of DG production as a signal initiated by apoA-I. This view is consistent with our previous findings that the differentiated rat vascular smooth muscle cells that produce cholesterol-poor HDL by apolipoproteins generate cholesterol-rich HDL after stimulation of protein kinase C by phorbol ester and that protein kinase C inhibitors decreased the apoA-I-mediated cholesterol release in macrophages (34). Further investigation is required to clarify whether the translocation of these signal-related molecules takes place to the same lipid-protein particle or to different particles that happen to have the same density.

In agreement with our previous finding that apoA-I induces the translocation of caveolin-1 and newly synthesized cholesterol to the CLPP fraction, this fraction may play a role in intracellular cholesterol transport to the plasma membrane when HDL is generated by apoA-I and may also provide a site for the initiation of signal transduction to induce such cholesterol trafficking. Interestingly, protein kinase Cα phosphorylated at serine-657 was mainly recovered from the free protein fraction in cytosol, although it is increased in the CLPP fraction also by apoA-I stimulation (28). This finding indicates the possibility that the enzyme is translocated to the CLPP and dissociated from the particle by serine phosphorylation. There is no further information for the reactions after the activation of protein kinase Cα.

This rapid initiation of the signaling cascade by apoA-I is apparently different from the relatively slower generation of DG by phosphatidylcholine-specific phospholipase C in the replenishment reaction for sphingomyelin when it is removed by the HDL assembly reaction by apoA-I with cellular lipid (21). This slower reaction is associated with the stabilization of ABCA1 (31). The rapid reaction seems to involve phospholipase Cγ and PI turnover, so that it should be initiated by the interaction of apoA-I with a receptor-like signal-mediating membrane protein, whether directly or indirectly. Although many reports indicated the initiation of the signaling cascade by apoA-I or HDL, there is no clear indication of the signal-mediating membrane protein that may directly interact with apolipoprotein or HDL (35–40). ABCA1 has been identified as a key protein for the generation of HDL by apolipoprotein from cellular lipid, but it is still unclear whether this protein interacts directly with apolipoprotein to generate HDL or plays an indirect role for the HDL assembly reaction (41–45). ABCA1 is an essential molecule for the reaction to generate HDL by apoA-I. Our preliminary experiments indicated the presence of ABCA1 in astrocytes but less stabilization effect by apoA-I. A recent report indicated that ABCA1 is required for the generation of apoE-HDL in the brain (46). However, it is unclear whether ABCA1 is a signal-mediating receptor in the reactions presented in this article.

This work was supported in part by grants-in-aid from the Ministry of Health, Labor, and Welfare and the Ministry of Education, Science, Culture, and Sports and by an International HDL Research Award.

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Biochemical and Biophysical Research Communications 321 (2004) 320–323
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Promoter polymorphism in fibroblast growth factor 1 gene increases risk of definite Alzheimer's disease

Hidehisa Yamagata [a,*], Yusen Chen [b], Hiroyasu Akatsu [c], Kouzin Kamino [d], Jin-ichi Ito [e], Shinji Yokoyama [e], Takayuki Yamamoto [c], Kenji Kosaka [c], Tetsuro Miki [b], Ikuko Kondo [a]

[a] *Department of Medical Genetics, Ehime University School of Medicine, Ehime, Japan*
[b] *Department of Geriatric Medicine, Ehime University School of Medicine, Ehime, Japan*
[c] *Choju Medical Institute, Fukushimura Hospital, Toyohashi, Japan*
[d] *Division of Psychiatry and Behavioral Proteomics, Department of Post-Genomics and Diseases, Osaka University Graduate School of Medicine, Suita, Japan*
[e] *Department of Biochemistry, Cell Biology and Metabolism, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan*

Received 10 June 2004

Abstract

Fibroblast growth factor 1 (FGF1, also known as acidic FGF) protects selective neuronal populations against neurotoxic effects such as those in Alzheimer's disease (AD) and HIV encephalitis. The FGF1 gene is therefore a strong candidate gene for AD. Using the promoter polymorphism of the FGF1 gene, we examined the relationship between AD and the FGF1 and apolipoprotein E (APOE) genes in 100 Japanese autopsy-confirmed late-onset AD patients and 106 age-matched non-demented controls. The promoter polymorphism (−1385 A/G) was significantly associated with AD risk. The odds ratio for AD associated with the GG vs non-GG genotype was 2.02 (95% CI = 1.16–3.52), while that of ε4 vs non-ε4 in APOE4 gene was 5.19 (95% CI = 2.68–10.1). The odds ratio for APOEε4 and FGF1 GG carriers was 20.5 (95% CI = 6.88–60.9). The results showed that the FGF1 gene is associated with autopsy-confirmed AD.

Keywords: Definite Alzheimer's disease; Fibroblast growth factor 1 gene; Promoter polymorphism; Association study; APOE; Risk factor

Alzheimer's disease (AD; MIM#104300) is the most common cause of dementia in mid- to late-life. Studying the factors that influence the risk of developing AD may lead to the identification of those at high risk for developing it, strategies for prevention or intervention, and clues to the cause of the disease. Both genetic and environmental factors have been implicated in the development of AD [1], but the cause of AD remains unknown, and no cure or universally effective treatment has yet been developed [2]. Even the diagnosis is difficult. A definitive diagnosis depends on analysis of neuritic plaques and neurofibrillary tangles found in brain tissue [3]. Given the recognition that AD constitutes a heterogeneous disorder, identification of established risk factors would be difficult using conventional methods.

Fibroblast growth factor 1 (FGF1, also known as acidic FGF) is a member of the fibroblast growth factor family that possesses broad mitogenic and cell survival activities and is involved in a variety of biological processes [4]. FGF1 protects selective neuronal populations against neurotoxic effects such as those in Alzheimer's disease [5,6] and HIV encephalitis [7]. Immunohistochemical examination of postmortem brain tissue of AD revealed that FGF1 was specifically expressed in a subpopulation of reactive astrocytes surrounding senile

* Corresponding author. Fax: +81-89-960-5279.
E-mail address: hideyama@m.chime-u.ac.jp (H. Yamagata).

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doi:10.1016/j.bbrc.2004.06.142

plaques. Such upregulation of FGF1 expression might be related to the presence of reactive astrocytes rather than β-amyloid protein deposition [8,9]. Recent studies suggest that FGF1 upregulates APOE synthesis and subsequently HDL production in reactive astrocytes in an autocrine or paracrine manner, and exerts its effect after central nervous system (CNS) damage through APOE secretion [10,11]. Besides, the fact that FGF1 expression is lower in the hippocampal formation than in motoneurons suggests that FGF1 contributes to the selective vulnerability of neurons in the entorhinal cortex in AD, and altered patterns of FGF1 immunoreactivity may play an important role in the pathophysiological processes of AD [6,12]. The FGF1 gene is therefore a strong candidate gene for AD. However, there are no reports regarding the association of FGF1 gene polymorphism with AD. Therefore, we investigated whether FGF1 gene polymorphism could contribute to risk in a limited subgroup of AD (autopsy-confirmed AD).

Subjects and methods

The Ethics Committee of Ehime University School of Medicine approved the study protocol. Patients were selected based on the NINCDS-ADRDA criteria for definite AD, and non-demented controls were rigorously evaluated for cognitive impairment using the Mini-Mental State Examination [3,13]. Brain and blood samples were obtained with informed consent from subjects in the Chubu and Kansai areas of Japan. A total of 100 unrelated late-onset AD (LOAD) patients had been diagnosed previously, and 106 controls (outpatients or healthy volunteers) were selected and matched for age and place of residence of the patients as described elsewhere [14,15]. The mean age ± SD (years) at the time of this study was as follows: 85.3 ± 6.0 for LOAD, 83.0 ± 4.9 for controls. Genomic DNA was extracted from the brain or peripheral blood using the phenol–chloroform method [16].

During screening for FGF1 gene mutation and polymorphism, we detected a common single nucleotide polymorphism (SNP) of −1385 G/A (C/T) (rs34011) in the promoter region. This polymorphism could easily be detected by PCR-RFLP using the restriction enzyme *Hha*I, where G and A, with respective frequencies of 0.65 and 0.35, were observed in our Japanese control population. The polymorphic region was amplified by PCR with the primers FGF1-F (5′-TCAAGC AATTCTCCTGCCTT-3′) and FGF1-R (5′-CCACTTCAAGGGATT ATGGTG-3′). PCR was carried out in a 25-μl reaction volume containing standard reaction buffer (1.5 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris–HCl, pH 8.3), 200 μM each dNTP, 5 μM each primer, 0.5 U *Taq* DNA polymerase and 50 ng genomic DNA as a template with 35 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. PCR product size was 355 bp, and the G allele was digested by *Hha*I to 53 + 141 + 161 bp, and the A allele to 53 + 302 bp. DNA was electrophoresed on 2% agarose gels and visualized with ethidium bromide staining under UV light (Fig. 1). To investigate the contribution of the gene to sporadic LOAD, we compared allele frequencies between LOAD and normal control subjects. Because APOEε4 is a risk factor for AD, we stratified the population by ε4 carrier status. APOE genotyping was performed as described previously. Allelic and genotypic distribution were analyzed by the usual χ^2 test of association. The genotypic frequencies were compared by χ^2 test with the values predicted by the assumption of Hardy–Weinberg equilibrium in the sample. Values of $p < 0.05$ were considered significant. Odds ratios were calculated with two-tailed p values and 95% confidence intervals.

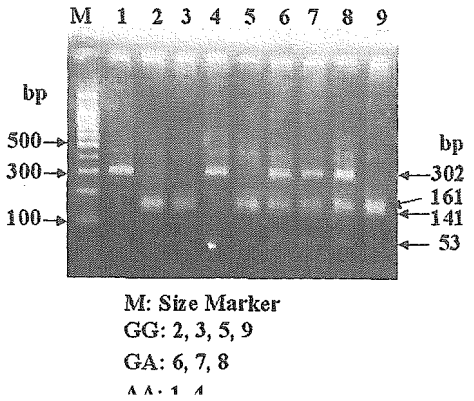

Fig. 1. Promoter polymorphism of FGF1. After amplification, PCR products were digested with *Hha*I and DNA was detected after electrophoresis on 2% agarose gels. Three genotypes of −1385 G/A (*Hha*I polymorphism) are shown: genotypes GG (lanes 2, 3, 5, and 9), GA (lanes 6–8), and AA (lanes 1 and 4).

