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The micro domain responsible for ligand-binding of guanylyl cyclase C

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Abstract **Guanylyl cyclase C (GC-C), a member of membrane-bound guanylyl cyclases, is a receptor protein for guanylin and uroguanylin. The binding of a ligand to the extracellular domain of GC-C (ECD_{GC-C}) triggers signal transduction, resulting in the regulation of intestinal fluids and electrolytes. A previous study proposed that a ligand-binding site on GC-C is localized near the transmembrane region. To further investigate the mechanism by which GC-C is activated, the C-terminal polypeptide (Met341–Gln407) of ECD_{GC-C} (the micro domain), which includes the ligand-binding site, was over-expressed in *Escherichia coli* and its ligand-binding ability was examined. The micro domain showed ligand-binding activity ($IC_{50} = 1\times10^{-8}$ M). This result clearly indicates that a ligand-binding site is located in close proximity to the membrane-bound region, and that the micro domain is capable of independently binding the ligand, without assistance from other domains. The use of this micro binding domain in the study of interactions between GC-C and ligands could be a useful tool and could lead to a better understanding of GC-C signal transduction.**

Key words: Guanylyl cyclase; Heat-stable enterotoxin; Binding domain; Guanylin; Uroguanylin

1. Introduction

Guanylyl cyclase C (GC-C) is a membrane-bound protein, which consists of an N-terminal extracellular domain (ECD_{GC-C}), a transmembrane domain, and a C-terminal intracellular domain [1,2]. ECD_{GC-C} is responsible for the ligand binding, resulting in the activation of the catalytic domain and, ultimately, the regulation of intestinal fluids and electrolytes, via the cystic fibrosis transmembrane conductance regulator protein [3]. GC-C is stimulated by several ligands, including the endogenous peptide hormones, guanylin and uroguanylin, and heat-stable enterotoxin (STa), the exogenous ligand [4–10]. Previously, we reported that a ligand-binding site is located at the region between Ser387 and Lys393, near the transmembrane domain [11]. This result poses the question of the minimum peptide region required to recognize the ligand.

Guanylyl cyclase A (GC-A), which is thought to have a tertiary structure similar to GC-C, is a member of the membrane-bound guanylyl cyclase family and is a receptor protein for atrial natriuretic peptide [12]. Recently an analysis of the crystal structure of the extracellular domain (ECD_{GC-A}) of GC-A revealed that ECD_{GC-A} consists of two subdomains, the N-terminal α-helical and the C-terminal β-sheet domains [12]. The identity of the region of the amino acid residues between GC-A and GC-C is about 10%, but that of the C-terminal regions of ECD_{GC-C} and ECD_{GC-A}, which corresponds to the β-sheet domain of ECD_{GC-A}, is relatively high (23%), as shown in Fig. 1. In addition, a secondary structure prediction of ECD_{GC-C} implies that the secondary structure of the C-terminal region of ECD_{GC-C} consists of β-strands, which contain the ligand-binding site and is located in the same relative region as that of the C-terminal region of ECD_{GC-A}, as shown in Fig. 1 [13]. Therefore, we speculate that the tertiary structure of the C-terminal region of ECD_{GC-C}, referred to as the micro domain, is similar to that of the C-terminal region of ECD_{GC-A} and that the micro domain might possess the ability to bind the ligand, without assistance from other domains.

Based on this hypothesis, the C-terminal polypeptide of ECD_{GC-C} (the micro domain) was prepared and its ligand-binding ability examined. Based on the findings herein, the micro domain itself has a tertiary structure that is capable of binding the ligand. The data obtained permit a better understanding of the nature of signal transduction of GC-C.

