

Distribution of membrane bound guanylyl cyclases in human intestine

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Abstract

The quantification and distinction of particulate guanylyl cyclases in the human intestine were considered by an enzymatic approach, which comprised the signal transduction from receptor binding to cGMP formation, and, in addition, by showing the expression of an intracellular portion of these transmembrane proteins. Basal guanylyl cyclase (GC) activities were 50 to 80 pmol cGMP formation/min/mg protein and were stimulated up to twofold by heat stable enterotoxin, but were not significantly influenced by atrial natriuretic factor. Enzymatic analysis of colonoscopic specimens pointed to the prevalence of guanylyl cyclase C in the terminal ileum and in the large bowel including colon ascendens, colon descendens, sigmoid, and rectum. The availability of sequence information on human guanylyl cyclases permitted the development of a polymerase chain reaction approach for distinguishing the expression of GC-A and GC-C in human tissue samples. The expression levels of particulate guanylyl cyclases found by polymerase chain reaction in surgical biopsy specimens confirmed the enzymatic data, in that substantial expression of GC-C was found not only in the small intestine but also in the

large bowel. According to the restriction mapping of amplicates, GC-C prevailed over GC-A throughout the human intestine, particularly in the mucosal layers.

(Gut 1994; 35: 1250-1257)

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Accepted for publication
14 December 1993

Travellers' diarrhoea is one well known example of acute bacterial disease, which reportedly accounts for up to 50% of infant death in developing countries.^{1,2} The heat stable enterotoxins produced by several enterobacterial species cause diarrhoeal disease by increasing the cGMP concentrations in intestinal tissue.^{3,4} The increase of intracellular cGMP elicited by heat stable enterotoxins decreases water and sodium absorption and enhances chloride secretion by, as yet, not fully understood mechanisms entailing an intestinal isoform of cGMP dependent protein kinase.^{1,5} The second messenger cGMP is produced by soluble and membrane bound enzymes.⁶⁻⁸ The particulate guanylyl cyclases (GC), for example, GC-A and GC-B, are activated in response either to natriuretic peptides, or, in the case of GC-C, to heat stable enterotoxin (ST_a).

We have recently isolated a cDNA encoding a guanylyl cyclase coupled enterotoxin receptor from the human colonic cell line T84.⁹ Sequence analysis of this cDNA clone showed the domain division also seen in previously known particulate guanylyl cyclases with comparatively high homologies in the intracellular regions (Fig 1).

While little is known about the distribution of particulate guanylyl cyclases in the intestine of human adults, it has been reported that the increased susceptibility of newborns to diarrhoeal disease parallels the age dependency of GC-C activity¹⁰ and ST_a receptor density¹¹ in intestine specimens from children. This study was designed to clarify the distribution of particulate guanylyl cyclases, especially GC-A and GC-C, in the intestine of adults. As current textbooks identify the small intestine as the main site for the origin of heat stable enterotoxin induced diarrhoea, we were interested in the occurrence of GC-C also in the colon in comparison with the terminal ileum. The recent discovery of guanylin, a peptide with a high degree of sequence homology with heat stable enterotoxins and stimulatory effects on GC-C,¹² together with the fact that this peptide was purified from the intestine, suggests that guanylin is possibly a physiological regulator of intestinal function and made us investigate the distribution in the intestine of its receptor. The possible presence of GC-A in intestinal mucosa

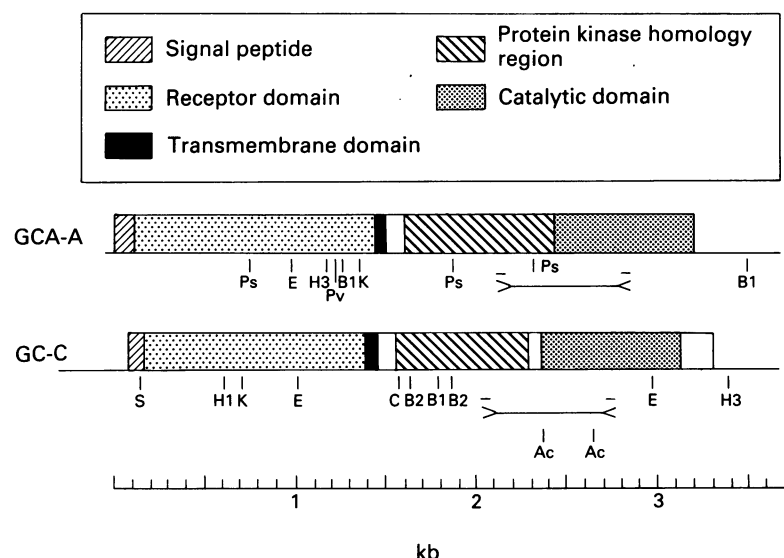


Figure 1: Physical maps of human GC-A and GC-C cDNA. The functional domains postulated according to sequence homologies were assigned to the published cDNA sequences for human GC-A²⁵ and GC-C.⁹ The positions of the binding regions of the common primer pair and the resulting amplification products of about 640 bp are shown below the maps. Additionally some prominent restriction sites are shown and abbreviated as: Ac: *AccI*, B1: *BamHI*, B2: *BgIII*, C: *C1aI*, E: *EcoRI*, H1: *HpaI*, H3: *HindIII*, K: *KpnI*, Ps: *PstI*, Pv: *Pvu I*, S: *SmaI*. *AccI* sites are shown on the polymerase chain reaction products only.

