Glutamine Metabolism Stimulates Intestinal Cell MAPKs by a cAMP-Inhibitable, Raf-Independent Mechanism

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Background & Aims: Infectious diarrhea caused by viruses plus enterotoxigenic bacteria is often more severe than diarrhea induced by either pathogen alone. We postulated that the increased cell adenosine 3',5'-cyclic monophosphate (cAMP) concentration observed during infection by enterotoxigenic organisms retards the intestinal repair process by blocking activation of mitogen-activated protein kinases (MAPKs) in proliferating intestinal cells. **Methods:** We evaluated the effects of glutamine on MAPK activity, thymidine incorporation, and cell number in glutamine-starved and -sufficient rat intestinal crypt cells (IEC-6). **Results:** In glutamine-starved cells, 10 mmol/L glutamine in the absence of serum stimulated [³H]thymidine incorporation 8-fold. This effect was inhibited by 60% with 8-(4-chlorophenylthio) (8-CPT)-cAMP (100 μ mol/L) + isobutyl methylxanthine (100 μ mol/L). In cells not starved of glutamine, glutamine stimulated thymidine incorporation by 3-fold, and 8-CPT-cAMP completely blocked the mitogenic effect. Inhibition of proliferation by cAMP persisted for at least 68 hours after cAMP removal. In vitro kinase assays showed that glutamine signaling requires an intact ERK (extracellular signal-related kinase) pathway in unstarved cells. In starved cells, at least one other pathway (JNK) was activated by glutamine, and the mitogenic inhibition by 8-CPT-cAMP was incomplete. Other intestinal fuels (glucose and acetate) were not mitogenic. **Conclusions:** Increased levels of intracellular cAMP inhibit ERKs but only partially reduce glutamine-stimulated proliferation in enterocytes adapted to low glutamine.

Viruses induce diarrhea by damaging the intestine, whereas enterotoxigenic bacteria elaborate toxins that induce intestinal salt and water secretion without injuring the mucosa. Diarrhea caused by viruses plus enterotoxigenic *Escherichia coli* is more severe than diarrhea induced by either agent alone.¹⁻⁴ Repair of the intestinal epithelium is facilitated by luminal growth

factors, such as epidermal growth factor (EGF), transforming growth factor (TGF)- α , and insulin-like growth factor I, which are present in breast milk and interact with specific membrane receptors.⁵ Many of these factors activate protein kinases (MAPKs), such as the extracellular signal-related kinases (ERKs) ERK-1 and ERK-2, as the initial step in signal transduction.^{6,7} Another member of the MAPK family that may participate in inflammation and repair is c-Jun nuclear kinase (JNK, also called stress-activated protein kinase, SAPK). JNK phosphorylates and activates the nuclear transcription factor c-Jun. ERKs phosphorylate and activate Elk-1 and other transcription factors.^{6,8,9}

Not only is there good evidence that MAPKs are activated by many types of mitogenic signals, but dominant mutations of Raf-1,¹⁰ MEK-1, and ERKs inhibit NIH-3T3 cell proliferation. Activating mutations of MEK-1 produced accelerated proliferation.¹¹ Adenosine 3',5'-cyclic monophosphate (cAMP) has inhibitory or stimulatory effects on MAPK level and proliferation in a cell-type specific manner (reviewed by Graves and Lawrence¹²). cAMP may inhibit signal transduction through the MAPK pathway (Rat-1 cells)¹³⁻¹⁵ or the JNK pathway (T lymphocytes).¹⁶ We hypothesized that cAMP would also inhibit crypt cell proliferation, and we tested the ability of the trophic nutrient glutamine (GLN) to stimulate enterocyte proliferation in the presence of increased cAMP concentrations. The results of these studies have been presented in part previously.¹⁷

Abbreviations used in this paper: AOA, aminoxyacetate; cAMP, adenosine 3',5'-cyclic monophosphate; 8-CPT, 8-(4-chlorophenylthio); DMEM, Dulbecco's modified Eagle medium; DMEM-L, DMEM with low glucose; EGF, epidermal growth factor; ERK, extracellular signal-related kinase; EtOH, ethyl alcohol; GLN, glutamine; GST, glutathione S-transferase; JNK, c-Jun nuclear kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; SDS, sodium dodecyl sulfate; TGF- α , transforming growth factor α ; QO₂, oxygen consumption.

Materials and Methods

Materials

Acrylamide and bisacrylamide were obtained from National Diagnostics (Atlanta, GA), glutathione-Sepharose from Pharmacia Biotechnology (Piscataway, NJ), and anti-ERK antibody from Santa Cruz Biotechnology (Santa Cruz, CA). Protease and phosphatase inhibitors (aprotinin, leupeptin, bestatin, 4-nitrophenyl phosphate, pepstatin, Pefabloc, dithiothreitol, and Nonidet P-40 [NP-40]) were from Boehringer Mannheim (Indianapolis, IN). PD98059 was from Calbiochem-Novabiochem (San Diego, CA). [^3H]Thymidine was obtained from Amersham (Arlington Heights, IL). [$\gamma\text{-}^{32}\text{P}$]Adenosine triphosphate (ATP) was purchased from DuPont (Boston, MA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Cells

We studied the nontransformed crypt cell line IEC-6 (ATCC, Gaithersburg, MD) at passage 9–20. Cells were grown in Dulbecco's modified Eagle medium (DMEM) with 4.5 g/L glucose, 0.5 U/mL bovine insulin, and 5% fetal calf serum (Atlanta Biologicals, Norcross, GA). Cells were passaged every 3–4 days.

