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# Role of the prosequence of guanylin

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**Abstract**

Guanylin is a guanylyl cyclase (GC)-activating peptide that is mainly secreted as the corresponding prohormone of 94 amino acid residues. In this study, we show that the originally isolated 15-residue guanylin, representing the COOH-terminal part of the prohormone, is released from the prohormone by cleavage of an Asp-Pro amide bond under conditions applied during the isolation procedures. Thus, the 15-residue guanylin is probably a non-native, chemically induced GC-activating peptide. This guanylin molecule contains two disulfide bonds that are absolutely necessary for receptor activation. We demonstrate that the folding of the reduced 15-residue guanylin results almost completely in the formation of the two inactive disulfide isomers. In contrast, the reduced form of proguanylin containing the entire prosequence folds to a product with the native cysteine connectivity. Because proguanylin lacking the 31 $NH_2$-terminal residues of the prosequence folds only to a minor extent to guanylin with the native disulfide bonds, it is evident that this $NH_2$-terminal region contributes significantly to the correct disulfide-coupled folding. Structural studies using CD and NMR spectroscopy show that native proguanylin contains a considerable amount of $\alpha$-helical and, to a lesser extent, $\beta$-sheet structural elements. In addition, a close proximity of the $NH_2$- and the COOH-terminal regions was found by NOESY. It appears that this interaction is important for the constitution of the correct conformation and provides an explanation of the minor guanylyl cyclase activity of proguanylin by shielding the bioactive COOH-terminal domain from the receptor.

**Keywords:** disulfide-coupled folding; disulfides; folding; guanylin; proguanylin; prohormone; propeptide; prosequence

Guanylin is an intestinal peptide hormone of 15 amino acids that activates a transmembrane guanylyl cyclase (GC) (Currie et al., 1992). It contains two disulfide bonds with a 1–3 and 2–4 cysteine connectivity, which is crucial for biological activity. GC signaling comprises the production of the second messenger cyclic 3′,5′-guanosine monophosphate (cGMP) and subsequent activation of the cystic fibrosis transmembrane conductance regulator chloride channel (CFTR) (Chao et al., 1994; Vandraager et al., 1997). Thus, guanylin was postulated to be an autocrine/paracrine regulator of the intestinal electrolyte and fluid transport (Forte et al., 1993; Forte & Currie, 1995; Hamra et al., 1997). Uniquely with mammalian peptides containing multiple disulfides, bioactive guanylin forms two topological stereoisomers that differ in the backbone conformation, while containing the same cysteine connectivity (Fig. 1A; Skelton et al., 1994). These topoisomers are in dynamic equilibrium and interconvert without disulfide opening and rearrangement. It was shown that only one guanylin isomer significantly elevates the level of intracellular cGMP, thus revealing that cGMP formation induced by GC activation requires a highly defined ligand structure (Schulz et al., 1998). Nothing is known about a possible in vivo effect of the inactive topoisomer.

Unlike most other regulatory peptides, guanylin occurs predominantly as the corresponding prohormone of 94 amino acid residues (sequence positions 22–115 according to the preprohormone, Fig. 1B) and is secreted in this molecular form of negligible GC-activating potency into the intestinal mucosa and blood (Kuhn et al., 1993; Yamaguchi et al., 1995; Hamra et al., 1996). The GC-activating guanylin of 15 amino acids, guanylin-(101–115), forms the COOH-terminal portion of the prohormone. Although cleavage sites for different endoproteases such as trypsin and chymotrypsin were identified (Garcia et al., 1993), nothing is known about the in vivo processing of proguanylin. As proguanylin con-

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*Abbreviations:* cGMP, cyclic 3′,5′-guanosine monophosphate; CD, circular dichroism; CFTR, cystic fibrosis transmembrane conductance regulator chloride channel; COSY, correlated spectroscopy; $Cys_2$, cystine; DTT, dithiothreitol; $DTT_{ox}$, oxidized dithiothreitol; EDTA, ethylenediaminetetracetic acid; ESMS, electrospray mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; GC, guanylyl cyclase; GnHCl, guanidinium hydrochloride; GSH, reduced glutathione; GSSG, oxidized glutathione; HPLC, high-performance liquid chromatography; MeCN, acetonitrile; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect, also used for NOESY cross peak; NOESY, NOE spectroscopy; ST, *Escherichia coli* heat-stable enterotoxin; TFA, trifluoro-acetic acid; TOCSY, total correlation spectroscopy.

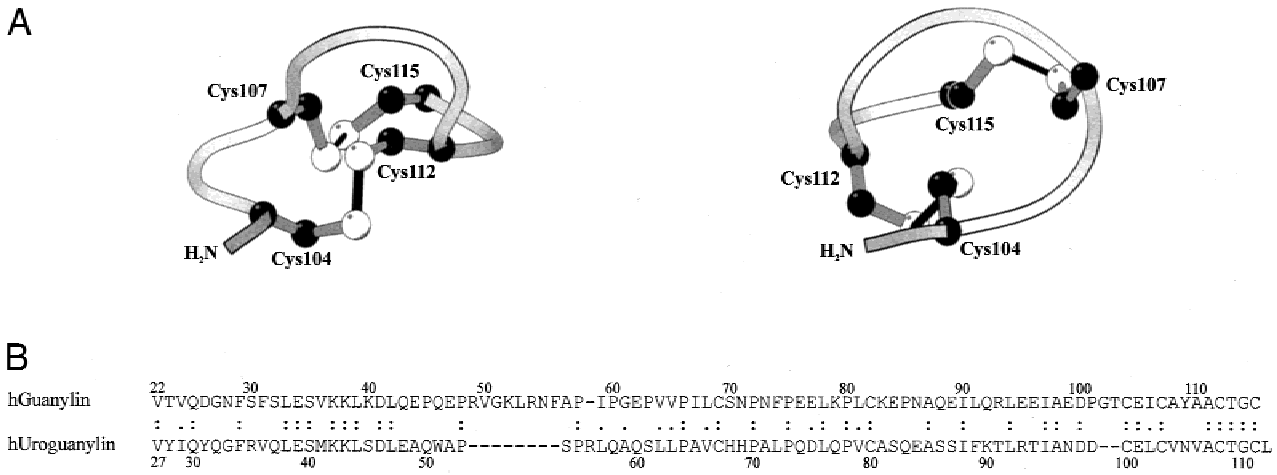



**Fig. 1. A:** MOLSCRIPT diagrams of the two topological stereoisomers of human guanylin-(103–115) (Brookhaven PDB, accessions 1GNA and 1GNB; Skelton et al., 1994); left: GC-activating isomer A; right: GC-inactive isomer B; white circles: sulfur atoms; black circles: C$\alpha$ and C$\beta$ of cysteine residues. **B:** Amino acid sequence alignment of human proguanylin and prouroguanylin. Identical sequence positions are indicated by a colon, chemically similar residues by a single dot. The numbering refers to the respective preproproteins.

