Inhibition of Glutamine Synthetase Decreases Proliferation of Cultured Rat Intestinal Epithelial Cells

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ABSTRACT: The importance of glutamine synthetase (GS) for cell proliferation was examined in rat intestinal crypt cells (IEC-6) by inhibiting its activity with 10 mmol/L methionine sulfoximine (MS) at varying extracellular glutamine (Q) concentrations. In uninhibited cultures, cell number, protein, and DNA accumulation and synthesis showed a dependence on extracellular Q over a concentration range of 0.06 to 1.06 mmol/L, with apparent half-maximal responses of 0.46 mmol/L extracellular Q. In contrast, proliferation of GS-inhibited cultures required ≥1.06 mmol/L extracellular Q, with an apparent half-maximal response of 2 mmol/L. MS inhibited GS activity >97% in extracts of washed cells and appeared to be specific because its effects on proliferation were overcome by 4.06 mmol/L Q and were reversible. The increased dependence of IEC-6 cells on extracellular Q when GS was inhibited suggests that Q derived from GS (GS-Q) contributes importantly to cell proliferation at physiologic levels of extracellular Q (0.6 mmol/L). The unexpectedly high concentration of extracellular Q required to rescue maximal proliferation during GS-inhibition, relative to a reported K_m for Q-transport into the cell, indicates that intracellular Q derived from the extracellular medium (exo-Q) is inefficiently utilized. In a previous study, we found that GS-protein and mRNA are concentrated in the proliferative crypt region of the small intestine in vivo, and predicted that GS activity is important for crypt cell proliferation. Here, we show that enzyme activity is important for cell proliferation at physiologic concentrations of Q in this cell culture model. Finally, we speculate that exo-Q and GS-Q are utilized differently in the cell.

KEY WORDS: • endogenous glutamine production • IEC-6 cells • rats • small intestine • crypt cells

The mature epithelial mucosal cells lining the intestinal tract of many animals are replaced completely every 2–3 d (Cairnie et al. 1965a and 1965b, Cheng and Leblond 1974). The loss of these cells, which occurs at the villous tips, is balanced by continuous replication of cells, a process that is restricted to the lower two thirds of the crypt region (Cheng and Leblond 1974). The constant renewal of the epithelium of this highly metabolically active tissue is a process that requires a ready supply of nutrients and energy. The amino acid glutamine (Q) is important in this regard. Glutamine is critical for the growth and function of the gastrointestinal epithelium. Evidence that Q is the preferred oxidative substrate for enterocytes comes from studies of Q metabolism in the isolated perfused rat intestinal preparation (Windmueller 1982) as well as in neonatal pig enterocytes (Wu et al. 1995). Glutamine also acts as a precursor for the synthesis of purines and pyrimidines, amino sugars, asparagine and other amino acids (Kitos et al. 1962). Glutamine has a proliferative effect throughout the rat intestine (Jacobs et al. 1988) and mediates proliferation stimulated by other growth factors (Ko et al. 1993). Circulating glutamine concentration in the mammalian bloodstream is maintained at ~0.6 mmol/L, which is the highest among the amino acids (Soubra 1992). In the postabsorptive state, the small intestine extracts 20–30% of the circulating glutamine with each pass through the intestinal vasculature (Windmueller and Spaeth 1974, Windmueller 1982).

