

# Guanylyl cyclase C agonists regulate progression through the cell cycle of human colon carcinoma cells

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The effects of *Escherichia coli* heat-stable enterotoxin (ST) and uroguanylin were examined on the proliferation of T84 and Caco2 human colon carcinoma cells that express guanylyl cyclase C (GC-C) and SW480 human colon carcinoma cells that do not express this receptor. ST or uroguanylin inhibited proliferation of T84 and Caco2 cells, but not SW480 cells, in a concentration-dependent fashion, assessed by quantifying cell number, cell protein, and [<sup>3</sup>H]thymidine incorporation into DNA. These agonists did not inhibit proliferation by induction of apoptosis, assessed by TUNEL (terminal deoxynucleotidyl transferase-mediated dNTP-biotin nick end labeling of DNA fragments) assay and DNA laddering, or necrosis, assessed by trypan blue exclusion and lactate dehydrogenase release. Rather, ST prolonged the cell cycle, assessed by flow cytometry and [<sup>3</sup>H]thymidine incorporation into DNA. The cytostatic effects of GC-C agonists were associated with accumulation of intracellular cGMP, mimicked by the cell-permeant analog 8-Br-cGMP, and reproduced and potentiated by the cGMP-specific phosphodiesterase inhibitor zaprinast but not the inactive ST analog TJU 1-103. Thus, GC-C agonists regulate the proliferation of intestinal cells through cGMP-dependent mechanisms by delaying progression of the cell cycle. These data suggest that endogenous agonists of GC-C, such as uroguanylin, may play a role in regulating the balance between epithelial proliferation and differentiation in normal intestinal physiology. Therefore, GC-C ligands may be novel therapeutic agents for the treatment of patients with colorectal cancer.

**E***scherichia coli* heat-stable enterotoxins (STs) are a family of homologous peptides produced by bacteria that cause diarrhea in travelers, people in underdeveloped countries, and farm animals (1–4). ST induces intestinal secretion by binding to guanylyl cyclase C (GC-C), a single transmembrane protein that is expressed exclusively in the brush border of intestinal epithelial cells from the duodenum to the rectum in adult humans (5–10). Toxin interaction with the extracellular domain activates the cytoplasmic catalytic domain of GC-C, inducing accumulation of intracellular cGMP ([cGMP]; ref. 11). This cyclic nucleotide activates cGMP-dependent protein kinase II (PKG II), which phosphorylates the cystic fibrosis transmembrane conductance regulator, increasing chloride transport, and inhibits electroneutral sodium absorption, resulting in fluid and electrolyte secretion in the intestine and diarrhea (11–13).

STs are an example of molecular mimicry wherein bacteria have developed an evolutionarily advantageous strategy that exploits mechanisms regulating normal intestinal physiology. STs are members of a larger family of peptides that include guanylin and uroguanylin, GC-C agonists produced endogenously in mammalian gut (14–17). These peptides share sequence homology, have a tertiary structure stabilized by intrachain disulfide bonds, and exert their (patho)physiological effects by binding to GC-C and inducing cGMP accumulation. Uroguanylin, which is highly expressed in stomach, duodenum,

and jejunum, is 100-fold more potent than guanylin at acidic pH (18, 19). In contrast, guanylin is more abundant in ileum and colon and is 4-fold more potent than uroguanylin at a pH of 8.0 (18, 19). These endogenous GC-C agonists may regulate physiological processes in distinct regions of the intestine, modulated by local pH.

Although guanylin-like peptides and GC-C appear to regulate fluid and electrolyte balance in intestine, the precise role of this receptor in normal intestinal physiology remains undefined. GC-C exhibits broad phylogenetic expression, suggesting the existence of an evolutionary pressure mediating its conservation (20, 21). In intestine, GC-C is expressed along a crypt-to-villus gradient, with the greatest expression in the mid-villus where enterocytes transition from proliferation to differentiation, suggesting that GC-C may play a role in regulating that transition (22, 23). Also, expression of GC-C is highly conserved, whereas that of guanylin is significantly reduced, in proliferating colorectal cancer cells and tumors (10, 24–27). In addition, oral uroguanylin reduced the formation of polyps in the Min/+ mouse model of colon cancer (27). Taken together, these observations suggest an association between GC-C and the regulation of enterocyte proliferation.

In this study, regulation of human colon cancer cell proliferation by GC-C was examined *in vitro*. ST and uroguanylin inhibited the proliferation of human colon cancer cells by activating GC-C and stimulating accumulation of cGMP. Inhibition of intestinal cell proliferation by GC-C agonists reflected prolongation of the cell cycle in the absence of cell death.

