Glutamine Stimulates Argininosuccinate Synthetase Gene Expression through Cytosolic O-Glycosylation of Sp1 in Caco-2 Cells*

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Glutamine stimulates the expression of the argininosuccinate synthetase (ASS) gene at both the level of enzyme activity and mRNA in Caco-2 cells. Searching to identify the pathway involved, we observed that (i) the stimulating effect of glutamine was totally mimicked by glucosamine addition, and (ii) its effect but not that of glucosamine was totally blocked by 6-diazo-5-oxo-l-norleucine (DON), an inhibitor of amidotransferases, suggesting that the metabolism of glutamine to glucosamine 6-phosphate was required. Moreover, run-on assays revealed that glucosamine was acting at a transcriptional level. Because three functional GC boxes were identified on the ASS gene promoter (Anderson, G. M., and Freytag, S. O. (1991) Mol. Cell Biol. 11, 1935–1943), the potential involvement of Sp1 family members was studied. Electrophoretic mobility shift assays using either the Sp1 consensus sequence or an appropriate fragment of the ASS promoter sequence as a probe demonstrated that both glutamine and glucosamine increased Sp1 DNA binding. Immunoprecipitation-Western blot experiments demonstrated that both compounds increased O-glycosylation of Sp1 leading to its translocation into nucleus. Again, the effect of glutamine on Sp1 was inhibited by the addition of DON but not of glucosamine. Taken together, the results clearly demonstrate that the metabolism of glutamine through the hexosamine pathway leads to the cytosolic O-glycosylation of Sp1, which, in turn, translocates into nucleus and stimulates the ASS gene transcription. Collectively, the results constitute the first demonstration of a functional relationship between a regulating signal (glutamine), a transcription factor (Sp1), and the transcription of the ASS gene.

It is now well established that amino acids may regulate the expression of mammalian genes when added or suppressed in the medium of cultured cells (for review, see Refs. 1 and 2). Among amino acids, glutamine, which is the most abundant circulating amino acid, stimulates or inhibits the expression of various genes. This includes (i) intracellular proteins such as transcription factors (3, 4), constitutive proteins of the cytoskeleton (5, 6) and of the extracellular matrix (7), heat shock proteins (8) or metabolic enzymes (9, 10); (ii) membrane proteins such as the transport system (11); and (iii) secreted proteins such as acute phase proteins (12), cytokines (13, 14), and growth factor (15). However, the molecular mechanism(s) involved in the glutamine effect is not totally understood and may depend on the cell type studied. For example, glutamine metabolism was involved in the stimulation of phosphoenolpyruvate carboxykinase gene expression in isolated rat hepatocytes (16) and c-jun gene expression in enterocytes (3), but amino acid-induced cell swelling was also identified as the inducing pathway on gene expression in hepatocytes (5, 6).

Moreover, studying the regulation of the expression of argininosuccinate synthetase (ASS) (EC 6.3.4.5.), the key enzyme of arginine synthesis, we observed that the mechanism involved might depend on the cell type studied. Indeed, we previously reported that the glutamine-induced cell swelling was involved in hepatocytes because hypo-osmolarity totally mimicked the glutamine stimulating effect on the mRNA level (10); but this was not the case in Caco-2 cells, a human intestinal cell line (17). The present work was therefore begun to specify the molecular mechanism involved in the stimulating effect of glutamine on ASS gene expression in Caco-2 cells.

The results obtained clearly demonstrate that the metabolism of glutamine through the hexosamine pathway is required and leads to the cytosolic O-glycosylation of Sp1, which, in turn, stimulates the transcription of the ASS gene. To our knowledge, this is the first report for the ASS gene of a functional relationship among a regulating signal (glutamine), a transcription factor (Sp1), and the transcription rate of the gene.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium, non-essential amino acids, and fetal calf serum were purchased from Eurobio (France); L-glutamine, glucosamine, mannosamine, 6-diazo-5-oxo-l-norleucine (DON), antibiotics, protease inhibitor mixture (P8340), and phosphatase inhibitor mixture 1 (P2850) were obtained from Sigma Chemical Co. Hybond-N Nylon membranes, radiolabeled elements [α-32P]dCTP (3000 Ci/mmol), [γ-32P]ATP (3000 Ci/mmol), and [α-32P]UTP (3000 Ci/mmol), the Megaprime kit, ECL Western blotting detection system, poly(dI-C), and Hyperfilm were provided by Amersham Biosciences. Human ASS cDNA (pAS419) was kindly provided by Dr. A. L. Beaudet, Houston, TX (18). Horseradish peroxidase and Sp1, Sp2, and Sp3 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and O-linked N-acetylglucosamine (O-GlcNac; RL2) from Affinity BioReagents. The Sp1 consensus oligonucleotide probe (5′-ATTGATCGGCGCCGGGCAG-3′) and polynucleotide kinase T4 were

