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Processing and Characterization of Human Proguanylin Expressed in *Escherichia coli**

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Guanylin is a 15-amino acid peptide hormone that was originally isolated from the jejunum of the rat small intestine and shown to be an endogenous activator of the intestinal heat-stable enterotoxin receptor-guanylyl cyclase. Guanylin is synthesized as a 115-amino acid prohormone, proguanylin, which is processed at a site yet to be determined, into a C-terminal bioactive fragment(s). In order to examine the processing of proguanylin *in vitro*, we have generated large quantities of the properly folded prohormone by constructing an expression vector that directs its secretion into the periplasmic space of *Escherichia coli*. The bacterially expressed human proguanylin was then processed to smaller C-terminal fragments by protease digestion. Digestion with trypsin or lysine-C generated C-terminal peptides of different length, which have been purified and characterized. Guanylin-22 and guanylin-32 have binding affinities and biological activities similar to guanylin-15, while guanylin-63 and the entire proguanylin have only minimal bioactivity. Circular dichroism spectroscopy reveals that proguanylin is a stably folded protein containing mostly β-sheet and β-turn structure.

Heat-stable enterotoxins (STa)[1] are small peptides of 18 or 19 amino acids that are secreted into the intestine by enterotoxigenic strains of *Escherichia coli* (Chan and Giannella, 1981). The 13 amino acids necessary for the toxic activity of the peptide include 6 cysteines that form three disulfide bridges (Yoshimura *et al.*, 1985). STa exerts its actions by binding and activating a member of the receptor-guanylyl cyclase family that is preferentially expressed in the intestine and is called the heat-stable enterotoxin receptor (STaR) (Schulz *et al.*, 1990; de Sauvage *et al.*, 1991). Binding of STa to STaR induces a dramatic increase of the cyclic guanine monophosphate (cGMP) content of the cell (Field *et al.*, 1978; Hughes *et al.*, 1978). The cGMP increase inhibits salt absorption and stimulates chloride secretion into the gut. The imbalance of ions is accompanied by a massive accumulation of water in the gut that gives rise to diarrhea and dehydration characteristic of enterotoxin activity.

A search for an endogenous activator of STaR resulted in the isolation from the rat small intestine of a 15-amino acid peptide called guanylin (Currie *et al.*, 1992). Guanylin shares structural similarities to STa, including 4 cysteine residues. Guanylin is also able to compete for ^{125}I-STa binding and stimulate cGMP production in cell lines expressing the STaR (Currie *et al.*, 1992; de Sauvage *et al.*, 1992a). Recent cDNA cloning experiments showed that guanylin is synthesized as a 10-kDa precursor called proguanylin (de Sauvage *et al.*, 1992a; Wiegand *et al.*, 1992; Schulz *et al.*, 1992) (Fig. 4). Upon trypsin or acid treatment of proguanylin, a C-terminal fragment is released that binds to and activates the STaR (de Sauvage *et al.*, 1992a). While the site(s) of physiological processing is unknown, there are several basic residues in proguanylin that could potentially fill this role.

Functional studies aimed at determining the size of a guanylin molecule with maximal activity require large quantities of the prohormone. Correct disulfide pairing of the 4 cysteines in the C-terminal fragment of proguanylin may require the membrane translocation of the entire molecule through the endoplasmic reticulum. Therefore, we have secreted the entire prohormone into the periplasm of *E. coli*. The soluble proguanylin has been purified and processed enzymatically and chemically to yield fully active mature hormone. Additionally, the high level expression has enabled us to begin structural characterization of both proguanylin and guanylin.