Results

The PCR results were scored by two independent investigators who did not know whether each sample was from a case patient or a control. No intraobserver variability was found on repeated readings of the same gel, and the interobserver variability was less than 1%. All ambiguous samples were analyzed a second time.

The distribution of the three genotypes (GG, GA, and AA) reached Hardy–Weinberg equilibrium. The G allele was found in 75% of the 100 LOAD patients and 63% of the 106 control subjects. A significant association was observed between the −1385 G/A polymorphism and LOAD ($p < 0.03$; Table 1). We then examined the GG genotype as a risk factor for AD, considering the APOE status. As expected, APOEε4 conferred an increased risk for AD [odds ratio (OR) = 5.19]. OR in homozygotes for the G allele was 2.02 [95% confidence interval (CI) = 1.16–3.52]. However, the risk-increasing effect was smaller for −1385 G than for APOEε4 (Table 2). Four categories were defined by the presence (+) or absence (−) of a ε4 or GG genotype. The GG genotype alone showed an increased risk (95% CI: 1.81–7.69), and OR for APOEε4 and the GG genotype was 20.5 (95% CI: 6.88–60.9).

Discussion

To date, some polymorphisms of the FGF1 gene have been reported to associate with intracranial aneurysm [17]. However, functional role of the haplotype in its pathophysiology remains unclear. As the FGF1 gene contains alternative 5′-untranslated exons, the transcription is controlled by at least four distinct promoters in a tissue-specific manner [18–20]. Payson et al. [19] have reported that the sequence from −1614

Table 1
Genotype and allele numbers and frequencies for G/A polymorphism in promoter of FGF1

Group	Genotype (frequency)				Allele (frequency)	
n	AA	GA	GG	AA + GA	G	A
LOAD (100)	6 (0.06)	38 (0.38)	56 (0.56)*	44 (0.44)**	150 (0.75)	50 (0.25)***
Control (106)	14 (0.13)	51 (0.48)	41 (0.39)	65 (0.61)	133 (0.63)	79 (0.37)

LOAD, late-onset AD.
* $p < 0.03$.
** $p < 0.02$.
*** $p < 0.01$.

Table 2
Relative risk for interaction between APOEε4 and −1385 GG

		LOAD cases	Controls	Odds ratio	95% CI
	−1385 G/A				
	non-GG	44	65	Reference	
	GG	56	41	2.02	1.16–3.52
APOEε4					
–		52	90	Reference	
+		48	16	5.19	2.68–10.1
APOEε4	−1385 GG				
–	–	17	58	Reference	
–	+	35	32	3.73	1.81–7.69
+	–	18	11	5.58	2.21–14.1
+	+	30	5	20.5	6.88–60.9

APOEε4 (+), one or two copies of ε4: APOEε4 (−), no copies of ε4, 95% CI, confidence interval at 95% level.

to the FGF1 start site is sufficient to stimulate promoter activity. Therefore, it is reasonable to think that −1385 G/A polymorphism in the FGF1 promoter region can contribute the promoter activity. We performed an association study of the promoter polymorphism of the FGF1 gene.

We have evaluated definite LOAD as a relatively homogeneous case group. Our preliminary data suggest that the FGF1 gene, or a nearby gene, is an additional risk factor, independent of the APOE gene. Association studies often produce conflicting results. There are three possible reasons. First, this might be due to a type I statistical error, where there is a weak association between the polymorphism and the disease. Second, it might arise from the difference in genetic background between the American, French, Asian, and Japanese populations. In some studies, the AD group was made up of a mixture of familial and sporadic patients. We therefore tried to choose homogeneous subjects (autopsy-confirmed and late-onset AD) as much as possible. A third possibility could be linkage disequilibrium with other causative polymorphisms.

Patients with the GG genotype in this study had a higher risk of AD than those with the A allele. This indicates that the GG genotype in the promoter may influence the expression of FGF1 and could be involved in the selective vulnerability of neurons in AD. The results of this study support the hypothesis that FGF1 contributes to the selective vulnerability of neurons in the entorhinal cortex in AD, and altered patterns of FGF1 immunoreactivity may play an important role in the pathophysiological processes of AD [11,6,12]. This hypothesis should be further examined by functional analysis of FGF1 polymorphisms.

Acknowledgments

We are most grateful to all participants in the study. We thank Drs. Masaki Imagawa, Hideki Yamamoto, Hirotaka Tanabe, Yasuhiro Nonomura, Hiroshi Yoneda, Tsuyoshi Nishimura, Toshiaki Sakai, and Masatoshi Takeda for their help in data collection. We are indebted to Dr. Wendy Gray for revising the manuscript. This work was supported by a grant from the Japanese Millennium Project.

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Journal of Atherosclerosis and Thrombosis Vol. 11, No. 4

Reviews

The Very Low-density Lipoprotein (VLDL) Receptor: Characterization and Functions as a Peripheral Lipoprotein Receptor

Sadao Takahashi[1], Juro Sakai[2, 3], Takahiro Fujino[4, *], Hiroaki Hattori[5], Yasuo Zenimaru[1], Jinya Suzuki[1], Isamu Miyamori[1], and Tokuo T. Yamamoto[4, **]

[1] *The Third Department of Internal Medicine, University of Fukui, Fukui, Japan.*
[2] *Laboratory for Systems Biology and Medicine, Research Center for Advanced Science and Technology, University of Tokyo, Tokyo, Japan.*
[3] *Exploratory Research for Advanced Technology of Japan Science and Technology Corporation, Tokyo, Japan.*
[4] *Tohoku University Gene Research Center, Miyagi, Japan.*
[5] *Department of Advanced Medical Technology and Development, BML, Inc, Saitama, Japan.*
* *(Present) Department of Bioscience, Integrated Center for Science, Ehime University, Ehime, Japan.*
** *(Present) Center for Advanced Genome Research, Institute of Aging, Development and Cancer, Tohoku University, Miyagi, Japan.*

The very low-density lipoprotein (VLDL) receptor is a member of the low-density lipoprotein (LDL) receptor family. *In vitro* and *in vivo* studies have shown that VLDL receptor binds triglyceride (TG)-rich lipoproteins but not LDL, and functions as a peripheral remnant lipoprotein receptor. VLDL receptor is expressed abundantly in fatty acid-active tissues (heart, skeletal muscle and fat), the brain and macrophages. It is likely that VLDL receptor functions in concert with lipoprotein lipase (LPL), which hydrolyses TG in VLDL and chylomicron. In contrast to the LDL receptor, VLDL receptor binds apolipoprotein (apo) E2/2 VLDL particles as well as apoE3/3 VLDL, and the expression is not down-regulated by intracellular lipoproteins. Recently, various functions of the VLDL receptor have been reported in lipoprotein metabolism, metabolic syndrome/atherosclerosis, cardiac fatty acid metabolism, neuronal migration and angiogenesis/tumor growth. Gene therapy of VLDL receptor into the liver showed a benefit effect for lipoprotein metabolism in both LDL receptor knockout and apoE mutant mice. Beyond its function as a peripheral lipoprotein receptor, possibilities of its physiological function have been extended to include signal transduction, angiogenesis and tumor growth. *J Atheroscler Thromb, 2004; 11: 200–208.*

Key words: VLDL receptor, LDL receptor family, Lipoprotein metabolism, Signal transduction

Introduction

The low-density lipoprotein (LDL) receptor family is a growing receptor family composed of more than ten receptors. LDL receptor, a genetic defect of which induces familial hypercholesterolemia (FH), has been discovered and elucidated as an LDL receptor pathway (1, 2). Plasma LDL particles are recognized, internalized and degraded by the coated pit-located hepatic LDL receptors. FH heterozygotes (about 1 per 500 people) express half of the normal number of functional LDL receptors, and rare FH homozygotes (about 1 per million people) express few to no functional LDL receptors on their cell surface. Their plasma cholesterol levels rise from 300 to 500 mg/dl, and 600 to 1200 mg/dl, respectively. Even though the physiological functions of LDL receptor have been established, the functions of other LDL receptor family members are under investigation. In the LDL receptor family, the very

Address for correspondence: Sadao Takahashi, The Third Department of Internal Medicine, University of Fukui, 23–3, Matsuoka-cho, Fukui, 910–1193, Japan.
E-mail: sadaost@fmsrsa.fukui-med.ac.jp
Received March 15, 2004.
Accepted for publication March 23, 2004.

low-density lipoprotein (VLDL) receptor and apolipoprotein (apo) E receptor 2 (apoER2) are most similar to the LDL receptor structurally (3). In this review, we describe a special feature of the VLDL receptor as a peripheral lipoprotein receptor.

Characterization of the VLDL Receptor

Cloning of the VLDL receptor

In 1998, Herz *et al.* cloned LDL receptor-related protein-1 (LRP-1), which is the second protein of the LDL receptor family from the human liver cDNA library (4). Its protein is a large cell surface protein containing 4,544 amino acids (to 600 kDa) and consists of five common structural domains resembling those of LDL receptor: (i) ligand-binding type cysteine-rich repeats (ii) epidermal growth factor (EGF) receptor-like cysteine-rich repeats (iii) YWTD domains (iv) a single membrane-spanning segment, and (v) a cytoplasmic domain. LRP-1 binds apoE-containing lipoproteins but not LDL, and functions as a remnant lipoprotein receptor in the liver in cooperation with LDL receptor. Recently, novel functions on amyloid β-protein and tissue-type plasminogen activator (tPA) in the brain also have been elucidated (5, 6).

