Rat Guanylyl Cyclase C Expressed in COS-7 Cells Exhibits Multiple Affinities for *Escherichia coli* Heat-Stable Enterotoxin†

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ABSTRACT: Intestinal cells exhibit binding sites with different affinities for *Escherichia coli* heat-stable enterotoxin (ST) and guanylin, suggesting the existence of different receptors for these peptides. Guanylyl cyclase C from intestinal cells has been identified as one receptor for these peptides. Equilibrium and kinetic binding characteristics of rat guanylyl cyclase C expressed in COS-7 cells were examined, employing ST, to determine if this receptor exhibited multiple affinities. Scatchard analysis of equilibrium binding yielded curvilinear isotherms consistent with the presence of high (pM) and low (nM) affinity sites. Kinetic analysis of binding demonstrated that these sites exhibited similar dissociation but different association kinetics. In addition, two distinct affinity states of low affinity sites were identified with dissociation constants of 0.15 and 5.85 nM. Association of ST and low affinity sites was biphasic, while dissociation from these sites was unimodal. Close agreement of equilibrium and kinetic dissociation constants suggested that low affinity sites were in the lowest affinity state at equilibrium. Comparison of the ligand dependence of guanylyl cyclase activity (EC_{50} = 110 nM) with receptor occupancy revealed that binding of ST to the lowest affinity state of low affinity sites (EC_{50} = 80 nM) is directly coupled to catalytic activation. These studies suggest that binding sites with different affinities for ST exhibited by intestinal cells reflect the expression of a single gene product, guanylyl cyclase C, rather than different receptors for the ligand. The shift in affinity state of low affinity sites and its correlation with catalytic activation suggest a central role for this phenomenon in mechanisms mediating receptor−effector coupling of membrane guanylyl cyclases.

Escherichia coli secrete a heat-stable enterotoxin (ST) that produces intestinal secretion by binding to specific receptors localized in apical membranes of intestinal mucosal cells (Gianella et al., 1983; Dreyfus et al., 1984; Frantz et al., 1984; Kuno et al., 1986; Cohen et al., 1987; Guarino et al., 1987). A homologue of this protein, guanylin, is produced by Paneth cells in intestinal crypts and likely plays a role in regulating intestinal fluid and electrolyte homeostasis (Currie et al., 1992; de Sauvage et al., 1992a; Schulz et al., 1992). Receptors for these ligands, guanylyl cyclase C (GCC), have been identified in intestinal cells which belong to the family of proteins possessing peptide binding and guanylyl cyclase catalytic activities (Schulz et al., 1990). Ligand−receptor interaction results in the activation of the catalytic domain and accumulation of intracellular cGMP. It is this cyclic nucleotide that directly mediates increases in intestinal secretion by activating cyclic AMP-dependent protein kinase, phosphorylating the cystic fibrosis transmembrane regulator, and increasing the efflux of chloride into the intestinal lumen (Chao et al., 1994; Tien et al., 1994).

Receptors for ST and guanylin exhibit significant structural and functional heterogeneity, suggesting that multiple receptors for these peptides representing different gene products may exist (Kuno et al., 1986; Waldman et al., 1986, 1994; Ivens et al., 1990; Thompson & Gianella, 1990; Hugues et al., 1991, 1992; Katwa et al., 1991; Hirayama et al., 1992; Crane et al., 1992; Forte et al., 1993; Hakki et al., 1993a,b; Mann et al., 1993; Cohen et al., 1993; Carrithers et al., 1994). Affinity labeling of receptors with ligand identified multiple high and low molecular weight proteins that specifically bind to ST in intestinal cells (Kuno et al., 1986; Ivens et al., 1990; Thompson & Gianella, 1990; Hakki et al., 1993a,b; Carrithers et al., 1994). Similarly, ST binding proteins with different structural characteristics have been purified from the lipid bilayer and the cytoskeletal components of intestinal membranes (Waldman et al., 1986; Hakki et al., 1993a,b). Also, ST receptors with distinctly different binding properties have been identified in intestinal membranes isolated from rodents and humans (Hugues et al., 1991; Crane et al., 1992; Carrithers et al., 1994; Waldman et al., 1994). Finally, ST binding sites which are not coupled to guanylyl cyclase have been identified in cells derived from intestinal mucosa (Mann et al., 1993).

Although there is significant heterogeneity of ST binding proteins in intestinal cells, much of this heterogeneity can be ascribed to differential post-translational processing of GCC. Multiple ST binding proteins which correlate closely in size to those in intestinal cells have been identified in mammalian cells expressing exogenous rat or human GCC (de Sauvage et al., 1992b; Vaandrager et al., 1993a).

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Multiple ST binding proteins in cloned systems have been demonstrated to reflect, at least in part, differential proteolysis and glycosylation of GCC (de Sauvage et al., 1992b; Vaandrager et al., 1993a,b). However, binding in mammalian cells expressing exogenous GCC exhibits only a single population of functional binding sites (de Sauvage et al., 1992b). These observations suggest that the different populations of ST binding sites identified in intestinal cells may reflect the function of receptors other than those coupled to GCC. In the present studies, the binding characteristics of rat GCC exogenously expressed in COS-7 monkey kidney cells were examined to determine if heterogeneity of receptor function reflected multiple populations of ST receptors encoded by different genes or different activities of a single gene product, GCC.