## Materials and Methods

**Reagents.** DMEM, Eagle's minimal essential medium (EMEM) containing Earle's salts but not L-glutamine, L-glutamine, and other reagents for cell culture were obtained from Life Technologies (Rockville, MD). FBS and DMEM/F12 were from Mediatech (Herndon, VA). Native ST and the inactive analog ST(5-17) Ala<sup>9,17</sup>,Cys(Acm)<sup>5,10,6-14</sup> disulfide (TJU 1-103) were prepared by solid phase synthesis and purified by reverse phase HPLC, their structure confirmed by mass spectrometry, and their activities confirmed by examining competitive ligand binding and guanylyl cyclase activation. Uroguanylin was obtained

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Abbreviations: [cGMP]<sub>i</sub>, intracellular cGMP; GC-C, guanylyl cyclase C; IBMX, 3-isobutyl-1-methylxanthine; PI, propidium iodide; PDE, phosphodiesterase; PKG II, cGMP-dependent protein kinase II; ST, *Escherichia coli* heat-stable enterotoxin; TUNEL, terminal deoxynucleotidyltransferase-mediated dNTP-biotin nick end labeling of DNA fragments; EMEM, Eagle's minimal essential medium.

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from Peninsula Laboratories. [*methyl*-<sup>3</sup>H]Thymidine (1 mCi/ml) was obtained from Amersham Pharmacia Biotech. ScintiVerse and DMSO were obtained from Fisher Scientific. 3-isobutyl-1-methylxanthine (IBMX), zaprinast, 8-Br-cGMP, propidium iodide (PI), and all other chemicals were obtained from Sigma.

**Cell Culture.** Cell lines were obtained from the American Type Culture Collection and grown in DMEM/F12, containing 2.5 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% FBS. Cells were maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>, fed with fresh medium every third day, and split when subconfluent. Caco2 cells were used at passages 25–35, T84 at passages 45–60, and SW480 at passages 100–110. Cells were used during their logarithmic growth phase.

**Cell Proliferation.** Cell number was quantified on a hemocytometer after trypsinization and staining with trypan blue. Protein concentrations were quantified by using BCA reagent (Pierce). [<sup>3</sup>H]Thymidine incorporation into DNA was quantified by incubating cells in 96-well plates with 0.2 μCi/well of [<sup>3</sup>H]thymidine. After incubation, media were aspirated, and cells were incubated for 15 min with ice-cold 10% TCA and rinsed sequentially with 10% TCA and 100% methanol. The acid-insoluble material containing <sup>3</sup>H-labeled DNA was solubilized in 100 μl of 0.2 M NaOH, 80-μl aliquots were dissolved in 1 ml ScintiVerse, and radioactivity was quantified in a Packard β-scintillation spectrometer. In experiments examining the effects of FBS stimulation on cell proliferation, cell numbers were quantified on 60-mm dishes of exponentially growing cells (≈60% confluent) at time 0 (*t*<sub>0</sub>) and after 48 h of treatment with ST (1 μM) or PBS. Proliferation of cells stimulated with FBS was quantified in 96-well plates at a density of ≈50,000 cells/well. Six hours after seeding, cells were synchronized by FBS starvation in DMEM for 18 h, followed by stimulation for 24 h in media containing 10% FBS, in the presence or absence of the indicated reagents. [<sup>3</sup>H]Thymidine was added during the last 3 h of treatment and incorporation into DNA quantified as described above. In experiments examining the effects of L-glutamine, exponentially growing T84 cells (≈60% confluent) in 60-mm dishes (cell numbers) or in 96-well plates ([<sup>3</sup>H]thymidine uptake) were starved in EMEM for 24 h. At this point (*t*<sub>0</sub>), fresh EMEM containing 10 mM L-glutamine was added, with ST (1 μM) or PBS. Cell numbers were quantified at *t*<sub>0</sub> and after 48 h of treatment. Proliferation was assessed by quantifying [<sup>3</sup>H]thymidine incorporation after 12, 24, and 48 h.

**Cell Cycle Kinetics.** For flow cytometry, T84 cells were plated in 6-well plates (≈10<sup>6</sup> cells per well). At 24, 48, and 72 h, cells were placed in suspension by trypsinization, pelleted by centrifugation, washed with PBS, and fixed in 500 μl ice-cold 75% ethanol for 30 min. After another wash with PBS, cell were resuspended in 500 μl of staining solution (50 μg/ml PI/100 μg/ml RNase A/1 mM EDTA/0.1% Triton X-100), and analyzed on a Coulter EPICS XL-MCL flow cytometer. Distribution in different phases of the cell cycle was analyzed by using WINMDI software (version 2.8) provided by Joseph Trotter, Scripps Research Institute (La Jolla, CA). Twenty thousand cells, cleared from doublets, were analyzed from each sample. The influence of ST on the S phase of the cell cycle was investigated employing exponentially growing cells (≈60% confluent, in 96-well plates) that were synchronized in EMEM for 48 h and then stimulated with 10 mM L-glutamine (in EMEM) for the indicated times, in the presence of ST (1 μM) or PBS. [<sup>3</sup>H]Thymidine (0.2 μCi/well) incorporation into DNA during the last 2 h of incubation was assessed as described above. In experiments examining the latency of the ST effect, T84 cells were pulse-labeled with [<sup>3</sup>H]thymidine for the last 3 h of a 24-h period of stimulation with L-glutamine. ST (1 μM) or PBS was added 15 min before

[<sup>3</sup>H]thymidine to investigate the impact of short treatment duration on the proliferative fraction of the cell population.

