GCAP-II: isolation and characterization of the circulating form of human uroguanylin

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Abstract The systematic isolation of circulating regulatory peptides which generate cGMP as second messenger resulted in the identification of a novel member of the guanylin family. In the present study we describe the purification and amino acid sequence of a new guanylate cyclase C activating peptide (GCAP-II). GCAP-II contains 24 amino acids in the following sequence: FKTLRTIANDDCELCVNVACTGCL. Its molecular mass is 2597.7 Da. The 16 C-terminal amino acids are identical to uroguanylin from human urine. Native and synthetic GCAP-II activate GC-C, the specific guanylate cyclase receptor, of cultured human colon carcinoma (T84) cells. GCAP-II stimulates chloride secretion in isolated human intestinal mucosa mediated by intracellular cGMP increase. GCAP-II specific antibodies were used to localize the peptide by immunohistochemistry in entero-endocrine cells of the colonic mucosa.

Key words: Guanylate cyclase activating peptide II; Uroguanylin; Guanylin; cGMP; Intestinal chloride secretion; Entero-endocrine cell

1. Introduction

The second messenger cGMP constitutes an important signalling system. Among the cellular functions regulated through this pathway is the electrolyte transport of various epithelia. In the intestinal epithelium this system is coupled to a particulate guanylate cyclase (GC-C) which is abundantly localized in the apical membrane of the enterocytes [1]. Intestinal GC-C is activated by heat-stable enterotoxins (ST) which are small peptides secreted by several pathogenic bacteria [1]. Subsequent increases in epithelial cGMP mediate the activation of cystic fibrosis transmembrane conductance regulator (CFTR) in the apical membrane which in turn results in chloride and water secretion and secretory diarrhea [2]. Recently, guanylin, the first endogenous ligand to this GC-C receptor was discovered by Currie and coworkers [3]. Northern blot and immunohistochemical analysis have revealed that guanylin occurs in the mucosa of the mammalian intestine (see reviews [4] and [5]). The peptide is probably released from entero-endocrine cells [6,7] into the intestinal lumen to activate the GC-C and consequently the chloride transport in enterocytes by means of a paracrine interaction [5]. However, several data indicate that the guanylin/GC-C system is not exclusively confined to the intestine. Low levels of GC-C have been detected in extra-intestinal tissues, such as kidney, liver, reproductive tract, adrenals, airway epithelia, and pancreas [4,5]. In addition, a larger molecular form of this peptide, namely guanylin-22–115, circulates as a bioactive peptide in human blood, suggesting that this peptide regulates the function of different target organs by an endocrine interaction [8].

Uroguanylin, a second ligand of the GC-C receptor, has been recently purified from human and opossum urine [9,10]. The structural homology and the similar biological activity of guanylin and uroguanylin suggest that they are members of a peptide family, the main function of which is the activation of GC-C in the intestine and in other tissues. With regard to putative endocrine interactions of this system we initiated a systematic search for further endogenous activators of GC-C circulating in human blood. Here we report the isolation, the biochemical and functional characterization, and the immunohistochemical localization of a new GCAP related to uroguanylin.

2. Materials and methods

2.1. T84 cell cGMP bioassay

The bioassay for the detection of GC-C activating peptides was carried out as described in detail [3,8]. Human colon carcinoma (T84) cells were used at passages 34–45. The cells were incubated for 60 min with aliquots derived from the chromatographic fractions, in the presence of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX, 1 mM; Sigma, Deisenhofen, Germany). The effects on intracellular cGMP content were compared with those of synthetic human guanylin (IPF, Hannover, Germany) and *E. coli* heat-stable enterotoxin (STa; Sigma, Deisenhofen, Germany). cGMP concentrations were measured using a specific radioimmunoassay [11].