MATERIALS AND METHODS

Construction of Plasmid. Rat GCC cDNA cloned in pBluescript SK (Schulz et al., 1990) was cut with *Eco*RI, and a 3.7 kb DNA fragment was isolated and subcloned into the *Eco*RI-cut vector pMT2 (Shaw et al., 1991; Wong et al., 1985). This vector permits high levels of gene expression transiently in mammalian cells, reflecting its strong adenovirus late promoter, SV 40 origin of replication, and SV 40 T antigen-encoding sequence (Shaw et al., 1991; Wong et al., 1985).

Cell Culture and Transfections. COS-7 cells were maintained in DMEM Ham's F12 media with 10% fetal bovine serum and penicillin and streptomycin (10 units/mL and 10 μg/mL, respectively). Rat GCC cDNA (Schulz et al., 1990) was subcloned into pMT2 and employed to transfect COS-7 cells using DEAE-dextran and chloroquine (Cullen, 1987). Ten micrograms of DNA was employed to transfect cells in 75 cm^2 flasks, and transfected cells were grown to 80% confluence, trypsinized, split 1:3, and harvested after 72 h. Transfection was confirmed employing ST binding and guanylyl cyclase assays.

Preparation of Membranes. Cells expressing GCC were washed 3 times with ice-cold buffer containing 50 mM Tris-HCl, pH 7.6, 1 mM EDTA, 1 mM PMSF, and 1 mM dithiothreitol (TED), scraped, and resuspended in TED. Cell suspensions were homogenized with 15 strokes of a Teflon-on-glass homogenizer. Where indicated, homogenates were centrifuged at 105000*g* × 30 min and the resulting membranes suspended in TED.

ST Receptor Binding. ST receptor binding to membranes was quantified as described previously (Crane et al., 1992; Carrithers et al., 1994). COS-7 membranes were incubated with ^{125}I-ST in binding buffer containing 50 mM Tris-HCl, pH 7.6, 0.66 mM cystamine, 0.1% bacitracin, 450 mM NaCl, and 1 mM EDTA at 37 °C. Binding was terminated by vacuum filtration of the reaction mixture through Whatman GF-B filters presoaked in 0.3% poly(ethylenimine). Equilibrium binding studies were performed by incubating 30 μg of membrane or homogenate protein with concentrations of ^{125}I-ST ranging from 0.122 pM to 20 nM at 37 °C for 120 min. Association and dissociation of labeled ST and receptors were examined at 37 °C using either 10 pM ^{125}I-ST and 60 μg of protein (high affinity sites) or 2.9 nM ^{125}I-ST and 30 μg of protein (low affinity sites). The assay volume used for high affinity association and dissociation was 300 μL, and that used for low affinity association and dissociation was 100 μL. Dissociation of ligand−receptor complexes was initiated with an excess of unlabeled ST (5 μM; Hugues et al., 1991; Crane et al., 1992).

Guanylyl Cyclase. Guanylyl cyclase activity was measured as described previously (Crane et al., 1992). Briefly, incubations contained 30 μg of membrane protein, 1.2 mM isobutylmethylxanthine, 50 mM Tris-HCl, pH 7.6, a regenerating system composed of 15 mM creatine phosphate and 2.7 units of creatine phosphokinase, and various concentrations of ST. Incubations were conducted for 5 min at 37 °C and terminated by the addition of ice-cold 50 mM sodium acetate, pH 4.0, followed by immersion in a boiling water bath for 3 min. Cyclic GMP produced was quantified by radioimmunoassay (Patrinellis & Waldman, 1995).

Accumulation of Cyclic GMP in Whole Cells. COS-7 cells in 35 × 10 mm dishes transfected with pMT2 alone or that vector containing rat GCC were washed 3 times with serum-free DMEM, containing 25 mM HEPES, pH 7.3, and 0.5 mM isobutylmethylxanthine, and then incubated in that medium containing 1×10^{-6} M unlabeled ST for 10 min at 37 °C. Incubations were terminated by the addition of ice-cold 95% ethanol and centrifuged to remove the protein pellet, and resulting supernatants were dried under vacuum. Residues were resuspended in 500 μL of 0.1 M HCl, and cyclic GMP was determined by ELISA. The cyclic GMP ELISA was conducted by a modification of a procedure described previously (Linden et al., 1992). Briefly, 2′-*O*-monosuccinylguanosine 3′,5′-cyclic monophosphate (ScG-MP; Sigma Chemical Co., St. Louis) was conjugated to poly(L-lysine) (Linden et al., 1992), and this conjugate used to coat 96-well microtiter plates. Standards and samples to be assayed for cGMP were prepared in 0.1 M HCl and acetylated employing triethylamine and acetic anhydride (4.5:1 v/v). Acetylated samples and goat anti-cGMP polyclonal antibody (1:10 000) were added to wells, incubated overnight (4 °C), washed in phosphate-buffered saline (pH 7.4) containing 0.1% Tween-20, and incubated with rabbit anti-goat IgG coupled to horseradish peroxidase for 1 h at 37 °C. Plates were developed using 2.2-azinobis[3-ethylbenzthiazolinesulfonate(6)] as substrate, incubating 30 min at room temperature, and absorbance quantified at 405 nm in a Bio-Tek Model EL308 microtiter plate reader. Employing this ELISA, the linear range of cyclic GMP quantification was 10−500 fmol, and interassay variability was routinely <10%.

Miscellaneous. Protein concentration was quantified by the method of Bradford (Bio-Rad, Richmond, CA; Bradford, 1976). ST was purified from culture broth and monoiodinated to a final specific activity of 2000 Ci/mmol as described previously (Thompson et al., 1985). Equilibrium binding curves were fitted and constants obtained employing Cigale on a Macintosh IIsi. Cigale was written by M. Bordes (Institute de Pharmacologie Cellulaire et Moleculaire, Universite de Nice, Sophia Antipolis, France; Hugues et al., 1991; Crane et al., 1992; Waldman et al., 1994). Linear regression to obtain association and dissociation constants was performed employing Cricket Graph (Cricket Software, Malvern, PA) on a Macintosh IIci. All assays were performed in duplicate or triplicate; data presented are representative of at least 3 experiments, values presented are means + SEM, and n = number of observations.