2. Materials and methods

2.1. Materials

Restriction enzymes were purchased from Toyobo (Osaka, Japan) and New England BioLabs, Inc. (Beverly, MA, USA). Taq polymerase and T4 DNA ligase were obtained from Takara Shuzo Co. (Kyoto, Japan). STp(4–17) (porcine STa with the amino acid sequence from positions 4 to 17) and ^{125}I-ANB-STp(4–17) were prepared according to a previously described procedure [14,15]. All other chemicals and solvents were of reagent grade. Polymerase chain reaction (PCR) was carried out using a Sanyo DNA amplifier MIR-D30 (Osaka, Japan).

2.2. Construction of the expression vector of the micro domain for the expression by Escherichia coli cells

The cDNA encoding the extracellular domain (Ser1–Gln407) of porcine GC-C(C349A), in which the Cys349 residue was mutated to an Ala residue, was prepared according to a previously reported method [14,15] using primers for the mutation (sense, ACTTTGGACAACGCCGGGGATATTGAC; antisense, GTCAATATCCCCGGCGTTGTCCAAAGT). The cDNA was inserted into pVL1392

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Abbreviations: PCR, polymerase chain reaction; TFA, trifluoroacetic acid; HPLC, high performance liquid chromatography; Tris–HCl, tris(hydroxymethyl)aminomethane hydrochloride; IPTG, isopropyl β-thiogalactopyranoside; GC-C, guanylyl cyclase C; STa, heat-stable enterotoxin; STp(4–17), porcine STa with the amino acid sequence from positions 4 to 17; PAGE, polyacrylamide gel electrophoresis; ECD_{GC-C}, extracellular domain of GC-C; ECD_{GC-A}, extracellular domain of GC-A

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(Invitrogen) at the *Pst*I and the *Bam*HI sites, resulting in the construction of pVL-ECD(C349A). The cDNA encoding the micro domain (Met341–Gln407) was subcloned into the pET17b expression vector (Novagen), following the introduction, by means of PCR, of an *Nde*I site at its 5′ end and a *Xho*I site at its 3′ end using pVL-ECD(C349A) as a template. The resulting expression vector, referred to as pY42, contained the cDNA which encoded the extra amino acid residues GHHHHHH at the C-terminus of the micro domain (Met341–Gln407). The cDNA sequences of the vectors were confirmed by analysis using the ABI PRISM™310 Genetic Analyzer (PE Applied Biosystems).

2.3. Expression of the micro domain by E. coli cells

E. coli BL21(DE3) cells, transformed with the expression vector pY42, were cultured at 37°C in Luria broth supplemented with ampicillin (50 mg/l). Expression of the mutant protein was induced by the addition of 1 mM isopropyl β-thiogalactopyranoside (IPTG) at the mid-log phase of cell growth. After incubation at 37°C for 3 h, the cells were harvested and washed with PBS(−) containing 1 mM phenylmethylsulfonyl fluoride. The cells were resuspended in the same buffer, sonicated on ice, and centrifuged (15 000×*g*, 20 min). The resulting precipitates were incubated with 10 mM dithiothreitol in 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris–HCl) (pH 8.0) containing 6 M guanidine–HCl at 50°C for 30 min. The protein was first purified by means of Cosmosil 140C_{18}-OPN (Nacarai Tesque Inc., Kyoto, Japan), as described previously [9]. The mutant protein was finally purified by high performance liquid chromatography (HPLC), as described previously [9], and lyophilized. The protein thus prepared was characterized by mass spectrometry and amino acid analysis, as described previously [7].

2.4. In vitro refolding of the mutant protein

The purified protein (1 nmol) was dissolved in 0.05% trifluoroacetic acid (TFA) (20 μl) and mixed with nine volumes of 50 mM Tris–HCl (pH 7.4) or 50 mM AcONa (pH 4.7), followed by incubation at room temperature for 1 h. The reaction mixture was centrifuged (15 000×*g*, 20 min), and the resulting precipitants and supernatants were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE). The reaction mixture was also analyzed by HPLC, as described previously [9].

