Identification of Glucose Transporter 4 Knockdown-dependent Transcriptional Activation Element on the Retinol Binding Protein 4 Gene Promoter and Requirement of the 20 S Proteasome Subunit for Transcriptional Activity*

Erina Inoue†, Aoi Yamashita†, Hirofumi Inoue†, Mariko Sekiguchi†, Asuka Shiratori§†, Yuji Yamamoto§, Tadahiro Tadokoro†, Yoshiko Ishimi¶, and Jun Yamauchi‡†§¶

From the †Nutritional Epidemiology Program and §Food Function and Labeling Program, National Institute of Health and Nutrition, 1-23-1 Toyama, Shinjyuku, Tokyo 162-8636, Japan and the ‡Department of Applied Biology and Chemistry, Tokyo University of Agriculture, 1-1-1 Sakuragaoka, Setagaya, Tokyo 156-8502, Japan

Retinol binding protein 4 (RBP4) is the transport protein that carries retinol in blood. RBP4 was described recently as a new adipokine that reduced insulin sensitivity. Mice lacking Rbp4 gene expression; however, the molecular mechanism is unknown. We found a G4KA (GLUT4 knockdown-dependent transcriptional activation) element located ~1.3 kb upstream of the Rbp4 promoter. Mutations within the G4KA sequence significantly reduced expression of the Rbp4 promoter-reporter construct in G4KD-L1 (GLUT4 knockdown 3T3-L1) adipocyte cells. In a yeast one-hybrid screen of a G4KD-L1 cell cDNA library, using the G4KA element as bait, we identified subunits of the 20 S proteasome, PSMB1 and PSMA4, as binding partners. In chromatin immunoprecipitation assays, both subunits bound to the G4KA element; however, only PSMB1 was tightly bound in the GLUT4 knockdown model. PSMB1 RNA interference, but not PSMA4, significantly inhibited Rbp4 transcription. Nuclear transportation of PSMB1 was increased in G4KD-L1 cells. These results provide evidence for an exclusive proteasome subunit-related mechanism for transcriptional activation of RBP4 within a GLUT4 knockdown model.

Retinol binding protein 4 (RBP4) is a protein with the specific function of transporting retinol in blood. In addition, RBP4 serves to solubilize lipid-soluble retinol in aqueous serum, protecting it from oxidative destruction and maintaining it at a constant concentration in circulation (1). RBP4 is synthesized in the liver and adipocytes and is secreted into the circulation, carrying one molecule of retinol per molecule of RBP4. RBP4 circulates attached to the serum thyroid binding protein transthyretin in a 1:1 molar ratio (1). The retinol-RBP4-transthyretin complex breaks up as retinol is released from RBP4 at the target tissue. Retinol then enters the target cell through a supposed cell surface RBP4 receptor (2). Thus, RBP4 has crucial roles in retinol concentration, transport, and uptake by target cells (3).

A major cause of type 2 diabetes is impaired insulin action in adipose tissue, skeletal muscle, and liver. Even in the absence of diabetes, insulin resistance is a major risk factor in cardiovascular disease and early mortality (4). Resistance to insulin-stimulated glucose transport in adipose tissue and skeletal muscle is one of the earliest defects detected in an insulin-resistant status (5). Transport of glucose by glucose transporter 4 (GLUT4) is the rate-limiting step in glucose use by muscle and adipose tissue (5). With the development of insulin resistance, GLUT4 expression is selectively down-regulated in adipose tissue but not in skeletal muscle (5, 6). Down-regulation of GLUT4 expression in adipose tissue is an almost universal factor in diseases with insulin resistant status, including obesity, type 2 diabetes, and metabolic syndrome (5).

Genetic knock-out of GLUT4 in adipocytes of mice (7) is associated with increased serum levels of RBP4 (8). Injection of RBP4 or transgenic overexpression in mice impairs insulin signaling in skeletal muscle and induces the expression of gluconeogenic enzymes in the liver (8). Elevated serum concentrations of RBP4 have been reported in subjects with obesity and type 2 diabetes and are associated with insulin resistance and cardiovascular risk factors (9–13). However, it is unclear how GLUT4 down-regulation leads to RBP4 up-regulation in adipocytes.

