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Epitope Conservation and Immunohistochemical Localization of the Guanylin/Stable Toxin Peptide Receptor, Guanylyl Cyclase C

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Abstract The heat-stable enterotoxins (ST) are a family of cysteine-rich low-molecular weight peptides produced by pathogenic bacteria, and are one of the major causes of watery diarrhea all over the world. These toxins mediate their action by binding to an intestinal cell surface receptor that is a membrane-associated guanylyl cyclase (GCC). This receptor also serves as the receptor for the recently characterised endogenous ligand, guanylin. We have expressed various domains of the receptor in *Escherichia coli* and used purified proteins for the generation of both polyclonal and monoclonal antibodies. While polyclonal antibodies were able to partially inhibit ST binding to the native receptor present in the T84 human colonic cell line, GCC:B10 monoclonal antibody did not interfere with ligand binding. Western blot analysis, using membranes prepared from human colonic T84 cells, detected two bands of size 160 and 140 kDa, representing alternately glycosylated forms of the receptor. Using the recombinant proteins, we could map the epitope of GCC:B10 monoclonal antibody to the intracellular domain of the receptor. We used the antibody to localize the receptor throughout the rat intestine, and in the porcine and bonnet monkey colon. We could detect receptor expression in the villus and the crypts of the duodenum, jejunum, ileum, and caecum, and in the crypts of the colon. Receptor expression was observed in cells that had earlier been shown to express cGMP-dependent kinase, but not the cystic fibrosis transmembrane regulator, a known downstream target of cGMP/G-kinase, which suggests that GCC/cGMP could regulate additional cellular signal transduction machinery. J. Cell. Biochem. 66:500–511, 1997.

Key words: heat-stable enterotoxin; guanylyl cyclase C; monoclonal antibody; immunohistochemical localization

Cyclic GMP is increasingly being recognized as an important second messenger in mediating cellular responses to both extracellular and intracellular signals [Nakane and Murad, 1994]. Guanylyl cyclases are present in two forms in a cell; one is cytosolic, and therefore referred to as soluble guanylyl cyclase [Schmidt et al., 1993] and the other form is associated with the cell surface and hence particulate in nature [Garbers, 1992; Drewett and Garbers, 1994]. The soluble guanylyl cyclases are heterodimeric proteins and are activated by nitric oxide. In contrast, the particulate cyclases serve as distinct receptors for a number of peptides that mediate their action through the elevation of cGMP in cells [Garbers, 1992; Singh et al., 1991]. From an analysis of the DNA sequence of cloned particulate guanylyl cyclases, these receptors possess an extracellular ligand binding domain, a single transmembrane domain, and a cytoplasmic domain that is composed of two regions; one region immediately following the transmembrane domain bears some homology to protein tyrosine kinases, and the second region present towards the C-terminus is the most highly conserved domain across the members of the family [de Sauvage et al., 1991; Chinkers et al., 1989; Singh et al., 1991; Wada et al., 1994] and harbors the guanylyl cyclase catalytic core [Thorpe and Morkin, 1993].

The heat-stable enterotoxins (ST) are a family of cysteine rich, low molecular weight peptides, produced by a variety of pathogenic bacteria, and are one of the major causes of watery

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diarrhea in infants the world over [Gianella, 1981; Levine, 1987]. These toxins bind to a cell surface receptor present in intestinal cells and elevate intracellular levels of cGMP, leading to increased chloride secretion and the ensuing diarrhea [Chao et al., 1994]. The T84 cell line, derived from a human colonic carcinoma, has served as a model to study ST binding and action [Huott et al., 1988; Visweswariah et al., 1992], and evidence obtained suggests that high levels of intracellular cGMP cross-activate cAMP-dependent protein kinase, leading to phosphorylation of the cystic fibrosis transmembrane regulator (CFTR), and hence increased chloride secretion [Chao et al., 1994]. Biochemical characterization [Visweswariah et al., 1994] as well as cloning and sequencing of the ST receptors (GCC) from the rat [Schulz et al., 1991], human [de Sauvage et al., 1991; Singh et al., 1991], and porcine [Wada et al., 1994] intestine, indicated that the receptors are membrane-associated forms of guanylyl cyclase. Recently, endogenous ligands for the ST receptors have been characterized and purified from the intestinal mucosae and urine of the rat, human, and North American opossum [Currie et al., 1992; Garbers, 1992; Hamra et al., 1996], and have been named guanylin and uroguanylin. The function of these peptides in normal intestinal physiology and/or in regulating ionic balance in the intestine is still under investigation. GCC has been shown to be expressed in extraintestinal tissues in both the rat [Laney et al., 1992] and the opossum [Forte et al., 1989], but the role of the receptor in these tissues is still unknown.

We have earlier described the biochemical characterization of the human receptor present in T84 cells [Visweswariah et al., 1994] as well as the expression of the extracellular ligand binding domain of the receptor as a fusion protein with glutathione S-transferase (GST) in *E. coli* [Nandi et al., 1996]. We have generated polyclonal antibodies to the receptor, which were able to neutralize the activity of ST in T84 cells, by inhibiting ligand binding to the receptor [Nandi et al., 1996]. We report here the generation of the first monoclonal antibody to GCC, which, on epitope analysis, is specific for GCC receptors. We have used this antibody for the immunohistochemical localization of the receptor in the rat intestine, as well as in the porcine and monkey colon, and the distribution of receptor expression suggests that GCC may be involved in regulating a variety of cellular responses.

