Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 330 (2005) 88–96
BBRC
www.elsevier.com/locate/ybbrc

Caveolin-2 regulation of the cell cycle in response to insulin in Hirc-B fibroblast cells

Sangmin Kim, Yunbae Pak *

Department of Biochemistry, Division of Life Science, College of Natural Sciences, Graduate School of Applied Life Science, Gyeongsang National University, Jinju 660-701, Republic of Korea

Received 18 February 2005

Abstract

The regulatory function of caveolin-2 in cell cycle regulation by insulin was investigated in human insulin receptor-overexpressed rat 1 fibroblast (Hirc-B) cells. Insulin increased induction of the caveolin-2 gene in a time-dependent manner. Direct interaction between ERK and caveolin-2 was confirmed by immunoprecipitation and phosphorylated ERK increased the specific interaction in response to insulin. That insulin induced their nuclear co-localization over time was demonstrated by immunofluorescence microscopy. Insulin increased the S phase in the cell cycle by 6-fold. When recombinant caveolin-1 was transiently expressed, a decrease in the S phase was detected by flow-cytometry. The results indicate that the up-regulation of caveolin-2 in response to insulin activates the downstream signal cascades in the cell cycle, chiefly the increased phosphorylation of ERK, the nuclear translocation of phosphorylated ERK, and the subsequent activation of G0/G1 to S phase transition of the cell cycle. The results also suggest that DNA synthesis and the activation of the cell cycle by insulin are achieved concomitantly with an increase in the interaction between caveolin-2 and phosphorylated ERK, and the nuclear translocation of that complex. Taken together, we conclude that caveolin-2 positively regulates the insulin-induced cell cycle through activation of and direct interaction with ERK in Hirc-B cells.

Keywords: Caveolin-2; Insulin; ERK; Cell cycle

It has been well established that insulin signaling in cellular mitogenesis and differentiation is transmitted via mitogen-activated protein kinase (MAPK) pathway [1]. The highly conserved MAPK cascades are found in all eukaryotic organisms and consist of a three-kinase module that includes extracellular signal-regulated protein kinases (ERK-1/2), which in turn are activated by MEK and Raf [2]. The MAPK cascade plays a pivotal role in the re-entry of fibroblasts into the cell cycle for mitogenesis and in differentiation of cells in response to various growth factors [3]. Interestingly, ERK-1/2 or p42/p44 MAPK was re-localized from cytoplasm to nucleus in fibroblasts upon growth factor stimulation with a strong correlation between the mitogenic potency of a stimulus and its ability to trigger ERK translocation [4]. Furthermore, comparison of the kinetics of ERK activation and nuclear translocation suggests that it is the active phosphorylated form of ERK that translocates into the nucleus [5].

The progression of quiescent cells from the G0 through the G1 phase of the cell cycle is orchestrated by interaction between components of the cell cycle regulatory apparatus [6]. The induction of G1 phase regulatory cyclins, cyclin-dependent kinases (cdks), and E2F-responsive genes contributes to continued passage of cells through G1 and into S phase as cells respond to extracellular signals by transmitting intracellular instructions to coordinate appropriate responses [7]. In eukaryotes, a large family of cdks have been identified

* Corresponding author. Fax: +82 55 752 7062.
E-mail address: ybpak@nongae.gsnu.ac.kr (Y. Pak).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.02.130

[8] each of which coordinates one or more cell cycle checkpoints. A series of biochemical pathways that ensure the initiation of particular cell cycle events occurs only after successful completion of others. Passage from G1 into S phase requires the activation of cyclin D1-associated cdk4 and cdk6 [9], which contributes to phosphorylation of the retinoblastoma protein, pRB [7,10].

Caveolin, an integral membrane protein, is a principal component of caveolae membranes in vivo [11]. Caveolin is expressed by at least three genes whose products are referred to as caveolin-1, caveolin-2, and caveolin-3. Recent studies suggest that caveolin family members function as scaffolding proteins to organize and concentrate cholesterol, glycosylphosphatidylinositol (GPI), glycolipid, and sphingolipid, and interact with signaling molecules like Src-like kinases, H-Ras, eNOS, G-proteins, and ERK within caveolae [12]. Therefore, various functions of caveolae and regulatory roles of caveolin have been proposed [11–14].

Recently, Hulit et al. [15] reported that the cyclin D1 gene is inhibited during overexpression of caveolin-1 as a result of repression of the cyclin D1 promoter. The repression of the cyclin D1 gene by caveolin-1 contributes to the inhibition of cellular transformation [15,16]. The expression of caveolin-1 is significantly reduced in human breast cancer cells as compared to their normal mammalian epithelial counterparts [16,17]. Caveolin-1, thus, affects the regulatory mechanism of cell growth and reduces not only cell growth but also tumorigenicity. This observation is consistent with the oncogenic transformation of the cells causing a lack of caveolin-1 expression, and further the transcriptional inhibition is related functionally to the tumor phenotype [15–18].

