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BASIC—ALIMENTARY TRACT

DNA Hypermethylation Contributes to Incomplete Synthesis of Carbohydrate Determinants in Gastrointestinal Cancer

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See editorial on page 305.

Background & Aims: It has long been known that malignant transformation is associated with abnormal expression of carbohydrate determinants. The aim of this study was to clarify the cause of cancer-associated abnormal glycosylation in gastrointestinal (GI) cancers. ***Methods:*** We compared the expression levels of "glyco-genes," including glycosyltransferases and glycosidases, in normal GI mucosa and in gastric and colorectal cancer cells. To examine the possibility that DNA hypermethylation contributed to the down-regulation of these genes, we treated GI cancer cells with 5-aza-2′-deoxycytidine (5-aza-dC), an inhibitor of DNA methyltransferase. ***Results:*** The silencing of some of these glyco-genes, but not up-regulation of certain molecules, was observed. The Sd^a carbohydrate was abundantly expressed in the normal GI mucosa, but its expression was significantly decreased in cancer tissues. When human colon and gastric cancer cells were treated with 5-aza-dC, cell surface expression of Sd^a and the transcription of *B4GALNT2*, which catalyzes the synthesis of the Sd^a, were induced. The promoter region of the human *B4GALNT2* gene was heavily hypermethylated in many of the GI cancer cell lines examined as well as in gastric cancer tissues (39 out of 78 cases). In addition, aberrant methylation of the *B4GALNT2* gene was strongly correlated with Epstein-Barr virus-associated gastric carcinomas and occurred coincidentally with hypermethylation of the *ST3GAL6* gene. ***Conclusions:*** Epigenetic changes in a group of glycosyltransferases including *B4GALNT2* and *ST3GAL6* represent a malignant phenotype of gastric cancer caused by silencing of the activity of these enzymes, which action may eventually induce aberrant glycosylation and expression of cancer-associated carbohydrate antigens.

It has long been known that malignant transformation is associated with abnormal expression of carbohydrate determinants.[1] Many glycosyl epitopes such as sialyl Tn, Tn, T, and sialyl Lewis x/a ($sLe^{x/a}$) have been reported to be cancer-associated antigens. Some of them show statistically significant correlations between the degree of their expression in cancer tissues and the postoperative prognosis of patients with many types of human cancers.[2–4] In addition, $sLe^{x/a}$ determinants are known to serve as ligands for E-selectin, which is inducibly expressed by endothelial cells, in hematogenous metastasis of cancers.[5,6] A long-standing debate is which is more important in understanding cancer-associated carbohydrate antigens, "neo-synthesis" or "incomplete synthesis." To verify the former hypothesis, the levels of many glycosyltransferases involved in "neo-synthesis" of tumor-related glycosyl epitopes and their mRNA expression have been studied; however, no conclusive results have been obtained to date.[7–9]

On the other hand, there is a group of carbohydrate determinants that is less expressed in cancer tissues when compared with their level in normal tissues. Because their structures are commonly more complicated, the concept of "incomplete synthesis," that the synthesis of complex carbohydrate determinants in nonmalignant cells might be impaired upon malignant transformation, has been proposed as an important cause of cancer-associated abnormal glycosylation.[10] The blood group Sd^a carbohydrate antigen serves as a typical example among the latter

Abbreviations used in this paper: 5-aza-dC, 5-aza-2′-deoxycytidine; COBRA, combined bisulfite restriction analysis; DNMT, DNA methyltransferase; EBV, Epstein-Barr virus; FUT, fucosyltransferase; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GALNT, *N*-acetylgalactosaminyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GI, gastrointestinal; mAb, monoclonal antibody; HP, *Helicobacter pylori*; Sd^a-β1, 4GalNAcT, β1,4*N*-acetylgalactosaminyltransferase, which forms Sd^a carbohydrate determinants; ST, sialyltransferase; type II precursor, Galβ1,4GlcNAc-R.

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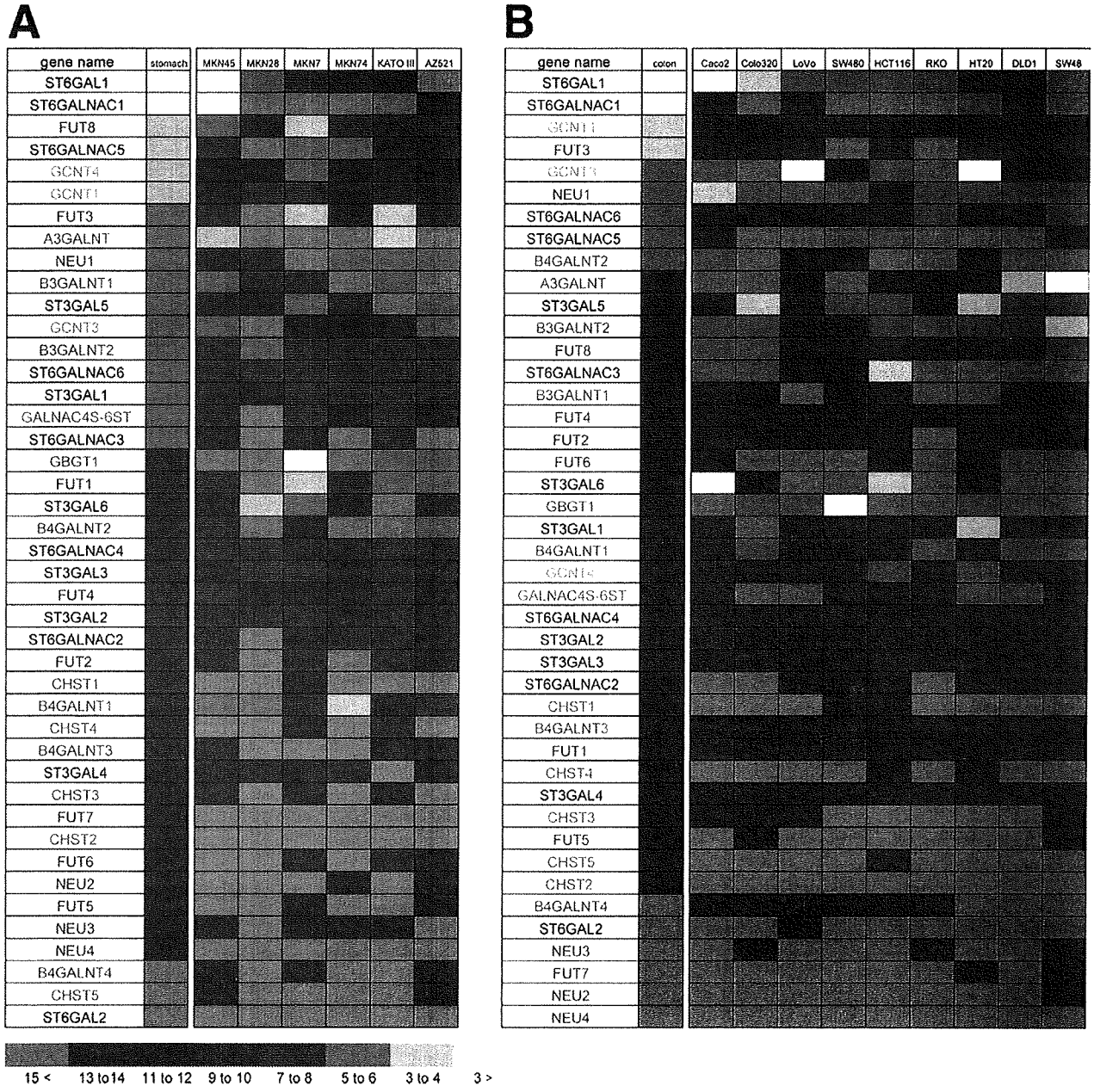

Figure 1. Profiles of expression of glycosylation-related genes in human GI tissues and cancer cell lines. Quantitative PCR analysis was carried out on normal human stomach and gastric cancer cell lines (*A*) and on normal human colon and CRC cell lines (*B*). Human glyco-genes, encoding 8 fucosyltransferases (classified by *blue*), 8 *N*-acetylgalactosaminyltransferase genes (*red*), 3 *N*-acetylglucosaminyltransferase genes (*orange*), 14 sialyltransferase genes (*black*), 6 sulfotransferase genes (*green*), and 4 sialidase genes (*purple*) were examined. Expression levels of each gene were sorted according to ΔCt (see Materials and Methods) calibrated by using GAPDH and visualized by color as indicated by the bar below.

group. This carbohydrate determinant is abundantly expressed on glycolipids and glycoproteins in the normal gastrointestinal (GI) tract mucosa in the majority of humans; however, its expression in cancer tissue is strikingly reduced or absent.[11,12] The last step in the biosynthesis of Sd^a is catalyzed by β1,4-*N*-acetylgalactosaminyl-transferase (β1,4GalNAcT). The activity of the β1,4GalNAcT responsible for synthesizing the Sd^a determinant (Sd^a-β1,4GalNAcT) also dramatically decreases in gastric and colonic cancer tissue.[13,14] Recently we reported that forced expression of Sd^a-β1,4GalNAcT in GI cancer cells reduced their expression of $sLe^{x/a}$ carbohydrates and decreased their metastatic potential in nude mice, probably owing to competition with $sLe^{x/a}$ synthases for acceptor carbohydrate.[15] Thus, the lack of Sd^a antigens in cancer cells is functionally important;

however, very little is known about the molecular mechanism underlying the regulation of Sd^a expression.

In line with these hypotheses of "neo-synthesis" and "incomplete synthesis," we compared the expression of "glyco-genes," including glycosyltransferases and glycosidases, in normal GI mucosa with that in gastric and colorectal cancer (CRC) cells in this study. Recently, epigenetic changes, such as DNA hypermethylation, have been recognized as one of the important mechanisms for gene inactivation.[16] In this study, we investigated the possible role of aberrant methylation in the glycosyltransferase gene promoter region in human GI cancer cells. We also examined epigenetic changes in a group of glycosyltransferases in human gastric cancer tissues and analyzed their relation to clinicopathologic features of the cases.

Materials and Methods

Cell Lines and Specimens

The gastric and colon carcinoma cell lines that were used in this study were obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan) or the American Tissue Type Collection (Manassas, VA). Human CRC cell line HCT116 with genetic disruption of DNMT1 (DNMT1 KO) or both DNMT1 and DNMT3b (DKO) were established as described previously.[17] The 78 gastric tumor specimens and their paired normal tissue specimens were obtained from 78 randomly selected Japanese patients. Informed consent was obtained from all patients before the samples were collected.

