3 for porcine uroguanylin. cDNA first strands were then purified using Glassmax spin colums as described above. Half of each of the cDNA first strands were 3′-oligo(dA)-tailed according to the standard procedure (17). 1/5 of these tailing reaction mixtures were subsequently used for the first amplification step under standard conditions (17): Primers GSP-2/UNIP-5, 5× (95°C, 20 sec; 50°C, 20 sec; 70°C, 1 min); 4× (95°C, 20 sec; 44°C, 20 sec; 70°C, 1 min); and 1× (95°C, 20 sec; 44°C, 1 sec; 70°C, 3 min).

1/20 of each reaction mixture from the preceding amplification steps was subjected as a template to each of the following amplification steps. Second amplification step: Primers GSP-2/UNIP-6, 24× (95°C, 20 sec; 44°C, 20 sec; 70°C, 1 min) and 1× (95°C, 20 sec; 44°C, 20 sec; 70°C, 3 min).

Third amplification step: Primers GSP-3/UNIP-6, 19× (95°C, 20 sec; 44°C, 20 sec; 70°C, 1 min) and 1× (95°C, 20 sec; 44°C, 20 sec; 70°C, 3 min).

Fourth amplification step: Primers GSP-3/UNIP-6, 29× (95°C, 20 sec; 44°C, 20 sec; 70°C, 1 min) and 1× (95°C, 20 sec; 44°C, 20 sec; 70°C, 3 min).

For porcine guanylin, GSP-2 and GSP-3 were OHI-3 and OHI-1, respectively. In the case of porcine uroguanylin, GSP-2 and GSP-3 were HUGU-15 and SDII-2, respectively.

Peptide synthesis. To assess the biological activity, the following peptide fragments deduced from the corresponding porcine cDNA sequences were chemically synthesized: PSTCEICAYAACAGC (porcine guanylin$_{95\text{-}109}$ = $_{p}$guanylin$_{95\text{-}109}$) and FQALRTIAGDDCELCVNVACTGCS (porcine uroguanylin$_{90\text{-}113}$ = $_{p}$uroguanylin$_{90\text{-}113}$). Solid phase peptide synthesis was performed according to the previously published procedure (18). The correct pairings of cysteines crucial for the biological activity were selectively introduced by the use of mixed acetamidomethyl- and trityl-protected cysteine residues. Purity and identity of the peptides were checked by reverse-phase HPLC, electrospray mass spectrometry and Edman degradation. The peptides were used for the bioassays according to the net peptide content as determined by amino acid analysis.

cGMP assays with T84 cells. Effects of human guanylin, $_{p}$guanylin$_{95\text{-}109}$, and $_{p}$uroguanylin$_{90\text{-}113}$ on cGMP increase in human T84 colon carcinoma cells were measured as described previously (8).

Ussing chamber experiments. Pigs from the slaughterhouse were killed, the ascendic colon was immediately removed, opened longitudinally and stripped of the serosal and muscular layers. The mucosal sheets were mounted in modified Ussing chambers (19) with an exposed surface area of 1 cm^2. Tissues were bathed in Krebs-Ringer solution in both the mucosal and serosal reservoirs (8 ml volume). Both buffer solutions were mixed and oxygenated by a gas-lift system with 95% O_2/5% CO_2 and maintained at 37°C. The Krebs-Ringer bicarbonate solution contained 140 mM Na^+, 123.4 mM Cl^-, 5.4 mM K^+, 1.2 mM Ca^{2+}, 1.2 mM Mg^{2+}, 2.4 mM HPO_4^{2-} 0.6 mM $H_2PO_4^-$, 21 mM HCO_3^-, and 10 mM glucose. Osmolarity was adjusted to 300 mosm/l with mannitol and the pH was adjusted to 7.4. Each chamber was connected to voltage clamp amplifiers (VCC 600 or VCCMC 6; Physiological Instruments, San Diego, USA). Transepithelial potential differences (Vt) were measured using Ringer-agar bridges connected to calomel half-cells with reference to the mucosal solution. The tissues were voltage-clamped using Ag-AgCl electrodes in 3 M KCl. The short-circuit current (I_{SC}, expressed in $\mu A/cm^2$) was considered positive for cation flow from the mucosal to the serosal side.