2.5. Photoaffinity labeling of the micro domain with ^{125}I-ANB-STp(4–17) and a binding assay

The refolded protein (0.1 nmol) was mixed with ^{125}I-ANB-STp(4–17) (4×10^4 cpm) in the presence or absence of STp(4–17) (final concentration, 10^{-5} M), which showed the same GC-C binding affinity as native STp [2], and incubated at 37°C for 1 h in a final volume of 60 μl of PBS(−), using a previously reported method [14,15]. The solution, in an ice bath, was then exposed to UV radiation (302 nm, model UVM-57, UVP Inc., CA, USA) for 30 min. The reaction mixture was subjected to SDS–18% PAGE. The photoaffinity-labeled protein was analyzed by autoradiography on a Fujix Bio-image analyzer BAS 2000 (Fuji film, Tokyo, Japan).

To estimate the ligand-binding affinity of the micro domain, we attempted to separate free ^{125}I-ANB-STp(4–17) and ^{125}I-ANB-STp(4–17), which binds to the micro domain, by gel filtration or Ultrafree filter (Millipore). However, it was difficult to completely separate the free ligand from the micro domain using our system. Therefore, the ligand-binding ability was estimated by the autoradiography on SDS–PAGE. A competitive binding assay was performed in the presence of several concentrations of STp(4–17) under the conditions described above. The reaction mixture was separated by SDS–PAGE, and the radioactivity of the photoaffinity-labeled micro domain on the gel was quantified by the Fujix Bio-image analyzer BAS 2000.

3. Results and discussion

Ligand binding to the ECD_{GC-C} triggers the signal transduction of GC-C, resulting in the activation of the intracellular catalytic domain of GC-C. In our previous study, we proposed that the ligand-binding site is located in the region between Ser387 and Lys393 in ECD_{GC-C}, near the transmembrane domain [11]. Therefore, this region should supply the specific tertiary structure required for the ligand binding. However, the tertiary structure of the entire and the essential ligand-binding domain of ECD_{GC-C} has not yet been reported.

Previously, ECD_{GC-C} was prepared using a system consisting of baculovirus and insect cells in order to obtain structural information on the ligand binding of ECD_{GC-C} [14]. However, the expression efficiency of the recombinant ECD_{GC-C} by the baculovirus expression system was low, and the protein aggregated readily after purification. In addition, it is generally known that small peptide fragments of receptor proteins, such as the peptide fragment consisting of seven amino acid residues from Ser387 to Lys393 of ECD_{GC-C}, cannot fold into the native conformation sufficient to bind to the ligand. Therefore, the minimum functional binding domain of ECD_{GC-C} was explored in order to further study the interaction between ECD_{GC-C} and ligands.

A recent X-ray crystallographic structural analysis of ECD_{GC-A} indicated that the C-terminal region of ECD_{GC-A} is folded into a β-sheet structure [12]. The sequence alignment of ECD_{GC-C} and ECD_{GC-A} showed that these proteins shared a 23% homology at the C-terminal region (from Met341 to Gln407 of GC-C), as shown in Fig. 1, implying that the tertiary structure of the C-terminal region of ECD_{GC-C}, referred to as the micro domain, might be the same as that of ECD_{GC-A}. The region between the Phe376 and the Ala403 residues of ECD_{GC-A}, which corresponds to the region between the Met356 and the Pro383 residues of ECD_{GC-C} (Fig. 1), is comprised of a β-sheet domain in the crystal structure of ECD_{GC-A}. In the case of ECD_{GC-C}, this region contains the ligand-binding site, suggesting that this domain should be included as a ligand-binding unit of GC-C. In addition, our previous study indicated that the Asp347 and Asn348 residues of GC-C play an important role in the ligand binding of GC-C [16]. Moreover, the sequence between Gly342 and Leu346 is completely conserved in GC-Cs from several species, also implying that it plays a significant role in ligand binding. The N-terminal Met341 residue is also included since the Met residue is generated by the starting codon in the *E. coli* expression system used in this study. Therefore, the micro domain (Met341 to Gln407) was prepared and its ligand-binding ability examined, in an attempt to better understand the nature of the essential ligand-binding domain.