At that time, we considered that LRP-1 was too large of a protein and speculated that another lipoprotein receptor would have a sequence and structure more similar to those of LDL receptor. To exclude the rabbit LDL receptor, the entire pooled cDNA library was digested with Sal I and recircularized with T4 DNA ligase. The presence of a unique Sal I site in the rabbit LDL receptor cDNA and the Okayama-Berg vector resulted in the loss of any LDL receptor cDNAs after recircularization and retransformation. The resulting LDL receptor-subtracted cDNA library was screened with the 1.9-kb Sma I-Sal I fragment from the rabbit LDL receptor cDNA under low-stringency hybridization conditions (7, 8). Surprisingly, a cloned new cDNA encoded a protein with striking homology to the LDL receptor (Fig. 1). We considered that the new gene was a brother of the LDL receptor but LRP-1 was a distant relative. The mature protein consisted of five domains spanning 846 amino acids: 328 N-terminal amino acids including an 8-fold repeat of 40 amino acids homologous to the ligand binding repeat of the LDL receptor; 396 amino acid residues homologous to the epidermal growth factor precursor including three cysteine-rich repeats; a region immediately outside of the plasma membrane, rich in serine and threonines; 22 amino acids transversing the plasma membrane; and 54 amino acids including the NPVY sequence required for clustering of the LDL receptor in coated pits and that projects into the cytoplasm. Following the rabbit VLDL receptor cDNA cloning, we also cloned the human VLDL receptor cDNA from the THP-1 monocytic leukemia cell cDNA library (9). The human VLDL receptor gene contains 19 exons spanning approximately 40 kb. The exon-intron organization of the gene is almost the same as that of the LDL receptor gene, except for an extra exon that encodes an additional repeat in the ligand-binding domain (LDL receptor contains a 7-fold repeat and VLDL receptor has an 8-fold repeat). The VLDL receptor mRNAs produce two kinds of VLDL receptor proteins by alternative splicing type 1 VLDL receptor and type 2 VLDL receptor, which lacks the O-linked sugar domain encoded by exon 16. Although the structure and organization of the VLDL receptor gene is highly similar to those of the LDL receptor gene, the two genes are located on different chromosomes: the LDL receptor gene is on chromosome 19 and the VLDL receptor gene on chromosome 9.

Tissue distribution

The VLDL receptor mRNAs are highly abundant in the heart, muscle, adipose tissue, and brain, and are barely detectable in the liver, in which the LDL receptors are expressed abundantly (7). We also confirmed the VLDL receptor expression in THP-1 cells, PMA-induced (THP-1) macrophages, HL-60 cells, human monocyte-derived macrophages, rabbit alveolar macrophages and rat cultured cardiomyocytes (9–13). Immunoreactive VLDL receptor protein was detected in the endothelium of the capillaries and small arterioles but not in the veins or venules of bovine skeletal muscle, heart, ovary and brain. The VLDL receptor was also detected at high levels on the endothelial surface of bovine coronary arteries but not in the aortic endothelium (14). Interestingly, the type 1 VLDL receptor containing the O-linked sugar domain was preferentially expressed in the heart, brain, and skeletal muscle, whereas bovine aortic endothelial cells expressed only type 2 VLDL receptor (15). In human and

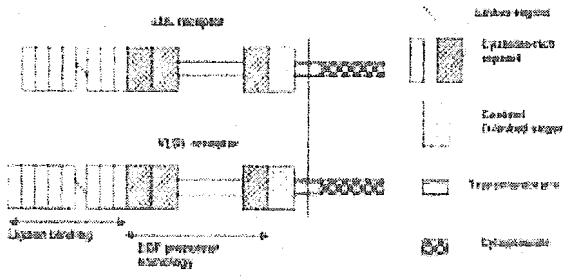

Fig. 1. Structure of LDL receptor and VLDL receptor.
The number of cysteine-rich repeats in the ligand-binding domain is different between the two receptors. LDL receptor contains a 7-fold repeat and VLDL receptor has an 8-fold repeat. The two receptors have eight and ten residues in the linker region, in the LDL receptor between repeats 4 and 5, and in the VLDL receptor between repeats 5 and 6.

rabbit atherosclerotic lesions, the VLDL receptor was expressed in macrophages and smooth muscle cells, and its expression was highly induced in atherosclerotic lesions (16–19). The VLDL receptor is most abundantly expressed in the heart. An in situ hybridization study revealed that VLDL receptor mRNA was detected within the human myocardium, and the hybridization pattern corresponded to that of myofibrils. In the human liver, the presence of VLDL receptor mRNA was only detected within vascular structures and sinusoidal lining cells (Kuppfer cells), with no signal detected in hepatocytes (16). In the human brain, the VLDL receptor was present on resting and activated microglia associated with senile plaques and cortical neurons. A novel splicing variant lacking exon 4 in the cysteine-rich repeats region was discovered by RT-PCR more often than type 1 and type 2 VLDL receptors, only in the brain (20).

Ligand-binding specificity of the VLDL receptor as a lipoprotein receptor

To confirm the ligand-binding specificity of the new cloned gene that was similar to the LDL receptor structurally, we transfected the cloned cDNA into ldlA-7 cells (LDL receptor-deficient CHO cells) and examined the ligand-binding specificity compared to that of LDL receptor transfectants. The new gene produced proteins that bound apoE-containing lipoproteins including VLDL, intermediate density lipoprotein (IDL) from Watanabe heritable hyperlipidemic (WHHL) rabbits and β-migrating VLDL (β-VLDL) from cholesterol-fed rabbits, but did not bind LDL from WHHL rabbits, whereas CHO cells transfected with the human LDL receptor cDNA bound both apoB- and apoE-containing lipoproteins including VLDL, IDL, LDL from WHHL rabbits and β-VLDL from cholesterol-fed rabbits. ^{125}I-labeled β-VLDL binding to the transfected cells was inhibited by unlabeled apoE-liposomes, indicating that the receptor recognizes apoE (7, 8). These binding properties were confirmed by a more detailed examination which indicated that the VLDL receptor never bound LDL. The VLDL receptor was more slowly processed than the LDL receptor, and did not show the increase in affinity and decrease in binding of β-VLDL on cooling to 4 degrees that was exhibited by the LDL receptor (21). On the other hand, the VLDL from fasted normal human subjects bound with lower affinity than the VLDL prepared from WHHL rabbits or the β-VLDL from cholesterol-fed rabbits. A ligand blot study clearly showed that human VLDL receptor bound β-VLDL from fasted cholesterol-fed rabbits with higher affinity than human fasted VLDL particles (Fig. 2A), and cold LDL completed ^{125}I-β-VLDL binding to the LDL receptor protein but not to the VLDL receptor protein (Fig. 2B). The low affinity binding of fasted human VLDL to the VLDL receptor could be overcome by enriching VLDL with either apoE or lipoprotein lipase (LPL) (22). There are three mechanisms between LPL and the VLDL receptor: (i) directly binding to the VLDL receptor through LPL, (ii) mediating the TG-rich lipoprotein particles through heparan sulfate proteoglycans, and (iii) its lipolytic activity, converting VLDL particles to smaller remnants (apoE-rich particles) before they can become endocytosed by VLDL receptor through apoE. Fluorescence microscopic examination indicated that Pseudomonas LPL that was dissimilar to human LPL structurally, but had a lipolytic activity, directly enhanced the binding of the fasted human VLDL to the VLDL receptor (Fig. 3). We consider that the third mechanism is the most important factor in VLDL receptor binding specificity. Niemeier A *et al.* showed the same mechanism for chylomicron particles. The VLDL receptor mediated the uptake of chylomicron remnant (CR), and this uptake was further increased by the addition of apoE and inactivated LPL (23). Taking into account that the VLDL receptor and LPL are expressed in

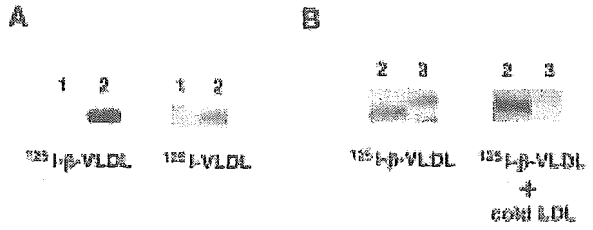

Fig. 2. Ligand blots study.
A: Human VLDL receptor over-expressing CHO cells recognized ^{125}I-rabbit β-VLDL with a higher affinity than ^{125}I-human fasted VLDL. (1: mock transfectants, 2: VLDL receptor transfectants)
B: Both VLDL receptor and LDL receptor recognized ^{125}I-rabbit β-VLDL. Only LDL receptor band disappeared by the addition of a 15-fold concentration of cold LDL, indicating that LDL was not a ligand of the VLDL receptor. (2: VLDL receptor transfectants, 3: LDL receptor transfectants)

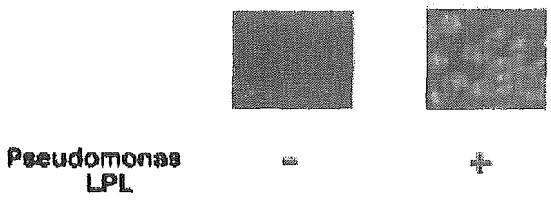

Fig. 3. Fluorescence microscopic examination in VLDL receptor transfectants.
Fluorescence (DiI)-labeled fasted human VLDL (1.5 μg/ml) was not recognized by human VLDL receptor, but treatment of Pseudomonas LPL (5.0 μg/ml) on VLDL accelerated the binding of human VLDL to VLDL receptor.

the same tissues, these findings suggest that the metabolism of IDL (VLDL remnant) and CR could be mediated by the VLDL receptor in peripheral fatty acid-active tissues, in concert with LPL (3).

Type III hyperlipoproteinemia is a genetic lipid disorder that is characterized by the accumulation of cholesterol-rich CR and hepatic β-VLDL. Most patients of type III hyperlipoproteinemia are homozygous for a mutant form of apoE (apoE2/2, Arg_{158}-Cys) that is not recognized by hepatic LDL receptors. We performed a competition study for binding with β-VLDL using VLDL derived from normolipidemic human subjects, homozygous for either apoE3/3 or apoE2/2, in LDL receptor-deficient CHO cells expressing the human VLDL receptor. In contrast to the LDL receptor, VLDL receptor bound apoE 2/2 VLDL or apoE 3/3 VLDL identically *in vitro* (24). Furthermore, adenovirus-mediated VLDL receptor expression in the liver of apoE2/2 and apoE3-Leiden transgenic mice lowered cholesterol levels, indicating that the VLDL receptor recognized apoE2/2 and apoE3-Leiden. The reduction in plasma cholesterol was mainly due to a reduction in the VLDL levels (25).