Tissues were allowed to establish a stable I_{SC} for at least 30 minutes prior to any drug addition. Peptides were added to solutions at the mucosal (apical) side of the chamber. Responses (ΔI_{SC}) were determined as the difference between the basal I_{SC} and the maximal change in I_{SC} observed in response to substances tested. The tissue conductance (Gt) was measured every minute. At the end of each experiment the cholinomimetic agent carbachol ($10^{\times 4}$ M) was applied to the serosal side of the preparation to confirm tissue viability. The following substances were tested: $_{p}$guanylin$_{95\text{-}109}$, $_{p}$uroguanylin$_{90\text{-}113}$ and carbachol. Peptides were tested in doses ranging from 10^{-6} – 10^{-8} M and carbachol was tested at a dose of 10^{-4} M.

RESULTS

Molecular Cloning of the Porcine Guanylin and Uroguanylin cDNA

Primary PCR amplifications led to the amplification of a 227 bp fragment from porcine jejunum cDNA using the guanylin-specific primer pair GUUP-1/GUUP-2. From the same cDNA, a 145 bp fragment was obtained by priming with the uroguanylin-specific primer pair HUGU-11/GUUP-3. The fragments were cloned in pGEM 5Zf T-vector and sequenced. As expected, comparison with the already known guanylin and uroguanylin cDNA sequences revealed highest sequence identity of the GUUP-1/GUUP-2-priming product with the known guanylin-specific cDNAs and highest sequence identity of the HUGU-11/GUUP-3 priming product with the known uroguanylin-specific cDNAs. From the nucleotide sequences obtained, highly specific PCR primers were constructed and used for amplifications