Previous studies have suggested that enterocytes, i.e., the mucosal epithelial cells lining the gastrointestinal tract, have a relatively small free glutamine pool, little capacity for endogenous glutamine production and a high rate of protein synthesis and cell turnover (Windmueller and Spaeth 1974). As such, these cells are dependent on an external supply of glutamine for normal function (Windmueller 1982). These cells represent >80% of the mucosal epithelial cell population and are probably the major consumers of luminal and arterial glutamine. Recently, we demonstrated the presence of glutamine synthetase (GS), the enzyme that catalyzes the production of glutamine from glutamate and ammonia, in the rat small intestine and showed that the in vitro activity of this enzyme is dependent upon stage of development (Shenoy et al. 1996). Glutamine synthetase activity was elevated around the time pups were weaned and shortly thereafter, which corre-
responds to a period of increased intestinal epithelial cell proliferation and differentiation. Furthermore, we determined that the mRNA coding for GS and the enzyme protein itself were concentrated in the crypt region of the rat small intestine (Roig et al. 1995). These observations suggested that proliferating, undifferentiated stem cells can produce their own Q from glutamate and NH₃. Roig et al. (1995) postulated that crypt cell GS is important for the continuous proliferation of intestinal epithelial cells, but this has not yet been established. Because glutamine is an important biosynthetic precursor, local production of glutamine may be critical for helping to maintain the capacity for continual cell proliferation. If so, then specific pharmacologic or molecular blockade of GS should cause reductions in cell proliferation.

Here we test the hypothesis that endogenous production of glutamine is important for proliferation of IEC-6 cells, a cell line originally isolated from rat intestinal crypt cells. This in vitro model system was chosen because IEC-6 cells proliferate rapidly (on tissue slices, 0.003 or 0.001 L of media), so a confluent monolayer in which proliferation continues at a reduced rate (see below), and terminal differentiation does not occur. Thus the cells sustain a histotypic architecture not unlike that seen in the crypt region. Under these conditions, the cells are accessible to pharmacologic blockade by methionine sulfoximine (MS), a specific and irreversible inhibitor of GS activity (Ronzi et al. 1969). It has been shown that 1 mol of GS can bind up to 8 mol of MS, which competes with the binding of glutamate and ammonia (Ronzi et al. 1969). Complete irreversible inhibition of sheep brain GS occurs when the reaction mixture contains between 5 and 15 mmol/L MS (Ronzi et al. 1969). These observations suggested that proliferative inhibition of sheep brain GS occurs when the reaction mixture contains between 5 and 15 mmol/L MS (Ronzi et al. 1969). This has permitted an analysis of the dependence of IEC-6 cell proliferation on GS activity.

**MATERIALS AND METHODS**

**Chemicals.** [Methyl-³H] thymidine [185 GBq/(mmol·L)] was from Amersham (Arlington Heights, IL). Trypsin, Dulbecco’s minimum essential medium (DMEM), fetal bovine serum and an antibiotic antimycotic solution (ABAM) were from GIBCO BRL (Grand Island, NY). Methionine sulfoximine (L-³-[3-amino-3-carboxypropyl]³S-methylsulfoximine), DABA (3,5-diaminobenzoic acid) and all other reagents were from Sigma Chemical (St. Louis, MO).

**Cell culture.** Rat intestinal epithelial cells (CRL 1592, IEC-6) from the American Type Culture Collection (Rockville, MD) were incubated at 37°C in 5% CO₂ and 95% air. This cell line was originally isolated from the crypt region of the rat small intestine (Quaroni et al. 1979). Under normal culture conditions, cells rapidly divide and form adherent monolayers of undifferentiated cells. Cells were collected after trypsinization of a confluent stock culture, counted using a hemacytometer, and subcultured at 10,000 cells/cm² into 100-mm diameter tissue culture plates, or 6- or 24-well plates and were fed 0.012, 0.003 or 0.001 L of media twice daily. Culture media were replaced every other day. Media consisted of 9.1 glutamine-free DMEM and FBS, 200 U/L penicillin, 200 mg/ml streptomycin and 0.05 mg/L amphotericin B. The free glutamine concentration of this solution was assumed to be 0.06 mmol/L, or 10% of the mammalian blood concentration, which is consistent with the glutamine concentration of similar media formulations (Tadros et al. 1993). IEC-6 cells were cultured in media containing no supplemental glutamine (0.06 mmol/L extracellular Q) or media containing 0.06, 0.26, 0.46, 1.06, 2.06, 4.06 or 8.06 mmol/L extracellular Q. These final concentrations account for the concentration of glutamine contained in FBS.

