Determination of the Binding Site on the Extracellular Domain of Guanylyl Cyclase C to Heat-stable Enterotoxin*

(Received for publication, May 24, 1999, and in revised form, August 10, 1999)

Makoto Hasegawa‡, Yuji Hidaka, Yoshiko Matsumoto, Toshifumi Sanni, and Yasutsugu Shimonishi§

From the Division of Protein Organic Chemistry, Institute for Protein Research, Osaka University, Yamadaoka 3-2, Suita, Osaka 565-0871, Japan

Guanylyl cyclase C, one of the family of membrane-bound guanylyl cyclases, consists of an extracellular domain and an intracellular domain, which are connected by a single transmembrane polypeptide. The extracellular domain binds unique small polypeptides with high specificity, which include the endogenous peptide hormones, guanylin and uroguanylin, as well as an exogenous enterotoxigenic peptide, heat-stable enterotoxin, secreted by pathogenic Escherichia coli. Information on this specific binding is propagated into the intracellular domain, followed by the synthesis of cGMP, a second messenger that regulates a variety of intracellular physiological processes. This study reports the design of a photoaffinity labeled analog of heat-stable enterotoxin (biotinyl-(AC5)2-[Gly4,Pap11]STp(4–17)), which incorporates a Pap residue (p-azidophenylalanine) at position 11 and a biotin moiety at the N terminus, and the use of this analog to determine the ligand-binding region of the extracellular domain of guanylyl cyclase C. The endoproteinase Lys-C digestion of the extracellular domain, which was covalently labeled by this ligand, and mass spectrometric analyses of the digest revealed that the ligand specifically binds to the region (residue 387 to residue 393) of guanylyl cyclase C. This region is localized close to the transmembrane portion of guanylyl cyclase C on the external cellular surface. This result was further confirmed by characterization of site-directed mutants of guanylyl cyclase C in which each amino acid residue was substituted by an Ala residue instead of residues normally located in the region. This experiment provides the first direct demonstration of the ligand-binding site of guanylyl cyclase C and will contribute toward an understanding of the receptor recognition of a ligand and the modeling of the interaction of the receptor and its ligand at the molecular level.

Guanylyl cyclase C (GC-C),1 a member of the growing family of membrane-bound guanylyl cyclases, catalyzes the synthesis of cGMP as a second messenger in intestinal and kidney cortex epithelial cells in response to stimulation by a ligand (1–3). It is generally thought that this process is involved in the regulation of intestinal and kidney fluids and electrolytes, via the endogenous peptide hormone, guanylin or uroguanylin (4, 5). It is also noteworthy that GC-C greatly increases cGMP levels in intestinal epithelial cells, on interaction with an exogenous peptide, heat-stable enterotoxin (STa), which is produced by enteric bacteria such as enterotoxigenic Escherichia coli. This results in an efflux of watery electrolytes into the intestinal lumen, which leads to, in turn, acute diarrhea in human and domestic animals (6).

GC-C is a single subunit protein molecule (1050 residues) with a unique structure consisting of an N-terminal extracellular domain (ECD, ~407 residues), which is responsible for ligand binding, and a C-terminal intracellular domain (~619 residues), for the catalysis of cGMP synthesis, which are connected through a single transmembrane polypeptide (~24 residues) (1, 7, 8). The C-terminal intracellular domain is comprised of two functional regions, a kinase homology region and a guanylyl cyclase catalytic region. The N-terminal domain is structurally specific for a ligand such as guanylin and uroguanylin, and the resulting information is then transferred to the cytoplasmic intracellular domain via the transmembrane polypeptide, resulting in the stimulation of the guanylyl cyclase catalytic region in the intracellular domain. The molecular mechanism for recognizing the structure of a ligand and leading to signal transduction by GC-C currently is unknown, but one plausible scenario is that GC-C induces a change in conformation or topology between each subunit when ligand binding occurs, resulting in the removal of a negative regulatory effect of the kinase homology region on the activation of the guanylyl cyclase catalytic region and, thus, creating a favorable environment for the synthesis of cGMP (9). GC-C exists as an oligomer in cultured cells irrespective of the presence or absence of a ligand (9, 10). On the contrary, the ECD molecule forms an oligomer in a ligand-dependent manner (11). Thus, the relationship of the oligomeric state of GC-C to the binding to a ligand remains unclear.

