Expression of Inducible 6-Phosphofructo-2-Kinase/ Fructose-2,6-Bisphosphatase/PFKFB3 Isoforms in Adipocytes and Their Potential Role in Glycolytic Regulation

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6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase) catalyzes the synthesis and degradation of fructose 2,6-bisphosphate (F2,6BP), which is a powerful activator of 6-phosphofructo-1-kinase, the rate-limiting enzyme of glycolysis. Four genes encode PFK-2/FBPase (PFKFB1–4), and an inducible isoform (iPFK-2/PFKFB3) has been found to mediate F2,6BP production in proliferating cells. We have investigated the role of iPFK-2/PFKFB3 and related isoforms in the regulation of glycolysis in adipocytes. Human visceral fat cells express PFKFB3 mRNA, and three alternatively spliced isoforms of iPFK-2/PFKFB3 are expressed in the epididymal fat pad of the mouse. Forced expression of the iPFK-2/PFKFB3 in COS-7 cells resulted in increased glucose uptake and cellular F2,6BP content. Prolonged insulin treatment of 3T3-L1 adipocytes led to reduced PFKFB3 mRNA expression, and epididymal fat pads from db/db mice also showed decreased expression of PFKFB3 mRNA. Finally, anti-phospho-iPFK-2(Ser461) Western blotting revealed strong reactivity in insulin-treated 3T3-L1 adipocyte, suggesting that insulin induces the phosphorylation of PFKFB3 protein. These data expand the role of these structurally unique iPFK-2/PFKFB3 isoforms in the metabolic regulation of adipocytes. Diabetes 54:3349–3357, 2005
Alternative splicing of the variable, COOH-terminal region of the PFKFB3 gene can lead to the expression of six structural isoforms in human brain (17). Although these isoforms have been reported to differ in the structure of their COOH termini, their precise physiological roles are unknown. In the present report, we have explored the expression and activity of different PFKFB3 isoforms in adipose tissue with the goal of initiating an investigation of the potential role of these enzymes in the metabolic changes underlying obesity.

**RESEARCH DESIGN AND METHODS**

Insulin, dexamethazone, and 3-isobutyl-1-methylxanthine (IBMX) were obtained from Sigma-Aldrich (St. Louis, MO). Trogloxitane was a gift from Sankyo (Tokyo, Japan). A rabbit polyclonal anti-phospho-iPFK-2 (Ser461) antibody was raised against the phosphorylated peptide RBN(Sp)YTP (corresponding to residues 458–463 of human iPFK-2 in which Ser461 was phosphorylated) (18). Goat polyclonal antibody (anti-PFK-2-bp) N-11 antibody, which reacts with the mouse iPFK-2/PFKFB3 isoform, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Formalin-fixed, paraffin-embedded human fat tissue was obtained from Novagen (Madison, WI).

3T3-L1 cells and COS-7 cells were obtained from American Type Cell Culture (Manassas, VA). Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT) at 37°C in a humidified 5% CO₂ incubator. For adipocyte differentiation, 3T3-L1 cells were stimulated after 2 days of confluence with 10 μg/ml insulin, 0.5 mmol/l IBMX, and 1.0 mmol/l dexamethazone. After 48 h, the medium was replaced with DMEM supplemented with 10% FBS. Fresh medium was added every 48 h.

C57BL/KsJ-db/db/db Jcl mice and C57BL/KsJ-t/m+ t/m+ Jcl mice were obtained fromCLEA Japan (Tokyo, Japan). This study was approved by the Animal Experiment Ethics Committee of the Graduate School of Medicine of Hokkaido University.

**In situ hybridization.** PFKFB3 is distinguished from other members of the PFKFB family by the presence of AU-rich element in 3’ UTR of its mRNA. Therefore, we designed a specific probe for PFKFB3 mRNA that included the AU-rich element. The procedure of in situ hybridization is described in a previous report (14).