To determine time to achieve confluence in media containing no supplemental extracellular Q (0.06 mmol/L) or media containing a high extracellular Q concentration (4.06 mmol/L), IEC-6 cells were plated on 6-well plates. Plates were checked once in the morning and late afternoon for confluency, which was established when there were no visible gaps between cells anywhere on the bottom surface of the plate.

To determine cell number, total protein and total DNA, cells were seeded into 100-mm diameter tissue culture plates and fed media containing varying concentrations of extracellular Q (0.08, 0.26, 0.46, 1.06, 2.06, 4.06 and 8.06 mmol/L). A replicate set contained 10 mmol/L MS. The concentration used was similar to that used for complete, irreversible inhibition of sheep brain GS (Ronzi et al. 1969). On d 4, cells were recovered, counted in a hemacytometer and cell pellets prepared as below. The entire experiment contained three independent replicates from three consecutive cell passages. To determine whether cells recover from MS inhibition, a separate series of plates was used in which cells were grown in 1.06 mmol/L extracellular Q plus 10 mmol/L MS (n = 10). On d 4, half of the cultures were switched to 1.06 mmol/L extracellular Q alone (n = 5). Cells were counted on d 7.

To recover cells, cultures were trypsinized, washed three times in PBS and centrifuged (200 x g) to obtain a cell pellet, which was frozen (−20°C) or used immediately.

**Glutamine synthetase specific activity of IEC-6 cells.** IEC-6 cells were plated on 6-well plates containing media with 4.06 mmol/L extracellular Q. To determine the extent to which 10 mmol/L MS decreases GS activity, 6-d cultures (1 d postconfluent) were exposed to 10 mmol/L MS (n = 6) or an equivalent volume of sterile PBS (n = 6) for 24 h. Additional cells were cultured to determine whether extracellular Q concentration affects GS activity. On d 2 after establishment of confluency (d 7), one half of the cultures received fresh media with 0.06 mmol/L extracellular Q (n = 6); the other half received 0.6 mmol/L extracellular Q (n = 6). After 24 h, cell pellets were prepared as described above. Fresh cell pellets were resuspended in chilled homogenate buffer (330 mmol/L sucrose, 50 mmol/L Tris-HCl (pH 7.3), 5 mmol/L MgCl₂, 1 mmol/L phenyl methyl sulfonyl fluoride and 1.2 μmol/L leupeptin), sonicated for 5 s on ice and assayed immediately. GS, which converts glutamate and ammonia into glutamine in the presence of ATP, was assayed indirectly on the basis of the formation of L-γ-glutamylhydroxyamic acid from glutamate and hydroxylamine. 1-γ-Glutamylhydroxyamic acid was assayed colorimetrically after reaction with Fe(NO₃)₃ (Iqbal and Ottaway 1970). All GS activities were normalized to the amount of protein in cell homogenates.

**Glutamine synthetase specific activity of Caco-2 cells.** To compare glutamine synthesizing capabilities of a crypt cell line (IEC-6 cells) with that of a mature differentiated cell line, we also measured GS activities of postconfluent Caco-2 cells. These cells, derived from a human colon carcinoma cell line, have been used as a model of the mature differentiated intestinal epithelium because, like mucosal epithelial cells, they form a polarized epithelial layer with a distinct brush border membrane that contains hydrolases such as sucrase isomaltase (Chantret et al. 1988). In an earlier study, we determined that GS protein and mRNA are preferentially expressed in the crypt region of the small intestine (Roig et al. 1995). It was anticipated, therefore, that GS activities would be greater in the crypt cell line. Under normal culture conditions, Caco-2 cells rapidly divide and form adherent monolayers of undifferentiated cells. After establishment of confluency, the process of differentiation takes place and is maximal within 2 wk. Cells were seeded onto twelve 100-mm diameter plates at a density of 11,500 cells/cm². The medium was similar to that used for IEC-6 cells, except that 20% instead of 10% FBS was used. Six cultures received 0.6 mmol/L extracellular Q (physiologic) and six received 6 mmol/L extracellular Q. Culture media were replaced every other day. Cells were harvested 2 wk after establishment of confluency and processed for GS activity in the same manner as described for IEC-6 cells.

