Identification and characterization of peroxisome proliferator response element in the mouse GLUT2 promoter

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Abstract

In the present study, we show that the expression of type 2 glucose transporter isoform (GLUT2) could be regulated by PPAR-γ in the liver. Rosiglitazone, PPAR-γ agonist, activated the GLUT2 mRNA level in the primary cultured hepatocytes and Alexander cells, when these cells were transfected with PPAR-γ/RXR-α. We have localized the peroxisome proliferator response element in the mouse GLUT2 promoter by serial deletion studies and site-directed mutagenesis. Chromatin immunoprecipitation assay using *ob/ob* mice also showed that PPAR-γ rather than PPAR-α binds to the -197/-184 region of GLUT2 promoter. Taken together, liver GLUT2 may be a direct target of PPAR-γ ligand contributing to glucose transport into liver in a condition when PAPR-γ expression is increased as in type 2 diabetes or in severe obesity.

Keywords: GLUT2; liver; promoter; PPAR-γ; rosiglitazone; type 2 diabetes

Introduction

Recently, peroxisome proliferator-activated receptor (PPAR) family draws much attention in the treatment of type 2 diabetes. Three isoforms, encoded by separate genes, have been identified so far: PPAR-α, PPAR-β(δ), and PPAR-γ (Sher *et al.,* 1993). PPAR-α is highly expressed in hepatocytes, enterocytes, proximal tubular epithelium of kidney, and cardiac muscle, and the expression pattern parallels, to a certain extent, the sensitivity of various tissues to the β-oxidation induction by synthetic peroxisome proliferators (Spiegelman, 1998; Guillemain *et al.,* 2000). PPAR-γ is known to be involved in adipogenesis, and maintenance of differentiation is important in breast, colon, and urinary bladder function. PPAR-γ is a member of the nuclear hormone receptors that contains the ligand-dependent activation domain (AF-2) (Picard *et al.,* 2002). Upon ligand binding, PPAR-γ heterodimerized with retinoid X receptor (RXR)-α binds to the PPAR response element (PPRE) and activates target gene transcription. Thiazolidinediones (TZDs) are a class of antidiabetic agents that improve insulin sensitivity in various animal models of diabetes (Cavaghan *et al.*, 1997; Rahimian *et al.*, 2001). Patients with a dominant-negative mutation in the PPAR-γ gene show severe hyperglycemia, which provides a genetic link between PPAR-γ and type 2 diabetes (Barroso *et al.,* 1999). So far, a mechanism of antihyperglycemic effect of TZDs on the liver is considered to be rather indirect (Jiang *et al.,* 2002), partly because hepatic expression of PPAR-γ is rather low, and adipocytes specific deletion of the PPAR-γ gene affects the blood glucose level (He *et al.,* 2003). However, in the pathological state, as in obesity and type 2 diabetes patients, the expression of PPAR-γ is known to be increased (Rahimian *et al.,* 2001). Thus, the direct role of PPAR-γ ligand on the genes involved in hepatic glucose metabolism cannot be ruled out. Previously, we have characterized the peroxisom proliferator-activator response element (PPRE) in the promoter regions of rat GLUT2 (Kim *et al.,* 2000) and glucokinase (Kim *et al.,* 2002) gene and reported their physiological implications in the insulin secretion in response to changing blood glucose level in the pancreas of type 2 diabetic Zucker diabetic fatty (ZDF) rats. We also showed that liver type glucokinase (L-GK) was directly upregulated by TZDs in its promoter (Kim *et al.,* 2004). Because L-GK functions very closely with GLUT2 in transporting glucose into liver, it is possible that TZDs can activate both GLUT2 and L-GK gene in a coordinate manner.

To explore this possibility, we have dissected mouse GLUT2 promoter and identified a PPRE in the -197/-184 region. We also demonstrated that PPAR-α

is not a good inducer of GLUT2 expression whereas PPAR-γ stimulated the endogenous GLUT2 mRNA expression in primary mouse hepatocytes when PPAR-γ was ectopically expressed. Also, in the liver of *ob/ob* mice, where PPAR-γ expression is increased (Edvardsson *et al.,* 1999), the PPAR-γ ligand upregulated the GLUT2 expression whereas PPAR-α ligand did not affect its expression, indicating that PPAR-γ may contribute to lowering blood glucose level by activating GLUT2 gene expression in the diabetic liver.

Materials and Methods

Materials

Wy14,643 (20 mM in 19% BSA and 5% dimethyl sulfoxide), 9-*cis* retinoic acid (2 mM in 50% ethanol and 50% dimethyl sulfoxide), and rosiglitazone (2 mM in 5% BSA and 5% dimethly sulfoxide) were diluted to the final concentration of 20 μM, 1 μM, and 2 μM respectively. Wy14,643 and 9-*cis* retinoic acid were purchased from Sigma-Aldrich (St. Louis, MO). Rosiglitazone was kindly donated by GlaxoSmithKline, UK. PPAR-α and PPAR-γ antibodies were purchased from PPMX Perseus Proteomics Inc, Japan.