[^3H]Thymidine Incorporation Rates of Cultured Enterocytes

IEC-6 cells were plated in 24-well plates at a seeding density of 5×10^4 cells per well. Six hours after plating, cells were synchronized by serum starvation in DMEM–low glucose (DMEM-L) with no serum, low glucose (5 mmol/L), and “serum” levels of GLN (0.7 mmol/L) or no GLN for 18 hours. Cells were then treated for 24 hours. [^3H]Thymidine (2 μCi) was incubated with treatments. Perchloric acid in methanol (0.5 mL of 4 mol/L) was then added, and cellular proteins were allowed to precipitate overnight. The pellet was dissolved in Scintisafe Econo2 (Fisher Scientific, Fair Lawn, NJ) and counted in a β -scintillation counter (Beckman, Fullerton, CA).

Raf Kinase, MEK, and ERK Immune Complex Assay

IEC-6 cells (70% confluent) were starved of serum and GLN overnight. After exposure to GLN or inhibitors, cells were washed twice with cold phosphate-buffered saline (PBS). Lysis buffer (20 mmol/L Tris, pH 7.5, 10% glycerol, 1% Triton X-100, 137 mmol/L NaCl, and 2 mmol/L EDTA) was added. Cells were scraped and collected in a microcentrifuge tube, centrifuged, and transferred to new tubes. Two micrograms of monoclonal antibody (anti-Raf C-12, rabbit polyclonal anti-MEK-1, or goat polyclonal anti-ERK2; Santa Cruz Biotechnology) was added, and the tubes were rolled at 4°C for 1.5 hours. Protein A (Santa Cruz Biotechnology) slurry was added into each tube, and tubes were again rolled for 40 minutes. Beads were washed twice with cold lysis buffer and twice with cold PBS. For the Raf assay, 5 μL recombinant MEK (100 $\mu\text{g}/\text{mL}$)

was added, along with 0.3 $\mu\text{mol}/\text{L}$ ATP and 6 μg bovine serum albumin. The mixture was shaken at 30°C for 10 minutes.

For both the Raf and MEK assays, ERK-2 (20 $\mu\text{g}/\text{mL}$, 5 μL) was added for 10 minutes. For the ERK assay, 20 μL of 20 mmol/L HEPES, pH 7.5, was added to the beads. Finally, 20 μL of a mixture containing 75 $\mu\text{mol}/\text{L}$ cold ATP, 2 $\mu\text{Ci}/\text{mL}$ hot ATP, and 10 μg of myelin basic protein was added, and the mixture was incubated for an additional 10 minutes. The reaction was stopped by adding 20 μL EDTA (100 mmol/L, pH 7.5). Tubes were centrifuged briefly, and 40 μL was spotted onto P-81 filter paper (Whatman distributor; Hillsboro, OR). Papers were washed in 10% phosphoric acid for 10 minutes 6 times. Papers were rinsed in 100% ethyl alcohol (EtOH) and dried with a hair drier before liquid scintillation counting.

In Vitro Assays of Cellular JNK and ERK Activity

Cells were collected with a cell lifter in Dignam C buffer (420 mmol/L NaCl, 1.5 mmol/L MgCl_2 , 20 mmol/L HEPES [pH 7.0], 0.2 mmol/L EDTA, 25% glycerol, and 0.5 mmol/L dithiothreitol) plus protease/phosphatase inhibitors (0.5 mmol/L Pefabloc, 0.1 mmol/L 4-nitrophenyl phosphate, 0.04 mmol/L β -glycerophosphate, 0.05 mmol/L Na_3VO_4 , 40 $\mu\text{g}/\text{mL}$ bestatin, 2 $\mu\text{g}/\text{mL}$ aprotinin, 0.54 $\mu\text{g}/\text{mL}$ leupeptin, and 0.7 $\mu\text{g}/\text{mL}$ pepstatin A). We used a solid-phase assay with a glutathione *S*-transferase (GST)–c-Jun or GST-Elk fusion protein as the substrate.¹⁸ GST-Elk plasmid was a gift of R. Treisman (Transcription Laboratory, Imperial Cancer Research Fund, London, England); GST-cJun plasmid was a gift of M. Karin (University of California, San Diego). Substrate (1 μg) was linked to GLN-Sepharose. For the JNK assay, 25 μg of whole cell extract was mixed with the substrate, 1 μg of GST–c-Jun, in 300 μL HBB (20 mmol/L HEPES, pH 7.7, 50 mmol/L NaCl, 0.1 mmol/L EDTA, 2.5 mmol/L MgCl_2 , 0.5% Triton X-100, plus protease/phosphatase inhibitors), at 4°C for 3 hours. The GST-bound proteins were then washed 4 times with HBB and incubated with 4 mmol/L HEPES, 2 mmol/L MgCl_2 , 4 mmol/L β -glycerophosphate, 0.2 mmol/L dithiothreitol, 10 $\mu\text{mol}/\text{L}$ Na_3VO_4 , 2 mmol/L *p*-nitrophenylphosphate, and 2 $\mu\text{mol}/\text{L}$ ATP (with [$\gamma\text{-}^{32}\text{P}$]ATP > 4000 Ci/mmol), at 30°C for 30 minutes. The phosphorylated c-Jun fusion protein was boiled in sodium dodecyl sulfate (SDS) sample buffer and loaded onto a 10% SDS–polyacrylamide gel with molecular-weight standards. Equal loading was verified by Coomassie blue staining. Autoradiography/densitometry was used to quantify labeled GST-Jun.