Reverse Transcription-Polymerase Chain Reaction

Quantitative polymerase chain reaction (PCR) of glyco-genes was performed by using ABI TaqMan probes (Applied Biosystems, Foster City, CA) as described previously.[18,19] Threshold cycle numbers (Ct) were determined with Sequence Detector software and transformed by using the ΔCt method as described by the manufacturer, with *glyceraldehyde-3-phosphate dehydrogenase* (GAPDH) used as the calibrator gene. Human glyco-genes examined in this study, 8 genes encoding fucosyltransferases (*FUT1, FUT2, FUT3, FUT4, FUT5, FUT6, FUT7,* and *FUT8*), 8 *N*-acetylgalactosaminyltransferase genes (*A3GALNT, GBGT1, B3GALNT1, B3GALNT2, B4GALNT1, B4GALNT2, B4GALNT3,* and *B4GALNT4*), 3 *N*-acetylglucosaminyltransferase genes (*GCNT1, GCNT3,* and *GCNT4*), 14 sialyltransferase genes (*ST3GAL1, ST3GAL2, ST3GAL3, ST3GAL4, ST3GAL5, ST3GAL6, ST6GAL1, ST6GAL2, ST6GALNAC1, ST6GALNAC2, ST6GALNAC3, ST6GALNAC4, ST6GALNAC5,* and *ST6GALNAC6*), 6 sulfotransferase genes (*GALNAC4S-6ST, CHST1, CHST2, CHST3, CHST4* and *GCNT5*), and 4 sialidase genes (*NEU1, NEU2, NEU3,* and *NEU4*), and TaqMan probe kits used in this study are summarized in Supplementary Table 1 (see supplementary material online at www.gastrojournal.org). Human stomach and colon total RNA (BioChain, Hayward, CA) were used as normal controls; they were prepared from normal stomachs and colon mucosae pooled from healthy subjects.

Flow Cytometry

Flow cytometry was performed with a FACScan (BD Bioscience, Franklin Lakes, NJ). Monoclonal antibody (mAb) KM694 (directed against Sd^a) was provided by Tokyo Research Laboratories (Kyowa Hakko Kogyo Co, Ltd. Tokyo, Japan).

Combined Bisulfite Restriction Analysis and Bisulfite Sequencing

We assessed gene methylation by using primers that were designed to amplify both the methylated and unmethylated alleles.[20] Bisulfite modification was carried out by using an EpiTect Bisulfite Kit (Qiagen, Tokyo, Japan). For combined bisulfite restriction analysis (COBRA), the PCR primers used for B4GALNT2 were 5′-ATTGGTTTTTYGTATAGGTGGTTG-3′ and 5′-CCRAACCRATTCCCACACTC-3′, yielding a PCR product of 174 bp. Primers for ST3GAL6[21] were 5′-GTTTGTTATATYGGGTYGTAGAAG-3′ and 5′-AATTAAAACTAACRAAAACCTAAAACT-3′ (162 bp). The products were then digested with the restriction endonuclease Hha*I* (for *B4GALNT2*) or AfaI (for *ST3GAL6*), which cleave only methylated CpG sites. For bisulfite sequencing, the PCR primers used for B4GALNT2 were 5′-GAGAGGTGAAATTTYGGGAGTA-3′ and 5′-RACTATCCACAACCCRCAATC-3′ (430 bp). For sequencing of the bisulfite-PCR product, the DNA fragment was purified and cloned into pCR4-TOPO vector (Invitrogen, Carlsbad, CA). Clones for subsequent sequencing were randomly picked up.

Detection of the Epstein–Barr Virus Genome and Helicobacter pylori

To detect the Epstein–Barr virus (EBV) genome in gastric tumors, we performed real-time PCR using 2 sets of primers as described previously.[22] Consistent results were obtained with both systems. *Helicobacter pylori* (HP) infection was identified by conducting histologic review of hematoxylin and eosin-stained tissue specimens and PCR assays as described by Clayton et al.[23]

Mutation Analysis

Genomic DNA was amplified by using exon-specific primers for p53 exons 2–11 and the mutations were examined as described previously.[24]

Immunohistochemical Analysis

Frozen sections of 8-μm thickness were prepared from a surgical specimen. After blocking sections with 3% bovine serum albumin in phosphate-buffered saline and then incubating them with mAb KM694, bound mAbs were detected with fluorescein isothiocyanate–conjugated goat anti-mouse immunoglobulin M (Southern Biotechnology Associates, Inc, Birmingham, AL).

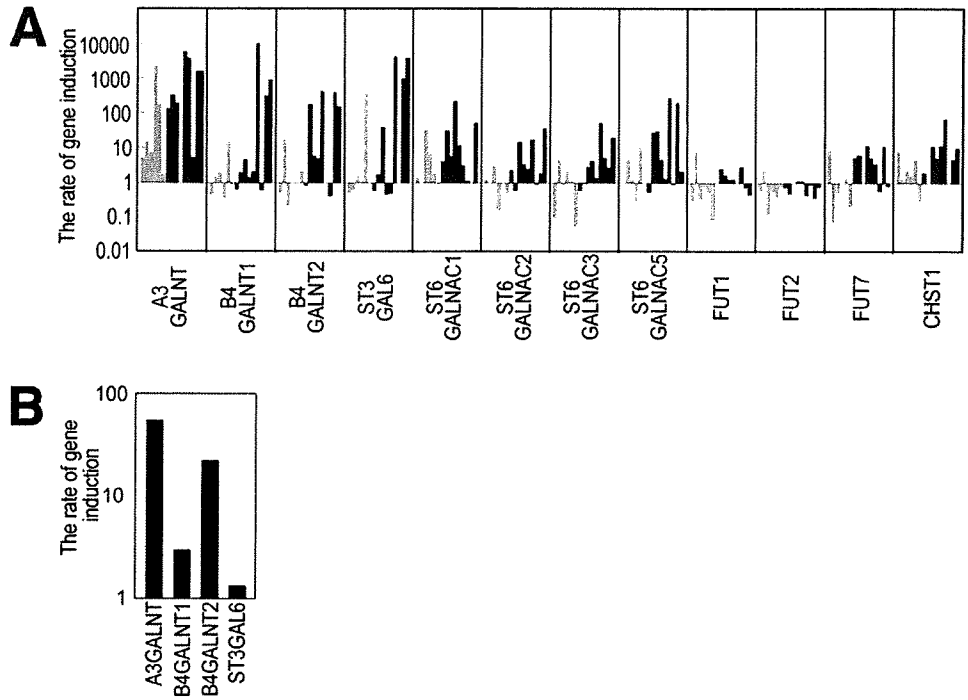

Figure 2. Expression of glycosylation-related genes in DNA methyltransferase-inhibited cells. (*A*) Six human gastric cancer (MKN45, MKN28, MKN7, MKN74, KATO III, and AZ521, as depicted in order from left to right by the *gray bars*) and 9 CRC (Caco2, Colo320, LoVo, SW480, HCT116, RKO, HT29, DLD1, and SW48, as depicted in order from left to right by the *black bars*) cell lines were treated with 2 μmol/L 5-aza-dC for 72 hours, and the expression level of each gene was then assessed by RT-PCR. The rate of induction is expressed as the ratio of treated to untreated cells. The genes that were analyzed are shown at the bottom of the bar graph. (*B*) RNA was harvested from HCT116 and DKO cells, and the expression level of each of the indicated genes was assessed by RT-PCR. The rate of induction is expressed as the ratio of induction in DKO cells to that in the parental HCT116 cells.

Statistical Analysis

Each tumor was classified based on tumor location; macroscopic type; lymphatic invasion; venous invasion (Japanese Gastric Cancer Association)[25]; pathologic tumor, lymph node, metastasis (pTMN) classification[26]; and the Lauren classification.[27] Methylation of *B4GALNT2* was compared by using the Student *t*-test for age; the Mann-Whitney *U* test for tumor size, pT status, pN status, and disease stage; and the Fisher exact test for gender, tumor location, macroscopic type, histology, lymphatic invasion, venous invasion, pM status, EBV association, HP status, p53 mutation, and methylation of ST3GAL6. The Fisher exact test was carried out by using SAS (SAS Institute Inc, Cary, NC), and other statistical analyses were made with SPSS software (version 11.0; SPSS Inc, Chicago, IL). All tests were 2-tailed, and values of $P < .05$ were considered significant.

Results

Expression of Genes Involved in the Synthesis and Modification of Carbohydrate Determinants in Human GI Tissue and Cancer Cells

It has been known that carbohydrate structures in GI cancers are quite different from those in normal GI epithelium. To clarify the cause of this abnormal glycosylation in GI cancer cells, we first examined the expression levels of 43 "glyco-genes," including 8 genes encoding fucosyltransferases, 14 sialyltransferase genes, 8 *N*-acetylgalactosaminyltransferase genes, 3 *N*-acetylglucosaminyltransferase genes, 6 sulfotransferase genes, and 4 sialidase genes (Supplementary Table 1). There was no gene whose expression was universally up-regulated in the GI cancer cell lines examined when compared with normal tissues. On the other hand, we found approximately one third of glycosyltransferase genes that were expressed in normal GI mucosa but whose expression levels were decreased in many GI cancer cell lines (Figure 1*A* and *B*). This silencing of glycosyltransferases was the major cancer-associated change detected in glyco-gene expression. To examine the possibility that DNA methylation contributed to the low expression levels of these genes, we chose 12 genes containing CpG islands in their promoter region from among cancer-associated down-regulated glyco-genes. When GI cancer cells were treated with 5-aza-2′-deoxycytidine (5-aza-dC), a DNA methyltransferase inhibitor, the mRNA expression of glycosyltransferases was significantly induced in many of them (Figure 2*A*). On the other hand, the expression of ≥2 glycosyltrans-

ferases (*FUT1* and *FUT2*) was not recovered by the 5-aza-dC-treatment, implying there might be certain glyco-genes whose expression was not controlled by DNA hypermethylation despite the presence of CpG islands. In the human CRC cell line HCT116 with genetic disruption of both *DNMT1* and *DNMT3b*,[17] in which genomic DNA methylation was nearly eliminated, the expression of *A3GALNT* and *B4GALNT2* was rescued (Figure 2*B*). Because it has been reported that promoter hypermethylation of the *A3GALNT* gene is associated with the loss of blood group A antigen expression in bladder cancer, oral squamous cell carcinoma, and gastric cancer cell lines,[28-30] our results suggest that aberrant methylation of the *A3GALNT* gene may lead to a cancer-associated reduction in the level of A antigen in colon cancers. Although remarkable induction of *B4GALNT1* mRNA was observed after 5-aza-dC-treatment, we excluded the *B4GALNT1* gene from subsequent analysis; because the expression of *B4GALNT1* and GM2 gangliosides synthesized by *B4GALNT1* is already known to be increased in GI cancers.[31] In any case, these results strongly suggest that down-regulation of glycosyltransferases might be the leading cause of cancer-associated abnormal glycosylation and that the *B4GALNT2* gene is a good representative of gene silencing by hypermethylation.