Lp (a) is a major inherited risk factor associated with premature heart disease and stroke. Strickland and colleagues (18) demonstrated that Lp (a) was recognized by the VLDL receptor, and clearance of Lp (a) was delayed in VLDL receptor-deficient mice. At the moment, the lipoproteins recognized by the VLDL receptor are VLDL, IDL (VLDL remnants), CR, and Lp (a).

Regulation of the VLDL receptor expression

When a cell takes up LDL particles into the cytoplasm through LDL receptor, three steps act to stabilize the cell's cholesterol content: (i) a suppression of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase gene (a key enzyme for de novo cholesterol synthesis) and acceleration of the degradation of the enzyme protein, (ii) an activation of Acyl-CoA: cholesterol acyltransferase (ACAT), a cholesterol-esterifying enzyme, to protect cells from free cholesterol, and (iii) a suppression of the LDL receptor own gene (a key lipoprotein receptor taking up plasma cholesterol). It has been accepted that the LDL receptor is down-regulated by intracellular lipoproteins. We first reported that the VLDL receptor is not down regulated by sterols in THP-1 and rabbit resident alveolar macrophages (9, 10). Western blots also showed that the LDL receptor protein disappeared when 100 μg/ml of β-VLDL was added to the medium for 48 hours, but the VLDL receptor protein level was not changed in THP-1 cells (Fig. 4). There is a sterol regulatory element (SRE)-1 in the human LDL receptor gene and two SRE-1-like sequences in the VLDL receptor gene (9). SRE-1 contains a direct repeat of the nucleotide sequence CAC on the same DNA strand, separated by two Cs. The two CAC sequences are considered to be the target of SRE-binding protein 1, which controls transcription of the LDL receptor gene. The SRE-1-like sequences in the VLDL receptor contain single nucleotide substitutions that disrupt the direct CAC repeats. This might be the reason why VLDL receptor expression is not regulated by intracellular lipoproteins.

In addition, a fasted state induced high VLDL receptor expression in the heart and low expression in the fat in mice, even though the expression in rats was not changed by a 48-hour fasting state (26, 27). We also confirmed that, in Balb/c mice fasted for 48 hours, VLDL receptor as well as LPL, fatty acid translocase (FAT)/CD36, heart-type fatty acid-binding protein (H-FABP), acyl-CoA synthetase (ACS) and long chain acyl-CoA dehydrogenase (LCAD) mRNAs, as fatty acid metabolism indicators, increased. Pyruvate kinase (PK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs, as glucose metabolism indicators, did not change. Electron microscopic examination indicated that the lipid droplets accumulated in the hearts of Balb/c mice fasted for 48 hours. During the development of SD (Sprague-Dawley) rats, VLDL receptor, LPL, FAT/CD36, H-FABP, ACS and LCAD mRNAs increased gradually with growth. However, PK and GAPDH mRNAs did not show this tendency. In cultured neonatal rat cardiomyocytes, VLDL receptor expression increased with days in culture, which was compatible with the *in vivo* results. Oil red-O staining showed cardiomyocytes after 7 days in culture (when the VLDL receptor protein is present) had accumulated β-VLDL. There were no detectable LDL receptor mRNAs in cultured neonatal rat cardiomyocytes, showing that remnant lipoproteins (β-VLDL) are taken up by the myocardium through the VLDL receptor (13). Triglyceride hydrolysis by LPL results in the generation of free fatty acids, 2-monoglycerol and remnant lipoprotein particles (IDL and CR). The resultant fatty acids are transported across the plasma membrane into the heart by simple

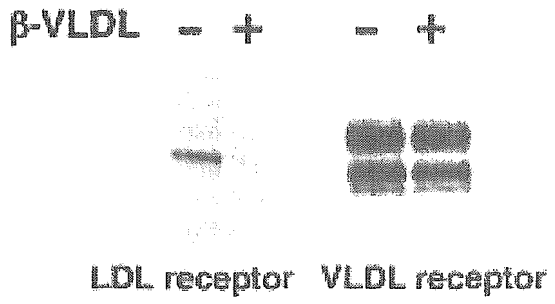

Fig. 4. Western blots study in THP-1 cells.
LDL receptor proteins virtually disappeared by the addition of β-VLDL (100 μg/ml) for 48 hours. VLDL receptor proteins remained.

diffusion and membrane-associated transporters (FAT/CD36, FATP and FABPpm). On the other hand, remnant lipoprotein particles are also taken up into the heart by the VLDL receptor.

Thyroid hormone was a positive regulator of the VLDL receptor in rat skeletal muscle but not in the fat or heart (27). VLDL receptor expression in rabbits is up-regulated by estrogen in the heart and down-regulated by granulocyte-macrophage colony-stimulating factor (GM-CSF) in muscle (28, 29). In JEG-3 and BeWo choriocarcinoma cells, two trophoblast-derived cell lines, 8-bromo-cAMP suppressed VLDL receptor expression. On the other hand, insulin and clofibrate (hypolipidemic drugs) up-regulated VLDL receptor expression. 25-hydroxycholesterol did not lead to sterol negative feedback on VLDL receptor expression (30). The VLDL receptor mRNA in adipose tissue and muscle was increased by 14-day gemfibrozil treatment in rabbits, indicating that the enhanced VLDL receptor expression was one of the mechanisms of the lipid-lowering effect of fibrate (31). IFN (Interferon)-γ inhibited VLDL receptor expression in PMA-treated THP-1 cells (monocytes), HL-60 cells and human monocyte-derived macrophages. However, this effect was not observed in normal THP-1 cells (12).

In contrast with the non-sterol regulation of VLDL receptor expression *in vitro*, Tiebel *et al.* (32) examined dietary regulation of the VLDL receptor in C57BL/6, LDL receptor knockout (LDL-R-/-), apoE knockout (apoE-/-) and LDL receptor/apoE double knockout (LDL-R-/-; apoE-/-) mice. VLDL receptor mRNA expression was down-regulated 3-fold by administrting an atherogenic diet, in the heart and skeletal muscle only, in LDL-R-/- mice. VLDL receptor mRNA was up-regulated by an atherogenic diet in the adipose tissue in all models except LDL-R-/-; apoE-/- mice. These findings suggest that SRE-1-like sequences in the VLDL receptor gene may be functional in the heart and skeletal muscle when LDL receptor is absent, and that apoE is required for regulation of VLDL receptor expression.

Reliable evidence of VLDL receptor as a lipoprotein receptor *in vivo*

In 1996, a conflicting report using VLDL receptor knockout (VLDL-R-/-) mice to study VLDL receptor as a lipoprotein receptor was published. VLDL-R-/- mice did not show any lipoprotein abnormality, although the adipose tissue mass of VLDL-R-/- mice was reduced (33). On the other hand, gene expression of the VLDL receptor in the liver declined plasma cholesterol levels in various laboratories (25, 34–36). We suggested that the tissue distribution of VLDL receptor might be a reason why VLDL receptor deficiency did not induce lipoprotein abnormality and why the plasma lipoprotein level was controlled by lipoprotein receptors in the liver. In 2000, Tacken *et al.* (37) elegantly solved this problem and demonstrated that the VLDL receptor is a peripheral lipoprotein receptor for VLDL triglycerides *in vivo*. They created VLDL receptor/LDL receptor double knockout (VLDL-R-/-; LDL-R-/-) mice and VLDL receptor over-expressing on LDL receptor knockout (LDL-R-/-) mice. When the mice were fed a high-fat diet, a lack of the VLDL receptor (VLDL-R-/-; LDL-R-/-) resulted in a significant increase in the serum triglyceride levels, and over expression of the VLDL-R resulted in a significant decrease in the serum triglyceride levels, compared to those in the LDL-R-/- mice. Furthermore, a period of prolonged fasting with a chow diet showed a significant increase in serum triglyceride levels in VLDL-R-/-; LDL-R-/- mice compared to those in LDL-R -/- mice. These data indicate that the plasma lipoprotein level in mice strongly depends on LDL receptor in the liver, and LDL receptor disguised the effect of VLDL receptor on lipoprotein metabolism.

Multiple Functions

Gene therapy for familial hypercholesterolemia and apoE mutants

The adenovirus-mediated transfer of the VLDL receptor gene into LDL receptor knockout mice liver greatly enhanced the ability to clear the IDL fraction, resulting in a marked lowering of the plasma IDL/LDL fraction (34, 35). Moreover, helper-dependent adenovirus-mediated delivery of VLDL receptor into hepatocytes produced the long-term lowering of plasma cholesterol and prevented atherosclerosis development in LDL receptor knockout mice (36). Gene therapy using VLDL receptor instead of LDL receptor for familial hypercholesterolemia (FH) is reasonable, because the normal LDL receptor might be recognized as a foreign protein in FH patients. VLDL receptor is non-immunogenic because it is normally expressed in fatty acid-active tissues, even in FH patients. Gene therapy using VLDL receptor has a possibile use in the treatment of apoE mutants, because VLDL receptor binds apoE2/2 and apoE3-Leiden (25). As yet there has been no report of gene therapy using VLDL receptor for human FH or apoE mutants.

Metabolic syndrome/atherosclerosis and the VLDL receptor

Metabolic syndrome is characterized by a cluster of obesity, insulin resistance, hypertension, and atherogenic dyslipidemia, and is associated with increased risk of coronary heart disease. It seems that VLDL receptor is involved in the mechanism of metabolic syndrome because VLDL receptor knockout (VLDL-R-/-) mice revealed a modest decrease in adipose tissue mass (33), and a higher affinity ligand of the VLDL receptor is remnant lipoprotein (3). Recently, Goudriaan *et al.* (38) reported that VLDL-R-/- mice remained lean and did not have insulin resistance after 17 weeks of a high-fat, high-calorie

(HFC) diet compared to wild-type mice, and the weight gain of VLDL-R-/- on ob/ob mice was less profound compared with that of ob/ob mice. Moreover, VLDL receptor deficiency led to increased plasma triglyceride after HFC feeding. These data indicated that inhibition of VLDL receptor expression in adipose tissue may be a therapeutic strategy for obesity.