2.2. Purification of GCAP-II

Hemofiltrate (HF) from patients with chronic renal insufficiency was obtained at a local nephrology center (Niedersächsisches Zentrum für Nephrologie, Hannoversch-Münden, Germany). Freshly collected HF was immediately acidified with hydrochloric acid (HCl) to pH 3.0 and cooled to 4°C. Batches of 750 liters were diluted 1:3 (v/v) with deionized water and applied to a cation-exchange column (Fractogel TSK SP 650 (M), Merck, Darmstadt, Germany). The peptides bound to the column were eluted with 0.5 M ammonium acetate and the eluate was lyophilized. Peptide extracts of batches of 3000 liters of HF were resuspended in deionized water and subjected to an ultrafiltration using a membrane with a cut-off of 20 kDa (cellulose triacetate membrane; Sartorius, Göttingen, Germany) to eliminate most of the remaining plasma proteins such as albumin. The filtrate was then acidified with HCl (pH 3.0) and subjected to seven subsequent chromatographic purification steps. After each step, aliquots of the fractions were assayed for cGMP generation in T84 cells, and the active fractions were pooled for further purification.

Step 1: Preparative reversed phase (RP) C18 chromatography (300 Å, 15–20 μm, 47 × 300 mm; Vydac, Hesperia, CA, USA). The peptides

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were eluted with a flow rate of 40 ml/min and fractionated with the following gradient: 10 to 60% eluent B in 30 min (eluent A: 0.01 N HCl; eluent B: 80% acetonitrile, 0.01 N HCl).

Step 2: RP C18 (300 Å, 15–20 μm, 47 × 300 mm; Vydac, Hesperia, CA, USA); gradient from 32 to 48% eluent B in 42.5 min (eluent A: 0.01 N HCl; eluent B: 80% acetonitrile, 0.01 N HCl); flow rate, 40 ml/min.

Step 3: Strong cation-exchanger (300 Å, 5 μm, 10 × 50 mm; Pepkat, Biotek, Östringen, Germany); gradient from eluent A (25 mM Na_2HPO_4, pH 3.0) to 40% eluent B (1.5 M NaCl, 25 mM Na_2HPO_4, pH 3.0) in 60 min and to 100% eluent B in 5 min; flow rate, 3 ml/min.

Step 4: RP C4 (300 Å, 5 μm, 4 × 250 mm; ProRP, Biotek, Östringen, Germany); gradient from 25 to 45% eluent B in 60 min (eluent A: 0.1% trifluoroacetic acid (TFA), eluent B: 80% acetonitrile, 0.1% TFA); flow rate, 0.7 ml/min.

Step 5, 6 and 7: The last three steps were carried out with the same RP C18 column (300 Å, 5 μm, 4.6 × 250 mm; Vydac, Hesperia, CA, USA). The peptides were eluted at a flow rate of 0.7 ml/min using as eluent A 0.01 N HCl and as eluent B 80% acetonitrile, 0.01 N HCl. The gradient was 25 to 45% eluent B in 60 min (*step 5*), 25 to 45% eluent B in 80 min (*step 6*) and 28 to 45% eluent B in 51 min (*step 7*).

2.3. Sequence analysis

The amino acid sequence of the purified peptide was determined by Edman degradation using an ABI 473A gas-phase sequencer with standard program and gradient (Applied Biosystems, Weiterstadt, Germany). 50 to 100 pmol of the purified bioactive peptide were applied onto the Bioprene-coated glass fibre disc (Applied Biosystems, Weiterstadt, Germany).

2.4. Mass spectrometry (MS)

The molecular weight of the purified peptide was determined by microbore-HPLC-MS coupling using the dual syringe solvent delivery system ABI 140A (Applied Biosystems, Weiterstadt, Germany) with an AQS 1.0 × 250 mm RP C18 column (YMC, Schernbeck, Germany) and the API *III* biomolecular mass analyser (Sciex, Perkin Elmer, Langen, Germany) equipped with articulated ion spray inlet. Single scans from 400 to 2400 atomic mass units were recorded every 7 s during separation time. The column outlet was directly transferred to the electrospray unit. Molecular masses were determined in positive ion mode.

