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Regulation of Insulin Response in Skeletal Muscle Cell by Caveolin Status

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Abstract Recent studies on the role of caveolin-1 in adipocytes showed that caveolin has emerged as an important regulatory element in insulin signaling but little is known on its role in skeletal muscle cells. In this study, we demonstrate for the first time that caveolin-1 plays a crucial role in insulin dependent glucose uptake in skeletal muscle cells. Differentiation of L6 skeletal muscle cells induce the expression of caveolin-1 and caveolin-3 with partial colocalization. However in contrast to adipocytes, phosphorylation of insulin receptor β (IRβ) and Akt/Erk was not affected by the respective downregulation of caveolin-1 or caveolin-3 in the muscle cells. Moreover, the phosphorylation of IRβ was detected not only in the caveolae but also in the non-caveolae fractions of the muscle cells despite the interaction of IRβ with caveolin-1 and caveolin-3. These data implicate the lack of relationship between caveolins and IRβ pathway in the muscle cells, different from the adipocytes. However, glucose uptake was reduced specifically by downregulation of caveolin-1, but not that of caveolin-3. Taken together, these observations suggest that caveolin-1 plays a crucial role in glucose uptake in differentiated muscle cells and that the regulation of caveolin-1 expression may be an important mechanism for insulin sensitivity, implying the role of muscle cells for type 2 diabetes. J. Cell. Biochem. 99: 747–758, 2006.

Key words: caveolin; skeletal muscle; insulin sensitivity

Insulin-stimulated glucose uptake in adipose tissue and striated muscle is critical for reducing blood glucose levels. A failure in the uptake of glucose due to decreased insulin sensitivity in fat or muscle leads to the development of type 2 diabetes. It has been suggested that insulin-stimulated glucose uptake occurs primarily via the translocation of GLUT-4 glucose transporter to the plasma membrane [Galante et al., 1994; Watkins et al., 1997; Shepherd and Kahn, 1999; Zhou et al., 2000].

GLUT-4 translocation seems to be dependent on insulin-mediated signaling pathways. This pathway involves phosphorylation of insulin receptor β (IRβ) and insulin receptor substrates (IRS-1/2) and the subsequent activation of PI3-kinase [Saltiel and Kahn, 2001; Kanzaki and Pessin, 2003]. The exact mechanism by which these effector molecules lead to GLUT-4 translocation is unclear, but it is thought that the translocation of GLUT-4 protein to the plasma membrane by insulin stimulation is regulated via a dynamic membrane trafficking system [Kanzaki and Pessin, 2003]. Recently, it was reported that caveolin-1 and caveolae play a dual role in the regulation of glucose homeostasis, both through a direct interaction between caveolin-1 and IRβ and indirectly by promoting GLUT-4-mediated glucose uptake in adipocyte [Cohen et al., 2003a].

Yoon Sin Oh and Kyung A Cho contributed equally.

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Caveolae are 50–100 nm small invaginations in the cells which play a role in numerous cell functions, including lipid transport, membrane traffic, and cell signaling. A wide variety of signaling molecules are found to be enriched in caveolae, recruited there following activation of various receptors, such as protein kinase C, G proteins, or receptor tyrosine kinases. Caveolin, the principal component of caveolae, is a 21–24-kDa integral membrane protein [Sargiacomo et al., 1995; Schlegel et al., 1998]. The caveolin gene family consists of caveolin-1, -2, and -3. Caveolin-1 and -2 are co-expressed in many cell types to form a hetero-oligomer in the plasma membrane [Monier et al., 1996; Schlegel and Lisanti, 2000], the expression of caveolin-3 is muscle-specific [Tang et al., 1996; Song et al., 1996b; Venema et al., 1997; Hagiwara et al., 2000]. Caveolin-1 has a scaffolding domain within its NH2-terminal region. Through this domain, caveolin-1 interacts with G-protein α-subunits, H-Ras, Src-family tyrosine kinases, PKC isoforms, EGF-R, Neu, and eNOS and regulates their activities [Kim et al., 1999; Bucci et al., 2000; Razani and Lisanti, 2001; Lin et al., 2003; Abulrob et al., 2004; Cao et al., 2004]. Previously we reported that caveolin-1 plays an important role in the senescent phenotype [Park et al., 2000], which can be reversed by regulating caveolin-1 expression in senescent cells [Cho et al., 2003, 2004; Park et al., 2004]. Moreover, mice with the disrupted caveolin-1 exhibited a lean body mass, most likely due to a decreased stability of IRβ [Cohen et al., 2003a]. In adipocytes, caveolin-1 colocalizes with IRβ and positively regulates the activation of IRβ upon insulin stimulation [Yamamoto et al., 1998]. In addition, it has been reported that GLUT-4 is also localized in caveolae in adipocytes [Karlsson et al., 2002; Sun et al., 2006].

Until now, the role of caveolin-1 in insulin signaling in muscle tissue has received little attention, probably because caveolin-3 is expressed in a muscle-specific manner. But it was reported that caveolin-1 was also expressed in skeletal muscle [Kawabe et al., 2001; Li et al., 2001] and interaction between caveolin-1 and caveolin-3 was muscle cell specific [Capozza et al., 2005]. While it was reported that caveolin-1 and caveolin-3 proteins were expressed in this cell type, the functional significance of this phenomenon remained elusive. When the glucose metabolism is compared, the muscle tissue is much more important than the fat tissue on the basis of mass amount. Therefore, the molecular mechanism for insulin sensitivity in the muscle cells triggers to elucidating the pathogenesis of type 2 diabetes, especially of non-obese lean type. This study deals with the characterization of insulin sensitivity of muscle cells in connection with caveolin status.