VLDL receptor expression, primarily in macrophages, has been confirmed in human and rabbit atherosclerotic lesions (16–19). In addition, we reported that IFN-γ inhibited VLDL receptor expression and foam cell formation in three human macrophages (PMA-induced THP-1, PMA-induced HL-60 and human monocyte-derived macrophages) by β-VLDL, which is a representative lipoprotein in metabolic syndrome and type III hyperlipoproteinemia (12). These data showed that VLDL receptor might be a macrophage β-VLDL receptor, which is one of the receptors for macrophage foam cell formation. However, controversial findings using a mouse model *in vivo* have been reported. Yagyu *et al.* (39) showed that atherosclerosis was not different between HuB (human apo B) transgenic mice and VLDL receptor-deficient HuB transgenic mice after 4 months of an atherogenic diet. Tacken *et al.* (40) also showed that neither VLDL receptor deficiency nor endothelial VLDL receptor over expression affected the atherosclerotic lesion size. Interestingly, they indicated that deficiency for the VLDL receptor profoundly increased intimal thickening after vascular injury. We were not able to detect a sufficient amount of VLDL receptor expression in mouse peritoneal macrophages or J774 mouse macrophage cells (data not shown). At this time, we do not have any data to explain this difference between humans and mice.

Reelin signaling and the VLDL receptor

In 1999, we encountered an impact paper from Herz and colleagues (41) showing that the absence of both apoER2 and VLDL receptor in mice led to an inversion of cortical layers and the absence of cerebellar foliation. This phenotype is similar to that seen in animals carrying a mutation in either the reelin gene or the disabled-1 (Dab-1) gene. Both VLDL receptor and apoER2 can bind reelin on their extracellular domains, which subsequently induces the tyrosine phosphorylation of Dab-1 (42, 43). Dab-1 not only binds to the Asn-Pro-Xxx-Tyr (NPxY; where x denotes any amino acid) sequences in the cytoplasmic domain of both apoER2 and VLDL receptor, but also binds to those of the LDL receptor and LRP-1 (41). The NPVY sequence in LDL receptor has been accepted as a coated pit signal sequence, but these findings opened a new era of signaling research on the LDL receptor family (44).

Novel functions of VLDL receptor

In addition to apoE and LPL (7, 8, 22, 45), VLDL receptor binds receptor-associated protein (RAP) (46), thrombospondin-1 (47), urokinase plasminogen activator (uPA)/plasminogen activator inhibitor-1 complex (45, 48), several other proteinase-serpin complexes (49), and tissue factor pathway inhibitor (TFPI) (50). More recently, it has been reported that a 23-amino acid fragment of TFPI localized to the C-terminus mediated the VLDL receptor, and they showed that the VLDL receptor might be involved in angiogenesis and tumor growth (51). Early study using VLDL receptor-transfected COS-7 cells indicated that cell growth was inhibited by the VLDL receptor, and this growth inhibition was ligand-independent (52). VLDL receptor knockout mice were always segregated with the retinal angiogenesis and subretinal neovascularization (53). On the other hand, it seems that endothelial cells are important sites for VLDL receptor because the movement of active LPL across endothelial cells involves both heparan sulfate proteoglycan and VLDL receptor, and VLDL receptor knockout mice showed lower plasma LPL activity, though the exact mechanism is under investigation (37, 54).

Beyond the function of VLDL receptor as a peripheral lipoprotein receptor, the possibilities of its physiological function have been extended to include signal transduction, angiogenesis and tumor growth.

Acknowledgement: This research was supported in parts\ by research grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 17, Issue of April 23, pp. 16954–16962, 2004
Printed in U.S.A.

A Krüppel-like factor KLF15 Contributes Fasting-induced Transcriptional Activation of Mitochondrial Acetyl-CoA Synthetase Gene *AceCS2**

Received for publication, November 4, 2003, and in revised form, February 4, 2004
Published, JBC Papers in Press, February 10, 2004, DOI 10.1074/jbc.M312079200

Joji Yamamoto,[a,b] Yukio Ikeda,[a,b] Haruhisa Iguchi,[b] Takahiro Fujino,[c] Toshiya Tanaka,[d] Hiroshi Asaba,[d] Satoshi Iwasaki,[b] Ryoichi X. Ioka,[b] Izumi W. Kaneko,[c] Kenta Magoori,[d] Sadao Takahashi,[e] Toshiyuki Mori,[f] Hiroshi Sakaue,[f] Tatsuhiko Kodama,[d] Masashi Yanagisawa,[b,g,h] Tokuo T. Yamamoto,[c] Sadayoshi Ito,[a] and Juro Sakai[b,d,i]

From the [a]Division of Nephrology, Endocrinology, and Vascular Medicine, Department of Medicine, the Tohoku University Graduate School of Medicine, Sendai 980-8574, Japan, the [b]Yanagisawa Orphan Receptor Project, Exploratory Research for Advanced Technology, Japan Science and Technology Corporation, Tokyo 135-0064, Japan, the [c]Tohoku University Gene Research Center, Sendai 981-8555, Japan, the [d]Laboratory for Systems Biology and Medicine, Research Center for Advanced Science and Technology, University of Tokyo, Tokyo 153-8904, Japan, the [e]Third Department of Internal Medicine, Fukui Medical University, Fukui, 910-1193, Japan, the [f]Division of Diabetes and Digestive and Kidney Diseases, Department of Clinical Molecular Medicine, Kobe University Graduate School of Medicine, Kobe 650-0017, Japan, and the [g]Howard Hughes Medical Institute, Department of Molecular Genetics, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235-9050

Acetyl-CoA synthetase 2 (AceCS2) produces acetyl-CoA for oxidation through the citric acid cycle in the mitochondrial matrix. AceCS2 is highly expressed in the skeletal muscle and is robustly induced by fasting. Quantification of AceCS2 transcripts both in C2C12 and human myotubes indicated that fasting-induced *AceCS2* gene expression appears to be independent on insulin action. Characterization of 5′-flanking region of the mouse *AceCS2* gene demonstrates that Krüppel-like factor 15 (KLF15) plays a key role in the trans-activation of the *AceCS2* gene. Deletion and mutation analyses of *AceCS2* promoter region revealed that the most proximal KLF site is a curtail site for the trans-activation of the *AceCS2* gene by KLF15. Using Sp-null *Drosophila* SL2 cells, we showed that the combination of KLF15 and Sp1 resulted in a synergistic activation of the *AceCS2* promoter. Mutation analyses of three GC-boxes in the *AceCS2* promoter indicated that the GC-box, located 8 bases downstream of the most proximal KLF15 site, is the most important GC-box in the synergistic trans-activation of the *AceCS2* gene by KLF15 and Sp1. GST pull-down assays showed that KLF15 interacts with Sp1 *in vitro*. Quantification of various KLF transcripts revealed that 48 h fasting robustly induced the KLF15 transcripts in the skeletal muscle. Together with the trans-activation of the *AceCS2* promoter, it is suggested that fasting-induced AceCS2 expression is largely contributed by KLF15. Furthermore, KLF15 overexpression induced the levels of AceCS2 transcripts both in myoblasts and in myotubes, indicating that *AceCS2* gene expression *in vivo* is indeed induced by KLF15.

Acetyl-CoA is an important intermediate in various metabolic pathways including fatty acid and cholesterol biosynthesis and the energy production by the citric acid cycle. There are several enzymes that generate acetyl-CoA in the mammals, including pyruvate dehydrogenase, which converts pyruvate to acetyl-CoA without generating free acetate. The degradation of fatty acid via β-oxidation system also produces acetyl-CoA as an end product. Although acetate is not an essential source of acetyl-CoA in animals, the enzymatic ligation for the production of acetyl-CoA from acetate and CoA is a key reaction in the catabolism of acetate formed by several conditions including bacterial fermentation in the colon, oxidation of ingested ethanol in the liver. Acetyl-CoA synthetase (AceCS,[1] EC 6.2.1.1) is an enzyme that catalyzes the production of acetyl-CoA from acetate and CoA. In particular, this enzyme plays a key role in the nervous system for recycling of acetate released by acetylcholine esterase for the formation and release of acetylcholine in cholinergic nerve terminals.

There are two AceCSs with similar enzymatic properties in mammals: one designated AceCS1 is a cytosolic enzyme and the other, designated AceCS2, is a mitochondrial matrix enzyme (1). Localized in the cytoplasm, AceCS1 provides acetyl-CoA for the synthesis of fatty acids and cholesterol. In contrast, AceCS2 produces acetyl-CoA for oxidation through the citric acid cycle to produce ATP and CO_2 in the mitochondrial matrix.

* This work was supported through Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government, and Exploratory Research for Advanced Technology/ Japan Science and Technology Corporation (Yanagisawa orphan receptor project). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[h] An Investigator of the Howard Hughes Medical Institute.

[i] To whom correspondence should be addressed: Laboratory for Systems Biology and Medicine, Research Center for Advanced Science and Technology, University of Tokyo, Tokyo 153-8904, Japan. Tel.: 81-3-5452-5472; Fax: 81-3-5452-5429; E-mail: jmsakai-tky@umin.ac.jp.

[1] The abbreviations used are: AceCS, acetyl coenzyme A synthetase; AFX, ALL1-fused gene from X chromosome; CMV, cytomegalovirus; FKHR, forkhead box O1a; GFP, green fluorescent protein; GLUT4, glucose transporter 4; GST, glutathione *S*-transferase; HSkMC, human skeletal muscle cell; HNF, hepatocyte nuclear factor; KLF, Krüppel-like factor; MEF2, myocyte enhancer factor 2; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1α; PPAR, peroxisome proliferator-activated receptor; RACE, rapid amplification of cDNA end; SREBP, sterol regulatory element-binding protein; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility shift assay; GFP, green fluorescent protein.