tains a potential acid-labile Asp-Pro site (Kowit & Maloney, 1982; Rittenhouse & Marcus, 1984), which may liberate the bioactive guanylin-(101–115) during isolation procedures (Schulz et al., 1992), it remained unclear whether guanylin-(101–115) is actually a native peptide. Although the topoisomerism of the 15-residue guanylin has been investigated, nothing is known about the structure of this peptide as the COOH-terminal part of the prohormone.

In a recent study, it was demonstrated for the closely related peptide uroguanylin (Fig. 1B) that its corresponding prosequence is crucial for the disulfide-coupled folding of the reduced precursor (Hidaka et al., 1998). It was found that uroguanylin requires the prosequence to fold to a protein containing the native disulfide pattern. In contrast, the oxidative folding of 24-residue uroguanylin, corresponding to the molecular form isolated from human blood, resulted in the two possible non-native and biologically inactive disulfide isomers. Thus, it was concluded that the prosequence of uroguanylin plays a functional role as an intramolecular chaperone in the formation of the native disulfide pattern during biosynthesis of uroguanylin.

The current study addresses a functional role of the prosequence of guanylin and the acid lability of proguanylin. We present evidence that the prosequence of proguanylin is required during in vitro folding to obtain bioactive guanylin with a native disulfide bond pattern. Structural studies, in particular NMR spectroscopical investigations, are used to show that the $NH_2$-terminal region of proguanylin is in close relation to the COOH-terminus that contains the guanylyl cyclase-activating fragment. The data obtained are used to explain multiple functions of a peptide hormone's prosequence during its intracellular maturation and by influencing the extracellular bioactivity.

## Results

### *Stability of proguanylin toward acetic acid*

Proguanylin contains an acid-labile Asp-Pro site (positions 100–101), which separates the prosequence from the bioactive guanylin-(101–115). To analyze the stability of this amide bond, the native proguanylin was incubated under conditions that allowed the isolation of guanylin-(101–115) from intestinal mucosa, i.e., 1 M HOAc at 95 °C. Under these conditions, proguanylin generates quantitatively two fragments—guanylin-(22–100) and guanylin-(101–115)—within 60 min. Figure 2 shows the HPLC analysis of this cleavage, which was verified by ESMS. The generated guanylin-(101–115) could be easily identified by the characteristic HPLC pattern, exhibiting two peaks corresponding to its rapidly interconverting topoisomers, and by comparison with the synthetic reference peptide (Klodt et al., 1997). The complete disappearance of proguanylin coeluting with the fragment guanylin-(22–100) was confirmed by ESMS. The kinetics of HOAc-induced proguanylin cleavage was also analyzed by detection of intracellular cGMP upon stimulation of human T84 cells. Starting with proguanylin of only minor GC-activating potential, the intracellular cGMP level increased, induced by HOAc-treated proguanylin mixtures with a total peptide concentration of $10^{-7}$ M (Fig. 3). The quantitative evaluation of the time-dependent course of the formation of cGMP is in agreement with the cGMP level induced by synthetic guanylin-(101–115) and confirms the formation of the carboxy-terminal fragment guanylin-(101–115). The half-life of proguanylin in boiling 1 M HOAc, as estimated from T84 cell bioassay and HPLC analysis, is in a range of 15–20 min. Although proguanylin was completely stable toward 1 M HOAc at an ambient temperature over three days, it also generated the fragments guanylin-(101–115) and guanylin-(22–100) during treatment with water at 95 °C. However, the kinetics of the cleavage of the Asp100–Pro101 amide bond in aqueous solution is significantly slowed down and a fourfold increase of the half-life of proguanylin can be estimated. It appears that after 10 min an amount of bioactive guanylin-(101–115) is formed, large enough to be detected by the cGMP/T84 assay. The guanylin fragment-(22–100) alone did not elevate cGMP (data not shown).

### *Folding of guanylin-(101–115)*

It has long been known that reduced guanylin-(101–115) subjected to air oxidation at alkaline pH leads to a product of very little

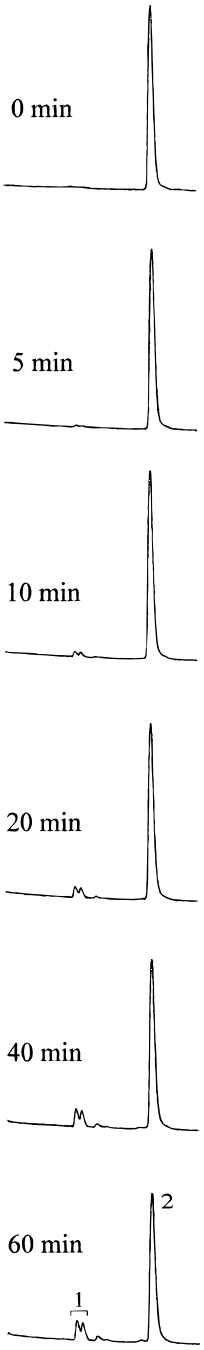



**Fig. 2.** Analytical HPLC profile of acetic acid cleavage of proguanylin. Peak 1: guanylin-(101–115), A- and B-topoisomers; peak 2: guanylin-(22–100) coeluting with proguanylin. Half-life of proguanylin (about 20 min) was estimated by the ratios of relative peak areas corresponding to proguanylin/guanylin-(22–100) and guanylin-(101–115) after 20 min (ratio 13.2) and 60 min (ratio 6.5). After 60 min, proguanylin was completely cleaved.