MATERIALS AND METHODS

Reagents

The reagents used in this study and the sources from which they were purchased were as follows: Monoclonal anti-caveolin-1, -2, and -3 antibodies (BD Transductions, Palo Alto, CA); anti-Akt, phospho Akt, 42/44 MAP kinase (ERK-1/2) and phospho 42/44 MAP kinase antibodies (Cell Signaling Technology, Berverly, MA); antibodies against IRβ and IRS-1 were from (Upstate, Lake Placid, NY), anti-GLUT-4 antibody (Santa Cruz, CA); anti-β-actin antibody, sucrose and phloretin (Sigma, St. Louis, MO); 2-deoxy-D-[1-^{3}H]glucose (Amersham Pharmacia, Buckinghamshire, England); and insulin (Roche Incorporation). Cavolin siRNAs (Dharmacon Incorporation, Dallas, TX); secondary horseradish peroxidase-conjugated anti-rabbit, anti-mouse and anti-goat antibodies (Zymed, San Francisco, CA); Texas red conjugated anti-rabbit, FITC conjugated anti-mouse, Rhodamine conjugated anti-goat secondary antibodies (Molecular Probes, Inc., Eugene, OR); and chemiluminescent detection systems (Pierce, Rockford, IL) and 21-nucleotide siRNAs from Dharmacon Research (Lafayette, CO) as duplexed 2′-unprotected, desalted, and purified siRNAs.

Cell Culture and Differentiation of L6 Skeletal Myoblasts

Undifferentiated myoblast L6 cells purchased from the ATCC (Manassas, VA) and maintained in DMEM/F12 containing 10% FBS containing 100 U/ml penicillin and 100 μg/ml streptomycin, were plated in 6 well plates at 1×10^6 cells/well in DMEM/F12 containing 10% FBS. They were grown to 90% confluence, and the medium replaced with DMEM supplemented with 2% FBS and antibiotics. The cells were allowed to differentiate for 9–12 days.

Western Blotting

Cells and tissues were solubilized with lysis buffer containing 1% SDS. Fifty micrograms

of proteins from the lysates were resolved by 12% SDS–PAGE, transferred to nitrocellulose membranes, incubated with specific antibodies and visualized by blotting with horseradish peroxidase-conjugated secondary antibodies.

Immuno Precipitation

Cells were treated with insulin (100 nM) in the DMEM media for 30 min, washed twice with phosphate-buffered saline and lysed in a buffer containing 1% triton X-100, 1% cholic acid, 25 mM Tris pH 7.4, 10 mM NaCl, 1 mM protease inhibitor, 1 mM PMSF, 1 mM NaF, 1 mM sodium orthovanadate. Insoluble material was removed by centrifugation at 10,000 rpm for 10 min, and 2.5 μg/ml caveolins or IRβ antibody was added and left overnight at 4°C. Immune complexes were collected on protein A/G agarose and washed three times in ice-cold wash buffer. Immunoblotting was performed using an antibody against phosphotyrosine, IRβ, caveolin-1 and caveolin-3.

Electron Microscopic Analysis

Fully differentiated cells or siRNA treated cells were fixed in 2.5% glutaraldehyde, and then in 1% buffered osmium tetroxide. After dehydration they were treated with graded series of ethanol, emdedded Epoxy resin (Poly bed 812 kit). Their ultra-thin sections were obtained by ultramicrotome (RMC products-MTXL) with diamond knife. Heavy metal stain was done with 4% uranyl acetate and lead citrate. The sections were examined in an electron microscope (JEM-100CX: Tokyo, Japan) at 60 kV.

2-Deoxyglucose Uptake Assay

Insulin stimulated glucose transport in L6 cells was estimated by measuring 2-deoxyglucose uptake. In brief, cells were washed twice with DMEM then incubated in DMEM containing for 3 h at 37°C (serum starvation), washed twice with HBS buffer (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 5 mM glucose, pH 7.4). Glucose uptake was initiated by adding of [^{3}H]-2-deoxy-D-glucose to a final concentration of 100 nM for 30 min at 37°C. Glucose transport was terminated by two ice-cold PBS washes, and the cells were solubilized with 0.5% SDS, 0.5% NaOH. Tritium levels were determined by scintillation counting. Non-specific deoxyglucose uptake was measured in the presence of 5 mM phloretin and subtracted from each determination to obtain specific uptakes.

Design and Synthesis of siRNA Duplexes

Synthetic siRNA duplexes were used as described by Elbashir et al. [2001]. Double-stranded siRNA molecules were designed to target rat caveolin-1 and caveolin-3 mRNA. The target sequence of caveolin-1 was AACAUCUACAAGCCCAACAAC and that of caveolin-3 AATCAAGGACATTCACTGCAA. A GL luciferase oligonucleotide was used as a negative control.

Electroporation of L6 Muscle Cells

L6 muscle cells were transfected with siRNA duplexes by electroporation. In brief, myocytes at day 12 of differentiation were detached from culture dishes with trypsin in PBS. Approximately, 1×10^6 cells were then mixed with siRNA duplexes, which were delivered to the cells by pulse electroporation using a BioRad gene pulser II system at 1.0 kV and 10 μF. After electroporation, cells were immediately mixed with fresh medium for 10 min and reseeded onto dishes designed for deoxyglucose uptake assay, Western blotting, and immunofluorescence microscopy.

Sodium Carbonate Extraction and Sucrose Density Gradient Fractionation of Caveolae

Experiments were carried out following the detergent-free protocol developed by Song et al. [1996a]. Briefly, L6 cells were washed with ice-cold phosphate-buffered saline, scraped into 1 ml of 0.5 M Na_2CO_3, pH 11.0, and homogenized. Sonicated cell samples were mixed with equal volumes of 80% sucrose solution in MES-buffered saline (25 mM MES, pH 6.5, 0.15 M NaCl), placed in an ultracentrifuge tube, and overlaid with 4 ml of 30% sucrose and 4 ml of 5% sucrose in MES-buffered saline containing 0.25 M Na_2CO_3. Gradients were generated by centrifugation at 39,000 rpm for 18 h in a SW41 rotor (Beckman). Fractionations were collected from the top of the gradient, and dissolved in $1 \times$ Laemmli SDS sample buffer prior to Western blotting.

