

Heat-stable Enterotoxin Receptor/Guanylyl Cyclase C Is an Oligomer Consisting of Functionally Distinct Subunits, Which Are Non-covalently Linked in the Intestine*

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Guanylyl cyclase (GC) C is a heat-stable enterotoxin (STa) receptor with a monomeric M_r of approximately 140,000. We calculated from its hydrodynamic parameters that an active GC-C complex has a M_r of 393,000, suggesting that GC-C is a trimer under native conditions. Both trimeric and dimeric GC-C complexes were detected by ¹²⁵I-STa binding and SDS-polyacrylamide gel electrophoresis under non-reducing conditions. The GC activity and STa binding from intestinal brush border membranes comigrated in gel filtration and velocity sedimentation with recombinant GC-C. However, ¹²⁵I-STa cross-linking demonstrated that STa receptors with molecular masses of 52 and 74 kDa are non-covalently attached to GC in the intestine. Radiation inactivation revealed different functional sizes for basal GC activity, STa-stimulated GC activity, and STa binding (59, 210–240, and 32–52 kDa, respectively). At low radiation doses, basal GC activity was stimulated, suggesting that GC-C is inhibited by a relatively large, probably internal structure. These results suggest that STa may activate GC-C by promoting monomer-monomer interaction (internal “dimerization”) within a homotrimeric GC-C complex, and that GC-C is proteolytically modified in the brush border membrane but retains its function.

Guanylyl cyclase (GC)¹ C is a recently cloned member of the family of receptor guanylyl cyclases (1–4). This class of transmembrane proteins contains an intracellular guanylyl cyclase and a kinase homology domain, whereas the extracellular portion consists of a ligand binding domain. A heat-stable enterotoxin (STa) from *Escherichia coli*, associated with severe secretory diarrhea in mammals, acts as a potent activator of GC-C (5). The natural ligand of GC-C is thought to be guanylin, a peptide derived from a larger precursor protein that is synthesized in the intestinal mucosa (6, 7). The GC-C isozyme is also expressed most abundantly, although not exclusively, in the intestine (1, 7). The function of the guanylin/GC-C system is presumably to maintain a proper fluidity of the intestinal lu-

men; the GC-C product, cGMP, is known to play an important role as a regulator of salt and water transport in the gut by inhibiting NaCl absorption and by stimulating chloride secretion through activation of the cystic fibrosis-related chloride channel (8, 9). In both processes an intestine-specific type II cGMP-dependent protein kinase is probably involved (9, 10).

Studies with recombinant GC-C stably expressed in 293 human embryonic kidney cells showed that GC-C is an N-linked glycoprotein that is not associated with other proteins after solubilization (11, 12). Furthermore, solubilized GC-C does not require other specific proteins for its activation by STa, provided that it is stabilized by ATP (13). The adenine nucleotides presumably interact with the kinase homology domain (13). In SDS-PAGE under non-reducing condition, GC-C behaved as a dimeric form that displayed a decreased mobility after binding of STa (12, 13).

In this study we determined the molecular mass of GC-C under more native conditions by size exclusion chromatography and velocity sedimentation analysis and by SDS-PAGE after ¹²⁵I-STa cross-linking. We further determined the functional size of the different activities of GC-C in membranes by radiation inactivation (14). Because GC-C has been suggested to exist in intestinal brush border membranes as a proteolyzed form (11, 12, 15), we compared the characteristics of GC from these membranes with recombinant GC-C stably expressed in 293 cells (12).

We report here that the M_r of GC-C closely matches that of a homotrimer, and that the interaction between two of the three subunits is increased upon binding of STa. Although GC-C is cleaved in brush border membranes, resulting in apparent lower M_r STa-binding proteins, its properties were similar to those of full-length GC-C. We also observed that STa binding and cyclase activity reside on relatively small subdomains within GC-C, as suggested by its primary structure (1).

EXPERIMENTAL PROCEDURES

Materials—*E. coli* heat-stable enterotoxin (STa) and 3-isobutyl-1-methylxanthine were obtained from Sigma, and bis(sulfosuccinimidyl)-suberate from Pierce Chemical Corp. Protein standards for SDS-PAGE (thyroglobulin subunit, M_r 330,000; myosin, M_r 200,000; β -galactidase phosphorylase b, M_r 97,000; bovine albumin, M_r 67,000; egg albumin, M_r 45,000) were purchased from Pharmacia Biotech.

Preparation of 293-rGC-C Membranes, Intestinal Brush Border Caps, and Brush Border Membrane Vesicles—Human embryonic kidney 293 cells, which stably express cDNA coding for rGC-C, were a gift from Dr. S. Schulz (Dallas, TX) and were cultured as described previously (12). Crude membranes were prepared by homogenization of the 293-GC-C cells followed by centrifugation at 20,000 $\times g$ for 15 min (12).

Non-vesiculated brush border caps were prepared from jejunum and ileum of male Wistar rats by vibration of everted intestine in hypotonic EDTA buffer and low speed centrifugation, essentially as described previously (16).

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¹ The abbreviations used are: GC, guanylyl cyclase; STa, *E. coli* heat-stable enterotoxin; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; Gy, gray(s).

Brush border membrane vesicles were made from isolated intestinal cells by freeze-thawing, followed by magnesium precipitation according to Ref. 17, and resuspended in buffer B (300 mM mannitol, 10 mM Tris/HCl, pH 7.4). This preparation was used in the radiation inactivation experiments, because its GC activity is more resistant to freeze-thawing compared to the enzyme activity in brush border caps.