**Tissue glutamine synthetase specific activity.** The in vitro GS activities of fresh homogenates of lung, skeletal muscle (major leg muscles above the knee) and small intestine (jejunum) from six normal female adult rats were measured. Rats were given an overdose of sodium pentobarbital (50 mg/kg) and tissues were dissected free, rinsed in ice-cold PBS and placed in 5 volumes of chilled GS buffer (see above). Tissues were homogenized with a polytron and immediately assayed for GS activity.

**Total protein.** Total protein was assayed by the method of Lowry et al. (1951).
GLUTAMINE SYNTHETASE INHIBITION

Total DNA. Frozen cell pellets were thawed in chilled PBS and sonicated briefly on ice. Total DNA per culture was measured using a fluorescent assay based on a reaction product between diaminobenzoic acid (DABA) and the deoxyribose sugars released by acid hydrolysis (Puzas and Goodman 1978).

DNA synthesis assay. Cells were cultured in 24-well plates in DMEM containing 2.06 mmol/L extracellular Q. One-day preconfluent (d 4) and 3-d postconfluent (d 8) cultures were then treated with media containing 0.06, 0.46, or 2.06 mmol/L extracellular Q = 10 mmol/L MS. After 20 h, they were labeled with 74 MBq/L [3H]-thymidine for 4 h. Cell pellets were prepared as above, immediately resuspended in PBS, briefly sonicated and processed using a modification of the method of Bollum. In brief, duplicate 10-μL samples of the sonicated cell solution were spotted onto glass fiber filter papers and allowed to air dry. The filter discs were placed in tubes containing 0.6 mol/L ice cold trichloroacetic acid (TCA) to precipitate DNA, once in 0.3 mol/L TCA, once in 100% ethanol, once in acetone, allowed to air dry and transferred to vials containing scintillation fluid. Radioactivity was measured using a Beckman liquid scintillation counter (Model LS 3801, Norcross, GA). Activity counts were adjusted for background using the average activity counts obtained from four control filter discs spotted with 10 μL of PBS and treated in the same manner as discs spotted with sample solution. For each treatment group, n = 3.

Measurement of media-free glutamine concentration. Freshly prepared culture media containing 0.46 mmol/L or 1.06 mmol/L extracellular Q, or media from two 6-d old cultures grown in the presence of 0.46 and 1.06 mmol/L extracellular Q were ultrafiltered through a 3000 MW cutoff Centricon cartridge (Amicon, Beverly, MA) and analyzed for total free amino acids by ion exchange chromatography with ninhydrin detection on a Beckman 6300 Amino Acid Analyzer. Because glutamine co-eluted with serine, the glutamine concentration was calculated after subtracting the level of serine used to prepare DMEM. To verify the results, parallel samples were acid-hydrolyzed in 6 mol/L HCl for 15 h at 110°C, and glutamine was calculated as glutamate after hydrolysis minus the level present before hydrolysis. Results from the two methods were in agreement.

Statistical analysis. Results are reported as means ± SD for n independent cell cultures. Student’s t test was used to determine differences between two sample means and significant difference was established at α = 0.05. Although the outcomes (cell number, total protein and total DNA) of the proliferation experiments were observed in treatment groups organized by two completely crossed experimental factors (MS status and glutamine dose), two-way ANOVA could not be used to analyze treatment group means because of the extreme heterogeneity of treatment group variances. Neither logarithmic nor square root transformation of responses was able to improve this heterogeneity. The independent-sample t test for samples with unequal variances was therefore used to compare MS− and MS+ mean responses locally at each glutamine dose. The statistical significance of these comparisons was assessed at α = 0.01. Linear glutamine dose-response relationships were assessed separately for MS− and MS+ groups using the Spearman rank correlation coefficient. Two-way ANOVA was used to determine glutamine dose and MS status effects on [3H]-thymidine incorporation in pre- and postconfluent cells. A t test was used to compare MS absent cells to MS present cells for each level of glutamine and significant difference was assessed at α = 0.01.