Immunofluorescence Microscopy

Cells cultured on glass coverslips were fixed with 4% formaldehyde and permeabilized with PBS containing 0.5% Triton X-100 and blocked with 2% BSA in PBS for 1 h. They were then

incubated with rabbit polyclonal antibody against caveolin-1, mouse monoclonal anti body against caveolin-3 or goat polyclonal antibody against GLUT-4 overnight. After washing, the coverslips were incubated with Texas red-conjugated anti-rabbit, FITC-conjugated anti-mouse, or Rhodamine conjugated anti-goat secondary antibodies. The labeled cells were observed under a confocal microscope.

RESULTS

Tissue-Specific Expression of the Caveolins

Caveolin-1 was abundantly expressed in endothelial cells, fibroblasts, and adipocytes while caveolin-3 was expressed in cardiac, skeletal and smooth muscle cell myocytes. Caveolin-2 was reported to be co-expressed with caveolin-1 in general, and form hetero-oligomeric complexes [Rothberg et al., 1992]. To check the tissue distribution of caveolin proteins, we prepared extracts from a number of different rat tissues (Fig. 1). Caveolin-1 was most widely expressed in adipose and lung tissue and expression of caveolin-2 protein most closely parallels the distribution of caveolin-1. Caveolin-3 was expressed only in skeletal muscle and heart tissues. Interestingly, caveolin-1 and -2 were also co-expressed with caveolin-3 in muscle and heart.

Expression of Caveolins and Caveolae Structures During Differentiation

To identify the role of caveolin-1 in muscle, we studied its expression and functions in skeletal muscle cells (L6 myoblasts). Capozza et al. [2005] reported that caveolin-1 and caveolin-3 were colocalized and interacted in L6 myoblasts, but not in fibroblasts. Galbiati et al. [2001] also reported that during differentiation of C2C12 cells, in other skeletal muscle cells, caveolin-1 and caveolin-3 proteins are detectable. Therefore, we used differentiated L6 cells induced by stimulating with 2% FBS for 9 days. L6 myoblasts showed myotubule formation after differentiation (Fig. 2A) and during differentiation the level of myogenin, a differentiation marker protein, was increased as well as GLUT-4 (Fig. 2B). Also, caveolin-1, caveolin-2 and caveolin-3 expressions increased whereas IRβ expressions were unchanged during differentiation (Fig. 2B).

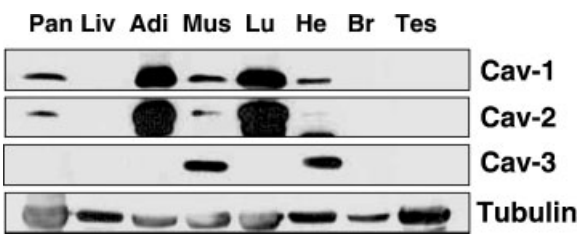

Fig. 1. Tissue-specific expression of the caveolins. Major organs from Fisher 344 rats were isolated and analyzed for the expression levels of caveolin proteins by Western blotting using anti-caveolin-1, caveolin-2, or caveolin-3 antibodies. Antibody to the ubiquitously expressed tubulin was used to confirm equal loading. Pan, Pancreas; Liv, Liver; Adi, Adipose tissue; Mus, Skeletal muscle; Lu, Lung; He, Heart; Br, Brain; Tes, Testis.

Next, we examined caveolae structures by transmission electron microscopy in differentiated L6 cells. As expected, caveolae structures, (50–100 nm invaginations, in the membrane) increased in differentiated L6 (Fig. 2C, arrows, 2D) but not in L6 myoblasts.

Downregulation of Caveolins and Caveolae Structures by siRNA

To examine the exact role of caveolin-1 and caveolin-3 in the muscle, we downregulated caveolin-1 or caveolin-3 using 21-nucleotide siRNA in differentiated L6 cells. When caveolin-3 was downregulated with a specific siRNA, the expression of caveolin-1 was also slightly downregulated. These results caused by that two proteins co-interacted in differentiated L6 cells (Fig. 5B). However, as shown in Figure 3A, caveolin-1 and -3 were effectively downregulated by siRNA through electroporation. Also, caveolae structures were disrupted by downregulation of caveolin-1 or -3 (Fig. 3B). Quantitative analysis of caveolae structures revealed that siRNA-treated cells contained over five times less caveolae structure than GL siRNA-treated cells (Fig. 3C). From these results, the siRNAs to caveolin-1 and -3 were found to be active not only in terms of caveolin reduction but also in terms of the inhibition of caveolae structures in L6 cells.

Roles of Caveolin-1 and Caveolin-3 on Myotubule Formations

It was suggested that caveolin-3 plays a role in myotube fusion and muscle formation [Galbiati et al., 1999], but the role of caveolin-1 in skeletal muscle cell is not clear. To determine the role of caveolins on myotubule formation, we induced the differentiation after siRNA treatment in L6 myoblasts. After 9 days, L6 myoblasts were differentiated. As shown in