MATERIALS AND METHODS

Vector Construction and Expression—The cDNA encoding the 99-amino acid human proguanylin (de Sauvage *et al.*, 1992a) was subcloned into the expression vector pAK19 (Carter *et al.*, 1992) following introduction by polymerase chain reaction of an *Mlu*I site at its 5′ end and an *Sph*I site at its 3′ end. pAK19 contains the *E. coli* alkaline phosphatase promoter, the heat-stable enterotoxin II (STII) Shine-Dalgarno sequence, and sequence that encodes STII signal peptide. The 5′ *Mlu*I site places the proguanylin cDNA insert, minus its signal peptide, in frame with amino acid 23 of the STII signal peptide (Carter *et al.*, 1992). The resulting expression construct, named pHPG2, was transformed into competent *E. coli* strain W3110 ton A and grown overnight in Luria broth (LB) media supplemented with 50 μg/ml carbenicillin. At this point, induction media (final concentrations: 16.4 mM K_2HPO_4, 9.2 mM NaH_2PO_4, 47.4 mM $(NH_4)_2SO_4$, 3.7 mM sodium citrate, 22 mM KCl, 7.7 mM $MgSO_4$, 11 g/liter casein hydrolysate, and 11 g/liter yeast extract) was inoculated with the saturated culture at a ratio of 1 volume of saturated culture to 100 volumes of induction media and grown at 37 °C for up to 32 h. Expression was checked by osmotically shocking the cell pellet contained in 1 ml of a 1 O.D. (A_{600}) *E. coli* culture with 0.1 volume of ice-cold H_2O, and analyzing protein content of the supernatant by 18% SDS-polyacrylamide gel electrophoresis. Fermentations were carried out in 10-liter Biolaffitte fermentors.

Proguanylin Purification—The media from the induced culture contained substantial amounts of the expressed proguanylin, as did the periplasmic space of the induced *E. coli*. To purify the proguanylin in the periplasmic fraction, 100 g of cell paste was osmotically shocked by the addition of 500 ml of ice-cold distilled H_2O containing 10 mM EDTA (Carter *et al.*, 1992). The suspension was stirred at 4 °C for 1 h, centrifuged at 5000 × *g* for 30 min, and the supernatant from the centrifugation clarified by filtration through a 0.2-μm filter. The clarified su-

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[1] The abbreviations used are: STa, heat-stable enterotoxin; STaR, heat-stable enterotoxin receptor; HPLC, high performance liquid chromatography; RP-HPLC, reverse-phase HPLC; PAGE, polyacrylamide gel electrophoresis; LC-MS, liquid chromatrography-mass spectrometry; HIC, hydrophobic interaction chromatography.

pernatant was acidified with 0.1% trifluoracetic acid, brought to 15% acetonitrile, and allowed to sit overnight at 4 °C. The turbid solution was centrifuged for 30 min at 5000 × *g*, the supernatant filtered through a 0.2-μm filter, and pumped onto a 105 Amicon C8 200 column (0.9 × 50 cm) at a flow rate of 9 ml/min. The column was washed with 15% acetonitrile, 0.1% trifluoracetic acid until the absorbance base line (measured at 214 nm) reached zero. The mobile phase was increased to 33% acetonitrile, and a gradient was run from 33 to 48% acetronitrile over 60 min. Fractions were collected at 1-min intervals, and small aliquots from each fraction were analyzed by reverse-phase (RP)-HPLC column (105 Amicon C8 4.6 × 250 mm) using an isocratic mobile phase of 40% acetonitrile, 0.1% trifluoracetic acid. Fractions were collected and lyophilized, and the composition was verified by mass spectrometry and/or peptide sequencing. Fractions from the RP-HPLC that contained the correct peptide were pooled and further purified by hydrophobic interaction chromatography using a phenyl-Sepharose 10/10 Pharmacia FPLC column. Buffer A was 50 mM Na_2PO_4, 2 M $(NH_4)_2SO_4$, pH = 7.8; buffer B was 50 mM Na_2PO_4, pH = 7.8. A gradient of 75% A to 25% B at a flow rate of 1 ml/min eluted proguanylin at approximately 50% A. The pure proguanylin peak was collected, desalted by RP-HPLC, and lyophilized.

To purify the proguanylin from the induction media, the exact same procedure was followed as for the purification of the periplasmic fraction, except that no osmotic shock step was necessary and the media were acidified with 0.1% trifluoracetic acid and brought to 15% acetonitrile as the first step. Subsequent steps were the same as described in the preceding paragraph for the periplasmic fraction.

Trypsin Digestion and Purification—2.5 mg of proguanylin in 0.1 M Tris-HCl (pH 8.0) was incubated overnight at 37 °C with 2% trypsin (w/w) (Sigma) in a total reaction volume of 0.5 ml. The total digest was injected on a Sychron C4 (2 × 160 mm) column. Peptides were separated using a linear gradient of 0.1% trifluoracetic acid to 70% acetonitrile in 30 min at a flow rate of 0.2 ml/min. Peaks were detected by monitoring absorbance at 214 and 280 nm.