Recovery of Sd^a Carbohydrate Determinant in CRC Cells by Suppression of DNA Methyltransferases

The human *B4GALNT2* gene encodes a β1,4GalNAcT that is responsible for the synthesis of Sd^a carbohydrate antigen (Sd^a-β1,4GalNAcT). A noteworthy characteristic of the Sd^a carbohydrate determinant is that its expression is restricted to normal GI mucosa and is strikingly reduced or absent in GI cancer tissue.[11,12] So we asked if the membrane Sd^a structure could be detected in human CRC cell lines in which DNA methylation was suppressed. Treatment of T84 and HT29 human colonic cancer cell lines, which originally lacked the Sd^a carbohydrate, with 5-aza-dC resulted in an obvious increase in cell-surface expression of Sd^a along with the concomitant induction of *B4GALNT2* expression (Figure 3*A* and *B*). When these cells were treated with butyrate, a histone deacetylase inhibitor, neither expression of Sd^a antigen nor *B4GALNT2* mRNA was induced. We found that DNMT1 KO cells strongly expressed Sd^a determinants, whereas the parental HCT116 cells only weakly expressed it (Figure 3*C*). Furthermore, transcripts of *B4GALNT2* were detected in DNMT1 KO cells, but not in the parental HCT116 cells. These results suggest collectively that DNA hypermethylation rather than histone deacetylation may contribute to the down-regulation of *B4GALNT2* expression in cancer cells.

Methylation Status of B4GALNT2 *Gene Promoter Region in GI Cancer Cell Lines and Primary Gastric Carcinomas*

Next, we examined the methylation status of the upstream of the *B4GALNT2* gene in gastric cancer cell lines by COBRA. Hypermethylation in the *B4GALNT2* gene was detected in 5 of 6 human gastric cancer cell lines tested (Figure 4*A*, *left*). Atypical methylation in the *B4GALNT2* in a primary gastric carcinoma but not in the normal gastric mucosa adjacent to it was also found (Figure 4*A*, *right*). Because COBRA reflects the methylation status of only 2 adjoining CpG motifs, PCR products, extending from 169 bp upstream to 217 bp downstream from the translation start site and containing 39 CpGs, were subjected to bisulfite sequencing. Most of the CpGs examined were methylated in gastric cancer cells except in MKN45 cells, which were methylation negative by COBRA (Figure 4*B*). We also examined the methylation status of the *B4GALNT2* gene in DNMT1/

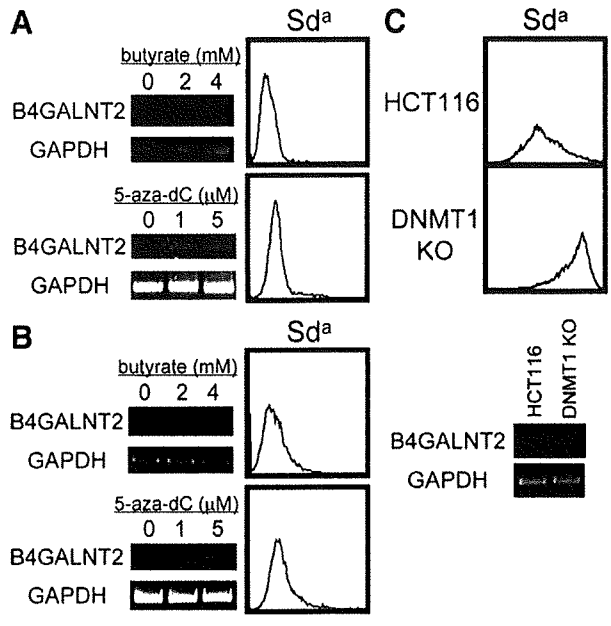

Figure 3. Effects of inhibitors on cell-surface Sd^a antigen and *B4GALNT2* mRNA expression in human CRC cells. Human CRC cell line T84 (*A*) and HT29 (*B*) were treated with the histone deacetylase inhibitor butyrate (*upper panels*) or the methyltransferase inhibitor 5-aza-dC at the concentrations indicated (*lower panels*). For RT-PCR analysis, cells were collected after 3 days of treatment, and expression levels of *B4GALNT2* and *GAPDH* were then assessed. For flow cytometric analysis, cells were treated with 4 μmol/L butyrate (*upper panels*) or 5 μmol/L 5-aza-dC (*lower panels*) for 6 days and then were stained with mAb KM694 (specific for Sd^a). Filled histograms represent the control staining of untreated cells. (*C*) Human CRC cell line HCT116 and DNMT1 KO cells were stained with mAb KM694 and then analyzed by flow cytometry. Filled histograms represent the control staining without mAb (*upper panels*). HCT 116 and DNMT1 KO cells were also assessed for expression levels of *B4GALNT2* and *GAPDH* by RT-PCR (*lower panels*). The data are representative of 3 separate experiments, which gave similar results.

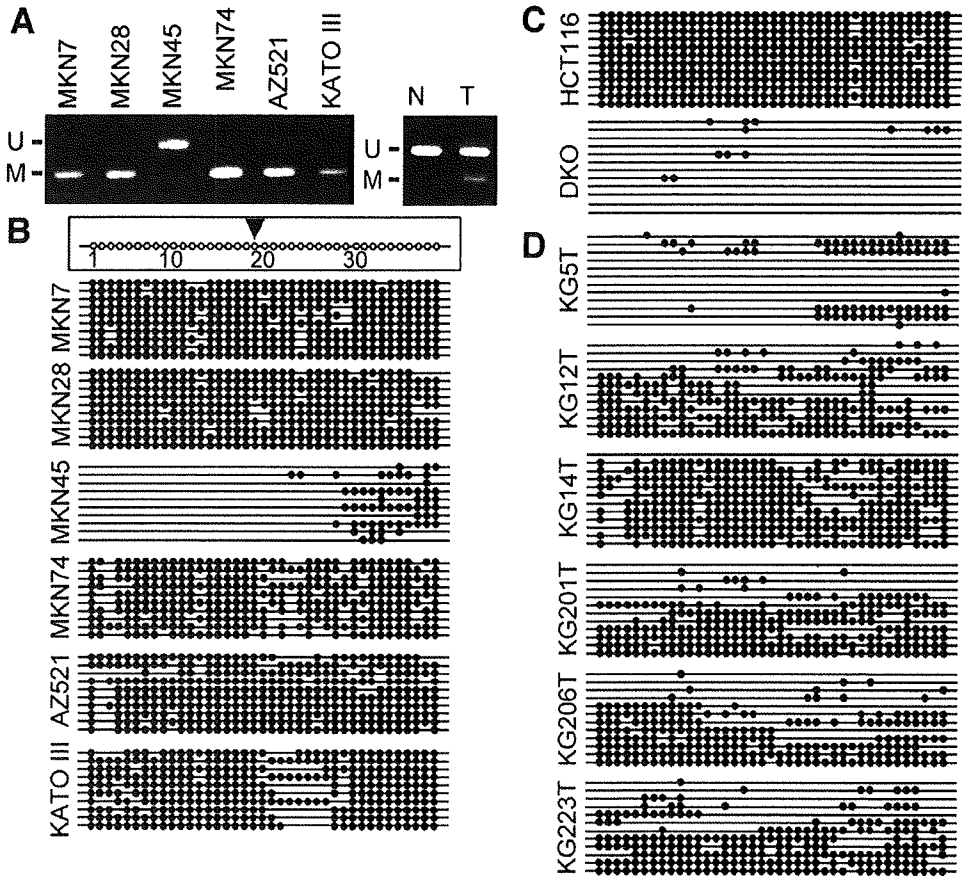

Figure 4. Methylation of the *B4GALNT2* gene in gastric cancer cells and primary gastric carcinomas. (*A*) Combined bisulfite restriction analysis (COBRA) of human gastric cancer cell lines (*left*) and representative results for a primary gastric carcinoma (*right*). M, methylated alleles; N, gastric normal mucosa adjacent to the tumor; T, gastric tumor; U, unmethylated alleles. (*B, C* and *D*) Methylation status of individual CpG residues in the *B4GALNT2* gene in human gastric cancer cell lines (*B*), human CRC cell line HCT116 and DKO cells (*C*), and primary gastric carcinomas (*D*) assessed by bisulfite sequencing. Bisulfite-PCR products cloned into the pCR4-TOPO vector were randomly picked up for sequencing. As illustrated in the box at the top of *B*, the line indicates an independent clone of bisulfite-PCR products; it contains 39 consecutive CpGs (*open circles*). For sequencing of the bisulfite–PCR product, the DNA fragment was purified and cloned into the pCR4-TOPO vector (Invitrogen). The start site of translation is indicated by the *arrowhead*. In the results shown below this box, the *filled circles* on the lines for each clone appear only when CpGs are methylated. Cell lines and case ID of tumors are shown at the left in *B* and *C*, respectively.

DNMT3b DKO cells. As expected, methylated CpGs were hardly seen in DKO cells, whereas most of the CpGs examined were methylated in the parental HCT116 cells (Figure 4*C*). Furthermore, it was clearly evident that the upstream of the *B4GALNT2* gene was frequently hypermethylated in human gastric cancer tissues (Figure 4*D*). Sample KG5T, methylation negative by COBRA, and MKN45 cells looked less methylated but included apparently hypermethylated clones. These results imply that DNA hypermethylation in the promoter region of the *B4GALNT2* gene may have contributed to the down-regulation of *B4GALNT2* expression in gastric cancers.