and the direct participation of atrial natriuretic factor in the origin of diarrhoea has repeatedly been claimed¹³⁻¹⁵ or challenged.¹⁶⁻¹⁸ To clarify this point, we examined the occurrence of atrial natriuretic factor stimulated guanylyl cyclase activities and the expression levels of the known GC-A gene in human tissue specimens representing different regions and layers of the intestine.

The aim of this study was therefore to complement and extend current knowledge of the distribution of particulate guanylyl cyclases along the intestine of human adults. Accordingly, the occurrence and frequency of two guanylyl cyclase isoforms, namely GC-A and GC-C, was investigated in the same study. While most previous histochemical studies on particulate guanylyl cyclases used rat tissue, our experiments were conducted on specimens obtained from human adults, an age group that previously has not been examined in this context. For this purpose, biopsy specimens obtained during routine colonoscopies were used for guanylyl cyclase activity assays. To assess clearly the guanylyl cyclase expression pattern in mucosal specimens, contamination of this epithelial layer by blood vessels and smooth muscle from adjacent tissue layers was carefully avoided. Finally, the enzymatic findings were consistently confirmed at the level of gene expression. Guanylyl cyclase basal activities in colonoscopic specimens were monitored and differentially stimulated by addition of either atrial natriuretic factor or heat stable enterotoxin. The molecular expression of particulate guanylyl cyclase genes was investigated by polymerase chain reaction amplification of tissue derived cDNA with subsequent restriction mapping.

Methods

CHEMICALS AND MOLECULAR BIOLOGY REAGENTS

For stimulation of GC-A, rat atrial natriuretic factor (99-126) purchased from the Institut Frappier, Canada, was used. *Escherichia coli* heat stable enterotoxin (ST_a) was obtained from Sigma, St Louis, MO, USA. The Bradford reagent used for protein determinations was obtained from BioRad, Munich, Germany. All other chemicals were of analytical grade and mainly obtained from Sigma.

Magnetic microspheres with covalently attached dT₂₅ residues for the purification of mRNA were obtained from Dynal, Oslo, Norway. Custom synthesis of oligonucleotides was performed by MWG, Ebersberg, Germany. Reverse transcriptase from Moloney murine leukaemia virus, Taq-DNA polymerase, and restriction enzymes were purchased from Pharmacia, Freiburg, Germany, Perkin-Elmer, Überlingen, Germany, and Boehringer Mannheim, Germany, respectively.

TISSUE SPECIMENS

Tissue specimens of about 50 mg each representing different sections of the lower

intestine were obtained from four patients between 54 and 62 years of age, who had surgery because of intestinal tumours. Unaffected tissue surrounding the tumours was used. In addition to the mucosal cell layer, which was scraped off before freezing in liquid nitrogen, samples were also collected from the corresponding submucosal muscular layer. The enzymatic studies were based on specimens obtained during colonoscopy from various patients in the age range from 20 to 70 years. Average values of several independent experiments are reported and error bars represent standard errors of the mean.

MEASUREMENT OF GUANYLYL CYCLASE ACTIVITY

Sample preparation for guanylyl cyclase activity assays entailed cell disruption and isolation of membranous cell components. Three colonoscopy samples per membrane preparation were taken up to 500 µl of 20 mM hydroxyethylpiperazine-ethanesulphonic acid (HEPES) buffer pH 7.4, containing 5 mM EDTA, and homogenised with a Potter-Elvehjem homogeniser. Larger surgical samples were suspended in five times their wet weight of the same buffer. Homogenised tissue suspensions were sonicated for 10 seconds and cell debris was removed by low speed centrifugation. Soluble and particulate cell components were separated by 30 minutes of centrifugation at 18 000×g. The sediment was resuspended in the original volume of 20 mM HEPES buffer containing 0.5 mM EDTA. Aliquots were frozen in liquid nitrogen and were stored at -70°C. Protein concentrations were measured photometrically at 595 nm according to Bradford.¹⁹

