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The circulating bioactive form of human guanylin is a high molecular weight peptide (10.3 kDa)

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Guanylin is a peptide isolated from rat intestine that stimulates intestinal guanylate cyclase. We describe here the purification of circulating guanylin from human hemofiltrate. By N-terminal protein sequence analysis 47 amino acids were determined. This sequence corresponds to the positions 22 to 68 of the prohormone deduced from the cDNA sequence of human proguanylin. Mass spectral analysis of the circulating peptide showed the molecular weight to be 10,336 Da, which corresponds to the mass calculated from position 22 to the C-terminus of the peptide predicted from the cDNA sequence. Circulating guanylin markedly increased the cyclic GMP content of T84 cells. Our data show that the hormonal form of guanylin is circulating as a 10.3-kDa peptide in human blood.

Guanylin; Guanylate cyclase; Cyclic GMP; Peptide hormone; Hemofiltrate

1. INTRODUCTION

Guanylin is a 15-amino acid peptide isolated from rat intestine which stimulates intestinal guanylate cyclase (GC-C) [1]. Subsequent increases in epithelial cyclic GMP levels inhibit intestinal fluid absorption and increase chloride secretion [2].

Recently the human cDNA encoding this hormone was cloned [3,4]. The cDNA sequence revealed that the isolated peptide is the C-terminal end of a longer proguanylin molecule and possibly represents a truncated fragment of previous acetic acid extraction methods during the purification procedure. To our knowledge the correct processing site of proguanylin to the mature hormone has not been described.

In-situ hybridization suggested that guanylin may be expressed in the Paneth cells of the small intestine and that the peptide acts as a local paracrine modulator of intestinal fluid transport [3]. However, at least in rat, guanylin-mRNA was also found in low amounts in other tissues, such as adrenal gland, kidney and uterus [5]. The same group reported for several species that the mRNA for GC-C, the apparent guanylin receptor, exists not only in the epithelium of the gastrointestinal tract but also in other organs [5]. Thus, the actions of guanylin may ultimately extend to other tissues. The existence of guanylin in human blood plasma has not been described yet. However, the possibility exists that guanylin is a circulating hormone modulating the function of various organs by an endocrine mode.

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2. MATERIALS AND METHODS

2.1. *Bioassay for guanylin activity*

Guanylin-like bioactivity was assayed as described by Currie et al. [1]. In brief, T84 cells (human colon carcinoma cells; ATCC, Rockville, MD) were cultured in a 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 50 U/ml of penicillin and 50 μg/ml of streptomycin (Gibco).

Experiments were performed with T84 cells at passages 59–68, grown to confluence in 24-well plates. Before starting the experiments the cells were washed three times with 0.5 ml DMEM and then incubated at 37°C for 10 min with 0.25 ml DMEM containing 1 mM isobutylmethylxanthine (IBMX, Sigma), a cyclic nucleotide phosphodiesterase inhibitor. Agents and fractions derived from the chromatographic steps were lyophilized, dissolved in 0.1 ml DMEM, and added to the wells for 60 min. Then the incubation medium was aspirated and the reaction was terminated by addition of 0.1 ml ice-cold 70% ethanol. After freeze–thawing, the wells were dried by evaporation and 1 ml of 50 mM sodium acetate buffer (pH 6.0) was added to each well. Supernatant fractions were acetylated and cyclic GMP was measured by radioimmunoassay [6].

2.2. *Purification of guanylin*

As a source for human plasma peptides we used hemofiltrate, collected from patients with chronic renal failure. This material offers several advantages: (1) it is available in large quantities; and (2) hemofiltration constitutes a first purification step of plasma peptides, since the cutoff of the hemofilter, 20,000 Da, reduces the amount of filtered proteins to a minimum. Hemofiltrate was obtained from renal insufficient patients at a local hospital (Niedersächsisches Zentrum für Nephrologie, Hannoversch-München, Germany). The first step of