**Cloning of the mouse adipocyte PFKFB3.** Epididymal fat pads from male C57BL6 mouse (10 weeks of age) were harvested and frozen in liquid nitrogen. The frozen tissue was homogenized, and the total RNA was extracted using the RNeasy Mini kit (Qiagen). Total-length mouse adipocyte cDNA was amplified with specific primers coding for mouse PFKFB3 (5’-ATGCCGTTGGAACTGA-CCA-3’ and 5’-GTTGCTCTTGGAAGAGTCGGCAC-3’). The PCR conditions were initial denaturation for 3 min at 95°C, 35 cycles of 90 s at 95°C, 30 s at 61°C, and 1 min at 72°C, and finally 10 min at 72°C. The PCR products were separated by electrophoresis on a 5% polyacrylamide gel. PCR was performed in a Perkin-Elmer model 2400 thermal cycler (Applied Biosystems) In accordance with the previous findings, the length of the resultant PCR product enabled us to distinguish between the different PFKFB3 splicing variants. Amplified fragments from each PCR were cloned into pCR2.1 vector (Invitrogen) and sequenced using the dye terminator cycle sequencing kit (Applied Biosystems).

**Quantitative real-time RT-PCR.** Quantitative real-time RT-PCR for PFKFB3 mRNA was performed using the ABI 7000 Sequence Detector (Applied Biosystems) using a Quantitect SYBR green RT-PCR kit (Qiagen). The following primers were used for specific amplification for mouse PFKFB3: 5’-AGAACCTTCCAATCCTCCCCACCAA-3’ and 5’-AGGGATGATGTCCCATGTTGAAAGA-3’ (GenBank accession no. AF294617). The specificity of each PCR product was routinely checked by melting curve analysis and by agarose gel electrophoresis.

**Northern blot analysis.** As described before, PFKFB3 is distinguished by the presence of AU-rich element in its 3’ UTR. Therefore, mouse PFKFB3 3’ UTR containing the AUUUA motif (corresponding to mouse PFKFB3 3669–3688) was cloned into the pCB3 vector (Invitrogen), and antisense RNA probes were synthesized with the DIG RNA Labeling kit (Roche Diagnostics). This probe is specific for PFKFB3 and does not react with other members of the PFKFB family. Northern blot procedures are described in the previous report (14).

**Transfection of COS-7 cells.** Cells were cultured in 6-well plates in DMEM/10% FBS and transfected PFKFB3 mRNA in pCDNA3.1/V5-His vector using a FuGENE6 reagent (Roche Diagnostics) according to the manufacturer’s protocol. After 48 h, the cells were used for experiments. The expression of recombinant protein was confirmed by Western blotting using an anti-V5 antibody, which recognizes short amino acid sequences fused to the recombinant gene within the cloning vector.

**Glucose transport assay.** Cells plated in 6-well culture dishes were washed with glucose transport solution (140 mmol/l NaCl, 20 mmol/l HEPES/Na, 5 mmol/l KCl, 2.5 mmol/l MgSO₄, and 1 mmol/l CaCl₂) and incubated for 10 min with 2-deoxy-D-glucose. The cells then were washed three times with transport solution and lysed with 0.8 ml 50 mmol/l NaOH. The lysates then were collected, and the radioactivity was measured by scintillation counting.

**Measurement of F2,6BP.** Intracellular F2,6BP content was measured using Van Schaftingen’s method after the disruption of cells in 0.8 ml 50 mmol/l NaOH (8).

**Statistical analysis.** Results are expressed as means ± SD. Statistical significance of difference was assessed by Student’s t test.

**RESULTS**

**Detection of PFKFB3 mRNA in human visceral fat tissue.** To examine the expression of PFKFB3 mRNA in human visceral fat, we designed an RNA probe that was complementary to the 3’ UTR, including the AU-rich element, of PFKFB3 and developed an in situ hybridization method. This probe is specific for PFKFB3 mRNA and does not cross-react with other members of the PFKFB family (14). We also confirmed the detection of a single, expected band corresponding to the PFKFB3 5.4-kb mRNA using this probe in Northern blot analysis of different human tumor tissues (data not shown). As shown in Fig. 1, PFKFB3 mRNA is readily detectable within adipocytes, and no cell-associated signals were detected with the PFKFB3 sense probe.