Gel Filtration—Intestinal brush border caps (2–3 mg/ml protein) or membranes from 293-GC-C cells (1–2 mg/ml) were suspended in buffer A (100 mM NaCl, 50 mM Tris/HCl, pH 7.4) and solubilized with 0.5% Triton X-100 in the presence or absence of 1 mM ATP by incubation on ice for 5 min. The preparations were subsequently centrifuged at $20,000 \times g$ for 40 min, and 200 μ l of the supernatant fluids containing the solubilized proteins were injected on a Superose 6 HR 10/30 analytical gel filtration column (Pharmacia Biotech). The column was equilibrated and subsequently eluted with buffer A plus 0.1% Triton X-100 and for some experiments 1 mM ATP at 0.2 ml/min. Fractions of 400 μ l were collected and immediately assayed for GC activity and 125 I-STa binding. For each experiment the column was calibrated with thyroglobulin (Stokes radius, $a = 8.5$ nm), ferritin ($a = 6.1$ nm), immunoglobulin G ($a = 5.2$ nm), and cytochrome *c* ($a = 1.0$ nm). The Stokes radius of the GC and STa binding activities present in the samples were calculated as described (18).

Sucrose Density Gradient Centrifugation—Linear 7.5–25% sucrose gradients in buffer A containing 0.1% Triton X-100 and 1 mM ATP were prepared by a gradient mixer in 13-ml ultracentrifuge tubes. On top of the gradients, 300 μ l of freshly solubilized membrane proteins, prepared similarly as for the gel filtration, were layered. After centrifugation for 17 h at $100,000 \times g$ in a swing-out rotor, 500- μ l fractions were collected from the bottom of the gradient by a needle connected to a peristaltic pump. Apparent sedimentation coefficients ($s_{20,w}$) of the GC and STa binding activities in the fractions were calculated by plotting the distance from the top of the gradient against the position of the following standards: catalase (11.3 S), bovine serum albumin (4.9 S), and cytochrome *c* (1.9 S).

Hydrodynamic Calculations—The molecular mass (m) was calculated from: $m = 6\pi\eta N a s_{20,w} / (1 - v\rho)$ where η = viscosity of medium, N = Avogadro's number, a = Stokes radius, $s_{20,w}$ = sedimentation coefficient, v = partial specific volume, and ρ = density of medium (18, 19). We assumed v for proteins to be 0.73 cm³/g (19). Corrections for bound detergent were performed by repeating the velocity sedimentation experiments in deuterated buffers as described in Ref. 19, assuming a v for Triton X-100 of 0.94 cm³/g.

Radiation Inactivation and Dosimetry—Aliquots (400 μ l) of brush border membrane vesicles in buffer B (5–10 mg/ml protein) or 293-GC-C cells in buffer A (10–15 mg/ml protein) were sealed in 1-ml glass ampules and frozen in liquid N₂. Before the functional assays, the 293 cells were thawed and membranes were prepared as described above and resuspended in 400 μ l of buffer A. Samples were irradiated in a cryostat at -135°C using the 3-MeV Van de Graaff electron accelerator of the Interfaculty Reactor Institute with 250-ns duration electron pulses and 2 A peak current, as described before (20). To improve dose homogeneity, a 0.3-mm-thick aluminum scatterplate was used on top of the beam window of the cryostat. Dosimetry with calibrated 1×1 -cm FWT Radiachromic nylon film dosimeters between 3-mm-thick nylon plates was performed before each irradiation experiment. The dose obtained was approximately 0.1 Gy/nanocoulomb with an accuracy of $\pm 5\%$. Electron pulses were given at a repetition rate of 5 Hz to maintain proper temperature stability. The charge per pulse was determined at least before and after each irradiation and was found to be stable within 1%.

The molecular mass (m) in kDa of the inactivated targets was calculated using the relation: $m = c S_0 / D_{37}$ (14), where $c = 6400$ kGy/kDa, $S_0 = 2.88$ (i.e. the correction factor for the irradiation temperature of -135°C ; Ref. 14) and D_{37} = the dose in kGy required for inactivation to 37% survival as obtained from the monoexponential phase of the inactivation curve by linear regression analysis.

The validity of our method was verified by determination of the target size of yeast glucose-6-phosphate dehydrogenase (21). We observed a monoexponential inactivation for this protein with a D_{37} of 187 ± 11 kGy, corresponding to a target size of 99 ± 6 kDa, which is in close agreement with the biochemically determined value of 104 kDa (21).

Guanylyl Cyclase Assay and Cyclic GMP Determinations—Samples (50 μ l) were incubated at 37°C for 5–10 min in a final volume of 0.1 ml containing 100 mM NaCl, 50 mM Tris/HCl, pH 7.4, 0.25 mM 3-isobutyl-1-methylxanthine, 1 mM GTP, 1 mM ATP, and 3 mM MnCl₂, or 10 mM MgCl₂ plus an ATP/GTP regenerating system consisting of 5 mM creatine phosphate and 3–5 units of creatine phosphokinase. Brush border membrane vesicles were permeabilized by addition of 0.1% Triton X-100. The reaction was stopped by the addition of 1 ml of 0.1 M HCl.

After centrifugation (10 min, $20,000 \times g$), the supernatant fluid was neutralized with an equal volume of 0.09 M Tris, 2 mM EDTA. The samples were acetylated, and cGMP was determined by radioimmunoassay (22).