Guanylyl cyclase activity was determined essentially as described previously.²⁰ Briefly, 40 µl of reaction buffer were added to 20 µl of appropriately diluted sample for a preincubation at 37°C for three minutes. The guanylyl cyclase reactions were started by the addition, in a volume of 20 µl, of substrate, and stopped after a defined reaction time by addition of 1 ml of a boiling solution of 30 mM EDTA. The final reaction mixture contained 1 mM 1-methyl-3-isobutyl-xanthine, 5 mM creatine phosphate, 500 µg/ml creatine phosphokinase, 0.1 mM amiloride, 1 mM GTP, and 3 mM MnCl₂ in 50 mM triethanolamine/HCl buffer, pH 7.4. For differential stimulation of GC-A or GC-C, either 10⁻⁶ M of atrial natriuretic factor or 10⁻⁷ M of ST_a were included in the reaction mixture. The amounts of cGMP formed during an appropriate incubation time of usually 30 minutes at 37°C were determined by radioimmunoassay as described previously.²¹ Routinely, the membrane preparations from colonoscopies were prediluted for the guanylyl cyclase assay by a factor of 10, and those from surgical biopsies by a factor of three, to yield protein concentrations in the range of 2.5 to 4 mg/ml. Bovine adrenal cortex and porcine ileum served as reference tissues containing atrial natriuretic factor or ST_a stimutable guanylyl cyclases, respectively,

and were used for assessing the methods of sample preparation and activity assay. Replacing magnesium for manganese in the guanylyl cyclase assay resulted in the high sensitivity for basal activities required for measurements in colonoscopic specimens. The membrane preparations were devoid of soluble guanylyl cyclase because the basal activities in the specimens were not stimulated by 100 μ M sodium nitroprusside.

CDNA PREPARATION

Tissue specimens of about 50 mg were ground under liquid nitrogen and lysed in 5 M guanidinium isothiocyanate, 50 mM TRIS-HCl, pH 7.5, 10 mM EDTA, 5% β -mercaptoethanol. Preparation of total RNA was largely according to Chirgwin *et al.*,²² and included the addition of sarkosyl to a final concentration of 2% and heating at 65°C for two minutes. After isopropanol precipitation poly-A⁺ RNA was purified using oligo-dT residues coupled to magnetic beads.²³ cDNA was obtained by reverse transcription using random hexamers as primers and Moloney murine leukaemia virus reverse transcriptase at 37°C for one hour in 45 mM TRIS-HCl, pH 8.3, 68 mM KCl, 15 mM dithiothreitol, 9 mM MgCl₂, 0.08 mg/ml bovine serum albumin and 1.8 mM of each deoxynucleotriphosphate.²⁴

POLYMERASE CHAIN REACTION

For the polymerase chain reaction amplification of a stretch of cDNA common to the three known human particulate guanylyl cyclases^{9 25 26} a primer pair flanking a 600 bp section of the putative intracellular domain was designed and synthesised chemically. The oligonucleotide sequences were 5' TG TAC AGC TA/TT GGI ATC ATC 3' for the upstream primer and 5' C CAC CAT GTA A/CGC ATC ACC G 3' for the downstream primer, respectively.

The criteria applied for the design of a primer pair for performing polymerase chain reactions included the specificity for guanylyl cyclases compared with adenylyl cyclases, equal matching to all three known particulate guanylyl cyclases, appropriate distance from one another, and minimised secondary structure of both oligonucleotides. The selected primers define an amplicon of 640 bp, which bears different restriction site markers for identifying the three known types of human particulate guanylyl cyclases. The upstream primer confers specificity for particulate guanylyl cyclases and does not match to the known sequences of soluble guanylyl cyclase cDNAs.

The cDNAs encoding human GC-C and rat GC-A were used as test templates for adjusting optimal amplification conditions. Taq-DNA polymerase was used at 1.25 U/50 μ l. Unless stated otherwise, the amplification mix contained 10 mM TRIS-HCl, pH 8.3, 50 mM KCl, 1.25 mM MgCl₂, 5% glycerol, 200 μ M each of the four deoxynucleotridiphosphates,

and 100 nM of each primer. Amplifications were run for 35 cycles consisting for one minute each of denaturation at 94°C, of primer annealing at 56°C, and of DNA synthesis at 72°C. To exclude artefacts in the cDNA amplification three kinds of control polymerase chain reactions were run. Firstly, the addition of template was omitted; secondly, the cDNA template was produced in a mock reverse transcription reaction without tissue RNA; thirdly, to exclude the possibility of DNA contamination in the poly-A⁺ RNA preparation, Moloney murine leukaemia virus reverse transcriptase was omitted in a mock first strand cDNA synthesis. None of these controls yielded any amplification products.

Results

GUANYLYL CYCLASE ACTIVITIES IN HUMAN INTESTINE

Membrane preparations from colonoscopies contained 2.5 to 4.0 mg protein per ml and exhibited a linear correlation between the amount of sample and the corresponding guanylyl cyclase activity at a range of total protein concentrations from 50 μ g/ml to 1 mg/ml (not shown). The guanylyl cyclase activities of colonoscopic or surgical biopsy specimens in the described assay system behaved in a linear fashion for at least 2.5 hours (not shown).