**Cloning of the PFKFB3 from mouse adipose tissue.** A previous report has provided evidence for six distinct isoforms of the PFKFB3 gene product in human brain (17). Alternatively spliced isoforms of PFK-2/FBPase also were reported in a study of the rat brain (19,20). To examine the expression pattern of the PFKFB3 in mouse adipocytes, total RNA was extracted from mouse (C57BL/6) epididymal fat pads, the cDNA was synthesized by reverse transcriptase, and the PFKFB3 cDNA was amplified and cloned into the pcDNA3.1/V5-His vector. Three different isoforms thus were identified, designated PFKFB3-ABCG,
PFKFB3-ACG, and PFKFB3-AG (Fig. 2A). These sequences differ in the predicted structure of their COOH termini and in their overall exonic organization. The variable region of COOH termini of PFKFB3 in human and rat brain is derived from seven exons, as described previously, whereas the 3'-variable region of mouse adipocyte PFKFB3 is encoded by four exons (Fig. 2B). The structures of exons A, C, and G in mouse PFKFB3 gene are homologous to those of the rat and human PFKFB3 gene. The shortest isoform, PFKFB3-AG, contains exons A and G. The exon-intron structure of PFKFB3-AG has not been identified in rat brain, but it has been described in human brain PFK-2 (17). The expression of PFKFB3 isoforms are induced during 3T3-L1 adipocyte differentiation. Numerous genes are induced as part of the adipocyte differentiation program. We next investigated the expression of PFKFB3 in cultured mouse 3T3-L1 cells induced to undergo adipocyte differentiation. We designed specific primers for amplification of PFKFB3 and an antisense RNA probe that is specific for PFKFB3 mRNA 3' UTR, as described in RESEARCH DESIGN AND METHODS. Quantitative real-time RT-PCR

FIG. 1. Analysis of PFKFB3 mRNA expression in human visceral fat. A: Tissue sections were examined by hematoxylin and eosin. Tissue sections were examined by in situ hybridization using antisense RNA probe (B) and sense RNA probe (C). Magnification ×400.

FIG. 2. Comparison of the COOH-terminal variable regions of the mouse PFKFB3 in adipocytes. Alignment analysis of the nucleotide sequences of different PFKFB3 isoforms in mouse adipocytes. The alignment is shown downstream of the nucleotide 1346 of the mouse PFKFB3. Residues within the solid boxes indicate identities. A: Gaps are shown as dashes. B: Schematic diagram shows the intron-exon organization of the variable region of the mouse PFKFB3 in adipocytes.
compared with the control value. IB, immunoblot.

Point represents the means ± SD of three different samples. *P < 0.05 compared with the control value. IB, immunoblot.

analysis and Northern blot analysis of 3T3-L1 cells showed that the expression of PFKFB3 mRNA was significantly increased during differentiation (Fig. 3A and B). Because of the low sensitivity of antisense RNA probe used in Northern blot analysis, which is unavoidable given the close sequence homology among the different isoforms, the induction of PFKFB3 mRNA in day 4 is not clearly defined by Northern blotting. Nevertheless, there is clear and apparent induction of PFKFB3 mRNA observed by real-time RT-PCR analysis. As shown in Fig. 3C, the production of PFKFB3 protein increased during adipocyte differentiation, as assessed by Western blot analysis. The induction and increase in the level of PFKFB3 protein was similar to that observed for peroxisome proliferator-activated receptor (PPAR)-γ, which is a well-characterized feature of adipocyte differentiation. Intracellular F2,6BP levels also increased significantly during adipocyte differentiation (Fig. 3D).

The expression pattern of splicing variants for PFKFB3 during 3T3-L1 adipocyte differentiation. We analyzed the expression pattern of the splicing variants of PFKFB3 during 3T3-L1 adipocyte differentiation by RT-PCR analysis. As described in the Research Design and Methods, we selected a forward primer that was complimentary to a sequence in the constant region of PFKFB3 cDNA and a reverse primer that was located close to the stop codon (17). Amplification with these primer pairs revealed three PCR products of sizes 345, 270, and 96 bp that correspond to the designated PFKFB3 isoforms: PFKFB3-ACG, PFKFB3-AG, and PFKFB3-AG. Sequence analysis also confirmed that each isoform contained the COOH-terminal variable region of PFKFB3 (data not shown). The expression of these isoforms, in particular PFKFB3-ACG and PFKFB3-AG, was significantly increased during 3T3-L1 adipocyte differentiation (Fig. 4). Taken together, these findings indicate that the increase in glycolytic flux that accompanies adipocyte differentiation is associated with PFK-2 expression: an increase in the level of the mRNAs for PFKFB3 and in particular the PFKFB3-ACG and PFKFB3-AG.