Methylation Status of B4GALNT2 *Gene and Clinicopathologic Characteristics in Primary Gastric Carcinomas*

To understand the significance of hypermethylation in the *B4GALNT2* gene, we analyzed the methylation status of the *B4GALNT2* gene and clinicopathologic characteristics of patients with gastric carcinomas. We deemed that the *B4GALNT2* gene was methylated when the percentage of methylated DNA was ≥10% by COBRA. Of the 78 primary gastric tumors studied, 39 were classified as methylation positive (Table 1). Univariate analysis revealed no difference between the methylation-positive and -negative groups with respect to age, gender, tumor location, macroscopic type, lymphatic invasion, venous invasion, or pT, pN, or pM status. However, there were significant differences between patients in the methylation-positive and -negative groups with respect to histology (P = .012) and EBV status (P = .001). EBV was detected in 10 of the 78 tumors, and all EBV-associated tumors were methylation-positive ones. No difference was noted in the frequency of *p53* mutation or the infection of HP between the methylation-positive and -negative groups. To examine the correlation between the

Table 1. Clinicopathologic Features of Gastric Cancer With or Without Methylation of *B4GALNT2*

Characteristics	Total	Number of patients (%)		*P*-value
		Methylated	Unmethylated	
Number of patients	78	39 (50.0)	39 (50.0)	
Mean age ± SD (*y*)		63.6 ± 13.6	65.4 ± 10.3	.531
Gender				
Male	52 (66.6)	24 (61.5)	28 (71.8)	.472
Female	26 (33.3)	15 (38.5)	11 (28.2)	
Tumor location				
Upper one third	22 (28.2)	14 (35.9)	8 (20.5)	.279
Middle one third	23 (29.5)	9 (23.1)	14 (35.9)	
Lower one third	33 (42.3)	16 (41.0)	17 (43.6)	
Macroscopic type				
0	4 (5.1)	2 (5.1)	2 (5.1)	.98
1	6 (7.7)	3 (7.7)	3 (7.7)	
2	30 (38.5)	14 (35.9)	16 (41.0)	
3	30 (38.5)	15 (38.5)	15 (38.5)	
4	8 (10.3)	5 (12.8)	3 (7.7)	
Histology (Lauren)				
Intestinal	36 (46.2)	12 (30.8)	24 (61.5)	.012
Diffuse	42 (53.8)	27 (69.2)	15 (38.5)	
Lymphatic invasion				
Negative	20 (25.6)	9 (23.1)	11 (28.2)	.78
Positive	58 (74.4)	30 (76.9)	28 (71.8)	
Venous invasion				
Negative	37 (47.4)	20 (51.3)	17 (43.6)	.651
Positive	41 (52.6)	19 (48.7)	22 (56.4)	
Pathologic tumor classification				
pT1	5 (6.4)	3 (7.7)	2 (5.1)	.407
pT2	43 (55.1)	19 (48.7)	24 (61.5)	
pT3	28 (35.9)	15 (38.5)	13 (33.3)	
pT4	2 (2.6)	2 (5.1)	0 (0.0)	
Pathologic lymph node status				
pN0	22 (28.2)	9 (23.1)	13 (33.3)	.373
pN1	28 (35.9)	15 (38.5)	13 (33.3)	
pN2	16 (20.5)	8 (20.5)	8 (20.5)	
pN3	12 (15.4)	7 (17.9)	5 (12.8)	
Pathologic metastasis status				
pM0	66 (84.6)	36 (92.3)	30 (76.9)	.114
pM1	12 (15.4)	3 (7.7)	9 (23.1)	
Stage (pTNM)				
I	18 (23.1)	8 (20.5)	10 (25.6)	.804
II	16 (20.5)	8 (20.5)	8 (20.5)	
III	21 (26.9)	12 (30.8)	9 (23.1)	
IV	23 (29.5)	11 (28.2)	12 (30.8)	
Helicobacter pylori				
Positive	65 (83.3)	33 (84.6)	32 (82.1)	.999
Negative	13 (16.7)	6 (15.4)	7 (17.9)	
Epstein–Barr virus				
Positive	10 (12.8)	10 (25.6)	0 (0.0)	.001
Negative	68 (87.2)	29 (74.4)	39 (100.0)	
p53 mutation				
Positive	19 (24.4)	6 (15.4)	13 (33.3)	.112
Negative	59 (75.6)	33 (84.6)	26 (66.7)	

SD, standard deviation; pTNM, pathologic tumor, lymph node, metastasis status according to the International Union Against Cancer classification system.

Sd^a expression and DNA hypermethylation of *B4GALNT2*, we determined the expression levels of Sd^a carbohydrates in freshly frozen gastric cancers, because Sd^a antigen is expressed as a glycolipid in the stomach; its reactivity to antibodies was lost in formalin-fixed paraffin-embedded samples that we used for our clinicopathologic analysis. Of the 15 freshly frozen gastric cancers studied, the expression of Sd^a determinants was totally lost in all cases as determined by immunohistologic staining; 7 cases were methylation positive by COBRA (data not shown).

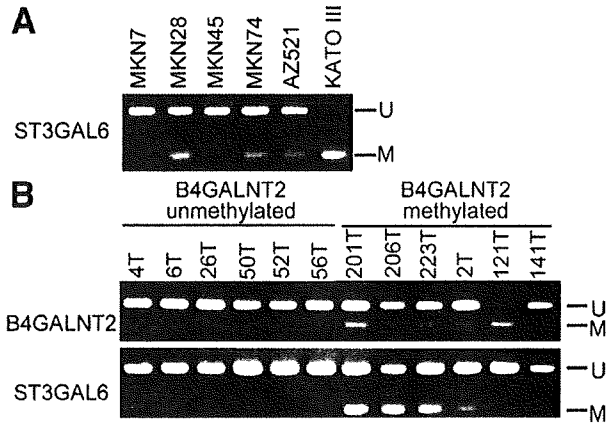

Figure 5. Methylation of *B4GALNT2* and *ST3GAL6* genes in gastric cancer cells and primary gastric carcinomas. Representative results are shown from COBRA using human gastric cancer cell lines (*A*) and primary tumors (*B*). M, methylated alleles; U, unmethylated alleles. The genes that were analyzed are shown on the left.

Methylation Status of Glyco-Genes in Gastric Cancer Cell Lines and Primary Gastric Carcinomas

Finally, we examined whether epigenetic changes occurred in the *ST3GAL6* gene together with those in the *B4GALNT2* gene in human gastric cancer cells. Of the cancer-associated down-regulated glyco-genes that we found in the present study, the *ST3GAL6* was hypermethylated in concurrence with methylation of the *B4GALNT2* in many of the gastric cancer cell lines as well as in gastric cancer tissues (Figure 4*A* and Figure 5). As shown in Table 2, aberrant methylation in the *ST3GAL6* was detected in 24 of 32 primary gastric tumors with statistically significant correlation with the methylation of *B4GALNT2* and EBV status ($P < .01$). No difference was noted in the frequency of *p53* mutation between the *ST3GAL6*-methylated and -unmethylated groups. These results strongly suggest that epigenetic changes may occur in a group of glyco-genes including *B4GALNT2* and *ST3GAL6* in gastric cancer tissues, which may eventually induce aberrant glycosylation and expression of cancer-associated carbohydrate antigens by silencing the enzyme activity responsible for antigen expression.

Discussion

Aberrant glycosylation, which would be expected to eventually induce the expression of cancer-associated carbohydrate antigens, has been observed in many types of tumors. In the aspect of carbohydrate synthesis, here we clearly demonstrated that the down-regulation of a set of glyco-genes involved in carbohydrate biosynthetic pathways is a major event in the cancer, rather than the up-regulation of certain glycosyltransferases. Cancer-specific DNA hypermethylation played a significant role in this gene silencing. We further extended the analysis to 93 gastric cancer tissues and, for the first time, found a high frequency of DNA hypermethylation in glyco-genes. The significance of these issues is discussed below.

Recent studies including ours suggest clearly that the precise mechanism for up-regulation of cancer-associated carbohydrate antigens revolves not necessarily around the enhancement of glycosylation in tumors, but rather around the down-regulation of glyco-genes that are involved in the synthesis of normally expressed determinants, such as Sd^a, disialyl $Lewis^a$, and so on.[15,32-34] In our results, the CpG islands of the *B4GALNT2* gene encoding Sd^a-β1,4GalNAcT, the enzyme responsible for the synthesis of the Sd^a structure, were densely methylated; and this methylation was closely correlated with the transcriptional silencing of the *B4GALNT2* gene. Because DNA hypermethylation of the *B4GALNT2* gene was detected in 50% of our gastric cancer cases examined by COBRA, this hypermethylation seems to be an important molecular mechanism well explaining the down-regulation of Sd^a. Our present study using freshly frozen samples reconfirmed our previous report that nearly 100% of gastric cancers showed loss of Sd^a antigen.[12] We believe that the difference in frequency between DNA hypermethylation and loss of Sd^a may be attributed to the rather dull sensitivity of COBRA. For example, MKN45 cells were methylation negative by COBRA, despite the apparent hypermethylation of certain areas in the promoter region as assessed by bisulfite sequencing (Figure 4*A*, *B*). Besides, in the case of the *B4GALNT2* gene and Sd^a antigens, the methylation status of the *A3GALNT* gene, encoding the enzyme responsible for the synthesis of the blood group A and B determinants, correlates well with the expression of the blood group A and B determinants in gastric

Table 2. Methylation Status of *B4GALNT2*, EBV Infection, and *P53* Mutation of Gastric Cancer With or Without Methylation of *ST3GAL6*

	Number of patients (%)		
		ST3GAL6	
Characteristic	Total	Methylated	Unmethylated
Number of patients	63	28 (44.4)	35 (55.6)
B4GALNT2[a]			
Methylated	32 (50.8)	24 (85.7)	8 (22.9)
Unmethylated	31 (49.2)	4 (14.3)	27 (77.1)
Epstein-Barr virus[a]			
Positive	9 (14.3)	8 (28.6)	1 (2.9)
Negative	54 (85.7)	20 (71.4)	34 (97.1)
p53 mutation			
Positive	15 (23.8)	6 (21.4)	9 (25.7)
Negative	48 (76.2)	22 (78.6)	26 (74.3)

NOTE. Methylation of *ST3GAL6* was compared by using the Fisher exact test for methylation of *B4GALNT2*, EBV association, and p53 mutation.
[a]Statistically significant ($P < .01$).

cancer cell lines including MKN28, MKN45, and KATO III cells.[30] We reported earlier that forced expression of Sd^a-β1,4GalNAcT resulted in a marked increase in cell-surface expression of Sd^a along with a concomitant loss of cancer-associated $sLe^{x/a}$ carbohydrate antigens.[15] Of note, DNA hypermethylation of *B4GALNT2* gene may be associated with the concomitant increase in $sLe^{x/a}$ in GI cancers as a typical example of "incomplete synthesis" for abnormal expression of carbohydrate determinants. In addition, we reaffirmed that DNA methylation contributes to the cancer-associated silencing of the *A3GALNT* gene in GI cancer cells. In bladder cancers and oral squamous cell carcinomas, a relationship between decreased expression of the blood group A and B determinants and allelic loss and/or hypermethylation of the *A3GALNT* gene has been reported.[28,29,35] DNA hypermethylation of this gene in GI cancers may also lead to enhanced expression of $sLe^{x/a}$ consequent to a reduction in blood group A antigen.