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1 The abbreviations used are: ASS, argininosuccinate synthetase; C/EBP, CCAAT/enhancer-binding protein; DON, 6-diazo-5-oxo-L-norleucine; EMSA, electrophoretic mobility shift assay; GFAT, L-glutamate:O-fructose-6-phosphate amidotransferase.
purified from Promega. Purification columns and polyvinylidene difluoride membranes were obtained from Bio-Rad Laboratories.

Cell Culture—Caco-2/TC7 cells, a human colon adenocarcinoma cell line, were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were grown at 37°C in a 5% CO2, 95% air incubator. Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 1% non-essential amino acids, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin was used. Cells were grown for 2 days after confluence, and 24 h before the experiments, the medium was changed to serum-free Dulbecco's modified Eagle's medium. They were then cultured in the absence or presence of glutamine or hexosamine as indicated.

Preparation of the nuclei and RNA—ASS activity was measured at 37°C according to McLean et al. (19), and the results were expressed as units (μmol/h/mg protein with bovine serum albumin as standard.

Nuclear Run-on Transcription Assays—Preparation of the nuclei and RNA polymerase elongation reaction was performed as described (22). For transcription, 107 nuclei were incubated for 30 min at 28°C in 20 μl of reaction mixture containing 15% glycerol, 50 mM Hepes, pH 8.0, 150 mM KCl, 1 mM dithiothreitol, 2.5 mM magnesium acetate, 1 mM MnCl2, 0.5 mM EDTA, 4 mM creatine phosphate, 15 units/ml creatine kinase, 0.5 mM each ATP, CTP, and GTP, and 500 units/ml RNasin in the presence of 100 μCi of [α-32P]UTP. Then, 40 units of DNase I were added, and the samples were incubated at 37°C for 15 min before RNA extraction. Denatured plasmids (5 μg) were spotted onto nylon membrane, and each DNA-bearing filter was prehybridized and then hybridized with 5 x 106 cpm of labeled RNA at 42°C for 3 days. Hybridization was carried out with three plasmids including pAS419, pBR322 as control for nonspecific binding, and an 18 S rRNA probe as internal control. Filters were then washed and exposed to Hyperfilm at -80°C with intensifying screens. The relative amount of labeled nuclear transcripts hybridized to plasmids was determined by densitometric scanning of autoradiograms.

Preparation of Nuclear Fractions—Caco-2 cells were rinsed and scraped from the culture dishes in cold phosphate-buffered saline and pelleted. The pellet was lysed in buffer containing 10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.1 mM EDTA, 1 mM dithiothreitol, 0.25% Nonidet-P40, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor mixture (P8340), and phosphatase inhibitor mixture 1 (P2850). After a 5-min incubation on ice, the nuclear pellet was isolated by centrifugation (2 min; 12,000 g). The supernatant was saved as the cytosolic extract. The nuclear pellet was then suspended in ice in nuclear extract buffer containing 20 mM Hepes, pH 7.9, 420 mM NaCl, 1.5 mM MgCl2, 0.1 mM EDTA, 1 mM dithiothreitol, 25% glycerol, 1 mM phenylmethylsulfonyl fluoride, and P8340 and P2850 and incubated for 15 min. Samples were then centrifuged at 16,000 x g for 20 min, and the supernatant was saved as the nuclear extract. Protein concentrations were determined using Bradford assay (23).