**Cell Death.** Exponentially growing T84 cells in 60-mm dishes were starved in EMEM for 24 h. At this point, fresh EMEM containing 10 mM L-glutamine was added, together with either 1 μM ST, 1 μM uroguanylin, or PBS. After 24 h, cells were collected by trypsinization and pelleted, and apoptotic cell death was determined by TUNEL (terminal deoxynucleotidyltransferase-mediated dNTP-biotin nick end labeling of DNA fragments) analysis, employing the Flow-TACS Kit (R & D Systems). This system is optimized for flow cytometry compared with TUNEL analysis employing BrdUTP. One million cells per condition were fixed in 3.7% formaldehyde solution, and biotinylated dNTPs incorporated into the 3' ends of fragmented DNA (28) were stained with FITC-conjugated streptavidin. Cells were costained with PI and analyzed by flow cytometry within 1 h. In some experiments, apoptosis was assessed by DNA fragmentation analysis (27). Briefly, 2 × 10<sup>5</sup> T84 cells were seeded into 35-mm dishes and cultured for 7 days in DMEM/F12 containing 10% FBS. Preconfluent monolayers were washed with serum- and antibiotic-free DMEM, incubated in that media for 16 h, washed, and then incubated for 2 h in DMEM supplemented with either PBS, DMSO, 10 μM uroguanylin, 1 μM ST, 1 mM IBMX, 10 μM uroguanylin plus 1 mM IBMX, or 1 μM ST plus 1 mM IBMX. DNA was isolated from cells collected by trypsinization, washed twice with PBS, and resuspended in 200 μl PBS (DNA Fragmentation Analysis Kit; Roche, Indianapolis, IN). DNA was analyzed by electrophoresis in 1.8% NuSieve 3:1 agarose (BMA Biomedicals) with ethidium bromide. Cell death mediated by necrosis was assessed by flow cytometry, as outlined above, trypan blue exclusion, and lactate dehydrogenase release.

**Cyclic Nucleotide Assays.** Accumulation of cGMP and cAMP were determined in exponentially growing T84 cells (≈60% confluent, 96-well plates) after 3 h of exposure to 1 μM ST or PBS. Briefly, cells were starved in EMEM for 24 h and stimulated with 10 mM L-glutamine (EMEM) for 21 h; ST (1 μM) or PBS was added and the cells incubated for an additional 3 h at 37°C. The media were aspirated and reactions were terminated by the addition of 200 μl/well of a lysis buffer containing 0.5% dodecyltrimethylammonium bromide. Aliquots (100 μl) of each lysate were processed for quantification of cGMP or cAMP by enzyme-immunoassay (Amersham Pharmacia Biotech).

**Statistics.** All determinations were performed at least in duplicate, experiments were performed at least in triplicate, and data are expressed as mean ± SEM. Data were analyzed employing the paired two-tailed Student *t* test, and significance was assumed at *P* ≤ 0.05.

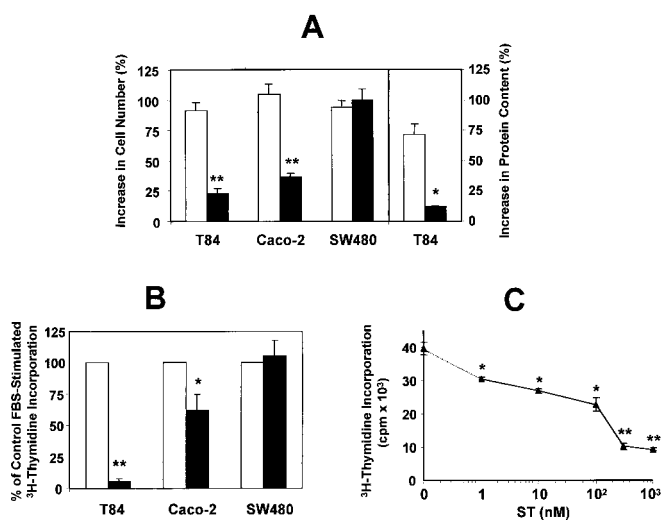
## Results

**ST Inhibits Proliferation of Human Colon Carcinoma Cells Induced by Serum.** ST (1 μM) reduced proliferation induced by serum ≈75%, quantified by protein content and/or cell number, of T84 (protein content: PBS, 71.56% ± 9.29 vs. ST, 12.29% ± 1, *P* < 0.05; cell number: PBS, 91.49% ± 7.12 vs. ST, 23.4% ± 3.63, *P* < 0.01) and Caco2 (cell number: PBS, 105.26% ± 8.1 vs. ST, 36.84% ± 2.83, *P* < 0.01) human colon carcinoma cells, which express GC-C (Fig. 1*A*). Proliferation induced by serum of SW480 human colon carcinoma cells, which do not express GC-C (29), was not affected by ST. Similarly, ST (1 μM) reduced [<sup>3</sup>H]thymidine incorporation into the DNA of T84 cells to 5.94% ± 2.13 of controls (*P* < 0.001) and Caco2 cells to 62.2% ± 12.43 of controls (*P* < 0.05), but not that of SW480 cells, after serum stimulation (Fig. 1*B*). The cell permeant analog of cGMP, 8-Br-cGMP, the downstream effector of ST, inhibited prolifer-