We report the identification of a core DNA element (G4KA) in the RBP4 promoter of 3T3-L1 adipocytes, using a stable lentiviral shRNA-mediated knockdown of GLUT4 3T3-L1 (G4KD-L1), and the identification of 20 S proteasome subunits, PSMB1 and PSMA4, which bind to this element. RNA interference knockdown of PSMB1 inhibited the expression of Rbp4 promoter-reporter constructs, but knockdown of PSMA4 did not. Nuclear localization of PSMB1 was enhanced in G4KD-L1 cells. Our results suggest a proteasome subunit-related mech-
anism for transcriptional activation of RBP4 within adipocyte-specific GLUT4 knock-out mice (8).

**EXPERIMENTAL PROCEDURES**

**Materials**—Antibodies to PSMB1 and PSMA4 were obtained from Santa Cruz Biotechnology, and antibody to PSMC1 from Abcam. Predesigned shRNA plasmids for PSMB1 and PSMA4 were from Santa Cruz Biotechnology. The proteasome inhibitor, MG-132, was from Sigma.

**Plasmids**—The expected entire region of the mouse Rbp4 promoter was amplified by PCR using a BAC clone (RP24-93G11; BACPAC Resources). Internal and 5′-deletion mutants were made using individual restriction enzymes and PCR with specific primers. Constructs of a chimeric gene in which the entire promoter of mouse Rbp4 promoter and its deletion mutants were inserted into the pGL4.17 [luc2/Neo] luciferase reporter plasmid (Promega) were made. Mouse PSMB1 and PSMA4 cDNA were inserted into the pcDNA3.1D/V5-His-TOP vector (Invitrogen).

**shRNA**—shRNA for mouse GLUT4 oligonucleotide pairs were as follows (sense and antisense strands are underlined; italic type indicates loops; designed by B-Bridge; only the upper strand is shown): G↓GATCCGGCACACGGTCTGGGT GATTCAGAGATCCACCCAGCTGTGTGGTTTTTG.

**Oligonucleotides** were annealed and inserted into BamHI-GATTCAAGAGATCACCCAGAGCCTGTGTGGTTTTTG.

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**Oligonucleotides** were annealed and inserted into BamHI-EcoRI sites of the pSIF-H1-puro vector (System Bioscience).

**Lentivirus Production and Injection of 3T3-L1 Adipocytes**—Recombinant lentivirus was produced according to the manufacturer’s instructions (System Bioscience). The infection of 3T3-L1 cells was performed as described previously (14). The infected 3T3-L1 cells were selected by puromycin (Invitrogen).

**Cell Culture and Transient Transfection**—3T3-L1 cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) at 37 °C in 5% CO2. Transfections were performed using Lipofectamine Plus (Invitrogen) as described previously (15). Dual-Luciferase reporter assays were performed according to the manufacturer’s instructions (Promega).

**Glucose Uptake Analysis**—The procedure for 2-[3H]deoxyglucose uptake was described previously (16). In brief, 3T3-L1 adipocytes were serum-starved for 3 h, and the cells were stimulated with insulin for 20 min at 37 °C. Glucose uptake was determined after the addition of 2-[3H]deoxyglucose (0.1 μCi, final concentration, 0.1 mm) in KRP-HEPES buffer for 10 min at 37 °C (121 mM NaCl, 4.9 mM KCl, 1.2 mM MgSO4, 0.33 mM CaCl2, and 12 mM HEPES) (pH 7.4).

**Yeast One-hybrid System**—The yeast strain YM4271, transformed with the yeast expression plasmid pHi5i containing 3XG4KA sequence, was used to screen a yeast GAL4 activation domain fusion G4KD-L1 cDNA library. Positive clones were selected on SD medium (Clontech) that lacked Leu and His but contained 100 mM 3-amino-triazol (Sigma). Surviving colonies were restreaked on selection medium. All assays using this system were performed according to the manufacturer’s instructions (Clontech). cDNA from positive clones were sequenced.

**Gel Electrophoresis Mobility Shift Assay (EMSA)**—PSMB1 and PSMA4 were in vitro-translated using the TNT T7 polymerase quick-coupled transcription/translation system (Promega). A double-stranded oligonucleotide (G4KA, 5′-TTCTCTAAACA-3′; only the upper strand is shown) was end-labeled using [γ-32P]ATP (Amersham Biosciences) and T4 polynucleotide kinase (Invitrogen) (17). Samples were incubated for 30 min at room temperature and resolved on 5% polyacrylamide gels. Gels were then dried and subjected to autoradiography (BAS-1800, FujiFilm).

**Cell Extractions and Western Blot Analysis**—Cell extractions were performed using NE-PER nuclear and cytoplasmic extraction reagents (Pierce). Western blot analysis was as described previously (17).