MATERIALS AND METHODS

Materials

All general laboratory chemicals were obtained from Sigma (St. Louis, MO) or locally procured. Restriction enzymes were from Boehringer Mannheim (Mannheim, Germany) and Bangalore Genei Pvt. Ltd. (Bangalore, India). Primers for polymerase chain reaction (PCR) amplification were obtained from Bangalore Genei. Iscove's Modified Dulbecco's Medium (IMDM), adjuvants and fetal calf serum (FCS) were from Gibco (Gaithersburg, MD) and Life Technologies (Bethesda, MD). Anti-mouse IgG conjugated to HRP was obtained from Amersham (Buckinghamshire, UK). Stable toxin of the human variety (STh) and a mutant form of the STh peptide (ST_{Y72F}) were purified from strains hyperexpressing the peptides, as described earlier [Dwarkanath et al., 1989; Visweswariah et al., 1994]. T84 cells were obtained from ATCC (Rockville, MD) (CCL 247) and cultured as previously described [Visweswariah et al., 1992] in Dulbecco's modified Eagle's Medium (DMEM:F12) mixture containing 5% newborn calf serum.

Generation of Plasmids Expressing Various Domains of GCC

The cDNA for GCC was a kind gift of Dr. J.-M. Heim, Munich, Germany. Figure 1 represents schematically the various domains of the receptor that we have expressed and employed in this study. GCC-ED1 and GCC-ED2, both fusion proteins with glutathione S-transferase, have been described in detail earlier [Nandi et al., 1996]. GCC-ED1 is a 57-kDa protein that corresponds to the amino terminal half of the extracellular domain. GCC-ED2, 81 kDa in size, corresponds to the entire extracellular domain of the receptor and includes 63 amino acids of the intracellular domain. To generate a protein corresponding to the C-terminal region of GCC-ED2, the Kpn I-Hind III fragment of the GCC cDNA was cloned into the pRSETa vector (Invitrogen, La Jolla, CA) to generate the plasmid pRSET-ED5. This encoded a protein of 43 kDa, which we called GCC-ED5.

A DNA fragment corresponding to the entire intracellular domain of GCC was obtained by PCR amplification. The primers used for ampli-

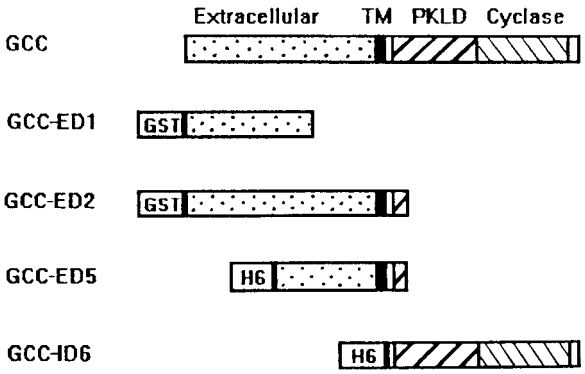

Fig. 1. Schematic representation of the structure of various domains of GCC. TM, transmembrane doman; PKLD, protein kinase like domain; GCC-ED, guanylyl cyclase C extracellular domain; GCC-ID, guanylyl cyclase C intracellular domain; GST, glutathione S-transferase; H6, hexahistidine tag.

fication were (1) sense strand: 5′ AGA AAA TAG GTA CCA GAT TAT GAA CTT CGT 3′ and (2) antisense strand: 5′ CAT CTG CAG TTA AAA ATA GGT GCT 3′. The 1.8-kb amplified product was cloned into pTZ-18R (Pharmacia, Gaithersburg, MD) in Kpn I-Pst I sites to generate a plasmid named pTZ-ID5. The cloned fragment was sequenced in the region that had an overlap with pRSET-ED5 and no mutation was detected. The Kpn I-Hind III fragment from pTZ-ID5 was then cloned into the pRSETc vector to generate pRSET-ID6 plasmid. This encoded a protein of molecular mass 70 kDa, which we named GCC-ID6. Both GCC-ED5 and GCC-ID6 have hexahistidine stretches at the N-terminus, which originate from the pRSET vectors.

Purification of the Recombinant Proteins

GCC-ED1 and GCC-ED2 were purified using glutathione agarose affinity chromatography and bound protein eluted with free glutathione as described earlier [Nandi et al., 1996]. Expression of the recombinant proteins GCC-ED5 and GCC-ID6 was performed in the host strain BL21(DE3)pLysS [Studier et al., 1990]. On induction with 500 µM of isopropyl β-D-thio galactopyranoside (IPTG) at 37°C, both the proteins were localized in inclusion bodies, and therefore purified to homogeneity by preparative SDS gel electrophoresis followed by electroelution using the Bio-Rad (Richmond, CA) electroelution apparatus.

Polyclonal antibodies to GCC-ED5 were raised by injecting 200 µg of the protein in Freund's complete adjuvant at multiple sites intradermally in rabbits. The animals were boosted with 200 µg of incomplete Freund's adjuvant at intervals of 20 days. Antiserum was collected and IgG purified by ammonium sulphate precipitation [Harlow and Lane, 1988].

Generation of Monoclonal Antibody GCC:B10

Balb/c mice were immunized intradermally with 100 µg of the fusion protein GCC-ED2 in 1:1 ratio of Freund's complete adjuvant to protein solution and boosted three times with 50 µg of GCC-ED2 in Freund's incomplete adjuvant at intervals of 1 month. Four days prior to fusion, the mice were boosted everyday intraperitoneally with 25 µg of GCC-ED2 in saline. Cell fusion was performed with the myeloma line Sp 2/O Ag-14 [Shulman et al., 1978] according to previously described procedures [Visweswariah et al., 1987]. Wells containing hybrid clones were screened for the presence of antibodies between 12 and 15 days following fusion by enzyme-linked immunosorbent assays (ELISA), as described below. Hybrid cells in the antibody positive wells were subcloned and monoclonality established using limiting dilution.

