Tissue-Specific and De Novo Promoter Methylation of the Mouse Glucose Transporter 2

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Received April 19, 2005; accepted August 22, 2005

Glucose transporter 2 (GLUT2) is tissue-specifically expressed in liver and kidney, and reduced in neoplastic hepatic lesions and in most hepatoma cell lines. Here we examined the involvement of epigenetic modifications in the regulation of GLUT2. Four CpGs in the GLUT2 promoter were undermethylated in GLUT2-expressing tissues. In isolated hepatocytes, GLUT2 expression declined and the promoter was methylated de novo. This de novo methylation occurred with a similar time-course in hepatocytes cultured in a high-glucose medium that induced GLUT2 expression, suggesting that de novo methylation can be induced independently of GLUT2 expression. GLUT2 was reactivated in hepatocytes following exposure to the methylation inhibitor 5-aza-2’-deoxycytidine (AzaC) but only after the methylation had occurred. In p53-deficient mouse liver, the CpGs were methylated de novo; the GLUT2 expression declined. The GLUT2 promoter was hypermethylated in Hepa1c1c7 cells, but expression could be rescued by AzaC. Thus, it is proposed that DNA methylation has an important role in the regulation of GLUT2 in mouse tissues and liver-derived cells.

Key words glucose transporter 2 (GLUT2); DNA methylation; hepatocyte; liver; mouse

Methylation is one of the major epigenetic modifications of DNA in mammalian cells, regulating tissue-specific genes as well as housekeeping genes.1) This process usually silences gene transcription, either by preventing protein binding or by indirect mechanisms involving changes in chromatin structure.

GLUT2, a member of the glucose transporter (GLUT) family, is involved in glucose homeostasis through its role in glucose uptake from the intestine, reabsorption by the kidney, sensing in the pancreatic beta cells, and uptake and release by the liver.2) Animal studies suggest that GLUT2 expression in the liver increases in response to hyperglycemia and is suppressed by hyperinsulinemia.3) GLUT2 expression is also stimulated by high glucose concentrations in primary cultures of rat hepatocytes.3) However, the glucose responsiveness of glucose-dependent genes such as GLUT2 is lost in most hepatoma cell lines.4)

In the present study, the involvement of DNA methylation in the regulation of GLUT2 was examined using in vitro and in vivo models, with a focus on tissue-specific differences in DNA methylation as well as the cause-and-effect association between GLUT2 down-regulation and the de novo methylation.

MATERIALS AND METHODS

Chemicals All chemicals were reagent grade or higher and were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.), unless otherwise specified.

Mice Four-week-old C57Bl/6 male mice were purchased from Samtaco BioKorea (Osan, Korea). p53-deficient mice5) were obtained from the Jackson Laboratories (Bar Harbor, ME, U.S.A.), and the mutants were crossed at least 5 times with C57Bl/6 mice. C57Bl/6 mice were used as wild-type controls.

Primary Hepatocytes Mice were anesthetized and their livers were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.), and the mutants were crossed at least 5 times with C57Bl/6 mice. p53-deficient mice5) were obtained from the Jackson Laboratories (Bar Harbor, ME, U.S.A.), and the mutants were crossed at least 5 times with C57Bl/6 mice. C57Bl/6 mice were used as wild-type controls.

Primary Hepatocytes Mice were anesthetized and their hearts were perfused with EGTA solution (HBSS without Ca2+ and Mg2+, with 0.5 mM EGTA and 10 mM HEPES, pH 7.35) followed by collagenase solution (Williams’ medium E [WME] medium with Ca2+, Mg2+, 0.025% [w/v] collagenase type I, 10 mM HEPES, pH 7.35). Hepatocytes were then washed by centrifugation at 50×g for 5 min, and resuspended in WME medium containing 2 g/l d-glucose and 5% calf serum. In order to induce GLUT2 in hepatocytes, we used WME media that contained a higher level of glucose. For the preparation of high-glucose media, we added glucose to WME media to a final concentration of 4.0 g/l. This concentration 4.0 g/l was used because glucose concentrations in common mammalian cell culture media range up to 4.5 g/l, e.g. Dulbecco’s modified Eagle’s media, 1.0—4.5 g/l6); Iscove’s modified Dulbecco’s media, 4.5 g/l7). The isolated hepatocytes were cultured for 6 h at 37°C in a 5% CO2 atmosphere to facilitate attachment, after which the medium was exchanged every 48 h.