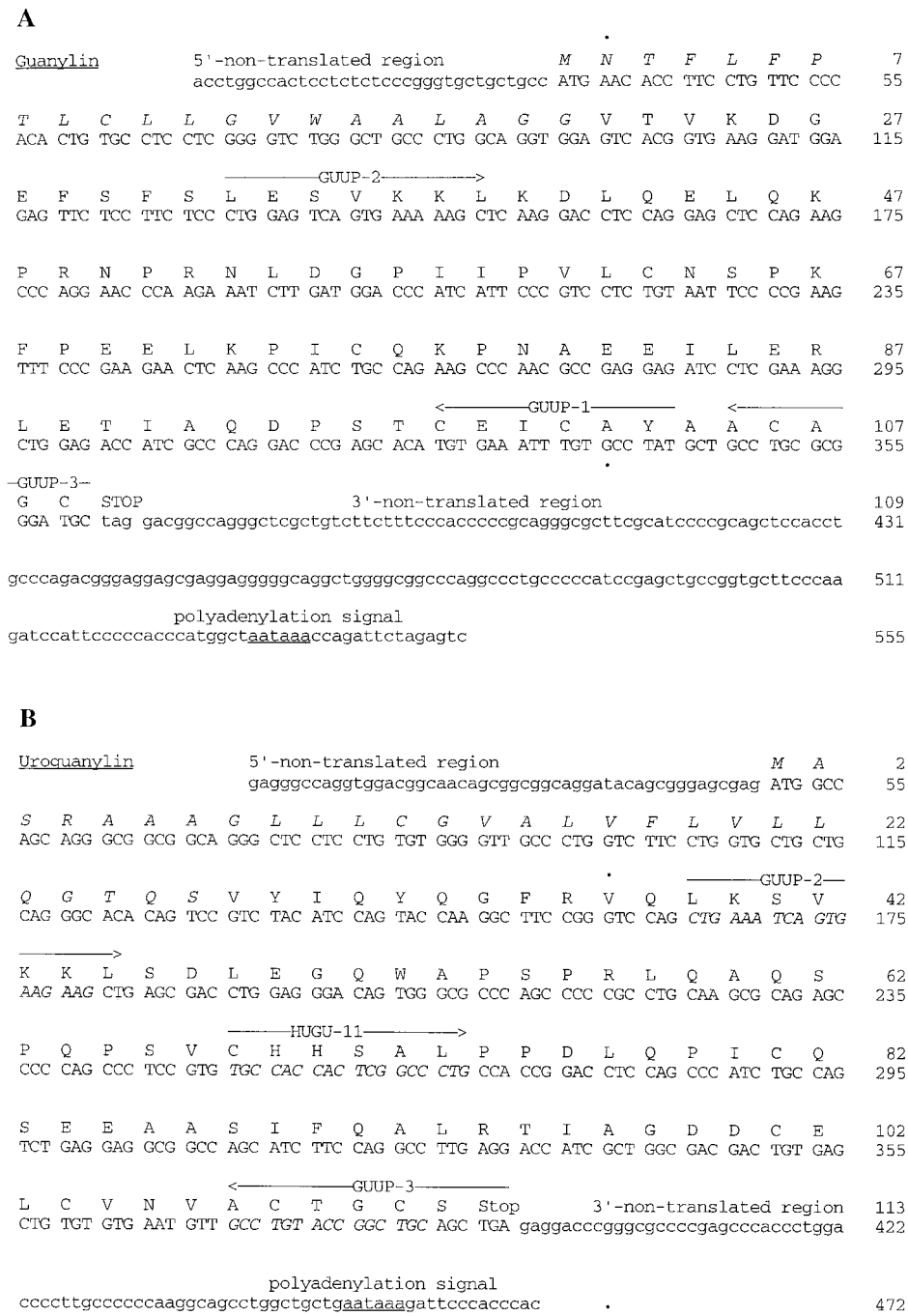

FIG. 1. cDNA sequences and derived amino acid sequences of the porcine guanylin (A) and uroguanylin (B) precursors. The positions of the primers used for amplification of the first partial cDNA fragments are indicated by arrows. The parts of the precursor proteins representing the putative secretory signal peptides are in italics. Polyadenylation signals are underlined. The porcine guanylin cDNA is 555 bp in size and codes for a 109-amino acid precursor protein, whereas the porcine uroguanylin cDNA spans 472 bp and codes for a 113-amino acid precursor protein.

of the 5′- and 3′-termini of the cDNAs as described above. Finally, from all partial cDNA sequences obtained the entire cDNA sequences of the porcine guanylin and uroguanylin precursors were assembled. The porcine guanylin precursor cDNA sequence spans 555 bp and codes for a 109-amino acid precursor protein (Fig. 1A). As determined by sequence comparisons, within the entire cDNA sequence the 5′-non-translated region is 34 bp in size, the coding region (including the translational stop codon) 330 bp and the 3′-non-translated region 191 bp. The 3′-non-translated region contains a typical AATAAA

polyadenylation signal at the expected distance from the 3′-terminus.

In a similar way, the entire cDNA sequence for the porcine uroguanylin precursor was assembled. It is 472 bp in size and codes for a 113-amino acid precursor protein (Fig. 1B). The 5′-non-translated region is 49 bp in size, the coding region 342 bp and the 3′-non-translated region 81 bp. Also in this case, a typical AATAAA polyadenylation signal appears at the expected distance from the 3′-terminus.

Expression Pattern of the Porcine Guanylin and Uroguanylin Gene

In order to confirm the cloning of porcine guanylin and uroguanylin cDNA and to compare the expression patterns of the corresponding genes, we performed Northern hybridization and RT-PCR analysis with porcine RNA from different tissues, especially of the gastrointestinal tract. As a hybridization probe for guanylin gene-specific transcripts, we used a 139 bp porcine cDNA fragment (pos. 152-290 of the cDNA sequence, presented in Fig. 1A) generated by PCR with the primers OHI-1/OHI-2 and the entire porcine guanylin cDNA as a template. For detection of uroguanylin gene expression, we used a 260 bp RT-PCR fragment (pos. 131-390 of the cDNA sequence, presented in Fig. 1B) which was generated similarly by use of the entire porcine uroguanylin cDNA and the primer pair HUGU-14/SDII-4.

