Design and characterization of a soluble fragment of the extracellular ligand-binding domain of the peptide hormone receptor guanylyl cyclase-C

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The intestinal guanylyl cyclase-C (GC-C) was originally identified as an Escherichia coli heat-stable enterotoxin (STa) receptor. STa stimulates GC-C to much higher activity than the endogenous ligands guanylin and uroguanylin, causing severe diarrhea. To investigate the interactions of the endogenous and bacterial ligands with GC-C, we designed and characterized a soluble and properly folded fragment of the extracellular ligand-binding domain of GC-C. The membrane-bound guanylyl cyclases exhibit a single transmembrane spanning helix and a globally folded extracellular ligand-binding domain that comprises about 410 of 1050 residues. Based on the crystal structure of the dimerized-binding domain of the guanylyl cyclase-coupled atrial natriuretic peptide receptor and a secondary structure-guided sequence alignment, we generated a model of the extracellular domain of GC-C comprised of two subdomains. Mapping of mutational and cross-link data onto this structural model restricts the ligand-binding region to the membrane proximal subdomain. We thus designed minIGC-C, a 197 amino acid fragment that mimics the ligand-binding membrane proximal subdomain. Cloning, expression and spectroscopic studies reveal minIGC-C to be a soluble and properly folded protein with a distinct secondary and tertiary structure. MinIGC-C binds STa with nanomolar affinity.

Keywords: guanylate cyclase-C/heat-stable enterotoxin STa/membrane protein/peptide hormone receptor/uroguanylin

Introduction

Infections with enterotoxigenic strains of Escherichia coli are not only the cause of traveler’s diarrhea, but are also a major cause of infant mortality in many developing countries (Levine et al., 1977; Field and Semrad, 1993; Farthing, 1994; Orndorff et al., 1996; WHO Fact Sheets, 1996; Qadri et al., 2005). These bacteria produce peptidogenic enterotoxins such as heat-stable enterotoxins (STa) that bind to a receptor found in the microvillus membranes of the intestine, stimulating uncontrolled fluid secretion by receptor hyperactivation (Thompson, 1987; Qadri et al., 2005). The specific search for the receptor of heat-stable enterotoxins led to the discovery and identification of the intestinal guanylyl cyclase-C (GC-C) (Schulz et al., 1990). Activation of GC-C in the intestinal mucosa stimulates transmucosal Cl− and HCO3− secretion via cGMP as a second messenger through the opening of apical CFTR (cystic fibrosis transmembrane conductance regulator) Cl− channels, and also causes inhibition of Na+ absorption (Gianella, 1995; Vaandrager, 2002). These effects are accompanied by an enhanced secretion of fluid into the intestinal lumen, providing an explanation for the severe diarrhea caused by bacterial STas, which stimulate GC-C to much higher activity than the endogenous ligands guanylin and uroguanylin (Currie et al., 1992; Carpick and Gariepy, 1993; Kloidt et al., 1997; Forte, 1999; Qadri et al., 2005).

In order to study the interactions of the ligands guanylin, uroguanylin and STa with GC-C by spectroscopic methods and to provide a model for drug design and drug-binding studies, a soluble and properly folded fragment of the extracellular ligand-binding domain of GC-C (GC-C_ECD) with the ability to bind these ligands is required. Rat, pig and human GC-C consist of 1050 amino acid residues of which the NH2-terminal globularly folded extracellular ligand-binding domain comprises 407 residues, followed by a single transmembrane spanning helix of 24 residues. A total of 619 COOH-terminal residues form the intracellular kinase homology and guanylyl cyclase catalytic domains (Schulz et al., 1990; de Sauvage et al., 1991; Wada et al., 1994). The isolated, membrane-free GC-C_ECD possesses similar binding affinity to heat-stable enterotoxin as the complete biologically active GC-C receptor (KD[GC-C_ECD] = 0.4 nM; KD[GC-C] = 0.6 nM; Hasegawa et al. (1999a)). Using cross-linking as well as site-directed mutagenesis studies, the ligand-binding site on the GC-C_ECD has been restricted to a sequence of 10 amino acid residues positioned near the predicted transmembrane portion of GC-C (Wada et al., 1996; Hasegawa et al., 1999b).