Hepa1c1c7 Cell Line Mouse hepatoma Hepa1c1c7 cells were purchased from the Korean Cell Line Bank (Seoul, Korea) and cultured in Eagle’s minimum essential medium containing 5% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine.

Cell Treatments Cultured cells were treated with 5-aza-2’-deoxycytidine (AzaC), up to 50 µM, for 72 h prior to harvesting. After harvesting, cells were snap-frozen in liquid nitrogen and stored at −80°C until use.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Total RNA was extracted from cells and tissues using Tri-reagent. Reverse transcription was performed using a 1st strand cDNA synthesis kit (Roche, Mannheim, Germany). Thermocycling conditions are available upon request. β-Actin was amplified as a control for RNA integrity. The following primer sequences were used for PCR: GLUT2, sense 5’-TTG CTG GCC TCA GCT TTA TT-3’ and antisense 5’-TGC CAG CTG TCT GAA AAA TG-3’; Dnmt1, sense 5’-GCC CAG CAA AGA GTA TGA GC-3’ and antisense 5’-TTC TTG GTT GCT TCG TAA CT-3’; Dnmt3a, sense 5’-TGG AGC TGC AAG AGT GTC TG-3’ and antisense 5’-GGT GCC AGG AAA GAT TGC AAT-3’; β-Actin, sense 5’-GCC AGG AAA GAT TGC AAT-3’ and antisense 5’-GGT GCC AGG AAA GAT TGC AAT-3’; β-Actin,
sence 5′-GTG GGC CGC TCT AGG CAC CAA-3′ and antisense 5′-CTC TTT GAT GTC ACG CAC GAT TTC-3′.

Bisulfite Sequencing Analysis  SacI-digested genomic DNA was denatured in a 0.3 M NaOH solution and incubated in 2.5 M sodium bisulfite/20 mM hydroquinone at 55 °C for 16 h. The DNA was desalted, desulfonated in 0.3 M NaOH at 37 °C for 15 min, and then neutralized in 10 M ammonium acetate. The samples were precipitated and resuspended in Tris–EDTA. Bisulfite-modified DNA was used for semi-nested PCR using primers specific for the modified DNA. First, modified DNA was subjected to PCR using sense (A1, 5′-TTC CCT TAA TCC CCTCAA CA-3′) and antisense (B1, 5′-TGT GGT AGA AAT GGG ATA GGG-3′) primers. The PCR conditions are available upon request. Two microliters of the resultant PCR products was subjected to 35 cycles of PCR under the same conditions using the A1 primer and another antisense primer (B2, 5′-GGG ATA GTT GAG -2(I) collagen, that are frequent in de-differentiated liver cells.11) The methylation of the four CpG sites varied among the tissues studied (Fig. 1). The CpGs in the GLUT2-expressing tissues liver (8/40 CpGs, 20.0%) and kidney (11/40, 27.5%) were undermethylated compared to the non-expressing tissues lung (34/40, 85.0%), cerebrum (33/40, 82.5%), and skeletal muscle (31/40, 77.5%), suggesting that the methylation of the GLUT2 promoter in mouse tissues is involved in the tissue-specific expression of GLUT2. The functional role for the CpG demethylation in GLUT2 expression, however, remains to be studied. Alleles that were unmethylated at all four CpG sites were observed at frequencies of 6/10 and 3/10 in liver and kidney, respectively, whereas no such allele was found in the other tissues examined. The methylation status of these tissues is consistent with the many examples of genes for which a correlation has been found between tissue-specific expression and demethylation.8)

Regardless of their tissue-specific expression, the 5′ flanking regions of the GLUT1 (16 CpGs between −191 and +26) and GLUT4 (10 CpGs between −141 and +17) genes were unmethylated in mouse liver, kidney, cerebrum, lung, and skeletal muscle (data not shown). This is similar to other genes, such as human α-globin and α-2(I) collagen, that are expressed tissue-specifically but which remain unmethylated in all the tissues examined.9,10)

