Glutamine and KGF each regulate extracellular thiol/disulfide redox and enhance proliferation in Caco-2 cells

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Departments of 1Medicine and 4Biochemistry, 2Graduate Program in Molecular and Systems Pharmacology and 3Center for Clinical and Molecular Nutrition, Emory University School of Medicine, Atlanta, Georgia 30322; and 5Department of Surgery, University of Rochester Medical Center, Rochester, New York 14642

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Jonas, Carolyn R., Li H. Gu, Yvonne S. Nkabayo, Yanci O. Mannery, Nelly E. Avissar, Harry C. Sax, Dean P. Jones, and Thomas R. Ziegler. Glutamine and KGF each regulate extracellular thiol/disulfide redox and enhance proliferation in Caco-2 cells. Am J Physiol Regul Integr Comp Physiol 285: R1421–R1429, 2003. First published August 28, 2003; 10.1152/ajpregu.00702.2002.—Glutamine (Gln) and keratinocyte growth factor (KGF) each stimulate intestinal epithelial cell growth, but regulatory mechanisms are not well understood. We determined whether Gln and KGF alter intracellular glutathione/glutathione disulfide (GSH/GSSG) or extracellular cysteine/cystine (Cys/CySS) concentrations in cell medium. Cell proliferation was determined by 5-bromo-2-deoxyuridine (BrdU) incorporation. Gln (10 mmol/l) or KGF (10 μg/l) did not alter BrdU incorporation at reducing extracellular Eh (10 to 150 mV), but significantly increased incorporation at more oxidizing Eh (Gln at 0 to −109 mV; KGF at −46 to −80 mV). Cellular glutathione/glutathione disulfide (GSH/GSSG) Eh was unaffected by Gln, KGF, or variations in extracellular Cys/CySS Eh. Control cells largely maintained extracellular Cys/CySS Eh at initial values after 24 h (−36 to −136 mV). However, extracellular Eh shifted toward a narrow physiological range with Gln and KGF treatment (Gln −56 to −88 mV and KGF −76 to −92 mV, respectively; P < 0.05 vs. control). The results indicate that thiol/disulfide redox state in the extracellular milieu is an important determinant of Caco-2 cell proliferation induced by Gln and KGF, that this control is independent of intracellular GSH redox status, and that both Gln and KGF enhance the capability of Caco-2 cells to modulate extremes of extracellular redox.

cysteine; glutathione; intestine; oxidation-reduction; keratinoctye growth factor

INTRACELLULAR CONCENTRATIONS of low molecular weight thiols and the redox state of intra- and extracellular thiol pools are known to regulate cellular proliferation, differentiation, and apoptosis (17, 18, 22, 33–35). The cysteine (Cys) or cystine (CySS) couple is the predominant extracellular low molecular weight thiol/disulfide redox (23–24). In HT-29 cells, Cys depletion in cell medium causes a rapid and marked oxidation of intracellular glutathione/glutathione disulfide (GSH/GSSG) redox, which is rapidly reversed with repletion of Cys (32). In rat jejunum, luminal GSSG is partially reduced to GSH by a mechanism using Cys released directly from the epithelium or derived from breakdown of GSH released from the epithelium (8). We recently showed that extracellular Eh regulates both spontaneous and EGF- or insulin-like growth factor-1 (IGF-I)-stimulated Caco-2 cell proliferation (22). Furthermore, these specific growth factors enhanced the ability of Caco-2 cells to return the extracellular Cys/CySS pool toward a physiological range when initially cultured in oxidizing or reducing Cys/CySS Eh (22). Therefore, changes in extracellular Cys/CySS redox status may play a fundamental role in intestinal cell responses to certain gut trophic agents.

GSH, the major low molecular weight intracellular thiol, plays a critical role in maintenance of intracellular thiol/disulfide redox balance in gut and other tissues (25). Decreased concentrations of GSH and/or increased concentration of GSSG, impair cell proliferation in several cell types (17, 18, 25, 34). In human colonic epithelial cell lines, intracellular GSH/GSSG redox potential (Eh) becomes oxidized as cells transition from the proliferative to the differentiated state, either spontaneously in Caco-2 cells (33) or with induction by differentiation inducers in HT-29 cells (26).