TABLE I
Primers for quantitative real-time RT-PCR

Gene	Sequence of forward and reverse primers (5′-3′)	Accession no.
Cyclophilin	CAAAGACACCAATGGCTCACAG CCACATCCATGCCCTCTAGAA	M60456
Human GAPDH	GCCATCAATGACCCCTTCATT TCTCGCTCCTGGAAGATGG	NM_002046
AceCS2	TCGTTCATACACAGGCAGGCTAT ACACGAGCTTGTGCGTCATG	NM_080575
Human AceCS2	GAGGATCAGAGGCCACATTTTT CACAGGAGAAACACTCACCAGG	NM_032501
Hexokinase II	CCTGCTTATTCACGGAGCTCA ATACTGGTCAACCTTCTGCACTTG	NM_013820
PDK4	GTGAACACTCCTTCGGTGCA TCAGGCTCTGGATATACCAGCTC	NM_013743
GLUT4	GACCTGTAACTTCATTGTCGGCA ATAGCATCCGCAACATACTGGA	NM_009204
KLF1	CTGACCAGAAGACCATACAAAGGA TGAGGACATGTGAGGTTTGTCC	NM_010635
KLF2	AGAATGCACCTGAGCCTGCTAG AATTTCCCCGAAAGCCTGC	ENSMUST00000006440
KLF3	CACCCCCTTTAATGAACCCAG TGGTTCTCTTGC AACAAGGCT	NM_008453
KLF4	CACAGGCGAGAAACCTTACCA AATTTCCACCCACAGCCGT	NM_010637
KLF5	TTCCAAACTGGCGATTCACAA ATTAACTGGCAGAGTGGCAGGTAA	NM_009769
KLF6	CCGGTGCCAAGCCTTTTAA GGTCAGACCTGGAGAAACACCT	NM_011803
KLF7	GTTTTGCACGGAGCGATGA AGACCTGGAGAAACACCTGTCG	NM_033563
KLF8	CTGTCCCACTCATTGGAGGAGA CAACTTTGACCGATCCAGCACT	XM_142240
KLF9	ATACAGGTGAACGGCCCTTTC TCCGAGCGCGAGAACTTTT	NM_010638
KLF10	TTCTCTCCAGCAAGCTTCGGA TCACTCTGCTCAGCTTTGTCCC	NM_013692
KLF11	CCCACCTTAAAGCTCATCTTCG GCAGGTAAAAGGCTTCTCCCCT	AK084913
KLF12	CAGTCAGTGCCGGTTGTCTACA CTCCATTTGTGCTTTGCCATG	NM_010636
KLF13	CCTCACCCCTTTGGTAATAGGA GCTGTGGACTTCTCAAGTTCGC	NM_021366
KLF14	CAGAGCTGGATGGCATAGAGGA TGAAAGGAGCGGCTAGCTCTT	AJ275988
KLF15	ACCGAAATGCTCAGTGGGTTACCTA GGAACAGAAGGCTTGCGAGTCA	NM_023184
KLF16	TGAGTTTGGCCTTTGTGGCAT TGTTCCCAATCCTGGAGTCCA	NM_078477

AceCS1 is a member of a family of genes whose transcription is regulated by sterol regulatory element-binding proteins (SREBPs), a basic helix-loop-helix leucine zipper transcription factor that activates multiple genes for cholesterol and fatty acid metabolism (2, 3). The levels of AceCS1 mRNA was induced when cultured cells were deprived of sterols and negatively regulated by sterol addition. In our previous study, we have shown that the *AceCS1* promoter region consists of multiple clustered SREBP binding sites and immediately downstream from this region there is a cluster of multiple GC-boxes (3). All these SREBP binding sites are bound with purified SREBP-1a and are required for a maximal response to cotransfected SREBP. The sterol regulation of the *AceCS1* gene was critically dependent on three closely spaced SREBP binding sites and an adjacent GC-box. We also showed that SREBP synergistically activated the *AceCS1* promoter along with Sp1 or Sp3 but not with nuclear factor Y (NF-Y).

AceCS2 is highly expressed in the cardiac and skeletal muscle and its regulation is completely different from that of AceCS1. A marked induction of AceCS2 mRNA is seen in the heart and skeletal muscle when animals are fasted (1). The levels of AceCS2 mRNA in the skeletal muscle of Zucker diabetic fatty rats were also increased compared with those in the normal littermates. These data indicated that the AceCS2 transcripts are induced in the heart and skeletal muscle under ketogenic conditions including prolonged fasting and diabetes.

During fasting, the expression of more than 94% of genes is not altered in the skeletal muscle. Among genes that are differentially expressed during fasting, genes involved in protein breakdown (components of the ubiquitin-proteasome pathway), fatty acid oxidation, and pyruvate dehydrogenase kinase 4 (PDK4) that suppresses glucose oxidation by inhibiting pyruvate dehydrogenase complex activity, are up-regulated (4, 5). In contrast, genes encoding glycolytic enzymes are down-regulated by fasting. Induction of genes involved in fatty acid oxidation is also seen in the skeletal muscle of diabetic mice (6). Although these changes indicate a complex adaptive program for sparing glucose, the mechanism underlining transcriptional regulation is not fully understood.

To define the mechanism of transcriptional induction of AceCS2 by fasting, we isolated and characterized 5′-flanking region of the mouse *AceCS2* gene. In this article, we describe that fasting induced transcriptional activation of the *AceCS2* gene in the skeletal muscle is largely contributed by a unique transcription factor, KLF15, a Krüppel-like factor. Our current data indicate a unique role of KLF15 in the activation of genes induced by prolonged fasting.

EXPERIMENTAL PROCEDURES

Materials—We obtained mouse Genome Walker kits and luminescent β-galactosidase reporter system from Clontech; the dual luciferase reporter assay system, pGL3 Basic, pRL-TK, and TnT Quick Coupled Transcription/Translation system from Promega Inc.; pcDNA3, pcDNA3.1, TOPO TA Cloning™ kit, TRIzol™ reagent, SuperScript II™, GeneRacer™ kit, LipofectAMINE PLUS, Dulbecco's modified Eagle's medium (DMEM), and Schneider's medium from Invitrogen; [γ-^{32}P]ATP (6000 Ci/mmol), and L-[^{35}S]methionine (1000 Ci/mmol), GST Gene Fusion System and Bulk GST Purification Module from Amersham Biosciences; QuikChange™ site-directed mutagenesis kit and MBS mammalian transfection kit from Stratagene; Oligotex-dT30 mRNA purification kit from TaKaRa (Shiga, Japan); nuclear extract kit from Active Motif (Carlsbad, CA); anti-human KLF15 polyclonal antibody from Abcam (catalog no. ab2647; Cambridge, UK); and oligonucleotides from Qiagen. Unless otherwise indicated, all restriction and DNA-modifying enzymes were obtained from Toyobo (Tokyo, Japan).

Expression Plasmids—To create a KLF15 expression plasmid, a full-length cDNA for rat KLF15 (provided by Dr. R. Hiramatsu, Genomics Science Laboratories, Sumitomo Pharmaceuticals Co. Ltd., Takarazuka, Japan) was inserted into pcDNA3. We obtained pcDNA1 vector-based plasmids for human MEF2A, mouse MEF2B, mouse MEF2C, and mouse MEF2D from Dr. E. N. Olson (Department of Molecular Biology, University of Texas Southwestern Medical Center) (7, 8); pSV-SPORT-PGC-1α, an SV40-driven plasmid containing mouse PGC-1α from Dr. B. M. Spiegelman (Dana-Farber Cancer Institute and the Department of Cell Biology, Harvard Medical School) (9); pcDNA3-based plasmid for mouse forkhead box O1a (FKHR) and mouse ALL1-fused gene from X chromosome (AFX), and pIRS-MLP-luc, a luciferase reporter plasmid driven by a promoter consisting of three tandem copies of a insulin responsive sequence (IRS) plus the adenovirus major late promoter (MLP) from Dr. A. Fukamizu (Center of Tsukuba Advanced Research Alliance, Institute of Applied Biochemistry, University of Tsukuba) (10, 11); pcDNA3.1-based plasmid for human c-Ets-1 from Dr. T. Minami (Laboratory for Systems Biology and Medicine, University of Tokyo, Japan) (12); and pcDNA3-based plasmids for mouse hepatic nuclear factor 1α (HNF1α) and mouse hepatocyte nuclear factor 1β (HNF1β) from Dr. K. Yamagata (Second Department of Internal Medicine, Osaka University Medical School, Suita, Japan) (13, 14). pCMV-MyoD, a CMV-driven plasmid for mouse MyoD, was constructed inserting the reverse transcription-PCR products of C2C12 myotube RNA into the pcDNA3 vector. pPac, a *Drosophila* actin 5C promoter-driven expression vector, pPacβ-gal containing an *Escherichia coli* β-galactosidase, and pPacSp1 and pPacSp3, respectively, containing Sp1 and Sp3, were provided by Dr. G. Suske (Philipps-Universität Marburg, Germany). pPac-based plasmid encoding rat KLF15 was generated by inserting the KLF15 cDNA into the pPac vector. An Sp1-GST fusion construct, pGEX-Sp1, was prepared by inserting the Sp1 cDNA fragment into the XhoI site of pGEX-4T-2.

Cloning of 5′-Flanking Region of Mouse AceCS2 Gene—The 5′-flanking region of the mouse *AceCS2* gene was cloned by PCR using Mouse Genome Walker kits (Clontech) as described previously (3). Briefly, the first PCR was conducted with adaptor primer 1 (provided by the supplier) and AceCS2 primer 1 (5′-TGACCACCCCGATTGTCCAGAG-3′) on a mouse genomic library (provided by the supplier). Nested PCR was then carried out with adaptor primer 2 (provided by the supplier) and AceCS2 primer 2 (5′-CGCAGCAGTCGCCCCACACCGCTGC-3′) on the first PCR products. The resulting 2.4-kb PCR product was subcloned into the pGEM-T easy cloning vector (Promega) to create pGEM-5′FL-AceCS2. Sequencing of the insert of pGEM-5′FL-AceCS2 was performed in both directions by the PCR cycle sequence method with an automatic sequence analyzer (Beckman Coulter CEQ 2000XL DNA Analysis System).