2.5. Peptide synthesis

Peptide synthesis was carried out on a 9050 peptide synthesizer using a preloaded Fmoc-L-Leu-PEG-PS resin (PerSeptive/Biosearch, Freiburg, Germany) as described [12]. Disulfide bridges were introduced selectively using Fmoc-L-Cys(Trt)-OH and Fmoc-L-Cys(Acm)-OH with subsequent air and iodine oxidation procedures. Purity and identity of the bicyclic peptide were checked by HPLC, capillary zone electrophoresis (Biofocus 3000, Bio-Rad, München, Germany), and electrospray mass spectrometry (Sciex API *III*, Perkin Elmer, Langen, Germany).

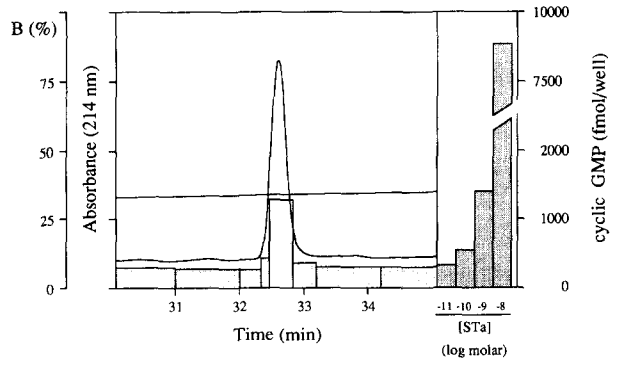

Fig. 1. Final purification of GCAP-II by RP C18 chromatography. HPLC conditions are given in section 2. B: eluent B (%, v/v). Shaded columns indicate the bioactivity. On the right side of the graph, concentration-dependent activation of T84 cGMP by enterotoxin (STa: 10 pM to 10 nM).

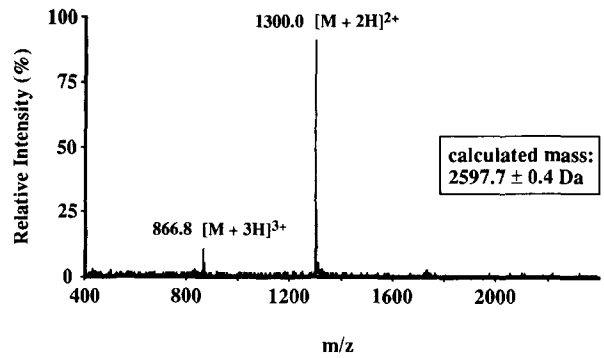

Fig. 2. Electrospray mass spectrum of the purified peptide GCAP-II. Mass was calculated from the multiple charge ions $[M + 2H]^{2+}$ and $[M+3H]^{3+}$.

2.6. Ussing chamber experiments

Specimens of jejunum were obtained from patients undergoing abdominal surgery at Hannover Medical School. The use of tissue was approved by the Human Ethics Committee of Hannover Medical School. The clinical conditions requiring removal of intestine were carcinomas of the gut or pancreas. Tissues used for experimental studies were taken from macroscopically normal areas distant from the pathologically affected tissue. After dissection of muscle layers, sheets of isolated mucosa were mounted in Ussing chambers and automatically voltage-clamped as described previously [13]. Short-circuit current (Isc) was continuously recorded. The transepithelial conductance was determined each minute by bipolar voltage pulses of 1 mV. After an equilibrium period of 30 min cumulative concentrations of synthetic peptide were added to the mucosal reservoir.

2.7 Immunohistochemistry

A multiple antigenic peptide (MAP) consisting of a branched heptalysine core with eight copies of the N-terminal domain of GCAP-II (FKTLRTIANDD) covalently bound was synthesized [12,14]. Antisera were raised in rabbits and used to localize the peptide in various tissues such as colon, liver, kidney, and stomach. Controls were carried out as routinely [6], for preabsorption tests the entire synthetic GCAP-II as well as the MAP and the linear undecapeptide were used in excess.