The region between the Met341 and the Gln407 residues of ECD_{GC-C} contains one cysteine residue at position 349. This

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     361                                                                  435
GC-A TGYLKIDRNG DRDTDFSLWD MDPETGAFRV VLNYNGTSQE LMAVSEHKLY -WPLGYPPPD VPKCGFDNED PACNQD
 ST  teeeee tt       eeeee e ttt  eee eeeeetttte eee tt      tt         tt

     341                                                                  407
GC-C MGPVTLDNCG DIDNTMFLLY TSVDTSKYKV LLTYDTRKNY TNPVDKSPTF IWKNHKLPND IP--GRGPQ
 ST  ttteeetttt ttteeeeeee ettttteeee eeettteee ettttteee ettttctttt
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Fig. 1. Primary and secondary structures of the C-terminal region of ECD_{GC-A} and ECD_{GC-C}. Completely matched amino acid residues are shown as dotted. ST denotes the secondary structure. The e and t in the secondary structure represent the β-sheet and the β-turn, respectively. The secondary structure of GC-A was inferred from PDB and that of GC-C was predicted by the Chou–Fasman's method [13].

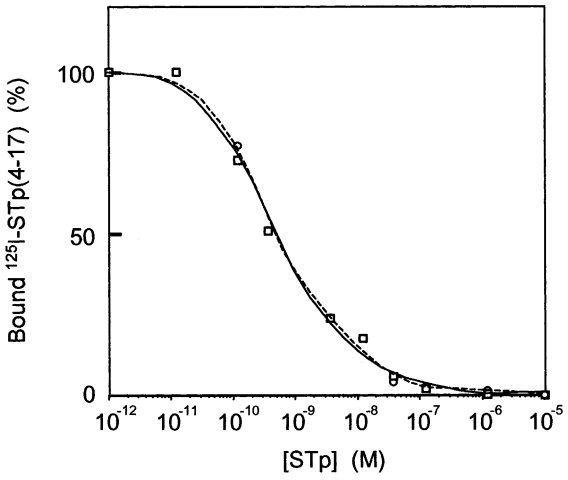

Fig. 2. Curves for the inhibition by STp of the binding of ^{125}I-ANB-STp(4–17) to wild-type and mutant C349A of ECD. The binding assay was performed using a previously reported method [14]. ECD, open squares with a dotted line; mutant C349A, open circles with a solid line.

Cys349 residue is not found in other species of GC-C, suggesting that it may not play a role in ligand binding. To avoid the formation of an intermolecular disulfide bond at the Cys349 residue, it was mutated to an Ala residue. For this purpose, mutant C349A of the entire ECD_{GC-C} was first prepared and its ligand-binding ability examined, using a previously reported method [14]. The mutant C349A of ECD_{GC-C} showed a ligand-binding ability identical to that of wild-type ECD_{GC-C} (Fig. 2), indicating that the Cys349 is not absolutely required for the binding of GC-C with ligands.

The cDNA encoding the micro domain, in which the Cys349 residue was mutated to an Ala residue, was generated by PCR using the cDNA encoding the mutant C349A of ECD_{GC-C} as a template, and was inserted into the expression vector, pET17b (Novagen), resulting in the construction of the expression vector pY42. The micro domain was expressed as an inclusion body in *E. coli* BL21(DE3) cells transformed with pY42, as shown in Fig. 3A. Therefore, the micro domain was purified by HPLC in a denatured form (Fig. 3B). The purified micro domain showed a signal at $m/z = 8498.4$, consistent with its mass value (8498.1) calculated from the amino acid sequence, in a mass spectrometric analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

The solubility of the purified micro domain, which is in a denatured form, was poor at neutral pH, but it was soluble in 0.05% TFA (pH 2.3). It has been reported that GC-C binds to the ligand at pH 4–8 [17]. Therefore, the purified micro domain was refolded by the dilution method under various conditions, followed by altering the solution pH. The purified micro domain was dissolved in 0.05% TFA and then mixed with nine volumes of 50 mM Tris–HCl (pH 7.4) or 50 mM AcONa (pH 4.7). The micro domain existed as a soluble form at neutral pH after this procedure as well as under acidic conditions, implying that the micro domain was folded into the native conformation.