Studies of the site-directed mutagenesis of porcine GC-C revealed two regions in the ECD that are sensitive to point mutations (12); one is a highly conserved region from residue 91 to residue 155 (numbers denote the positions of amino acid residues relative to the N terminus of porcine GC-C) in the amino acid sequences of GC-Ca determined thus far, and the other is in the sequence from residue 274 to residue 409 which is close to the transmembrane portion. A mutation in the region from residue 347 to residue 401 in the latter, which is positively charged at the C terminus; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ Supported by a Research Fellowship of the Japan Society of the Promotion of Science for Young Scientists.
§ To whom correspondence should be addressed: Division of Protein Organic Chemistry, Institute for Protein Research, Osaka University, Yamadaoka 3-2, Suita, Osaka 565-0871, Japan. Tel.: +81-6-6879-8601; Fax: +81-6-6879-8603; E-mail: simonis@protein.osaka-u.ac.jp.
1 The abbreviations used are: GC, guanylyl cyclase; STa, heat-stable enterotoxin produced by enterotoxigenic E. coli; ECD, the extracellular domain of GC-C; STp(4–17), porcine STa with the amino acid sequence from position 4 to 17; Pap, p-azido-L-phenylalanine; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; ANB, 5-azido-2-nitrobenzyl; HPLC, high performance liquid chromatography; AC5, -NH(CH2)5CO-; AC, enterotoxin produced by enterotoxigenic E. coli.
tioneer near the transmembrane portion, causes a significant reduction in both ligand binding activity and guanylyl cyclase catalytic activity. These results suggest that the region on the ECD for interacting with a ligand is focused on or located within these regions, the mutation of which strongly affects the biological properties of GC-C.

We describe herein the identification of the specific region on the ECD of porcine GC-C bound to a ligand. For defining the region on the ECD that confronts a ligand, we designed and synthesized a photoaffinity labeling analog of STa (biotinyl-(AC5)2-[Gly4,Pap11]STp(4–17)) (Fig. 1) with a Pap residue (p-azido-i-phenylalanine) at position 11 and a biotin moiety at the N terminus. This analog of STa would be expected to undergo UV-induced binding to the ECD, and a peptide fragment bound to this analog could then be salvaged from the enzymatic digest of the photoaffinity labeled ECD by using an avidin-immobilized matrix. MALDI-TOF mass spectrometry was used to map a peptide on ECD for biotinyl-(AC5)2-[Gly4,Pap11]STp(4–17), which has been specifically limited to the amino acid sequence from residue 387 to residue 393, close to the transmembrane portion. This finding was confirmed by site-directed mutagenesis because substitution by an Ala residue for Thr-389, Phe-390, or Trp-392 in the sequence, respectively, greatly affects binding ability to the ligand, as well as cyclase catalytic activity of GC-C. These results provide novel insights into the elucidation of the recognition and activation mechanism of GC-C by a ligand.

EXPERIMENTAL PROCEDURES

Materials—The reagents used for peptide synthesis were purchased from the Peptide Institute Inc. (Minoh, Japan) and Nacalai Tesque, Inc. (Kyoto, Japan). T4 DNA ligase and restriction enzymes were from Takara Shuzo Co. (Kyoto, Japan) and New England Biolabs, Inc. (Bev- lington, MA), respectively. Na125I (carrier free) was purchased from NEN Life Science Products and used for the iodination of STp (4–17). Other reagents and solvents were purchased from Sigma and Katayama Chemicals Inc. (Osaka, Japan), all of which were reagent grade.

Synthesis of Biotinyl-(AC5)2-[Gly4,Pap11]STp(4–17)—Peptides were synthesized using standard solid-phase procedures using tert-butyloxycarbonyl chemistry (13). STp(4–17) and ANB-STp(4–17) were synthesized as described previously (14). Details of the procedures for synthesis of [Gly4,Pap11]STp(4–17) in which the amino acid residues at positions 4 and 11 of STp(4–17) were replaced by Gly and Pap, respectively, that is, synthesis of intermediates, protection of the functional side chains of amino acid residues, removal of peptides from the resin, conversion from linear forms of peptides to their oxidized forms, conversion of amino-1-phenylalanine to Phe, and purification of peptides on reversed-phase HPLC were described as previously (15). In the final step of the synthesis, the biotin group was attached to the N terminus of [Gly4,Pap11]STp(4–17) by the following procedure: [Gly4,Pap11]STp(4–17) (100 nmol) was mixed with biotinyl-(AC5)2-[Gly4,Pap11]OSu (Dojin Chemical Inc., Kumamoto, Japan) and triethylamine (50 eq each of the (AC5)2-[Gly4,Pap11]STp(4–17)) was isolated by reversed-phase HPLC. Other iodination procedures yielded about 260 pmol of the labeled ECD6H, based on the starting crude ECD6H (800 pmol), as judged from the ligand-binding capacity of the ECD6H treated without photoaffinity labeling (control experiments).