Rapid Amplification of cDNA End (RACE)—5′-RACE was performed using GeneRacer™ Kit (Invitrogen) according to the manufacturer's protocol. Briefly, 750 ng of poly(A)$^+$ RNA from quadriceps muscles of fasted mice was first treated with calf intestinal phosphatase to remove the 5′-phosphates. This eliminates truncated mRNA and non-mRNA from subsequent ligation with the GeneRacer™ RNA Oligo. The dephosphorylated RNA was then treated with tobacco acid pyrophosphatase to remove the 5′ cap structure from intact, full-length mRNA, and was ligated with 0.25 μg of the GeneRacer™ RNA Oligo using T4 RNA ligase at 37 °C for 1 h. The resulting products were then hybridized with random hexamer, reverse transcribed with SuperScript II reverse transcriptase, and subjected to PCR with a primer specific for the RNA Oligo (5′-CGACTGGAGCACGAGGACACTGA-3′) and an AceCS2 specific antisense primer (5′-CTGCACATGCTGATCCAGGCAGTTGA-3′). PCR parameters were 94 °C for 30 s and 68 °C for 1 min for 35 cycles. A major product of ~400 bp was cloned into the pCR2.1 vector (Invitrogen): eleven clones were isolated for sequencing analysis.

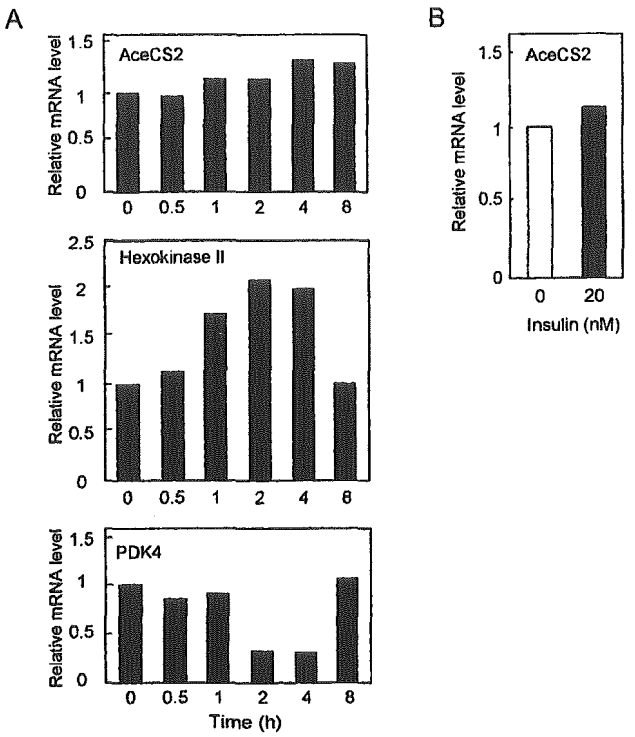

FIG. 1. **Effects of insulin on the levels of AceCS2 transcripts in cultured myotubes.** On day 0, C2C12 cells (*A*) and HSkMCs (*B*) were plated in 6-well plates at a density of 1 × 10^5 cells/well and grown in medium A. On day 2, cells were induced to differentiate into myotubes by incubating in medium B. Medium was changed every other day. On day 6, medium B was replaced by serum-free DMEM supplemented with 0.1% horse serum, 100 units/ml penicillin, and 100 μg/ml of streptomycin sulfate. C2C12 myotubes were treated with 200 nM bovine insulin for the indicated time periods (*A*), and human myotubes (HSkMCs) were cultured in the absence or presence of 20 nM bovine insulin for 16 h (*B*). Total RNA from C2C12 and human myotubes (*A* and *B*) were extracted, reverse-transcribed, and subjected to quantitative real time PCR as described under "Experimental Procedures." The levels of AceCS2, Hexokinase II, and PDK4 mRNA treated with insulin are shown in *panel A*. AceCS2 mRNA levels in HSkMCs cultured in the absence or presence of 20 nM bovine insulin are compared in *panel B*. Mouse cyclophilin or human GAPDH mRNA was used as the invariant control. Values represent the amount of mRNA in insulin-treated cells relative to that in the cells incubated without insulin, which is arbitrarily defined as 1.

AceCS2 Promoter-Reporter Constructs—pAceCS2(2347) is the mouse *AceCS2* promoter-luciferase reporter gene that spans −2347 to −1 relative to translation initiation site. pAceCS2(1928), pAceCS2(1528), pAceCS2(1383), pAceCS2 (940), pAceCS2 (654), pAceCS2 (110), and pAceCS2 (77), are 5′-deletion mutants of pAceCS2(2347), each contains a deletion with the 5′-end denoted in each parentheses and the same 3′-end point at −1. pAceCS2(2347) was constructed by PCR of pGEM-5′FL-AceCS2 using a forward primer starting from −2347 and a reverse primer OGY3as (5′-CTCGCCCGACGCCCCACGCG-3′). The PCR product was cloned into the SmaI site of pGL3 basic. pAceCS2(1928), pAceCS2(1528), pAceCS2(1383), pAceCS2 (940), pAceCS2 (654), pAceCS2 (110), and pAceCS2 (77) were constructed in a similar manner to pAceCS2(2347) using respective forward primers starting from the position −1928, −1528, −1383, −940, −654, −110, and −77, respectively, and coupled with a common reverse primer OGY3as. Base substitution mutants were generated in pAceCS2(1928) and pAceCS2 (654) using the QuikChange™ site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol. Oligonucleotides designed to mutate each element were as follows: the KLF site at −91, GGGTGGTG → GCTGCAGG; GC-box A at −132, AGGGCAGGC → AGACTAGTC; GC-box B at −76, GGGGCGGAG → GGAATTCAG; and GC-box C at −65, GGGGCGGGG → GGATATCGG.

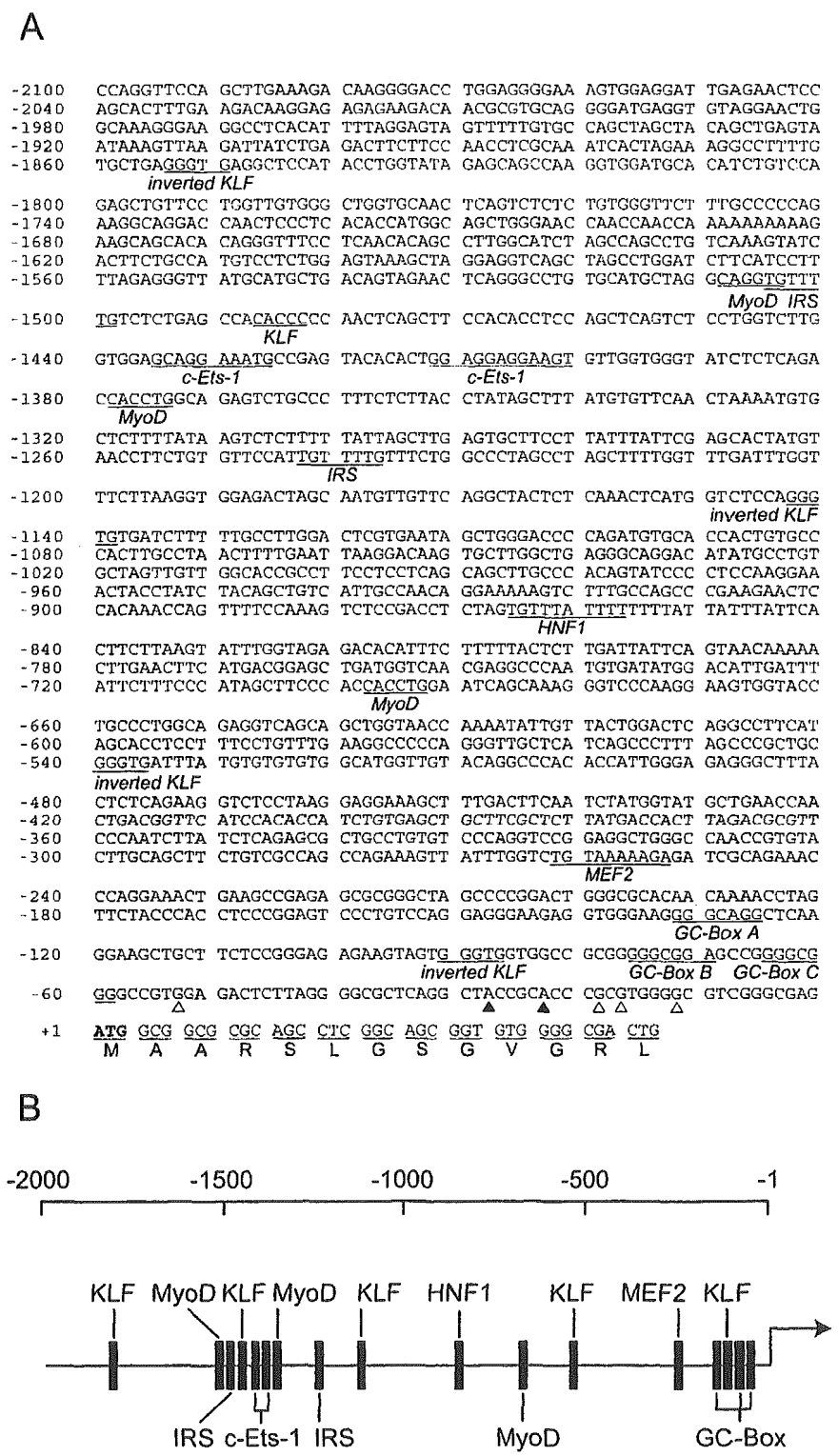

FIG. 2. **Mouse *AceCS2* Promoter.** *A*, nucleotide sequence of the 5′-flanking region of the mouse *AceCS2* gene. Nucleotide position +1 is assigned to the A of the ATG initiator codon (*boldface*), and the residues preceding it are indicated by *negative numbers*. The major and minor transcription initiation sites determined by 5′-RACE are indicated by *closed* and *open triangles*, respectively. Potential sites for KLF, MyoD, IRS, c-Ets-1, HNF1, MEF2, and GC-boxes are *underlined*. *B*, schematic diagram of the mouse *AceCS2* promoter. Potential elements are indicated by *closed boxes* and *labeled*.