Lysine-C Digestion and Purification—For the complete digest, proguanylin (2 nmol) was digested with 5% (w/w) of Lys-C (Worthington) in 100 μl of 0.1 M Tris-HCl, (pH 8.0), at 37 °C for 17 h. For the partial digest, proguanylin (2 nmol) was digested with 5% (w/w) of Lys-C in 100 μl of 0.1 M Tris-HCl, (pH 8.0), at 23 °C for 5 min. The Lys-C digests was injected on a Sychron C4 (2 × 160 mm) column. Peptides were separated using a linear gradient of 0.1% trifluoracetic acid to 70% acetonitrile in 30 min at a flow rate of 0.2 ml/min. Peaks were detected by monitoring absorbance at 214 and 280 nm.

Mass Spectrometry and Peptide Sequencing—Automated Edman degradation was performed on a model 477 Applied Biosystems Sequencer equipped with a 120A phenylthiohydantoin-derivative analyzer. Electrospray spectra were obtained on a Sciex APII triple quadrapole mass spectrometer. Spectra were obtained by direct infusion at a flow rate of 1.5 ml/min.

Binding and cGMP Assay—^{125}I-STa binding assays and guanylylcyclase stimulation assays were performed using 293-STaR cells as described (de Sauvage *et al.*, 1991).

Circular Dichroism—Circular dichroism (CD) spectra were obtained for proguanylin at 0.1 mg/ml in 10 mM $NaCH_3CO_2$ (pH 6.0). Spectra were measured from 250 to 190 nm (far UV) in an Aviv/Cary 60-DS spectropolarimeter with the use of a 0.01-cm cell. Five scans were made over the wavelength range at a time constant of 0.3 s, and the values were averaged. Spectra were corrected by subtraction of CD spectra obtained with buffer alone in the cell. Data are reported as mean residue ellipticity by taking 111 g/mol as the mean residue molecular weight.

RESULTS AND DISCUSSION

In order to generate large quantities of bioactive proguanylin for functional and structural studies, we have expressed in *E. coli* a fusion protein consisting of the bacterial STII signal peptide and human proguanylin. It was hoped that the STII signal peptide would lead to the translocation of the fusion protein through the inner bacterial membrane to the periplasmic space, accompanied by cleavage of the STII signal peptide. There are many examples where such membrane translocation to the net oxidizing environment of the periplasm results in controlled and correct folding of proteins containing multiple disulfide bonds (Carter *et al.*, 1992; Stader and Silhavy, 1990). control of the *E. coli* alkaline phosphatase promoter, which is induced under conditions of phosphate starvation. The media are designed so that phosphate is depleted as the cells achieve a high density, leading to induction of this promoter. The advantage of this system is that induction occurs at a high cell density, and the expression of the protein is gradual enough that there is time for the STII fusion protein to be correctly translocated to the periplasm (Carter *et al.*, 1992). SDS-PAGE analyses of cell paste and supernatant during a 10-liter fermentation are shown in Fig. 1. The bacteria secreted proguanylin from about 12 to 32 h, at which point the induction was terminated. Substantially more proguanylin leaked out through the cell membrane into the supernatant (Fig. 1*A*) than remained in the cells (Fig. 1*B*). From a 10-liter fermentation, approximately 3.2 g of proguanylin were found in the 8 liters of supernatant, and 0.5 g of proguanylin in the 1 kg of cell paste.

The proguanylin was purified from both the induction media supernatant and the periplasmic fraction of the cell paste. The periplasmic fraction is obtained by an osmotic (hypotonic) lysis of the *E. coli*. For both the induction media supernatant and the periplasmic fraction of the cell paste, a substantial first-step purification was achieved by an acid precipitation followed by a 15% acetonitrile precipitation. The proguanylin remains in the supernatant and is approximately 50% pure, as judged by HPLC. LC-MS analysis gave a molecular mass of 10,707 Da for the proguanylin. This is the mass expected if the STII signal is properly removed. The supernatant of the acid-precipitated osmotic shock fraction was analyzed by analytical RP-HPLC (Fig.