A growing number of investigations indicate that supplementation with the amino acid glutamine (Gln) stimulates growth, repair, and barrier function of the gut epithelia (30, 42). Gln is a major respiratory fuel for intestinal mucosal cells in vivo and is required for optimal cell proliferation in vitro (27, 38, 41). The Gln product glutamate is a constituent of GSH; in rats, enteral Gln supplementation increases splanchnic GSH production (4), whereas Gln-enriched nutrition in catabolic animal models increases tissue GSH concentrations (13, 16). Thus control of GSH redox status by Gln represents a potential mechanism for the proliferative effects of this nutrient in intestinal epithelial cells.

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Keratinocyte growth factor (KGF) is an epithelial cell-specific growth factor with marked stimulatory effects on gut mucosal cell lineages (3, 10, 11, 19). We recently found that KGF prevents the decrease in GSH and oxidation of the GSH/GSSG pool in rat intestinal mucosa induced by malnutrition (20). Modulation of GSH redox status by KGF may be an important determinant of the gut-trophic effects of this agent, because redox reactions are known to control growth factor-stimulated cell proliferation (1, 5, 14, 15, 36, 39), and depletion of intracellular GSH inhibits cell proliferation in response to specific growth factors (1, 5, 36).

The current study was designed to test whether treatment with Gln alters extremes of extracellular redox in Caco-2 cells and whether initial extracellular redox conditions determine proliferative responses to this nutrient. In light of our data showing extracellular redox regulation in Caco-2 cells treated with EGF and IGF-I (22), we also compared the effects of Gln with administration of KGF, an epithelial-specific growth factor. Our results show that the thiol/disulfide extracellular redox state is a major determinant of cell proliferation induced by both Gln and KGF. In addition, treatment with both Gln and KGF enhanced the ability of Caco-2 cells to adjust extremes of extracellular redox toward a range similar to that found during health in vivo (23, 24).

**METHODS**

**Chemicals.** KGF was a gift from Amgen, Thousand Oaks, CA. All other chemicals and reagents were purchased from Sigma (St. Louis, MO).

**Cell culture.** The Caco-2 cell line was obtained from American Type Culture Collection (ATCC, Gaithersburg, MD) at passage 19. Cells were maintained as monolayer cultures under 5% CO2 at 37°C in MEM and were supplemented with 2 mM Gln, 10 U/ml penicillin and 10 μg/ml streptomycin, and 10% fetal calf serum (GIBCO BRL, Rockville, MD). Cells were passaged every 5 to 7 days (when 80–90% confluent). All experiments outlined below were performed on cells from passages 25 to 32. During serum starvation, cells were cultured in serum-free MEM. Gln was also excluded during serum starvation to limit cell proliferation. There was no visual evidence of cell death (increased number of detached cells, Trypan blue staining) after serum starvation or any of other manipulations performed in this study.

**Extracellular thiol/disulfide redox conditions.** Experiments were designed to generate a range of oxidizing to reducing extracellular redox conditions by controlled manipulation of the Cys/CySS redox pool. In our previous studies, the Cys/CySS Eh in plasma of healthy adults was 80 ± 9 mV (24); thus we designed the current in vitro studies to test oxidizing to reducing redox states around this physiologic Eh range. The extracellular thiol pool was altered by varying concentrations of CySS and CySS added to cyst(e)ine-free DMEM. Concentrations for Cys and CySS required to achieve initial redox states (Eh) of 0, -46, -80, -109, -131, and -150 mV were calculated using the Nernst equation: $E_{n} = E_{o} + RT2F \ln \left(\frac{[Cys]}{[CySS]}\right)$. The Eh (expressed in V or mV) represents the electromotive force relative to a standard hydrogen electrode where R is the gas constant, T is the absolute temperature, F is Faraday’s constant, and Eo is the standard electrode potential for the Cys/CySS redox couple.

Concentrations of CySS/Cys added to modified DMEM were as follows (in μmol/l): 99.75:0.5 (0 mV, most oxidizing condition), 98.4 (-46 mV), 93.14 (-80 mV), 80.40 (-109 mV), 60.80 (-131 mV), 10.180 (-150 mV, most reducing condition). All conditions contained the same molar equivalents of Cys. Calculated Eh values included Eo of -0.250 V, accounting for the medium pH adjustment to 7.4 after CySS and CySS addition. This value is in accordance with that reported from studies in the human colonic epithelial cell line HT-29 (26). Medium pH was adjusted to 7.4 after CySS and CySS addition and was filter sterilized before use.