For the ERK assay, 5 μg anti-Erk antibody mixture with protein A–Sepharose in RIPA (150 mmol/L NaCl, 50 mmol/L Tris-Cl [pH 7.2]), 1% deoxycholate, 1% Triton X-100, and 0.1% SDS (plus protease/phosphatase inhibitors) were rotated for 2 hours at 4°C. The mixture was sequentially washed once with 10% sucrose RIPA, HBNP (20 mmol/L HEPES [pH 7.5], 50 mmol/L NaCl, 0.1 mmol/L EDTA, 2.5 mmol/L MgCl_2 , and 0.5% NP-40), HB (HBNP without NP-40), and kinase reaction buffers (20 mmol/L HEPES [pH 7.5], 10 mmol/L MgCl_2 , 2 mmol/L dithiothreitol, 0.1 mmol/L Na_3VO_4 , 25

mmol/L β -glycerophosphate, and 10 mmol/L 4-nitrophenyl phosphate) and then incubated with 1 μ g substrate in 25 μ L of kinase reaction buffer for 30 minutes at 30°C and washed 3 times with 1 $\times$ RIPA. The phosphorylation products were run on a 12.5% SDS-polyacrylamide gel. The gel was dried, and autoradiography with Phosphorimager analysis was used to quantify density of the bands.

Oxygen Consumption

After 16 hours of serum starvation, IEC-6 cells were trypsinized and collected in DMEM-L medium. We incubated 5×10^6 cells in 1 mL DMEM-L medium in water-jacketed vials with continuous stirring at 37°C. After 2 minutes' equilibration with air and 10 μ L GLN with or without 8-(4-chlorophenylthio) (8-CPT)-cAMP, PD98059, or aminooxyacetate (AOA), the system was sealed and oxygen consumption (QO_2) was measured by a polarographic Clarke electrode for 10 minutes. The preliminary data showed that cell QO_2 was stable for 15 minutes and then decreased slowly. Results are expressed as percent oxygen consumed per million cells per minute.

Cell Cycle Study

Cell culture and treatment followed the [3 H]thymidine incorporation protocols. After treatment, cells were washed with cold PBS twice and then were trypsinized, centrifuged, and resuspended in a small volume of PBS. Ice-cold EtOH (700 μ L) was added for 5 minutes, and the cells were then washed twice with cold PBS. Cold propidium iodide solution (500 μ L, concentration of 50 mg/mL with 2 μ g/mL ribonuclease and 0.1% Triton X-100) was added to 10^7 cells. Fluorescence was analyzed with a Becton Dickinson FACScan flow cytometer (San Jose, CA). Single-perimeter histograms were collected on 100,000 single cells. Doublets were eliminated by pulse analysis.

Cell Counting Study

Cells were grown in culture, starved, and treated for these studies in the same manner as described in the [3 H]thymidine experiments. Cells were counted after 10 minutes of trypsinization in a hemacytometer. Cells in each well were counted twice.

Statistics

Results are expressed as means $\pm$ SE. Effects of treatment were assessed by the 1-way analysis of variance. Individual groups were compared with the post hoc Tukey test (SigmaStat; Jandel Scientific, San Rafael, CA).

Results

Effect of GLN and 8-CPT-cAMP on DNA Synthesis Rate of IEC-6 Cells

Cells were preincubated overnight in either a "starvation medium" without serum or GLN (to maximize the response to GLN¹⁹) or in serum-free medium with 0.7 mmol/L GLN to mimic the GLN concentration

in plasma ("sufficient"). GLN-sufficient cells responded to 10 mmol/L²⁰ GLN with a 3.8 ± 0.3 -fold increase in [3 H]thymidine incorporation, whereas in starved cells thymidine incorporation increased 8.5 ± 0.2 -fold ($n = 3$ –4 observations). Total thymidine incorporation 24 hours after addition of 10 mmol/L GLN was similar in both GLN-starved or -sufficient cells: about 8-fold greater than thymidine incorporation in GLN-starved cells.¹

We hypothesized that elevated 8-CPT-cAMP levels would inhibit MAPKs and thereby reduce the intestinal cell proliferative response to GLN.¹⁹ cAMP inhibited the thymidine incorporation of IEC-6 cells after addition of 10 mmol/L GLN in a concentration-dependent manner (Figure 1A). The slopes of the cAMP dose-effect relationships for starved and sufficient cells showed that cAMP inhibition was greater in the sufficient cells, with 100% inhibition of GLN-stimulated thymidine incorporation at 100 μ mol/L 8-CPT-cAMP.

We compared the effects of GLN with EGF on [3 H]thymidine incorporation with or without cAMP (Figure 1B). EGF (without GLN) stimulated [3 H]thymidine incorporation 2.2 ± 0.2 -fold above rate of incorporation in starved cells; this effect was blocked (97% inhibition) by 8-CPT-cAMP ($n = 11$ observations from 3 passages of cells; not shown). In GLN-sufficient (0.7 mmol/L) cells, EGF stimulated [3 H]thymidine incorporation 3.8-fold; this effect was also completely inhibited by 8-CPT-cAMP.

Effect of cAMP on MAPK Induction by GLN and EGF

We compared the effects of GLN with or without 8-CPT-cAMP on ERK and JNK induction. cAMP has been shown to inhibit their activation in many cell types.^{13–15,21} For the ERK assay, we used an in vitro kinase assay of whole-cell extracts, with the nuclear target of ERK-1 and -2, GST-Elk, as the substrate. In these studies, cells were preincubated in nonstarvation media with 0.7 mmol/L GLN and subsequently exposed to GLN or a positive control, EGF, for 5, 15, or 30 minutes. Other cells were exposed to GLN or EGF, plus 8-CPT-cAMP (300 μ mol/L) with isobutyl methylxanthine (IBMX; 100 μ mol/L).

GLN signals through ERKs in IEC-6 cells¹⁹ (Figure 2A). Elk phosphorylation increased significantly to 3.1 ± 0.3 of control levels at 5 minutes after GLN exposure ($P < 0.05$). This effect was significantly reduced in cells exposed to 8-CPT-cAMP ($\sim 40\%$ inhibition; $P < 0.05$). At 15 and 30 minutes, the level of ERK activity declined and was only slightly affected by 8-CPT-cAMP.