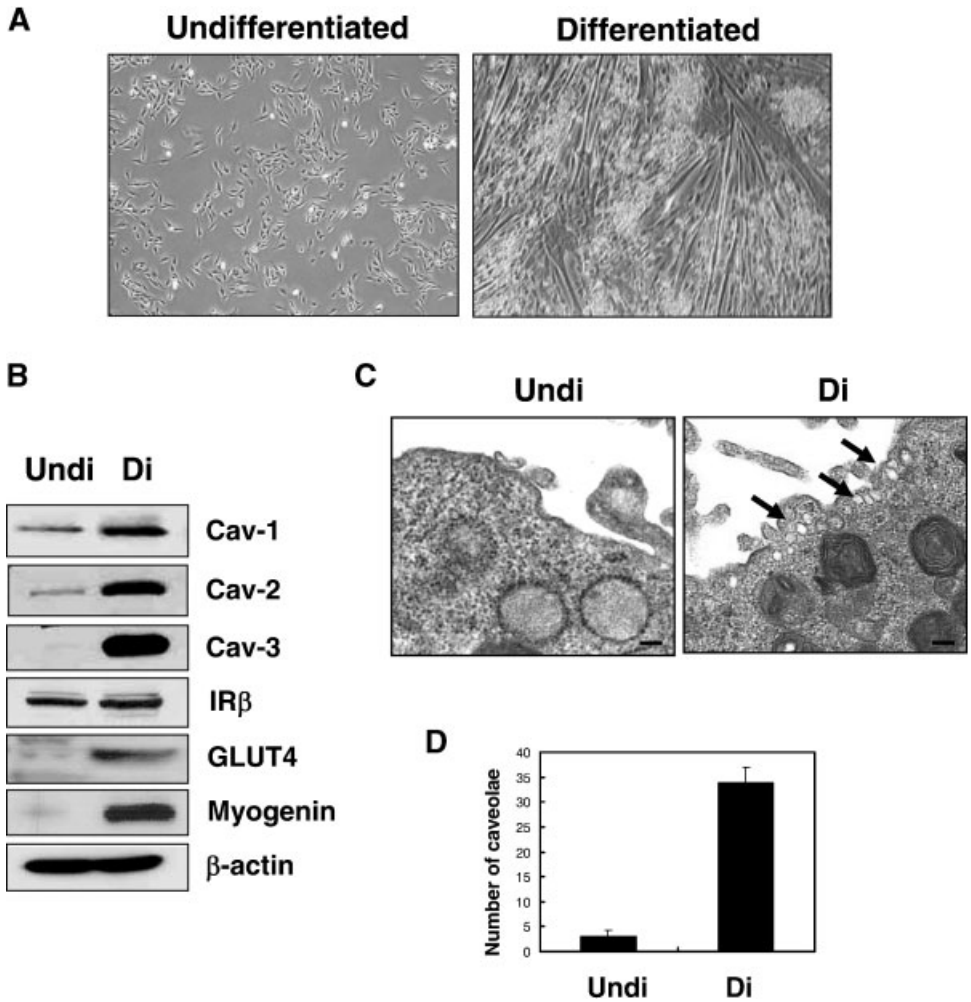

Fig. 2. Expression of caveolins and caveolae structures during differentiation. Myoblasts (L6) were cultured in 10% FBS/DMEM, 100 U/ml antibiotics, and when cells were over confluent, the medium was changed to one containing 2% FBS/DMEM. After 9 days the cells showed multinucleated myotubules. **A**: Morphology of undifferentiated (Undi) and differentiated cells (Di) observed by light microscopy. **B**: Expression levels of caveolins and insulin signaling molecules in Undi and Di cells were analyzed by Western blotting. **C**: Undi and Di cells were grown on 100-mm dishes and examined under transmission electron microscopy. All analyses were performed at a magnification of ×14,000. Arrows indicate caveolae structures (Bar = 100 nm). **D**: Numeric counts of caveolae-like vesicles were statistically analyzed in three independent cells.

Figure 4A, differentiation of L6 myoblasts and myotubule formation were not induced by downregulation of caveolin-3, in contrast to caveolin-1 or control siRNA. These results were confirmed by myogenin expression (Fig. 4B).

Role of Caveolin-1 and Caveolin-3 in IRβ Activation

To define the role of caveolin-1 in differentiated muscle cells, we focused on the role of caveolin-1 in insulin signaling. Previous studies showed that ligand-bound IRβ localized to caveolae in adipocytes [Scherer et al., 1994], and that caveolin-1 plays an essential role in IRβ activation leading to the phosphorylation of IRS-1, Akt and Erk in vivo [Yamamoto et al., 1998]. We, therefore, examined the phosphorylation of Akt and Erk (activated through IRβ phosphorylation by insulin stimulation) after transfecting the siRNAs of caveolin-1 or -3 in differentiated L6 cells. Neither caveolin-1 nor caveolin-3 affected the insulin-dependent activation of Akt/Erk (Fig. 5A). Figure 5B shows that IRβ interacted with caveolin-1 or caveolin-3 and was phosphorylated by insulin stimulation. To elucidate whether caveolin-1 or caveolin-3 affect the activation of IRβ, we monitored the phosphorylation of IRβ by insulin stimulation after downregulation of caveolin-1 or caveolin-3. Phosphorylation of IRβ was not affected by downregulation of caveolin-1 or caveolin-3