**Gln and KGF treatment protocols.** Caco-2 cells were plated in 96-well plates at a seeding density of 10⁵ cells/well. After 48 h (-60% confluence), cells were cultured in MEM without serum or Gln for 24 h. Caco-2 cell proliferative responses to different Gln and KGF doses were confirmed using cells treated for the subsequent 24 h in serum-free MEM, with or without increasing concentrations of added Gln or KGF. To determine Caco-2 proliferation responses to altered extracellular Eh, alone, cells were similarly serum- and Gln-starved for 24 h and during the subsequent 24 h period cultured in cyst(e)ine-free DMEM (containing 4 mM/l Gln) with varying amounts of Cys and CySS, as described above. To determine responses to Gln and KGF treatment during altered extracellular thiol/disulfide redox, cells were serum- and Gln-starved for 24 h, and during the subsequent 24-h period cultured in MEM supplemented with 1-mM [total dose] or KGF (10 μg/l]) for 24 h, with or without Cys and CySS additions to give the range of initial Eh values outlined previously. Parallel experiments were conducted in which cells were treated with 1-alanine (Ala; 10 mM/l) to evaluate responses to an amino acid that is not used as a source of ATP production by gut epithelial cells (28).

**5-Bromo-2-deoxyuridine incorporation assay.** For measurement of 5-bromo-2-deoxyuridine (BrdU) incorporation into DNA, cells were incubated in the presence of BrdU for 2 h. After being labeled, BrdU incorporation was measured with a cell proliferation ELISA (Boehringer Mannheim, Indianapolis, IN). The absorbance from peroxidase reaction with the substrate tetramethylbenzidine (TMB) was measured by a scanning multi-well spectrophotometer at 450 nm (reference wavelength at 650 nm) (Molecular Devices, Sunnyvale, CA). Increases in BrdU incorporation were expressed as fold changes relative to controls. BrdU quantitation was by integration relative to the internal standard, 5-bromo-2-deoxyuridine-d5 (BRDU-d5) as the internal standard. Controls were cells grown in the 0 mV condition without serum or added Gln or KGF. [3H]Thymidine incorporation was used to confirm dose responses to Gln measured using BrdU.

**Determination of cellular and extracellular thiols and disulfides by HPLC.** Cell medium was added 1:1 to ice-cold 100 g/l perchloric acid, 0.2 mol/l boric acid, and 10 μmol/l γ-glutamyl-glutamate (γ-Glu-Glu) as the internal standard. Cells were rinsed with 500 μl (1×) PBS and then an ice-cold solution (500 μl) containing 50 g/l perchloric acid, 0.2 mol/l boric acid, and 10 μmol/l γ-Glu-Glu was added directly to the cells. Cells were scraped and transferred to microcentrifuge tubes; precipitated protein was separated by centrifugation. GSH, GSSG, CySS, and the disulfide of Cys and GSH, CySSG, were determined from the acid-soluble supernatant as N,N-bis-dansyl or S-carboxymethyl-N-dansyl derivatives by HPLC with fluorescence detection as described (22, 26). Quantitation was by integration relative to the internal standard, expressed as nanomoles per milligram protein. The Eh values for the intracellular GSH/GSSG and extracellular GSH/CySS (mV) pools were calculated using the Nernst equation with combined constants for a two-electron transfer $E_{n} = E_{o} + 30 \log([\text{disulfide}]/[\text{thiol}])^{2}$, using Eo = -0.264 mV for
GSH/GSSG and −0.250 mV for Cys/CySS, respectively (6, 26).

CySS transport studies. Caco-2 cells were plated in six-well plates at a density of 3 × 10^4/well as above. After 48 h, cells were cultured in MEM without serum or Gln for 24 h, then incubated at 37°C in oxidizing medium (extracellular Eₜ₀ = 0 mV) for 20 min. Additional medium (0 mV) containing 2 μCi [35S]CySS (Amersham Biosciences, Piscataway, NJ) and either Gln (10 mmol), KGF (10 μg/l), or no treatment (control), was then added to each well. The cells were incubated for 2–10 min, washed twice in cold PBS, and harvested using 2% Triton-X. An aliquot of cellular material from each well (100 μl) was quantitated by scintillation counting using parallel values obtained from counting of 1 nM of [35S]CySS in each experiment. The rate of CySS transport into cells was normalized to intracellular protein content (37).

Statistical analysis. Results are expressed as means ± SE. Differences in BrdU incorporation, measures of GSH and Cys redox, and CySS transport were compared across groups by one-way ANOVA. Specific treatment effects were compared post hoc using the Fisher’s protected least-significant difference test. Data comparisons with P < 0.05 were considered statistically significant.