Construction of plasmids

The promoter region (-1112/+1) of the mouse GLUT2 gene was cloned into the *Kpn*I/*Xho*I site of the pGL3-basic vector (Promega, Madison, WI) to generate pMGT2-1112. Deletion constructs of mouse GLUT2 promoter were prepared from pMGLUT2 (pMGT2-1112/+1). pMGT2d-890 was prepared by excising out 222 bp fragment of *Sal*I and *EcoR*I digestion. pMGT2d-389 was constructed by digesting with *Sal*I and *Pst*I from the pMGT2-1112/+1. Other deletion constructs of mouse GLUT2 promoter were prepared by digesting pMGT2-1112/+1 after *EcoR*I sites was generated by PCR-based site-directed mutagenesis kit (Stratagene, La Jolla, CA), and named pMGT2d-283, pMGT2d-166, and pMGT2d-57, respectively. The PPRE truncated construct, pMGT2d-283/-166, was also prepared from pMGT2d-389 with the same method described above. Mutant constructs pMGT2m-389mut1 (mut1), pMGT2m-389mut2 (mut 2), and pMGT2m-389mut1+2 (mut1+2) were produced by introducing substitution mutations into pMGT2d-389 using site-directed mutagenesis. The sequences of constructs were confirmed by dideoxy-DNA sequencing method. Expression plasmids, pCMX-mPPAR-α, pCMX-mPPAR-γ and pCMX-mRXR-α were kind gifts from Drs. R. M. Evans and D. J. Mangelsdorf (Mangelsdorf *et al.,* 1992; Kliewer *et al.,* 1994). Control vector, pCMX was prepared from pCMX-mRXR-α by excising out the 1.5 kb fragment of mRXR-α cDNA. The dominant negative plasmid of NF-Y (△4NF-YA13m29; NF-Ym) was provided by Dr. R. Mantovani (Gurtner *et al.,* 2003).

Primary hepatocytes preparation from mouse liver

Hepatocytes were isolated from male ICR mouse (approximately 30 g) by the collagenase perfusion method (Seglen, 1972). Dissociation into individual hepatocytes was performed in Dulbecco's modified Eagles' medium (DMEM) containing 10% (v/v) heat inactivated fetal calf serum (FCS), 0.1 IU/ml insulin, 10 nM dexamethasone, 25 mM glucose, 100 U/ml penicillin G, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B. For each hepatocyte preparation, cell viability was checked by the exclusion of trypan blue.

Cell culture and transient transfection

Alexander cells (Human epithelial hepatoma cell lines; American Type Culture Collection number CRL-8024) were maintained as monolayer cultures and grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Plasmid DNAs were purified using Qiagen Midiprep kit columns (Qiagen, Valencia, CA). Transient transfection and luciferase assays were performed as described previously (Cha *et al.,* 2001). Briefly, cells were plated in six-well tissue culture plates at a density of 1×10^6 cells/well in 2 ml of medium. After a 20 h attachment period, transfections with 0.5 μg of each construct of GLUT2 promoter and control vector were performed with LipofectAMINE PLUS reagent (Life Technologies, Carlsbad, CA), according to the manufacturer's protocol. After 24 h transfection, the medium was replaced by a medium containing Wy 14,643 (20 μM, final concentration), rosiglitazone (2 μM, final concentration) and 9-*cis* retinoic acid (1 μM, final concentration) respectively. Cells were cultured further for 24 h and harvested in reporter lysis buffer (Promega). Luciferase data were expressed as luciferase activity corrected by β-galactosidase activity in the cell lysate. Each transfection was performed in triplicate and repeated three to five times.

Nuclear extracts preparation

Nuclear extract from liver of male Sprague-Dawley rats was prepared as described by Gorski *et al.* (Gorski *et al.,* 1986). Protein concentration was determined by the method of Bradford (Bradford, 1976).

Electrophoretic mobility shift assay (EMSA)

The oligonucleotide probe GLUT2-PPRE was labeled as described previously (Kim *et al.,* 2002b). Ten pmoles of single stranded sense oligonucleotide were labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase and annealed with 50 pmoles of unlabeled antisense oligonucleotides. The resulting double stranded oligonucleotides were purified by Sephadex G50 spin column. The labeled probe (10,000 cpm) was incubated with 5 μg of nuclear extract from rat liver for 30 min on ice. The binding reactions were same as described (Kim *et al.,* 2000). To perform supershift

assay, the binding mixtures were incubated for 10 min at room temperature in the presence of 1 μl of anti-PPARγ (Kim *et al.,* 2002a). Protein-DNA complexes were resolved from the free probe by electrophoresis at 4°C on a 4% polyacrylamide gel in 0.25 × TBE buffer (1 × TBE contained 9 mM Tris, 90 mM boric acid, 20 mM EDTA, pH 8.0). The dried gels were exposed to X-ray film at -70°C with an intensifying screen. The oligonucleotide sequences used in EMSA were as follows: GLUT2-PPRE, 5'-CTGTGCTCAAGC CACAAGTCATTGGGGTAAAGGGT-3'; mut1, 5'-CTG TGCTCAAGCCACtaccgtTTGGGGGTAAAGGGT-3'; mut2, 5'-CTGTGCTCAAGCCACAAGTCATtcagtgAAA-GGGT-3'; mut1+2, 5'-CTGTGCTCAAGCCACtaccgtT-tcagtgAAAGGGT-3'. Mutated bases are shown in lower-case letters. The oligonucleotide for CYP4A6-PPRE (PPRE of cytochrome p450 gene) (Muerhoff *et al.,* 1992) was synthesized.

Animals and drug administration

5-week-old male *ob/ob* mice and lean littermates (Jackson Laboratories, Bar Harbor, ME) were housed in plastic cages on a 12:12 light/dark cycle with free access to water and food (Purina 5008). After 2 weeks of acclimation, animals were weighed, bled via the tail vein, and assigned to treatment groups based on starting glucose values (first criterion) and initial body weights (second criterion). Diabetes were confirmed (glucose level; 296 mg/dl) by checking the plasma glucose levels. Before treatment of drug, oral glucose tolerance test (OGTT) was performed (0-day), and mice were gavaged once daily with vehicle, rosiglitazone (30 mg/kg) for 7 days (7-day). Animals were weighed and performed oral glucose tolerance test again. At the end of the experiments, animals were sacrificed by cervical dislocation under isoflurane anesthesia, and livers were rapidly excised, flash frozen in liquid nitrogen, and stored at -70°C for RNA analysis.