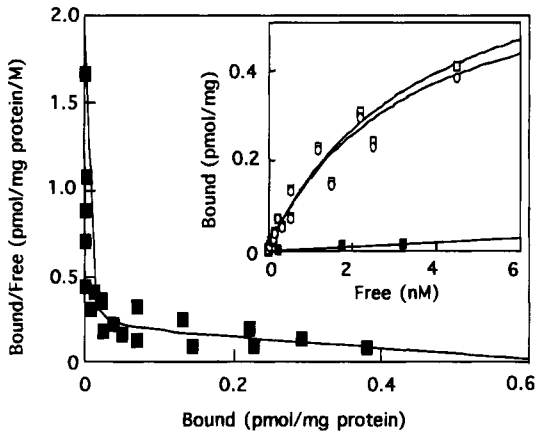

FIGURE 1: Scatchard analysis of direct equilibrium binding of labeled ST using membranes from COS-7 cells expressing rat GCC. Experiments were performed as described in Materials and Methods. Inset: direct equilibrium binding. Open squares, total binding; open circles, specific binding; closed squares, nonspecific binding.

RESULTS

Expression of Rat GCC in COS-7 Cells. Membranes prepared from COS-7 cells transfected with pMT2 containing the cDNA for rat GCC specifically bound ^{125}I-ST (386 ± 26 fmol/mg of protein, $n = 3$) compared to control cells transfected with vector only (1.0 ± 2.0 fmol/mg of protein, $n = 3$) when binding was performed using 10 pM labeled ligand under equilibrium conditions. Also, addition of unlabeled ST (10^{-6} M) to cells transfected with the vector containing GCC induced an increase in intracellular cyclic GMP (44 ± 9 pmol/(min·mg of protein), $n = 3$ to 764 ± 108 pmol/(10 min·mg of protein), $n = 4$) compared to cells transfected with vector alone (43 ± 9 pmol/(10 min·mg of protein) to 38 ± 7 pmol/(10 min·mg), $n = 3$). In addition, unlabeled ST (10^{-6} M) activated guanylyl cyclase in the presence of adenine nucleotides in membranes prepared from cells transfected with the vector containing GCC (4.09 ± 0.43 pmol/(min·mg of protein) to 14.95 ± 0.58 pmol/(min·mg of protein), $n = 3$) but was without effect on enzyme activity in cells transfected with vector alone (4.33 ± 0.28 pmol/(mg·min) to 4.89 ± 0.12 pmol/(mg·min), $n = 3$). These data demonstrate that COS-7 cells transfected with pMT2 containing rat GCC expressed the functionally coupled protein.

Direct Equilibrium Binding Studies. Radiolabeled ST specifically bound to membranes prepared from COS-7 cells transfected with pMT2 containing GCC in a concentration-dependent and saturable fashion (Figure 1, inset). Analysis of equilibrium binding by the method of Scatchard yielded curvilinear isotherms, suggesting the presence of two populations of binding sites with high (pM) and low (nM) affinities for ST (Table 1). Variability of B_{max} values for high and low affinity sites likely reflects variability of transfection efficiencies between preparations (Table 1). The equilibrium binding characteristics of high and low affinity ST binding sites exhibited by rat GCC expressed in COS-7 cells compares closely with those reported previously for this protein in rat intestine (Hugues et al., 1991; Crane et al., 1992; Hakki et al., 1993a,b), suckling mouse intestine (Waldman et al., 1994), and human colorectal tumors and cell lines (Forte et al., 1993; Carrithers et al., 1994).

Kinetic Binding Studies. (A) Association Kinetics of High Affinity Sites. The presence of two populations of ST binding sites in membranes prepared from COS-7 cells expressing rat GCC was confirmed by analyses of the kinetics of association and dissociation. Association kinetics of high affinity binding sites were examined employing 10 pM of ^{125}I-ST (Figure 2A). Employing this ligand concentration, the relative contribution to occupancy of high and low affinity sites at equilibrium may be calculated by employing the equation:

$$B = (B_{max}L)/(K_D + L) \quad (1)$$

where B represents ligand bound at equilibrium, B_{max}, the maximum number of binding sites of the family of receptors being studied, L, the ligand concentration, and K_D, the dissociation constant of the ligand−receptor complex (Mazella et al., 1983). Employing 10 pM labeled ligand, >75% of the observed binding in these studies was contributed by high affinity compared to low affinity sites. Over the time course of these experiments, free labeled ST varied less than 30%. Association performed under these conditions fulfilled the criteria for being pseudo first order since ligand concentrations remained essentially constant and only a single class of receptors was visualized at a given ligand concentration (Molinoff et al., 1981; Mazella et al., 1983). ^{125}I-ST bound in a time-dependent fashion to high affinity sites in membranes prepared from COS-7 cells transfected with rat GCC, achieving equilibrium after approximately 60 min at 37 °C (Figure 2A). Semilogarithmic transformation of association binding data yielded linear isotherms, confirming the assumption that only a single class of high affinity receptors was visualized at the ligand concentration employed (Figure 2A, inset). The slopes of the semilogarithmic transformations of association yielded an observed association rate constant for high affinity receptors ($k_{obs\text{-}H}$) of (1.92 ± 0.80) × 10^{-2}.