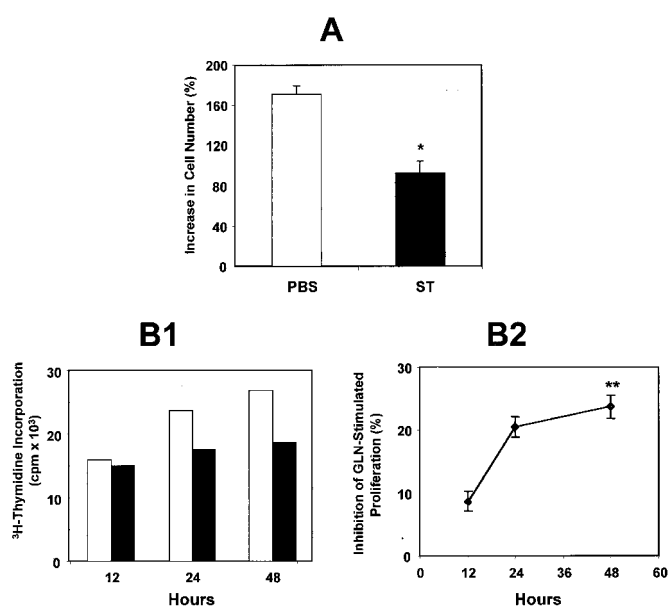
ation of SW480 cells stimulated by FBS to  $75.78\% \pm 0.64$  of the control ( $P < 0.01$ , data not shown). The differential effect of ST on [ $^3\text{H}$ ]thymidine incorporation likely reflects the greater density of GC-C on T84 compared with Caco2 cells (29). ST inhibited [ $^3\text{H}$ ]thymidine incorporation in T84 cells in a concentration-dependent fashion with a  $K_i$  ( $14.4 \pm 1.6$  nM; Fig. 1C), comparable to the  $K_a$  of that ligand for GC-C (30, 31).

**ST Inhibits Proliferation of T84 Cells Induced by L-Glutamine.** L-glutamine induces human intestinal cells to proliferate (32, 33). Proliferation of T84 cells stimulated by 10 mM L-glutamine, quantified by cell number, was inhibited by 1  $\mu\text{M}$  ST (PBS,  $171.41\% \pm 7.98$  vs. ST,  $92.74\% \pm 11.54$ ,  $P < 0.05$ ). ST inhibition of proliferation induced by glutamine was comparable to that observed with cells stimulated by serum (Fig. 2A). ST inhibition of proliferation induced by glutamine was associated with a time-dependent reduction in DNA synthesis, quantified by assessing [ $^3\text{H}$ ]thymidine incorporation (Figs. 2B and 2C).

**ST Reduces the Rate of DNA Synthesis in T84 Cells.** Progression through the cell cycle, assessed by flow cytometry, of T84 cells synchronized by starvation and subsequently induced to proliferate by FBS (24 h) was not altered by ST (Fig. 3A). Identical results were obtained with T84 cells growing asynchronously, or synchronized by starvation and induced to proliferate by L-glutamine for 12, 24, 48, and 72 h (data not shown). It is particularly noteworthy that the proportion of cells identified by flow cytometry in the sub- $G_1$  fraction, which reflects cells undergoing apoptosis or necrosis, was identical to incubations containing ST or PBS (Fig. 3A). However, ST shifted to the right the time course of [ $^3\text{H}$ ]thymidine incorporation into



**Fig. 1.** (A) T84, Caco2, or SW480 cells, stimulated with 10% FBS, were incubated for 48 h with 1  $\mu\text{M}$  ST (■) or PBS (□), and then protein and/or cells were quantified as described in *Materials and Methods*. Values are expressed as the percentage increase in cell number or protein stimulated by FBS relative to those values at  $t_0$  (baseline). Total cells at  $t_0$  were: T84,  $4.7 \times 10^6 \pm 0.2$ ; Caco2,  $1.8 \times 10^6 \pm 0.4$ ; and SW480,  $3.3 \times 10^6 \pm 0.4$ . T84 protein content at  $t_0$  was  $0.5 \pm 0.02$  mg/ml. (B) T84, Caco2, and SW480 cells were synchronized 6 h after seeding by starvation for 18 h and then stimulated with 10% FBS for 24 h in the presence of 1  $\mu\text{M}$  ST (■) or PBS (□). After 21 h, [ $^3\text{H}$ ]thymidine was added to the media, and incubation was continued for another 3 h. [ $^3\text{H}$ ]thymidine incorporation into DNA was quantified as described in *Materials and Methods*. Values are expressed as a percentage of the [ $^3\text{H}$ ]thymidine incorporation stimulated by FBS in the presence of PBS. (C) T84 cells were synchronized by starvation, stimulated to proliferate by addition of 10% FBS, and exposed to [ $^3\text{H}$ ]thymidine and the indicated concentrations of ST, as described in B. After 24 h of incubation, [ $^3\text{H}$ ]thymidine incorporation into DNA was quantified as described in *Materials and Methods*. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