isolation was carried out according to a modification [7] of the polypeptide isolation by Mutt [8]. After its collection hemofiltrate was immediately acidified with pure hydrochloric acid (HCl) by pH 3.5. A batch of 720 liters was diluted 1:1 (v/v) with water, adjusted to pH 2.7 by concentrated HCl, and stirred for 18 h after adding 2.5 kg of alginic acid. The batch was then filtered on a Büchner funnel to obtain the dry alginic acid with absorbed peptides. Subsequently the alginic acid was washed with 98% ethanol (v/v) and 0.005 N HCl. The polypeptides were then eluted with ice cold 0.2 N HCl. After lyophilization, 6.5 g of the extract (total weight: 46.5 g) which corresponds to 100 liters of hemofiltrate were resuspended in 100 ml of 0.01 N HCl and centrifuged at 2,500 × *g* for 20 min at 4°C. The supernatant was filtered and applied in 4 ml fractions to a preparative reversed-phase (RP) C4 column (300 Å, 20–45 μm, 30 × 125 mm) (Parcosil, Biotek, Heidelberg, Germany). The samples were eluted with a flow rate of 5 ml/min and fractionated with the following gradient: 0.01 N HCl (buffer A) to 80% acetonitrile (v/v), 0.01 N HCl (buffer B) in 120 min (Step 1). An aliquot of each fraction was tested in the bioassay with T84 cells. The bioactive fractions were pooled and submitted to six RP-HPLC steps. After each step aliquots of all fractions were tested in the bioassay, and the active fractions were processed for further purification. The following HPLC conditions were used for the purification of guanylin bioactivity: Step 2, column RP C4, Parcosil, 250 Å, 20–45 μm, 20 × 125 mm: gradient from 0.1% trifluoroacetic acid (TFA) to 80% 2-propanol, 0.1% TFA in 120 min; flow rate, 3 ml/min; Step 3, column RP C4, Parcosil, 300 Å, 5 μm, 10 × 125 mm; gradient from 30% acetonitrile, 0.1% TFA to 80% acetonitrile, 0.1% TFA in 150 min; flow rate, 2 ml/min; Step 4, column RP C18, Parcosil, 100 Å, 5 μm, 4 × 250 mm; gradient from 20% acetonitrile, 0.1% TFA to 60% acetonitrile, 0.1% TFA in 80 min; flow rate, 0.7 ml/min. The last three steps of purification were carried out on a microbore RP C18 column (300 Å, 5 μm, 2.1 × 100 mm) (Vydac, Hesperia, CA, USA) with a flow rate of 0.2 ml/min. The following gradients were used: Step 5, 30% acetonitrile, 0.01 N HCl to 60% acetonitrile, 0.01 N HCl in 60 min; Step 6, 0.08% heptafluorobutyric acid to 30% methanol, 50% 2-propanol, 0.08% heptafluorobutyric acid in 80 min; Step 7, 20% methanol, 0.1% TFA to 100% methanol, 0.08% TFA in 100 min. In the last purification step a single bioactive fraction was obtained. Capillary zone electrophoresis of an aliquot of this fraction showed a single peak. This highly purified material was submitted to amino acid sequence analysis and mass spectrometry.

2.3. *Sequence analysis*

Amino acid sequencing was carried out on an ABI 470A gas-phase sequencer equipped with an ABI 120A PTH-amino acid analyser (Applied Biosystems, Weiterstadt, Germany) using the 03CPTH program, the standard gradient and a Kontron data system. 20 to 500 pM of the peptide or the fragments were applied onto the BIOPRENE-coated glass fibre discs.

2.4. *Mass spectrometry*

Mass determination of the purified peptide was carried out on a Finnigan TSQ700 triple stage quadrupol mass spectrometer in single MS mode with ESI interface. Sample introduction of the peptide was performed via a Harvard syringe pump with a flow rate of 2 μl/min.

2.5. *Peptide synthesis*

Synthesis of rat guanylin [1] was performed on a continuous-flow synthesis using standard Fmoc-protocols for activation and cleavage reactions [9]. The disulphide bridges were introduced selectively by using Fmoc-Cys (Trt) and Fmoc-Cys (Acm) with subsequent air and iodine oxidation procedures. After purification (C18-RP chromatography) purity and identity were checked by HPLC, CZE and mass analysis.

2.6. *Capillary zone electrophoresis*

CZE was carried out with a P/ACE 2000 (Beckman, München, Germany). Conditions were: buffer, 0.1 M phosphate, pH 2.5; constant current 80 mA, detection 200 nm, fused silica capillary 50 cm × 75 μm [10].