Troglitazone induces the expression of PFKFB3 mRNA and protein in 3T3-L1 cells. Thiazolidinedione is a ligand for PPAR-γ (21), and it increases the number of small adipocytes in white adipose tissue (22). We examined the effect of troglitazone, one of thiazolidinediones, on the expression of iPFK-2/PFKFB3 in 3T3-L1 cells. As shown in Fig. 5A and B, the expression of PFKFB3 mRNA increases after treatment of troglitazone, as analyzed by quantitative real-time RT-PCR analysis and Northern blotting, and this finding is accompanied by a corresponding increase in immunoreactive PFKFB3 protein (Fig. 5C). The induction and increase in the level of PFKFB3 expression was similar to that observed for PPAR-γ, suggesting a potential role for PFKFB3 in the regulation of glycolysis during triacylglycerol synthesis. We also examined the expression of different splicing variants of PFKFB3 in 3T3-L1 cells stimulated with troglitazone. As shown in Fig. 5D, the expression of PFKFB3-ACG, PFKFB3-AG, and PFKFB3-AG also increased after the administration of troglitazone.

Overexpression of the PFKFB3 results in increased glycolysis. To validate the enzymatic activity and potential physiological role of PFKFB3-ACG and PFKFB3-AG in glycolytic flux, we cloned both isoforms into the pcDNA3.1/V5-His vector and transfected them into COS-7 cells. Transfection efficiencies were calculated by determining the number of transfected cells using β-Gal staining kit (Invitrogen), and 70% of COS-7 cells expressed reporter gene protein. Transfection of each isoform in COS-7 cells resulted in a significant increase in PFKFB3 protein, as revealed by Western blot analysis using an anti-V5 antibody, which recognizes the short amino acid sequence fused to the recombinant gene encoded within the cloning vector (Fig. 6A). Overexpression of PFKFB3 protein also was confirmed by Western blot analysis using an anti–PFK-2 br/pl antibody. PFKFB3-AG and PFKFB3-AG were expressed in equivalent levels in the COS-7 transfection system. As mentioned previously, 1PFK-2/PFKFB3 is phosphorylated at the Ser461 residue, which is located in exon C of COOH-terminal variable region. Accordingly, immunoblotting with an anti–phospho-iPFK-2(Ser461) anti-
body revealed a prominent band corresponding to the 60-kDa iPFK-2/PFKFB3 in the lysates of COS-7 cells expressing PFKFB3-ACG but at significantly reduced levels in COS-7 cells transfected with an PFKFB3-AG. The intracellular content of F2,6BP was significantly increased after transfection by both isoforms (Fig. 6B). Note that COS-7 cells express endogenous PFKF3, which is normally phosphorylated under serum culture conditions (18). The uptake of 2-deoxyglucose into cells also was increased in both cases, indicating that the increased production of these proteins produced a physiologically meaningful increase in glycolytic flux (Fig. 6C).

**Prolonged insulin stimulation results in a reduction of PFKFB3 mRNA.** The physiological impact of insulin on glucose metabolism changes critically with the duration of stimulation of the insulin receptor. We next examined the effect of prolonged insulin treatment on the expression of PFKFB3 mRNA in differentiated adipocytes. 3T3-L1 adipocytes were treated with insulin for 18 h, and the expression of PFKFB3 mRNA was measured by quantitative real-time RT-PCR analysis. As shown in Fig. 7A, the expression of PFKFB3 mRNA in 3T3-L1 adipocytes decreased significantly after long-term insulin stimulation, whereas similar conditions caused no significant change of PFKFB3 mRNA expression in 3T3-L1 preadipocytes, which have less insulin binding activity and less insulin receptor expression when compared with 3T3-L1 adipocytes (data not shown) (23,24). These data suggest a cell-specific mode of regulation of the expression of PFKFB3 mRNA by insulin in adipocytes.