Further, our report provides the first description of a relationship between the methylation status of glycosyltransferase and clinicopathologic features. Throughout the present study, we observed a strong correlation between promoter methylation of the *B4GALNT2* gene and EBV-associated gastric carcinoma (Table 1). The frequency of *B4GALNT2* methylation in EBV-associated tumors was 100%. EBV is a ubiquitous herpes virus that infects most children during early childhood and is involved in a subset of gastric carcinomas, although its specific role in carcinogenesis remains unclear. It has been shown that the expression of tumor-suppressor genes, such as p16 cyclin-dependent kinase 4A inhibitor ($p16^{INK4A}$), is absent significantly more often in EBV-associated gastric carcinomas than in EBV-negative ones and that their loss is associated with their methylation.[36] Our finding is consistent with former reports indicating that carcinogenesis of EBV-associated gastric tumors commonly involves hypermethylation of multiple genes. Extensive studies on the molecular mechanism underlying oncogenic virus-related aberrant methylation have been carried out. It was reported that oncogenic virus-related aberrant methylation was caused by DNMT3b up-regulation via Ras activation.[37] It is plausible that aberrant methylation seen in EBV-associated gastric tumors may be based on the same molecular machinery. Furthermore, in the present study, approximately three fourths of the *B4GALNT2* methylation-positive carcinomas were EBV negative; therefore, increased methylation of this gene in tumors without EBV association might be mediated by some different, as yet unknown mechanism. HP infection of the stomach is also a significant factor related to carcinogenesis.[38] No correlation was observed between hypermethylation of *B4GALNT2* and HP status, although HP infection is extraordinarily common (83.3% in Table 1). These results suggest collectively that aberrant methylation of *B4GALNT2* might be induced by factors independent of those related to HP infection.

Another important finding of this study was that the hypermethylation occurred coincidentally in *B4GALNT2* and *ST3GAL6* genes, as was clearly shown in Figure 5 and Table 2. Because the human *ST3GAL6* gene encodes the α2,3-sialyltransferase responsible for the synthesis of type II precursor, the suppression of this gene seems to result in a lesser amount of the precursor for the biosynthesis of the Sd^a determinant. These observations allow us to suppose that epigenetic suppression of multiple glyco-genes, including glycosyltransferases, glycosidases, and mucins in tumors, may not occur in a random manner but in a certain set of them and other genes. We can add on yet the fact that some glyco-genes, whose expression is decreased in cancers and whose promoter regions contain CpG islands, seemed to be controlled epigenetically. Taken together, our data suggest that there might be a certain group of glyco-genes whose expression in cancers is controlled together by DNA hypermethylation. Although more studies on individual glyco-genes are required to support this hypothesis, simultaneous silencing of glycosyltransferases might eventually result in the induction of aberrant glycosylation and expression of cancer-associated carbohydrate antigens by inactivating their enzyme activity. In conclusion, we propose that an epigenetic change such as DNA hypermethylation is one of the major mechanisms causing cancer-associated changes in carbohydrate determinants by silencing normal glycosylation, especially being a part of the mechanism referred to previously as incomplete synthesis.

BASIC—ALIMENTARY TRACT

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2008.03.031.

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Supplementary Table 1. Human glyco-genes.

Genes	Accession No.	ID of TaqMan probe kits	Enzyme	References
FUT1	NM000148	Hs00355741_m1	α1,2-fucosyltransferase, H blood group α1,2-fucosyltransferase	1, 2
FUT2	NM000511	Hs00704693_s1	α1,2-fucosyltransferase, Se blood group α1,2-fucosyltransferase	3
FUT3	NM000149	Hs00356857_m1	α1,3/4-fucosyltransferase, Lewis blood group α1,3/4-fucosyltransferase	4
FUT4	NM002033	Hs00275643_s1	α1,3-fucosyltransferase	5, 6
FUT5	NM002034	Hs00704908_s1	α1,3-fucosyltransferase	7
FUT6	NM000150	Hs00173404_m1	α1,3-fucosyltransferase	8
FUT7	NM004479	Hs00237083_m1	α1,3-fucosyltransferase	9, 10
FUT8	NM178154	Hs00189535_m1	α1,6-fucosyltransferase	11
A3GALT/A3GALNT	NM020469	Hs00220850_m1	α1,3-*N*-galactosyltransferase, α1,3-*N*-acetylgalactosaminyltransferase	12, 13
GBGT1	NM021996	Hs00222752_m1	globoside α1,3-*N*-acetylgalactosaminyltransferase, Forssman synthetase	14
B3GALNT1	NM003781	Hs00364202_s1	β1,3-*N*-acetylgalactosaminyltransferase 1, globoside synthase	15
B3GALNT2	NM152490	Hs00380823_m1	β1,3-*N*-acetylgalactosaminyltransferase 2	16
B4GALNT1	NM001478	Hs00155195_m1	β1,4-*N*-acetylgalactosaminyltransferase 1, GM2/GD2 synthase	17
B4GALNT2	NM153446	Hs00396440_m1	β1,4-*N*-acetylgalactosaminyltransferase 2 Sd[a] synthase	18
B4GALNT3	NM173593	Hs00403843_m1	β1,4-*N*-acetylgalactosaminyltransferase 3	19
B4GALNT4	NM178537	Hs00331790_m1	β1,4-*N*-acetylgalactosaminyltransferase 4	20
GCNT1	NM001490	Hs00155243_m1	gulcosaminyl (*N*-acetyl) transferase 1, core 2 (β1,6-*N*-acetylglucosaminyltransferase)	21
GCNT3	NM004751	Hs00191070_m1	gulcosaminyl (*N*-acetyl) transferase 3	22
GCNT4	NM016591	Hs00275464_s1	gulcosaminyl (*N*-acetyl) transferase 4, core 2 (β1,6-*N*-acetylglucosaminyltransferase)	23
ST3GAL1	NM003033	Hs00161688_m1	CMP-NeuAc:β-galactoside α2,3-sialyltransferase 1	24
ST3GAL2	NM006927	Hs00199480_m1	CMP-NeuAc:β-galactoside α2,3-sialyltransferase 2	25
ST3GAL3	NM006279	Hs00196718_m1	CMP-NeuAc:Galβ1,3/4GlcNAc α2,3-sialyltransferase	26
ST3GAL4	NM006278	Hs00272170_m1	Galβ1,3/4GlcNAc α2,3-sialyltransferase	27
ST3GAL5	NM003896	Hs00187405_m1	CMP-NeuAc:lactosylceramide α2,3-sialyltransferase	28
ST3GAL6	NM006100	Hs00196086_m1	CMP-NeuAc:α2,3-sialyltransferase	29
ST6GAL1	NM003032	Hs00949382_m1	CMP-NeuAc:galactoside α2,6-sialyltransferase	30
ST6GAL2	NM032528	Hs00293264_m1	CMP-NeuAc:galactoside α2,6-sialyltransferase	31
ST6GALNAC1	NM018414	Hs00300842_m1	GalNAc α2,6-sialyltransferase 1	32
ST6GALNAC2	NM006456	Hs00197670_m1	GalNAc α2,6-sialyltransferase 2	33
ST6GALNAC3	NM152996	Hs00541761_m1	GalNAc α2,6-sialyltransferase 3	34
ST6GALNAC4	NM014403	Hs00205241_m1	GalNAc α2,6-sialyltransferase 4	35
ST6GALNAC5	NM030965	Hs00229612_m1	GalNAc α2,6-sialyltransferase 5	36
ST6GALNAC6	NM013443	Hs00203739_m1	GalNAc α2,6-sialyltransferase 6	37
GALNAC4S-6ST	NM015892	Hs00248144_m1	*N*-acetylgalactosamine 4-sulfate 6-*O*-sulfotransferase	38
CHST1	NM003654	Hs00186341_m1	galactose-6-*O*-sulfotransferase	39
CHST2	NM004267	Hs00358839_g1	carbohydrate *N*-acetylgulcosamine-6-*O*-sulfotransferase 2	40
CHST3	NM004273	Hs00427946_m1	chondroitin 6-sulfotransferase	41
CHST4	NM005769	Hs00428480_m1	*N*-acetylgulcosamine–6-*O*-sulfotransferase 2, HEC-specific *N*-acetylgulcosamine–6-*O*-sulfotransferase	42
CHST5	NM012126	Hs00201677_m1	*N*-acetylgulcosamine–6-*O*-sulfotransferase 3, intestinal *N*-acetylgulcosamine–6-*O*-sulfotransferase	43
NEU1	NM000434	Hs00166421_m1	sialidase 1, lysosomal sialidase	44
NEU2	NM005383	Hs00193573_m1	sialidase 2, cytosolic sialidase	45
NEU3	NM006656	Hs00198406_m1	sialidase 3, plasma membrane-associated sialidase	46
NEU4	NM080741	Hs00293852_m1	sialidase 4, glyceraldehyde-3-phosphate dehydrogenase	47
GAPDH	NM002046	Hs00266705_gl		

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Glutamine Increases Autophagy Under Basal and Stressed Conditions in Intestinal Epithelial Cells

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Background & Aims: **Glutamine plays a protective role in intestinal cells during physiologic stress; however, the protection mechanisms are not fully understood. Autophagy functions in bulk degradation of cellular components, but has been recognized recently as an important mechanism for cell survival under conditions of stress. We therefore sought to see if glutamine's actions involve the induction of autophagy in intestinal cells and, if so, the mechanisms that underlie this action.** ***Methods:*** **Formation of microtubule-associated protein light chain 3 (LC3)-phospholipid conjugates (LC3-II) in rat intestinal epithelial IEC-18 cells and human colonic epithelial Caco-2_{BBE} cells was determined by Western blotting and localized by confocal microscopy. Activation of mammalian target of rapamycin (mTOR) pathway, mitogen-activated protein (MAP) kinases, caspase-3, and poly (ADP-ribose) polymerase were monitored by Western blotting.** ***Results:*** **Glutamine increased LC3-II as well as the number of autophagosomes. Glutamine-induced LC3-II formation was paralleled by inactivation of mTOR and p38 MAP kinase pathways, and inhibition of mTOR and p38 MAP kinase allowed LC3-II induction in glutamine-deprived cells. Under glutamine starvation, LC3-II recovery after heat stress or the increase under oxidative stress was blunted significantly. Glutamine depletion increased caspase-3 and poly (ADP-ribose) polymerase activity after heat stress, which was inhibited by treatment with inhibitors of mTOR and p38 MAP kinase.** ***Conclusions:*** **Glutamine induces autophagy under basal and stressed conditions, and prevents apoptosis under heat stress through its regulation of the mTOR and p38 MAP kinase pathways. We propose that glutamine contributes to cell survival during physiologic stress by induction of autophagy.**

Glutamine is the most abundant free amino acid in the body and is a major respiratory fuel and metabolic precursor for many cell types including intestinal epithelial cells (IECs) and immune cells.[1–3] Although considered nonessential, glutamine becomes conditionally essential during severe catabolic stress such as major surgery, trauma, or sepsis in which intracellular and plasma glutamine levels decrease rapidly.[4,5] Recent studies have shown supplementation of parenteral or enteral glutamine reduces complication rates in critically ill and postoperative patients.[6–8]

The mechanisms underlying glutamine's protective and trophic actions are incompletely understood. The possibility that it might be essential for the autophagic response of intestinal epithelial cells was therefore considered by this study. Autophagy is a catabolic process that recycles cellular proteins and organelles, an evolutionarily conserved response to metabolic stress.[9–11] Autophagy is recognized as a cell survival mechanism during periods of nutrient deprivation in which the bulk degradation of cytoplasmic proteins and nonessential organelles provides an alternative energy source. The process of autophagy is characterized by the formation of double-membrane vesicles known as autophagosomes, which is mediated by the Atg12-Atg5-Atg16 complex and microtubule-associated protein light chain 3 (LC3)-phospholipid conjugates (LC3-II).[12,13] The outer membrane of the autophagosome fuses with the lysosome, and cytoplasm-derived materials are degraded in autolysosome.