125 I-STa Binding and Cross-linking—STa was radioiodinated to approximately 600 Ci/mmol with chloramine T using a procedure described for succinyl cGMP tyrosine methyl ester (22) and was partially purified on a Sep-Pak C18 column (Waters, Millipore Corp.) by elution between 15 and 50% acetonitrile in 10 mM ammonium acetate, pH 5.8. For binding experiments, samples were incubated for 45 min at 37°C in a final volume of 100 μ l of PBS containing 0.1% bovine serum albumin and $10\text{--}20 \times 10^3$ cpm of 125 I-STa. Binding was terminated by the addition of 0.9 ml of ice-cold PBS. Bound 125 I-STa was separated from the free STa by filtration on GF-F filters (Whatman) pretreated with 0.3% polyethylenimine. For cross-linking, 50 μ l of sample in PBS was incubated for 45 min with 0.5 μ Ci of 125 I-STa at 37°C . Subsequently either 25 μ l of a 3-fold concentrated SDS sample buffer without β -mercaptoethanol was added (non-reducing conditions) or 5 μ l of the cross-linker bis(sulfosuccinimidyl)suberate to a final concentration of 0.5 mM, followed, after a 30-min incubation at room temperature, by 25 μ l of the SDS sample buffer containing 2 M β -mercaptoethanol (reducing conditions). Non-reduced samples were heated for 10 min at 60°C , whereas reduced samples were boiled for 3 min prior to SDS-PAGE (12, 13).

RESULTS

Size Determination of GC-C by Gel Filtration and Velocity Sedimentation—GC activity solubilized from 293-GC-C membranes in the presence of 1 mM ATP was recovered after gel filtration as a single peak with a Stokes radius of 8.5 ± 0.3 nm (see Fig. 1A and Table I). The GC-C in this peak could be activated 2.8 ± 0.4 -fold by STa in the presence of MgGTP ($n = 4$). However, STa binding showed two peaks of similar magnitude, designated I and II. Peak I comigrated with the GC activity, whereas peak II had a smaller size (see Fig. 1A and Table I). Inactivation of GC-C by incubation of the solubilized proteins for 10 min at 37°C in the absence of ATP (13) resulted in a large reduction in the amplitude of peak I, but not in its position. Peak II was hardly affected by this treatment, which therefore resulted in an apparent separation of GC-C activity and STa binding (Fig. 1B). Both GC activity and most of the STa binding originating from freshly isolated intestinal brush border caps coeluted with peak I from 293-GC-C membranes (see Fig. 1 and Table I). Inactivation of brush border GC activity resulted in a relatively minor lowering of the STa-binding peak as compared to the GC activity peak and caused no change in its position (see Fig. 1D).

As shown in Fig. 2 and Table I, velocity sedimentation analysis on a 7.5–25% sucrose gradient revealed a qualitatively similar behavior of brush border and recombinant GC activity and STa binding as observed in gel filtration. We could calculate from the hydrodynamic data that the M_r of a native GC-C complex is 373,000, assuming that the detergent content is negligible. The amount of bound Triton X-100 was estimated by repeating the sucrose density centrifugation in D₂O-containing buffers (19). The apparent sedimentation coefficient of recombinant GC-C in peak I in D₂O buffers was found to be 4–5% lower as compared to the $s_{20,w}$ in H₂O buffers, whereas no significant difference was observed for peak II ($n = 2$). We calculated that GC-C contained only $\sim 8\%$ detergent under our conditions, which results in a 6% increase in the M_r of the protein part of the GC-C complex, resulting in a M_r of almost 400,000 (Table I).

Molecular Mass Determination by SDS-PAGE—In both intestinal brush borders and 293-GC-C membranes STa-binding proteins with low M_r relative to full-length GC-C, were observed by SDS-PAGE after chemical cross-linking (11, 12, 23, 24). As shown in Fig. 3, predominantly 74- and 52-kDa 125 I-STa-binding proteins were present in the large guanylyl cyclase/STa-binding complex from brush borders. Solubilized 293-GC-C membranes were found to contain both high M_r STa receptors

FIG. 1. Gel filtration profiles of GC activity and STa binding from solubilized brush border and 293-GC-C membranes. Brush border caps (BBM; panels C and D) or 293-GC-C membranes (panels A and B) were solubilized in 0.5% Triton X-100 in the presence or absence of 1 mM ATP. The samples plus ATP (panels A and C) were directly applied to the gel filtration column and eluted in the presence of ATP, whereas the samples minus ATP (panels B and D) were incubated for 10 min at 37 °C prior to gel filtration and eluted in the absence of ATP. GC activity (●), determined with 1 mM MnGTP in the presence of 1 mM ATP in all fractions, and STa binding (▲) were assayed in 50- μ l aliquots and expressed as the activity present in the total fraction (400 μ l). Arrows indicate position of markers, from left to right: thyroglobulin, ferritin, and immunoglobulin G.