Electrophoretic Mobility Shift Assay (EMSA)—Single-stranded complementary oligonucleotides of ASS promoter (38 pb) were prepared and annealed by custom oligonucleotide synthesis (Eurogentec). The probe corresponded to nucleotides -121 to -84, a sequence containing two functional GC boxes (24). The double-stranded oligonucleotide (200 ng) and Sp1 consensus oligonucleotide probe (3.75 pmol) were labeled with [γ-32P]ATP using polynucleotide kinase T4. Labeled probes were purified from unincorporated [γ-32P]ATP using a purification column and recovered in Tris-EDTA buffer, pH 7.8. An aliquot of nuclear proteins (15 μg) was prepared in buffer containing 5% glycerol (v/v), 12.5 mM Hepes, pH 7.9, 0.05 mM EDTA, 50 mM KCl, 0.5 mM MgCl2, 0.05% Nonidet P-40, and 1 μg of poly(dI-C)). Labeled probe (50,000–
Glutamine Stimulated ASS Gene Expression through the Hexosamine Pathway in Caco-2 Cells—As shown on Fig 1A, culturing the cells with 2 mM glutamine for 24 h induced a 1.6-fold increase in ASS activity, and the stimulatory effect was significantly higher at 10 mM than at 2 mM glutamine. Such a 1.6-fold increase in ASS activity, and the stimulatory effect was with a similar increase in the ASS mRNA level (Fig. 1).

The half-maximum stimulatory effect was obtained at about 1.5 mM C. Subsequently, nuclear or cytosolic proteins (500 μg) from Caco-2 cells were added in binding buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 10 mM EDTA, 100 mM NaF, 2 mM orthovanadate) for 2 h at 4°C. The mixture was pelleted, and the pellet was washed twice in the binding buffer and twice in buffer containing 50 mM Hepes, pH 7.5, 150 mM NaCl, and 1% Triton X-100. Immunoprecipitated Sp1 proteins were suspended in Western sample buffer (100 mM Hepes, pH 6.8, 10% β-mercaptoethanol, 20% SDS), boiled, subjected to electrophoresis on 7.5% SDS-PAGE, and then electroblotted onto polyvinylidene difluoride membranes. After blocking for 1 h with Tris-buffered saline containing 0.1% Tween 20 and 5% nonfat dry milk, the membranes were incubated with O-linked GlcNAc antibody (RL2; dilution 1:1,000) or Sp1 antibody (1:750) in blocking buffer overnight at 4°C. Then they were washed and incubated with a secondary antibody conjugated to horseradish peroxidase at room temperature for 1 h. Peroxidase labeling was detected using the ECL Western blotting detection system.

**RESULTS**

Glutamine Stimulated ASS Gene Expression through the Hexosamine Pathway—Glutamine and Sp1 Glycosylation

Among amidotransferases, DON is known to inhibit L-glutamine amidotransferase (EC 2.5.1.16) (25), the key enzyme of the hexosamine pathway, which produces glucosamine 6-phosphate. To assess the involvement of this pathway, we tested the influence of glucosamine on ASS activity (control: 0.33 ± 0.08 unit/mg = 100%; + GlcN: 191 ± 14% (significantly different from control cells); + Gln + DON: 109 ± 31%; n = 3, p < 0.05). This therefore demonstrated that the metabolism of glucosamine was required. Among amidotransferases, DON is known to inhibit 1-glutamine:fructose-6-phosphate amidotransferase (EC 2.5.1.16) (25), the key enzyme of the hexosamine pathway, which produces glucosamine 6-phosphate. To assess the involvement of this pathway, we tested the influence of glucosamine on ASS activity (Fig. 1D; also see Fig. 1C for mRNA). A dose-response study confirmed that the effect of glutamine on ASS was dose-dependent and the half-maximum stimulatory effect was obtained at about 1.5 mM (Fig. 1D). To check if glutamine metabolism was involved, we tested the influence of DON, an inhibitor of glutamine amidotransferases (25). Culturing the cells in the presence of 40 μM DON totally inhibited the stimulatory effect of 10 mM glutamine on ASS activity (control: 0.33 ± 0.08 unit/mg = 100%; + Gln: 191 ± 14% (significantly different from control cells); + Gln + DON: 109 ± 31%; n = 3, p < 0.05). This therefore demonstrated that the metabolism of glucosamine was required. Among amidotransferases, DON is known to inhibit 1-glutamine:fructose-6-phosphate amidotransferase (EC 2.5.1.16) (25), the key enzyme of the hexosamine pathway, which produces glucosamine 6-phosphate. To assess the involvement of this pathway, we tested the influence of glucosamine, a compound entering the hexosamine pathway downstream of GFAT. Culturing the cells with 10 mM glucosamine for 24 h induced an ~2-fold increase in the ASS activity, which was associated with a similar increase in the ASS mRNA level (Fig. 2A; also see Fig. 2B for mRNA) as observed with glutamine. A time-course study of the effect of 10 mM glucosamine on the mRNA level revealed that the effect appeared as soon as 2 h and increased for up to 6 h, remaining at the maximal value up to 24 h (data not shown). Moreover, the half-maximum effect of glucosamine was obtained at about 2.0 mM on ASS activity (Fig. 2C). Then, we assessed the specificity of the effect of glucosamine. By culturing the cells in the presence of 10 mM mannosamine, we were unable to detect any stimulating effect on either ASS activity or mRNA level (Fig. 2A).
Glucosamine and Glutamine Increased Sp1 Binding on the ASS Gene Promoter—Caco-2 cells were cultured with or without 10 mM glucosamine for different periods of time, as indicated, and EMSAs were performed. As shown in Fig. 3A by using the consensus sequence of the Sp1-binding site, glucosamine induced a progressive increase in a major band, attaining a 2-fold increase at 6 h (2.17 ± 0.26; n = 3; p < 0.05). Such a band corresponded to the Sp1-binding complex because the band totally disappeared when a 100-fold molar excess of unlabeled Sp1 oligonucleotide was added (Fig. 3A, lane 10) and...
supershifted when anti-Sp1 antibody was added (Fig. 3A, lane 8); the complex was not competed away by an excess of unlabelled nonspecific DNA sequence (lane 11). Besides the major band, the figure also shows that glucosamine induced a slight increase in another minor band. This minor but specific band, which was not supershifted by adding anti-Sp1 antibody, was then identified as Sp3 (see below). To ascertain that Sp1 was also involved in the glutamine effect, we performed EMSA experiments from cells cultured in the presence of 10 mM glutamine. As shown in Fig. 3B, glutamine induced a significant increase in Sp1 binding, persisting up to 20 h and corresponding to a 1.5-fold increase (1.50 ± 0.01; n = 3; p < 0.05). Finally, and to verify that the hexosamine pathway was involved, we tested the influence of DON on the effect of both glutamine and glucosamine. The addition of DON totally inhibited the increasing effect of glutamine on Sp1 binding but was without any effect on the binding induced by glucosamine (Fig. 3B). Taken together, these results clearly demonstrated that glutamine stimulated the DNA binding activity of Sp1 through the hexosamine pathway. In addition, we were unable to detect any stimulating effect of glutamine on Sp1 DNA binding by culturing the cells in a glucose-deprived medium (data not shown).