3. RESULTS

Initial characterization of the T84 cells revealed that nitric oxide donors like SIN-1 (Cassella Riedel, Germany) and sodium nitroprusside (Sigma) have no effect on their cyclic GMP content. Incubation of T84 cells with natriuretic peptides like CDD/ANP-99–126 (αhANP) and CDD/ANP-95–126 (urodilatin) (both Saxon Biochemicals, Germany), induced a small increase in cyclic GMP to maximally 4-fold at 0.1 μM of

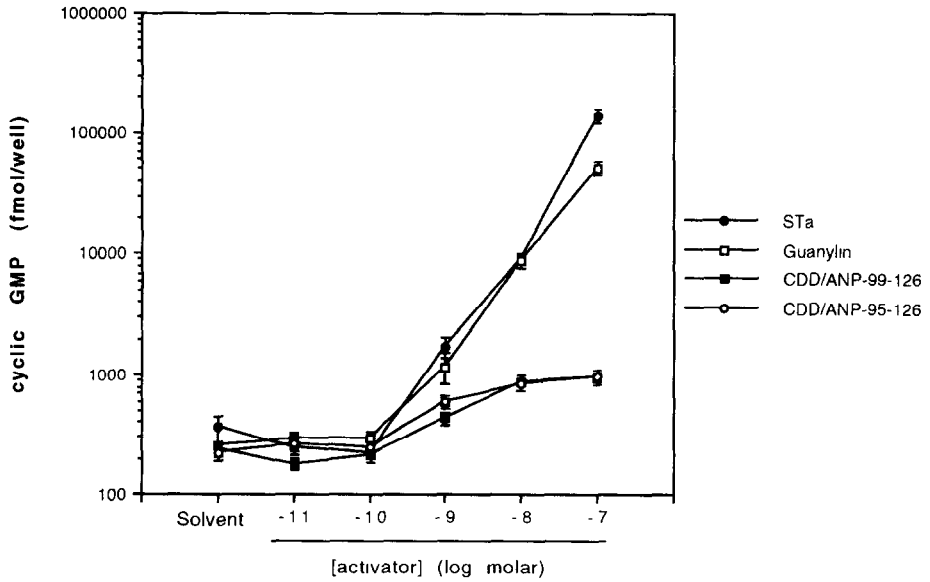

Fig. 1. Effect of stimulators of particulate guanylate cyclase GC-C (guanylin and STa) and GC-A (CDD/ANP-99–126 and CDD/ANP-95–126) on cyclic GMP content in T84 cells. Cells were incubated with the indicated concentrations during 60 min in the presence of 1 mM IBMX. Values represent means ± S.E. ($n = 4$).