RESULTS

Dependence of IEC-6 cell proliferation on extracellular Q. IEC-6 cells plated at 10,000 cells/cm² and cultured in 4.06 mmol/L Q, a supraphysiologic concentration, achieved confluency in 5 d. In contrast, those cultured in 0.06 mmol/L extracellular Q, a subphysiologic concentration, required 14 d. To quantify cell proliferation over a range of extracellular Q concentrations, cell number, total protein and total DNA per well were determined on d 6, just when cells raised in 4.06 mmol/L extracellular Q reached maximal density. Increasing concentrations of extracellular Q supported increased cell numbers up to 1–2 mmol/L Q (Fig. 1A), resulting in a 10-fold increase in cell number between 0.06 and 1.06 mmol/L extracellular Q. No further increase was observed up to 8 mmol/L extracellular Q. A similar result was seen when total protein and total DNA were measured (Figs. 1B and C). The slight increase in cell number seen between 1.06 and 2.06 mmol/L extracellular Q probably reflected cells in the G2 phase of the cell cycle completing mitosis because no increase in DNA was seen in this interval. In addition, the gradual increase in total protein seen at increasing extracellular Q concentrations at the high end of the range probably reflected accumulation of extracellular matrix protein. The apparent half-maximal proliferation response with respect to extracellular Q was 0.46 mmol/L.

The increased rate of cell proliferation at higher concentrations of extracellular Q was corroborated by an experiment that measured the uptake of [3H]-thymidine into DNA of preconfluent cells. Cells were grown for 4 d on 2.06 mmol/L extracellular Q, shifted to a new concentration of extracellular Q for 24 h and supplemented with [3H]-thymidine for the final 4 h. A fivefold greater incorporation of [3H]-thymidine was observed in cultures shifted to 0.46 mmol/L compared with cells shifted to 0.06 mmol/L Q (Fig. 2A). This was consistent with the approximately sixfold higher level of DNA accumulation over this range of extracellular Q seen in the DNA content experiment (Fig. 1C). Cultures maintained at 2.06 mmol/L extracellular Q for the last day showed less than a 1.5-fold additional increase of incorporation relative to those shifted to 0.46 mmol/L Q. Thus incorporation reached a plateau at this higher extracellular Q concentration, as was also seen in the DNA accumulation study.

A similar [3H]-thymidine incorporation study was done on d-8 cultures that were 3 d postconfluent. These confluent cultures incorporated substantial levels of [3H]-thymidine, which was 30% that of preconfluent cultures at 2 mmol/L extracellular Q, but 150% that of preconfluent cultures at 0.06 mmol/L extracellular Q (Fig. 2B). Incorporation into postconfluent cultures nevertheless still exhibited a dependence upon extracellular Q, although this dependence was not as pronounced as that seen in the preconfluent cultures. Thus, cell proliferation appeared to continue after confluence, and this process was also dependent on extracellular Q. Continued proliferation did not result in an increased cell density, which was consistent with the appearance in the culture of floating cells that were washed away before analysis.