**ChIP Assay**—ChIP analyses were performed using a ChIP assay kit (Upstate Biotechnology). Cells were incubated on 10% formaldehyde for 15 min at 37 °C. The cells were then lysed, sonicated, and chromatin immunoprecipitated using antibodies against the indicated proteins. Specific primer pairs were designated as follows: G4KA, (−1149) 5′-CAGCCTTCCCTCAGTTGTTG-3′ and (−1396) 5′-CACCCTTGGTGTATTTAGTCTC-3′; and distal (−9644) 5′-AGACGACCACTTTCGATCTTT-3′ and (−9883) 5′-CCGGTTAAATGCAGAGGTA-3′, and PCR conditions were optimized to allow semiquantitative measurements. Conditions used were 25 cycles of 30 s at 90 °C, 15 s at 58 °C, and 1 min at 72 °C. PCR products were visualized on 2% agarose/Tris acetate EDTA gels.

**RNA Isolation and Analysis**—Total RNA was isolated from cultured cells using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Northern blot and real-time quantitative reverse transcription-PCR analysis were as described previously (17). Specific primer pairs for PCR were designed as follows: RBP4, 5′-CAGCCTTCCCTCAGTTGTTG-3′ and 5′-AGACGACCACTTTCGATCTTT-3′; and distal (−9644) 5′-AGACGACCACTTTCGATCTTT-3′ and (−9883) 5′-CCGGTTAAATGCAGAGGTA-3′, and PCR conditions were optimized to allow semiquantitative measurements. Conditions used were 25 cycles of 30 s at 90 °C, 15 s at 58 °C, and 1 min at 72 °C. PCR products were visualized on 2% agarose/Tris acetate EDTA gels.

**Immunostaining**—Cells were fixed with 4% formaldehyde in phosphate-buffered saline for 10 min. Nonspecific binding of antibodies was blocked by 5% sheep serum for 60 min, after which cells were incubated with primary antibody in 5% sheep serum for 60 min. Bound primary antibodies were visualized with a secondary antibody (18). After extensive washing with an ECL wash, slide glasses were mounted with IMMU-Mount (Thermo Fisher Scientific). Cells were observed using confocal fluorescence microscopy (OLYMPUS).

**Statistical Analysis**—Statistical analysis was performed using two-tailed paired t tests; p < 0.05 was considered statistically significant.

**RESULTS**

**Lentiviral shRNA-mediated Knockdown of GLUT4 in 3T3-L1 Adipocytes**—Currently, small interfering RNA (siRNA) is the most potent and widely used method for inhibiting gene expression in cell cultures. siRNA can be synthesized chemically or expressed from plasmid vectors. Although siRNAs are powerful tools for targeted inhibition of gene expression, the transfection efficiency of siRNA can be limiting in some cell types, including 3T3-L1. Even with successful siRNA delivery, knockdown effects are generally short-lived. Therefore, we
used the lentiviral shRNA method to delete GLUT4 from 3T3-L1 adipocytes (14).

We subcloned shRNA against GLUT4 into the lentivirus expression plasmid and transfected them into 293T cells. Control cells were transfected with the expression plasmid alone. After 48 h post-transfection, pseudovirus-containing supernatants were collected. 3T3-L1 preadipocytes were infected with pseudovirus and then differentiated into adipocytes to assess the role in glucose transport. As shown in Fig. 1A, infected cells (GLUT4 knockdown 3T3-L1; G4KD-L1) showed markedly reduced endogenous GLUT4 expression, other proteins such as GLUT1, peroxisome proliferator-activated receptor γ, C/EBP, and β-actin were not affected however, RBP4 was affected as described (7–13). RBP4 mRNA was significantly increased in G4KD-L1 cells compared with the control (Fig. 1B); suggesting Rbp4 gene expression was up-regulated in response to GLUT4 knockdown. Insulin-stimulated 2-[3H]deoxyglucose uptake was reduced by ~70% in G4KD-L1 cells compared with the control cells (Fig. 1C). Thus, the lentiviral shRNA-mediated system provided efficient knockdown of endogenous GLUT4 in 3T3-L1 adipocytes.