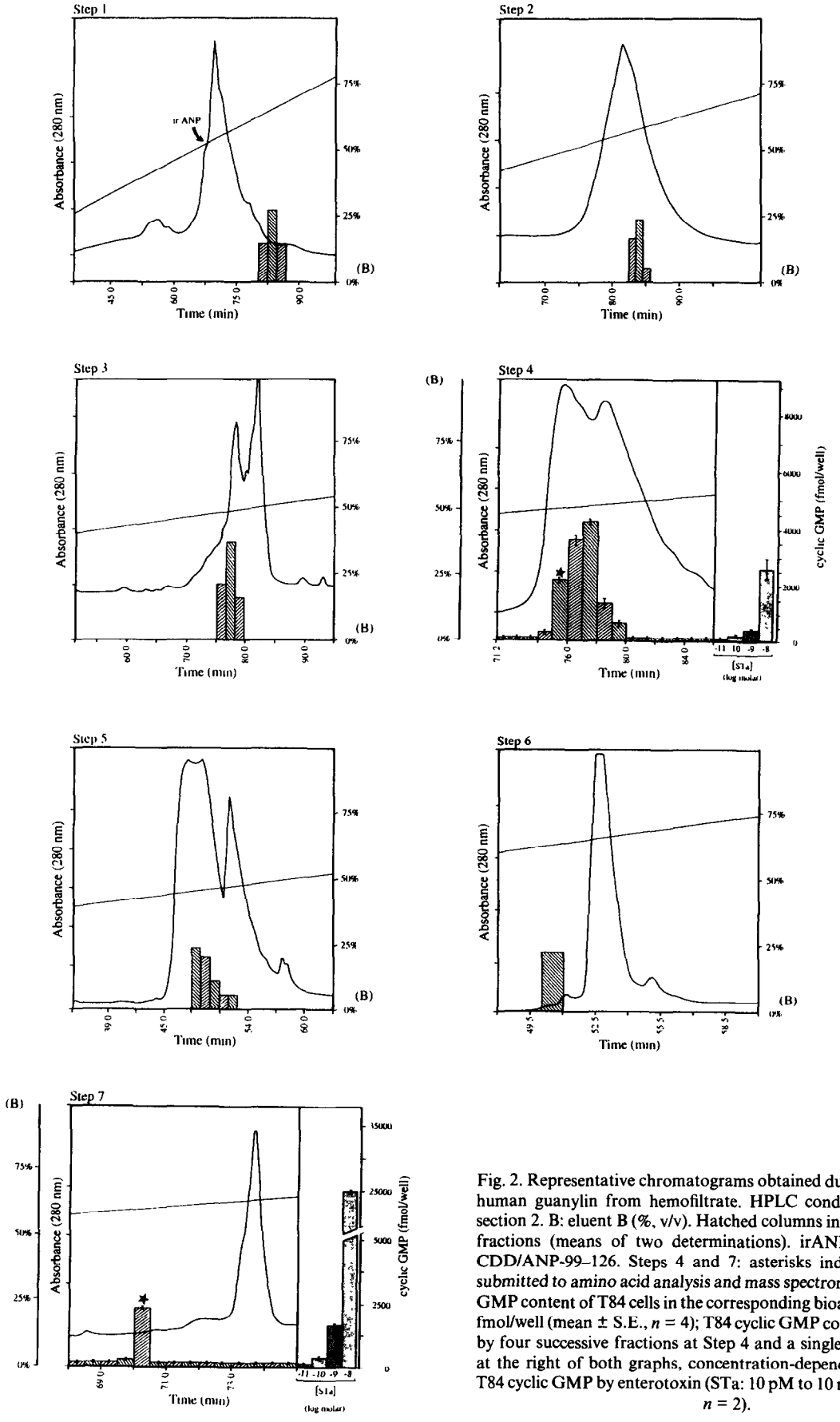

Fig. 2. Representative chromatograms obtained during purification of human guanylin from hemofiltrate. HPLC conditions are given in section 2. B: eluent B (%, v/v). Hatched columns indicate the bioactive fractions (means of two determinations). irANP: immunoreactive CDD/ANP-99–126. Steps 4 and 7: asterisks indicate the fractions submitted to amino acid analysis and mass spectrometry; resting cyclic GMP content of T84 cells in the corresponding bioassays was 178 ± 22 fmol/well (mean ± S.E., n = 4); T84 cyclic GMP content was increased by four successive fractions at Step 4 and a single fraction at Step 7; at the right of both graphs, concentration-dependent stimulation of T84 cyclic GMP by enterotoxin (STa: 10 pM to 10 nM) (means ± S.E., n = 2).

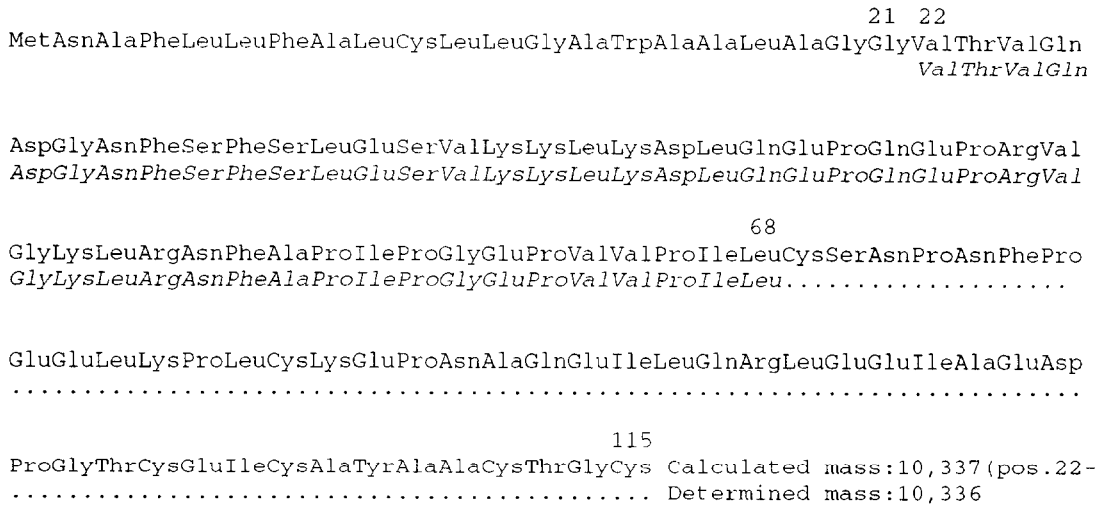

Fig. 3. Comparison between the amino acid sequence determined for the peptide purified from hemofiltrate (*italic*) and the sequence of human proguanylin deduced from the cDNA sequence [3,4]. Numbers above the sequence refer to the amino acid position.