Dependence of in vitro GS activity on extracellular Q. IEC-6 cells grown for 7 d (2 d postconfluent) in 4.06 mmol/L extracellular Q exhibited in vitro GS activity of 7.5 ± 1.4 μmol/(mg protein·h). A parallel confluent culture, which was switched for the final day to 0.06 mmol/L Q, exhibited a higher in vitro GS activity of 14.2 ± 1.4 μmol/(mg protein·h). Thus activity appeared to depend on the concentration of extracellular Q, as if the cells compensated for the shift to decreased extracellular Q with increased GS activity. The in vitro GS activities of IEC-6 cells were significantly higher than those seen in fully differentiated Caco-2 cells. Caco-2 cells grown in 6 mmol/L extracellular Q exhibited an in vitro activity of 0.78 ± 0.09 μmol/(mg protein·h), whereas cells grown in 0.6 mmol/L extracellular Q (physiologic) exhibited a higher in vitro activity of 2.09 ± 0.25 (n = 6). Thus, differentiated Caco-2 cells grown at physiologic and supraphysiologic concentrations possessed less GS activity than undifferentiated IEC-6 cells in extracts, which was consistent with the different levels of GS mRNA and protein seen in their tissue section counterparts, the villous and crypt epithe-
lial cells (Roig et al. 1995). In addition, the in vitro GS activities of both Caco-2 and IEC-6 cells were affected by the level of extracellular Q in a potentially compensatory fashion. The in vitro GS activities of fresh homogenates of normal adult rat lung, skeletal muscle and small intestine were also significantly lower than those of the IEC-6 cells, 2.02 ± 1.43, 1.06 ± 0.72 and 0.58 ± 0.09 μmol/(mg protein · h), respectively (n = 6 for each tissue type). Altogether, the in vitro enzyme activities corresponded to the finding that GS protein is elevated in intestinal crypt cells in vivo relative to villous mucosa and other tissues, and the changes in in vitro GS activities in response to extracellular Q concentrations suggest that GS is active in these cells.

**Effect of MS on GS activity.** To determine the effect of MS on GS activity, confluent d-6 IEC-6 cells, grown in 4.06 mmol/L extracellular Q, were treated for the final 24 h in the presence or absence of 10 mmol/L MS. Cells were washed free of MS, and extracts prepared from the MS-treated cells contained an activity of 0.33 ± 0.08 μmol/(mg protein · h), compared with 12.35 ± 1.28 μmol/(mg protein · h) from the untreated cells. Thus, this concentration of MS appeared to inhibit in vivo GS by >97%.

**Effect of MS on IEC-6 cell proliferation.** The above studies showed that IEC-6 cell proliferation was dependent on the concentration of extracellular Q in the physiologic range and suggested that GS activity might be regulated to contribute more GS-Q at lower levels of extracellular Q. To more directly assess the role of GS and Q derived from GS (GS-Q), the studies described above were repeated, in parallel, in the presence of 10 mmol/L MS. Although MS-treated cultures could achieve the same cell numbers as untreated cells within 6 d, this required a minimum of 4.06 mmol/L Q compared with 1 mmol/L for untreated cells (Fig. 1A). Little or no proliferation occurred in MS-treated cells below 1 mmol/L extracellular Q. The apparent half-maximal response with respect to Q was 2 mmol/L, and the slope of the relationship between cell number and extracellular Q concentration was less than that in the absence of MS. Similar effects were observed on total protein and total DNA (Figs. 1B and C), and [3H]thymidine incorporation (Figs. 2A and B). Lack of proliferation below 1 mmol/L extracellular Q did not result from depletion of extracellular Q, because near confluent cultures fed 0.46 and 1.06 mmol/L extracellular Q still contained 0.21 and 1.0 mmol/L free Q, respectively, on d 6 as determined by amino acid analysis. Thus, the effect of MS on cell proliferation appeared to be specific for its inhibition of GS because excess product of GS activity, Q, was able to completely override its effect when presented extracellularly. However, the high level of extracellular Q required, and the shape of the dose-response curve, suggested that this rescue mechanism was inefficient, which will be discussed below.

To determine whether the effect of MS was reversible, cultures were exposed to media containing 1.06 mmol/L extracellular Q in the presence of MS for the first 3 d, and were subsequently fed 1.06 mmol/L extracellular Q in the presence or absence of MS for 4 additional days. MS-reversed cultures contained 5.46 ± 0.50 × 10^6 cells compared with 1.89 ± 0.35 × 10^6 cells in cultures sustained in MS for the entire 7-d period (P < 0.05). Thus, cells were able to reinitiate proliferation after release from the MS blockade, suggesting that inhibition of proliferation was not due to a toxic effect on the cells.