Amino acids have long been known to be regulators of autophagy.[14] The signaling mechanism by which amino acids regulate autophagy appears to involve stimulation of mammalian target of rapamycin (mTOR) kinase,[15–17] although recent studies showed that they also may use mTOR-independent pathways.[18,19] Only certain amino acids are capable of modulating autophagy and their actions are highly cell-specific.[20]

In the present study, we show that glutamine induces autophagy in rat intestinal epithelial IEC-18 cells and human colonic epithelial Caco-2_{BBE} cells, a process that is mediated by inhibition of the mTOR and p38 mitogen-activated protein (MAP) kinase pathways. In IEC-18 cells, glutamine also maintains autophagy under heat- and oxidative-stressed conditions and prevents apoptosis under heat-stressed conditions. Our data suggest that glutamine contributes to cell survival during

Abbreviations used in this paper: BBE, brush border expression; DMEM, Dulbecco's modified Eagle medium; Hsp, heat shock protein; IEC, intestinal epithelial cell; LC3, microtubule-associated protein light chain 3; MAP, mitogen-activated protein; mTOR, mammalian target of rapamycin; PARP, poly (ADP-ribose) polymerase; SAPK, stress-activated protein kinase; siRNA, short interfering RNA; S6K, p70 S6 kinase; TBS, Tris-buffered saline; TBST, Tris-buffered saline with Tween.

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physiologic stress by induction of autophagy through its regulation of the mTOR and p38 MAP kinase pathways.

Materials and Methods

Chemicals

Chemicals were obtained from Fisher Scientific (Hanover Park, IL) unless otherwise stated. Media and all cell culture supplements were obtained from Invitrogen (Grand Island, NY), L-leucine and 3-methyladenine were obtained from Sigma-Aldrich (St. Louis, MO), and rapamycin and SB203580 were obtained from Axxora (San Diego, CA).

Cell Culture

The diploid nontransformed rat small intestinal epithelial IEC-18 cell line (ATCC, Manassas, VA; CRL-1589) was used between passages 20 and 35. IEC-18 cells were grown in high-glucose (4.5 g/L) Dulbecco's modified Eagle medium (DMEM) containing 2 mmol/L L-glutamine, 5% vol/vol fetal bovine serum, 50 U/mL penicillin, 50 μg/mL streptomycin, and 0.1 U/mL insulin, and cultured to 90% confluence. The human colon carcinoma cell line Caco-2_{BBE} (brush border expressor) was used between passages 50 and 75. Caco-2_{BBE} cells were seeded onto cell culture inserts at a density of 10^5 cells/cm^2, grown in high-glucose DMEM containing 2 mmol/L L-glutamine, 10% vol/vol fetal bovine serum, 50 U/mL penicillin, 50 μg/mL streptomycin, and 10 μg/mL transferrin, and allowed to differentiate for 14 days. Both cell lines then were incubated for 24 hours in reduced-serum (1% vol/vol fetal bovine serum) DMEM containing indicated concentrations of glutamine with all other supplements. Cell cultures were maintained in a humidified 5% CO_2 incubator at 37°C. Heat shock was achieved by sealing dishes and immersing them in a 42°C water bath for 15 or 30 minutes. For confocal microscopy, cells were grown in complete growth medium on glass coverslips. IEC-18 cells were cultured to 90% confluence, and Caco-2_{BBE} cells were cultured for 14 days. Cells then were incubated for 24 hours in reduced-serum DMEM containing either 0 or 0.7 mmol/L glutamine with all other supplements.

RNA Silencing of mTOR and p38 MAP Kinase

To silence mTOR or p38 MAP kinase, predesigned short interfering RNA (siRNA) specific for rat mTOR (NM_019906, bases 710-728, ID number: 132719) or p38 MAP kinase (NM_031020, bases 666-684, ID number: 135448) were purchased from Ambion (Austin, TX). As a negative control, AllStars Negative Control siRNA (Qiagen, Valencia, CA) was used. siRNA (final concentration, 5 nmol/L) was mixed with siLentFect lipid reagent (Bio-Rad, Hercules, CA) in Opti-MEM (Invitrogen) and allowed to form complexes for 30 minutes at room temperature. Complexes were added to 70% confluent IEC-18 cells, in which 15 minutes earlier the complete medium had been replaced with Opti-MEM. The cells were incubated at 37°C for 30 minutes and then complete medium with 10% vol/vol fetal bovine serum was added. Twenty-four hours after the transfection, cells were incubated for 24 hours in reduced-serum DMEM without glutamine.

Western Blot Analysis

Cells were scraped and disrupted in lysis buffer (composition: 50 mmol/L Tris pH 7.4, 150 mmol/L NaCl, 1% vol/vol NP-40, 1 mmol/L Na_3VO_4, 1 mmol/L NaF, and the complete protease inhibitor cocktail [Roche Molecular Biosciences, Indianapolis, IN]). An aliquot was removed and protein concentrations were measured using bicinchoninic acid. Laemmli sample buffer was added to the remainder (composition: 250 mmol/L Tris pH 7.4, 2% wt/vol sodium dodecyl sulfate, 25% vol/vol glycerol, 10% vol/vol 2-mercaptoethanol, and 0.01% wt/vol bromophenol blue) and samples were heated to 70°C for 10 minutes and stored at −80°C until analysis. Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred immediately onto polyvinylidene difluoride membranes (Polyscreen; Perkin-Elmer, Boston, MA) using 1× Towbin buffer (25 mmol/L Tris pH 8.8, 192 mmol/L glycine, 15% vol/vol methanol). Membranes subsequently were incubated in 3% wt/vol bovine serum albumin in Tris-buffered saline (TBS with Tween [TBST]; composition: 140 mmol/L NaCl, 5 mmol/L KCl, 10 mmol/L Tris pH 7.4, with 0.1% vol/vol Tween 20) at room temperature for 1 hour. Antibodies were added and incubated overnight at 4°C in TBST. Polyclonal rabbit antibodies against LC3 (2775), phospho-mTOR (2971), mTOR (2972), phospho-p70 S6 kinase (9205), phospho-p38 MAP kinase (9211), p38 MAP kinase (9212), phospho-p44/42 MAP kinase (extracellular signal-related kinase) (9101), phospho-SAPK (stress-activated protein kinase)/c-Jun amino-terminal kinase (9251), caspase-3 (9662) and poly (ADP-ribose) polymerase (PARP) (9542), and monoclonal mouse antibody against phospho-Akt (4051) were purchased from Cell Signaling (Danvers, MA). Polyclonal rabbit antibody against heat shock protein (Hsp)25 (SPA-801) and monoclonal mouse antibody against Hsp70 (SPA-810) were purchased from Stressgen/Assay Designs (Ann Arbor, MI). Monoclonal mouse antibody against β-actin (AAN01) was purchased from Cytoskeleton (Denver, CO). Membranes were washed 3 times in TBST and subsequently incubated with species-appropriate, peroxidase-conjugated secondary antibodies (1 h; Jackson Immunoresearch, West Grove, PA). Blots were washed 3 times with TBST and once with TBS without Tween and developed using an enhanced chemiluminescence system (Supersignal; Pierce Chemical, Rockford, IL).

Confocal Microscopy of Autophagosomes

After treatment with 0 or 0.7 mmol/L glutamine in reduced-serum DMEM for 24 hours, cells were washed in phosphate-buffered saline (PBS) and then fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield,

PA) for 15 minutes at room temperature. Fixed cells were washed with PBS, permeabilized in 100% methanol for 10 minutes at −20°C, washed in PBS, and blocked in blocking buffer (X909; DAKO, Carpinteria, CA) for 1 hour at room temperature. Cells subsequently were incubated with anti-LC3 antibody in antibody diluent (S3022; DAKO) for overnight at 4°C. After 3 TBST washes, cells were incubated with Cy2-anti-rabbit antibody (Jackson Immunoresearch) in antibody diluent for 2 hours at room temperature and then washed 3 times in TBST. Coverslips were mounted on slides using SlowFade Gold antifade reagent with 4′,6-diamidino-2-phenylindole (Invitrogen). Cells were observed with a Leica TCS SP2 Laser Scanning Confocal Microscope, photographed at a magnification of 400×, and analyzed using Leica Confocal Software (Leica Microsystems Inc, Bannockburn, IL).

Data Analysis

All experiments were repeated at least 3 times with cells of different passage numbers. Densitometry of autoradiography images was performed using ImageJ (Research Services Branch, National Institute of Mental Health, Bethesda, MD) and normalized to the signal intensity of β-actin for equal protein loading control for each sample in each experiment. This quantitation was performed within the linear range of the standard curve defined by the standard sample, β-actin, for all densitometry analysis. For confocal microscopy experiments, the number of LC3 punctate dots and nuclei were counted using ImageJ software. Statistical analysis (analysis of variance [ANOVA]) was performed by using a Bonferroni correction with StatView 5.0 (SAS Institute, Cary, NC). Data were expressed as means ± SE.

Results

Glutamine Increases Autophagy in IEC-18 and Caco-2$_{BBE}$ Cells

Autophagosome formation is a complex, multistage process that involves many proteins.[9–11] The process can be assessed most readily by following the phospholipid conjugation of the protein LC3-I (cytosolic form) to LC3-II (autophagosomal membrane-bound form), which has an increased gel mobility on sodium dodecyl sulfate–polyacrylamide gel electrophoresis.[12,13] Autophagosome formation also can be visualized because LC3-II accumulates in this organelle. To determine if glutamine is required for autophagosome formation, IEC-18 and Caco-2$_{BBE}$ cells were incubated with varying concentrations of glutamine for 24 hours. The concentration range of glutamine ranged from the physiologic concentration normally found in plasma (0.7 mmol/L) to concentrations that were pharmacologic (2.8 mmol/L). In the absence of glutamine, a small degree of LC3-II expression could be detected by Western blotting (Figure 1*A* and *B*), which additionally was reflected by a small number of autophagosomes observed by confocal microscopy (Figure 1*C* and *D*). However, with increasing glutamine concentration, a significant increase in both the phospholipid-conjugated LC3-II as well as the number of autophagosomes is observed (Figure 1*A–D*, autophagosome number quantified in Figure 1*E* and *F*).