both peptides (Fig. 1). In contrast, heat-stable enterotoxin (STa, Sigma) and synthetic guanylin induced large increases in cyclic GMP level, up to 555-fold at 0.1 μM STa and 202-fold increase from basal levels at 0.1 μM synthetic rat guanylin (Fig. 1). Thus, T84 cells are sensitive and rather specific detector cells for guanylin activity in human plasma.

Purification of bioactive material was accomplished as described in section 2. After polypeptide extraction with the alginic acid method, the material was chromatographed on a preparative C4-RP column (Step 1). The fractions with maximal bioactivity eluted between 69 and 70% buffer B (Fig. 2). As a control, an aliquot of each fraction was also tested for circulating CDD/ANP-99–126 by radioimmunoassay. Fractions containing immunoreactivity eluted at 46% buffer B. The bioactive fractions were then purified to homogeneity by a series of subsequent RP-HPLC steps (see section 2 and Fig. 2). During chromatographic Step 4, four successive fractions contained bioactivity. CZE of an aliquot of each fraction revealed that one of them consisted of almost pure material. This fraction was subjected to N-terminal protein sequence analysis and to electrospray mass spectrometric analysis. The amino acid sequence as indicated in Fig. 3, is homologous with positions 22 to 68 of the guanylin precursor deduced from the human cDNA sequence [3,4]. The further amino acid sequence is suggested from mass spectrometry of the extracted peptide revealing a molecular weight (MW) of 10,336 Da. This MW corresponds exactly to the calculated mass for the positions 22 up to the C-terminus (position 115) of proguanylin deduced from human cDNA. This active fraction contained approximately 500 pmol of the peptide, in a total fraction-volume of 700 μl (concentration: 0.7 μM). An aliquot of 25 μl of this sample (approximately 18 pmol of the bioactive material: 9 pmol/well, corresponding to a concentration of 26 nM) stimulated the cyclic GMP content of T84 cells by 9-fold. For comparison, in the same experiment, 10 nM STa (5 pmol/well) induced a 14-fold increase in cyclic GMP content of T84 cells (Fig. 2, Step 4).

The other three bioactive fractions of this chromatographic step were refractionated on a microbore C18-HPLC column (purification Steps 5–7, section 2). Finally, a single bioactive fraction was obtained. CZE of an aliquot of this fraction resulted in a single peak. N-terminal protein sequence analysis resulted again in the sequence starting with: Val-Thr-Val-Gln-Asp-Gly-Asn-.... These N-terminal amino acids indicated that this material was identical to the material sequenced in the chromatographic Step 4. In the bioassay, a 5-μl aliquot of this sample (this is, 5% volume of the whole sample volume) led to a 10-fold stimulation of cyclic GMP content in T84 detector cells. For comparison the same activity was yielded by 1 nM STa (0.5 pmol/well) (Fig. 2, Step 7). As illustrated in Fig. 2, the responsiveness of T84 cells to STa was markedly greater in the bioassay performed at Step 7 as compared to Step 4. The difference is probably attributable to the fact that the cells were used at different passages (Step 4, passage 64; Step 7, passage 61).

4. DISCUSSION

In the present study we describe the purification and sequence of the circulating form of human guanylin. This is for the first time that the existence of guanylin in human blood has been proven including its exact molecular form.

Hemofiltrate was purified by a series of reverse-phase HPLC fractionation steps using T84 cells as sensitive

detector cells of guanylin activity. This procedure led to the final isolation of a 10,336-Da peptide. By N-terminal protein sequence analysis 47 amino acids of the circulating peptide could be determined. This sequence is in accordance with the amino acids in positions 22 to 68 deduced recently for human intestinal proguanylin from the cDNA sequence [3,4]. The mass determined for the circulating peptide corresponds exactly to that calculated for the positions 22 up to the C-terminus of proguanylin deduced from the cDNA. Our data strongly suggest that a high molecular form of guanylin is circulating in human blood. However, since the complete sequence of the circulating peptide was not obtained, the possibility exists that its C-terminus is different to that deduced from human guanylin cDNA [3,4].