The Mapped Core Element, G4KA Sequence, in Response to GLUT4 Knockdown-dependent Transcriptional Activation in Adipocytes—To identify the core element in response to GLUT4 knockdown, functional analysis was performed using the entire sequence of the Rbp4 promoter and a series of promoter deletion mutants in transient transfection assays using G4KD-L1 and control cells. Although almost the entire region of the Rbp4 promoter-reporter construct (Fig. 2A, FULL; ~6.5 kb) was inactivated during transcription, the R9 (~1660 to +183) promoter-reporter construct was significantly up-regulated in G4KD-L1 cells compared with control cells (Fig. 2A, lanes 9 and 10). Transcriptional activities of the R10 (~1059 to +183) construct did not differ between the G4KD-L1 and control cells. Suggesting the R9-specific 600-bp sequence (~660 to ~1060) contained the core element. Even though the FULL (Fig. 2A, lanes 1 and 2) and RV (Fig. 2A, lanes 7 and 8) constructs also contained this 600-bp sequence, additional upstream DNA sequences may include negative DNA elements for transcriptional activation of Rbp4 (see “Discussion”).

We then investigated an R9-specific 600-bp deletion using reporter plasmids containing a thymidine kinase (TK) TATA box to potentiate basal transcriptional activity. As shown in Fig. 2B, a 60-bp DNA sequence within R9 (~1300 to ~1241) is sufficient for activation in G4KD-L1 adipocytes (lanes 13 and 14). Further deletion experiments indicated only 10 bp of the sequence (R9-f-2; ~1290 to ~1281) is essential for activation in G4KD-L1 adipocytes (Fig. 2C, lane 3). Mutational analysis showed that any nucleotides changes to the 10 bp resulted in <50% transcriptional activity when compared with the wild-type (Fig. 2D, lanes 1 to 8). Disruption (deletion and mutation) of the R9-f-2 sequence, in the context of the full-length promoter construct, significantly reduced transcriptional activity in G4KD-L1 and control cells (supplemental Fig. S1). These results demonstrate that the 10-bp R9-f-2 sequence is required for full transcriptional activity. We designated the R9-f-2 sequence as G4KA.

Molecular Cloning of 20 S Proteasome Subunits, PSMB1 and PSMA4, as Binding Partners for G4KA—To isolate and identify the G4KA binding factor, a yeast one-hybrid assay using the G4KA sequence was employed to screen a yeast expression library derived from G4KD-L1 adipocytes. Of 2.6 × 10^7 colonies, five yeast colonies were identified that continued to grow in the presence of 100 mM 3-amino-triazol and contained integrated library inserts. Three independent yeast colonies contained nucleotide sequence consisting of 240 amino acid residues of the mouse 20 S proteasome β subunit PSMB1 (GenBank™ accession no. NP035315.1). A further two independent positive clones contained a sequence consisting of 261 amino acid residues of the 20 S proteasome α subunit, PSMA4 (GenBank™ accession no. NP036096.1). The 20 S proteasome is the central enzyme involved in nonlysosomal protein degradation in both the
cytosol and nucleus. The two subunits are members of the N-terminal nucleophile-hydrolase superfamily. Although both 20 S Proteasome Subunits Bind to the G4KA Element in Vitro and in Vivo, PSMB1 Had an Enhanced Binding Efficiency in GLUT4 Knockdown—To investigate the ability of PSMB1 and PSMA4 to bind to the G4KA sequence, we performed EMSA using in vitro-translated PSMB1 and PSMA4 and a 32p-labeled G4KA oligonucleotide probe. We detected DNA-protein complexes whose mobility shifted upon incubation with PSMB1 and PSMA4 proteins (Fig. 3A, lanes 3 and 5). The complexes disappeared when cold G4KA (wild-type) competitor was added (Fig. 3A, lanes 4 and 6). When PSMB1 or PSMA4 proteins were incubated in EMSAs with a G4KA probe, the binding protein complex was supershifted after incubation with an antibody to PSMB1 (Fig. 3A, lane 7) or to PSMA4 (lane 8), suggesting PSMB1 and PSMA4 can bind to the G4KA sequence in vitro. Next, we performed EMSA using cytoplasmic and nuclear extracts from control and G4KD-L1 cells, respectively. Although we detected DNA-protein complexes after incubation with cell extracts from both control and G4KD-L1, stronger binding was observed using nuclear extracts from G4KD-L1 (Fig. 3B, compare lane 2 to 5). Incubation of excess unlabeled G4KA wild-type competitor in the EMSA reactions caused the probe signal to disappear (Fig. 3B, lane 11), but the probe signal did not disappear completely after incubation with excess unlabeled mutated competitors (sequences are shown in Fig. 2D, M1, M2, M3, M5, and M6) (Fig. 3B, lanes 6–10). A trace level of probe signal was observed when the mutated oligonucleotide was used as a probe (Fig. 3B, lanes 12–14). These results suggest that there are nuclear proteins that bind to the G4KA sequence in a G4KA sequence-specific manner. It has been demonstrated that PSMB1 also binds to a gastrin response element (GRE) in the vascular monoamine transporter type 2 promoter (19). The GRE of this promoter consists of a 5′-CCCTCCGCCC-3′ sequence, which is different from the G4KA sequence 5′-TTCTCTAAAC-3′. We investigated the binding properties of PSMB1 to these DNA sequences. Examination by EMSA revealed that the G4KA-pro-
tein complex (containing PSMB1) (Fig. 3C, lane 1) gradually disappeared when an unlabeled G4KA competitor was added in a dose-dependent manner (Fig. 3C, lanes 2–6); however, the G4KA-PSMB1 complex was not affected by unlabeled G4KA even though the same amount of GRE competitor was used (Fig. 3C, lanes 7–11). Conversely, the GRE-PSMB1 complex was not affected by unlabeled G4KA (Fig. 3C, lanes 13–17). Sequence specific binding of PSMB1 to GRE was observed (Fig. 3C, lanes 18–22); however, the PSMB1-GRE complex was not competed by G4KA (lanes 13–17). These observations suggested that PSMB1 binds both G4KA and GRE by different mechanisms. In agreement with this idea, binding of the GRE-PSMB1 complex was disrupted by incubation of an antibody to PSMB1 (19). This was reproduced in our assay (Fig. 3D). In contrast, as shown previously in Fig. 3A, the G4KA-PSMB1 complex was supershifted by a PSMB1 antibody. These results suggest that PSMB1 binds both sequences of G4KA and GRE at different sites within PSMB1.