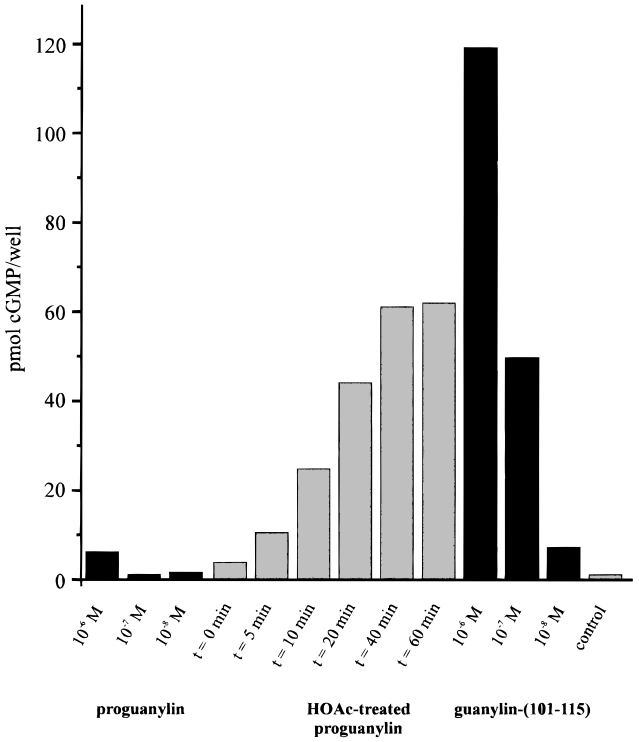



**Fig. 3.** cGMP elevation in T84 cells by native proguanylin and guanylin-(101–115) in a concentration range between $10^{-6}$ to $10^{-8}$ M, and acetic acid cleavage mixtures of native proguanylin with a total peptide concentration of $10^{-7}$ M. Experiments were carried out in duplicate for each sample. Data are expressed as the mean.

bioactivity (Forte & Currie, 1995). As shown by HPLC analysis, all three possible disulfide isomers were formed (Fig. 4A). However, only as a minor product, guanylin with the native disulfide bonds (relative 1–3/2–4 cysteine connectivity) was identified by its particular appearance as two topological isomers and by comparison with a chemically synthesized standard. The later eluting products corresponding to peaks 2 and 3 of the oxidation product were cleaved with chymotrypsin at Tyr109 to assign them to guanylin with either 1–2/3–4 or 1–4/2–3 disulfides (Badock et al., 1998). As is evident from ESMS analysis of the cleavage products, peak 2 corresponds to the isomer with a relative 1–4/2–3 disulfide pattern, and peak 3 corresponds to the 1–2/3–4-bridged guanylin isomer. Testing various conditions for the folding of guanylin-(101–115), the ratio of the three possible guanylin isomers contained in the product showed no principal differences (Table 1). Guanylin, containing the native disulfides, was about 5–10% at most, and the non-native isomers were by far the major products. Although the isomer with a 1–3/2–4 pattern of disulfides was slightly preferred using a Cys/$Cys_2$-containing redox buffer (Fig. 4B), the ratio between guanylin disulfide isomers could not be shifted toward the isomer with the native disulfide bonds by varying the temperature or by the use of additives such as GnHCl and redox buffers.

*Folding of proguanylin and guanylin-(53–115)*

To investigate the folding of proguanylin, the native protein was reduced by treatment with DTT and then subjected to the folding reaction under various conditions. As refolded proguanylin eluted as a single broadened peak during HPLC analysis (Fig. 4C) and disulfide isomers could not be directly detected, we made use of the particular sensitivity of proguanylin toward acetic acid. Re-

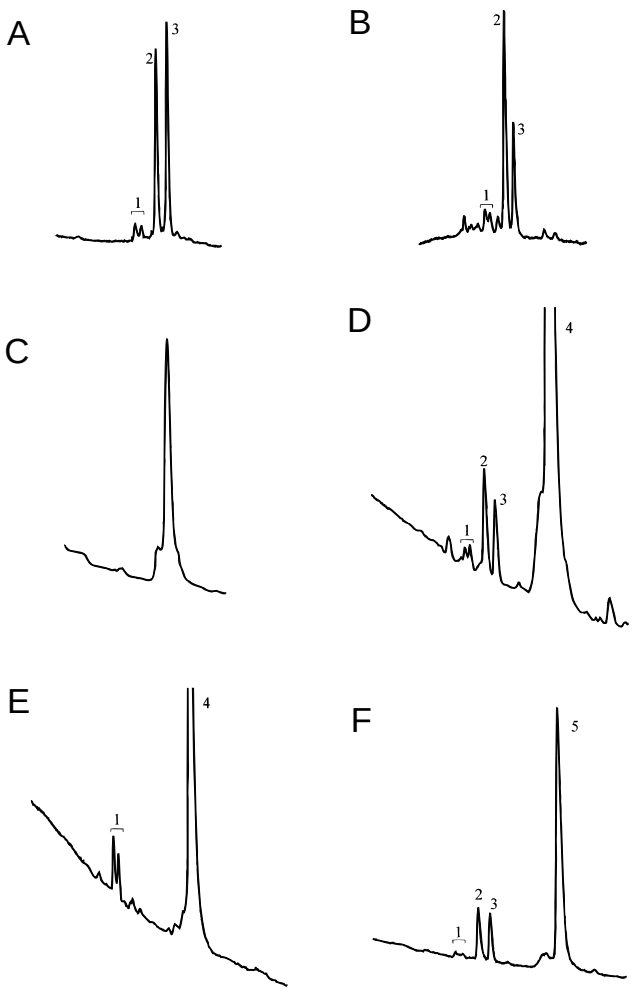



**Fig. 4.** HPLC profiles of oxidative folding products of reduced guanylins (100 mM $NH_4HCO_3$, 2 mM EDTA, pH 8.3). (**A**) Air oxidation of guanylin-(101–115), (**B**) oxidation of guanylin-(101–115) in the presence of Cys/$Cys_2$, (**C**) air oxidation of proguanylin, (**D**) air oxidation of proguanylin followed by HOAc cleavage, (**E**) oxidation of proguanylin in the presence of Cys/$Cys_2$ followed by HOAc cleavage, (**F**) oxidation of guanylin-(53–115) in the presence of Cys/$Cys_2$ followed by HOAc cleavage. Peak 1: Topoisomers of 1–3/2–4 disulfide-bonded guanylin-(101–115); peak 2: 1–4/2–3 disulfide isomer; peak 3: 1–2/3–4 disulfide isomer; peak 4: guanylin-(22–100); peak 5: guanylin-(53–100). The experimental conditions are described in detail in Table 1.