Figure 2 shows the dose response curves for atrial natriuretic factor and ST_a. These were obtained from membrane preparations derived from colon samples after 30 minutes of incubation in the activity assay. While ST_a provoked a clear rise in cGMP formation, atrial natriuretic factor failed to stimulate the basal guanylyl cyclase activity of this tissue. Similar results were obtained from surgical colon biopsy specimens even after prolonged incubation (not shown). With porcine ileum as the source for ST_a stimulatory guanylyl cyclase a similar concentration response curve as with the biopsy specimens under

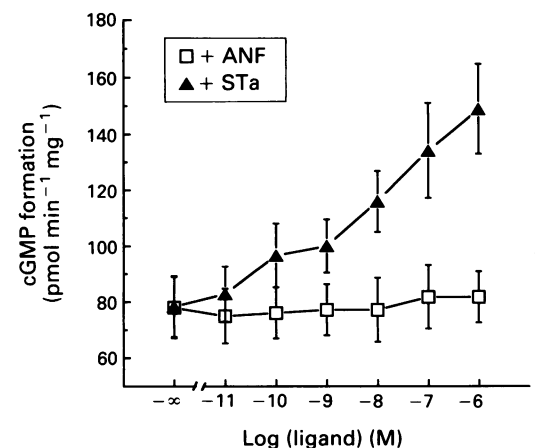


Figure 2: Stimulation of intestinal particulate guanylyl cyclase activities by heat stable enterotoxin and atrial natriuretic factor. Membranous cell components were isolated from colonoscopic specimens taken from the colon descendens. Their specific guanylyl cyclase activities with and without addition of increasing amounts of the ligands atrial natriuretic factor and ST_a were determined from triplicate measurements as described in methods section.

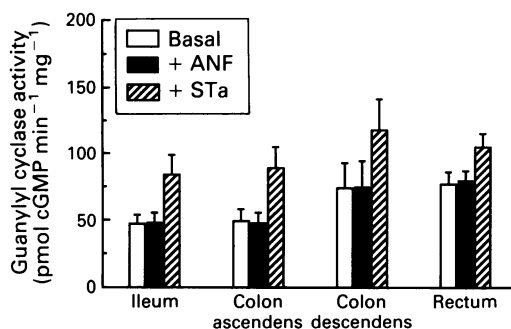


Figure 3: Activities of particulate guanylyl cyclases in different regions of human intestine. The membrane bound enzymes were prepared from colonoscopic specimens taken from the terminal ileum ($n=9$), colon ascendens ($n=9$), colon descendens ($n=7$), and rectum ($n=7$). Basal as well as atrial natriuretic factor and ST_a stimulated guanylyl cyclase activities were determined after one hour of incubation at the described assay conditions. The particulate guanylyl cyclase activities measured in the colonoscopic specimens were averaged for each of the four different intestine regions.

examination was obtained (not shown). In contrast, GC-A contained in the membrane fraction of bovine adrenal cortex started to respond to atrial natriuretic factor at a ligand concentration of about 1 nM and attained the saturation level of stimulation at about 100 nM atrial natriuretic factor (not shown).

To determine the distribution of particulate guanylyl cyclases in the lower intestine of adult humans, 32 specimens belonging to four different intestine regions were examined enzymatically. Membrane preparations derived from colonoscopic specimens taken from terminal ileum, colon ascendens, colon descendens, and rectum were subjected to guanylyl cyclase activity assays using differential stimulation. Figure 3 shows the basal guanylyl cyclase activities and stimulation by atrial natriuretic factor and ST_a .

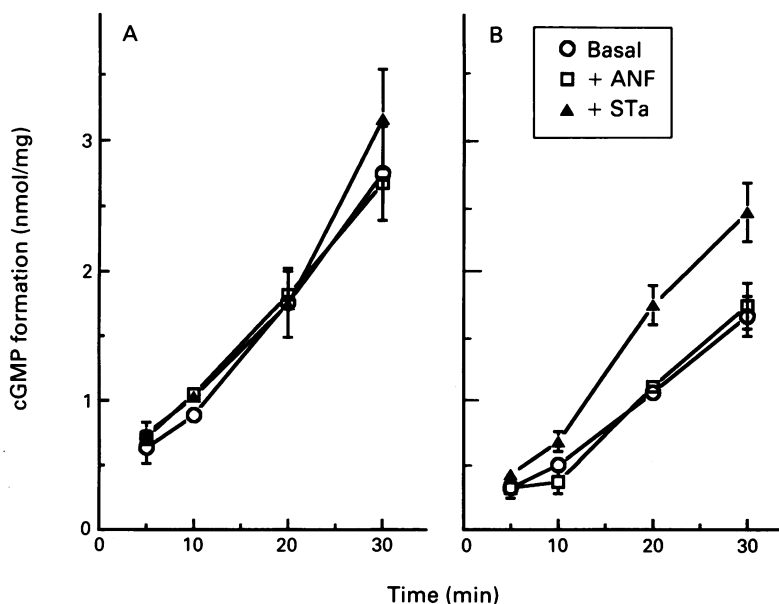


Figure 4: Comparison between the cGMP formation by particulate guanylyl cyclases from mucosal and submucosal cell layers. Tissue samples from the colon descendens were assayed for cGMP formation with or without stimulation by atrial natriuretic factor or ST_a at several time intervals. Specimens obtained after surgery represent submucosal tissue layers (A) and mucosa (B). The time curves show basal activities and guanylyl cyclase activities stimulated by 10^{-6} M atrial natriuretic factor or 10^{-7} M ST_a and obtained from triplicate assays.