RNA preparation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from primary hepatocytes using TRIzol reagent by manufacturer's protocol (Life Technologies). For RT-PCR, first strand cDNA was synthesized from 3 μg of total RNA using random hexamer and Superscript II reverse transcriptase (Life Technologies). One μl of the reverse transcription reaction mixture was amplified with primers specific for GLUT2 and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) in the total volume of 50 μl. Linearity of the PCR was tested by amplification cycles between 20-30. According to test amplification profile, samples were amplified using the following parame-
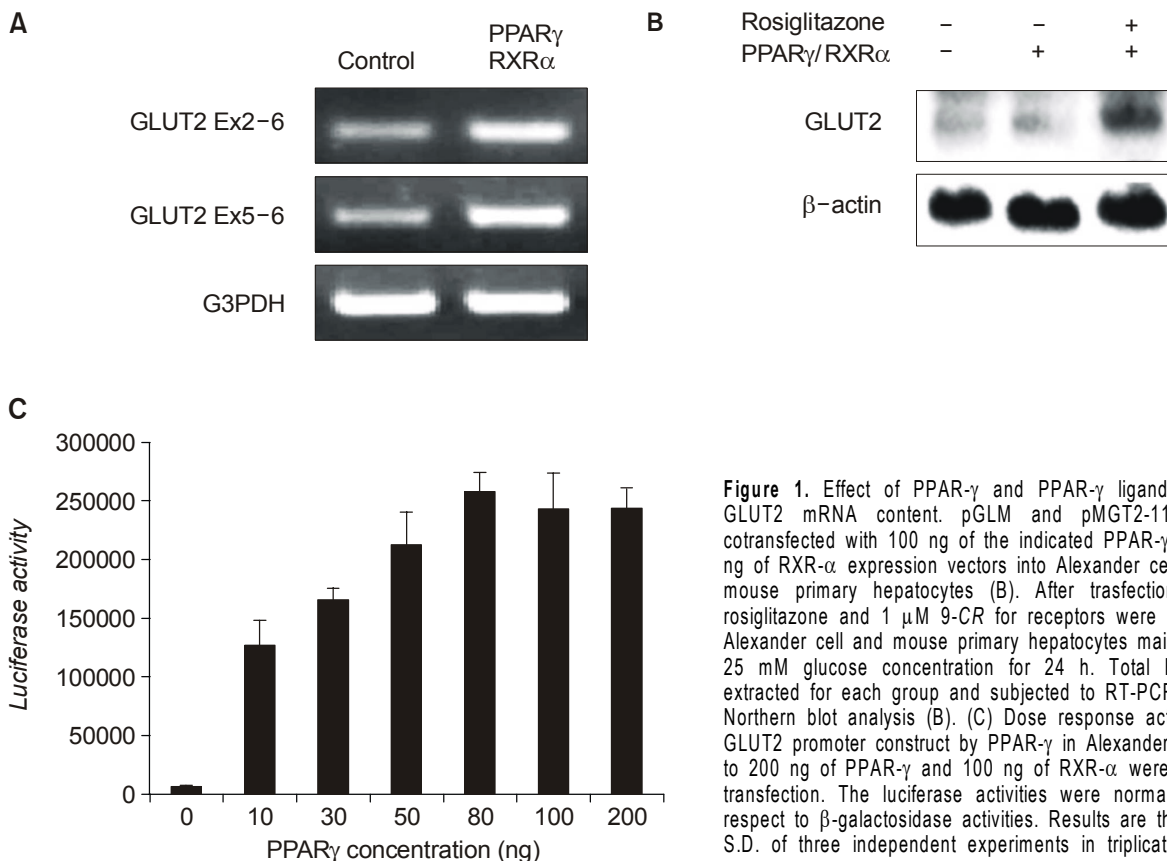
Figure 1. Effect of PPAR-γ and PPAR-γ ligands on the GLUT2 mRNA content. pGLM and pMGT2-1112 were cotransfected with 100 ng of the indicated PPAR-γ and 100 ng of RXR-α expression vectors into Alexander cell (A) and mouse primary hepatocytes (B). After trasfection, 2 μM rosiglitazone and 1 μM 9-*CR* for receptors were treated to Alexander cell and mouse primary hepatocytes maintained at 25 mM glucose concentration for 24 h. Total RNA was extracted for each group and subjected to RT-PCR (A) and Northern blot analysis (B). (C) Dose response activation of GLUT2 promoter construct by PPAR-γ in Alexander cell. Ten to 200 ng of PPAR-γ and 100 ng of RXR-α were used for transfection. The luciferase activities were normalized with respect to β-galactosidase activities. Results are the mean± S.D. of three independent experiments in triplicate.

ters: 94°C for 30 s, 58°C for 30 s, 72°C for 30 s. G3PDH was used as an internal control for quality and quantity of RNA. Primers used in PCR were follows: GLUT2-Ex2-sense, 5'-ATCACCGGAACCTTGG-CTTTCACT-3', GLUT2-Ex5-sense, 5'-GGCTAATTTC-AGGACTGGTT-3'; GLUT2-Ex6R-antisense, 5'-TTTC TTTGCCCTGACTTCCT-3'; G3PDH-sense, 5'-ACCAC AGTCCATGCCATCAC-3'; G3PDH-antisense, 5'-TCC ACCACCCTGTTGCTGTA-3'.