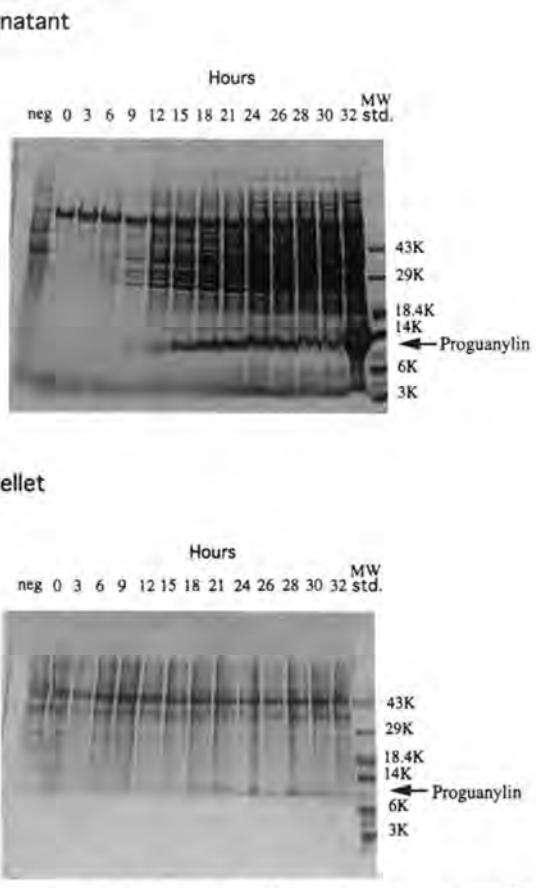

FIG. 1. **Time course of induction and secretion of proguanylin by *E. coli*.** *E. coli* were transformed with plasmid pHPG2 and grown in a 10-liter fermentor as described under "Materials and Methods." Aliquots of media supernatant (*A*) or cells (*B*) were removed and analyzed by SDS-PAGE (18%) at indicated intervals. Gel was stained with Coo-

2A) and the peak eluting at 40% acetonitrile was confirmed by LC-MS to be proguanylin. For preparative purification, 500 ml of the acid-precipitated osmotic shock supernatant (Fig. 2B) or 1-liter batches of the acid-precipitated induction media (not shown) were loaded onto a preparative RP-HPLC and eluted with a gradient of 30–50% acetonitrile, 0.1% trifluoracetic acid over 80 min. The proguanylin-containing fractions were determined by analytical RP-HPLC of fractions from the preparative column. An analytical RP-HPLC profile of the final proguanylin pool is shown in Fig. 2C.

Although the proguanylin appeared to be >99% pure by RP-HPLC, isocratic elution analysis of the material indicated a slight nongaussian shape to the peak. The only chromatographic technique that was successful in separating contaminants from the proguanylin peak was hydrophobic interaction chromatography (HIC). The major eluting peak from the HIC column was proguanylin, but a number of contaminants were separated (Fig. 3).