3. Results

In initial experiments, the peptide fractions from human hemofiltrate separated via RP-HPLC were assayed for the increase of cGMP in T84 cells. Two major GC-C stimulating activities eluted from the RP C18 column (data not shown). Isolation of the second activity led to the purification of the high molecular weight guanylin (guanylin-22–115) [8]. To characterize the first activity, 3000 liters HF were used to purify the corresponding bioactive peptide to homogeneity by a series of subsequent HPLC fractionations. After six chromatographic purification steps, three bioactive fractions were obtained. Rechromatography of the fraction showing the strongest bioactivity, a single peak was detected, revealing that the corresponding fraction consisted of purified material (Fig. 1). Electrospray mass spectrometric analysis showed that the molecular weight of the corresponding peptide is 2597.7 Da (Fig. 2).

Sequence analysis of the purified peptide using Edman degradation resulted in the following sequence: FKTLRTIANDD-XELXVNVAXTGXL. Comparison of the sequence of this second circulating guanylate cyclase C activating peptide (GCAP-II) with other proteins in the SwissProt and EMBL databases showed that the sequence is new and that the 16 amino acids from position 9 to position 24 of GCAP-II are identical to those

of human uroguanylin, the peptide isolated from urine by Kita and coworkers [10] (see Fig. 3). The molecular weight determined by MS corresponds to the theoretical molecular weight calculated from the sequence supposed that the non-detectable amino acids (X) are cysteines and that two disulfide bonds are formed. Thus, GCAP-II represents an N-terminally extended form of uroguanylin. In addition to the structural similarity with guanylin, a corresponding biological activity was revealed. The striking characteristics of these peptides include the four conserved cysteine residues (for comparison, see Fig. 3) and the fact that they both circulate as bioactive peptides in human blood.

The other two bioactive fractions of chromatographic step 6 were separately subjected to HPLC-MS coupling. The main constituent of both was the 2597.7 Da peptide, indicating that GCAP-II was also responsible for the bioactivity of these fractions.

The disulfide bridges of the chemically synthesized GCAP-II were linked between the cysteines in positions 12–20 and 15–23, as previously suggested for guanylin or uroguanylin (Fig. 3).

The biological activity of synthetic GCAP-II was assessed by evaluating its effects on T84 cell cGMP content as well as on electrolyte transport in isolated human intestinal mucosa. Synthetic GCAP-II causes a concentration-dependent increase in cGMP levels in T84 cells, with a threshold concentration of 10 nM. Its effective concentration range appears so far similar to that of synthetic human guanylin (guanylin-101–115) (Fig. 4). The effect of GCAP-II on intestinal electrolyte transport was analyzed by Ussing chamber experiments with isolated mucosal strips of human jejunum using synthetic GCAP-II. Addition of GCAP-II to the luminal compartment induces a concentration-dependent increase in Isc, again with a threshold concentration of 10 nM (Fig. 5A). This effect is very similar to that of human guanylin, when tested in parallel chambers (Fig. 5B). The Isc increases are in the same order of magnitude as the rise in cGMP in the T84 cell assay. The maximal Isc increases evoked by GCAP-II or guanylin (1 μM) are rapidly and completely reversed by the subsequent addition of 0.1 mM bumetanide, an inhibitor of the basolateral Na^+-K^+-$2Cl^-$ cotransporter, to the serosal solution. This indicates that both peptides stimulate electrogenic chloride secretion in human intestine.

The immunohistochemical analysis of the colonic mucosa revealed that GCAP-II immunoreactivity is localized exclusively in entero-endocrine cells (Fig. 6). Other cells in the epithelium or in the lamina propria or in the other organs investigated are devoid of immunoreactivity for GCAP-II.