Information about detailed structural properties of the family of membrane-bound guanylyl cyclases is limited and only the crystal structure of the dimerized extracellular domain of the atrial natriuretic peptide receptor [ANP receptor, guanylyl cyclase-A (GC-A)] in the absence and presence of ligand is available (van den Akker et al., 2000; Ogawa et al., 2004). Also, the three-dimensional structure of the natriuretic peptide clearance receptor C (NPR-C) in the presence and absence of ligand has been determined (He et al., 2001). This receptor is not a guanylyl cyclase but shows significant sequence homology to the extracellular domain of the ANP receptor. While the intracellular domains throughout the seven members of the family of membrane-bound guanylyl cyclases are quite similar with a sequence identity of ~55%, their extracellular
domains differ notably. The sequence identity between the extracellular domains of GC-C and GC-A is only 19%.

A sequence alignment based on the experimentally determined secondary structure of GC-A (van den Akker et al., 2000) and the predicted secondary structures of GC-C and the remaining guanylyl cyclases, however, reveals that insertions and deletions only occur in loop regions, thus leading to the suggestion of a similar fold for all extracellular domains of membrane-bound guanylyl cyclases (van den Akker, 2001). Based on such a structure-guided sequence alignment and the crystal structure of GC-A, we have created a model structure of the GC-C_ECD. This model structure clearly shows two subdomains with the membrane proximal subdomain containing the ligand-binding region. Based on our model structure of the GC-C_ECD, we have designed, constructed, overexpressed and characterized a soluble and properly folded receptor fragment (miniGC-C, 197 amino acid residues) that is able to bind the ligand StAa(5-17) with nanomolar affinity. These results provide the basis for the investigation of a peptide hormone/receptor system under membrane-free conditions.

Materials and methods

Homology modeling of the extracellular domain of GC-C

For the generation of a structural model of the GC-C_ECD, the programs SWISS-PDBViewer and SWISS-MODEL (Gues and Peitsch, 1997) available at the ExPASy Molecular Biology Server (http://www.expasy.org) were used. Template coordinates were taken from the known crystal structure of the extracellular domain of GC-A (pdb code: 1dp4; van den Akker et al., 2000). The sequence alignment of the extracellular domains of GC-C and GC-A was based on secondary structure prediction and on the results from 3D-PSSM (Kelley et al., 2000), a program that is able to recognize remote protein sequence homologues as well as structural relationships. The experimentally determined disulfide bonds (Hasegawa et al., 2005) were introduced using the program SYBYL (Version 6.5; Tripos Inc., St Louis, MO, USA) followed by energy minimization (100 steps using the Tripos force field of SYBYL). Based on this model structure of the GC-C_ECD, the structure of miniGC-C was modeled using the same programs.

Construction of the expression vector pET-32a-pres-miniGC-C

The cDNA encoding the complete extracellular domain of porcine GC-C (Wada et al., 1994) was used as a template for site-directed mutagenesis by overlap extension (Ho et al., 1989). The forward primer for amplification of the 5‘ end of the miniGC-C gene (primer a) introduces a XbaI and BglII site and a sequence encoding a PreScission proteinase cleavage site in this region (primer a: GGA GGA TCT AGA A GAT CTG GGT CTG GAA GTT CTG TTC CAG GGG CCC TCT CCA GCA AGG AAC ATG AGC TTC CAG AAC ATT TTT CAT ATA GTC). The reverse primer (primer b) contains the loop sequence AlaProGlyAsp (primer b: CAT AGT ATC GCC CGG CGC CAG TGT CAG AAC CAG AAC ATG TTT CAT ATA GTC). For amplification of the 3‘ end of the miniGC-C gene, the forward primer (primer c: ACA CTG GCG CCG GGC GAT ACT ATG TTT CTC CTG TAC ACC TCT GTG) also contains the loop sequence and the reverse primer (primer d) introduces an BamHI site at its 5‘ end (primer d: GGA GGA GGA TCC TTA TTG AGG GCC CGG GCC TGG AAT ATC). Following annealing of complementary regions of the two PCR fragments corresponding to parts of primers b and c, i.e. the loop sequence, and synthesis of complementary strands, the mutated DNA was amplified using primers a and d as forward and reverse primers, respectively. This PCR fragment was inserted into the T7 RNA polymerase-based expression vector pET-32a (Novagen, Madison, WI, USA).