Digestion of Photoaffinity Labeled ECD6H—The solution that contained the photoaffinity labeled ECD6H, described above, was directly diluted with 1 ml of buffer (100 mTris (pH 9.0) and 8 m urea) and incubated at 4 °C for 2 h. The solution was then diluted with 100 mTris buffer (pH 9.0) (5 ml) containing 0.1% SDS and concentrated to 0.5 ml on a Centricon-10 (Amicon Inc.). Peptide N-glycosidase F (0.4 units) was added to the solution for removal of carbohydrate chains at the N-linked glycosylation sites and allowed to incubate at 37 °C for 2 h. The resulting solution was then incubated with endoproteinase Lys-C (3) overnight at 37 °C for 16 h.

Isolation of a Photoaffinity Labeled Peptide—The above digested ECD6H solution was mixed with avidin-immobilized agarose (50-μl bed volume) and incubated at room temperature for 30 min. The avidin-immobilized agarose was collected on an Ultrafree-MC 0.1 μm filter unit (Millipore Inc.). The agarose was washed twice with 100 ml Tris (pH 9.0) (300 μl) containing 100 mM NaCl and then with sufficient 0.05% trifuluoroacetic acid to remove salts. The peptides were removed from the agarose by heating in a mixture of 0.05% trifluoroacetic acid and 50% acetonitrile at 100 °C for 15 min. The supernatant, which contained the peptides, was concentrated in vacuo and subjected to mass measurements with an Voyager Elite-XL MALDI-TOF mass spectrometer (Japan Perceptive Biosystems, Inc., Tokyo, Japan) using α-cyano-4-hydroxycinnamic acid as a matrix (16, 17). Site-directed Mutagenesis of Porcine GC-C—The preparation of the constructs of the recombinant GC-C and its mutant proteins was carried out according to procedures reported previously (12), with minor modifications. These recombinant proteins were transiently expressed in 293T human embryonic kidney cells, which were grown in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum and supernatant as a buffer for the reaction solution (16, 17). Production of the mutant proteins of GC-C, expressed on 293T cells, was assayed as described previously (8). Specifically, the 293 cell membrane fraction was used to measure the production of the mutant proteins of GC-C, expressed on 293T cells, was assayed as described previously (8). Specifically, the 293 cell membrane fraction was used to measure the production of the mutant proteins of GC-C, expressed on 293T cells, was assayed as described previously (8). Specifically, the 293 cell membrane fraction was used to measure the production of the mutant proteins of GC-C, expressed on 293T cells, was assayed as described previously (8). Specifically, the 293 cell membrane fraction was used to measure the production of the mutant proteins of GC-C, expressed on 293T cells, was assayed as described previously (8). Specifically, the 293 cell membrane fraction was used to measure the production of the mutant proteins of GC-C, expressed on 293T cells, was assayed as described previously (8). Specifically, the 293 cell membrane fraction was used to measure the production of the mutant proteins of GC-C, expressed on 293T cells, was assayed as described previously (8).
Fig. 1. Amino acid sequences of STp produced by a porcine strain of enterotoxigenic E. coli (A) and biotinyl-(AC5)2-[Gly4,Pap11]STp(4–17) (B). Six Cys residues are intramolecularly linked by disulfide linkages between Cys8 and Cys10, Cys6 and Cys14, and Cys9 and Cys17.

RESULTS

Synthesis of Biotinyl-(AC5)2-[Gly4,Pap11]STp(4–17)—A novel STA analog (biotinyl-(AC5)2-[Gly4,Pap11]STp(4–17), Fig. 1) was synthesized as a probe for mining a peptide fragment that encompasses the ligand-binding site on the ECD of GC-C. The synthetic ligand contained two functional residues as follows: one was a photosensitive amino acid (Pap) with azido group, which is easily converted to nitrene by radiation with UV light (300 nm) and covalently anchored to electron-rich groups such as N-H, O-H, etc. on the receptor molecule (17); and the other was a biotin moiety, which noncovalently binds to avidin with an extremely high affinity ($K_D$ < 10$^{-15}$ M) and is widely used as a carrier in purification of proteins using avidin-based affinity chromatography. This synthetic compound was confirmed not only to have the same conformation as that of STp(4–17) by comparison of their CD spectra but also to be activated by UV radiation, showing it to be useful as a ligand for ECD. In addition, this ligand was confirmed to be efficiently adsorbed on avidin-immobilized agarose and easily removed from the avidin-agarose by heating at 100 °C for 10 min (data not shown).