**FIGURE 1** Cell proliferation as a function of extracellular Q of IEC-6 cultures, with or without glutamine synthetase (GS) inhibition. Cells were subcultured at low density and grown for 6 d at the indicated concentration of extracellular Q, in the presence (MS+) or absence (MS−) of 10 mmol/L methionine sulfoximine (MS). (A) Total cell number per plate. (B) Total protein per plate. (C) Total DNA per plate. Each point represents the mean ± SD of three independent cell cultures. Asterisks indicate that the MS− and MS+ cultures differ (P < 0.01) at a given extracellular Q concentration.
Thymidine was provided during the final 4 h. In both groups, incorporation was maximal, to model the proliferative activity of crypt cells, based on labeling of an intracellular Q pool from [15N]-ammonium acetate provided in the culture medium (unpublished data).

However, the high concentrations of extracellular Q required for uptake of [3H]thymidine into confluent cells, which was probably reflective of continued proliferation. The extracellular Q concentration that drives half-maximal proliferation was about four times higher than in the absence of MS, 2 mmol/L vs. 0.46 mmol/L. A similar requirement for extracellular Q could be reversed by the removal of MS. The effect of MS on GS activity seemed to be nearly complete because half-maximal proliferation is similar to the physiologic serum level of Q, ~0.6 mmol/L (Souba 1992), and is consistent with a reported Km for uptake of Q into cultured Caco-2 cells of ~0.25 mmol/L (Souba et al. 1992). The limitation of proliferation at lower concentrations of extracellular Q did not appear to be due to exhaustion of this amino acid. Amino acid analysis of media sampled from cultures treated with either 0.46 or 1.0 mmol/L extracellular Q showed that substantial levels of this amino acid remained at the time of media change. A similar result was reported in transformed and untransformed fibroblast cell lines, in which extracellular glutamine was not depleted at low, growth-limiting concentrations of this amino acid (Nomura and Rubin 1988). It was suggested that glutamine or some reaction in which it participates, e.g., purine and pyrimidine synthesis, is regulatory for cell proliferation.

Cultures treated with 10 mmol/L MS also exhibited a dependence on extracellular Q, shifted to a higher concentration range. This effect was seen in both preconfluent cultures and confluent cultures analyzed by incorporation of [3H]thymidine. The extracellular Q concentration required to support half-maximal proliferation was about four times higher than that in the absence of MS, 2 mmol/L vs. 0.46 mmol/L, and the slope of the dose-response curve was markedly reduced. The effects of MS appeared to be specific because they could be completely overcome by higher concentrations of extracellular Q and could be reversed by the removal of MS. The effect of MS on GS activity seemed to be nearly complete because >97% of the activity in in vitro extracts of confluent cells was blocked by prior treatment of the cells with MS, as seen in other cell types (Kvidera and Carey 1994, Miller and Carrino 1981, Rowe 1985, Tadros et al. 1993). These results suggest that in the physiologic range of extracellular Q concentrations, optimal IEC-6 cell proliferation, both before and after confluence, was dependent on the activity of GS. Presumably the product of GS activity, Q, jointly contributes with Q taken up from the extracellular medium, extracellular Q, to meet the Q needs of the cell. This conclusion is supported by the finding that GS activity in cell extracts is greater when taken from cells grown at lower concentrations of Q, as if the cells were raising GS activity in an effort to produce GS-Q to overcome the deficit. This effect has also been seen in cultured muscle cells (Feng et al. 1990). In addition, we have recently found that GS is active in IEC-6 cells, based on labeling of an intracellular Q pool from [15N]-ammonium acetate provided in the culture medium (unpublished data).

