

Disruption of the Guanylyl Cyclase-C Gene Leads to a Paradoxical Phenotype of Viable but Heat-stable Enterotoxin-resistant Mice

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Abstract

Heat-stable enterotoxins (STa), which cause an acute secretory diarrhea, have been suggested to mediate their actions through the guanylyl cyclase-C (GC-C) receptor. The GC-C gene was disrupted by insertion of *neo* into exon 1 and subsequent homologous recombination. GC-C null mice contained no detectable GC-C protein. Intestine mucosal guanylyl cyclase activity was ~ 16-fold higher in wild-type mice than in the GC-C null mice, and STa-stimulable guanylyl cyclase activity was absent in the null animals. Thus, GC-C is the major cyclase activity present in the intestine, and also completely accounts for the STa-induced elevations of cGMP. Gavage with STa resulted in marked fluid accumulation within the intestine of wild-type and heterozygous suckling mice, but GC-C null animals were resistant. In addition, infection with enterotoxigenic bacteria that produce STa led to diarrhea and death in wild-type and heterozygous mice, while the null mice were protected. Cholera toxin, in contrast, continued to cause diarrhea in GC-C null mice, demonstrating that the cAMP signaling pathway remained intact. Markedly different diets (high carbohydrate, fat, or protein) or the inclusion of high salt (K⁺, Na⁺) in the drinking water or diet also did not severely affect the null animals. Given that GC-C is a major intestinal receptor in all mammals, the pressure to retain a functional GC-C in the face of diarrhea-inflicted mortality remains unexplained. Therefore, GC-C likely provides a protective effect against stressors not yet tested, possibly pathogens other than noninvasive enterotoxigenic bacteria. (*J. Clin. Invest.* 1997. 100:1590–1595.) Key words: *Escherichia coli* • intestine • cyclic GMP • diarrhea • disease models, animal

Introduction

Guanylyl cyclase-C (GC-C)¹ is a member of a transmembrane receptor family that transduces an extracellular signal to increase production of intracellular cGMP (1). Other members

of the family include receptors for egg and natriuretic peptides, and a subfamily of orphan receptors expressed in sensory tissues (1–5). GC-C has been suggested as the receptor for *Escherichia coli* heat-stable enterotoxin (STa) (6–8). Two endogenous peptides have been recently suggested as the normal ligands for GC-C, and, although there are no direct binding studies with guanylin or uroguanylin to determine whether GC-C is the principal or only protein to which these peptides bind, the two peptides stimulate cyclase activity and compete for STa binding to GC-C (9, 10). The cyclase is expressed in high amounts in mammalian intestine and, dependent upon the species, in other tissues as well (6, 11–15). Since all mammals appear to express GC-C and are susceptible to STa-induced acute secretory diarrhea, it is assumed that the evolutionary pressure to retain a functional GC-C receptor resides in its having an essential role in the normal regulation of the intestine (or other tissues) and/or its requirement in an essential protective role against, for example, certain enteric infections.

The gene encoding GC-C, *gucy2c*, has been mapped to distal chromosome 6 in the mouse, and chromosome 12p12 in the human (16), where no genetic diseases have yet been described. Therefore, targeted gene disruption in the mouse appeared to represent one of the most powerful methods available to delineate the expected critical functions of GC-C. Here, we report the production of a mouse line in which the gene encoding GC-C is disrupted, and in which no protein for GC-C is detected. The mice are viable, fertile, and develop normally, but are resistant to STa-induced diarrhea and to enterotoxigenic bacteria that produce STa. Furthermore, marked variation in the diet or in the salt content of the food or drinking water does not adversely affect the null mice. Thus, the stressors that are likely more severe than STa-induced diarrhea have not yet been discovered.

Methods

Targeting vector construction. A murine 129/Sv genomic library in λFIXII (Stratagene, La Jolla, CA) was probed with the 5' 0.9 kb of rat GC-C. One positive clone (1a) of ~ 14.5 kb was identified. Restriction mapping and partial sequencing revealed that clone 1a contained exons 1–4 of the GC-C gene, with ~ 1 kb of 5' flanking sequence. When the library was again probed with the 5' end of clone 1a, in addition to clones with similar restriction patterns to clone 1a, several clones containing additional 5' sequence were identified. Clone 7a contained ~ 6 additional kb of 5' flanking sequence and was used for construction of the targeting vector.