To specify whether Sp1 was actually involved in the increase in the rate of transcription of the ASS gene, we also performed EMSA experiments by using an oligonucleotide probe corresponding to a fragment of the ASS gene promoter containing two functional GC boxes (24). As shown in Fig. 3C, glutamine and glucosamine increased the binding of Sp1 (lanes 2–7 and lane 11 for supershift experiments), and also Sp3, because a
very slight increase in Sp3 binding could be observed (lanes 12 and 13 for supershift experiments). Finally, the addition of anti-Sp2 antibody was unable to induce any supershift (Fig. 3C, lane 14) demonstrating that, among the Sp family, Sp2 was not involved in the glutamine effect. Thus, Sp1 was responsible for the stimulating effect of glutamine on ASS gene expression, a result that demonstrates the physiological role of the GC boxes previously identified in the promoter fragment (24). Because glutamine acts through the hexosamine pathway, we then investigated the possibility that glutamine may activate the DNA binding activity of Sp factors via their glycosylation, focusing on Sp1 as the main factor involved.

Glucosamine and Glutamine Increased the Cytosolic O-glycosylation of Sp1—Caco-2 cells were cultured with or without glucosamine or glutamine (10 mM each) as indicated, and the glycosylation status of Sp1 was measured on nuclear extracts by using the RL2 antibody recognizing N-acetylglucosamine attached to Ser or Thr. As shown in Fig. 4A, the addition of glucosamine for 4 h induced an increase in both the glycosylation status and the level of Sp1, corresponding to a 2-fold increase (1.86 ± 0.15 and 1.80 ± 0.16, respectively; n = 4; p < 0.05). Similarly, culturing the cells for 20 h with glutamine also induced an increase in both the level and the glycosylation status of Sp1 (Fig. 4A). Because glucosamine and glutamine increased the level of Sp1 and its glycosylation status with a similar order of magnitude, this strongly suggested that both compounds acted by increasing the amount of Sp1 without any change in its glycosylation status into the nucleus. Experiments were therefore conducted to study the glycosylation status of Sp1 measured on cytosolic extracts from Caco-2 cells cultured for 20 h with or without glutamine or glucosamine (10 mM each). As shown in Fig. 4B, the addition of glutamine induced an increase in the glycosylation status of Sp1, and the addition of DON totally inhibited the increase. Moreover, such an increase was also observed in the presence of glucosamine but was not inhibited by the addition of DON. Taken together, these results suggested that glutamine stimulates the cytosolic O-glycosylation of Sp1 leading to its translocation from cytosol to nucleus. To ascertain such a phenomenon, Caco-2 cells were cultured for various periods of time with or without glucosamine, and the glycosylation status of Sp1 was measured on both cytosolic and nuclear extracts from the same batch of cells (Fig. 5). As shown in Fig. 5B, the addition of glucosamine induced a progressive increase in the glycosylation status of Sp1 in the cytosol, which was associated with a parallel decrease in the level of Sp1 (Fig. 5D). Concomitantly, we observed a progressive increase in the level of Sp1 into the nucleus of the same cells in the presence of glucosamine (Fig. 5E) but without any change in its glycosylation status (Fig. 5C). Thus, these results clearly demonstrated that glucosamine induced the O-glycosylation of Sp1 in the cytosol leading to its translocation into the nucleus.