Proguanylin is inactive with respect to binding or activating

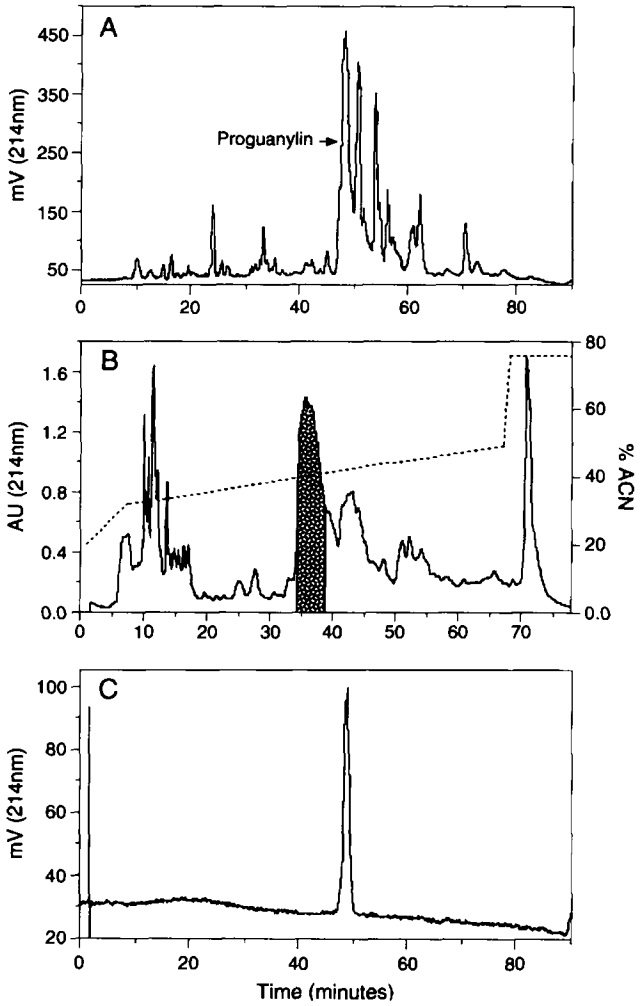

FIG. 2. **Reverse-phase high performance liquid chromatography analysis and purification of proguanylin.** The samples were chromatographed using an acetonitrile, 0.1% trifluoracetic acid gradient (33–48%) and a C8 column as described under "Materials and Methods." *A*, analytical RP-HPLC of osmotic shock supernatant after acid precipitation. Column is a 105 Amicon C8 4.6 × 250 mm at 1 ml/min. *B*, preparative RP-HPLC of 500 ml of osmotic shock supernatant after acid precipitation. Proguanylin peak is highlighted. Mobile-phase gradient is indicated by the *dashed line* and *scale* at *right*. Column is a 105 Amicon C8 200 column (0.9 × 50 cm) at a 9 ml/min flow rate. *C*, analytical RP-HPLC of purified proguanylin using a 105 Amicon C8 column (4.6 × 250 mm). Absorbance for the indicated chromatograms was monitored at 214 nm.

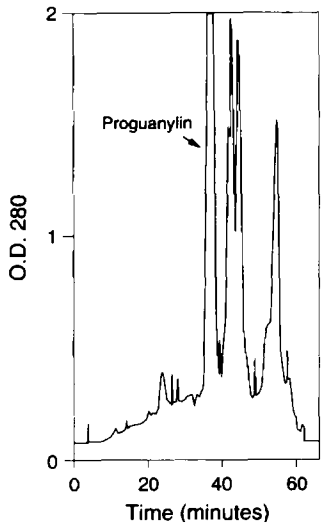

FIG. 3. **Hydrophobic interaction chromatography of RP-HPLC-purified proguanylin.** Proguanylin peak is indicated by the *arrow*. Buffer A was 50 mM Na_2PO_4, 2 M $(NH_4)_2SO_4$ (pH 7.8); buffer B was 50 mM Na_2PO_4 (pH 7.8). A gradient of 75% A to 25% B at a flow rate of 1 ml/min eluted proguanylin at approximately 50% A. Absorbance was monitored at 214 nm.

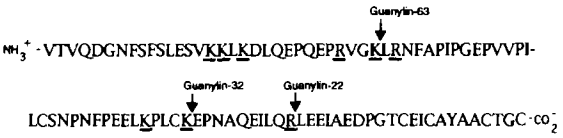

FIG. 4. **Amino acid sequence of human proguanylin showing 3 potential proteolytic processing sites.** Guanylin-63, -32, and -22 are the C-terminal peptides that would result from cleavage at residues 52, 83, and 93, respectively. Alternative basic amino acid residues that are possible processing sites are *underlined*.

STaR (de Sauvage *et al.*, 1992a; Schulz *et al.*, 1992). Prohormone processing enzymes are known to cleave primarily at dibasic residues, such as arginine and lysine (Barr, 1991). In order to study the biological activity of fragments of the material purified from *E. coli*, proguanylin was completely or partially digested with the proteases trypsin or lysine-C, respectively. While these are not the physiological processing enzymes, they are convenient tools to generate smaller fragments since they cleave at similar residues as most known processing enzymes. The cleavage sites from which fragments were generated for our studies are indicated in Fig. 4. Complete digestion of proguanylin with trypsin is expected to cleave at Arg-93 and yield a 22-amino acid C-terminal fragment. Complete digestion with Lys-C is expected to yield a 32-amino acid C-terminal fragment resulting from cleavage at Lys-83. Partial digestion with lysine-C should also generate a 63-amino acid fragment resulting from cleavage at Lys-52 of proguanylin.

The trypsin digestion was injected onto an RP-HPLC column (Fig. 5A), and the peaks were analyzed by mass spectrometry and amino acid analysis. The peak corresponding to the 22-amino acid fragment was re-injected onto the RP-HPLC and found to be homogeneous (Fig. 5B). Mass spectrometry analysis indicates a molecular mass of 2258 Da as predicted for the completely oxidized peptide.