RESULTS

Gln and KGF induce Caco-2 cell proliferation. Initial experiments were performed to define doses of Gln and KGF required to stimulate Caco-2 cell proliferation. Gln at doses of 1.0, 10, and 100 mmol/l each significantly increased BrdU incorporation by 40–50% compared with control (Fig. 1A). To determine an optimal Gln dose for these experiments, DNA synthesis was also measured using [3H]thymidine incorporation and the same Gln doses. Results showed a similar increase (132%) with 10 mmol/l Gln (P < 0.05). KGF stimulated BrdU incorporation in a threshold dose-related manner (Fig. 1B). No additive or synergistic effect on Caco-2 cell proliferation occurred when cells were cultured in Gln (10 mmol/l) combined with KGF (10 μg/l) (data not shown). On the basis of these data and earlier studies on dose-related intestinal epithelial cell proliferation with Gln (38), doses of 10 mmol/l Gln and 10 μg/l KGF (10 μg/l) were used in subsequent experiments.

Caco-2 cell proliferation is modulated by altered extracellular thiol/disulfide redox. Our previous clinical studies indicate that CySS and Cys concentrations in plasma of young healthy adults and in adults with hematologic malignancies are ~50–60 μmol/l CySS and 8–16 μmol/l Cys, respectively (21, 24). These values are considerably less than the 200 μmol/l CySS (400 μmol/l equivalents of Cys) present in standard DMEM. To more closely approximate plasma thiol/disulfide pools and allow for cellular utilization, the experiments were designed with a total concentration in Cys moieties of 200 μmol/l, i.e., [Cys] + 2·[CySS] = 200 μmol/l (4). CySS and Cys concentrations were varied with this constant total pool size to achieve predicted initial extracellular Eₜ₀ values from 0 to −150 mV. HPLC analysis confirmed these values and that a relevant 100 mV range of extracellular Cys/CySS Eₜ₀ in cell medium (~36 to −136 mV) was maintained for 24 h after initial culture in redox-modified DMEM.
cellular \( E_h \) (0 and \(-46 \text{ mV} \)) exhibited the greatest stimulation of DNA incorporation with Glu supplementation (2.5-fold increase compared with respective control values; Fig. 2). An intermediate, but still significant, increase in Caco-2 proliferation occurred at initial \( E_h \) conditions of \(-80 \text{ and } -109 \text{ mV} \) (1.7- and 1.6-fold increase, respectively). Thus treatment with 10 mmol/l Glu resulted in different pattern of Caco-2 cell proliferation than observed in control cells cultured at the same medium \( E_h \).

To test whether results with Glu could be reproduced with an amino acid not used for energy by gut epithelial cells, parallel experiments were performed at initial extracellular \( E_h \) conditions of 0 mV, -80 mV, and -150 mV with Ala treatment (10 mmol/l). In contrast to the proliferative responses induced with equimolar concentrations of Glu (Fig. 2), Ala did not stimulate Caco-2 proliferation vs. control values at any of the initial extracellular \( E_h \) conditions (0 mV, 129 ± 17; -80 mV, 135 ± 15; and -150 mV, 159 ± 28; \( P = \text{not significant} \)).

Effects of extracellular thiol/disulfide redox on KGF-induced Caco-2 cell proliferation. We performed parallel studies using KGF to determine whether Caco-2 cell responses to the nutrient Glu are similar to those after treatment with KGF. Cells were treated with KGF over the same range of \( E_h \) as used in the Glu supplementation studies. At initial \( E_h \) conditions of -109, -131, and -150 mV, KGF treatment did not alter BrdU incorporation compared with control values (Fig. 3). However, KGF stimulated DNA synthesis at the most oxidizing extracellular thiol redox states (1.7-fold stimulation at 0 mV, \( P < 0.06 \) and 1.9- and 2.0-fold stimulation at -46 mV and -80 mV \( E_h \), respectively, each \( P < 0.05 \)). The pattern of KGF-induced cell proliferation by changes in extracellular redox was thus similar to that observed with Glu supplementation (stimulation at more oxidizing states, no change at the most reducing states).