Northern blot hybridization of mRNA

Aliquots of total RNA (20 μg) from each sample were denatured with RNA sample loading buffer (20 mM MOPS, pH 7.0, 2 mM sodium acetate, 1 mM EDTA, 8% formaldehyde, 50% formamide), and subjected to electrophoresis in a 0.9% denaturing formaldehyde a-garose gel, and transferred to Nylon membrane. The cDNA of mouse GLUT2 was labeled with [α-^{32}P] dCTP using Rediprime Labeling Kit (Amersham Biosciences) and used as a probe. The membranes were hybridized with the probe for 2 h at 65°C with Rapid-Hybrid buffer (Amersham Biosciences). After hybridization, the membrane was washed twice with high salt washing buffer (0.1% SDS, 2 X SSC) at room temperature for 30 min followed by low salt washing buffer (0.1% SDS, 0.1 X SSC) at 65°C for 15 min. The membrane was exposed to X-ray film with intensifying screen at -70°C.

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay protocol was adapted from methods as described by Duong et al. (Duong et al., 2002). Adult male lean (+/+) and diabetic (*ob/ob*) mice (blood glucose level; > 240 mg/dl) had free access to Purina chow. The livers were perfused with serum- free Dulbeco's modified Eagle's medium (DMEM) for 5 min and cross-linked with 5% formaldehyde in serum-free DMEM for 5 min. The livers were homogenized, pelleted by centrifugation at 2000 rpm for 4 min at 4°C, and resuspended in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0). The lysate was sonicated on ice for 3 min at a setting of cycle 0.8, amplitude 80 and sheared to 100-600 bps. To provide a positive control (input) for each condition, one undiluted aliquot was retained for further processing in parallel with all the other samples at the reversal of cross-linking step. To reduce nonspecific background, each chromatin sample (1 ml) was precleared with 60 μl of protein A/G-agarose (Santa Cruz Biotech, CA), supplemented with 200 μg/ml sonicated salmon s-perm DNA (Stratagene), and the beads were pelleted. Chromatin complexes in the supernatant were immunoprecipitated overnight at 4°C using 30 μg of primary antibody, or without antibody. Immune complexes were collected with 60 μl of protein A/G-aga-rose including 200 μg/ml of salmon sperm DNA. Promoter-specific PCR primers were as follows: GLUT2 promoter (sense, 5'-TGCCTCGGCTTCCACAAAAG-3', antisense, 5'-GTGGATGAATAGCTGACCAG-3').

Statistics

All transfection studies were carried out in three separate experiments, where triplicate dishes were transfected. Data are expressed as means ± S.D. Statistical analysis was carried out using Microsoft Excel software (Microsoft, Redmond, WA).

Results

Effect of PPAR-γ on the GLUT2 expression

As shown in Figure 1A, RT-PCR of the exons of GLUT2 revealed that transfection of PPAR-γ/RXR-α with rosiglitazone induced the GLUT2 expression when compared to untransfected Alexander cell line (control), whch was maintained at the 25 mM glucose concentration. Northern blot analysis showed that GLUT2 expression was not increased by Wy14,643/ 9-*cis* retinoic acid treatment at 25 mM glucose concentration in mouse primary hepatocytes (data not shown). However, Rosiglitazone/9-*cis* retinoic acid treatment induced GLUT2 mRNA level. GLUT2 mRNA was not increased by transfecting PPAR-γ/RXR-α when their ligands were not added to the culture me-
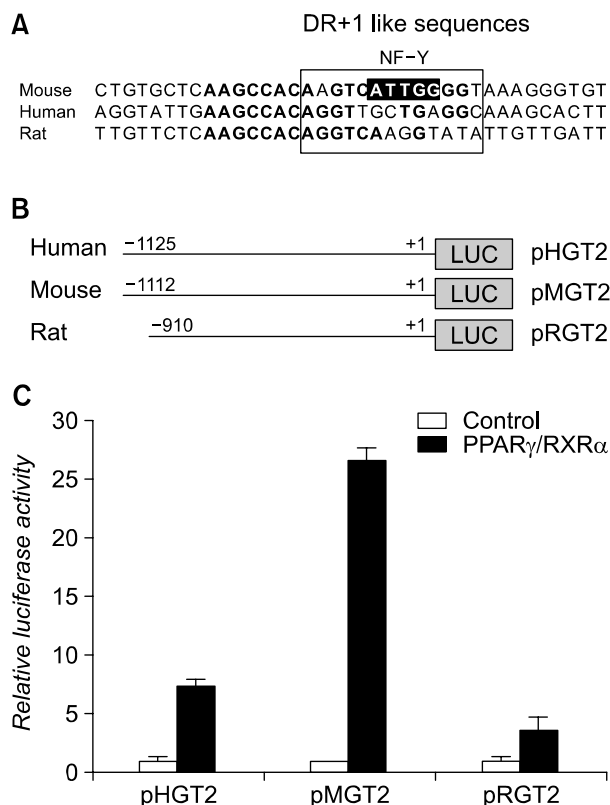
Figure 2. GLUT2 promoters are responding to PPAR-γ. (A) Sequence comparison of the proximal region of GLUT2 gene. (B) Schematic diagram of GLUT2 promoter constructs from human, mouse, and rat. (C) Responsiveness of GLUT2 promoter constructs to PPAR-γ. Detailed experimental conditions are the same as described in Figure1.

dium (Figure 1B). To determine whether PPAR-γ re-
gulates GLUT2 promoter activity, we constructed
mouse GLUT2 promoter construct (pmGT2-1112) and
observed their responsiveness (Figure 1C). The GLUT2
promoter construct was activated by transfected
PPAR-γ expression vector from 10 ng to 80 ng) in
a dose dependent manner, suggesting that the pro-
moter is responding to PPAR-γ specifically (Figure
1C).