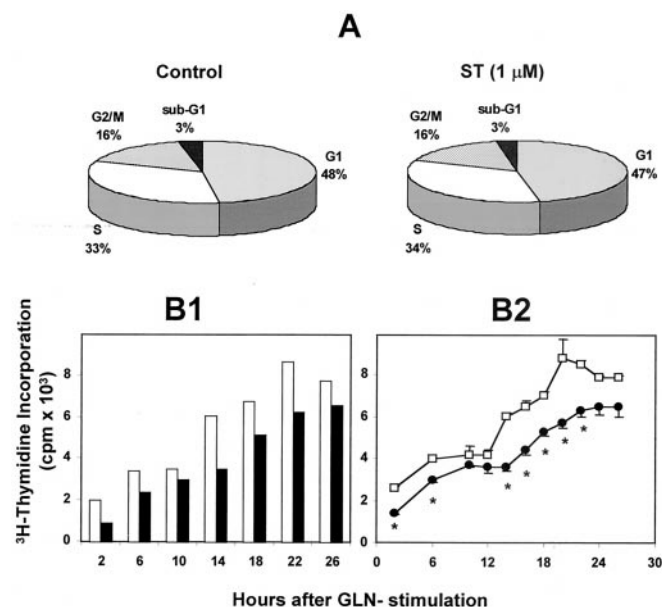


**Fig. 2.** (A) T84 cells were synchronized by starvation in EMEM depleted of L-glutamine for 24 h. Proliferation was stimulated with EMEM containing 10 mM L-glutamine; cells were incubated for 48 h with 1  $\mu\text{M}$  ST or PBS, and quantified as described in Fig. 1A. Values are expressed as the percentage increase in cell number stimulated by L-glutamine relative to those values at  $t_0$ . Total cells: at  $t_0 = 0.4 \times 10^6 \pm 0.07$ ; at 48 h, control =  $1.4 \times 10^6 \pm 0.2$ ; ST =  $0.9 \times 10^6 \pm 0.06$ . (B) Cells were synchronized and stimulated to proliferate by glutamine (as described in A), and [ $^3\text{H}$ ]thymidine and 1  $\mu\text{M}$  ST (■) or PBS (□) was added and incubated for the indicated times. At the conclusion of incubations, [ $^3\text{H}$ ]thymidine incorporation into DNA was quantified as described in *Materials and Methods*. Values reflect a representative experiment. In B2, data obtained in B1 are expressed as  $\{100 - [([\mathbf{^3H}]thymidine\ incorporation\ in\ ST-treated\ incubations) / ([\mathbf{^3H}]thymidine\ incorporation\ in\ control\ incubations)] \times 100\}$ . \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

DNA of T84 cells stimulated by L-glutamine, and caused a decrease in its maximum incorporation (Figs. 3B1 and 3B2). Double reciprocal analysis of these data revealed that ST delayed [ $^3\text{H}$ ]thymidine incorporation, and consequently synthesis of DNA, by  $\approx 4$  h (Fig. 3B2).

**The Antiproliferative Effect of ST Does Not Reflect Cell Death.** Inhibition by 1  $\mu\text{M}$  ST or uroguanylin of proliferation induced in synchronized T84 cells by L-glutamine was not associated with DNA fragmentation, assessed by TUNEL analysis (Fig. 4A). There were no differences in the percentages of apoptotic cells in cultures incubated with ST or uroguanylin compared with PBS (Fig. 4B). A recent report suggested that uroguanylin induced apoptosis in T84 cells (27). However, there were no differences in DNA fragmentation in T84 cells processed as described in that earlier report and exposed to DMSO, 10  $\mu\text{M}$  uroguanylin, 1  $\mu\text{M}$  ST, 1 mM IBMX, 10  $\mu\text{M}$  uroguanylin plus 1 mM IBMX, or 1  $\mu\text{M}$  ST plus 1 mM IBMX (Fig. 4C), in close agreement with results obtained in the present study by TUNEL analysis. Examination of trypan blue exclusion and lactate dehydrogenase release confirmed that the antiproliferative effect of GC-C agonists in T84 cells was not mediated by cell necrosis (data not shown).

**The Antiproliferative Effects of ST Are Mediated by cGMP.** The concentration-dependence of inhibition of [ $^3\text{H}$ ]thymidine incorporation into T84 cells was identical after incubation with ST for 15 min or 21 h before pulse-labeling with [ $^3\text{H}$ ]thymidine, consistent with the hypothesis that the antiproliferative effect of that ligand is an immediate response (compare Figs. 1C and 5A). Also, uroguanylin, an agonist with lower potency for