Screening of Hybridoma Supernatants by ELISA

GCC-ED2 or GST (100 ng/well) was added in phosphate buffered saline (PBS) and protein coated at 4°C for 16 h. Wells were blocked with 0.2% bovine serum albumin (BSA) in PBS, and 50 µl of culture supernatant was added. The plates were incubated at 37°C for 2 h and washed with PBS containing 0.1% Tween 20. Suitably diluted anti-mouse IgG conjugated to horse radish peroxidase (HRP) was added and further incubated for 1 h at 25°C. The plates were washed and color was developed using 100 µl of 3,3′,5,5′ tetramethyl benzidine substrate (Bangalore Genei). The reaction was stopped using 50 µl of 3N H_2SO_4 and the absorbance monitored at 450 nm. The GST specific clones were eliminated and we thus obtained a monoclonal antibody to the receptor, which we designate as GCC:B10. Ascites were raised in pristane primed Balb/c mice, following intraperitoneal injection of approximately 5 million cells. Ascitic fluid was obtained and IgG purified by protein A Sepharose affinity chromatography [Harlow and Lane, 1988]. Isotyping was performed using an ELISA based isotyping kit (Sigma). GCC:B10 monoclonal antibody is of IgG 2a subtype with k light chain.

Western Blot Analysis of Recombinant Receptor Proteins

Purified recombinant proteins (10 µg) were resolved by SDS gel electrophoresis. The proteins were transferred to poly(vinylidine difluoride) membrane in 3-(cyclohexylamino) propanesulphonic acid buffer, pH 11.0, containing 10% methanol at 200 mA constant current for 1 h. The membrane was blocked with PBS containing 2% BSA and blots probed with GCC:B10 monoclonal IgG (100 ng/ml) diluted in PBS containing 0.1% Tween 20 and 0.2% BSA. Membranes were washed in PBS containing 0.1% Tween 20 and the bound antibody detected using anti-mouse antibody conjugated to horse radish peroxidase (Amersham) using 0.04% 3-amino 9-ethyl carbazole as the substrate.

Binding Analysis on T84 Membranes

Binding was performed as described earlier [Visweswariah et al., 1994]. Briefly, T84 membranes (30 to 50 µg) were incubated with 400 µg of polyclonal IgG or normal rabbit IgG or 10 µg of GCC:B10 monoclonal antibody for 16 h at 4°C following which ^{125}I-labelled ST_{Y72F} was added [Visweswariah et al., 1994]. The mixture was incubated at 37°C for 1 h in the presence or absence of unlabelled STh (10^{-7}M) followed by filtration through glass fibre filters.

Western Blot Analysis of the Membranes Prepared From T84 Cells

Membranes were prepared as described by Visweswariah et al. [1994]. Membrane protein (100 µg) were resolved by SDS PAGE (7.5% acrylamide, 0.125% N,N′-methylene-bis-acrylamide) gels and blotted to nitrocellulose membranes (Hybond ECL, Amersham) in 50 mM Tris-glycine buffer, pH 8.5, containing 20% methanol. Nitrocellulose membranes were blocked with blocking reagent (Amersham) and probed with GCC:B10 monoclonal antibody (0.33 µg/ml). Bound IgG was detected using anti-mouse IgG conjugated to horse radish peroxidase by enhanced chemiluminescence (ECL reagents, Amersham) following the manufacturer's instructions.

Immunoprecipitation of the solubilized T84 membrane was performed using 300 µg of the T84 membrane protein solubilized in RIPA buffer, pH 8.0 (50 mM Tris, pH 8.0, 1% NP40, 0.1% SDS, 0.5% DOC, and 150 mM NaCl). The solubilized membrane fraction was treated with 20 µg/ml of normal rabbit serum IgG and 10 µl of protein A Sepharose for 2 h at 4°C. The supernatant was incubated for 2 h at 25°C with 20 µg/ml of GCC-ED2 polyclonal IgG and a further 1 h with protein A Sepharose. The protein A beads were washed with RIPA buffer, boiled in SDS sample buffer, and subjected to SDS gel electrophoresis followed by Western blotting as described above.

Immunohistochemical Localization of GCC in the Intestine

An adult female Wistar rat (150 g) was starved for 24 h, killed by cervical dislocation, and the viscera exposed. Sections were collected, as described earlier [Li and Goy, 1993] and fixed in Bouin's fixative (75 ml picric acid, 25 ml formaldehyde, 5 ml acetic acid) for 8 h. Porcine colon and *Macaca radiata* colon tissues were collected freshly fixed and processed similarly. Paraffin blocks were made and 5-µm serial section were cut and spread on albumin coated slides. The sections were deparaffinized in xylene for 1 h and hydrated in various grades of alcohol (absolute, 70, 50, 30%) in PBS, pH 7.2, for 20 min in each grade. The sections were treated with 90% methanol containing 0.03% H_2O_2 for 30 min and rehydrated for another 30 min in PBS. Sections were treated with 5% goat serum for 1 h, and washed with PBS containing 0.1% Tween 20 followed by PBS alone. GCC:B10 monoclonal IgG or normal mouse IgG (10 µg/ml) diluted in PBS containing 5% goat serum was added and incubated in a humidified chamber. The sections were washed with PBS containing 0.1% Tween 20 and incubated with 1:500 diluted anti-mouse HRP conjugate (Amersham) for 2 h. Sections were washed as above and color developed using 0.6 mg/ml solution of 3,3′diaminobenzidine containing 0.03% H_2O_2. The sections were counterstained with Meyer's hematoxylin for 60 s, dehydrated in alcohol and xylene, mounted in DPX mountant, and observed in bright light in an Olympus microscope (Model no. CH2).