Localization and identification of PPRE in the GLUT2 promoter

Figure 2A shows the sequence comparison of the
proximal regions of GLUT2 gene between species.
Unlike human and rat GLUT2 promoter, mouse pro-
moter seems to contain NF-Y binding sites in the
DR+1 consensus sequence (Boxed region). For the
comparison of the GLUT2 promoter activity to
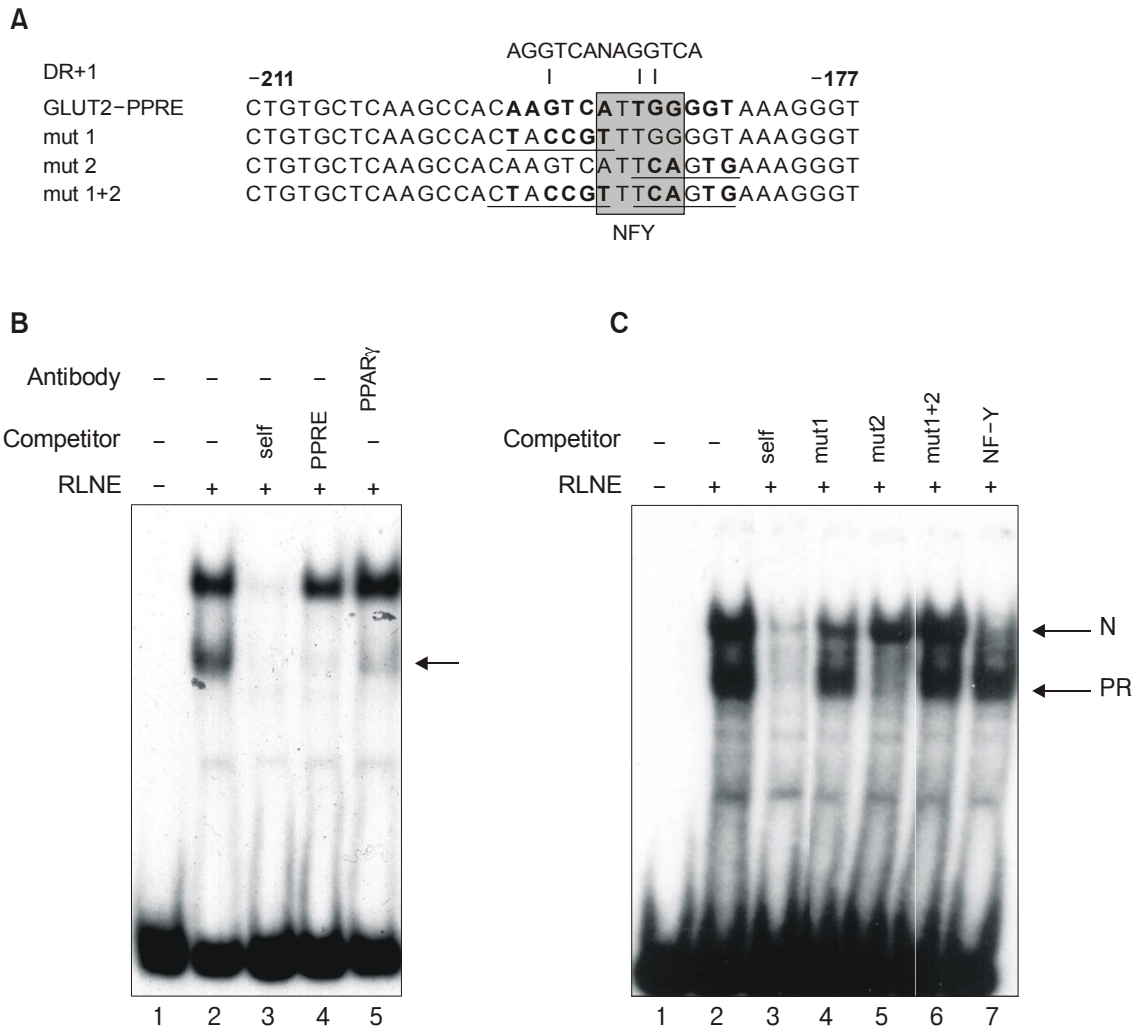PPAR-γ, part of the proximal regions of GLUT2 gene