Binding Affinity of Biotinyl-(AC5)2-[Gly4,Pap11]STp(4–17) to ECD6H—To confirm whether biotinyl-(AC5)2-[Gly4,Pap11]STp(4–17) can efficiently bind to ECD6H, we examined the binding activity of biotinyl-(AC5)2-[Gly4,Pap11]STp(4–17) to ECD6H using a competitive ligand binding assay in the presence of a constant amount of $^{125}$I-TyrSTp(4–17) or STp(4–17) with $IC_{50}$ values of 8 × 10$^{-7}$ and 2 × 10$^{-6}$ M, respectively, indicating that the binding potency of biotinyl-(AC5)2-[Gly4,Pap11]STp(4–17) to ECD6H is 40-fold lower than that of STp(4–17). Previous experiments demonstrated that [Pap11]STp(4–17), in which a Pap residue was introduced at position 11 in STp(4–17), retained a high efficiency of photoaffinity labeling to GC-C, although it diminished the binding potency to GC-C to a level about 30-fold lower than that of STp(4–17) (15). Therefore, we concluded that biotinyl-(AC5)2-[Gly4,Pap11]STp(4–17) has the same level of the binding potency as [Pap11]STp(4–17) and is a good probe for the efficient photoaffinity labeling of ECD6H. On the basis of the finding shown in Fig. 2, we determined that the optimum concentration of the ligand for a photoaffinity labeling experiment is 5 × 10$^{-6}$ M.

Photoaffinity Labeling of ECD6H with Biotinyl-(AC5)2-[Gly4,Pap11]STp(4–17) and Isolation of a Labeled Peptide Fragment—UV radiation of the purified ECD6H in the dark, in the presence of biotinyl-(AC5)2-[Gly4,Pap11]STp(4–17), resulted in extensive and irreversible binding of the ligand to ECD6H. Approximately 50% of the ECD6H was occupied with the ligand, and the remaining 50% was free, in comparison with the ECD6H treated under the same conditions except for the absence of biotinyl-(AC5)2-[Gly4,Pap11]STp(4–17) (data not shown). This suggested that half of the ECD6H was involved in cross-linking with biotinyl-(AC5)2-[Gly4,Pap11]STp(4–17).

The photoaffinity labeled ECD6H was treated with peptide N-glycosidase F for removal of carbohydrate moieties and then digested with endoproteinase Lys-C. The digest was passed through a column of the avidin-immobilized matrix to adsorb the photoaffinity labeled peptide fragments, which were then removed from the avidin-immobilized matrix by heating. The eluate from the avidin matrix was analyzed by direct MALDI-TOF mass spectrometry. Fig. 3 shows a typical mass spectrum of the eluate from the avidin-immobilized matrix. The mass values of two major signals, observed at $m/z$ = 878.6 and 2716.4, were in complete agreement with the calculated mass values for the peptide fragment (residue 387 to residue 393) of ECD (calculated mass value for SPTFIWK, 878.5) and the same peptide fragment bound to biotinyl-(AC5)2-[Gly4,Pap11]STp(4–17) (calculated mass value for SPTFIWK + biotinyl-(AC5)2-[Gly4,Pap11]STp(4–17), 2716.1), respectively. Moreover, the mass values of the other two signals observed at $m/z$ = 2316.1 and 2430.4 were quite close to the values of PTFI (residue 388 to residue 391) and FIWK (residue 390 to residue 393), bound to biotinyl-(AC5)2-[Gly4,Pap11]STp(4–17) (calculated mass values, 2314.9 and 2430.9, respectively). These fragments could arise via the unexpected cleavage of the labeled peptide, detected at $m/z$ = 2716.4. The mass values of the signals observed in Fig. 3 conform only to peptides that encompass residues 387–393, bound to biotinyl-(AC5)2-[Gly4,Pap11]STp(4–17) but did not fit those of any other sequences (Fig. 4). These results are also supported by the observation that the signals (Fig. 3) were not detected in the digest of ECD6H that was treated in the absence of biotinyl-(AC5)2-[Gly4,Pap11]STp(4–17) by the same procedure as that described above (control experiment) (not shown).