DISCUSSION

The present results clearly demonstrate that glutamine may regulate the expression of the ASS gene through the hexosamine pathway leading to the glycosylation of Sp1, which in turn stimulates the transcription of the ASS gene, as illustrated in Fig. 6. To our knowledge, this is the first report proposing a complete scheme for the regulation of the expression of a gene by glutamine and also the first demonstration of a link between a transcription factor, namely Sp1, and a signal molecule for the regulation of the expression of the ASS gene. Indeed, concerning the former point, Sp1 was the only factor shown to interact with three proximal promoter sequences (GC boxes) of the ASS gene 12 years ago (24), but no link between Sp1 and various signal molecules including hormones, cytokines, and amino acids has been reported up to the present. Moreover, in the context of the effect of amino acids on the regulation of gene expression, it should be pointed out that neither amino acid
response elements nor nutrient-sensing response elements could be identified on the ASS proximal promoter (17) in contrast to CHOP (C/EBP homology protein) and asparagine synthase genes (27, 28). Additionally, a number of transcription factors, namely activator protein-1 (AP-1 (29)), hepatocyte nuclear factor-1 (HNF-1 (30)), activating transcription factor-4 (ATF-4) (28) and -2 (31), and C/EBPβ (32), were also involved in the regulation of the expression of genes by amino acids, but again the responsive elements to these transcription factors were not identified on the ASS gene promoter. Thus, our results focused on the importance of Sp1 to regulate ASS gene expression by amino acids, and we observed that this factor could bind the GC boxes of the ASS promoter under the influence of glutamine. Moreover, we also observed a weak increase in Sp3 binding activity suggesting that, beside Sp1, the ASS gene might be regulated by Sp3 but to a lesser extent than Sp1. Interestingly, studying the expression of the asparagine synthase gene, histidine limitation was recently shown to stimulate the role of glycosylation in Sp1 activation, our data demonstrated that beside its involvement in the stimulation of transcriptional activity, O-glycosylation of Sp1 may play a key role in its translocation into the nucleus because the Sp1 nuclear protein level/activation status ratio was unchanged under the influence of both glutamine and glucosamine (see Figs. 4 and 5). Thus, this shows that beside its involvement in the stimulation of transcriptional activity, O-glycosylation of Sp1 may play a key role in its translocation. Interestingly, such a role of O-glycosylation of Sp1 has been reported very recently in a study of the effect of insulin on liver cells (45). Moreover, it also should be underlined that O-glycosylation of Sp1 was reported to prevent its degradation (34).

Concerning the hexosamine pathway, glutamine acts through the reaction catalyzed by GFAT; and it must be remembered that GFAT requires two substrates, namely fructose 6-phosphate and glutamine. Although this pathway was reported to mediate many of the effects of high glucose level via the synthesis of fructose 6-phosphate (26, 40, 46), the importance of glutamine has been neglected until now. Indeed, to our knowledge, the involvement of the hexosamine pathway in the effect of glutamine has not been studied on gene expression. Moreover, we observed that glutamine addition in a glucose-deprived medium remained without any effect on either Sp1 binding or ASS gene expression in Caco-2 cells; conversely, it was very recently reported that glutamine addition to a glutamine-deprived medium exerted only a slight stimulatory effect on gene expression in cultured adipocytes (47). Taken together, these results therefore underline the importance of glutamine in the regulation of gene expression through the hexosamine pathway.