RESULTS

Bacterial Expression of Various Domains of GCC

We have earlier reported the cloning and expression of two domains of human GCC as fusion proteins with glutathione S-transferase [Nandi et al., 1996]. We observed that the fusion protein of the entire extracellular domain

was able to bind ST peptides with an affinity comparable to the native receptor, whereas the truncated protein comprising only two thirds of the extracellular domain from the N-terminus was unable to interact with radiolabelled ST [Nandi et al., 1996]. We have generated two additional clones, one representing the C-terminal half of the extracellular domain, called GCC-ED5, and another clone corresponding to the entire intracellular domain of the receptor, called GCC-ID6 (Fig. 1). Both these clones proved useful in identifying the epitope of the monoclonal antibody generated in the course of this study, as described later.

Expression of all these proteins at 37°C led to accumulation of the recombinant proteins in inclusion bodies. Purification of the proteins was, therefore, carried out by electroelution from preparative SDS polyacrylamide gels, and purified proteins were used for the production of polyclonal antibodies. The polyclonal antibodies raised to GCC-ED5 were tested for their ability to inhibit ST binding to membranes prepared from T84 cells. As can be seen in Figure 2, antibodies to GCC-ED2 and GCC-ED5 were able to partially inhibit ^{125}I-labelled ST_{Y72F} binding to membranes prepared from T84 cells. However, the extent of inhibition brought about by the GCC-ED2 antibodies indicated that regions of the receptor involved in ligand binding could be present in the amino terminal as well as the C-terminal of the extracellular domain of the receptor.

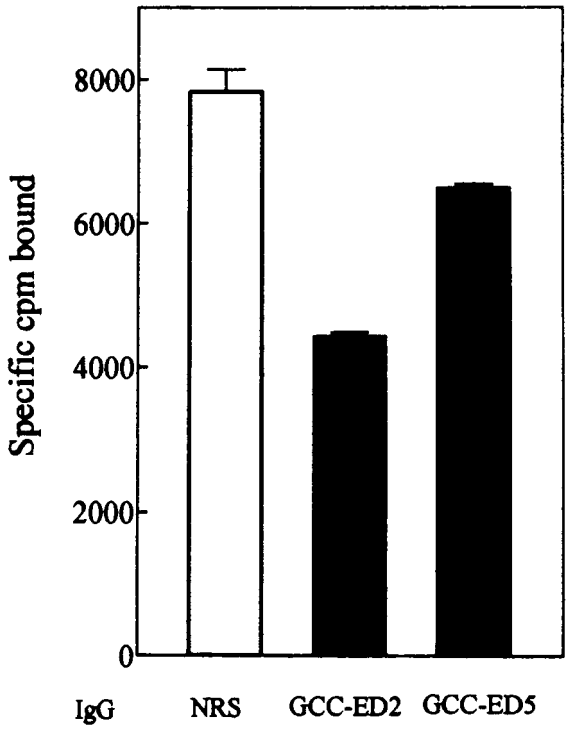

Fig. 2. Effect of polyclonal antibodies to GCC on ST binding to the receptor in T84 cells. T84 membrane protein (30 to 50 µg) was incubated with 400 µg of antibodies to GCC-ED2 and GCC-ED5 for 16 h at 4°C and then incubated with 50,000 cpm of ^{125}I-labelled ST_{Y72F} in the presence or absence of 5×10^{-8} M STh. The complex was then filtered under vacuum through glass fibre filters and the receptor bound radioactivity was counted. Values represent mean ± SD of triplicate determinations, with each experiment repeated three times.

Generation of Monoclonal Antibody GCC:B10

We used electroeluted GCC-ED2 as the antigen for immunizing mice and antibodies to the extracellular domain were detected by ELISA in the sera of the mice prior to fusion (data not shown). One strongly secreting hybridoma was subcloned to monoclonality and designated GCC:B10.

Incubation of membranes prepared from T84 cells with purified GCC:B10 monoclonal IgG, prior to addition of radiolabelled ST_{Y72F}, did not significantly inhibit binding of ST_{Y72F} to the receptor, indicating that this antibody was directed to regions of the receptor not involved in direct ligand interaction (Fig. 3a).

The antibody was able to react with GCC-ED2 on a Western blot analysis (Fig. 3b, lane 3), but did not show any reactivity with GCC-ED1 (Fig. 3, lane 2) or GST (Fig. 3, lane 1), suggesting that it was directed to a region of the recombinant protein towards the C-terminus of the extracellular domain of the receptor. This was confirmed by its reactivity with GCC-ED5 (Fig. 3, lane 4), which comprised the C-terminal domain of the protein GCC-ED2 (see Fig. 1).

It is important to note that the transmembrane domain as well as a short stretch of the intracellular domain of the receptor are also contained in GCC-ED2 and GCC-ED5 (Fig. 1). When Western blot analysis was performed using purified GCC-ID6, we could detect reactivity with GCC:B10 monoclonal antibody, indicating that the antibody was directed to the small stretch of 63 amino acids present in the intracellular domain of the receptor (Fig. 3c). This is in good agreement with our earlier observation that GCC:B10 monoclonal antibody did not inhibit ligand binding to the receptor, which occurs in the extracellular domain (Fig. 3a). A theoretical analysis for the detection of antigenic epitopes in the sequence of GCC-ED2 indicated that this region was strongly anti-