Intracellular thiols and disulfides during Gln- and KGF-stimulated Caco-2 cell proliferation. GSH, GSSG, Cys, and CySS content in Caco-2 cells was analyzed by HPLC to determine whether the redox state of intracellular thiol antioxidants was changed in response to varied extracellular \( E_h \), in the presence or absence of Gln and KGF. The initial extracellular \( E_h \) conditions 0, -80, and -150 mV did not affect cellular GSH or GSSG concentrations or GSH/GSSG \( E_h \) at 24 h in the absence of Gln or KGF (Table 1). These data indicate that changes in extracellular Cys/CySS that influence Caco-2 cell BrdU incorporation do not cause a concomitant change in intracellular GSH/GSSG redox. In control cells, Cys concentrations were below the limit of HPLC detection (<0.017 mmol/l) at each of the initial extracellular Cys/CySS \( E_h \) conditions, whereas CySS concentrations did not significantly change (Table 1).

As in control cells, Glu supplementation at initial extracellular \( E_h \) of 0, -80, or -150 mV did not alter intracellular GSH redox (Table 1). Intracellular GSH/GSSG redox examined within each of the three \( E_h \) conditions was not different with Glu supplementation or KGF treatment at 0 mV (i.e., GSH, GSSG, or GSH/GSSG \( E_h \)). At both -80 mV and -150 mV, Glu supplementation and KGF treatment were each associated with a modest decrease in cellular GSH concentration vs. control values (Table 1). However, this was associated with a tendency for GSSG to decrease [not significant (NS)], and cellular GSH/GSSG \( E_h \) was ultimately the same as the controls at each extracellular \( E_h \) condition (Table 1). In contrast to control cells, intracellular Cys was detectable in cells treated either with Glu or KGF. CySS values were similar to control values across each initial extracellular \( E_h \). Thus, although
intracellular Cys/CySS $E_h$ could not be calculated in control cells due to undetectable Cys levels, the increase in Cys with Gln and KGF resulted in an increased ratio of intracellular Cys/CySS relative to control cells at each $E_h$ (Table 1). Thus in these Caco-2 cell models, significant changes in the intracellular GSH/ GSSG pool redox did not occur with growth regulation by Gln or KGF at any extracellular $E_h$ condition, but each of these agents appears to increase intracellular Cys and the Cys/CySS ratio.

Glutamine and KGF modulate Caco-2 cell extracellular redox state. Concentration of thiols and the disulfide of GSH plus Cys (CySSG) in cell medium were measured to determine regulation of extracellular Cys/CySS redox over time and with Gln and KGF treatment. In control cells and cells treated with Gln or KGF, a substantial fraction of the total added Cys + CySSG was lost from the culture medium at 24 h under all initial redox conditions (Table 2), presumably due to intracellular amino acid transport and support of cell growth-associated protein synthesis and other metabolic pathways (25, 37). In control cells, extracellular Cys/CySS $E_h$ ranged from −36 to −136 mV after 24 h in culture (compared to the initial $E_h$ values ranging from 0 to −150 mV) (Fig. 4). Although still representing a substantial range of oxidized to reduced $E_h$ (100 mV range), this finding indicates that Caco-2 cells have the ability to regulate their extracellular redox milieu when cultured at extremes of oxidizing and reducing conditions (22).

The addition of either Gln or KGF resulted in marked shifts in the extracellular redox environment to values closer to that found for Cys/CySS redox in human plasma (approximately −80 mV) (23, 24). Extracellular redox with Gln supplementation at 24 h was −56 mV compared with the initial $E_h$ of 0 mV and −88 mV compared with initial $E_h$ of −150 mV, respectively (Fig. 4). Similarly, with KGF treatment, extracellular redox at 24 h was −76 mV compared with initial $E_h$ of 0 mV and −92 mV compared with initial $E_h$ of −150 mV, respectively (Fig. 4). $E_h$ values with both Gln and KGF were similar at the three initial redox conditions and were significantly different at 24 h than the corresponding control values (Fig. 4).

Because GSH was not initially present in the culture medium, measurable concentrations of GSH and mixed disulfide CySSG (GSH equivalents) in medium after 24 h are an index of GSH release from the Caco-2 cells. In all treatment groups, the extracellular GSH concentration was significantly higher in cells cultured in more oxidizing initial medium $E_h$ (0 mV and −80 mV) compared with cells cultured in the most reducing initial medium $E_h$ (−150 mV); CySSG concentrations exhibited a similar pattern (Table 2). This suggests that increased GSH release occurs after 24 h of culture at the 0 and −80 mV conditions relative to the −150 mV condition, because concentrations of both GSH and CySSG in medium were similar in control cells and Gln- and KGF-treated cells across the imposed initial extracellular redox states (Table 2). Thus changes in GSH efflux by Caco-2 cells do not appear to be a mechanism for the control of extracellular redox by Gln or KGF treatment (Fig. 4).