The resulting vector pET-32a-pres-miniGC-C is able to produce the protein miniGC-C NH₂-terminally linked to an E. coli thioredoxin fusion (Trx-tag, Novagen) by a 46-amino-acid linker containing six histidine residues and a PreScission proteinase cleavage site (LEVLFQ squarely) enabling the purification of the fusion protein by one-step metal chelating chromatography followed by proteinase cleavage to remove the Trx-tag.

Expression and purification of miniGC-C

A starter culture of 50 ml of LB containing 100 μg/ml ampicillin and 15 μg/ml kanamycin was inoculated with the E. coli strain AD494(DE3)/pET-32a-pres-miniGC-C and grown overnight at 37°C. The starter culture was used to inoculate 1.5 l of LB medium (containing 100 μg/ml ampicillin, and 15 μg/ml kanamycin) at an OD₆₀₀ of 0.1. Cultures were grown to an OD₆₀₀ of 0.8, and expression of the recombinant protein was induced by addition of IPTG to a final concentration of 1 mM. For protein expression, the temperature was reduced to 25°C. After 20 h, cells were harvested and resuspended in 10 ml of buffer A (100 mM sodium phosphate, pH 8.0, 300 mM NaCl) per gram wet weight cells with 1% N-lauryl sarcosinate, 10% of an EDTA-free proteinase inhibitor pill (Roche Biochemicals, Mannheim, Germany), 0.2 mg/ml lysozyme, 0.2 mg/ml DNaseI and 2 mg/ml benzamidine. The cells were lysed by five 30 s sonication-steps (T = 4°C; Labsonic U sonicator at 150 W intensity) followed by freezing at −80°C for 1 h. After thawing, sonication was repeated, and the extract was clarified by centrifugation for 45 min at 19 000 rpm, at 4°C. The pellet was resuspended in the same volume of buffer A with 0.5 mM PMSF, 0.2 mg/ml lysozyme, 0.2 mg/ml DnaseI, 2 mg/ml benzamidine and 1% (v/v) sodium-N-lauryl-sarcosinate and was then treated again with five 30 s sonication-steps and clarified as above. The supernatants were pooled. After filtration, the soluble extract (50 ml) was loaded onto a Co²⁺-Talon superflo column (15 ml bed volume, Clontech, Palo Alto, CA, USA) that was pre-equilibrated in buffer A. The column was washed with buffer A and buffer A containing 40 mM imidazole, and the absorbed proteins were eluted with buffer A containing 100 mM imidazole. Fractions containing the fusion protein (as verified by SDS-PAGE) were dialyzed against 100 mM sodium phosphate and 300 mM sodium chloride, pH 8.0, to reduce the high imidazole concentrations.

The diazylated protein solution was incubated with PreScission proteinase (Amersham Biosciences Inc., Uppsala, Sweden; according to the manufacturer’s instructions) for 16 h at 4°C, cleaving the fusion protein to miniGC-C and Trx-tag. The sample solution was again loaded onto a Co²⁺-Talon superflo column pre-equilibrated in buffer A. The column was washed with buffer A, and the miniGC-C containing
fractions were pooled and dialyzed against 50 mM sodium phosphate, 100 mM sodium chloride, pH 7.4. Ultrafiltration was performed to concentrate the protein solution to a final concentration of \( \sim 300 \mu \text{M} \) (Amicon Ultra-15, pore size 10 kDa, sample volume 15 ml; Millipore, Amicon Bioseparations, Billerica, MA, USA). After estimating protein concentration using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA; Smith et al., 1985) purified miniGC-C was subjected to analytical RP-HPLC, amino acid sequencing and mass spectrometry, which confirmed the expected mass and NH2-terminal sequence of miniGC-C.