The targeting vector was constructed such that a neomycin resistance gene, under the control of the RNA polymerase II promoter, interrupted the coding region of the GC-C gene in exon 1 at a unique

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1. **Abbreviations used in this paper:** cGKII, cGMP-dependent protein kinase; ES, embryonic stem; ETEC, enterotoxigenic *Escherichia coli*; GC-C, guanylyl cyclase-C; STa, heat-stable enterotoxin.

BglII site. A 3.5-kb XbaI/SacI fragment of clone 7a was ligated to a 1.4-kb SacI/EcoRI fragment of clone 1a, and subcloned into the Bluescript plasmid (Stratagene). The resulting plasmid, which included exon 1 and ~4 kb of 5' flanking sequence, was used as a template for PCR to generate one arm of the targeting construct. Primers with 5' Sall sites were used to amplify a 2.4-kb fragment (nucleotides -2317 to +94, numbering from the initiating Met codon), which was then gel purified and trimmed with Sall. Due to the low efficiency of Sall digestion, the PCR product was first cloned into Bluescript and then into XhoI-digested pTKneo to generate pTKneo/short arm. The other arm of the targeting construct was produced by subcloning a 7-kb Sall/XhoI fragment of clone 1a into XhoI-digested Bluescript. The unique Sall site in the Bluescript multiple cloning site was deleted by digestion with XhoI and EcoRV, followed by partial fill-in with Klenow and religation. A 6-kb BglII/NotI fragment, including the 3' end of exon 1, was excised from Bluescript and cloned into a NotI-digested pTKneo/short arm, using a BglII/NotI adapter, to generate the complete targeting construct. Orientation of both arms relative to the neomycin cassette was confirmed by restriction digest.

Gene disruption in embryonic stem (ES) cells. SM-1 ES cells (kindly provided by Dr. R. Hammer and S. Maika, University of Texas Southwestern Medical Center) were maintained on irradiated feeder fibroblasts using recommended conditions (17). Approximately 10^7 exponentially growing ES cells (passage 5) were electroporated (230 V/500 μ F) (Gene Pulser; Bio-Rad Laboratories, Richmond, CA) with 25 μ g Sall-linearized targeting construct on day 0. Targeted cells were selected in the presence of 180 μ g/ml G418 (days 1-8) and 0.2 mM ganciclovir (days 3-5). Individual colonies were isolated on day 7, and expanded for Southern blot analysis (see below).

Generation of GC-C targeted mice. ES cells from five clones displaying homologous GC-C recombination were injected into day 3.5 postcoitum blastocysts derived from C57BL/6 mice. Healthy blastocysts were reintroduced into the oviducts of pseudopregnant BALB/c foster mothers. Two independent lines of mice with high percent coat color chimerism were established. Male chimeras with > 95% agouti coat were crossed with C57BL/6J or BLSW females. Agouti F1 progeny were genotyped by Southern blot of tail DNA, and animals heterozygous for the targeted disruption of GC-C were crossed. All animals were maintained and handled using guidelines and protocols established or approved by the University of Texas Southwestern Medical Center Animal Resources Center.

Southern blot analysis. Approximately 15 μ g DNA, prepared from ES cells or mouse tail, was digested overnight with the appropriate restriction enzyme and analyzed by Southern blot. For routine genotyping, DNA was digested with XbaI and probed with a 2.5-kb XbaI/BglII fragment that recognizes a sequence 5' to that included in the targeting vector (expected band: +/+ 7.5 kb; -/- 4.5 kb). In some instances genotyping was confirmed by digesting DNA with BamHI/HindIII and probing the digest with the *neo* cassette (expected band: 9 kb). The *neo* probe was also used to ensure that targeted ES cells integrated only one copy of the disrupted GC-C gene.

Suckling mouse assay. 3-d-old pups were given an oral inoculum of 0.1 ml PBS or PBS with either 5×10^{-7} M STa, or 5 μ g cholera toxin. Evans Blue dye (0.1%) was included in the inoculum to monitor its progression through the gut in methods similar to those previously described (18). Mice were kept at ambient temperature for 2 h and then were killed by chloroform vapor inhalation. The intestinal tract (small and large intestines plus rectum) was removed and weighed, as was the remaining carcass. Results of the assay were expressed as the ratio of the gut to carcass weight. Pups were subsequently genotyped by tail DNA analysis.