(B) Association Kinetics of Low Affinity Sites. Association kinetics of low affinity sites were examined employing 2.9 nM of ^{125}I-ST, wherein >95% of the observed binding was contributed by low affinity compared to high affinity sites (Figure 2B). Over the time course of these experiments, free labeled ST varied less than 10% and association performed under these conditions fulfilled the criteria for being pseudo first order (Molinoff et al., 1981; Mazella et al., 1983). ^{125}I-ST bound in a time-dependent fashion to low affinity sites in membranes prepared from COS-7 cells transfected with rat GCC, achieving equilibrium after about 30 min at 37 °C (Figure 2B). Semilogarithmic transformation of these data yielded curvilinear isotherms, indicating a complex reaction with at least two association events occurring at different rates (Figure 2B, inset). Individual rate constants were determined by resolving the curvilinear semilogarithmic transformation into two linear components (Figure 2B, inset). The rate constant of the lowest affinity state of the low affinity site, $k_{obs\text{-}L2}$, was determined from the slope of linear regression of the semilogarithmic plot of time points greater than 10 min. At those time points, there was virtually no contribution from the more rapidly associating component, R_{L1}, to the overall rate of association. The rate constant for the more rapidly associating component, $k_{obs\text{-}L1}$, was calculated by subtracting the contribution of binding to R_{L2} from total specific binding, at each time point, before semilogarithmic transformation (Crane et al., 1992). The values obtained for the observed association rate constants of the different affinity states of the low affinity receptor were as

Table 1: Binding Characteristics of Rat GCC Exogenously Expressed in COS-7 Cells[a]

binding characteristics	high affinity sites	low affinity sites	
		higher affinity state	lower affinity state
K_D (M) (equilibrium)	$(0.42 \pm 1.53) \times 10^{-11}$	ND	$(2.2 \pm 1.08) \times 10^{-9}$
B_{max} (pmol/mg)	$(4.07 \pm 3.02) \times 10^{-2}$	ND	2.09 ± 2.9
k_{obs} (min^{-1})	$(1.92 \pm 0.80) \times 10^{-2}$	$(1.97 \pm 0.85) \times 10^{-1}$	$(1.02 \pm 0.69) \times 10^{-2}$
k_a (M^{-1} min^{-1})[b]	$(1.16 \pm 0.53) \times 10^{9}$	$(7.0 \pm 1.6) \times 10^{7}$	$(1.76 \pm 1.0) \times 10^{6}$
k_d (min^{-1})[b]	$(1.38 \pm 0.4) \times 10^{-2}$	$(1.03 \pm 0.26) \times 10^{-2}$	$(1.03 \pm 0.26) \times 10^{-2}$
K_D (M) (kinetic)	$(1.19 \pm 0.46) \times 10^{-11}$	$(1.47 \pm 0.25) \times 10^{-10}$	$(5.85 \pm 0.4) \times 10^{-9}$

[a] Experiments were performed as described in Materials and Methods. The data represent the means of at least three experiments± SEM. [b] The binding constants k_a and k_d were calculated as described in Results.

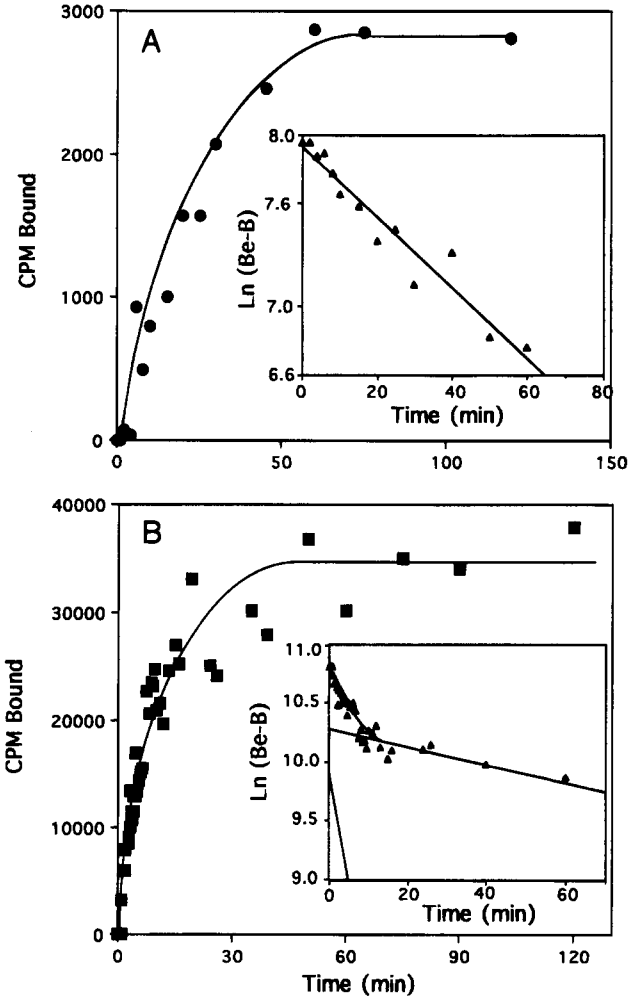

FIGURE 2: Time course of association of 10 pM (top panel) and 2.9 nM (bottom panel) ^{125}I-ST to membranes obtained from COS-7 cells expressing rat GCC. Reactions were initiated by the addition of membranes and aliquots removed at the indicated times. Inset, semilogarithmic transformation of the data: $y = (B_e - B)$, where B_e and B represent bound ligand at equilibrium and at time x, respectively. The curvilinear relationship exhibited in the inset of the bottom panel (closed triangles) was resolved into two linear components (solid lines). The semilogarithmic transformations of binding to the higher and lower affinity states of low affinity binding sites (bottom panel) are represented by the lines with the steeper and shallower slopes, respectively.