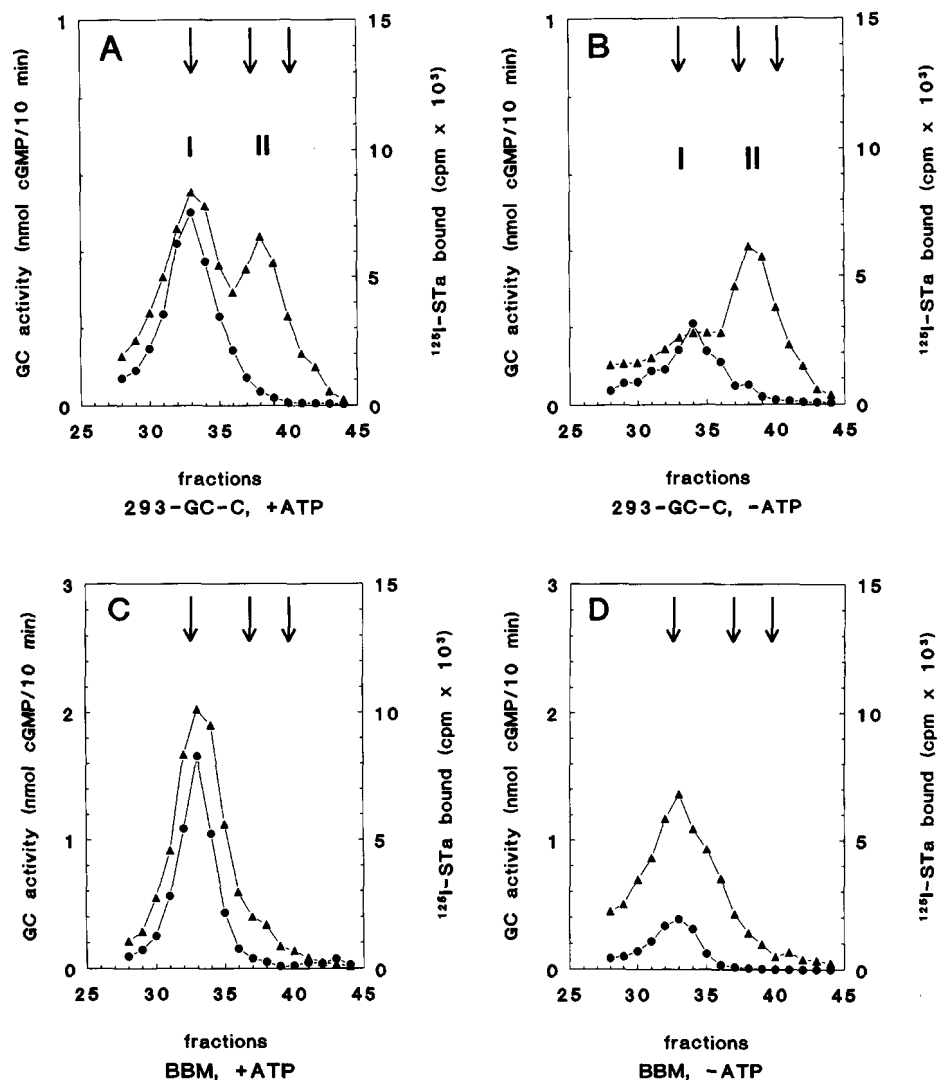


TABLE I

Hydrodynamic parameters and target sizes of STa binding and GC activity in brush border and 293-GC-C membranes

The hydrodynamic parameters were determined by gel filtration and sucrose density gradient centrifugation. The molecular masses were calculated from the Stokes radius and the $s_{20,w}$, and the target sizes were determined by radiation inactivation. The Stokes radius, $s_{20,w}$, and apparent mass represent values not corrected for possible detergent binding, in contrast to the corrected mass. Data are means \pm S.D. of three experiments with a few exceptions; the Stokes radius of 293-GC-C was of five, the $s_{20,w}$ of 293-GC-C of six, and the target sizes for STa binding of four experiments. BBM, brush border membranes; ND, not determined.

	BBM	293-GC-C	
		Peak I	Peak II
Stokes radius (nm)	8.0 \pm 0.3	8.5 \pm 0.3	6.0 \pm 0.3
Apparent $s_{20,w}$ (S)	11.3 \pm 1.1	10.4 \pm 0.7	6.5 \pm 0.7
Apparent mass (kDa)	380 \pm 39	371 \pm 30	164 \pm 18
Corrected mass (kDa)	ND	393 \pm 35	164 \pm 18
Target size (kDa)			
Basal GC	59 \pm 12	ND	
STa-stimulated GC	214 \pm 33	236 \pm 35	
STa binding	52 \pm 8	32 \pm 7	

(140 and 160 kDa), which are differently glycosylated forms of full-length GC-C (12), and multiple lower M_r STa-binding proteins (between 60 and 40 kDa). The high M_r STa receptors were enriched in peak I, whereas peak II consisted predominantly of lower M_r STa-binding proteins (Fig. 3).

We had noticed previously (12, 13) that in the absence of

reducing agents STa is apparently still bound to GC-C, as it causes a decrease in the mobility of a dimeric form of GC-C. We therefore directly applied the samples after incubation with ¹²⁵I-STa on a non-reduced SDS-PAGE gel, without prior chemical cross-linking. As shown in Fig. 3, a 120-kDa protein was the only STa receptor in total brush border preparation in the absence of reducing agent, which therefore is most likely composed of the 74- and 52-kDa STa receptors observed under reducing conditions. After separation by sucrose density centrifugation, the brush borders additionally contained a 170-kDa, 270-kDa, and a STa-binding protein just below the top of the gel. As these latter proteins were not observed in the original brush border preparation, they must have been generated as a consequence of the separation, presumably by oxidative cross-linking within the GC-C complex after the removal of stabilizing proteins. The 270-kDa STa-binding protein from brush borders probably represents a receptor dimer linked to two intracellular GC-C domains, as it was previously detected with an antibody against the C terminus of GC-C on immunoblots (12) and it runs in parallel with the 250–285-kDa STa receptors from 293-GC-C membranes (see Fig. 3), which are thought to represent GC-C dimers (13).