Infection with enterotoxigenic *E. coli* (ETEC). *E. coli* strain 1676 was obtained from Dr. Evelyn A. Nystrom (National Animal Disease Center, Ames, IA). ETEC strain H10407, which does not colonize and infect mice (provided by F. Cassels, Walter Reed Army Institute of Research, Washington, D.C.), or strain 1676 was grown overnight in trypticase soy broth supplemented with streptomycin and tetracycline (strain 1676) or on CYA plates (strain H10407). Cultures were har-

vested and diluted in PBS. Bacteria were counted using a $\times 40$ DIC objective and diluted to a final concentration of 10^7 per 50 μ l. Mouse pups (24 h in age) were given 10^7 bacteria by gavage and evaluated twice daily. All animals were genotyped at the conclusion of the experiment.

Guanylyl cyclase assay. The small intestine was removed from adult mice and the lumen flushed with ice-cold PBS/5 mM EDTA. After incision, the intestine was everted in 100 ml cold buffer, and the mucosa scraped from the underlying muscle with a glass slide. Mucosal scrapings were pelleted at 3,000 g, and washed three times with 25 ml ice-cold PBS/5 mM EDTA. The washed mucosa was resuspended in cyclase assay buffer (10% glycerol, 50 mM Hepes, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, and 1 μ g/ml pepstatin), and snap-frozen in liquid N₂. Guanylyl cyclase activity was assayed at 37°C in a 150- μ l reaction containing 50 mM Hepes, pH 7.5, 100 mM NaCl, 0.2 mM IBMX, 10 mM NaN₃, 1 mM GTP, and either 5 mM MgCl₂/1 mM ATP or 5 mM MnCl₂/1% Triton X-100. Reactions were initiated by the addition of 15 μ g mucosal protein, and terminated after 5 min by the addition of 0.5 ml each of 110 mM ZnOAc and 110 mM Na₂CO₃. cGMP was determined by radioimmunoassay (19).

Western blot analysis. 50 μ g of mucosal protein was fractionated by 7.5% SDS-PAGE, and transferred to an Immobilon P membrane (Millipore Corp., Bedford, MA). An antipeptide antibody raised against the carboxyl terminus of rat GC-C (20) was used to probe the membrane, and detection was with ECL reagent (Amersham Corp., Arlington Heights, IL).

Results

GC-C gene targeting. The gene encoding GC-C was disrupted by inserting a neomycin resistance gene cassette, in the opposite transcriptional orientation, in exon 1 (Fig. 1). Transcription of the targeted GC-C gene would result in the detection of an in-frame translation termination codon, and thus a transcript with a severely truncated open reading frame. Approximately 8.5 kb of GC-C sequence was included in the targeting vector, which resulted in a high frequency of homologous recombination in transfected ES cells. 25% of colonies screened after double selection in G418 and ganciclovir exhibited the Southern blot pattern expected for a correct targeting event when hybridized with a probe 5' to the site of targeting, and 12 colonies were expanded for further analysis. Five clones that showed the appropriate hybridization to both the 5' and *neo* probes were chosen for blastocyst injection. Three clones were injected individually, while two were pooled and injected together. Clone H8 and the mixture of clones B4 and H11 resulted in offspring with a high percentage of ES cell contribution as judged by agouti coat color. Male mice exhibiting > 90% coat color chimerism were bred with either C57BL6 or BLSW females. Germline transmission of the targeted GC-C gene disruption was confirmed by genotyping agouti F1 pups. Heterozygous F1 mice were crossed to produce the F2 generation. A representative Southern blot of F2 tail DNA is shown in Fig. 2. Genotyping of the GC-C locus of F2 pups revealed a ratio of +/+ : +/- : -/- that did not differ significantly from the 1:2:1 ratio expected for a nonlethal mutation (43 +/+, 84 +/-, 42 -/- mice). Homozygous null mice were phenotypically indistinguishable from wild-type or heterozygous littermates in the disease-free setting. Histological analysis of young adult mice revealed no gross abnormalities of intestine, liver, kidney, lung, heart, or brain.