From the cDNA the sequence of a 115 amino acid prohormone was predicted (see Fig. 3). The C-terminus contains the 15 amino acid sequence published for rat intestinal guanylin [1]. The correct processing site of the mature peptide has not been described until now. Our data suggest that the physiological cleavage site of the prohormone to a circulating peptide is between positions 21 and 22. However, other naturally occurring processing forms cannot be excluded. Transfection of a human embryonic kidney cell line with the full-length human cDNA led to the secretion of a 94 amino acid, 10,337 Da protein [3]. Obviously, this MW corresponds to that we have found for the circulating plasma peptide. In our hands 'high molecular weight' guanylin is very bioactive (as related to cyclic GMP formation in T84 cells). In contrast, the conditioned media of proguanylin-transfected cells apparently were not bioactive (no displacement of [^{125}I]STa binding) [3]. Possible explanations are that our assay is more sensitive than the one used by these authors or that the concentrations of guanylin in the supernatants of the transfected cells were too low to be effective. Also it cannot be excluded that interaction with T84 cells led to the proteolytic cleavage of the circulation peptide into an active guanylin.

The purified hemofiltrate fraction contained approximately 500 pmol of the bioactive peptide. At this stage of purification (Step 4) the sequenced fraction was one of four fractions with similar amount of bioactive material. Thus, a calculated amount of approximately 2 nmol guanylin was purified from 100 liters of hemofiltrate. Disregard to the inevitable loss of material during the purification, this concentration is rather high. Since hemofiltrate was obtained from renal insufficient patients we cannot exclude that the high circulating levels are due to impaired renal metabolism or decreased renal elimination of the peptide.

At the moment only speculations are possible about the cellular source of circulating bioactive guanylin. Northern analysis showed that guanylin-mRNA is prevalent in intestine and it was suggested that the peptide is a local regulator of intestinal fluid transport [3,4]. If this is true, plasma guanylin may just be a kind of 'overflow' of a local hormone. However at least in rat, low mRNA levels were found outside the intestine: in adrenal gland, kidney and uterus [5]. Moreover, Northern analysis of GC-C and binding studies with [^{125}I]STa suggest that the guanylin receptor is present in other epithelial cells [5,11]. Thus the actions of guanylin may ultimately extend to other tissues beside the intestine.

From the fact that high molecular weight guanylin circulates in human plasma in a biologically active form, a lot of questions arise. Future studies will clarify whether this is just an 'overflow' phenomenon of a local hormone system or whether guanylin forms part of an endocrine pathway that modulates fluid secretion of various organs.

REFERENCES

[1] 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.
[2] Kachur, J.F., Won-Kim, S., Moummi, C., Fok, K. and Currie, M.G. (1992) Gastroenterology 102, 738a.
[3] De Sauvage, F.J., Keshav, S., Kuang, W.-J., Gillett, N., Henzel, W. and Goeddel, D.V. (1992) Proc. Natl. Acad. Sci. USA 89, 9089–9093.
[4] Wiegand, R.C., Kato, J., Huang, M.D., Fok, K.F., Kachur, J.F. and Currie, M.G. (1992) FEBS Lett. 311, 150–154.
[5] Schulz, S., Chrisman, T.D. and Garbers, D.L. (1992) J. Biol. Chem. 267, 16019–16021.
[6] Kaever, V. and Resch, K. (1985) Biochim. Biophys. Acta 846, 216–225.
[7] Forssmann, K., Hock, D., Herbst, F., Schulz-Knappe, P., Talartschik, J., Scheler, F. and Forssmann, W.G. (1986) Klin. Wochenschr. 64, 1276–1289.
[8] Mutt, V., Hormone Isolation, in: Gut Hormones (S.R. Bloom, ed.), Churchill Livingstone, Edinburgh, London, New York, 1978, pp. 21–27.
[9] Atherton, E. and Sheppard, R.C., Solid Phase Peptide Synthesis, in: D. Rickwood and B.D. Hames, Eds., IRL Press at Oxford University Press, 1989.
[10] Karger, B.L., Cohen, A.S. and Guttman, A. (1989) J. Chromatography 492, 585–614.
[11] Krause, W.J., Freeman, R.H. and Fort, L.R. (1990) Cell Tissue Res. 260, 387–394.