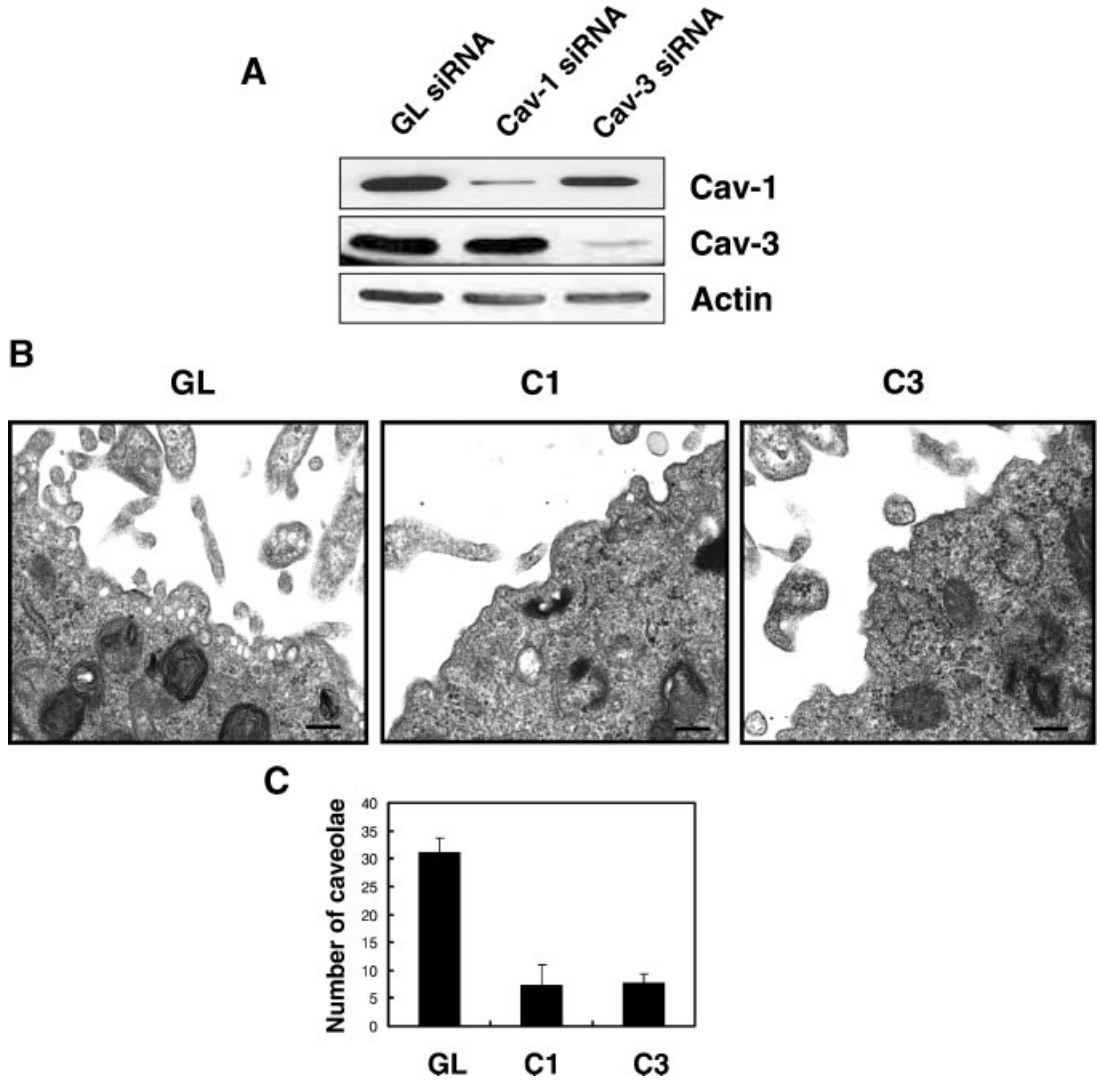

Fig. 3. Downregulation of caveolins and caveolae structures by siRNA. Fully differentiated L6 cells were transfected with the siRNA of caveolin-1 (0.4 nmol of siRNA duplexes/1 $\times$ 10^6 cells) and caveolin-3 (0.5 nmol of siRNA duplexes/1 $\times$ 10^6 cells) by electroporation, reseeded for 48 h. siRNA of GL was used as a control. **A**: The expression levels of caveolins were determined with anti-caveolin-1 and caveolin-3 antibodies. **B**: Electron microscopic analysis of differentiated L6 cells after siRNAs treatment. All analyses were performed at a magnification of $\times$14,000. **C**: Numeric counts of caveolae-like vesicles were statistically analyzed in five independent cells. (GL denotes that control siRNA for caveolins, whereas C1 and C3 denotes that siRNA for caveolin-1 and caveolin-3 were treatment.)

(Fig. 5C,D). In other words, caveolin-1 or caveolin-3 was not important to activation of IRβ by insulin stimulation. These results were confirmed by caveolae fractionation. We checked the localization and phosphorylation of IRβ by insulin stimulation. In contrast to adipocyte [Yamamoto et al., 1998], IRβ was localized in both caveolae and non-caveolae fraction and the distributions of IRβ were not changed by insulin stimulation in L6 cells (Fig. 5E). Also, phosphorylation of IRβ was detected in both non-caveolae and caveolae fractions. Unlike in adipocyte [Cohen et al., 2003b], although IRβ was bound with caveolin-1 or caveolin-3, the phosphorylation of IRβ was not affected in differentiated L6 cell.

Role of Caveolin-1 in Glucose Uptake Via GLUT-4 Translocation

Insulin increases glucose uptake by stimulating the translocation of GLUT-4 transporter from intracellular sites to the plasma membrane [Studelska et al., 1992; Galante et al., 1994; James et al., 1994]. Recently, caveolin-1 has emerged as a potential participant in the regulation of downstream insulin dependent