duced proguanylin was air oxidized and subsequently treated with 1 M acetic acid at 95 °C, thereby liberating the different disulfide isomers of guanylin-(101–115), which were easily detectable by HPLC analysis (Fig. 4D). The ratio of those disulfide isomers corresponded to the ratio of disulfide isomers that were formed during air oxidation of reduced guanylin-(101–115). Testing several folding conditions that were successful for other proteins, we found that proguanylin containing the native disulfide bonds can best be reconstituted by the use of redox buffers such as Cys/$Cys_2$ or GSH/GSSG, but without GnHCl (Table 1). From HPLC it was estimated that proguanylin with the native disulfide bond pattern was formed almost exclusively under these conditions (Fig. 4E).

To determine the possible importance of the $NH_2$-terminal part of the prosequence of proguanylin for the folding, we prepared guanylin-(53–115), which contains 63 amino acid residues and all disulfide bonds. This derivative was easily obtained by selective cleavage of proguanylin by endoprotease Lys-C as the Lys52 amide bond is of significantly higher proteolytic lability than Lys84. Folding reduced guanylin-(53–115) under conditions that were suitable for the proguanylin folding, i.e., in the presence of Cys/$Cys_2$, resulted in about 90% of the two non-native disulfide isomers and only a minor amount of guanylin with the native disulfide bonds was found (Fig. 4F). Thus, it is evident that the $NH_2$-terminal segment (22–52) of proguanylin contains molecular information that is crucial for the disulfide-coupled folding of the protein. We found no evidence for a cross reaction of the third disulfide bond formed by Cys69 and Cys82 with the COOH-terminal cysteine residues during the folding of proguanylin and guanylin-(53–115).

### *CD spectroscopy and secondary structure prediction*

The overall shape of the circular dichroism (CD) spectrum of proguanylin indicates the presence of $\alpha$-helical structure elements (Greenfield & Fasman, 1969; Schmid, 1989) (Fig. 5A). The quantitative evaluation of the secondary structure elements using Sreerama's method (Sreerama & Woody, 1993) shows approximately 33% $\alpha$-helix and 17% $\beta$-sheet. This experimental result corresponds with the secondary structure prediction according to several theoretical methods (Fig. 5B).

### *NMR spectroscopy of proguanylin*

Two-dimensional homonuclear $^1$H NOESY, COSY, and TOCSY spectra were recorded at 11 and 20 °C. The high dispersion of the amide proton chemical shifts and the upfield shift of single methyl group resonances as well as the overcrowded NOESY spectra suggest a well-structured protein. Helical structure is reflected by a high number of cross peaks in the backbone amide region of the NOESY spectra and by upfield-shifted C$\alpha$-proton resonances (Wishart et al., 1991) (Fig. 6). Downfield-shifted C$\alpha$-proton resonances suggest spin systems, which are either involved in a $\beta$-sheet structure or are followed by a proline (Wishart et al., 1991, 1995). Missing resonances in the correlation spectra (COSY, TOCSY) did not render the full sequence-specific assignment of the 94 spin systems possible. However, the very $NH_2$-terminal part, a small sequence in the central region and the COOH-terminus could be unambiguously assigned from their typical spin systems and their sequential connectivities.

Unambiguous long-range NOE interactions were found between the C$\alpha$-proton of Cys112 and the amide protons of Thr23 and Gln25 as well as between the amide protons of Thr23 and Thr113 (Fig. 6), indicating that proguanylin has a solution structure with a close proximity of the $NH_2$- and COOH-terminus. In agreement with the secondary structure predictions (Fig. 5B), the chemical shift data and the NOE pattern suggest a $\beta$-hairpin structure for the very $NH_2$-terminus followed by an $\alpha$-helix.

The determination of the oligomerization state by size-exclusion chromatography showed an apparent molecular weight of 18.6 Da for native proguanylin at pH 5 and 7.4, indicating that the protein probably forms a dimer in solution (data not shown). Therefore, it is possible that the long-range NOEs between Cys112 and Thr113 at the COOH-terminus with the $NH_2$-terminal residues Thr23 and Gln25 are due to an intra- or intermolecular interaction of the termini.

**Table 1.** *Conditions and ratio of disulfide isomers obtained after oxidative folding of guanylin peptides*

| Peptide | Folding conditions[a] | Ratio of isomers (%)[b] |
|---|---|---|
| Guanylin-(101–115) | Air oxidation, 20 °C | 5:45:50 |
| | 1 mM GSH:0.1 mM GSSG, 20 °C, 4 M GnHCl | 10:60:30 |
| | 1 mM Cys:0.05 mM $Cys_2$, 5 °C | 7:50:43 |
| Guanylin-(53–115) | 1 mM Cys:0.05 mM $Cys_2$, 5 °C | 3:50:47[c] |
| Proguanylin | Air oxidation, 20 °C, 4 M GnHCl | 7:50:43[c] |
| | Air oxidation, 5 °C | 3:47:50[c] |
| | 1 mM GSH:0.1 mM GSSG, 20 °C, 4 M GnHCl | 30:40:30[c] |
| | 1 mM GSH:0.1 mM GSSG, 5 °C, 4 M GnHCl | 50:27:23[c] |
| | 1 mM Cys:0.05 mM $Cys_2$, 5 °C, 4 M GnHCl | 40:35:25[c] |
| | 1 mM DTT:0.05 mM $DTT_{ox}$, 5 °C, 4 M GnHCl | 30:37:33[c] |
| | 1 mM GSH:0.1 mM GSSG, 5 °C | 70:17:13[c] |
| | 1 mM Cys:0.05 mM $Cys_2$, 5 °C | 80:12:8[c] |
| | 1 mM Cys:0.05 mM $Cys_2$, 5 °C | 95:3:2[c] |

[a] GnHCl, guanidinium hydrochloride; $Cys_2$, cystine; GSH, reduced glutathione; GSSG, oxidized glutathione; DTT, dithiothreitol; $DTT_{ox}$, oxidized dithiothreitol.
[b] Native:1–4/2–3:1–2/3–4.
[c] Determined by HPLC analysis after HOAc treatment.