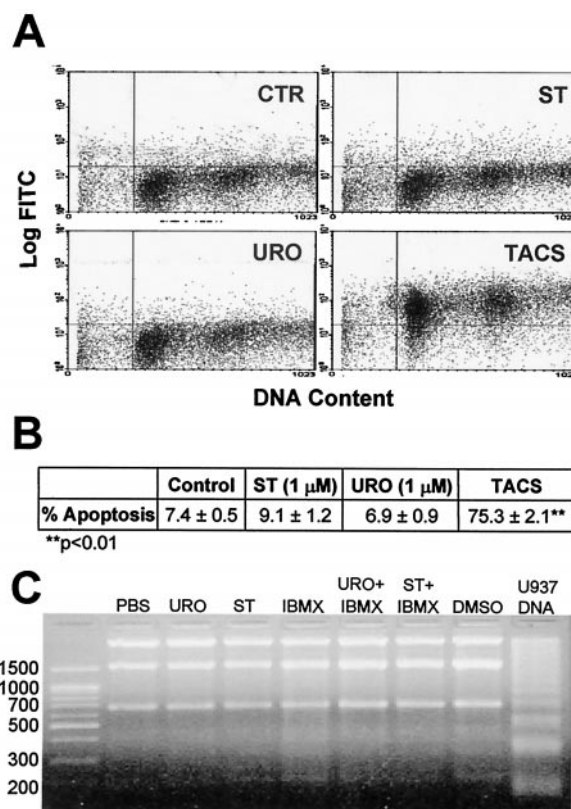


**Fig. 3.** (A) T84 cells were synchronized 6 h after seeding by starvation and then stimulated to grow by adding 10% FBS in the presence of 1  $\mu$ M ST or PBS. After 24 h, cells were trypsinized, pelleted, and then fixed and stained with PI. Fluorescence analysis of DNA content was performed as described in *Materials and Methods*, and the percentage of cells in each phase of the cell cycle is represented. Data are from a representative experiment. After synchronization by starvation ( $t_0$ ), 75.7%  $\pm$  4.5 of T84 cells were in the G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle. (B1) T84 cells were synchronized by starvation in EMEM depleted of L-glutamine for 48 h, stimulated to proliferate with EMEM containing 10 mM L-glutamine, and incubated for the indicated time points with 1  $\mu$ M ST (■) or PBS (□). [<sup>3</sup>H]Thymidine was added for the last 2 h of incubation, and the <sup>3</sup>H incorporated into DNA was measured as described in *Materials and Methods*. Data reflect a representative experiment. Results from three experiments performed as described in B1 are presented in B2 as mean  $\pm$  SEM. \*,  $P < 0.05$ .

binding to and activating GC-C compared with ST (18, 19), inhibited [<sup>3</sup>H]thymidine incorporation into DNA of T84 cells with a lower potency ( $K_i = 141 \pm 45$  nM) than ST ( $K_i = 13.7 \pm 5.2$  nM; Fig. 5A). In addition, 1  $\mu$ M ST induced accumulation of cGMP, but not cAMP, in T84 cells concurrently with the effects of that ligand on proliferation (Fig. 5B). Finally, 8-Br-cGMP (5 mM) and the cGMP-specific PDE5 inhibitor zaprinast (10  $\mu$ M), but not the inactive ST analog TJU 1-103 (1  $\mu$ M), mimicked the antiproliferative effect of 1  $\mu$ M ST or uroguanylin (Fig. 5C). Zaprinast (10  $\mu$ M) potentiated the antiproliferative effect of 1  $\mu$ M ST, presumably by increasing the accumulation of cGMP (34).

## Discussion

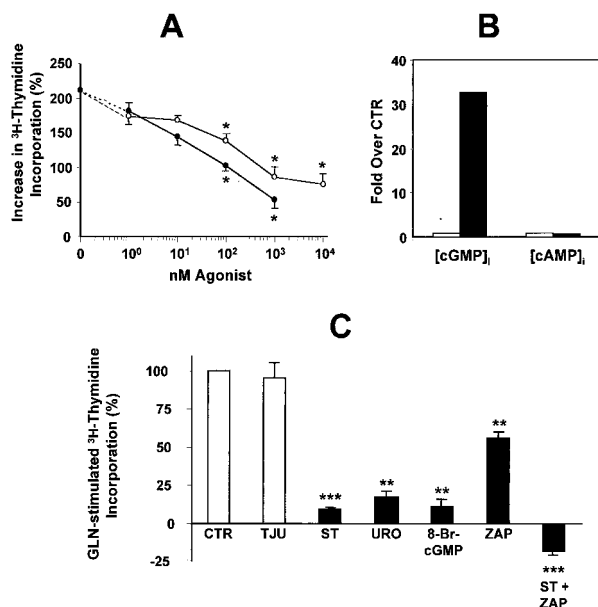
GC-C is the receptor for the family of homologous STs that mediate secretory diarrhea in travelers, people in underdeveloped countries, and farm animals worldwide (1–4). Expression of STs reflects molecular mimicry by bacteria, exploiting signaling pathways in the mammalian gastrointestinal tract to secure an evolutionary advantage. STs are structurally and functionally homologous to guanylin and uroguanylin elaborated in mammalian intestine (14–17). Although these peptides are endogenous GC-C agonists, their precise role in intestinal physiology has remained undefined. One hypothesis suggests a role for these peptides in the paracrine and autocrine regulation of fluid and electrolyte homeostasis in the intestine. Also, these peptides are expressed in extra-intestinal sites, including the kidney, suggesting that they may comprise one limb of an endocrine feedback loop that integrates the intestine into mechanisms regulating volume homeostasis (18,