To test for GLUT2 activity, primary hepatocytes freshly isolated from the livers of 4-week-old C57Bl/6 male mice were incubated in WME media with two different concentrations of d-glucose. The regular WME medium with 2 g/l d-glucose was supplemented with additional d-glucose to prepare high-glucose medium (4 g/l d-glucose). The accumulation of GLUT2 mRNA in hepatocytes was determined with RT-PCR up to 192 h post-plating (Fig. 2A). Under the conditions used, GLUT2 mRNA was detected in hepatocytes incubated in the regular WME at 12 h post-plating, but the mRNA level declined rapidly to an undetectable level at 24 h post-plating. Although the specific mechanism is not known, the loss of GLUT2 expression may be related to the de-differentiation of hepatocytes, because liver-specific genes are frequently lost in de-differentiated liver cells.11) The methylation level of the four CpGs in the GLUT2 promoter was almost unchanged during the first 96 h of culture in regular WME (Fig. 2B), but increased between 96 h and 192 h post-plating (from 12.5 to 50.0%). Thus, the CpG methylation was rather delayed compared to the rapid decline in the GLUT2 expression in hepatocytes cultured in low-glucose media.

To examine whether demethylation can reactivate GLUT2 expression, hepatocytes were exposed to AzaC for 72 h after various times in culture. AzaC induced GLUT2 at 192 h post-plating (Fig. 2C), but not earlier in culture. This time-dependent increase of GLUT2 inducibility by AzaC suggests that the pathways involved in the early down-regulation of GLUT2 are functionally altered over time, and that the influence of DNA methylation in GLUT2 regulation increases over time in cultured hepatocytes.

It has been suggested that DNA methylation does not intervene to silence active promoters, but affects genes that are already silent.12) Niwa et al.13) reported that retroviral transcription is repressed in embryonic cells at about 2 d after infection, whereas de novo methylation is delayed until about 15 d. The phosphoglycerate kinase gene is silent on the mammalian inactive X chromosome before methylation of its promoter occurs.14)

RESULTS AND DISCUSSION

The 5′ flanking region of the mouse GLUT2 gene between −321 and the transcription start site harbors four rather sparsely distributed CpGs and does not overlap with a CpG island, according to a sequence analysis using the Meth-Primer program (available at http://itsa.ucsf.edu/~urolab/methprimer/). The four CpGs, at −140, −158, −246, and −321, were analyzed by bisulfite sequencing to study the involvement of CpG methylation in the regulation of GLUT2.

The methylation of the four CpG sites varied among the tissues studied (Fig. 1). The CpGs in the GLUT2-expressing tissues liver (8/40 CpGs, 20.0%) and kidney (11/40, 27.5%) were undermethylated compared to the non-expressing tissues lung (34/40, 85.0%), cerebrum (33/40, 82.5%), and skeletal muscle (31/40, 77.5%), suggesting that the methylation of the GLUT2 promoter in mouse tissues is involved in the tissue-specific expression of GLUT2. The functional role for the CpG demethylation in GLUT2 expression, however, remains to be studied. Alleles that were unmethylated at all four CpG sites were observed at frequencies of 6/10 and 3/10 in liver and kidney, respectively, whereas no such allele was found in the other tissues examined. The methylation status of these tissues is consistent with the many examples of genes for which a correlation has been found between tissue-specific expression and demethylation.8)

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GLUT2 mRNA expression was much higher in hepatocytes incubated in high-glucose WME than in hepatocytes in regular WME (Fig. 2A). Although GLUT2 expression in high-glucose WME declined over time as it did in regular WME. In further support of the suggestion that there exist time-dependent alterations of the pathways involved in the early down-regulation of GLUT2, partial reactivation of GLUT2 in hepatocytes was observed at 192 h post-plating. However, the time-dependent increase in the promoter methylation of GLUT2 exhibited similar patterns irrespective of whether the hepatocytes were cultured in high-glucose or regular media (Fig. 2B). This finding is not consistent with the notion that DNA methylation affects genes that are already silent and provides new insight into the mechanism of de novo methylation in mammalian cells. We suggest that de novo methylation of GLUT2 promoter is affected by changes in the levels of cellular factors such as those associated with loss of differentiation, rather than by the gene silencing. Methylation-independent GLUT2 expression in hepatocytes cultured in high glucose medium may be due to heterogeneous populations of hepatocytes with varying degrees of methylation.