One-dimensional NMR spectra of proguanylin were identical in a concentration range from 0.032 to 2.4 mM. Furthermore, the relative diffusion coefficient measured by pulsed field gradient NMR spectroscopy (Gibbs & Johnson, 1991) was the same for peptide concentrations of 0.24 and 2.4 mM (data not shown). These results indicate that the oligomerization state of proguanylin does not change within this concentration range, but do not allow to distinguish between monomeric and dimeric state.

The COOH-terminal part of the prohormone contains the small GC-activating guanylin (positions 101–115), which is known to form two topological isomers as an isolated peptide (Skelton et al., 1994; Klodt et al., 1997). Up to now, only one set of spin systems for the COOH-terminal part of proguanylin could be detected and assigned from NMR spectra. Comparing the chemical shifts of the assigned COOH-terminal amino acids (data not shown) with those of the two isoforms of Leu-extended guanylin-(101–115) (Schulz et al., 1998) and guanylin-(103–115) (Skelton et al., 1994), they were found to be more similar to the A-form structure of guanylin. This isomer is the biologically active isoform and structurally resembles the heat-stable *E. coli* enterotoxin ST (Ozaki et al., 1991a, 1991b; Skelton et al., 1994; Schulz et al., 1998). However, the exact topology of this sequence as a part of the entire proguanylin has yet to be determined.

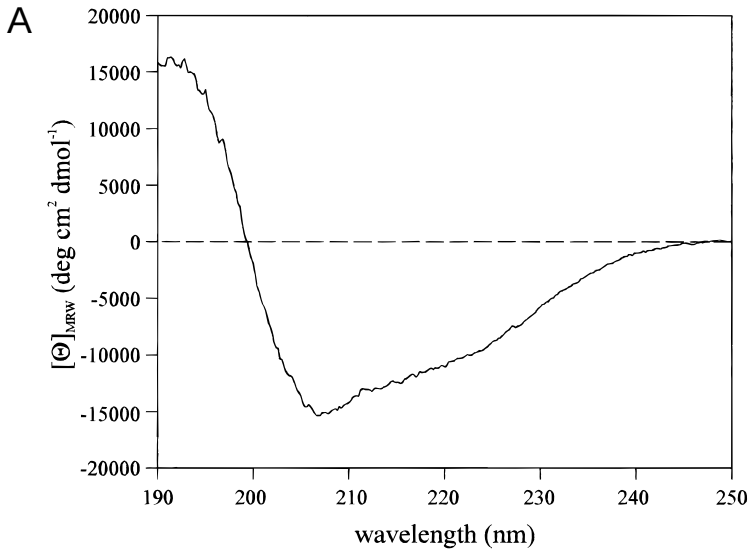
## Discussion

### *Proguanylin releases a bioactive fragment upon acid treatment*

Mimicking the conditions of the isolation of guanylin-(101–115) from intestinal mucosa (Currie et al., 1992; Yamaguchi et al., 1995; Hamra et al., 1996; Fan et al., 1997), our examination demonstrates that under these conditions proguanylin is cleaved at the Asp100–Pro101 amide bond, thereby generating guanylin-(101–115) and guanylin-(22–100). It appears that the cleavage kinetics is sufficient to produce an amount of bioactive guanylin-(101–115), which is detectable by the sensitive cGMP bioassay. This instability is not restricted to proguanylin, but applies to any other smaller guanylin molecule that contains this amide bond. Thus, for the isolation of a native form of guanylin, the inactivation of proteases by boiling in HOAc is not appropriate. We speculate that guanylin-(101–115) is rather a chemical artifact than an in vivo-processed peptide derived from proguanylin. Because the prohormone has only a minor GC-activating potential compared to guanylin-(101–115), the question of an actual native molecular form of guanylin that activates guanylyl cyclase has to be raised (Schulz et al., 1992). The identification of such a guanylin derivative would be of particular importance because the existence of multiple receptors for guanylin has been suggested (Giannella, 1995; Schulz et al., 1997). The isolations of guanylin-(100–115), which has an intact Asp-Pro site (Yamaguchi et al., 1995) as well as of a smaller guanylin containing 14 residues (Kita et al., 1995), strongly indicate that other forms of guanylin occur. Proguanylin contains two basic residues that represent potential enzymatic cleavage sites (Lys83 and Arg93). As guanylin-(84–115) is inactive in the T84/cGMP bioassay (data not shown) and, thus, is not a GC ligand, the GC-active guanylin-(94–115) is a good candidate for a native GC-active form of guanylin (de Sauvage et al., 1992).

### *The prosequence is required for the oxidative folding of guanylin*

Under any conditions used for the oxidative folding of guanylin-(101–115), only minor amounts of the product containing the native disulfides were obtained. This contradicts results that were reported for the folding of other small multiple disulfide-containing peptides such as *Escherichia coli* heat-stable enterotoxin ST (Y. Hidaka, unpubl. obs.), endothelin (Kumagaye et al., 1988), and conotoxins (Price-Carter et al., 1996). These peptides fold predominantly to products containing the native disulfide bonds. As

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                22          32          42          52          62          72
                |           |           |           |           |           |
Proguanylin     VTVQDGNFSFSLESVKKLKDLQEPQEPRVGKLRNFAPIPGEPVVPILCSNPNFPEELKPL
PHD              EEEE    EEEHHHHHHHHHHH                          E          EEEE
Gibrat          EEEECCCCHHHHHHHHHHHHHHCCCCHHHHHECCCCCCCCCCCCCEEEECCCCCCCCCHCHH
Levin           CCCCTSCHHCHHHHHHHHHHHTCCSTCTCCCCCCCCCCCCCCCCCEECCCCCSCCCHHHCHE
DPM             CCEETCCCCHHHHHHHHHHHHHHHCHTCCECCHHCHHCCCCCCEECEECCTCCTCCHHTCH
SOPMA           EEECCCCCCHHHHHHHHHHHHHHCCCCCCCEEEECCCCCCCCCCCCEEEEECCCCCCHHHHHH