DISCUSSION

GS protein is expressed at high levels in intestinal crypt epithelial stem cells relative to mature villous epithelial cells (Roig et al. 1995). To investigate the significance of high GS protein in crypt cells, the IEC-6 cell model was employed. IEC-6 cells were examined before confluence, when prolifera-

FIGURE 2 3H]thymidine incorporation in pre- or postconfluent IEC-6 cells exposed to varying concentrations of extracellular Q, with or without glutamine synthetase (GS) inhibition. (A) Preconfluent cultures. Values are means ± SD, n = 3. Cells were subcultured at low density, grown for 4 d at 2.06 mmol/L extracellular Q and shifted to the indicated concentration of extracellular Q in the presence (MS+) or absence (MS−) of 10 mmol/L methionine sulfoximine (MS), for 1 additional day. 3H]Thymidine was provided during the final 4 h. (B) Postconfluent cultures. Values are means ± SD, n = 3. Cells were subcultured at low density and grown for 8 d at 2.06 mmol/L extracellular Q and shifted to the indicated concentration of extracellular Q in the presence (MS+) or absence (MS−) of 10 mmol/L MS for 1 additional day. 3H]Thymidine was provided during the final 4 h. In both groups, incorporation was normalized to the amount of DNA present in the culture. Asterisks indicate that the control (MS−) and experimental (MS+) cultures differ (P < 0.01) at a given extracellular Q concentration.

* indicates that the control (MS−) and experimental (MS+) cultures differ (P < 0.01) at a given extracellular Q concentration.
uptake $K_{\text{m}}$, which would not be expected to be altered by the MS treatment. This suggests that extracellular Q was inefficient in replacing GS-Q. This would be expected if exo-Q and GS-Q belonged to different intracellular pools or were metabolically channeled along distinct pathways. Separate pools might be the result of different compartmentalization. In fact, a mitochondrial isosform of GS has been reported in liver and in other tissues (Caizzi et al. 1990, Laud and Campbell 1994, Smith and Campbell 1983 and 1987), suggesting the possibility that a proportion of GS-Q could be compartmentalized. Metabolic channeling might occur if enzymes that utilize Q were physically associated with GS in the cell. Q atoms are precursors for various biosynthetic pathways including purines, pyrimidines, sugars, amino acids and Krebs cycle intermediates, and the amide N of Q may be limiting for some of these pathways. If exo-Q and GS-Q preferentially contribute to different metabolic pathways, then the high concentrations of extracellular Q required to replace GS-Q might reflect an inefficiency of a hypothetical exo-Q pool to infiltrate a hypothetical GS-Q pool.

These findings in cultured IEC-6 cells may be relevant to cell proliferation in vivo. A motivating factor for this study was the finding that GS mRNA and protein are present at relatively high levels in intestinal crypt epithelial cells relative to villous epithelial cells (Roig et al. 1995). Here we found that a cell culture model for crypt cells, IEC-6 cells, exhibited higher GS activity in vitro than did a cell culture model for differentiated epithelial cells, Caco-2 cells, that mimics the distribution of GS protein in the tissue sections from which these cell lines presumably derived. Thus the different levels of GS protein correspond to different levels of activity in extracts. The relevance of reporting GS activities of whole-tissue homogenates (skeletal muscle, lung and small intestine) to GS activities of cultures consisting exclusively of IEC-6 cells is that these comparisons further emphasize the relatively high GS activity found specifically within crypt cells. Despite the fact that skeletal muscle and lungs are major glutamine synthesizing organs, they exhibit much less GS activity than IEC-6 cells. This may be due in part to the heterogeneity of cell types in whole tissues and cell-specific differences in amount of GS protein. Furthermore, the IEC-6 GS activity is functionally important as shown by the proliferation studies. In vivo, the crypt is the zone of maximal cell proliferation. The present results suggest, based on the importance of GS activity in culture, that it may also be required for proliferation of crypt cells in vivo. Although it is not known why crypt cells seem to have evolved a dependence on GS-Q, it is possible that this mechanism subserves an important need for metabolic channeling required for proper metabolic regulation. Further work is required to substantiate the metabolic channeling model and to establish the proposed role for GS in vivo. In this connection, recent unpublished studies suggest that Q derived from GS represents a substantial percentage of mucosal Q in vivo metabolic tracer labeling studies (P. Reeds, Baylor College of Medicine, Houston, TX, personal communication).

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LITERATURE CITED


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