### Table 1. Effects of variation in extracellular Cys/CySS redox state ($E_h$) and addition of KGF and glutamine on intracellular GSH, GSSG, GSH/GSSG $E_h$, Cys, and CySS in Caco-2 cells

<table>
<thead>
<tr>
<th>Control</th>
<th>$E_h$, mV</th>
<th>CyS, mmol/l</th>
<th>CySSG, mmol/l</th>
<th>CySS, mmol/l</th>
<th>CySSG, mmol/l</th>
<th>CySS, mmol/l</th>
<th>CySSG, mmol/l</th>
<th>CySS, mmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamine</td>
<td>0 mV</td>
<td>3.51 ± 0.25</td>
<td>3.40 ± 0.15</td>
<td>3.03 ± 0.20</td>
<td>0.12 ± 0.01</td>
<td>0.11 ± 0.06</td>
<td>0.14 ± 0.04</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>−80 mV</td>
<td>232.9 ± 2.0</td>
<td>2.70 ± 0.29</td>
<td>2.27 ± 0.13</td>
<td>0.06 ± 0.01</td>
<td>0.10 ± 0.03</td>
<td>0.07 ± 0.02</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>−150 mV</td>
<td>0.13 ± 0.02</td>
<td>0.12 ± 0.03</td>
<td>0.07 ± 0.01</td>
<td>&lt;0.01</td>
<td></td>
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</table>

Data are expressed as means ± SE. Results represent data from 3 separate experiments, each done in triplicate. Detectable limit for intracellular cystine (Cys) was 0.017 mmol/l under these experimental conditions. Values not sharing a letter are significantly different ($P < 0.05$) among 0 mV, −80 mV, and −150 mV $E_h$ conditions within the individual treatments (control, keratinocyte growth factor (KGF), and glutamine (Gln)). *$P < 0.05$ for Gln or KGF treatment vs. control within individual extracellular redox conditions (0 mV, −80 mV, and −150 mV, respectively). CySS, cystine.

### Table 2. Thiol and disulfide concentrations in culture medium with variation in Cys/CySS redox state ($E_h$) during glutamine or KGF treatment

<table>
<thead>
<tr>
<th>Value in Medium</th>
<th>Initial Extracellular Redox Potential ($E_h$, CyS/CySS)</th>
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<tbody>
<tr>
<td>Control</td>
<td>$E_h$, mV</td>
</tr>
<tr>
<td>Glutamine</td>
<td>0 mV</td>
</tr>
<tr>
<td></td>
<td>−80 mV</td>
</tr>
<tr>
<td></td>
<td>−150 mV</td>
</tr>
<tr>
<td>KGF</td>
<td>0 mV</td>
</tr>
<tr>
<td></td>
<td>−80 mV</td>
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<td></td>
<td>−150 mV</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE in mmol/l. Results represent data from 3 separate experiments, each done in triplicate. Values not sharing a letter are significantly different ($P < 0.05$) among 0 mV, −80 mV, and −150 mV $E_h$ conditions within the individual treatments (control, KGF, and Gln). *$P < 0.05$ vs. control within the extracellular redox conditions (0 mV, −80 mV and −150 mV, respectively).
Cys concentrations in the medium increased several-fold from the initial imposed level of 0.5 μmol/l at the 0 mV condition for all treatments; however, this increase was much greater with Gln or KGF treatment relative to controls (Table 2). Conversely, at 0 mV medium, CySS concentration decreased from the initial imposed level of 99.75 μmol/l to a lesser extent with Gln or KGF than in control cells. At the reducing −150 mV condition, cells induced a marked decrease in medium Cys concentrations from the initial imposed level of 180 μmol/l in all groups; however, this decrease was several-fold greater with Gln or KGF treatment relative to control cells. CySS concentrations were similar between groups (Table 2).

The change in total Cys equivalents (Cys + CySS concentrations) in cell medium with Gln and KGF treatment were similar (Table 2). At 0 and −80 mV, a higher concentration of Cys equivalents in cell medium relative to control values was induced by Gln or KGF treatment (i.e., less loss of Cys equivalents with each agent under more oxidizing conditions). At −150 mV, a lower concentration of Cys equivalents in cell medium relative to control values (i.e., greater loss of Cys equivalents with each agent under more reducing conditions) was induced in Gln- or KGF-treated cells.