EGF treatment produced a maximal (5.0 ± 1.5 -fold)

activation of kinase activity at 5 minutes. (Figure 2; $P < 0.05$). This stimulatory effect was inhibited 66% by coinubation of the cells with 8-CPT-cAMP ($P < 0.05$). The EGF treatment had a biphasic stimulatory effect. ERK activity was increased at 15 minutes by 3.9 ± 1.3 -fold and at 30 minutes by 4.8 ± 0.3 -fold ($P < 0.05$ at 30 minutes). At 15 minutes, 8-CPT-cAMP inhibited the EGF effect by 50%, whereas 8-CPT-cAMP inhibition was 100% at 30 minutes ($P < 0.05$). The means of 3 experiments at 5 and 30 minutes are shown in the bar graph in Figure 2C.

Effect of EGF and GLN on Raf Activity in IEC-6 Cells

Raf kinase is the tyrosine-threonine kinase in the ERK pathway that is activated by Ras after EGF and insulin receptor activation.²² Raf has been proposed to be a major site for regulation of ERK activity by cAMP (via protein kinase A) and is inhibited by cAMP in many cell

types.¹² In IEC-6 cells, EGF stimulated Raf kinase 14-fold, an effect that at 5 minutes was inhibited 75% by 8-CPT-cAMP (Figure 3). Raf continued to be activated 15 minutes after EGF treatment, by 9-fold, an effect blocked by 8-CPT-cAMP pretreatment (data not shown). Figure 3 shows that Raf phosphorylation/activation is not stimulated by GLN in IEC-6 cells.

Effect of GLN on MEK Activity in IEC-6 Cells

These results suggested that GLN activated an Raf-independent pathway; therefore, we examined MEK activation by GLN. MEK, also called mitogen-activated protein kinase kinase, is immediately "downstream" of Raf in the signaling cascade. Figure 4 shows that GLN activates MEK almost as effectively as EGF in IEC-6 cells, with a 2-fold activation at 5 minutes. As shown in the inset, the effect of GLN on MEK persisted with MEK stimulation by 1.6–1.7-fold at 15 and 40 minutes ($P < 0.05$). This effect of GLN was blocked by 8-CPT-cAMP (Figure 4).

Recently, a synthetic inhibitor, PD98059, has been developed that is specific for MEK. PD98059 does not block JNK or p38 MAPK activity in other cell types.²³ PD98059 fully inhibits phenotypic changes attributed to MAPK activation in other cell types. As shown in Figure 4, PD98059 (50 $\mu\text{mol/L}$) completely inhibited GLN-stimulated MEK at 5 minutes.

Figure 1. (A) Effects of GLN and 8-CPT-cAMP on 24-hour IEC-6 cell proliferation. Three hours after plating IEC-6 cells (~40% confluent) in 24-well plates, the cells were starved in serum-free DMEM-L and pretreated with GLN (0.7 mmol/L; ■) or without GLN (Δ) for 16 hours. Cells were then stimulated with 10 mmol/L GLN for 24 hours. 8-CPT-cAMP (0–100 $\mu\text{mol/L}$) with IBMX (100 $\mu\text{mol/L}$) was added to the medium 15 minutes before GLN treatment. [*Methyl*-³H]thymidine (2 μL ; sp act, 2.96 TBq/mmol, 80.0 Ci/mmol) was added at the same time. Counts incorporated into perchloric acid-insoluble material are shown as multiples of control group (group treated with 0 mmol/L 8-CPT-cAMP). (B) Effect of 8-CPT-cAMP on 24-hour IEC-6 cell proliferation stimulated by EGF. □, EGF; ▨, EGF + cAMP. Cells were either starved overnight of GLN or pretreated with 0.7 mmol/L GLN in serum-free DMEM-L, and the GLN concentration was maintained after 20 ng/mL EGF was added. Twenty-four hours later, [³H]thymidine incorporation into acid-insoluble material was quantified as above. In paired plates, cells were exposed to EGF + 8-CPT-cAMP (10^{-4} mol/L) + IBMX (100 $\mu\text{mol/L}$). Data are compared with control cells. On the left side, control cells were pretreated with 0.7 mmol/L GLN without EGF or 8-CPT-cAMP; thymidine incorporation rate was 19,800 cpm, and the rate increased 3.8 ± 0.6 -fold after EGF treatment. After EGF + 8-CPT-cAMP treatment, the rate decreased to 0.5 ± 0.02 -fold of control. $**P < 0.01$ compared with EGF alone. On the right side, control cells were treated overnight with 0 mmol/L GLN, and [³H]thymidine incorporation was 12,830 cpm. Treatment with EGF increased [³H]thymidine incorporation 2.01 ± 0.4 -fold, whereas treatment with EGF + 8-CPT-cAMP reduced [³H]thymidine incorporation to 0.6 ± 0.1 -fold the control value. $*P < 0.05$ compared with EGF alone; $n = 4$. Control cells had baseline [³H]thymidine cpm of 1000–7000 without GLN pretreatment and 5000–30,000 cpm with 0.7 mmol/L GLN pretreatment.

Figure 2. Effects of GLN, EGF, and cAMP on ERK activity of IEC-6 cells. Cells (~70 % confluent) in 100-mm plastic dishes were starved in DMEM-L without GLN or serum for 24 hours and then were treated with (A) 10 mmol/L GLN or (B) 20 ng/mL EGF with or without 8-CPT-cAMP (with IBMX) for 5, 15 or 30 minutes. Cell samples were collected after washing twice in Dignam C buffer and were allowed to phosphorylate GST-Erk beads in the presence of [32 P]ATP after immunoprecipitation with anti-ERK specific antiserum. Phosphorylated GST-Erk was heated and separated by 12.5% SDS-polyacrylamide gel electrophoresis. Equal loading was determined by Coomassie blue staining. Dried gel was exposed to film for 3 days. The gel is representative of 3 experiments. (C) Averaged densitometry values (means $20 \pm$ SEM). * $P < 0.05$ compared with control cell ERK activity. cAMP completely blocked ERK activation by GLN at 5 minutes and by EGF at 5 and 30 minutes.