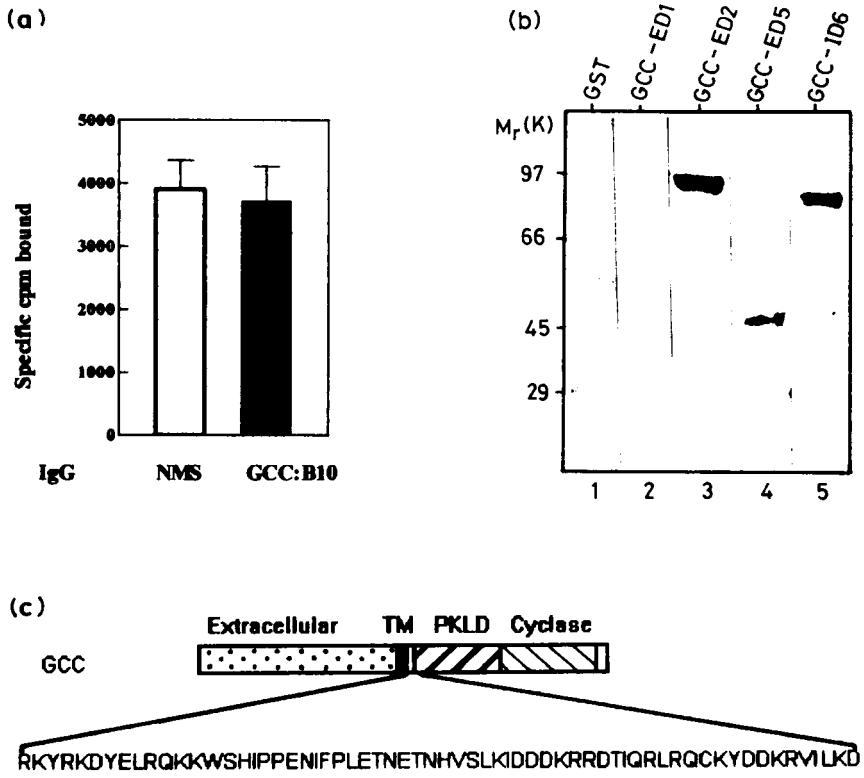

Fig. 3. Characterisation of GCC:B10 monoclonal antibody. **a:** T84 membranes were incubated with GCC:B10 monoclonal antibody or normal mouse IgG (10 μg each) followed by addition of labelled ST_{Y72F}. Bound radioactivity was monitored following filtration, and values represent the mean ± S.D. of triplicate determinations with each experiment repeated three times. **b:** Purified proteins (10 μg each) were resolved in a 10% SDS gels and blotted on to poly(vinylidine difluoride) membranes. The blots were probed with GCC:B10 monoclonal antibody (100 ng/ml), and the bound antibody was visualised using anti-mouse IgG conjugated to horse radish peroxidase with 3-amino 9-ethyl carbazole as the substrate. **c:** The 63 amino acids present in the intracellular domain common in GCC-ED5 and GCC-ID6 proteins.

genic (data not shown). This sequence of 63 amino acids had significant homology only with members of the heat-stable enterotoxin family of receptors.

The monoclonal antibody was tested for its ability to react in a Western blot analysis with membranes prepared from T84 cells (Fig. 4, lane 2). The monoclonal antibody GCC:B10 reacted with two proteins of Mr 140 kDa and 160 kDa, both of which bound to conconavalin A-Sepharose (data not shown), indicating that they were differentially glycosylated forms of the receptor as reported earlier [Vaandrager et al., 1993]. In addition, broader but less intense bands were seen as Mr 60–70 kDa, which could represent degraded fragments of the receptor, as described by others earlier [Vaandrager et al., 1993]. These bands reacted specifically with the GCC:B10 monoclonal antibody and no bands of corresponding size were detected with normal mouse IgG (Fig. 4, lane 1). Immunoprecipitation with polyclonal antibodies to GCC-ED2 of solubilized T84 membrane proteins, followed by Western blot analysis with GCC:B10 monoclonal antibody, again indicated reactivity with two bands of Mr 140 and 160 kDa. Lower molecular bands were not visible in the immunoprecipitate, perhaps indicating that the degraded fragments (seen in direct Western blots in Fig. 4, lane 2) did not cross-react with the extracellular domain polyclonal antibodies used for immunoprecipitation (Fig. 4, lane 3). The relative intensities of the two bands varied with different membrane preparations (data not shown) and, therefore, could represent differential glycosylation during T84 cell growth.

Immunohistochemical Localization of GCC

From available information to date, GCC appears to be localized along the avian [Katwa and White, 1992; Krause et al., 1995] and mammalian intestines [Li and Goy, 1993; Krause et al., 1994]. Most studies to date have utilized biochemical characterization of the receptor in

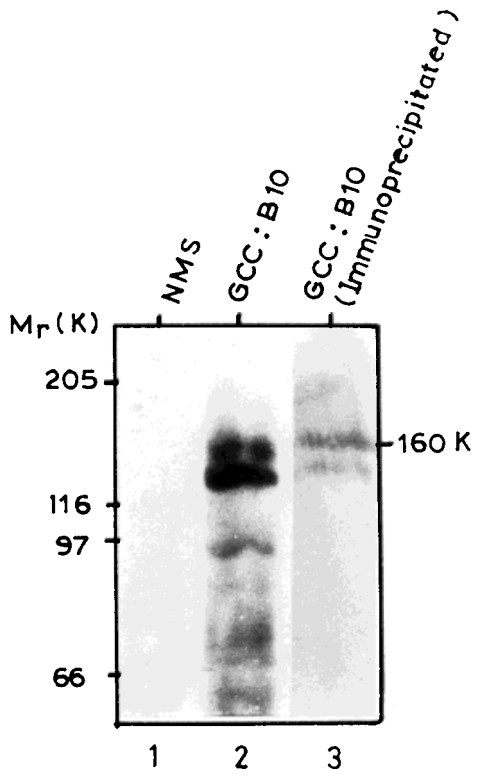

Fig. 4. Western blot analysis of membranes prepared from T84 cells. T84 membrane protein (100 µg) was subjected to SDS polyacrylamide gels and blotted to nitrocellulose membranes. Individual lanes were probed with normal mouse IgG (**lane 1**) or GCC:B10 monoclonal IgG (**lane 2**), and bound antibody detected by ECL. In **lane 3,** 300 µg of T84 membrane protein was solubilised in RIPA buffer, pH 8.0, and immunoprecipitated using polyclonal GCC-ED2 IgG and then probed with GCC:B10 monoclonal IgG as described in the text.