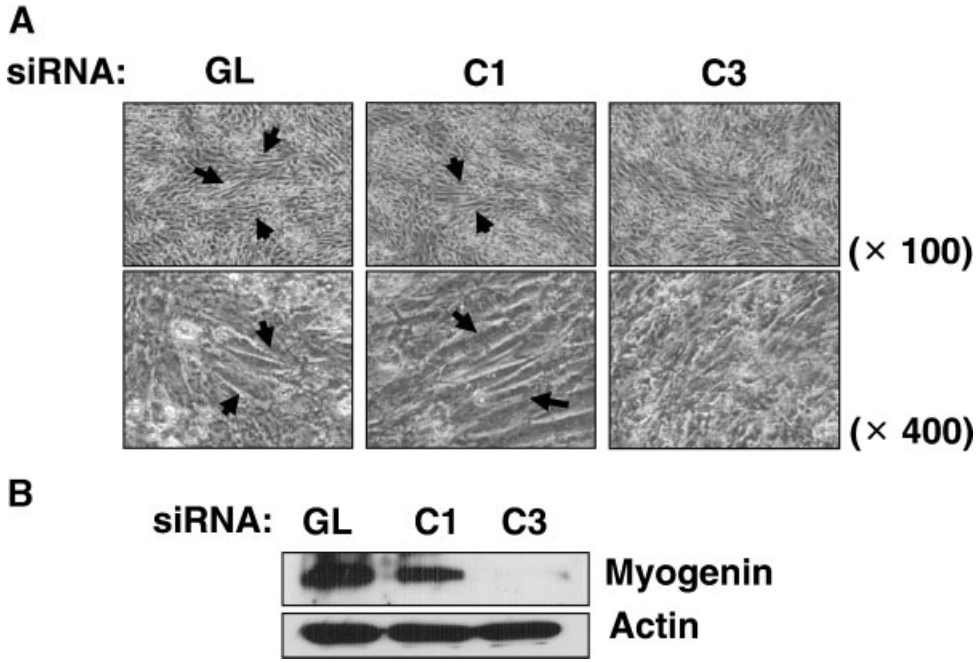

Fig. 4. Roles of caveolin-1 and caveolin-3 on myotubule formations. L6 cells were cultured as described under Figure 2 and when overconfluent, transfected with siRNA caveolin-1 and caveolin-3 by electroporation. Caveolins downregulated cells were replated and examined for 12 days. **A**: After 9 days, tubule formations of each siRNA treated cells were analyzed by light microscopy. (upper panel, ×100; lower panel, ×400) Arrows indicate the formation of myotubules. **B**: Total cell lysates from each of the cells were analyzed by Western blotting using anti-myogenin antibody. (GL denotes that control siRNA for caveolins, whereas C1 and C3 denotes that siRNA for caveolin-1 and caveolin-3 were treatment.)

cellular effect via a role in GLUT-4 mediated glucose transport in the adipocytes [Cohen et al., 2003a]. Therefore, we examined whether glucose uptake, the final output of IR stimulation, was modulated by caveolins in the muscle cells. Basal glucose uptake levels were not different between GL and siRNA of caveolin-1 and caveolin-3 but were significantly decreased upon insulin stimulation by the downregulation of caveolin-1, but not by caveolin-3 (Fig. 6A). Interestingly, GLUT-4 was totally enriched in caveolae fraction regardless of insulin treatment (Fig. 6B) and the translocation of GLUT-4 was failed by downregulation of caveolin-1 from cytosol to membrane (Fig. 6C). These results suggest that caveolin-1 might play an important role in glucose uptake via regulating GLUT-4 translocation upon insulin stimulation in differentiated L6 cells.

DISCUSSION

In this study, we have explored the differential roles of caveolins in skeletal muscle cells with special attention on their roles for muscle formation and insulin sensitivity. The key role of caveolin-1 in insulin signaling in adipocytes has been extensively examined, but not in muscle type cells. Caveolin-3 is well known as a muscle specific subtype; it forms a complex with dystrophin and its associated glycoproteins that are specific to muscle cells [Song et al., 1996b].

Recently, Capozza et al. [2005] demonstrated that caveolin-1 is expressed and colocalized with caveolin-3 in muscle cells. Interestingly, GLUT-4, a key molecule in insulin-dependent glucose uptake, was not expressed in L6 myoblasts but was increased with differentiation. Therefore, we analyzed the role of caveolin-1 and caveolin-3 in insulin signaling and insulin-dependent glucose uptake after differentiation of L6 myoblasts. Moreover, expression of caveolin-1 and caveolin-3 increased during differentiation in skeletal muscle cells (Fig. 2B) and they interacted with each other (Fig. 5B) to form caveolae structures in differentiated L6 cells (Fig. 2C). Caveolin-1 was also co-expressed with caveolin-3 in skeletal muscle and heart of rat (Fig. 1). These data suggested that caveolin-1 and caveolin-3 might play important roles in muscle cell development and its functions.

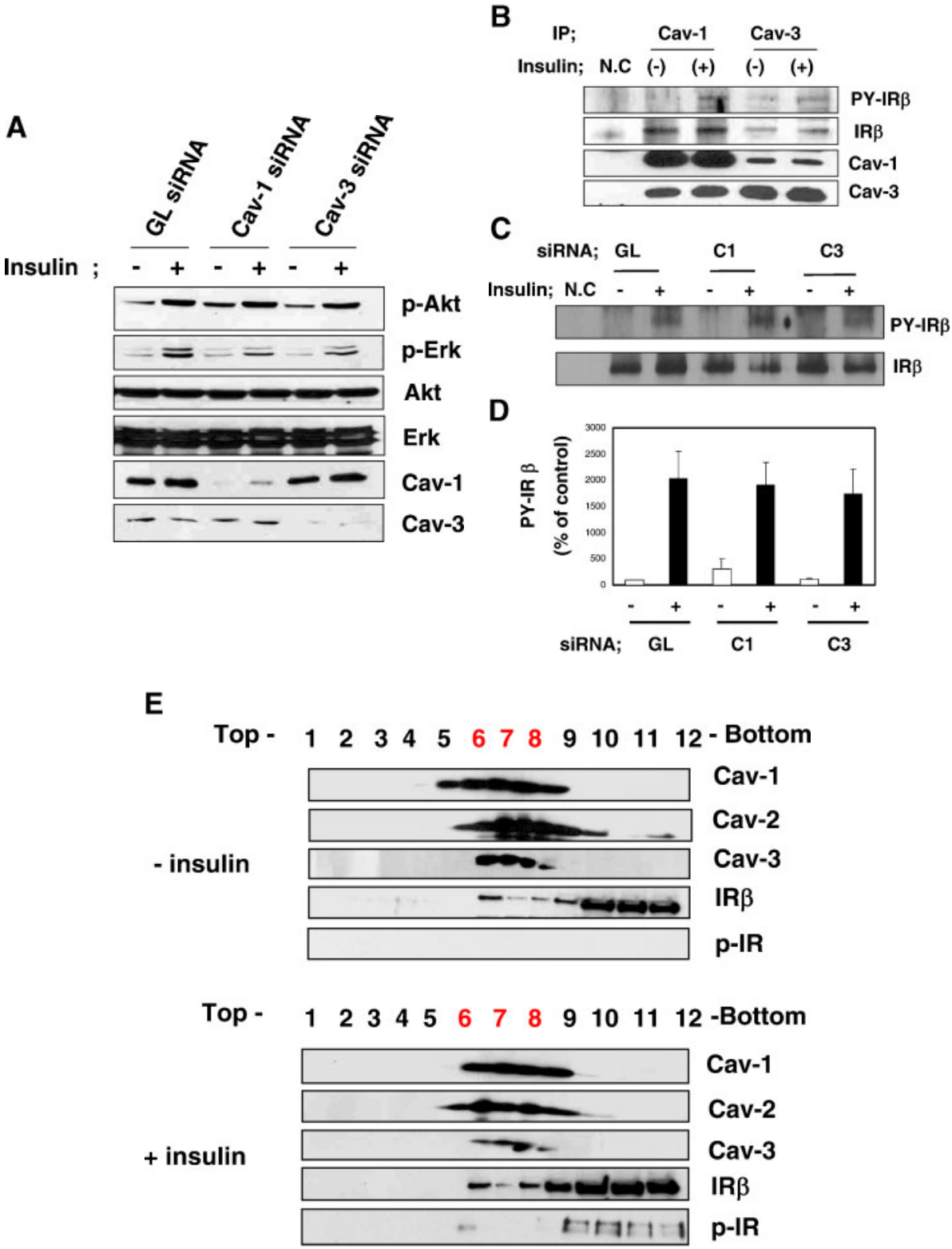
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Fig. 5.