**CySS transport into Caco-2 cells with glutamine and KGF at oxidizing extracellular Cys/CySS redox**. We performed transport studies using labeled CySS to examine the potential role of the Cys/CySS shuttle (8, 9) as a mechanism by which Gln and KGF enhance the capacity of Caco-2 cells to modulate an initially oxidizing extracellular Eh. Preliminary experiments at 0 mV demonstrated that inward CySS transport was linear at 2 min under all treatment conditions (control, Gln, and KGF; not shown). At 2 min, treatment with Gln resulted in a 34% increase in CySS transport into the cells that was not statistically significant vs. control values (Fig. 5). Treatment with KGF resulted in a significant 89% increase in inward CySS transport compared with control values (Fig. 5). Values between Gln and KGF treatment were not statistically different.

**DISCUSSION**

The current data suggest that maintenance of extracellular thiol-disulfide balance is physiologically important in the growth of Caco-2 cells, a human colon cancer-derived epithelial cell line. Our results also demonstrate for the first time that some specific nutrients (Gln) and epithelial cell-specific growth factors (KGF) are important for these cells to modulate extracellular Cys/CySS redox. The largest increase in BrdU incorporation after administration of these agents occurred under the most oxidizing conditions, suggesting that the balance of extracellular redox is a key determinant of proliferation induced by these agents in Caco-2 cells. In addition, when measured 24 h after either Gln supplementation or KGF treatment, the Caco-2 cell demonstrated an ability to modulate both extremes of experimental extracellular redox (oxidizing and reducing) toward a narrow physiological range. Thus the data provide an important link between proliferation and extracellular redox. Because redox status may modulate function of numerous cellular proteins via their disulfide moieties (25) and modification of several membrane-associated and/or intracellular proteins (e.g., receptors, signaling pathways) could affect the proliferative responses to Gln and KGF, the results do not establish a direct or specific relationship. However, the current results are similar to those in our previous study using the growth factors EGF and IGF-I, suggesting that generalized mechanism(s) of growth control are involved (22) and that a common feature involves control of extracellular redox state.

Gln is a preferred respiratory fuel and can contribute to cellular energy supply in intestinal epithelial cells (28, 30, 42). Ala was unable to substitute for Gln in stimulating Caco-2 cell BrdU incorporation. Because Gln is used by gut epithelial cells as a major energy
substrate, whereas Ala is not, it is possible that the proliferative response to Gln vs. Ala was a result of energy precursor supply. However, the redox-regulation experiments with Gln, Ala, and KGF were carried out in DMEM that contained a high concentration of the energy substrate glucose (25 mmol/l) with all treatments. Nonetheless, additional experiments to determine under oxidizing extracellular CySS/Cys E h conditions. Trans- 

tion of an initial oxidizing Cys/CySS extracellular redox in Caco-2 cells (22) and with the data of Noda et al. (35) in which exogenous Cys and CySS stimulated Caco-2 cell proliferation independent of an increase in cellular GSH. The reason for the lack of correlation between altered DNA synthesis rates and intracellular GSH redox indexes remains unclear. Our data are in contrast with other in vitro studies showing a positive association between intracellular GSH levels and rates of cell proliferation (26, 34, 36). We recently found that cellular GSH concentrations decline and GSH/GSSG E h becomes concomitantly oxidized as Caco-2 cells spontaneously progress from the proliferative to the differentiated phase over 25 days in culture (33). However, the current experiments were carried out in Caco-2 cells during the proliferative phase a few days after plating. In vivo studies in various catabolic rat models show that tissue GSH is increased by Gln supplementation in association with gut-trophic effects (13, 16). Also, in malnourished rats, KGF prevents the decrease in small bowel and colonic mucosal GSH and oxidation of the GSH/GSSG pool concomitant with stimulation of gut mucosal growth (20). Differences between our results and previous in vivo and in vitro studies with regard to cellular GSH may reflect differences in experimental models and cell types studied. It is also possible that small or transient changes in Caco-2 cellular thiol (GSH) status occur during proliferation, but were not detected, perhaps as a result of changes in cell synchronicity or due to the normalization of thiol levels by the time of assay (after 24 h). Others have observed thiol fluctuations limited to the rapid onset of proliferation characterized by transient increases in cellular GSH levels in human bronchial fibroblasts (1). It is thus possible that turnover of intracellular GSH or the GSH/GSSG ratio does play a role in proliferative responses in this cell line that may be detected by evaluation at earlier time points than in our experiments (34). Alternatively, Caco-2 cells may maintain a consistent intracellular redox balance during proliferation, possibly attributed to their transformed cell type. Fibrosarcoma (HT-1080) cells, which similarly exhibit unregulated growth, show no defined shift in intracellular redox balance with proliferation (17).