was subcloned into luciferase vector (Figure 2B) and
named pHGT2 (human), pMGT2 (mouse), and pRGT2
(rat), respectively. As shown in figure 2C, all the con-
structs showed good responsiveness to PPAR-γ and
pMGT2 showed the strongest promoter activity.
To identify and characterize a functional PPRE invol-
ved in regulating the mouse GLUT2 promoter activity,
we generated serially deleted 5' flanking regions (from
-1112, -890, 389, -283, -166, and -57 to +1) and
truncated PPRE (-283/-166) in pMGT2d-389 and
examined their responsiveness to PPAR-γ in Alex-
ander cells (Figure 3A). The promoter activity was
well maintained down to -283 region. Further deletion
to -166 region resulted in a dramatic drop in the lu-
ciferase activity which was assayed in Alexander cell
line (Figure 3B) and primary hepatocytes (Figure 3C),
suggesting that the PPRE might be present between
-283 and -166. Internal deletion between -283 and
-166 also diminished the promoter activity (Figure 3B).
   Computer-based analysis of the sequence sug-
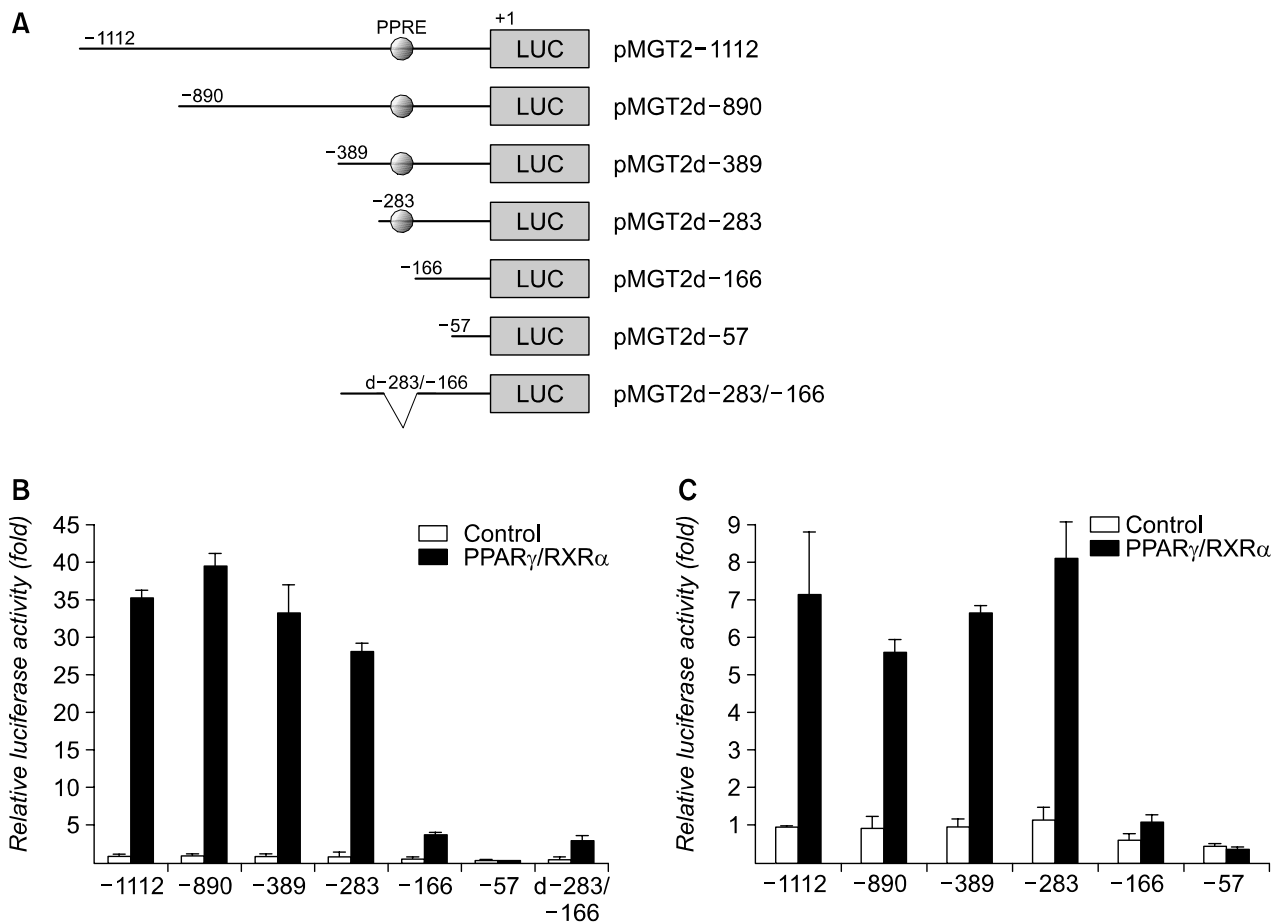
Figure 3. Localization of PPRE in the mouse GLUT2 promoter. (A) Structure of serial deletion and internal deletion (d-283/-166) constructs of pMGT2-1112. The indicated numbers represent bases from ATG codon as +1. Cells were cotransfected with each luciferase construct with or without PPAR-γ/RXR-α expression plasmids into Alexander cell (B) or mouse primary hepatocytes (C). All results were normalized with respect to β-galactosidase activities and were shown as relative fold increase in the luciferase activities compared to those of control. Normalized luciferase activities are shown as means ± S.D. of three independent experiments in triplicate.