Mice of the genotype db/db have a point mutation in the leptin receptor gene, and they are an established model of type 2 diabetes (25). We next examined the expression of PFKFB3 mRNA in epididymal fat pads obtained from db/db mice. We found that the expression of PFKFB3 mRNA was significantly reduced in the fat pads of db/db mice analyzed by quantitative real-time RT-PCR (Fig. 7B). The expression of PFKFB3 protein in adipose tissue was not significantly different between the db/db mice and control mice (data not shown).

**Insulin-induced serine phosphorylation of iPFK-2/PFKFB3 isoforms in 3T3-L1 adipocytes.** Insulin action is initiated by hormone binding to cell-surface insulin receptors, leading to the tyrosine phosphorylation of several intracellular mediators and downstream metabolic effects (26–29). Monocyte activation is known to occur by a glucose-dependent mechanism, and it is associated with an increase in iPFK-2 protein production and its phosphorylation (16,30–32). We next examined the ability of insulin to phosphorylate PFKFB3 isoforms in cultured adipocytes. The contribution of the phosphorylation of the COOH-terminal serine to the kinetic properties of PFK-2 is well-characterized in the cardiac, PFK-2 isoform. The insulin-induced increase in F2,6BP and heart PFK-2 activity occurs by the phosphorylation of the serine residues, Ser466 and Ser483 (33). Protein kinase B phosphorylates substrates at Ser/Thr in a conserved motif characterized by Arg at position −5 and −3. The amino acid sequences surrounding Ser466 of heart PFK-2 and Ser461 of iPFK-2/PFKFB3 are similar, although iPFK-2/PFKFB3 lacks Arg at position −5. In addition, Ser466 of heart PFK-2 can be phosphorylated in vitro and in vivo by a number of protein kinases (34). We hypothesized that its phosphorylation may similarly upregulate the kinase activity of iPFK-2/PFKFB3 protein. We generated anti—phospho-iPFK-2 (Ser461) antibody as described in RESEARCH DESIGN AND METHODS. Serum-starved, 3T3-L1 adipocytes were exposed to insulin for 5 min, and the proteins from cell extract were separated by SDS-PAGE. The phosphorylation of PFKFB3 isoforms was examined by Western blot analysis using recently characterized anti—phospho-iPFK-2 (Ser461)—specific antibody (18). Insulin significantly increased the serine phosphorylation of iPFK-2/PFKFB3 isoforms (Fig. 8A). Intracellular F2,6BP content increased in parallel with the phosphorylation of PFKFB3 isoforms (Fig. 8B). These data suggest that increased glycolytic flux induced by insulin in 3T3-L1 adipocytes is mediated by phosphorylation of PFKFB3 at position Ser461.

**DISCUSSION**

Insulin stimulates glucose transport via tyrosine phosphorylation of the insulin receptor and insulin receptor substrates (IRSs), leading to the downstream activation of phosphatidylinositol 3-kinase (35). The precise mechanisms responsible for the activation of glycolytic enzymes by insulin are of significant interest. Insulin has been shown to increase intracellular F2,6BP content by activating kinase activity of PFK-2/FBPase in adipocytes (36), but the particular enzymatic isoforms of PFK-2/FBPase that mediate increased glycolytic flux and triacylglycerol synthesis are unknown.

In the present study, we identified PFKFB3 as the isoform that is likely responsible for the activation of glycolysis in
adipocytes. Several lines of evidence support this conclusion. The expression of PFKFB3 mRNA in human visceral fat was confirmed by in situ hybridization, and the expression of both PFKFB3 mRNA and protein was significantly increased during 3T3-L1 adipocyte differentiation induced by insulin, dexamethasone, and IBMX. Further support for this observation is offered by the report that the PFKFB3 is induced by cyclic-AMP-dependent protein kinase signal activation (37). Troglitazone stimulated the induction of PFKFB3 mRNA and PFKFB3 protein expression in 3T3-L1 cells. Moreover, insulin stimulation was associated with phosphorylation of Ser461 residue of PFKFB3 protein. We also observed the coordinate expression of the PFKFB3-ACG and the PFKFB3-AG isoforms during 3T3-L1 adipocyte differentiation, and we confirmed the potential enzymatic activity of these isoforms by transfection studies in COS-7 cells.