Site-directed Mutational Analysis of the Ligand-binding Site—To investigate the issue of whether the ligand-binding region is truly localized within the amino acid sequence (resi-
due 387 to residue 393) in ECD, it was mutagenized using site-directed mutagenesis to give six mutant proteins (Fig. 5), in which each of the amino acid residues in the sequence (residue 387 to residue 392) were individually substituted by an Ala residue by employing a recombinant GC-C expression system using 293T human embryonic kidney cells. The expression of the mutant proteins was confirmed by Western blotting and staining with an anti-GC-C antibody raised against a synthetic peptide covering the C-terminal region (residue 1036 to residue 1050) of porcine GC-C (20), as shown in Fig. 5A. The recombinant GC-C gave two protein bands on SDS-PAGE (lane 1 in Fig. 5A), in which the heterogeneity of molecular weight of GC-C was likely caused by differences in the extent of N-linked glycosylation, as has been reported previously (20). The mutant proteins exhibited two protein bands on SDS-PAGE, which were stained by an antibody detection reagent, similar to the wild type of GC-C (lanes 2–7), suggesting that all the mutant proteins were expressed in the same quantity as that of the wild type GC-C.

The mutant proteins were photoaffinity labeled with ANB-[125I-Tyr4]STp(4–17), as shown in Fig. 5B. Among the six mutant proteins, one (S387A) showed a binding potency identical to that of the wild type GC-C. On the contrary, two mutant proteins (P388A and I391A) showed a reduced binding ability, and the remaining three mutant proteins (T389A, F390A, and W392A) were nearly completely devoid of binding ability to ANB-[125I-Tyr4]STp(4–17). These results suggest that substitution by an Ala residue of the amino acid residues at Thr-389, Phe-390, and Trp-392 resulted in a strong deficiency in ligand binding ability.

The response of the guanylyl cyclase activation of the mutant proteins for exposure to STp(4–17) was compared with that observed for the wild type GC-C by assaying the cGMP concentration in the 293T cells which express either these mutant proteins or the wild type GC-C (Fig. 5C). The formation of cGMP for each of the mutant proteins was correlated to the binding ability to STa, even though all the mutant proteins, when stimulated by STa, exhibited cGMP formation, and the other three mutant proteins (T389A, F390A, and W392A) showed only 0.5–1%, compared with the wild type GC-C, respectively. This again indicates that the mutant proteins (T389A, F390A, and W392A) have practically no ability to bind to STa (1 × 10⁻⁵ M).

The membrane-bound guanylyl cyclases represent a single transmembrane type receptor with distinct ligand specificities and are found in both mammals and lower eukaryotes (2, 3). The extracellular domains of these receptor proteins show sequence diversity, compatible with their ability to recognize and discriminate a diversity of ligand structures. On the contrary, the intracellular domains, in particular the guanylyl cyclase catalytic regions, have high sequence homology among the receptors, consistent with their unique function in the synthesis of cGMP. This suggests that the receptors have a similar structural topology and regulate diverse physiological processes through ligand-specific synthesis of intracellular cGMP as an intracellular message. The identification of the binding sites of ligands to the receptors on the external surfaces of membranes will lead to a better understanding of the molecular basis of ligand binding and receptor activation.

GC-C serves as a receptor protein for the polypeptide ligands: guanylin (4) and uroguanylin (5) for endogenous ligands as well as heat-stable enterotoxin (STa) (21) for an exogenous ligand produced by enteric bacteria, such as enterotoxigenic E. coli. Our previous experiments (15, 22, 23) demonstrated that [Pap₄]STp(4–17) substituted by a Pap residue in place of Asn¹¹ located near the key amino acid residue Ala¹³ in the context of binding to GC-C, is an efficient probe for photoaffinity cross-linking of STa to GC-C. In addition, we demonstrated that the ECD of GC-C, which is expressed in a system consisting of insect cells and a recombinant baculovirus, retains a ligand-binding ability that is similar to that of GC-C and could be prepared as a soluble, homogeneous protein (11). These data allowed us to examine the interaction of ECD with a ligand at the molecular level.