The basal activities in terminal ileum and colon ascendens were about 50 pmol cGMP formation/mg protein/min. In colon descendens and rectum the basal activities were around 75 pmol cGMP formation/mg protein/min. Atrial natriuretic factor did not show any stimulatory effect on the guanylyl cyclase activities in any of the tested intestine samples.

After stimulation by 10^{-7} M ST_a , the guanylyl cyclase activities in membrane preparations from terminal ileum and colon ascendens were increased maximally 1.8-fold compared with the basal activities. In colon descendens and rectum, a somewhat lower stimulation of guanylyl cyclase activity by ST_a was seen, namely 1.6-fold or 1.3-fold increases, respectively. The absolute difference between basal and ST_a stimulated specific guanylyl cyclase activities, however, was enhanced from 35 to 45 pmol cGMP formation per min and mg membrane protein from terminal ileum to colon descendens and, with 30 pmol, was slightly lower only in the rectum.

Essentially the same distribution patterns of particulate guanylyl cyclase activities were obtained when specimens representing all four intestinal regions were collected from the same subjects and evaluated for each patient separately (not shown).

COMPARISON OF GUANYLYL CYCLASE ACTIVITIES IN MUCOSAL AND SUBMUCOSAL CELL LAYERS

Surgically obtained tissue samples from colon descendens were used to show the horizontal distribution of particulate guanylyl cyclase activities in the mucosal and submucosal layers of a given intestine section. In Figure 4 the time courses of guanylyl cyclase activities with or without atrial natriuretic factor or ST_a stimulation in mucosal and submucosal membrane preparations are compared. Surprisingly, the basal activities in the submucosal sample surpassed those in the epithelial layer. On the other hand, the stimulation by ST_a after 30 minutes of incubation at 37°C was 1.5-fold in mucosa and only 1.1-fold in submucosa, while atrial natriuretic factor stimulation was negligible in both tissue layers.

AMPLIFICATIONS USING A NEWLY DESIGNED PRIMER PAIR

By sequence comparison we identified short regions common to human GC-A, GC-B, and GC-C in the intracellular portions of these membrane proteins and used these to design a primer pair specific for all known particulate guanylyl cyclase cDNAs (Fig 1).

Before using the respective polymerase chain reaction primers for cDNA amplification from intestine samples, a number of control experiments was performed to assure the specificity and sensitivity of the method. For adjusting optimal amplification conditions, recombinant plasmids containing cDNA encoding particulate guanylyl cyclases were

used as test templates in polymerase chain reaction. From human GC-C and rat GC-A cDNA templates, an amplicate of the correct size was obtained. Figure 5 shows that the specificity of amplification could be confirmed by restriction analysis of the polymerase chain reaction products, which exhibited the cleavage patterns expected from the known DNA sequences. The distinction between GC-C and GC-A by restriction mapping also worked, when mixtures of GC-C and GC-A templates were used (not shown). This showed that there was no bias for the amplification of either particulate guanylyl cyclase type. A rough quantification of the message for different particulate guanylyl cyclase types after 35 polymerase chain reaction cycles also was possible, as different template dilutions yielded different signal strengths and because the ratio of template mixtures was reflected in the portions of *AccI* and *PstI* cleavable amplicate. To assess the sensitivity of the polymerase chain reaction, recombinant phage lysates with known titres were used. Phage particles containing cloned particulate guanylyl cyclase cDNA were lysed by three freeze-thaw cycles. From as little as 100 plaque forming units of recombinant phage a discernible signal was obtained (not shown). Phage lysates representing cDNA libraries from rat jejunum or the human colonic cell line T84 also yielded amplicates of the size spanned by the particulate guanylyl cyclase primers after 35 cycles of amplification. By restriction analysis, the polymerase chain reaction products amplified from about 10^6 independent clones of these libraries could be assigned to GC-C (not shown).

AMPLIFICATION FROM INTESTINE SPECIMENS

mRNA was purified from about 50 mg of tissue and reversely transcribed. Portions of resulting cDNA were subjected to polymerase chain reaction and the amounts of amplified