Cells, Cell Culture, and Transfection—HEK293 cells (a line of human embryonic kidney cells) and C2C12 cells (a line of mouse myogenic cells) were obtained from Cell Resource Center for Biomedical Research at Tohoku University (Sendai, Japan). HSkMC (human skeletal muscle cells isolated from limbal skeletal muscle) were purchased from CELL APPLICATIONS, Inc. (San Diego, CA). HEK293 cells were maintained in medium A (DMEM containing 100 units/ml penicillin and 100 μg/ml of streptomycin sulfate, supplemented with 10% fetal bovine serum) at 37 °C in 5% CO_2. C2C12 cells and HSkMC were maintained in medium A and differentiated into myotubes in medium B (DMEM containing 100 units/ml penicillin, 100 μg/ml of streptomycin sulfate and 2% horse serum) for 5 days.

Schneider line 2 (SL2) cells, an Sp-null *Drosophila* cell line, were purchased from Invitrogen, maintained in Schneider's medium containing 100 units/ml penicillin and 100 μg/ml streptomycin sulfate, supplemented with 10% fetal bovine serum, and grown at 23 °C.

HEK293 and SL2 cells were transfected in 24-well plates using LipofectAMINE PLUS as described previously (15). After 24 h, the cells

were lysed with 0.1 ml of 1× passive lysis buffer (Promega), and aliquots were used for the measurement of firefly and *Renilla* luciferase activities as described below.

C2C12 cells were transfected exactly by the same method as that for HEK293 cells, except that the medium was switched to the differentiation medium (medium B) at 24 h post-transfection. After an additional incubation for 48 h, the cells were lysed and subjected to firefly and *Renilla* luciferase assays.

Enzyme Assays—Firefly and *Renilla* luciferase activities were measured according to the manufacturer's recommended protocol. Luciferase activities were determined in a Berthold Lumat Flash & Glow LB 955 luminometer. Firefly luciferase activities (relative light unit) were normalized by *Renilla* luciferase activities (relative light unit). For SL2 cells, β-galactosidase activities were determined using Luminescent β-galactosidase Reporter System (Clontech) and luciferase activities were normalized by the β-galactosidase activities (relative light unit).

Retroviral Vectors and Infection—The plat-E retroviral packaging cell line (16), and the retroviral vector pWZL containing the blasticidin S resistance gene were, respectively, provided by Drs. T. Kitamura (University of Tokyo, Tokyo, Japan) and G. P. Nolan (Stanford University, Palo Alto, CA). KLF15 cDNA was subcloned into the retrovirus vector, pWZL, to generate pWZL-KLF15.

To generate C2C12 cells stably expressing KLF15, the cells were infected with the retroviral vectors pWZL-KLF15 as described by Pear *et al.* (17) with the following modifications: Plat-E packaging cells were infected with plasmids using FuGENE 6 (Roche Applied Science) according to the manufacturer's instructions. After 24 h, cells were refed with fresh medium and cultured for an additional 24 h to obtain retroviral supernatants. For retroviral infection, C2C12 cells (50~60% confluence) were incubated with retroviral supernatants in the presence of 8 μg/ml polybrene for 24 h. Cells expressing KLF15 were selected for resistance to blasticidin S hydrochloride (10 μg/ml, Calbiochem, La Jolla, CA) and maintained in medium A. C2C12 cells stably expressing green fluorescent protein (GFP) were generated using a retrovirus vector encoding GFP (pWZL-GFP) and designated as C2C12/GFP.

Animal Manipulation—Male ICR mice were purchased from CLEA Japan, Inc (Tokyo, Japan). The mice were housed in a temperature- and humidity-controlled (26.5 °C and 35%, respectively) facility with a 12 h light/dark cycle (dark cycle was between 20.00–8.00). Mice were allowed to free access of water and a normal chow diet (CE-2, CLEA Japan) before experiments. Mice (7–8 weeks of age) were randomly divided into two groups: one group was fed *ad libitum* with regular diet and the other group was fasted for 48 h. All mice were sacrificed at the same time between 9.00 and11.00 AM. Quadriceps muscles were removed, weighed, and immediately frozen in liquid nitrogen and stored at −80 °C until RNA was extracted.

Quantitative Real Time PCR—First strand cDNA was synthesized from 5 μg of total RNA and oligo(dT) primers using SuperScript II reverse transcriptase. Specific primers for each gene transcript (listed in Table I) were designed using the Primer Express software (Applied Biosystems). Real time PCR contained, in a final volume of 20 μl, 3.125 ng of reverse-transcribed RNA, 167 nM of each primer, and 10 μl of 2× SYBR Green PCR Master Mix (catalog no. 4309155; Applied Biosystems). PCR was carried out in 384-well plates using the ABI *PRISM* 7900HT Sequence Detection System (Applied Biosystems). All reactions were performed in triplicate. The relative amounts of each transcript were calculated using the comparative C_T method (18). Mouse cyclophilin or human GAPDH mRNA was used as the invariant control.

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA)—For EMSA, nuclear extracts from C2C12 cells stably expressing KLF15 were prepared using Nuclear Extract Kit (Active Motif) according to the manufacturer's protocol. Complementary single-stranded oligonucleotides corresponding to nucleotides −70 to −105 were annealed and end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The labeled probe was incubated with 10 μg of nuclear extracts in a binding buffer containing 22.5 mM Hepes-KOH, pH 7.9, 2.6 mM $MgCl_2$, 13.3% glycerol, 50 mM KCl, 0.125 mM EDTA, 0.5 mM dithiothreitol, and 0.5 mg/ml poly(dI-dC) for 30 min at room temperature. For competition experiments, excess (100-fold) unlabeled double-stranded oligonucleotide was added to the binding mixture. In the supershift experiment, an antibody for KLF15 (2.5 μg) was added to the binding reaction. Protein-DNA complexes were analyzed by electrophoresis on 6% polyacrylamide gels as described previously (3).

GST Pull-down Analysis—^{35}S-labeled KLF15 was synthesized *in vitro* using pCMV-KLF15, TNT Quick Coupled Transcription/Translation system (Promega) and L-[^{35}S]methionine (Amersham Biosciences) according to the manufacturer's protocol. GST-Sp1 fusion protein was synthesized by the GST Gene Fusion System (Amersham Biosciences) and purified by the Bulk GST Purification Module (Amersham Biosciences). GST-Sp1 fusion protein and GST expressed in *E. coli* DH5α were isolated by using glutathione-Sepharose 4B beads. The immobilized GST protein beads were washed with phosphate-buffered saline and incubated with ^{35}S-labeled KLF15 at 4 °C for 1 h in the binding buffer (20 mM Hepes, pH 7.7, 75 mM KCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 0.05% Nonidet P-40, 2 mM dithiothreitol, and 10% glycerol). The beads were washed seven times with the same buffer, and subjected to SDS-polyacrylamide gel electrophoresis and autoradiography.

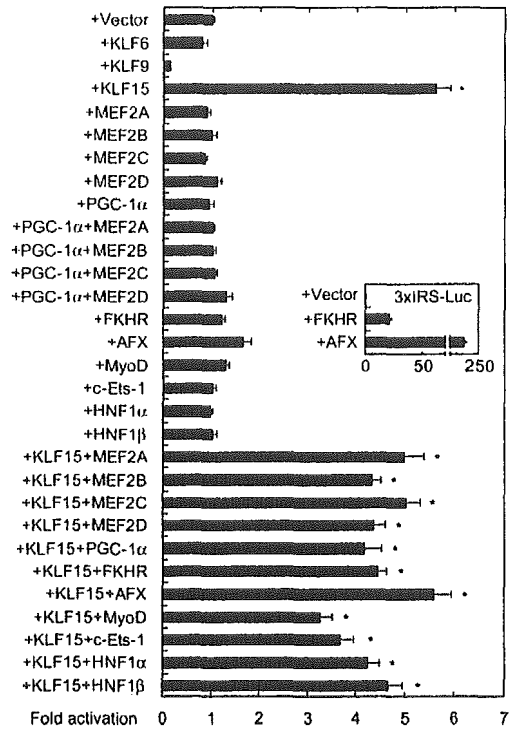

FIG. 3. **Trans-activation of *AceCS2* promoter by KLF15.** On day 0, HEK293 cells were plated at a density of 2 × 10^4 cells/well in 24-well plates. On day 1, the cells were transfected with 0.8 μg of pAceCS2(2347) along with 0.01 μg of the indicated expression plasmid(s). To normalize transfection efficiency, *Renilla* luciferase reporter (pRL-TK, 0.1 μg) was also included. On day 3, the cells were harvested for measurement of firefly and *Renilla* luciferase activities. *Renilla* luciferase activity was used as the internal reference to normalize transfection efficiency. The value for pAceCS2(2347)-luciferase activity co-transfected with pcDNA3.1 empty vector was arbitrarily set as 1. The -fold activation (luciferase activity co-transfected with each expression plasmid *versus* pcDNA3.1) is shown. The *error bars* represent mean ± S.E. of triplicate incubations. *Inset*, HEK293 cells were transfected with either 0.01 μg of pcDNA3.1 empty vector, pCMV-FKHR, or pCMV-AFX together with 0.8 μg of pIRS-luc-MLP and 0.1 μg of pRL-TK, and firefly and *Renilla* luciferase activities were determined as described above.

RESULTS

Effects of Insulin on Levels of AceCS2 Transcripts in Skeletal Muscle—We previously showed that fasting robustly increases the levels of AceCS2 mRNA in the skeletal muscle. There are several known fasting-inducible genes that are negatively regulated by insulin (19). These include genes encoding phosphoenolpyruvate carboxykinase (20), insulin-like growth factor-binding protein-1 (20), insulin receptor substrate-2 (21), and aquaporin adipose (22). To determine whether insulin negatively regulates the expression of AceCS2, we used C2C12 and human myotubes. As a positive control for the insulin response in C2C12 myotubes, we have analyzed the mRNA levels of two well known insulin responsive genes, hexokinase II and PDK4: hexokinase II and PDK4 are, respectively, up- and down-regulated by insulin. Cells were incubated with or without insulin for various time periods and the levels of AceCS2, hexokinase