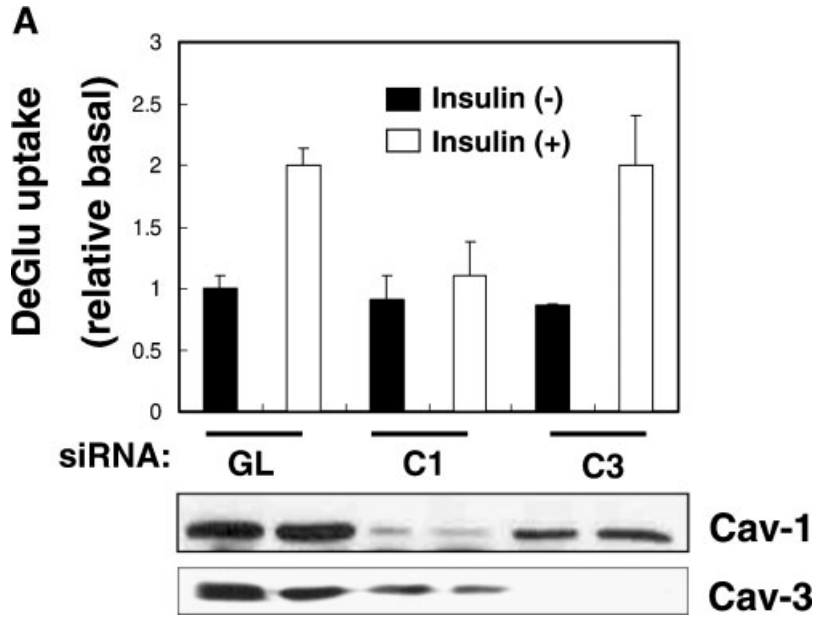

Fig. 6. Role of caveolin-1 in glucose uptake and GLUT-4 translocation. Fully differentiated cells were transfected with the siRNAs of caveolin-1, -3 or the siRNA of GL, as a control. After 48 h, the transfected cells were incubated in the absence or presence of insulin (100 nM). **A**: Glucose uptake levels by transfected cells were checked by adding 0.5 μCi/ml 2-deoxy-D-Glucose (deGlu). Results are expressed as fold stimulation over basal levels. Results shown are the means ± SE of three experiments. **B**: Differentiated L6 cells were treated as described under Figure 5E and the localizations of caveolins and of GLUT-4 were analyzed by Western blotting using each antibodies. **C**: GLUT-4 translocation was followed by confocal microscopy. GLUT-4 was detected by Rhodamine fluorescence (Red). Arrows indicate the localization of GLUT-4 in the membrane. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Caveolin-3 null mice show T-tubule abnormalities in muscle fibers [Galbiati et al., 2001] and mutation of caveolin-3 cause limb girdle muscular dystrophy leading to apoptosis of skeletal muscle [Smythe et al., 2003] and muscle diseases [Yabe et al., 2003]. These findings suggest that caveolin-3 plays an essential role in muscle development [Betz et al., 2001; Marx, 2001]. As shown in Figure 4, tubule formation was also negatively influenced by downregulation of caveolin-3 after differentiation (Fig. 4), but insulin-dependent IRβ activation and glucose uptake were not affected (Figs. 5 and 6). These results suggest that caveolin-3 would be involved in tubule formation rather than insulin sensitivities. Recently, it has been reported that caveolin-3 null mice show insulin resistance in skeletal muscle [Oshikawa et al., 2004]. In these null mice, IRβ and IRS-1/2 were not phosphorylated upon insulin stimulation in skeletal muscle, suggesting the role of caveolin-3 for insulin sensitivity. However, it was not clear whether insulin resistance was induced by caveolin-3 deficiency or by muscle abnormality.

Therefore, we compared the roles of caveolin-1 and caveolin-3 in insulin signaling in differentiated L6 skeletal muscle cells. In adipocytes, caveolin-1 plays a critical role in activation of

Fig. 5. Roles of caveolin-1 and caveolin-3 in insulin receptor β (IRβ) activation. Fully differentiated L6 cells and siRNA treated cells were starved for 3 h and incubated in the absence (−) or presence (+) of 100 nM insulin. **A**: Proteins (50 μg) in siRNA treated cell lysates were detected using anti-phospho specific Akt/Erk, Akt, Erk, and caveolins antibodies. **B**: Differentiated cell lysates were incubated with anti-caveolin-1 or caveolin-3 antibody for 18 h and protein A/G agarose was added. Complexes were detected by Western blotting with anti-phospho tyrosine antibody (PY-IRβ), IRβ, anti-caveolin-1, and caveolin-3. **C**: siRNA treated cell lysates were incubated with anti-IRβ antibody for 18 h and protein A/G agarose added. Complexes were detected by Western blotting with anti-phospho tyrosine antibody (PY-IRβ), IRβ. **D**: Quantitative analysis of Western blots. Relative abundance of each band was estimated by densitometric analysis. Each bar represents the mean ± SEM from three independent experiments. **E**: Differentiated cell lysates were homogenized without detergent, sonicated, and subjected to sucrose density gradient fractionation and the localizations of caveolins, IRβ and of phospho-IRβ were analyzed by Western blotting using each antibodies. GL denotes that control siRNA for caveolins, whereas C1 and C3 denotes that siRNA for caveolin-1 and caveolin-3 were treatment. NC, negative control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