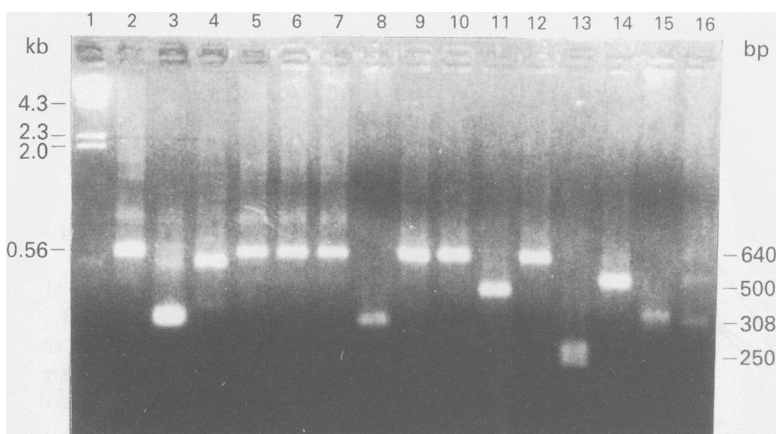


Figure 5: Restriction analysis of amplified particulate guanylyl cyclase cDNA. Plasmid DNA containing human GC-C (lanes 2 to 9) or rat GC-A cDNA (lanes 10 to 16) as inserts was used as the template in polymerase chain reactions under the conditions described. The amplicates were digested with a set of restriction enzymes selected by sequence comparison. Mapping the 640 bp amplicates with this range of restriction endonucleases permitted the two guanylyl cyclase forms to be distinguished from each other. Lanes 1 and 16 contain size markers, while uncleaved amplification product was separated on lanes 2 and 9. The polymerase chain reaction products were digested by the following restriction enzymes: *AccI* (lanes 3 and 10), *AvaII* (4, 11), *BanII* (5, 12), *HaeIII* (6, 13), *PstI* (7, 14), and *Sau3AI* (8, 15) and yielded the predicted fragment sizes.

DNA were estimated after gel electrophoresis. Using the cDNA derived from about 5 mg of tissue, sufficient DNA for a couple of restriction digests was amplified. A typical cDNA amplification experiment from the selected tissue specimens resulted in the electrophoretic banding patterns depicted in those gel lanes that contain uncleaved polymerase chain reaction product (Fig 6).

Whereas the submucosal samples yielded unambiguous DNA fragments of the expected size, additional bands developed in mucosal samples. The sizes of the background bands were smaller than the expected amplification product and common in all mucosal samples. The mucosal caecum sample contained an additional 700 bp band, which, however, was split by *AccI* into the 300 bp fragments typical for GC-C. The approximate sizes of the smaller fragments found besides the expected amplification product of 635 bp were 350 bp, 450 bp, and 500 bp, respectively. The contrasting electrophoretic appearance after cDNA amplification implies a profound difference between the expression of particulate guanylyl cyclases in mucosa and submucosa.

Because of the comparatively low cycle number and according to the control experiments the amounts of amplicate permit conclusions about the expression levels of particulate guanylyl cyclases in the tissue specimens. A minimum of particulate guanylyl cyclase expression was seen in the caecum. The highest amounts of amplicates from the submucosa preparations were found in the sigmoid and rectum regions. In the mucosal cell layers the highest expression levels were seen in ileum and sigmoid. The content of mRNA encoding particulate guanylyl cyclase in all intestinal specimens by far surpassed that of a control tissue, namely vena saphena magna.

DISTINCTION OF GUANYLYL CYCLASE FORMS IN AMPLIFIED CDNA

The ratio of different guanylyl cyclase forms in the amplicates was assessed by restriction digests with *AccI*, which specifically cleaves GC-C amplicates, and *PstI*, which has recognition sites only on amplicates from GC-A (Fig 1). The polymerase chain reaction products obtained from GC-B cDNA can be discriminated from GC-A and GC-C amplicates by digestion with *Sau3AI*, which shortens the GC-B amplicate by only 15 bp and *BanII* which results in 200 bp and 460 bp GC-B fragments. As shown in lanes 2 to 4 of Figure 6 (B), GC-B amplicates also were conspicuous in the present expression assay using the restriction enzymes used to distinguish GC-A and GC-C. Neither the digestion of selection amplicates with *Sau3AI* and *BanII*, nor characterisation of the available polymerase chain reaction products by *AccI* and *PstI* cleavage yielded any evidence for GC-B expression.