membranes prepared from various regions of the intestine, or employed in situ hybridization techniques with GCC specific probes [Li and Goy, 1993]. We attempted to use the monoclonal antibody that we have obtained to immunohistochemically localize the receptor in various regions of the rat intestine. The data shown in Figure 5 present the results that we have obtained.

In the duodenum, (Fig. 5a–d), we observe specific staining all along the villi, into the crypt regions, as well as the glands of Leiberkuhn (Fig. 5c), indicating that the receptor is present only in mucosal epithelia and absent in the lamina propria, submucosal and serosal layers. GCC was localized in the goblet cells and paneth cells. However, mitotic cells of the duodenum did not show immunostaining. The reactivity that we have observed was not detected when an equivalent concentration of normal mouse IgG was used (Fig. 5a) as well as when the antibody was preadsorbed with a large molar excess (10-fold) of GCC-ED2 protein (data not shown).

GCC expression could be detected in the jejunum in the plicae, which are a distinguishing feature of this region and the epithelial lining of the jejunal glands (Fig. 5f–h). The epithelia of the villus, which appear as uneven finger-like projections as opposed to the even size leaf-like projections of the duodenum, are stained positive for immunoreactivity using GCC:B10 monoclonal antibody (Fig. 5f). In the ileum, staining was detected along the villus, with an apparent equal intensity of staining along the crypt to the villus axis (Fig. 5i–k). The receptor could also be detected in goblet cells along the villus (Fig. 5k) and was expressed to lower levels in the mitotic cells. It is interesting to note that staining appeared to be localized in regions below the microvilli of the intestine, and this detail could not have been observed in earlier studies [Krause et al., 1994; Li and Goy, 1993].

In the caecum, staining appeared to be more superficial and localized along the villus and into the neck of the crypts (Fig. 5l and m). Most interesting was the localization observed in the colon (Fig. 5n–p), where specific immunostaining was detected in the crypts, with very little staining on the surface or in regions at the opening of the crypts. This is in contrast to studies performed by in situ hybridization, which indicated that the receptor appeared to be localized more uniformally in the colon [Li and Goy, 1993], and could represent a distinction between detection of the mRNA and actual expression of the protein.

Immunohistochemical Localization of the Receptor in the Colon of Different Species

As mentioned earlier, GCC has been identified in a number of species, including birds and pigs [Krause et al., 1995; Wada et al., 1994]. In view of the interesting localization of the receptor that we observed in the colon of the rat, we studied the localization of the receptor in colon sections prepared from the bonnet monkey, *Macaca radiata* (Fig. 6a and b) and the pig (Fig. 6c and d). We could detect significant reactivity in both the superficial regions of both the monkey and the porcine colon, as well as in the crypts, unlike the results that we obtained with the rat intestine (see Fig. 5p).