The ligand-binding ability of the refolded micro domain was examined using a photoaffinity-labeled STa analog, ^{125}I-ANB-STp(4–17), as shown in Fig. 4A,B. The negative control in this experiment was performed in the presence of 6 M guanidine–HCl (pH 7.4) (lanes 1 and 2 in Fig. 4A,B). The micro domain could not be photoaffinity-labeled with ^{125}I-ANB-STp(4–17) under denaturing conditions, indicating that non-specific binding was not significant. However, the refolded micro domain, refolded with 50 mM Tris–HCl (pH 7.4), was labeled with ^{125}I-ANB-STp(4–17) and the binding was specifically inhibited in the presence of unlabeled STp(4–17) (lanes 3 and 4 in Fig. 4A). Uroguanylin also inhibited the binding of ^{125}I-ANB-STp(4–17) to the micro domain (lanes 7 and 8 in Fig. 4B). However, the binding ability of uroguanylin was lower than that of STp(4–17) as well as in the case of GC-C [18], and uroguanylin could not completely inhibit the ^{125}I-ANB-STp(4–17) binding to the micro domain even in the presence of 10^{-5} M uroguanylin (lane 8 in Fig. 4B).

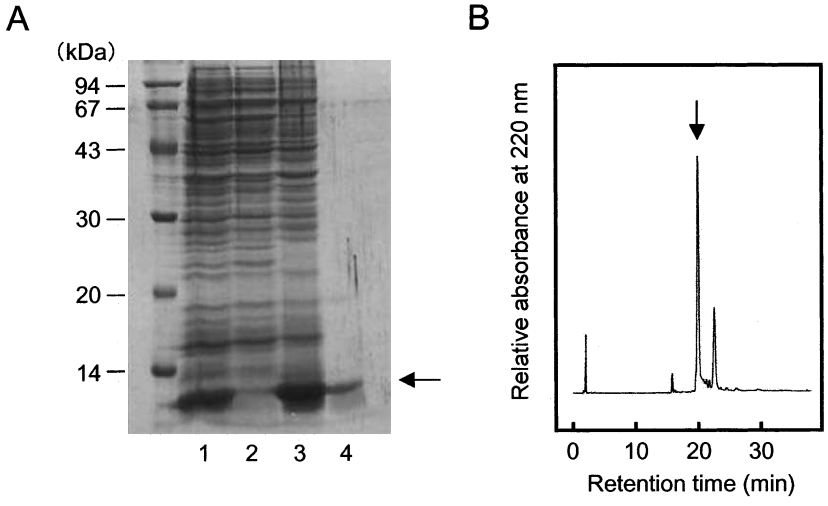

Fig. 3. SDS–PAGE (A) and HPLC (B) of the micro domain of ECD_{GC-C}. A: Proteins were separated on a 15% (w/v) polyacrylamide gel and visualized by staining with Coomassie brilliant blue: lane 1, total proteins expressed in *E. coli* cells after induction by IPTG; lane 2, supernatant of the cell lysates in lane 1; lane 3, cell lysate precipitates in lane 1; lane 4, the micro domain purified by HPLC in Fig. 2B. B: The precipitates in lane 3 were solubilized and treated with Cosmosil $140C_{18}$-OPN, as described in Section 2. The resulting materials were dissolved in 50% CH_3CN containing 0.05% TFA, and subjected to HPLC. The positions of the micro domain are indicated by arrows. HPLC was performed using a previously reported method [9].