The following tissues were analysed: mucosa from oesophagus, cardia, stomach fundus, stomach corpus, stomach antrum, and stomach pars pylorica, parotid gland, duodenum, jejunum, ileum, caecum, colon transversum, colon descendens, lung, and kidney. 50 μg total RNA of each tissue were used for Northern hybridization. The blots were first hybridized with the porcine uroguanylin-specific probe, then stripped and subsequently hybridized with the porcine guanylin-specific probe. Washing of the blots was perfomed under highly stringent conditions (final washing for 30 min with 0.1% SSC/0.1% SDS at 60°C). Autoradiographs obtained from blots containing RNA from different parts of the stomach did not show any guanylin or uroguanylin mRNA-specific signals, even after exposure for 10 days (data not shown). On the other hand, blots with intestinal RNA gave strong signals after exposure for two days using the guanylin- and the uroguanylin-specific probe (Fig. 2A). RNA from parotid gland, lung, and kidney revealed no signals.

Since the expression of the uroguanylin gene in the stomach was already demonstrated for humans, we analysed the RNA samples isolated from mucosa of different segments of the stomach by use of the more sensitive RT-PCR method (Fig. 2B). In this case, we obtained strong signals from mucosa of stomach fundus, corpus, antrum, and pars pylorica using the uroguanylin-specific primer pair. Homogeneous PCR fragments of the expected size of 805 bp were also generated from these tissues and additionally from mucosa of the cardia using primers specific for the particulate guanylate cyclase-C (20) which is a receptor of guanylin and uroguanylin. In contrast, with guanylin-specific primers we only obtained weak signals after 30 and 35 cycles of PCR from fundus, antrum, and pars pylorica. These findings confirm the data obtained from humans (11).

cGMP Assays with Human T84 Colon Carcinoma Cells

$_{p}$guanylin$_{95\text{-}109}$ and $_{p}$uroguanylin$_{90\text{-}113}$ were tested for their cGMP-increasing effect on human T84 colon carcinoma cells compared to human guanylin$_{101\text{-}115}$. The peptides caused a significant increase of intracellular cGMP starting at a concentration of 10^{-7} M or slightly lower (Fig. 3A). Among the peptide concentrations tested, the highest level of intracellular cGMP was reached at 10^{-6} M. Human guanylin revealed the maximum cGMP level of 7×10^{-8} M followed by $_{p}$uroguanylin$_{90\text{-}113}$ with 4.9×10^{-8} M and $_{p}$guanylin$_{95\text{-}109}$ with 3×10^{-8} M. The data obtained indicate the biological activity of porcine guanylin and uroguanylin in a cellular system with the human GC-C receptor.

Ussing Chamber Experiments

A total of 16 colonic tissue samples from 3 pigs were used for Ussing chamber experiments. After reaching stable conditions, the mean baseline I_{SC} was -31.17 ± 8.64 μA/cm^2 and resistance was 19 ± 4.91 mS. Response to carbachol was -103.63 ± 25.91 μA/cm^2. The effects of potential secretagogues on I_{SC} were assessed using a cumulative concentration response protocol. Addition of $_{p}$guanylin and $_{p}$uroguanylin to the mucosal solution induced an increase in I_{SC}. The increase of I_{SC} started at a minimum concentration of 10 nM for both peptides and subsequent addition of higher concentrations (100 nM and 1 μM) elicited further increases in I_{SC} (Fig. 3B).

DISCUSSION

Following the cloning strategy described above, we succeeded in cloning two porcine cDNAs from jejunum total RNA which may represent the porcine guanylin precursor cDNA (SDGCAP1) and the porcine uroguanylin precursor cDNA (SDGCAPII). Indeed, comparison of both nucleotide sequences with entries of the EBI/GenBank database revealed highest identities to the nucleotide sequences of guanylin (in the case of SDCAP1) and uroguanylin (in the case of SDGCAPII) precursor cDNA from species other than the pig (guinea pig, human, mouse, rat). This finding strongly indicates that SDGCAP1 rep-