A

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                                    AGGTCANAGGTCA
DR+1                    -211        I       I I            -177
GLUT2-PPRE     CTGTGCTCAAGCCACAAGTCATTGGGGTAAAGGGT
mut 1          CTGTGCTCAAGCCACTACCGTTTGGGGTAAAGGGT
mut 2          CTGTGCTCAAGCCACAAGTCATTCAGTGAAAGGGT
mut 1+2        CTGTGCTCAAGCCACTACCGTTTCAGTGAAAGGGT
                                           NFY
```



Figure 4. Effect of mutation at the putative PPRE of GLUT2 promoter. (A) Sequences of wild type (GLUT2-PPRE) and mutated oligonucleotides (mut1, mut2, and mut1+2) are shown. Putative GLUT2 PPRE sequence is compared with conventional DR+1. The mutated bases were represented by bold characters. Putative NF-Y consensus sequence is indicated by gray box. (B) EMSA of GLUT2-PPRE (from -211 bp to -177 bp) using rat nuclear extract. Thirty pmoles of [^{32}P]-labeled double-stranded oligonucleotide were incubated with 5 μg of rat liver nuclear extracts (RLNE) in the presence of 100-fold molar excess of the indicated cold competitors. For the supershift assay, 1 μg of anti-PPAR-γ antibody was used. Competitors are as follows: self; GLUT2-PPRE, PPRE; PPRE consensus sequence of CYP4A6 gene. The DNA-protein complexes are indicated by arrow. (C) Effect of mutation on the protein binding. Mutant probes and NF-Y consensus sequence were used as competitors. N; NF-Y, PR: PPAR/RXR-α complex.

gested that the region between -206 and -147 may contain a putative DR+1 element which is known to bind PPAR isoforms (Figure 2A). The binding of PPAR isoforms to putative GLUT2-PPRE was analyzed by EMSA using rat liver nuclear extract. Figure 4A shows the putative DR+1 sequence as well as NF-Y binding sites overlapped with DR+1 in the GLUT2 promoter. EMSA experiment using rat liver nuclear extract showed that faster moving protein-DNA complex (lower band) could be PPAR-γ because unlabeled CYP4A6-PPRE (Muerhoff *et al.,* 1992) efficiently competed with PPARs/RXR-α complex formation (Figure 4B, lane 4) and the complex was supershifted by PPAR-γ antibody (Figure 4B, lane 5).

To confirm the PPAR-γ binding sequence, competition assay using mutant versions (mut1, mut2, and mut 1+2, Figure 4A) of putative PPRE were performed (Figure 4C). Mut1+2 lost the ability to compete the formation of faster and slower DNA-protein complex (lane 6) whereas mut1 did not compete the formation of faster complex (lane 4) suggesting that mut1 region is critical for PPAR binding. Mut2 did not compete the formation of slower complex (lane 5) when compared to mut1. Instead, the slowly migrating band was competed by NF-Y consensus sequence (Figure 4C, lane 7) (Raymondjean *et al.,* 1991) and the addition of mut2 to reaction mixture failed to compete the slowly migrating DNA complex (Figure 4C, lanes 5). These

A

B

PPARγ   − − + +
NF−Ym   − + − +
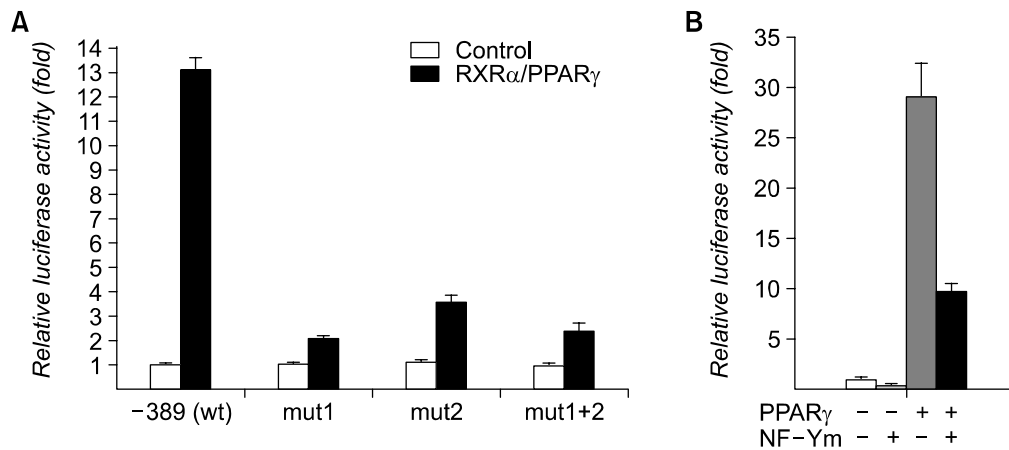
Figure 5. Effect of mutation on the GLUT2 promoter activity in the Alexander cells. (A) mut1, mut2, and mut1+2 were generated from pMGT2d-389 (wt) using site-directed mutagenesis. The reporter constructs containing a wild and mutant sequence of mouse GLUT2 promoter were transfected with or without expression vectors of PPAR-γ and RXR-α. 2 μM rosiglitazone and 1 μM 9-*CR* ligands for receptors were treated after transfection. (B) Role of NF-Y on the PPAR-γ driven GLUT2 promoter activity. The wild type pMGT2-1112 reporter construct was transfected into Alexander cells with or without expression vectors of PPAR-γ/RXR-α and NF-Ym as indicated. All results were normalized with respect to β-galactosidase activities. The results were shown as relative fold increase of luciferase activities compared to those of control. Results are the mean ± S.D. of three independent experiments in triplicate.
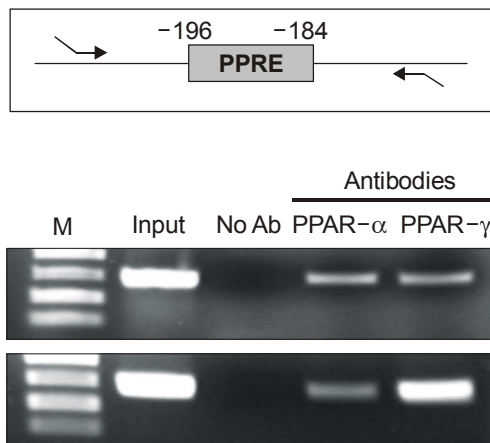
Figure 6. Chromatin immunoprecipitation (ChIP) assay. Equivalent amounts of DNA extracted from chromatin from lean and *ob/ob* mice, and chromatin were precipitated using 1 μg of PPAR-α or PPAR-γ antibody. The GLUT2-PPRE (-196/-184 bp) region was amplified by PCR. In order to normalize the amount of chromosomal DNA used in immunoprecipitation between groups, input chromatin (one hundredth of chromosomal DNA used for immunoprecipitation) was also used. Detailed PCR conditions and methods of sample treatment were described in the `Materials and Methods'. M, Marker.

data indicate, that NF-Y as well as RXR-α, a heteromeric partner of PPAR isoforms, may bind to the region (Figure 4A). The luciferase assay suggested the relative importance of mut1 and mut2 (Figure 5A). Both mut1 and mut1+2 showed reduced promoter activity, suggesting that mut1 region is important in PPAR-γ driven promoter activity. Mut2 region that contains NF-Y binding site may help exert promoter activity driven by PPAR-γ. This was further confirmed

by addition of dominant negative form of NF-Y (NF-Ym) which caused decreased PPAR-γ driven promoter activity (Figure 5B).

The binding of PPAR-γ to the GLUT2 promoter was increased in the liver of *ob/ob* mice