Next, we investigated the binding of these 20 S proteasome subunits to intact cells using ChIP assays. The binding of 20 S proteasome subunits to the G4KA element was monitored in G4KD-L1 and control 3T3-L1 adipocytes, using primers that encompass the G4KA element on the genome (Fig. 4, top). In G4KD-L1 and control cells, a similar level of PSMA4 binding to G4KA was measured. PSMB1 binding only was increased in G4KD-L1 cells (Fig. 4). A regulatory particle of the 19 S proteasome, PSMC1, did not bind to the G4KA element.

**PSMB1 Is a Transcription Factor for Rbp4**—Proteasome subunits are ubiquitously expressed. To determine the dependence of RBP4 transcription on individual proteasome subunits, we used RNA interference to knock down expression of PSMB1 and PSMA4 in 3T3-L1 cells. As shown in Fig. 5A, knockdown of PSMB1 markedly reduced luciferase activity (lane 2) but RNA interference of PSMA4 did not (lane 7), even though endogenous PSMB1 and PSMA4 protein levels were suppressed as a same degree (middle panels). Luciferase activities returned to control levels after co-transfection of a plasmid vector expressing PSMB1, in a dose-dependent manner (Fig. 5A, lanes 3–5). Next, we determined whether the knockdown and overexpression of PSMB1 alters endogenous RBP4 expression. Knockdown of PSMB1 markedly reduced RBP4 mRNA expression (Fig. 5B, lane 2). RBP4 mRNA returned to former levels after co-transfection of the PSMB1 expression vector, in a dose-dependent manner (Fig. 5B, lanes 3–5). Our results suggest PSMB1 is a transcription factor for Rbp4.

**Proteasome Activity Is Not Essential for RBP4 Transcription**—To establish whether the effect of transcriptional activation is proteasome-dependent, we examined G4KA (R9–f–2) TK-promoter-reporter construct transcription in the presence or absence of the proteasome inhibitor MG-132. When 3T3-L1 adipocytes were incubated in the presence of low dose MG-132, luciferase activity was raised slightly when compared with the control. There was no further luciferase activity increase in the presence of a high dose of MG-132, suggesting that the transcription-related effect is independent of proteasome activity (supplemental Fig. S2).