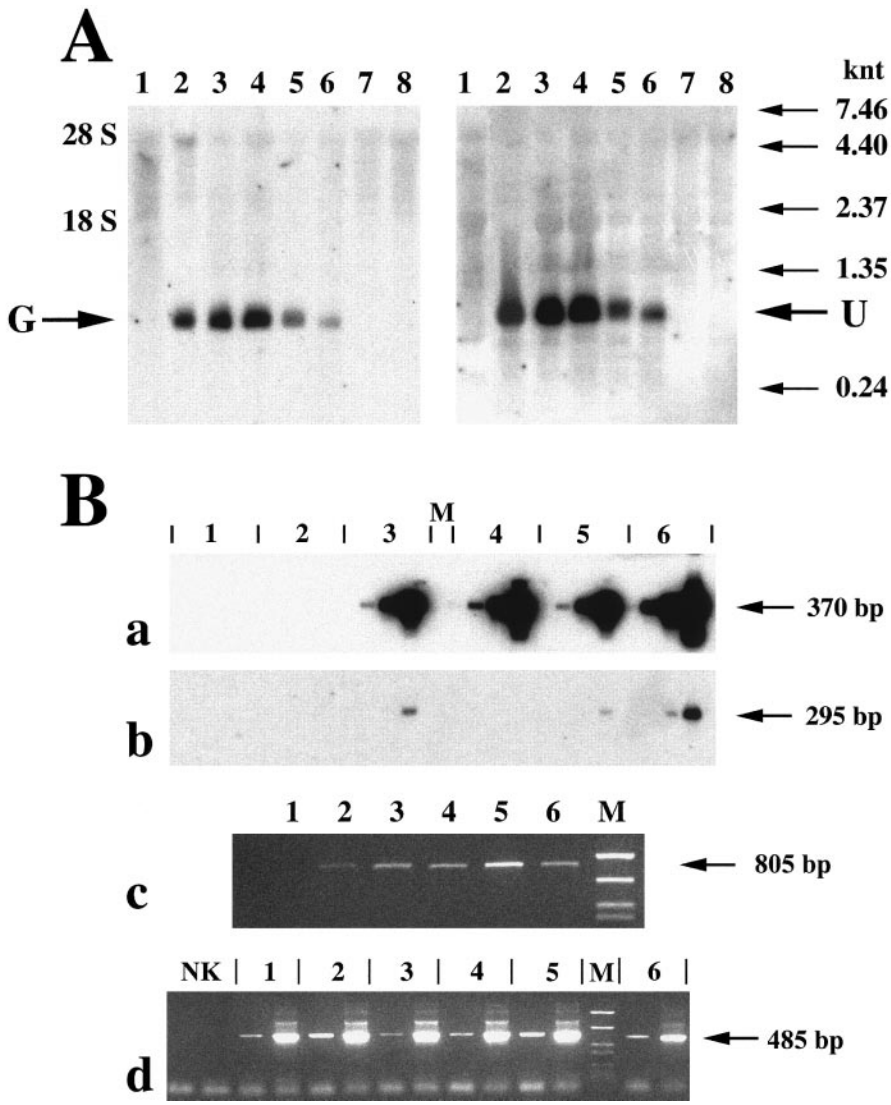

FIG. 2. Expression pattern of the porcine guanylin and uroguanylin genes. (A) Northern blot analysis was performed with 50 μg total RNA from porcine parotid gland (1), jejunum (2), ileum (3), caecum (4), colon transversum (5), colon descendens (6), lung (7), and kidney (8) and porcine guanylin (G) as well as uroguanylin (U)-specific cDNA fragments as hybridization probes. Strong guanylin (G) and uroguanylin (U)-specific hybridization signals are obtained from intestinal tissues (lanes 2-6) whereas parotid gland (1), lung (7), and kidney (8) do not show any signals. (B) RT-PCR analysis was performed using cDNA first strands from porcine mucosa of oesophagus (1), and of stomach cardia (2), fundus (3), corpus (4), antrum (5), and pars pylorica (6). The primer pairs used (see text) were specific for porcine uroguanylin (a), guanylin (b), GC-C (c), and β-tubulin (d) (universal). Guanylin and uroguanylin-specific RT-PCR samples were taken after 20, 25, 30, and 35 cycles (from left to right), Southern-blotted and subsequently hybridized with internally positioned, ^{32}P-labelled oligonucleotides (see text). GC-C-specific RT-PCR was performed for 35 cycles, β-tubulin-specific RT-PCR (control) for 20 and 30 cycles (from left to right). GC-C and β-tubulin-specific products were analysed on ethidium-bromide agarose gels without Southern hybridization. Strong uroguanylin-specific signals are obtained from fundus, corpus, antrum, and pars pylorica (3-6). Guanylin-specific RT-PCR shows only weak signals from fundus (3), antrum (5), and pars pylorica (6). GC-C-specific RT-PCR products are obtained from fundus, corpus, antrum, and pars pylorica (3-6). The comparable intensity of the β-tubulin-specific PCR fragments indicates a similar quality of all cDNA first strands used. All signals obtained were in the expected size range. Abbreviations: knt: kilonucleotides (10^3 nucleotides), M: size marker, NK: negative control.