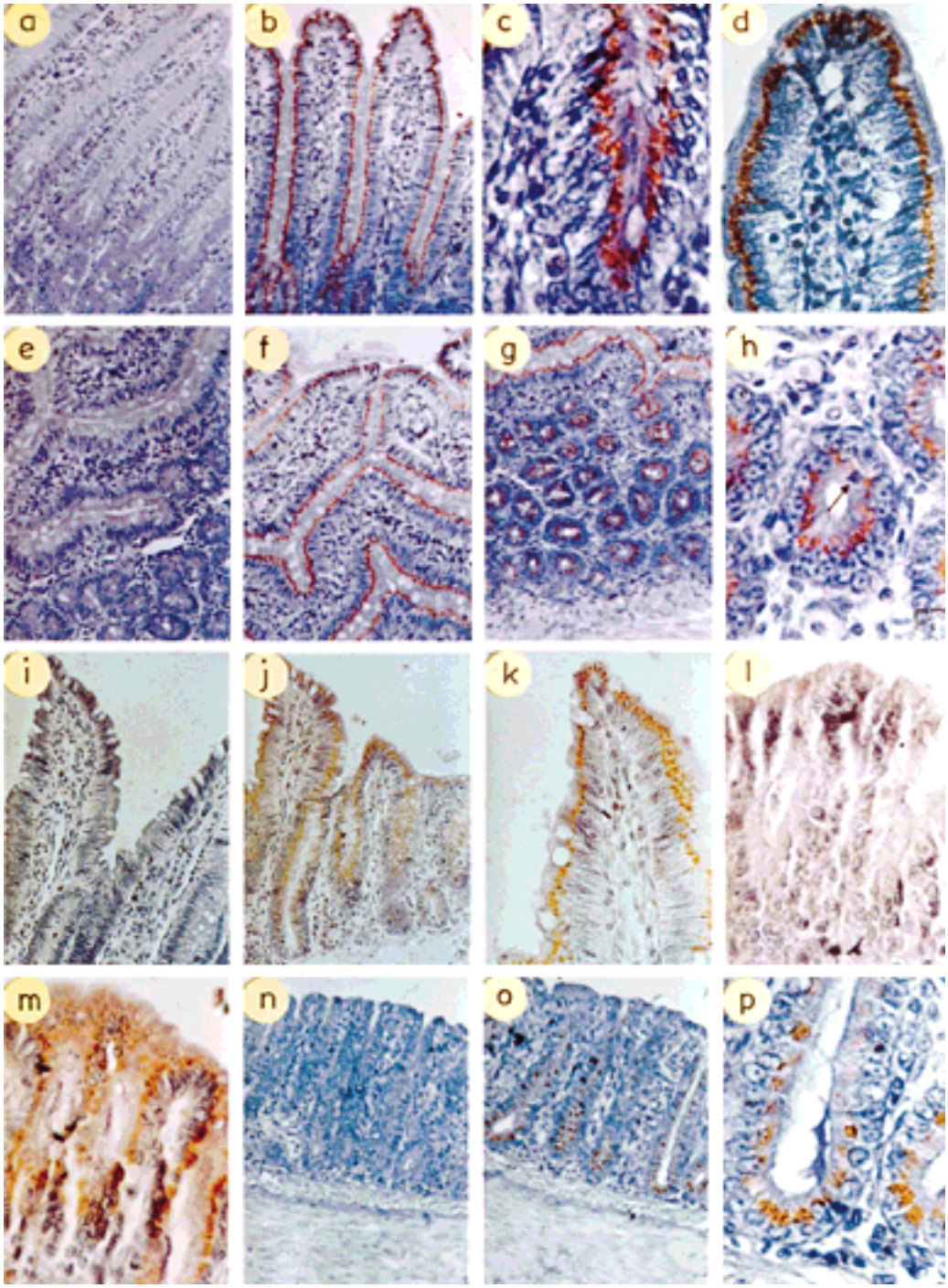

Fig. 5. Immunohistochemical localization of GCC in rat intestine. Sections were treated with GCC:B10 monoclonal antibody or normal mouse IgG (10 μg/ml) and bound antibody detected as detailed in the text. Duodenum (**a–d**) sections stained with GCC:B10 monoclonal antibody (b, c and d) or normal mouse IgG (a). Note that immunostaining is observed in goblet cells, and the cells of the mucosal epithelium and in the paneth cells of the deep crypts. (a, b, ×50; c, d, ×200). Jejunum (**e–h**) sections were treated with GCC:B10 monoclonal antibody (f, g, and h), or treated with normal mouse IgG (e). Arrows indicate mitotic cells (e–g, ×50; h, ×200). Ileum (**i–k**) sections treated with GCC:B10 monoclonal antibody (j and k) or with normal mouse IgG (i). Note that the numerous goblet cells of the ileum are immunoreactive with GCC:B10 monoclonal antibody. (i and j, ×50; k, ×200). Caecum (**l** and **m**) sections were treated with GCC:B10 monoclonal antibody (m) or normal mouse IgG (l). Note the superficial localization of the receptor and reduced expression in the crypts (×200). Colon (**n–p**) sections were treated with GCC:B10 monoclonal antibody (o and p) or normal mouse IgG (n). Note the reduction in the concentration of the receptor in the luminal epithelium and an increasing gradient of the receptor from villus to the crypt (n and o, ×50; p, ×200).

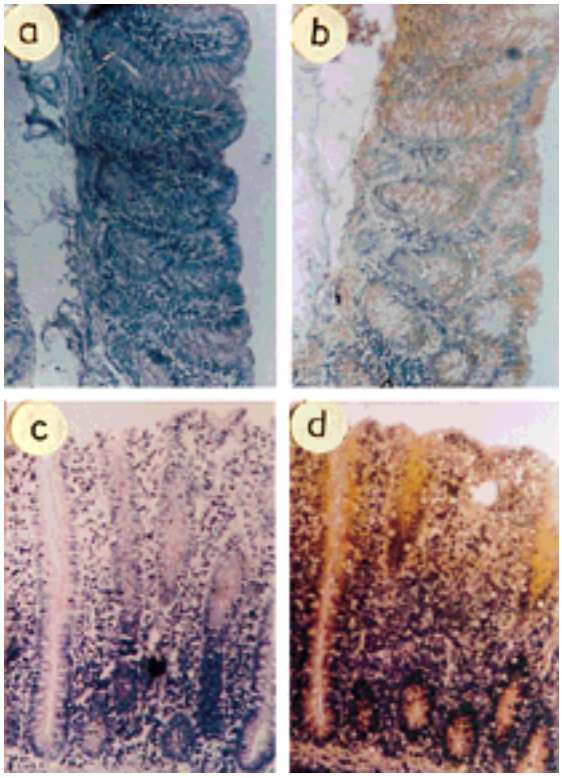

Fig. 6. Immunohistochemical localization of GCC in colon of the monkey and pig. Sections of the monkey colon were treated with GCC:B10 monoclonal antibody (**b**) or normal mouse IgG (**a**). Sections of the pig colon were treated with GCC:B10 monoclonal antibody (**d**) or normal mouse IgG (**c**). Note the uniform localization of the receptor in the crypts and the villus region in the pig and the monkey sections (×50).

DISCUSSION

We report here the generation of the first monoclonal antibody available to GCC, and this antibody, though raised against the human receptor, has been shown to recognize the rat, pig, and monkey receptors. We have also demonstrated that the antibody is able to react with the receptor in the porcine colon, where the epitope sequence is conserved, as well as in the monkey colon. We therefore suggest that this antibody would be useful for the detection of the receptor present in a variety of mammalian species such as the opossum, grey kangaroo, bettong [Krause et al., 1994] and birds [Krause et al., 1995], where ST-binding activity and guanylyl cyclase activation have been observed [Forte et al., 1989]. This antibody does also interact with the human receptor, since the antigen used for its production was the extracellular domain of the human guanylyl cyclase C receptor, expressed in *E. coli*.