The Lys-C digests were also analyzed by RP-HPLC (Fig. 6). After exhaustive digestion (17 h at 37 °C), a 32 amino acid fragment corresponding to residue Lys-83 of proguanylin was purified (Fig. 6A). After partial digestion (5 min at 23 °C), we could isolate a 63-amino acid C-terminal fragment resulting

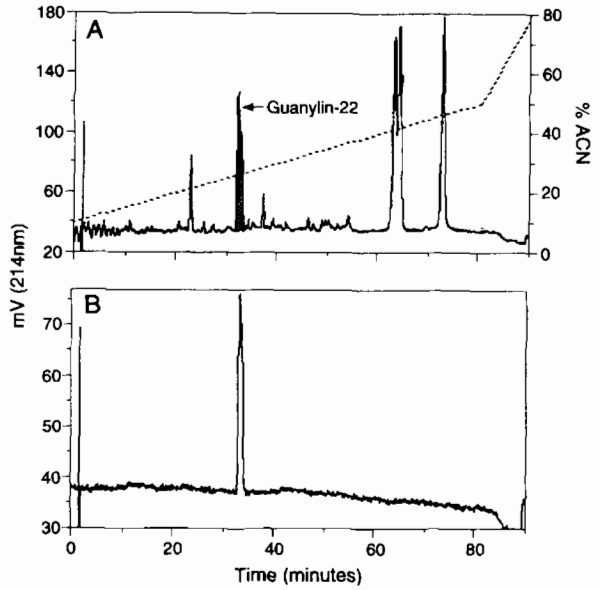

FIG. 5. **Trypsin digest of purified proguanylin.** *A*, RP-HPLC of total trypsin digest. *B*, RP-HPLC of purified C-terminal 22 residue fragment. Peptides were separated using a linear gradient of 0.1% trifluoracetic acid to 70% acetonitrile in 30 min at a flow rate of 0.2 ml/min on a Synchron C4 column. Other details of the chromatography are described under "Materials and Methods."

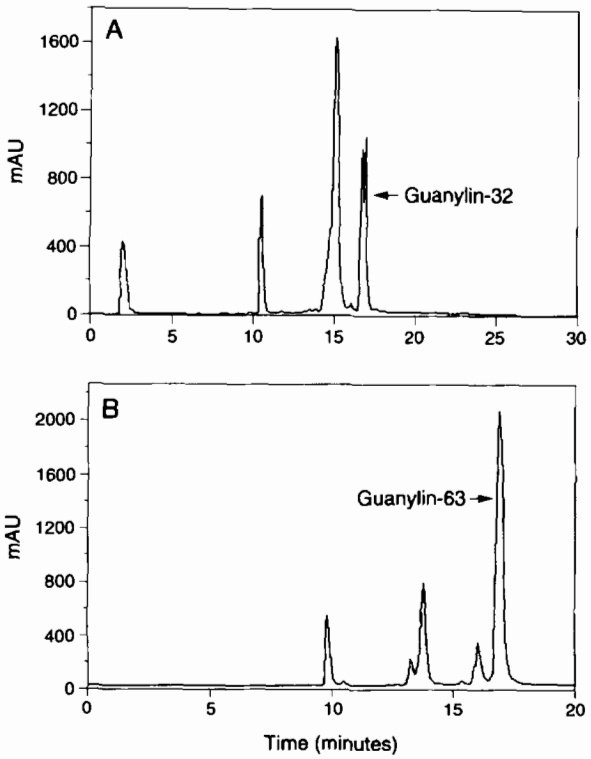

FIG. 6. **Lysine-C digest of purified proguanylin.** *A*, RP-HPLC of complete digest yielding a 32-amino acid fragment. *B*, RP-HPLC of partial digest yielding a 63-amino acid fragment. Details of the chromatography are identical to those described for guanylin-22 and are described under "Materials and Methods."

peptides were found to have the expected molecular mass.