Glutamine is essential for the metabolic and nutritional status of the intestinal epithelial cell,[2] but other amino acids also play a role in this regard. For instance, leucine is believed to be a potent inhibitor of autophagy.[15–17] Therefore, we examined the effects of leucine on glutamine-stimulated autophagy (supplementary Figure 1; see supplementary material online at www.gastrojournal.org). Because the concentration of leucine in DMEM is 0.8 mmol/L, this concentration was defined as *basal* in assessing LC3-II expression. IEC-18 and Caco-2$_{BBE}$ cells were treated for 24 hours with varying concentrations of leucine (0.8–6.4 mmol/L) with or without 0.7 mmol/L glutamine. Doubling the leucine concentration to 1.6 mmol/L reduced glutamine's induction of LC3-II by approximately 20% in both cells and increasing leucine further caused an even greater inhibition of glutamine LC3-II induction (supplementary Figure 1; see supplementary material online at www.gastrojournal.org).

Leucine has been shown to alter the activity of a number of proteins in the cell including mTOR.[20] mTOR is a kinase that phosphorylates a number of targets, including p70 S6 kinase (S6K). Because Akt (protein kinase B) is a serine/threonine kinase that regulates the activity of mTOR, we used phospho-specific antibodies to examine states of activation of Akt, mTOR, and S6K in conditions of glutamine starvation. Within 6 hours of glutamine depletion, LC3-II decreased, paralleled by increases in phosphorylated Akt, mTOR, and S6K in IEC-18 and Caco-2$_{BBE}$ cells (Figure 2*A* and *B*).

The identical samples also were analyzed for activation of certain MAP kinases because they also could be involved in the regulation of the autophagic response. In the presence of glutamine, p38 MAP kinase showed a low level of phosphorylation that increased within 6 hours of glutamine deprivation (Figure 2*A* and *B*), suggesting a role of glutamine to regulate this kinase. Minimal to no changes were observed in phosphorylation of extracellular signal-related kinase or c-Jun amino-terminal kinase upon glutamine deprivation (data not shown).

To confirm the role of mTOR and p38 MAP kinase in glutamine induction of LC3-II, pharmacologic inhibitors of mTOR (rapamycin) and p38 MAP kinase (SB203580), or siRNAs for mTOR and p38 MAP kinase, were used in IEC-18 cells. As previously shown, glutamine depletion decreased the level of LC3-II, which was paralleled by an activation of mTOR and p38 MAP kinase (Figure 3). Treatment of the cells with rapamycin (100 nmol/L for 24 hours) and SB203580 (5 μmol/L for 24 hours) leads to the induction of LC3-II, even under conditions of glutamine depletion. Silencing mTOR and p38 MAP kinase also restored LC3-II to near-normal levels in glutamine-deprived cells.

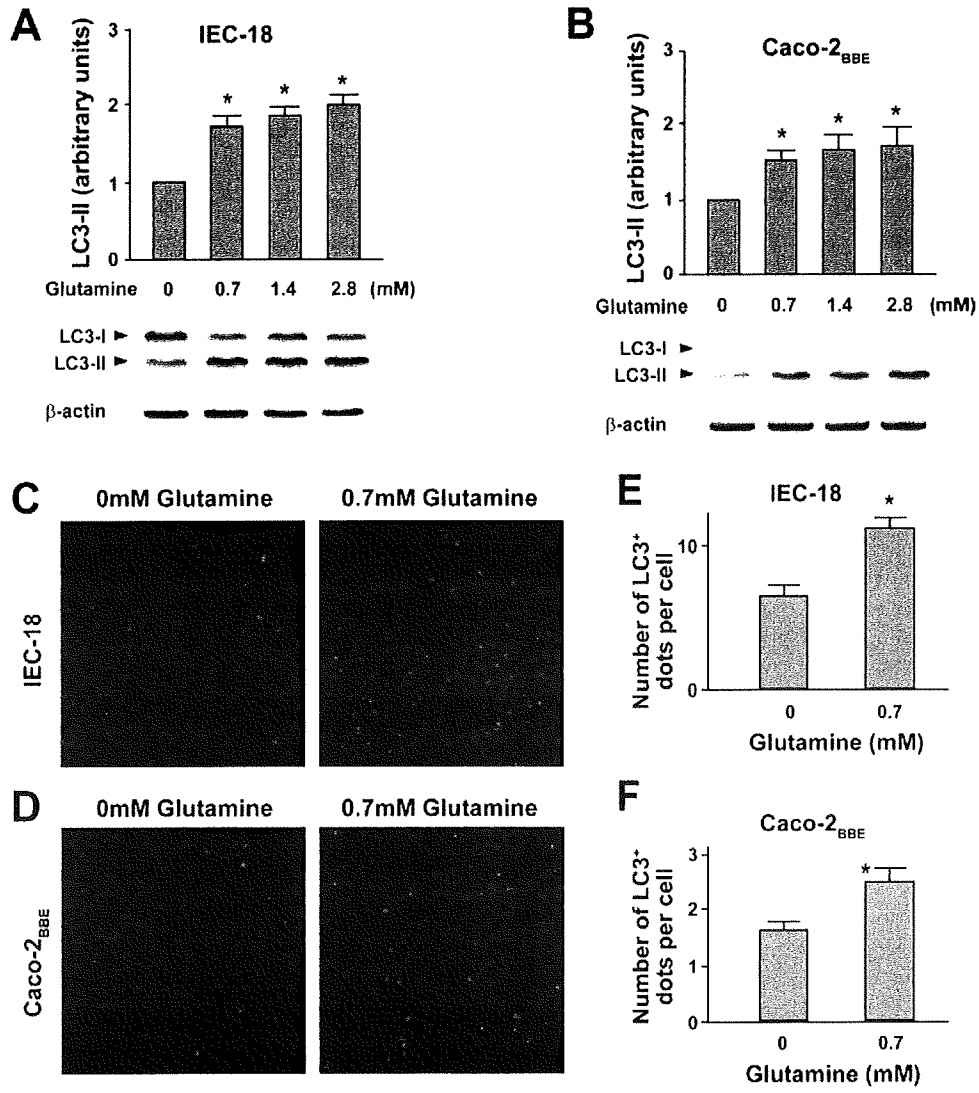

Figure 1. Glutamine enhances autophagy in IEC-18 and Caco-2_{BBE} cells. (*A*) IEC-18 cells were incubated with varying concentrations of glutamine for 24 hours and analyzed by Western blotting. The ratio of LC3-II to β-actin density was calculated using ImageJ software and set to 1 for the glutamine-deprived condition. Images are representative of 4 separate experiments. (*B*) Caco-2_{BBE} cells were incubated with varying concentrations of glutamine for 24 hours and analyzed by Western blotting. The ratio of LC3-II to β-actin density was calculated using ImageJ software and set to 1 for glutamine-deprived conditions. Images are representative of 4 separate experiments. (*C*) Autophagosome formation in IEC-18 cells was observed using confocal microscopy in the absence and presence of glutamine. IEC-18 cells were incubated with 0 or 0.7 mmol/L glutamine for 24 hours, fixed, and stained for LC3 (*green*), and nuclei were stained using 4′,6-diamidino-2-phenylindole (*blue*). Images are representative of 3 separate experiments. (*D*) Autophagosome formation in Caco-2_{BBE} cells was observed using confocal microscopy in the absence and presence of glutamine. Caco-2_{BBE} cells were incubated with 0 or 0.7 mmol/L glutamine for 24 hours, fixed, and stained for LC3 (*green*), and nuclei were stained using 4′,6-diamidino-2-phenylindole (*blue*). Images are representative of 3 separate experiments. Autophagosome formation, visualized by accumulation of LC3, was quantified and counted using ImageJ software in at least 300 cells in each experiment. Shown are the (*E*) number of autophagosomes per cell of IEC-18 cells, and the (*F*) number of Caco-2_{BBE} cells. Data are shown as means ± SE. $^*P < .05$ compared with 0 mmol/L glutamine by ANOVA using a Bonferroni correction.

Therefore, both the mTOR and p38 MAP kinase pathways appear to be involved in the regulation of autophagy by glutamine.

Glutamine Maintains Autophagy and Inhibits Apoptosis Under Stressed Conditions

Autophagy may play a role in cellular maintenance and protection, particularly under conditions of stress.[21] We therefore examined glutamine-dependent autophagic responses in cells subjected to thermal (heat) stress, a physiologic equivalent of fever. To determine if heat stress promotes an autophagic response, IEC-18 cells (grown in 0.7 mmol/L glutamine) were subjected to 42°C for up to 30 minutes. As shown in Figure 4*A*, heat stress caused a significant decrease in LC3-II, but, after heat stress and during