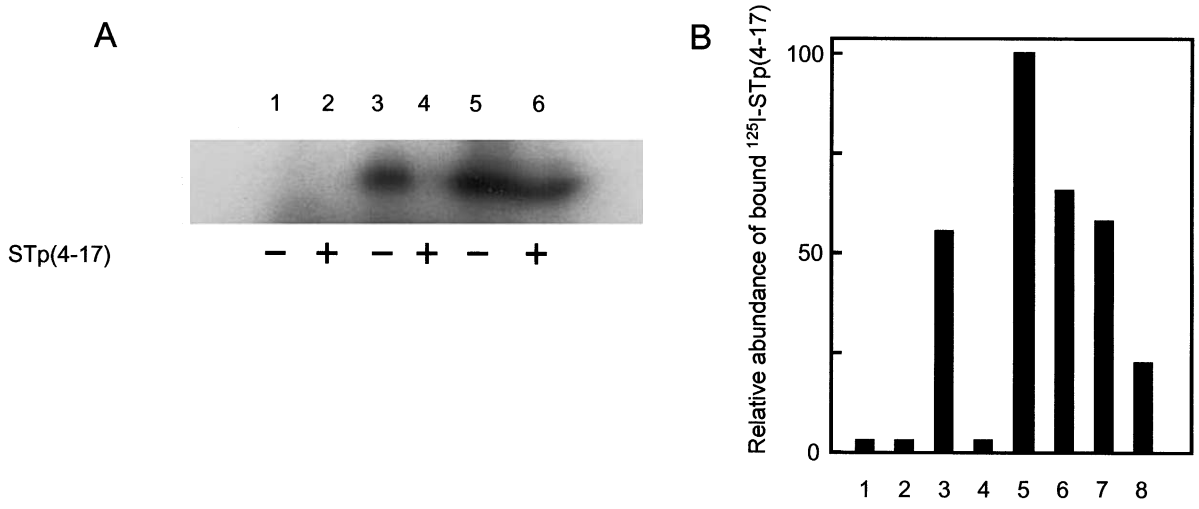

Fig. 4. Autoradiographic analysis of ligand-binding of the micro domain. A: Autoradiography of the micro domain on SDS–PAGE. B: Relative abundance of the bound ligand was measured by means of a Fujix Bio-image analyzer BAS 2000. The ligand-binding of the micro domain was examined by photoaffinity labeling with ^{125}I-ANB-STp(4–17) in the presence or absence of 10^{-5} M cold STp(4–17) (even- or odd-numbered lanes in A and B, respectively). The micro domain was refolded in 50 mM Tris–HCl (pH 7.4) containing 6 M guanidine–HCl (lanes 1 and 2), 50 mM Tris–HCl (pH 7.4) (lanes 3, 4, 7, and 8), and 50 mM AcONa (pH 4.7) (lanes 5 and 6) and the binding ability estimated in the same buffer. In lane 8, the binding ability of the micro domain was estimated in the presence of 10^{-5} M uroguanylin. The data present the average of two data sets.

Since GC-C is able to bind the ligand under acidic conditions [17], as described above, the ligand-binding ability of the micro domain was examined under acidic conditions. The refolded micro domain, which had been refolded with 50 mM AcONa (pH 4.7), was also able to bind STp(4–17) at pH 4.7, but non-specific binding was significant (lanes 5 and 6 in Fig. 4A,B). These data clearly show that the micro domain possesses the ability to bind to the ligand.