resents the porcine guanylin cDNA whereas SDGCAPII represents the porcine uroguanylin cDNA. The maximum total identities of both cDNA sequences with the corresponding cDNA sequences of other species are in the range of 65-70% which also fits with the values for maximum identities between guanylin or uroguanylin cDNA sequences of different species excluding pig. In contrast, the overall identity between the porcine guanylin and uroguanylin precursor cDNA is below 35%.

An important question arising in the context of guanylin/uroguanylin-related research concerns the possible existence of further peptides belonging to this novel family of gastrointestinal hormones. In order to

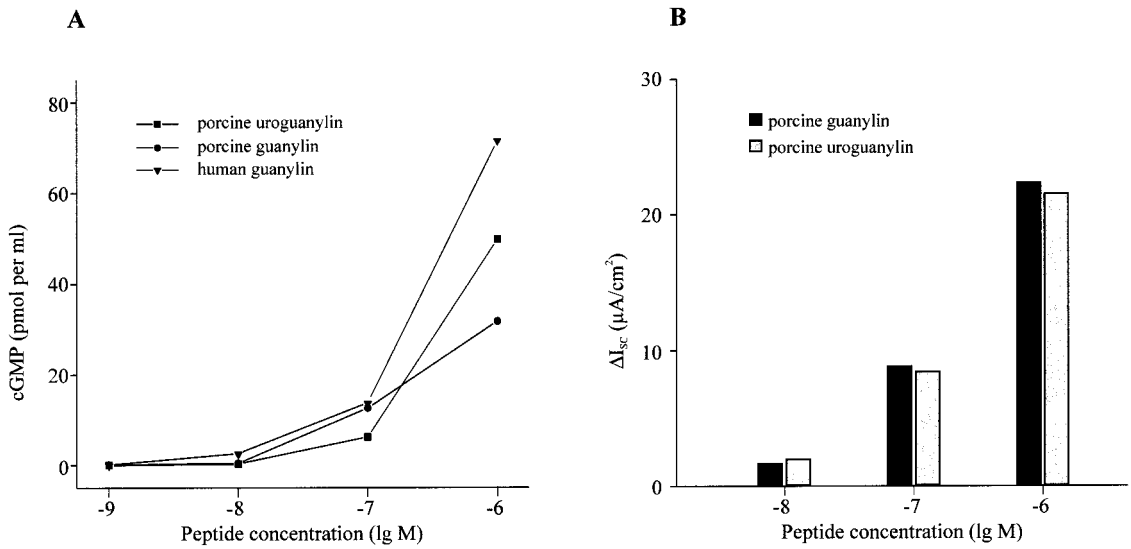

FIG. 3. Biological activity of porcine guanylin and uroguanylin. (A) Dose-dependent cGMP formation in cultured T 84 cells as a response to the stimulation by synthetic peptides. Experiments were carried out in duplicate for each peptide and the data shown represent the mean. (■) pUroguanylin$_{90\text{-}113}$, (●) pGuanylin$_{95\text{-}109}$, (▼) hGuanylin$_{101\text{-}115}$. (B) Comparison of the stimulatory effects of pGuanylin$_{95\text{-}109}$ and pUroguanylin$_{90\text{-}113}$ on the short circuit current in porcine colonic mucosa. Data are presented as mean ± SEM derived from 7-9 experiments each. Cumulative addition of the synthetic peptides to the mucosal solution of the Ussing chamber increases I_{SC}. pUroguanylin$_{90\text{-}113}$ is slightly less potent than pGuanylin$_{95\text{-}109}$.