Solubilized 293-GC-C membranes were also found to contain a STa-binding protein, which runs just below the upper edge of the gel under non-reducing conditions. As shown in Fig. 4, this very high M_r GC-C complex can be clearly separated on a 5% gel and was estimated to have a M_r of approximately 350,000.

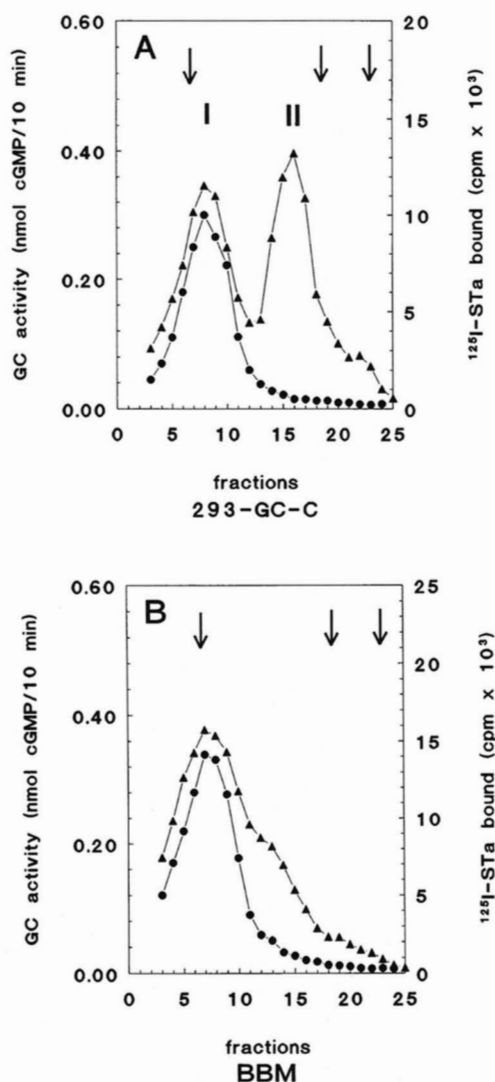


FIG. 2. Sucrose density centrifugation profiles of GC activity and STa binding from solubilized brush border and 293-GC-C membranes. Brush border caps (BBM; panel B) or 293-GC-C membranes (panel A), solubilized in 0.5% Triton X-100 in the presence of 1 mM ATP, were directly applied to 7.5–25% sucrose gradients. GC activity (●), determined with 1 mM MnGTP in the presence of 1 mM ATP, and STa binding (▲) were assayed in 50- μ l aliquots and expressed as the activity present in the total fraction (500 μ l). Arrows indicate position of markers, from left to right: catalase, bovine serum albumin, and cytochrome c.

As the dimeric forms of GC-C displayed an approximately 10–15% higher mobility on a 5% gel as compared to a 6% gel (*cf.* Figs. 3 and 4), the M_r of this complex might rather be around 400,000. This complex was also observed in immunopurified GC-C and most likely consists of a GC-C trimer (see Fig. 4). The lower M_r STa-binding proteins from 293-GC-C membranes, present in peak II, behaved as 110–75-kDa proteins under non-reducing conditions and are probably dimers of the 60–40-kDa STa-binding proteins observed under reducing conditions. As the M_r of peak II under native conditions was estimated to be 164,000 (see Table I), the lower M_r STa receptors might also form trimers under native conditions.

Radiation Inactivation Experiments—We further analyzed GC-C in native membranes by radiation inactivation, a technique that provides an estimate of the functional size of proteins (14). As shown in Fig. 5, basal GC activity from brush border membrane vesicles showed a complex response. Activity was stimulated at low dose, whereas a monoexponential decrease of the fractional activity was observed above 200 kGy.