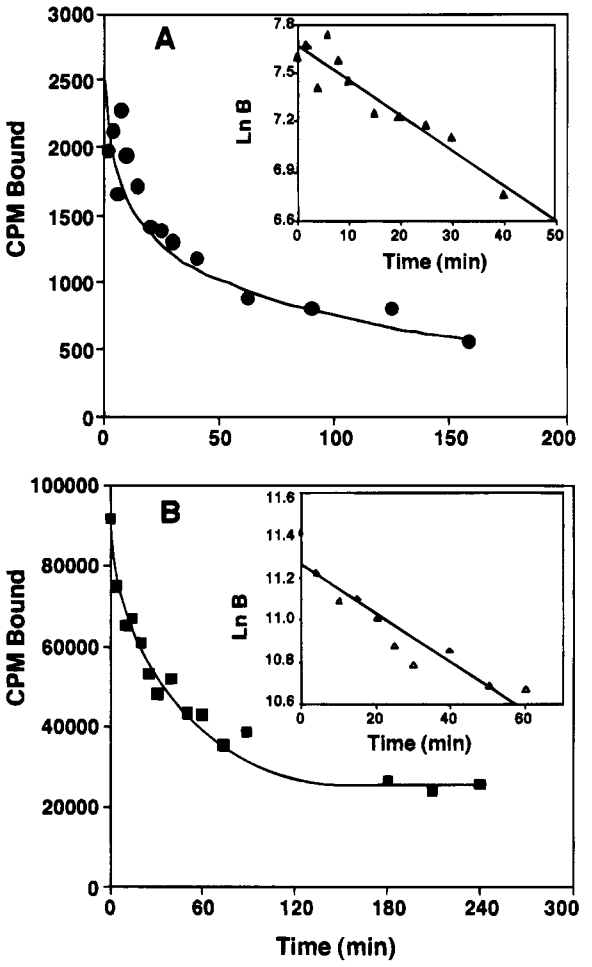

FIGURE 3: Time course of dissociation of labeled ST from membranes obtained from COS-7 cells expressing rat GCC. Dissociation was initiated by the addition of excess (5 μM) unlabeled ST to aliquots of association reactions conducted with 10 pM (upper panel) or 2.9 nM (lower panel) ^{125}I-ST after equilibrium had been achieved. Aliquots were removed and binding was quantified at the time points indicated as described in Materials and Methods. Insets, semilogarithmic transformations of the data: B = the amount of ligand bound at the indicated time.

follows: $k_{obs\text{-}L1} = (1.97 \pm 0.85) \times 10^{-1}$ min^{-1} and $k_{obs\text{-}L2} = (1.02 \pm 0.69) \times 10^{-2}$ min^{-1}.

(C) Dissociation Kinetics of High and Low Affinity Sites. Dissociation of labeled ST bound to high and low affinity receptors was examined by the addition of a large excess (5 μM) of unlabeled ligand. Dissociation was performed employing receptor−ligand complexes formed under equilibrium conditions in the presence of 10 pM or 2.9 nM ^{125}I-ST since, under these conditions, most of the dissociation visualized was contributed by high or low affinity binding sites, respectively (Figure 3). Binding to low affinity sites was only partially displaceable as described previously (Figure 3B; Frantz et al., 1984; Guarino et al., 1987; Hugues et al., 1991; Crane et al., 1992; Waldman et al., 1994). Indeed, about 25% of the associated ST remained bound to membranes after maximum dissociation was achieved (Figure 3B). In contrast, binding to high affinity sites could be nearly completely displaced by unlabeled ST, as demonstrated

previously (Figure 3A; Hugues et al., 1991). Presumably, binding remaining associated with low affinity sites after maximum dissociation reflects ST−receptor complexes that are nondissociable (Hugues et al., 1991). However, this interaction is not covalent since ST−receptor complexes can be completely dissociated by employing a variety of stringent conditions (Dreyfus & Robertson, 1984). The high concentration of unlabeled ST (5 μM) in dissociation incubations effectively prevented reassociation of labeled ST with receptors. Consequently, dissociation of labeled ST from high and low affinity sites was first order. Indeed, the semilogarithmic transformations of the dissociation data yielded linear isotherms whose slopes represent the dissociation rate constants of high ($k_{d\text{-}H}$ = (1.38 ± 0.40) × 10^{-2}min^{-1}) and low ($k_{d\text{-}L}$ = (1.03 ± 0.26) × 10^{-2}min^{-1}) affinity sites (Figure 3, panels A and B, insets). Rate constants for dissociation from low affinity sites were determined only for the dissociable fraction of the binding to those sites (Hugues et al., 1991). These data demonstrate that, for rat GCC expressed in COS-7 cells, the dissociation rate constant is independent of the binding site occupied.