**Fig. 4.** (A) T84 cells were synchronized and stimulated to proliferate by L-glutamine, as described in Fig. 2A, with simultaneous addition of 1  $\mu$ M ST, 1  $\mu$ M uroguanylin (URO), or PBS (CTR). Incubations were continued for 24 h, and cells were trypsinized and pelleted, divided in  $1 \times 10^6$  aliquots, fixed, and permeabilized by using Cytonin reagent. Biotinylated DNA was costained with both FITC-conjugated streptavidin and PI. The positive control (TACS) was generated by using the TACS-Nuclease provided with the FlowTACS kit. Flow cytometry analysis was performed as described in *Materials and Methods*, and data were plotted in two-dimensional format. Data are from a representative experiment. (B) Mean  $\pm$  SEM of the percentage of FITC-positive T84 cells (apoptotic/necrotic) from three experiments performed as in A. (C) T84 cells (seeded at a density of  $2 \times 10^5$  into 35-mm dishes) were cultured for 7 days in DMEM/F12, plus 10% FBS. Preconfluent monolayers were washed with DMEM (4.5 g/liter glucose, containing L-glutamine) and incubated in that media for 16 h. Cells were washed again in DMEM and then incubated for 2 h in that media supplemented as described in *Materials and Methods* (vehicle, PBS or DMSO; uroguanylin, URO). DNA was analyzed as described in *Materials and Methods*. The U937 positive control DNA was provided in the fragmentation analysis kit. Data are from a representative experiment.

19). This function for GC-C is analogous to that for GC-A and GC-B and their agonists, the natriuretic peptides, which regulate fluid and electrolyte secretion in the kidney and play a central role in volume homeostasis (11).

Also, GC-C may regulate processes other than fluid and electrolyte secretion in the intestine. Of significance, natriuretic peptides inhibit proliferation in human cell lines by interacting with guanylyl cyclase receptors and inducing accumulation of cGMP (35–37). Similarly, cGMP inhibits proliferation in several cell lines (38–41). cGMP delays the G<sub>1</sub>/S transition in human vascular smooth muscle cells (42). In addition, exisulind, which inhibits cGMP-specific phosphodiesterase (PDE), induces apoptosis in human colon cancer cells *in vitro* (43, 44). Furthermore, recent studies suggest that uroguanylin induces apoptosis in T84 and Caco2 human colon cancer cells (27). Thus, in some cell systems, including human intestine, guanylyl cyclases and cGMP may regulate cell proliferation and/or apoptosis (42, 45, 46).



**Fig. 5.** (A) Cells were synchronized, and proliferation was stimulated by L-glutamine, as described in Fig. 2A, and, 21 h later, cells were exposed to PBS or the indicated concentrations of ST (●) or uroguanylin (○). After 15 min of incubation, [<sup>3</sup>H]thymidine was added to the media, and incubation was continued for another 3 h. [<sup>3</sup>H]Thymidine incorporation into DNA was determined as described in *Materials and Methods*. (B) Cells were synchronized, stimulated to proliferate, and exposed to 1 μM ST (■) or PBS (□), as described in A. After 3 h of incubation, media was aspirated, and cGMP and cAMP were quantified as described in *Materials and Methods*. Data from a single experiment are expressed as fold accumulation in ST-treated compared with PBS-treated cells (control; CTR). (C) Cells were synchronized, stimulated to proliferate, and pulse labeled with [<sup>3</sup>H]thymidine, as described in A, and, 15 min later, T84 cells were exposed to PBS (CTR), 1 μM TJU 1-103 (TJU), 1 μM ST, 1 μM uroguanylin (URO), 5 mM 8-Br-cGMP, 10 μM zaprinast (ZAP), or 1 μM ST plus 10 μM zaprinast. After 3 h of incubation, [<sup>3</sup>H]thymidine incorporation into DNA was quantified as described in *Materials and Methods*. Results are the mean ± SEM of a representative experiment performed in triplicate. \*, *P* < 0.05; \*\*, *P* < 0.01; \*\*\*, *P* < 0.001.