It is instructive to compare the localization of the receptor that we have observed along the rat intestine with earlier studies using receptor autoradiography and in situ hybridization [Li and Goy, 1993; Krause et al., 1994, 1995]. The distribution of the receptor along the intestine parallels the results that have been obtained earlier in the human intestine [Krause et al., 1995], using radiolabelled ST peptide as a ligand to monitor receptor expression in sections of the human intestine [Krause et al., 1994]. From receptor autoradiography studies, receptor expression in rat was restricted to the intestinal epithelial cells covering the villi and the intestinal glands. Our immunohistochemical staining indicates similar results. No receptor activity was detected in the earlier report in the Brunner's glands [Krause et al., 1995] and our findings agree with this observation. We do not appear to see a marked gradation in the immunostaining along the villus to crypt axis in the small intestine, though a number of groups have suggested that maximum expression of the receptor is observed in the mid-villus region [Cohen et al., 1992; Krause et al., 1994].

Receptor autoradiography studies also indicated reduced receptor expression in the stem cells located within the intestinal glands [Krause et al., 1994], a detail which we have also observed in our studies. In the rat colon, we have localized the receptor in the crypts, and at reduced levels in the epithelium. In the rat, similar results were observed by in situ hybridization in the colon, with maximum reactivity observed in the crypts [Li and Goy, 1993]. However surface epithelia also indicated good expression of the receptor [Li and Goy, 1993], in contrast to our results where low expression is seen in the surface epithelium. It is relevant to mention here that reduced responsiveness to ST peptides has been observed in colonocytes as compared to enterocytes prepared from the small intestine of the rat, attributed to a lower level of receptor expression observed in the colonocytes [Cohen et al., 1992].

A peptide named guanylin has recently been shown to be the endogenous ligand for GCC, and acts by paracrine and/or autocrine mechanisms, in view of the peptide being produced by intestinal tissue [Forte and Hamra, 1996]. In situ hybridization using guanylin specific probes, detected the presence of guanylin mRNA in the middle to tip regions of the villus in the small intestine, with no signal detected in the

crypts [Li and Goy, 1993]. This is markedly different to the localization of GCC that we have observed in this study, where more uniform expression of the receptor is observed along the villus. Immunohistochemical localization of guanylin in the rat, using antibodies to the either the entire prohormone or regions from the mature peptide, indicated that guanylin peptide was expressed in the goblet cells present both in the villus and in the crypts of the small intestine [Cohen et al., 1995; Li et al., 1995]. We have observed GCC expression in goblet cells as well, suggesting that control of the hydration of mucus could be regulated in an autocrine manner by guanylin in the goblet cells. In the rat colon, guanylin expression was detected in the surface epithelium and in the neck regions of the crypts, but was absent from the deep crypts [Li and Goy, 1993]. This is in clear contrast to our results, where maximum receptor expression is detected in the colonic crypts. In the human intestine, a single report suggested that guanylin was expressed in the epithelium of the crypts of Leiberkühn in the secretory mucosa [Hill et al., 1995] and we do detect receptor expression in these cells in the rat.

The immediate downstream target of elevations in cGMP produced on GCC activation is the regulation of cystic fibrosis transmembrane regulator (CFTR). CFTR is phosphorylated by cGMP-dependent kinase leading to an increase in its chloride secreting ability. In cell lines such as the T84 cell line, which is deficient in protein kinase G, it has been suggested that cross-activation of protein kinase A is the mechanism by which CFTR is regulated [Forte et al., 1992]. We have compared the expression of GCC with that of CFTR in the intestine and find contrasting patterns of expression. Using in situ hybridization techniques, only the base of the villi in the small intestine expressed mRNA for CFTR [Trezise and Buchwald, 1991], but a more recent study using antibodies to CFTR suggested that CFTR was expressed all along the villus [Walker et al., 1995]. It is pertinent to mention here, however, that the antibody used for the latter study appeared to identify proteins unrelated to CFTR, since immunostaining was detected in cell lines not known to express CFTR [Walker et al., 1995]. Strong expression of CFTR was also seen in the crypts of the small intestine and the colon. A recent report, using antibody to CFTR, showed expression of CFTR in the goblet cells of the mouse and the pig intestine [Hayden and Carey, 1996]. Whatever the case, we have observed GCC expression in all the cells that have been shown by various investigators to express CFTR, suggesting that regulation of chloride secretion by CFTR could be the prime downstream effect of GCC activation. It is important to note, however, that GCC expression is observed in our study in cells that do not express CFTR, raising the possibility that the elevation of cGMP within these cells could lead to a number of responses independent of chloride secretion.

This hypothesis is strengthened when one compares distribution of GCC, which we have observed, with that of the recently identified intestinal cell-specific cGMP-dependent protein kinase II [Jarchau et al., 1994]. By in situ hybridization techniques using a probe specific for the Type II protein kinase G, expression of the enzyme was detected in the duodenum and the jejunum, with decreased expression along the villus to crypt axis [Markert et al., 1995]. Substantial amounts of the kinase were detected in the crypts of the caecum, and the enzyme is located in the apical membrane of the epithelial cells and in the brush border, when sections were stained with a specific antibody to the enzyme [Markert et al., 1995]. It therefore appears that the expression of protein kinase G parallels more closely the expression of the receptor than the earlier discussed expression of CFTR. Protein kinase G and GCC could therefore regulate ion transport through other mechanisms. More importantly, cGMP could bring about distinct responses in cells by the activation of cGMP-dependent protein kinase apart from its known effects on CFTR, and such studies should prove interesting lines of investigation in the future.

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