Guanylyl cyclase activity. Guanylyl cyclase activity was measured in a particulate preparation of mucosal scrapings from young adult mice. Total enzymatic activity was deter-

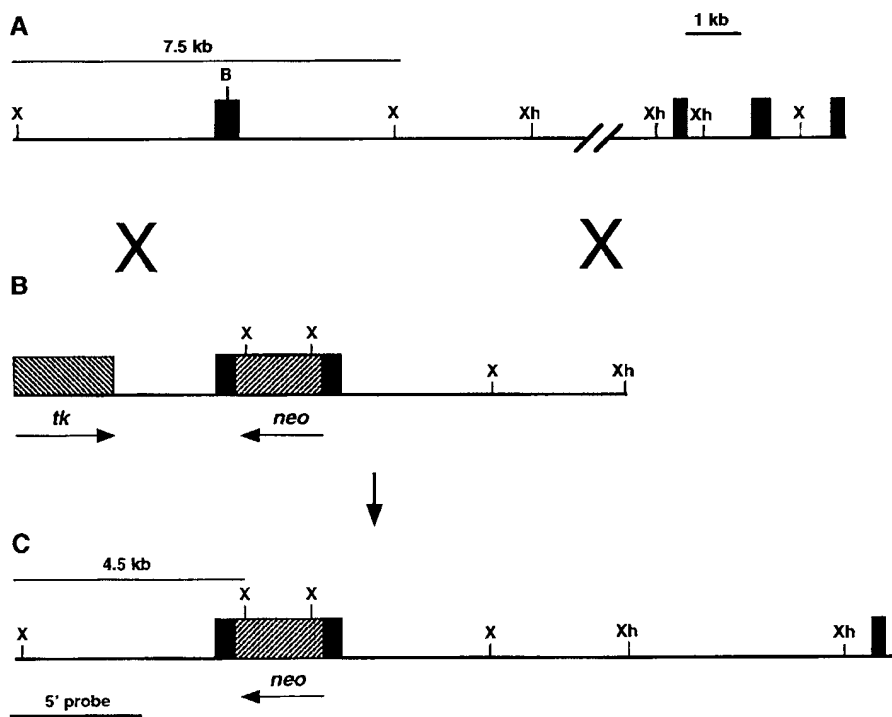


Figure 1. Targeted disruption of the GC-C gene. (A) Restriction map of the 5'-end of the GC-C gene showing the organization of the first three exons (filled boxes). (B) Structure of the targeting vector. A neomycin resistance gene cassette (*neo*) interrupts exon 1. (C) Predicted homologous recombination event. The altered XbaI restriction endonuclease digestion pattern produced by correct integration of the targeting vector is shown. The bar indicates the 5' probe used for genotyping. B, BglII; *tk*, Herpes simplex thymidine kinase gene; X, XbaI; Xh, XhoI.

mined in the presence of Mn^{2+} /Triton X-100, which maximally stimulates guanylyl cyclase activity (21). STA-stimulated guanylyl cyclase activity was determined with Mg^{2+} substituted as the metal cofactor. GC-C null mice contained no STA-stimulated guanylyl cyclase activity, while the activity in heterozygotes was intermediate between null (basal) and wild-type (Fig. 3). The null mice contained substantially reduced total guanylyl cyclase activity, as determined in the presence of Mn^{2+} /Triton X-100, suggesting that GC-C is the predominant particulate form of the enzyme in the intestinal mucosa. Western blot analysis confirmed the absence of GC-C receptor protein in the intestinal mucosa of null mice (Fig. 4). In addition, there was no specific binding of ^{125}I -STA to mucosa isolated from mice homozygous for the targeted GC-C disruption, while heterozygotes contained $\sim 50\%$ of the specific ^{125}I -STA binding of wild-type mice (data not shown). These results contradict early studies on the apparent STA receptor (22), but are in agreement with more recent work suggesting that GC-C is the sole high-affinity STA-binding protein in the intestine, and that smaller STA binding proteins identified by ligand cross-linking are derived from GC-C through proteolysis (7, 20).

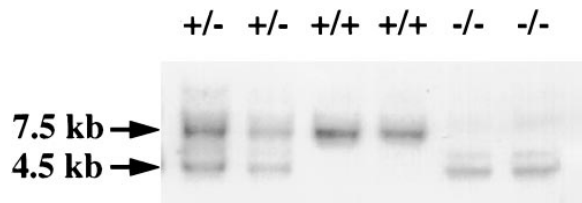


Figure 2. Southern blot analysis of an F2 litter. XbaI-digested genomic DNA was hybridized with the 5' probe described in Fig. 1. Disruption of exon 1 with the *neo* cassette results in the generation of a 4.5-kb XbaI fragment, whereas the wild-type fragment is predicted as 7.5 kb.