**Enhancement of PSMB1 Nuclear Transport in GLUT4 Knockdowns**—Fluorescent immunohistochemistry confirmed that PSMB1 was expressed in the nucleus and was also present within the cytoplasmic compartment in control 3T3-L1 cells (Fig. 6A, a–c). Interestingly, PSMB1 expression was markedly enhanced in the nucleus of G4KD-L1 cells compared with control 3T3-L1 cells (Fig. 6A, d–f). Biochemical analysis also showed accumulated PSMB1 protein in the G4KD-L1 nuclear fraction when compared with control 3T3-L1 cells. In agreement with enhancement of nuclear transport, cytoplasmic PSMB1 protein was slightly reduced in G4KD-L1 cells (Fig. 6B). PSMB1 expression levels (mRNA and protein) in whole cell lysates were slightly increased in G4KD-L1 compared with 3T3-L1 cells, but differences were not significant (data not shown). PSMA4 expression was observed in both nucleus and cytoplasm, and its distribution was the same between G4KD-L1 and control cells (Fig. 6A, g–l, and Fig. 6B). Our results suggest PSMB1 is expressed in the nucleus and cytoplasm and is predominantly imported into the nucleus in response to GLUT4 knockdown.

**DISCUSSION**

*Rbp4* expression has been well characterized (20, 21). Most of the RBP4 protein is synthesized in the liver and is secreted into the blood stream, where it functions as the transport protein for retinol (1). Studies on the *Rbp4* promoter have been performed using hepatic cell lines. In the present study, we have demonstrated, for the first time, systematic examination of *Rbp4* promoter analysis using adipocytes as target cells. Our results confirmed the G4KA element was transcriptionally active in GLUT4 knockdown adipocytes.

It has been demonstrated that tissue specific control of *Rbp4* expression is achieved through a mechanism in which negative regulation plays a major role (21). Two different elements within a region 334 bp upstream from the transcriptional start site of the human *RBP4* promoter were found to be necessary and sufficient for hepatocyte-specific expression. In addition to this proximal sequence, other promoter sequences within a 1200-bp region likely are to be involved in the interaction with repressor molecules (21). In agreement with this, our studies demonstrated low level expression when the entire sequence of the mouse *Rbp4* promoter was used in a reporter construct,

**FIGURE 2. Identification of an element in response to GLUT4 knockdown in the mouse *Rbp4* promoter.** A, luciferase assays using a series of mouse *Rbp4* promoter deletion mutants. The entire putative sequence of the *Rbp4* promoter (6.5 kb (FULL)) was deleted (5′–IR9, R10, R11) or internal (RI, NI, RV), and a luciferase-reporter gene was constructed. The 5′- and 3′-end points are indicated relative to the transcriptional start site (+1). These were transfected into G4KD-L1 or control cells. Results shown represent at least three independent experiments. Values are means ± S.D. * p < 0.05. A responsive region (−1660 to 1060) in G4KD-L1 cells in the R9 construct is indicated by a box. PDE6C, phosphodiesterase 6C; GPR120, G protein-coupled receptor 120. B, 600-bp of the G4KD-L1 responsive region was further deleted by 60 bp and linked to the TK-luc reporter plasmid. Transfections were performed as in A. A responsive element (−1300 to −1241) in G4KD-L1 cells in the R9–f–2 TK construct is indicated by a box. C, 60 bp of the G4KD-L1 responsive element was divided into six fragments (10-bp each) and linked to the TK-luc reporter plasmid. Transfections were performed as in A. 10 bp (G4KA element; see text) of the sequence responsive to GLUT4 knockdown is indicated by a box, n.s., not significant. D, mutational analysis of the G4KA sequence was performed. Mutated residues are indicated by italics. The nucleotide changes showed <50% transcriptional activity when compared with the control.
FIGURE 3. Binding properties of PSMB1 and PSMA4 to the G4KA sequence in vitro. A, PSMB1 and PSMA4 are sequence-specific binding factors for the G4KA element in vitro. EMSA was performed using in vitro-translated PSMB1 or PSMA4, together with a radiolabeled G4KA probe (5 ng). Unlabeled G4KA (wild-type) oligonucleotides (50 ng) were used as cold competitor (comp.). Supershift assays were performed using antibodies against PSMB1 and PSMA4. A nonspecific binding factor (indicated by asterisks) was present in the leticurocyte lysate (lys.). B1, anti-PSMB1 antibody; A4, anti-PSMA4 antibody; WT, wild-type. B, sequence-specific G4KA binding factors in cytoplasmic and nuclear extracts. EMSA was performed using radiolabeled probes (5 ng) with 20 μg of control cell cytoplasmic (cc), control cell nuclear (cn), G4KD-L1 cell cytoplasmic (gc), and G4KD-L1 cell nuclear (gn) extracts. 10 ng (2-fold excess of probe) of unlabeled oligonucleotides (sequences M1, M2, M3, M5, and M6 are shown in Fig. 2D) were used as cold competitors. C, sequence-specific binding properties of PSMB1 to G4KA and GRE. EMSA was performed using G4KD-L1 nuclear (gn) extract with 5 ng of G4KA (lanes 1–11) and 5 ng of GRE (lanes 12–22) probe. Unlabeled cold competitors, 1, 3, 5, 10, and 50 ng were incubated in the EMSA assays as indicated. D, binding of the GER-protein complex was disrupted by incubation of αB1. Arrows indicate specific DNA-protein complexes.
even when the G4KA element was included. In adipocytes, the transcriptional activation element of the Rbp4 promoter is located far upstream (1.3 kb) from the transcriptional start site; this is different in hepatocytes.