identify such peptides, we derived degenerate primers from highly conserved regions of both peptide hormones for the initial RT-PCR step (see above). Using these primers, we did not succeed in amplifying additional guanylin/uroguanylin-related cDNAs encoding any peptides other than the two described. This finding may indicate that guanylin and uroguanylin are the only two members of this family of gastrointestinal peptide hormones. However, the existence of additional members which occur in tissues other than those investigated as yet and which possibly share a lower sequence identity with guanylin and uroguanylin cannot be excluded.

As another proof of the identification of the porcine guanylin and uroguanylin precursor cDNAs, we analysed the expression of the corresponding genes in tissues already known to express the related genes of other species by use of Northern hybridization and RT-PCR analysis. As hybridization probes we used cDNA fragments spanning the nucleotides from positions 152-290 in the case of porcine guanylin and from positions 131-390 in the case of porcine uroguanylin. Because the maximum sequence identity of each partial cDNA fragment with the cDNA of the other porcine gastrointestinal peptide hormone (guanylin hybridization probe with uroguanylin cDNA and uroguanylin hybridization probe with guanylin cDNA) was lower than 22% and the hybridization experiments were performed under highly stringent conditions, cross-hybridization events were not to be expected. Similarly to our recent investigations in humans (11), we detected a strong expression of the genes for both peptide hormones within the intestine, whereas a significant expression within the stomach was only detectable for uroguanylin. An expression of the genes for GC-C and the CFTR channel (21, 22) within the stomach have also been described. In addition, a cGMP-stimulated bicarbonate excretion of the bile (23) as well as the stimulation of bicarbonate secretion in rat duodenum by guanylin (24) have been reported. Taking these findings all together, one may speculate that uroguanylin is possibly involved in a CFTR-mediated bicarbonate secretion of the stomach, generating a protective pH gradient within the mucin layer located between the lumen and the epithelial surface (25).

As a final proof of the identification of the porcine analogues, the functionality of synthetic peptides (see above) was tested due to their influence on mucosal ion transport utilizing Ussing chambers and in their potential to elevate the intracellular level of cGMP. By measuring the short-circuit current using porcine mucosa, both peptides exhibited a similar dose-dependent effect between 10^{-8} and 10^{-6} M. This quantitative efficacy is identical to that observed for the human or rat peptides with human or rat mucosa, respectively. The activation of intestinal guanylyl cyclase-C follows a similar dose-dependent order. However, porcine guanylin and uroguanylin were slightly less potent in the activation of the human receptor that is present on T84 cells. This may be explained by differences in amino acid sequences between the porcine and human ligands, thereby causing a less efficient interaction with the human receptor protein.

In summarizing, as presented within this paper we succeeded in cloning the cDNAs for the precursor proteins of porcine guanylin and uroguanylin and demonstrated the biological functionality of the putative mature forms. Our results verify the importance of both peptide hormones for a wide range of different, not only closely-related mammals. It will probably also enable the detection of highly conserved regions within the amino acid and nucleotide sequences of the different precursor proteins and the corresponding cDNAs, which may be helpful for the identification and investigation of related peptide hormones in species other than those already described.