**Analytical HPLC and mass spectrometry**

Analytical HPLC was carried out on a Jupiter C18 column (Phenomenex, 5 \( \mu \text{m} \), 300 \( \times \) 250 mm; solvent A, 0.1% TFA; solvent B, 0.1% TFA in MeCN/H2O 4:1; gradient, 10–70% solvent B in 60 min; flow rate, 0.2 ml/min; UV detection at 215 nm). Electrospray mass spectrometrical analysis was carried out on a Sciapi API III (Perkin Elmer).

**CD-spectroscopy**

Far UV CD spectra were recorded on a Jasco J-810 spectropolarimeter (Jasco Inc., Gross-Umstadt, Germany) at room temperature (i.e. 22°C) with 2 \( \mu \text{M} \) miniGC-C in a 0.1 cm cell. The buffer conditions were 20 mM sodium phosphate and 50 mM sodium chloride, pH 7.4. The spectrum was measured from 260 to 195 nm at 50 nm/2 min. The reference sample contained buffer without protein. Spectra were measured four times and averaged for sample and reference, respectively. The spectrum was smoothed using the JASCO Spectra Analysis Software and the secondary structure content was analyzed using the K2D algorithm (Andræ et al., 1993) available on the DICHROWEB webpage (Whitmore and Wallace, 2004).

**NMR-spectroscopy**

The one-dimensional NMR experiment was performed at 25°C on a Bruker DRX600 NMR spectrometer equipped with an inverse \( ^1\text{H}^{13}\text{C}/^{15}\text{N} \) triple resonance probe with pulsed-field gradient capabilities. The H2O resonance was suppressed using a binominal 3-9-19 watergate sequence (Sklar et al., 1993). \( ^1\text{H} \) chemical shifts were referenced with respect to external DSS. The protein concentration was \( \sim 300 \mu \text{M} \) in 50 mM sodium phosphate and 50 mM sodium chloride (10% D2O, v/v), pH 7.4. The spectrum was recorded with a spectral width of 14 ppm and 16,384 data points. Fourier transformation was performed without zero-filling, and an exponential window function with a line-broadening factor of 1 Hz was applied.

**Fluorescence emission spectroscopy**

Changes of the intrinsic tryptophan fluorescence of miniGC-C on addition of the porcine heat-stable enterotoxin STp-(5-17) were monitored using an F4500 Hitachi spectrofluorimeter. The 1.5 ml mirrored cell (path length 1 cm) was inserted into a thermostated cell holder that was connected to a circulating water bath. To determine the maximum difference of the fluorescence intensity upon complex formation, spectra of 25 nM miniGC-C in the absence and presence of 400 nM STp-(5-17) were recorded from 300 to 360 nm after excitation at 295 nm. The observed fluorescence reading at 328 nm for 25 nM miniGC-C was 113.9 in the absence of STp and 106.0 in the presence of 400 nM STp. For the measurement at 5 nM protein concentration, the slit width was increased in order to detect non-trivial changes in fluorescence intensity. The observed fluorescence reading at 328 nm was 206.0 in the absence and 198.8 in the presence of 80 nM STp. All measurements were carried out in 20 mM sodium phosphate pH 7.4, 50 mM sodium chloride at 25°C. To prevent adsorption of the protein or the peptide to the cell, polyoxyethylene-8-decylether was added to a final concentration of 0.3 mM. Titrations of miniGC-C with STp-(5-17) were observed at 328 nm at miniGC-C concentration of 5 or 25 nM. After each titration step, the sample was incubated for 2 min and the fluorescence signal was then recorded for 60 s and averaged.

The dissociation constant \( K_D \) for a one-site-binding equilibrium \( (A + B \rightleftharpoons AB) \) is defined as \( K_D = [A][B]/[AB] \) with [A] the concentration of free miniGC-C, [B] the concentration of free STp-(5-17) and [AB] the concentration of miniGC-C/STp-(5-17) complex. Free miniGC-C and free STp-(5-17) concentrations during the titration are given by \( [A] = [A]_0 - [AB] \) and \( [B] = [B]_0 - [AB] \) with \([A]_0 \) as the overall miniGC-C and \([B]_0 \) as the overall STp-(5-17) concentration, respectively. The fluorescence intensity difference \( \Delta F \) in each titration step is proportional to the concentration of miniGC-C/STp-(5-17) complex formed, and the maximum change in fluorescence intensity \( \Delta F_{\text{max}} \) is detected if the entire protein is complexed with STp-(5-17).