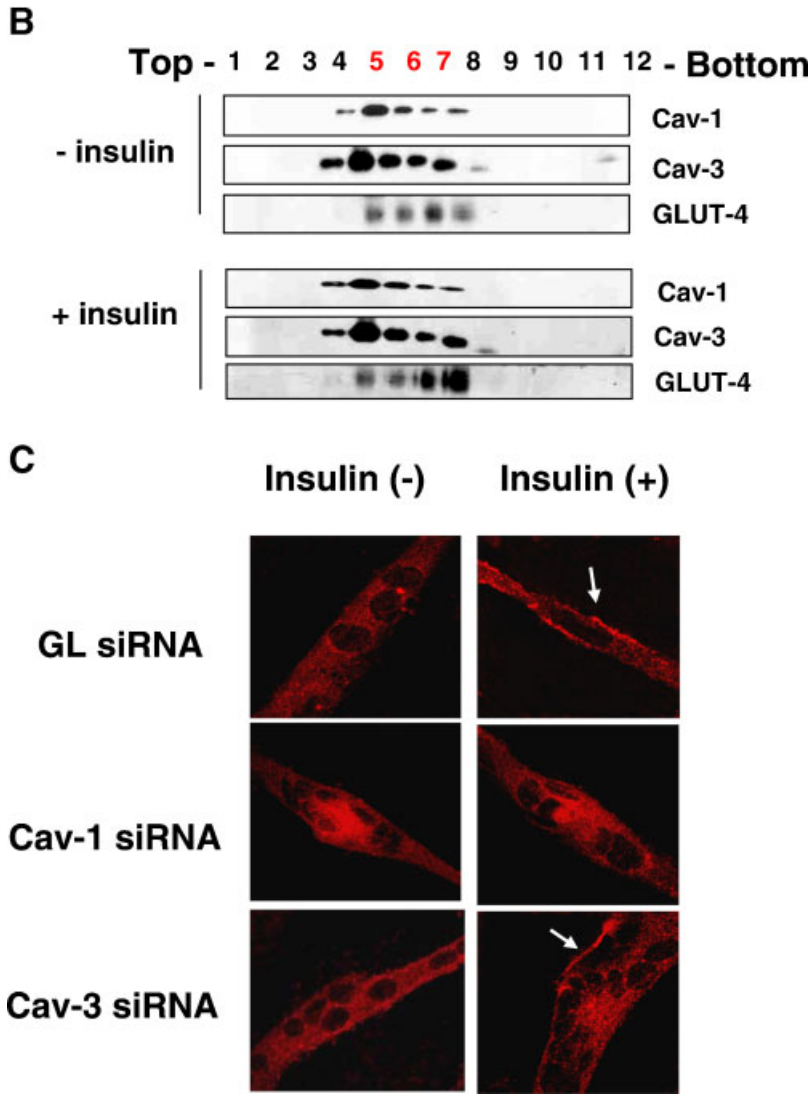

Fig. 6. (*Continued*)

IRβ via its direct interaction and GLUT-4 translocation upon insulin stimulation [Karlsson et al., 2002; Cohen et al., 2003b]. Although IRβ was directly interacts with caveolin-1 and caveolin-3 as in adipocyte, the phosphorylation of IRβ was not affected by downregulation of either caveolin-1 or caveolin-3 in L6 skeletal muscle cells. In addition, IRβ was localized not limitedly within the caveolin-enriched membrane domain but also in non-caveolae fractions and phosphorylation of IRβ was also detected in both fractions (Fig. 5E). These results suggest that the activity of IRβ was not tightly regulated by caveolin-1 or caveolin-3 in L6 skeletal muscle cells in contrast to adipocytes.

Although IRβ activation was not directly regulated by caveolin-1 (Fig. 5A,C), insulin-dependent glucose uptake was significantly decreased by downregulation of caveolin-1 in differentiated L6 cells (Fig. 6A). It is known that caveolin-1 participates in cellular trafficking and that it contains many molecules known to be involved in this process, including dynamin, and several SNARE proteins [Schnitzer et al., 1995; Liu et al., 2002]. SNARE proteins and their accessory proteins are required for the final fusion of GLUT-4-containing vesicles by integrating GLUT-4 protein into the plasma membrane [Saltiel, 2001]. Therefore, it is presumable that caveolin-1 would affect the GLUT-4 translocation after insulin stimulation. Although we did not obtain any direct evidence for a relationship between caveolin-1 and GLUT-4, we observed that GLUT-4 was

mainly localized in caveolin-rich membrane domain and GLUT-4 translocation was failed specifically by downregulation of caveolin-1 after insulin stimulation, but not by caveolin-3 (Fig. 6B,C). Therefore, it might be concluded that caveolin-1 would be involved in insulin-dependent glucose uptake via regulation of GLUT-4 translocation in muscle cells as well as adipocytes but by different mechanism for IRβ pathway activation. Our data suggest the possible linkage of muscle cells with type 2 diabetes pathogenesis through caveolin status for insulin sensitivity with different mode of action from that of fat cells. These results would contribute to the elucidation of the molecular mechanism of type 2 diabetes based on insulin sensitivity, especially of non-obese type, of which pathogenesis has not been well understood yet.

Taken together, this study demonstrates that caveolin-1 and caveolin-3 play specific roles in the maintenance and insulin response of skeletal muscle cells, with caveolin-1 emerging as a potential candidate regulator of insulin dependent glucose uptake in skeletal muscle cells.

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