Control of Caco-2 cell growth by extracellular thiol-disulfide balance suggests that a cell membrane-associated redox signal mechanism may be involved. Several lines of investigation demonstrate thiol-linked modulation of membrane-associated proteins or cytosolic and nuclear signaling proteins, including recep-
tor-associated protein complexes, protein tyrosine phosphorylation activity, and intracellular function of transcription factor association and transactivation pathways (2, 14, 15, 39). Recent in vitro studies suggest that intracellular generation of reactive oxygen species, including superoxide or hydrogen peroxide, may play an important role in growth factor signaling (2, 12, 40).

In a previous study, we showed that intact rat jejunum is able to control $E_h$ of luminal (extracellular) thiol-disulfide pools (Cys/CySS and GSH/GSSG) by altering epithelial cell thiol uptake and release in response to added luminal GSSG (8). The response to Gln in the present study is a novel finding regarding nutrient function, indicating that supplementation of a specific nutrient enables cells to modulate extracellular Cys/CySS redox under oxidizing or reducing conditions. Although underlying mechanisms for the ability of Caco-2 cells to control the extracellular redox environment remain unclear, the observed intra- and extracellular concentrations of GSH, GSSG, Cys, CySS and CySSG, and the CySS transport data shed light on possible mechanisms (Table 2). We determined inward transport of CySS into Caco-2 cells to investigate the potential role of the Cys/CySS shuttle (8, 9) as a potential mechanism for correction of initially oxidizing extracellular $E_h$. With the most oxidizing initial extracellular redox condition, control cell medium was reduced from the initial 0 mV to $-36$ mV at 24 h; this net reduction of extracellular thiol pools was enhanced by Gln ($-56$ mV) and KGF ($-76$ mV) (Fig. 4). GSH transport from cells to medium (Fig. 6A) cannot account for the change in medium $E_h$ given the modest and similar increase in GSH and mixed GSH disulfide (CySSG) concentrations in all experimental groups (Table 2). Gln and KGF treatment increased medium Cys concentrations relative to control without significant decrease in CySS. These treatments (KGF $>$ Gln) also increased CySS transport into the cells (Fig. 5) and were associated with detectable intracellular Cys and an increase in the Cys/CySS ratio (Table 1). Taken together, these data are consistent with activation of the Cys/CySS shuttle (Fig. 6B; Refs. 8, 9) as a control point to reduce the initial oxidizing extracellular $E_h$.

For the most reducing initial extracellular redox condition ($-150$ mV), the slight oxidation of the control extracellular thiol pool at 24 h (to $-136$ mV) was markedly enhanced toward a physiological level of $-80$ mV by both Gln ($-92$ mV) and KGF ($-88$ mV) (Fig. 4). The decrease in the medium GSH concentration at the reducing $-150$ mV initial redox condition relative to the oxidizing 0 mV condition is consistent with inhibition of cellular GSH release during all experimental treatments, but does not account for the differences between control cells and the Gln- or KGF-treated cells. The decreased extracellular Cys concentrations at 24 h relative to initial imposed Cys concentrations (180 $\mu$mol/l) is consistent with increased cellular Cys uptake and utilization/metabolism and was more pronounced in the Gln- and KGF-treated cells.

The lack of changes in CySS concentrations between groups relative to the initial imposed concentration (10 $\mu$mol/l) suggests that if plasma membrane thiol oxidases are involved in the Gln and KGF responses (31), Cys is oxidized to products other than CySS. These products could include pyruvate, sulfate, and taurine via upregulation of the Cys dioxygenase pathway, as previously shown to occur with Cys supplementation in rat hepatocytes and enterocytes (7, 29).

In summary, the extracellular thiol/disulfide redox milieu alters endogenous and Gln- and KGF-stimulated DNA synthesis in Caco-2 cells studied during the proliferative phase. Both Gln and KGF reverse the growth inhibitory effects of an oxidizing extracellular environment, potentially by activating the membrane Cys/CySS shuttle (8, 9). In addition, both Gln and KGF treatment enable Caco-2 cells to restore thiol-disulfide redox balance after imposed extremes of oxidizing or reducing extracellular redox. The data suggest that there could be an interaction between Gln- and KGF-activated pathways for cell growth and thiol/disulfide metabolism.

**DISCLOSURES**

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