In this work, we focused on the determination of the specific region of the ECD that is involved in binding to STa. For this purpose, we used the following four-step strategy: covalent binding of an STp analog with a photoaffinity functional group to ECD, digestion of the ECD which is covalently coupled with a photoaffinity labeled STp analog, isolation of the photoaffinity labeled fragment from the digest by affinity chromatography, and identification of the labeled peptide fragment by mass spectrometry. An STp analog (biotinyl-(AC₇)₂-[Gly⁴,Pap₄]STp(4–17)) was not only covalently linked to the ECD but permitted the isolation of a peptide fragment from the digest of the photoaffinity labeled ECD by affinity chromatography, a technique that takes advantage of the non-covalent binding between biotin and avidin. In general, the binding of a photoaffinity labeled ligand to its receptor protein appears to proceed with considerable low efficiency, as has been shown in other cases (24). Therefore, an expedient technique is needed, in order to detect the photoaffinity labeled receptor protein or its fragment from a photoaffinity labeled reaction mixture. In the present case, the photoaffinity labeled peptide fragment was successfully recovered by the attachment of a biotin moiety at the N terminus of the ligand from the digest of the photoaffinity labeled ECD6H but in only a tiny amount. Mass spectrometry identified the peptide fragment, which encompasses the amino acid sequence from residues 387 to 393 (SPTFIWK) (Fig. 4), positioned near the transmembrane portion of GC-C, in the digest of the ECD6H photoaffinity labeled with biotinyl-(AC₇)₂-[Gly⁴,Pap₄]STp(4–17). Moreover, the peptide fragments that are bound to the photoaffinity ligand with the sequence from residue 388 to residue 391 (PTFI) and that from residue 390 to residue 393 (FIWK) were observed by mass spectrometry (Fig. 3). These findings strongly suggest that the ligand binds to the amino acids, Phe or Ile, at positions 390 and 391, respectively, which are common in the three peptide fragments observed by
**Fig. 4.** Schematic representation of the ECD of porcine GC-C, displaying the subdomains based on its secondary structure predicted according to the method of Chou and Fasman (26). Vertical bars indicate the location of Cys residues, and the region bound to biotinyl-(AC5)2-[Gly4,Pap11]STP(4–17) is shown by the amino acid sequence, which is compared with those of other species. Asterisk shows identity of the amino acid sequence of the extracellular domain.

**Fig. 5.** Analysis of site-directed mutant proteins in the region (residues 387–392) of GC-C expressed in 293T cells. A, SDS-PAGE and Western blot analysis of the mutant proteins of GC-C contained in the membrane fractions of the 293T cells. Proteins were visualized by an anti-GC-C antibody raised against a synthetic peptide (residue 1036 to residue 1050 of GC-C). Lane 1, wild type; lane 2, S387A; lane 3, P388A; lane 4, T389A; lane 5, F390A; lane 6, I391A; lane 7, W392A. B, SDS-PAGE of the mutant proteins photoaffinity radiolabeled with ANB-[125I-Tyr](STP(4–17) in the absence or presence of 10 μM STP(4–17) (lanes of odd or even numbers, respectively). Lanes 1 and 2, wild type; lanes 3 and 4, S387A; lanes 5 and 6, F390A; lanes 7 and 8, T389A; lanes 9 and 10, F390A; lanes 11 and 12, I391A; lanes 13 and 14, W392A. C, cGMP production of 293T cells expressing the mutant proteins of GC-C in the presence of 10 μM STP(4–17). Lane 1, wild type; lane 2, S387A; lane 3, P388A; lane 4, T389A; lane 5, F390A; lane 6, I391A; lane 7, W392A.

Mass spectrometry. The result was again supported by site-directed mutagenesis of the amino acid residues in the binding region, which show that the substitution of an Ala residue at position Pro-388, Thr-389, Phe-390, or Trp-392 caused the complete loss of binding ability to the ligand (Fig. 5).

In our earlier work (12), the site-directed mutational analysis of the extracellular domain of porcine GC-C revealed that amino acid replacement (residues 347–348, 363–365, 373–375, and 389–401) in a region (residue 347–401), which is close to the transmembrane region and surrounds the peptide fragment found in this study, causes a strong reduction in both binding activity to STAs and guanylyl cyclase catalytic activity. In addition, the mutation of the Asn-379 residue to Ala, which is positioned at an N-glycosylation consensus site, lost the ability to contain an oligosaccharide at this residue and resulted in a strong reduction in binding ability of GC-C to STa (18). These previous results provide support for the conclusion that the region of GC-C involved in the binding to a ligand is located from Ser-387 to Lys-393, very near the transmembrane domain. It is also interesting to note that the amino acid sequence of the ligand-binding region that was found in porcine GC-C in this experiment is highly homologous to those not only of mammalian species but also of an amphibian (Xenopus laevis, African clawed frog) and a fish (Oryzias latipes, Japanese medaka), although this homology is not found in the amino acid sequences of the entire extracellular domains (1, 7, 8, 25)3–5 (Fig. 4).