Fluorescence intensities were corrected for buffer fluorescence and dilution due to the successive addition of 1.5 \( \mu \text{L} \) STp-(5-17) stock solution to the 1.5 ml sample. The total volume increase during the titration was \(< 2\%\). Plotting \( \Delta F \) versus the STp-(5-17) concentration reveals hyperbolic-binding isotherms, which were analyzed according to Eq. 1 (Lohman and Bujalowski, 1991):

\[
\Delta F = \Delta F_{\text{max}} \cdot \frac{b - \sqrt{b^2 - 4 \cdot [A]_0 \cdot [B]_0}}{2 \cdot [A]_0} \quad \text{with}
\]

\[
b = [A]_0 + [B]_0 + K_D.
\]

**Results**

**Modeling of the extracellular ligand-binding domain of GC-C and design of miniGC-C**

Experimentally determined structures of extracellular domains of receptors that are homologous to membrane-bound guanylyl cyclases include the dimerized extracellular ligand-binding domains of the ANP receptor (GC-A; van den Akker et al., 2000; Ogawa et al., 2004) and the NPR-C receptor (He et al., 2001) in the presence and absence of ligand. These structures can be used as a template for structural modeling of GC-C. The sequence identity between the extracellular domains of GC-C and GC-A or NPR-C is only 19% and 18%, respectively. Despite this low identity, a similar fold has been suggested for all extracellular domains of membrane-bound guanylyl cyclases (van den Akker, 2001). We have thus generated a structural model of the GC-C<sub>EXT</sub> based on a secondary structure-guided sequence alignment using the crystal structure of the dimerized extracellular ligand-binding domain of the guanylyl cyclase-coupled ANP receptor (GC-A) (van den Akker et al., 2000) as a template (Fig. 1A).
As for the homologous receptors (van den Akker et al., 2000; He et al., 2001; Ogawa et al., 2004), the GC-C_ECD model adopts a periplasmic-binding protein type-I like fold which is characterized by two subdomains: an NH$_2$-terminal membrane distal subdomain and a COOH-terminal membrane proximal subdomain both connected by a flexible hinge region of three polypeptide chains. Due to their linkage to neighboring antiparallel strands of the eight-stranded β-sheet of the membrane proximal subdomain, two of these chains are in close proximity (Fig. 1a). In order to design a smaller soluble and properly folded receptor fragment of the porcine GC-C ECD comprising residues Ser139-Leu276-(Ala-Pro-Gly-Asp)-Thr355-Gln407, we decided to term ‘miniGC-C’, represents the membrane proximal subdomain of the porcine GC-C_ECD comprising the four-residue sequence (Ala-Pro-Gly-Asp) that is characteristic for β-turns and that connects the two β-strands is indicated by a circle, the hormone-binding region is shown in magenta (figures generated with Molscript (Kraulis, 1991) and Raster3D (Merritt and Murphy, 1994)).

**Construction and bacterial overexpression of miniGC-C**

Based on the cDNA of the complete extracellular domain of porcine GC-C, the receptor fragment miniGC-C was cloned, fused with His$_6$- and Thioredoxin-tags, overexpressed in *E.coli* AD494(DE3) cultures possessing an oxidative cytoplasm, and purified via Co$^{2+}$ affinity chromatography before and after cleavage of tags with PreScission protease. The expression of miniGC-C has been carried out using an *E.coli* strain with an oxidative cytoplasm, and the purification was carried out under oxidative conditions. Subsequent characterization by HPLC, mass spectrometry and gel electrophoresis revealed the resulting protein of 197 amino acid residues to be a homogeneous product (Fig. 2). The additional two non-native NH$_2$-terminal residues (Gly and Pro) resulting from the PreScission protease cleavage site seem to have no impact on the biophysical and biochemical properties of miniGC-C, in line with the observation that no negative influence of these additional residues on the biochemical and biophysical properties of other proteins could be detected (Lauber et al., 2002, 2003; Vitzithum et al., 2008).