We have found that GLN has a much more potent impact on thymidine incorporation when cells are starved of serum and GLN overnight.¹⁹ Therefore, we determined if ERK signaling in GLN-“starved” cells is more effective or more resistant to PD98059 compared with control cells. Figure 5 shows that GLN and EGF activations of ERKs are similar in starved (left side) and

unstarved cells (right side). Furthermore, PD98059 is an effective inhibitor of MEK (and therefore the ERK pathway) regardless of GLN preexposure or starvation.

Effect of GLN Oxidation on MEK Activation in IEC-6 Cells

We used AOA (10 mmol/L), an inhibitor of transaminase reactions, to prevent conversion of GLN-derived glutamate to α -ketoglutarate and to inhibit the glutaminase reaction by producing a buildup of glutamate. We showed previously that AOA blocks GLN induction of ornithine decarboxylase, an enzyme implicated in cell proliferation.²⁰ Figure 6 shows that 10 mmol/L GLN stimulates QO_2 by IEC-6 cells by 2-fold. Stimulation was fully blocked by 10 mmol/L AOA. AOA also completely inhibited MEK activation by GLN at 5 minutes (Figure 4).

We also determined if inhibition of MAPK signaling interferes with GLN's oxidative metabolism. Figure 6 shows that GLN stimulation of QO_2 was not significantly reduced by 8-CPT-cAMP (300 μ mol/L) or PD98059 (50 μ mol/L).

PD98059 and 8-CPT-cAMP: Effects on GLN-Stimulated IEC-6 Cell Proliferation

We hypothesized that the early inhibition in ERK and MEK signaling in cells exposed to 8-CPT-cAMP would have a significant impact on subsequent DNA synthesis rate. 8-CPT-cAMP or PD98059 inhibited 10

Figure 3. Effect of EGF and GLN on Raf kinase activity in IEC-6 cells. Cells were starved as described in Figure 2. In 1 group of cells, cAMP (333 μ mol/L + 100 μ mol/L IBMX) was added 15 minutes before EGF (20 ng/mL). After 5 minutes, cell lysates were collected and used for an immune complex assay for Raf (described in Materials and Methods). The increase in kinase activity above that of unstimulated cells is shown. $n = 2-3$ except for EGF-stimulated cells, where $n = 1$. Control cpm, 3140.

Figure 4. Effects of GLN on MEK activity of IEC-6 cells: inhibition by 8-CPT-cAMP, MEK inhibitor PD98059, and GLN metabolism inhibitor AOA. MEK is the substrate of Raf and has been shown to phosphorylate ERK-1 and ERK-2. Cells were starved as described in Figure 2. PD98059 (50 μ mol/L), 8-CPT-cAMP (333 μ mol/L + 100 μ mol/L IBMX, "cAMP"), and AOA (10 mmol/L) were added 15 minutes before GLN (10 mmol/L) and EGF (20 ng/mL) were added. After 5 minutes of GLN or EGF treatment, collected cell lysates were used for an immune complex assay with myelin basic protein as the substrate (see Materials and Methods). $n = 2$. * $P < 0.05$ compared with control cells. $n = 3$ –4 observations for each treatment; cpm for control cells, 2460. Inset shows kinetics of GLN-stimulated MEK activation (fold of control level).

mmol/L GLN-stimulated thymidine incorporation to a similar extent (54% inhibition by 8-CPT-cAMP, 65% inhibition by PD98059) in GLN-starved IEC-6 cells (Figure 7, left panels). In GLN-sufficient enterocytes (0.7 mmol/L GLN pretreated), 8-CPT-cAMP or PD 98059 completely blocked the GLN stimulation and reduced DNA synthesis below basal levels (right panels).

To determine if inhibitors of cell proliferation had toxic effects on cultured intestinal cells, we treated cells with 8-CPT-cAMP + IBMX or PD98059 for 24 hours, and we counted nonviable cells that take up trypan blue (1:50 dilution, 5-minute exposure, $n = 500$ cells, 2 passages). Control cells deprived of GLN and serum had 94.5% viability. PD98059 (50 μ mol/L)-treated cells had

Figure 5. Effect of PD98059 on ERK activity of GLN-starved or GLN-sufficient cells. Whole-cell extracts were prepared from IEC-6 cells starved of GLN overnight or pretreated with 0.7 mmol/L GLN and subsequently exposed to GLN (G, 10 mmol/L) or EGF (E, 20 ng/mL) or no treatment (control, C) for 10 minutes. In vitro ERK activity was assayed with GST-EIk as the substrate. Some preparations were exposed to GLN or EGF combined with PD98059 (PD, 50 μ mol/L). Relative densitometry values are shown at the top. One of 2 similar blots.

92.3% viability. Of cells treated with 8-CPT-cAMP (300 μ mol/L) + IBMX (100 μ mol/L), 96.4% remained viable. In summary, we found no evidence that either 8-CPT-cAMP or PD98059 was toxic.