According to restriction analysis, amplicates from submucosa consisted of GC-A

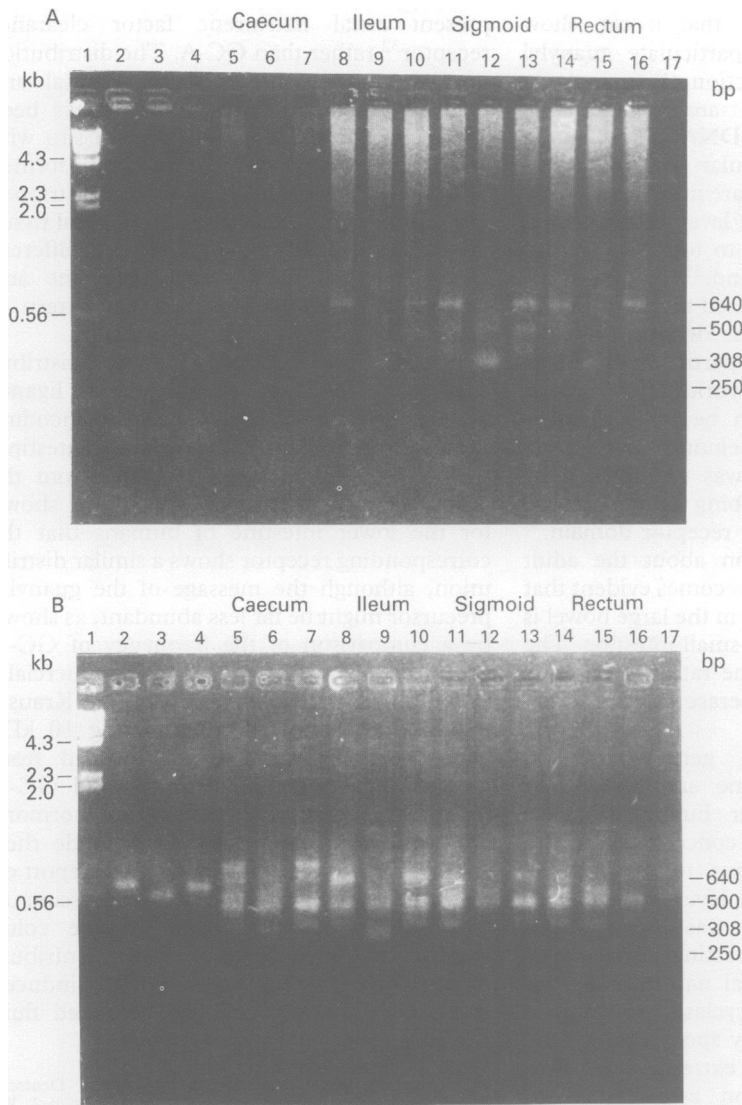


Figure 6: Determination of the prevailing particulate guanylyl cyclase forms in human intestine biopsy specimens cDNA corresponding to 5 mg of tissue was used as a template in the polymerase chain reaction samples, of which one fifth per lane was separated by gel electrophoresis. Amplification products from submucosal (A) and mucosal (B) cDNA represent the intestine regions ileum, caecum, sigmoid, and rectum. The polymerase chain reaction products were electrophoresed without cleavage (lanes 2, 5, 8, 11, 15) or after digestion with *AccI* (lanes 3, 6, 9, 12, 15) or *PstI* (lanes 4, 7, 10, 13, 16). (A) Additionally shows the restriction mapping of amplicates from blood vessel cDNA, while an amplicate isolated from an adrenal cortex cDNA library and preliminarily classified as GC-B was used in lanes 2 to 4 of (B). As size standards and as a reference for comparing DNA amounts, 500 ng of phage Lambda DNA digested with *Hind III* (lane 1) and polymerase chain reaction products of known size (lane 17) were included.

and GC-C sequences. Especially in the sigmoid region, GC-C clearly dominated the particulate guanylyl cyclase composition of the submucosa, but the share of GC-C found in submucosal biopsy specimens may result from attached mucosal material. The weak GC-A contribution in submucosal specimens may be attributed to blood vessels and smooth muscle.

Analysis of mucosal amplicates showed the prevalence of GC-C in all examined regions of the intestine, as the specific 640 bp band disappeared after *AccI* digestion and gave rise to bands corresponding to a size of about 300 bp. Despite the enormous sensitivity of detection provided by the polymerase chain reaction method, there is no indication of GC-A expression in intestinal mucosa as after cleavage by *PstI* the signal strengths of 640 bp bands fail to decrease and the amounts of

500 bp fragments are not augmented (Fig 6 (B)).

Discussion

A two pronged approach was used to describe the distribution of particulate guanylyl cyclases in human colon. Our results show that GC-C occurs in the large bowel in considerable amounts, with increasing concentrations from the terminal ileum to rectum. No evidence for the existence of GC-A in the mucosa of the lower intestine was detected. As a third interesting finding, we saw comparatively high levels of particulate guanylyl cyclase activity and message in the submucosal cell layers. With regard to the GC-C activities in the lower intestine, our data on colonoscopies for human adults are in good agreement with the values recently measured in the rat by Mezzoff *et al.*,²⁷ if the lower assay temperature of 32°C used by these authors is taken into account. The presence of GC-C in the lower intestine is not surprising, as the corresponding cDNA had been isolated from a colonic cell line,⁹ an acknowledged source of ST_a – receptor.¹² Our enzymatic results on the distribution of GC-A and GC-C in human tissue specimens are also in good agreement with recent data by Vaandrager *et al.*²⁸ using a similar approach of differential stimulation by atrial natriuretic factor and ST_a with freshly isolated rat intestinal crypt and villus cells, stripped mucosa of proximal rat colon, and various colon carcinoma and undifferentiated small intestinal cell lines.

Above all, in the rectum, but to a lesser extent also in the other intestinal sections, a high proportion of particulate guanylyl cyclase activity was seen, which was neither stimulated by atrial natriuretic factor or by ST_a. These high levels of non-stimulatable basal activities can be explained at least partly by the assay method. The choice of manganese as the divalent cation resulted in higher basal activities and less stimulation. This effect was most pronounced with the high basal guanylyl cyclase activities found in the rectum. The detection of low basal activities was a prerequisite for performing measurements on minute colonoscopic samples, but accounts, to some extent, for the weak stimulation. As GC-C clearly prevailed in the colonoscopic samples, receptor destabilisation by the ligand ST_a²⁹ offers an additional explanation for the high basal guanylyl cyclase activities.