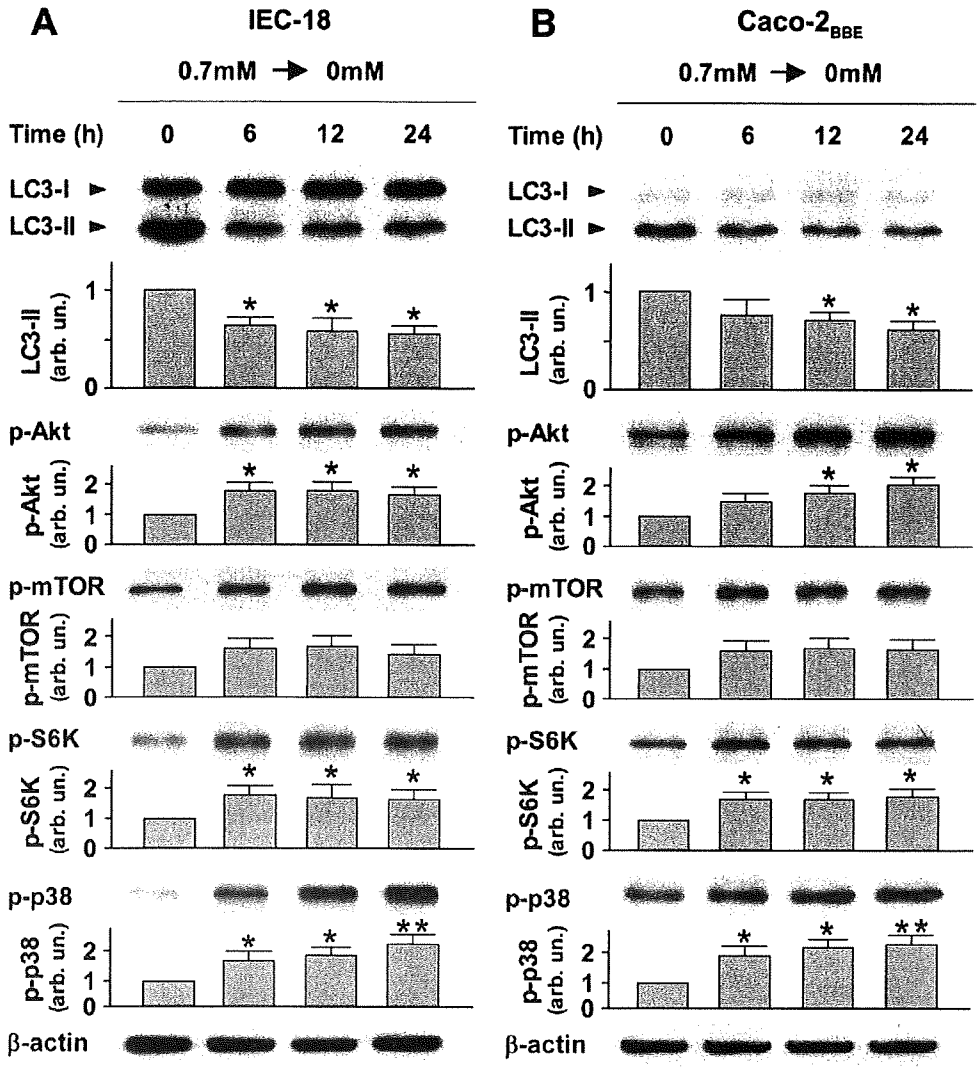

Figure 2. Glutamine inhibits mTOR and p38 MAP kinase activity in IEC-18 and Caco-2_{BBE} cells. (*A*) IEC-18 cells were incubated with 0.7 mmol/L glutamine for 24 hours. Cells subsequently were deprived of glutamine for varying times up to 24 hours. Samples were collected at designated times and analyzed by Western blotting. Images are representative of 3 separate experiments. (*B*) Caco-2_{BBE} cells were incubated with 0.7 mmol/L glutamine for 24 hours. Cells subsequently were deprived of glutamine for varying times up to 24 hours. Samples were collected at designated times and analyzed by Western blotting. Images are representative of 3 separate experiments. Densitometric values were obtained using ImageJ software and normalized to β-actin, set to 1 for conditions immediately after glutamine deprivation (0 hour). Data are shown as means ± SE. $^*P < .05$ and $^{**}P < .01$ compared with cells at time point 0 hour by ANOVA using a Bonferroni correction.

the recovery phase (37°C), this response attenuated and LC3-II abundance returned back to normal levels. Heat stress also stimulated phosphorylation of Akt and mTOR, the latter to a lesser extent. Both changes returned to a near-basal state after the heat stress removal during the recovery phase. The activity of p38 MAP kinase and expression of β-actin were not affected by heat stress. The induction of Hsp70 and Hsp25, 2 known heat-inducible stress proteins, could be seen clearly during the recovery phase. To determine if mTOR is responsible for the regulation of autophagy by heat stress, cells were pretreated with rapamycin (100 nmol/L for 24 hours). Rapamycin-treated cells did not show the LC3-II decrease during heat or the LC3-II increase upon return to 37°C (Figure 4*B*).

To determine whether glutamine plays a role in the autophagy response to heat stress, cells were incubated for 24 hours with 0.7 or 0 mmol/L glutamine. Under conditions of glutamine depletion, the autophagy response to heat stress was minimal and the LC3-II increase after heat stress was small, despite inactivation of mTOR (Figure 5*A*). In contrast, the robust activation of p38 MAP kinase in glutamine-deprived cells led us to postulate that p38 MAP kinase activation might be involved in inhibition of the normal autophagy response to heat stress. To test this hypothesis, glutamine-deprived cells were treated with the p38 MAP kinase inhibitor SB203580 (5 μmol/L for 24 hours). Treatment with SB203580 restored the LC3-II levels under basal as well as heat-stressed conditions (Figure 5*B*). Moreover, the changes of LC3-II levels during heat and after heat were similar to those in glutamine-treated cells (compare 0.7 mmol/L glutamine in Figure 5*A* with 0 mmol/L + SB203580 in Figure 5*B*). These results suggest that glutamine regulates autophagy by suppression of p38 MAP kinase activity under heat-stressed conditions.

We also examined the glutamine-dependent autophagic responses to oxidative stress (supplementary Figure 2; see supplementary material online at www.gastrojournal.org). When glutamine was present, 500 μmol/L H_2O_2 caused a significant increase in LC3-II, paralleled by a decrease in

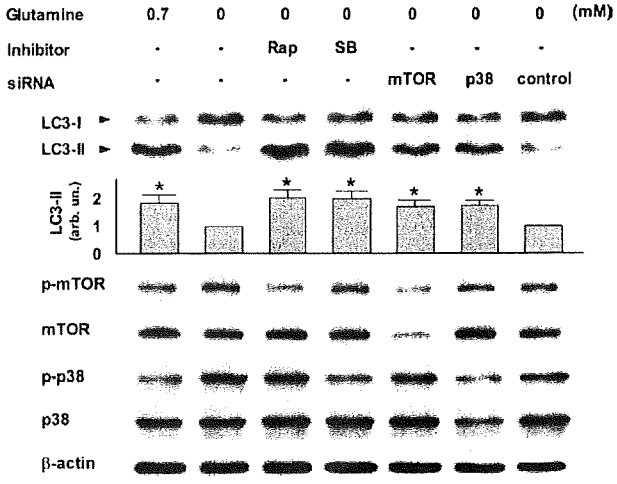

Figure 3. mTOR and p38 MAP kinase pathways are involved in glutamine's regulation of autophagy in IEC-18 cells. IEC-18 cells were incubated in 0.7 mmol/L glutamine-supplemented medium for 24 hours, or glutamine-deprived media without or with mTOR inhibitor rapamycin (Rap; 100 nmol/L) and p38 MAP kinase inhibitor SB203580 (SB; 5 μmol/L) for 24 hours. For silencing of mTOR and p38 MAP kinase with siRNA, silencing oligonucleotides or nonsilencing siRNA (control) were introduced into 70% confluent IEC-18 cells. Twenty-four hours after the transfection, cells were incubated for 24 hours in glutamine-deprived media. Total cell lysates were analyzed by Western blotting. Images are representative of 3 separate experiments. The ratio of LC3-II to β-actin density was calculated using ImageJ software, and set to 1 for no glutamine, inhibitors, and siRNA. Data are shown as means ± SE. *$P < .05$ compared with 0 mmol/L glutamine, no inhibitors, and siRNA by ANOVA using a Bonferroni correction.

phosphorylated mTOR. However, treatment with H_2O_2 stimulated the phosphorylation of p38 MAP kinase. Under glutamine deprivation, H_2O_2-induced LC3-II increase was minimal, as in the case of heat stress. In the absence of glutamine and despite decreased phosphorylation of mTOR after the addition of H_2O_2, p38 MAP kinase showed a strong activation (supplementary Figure 2*A*; see supplementary material online at www.gastrojournal.org). The restoration of the LC3-II level by the treatment with SB203580 (5 μmol/L for 24 hours) in glutamine-deprived cells suggests that glutamine also increases autophagy under oxidative stressed conditions through its inhibitory effect on p38 MAP kinase (supplementary Figure 2*B*; see supplementary material online at www.gastrojournal.org).

Heat stress can potentially stimulate an apoptotic response, but could depend on the metabolic state of the cell. In support of this, in the absence of glutamine, heat stress increased the production of cleaved caspase-3, a pivotal aspargine protease in the apoptotic response, and cleaved PARP, one of the main cleavage targets of caspase-3 (Figure 6, *middle lane*). Glutamine supplementation decreased cleaved caspase-3 and PARP (0.7 mmol/L; Figure 6, *far left lane*), indicating the presence of glutamine can direct cell fate and prevent apoptosis during heat stress recovery. Also notable is that when autophagy was blocked with 3-methyladenine (10 mmol/L for 24 hours) in glutamine-supplemented condition, cleaved caspase-3 and PARP increased, suggesting that in the absence of the autophagic response the cell defaults to apoptosis under conditions of heat stress. To

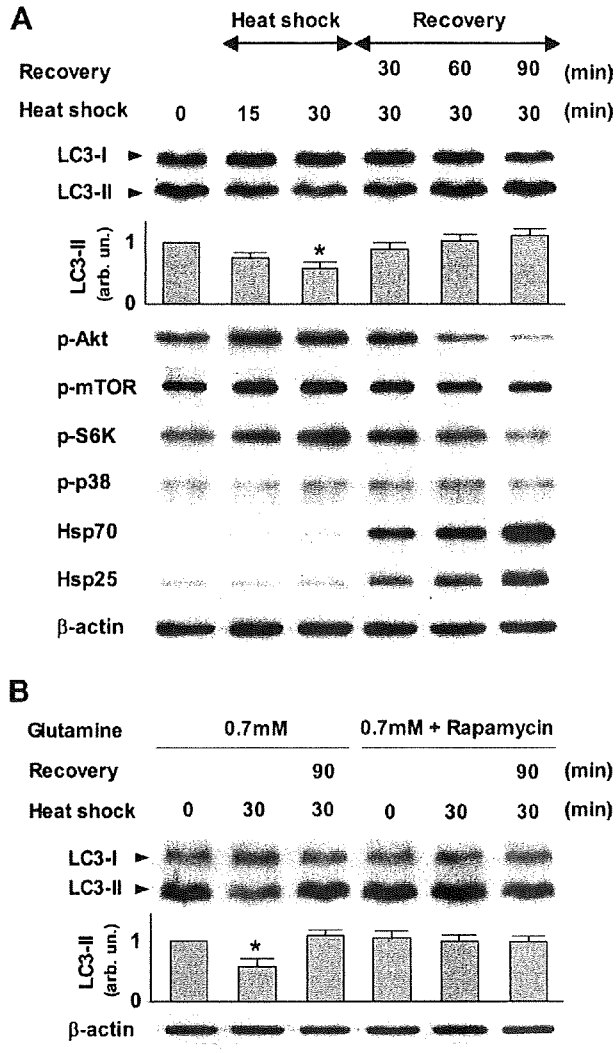

Figure 4. Heat stress regulates autophagy via the mTOR pathway. (*A*) IEC-18 cells were incubated with 0.7 mmol/L glutamine for 24 hours followed by heat shock at 42°C for 15 or 30 minutes, collected at designated times, and analyzed by Western blotting. The ratio of LC3-II to β-actin density was calculated using ImageJ software, set to 1 for the sample before heat shock. Images are representative of 3 separate experiments. Data are shown as means ± SE. *$P < .05$ compared with unstimulated conditions by ANOVA using a Bonferroni correction. (*B*) Inhibition of the mTOR pathway blocks the autophagy response to heat stress. IEC-18 cells were incubated in 0.7 mmol/L glutamine-supplemented media without rapamycin (*3 left lanes*) or with 100 nmol/L rapamycin (*3 right lanes*) for 24 hours followed by heat shock at 42°C for 30 minutes. Cells were collected before, immediately after, and 90 minutes after heat shock, and analyzed by Western blotting. The ratio of LC3-II to β-actin density was calculated using ImageJ software, set to 1 for unstimulated conditions without rapamycin. Images are representative of 3 separate experiments. Data are shown as means ± SE. *$P < .05$ compared with unstimulated conditions and no rapamycin by ANOVA using a Bonferroni correction.