                82          92          102         115
                |           |           |           |
Proguanylin     CKEPNAQEILQRLEEIAEDPGTCEICAYAACTGC
PHD                HHHHHHHHHHHHH       EEEEEE
Gibrat          HHCCCHHHHHHHHHHCCCCCCCCEEEECCCCCCC
Levin           ECCTCHHHHHHHHHHHHHCTTCCHHCHHHCCCCC
DPM             TCTCCHHHHHHHHHHHHHCCCCCEEEEEEHCCCC
SOPMA           CCCCCHHHHHHHHHHHHHCCCCCEEEEEEHCCCC
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**Fig. 5. A:** Far-UV CD spectrum of native proguanylin. **B:** Secondary structure prediction of proguanylin using PHDsec (Rost & Sander, 1993, 1994), Gibrat (Gibrat et al., 1987), Levin (Levin & Garnier, 1988), DPM (Deleage & Roux, 1987), and SOPMA (Geourjon & Deleage, 1995) methods (C, coil; E, extended; H, helix).

this is not the case for guanylin-(101–115), it can be suggested that this peptide is thermodynamically unfavored. It appears that an additional factor is required for the disulfide-coupled folding to the native conformation. Thus, an important characteristic point regarding guanylin is the non-Anfinsen type of the folding (Anfinsen, 1973).

Similar refolding experiments with proguanylin in the presence of a redox system such as GSH/GSSG or Cys/$Cys_2$ resulted in a product with the native disulfide bonding. Thus, it can be concluded that the prosequence of proguanylin functions as an intramolecular chaperone during the disulfide-coupled folding of proguanylin. Because mostly non-native disulfide isomers were formed in the presence of the denaturant GnHCl, it appears that noncovalent interactions between the prosequence and the COOH-terminal segment could influence the folding of proguanylin. Folding reduced guanylin-(53–115) under experimental conditions that were used for proguanylin led to almost no guanylin with the native disulfide bonds. Thus, the $NH_2$-terminal domain of proguanylin of 31 amino acid residues, or a part of it, is crucial for the disulfide-coupled folding of proguanylin.

The results obtained for proguanylin and guanylin-(101–115) are in agreement with a recent study of the folding of the related peptide uroguanylin and its prohormone (Hidaka et al., 1998). In that communication, it was reported that reduced prouroguanylin folds exclusively to the protein with 1–3/2–4 disulfide bonds in the COOH-terminal segment. In contrast, reduced uroguanylin of 24 residues did not refold to the peptide exhibiting the native disulfide bond pattern. The sequence alignment of human guanylin and uroguanylin shows some sequence similarity for the $NH_2$-terminal region and the COOH-terminus (Fig. 1B). This indicates a similar, chaperone-like function of the $NH_2$-terminal region of the prosequences of guanylin and uroguanylin for the correct disulfide-coupled folding of the bioactive COOH-terminal fragments. For only a few proteins, for example, subtilisin (Shinde & Inouye, 1993), it is known that a prosequence can guide the refolding. However, these examples differ from proguanylin in that