GLN is a precursor of purines and pyrimidines.²⁴ To confirm that GLN stimulates IEC-6 cell proliferation, rather than thymidine incorporation into DNA alone, we plated cells at a density of 10^5 cells/well and manually counted cells after trypsinization 24 hours later. Table 1 shows that GLN increased cell number by 68% ($P < 0.05$). This stimulation was blocked by cAMP and PD98059. Note that cells shifted from a glucose concentration of 5.5 mmol/L (DMEM-L) to a concentration of 25 mmol/L in the absence of GLN had no increase in cell number. Similarly, the addition of the intestinal fuel²⁵ acetic acid (10 mmol/L) did not stimulate mitogenesis. (Acetic acid at this concentration decreased the pH of the media by 0.4 pH units, which could have partially reduced a mitogenic effect.)

* Compared to Control, $P < 0.05$ ($n=3$ –5), (μ l O₂/ml/h / 10^6 IEC-6 cells)

Figure 6. Effect of AOA, cAMP, and PD98059 on GLN-stimulated QO₂ by IEC-6 cells. After 24-hour starvation, 5×10^6 IEC-6 cells in DMEM-L were equilibrated with air for 2 minutes in a continuously stirred chamber with a Clarke-type electrode for the detection of PO₂. Vehicle (control) or an agent in vehicle was added, and QO₂ was recorded for 8 minutes. Cells were exposed to vehicle or agent only once. Representative traces are shown for each treatment with means for 4 replications below. Treatments were as follows: Control, GLN-starved; GLN 10 mmol/L; GLN + 8-cpt-cAMP, 300 μ mol/L; GLN + PD98059, 50 μ mol/L; GLN + AOA, 10 mmol/L.

Figure 7. Effect of GLN, cAMP, and PD98059 on proliferation of GLN-starved and -sufficient IEC-6 cells. Cell starvation and thymidine incorporation assay were identical to those shown in Figure 1. cAMP (333 μ mol/L) and PD98059 (50 μ mol/L) were added 15 minutes before GLN (10 mmol/L); thymidine incorporation at 24 hours estimated the cellular DNA synthesis rate. On the left, the 3 bars (upper and lower) show results from GLN-starved cells before stimulation with GLN. On the right, the 3 bars show data from cells pretreated overnight with 0.7 mmol/L GLN. * P < 0.05 and ** P < 0.01 compared with control (DMEM-L without GLN). + P < 0.05 compared with 0.07 mmol/L GLN (n = 4). Control cpm is described in Figure 1.

Effect of 8-CPT-cAMP on GLN Activation of JNK

To determine if the 8-CPT-cAMP-resistant proliferation in starved IEC-6 cells could be related to stimulation of the stress-activated protein kinase (SAPK), Jun nuclear kinase (JNK), cells were treated with 10 mmol/L GLN for 30 minutes after overnight starvation

Table 1. Effect of cAMP and Intestinal Fuels on IEC-6 Cell Number ($\times 10^5$)

	GLN (10 mmol/L)	CAMP + GLN	PD98059 + GLN	D-Glucose (10 mmol/L)	Acetate (10 mmol/L)
Mean	1.82	3.06 ^a	1.95	2.35	1.88
SEM	0.24	0.23	0.20	0.17	0.18

NOTE. IEC-6 cells (10^5) were plated in 24-well plates overnight in DMEM-L without GLN or serum (DMEM-L, DMEM with 5.5 mmol/L glucose). The next day, nutrients with or without inhibitors were added for 24 hours, and cell number was counted 24 hours later. n = 6 for each treatment. 8-CPT-cAMP concentration is 10^{-4} mmol/L, combined with 100 μ mol/L IBMX. PD98059 concentration is 50 μ mol/L. n = 6 observations.

^a P < 0.05 compared with DMEM-L.

and JNK was determined. As a positive control, we used TNF- α (10 ng/mL) to enhance JNK activity. JNK activity was assessed by an in vitro kinase assay that uses GST-cJun N-terminus as the substrate (n = 2). Figure 8 shows that 10 mmol/L GLN with or without TNF- α increased JNK $\sim$ 4-fold. 8-CPT-cAMP did not inhibit either GLN or TNF- α -stimulated JNK activity.

Effect of 8-CPT-cAMP Inhibition on the G₁/S Transition of the Enterocyte Cell Cycle

We sorted cells by fluorescence-activation to determine the effect of 8-CPT-cAMP on the cell cycle. Cells were made quiescent by GLN/serum starvation overnight, and the next day the media was changed to media with GLN with or without 8-CPT-cAMP. As shown in Figure 9, 8-CPT-cAMP arrested progression of GLN-sufficient cells through the cycle, with accumulation in G₁ and depletion of cells in G₂ (P < 0.05; n = 3). Table 2 shows that GLN starvation also produces a G₁/S arrest.

Duration of cAMP and PD98059 Inhibition of Proliferation

To determine if cell proliferation inhibition by cAMP and PD98059 is reversible, IEC-6 cells were treated with cAMP or PD98059 for 4–24 hours; subsequently, cAMP and PD98059 were washed out (Figure 10). Cells were allowed to recover without GLN for 8–20 hours before GLN stimulation. Cells were then counted after GLN (10 mmol/L) treatment for 24 hours (Figure 10A) and 48 hours (Figure 10B). cAMP-treated cells did not recover despite washout of up to 20 hours and subsequent GLN treatment for 24 or 48 hours. PD98059-treated cells similarly did not begin to proliferate after up

Figure 8. Effects of GLN, TNF- α , and 8-CPT-cAMP on c-JUN kinase activity in IEC-6 cells. Cells in 100-mm dishes (70% confluent) were starved in DMEM-L without serum or GLN for 24 hours. They were subsequently treated with 10 mmol/L GLN. TNF- α (10 ng/mL) was added as a positive control. Cells in lanes 3 and 5 were exposed to 8-CPT-cAMP (333 μ mol/L) + IBMX (100 μ mol/L) for 10 minutes. Then the cells were washed and lysed by rotating in Dignam C buffer. Lysate (25 μ g) was coincubated with GST-cJun in the presence of [32 P]ATP. Labeled GST-cJun was separated by 10% SDS-polyacrylamide gel electrophoresis. Coomassie blue staining showed equal loading. Results are representative of 2 blots.