4. Discussion

The present study describes the isolation and the sequence analysis of a second GC-C-activating peptide circulating in human blood plasma: GCAP-II. It contains 24 amino acids and represents an N-terminally extended form of uroguanylin, the 16-amino acid peptide recently isolated from urine [10]. As a source for human plasma peptides we used hemofiltrate collected from patients with renal insufficiency, in analogy to our previous study demonstrating the molecular form of circulating guanylin [8]. Native GCAP-II induced marked increases in cGMP content of T84 cells, indicating that the circulating peptide is an active hormone and not merely a precursor form. This conclusion is confirmed using synthetic GCAP-II, that stimulates dose-dependent cGMP formation in T84 cells as well as chloride secretion from the isolated human intestinal mucosa. In both experimental models the effects of GCAP-II are comparable to those of synthetic human guanylin, suggesting similar affinities of both peptides for their common intestinal receptor, GC-C. In contrast, synthetic uroguanylin was somewhat more potent than guanylin in the stimulation of GC-C activity

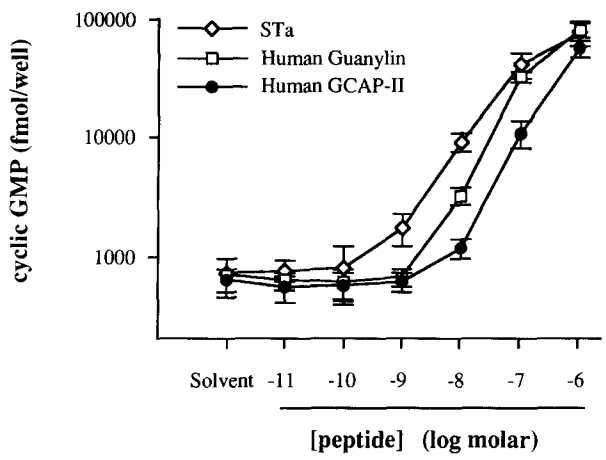

Fig. 4. Effects of activators of GC-C on intracellular cGMP levels in T84 cells. Cells were incubates with the indicated concentrations for 60 min in the presence of 1 mM IBMX. Values represent means ± S.D. (n = 4)

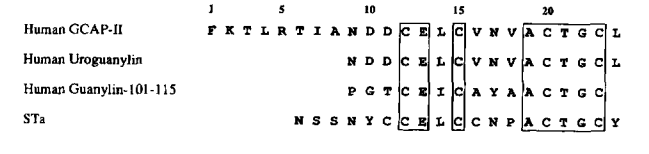

Fig. 3. Comparison of the amino acid sequences of human GCAP-II, human uroguanylin, human guanylin-101–115 and *E. coli* heat-stable enterotoxin (STa). Identical amino acids are boxed. Human GCAP-II represents the NH_2-terminal extended form of human uroguanylin.

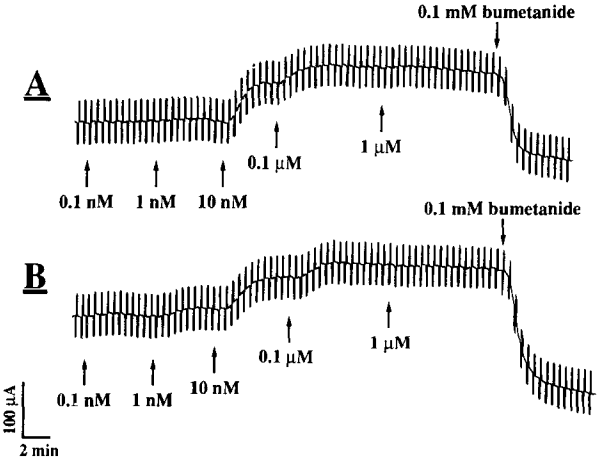

Fig. 5. Original tracing of an Ussing chamber experiment with isolated human jejunal mucosa. Cumulative addition of synthetic GCAP-II (A) and synthetic human guanylin-101–115 (B) to the luminal solution induced concentration-dependent increases in Isc. The increase in Isc was rapidly reversed by the subsequent addition of 0.1 mM bumetanide to the serosal solution.