(D) Determination of Association Rate Constants of High and Low Affinity ST Binding Sites and Comparison of Kinetic and Equilibrium Binding Parameters. Once $k_{obs\text{-}H}$, $k_{obs\text{-}L1}$, $k_{obs\text{-}L2}$, $k_{d\text{-}H}$, and, $k_{d\text{-}L}$ were defined, the second order rate constants of association of labeled ST with high affinity sites and with the high and low affinity states of low affinity sites were calculated using the relationship:

$$k_a = (k_{obs} - k_d)/[L] \quad (2)$$

where k_a and k_d represent the second order rate constant of association and first order rate constant of dissociation of the complex formed between ^{125}I-ST and receptors (Table 1; Mazella et al., 1983; Hugues et al., 1991). These rate constants for rat GCC expressed in COS-7 cells were closely comparable to those exhibited by native GCC in membranes obtained from rat intestine (Hugues et al., 1991; Crane et al., 1992). Determining k_a and k_d permitted calculation of K_D for high affinity sites and the high and low affinity states of low affinity sites (Table 1). Dissociation constants for high affinity sites and the low affinity state of low affinity sites determined by kinetic methods were in close agreement with those obtained employing equilibrium binding techniques. In addition, equilibrium and kinetic binding characteristics of rat GCC expressed in COS-7 cells were closely comparable to those obtained for these binding sites in native rat intestinal membranes (Hugues et al., 1991; Crane et al., 1992).

(E) Correlating Binding Site Occupancy with Enzyme Activation. The concentration dependence of guanylyl cyclase activation by ST was compared with that of fractional occupancy of high affinity binding sites and high and low affinity states of low affinity binding sites in membranes from COS-7 cells expressing rat GCC (Figure 4). Fractional occupancy of binding sites, f, was calculated from kinetic and equilibrium binding parameters empirically determined in the present studies, as a function of ligand concentration, according to the relationship (Boeynaems & Dumont, 1980):

$$f = B_e(1 - e^{-k_{obs}t})/B_{max} \quad (3)$$

where B_e represents bound ligand at equilibrium and is

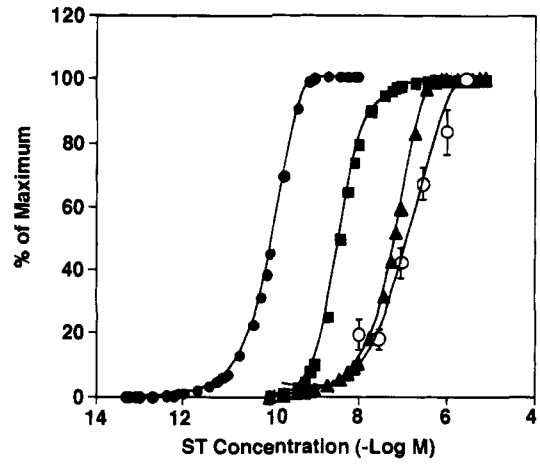

FIGURE 4: Relationship of guanylyl cyclase activation (open circles) to receptor occupancy of high affinity receptors (closed circles), high affinity state of low affinity receptors (closed squares), and low affinity state of low affinity receptors (closed triangles) in membranes from COS-7 cells expressing GCC. Guanylyl cyclase activity was estimated by radioimmunoassay as described in Materials and Methods and expressed as a percentage of maximum activity. Fractional receptor occupancy was calculated from kinetic and equilibrium binding data, as described in Results. The results represent at least three experiments. Error bars = SEM.

calculated according to the equation:

$$B_e = (B_{max}[L])/(K_D + [L]) \quad (4)$$

where [L] is ligand concentration and B_{max} and K_D were empirically determined from equilibrium binding analyses of each binding site and state. The observed association rate constant, k_{obs}, employed to determine fractional occupancy, was calculated according to the relationship:

$$k_{obs} = k_a[L] + k_d \quad (5)$$

where k_a and k_d were determined empirically for each binding site and state (Table 1). The time (t) employed for calculating fractional occupancy, 5 min, reflected the incubation time in studies of the concentration dependence of activation of guanylyl cyclase by ST. Employing these calculations, the concentration dependence of fractional occupancy of high affinity sites and the high and low affinity states of low affinity sites at 5 min was determined (Figure 4). The mean EC_{50}s of fractional receptor occupancy were 0.1 nM, 5.0 nM, and 80.0 nM, for high affinity sites and high and low affinity states of low affinity sites, respectively. ST activated GCC expressed in COS-7 cells in a concentration-dependent and saturable fashion (Figure 4). Guanylyl cyclase was activated a maximum of 4-fold by ST, which exhibited an EC_{50} of 110 nM, in close agreement with previous observations obtained by examining GCC in intestinal membranes (Carr et al., 1989; Hugues et al., 1991; Crane et al., 1992; Carrithers et al., 1994; Waldman et al., 1994). Activation of GCC by ST correlates closely with occupation of the low affinity state of low affinity sites, since the EC_{50} values for these events were nearly coincident. In contrast, the high affinity state of low affinity sites and high affinity sites are fully occupied by concentrations of ligand which have little effect on GCC catalytic activity. Activation of guanylyl cyclase by ST cannot be directly examined after 2 h of incubation because ligand−receptor interaction induces a rapid, time-dependent inactivation of this enzyme, after

which receptor−effector coupling cannot be detected (Waldman et al., 1984; Vaandrager et al., 1993a,b).

DISCUSSION

These studies are the first to fully define the binding characteristics of GCC exogenously expressed in mammalian cells. They demonstrate that cells expressing GCC exhibit both high and low affinity ST binding sites, as observed previously in intestinal cells (Hugues et al., 1991; Crane et al., 1992; Hakki et al., 1993a,b; Forte et al., 1993; Carrithers et al., 1994; Waldman et al., 1994). High and low affinity sites for ST were identified in COS-7 cells expressing GCC employing equilibrium and kinetic binding analyses. These different approaches yielded independent estimates of the dissociation constants of high and low affinity sites which were nearly identical. Also, the binding constants of ST receptor sites in membranes from COS-7 cells expressing rat GCC reported herein compare closely with those obtained previously in membranes from intestinal cells (Hugues et al., 1991, Crane et al., 1992, Hakki et al., 1993a; Carrithers et al., 1994; Waldman et al., 1994). In addition, the present studies demonstrate a ligand-dependent shift in affinity of low affinity receptors and correlation of the occupancy of the lowest affinity state of these sites with cyclase activation, as demonstrated previously in rat intestinal membranes (Crane et al., 1992).