By the described polymerase chain reaction approach, we have shown expression of particulate guanylyl cyclases in human intestine samples and additionally could determine the prevalence of one of the three forms. The primer binding regions as well as the cleavage patterns of the selected restriction enzymes turned out to be sufficiently conserved to make our approach applicable to several mammalian species. Therefore, in view of the small amounts of tissue required, the present polymerase chain reaction method provides an attractive alternative to a northern blot procedure. It is one of the strengths of the

present expression assay, that it can show the distinction between particulate guanylyl cyclase types by restriction mapping, as hybridisation procedures are not suitable for this purpose, when cDNA encoding the highly conserved intracellular portions of the transmembrane receptors are involved.

On the gene expression level, hybridisation of a GC-A cDNA probe to total RNA from human intestine was found.²⁵ As this large hybridisation probe contained the intracellular protein kinase and catalytic domains that are homologous in all known particulate guanylyl cyclases, the northern hybridisation signals from intestinal tissue can be interpreted as GC-C expression. In rat jejunum, ileum, and colon GC-C expression was also shown by northern hybridisation probing for the message encoding the extracellular receptor domain.³⁰ Extracting the information about the adult stage from this study,³⁰ it becomes evident that the GC-C expression level in the large bowel is about equal to that in the small intestine. The hybridisation data from the rat thus coincide with our finding by polymerase chain reaction for adult humans.

Enzyme activity and gene expression measurements support one another in two crucial points: the lower human intestine contains considerable concentrations of GC-C, while GC-A seems to be virtually absent from the mucosal layers. While ST_a stimulated GC-C activity was found in the colon at values even surpassing those in the ileum, no substantial atrial natriuretic factor stimulation of guanylyl cyclase activity was found in any of the biopsy specimens. In the mucosal layers even the extremely sensitive polymerase chain reaction assay failed to detect any expression of GC-A. This is in contrast with previous findings on the distribution^{13 14} and physiological role¹⁵ of GC-A in the intestine, but supports other previous data.^{16 17} In rat intestinal mucosa, a twofold increase of particulate guanylyl cyclase after atrial natriuretic factor application was reported,¹³ but these data have been contradicted by Tremblay *et al.*,¹⁶ who found no activation of guanylyl cyclase in isolated intestinal epithelial cells after incubation with atrial natriuretic factor. Reports on the location of GC-A and also the functional effect of atrial natriuretic factor in the intestine are also contradictory. Thus, a decrease of intestinal water absorption in response to atrial natriuretic factor application was found in rats by Moriarty *et al.*,¹⁵ whereas other researchers¹⁶⁻¹⁸ did not find any atrial natriuretic factor induced changes of the intestinal water balance in human subjects. There are at least three ways to explain these discrepancies. Firstly, a difference in atrial natriuretic factor receptor distribution may exist between species, in the sense that the situation in human intestine differs from that in other mammals.³¹ Secondly, the GC-A activities found by Waldman *et al.*¹³ might stem from submucosal tissue. Thirdly, the intestinal atrial natriuretic factor binding sites shown by Bianchi *et al.*¹⁴ might

present atrial natriuretic factor clearance receptor³² rather than GC-A. The distribution of GC-A and GC-C in the mucosal and submucosal layers of rat intestine has been shown by differential stimulation *in situ* with subsequent immunodetection of formed cGMP.²⁸ Our measurements with stripped mucosa and corresponding submucosal tissue specimens confirm these results for different regions of the human lower intestine and additionally establish a congruent pattern of particulate guanylyl cyclase expression.

It is tempting to compare the tissue distributions of GC-C and its endogenous ligand, guanylin.^{33 34} The levels of mRNA encoding the guanylin precursor in the gastrointestinal tract of the rat increase strikingly from the oesophagus to the colon.³⁴ We have shown for the lower intestine of humans that the corresponding receptor shows a similar distribution, although the message of the guanylin precursor might be far less abundant, as shown by a comparison of the frequency of GC-C and guanylin cDNA in a commercially available rat jejunum library (G Krause, unpublished data). As a circulating 10 kDa form of guanylin has been purified from human haemofiltrate,³⁴ guanylin and GC-C may be regarded as hormone and hormone receptor, with additional roles outside their common main site of synthesis. This report on the functional and molecular expression of particulate guanylyl cyclases in the colon suggests that the large bowel might contribute considerably to the origin of ST_a induced diarrhoea or to the guanylin regulated fluid balance.

This work was supported by the Deutsche Forschungsgemeinschaft grants Sc4/9-1 and Ge399/4-2. We thank Christine Grubmüller for excellent technical assistance. We are grateful to Drs Heldwein and Müller-Lissner, Medizinische Klinik Innenstadt der Universität München, who kindly provided colonoscopic tissue specimens. The support by Dr Danegger and his team at the district hospital Freising in procuring tissue samples after surgery is also gratefully acknowledged.

Parts of the results have been presented at the 8th Conference on Second Messengers and Phosphoproteins at Glasgow in August 1992. Some enzymatic data also are contained in Anja Bayerl's MD thesis.

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