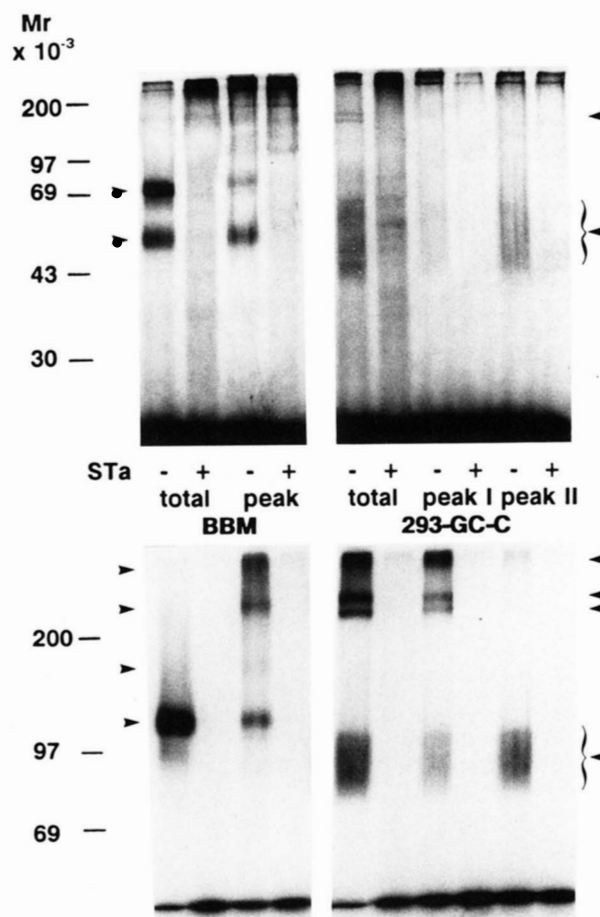


FIG. 3. ¹²⁵I-STa cross-linking of proteins from brush border and 293-GC-C membranes separated by sucrose density centrifugation. Solubilized brush borders (BBM) or 293-GC-C membranes were either separated on a 7.5–25% sucrose gradient or stored at 4 °C. After density centrifugation, peak fractions of STa binding (see Fig. 2) or stored preparations (total) were incubated for 45 min with ¹²⁵I-STa in the presence (+) or absence (–) of 1 μ M unlabeled STa at 37 °C. Upper panel, after ¹²⁵I-STa binding, samples were chemically cross-linked with 0.5 mM bis(sulfosuccinimidyl)suberate. Subsequently SDS-PAGE sample buffer containing β -mercaptoethanol was added, and samples were separated on a 10% polyacrylamide gel and autoradiographed for 30 days. Lower panel, after ¹²⁵I-STa binding, samples were treated directly with SDS-PAGE sample buffer without β -mercaptoethanol, separated on a 6% polyacrylamide gel, and autoradiographed for 4 days. Arrowheads indicate specific ¹²⁵I-STa-binding proteins, *i.e.* displaceable by unlabeled STa.

The D_{37} of this second phase was 312 ± 67 kGy ($n=3$), which reflects a target size of almost 60 kDa (see Table I). The GC activity determined in the presence of STa was also complex, as it was relatively more sensitive to low doses of radiation and became almost identical to the basal GC activity at doses above 200 kGy. However, the difference between the activity in the presence and absence of STa, which represents the STa-stimulated GC activity, decreased almost monoexponentially. The D_{37} of the STa-stimulated GC activity was 86 ± 11 kGy ($n=3$), whereas a similar D_{37} of 78 ± 10 kGy ($n=3$) was observed in 293-GC-C membranes, corresponding to target sizes of 210–240 kDa (see Fig. 5 and Table I). We were unable to determine the target size of basal GC-C activity in 293-GC-C membranes, as this activity was relatively low and was also attributable in part to a non-GC-C, azide-stimulatable GC activity (results not shown). Radiation analysis of the STa binding also revealed a complex behavior in brush border and in particular in 293-GC-C membranes (see Fig. 6). The D_{37} derived from the second monoexponential phase at doses above 200 kGy were 354 ± 49 and

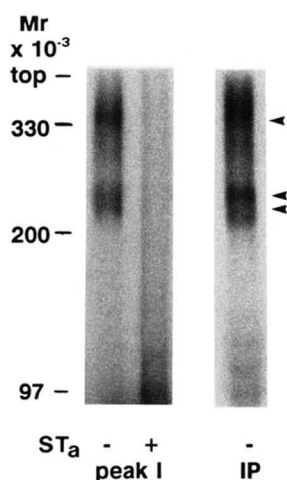


FIG. 4. Molecular weight determination of high M_r GC-C complexes by SDS-PAGE under non-reducing conditions. Solubilized proteins from 293-GC-C membranes were separated by sucrose density gradient centrifugation or immunoprecipitated with a GC-C specific antibody as described previously (13). After density centrifugation, peak fraction I of STa binding (see Fig. 2) or the immunopurified GC-C bound to protein A-Sepharose (IP) were incubated for 45 min with 125 I-STa in the presence (+) or absence (-) of 1 μ M unlabeled STa at 37 °C. After addition of SDS-PAGE sample buffer without β -mercaptoethanol, samples were separated on a 5% polyacrylamide gel. Arrowheads indicate specific 125 I-STa-binding proteins, i.e. displaceable by unlabeled STa. Top refers to the upper edge of the separation gel.

570 \pm 120 kGy ($n=4$) respectively for brush border and 293-GC-C membranes, corresponding to target sizes of 52 and 32 kDa (Table I).

DISCUSSION

Both gel filtration and velocity sedimentation experiments showed that STa-binding proteins from intestinal brush border membranes comigrated with GC activity at a position similar to that of recombinant GC-C. This seems in contradiction with earlier studies, reporting that the majority of the STa receptors are not coupled to GC activity (23, 24). However, in accordance with these studies we also found that the brush border STa receptors had relatively low M_r under denaturing conditions and are thus non-covalently coupled to the cyclase. The non-covalent coupling may have been disrupted in the earlier studies (23, 24), in which different isolation and solubilization methods were applied. As the crude brush border membrane preparation used by Hirayama *et al.* (24) contained a prominent 70-kDa high mannose type STa receptor, and the intracellular membranes of enterocytes were found to be enriched in the high mannose type of GC-C (12), their preparation may have been contaminated with intracellular membranes, in which GC-C may be cleaved differently as compared to brush border membranes. In accordance with this assumption, the low M_r STa receptors from the 293-GC-C membrane preparation, consisting of intracellular and plasma membranes could likewise be separated from the GC activity, in contrast to the STa binding activity in our brush border preparation. Since the latter fraction is almost devoid of intracellular membranes (16), and we applied mild solubilization conditions known to preserve the STa stimulation of GC-C, it is plausible that our brush border preparation resembles the physiological situation more closely.