The prediction of the secondary structure of ECD, as examined according to the method of Chou and Fasman (26), suggests that ECD is composed of two subdomains as follows: an α/β-rich region (residue 1 to residue 331) and a β-rich region (residue 332 to residue 401), as described in Fig. 4. The α/β region consists of alternating α-helices and β-strands and contains eight Cys residues, probably linked by intramolecular disulfide bridges, suggesting that this subdomain, which consists of 75% of the amino acid residues of the extracellular domain, functions as a determinant of the basic architecture of the entire molecule. In contrast, the β-rich region has a low propensity for α-helix in contrast to high propensities for a β-strand and turn and is comprised of hydrophilic amino acid residues, including a Cys residue which is not found in other species and perhaps is not involved in a disulfide linkage, suggesting that this subdomain forms a flexible conformation. The x-ray crystallography of the complex of human growth hormone and its receptor protein suggests that the ligand-binding region on the receptor protein is largely comprised of turn structures (27). The same may be inferred in the case of GC-C, because the sequence from Ser-387 to Pro-388 present in

---

2 R. T. MacFarland, DDBJ/GenBank™/EBI accession number D49837.
3 M. Kruhoeffer, Y. Cetin, U. Kaempf, and W.-G. Forssmann, DDBJ/GenBank™/EBI accession number P70106.
4 R. M. Goracznik, T. Duda, and R. K. Sharma, DDBJ/GenBank™/EBI accession number AF081464.
5 T. Mantoku and N. Suzuki, DDBJ/GenBank™/EBI accession number AB007192.
the ligand-binding region is assumed to form a turn structure, and in particular, Pro-388 could represent an element of a turn structure. Indeed Pro-388 was identified as a significant amino acid residue, as evidenced by site-directed mutational analysis of GC-C (Fig. 5). Furthermore, the data herein are in agreement with the conclusions that the region around this site points to a high content of hydrophilic amino acid residues, suggesting that this region is located on the surface of the molecule, thus facilitating interaction with a ligand. In addition, we recently found that the β-rich region has a binding ability to STα, when the interaction of a recombinant protein, comprising the β-rich region, with STp4–17 was examined. This experiment also provides evidence that the β-rich region near the transmembrane domain plays a role in ligand binding.

In this study, we identified a region of GC-C that is involved in ligand binding and that is critical for signaling via the ligand to the intracellular domain of GC-C, which is located near the transmembrane portion on the external surface of the cellular membrane. On the basis of the present finding, together with previously reported data, we hypothesize that the binding of a ligand to the extracellular domain induces a clustering (or oligomerization) of the extracellular domain and, in turn, modulates the topological relationship between each of the ligand-binding regions of ECD, as has been recently proposed in the case of the epidermal growth factor binding protein (28, 29). This process may facilitate a change of the conformation of the intracellular cytoplasmic domain, resulting in the activation of the catalytic domain. It would then be interesting to see whether a principle for the transmission of a ligand signal from an extracellular domain into an intracellular domain exists in other GCs, perhaps in a manner analogous to GC-C. The extracellular domain of GC-C activates the intracellular domain in the same manner as in other GCs, when generating cGMP, a common cytoplasmic second messenger, because the intracellular domain of GC-C is highly homologous to other GCs in terms of primary structure, although the extracellular domain of GC-C has a quite different amino acid sequence from those of other GCs (2). In any event, the determination of the binding site of GC-C for interacting with its ligand will provide new insights into our current understanding of the recognition and activation mechanism of GC-C by a ligand. A three-dimensional structural analysis of the extracellular domain and its complex with a ligand will be required, in order to answer the issue of how the extracellular domain propagates a signal to the intracellular cyclase catalytic domain on binding with a ligand.

Acknowledgments—The use of the facility at the Radioisotope Research Center of Osaka University is acknowledged. We thank Hiroko Sakamoto for preparing the manuscript.