The ability of the purified 22-amino acid tryptic peptide (guanylin-22) to compete with ^{125}I-STa for binding to the STaR was measured using 293-STaR cells (de Sauvage *et al.*, 1992b). As was found for guanylin-22 isolated from mammalian cells (de Sauvage *et al.*, 1992a), 100 nM guanylin-22 was needed to displace 50% of the bound ^{125}I-STa (Fig. 7*A*). This result is also

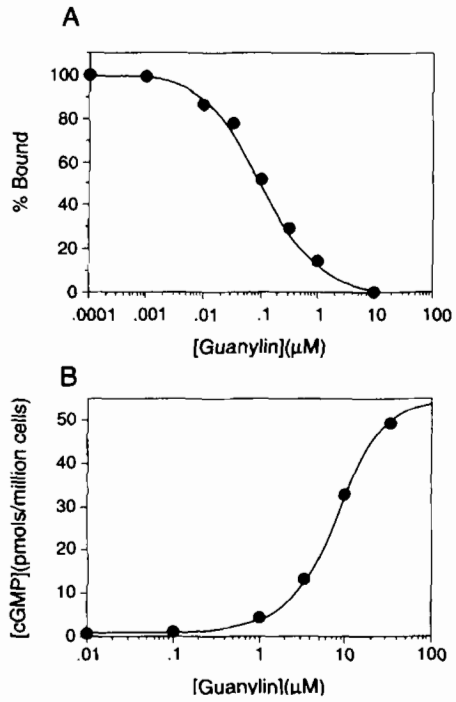

FIG. 7. **Functional analysis of guanylin-22.** *A*, displacement of ^{125}I-STa by guanylin-22 from 293-STaR cells expressing STaR. Various concentrations of guanylin-22 were incubated with 25 pM ^{125}I-STa and 2 × 10^5 293-STaR cells. Nonspecific binding was determined in the presence of a saturating concentration of guanylin-22 (5 μM). The percent specific binding is plotted *versus* the concentration of STa. Each *point* represents the mean of duplicate determinations. *B*, cGMP production stimulated by guanylin-22. 293-STaR cells were incubated with various concentrations of guanylin-22 for 30 min. Intracellular cGMP accumulation was then determined as described (de Sauvage *et al.*, 1991). Each *point* represents the mean of duplicate samples assayed in duplicate.

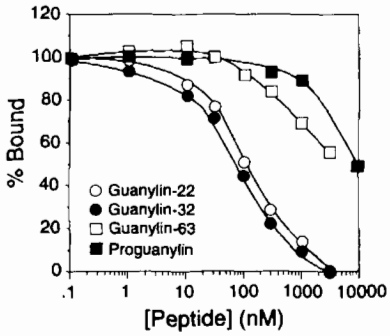

FIG. 8. **Displacement of ^{125}I-STa from 293-STaR cells by purified guanylin-22, -32, and -63.** Experiments were performed as in Fig. 7. Individual curves are identified by the *key* in the *inset*.

similar to the affinity measured for the 15-amino acid peptide generated by acid cleavage of proguanylin (Currie *et al.*, 1992). The ability of guanylin-22 to stimulate guanylyl cyclase activity was studied by incubating 293-STaR cells for 10 min in the presence of increasing concentrations of guanylin-22 and determining the intracellular concentration of cGMP (Fig. 7*B*). An 80-fold stimulation was observed at 30 μM guanylin-22, where 49 pmol of cGMP/10^6 cells were measured. This level of stimulation is similar to the 150-fold stimulation measured with STa at 100 nM (92 pmol of cGMP/10^6 cells) (de Sauvage *et al.*, 1991).

The discrepancy between the concentration of guanylin required for half-maximal cGMP elevation and the binding K_D is observed for all of the identified guanylyl cyclase receptors and is not yet understood (Schulz *et al.*, 1989; de Sauvage *et al.*,

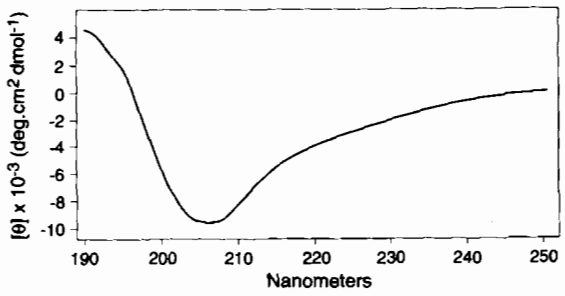

FIG. 9. **CD spectra of 0.1 mg/ml proguanylin.** Spectra were obtained at pH 6.0 and 23 °C in 10 mM $NaCH_3CO_2$. Recording of spectra is described under "Materials and Methods."