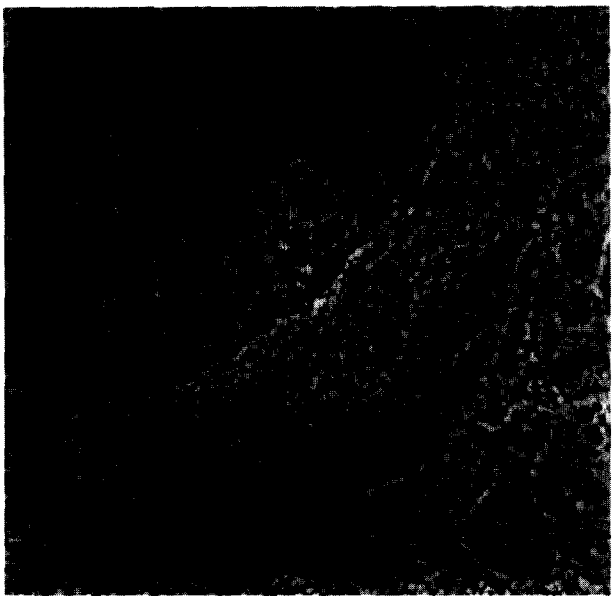

Fig. 6. Immunohistochemical demonstration of GCAP-II immunoreactivity in human colonic mucosa. Note the staining in a granulated cell type of the columnar epithelium characteristic for entero-endocrine cells. × 500.

in T84 cells [9,10]. Possible explanations for this discrepancy are passage-related differences in the responsiveness of T84 cells, the different incubation times used for experimentation (60 min in our study and 30 min in the study of Kita and coworkers [10]) and differences in the receptor affinity of GCAP-II and the shorter uroguanylin.

Recently, the uroguanylin cDNA has been cloned [15]. From the cDNA the sequence of a 112 amino acid prohormone can be predicted. The sequence of GCAP-II is contained in the C-terminal region from positions 89 to 112 of the precursor. Future studies will reveal whether uroguanylin, the 16-amino acid peptide excreted in urine, derives from an alternative processing of the precursor, or whether it constitutes an in vivo degradation product of circulating GCAP-II.

The tissue sites of guanylin production have been examined by Northern blot analysis and immunohistochemistry: both methods indicate that this peptide is produced mainly in the intestine and, at lower levels, in pancreas, adrenals, kidney, lung and uterus (see reviews [4,5]). The exact tissue distribution of GCAP-II or uroguanylin is not established. Northern blot analysis using total RNA showed a very high expression of the GCAP-II-gene in human colon [15]. With regard to the high levels of uroguanylin in urine it has been postulated that the kidney may be the main source of this peptide [10]. However, our attempts to demonstrate a significant gene expression in extraintestinal tissues, even in kidney, were unsuccessful. In the colon, GCAP-II may be released luminally, to reach the receptor (GC-C) in the brush-border membrane of the enterocytes (paracrine secretion). Also basolateral release may occur to contribute to circulating GCAP-II.

As we have already postulated for circulating guanylin [8], GCAP-II may serve as an endocrine link between the intestine and distant tissues involved in the regulation of fluid and electrolyte homeostasis. In particular, several experimental observations suggest that the kidney may be a main target tissue for circulating GCAP-II. White and coworkers [16] showed that renal tissue contains a functional receptor for the *E. coli* heat-stable enterotoxin (STa). Natriuretic effects of STa have been demonstrated both in vivo, in anaesthetized animals, and in vitro, in isolated perfused rat kidneys, in association with large increases in urine levels of cGMP [17,18]. Regarding the high urine levels of uroguanylin it is probable that circulating GCAP-II is filtered into the renal tubules. The effect may be mediated like the natriuresis induced by STa, either directly or after local cleavage forming the shorter uroguanylin. Northern analysis of GC-C and binding studies with [^{125}I]STa indicated the localization of the receptor in other extraintestinal tissues such as liver, reproductive tract, airways, and pancreas [4]. Thus, the circulating ligands guanylin-22–115 and GCAP-II may form part of an endocrine pathway for the regulation of multiple physiological processes.

In summary, the present study describes the isolation and the chemical and functional characterization of a second GC-C stimulating peptide (GCAP-II) from human hemofiltrate. GCAP-II represents an N-terminally extended form of uroguanylin and belongs to the guanylin/STa peptide family. Future studies will focus on the endocrine role of this circulating peptide family that might constitute a novel communication system between the intestine and extraintestinal tissues.

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