Figure 9. Effect of GLN and cAMP on the cell cycle of cultured enterocytes. After starvation and treatment as described in Figure 1, cells were fixed in EtOH, stained with propidium iodide, and analyzed on a fluorescence-activated cell scanner. Single-perimeter histograms of 100,000 events were collected on aliquots of cells treated with GLN + 8-CPT-cAMP or GLN. The coefficient of variance of the G_{0-1} ("resting phase") peaks for both conditions was <5.0 . S, S phase. An overlay of the 2 histograms shows the lack of progression through the cell cycle to the G_2 stage in cells treated with 8-CPT-cAMP + GLN ($n = 3$). No obvious evidence for apoptosis is shown.

to 20 hours of PD98059 washout, followed by 24 hours of GLN treatment. However, by 48 hours of GLN, cells treated 68 hours earlier with a 4-hour pulse of PD98059 did show recovery. Cells exposed to PD98059 56 hours earlier showed no significant increase in cell number compared with control cells (GLN-and serum-starved). In these studies, cell number never exceeded 3.3×10^5 , because cells had reached confluence at this density. These results were not biased by cells reaching confluence, because cells were only 80%–85% confluent at 24 hours and were ~95% confluent by 48 hours. The poor reversibility of the cAMP effect could be caused by sequestration of 8-CPT-cAMP within the cells, for example by cAMP-binding proteins.

Table 2. Effect of 8-CPT-cAMP on Cell Cycle Progression

Glutamine	–	+	+
8-CPT-cAMP	–	–	+
G_1	79 ± 1	73 ± 4	85 ± 0
S	10 ± 1	9 ± 1	7 ± 1
G_2	12 ± 2	18 ± 5^a	8 ± 1

NOTE. IEC-6 cells were grown to 80% confluence and 6 hours later were exposed overnight to "starvation" conditions (no GLN or serum), GLN (10 mmol/L), or GLN + 8-CPT-cAMP. Cells were then subjected to fluorescence-activated cell sorting. Data are means $\pm$ SEM of 3 observations.

^a $P < 0.05$ compared with GLN + 8-CPT-cAMP.

Figure 10. Determination of reversibility of cAMP inhibition and PD98059 inhibition of IEC-6 cell proliferation. Cells were plated in 24-well plastic plates at a density of 10^5 cells/well, washed, treated, and counted manually after trypsin treatment. Control cells were kept in DMEM-L. GLN was added at 10 mmol/L. The 3 numbers beneath each bar refer to (1) the time (hour) of cAMP/IBMX or PD98059 treatment; (2) the length of time of "washout"; and (3) the time (hour) of GLN treatment. Cells were counted (A) 24 hours and (B) 48 hours after GLN treatment ($n = 6$; $*P < 0.05$).

Discussion

The major new findings of this report are (1) L-glutamine stimulates intestinal cell proliferation in "starved" intestinal cells, and this mitogenic effect is partially inhibited by blocking of ERK signaling; (2) cAMP inhibits proliferation in cultured small intestinal cell lines and, like the MEK inhibitor PD98059, inhibits ERKs; (3) GLN stimulates ERK signaling through its metabolism; and (4) GLN signals through an Raf-independent mechanism.

GLN and Proliferation of Starved Cells

Intense interest has focused on GLN as a "nutraceutical" to enhance bowel function. Recent reports show GLN is trophic in the intestine,^{26,27} whereas others find no GLN effect on villus morphology.^{28–30} In general, minimal effects of GLN have been found in well-nourished animals with bowel resection and in well-nourished rats or humans receiving total parenteral nutrition supplemented with GLN.^{30,31} Trophic effects dominate reports about starved animals. For example, starved rats that are subsequently fed GLN-supple-

mented total parenteral nutrition³² and rats fed a GLN-supplemented diet after abdominal irradiation³³ have a more normal intestinal villous architecture.

GLN metabolism has myriad physiological effects in this tissue. GLN fuels intestinal oxidative metabolism; serves as a precursor for nucleotide, arginine, and polyamine biosynthesis; and enhances intestinal blood flow.²⁴ Our findings support the concept that EGF is much more potent than GLN in mitogenic signaling, whereas GLN is a mandatory cofactor for downstream effects (e.g., thymidine incorporation) of EGF. GLN is an essential substrate for carbamoyl phosphate synthetase II (CPS II), the enzyme that catalyzes conversion of GLN to carbamoyl phosphate, an initial step in pyrimidine synthesis.³⁴ Given that DMEM contains 10 mmol/L glucose but no nucleotides or nucleosides, we speculate that a primary effect of GLN is to provide nucleotides for DNA synthesis. However, GLN utilization for nucleotides would not explain GLN's rapid activation of the ERK or JNK pathways, or the ~50% increase in [³H]thymidine incorporation observed in GLN-sufficient (3.5 mmol/L) enterocytes when reexposed to GLN.¹⁹ These findings point to a nonnutritive "trigger" or signaling effect of GLN on intestinal cell proliferation.

cAMP Inhibits Intestinal Cell Proliferation

Our interest in 8-CPT-cAMP originated from the observation that increased intestinal mucosal 8-CPT-cAMP concentration is characteristic of not only toxicogenic diarrhea (e.g., cholera) but also inflammatory enteropathies such as salmonellosis, when mucosal prostaglandin levels are high.³⁵ In cholera toxin-exposed rabbit ileal loops, mucosal 8-CPT-cAMP content is increased to ~1 μ mol/L, compared with less than half this value in control tissues. These values appear lower than the maximal concentrations needed to block proliferation in crypt enterocytes, but 8-CPT-cAMP may have leaked rapidly from cells used for these assays. Levels in rapidly washed upper crypt cells in *Salmonella*-induced enteritis reached as high as 50 pmol/mg protein,³⁵ or about 5 μ mol/L, assuming mucosal protein content is about 10 g/dL. This value is well within the range that inhibits proliferation in IEC-6 cells (Figure 1). It would be interesting to know if intestinal mucosal 8-CPT-cAMP levels remain elevated in children with chronic enteropathy and delayed villus repair.