1991; Schulz *et al.*, 1990).

The two lysine-C-generated peptide fragments of proguanylin were compared to proguanylin and guanylin-22 in a ^{125}I-STa competition binding assay (Fig. 8). The ability of guanylin-32 to compete with ^{125}I-STa for binding to 293-STaR cells is comparable to guanylin-22 (IC_{50} = 100 nM), whereas guanylin-63 and intact proguanylin are far less effective (IC_{50} > 5 μM). These results suggest that processing of proguanylin at sites located upstream from Lys-52 is not likely to generate an active hormone. Therefore, the dibasic Lys-Lys site that is conserved between human and mouse at position 37–38 probably does not represent the physiological processing site of proguanylin.

Very little is known about the structure of prohormones. We were interested to see whether the intact prohormone, proguanylin, folds into a molecule with stable and definable structure in aqueous solution. CD spectroscopy will yield information concerning whether the proguanylin is folded into definable secondary structural elements, or exists as an unstructured random coil. CD spectra on the intact *E. coli* expressed proguanylin indicates a folded molecule, which has secondary structure (Fig. 9). The single minimum at 208 nm indicates strong β-sheet and β-turn structure, with negligible α-helix content. The minimum of the spectrum (208 nm) is shifted somewhat from classical β-sheet (Yoshimura *et al.*, 1985) CD minimum (218 nm), which is a reflection of short sheets connected by loops of random structure. Proguanylin is extremely proline-rich, with 5–7 amino acid residues separating the prolines, so a general topological model might be one in which there are numerous short β-sheets connected by reverse turns comprising prolines. Complete three-dimensional structure determination of the prohormone by NMR is under way, and should yield novel structural information on the role of the unprocessed, larger precursors of small peptide hormones.

REFERENCES

Barr, P. J. (1991) *Cell* **66,** 1–3
Carter, P., Kelly, R. F., Rodrigues, M. L., Snedecor, B., Covarubias, M., Velligan, M. D., Wong, W. L. T., Rowland, A. M., Kotts, C. E., Carver, M. E., Yang, M., Bourell, J. H., Shepard, H. M., and Henner, D. (1992) *BioTechnology* **10,** 163–167
Chan, S. K., and Giannella, R. A. (1981) *J. Biol. Chem.* **256,** 7744–7746
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. U. S. A.* **89,** 947–951
de Sauvage, F. J., Camerato, T. R., and Goeddel, D. V. (1991) *J. Biol. Chem.* **266,** 17912–17918
de Sauvage, F. J., Keshav, S., Kuang, W.-J., Gillett, N., Henzel, W., and Goeddel, D. V. (1992a) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 9089–9093
de Sauvage, F. J., Horuk, R., Bennett, G., Quan, C., Burnier, J. P., and Goeddel, D. V. (1992b) *J. Biol. Chem.* **267,** 6479–6482
Field, M., Graf, L. H., Laird, W. J., and Smith, P. L. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75,** 2800–2804
Hughes, J. M., Murad, F., Chang, B., and Guerrant, R. L. (1978) *Nature* **271,** 755–756
Saxena, V. P., and Wetlaufer, D. B. (1971) *Proc. Natl. Acad. Sci. U. S. A.* **68,** 969–972
Schulz, S., Singh, S., Bellet, R. A., Singh, G., Tubb, D. J., Chin, H., and Garbers, D. L. (1989) *Cell* **58,** 1155–1162
Schulz, S., Green, C. K., Yuen, P. S. T., and Garbers, D. L. (1990) *Cell* **63,** 941–948
Schulz, S., Chrisman, T. D., and Garbers, D. L. (1992) *J. Biol. Chem.* **267,** 16019–16021
Stader, J. A., and Silhavy, T. J. (1990) *Methods Enzymol.* **185,** 166–187
Wiegand, R. C., Kato, J., Currie, M. G., (1992) *Biochem. Biophys. Res. Commun.* **185,** 812–817
Yoshimura, S., Ikamura, H., Watanabe, H., Aimoto, S., Shimonishi, Y., Hara, S., Takeda, T., Miwatani, T., and Takeda, Y. (1985) *FEBS Lett.* **181,** 138–142