Suckling mouse assay. To assess the intestinal secretory response to *E. coli* STA, 3-d-old pups were given an oral inoculum of 10 U of STA. A unit of STA is defined as that amount of enterotoxin that yields a gut/carass ratio of > 0.83 in the suckling mouse (18). Although the inoculum is diluted two- to threefold by the stomach contents, a $100\text{-}\mu\text{l}$ dose of STA at 5×10^{-7} M is in the range of previously established EC_{50} values for stimulation of cGMP accumulation in cells expressing cloned

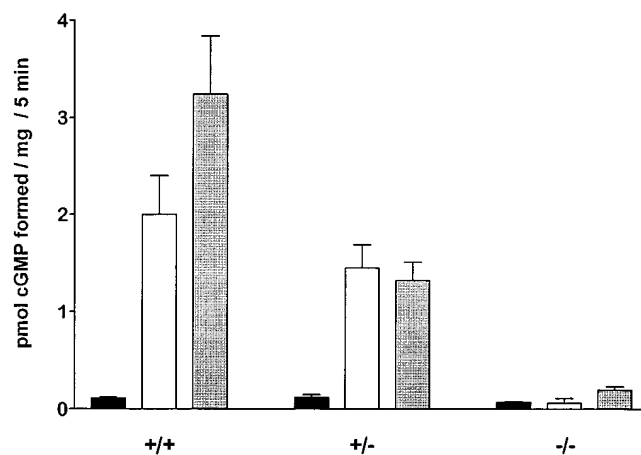


Figure 3. GC-C activity of intestinal mucosa as a function of genotype. Enzymatic activity is expressed as picomoles of cGMP formed per milligram of mucosal protein over 5 min. Basal and STA ($1 \mu\text{M}$)-stimulated activities were estimated in the presence of Mg^{2+} , and maximal activity (Triton X-100) was estimated in the presence of Mn^{2+} . The values presented represent activities from pooled, homogenized intestinal mucosal scrapings prepared from two adult mice of each genotype and assayed in duplicate. cGMP produced was measured by radioimmunoassay. Black bars, basal; white bars, STA 10^{-6} M; gray bars, Mn^{2+} /Triton X-100.

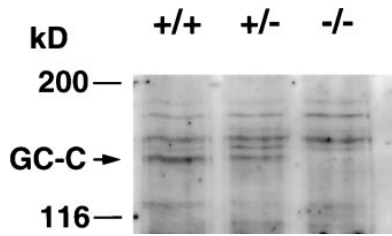


Figure 4. Immunoreactive GC-C as a function of genotype. Western blot analysis of intestinal mucosal preparations was performed using an antibody directed against the carboxyl-terminal decapeptide of GC-C. Mucosal

scrapings obtained as described in Methods were dissolved in SDS and electrophoresed on SDS-PAGE. The arrow indicates GC-C immunoreactivity which is about one-half of wild-type (+/+) in heterozygous (+/-) and absent in homozygous null (-/-) mice.

GC-C (6, 7). Wild-type or heterozygous mice responded to oral administration of STa with significant accumulations of fluid within the intestinal lumen (Fig. 5). However, homozygous null mice did not exhibit any fluid accumulation in response to STa, in that their gut/carcass ratio did not differ significantly from that of mice inoculated with PBS. To examine the specificity of the response, mice were also gavaged with cholera toxin, and both wild-type and null mice responded with an accumulation of fluid within the intestine (Fig. 5).

Infection with ETEC. In the initial study, 21 mice from four litters of heterozygous crosses were given an inoculum of *E. coli* strain 1676 by gavage. This strain of bacteria produces STa, expresses colonization factor F41, and has been shown to infect infant mice of multiple strains (23). Of the 21 mice, 4, all -/-, survived to weaning. 4 +/+, 10 +/-, and 2 additional -/- mice died within 72 h of administration of ETEC strain 1676. One mouse was lost during the study and not genotyped. Survival to the bacterial infection was significantly correlated with genotype (χ^2 , $P < 0.05$). Since the reason for two deaths in the null mice was not established, an additional litter of 11 homozygous null mice were given the same dose of bacteria, and 9 survived to weaning. The other two pups died within 24 h of