If the GLUT2 expression by PPAR-γ was activated in diabetic states, the physical contact between PPAR-γ and a putative PPRE in the GLUT2 promoter should be confirmed. To address this question, we performed chromatin immunoprecipitation assay (ChIP assay) using lean and *ob/ob* mice. As shown in Figure 6, PPAR-α and PPAR-γ bind to GLUT2-PPRE very weakly in the lean mice. However, the binding of PPAR-γ to the putative PPRE (-196/-84) on the GLUT2 promoter was increased in *ob/ob* mice, whereas the binding of PPAR-α was not increased, indicating that PPAR-γ may play a role as an activator of the GLUT2 gene expression in liver.

Discussion

PPAR-γ is more abundant in adipose tissue than in liver, or muscle (Tontonoz *et al.,* 1994; Braissant *et al.,* 1996). However, improvement in glucose homeostasis by PPAR-γ ligand treatment involves insulin sensitization in muscle and liver (Inzucchi *et al.,* 1998). Molecular mechanism of actions of TZDs in the glucose homeostasis occurring in tissues other than adipose tissues remains elusive. Direct effects of TZDs on glucose uptake in L6 myotubes have been identified (Ciaraldi *et al.,* 1990; Zhang *et al.,* 1994), which is supported by increased activity of the glucose transporter GLUT4 and of PI-3-kinase (Yone-

mitsu *et al.,* 2001). On the other hand, PPAR-γ sti-mulated the promoter activity of genes responsible for glucose sensing apparatus of pancreatic β-cells, sug-gesting that this ligand could also improve glucose-stimulated insulin secretion (Kim *et al.,* 2000; 2002b).

In the hyperglycemic states of type 2 diabetes, administration of TZDs causes amelioration of blood glucose level, suggesting that the liver glucose sensor can be a direct target of TZDs action. Previously, we have shown that LGK is transcriptionally activated by TZDs in liver (Kim *et al.,* 2004). In parallel with this observation, we have explored a possibility that one of the partner protein, GLUT2, of hepatic glucose sensor could be a transcriptional target of TZDs action in liver.

In this study, we have identified and localized the PPRE (-197 and -184 bp) in the region of mouse GLUT2 promoter. The mouse region is similar to authentic DR+1 sequence (Palmer *et al.,* 1995), and activated by TZDs treatment. Currently, the contri-bution of PPAR-γ in hepatic gene regulation in phy-siological state is challenged by the limited expression of PPAR-γ compared to that of PPAR-α (Way *et al.,* 2001). However, in pathological state of type 2 diabe-tes or severe obesity, the liver was shown to overex-press PPAR-γ isoforms (Rahimian *et al.,* 2001). Thus, the GLUT2 expression in liver could be activated by TZDs in the type 2 diabetes patients. Even in the pathological conditions, PPAR-α is more abundant than PPAR-γ. The DR+1 sequence in the GLUT2 may bind either PPAR-α/RXR-α or PPAR-γ/RXR-α. Then, why PPAR-α/RXR-α cannot activate the GLUT2 pro-moter? And, how the PPAR-γ/RXR-α can activate the GLUT2 promoter? To address these questions, we have performed the ChIP assay using mouse GLUT2 promoter. As shown, the binding of PPAR-γ to GLUT2 promoter is increased in the *ob/ob* mice, where PPAR-γ is overexpressed. From this experiment, it is concluded that the binding of PPAR-γ to GLUT2 promoter is increased in the diabetic or obese mice, where PPAR-γ is known to be overexpressed.

EMSA study suggested that mut1 and mut2 might constitute DR+1 site. However, competition and su-pershift experiment showed that the slow migrating band (upper band, Figure 4C) contains NF-Y. NF-Y binds CCAAT boxes located within 100 bp upstream from the transcription start sites, and these elements are known to be important for early functions in the formation of preinitiation complex (Dorn *et al.,* 1987; Milos *et al.,* 1992). NF-Y also has been reported to interact with transcription factors binding to upstream promoter *cis*-elements of gene. Among several CCAAT-binding proteins including Y-box factors, CTF/NF-1, NF-Y and C/EBP, NF-Y is the most ubiquitous and specific key proximal promoter factor in the trans-criptional regulation of different eukaryotic genes. Un-like other CCAAT-binding proteins, NF-Y has an absolute requirement of CCAAT pentanucleotide as well as a strong preference for specific flanking se-quences (Matuoka *et al.,* 1999). The mechanism of regulating the expression of these genes cannot be simply due to NF-Y DNA binding. It is likely that ad-ditional factors might be involved in the action of NF-Y. Indeed, previous studies from other groups have suggested that NF-Y interacts, either functionally or physically, with other transcription factors or n-uclear proteins both *in vitro* and *in vivo* (Framson *et al.,* 1993; Zwicker *et al.,* 1995; Ericsson *et al.,* 1996). In this system, the contribution of NF-Y transacting factor to PPAR-γ action was confirmed by the addition of dominant negative form of NF-Y (NF-Ym), which reduced the promoter activity by 65%. The contribu-tion of NF-Y to other transcription factors has been reported (Lee *et al.,* 2003). However, a molecular mechanism or interaction between NF-Y and transa-cting factors needs to be explored.

In conclusion, we have identified a functional PPRE in the GLUT2 promoter in liver, and showed that PPAR-γ could bind to this element, and increased the transcription of GLUT2 mRNA in the *ob/ob* mice where appropriate amount of PPAR-γ is expressed. Thus, it is assumed that PPAR-γ may have a direct activating role for the hepatic GLUT2 and LGK (Kim *et al.,* 2004), which help the transport of glucose into hepatocytes.

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