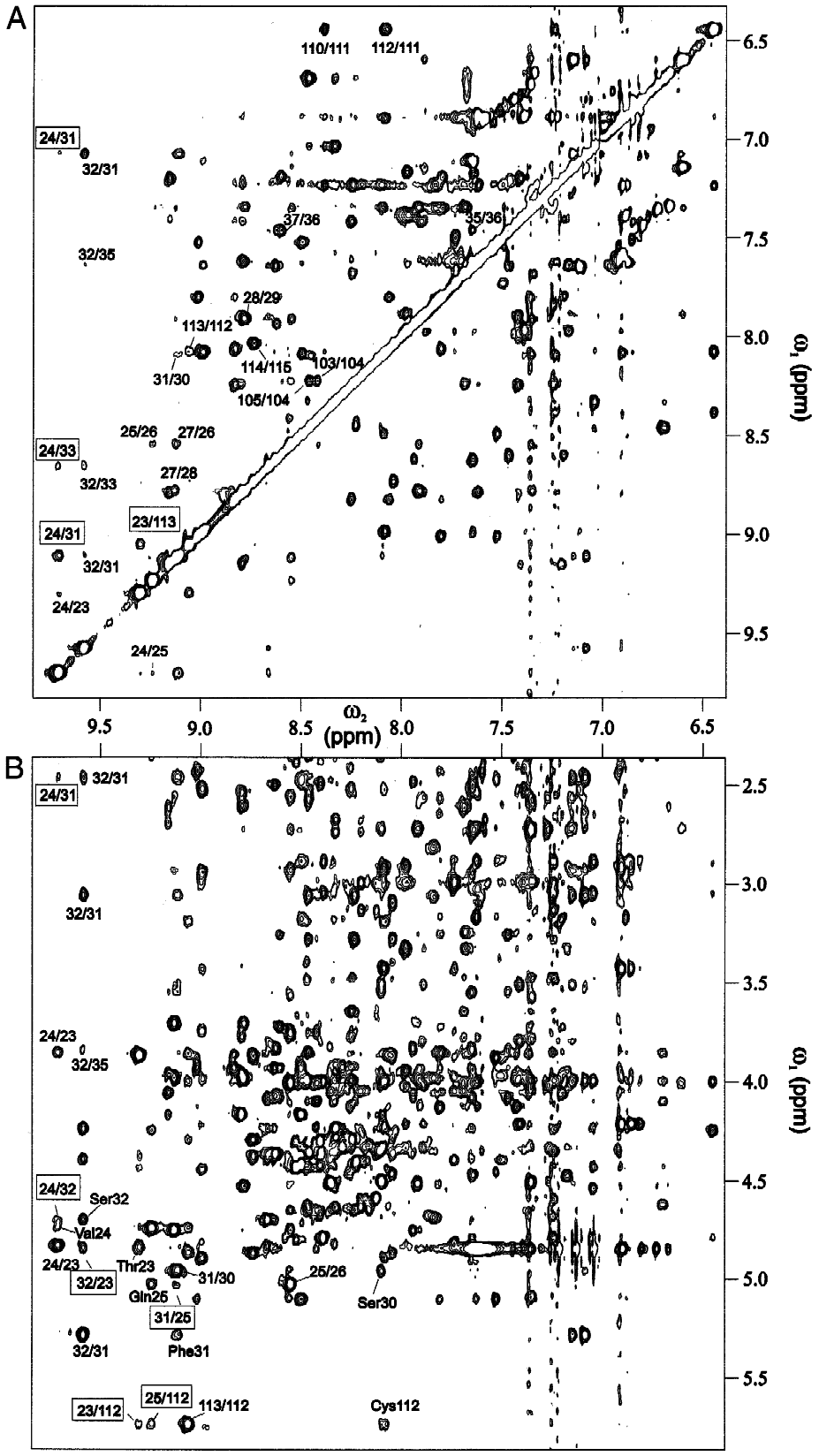


**Fig. 6.** Sections of the NOESY spectrum of proguanylin at 20 °C. The spectra were recorded with a mixing time of 200 ms. **A:** Cross peaks between amide protons are shown. The labels indicate interactions between assigned protons. The boxes indicate long-range NOEs. **B:** Cross peaks between amide and C$\alpha$-protons as well as few side-chain protons are shown. The labels indicate intraresidual backbone resonances of sequence specifically assigned amino acid spin systems as well as connectivities between different spin systems. Long-range interactions are indicated by boxes.

their prosequences are much smaller compared to the functional peptide.

### *Structural features of proguanylin*

Scanning the literature and databases such as the Brookhaven Protein Data Bank (PDB), it is remarkable that only a little information about structural properties of peptide hormones in their propeptide form is available. Using different threading methods (Jones et al., 1992; Fisher & Eisenberg, 1996; Rost et al., 1997), no structural homologs with a reliable score were found, reflecting that the structure of proguanylin may correspond to a new protein fold.

Contradictory to our results it was reported in an earlier study that human proguanylin expressed in *E. coli* contains mostly $\beta$-sheets and $\beta$-turns, but almost no $\alpha$-helix (Garcia et al., 1993). The recombinant proguanylin used for these experiments was, however, $NH_2$-terminally extended by five amino acids, resulting in a length of 99 amino acid residues. The discrepancy of the CD-spectroscopic results of native proguanylin of 94 residues in this study and the longer recombinant protein may indicate that the extension by five residues alters the secondary structure of the protein.

For 94-residue proguanylin, the participation of $NH_2$-terminal residues in intramolecular structure-stabilizing interactions can be supposed. Unambiguous long-range NOEs between $NH_2$- and COOH-terminal amino acids reveal a close proximity of the termini, providing a possible explanation for the different secondary structural characteristic of recombinant proguanylin of 99 residues. Furthermore, the established proximity of the $NH_2$- and COOH-terminus is in agreement with the results obtained from the folding experiments. It can be assumed that this interaction plays an important role in the disulfide-coupled folding of reduced proguanylin. A stabilizing effect during the folding between the termini would explain why guanylin-(53–115), which lacks this domain of the prosequence, does not fold to a protein containing the native disulfide pattern.

The interaction of the $NH_2$- and the COOH-terminus of proguanylin also provides an explanation for the minor GC-activating potency of proguanylin compared to guanylin-(101–115). Irrespective of the oligomerization state, an intra- or inter-molecular fixation of the $NH_2$-terminus at the potential GC-activating COOH-terminal segment would cause shielding of receptor and ligand. If the COOH-terminal binding site of the ligand is not completely covered by parts of the $NH_2$-terminal region of proguanylin, a residual activity of proguanylin may be detected. As concluded from a T84/cGMP bioassay, proguanylin is not completely GC inactive, but has an effect on cGMP decreased by two orders of magnitude compared to guanylin-(101–115).

Concerning the phenomenon of the formation of two topological stereoisomers of guanylin-(101–115) (Skelton et al., 1994; Klodt et al., 1997), only one set of spin systems was identified for the COOH-terminus of proguanylin. As the chemical shifts of the assigned COOH-terminal residues of proguanylin resemble more closely those of the A-isomers of guanylin (Skelton et al., 1994; Adermann et al., 1996; Schulz et al., 1998), we conclude that the COOH-terminal fragment corresponding to positions 101–115 contained in the propeptide adopts a structure similar to that determined for topoisomer A, i.e., the GC-activating isomer. It appears that the B-type topoisomer of guanylin may not be of physiological relevance. This hypothesis is strongly supported by the fact that the related prouroguanylin folds exclusively to the bioactive A-type isomer (Hidaka et al., 1998). However, the suggestion that proguanylin adopts one defined COOH-terminally conformation containing the topology of guanylin A-isomer remains to be proven by a complete determination of the structure of proguanylin, which is currently under investigation in our laboratory.

## Materials and methods

### *Preparation of guanylins*

Native proguanylin was isolated by HPLC from a large-scale peptide sublibrary, which was prepared from hemofiltrate collected from patients with chronic renal failure. A detailed description of the isolation of proguanylin was recently published (Schrader et al., 1997). The native proguanylin was purified by HPLC on a Vydac C18 column (The Separations Group, Hesperia, California; $10 \times 250$ mm, 5 $\mu$m, 300 Å; solvent A, 0.1% TFA; solvent B, 0.1% TFA in MeCN/$H_2O$ 4:1; gradient, 30–80% solvent B in 50 min; flow rate, 3.5 mL/min; ultraviolet (UV) detection at 230 nm). Purity and identity were checked by analytical HPLC, capillary zone electrophoresis, amino acid analysis, and Edman degradation. Electrospray mass spectrometry revealed a relative molecular weight of 10,336 Da for proguanylin (molecular weight calculated: 10,337 Da). Guanylin-(101–115) was synthesized using Fmoc chemistry on a preloaded Fmoc-Cys(Acm)-TentaGel S-Trt resin (Rapp Polymere, Tuebingen, Germany) followed by selective introduction of disulfides utilizing air and iodine oxidation (Klodt et al., 1997).

### *Analytical HPLC and mass spectrometry*

Analytical HPLC was carried out on a Nucleosil C18 PPN column (Macherey & Nagel, Dueren, Germany, 5 $\mu$m, 100 Å, $2 \times 250$ mm; solvent A, 0.1% TFA; solvent B, 0.1% TFA in MeCN/$H_2O$ 4:1; gradient, 20–80% solvent B in 30 min; flow rate, 0.2 mL/min; UV detection at 215 nm). Electrospray mass spectrometrical analysis (ESMS) was carried out on a Sciex API III (Perkin-Elmer, Langen, Germany).