Cell proliferation can be stimulated or inhibited by 8-CPT-cAMP in a cell-specific manner.¹² cAMP "blocked" small intestinal IEC-6 cells at the G₁/S transition, an observation previously reported in other epithelial cells.³⁶ A possible site of inhibition in our studies is at the level of Raf and/or MEK, and our studies showed these kinases

were inhibited by cAMP, as shown in previous investigations of mesenchymal cell lines.^{13,14}

cAMP and PD98059 Effects on ERKs and JNK

MAPKs are proline-directed kinases that are activated by phosphorylation of tyrosine and threonine residues. Three classes of MAPKs have been identified, which share considerable sequence homology and are often, but not always, regulated in parallel during mitogenesis or cell injury: the ERKs (extracellular signal-related kinases), the stress-activated protein kinase c-Jun N-terminal kinase (JNK), and p38 MAPK, which is also activated by osmotic and other cellular stresses and during cell motility. This and our previous studies showed that GLN stimulates ERK functional activity in enterocytes.¹⁹ It is likely that the ERKs were a major site of inhibition of proliferation in 8-CPT-cAMP-treated cells in the present studies. Additional sites of inhibition by 8-CPT-cAMP are possible, resulting in inhibition of transition into S-phase of the cell cycle.

Inhibition of proliferation by 8-CPT-cAMP could have been mediated by inhibition of cellular processes such as mitochondrial metabolism or cytoskeletal rearrangement (independent of 8-CPT-cAMP effects on the ERK pathway). However, mitochondrial QO₂ was not affected by 8-CPT-cAMP. Furthermore, PD98059, a highly specific inhibitor of MEK1 (the upstream activator of ERK-1 and -2)^{23,37} completely blocked GLN- or EGF-stimulated proliferation and inhibited Elk-1 phosphorylation in nonstarved IEC-6 cells, without altering QO₂. The inhibition of EGF-induced proliferation and ERK activity in IEC-6 cells by PD98059 has been demonstrated recently.³⁸

It is possible that cAMP inhibits proliferation through mechanisms in addition to ERK inhibition. cAMP has been shown to produce cell cycle arrest by inhibiting the action of cdk-activating kinase and by increasing the activity of the cdk inhibitor p27^{Kip1}.³⁹ However, in that study, murine BAC1.2F5A macrophages were arrested at both G₁/S and in G₂, whereas in our studies intestinal IEC-6 cells were arrested only in G₁/S. Also, the similarity between MEK inhibition by PD98059 and the effects of 8-CPT-cAMP suggests that a significant component of the cAMP inhibition is through MAP kinases. Additionally, we cannot rule out effects of 8-CPT-cAMP and PD98059 on cell viability, but general toxicity of these inhibitors was ruled out by exclusion of trypan blue by IEC-6 cells exposed to 8-CPT-cAMP or PD98059.

We could not find previous studies examining activation of JNKs in small intestinal cell lines. Our studies showed that JNK is activated by mitogenic stimuli.¹⁹

JNKs may be activated during hyperthermia and oxidant stress, and are activated during apoptosis.⁴⁰ Interestingly, recent studies from the laboratory of Ko et al.⁴¹ have shown that GLN starvation induces apoptosis in IEC-6 cells. In our GLN-starved cells, JNK was not activated initially, although there was activation after GLN treatment. Although there is no known specific inhibitor of the JNK pathway, the use of PD98059 allowed us to separate 2 MAPK pathways activated by GLN in proliferating IEC-6 cells. We found no significant effect of PD98059 on JNK activity (not shown) and only a partial effect of PD98059 in reducing [³H]thymidine incorporation response to GLN in "stressed" (starved) IEC-6 cells. While our studies demonstrate an effect of GLN on JNK in the starved cells, they do not rule out an effect of GLN on other pathways such as protein kinase C, phosphoinositol-3-kinase, or p38 MAPK.

GLN Metabolism Bypasses Raf to Stimulate MEK and ERK Induction

EGF, insulin, and other growth factors are known to activate MEK via Raf recruitment to membrane-bound ras, and Raf activation. There is evidence that growth signals may stimulate ERK activation in the absence of Raf activation (Figures 3 and 4). In fact, MEK is often activated without a corresponding increase in Raf kinase activity.¹² One example is in G protein-coupled signal transduction in vascular smooth muscle cells. In these cells, angiotensin II stimulates ERK activation by an Ras- and Raf-independent pathway requiring activation of protein kinase C.⁴²

We do not know the mechanism for initiation of GLN-stimulated mitogenesis. Research in other cell types suggests several possibilities. Kilberg et al.⁴³ showed that amino acid starvation induced derepression of system A amino acid transport activity within 2 hours. Amino acid refeeding (with A system amino acids or GLN) increased cellular proliferation when added after starvation, perhaps by an effect on intracellular amino acid "loading." Additionally, cytosolic GLN, aspartate, and aminoisobutyrate repressed ribosomal protein messenger RNA levels.⁴³ When cells were starved and subsequently refed, increased messenger RNA translation was observed. Other possibilities for how GLN signals mitogenesis include GLN-stimulated release of paracrine growth factors stored in a rapidly released pool, or a rapid increase in polyamine synthesis via ornithine decarboxylase induction.²⁰

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