The present study examined the role of GC-C and cGMP in regulating proliferation in human colon cancer cells. Proliferation was induced by serum (10%), and by L-glutamine (10 mM), a specific mitogen for intestinal cells (32, 33). ST inhibited proliferation of T84 and Caco2 cells in a concentration-dependent fashion with nanomolar potency. This effect was specifically mediated by GC-C, because proliferation was also inhibited by uroguanylin, a GC-C agonist, but not by an inactive analogue of ST. Similarly, ST did not affect proliferation of SW480 cells, colon carcinoma cells that do not express GC-C nor exhibit ST-induced accumulation of [cGMP]<sub>i</sub> (29). The effect of GC-C agonists on proliferation was an immediate response mediated by activation of the catalytic domain of GC-C and accumulation of cGMP. ST-induced inhibition of proliferation was graded with respect to the density of GC-C expressed on the cell surface and to the accumulation of [cGMP]<sub>i</sub> (29). Proliferation of T84 cells, which express the largest number of surface GC-C molecules, exhibited the greatest inhibition of proliferation compared with Caco2 cells, which express ≈80% fewer GC-C molecules on their surface and accumulate about ten times less cGMP after ST treatment (29). ST induced the accumulation of cGMP, but not cAMP, in target cells over the time course in which proliferation was inhibited. 8-Br-cGMP, the cell-permeant analogue of cGMP, had identical effects on T84 and Caco2 proliferation compared with ST and uroguanylin, and inhibited the proliferation of SW480 cells stimulated by FBS. Finally, the effects of GC-C agonists on proliferation were mimicked and potentiated

by zaprinast, a selective inhibitor of cGMP-regulated PDE5 that induces accumulation of [cGMP]<sub>i</sub>, potentiating ST stimulation of guanylyl cyclase activity in T84 cells (34, 47). Taken together, these data support the hypothesis that ST and uroguanylin regulate proliferation of human intestinal cells by binding to and activating GC-C, inducing the accumulation of cGMP.

Treatments that selectively raise [cGMP]<sub>i</sub>, including ST, uroguanylin, or PDE inhibitors, alone or in combination with GC-C agonists, did not induce apoptosis in human colon cancer cells, assessed by TUNEL and DNA fragmentation analyses during various phases of growth and employing different proliferative agents. These observations are in contrast to those reported recently concerning the induction of apoptosis in T84 and Caco2 cells by uroguanylin (27). However, the earlier study quantified apoptosis by manually counting small numbers of cells whereas the present study used flow cytometric analysis of ≥20,000 individual cells for each determination. In addition, these data contrast with those obtained with exisulind, which inhibits cGMP-specific PDEs and induces apoptosis in human colon carcinoma cells (43, 44). The inability to induce apoptosis of colon cancer cells by agonists that elevate [cGMP]<sub>i</sub>, cell-permeant analogs of that nucleotide, or inhibitors of cGMP-specific PDE5, demonstrated herein, suggests that exisulind may have multiple effects in tumor cells and may induce apoptosis through cGMP-independent mechanisms.

Whereas GC-C agonists did not induce apoptosis in colon cancer cells, these agonists delayed their progression through the cell cycle. Although ST inhibited proliferation assessed by a variety of measures, flow cytometry demonstrated that this agonist did not alter the fraction of cells in any phase of the cell cycle. Specific examination of S phase by pulse analysis of [<sup>3</sup>H]thymidine incorporation into DNA of synchronized colon cancer cells revealed that ST delayed DNA synthesis and prolonged that phase of the cell cycle. Taken together, these data suggest that GC-C agonists induce a generalized delay in the progression of colon carcinoma cells through, without arrest in a specific phase of, the cell cycle (48). Indeed, Eq. 1 (49):

$$N = N_0 2^{T/mgt}, \quad [1]$$

where *N* is the final cell number (PBS-treated cells:  $13.7 \times 10^5 \pm 4.57$ ; ST-treated cells:  $9.4 \times 10^5 \pm 1.42$ ), *N*<sub>0</sub> is the initial cell number ( $3.9 \times 10^5 \pm 1.57$ ), *T* is the elapsed time (44 h), and *mgt* is the mean generation time, supports the suggestion that GC-C agonists increase the duration of the cell cycle of T84 cells stimulated by FBS ≈40%, from  $26.9 \pm 9.52$  h to  $37.31 \pm 14.6$  h (*n* = 6, *P* < 0.05). These observations demonstrate that GC-C agonists are cytostatic, rather than cytotoxic, with respect to human colon carcinoma cells.

The precise mechanisms by which GC-C agonists delay progression of colon carcinoma cells through the cell cycle remain incompletely defined. Molecular targets for cGMP in those cells include PKG II (50), PDE3 (13), and cAMP-dependent protein kinase (PKA; ref. 51). Whereas activation of PKG II would mediate cGMP-selective regulation of fluid and electrolyte transport or phosphorylation of proteins involved in cell cycle regulation, inhibition of PDE3 or activation of PKA would ultimately result in functional transactivation of cAMP-regulated processes. The cytostatic effects of GC-C agonists, 8-Br-cGMP, and zaprinast described herein suggest that their actions are mediated through cGMP-specific downstream effectors rather than transactivation of cAMP-dependent mechanisms. The mechanisms by which cGMP regulates progression through the cell cycle, without specific effects on a particular phase of that cycle, remain undefined, although this phenomenon has been observed previously (48).

In conclusion, agonist activation of GC-C regulates the proliferation of colon carcinoma cells by slowing progression

through the cell cycle without inducing cell death. These data suggest that GC-C and its endogenous agonists, guanylin and uroguanylin, may play a role in regulating the transition between intestinal stem cell proliferation and their differentiation into mature enterocytes (52). Additionally, they support the suggestion that GC-C agonists may represent novel cytostatic agents for the prevention and treatment of colorectal cancer.

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