DNA methyltransferase 1 (Dnmt1) is considered a maintenance methylase, whereas Dnmt3a and 3b are thought to constitute the de novo methylases that affect the methylation status of normally unmethylated CpG sites. In the present study, Dnmt1, 3a, and 3b were found to be up-regulated in a time dependent manner in hepatocytes (Fig. 2C). Several studies have shown that Dnmts are frequently overexpressed in a coordinated fashion in a variety of human cancers that display hypermethylation of the promoter regions of tumor suppressor genes. We suggest that up-regulated Dnmt activity may have a role to play in the methylation of the GLUT2 promoter in hepatocytes.

In addition to the up-regulation of Dnmts, primary hepatocytes present several features that are potentially useful for the study of de novo methylation in liver tumors. Primary hepatocytes are de-differentiated like many tumor cells. In addition, the epigenetic changes occurring over time can be observed in freshly isolated cells, whereas tumor cells and cell lines have already a variety of epigenetic changes that reflect all the changes that occurred since the establishment of the cell line or tumor.

Mice deficient in the tumor suppressor protein p53 have a life expectancy of about 6 months and do not acquire spontaneous hepatocellular carcinomas within this life span. However, p53-deficient mouse liver cells exhibit enhanced cell proliferation rates. In addition, cell lines with tumorigenic potential have been established from the livers of p53-deficient mice. In the present study, the GLUT2 mRNA level in the livers of 4-week-old p53-deficient mice was similar to that of wild-type controls (data not shown), and the CpG methylation level (7/40, 17.5%) (Fig. 3A) was also similar. However, GLUT2 mRNA was down-regulated in the livers of 4-month-old p53-deficient mice (Fig. 3B), and the GLUT2 promoter was hypermethylated compared to age-matched controls (32.5% vs. 20.0%). In the livers of 7-month-old p53-deficient mice, the GLUT2 promoter was further methylated (82.5%). This result suggests that de novo methylation of the CpGs is accompanied by GLUT2 down-regulation in the cancer-prone liver cells of p53-deficient mice.

We found that Dnmt3b was up-regulated and Dnmt1 was down-regulated in the livers of 4-month-old p53-deficient mice (Fig. 3B), suggesting that Dnmt3b may be involved in the de novo promoter methylation of GLUT2 in p53-deficient mouse liver. In contrast, Dnmt3a expression was unaffected in the liver. p53 has recently been suggested to mediate repression of Dnmt1 in primary astrocytes and in human colon cancer cells, but in this study Dnmt1 was down-regulated compared to age-matched control tissues in the absence of p53. The underlying mechanism is, however, unclear.
It has been reported that the expression of GLUT2 is reduced in neoplastic hepatic lesions of the rat.\(^{20}\) In most hepatoma cell lines described to date, the glucose responsiveness of glucose-dependent genes such as GLUT2 is lost even when these cells remain well differentiated.\(^{21}\) In the present study, we tested whether the silencing of GLUT2 in mouse hepatoma Hepa1c1c7 cells is accompanied by changes of methylation status within the 5′-end of the gene. The bisulfite sequencing analysis showed that the four CpG sites were hypermethylated in the transformed cells (20/40, 50%) (Fig. 4A), relative to the mouse liver (8/40, 20%) (Fig. 1B). Treatment of Hepa1c1c7 cells with AzaC reactivated GLUT2 expression dose-dependently (Fig. 4B), indicating that DNA methylation is a cause of GLUT2 silencing in the hepatoma cells.

In summary, we demonstrated a correlation between the expression and the promoter methylation of GLUT2 in mouse tissues and liver-derived cells. The de novo promoter methylation of GLUT2 occurred independently of its expression in isolated hepatocytes, suggesting that de novo methylation of GLUT2 is not casually related to its expression. However, the down-regulation of GLUT2 expression could be rescued by demethylation in isolated hepatocytes and hepatoma cells, suggesting that methylation is responsible for gene regulation in liver-derived cells. Thus, we propose that DNA methylation has an important role in the tissue-specific expression of GLUT2 in the mouse and that it is also involved in gene regulation in mouse liver-derived cells.

Acknowledgements This study was supported in part by the Research Institute for Veterinary Science, Seoul National University, Seoul, Korea.

REFERENCES