Our finding that most of the low M_r STa-binding proteins in the brush border are coupled to guanylyl cyclase strongly suggests that they represent N-terminal fragments of GC-C. This conclusion is strengthened by the observation that GC activity and STa binding from brush borders behave functionally similar to recombinant GC-C (see also Refs. 11–13), and that most

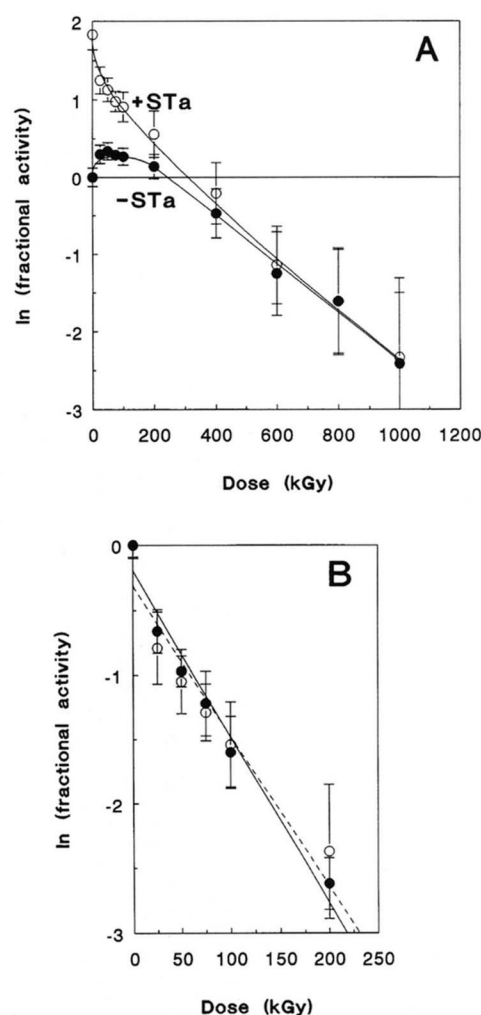


FIG. 5. Radiation inactivation of GC activity from brush border and 293-GC-C membranes. Brush border membrane vesicles or 293-GC-C cells were irradiated as described under "Experimental Procedures." Panel A, basal GC activity (●) and GC activity in the presence of 1 μ M STa (○) were determined in duplicate by incubation of the irradiated brush border membrane vesicles for 5 min at 37 °C with 1 mM MgGTP, 1 mM ATP, an ATP/GTP regenerating system, and 0.1% Triton X-100 to permeabilize the brush border membrane vesicles. Data are expressed as ln of the GC activity measured in the samples divided by the basal GC activity of non-irradiated brush border membrane vesicles. Panel B, STa-stimulated GC activity was determined by subtracting the basal GC activity from the GC activity in the presence of 1 μ M STa. GC activity was determined as described for panel A for brush border membrane vesicles (○, ---) or for 293-GC-C membranes (●, —), made from the irradiated cells. However, the 293-GC-C membranes were incubated for 10 min in the absence of Triton X-100. Data are expressed as ln of the STa-stimulated GC activity in the samples divided by the STa-stimulated GC activity of non-irradiated samples. Data are means \pm S.D. of three experiments.

of the immunodetectable GC-C in brush border membranes was identified previously as a 85-kDa C-terminal fragment (12). This concept is also corroborated by the observation of Cohen *et al.* (15), that low M_r STa receptors could be precipitated with an antibody directed against a N-terminal fragment of GC-C. The sizes of the STa receptors (74 and 52 kDa) and of the predominant C-terminal fragment of GC-C (85 kDa) indicate that GC-C is cleaved in the extracellular domain, presumably by an intestinal protease acting from the luminal side (see Fig. 7). Since GC-C was fully activatable in brush border membranes, the cleavage does not seem to impair its function.

The M_r of the solubilized GC-C complex, as calculated from the Stokes radius and the sedimentation coefficient, most closely matches that of a homotrimer, assuming a monomeric

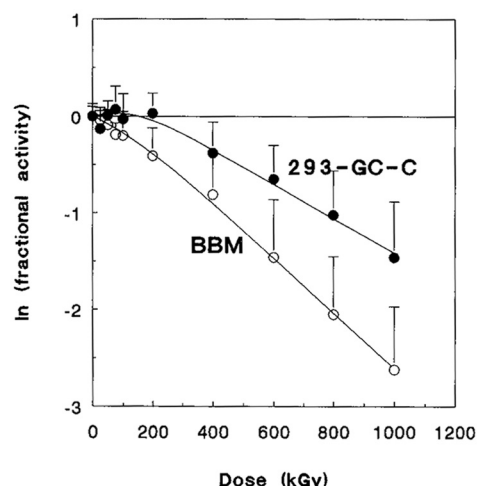


Fig. 6. Radiation inactivation of STa binding from brush border and 293-GC-C membranes. Brush border membrane vesicles (BBM) or 293-GC-C cells were irradiated as described under "Experimental Procedures." ^{125}I -STa binding was determined in brush border membrane vesicles (○) or 293-GC-C membranes (●), made from the irradiated cells. Data are expressed as the ln of the bound over free ^{125}I -STa (B/F) of the samples divided by the B/F of non-irradiated samples. Data are means \pm S.D. of four experiments.