REFERENCES

1. Schulz, S., Green, C. K., Yuen, P. S. T., and Garbers, D. L. (1990) Cell 63, 941–948
2. Garbers, D. L. (1992) Cell 71, 1–4
3. Wedel, B. J., and Garbers, D. L. (1997) FEBS Lett. 410, 29–33
4. 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
5. Hamra, F. K., Forte, L. R., Eber, S. L., Pidhorodeckyj, N. V., Krause, W. J., Freeman, R. H., Chin, D. T., Tompkins, J. A., Fok, K. F., and Smith, C. E. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10464–10468
6. Dupont, H. L., and Ericsson, C. D. (1993) Nature 364, 1821–1827
7. Singh, S., Singh, G., Heim, J.-M., and Gerzer, R. (1991) Biochem. Biophys. Res. Commun. 179, 1455–1463
8. Wada, A., Hirayama, T., Kitao, S., Fujisawa, J., Hidaka, Y., and Shimoniishi, Y. (1994) Microbiol. Immunol. 38, 535–541
9. Rudner, X. L., Mandel, K. K., De Sauvage, F. J., Kindman, L. A., and Almenoff, J. S. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5169–5173
10. Vaandragr, A. B., van der Wiel, E., Hom, M., Luthjens, L. H., and de Jonge, H. R. (1994) J. Biol. Chem. 269, 16409–16415
11. Hasegawa, M., Kawano, Y., Mateusoto, Y., Hidaka, Y., Fujii, J., Taniguchi, N., Wada, A., Hirayama, T., and Shimoniishi, Y. (1999) Protein Expression Purif. 15, 271–281
12. Wada, A., Hirayama, T., Kitaura, H., Fujisawa, J., Hasegawa, M., Hidaka, Y., and Shimoniishi, Y. (1996) Infect. Immun. 64, 5144–5150
13. Aimoto, S., Watanabe, H., Ikemura, H., Shimoniishi, Y., Takeda, T., Takeda, Y., and Miwatani, T. (1983) Biochem. Biophys. Res. Commun. 112, 320–326
14. Kuhota, H., Hidaka, Y., Oraki, H., Ito, H., Hirayama, T., Takeda, Y., and Shimoniishi, Y. (1989) Biochem. Biophys. Res. Commun. 161, 229–235
15. Hasegawa, M., Kawano, Y., Matsumoto, K., Hidaka, Y., Sato, T., and Shimoniishi, Y. (1997) Lett. Peptide Sci. 4, 1–11
16. Bayley, H., and Knowles, J. B. (1977) Methods Enzymol. 46, 69–114
17. Eberle, A. N., and de Graan, P. N. E. (1975) Methods Enzymol. 109, 129–156
18. Hasegawa, M., Hidaka, Y., Wada, A., Hirayama, T., and Shimoniishi, Y. (1999) Eur. J. Biochem. 236, 338–345
19. Takao, T., Hitojuji, T., Aimoto, S., Shimoniishi, Y., Hara, S., Takeda, T., Takeda, Y., and Miwatani, T. (1983) FEBS Lett. 152, 1–5
20. Vaandragr, A. B., Schulz, S., de Jonge, H. R., and Garbers, D. L. (1993) J. Biol. Chem. 268, 2174–2179
21. Aimoto, S., Takao, T., Shimoniishi, Y., Hara, S., Takeda, T., Takeda, Y., and Miwatani, T. (1982) Eur. J. Biochem. 129, 257–263
22. Yamasaki, S., Sato, T., Hidaka, Y., Oraki, H., Ito, H., Hirayama, T., Takeda, Y., Shugimura, T., Tait, A., and Shimoniishi, Y. (1990) Biochim. Biophys. Acta 1046, 2063–2070
23. Ozaki, H., Sato, T., Kubota, H., Hata, Y., Katsube, Y., and Shimoniishi, Y. (1991) J. Biol. Chem. 266, 5934–5941
24. Pandy, K. N., Inagami, T., and Misono, K. S. (1986) Biochemistry 25, 8467–8472
25. de Sauvage, F. J., Camerato, T. R., and Goeddel, D. V. (1993) J. Biol. Chem. 268, 17912–17918
26. Chou, P. Y., and Fasman, G. D. (1974) Biochemistry 13, 211–245
27. De Vos, A. M., and Kossiakoff, A. A. (1992) Science 255, 306–312
28. Livnah, O., Stura, E. A., Middleton, S. A., Johnson, D. L., Jolliffe, L. K., and Wilson, I. A. (1999) Science 283, 987–990