REFERENCES

1. Schulz, S., Green, C. K., Yuen, P. S. T., and Garbers, D. L. (1990) *Cell* **63,** 941–948.
2. Pfeifer, A., Aszodi, A., Seidler, U., Ruth, P., Hofmann, F., and Fassler, R. (1996) *Science* **274,** 2082–2086.
3. Chao, A. C., de Sauvage, F. J., Dong, Y. J., Wagner, J. A., Goeddel, D. V., and Gardner, P. (1994) *EMBO J.* **13,** 1065–1072.
4. Cuthbert, A. W., Hickman, M. E., MacVinish, L. J., Evans, M. J., Colledge, W. H., Ratcliff, R., Seale, P. W., and Humphrey, P. P. A. (1994) *Br. J. Pharmacol.* **112,** 31–36.
5. Currie, M. G., Fok, K. F., Kato, J., Moore, R. J., Hamra, F. K., Duffin, K. L., and Smith, C. E. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 947–951.
6. Hamra, F. K., Forte, L. R., Eber, S. L., Pidhorodeckyj, N. V., Krause, W. J., Freeman, R. H., Chin, D. T., Tompkins, J. A., Fok, K. F, Smith, C. E., Duffin, K. L., Siegel, N. R., and Currie, M. G. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 10464–10468.
7. Kita, T., Smith, C. E., Fok, K. F., Duffin, K. L., Moore, W. M., Karabatsos, P. J., Kachur, J. F., Hamra, F. K., Pidhorodeckyj, N. V., Forte, L. R., and Currie, M. G. (1994) *Am. J. Phys.* **266,** F342–348.
8. Kuhn, M., Raida, M., Adermann, K., Schulz-Knappe, P., Gerzer, R., Heim, J. M., and Forssmann, W. G. (1993) *FEBS Lett.* **318,** 205–209.
9. Hess, R., Kuhn, M., Schulz-Knappe, P., Raida, M., Fuchs, M., Klodt, J., Adermann, K., Kaever, V., Cetin, Y., and Forssmann, W. G. (1995) *FEBS Lett.* **374,** 34–38.
10. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York.
11. Mägert, H. J., Reinecke, M., David, I., Raab, H. R., Adermann, K., Zucht, H. D., Hill, O., Hess, R., and Forssmann, W. G. (1998) *Regul. Pept.* **73,** 165–176.
12. Hill, O., Kuhn, M., Zucht, H. D., Cetin, Y., Kulaksiz, H., Adermann, K., Klock, G., Rechkemmer, G., Forssmann, W. G., and Mägert, H. J. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 2046–2050.
13. Marchuk, D., Drumm, M., Saulino, A., and Collins, F. S. (1990) *Nucleic Acids Res.* **19,** 1154.
14. de Sauvage, F. J., Keshav, S., Kuang, W. J., Gillet, N., Henzel, W., and Goeddel, D. V. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 9089–9093.
15. Wiegand, R. C., Kato, J., Huang, M. D., Fok, K. F., Kachur, J. F., and Currie, M. G. (1992) *FEBS Lett.* **311,** 150–154.
16. Hill, O., Cetin, Y., Cieslak, A., Mägert, H. J., and Forssmann, W. G. (1995) *Biochim. Biophys. Acta* **1253,** 146–149.
17. Frohman, M. A., Dush, M. K., and Martin, G. R. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 8998–9002.
18. Klodt, J., Kuhn, M., Marx, U. C., Martin, S., Rösch, P., Forssmann, W. G., and Adermann, K. (1997) *J. Pept. Res.* **50,** 222–230.
19. Ussing, H. H., and Zehran, K. (1951) *Acta Physiol. Scand.* **23,** 110–127.
20. Wada, A., Hirayama, T., Kitao, S., Fujisawa, J., Hidaka, Y., and Shimonishi, Y. (1994) *Micobiol. Immunol.* **38,** 535–541.
21. Crawford, I., Maloney, P. C., Zeitlin, P. L., Guggino, W. B., Hyde, S. C., Turley, H., Gatter, K. C., Harris, A., and Higgins, C. F. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 9262–9266.
22. Strong, T. V., Boehm, K., and Collins, F. S. (1994) *J. Clin. Invest.* **93,** 347–354.
23. Myers, N. C., Grüne, S., Jameson, H. L., and Sawkat-Anwer, M. (1996) *Am. J. Physiol.* **270,** G418–424.
24. Guba, M., Kuhn, M., Forssmann, W. G., Classen, M., Gregor, M., and Seidler, U. (1996) *Gastroenterology* **111,** 1558–1568.
25. Wallace, J. L., and Granger, D. N. (1996) *FASEB J.* **10,** 731–740.