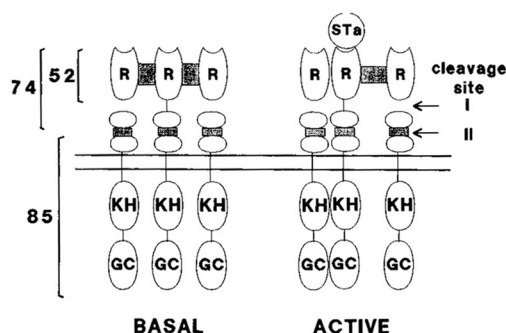


Fig. 7. Model for activation of GC-C and its proteolytic cleavage in brush border membranes. GC-C is a homotrimer in the basal state. After binding of presumably one STa molecule, the bond between two of the three subunits is stabilized. This internal dimerization is presumably a key step in the activation of the enzyme by STa. Gray boxes represent non-covalent bonds between protein subunits. This binding may become covalent by oxidation of SH groups and subsequent formation of intra- or intermolecular S-S bridges. Cleavage sites I and II are utilized by proteases in the intestinal lumen. The numbers on the left represent molecular masses (in kDa) of monomers of the various proteolytic fragments. R, receptor domain; KH, kinase homology domain; GC, guanylyl cyclase domain.

M_r of 140,000 (12). However, in SDS-PAGE in the absence of reducing agents, the apparent oligomeric state of GC-C was found to depend on the incubation conditions. A monomer was the predominant form of active GC-C in the absence of STa. A dimeric form was stabilized after incubation with STa, whereas a large aggregate, which we have now tentatively identified as a trimer, was observed after incubation under inactivating conditions (13). The target size of the STa-stimulated cyclase activity (210–240 kDa), derived from the radiation inactivation experiments, suggests that the functional unit of STa-activated GC-C is formed by a GC-C dimer. However, the active center of basal GC-C activity was found to reside on a much smaller unit of 60 kDa. This size would be consistent with a dimer of two adjacent cyclase domains (approximately 30 kDa each; Ref. 1). The radiation inactivation experiments further showed that the basal GC-C activity is increased at low doses. This can be explained by assuming that the cyclase activity is inhibited under basal conditions by a larger structure, which is more sensitive to radiation than the cyclase (25, 26). Since GC-C does

not depend on the presence of other proteins for its activation (13), this relatively large structure most likely resides within GC-C itself. A stimulation of basal activity by low radiation doses was also observed for the natriuretic receptor guanylyl cyclase (26, 27), suggesting the operation of similar activation mechanism within the receptor-GC family. The functional size of the STa binding site (52 and 32 kDa for brush border and 293-GC-C membranes, respectively) corresponds well with the size of the extracellular STa receptor domain of GC-C. Its slightly different size in brush border and 293-GC-C membranes might be caused by differences in proteolytically cleavage.

The various functional and physical sizes of GC-C revealed by our study can be explained most properly by the model depicted in Fig. 7. In the basal state, GC-C is a homotrimer in which the subunits are connected by relatively weak, SDS-disruptable bonds. This binding may become covalent by formation of intermolecular S-S bridges. After binding of STa to the extracellular domain, a conformational change is induced, which stabilizes the bond between two of the three subunits, and may cause an activation of the cyclase. The internal dimerization of the cyclase domains might be prevented in the basal state by the kinase homology and/or receptor domain, explaining the GC activation at low radiation doses. Following its activation, GC-C may gradually lose its activity, when there is no ATP bound to the kinase homology domain (13). It is tempting to speculate that the third subunit in the GC-C complex, which is not a part of the active dimer, plays a role in the inactivation of GC-C, since a trimeric form was stabilized after incubation under inactivating conditions.

A similar model was proposed for the activation of GC-A (4, 28), based on the observations that the cyclase domain of GC-A was active as a dimer (29), and that deletion of the kinase homology domain from GC-A resulted in a fully active cyclase (30). GC-A was also found to be an oligomer, presumably a tetramer, in the absence of ligand (31–33). However, the positions of the cross-linked GC-A complexes on 7.5% gels, identified as tri- and tetramers (33), look similar to those of di- and trimers of GC-C on our gels, suggesting that GC-A and GC-C might adopt a similar oligomeric state.

The formation of the GC-C trimer and the stabilization of the dimeric form after STa binding are probably functions of the extracellular domain, as the low M_r STa receptors from 293-GC-C membranes, which lack the intracellular domain, behaved as trimers on gel filtration and velocity sedimentation, and formed stable dimers after STa binding. The extracellular domains of GC-A expressed in 293 cells were also found to form trimeric complexes (33).

The observation that GC-C is a homotrimer raises the question of stoichiometry of the STa binding to the GC-C complex. The trace amount of ^{125}I -STa used in the cross-link experiments (*i.e.* far below saturation) makes it highly unlikely that the GC-C trimers bound more than one ^{125}I -STa molecule. Since all of the ^{125}I -STa-bound GC-C was observed to run at a similar position (with a lower mobility) as the "active" GC-C on SDS-PAGE under non-reduced conditions (13), we suggest that probably one molecule of STa is sufficient to convert the GC-C trimer into the lower mobility, presumably active conformation. In this model, however, it remains conceivable that the three subunits are not equivalent in the basal state, but have different affinities for STa, which could be modulated by binding of ATP or by binding of STa to the other subunits. This would result in complex binding kinetics, as indeed observed for both the intestinal STa receptor (34) and GC-A (28).

The model for the activation of GC-C proposed in this study shows some similarities with present models of growth hormone receptors, in particular the insulin receptor (35). Binding

of insulin to only one of the two putative insulin receptor subunits induces activation of a dimeric intracellular tyrosine kinase (36). The kinase homology domain of the membrane-bound guanylyl cyclases resembles the tyrosine kinase domain from the growth factor receptors (1, 4), making a comparison between these two classes of receptors attractive.

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