To identify factors which bind to the G4KA element, a yeast one-hybrid assay was performed using a G4KD-L1 cell cDNA library. We identified 20 S proteasome subunits, PSMB1 and PSMA4, as binding partners for the G4KA element. The ubiquitin-proteasome system plays a central role in modulating the intracellular levels of a wide range of regulatory molecules, generally those with short lives, including transcription factors (22), suggesting a mechanistic link between transcriptional activation and the turnover of the activator by the ubiquitin-proteasome system. A direct influence of proteasome-dependent proteolysis on transcription has been demonstrated: phosphorylation of the yeast transcription factor Gcn4 by the cyclin-dependent kinases Srb10 and Pho85 leads to proteasome-dependent degradation (23). Because Srb10 is a component of the RNA polymerase II holoenzyme, it was concluded that Gcn4 binds to the promoter region, activates Gcn4-dependent genes, and is destroyed by SCF\(^{Cdc4}\), the ubiquitin ligase for Gcn4. It is proposed that proteolysis is required to remove activators and reset the promoter. Confirming this idea, studies have shown that mutation of SCF\(^{Cdc4}\) and mutations in ubiquitin that prevent proteolysis impair the transcription of Gcn4 targets (24). Ubiquitin-mediated degradation of transcription factors, including MyoD (25), Sox9 (26), interferon regulatory factor-1 (27), and nuclear receptors (28–34) also are important for transcriptional regulatory mechanisms in mammalian cells.

**FIGURE 4. ChIP assays.** PSMB1 and PSMA4 also bind to the G4KA element in vivo, but PSMB1 binding efficiency was enhanced in G4KD-L1 cells when compared with control cells. Soluble chromatin was prepared from control and G4KD-L1 cells and immunoprecipitated (IP) with the indicated antibodies. Extracted DNA samples were amplified using primer pairs covering the G4KA region or a distal region (8.5 kb upstream of G4KA) as a control (Contl.). The top schematic diagram illustrates the G4KA-contained and distal regions amplified by PCR.

**FIGURE 5. RNA interference knockdown of proteasome subunits.** A (top), 3T3-L1 cells were transfected with shRNA expression plasmids (1 μg/well) for PSMB1 (lanes 2–5) or PSMA4 (lanes 7–10) together with a G4KA (R9-f-2) TK promoter-reporter construct. Luciferase assays were performed as in Fig. 2. I.B., immunoblot. Note that shRNA expressing PSMB1 (shPSMB1) reduced transcription; this was rescued by dose-dependent introduction of a PSMB1 expression vector, PSMB1-pcDNA3.1D/V5-His-TOPO (lane 3, 10 ng; lane 4, 50 ng; and lane 5, 100 ng). (middle) The knockdown efficiency and recombinant (**r**) protein expression were determined by Western blotting with the indicated antibodies. β-Actin was used as loading control values are means ± SD. *, p < 0.05. B, RNA interference knockdown of PSMB1 reduced endogenous Rbp4 mRNA expression. 3T3-L1 cells were co-transfected with shRNA expression plasmid for PSMB1 and a PSMB1 expression vector as described in A. Total RNA was extracted, and quantitative reverse transcription-PCR was performed using specific primers for RBP4 and β-actin.
Proteasome Subunit-dependent RBP4 Transcription