### *Size-exclusion chromatography*

Native proguanylin was loaded at 1 $\mu$M, 0.01 mM, 0.1 mM, and 1 mM on a Superdex 75 PC 3.2/30 column (Pharmacia, Freiburg, Germany) at a flow rate of 40 $\mu$L/min. The column was equilibrated either in 50 $\mu$M Tris-HCl buffer (pH 7.4, 0.2 M NaCl) or 0.1 M phosphate buffer (pH 5, 0.1 M NaCl) and was calibrated with bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa).

### *CD spectroscopy*

CD spectra were recorded at 19.8 °C in a 0.1-cm cell from 250 to 190 nm at 20 nm/min on a Jasco J 600A CD spectropolarimeter with a concentration of 7 $\mu$M native proguanylin in $H_2O$. Spectra were measured 10 times and averaged for proguanylin and the reference sample ($H_2O$), respectively.

### *NMR spectroscopy*

The proguanylin concentration used for NMR spectroscopy was 2.4 mM, pH 3.3, in $H_2O/D_2O$ (9:1, v/v, 500 $\mu$L). All NMR spectra were carried out on a Bruker DRX600 spectrometer at 284 and

293 K using standard methods (Wüthrich, 1986; Ernst, 1992). Water suppression was performed by excitation sculpting (Hwang & Shaka, 1995) for NOESY and z-filtered TOCSY spectra (Cavannagh & Rance, 1992), and by coherence selection with magic angle gradient for the COSY spectra (van Zijl et al., 1995). The spectra were recorded with a spectral width of 6,613.8 Hz in both dimensions and 4,096 × 512 or 700 data points, respectively, in the time domain. Quadrature detection was used in both dimensions with the time-proportional phase incrementation technique in $\omega_1$. Spectra were multiplied with a squared sinebell function phase-shifted by $\pi/4$ for the NOESY (mixing time 200 ms), the z-filtered TOCSY (mixing time 80 ms), and the DQF-COSY spectra prior to Fourier transformation. Application of zero filling resulted in 4 × 1 K data points in the frequency domain. Sixth-order baseline and phase correction were used. Data were evaluated on X-Window workstations with the NDee program package (SpinUp, Luenen, Germany).

### *Acetic acid cleavage of proguanylin*

Samples of native proguanylin (50 μg) were incubated in 100 μL 1 M HOAc at 95 °C for 5, 10, 20, 40, and 60 min. The reactions were stopped by freezing with liquid nitrogen, and the samples were lyophylized. The remaining material of each sample was dissolved in 50 μL $H_2O$ and subjected to cGMP bioassay, ESMS, and HPLC. For chromatography, a constant gradient from 20–80% solvent B in 30 min was used at a column temperature of 11 °C.

### *Reduction and refolding of guanylin and proguanylin*

Fully reduced samples of proguanylin, guanylin-(53–115) and guanylin-(101–115) were obtained by treatment of the disulfide-bonded precursor peptides with an excess of 500 equivalent DTT in 100 mM $NH_4HCO_3$ (pH 8.3) at 60 °C for 2 to 3 h. The reduced peptides were subsequently purified by HPLC (Vydac, C18, 10 × 250 mm, 5 μm, 300 Å, 5 μm; flow rate, 3.5 mL/min; UV detection at 215 nm). For the disulfide-coupled folding of guanylin-(101–115), 250 μg of the reduced peptide were dissolved in 5 mL of 100 mM $NH_4HCO_3$ buffer containing 2 mM EDTA at pH 8.3, resulting in a final peptide concentration of 50 μg/mL. Oxidation products were typically HPLC-analyzed after a reaction time of 18 h. Reduced proguanylin was folded using a 100 mM $NH_4HCO_3$ buffer at pH 8.3 containing 2 mM EDTA at a peptide concentration of 50 μg/mL in the presence or absence of GnHCl for 18 h. The resulting mixtures were analyzed by HPLC, lyophylized, and the COOH-terminal fragment of 15 residues, corresponding to guanylin-(101–115), was cleaved off in 50 μL 1 M HOAc as described above and analyzed by HPLC. Results and conditions of representative reactions are summarized in Table 1.

### *Generation of guanylin-(53–115) by endoprotease Lys-C cleavage of proguanylin*

To generate guanylin-(53–115), 1.5 mg of native proguanylin were incubated with 15 μg of endoprotease Lys-C (Boehringer-Mannheim, Mannheim, Germany) in 600 μL 0.1 M Tris-HCl (pH 8) at an ambient temperature. After 30 min, the COOH-terminal segment of proguanylin containing 63 amino acids was isolated by HPLC (Vydac, C18, 10 × 250 mm, 300 Å, 5 μm; solvent A, 0.1% TFA; solvent B, 0.1% TFA in MeCN/$H_2O$) using a gradient of 20–80% solvent B for 30 min. Product purity was checked by C18 HPLC and ESMS.

### *Chymotrypsin cleavage of guanylin disulfide isomers*

The guanylin-(101–115) isomers (Fig. 4A, peaks 2 and 3) obtained from the oxidation of the corresponding linear peptide were cleaved with 2.5 μg chymotrypsin in 50 μL of Tris-HCl buffer (0.1 M, pH 7.8) containing 10 mM $CaCl_2$ for 20 h and analyzed by C18 HPLC. To determine the disulfide pattern, the resulting peaks were separated and their molecular weights determined by ESMS.

### *cGMP assay*

The production of intracellular cGMP upon stimulation of cultured human colon carcinoma T84 cells was evaluated after incubation of these cells with pure peptides in a concentration range between $10^{-8}$ and $10^{-6}$ M and with HOAc-treated samples of proguanylin of a total concentration of $10^{-7}$ M for 45 min in the presence of isobutylmethylxanthine (IBMX, 1 mM) (Kuhn et al., 1993). The reaction was stopped by removal of the medium and addition of ice-cold ethanol. Subsequently, the cGMP content was measured using a specific radioimmunoassay (Kaever & Resch, 1985).

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