**Spectroscopic characterization of miniGC-C**

The overall shape of the far UV CD spectrum of miniGC-C is typical of a protein containing α-helix and β-sheet portions as well as unstructured regions, with a minimum between 205 and 210 nm and a positive band at 195 nm (Fig. 3). Secondary structure analysis using the K2D algorithm (Andräge et al., 1993) revealed 28% helix, 27% β-sheet and 45% random coil. This secondary structure content is very similar to the content calculated from our model structure (Fig. 1b) which comprises 25% helix, 22% β-sheet and 53% random coil. The one-dimensional $^1$H NMR spectrum of miniGC-C shows clear signal dispersion of the amide proton chemical shifts and upfield shifted methyl group resonances, suggesting a protein with a well-defined three-dimensional structure (Fig. 3).

**Ligand-binding of miniGC-C**

The ligand-binding sequence of GC-C as determined by Hasegawa et al. (1999b) contains a single tryptophan residue (W392 in the whole extracellular domain, W180 in miniGC-C), which involves in the ligand-binding process (Wada et al., 1996; Hasegawa et al., 1999b). Since the fluorescence emission of a fluorophor depends on its chemical environment, we expected changes in the intrinsic tryptophan fluorescence on ligand binding. The toxin-binding ability of miniGC-C was, therefore, probed by fluorescence emission spectroscopy with porcine heat-stable enterotoxin STp-(5-17) as a ligand without intrinsic fluorophor. Thus, changes in fluorescence intensity solely arise from specific interactions between receptor and ligand. Formation of the
The observed fluorescence is quenched upon the binding of STp. Titration of miniGC-C at elevated concentrations indicates a 1:1 stoichiometry of the complex. Analysis of the hyperbolic-binding isotherms at both miniGC-C concentrations (5 and 25 nM) according to the one-site-binding model [Eq. (1)] reveals an averaged-binding constant in the nanomolar range: $K_D = 5.9 \pm 1.4$ nM ($K_D = 7.2 \pm 1.0$ nM at 25 nM miniGC-C; $K_D = 4.5 \pm 0.5$ nM at 5 nM miniGC-C; Fig. 4).

Discussion

The structural characterization of the guanylin(uroguanylin/STa)/GC-C system is of high pharmacological interest. The design and successful expression of miniGC-C provides the basis for further structural studies of the ligand/GC-C system by different spectroscopic techniques under membrane-free conditions and will therefore assist in the development of medically applicable antagonistic GC-C ligands. MiniGC-C could also act as a general model system to study peptide hormone/receptor interactions.

The model structures of the GC-C<sub>ECD</sub> and miniGC-C

Due to its size, the complete extracellular domain of GC-C is not easily accessible for structural studies of the interaction with its ligands guanylin, uroguanylin and STa using NMR spectroscopy. Therefore, a smaller soluble and properly folded receptor fragment with the ability to bind these ligands was required. Based on the crystal structure of the extracellular domain of the homologous ANP-receptor, we initially generated a model structure of the GC-C<sub>ECD</sub>. Mapping of known mutational and cross-linking data (Wada et al., 1996; Hasegawa et al., 1999b) on the model structure of the GC-C<sub>ECD</sub> mainly restricts the ligand-binding region to a sequence around an exposed and accessible $\beta$-strand located in the membrane proximal subdomain (i.e. Ser387 to Lys393; numbering according to the complete extracellular domain; shown in magenta in the model structure in Fig. 1A). Despite the low sequence identity between both receptor domains of only 19%, the significance of this model structure is strongly supported by the designed receptor fragment: miniGC-C corresponds to the membrane proximal sub-domain and is a well-soluble protein with a distinct secondary and tertiary structure as well as a binding affinity to STp-(5-17) in the nanomolar range. This fragment contains the ligand-binding site and is therefore suitable for structural investigations on the guanylin(uroguanylin/STa)/GC-C system.

The guanylin/GC-C interaction

MiniGC-C binds STp-(5-17) with a very high affinity, but is still ~10-fold weaker than binding of STa to the complete receptor or the complete extracellular domain (Hasegawa et al., 1999a). As discussed below, the ligand-binding ability may be affected by the membrane distal subdomain of GC-C<sub>ECD</sub> which is not present in miniGC-C.