Thus, the proteasome is essential for effective transcriptional regulation. However, further studies on the subunits of the proteasome might suggest they have a direct role in transcription that is independent of their proteolytic function (35, 36). Moreover, it seems that the 19 S regulatory complexes can also target transcriptional co-activators to promoters (37). Both 19 S and 20 S proteasome subunits have been demonstrated to interact with chromatin at the sites of promoters (38, 39). The finding that the 19 S and 20 S subunits displayed differential patterns of binding in genome-wide ChIP assays in yeast supports the idea that they may have a degree of independent function during transcription (40). Consistent with this, our ChIP analysis shows that nuclear factors binding to the G4KA element are detectable by antibodies against PSMB1 and PSMA4 but not by an antibody against a regulatory subunit of the 19 S proteasome, PSMC1. A comparison between PSMB1 and PSMA4 confirms there are no similarities in amino acid sequence, and they have no presumed DNA binding motifs. It has been demonstrated that PSMB1 binds to a GRE in the vascular monoamine transporter type 2 promoter (19). The GRE consists of a 5'-'CCCTC-CGCC-3' sequence that is different from the G4KA sequence. PSMB1 binds independently to G4KA and GRE, suggesting there are at least two distinct DNA binding motifs in the PSMB1 molecule. In addition, interacting factor(s) (e.g. other proteasome subunits) influence the DNA binding ability to distinct DNA sequences. The precise mechanisms of how these proteasome subunits recognize their target sequence remains to be established, but they must physically interact with the promoter regions (22).

PSMB1 siRNA inhibits Rbp4 transcription, suggesting PSMB1 is a transcription factor for Rbp4. In support of this, exogenous overexpression of PSMB1 leads to a recovery from siRNA-inhibited transcription. A role for PSMA4 in Rbp4 expression is unclear. PSMA4 certainly binds to the G4KA element, both in vitro and in vivo, although it does not seem to be involved in transcription. The actual function of PSMA4 remains to be investigated. We cannot rule out nuclear functions, such as involvement in machineries at DNA-damaged and nuclear quality control sites, for example (22, 38).

The proteasome inhibitor MG-132 only moderately elevated RBP4 transcription, suggesting that activation of transcription is independent of proteasomal function. Proteasomes are reported to be transported unidirectionally from cytoplasm to nucleus (47), and functional nuclear localization signals have been identified on a number of subunits (43, 44). In PSMB1, there are sequences of basic amino acid residues, which are putative nuclear localization signals. In the present study, nuclear staining of PSMB1 was observed in 3T3-L1 adipocytes, together with punctuate staining throughout the cytoplasm. Further nuclear accumulation of PSMB1 was observed by immunohistochemical and biochemical analysis in G4KD-L1 adipocytes. PSMB1 expression levels (mRNA and protein) in whole cell lysate were slightly increased in G4KD-L1 adipocytes compared with 3T3-L1 cells, but the data were not significant (data not shown). Nuclear localization of the 20 S proteasome has been associated with particular diseases (45). A study group of severely obese adults with hypertension and diabetes had increased 20 S proteasome levels when compared with the just obese group (46). Our findings suggest GLUT4 knockdown leads to PSMB1 transportation into the nucleus and sequential activation of RBP4 transcription.

We demonstrated that the 20 S proteasome subunit PSMB1 enhanced Rbp4 expression in response to GLUT4 knockdown. Distribution of proteasomes in the cell changes during the cell cycle and development; therefore, their transport must be regulated. Proteasomes could remain in the cytoplasm, whereas the nuclear localization signal of the proteasomes is masked by an intramolecular interaction.
The proteasomes could become nuclear after a conformational change, induced by post-translational modifications, such as tyrosine phosphorylation (43). Tyrosine phosphorylation is known to act as an intracellular second messenger for transduction of external signals stimulated by various hormones and growth factors. Therefore, it may serve to initiate inducible or selective translocation of proteasomes (43). Domain research analysis (PhosphoSitePlus) illustrated that there is a putative tyrosine phosphorylation site (Tyr149) in the PSMB1 molecule (47). Whether this site is involved in PSMB1 function remains to be identified; we propose that modulation of PSMB1 function could be important in insulin resistance.

The molecular mechanism of insulin resistance and Rbp4 expression in adipocytes remains to be investigated (47). In the present study, we show the 20 S proteasome subunit PSMB1 has a crucial role in RBP4 expression. Serum RBP4 levels might contribute to systemic insulin resistance in mice (8) and humans (9–13). Therefore, we propose that modulation of PSMB1 function could be of importance in this subject.

Acknowledgments—We thank Professor K. Yamada (Kagawa Nutrition University) and Professor T. Esashi (Kanagawa Institute of Technology) for support and encouragement.

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Proteasome Subunit-dependent RBP4 Transcription