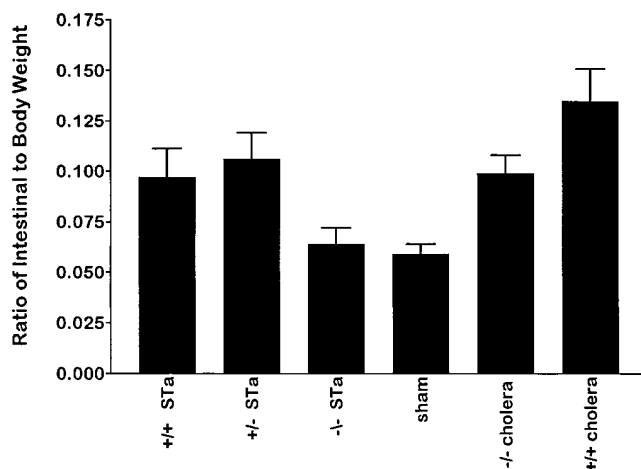


Figure 5. STa-induced fluid accumulation in suckling mice as a function of genotype. 2 h after oral administration of vehicle (PBS), 10 U of STa, or 5 μ g of cholera toxin (*cholera*), the gut/carcass weight ratio was determined. ANOVA analysis, $P = 8.75 \times 10^{-13}$; Tukey's test of means shows that nulls differ significantly from wild-type or heterozygous mice in response to STa, but not from the PBS control. The mice continued to respond to cholera toxin.

the gavage. One of the -/- pups was a runt and the second appeared to be rejected by the mother. There was no evidence of diarrhea in any of the pups. To evaluate nonspecific effects of ETEC administration on mouse survival, 26 pups (24 h old) of heterozygous pairings were given 10^7 CFU of a human ETEC strain H10407 which does not colonize and infect mice (23). 3 mice were lost during the first night after gavage of the inoculum, and the remaining 23 survived to weaning.

Diet and salt intake. Animal weight and survival on different diets and salt intakes was analyzed over an 8-wk period in mice starting at the age of 3–4 mo. The different diets were modified from the basal diets as follows: (a) protein 40 g/100 g diet (basal: 17 g/100 g diet); (b) fat 15.25 g/100 g diet (basal: 5 g/100 g diet); (c) sucrose 50 g/100 g diet (basal, none); and (d) fat 1.76 g/100 g diet (basal: 5 g/100 g diet). High NaCl diets had 8% NaCl while the basal diet normally contains 0.7% NaCl. The NaCl-deficient diet contained 0.008% NaCl. KCl was included in the drinking water at a concentration of 1%. The basal diet remained unchanged in this case. Despite the marked variation in diet and salt intake, there were no animal deaths in either wild-type or GC-C null mice. Animals weights also did not vary as a function of genotype (data not shown).

Discussion

Diarrhea resulting from infection with *E. coli* is a world-wide health problem (causing up to 800,000 deaths per year), and is induced by *E. coli* through at least five different pathogenic mechanisms (24). Enterotoxigenic and enteropathogenic strains (ETEC and EPEC) produce a noninflammatory diarrhea, while the more severe enteroinvasive, enterohemorrhagic, and enteroaggregative strains (EIEC, EHEC, and EAggEC) cause inflammatory diarrhea. The various strains may elaborate heat-stable and/or heat-labile enterotoxins, or may invade the intestinal mucosa, causing necrosis. Although infections with enterotoxigenic and enteroaggregative *E. coli* are a major cause of infant and domestic livestock morbidity and mortality, adults are not immune. Traveler's diarrhea, which affects up to half of those traveling from developed to underdeveloped countries, can be caused by ETEC (40–70% of the cases of traveler's diarrhea reported; 25). The low molecular weight STa produced by ETEC and EAggEC possess two to three disulfide bonds, which are required for biological activity.