From the model structure, the detailed geometry of the ligand-binding sequence cannot be assessed exactly, but it is located close to an exposed and accessible $\beta$-strand (Fig. 1). The experimentally determined three-dimensional structure of the guanylin-prohormone (Lauber et al., 2003) provides clues for the mode of interaction and the structure of the binding sequence. In the structure of the guanylin-prohormone, the sequence corresponding to guanylin is involved in interactions with the NH<sub>2</sub>-terminal $\beta$-hairpin by providing the third strand of a small triple-stranded antiparallel $\beta$-sheet (Lauber et al., 2003). Guanylin and uroguanylin are known to occur as two topological isoforms (Skelton et al., 1994; Marx et al., 1998), with only one of the isoforms being biologically active and able to interact with GC-C (Marx et al., 1998; Schulz et al., 1998). In the guanylin-prohormone structure, the peptide corresponding to guanylin...
adopts the biologically active topology (Lauber et al., 2003), probably due to the stabilizing interactions via the β-strand which may mimic the receptor interaction. These intramolecular interactions observed between guanylin and its prosequence suggest a similar motif for the guanylin/GC-C interaction. Supporting this assumption, the secondary structure prediction (Jones, 1999) of the ligand-binding region of GC-C_{ECD} reveals a short β-strand for residues Phe390 and Ile391. This β-strand, however, is not identifiable in the model structure, but may well be present in miniGC-C or may be induced upon ligand binding.

Implications for GC-C activation
It is suggested that the ANP receptor is activated by an intermolecular twist between the two monomers of the dimeric extracellular domain which involves very little intramolecular conformational changes (Ogawa et al., 2004; Qiu et al., 2004). In contrast, other structurally homologous extracellular ligand-binding domains such as the NPR-C receptor or the metabotropic glutamate receptor mGluR seem to undergo a 13° intramolecular rearrangement of the two subdomains within one monomeric unit relative to each other (Armstrong et al., 1998; Kunishima et al., 2000; He et al., 2001). In this case, the flexible linker region between the two subdomains acts as a hinge. However, for both the ANP and the NPR-C receptor, the ligand-binding site is located between the two monomeric units of the dimerized extracellular domain (He et al., 2001; Ogawa et al., 2004). In contrast, all mutational data available suggest that the ligand-binding domain of GC-C is located at the outside of the dimer in the membrane proximal subdomain in the sequence stretch referred to as micro domain (Hidaka et al. 2002; shown in blue on the

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**Fig. 3.** One-dimensional ^1^H NMR spectrum of miniGC-C at a protein concentration of 300 µM, 25°C, in 50 mM sodium phosphate and 50 mM sodium chloride (10% D_2O, v/v), pH 7.4. Inset: Far UV CD spectrum of miniGC-C. The spectrum was recorded on a Jasco J-810 spectropolarimeter at room temperature (22°C) at a protein concentration of 2 µM in 20 mM sodium phosphate buffer, pH 7.4, and 50 mM sodium chloride.

**Fig. 4.** Fluorescence emission spectroscopy. Change of the miniGC-C tryptophan fluorescence at 328 nm (ΔF_{328}) on addition of STp-(5-17) after excitation at 295 nm. The miniGC-C concentration was 5 nM in 20 mM sodium phosphate pH 7.4, 50 mM sodium chloride and 0.3 mM polyoxyethylene-8-decylether. The solid line represents the fit of Eq. 1 to the hyperbolic-binding isotherm, which revealed a K_D of 4.5 ± 0.5 nM for this data set.
A ligand binding fragment of GC-C

model structure in Fig. 1a). This sequence is mainly located in the proximal subdomain but also covers a short stretch on the distal subdomain located in the cleft formed by the two subdomains (Fig. 1a). It is therefore likely that GC-C activation requires a conformational change of the two subdomains similar to that observed for NPR-C (He et al., 2001). The possible involvement of residues located on the membrane distal subdomain of GC-C_ECD might also explain the ~10-fold weaker ligand-binding affinity of miniGC-C compared with the complete extracellular domain (Hasegawa et al., 1999a).

Further structural investigations are required to shed light on the mode of interaction between GC-C and its ligands, and miniGC-C provides a starting point for these investigations.

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