To estimate the binding capacity of the micro domain, a competitive binding assay was performed in the presence of several concentrations of cold STp(4–17) and the ligand-binding ability was estimated by autoradiographic analysis. Fig. 5 shows the competitive binding curve between the micro domain and ^{125}I-ANB-STp(4–17) at equilibrium with an IC_{50} value of 1×10^{-8} M. This result clearly shows that the micro domain binds the ligand in the absence of other domains of GC-C. In order to determine the K_d value of the micro domain for ligand binding, we attempted to use a gel filtration method to separate free and bound ligands, as described in Section 2. However, it was difficult to completely separate these peptides. Therefore, it would be necessary to construct a new assay system for the determination of the K_d value of the micro domain. However, the binding affinity of the micro domain was lower than that of ECD_{GC-C} (IC_{50} value $=1\times10^{-9}$ M) in our assay system, as shown in Fig. 5 [14]. The K_d value of ECD_{GC-C} was 4×10^{-10} M, as previously reported [14]. Therefore, the K_d value of the micro domain might be one order lower than that of ECD_{GC-C}. The lower affinity of the micro domain is not dependent on the lack of the sugar chain at the Asn379 residue, since the presence of glycosylation at the Asn residue had no effect on the ligand-binding affinity of ECD_{GC-C} [15]. These results indicate that other domains of GC-C may cooperatively aid in ligand binding at the micro domain. Indeed, a preliminary structural analysis of the micro domain, based on circular dichroism (CD) measurements, indicates that the micro domain consists of small amounts of α-helix and β-sheet (data not shown) although the predicted secondary structure is the β-sheet, suggesting that the micro domain may need assistance from other domains of GC-C to sufficiently fold into the native conformation so as to bind the ligand.

Previously Nandi et al. reported on the expression of the N-terminal domain of GC-C by *E. coli* [19]. They prepared the entire ECD_{GC-C}, including the transmembrane domain, and a truncated form of ECD_{GC-C}, which corresponds to the region 1–304. They reported that the entire extracellular domain possessed ligand-binding ability but that the truncated form did not. These results are consistent with the results reported here, since the micro domain corresponds to the region Met341–Gln407. Therefore, it can be concluded that the critical ligand-binding site is located at the micro domain.

The micro domain of ECD_{GC-C} corresponds, relatively, to the region 361–428 of ECD_{GC-A}. The putative ANP-binding sites on GC-A were assigned to regions 173–188 and 191–198 [20]. Although the tertiary structure of GC-C has not yet been elucidated, ECD_{GC-C} might have a tertiary structure similar to that of ECD_{GC-A}. Based on this hypothesis, the identified

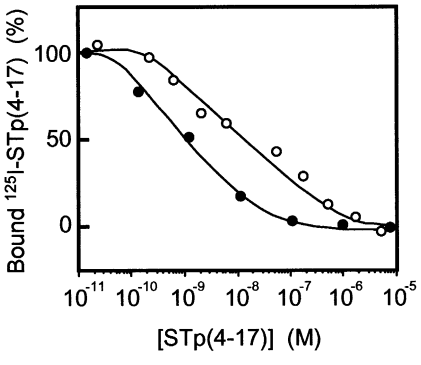

Fig. 5. Competitive binding assay of the micro domain and ECD_{GC-C}. The curves show the inhibition of binding of ^{125}I-ANB-STp(4–17) to the refolded micro domain (pH 7.4) (open circles) and ECD_{GC-C} (closed circles) at several concentrations of STp(4–17).

STa-binding site (Ser387–Lys393) on the micro domain would be located at the opposite site of the ANP-binding site on the β-sheet domain of ECD_{GC-A}, suggesting that the ligand-binding site of GC-C is located in a different region from that of GC-A or that GC-C may possess another ligand-binding site. Indeed, the mutant micro domain, in which the Trp392 in the STa-binding site of the micro domain was mutated to an Ala residue, showed a lower ligand-binding affinity than that of the micro domain (data not shown). Therefore, it is possible that the ligand-binding mode of GC-C may be different from that of GC-A.

In conclusion, the micro domain of ECD_{GC-C} possesses the ability to bind ligands in the absence of other domains of GC-C. This micro domain should be a powerful tool to further investigate interactions between GC-C and ligands, and will provide new insights into the signal transduction of GC-C.

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