The present studies suggest that ST binding sites with different affinities in intestinal cells reflect the expression of a single gene product, GCC, rather than multiple gene products encoding different binding proteins, since COS-7 cells do not possess endogenous ST binding nor ST-coupled guanylyl cyclase. Alternatively, transfection of COS-7 cells with exogenous cDNA may induce the expression of a binding site for ST in addition to GCC. Transfection of COS-7 cells and 293 human embryonic kidney cells with human cDNA containing a specific Alu repeat sequence induced the expression of an apparent low affinity binding site for ST that was not coupled to activation of guanylyl cyclase nor associated with the expression of GCC (Almenoff et al., 1994). It was suggested that this cDNA induced the expression of low affinity ST binding sites by a *trans*-acting mechanism possibly involving RNA−RNA or RNA−protein interactions (Almenoff et al., 1994). However, it is unlikely that the results presented herein reflect the activation of a similar binding site since, in those earlier studies, direct binding of labeled ST to transfected cells could not be detected (Almenoff et al., 1994). In contrast, high and low affinity ST binding sites in membranes from transfected COS-7 cells were quantifiable in direct binding studies by equilibrium or kinetic analysis.

It was suggested previously that identification of high and low affinity sites supports the hypothesis that different ST-binding proteins may be expressed by intestinal and other cells (Kuno et al., 1986; Waldman et al., 1986, 1994; Ivens et al., 1990; Thompson & Gianella, 1990; Hugues et al., 1991, 1992; Katwa et al., 1991; Hirayama et al., 1992; Crane et al., 1992; Forte et al., 1993; Hakki et al., 1993a,b; Mann et al., 1993; Cohen et al., 1993; Carrithers et al., 1994). Indeed, significant heterogeneity of structure and subcellular localization of ST binding proteins have been observed in intestinal cells, only some of which reflect post-translational processing of GCC (Kuno et al., 1986; Waldman et al., 1986; Ivens et al., 1990; Thompson & Gianella, 1990; Katwa et al., 1991; Hugues et al., 1992; Hirayama et al., 1992; Hakki et al., 1993 a,b; Cohen et al., 1993). Also, ST binding activity in cells which is not associated with guanylyl cyclase activation or GCC expression has been described (Hugues et al., 1992; Hirayama et al., 1992; Hakki et al., 1993b; Mann et al., 1993; Almenoff et al., 1994). In addition, ST may induce responses in extraintestinal organs, such as kidney, in which GCC is not expressed, suggesting that other receptors to which that peptide binds may exist (Lima et al., 1992; Schulz et al., 1992). Furthermore, atrial natriuretic peptides, which bind to members of the membrane receptor−guanylyl cyclase family of proteins, guanylyl cyclases A and B, also bind to other receptors which are different gene products and not coupled to guanylyl cyclase (Garbers & Lowe, 1994). The present studies suggest that high and low affinity sites for ST and guanylin observed in cells of intestinal origin reflect the activity of a single gene product, GCC, rather than multiple gene products. However, these studies do not eliminate the possibility that receptors for ST and homologous ligands which are products of different genes may be expressed in intestinal and other cells.

Human GCC expressed in 293 human embryonic kidney cells exhibited a single class of ST binding sites with intermediate affinity (97 pM) under equilibrium conditions, and linear association kinetics, in contrast to results presented herein (deSauvage et al., 1992b). The contrast between those and the present results do not reflect differences in the mammalian cell expression systems employed since rat GCC stably expressed in 293 cells exhibits binding characteristics which are identical to those obtained with rat GCC transiently transfected in COS-7 cells (data not shown). Differences in binding characteristics may reflect the species of origin of the GCC employed in these studies, although ST binding in human cells derived from intestine exhibits high and low affinity sites with binding characteristics similar to those presented herein (Forte et al., 1993; Carrithers et al., 1994). In earlier studies of exogenously expressed human GCC, the lowest concentration of labeled ST employed in equilibrium binding analyses appeared to be 10 pM (deSauvage et al., 1992b). At this ligand concentration, most (>70%) high affinity sites are occupied, rendering them inapparent by Scatchard analysis. Also, equilibrium binding characteristics in those earlier studies were defined by employing competitive binding analysis which is less sensitive than direct binding for detecting sites with different affinities representing a small proportion of the total receptors. Furthermore, in those earlier studies, association of ST and GCC was examined employing 25 pM of labeled ST. At this concentration of ligand, the majority (>60%) of binding is contributed by high affinity sites which exhibit linear association kinetics. In addition, in those studies association was with at relatively infrequent intervals at times >10 min. The shift from higher to lower affinity of low affinity sites can be observed only when binding is quantified early and frequently during the time course of association (Figure 2, inset; Crane et al., 1992).