Through purification, cDNA cloning and expression, and disruption of genes involved in the regulation of intestinal secretion, a signaling pathway for STa has been delineated. STa was known for some years to activate a particulate form of guanylyl cyclase found on the apical membrane of enterocytes (15, 26). The mechanism by which STa activated the cyclase was not established until the guanylyl cyclase activated by STa (GC-C) was cloned and expressed in cultured cells (6–8). The structure of GC-C immediately suggested that it was the receptor itself, since it closely resembled known guanylyl cyclase receptors, diverging only in primary sequence within the extracellular domain. STa, in fact, bound and markedly stimulated the recombinant GC-C (6, 7). The generation of antibodies to GC-C furthermore suggested that low molecular weight STa binding proteins were proteolytic fragments of GC-C (7, 20). Thus, the initial step of the STa-induced secretory response focused to a single event, the binding of STa to one protein (GC-C) and the subsequent increased production of the second messenger, cGMP. It was also speculated that pathogenic bacteria

producing STa were taking advantage of a normal endogenous signaling pathway, and subsequently two peptides were isolated from mammals that are capable of stimulating GC-C (9, 10). Although it still remains unclear if these two peptides (guanylin and uroguanylin) normally signal through GC-C, given the mortality rates due to diarrhea, it seems necessary to assume that the normal functions of GC-C are critical for survival at some stage before reproduction, since otherwise considerable selective pressure should have resulted in an STa-insensitive form of GC-C. Downstream from the receptor, a special isoform of cGMP-dependent protein kinase (cGKII; 27, 28) appears to be the critical cGMP target with respect to the intestinal secretory response. The recent disruption of the cGKII gene, in fact, leads to resistance to STa-induced diarrhea strongly implicating cGKII as the first step downstream from receptor activation (29). It is speculated that cGKII then phosphorylates the cystic fibrosis transmembrane conductance regulator (CFTR), thereby opening this chloride channel. Both the cGMP- and cAMP-induced diarrhea, then, are thought to ultimately be due to chloride efflux accompanied by impaired sodium absorption (15, 30). Guanylin also has been shown to reproduce the effects of STa, presumably through activation of CFTR (30–32). However, guanylin causes diarrhea in infant mice only at doses at least four orders of magnitude higher than that of STa (33).

In terms of elucidating the predicted critical functions of GC-C, other tissues must also be considered. STa binding has been detected in many extraintestinal organs of the North American opossum, including liver and kidney, although GC-C has not been directly demonstrated as the binding protein (14). In the rat, GC-C mRNA has been reported in the testis, placenta, and fetal, neonatal, and regenerating liver (11, 12). Using RT-PCR, however, Carrithers et al. (34) detected human GC-C mRNA only in the intestinal tract and in tumors derived from intestinal tissues. Gao and Garbers (35) found mRNA for GC-C in the rat pancreas and particularly high expression in cultured insulinoma cells. Thus, the expected critical functions of GC-C could be a result of its importance in tissues outside the intestine.

As seen here, however, gene disruption of GC-C failed to apparently adversely affect mice, at least animals raised in a disease-free environment. Furthermore, the GC-C null mice were now resistant to STa-induced diarrhea. Dramatic changes in diet or salt intake also did not adversely affect the null mice. Therefore, the GC-C gene disruption has resulted in an apparent paradox. A receptor which accounts for significant mortality in young animals due to diarrhea is highly conserved in all mammals. In the absence of a critical survival benefit of the receptor, there would be strong evolutionary pressure for a mutation to block the diarrhea, but animals with such mutations in GC-C, and therefore demonstrating resistance to STa, have not been described. The likely explanation for the results is that we have yet to test the appropriate stressors, stressors that would likely be common to all mammals. Since animals eat markedly different foodstuffs, it is reasonable to argue that diet will not provide the stress, and in fact in these studies, mice on high carbohydrate, protein, or fat diets were not severely affected as a function of genotype.

Another possibility is salt intake. For example, if K⁺ uptake were critically regulated by GC-C, then high K⁺ intake in the absence of GC-C could lead to cardiac arrhythmias and death. However, we found no apparent effects of diet or salt

intake on survival. Another stressor could be pathogens more severe than the *E. coli* tested. Could GC-C protect against bacterial invasion? It is now known that if the epithelial cell responds to bacterial binding with phosphorylation of proteins and movement of actin (36), but whether cGMP plays a role in such signaling has not yet been determined.

Based on the mouse model, it then appears that a symbiotic relationship has developed between enterotoxigenic *E. coli* and mammals. Mammals are unable to live without GC-C, and therefore must accommodate the diarrhea and mortality associated with such bacteria. Finding the predicted stressors which will cause animal death in the absence of GC-C may now require introduction of such mice into an open environment.

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