Marsin et al. (16) recently demonstrated that the mechanism of the activation of iPFK-2/PFKFB3 involves phosphorylation of Ser461 residue in the COOH-terminal region by AMP-activated protein kinase. The contribution of this phosphorylation event to the kinetic properties of PFK-2 has been well-characterized in heart PFK-2 (33,38–40). However, the expression of heart PFK-2/PFKFB2 in adipocytes is low when compared with iPFK-2/PFKFB3. The expression of the heart PFK-2/PFKFB2 isoform also is not induced during adipocyte differentiation (data not shown), whereas the mRNA for PFKFB3 is significantly increased during this process. These data, taken together, are con-

FIG. 5. The effect of troglitazone on the expression of PFKFB3 mRNA and protein. Quantitative real-time RT-PCR (A) and Northern blotting (B). Western blotting using an anti–PFK-2 br/pl antibody (C). RT-PCR analysis of splicing variants of PFKFB3 mRNA (D). Values are means ± SD for three different samples. *P < 0.05 compared with the control value.

FIG. 6. Effect of forced expression of the recombinant PFKFB3 protein. A: Western blot analysis of COS-7 cells transfected with plasmids encoding PFKFB3-ACG or PFKFB3-AG was carried out using anti–phospho-iPFK-2(Ser461) antibody, PFK-2 br/pl antibody, and anti-V5 antibody. Intracellular F2,6BP content (B) and 2-deoxyglucose (2-DG) uptake (C) were detected as described in RESEARCH DESIGN AND METHODS.*P < 0.05 compared with the control value. IB, immunoblot.
sistent with an important role for PFKFB3 in the synthesis of triacylglycerols in adipocytes.

The protein kinase that is responsible for the phosphorylation of Ser461 of iPFK-2/PFKFB3 has not been clarified. The Ser461 residue of brain PFK-2/PFKFB3 (which may be alternatively numbered as Ser460) is reported to be phosphorylated by protein kinase B and other protein kinases but without activation of PFK-2 in vitro (41). Instead, phosphorylation of Ser460 decreased the sensitivity of the PFK-2 to the potent allosteric inhibitor, phosphoenolpyruvate (41). These data support the notion that phosphorylation of the PFKFB3 protein regulates PFK-2 activity, which is important for glycolytic flux.

Chronic insulin treatment has been shown to decrease the half-life of IRS-1, which is consistent with a regulatory effect on IRS-1 protein degradation. We hypothesize that the expression of PFKFB3 mRNA in the fat tissue of \( db/db \) mice increases during adipogenesis and that a reduction of

A

IB; anti-phospho-iPFK-2
IB; anti PFK-2 br/pl

B

IB; anti-phospho-iPFK-2
IB; anti PFK-2 br/pl

FIG. 7. A: The effect of prolonged stimulation by insulin on the expression of PFKFB3 mRNA in 3T3-L1 adipocytes was analyzed by quantitative real-time RT-PCR. B: Quantitative real-time RT-PCR analysis of the expression of PFKFB3 in the epididymal fat pad of \( db/db \) mice. Values are the means ± SD for three different samples. * \( P < 0.05 \) compared with the control value.

FIG. 8. The effect of insulin on the serine phosphorylation of PFKFB3. Western blotting using anti–phospho-iPFK-2(Ser461) antibody. A: The bar graph represents quantitative analysis of the results means ± SD for three different samples. * \( P < 0.05 \) compared with the control value. IB, immunoblot.
PFKFB3 mRNA may then occur after prolonged hyperglycemia as a result of a negative feedback mechanism involving insulin. This is supported by the fact that prolonged treatment by insulin causes a reduction in the expression of PFKFB3 mRNA in 3T3-L1 adipocytes. Injection of insulin to fed rats has been shown to decrease F2,6BP content in white adipose tissue; however, such a decrease was not observed in fat pads from starved rats (42). These observations are consistent overall with the notion that the accumulation of F2,6BP via insulin in adipose tissue is regulated by negative feedback system.

In conclusion, we have identified the PFKFB3 structural isoform to be a potential regulator of glycolytic flux in adipocytes. The tissue-specific regulation of the expression and phosphorylation of PFKFB3 may represent novel targets for the treatment of metabolic disorders related to obesity.

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