The mechanisms by which expression of GCC results in the production of ST binding sites with different affinities remain unclear. Membrane receptor−guanylyl cyclases undergo ligand-independent oligomerization (Chinkers et al., 1992; Iwata et al., 1991; Vaandrager et al., 1994; Garbers & Lowe, 1994). In the absence of ligand, basal GCC appears

to be organized as trimers or tetramers in cell membranes (Vaandrager et al., 1994). The role of changes in oligomeric state in coupling ligand−receptor interaction to catalytic activation remains unclear. However, the presence of high and low affinity sites in cells expressing GCC could reflect different oligomeric states of this protein. Also, analysis of the primary structure of GCC reveals 8 potential glycosylation sites in the extracellular domain (Schulz et al., 1990). Rat GCC expressed in 293 cells undergoes differential glycosylation, resulting in proteins of 140 and 160 kDa (de Sauvage et al., 1992b; Vaandrager et al., 1993a,b). Differential glycosylation could result in GCC isoforms exhibiting different affinities for ligand. Indeed, differential glycosylation of GCA results in isoforms which exhibit greatly reduced catalytic activity (Koller et al., 1993). In addition, GCC undergoes significant post-translational proteolysis, and specific ST-binding subunits ranging from 160 to 50 kDa have been identified by ligand cross-linking or Western analysis in membranes from mammalian cells expressing GCC or intestinal mucosa cells (Ivens at al., 1990a; de Sauvage et al., 1992b; Hugues et al., 1992; Vaandrager et al., 1993a; Hakki et al., 1993a). Thus, high and low affinity ST binding sites may reflect post-translational proteolysis of GCC, as truncated products of this protein may bind differently than full length proteins.

The present studies demonstrate that low affinity ST binding sites of rat GCC undergo a ligand-induced shift from higher to lower affinity. Occupancy of the lowest affinity state of these receptors correlates directly with activation of guanylyl cyclase in rat intestine and exogenously expressed GCC (Figure 4; Crane et al., 1992). Similar observations have been obtained for exogenously expressed GCA stably expressed in 293 cells (Jewett et al., 1993). The shift in affinity of GCA was dependent upon the kinase homology domain, a signature domain of membrane receptor−guanylyl cyclases with significant homology to the catalytic domain of protein kinases (Jewett at al., 1993). Also, this shift in affinity was mediated by adenine nucleotides, which are required for receptor−cyclase coupling presumably by interacting with the kinase homology domain (Jewett at al., 1993). The precise mechanisms mediating this affinity shift exhibited by GCC remain unclear. However, GCC possesses a kinase homology domain that shares sequence homology with that of GCA. In addition, adenine nucleotides potentiate the activation of guanylyl cyclase by ST (Gazzano et al., 1991; Vaandrager et al., 1994). These observations support the hypothesis that receptor occupancy is coupled to catalytic activation of GCC through a mechanism involving adenine nucleotide interaction with the kinase homology domain, resulting in a shift in receptor binding function. The functional role of the ligand-induced shift in binding affinity in transmembrane signaling by receptor guanylyl cyclases remains unclear.

A general observation concerning guanylyl cyclases is that catalytic activation occurs at ligand concentrations that are higher than those required for receptor occupancy (Carr et al., 1989; Hugues et al., 1991; Crane et al., 1992; Garbers et al., 1994; Currie et al., 1992; Jewett et al., 1993). It has been suggested that this discrepancy in the concentration dependence of binding and activation implies that receptor−effector coupling may involve supramolecular mechanisms. For example, occupancy of all ligand binding sites in an oligomeric complex may be required to activate guanylyl cyclase catalytic domains (Garbers et al., 1994). An important consideration when comparing the concentration dependence of binding and catalytic activation is the conditions under which these activities are measured since they both increase in a time-dependent fashion. Previous studies compared the concentration dependence of guanylyl cyclase activation, measured over minutes, with that of receptor binding, measured at equilibrium (hours). Guanylyl cyclase activation by ligand is quantified after minutes of incubation because ligand−receptor interaction also induces a time-dependent, rapid inactivation of the enzyme, after which receptor−effector coupling cannot be detected (Waldman et al., 1984; Vaandrager et al., 1993a,b). At any given ligand concentration, receptor occupancy increases over time to a maximum at equilibrium (Boeynaems & Dumont, 1980; Molinoff et al., 1981). Thus, in those earlier studies, binding measured at equilibrium does not reflect receptor occupancy at the time enzyme activity was measured. Rather, receptor occupancy at those earlier times is substantially lower than at equilibrium. Since receptor occupancy is presumably coupled to catalytic activation, comparisons of their concentration dependence should be performed at the same time point. Indeed, comparison of the ligand concentration dependence of binding and catalytic activity measured at the same time point revealed that occupancy of the lowest affinity state of low affinity ST binding sites was coupled to activation of guanylyl cyclase in intestinal membranes (Crane et al., 1992). The present results confirm and extend those observations, demonstrating that occupancy of the lowest affinity state of GCC is coupled directly to catalytic activation, without requiring a supramolecular mechanism to explain the relationship between the concentration dependence of these functions.

In conclusion, equilibrium and kinetic analyses of rat GCC exogenously expressed in COS-7 cells revealed high and low affinity ST binding sites, suggesting that those sites observed previously in intestinal membranes obtained from several species reflect the expression of a single gene, GCC, rather than multiple genes encoding different binding proteins (Hugues et al., 1991; Hakki et al., 1993a,b; Forte et al., 1993; Carrithers et al., 1994; Waldman et al., 1994). Although the precise mechanisms by which expression of a single gene results in the production of binding sites with different affinities remains unclear, they may reflect differential post-translational processing or oligomerization. Kinetic binding analysis of low affinity binding sites demonstrates a ligand-dependent shift from higher to lower affinity, suggesting that a similar shift observed previously in intestinal membranes reflected the activity of GCC (Crane et al., 1992). Occupancy of the lowest affinity state of low affinity GCC binding sites is directly coupled to activation of guanylyl cyclase. The mechanisms underlying this affinity shift and its role in receptor−effector coupling are currently being examined.

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