Although there have been reports showing that the MAPK cascade is regulated by the relative abundance of caveolin proteins, chiefly by caveolin-1 [16,18,19], the molecular mechanisms by which caveolin regulates the cell cycle are not well established. Except for its structural role in the formation of caveolae, little is known concerning any role of caveolin-2, especially in the regulation of the cell cycle [20–23]. The present study was conducted to investigate the role of caveolin-2 in the regulation of the cell cycle by insulin and to assess whether caveolin-1 and -2 differently regulate the cell cycle in Hirc-B cells, which have caveolin-2 as an endogenous caveolin. By using co-immunoprecipitation, immunofluorescence microscopy, and flow-cytometry, we demonstrate that insulin increases the direct interaction of phosphorylated ERK and caveolin-2, nuclear translocation of the phosphorylated ERK, and subsequent activation of G0/G1 to S phase transition of the cell cycle. We also demonstrate that the endogenous caveolin-2 activates the insulin-induced cell cycle whereas caveolin-1 inhibits when Hirc-B cells were expressed with recombinant caveolin-1.

Materials and methods

Cell culture. Hirc-B fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco/BRL) (containing 5 mM D-glucose) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, Utah), 100 nM methotrexate (Sigma Chemical, St. Louis, MO), and 0.5% gentamicin (Sigma) in a 5% CO_2 incubator at 37 °C as previously described [24]. Rat-1 fibroblasts (Rat-1) were grown in DMEM supplemented with 10% FBS and 50 μg/ml each of streptomycin and penicillin (Sigma). Cells were routinely incubated for 16–24 h in serum-free DMEM before 100 nM insulin (Porcine, Sigma I0259) treatment.

Western blot analysis. For the protein extraction, cells were washed twice with cold phosphate-buffered saline (PBS) and lysed with RIPA buffer (50 mM Hepes, 150 mM NaCl, 100 mM Tris–HCl, pH 8.0, 1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, and 5 mM EDTA), containing 10 mM NaF, 5 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium vanadate, 20 μM leupeptin, and 100 μM aprotinin. The lysate was briefly sonicated on ice and microcentrifuged at 12,000 rpm for 10 min at 4 °C. Aliquots from clear supernatant were taken for protein quantification and determined by the BCA assay (Pierce, Rockford, IL). Equal amounts of samples (15–50 μg) were subjected to SDS–PAGE in 12.5% polyacrylamide gels [25] before proteins were electrotransferred to polyvinylidene difluoride (PVDF) membrane (Millipore: Bedford, MA, USA). After incubating with antibodies specific for caveolin-1 (Transduction Laboratories, C37120, C18360), caveolin-2 (Transduction Laboratories, C57820), insulin receptor β-subunit (IR-β) (Transduction Laboratories), ERK (Transduction Laboratories, M12320) or phosphospecific-ERK (Thr 202 and Tyr 204) (New England Biolabs., 9106S), the membranes were washed and antibody binding was detected by secondary antibodies conjugated to horseradish peroxidase and visualized by chemiluminescence (ECL Western Blotting Detection System, Amersham, Arlington Heights, IL, USA).

Northern blot analysis. Total RNA was isolated from cells using TRIZOL reagent (MRC, TR118) following supplier's protocol. Briefly, equal amounts of RNA (10 μg) were denatured in 68% formamide and 2.2 M formaldehyde at 65 °C, subjected to 1% agarose/formaldehyde gel electrophoresis, transferred to nylon membranes, and immobilized by UV cross-linking. Radioactively labeled cDNA probes of human caveolin-1 or caveolin-2 were generated by labeling with [α-^{32}P]dATP in a random primed reaction and Northern blotting was performed using the labeled cDNA probes. After UV cross-linking, membranes were sequentially hybridized and rehybridized with the cDNA probes. Hybridization was carried out for 24 h at 65 °C. Final blot washing conditions were 2× SSC (0.3 M NaCl/0.03 M sodium citrate, pH 7.0)/0.1% SDS at 37 °C for 10 min, 2× SSC/0.1% SDS at 65 °C for 10 min, and then 0.2× SSC/0.1% SDS at 65 °C for 10 min.

Immunohistochemistry. Detection of insulin receptor protein expression in Rat-1 and Hirc-B cells by immunohistochemistry was performed using an antibody specific for the IR-β. Cells were routinely cultured on glass coverslips in chamber slide. Cells on the coverslips were fixed for 20 min at 4 °C with 2% paraformaldehyde and 0.05% glutaldehyde, and permeabilized for 20 min in 0.05 M Tris-saline solution (TSS) containing 0.1% Triton X-100. The coverslips were exposed for 20 min at 25 °C to 3% hydrogen peroxidase in 60% methanol–40% TSS to quench endogenous peroxidase. To block nonspecific binding of antibodies, the coverslips were incubated in 0.1 M TSS containing 1% bovine serum albumin (BSA) (Sigma, fraction V) for 90 min at 25 °C. Primary antibodies for IR-β (10 μg/ml) were applied to cells overnight at 4 °C. The coverslips were further incubated for 40 min at 25 °C with biotinylated secondary antibodies (Santa Cruz, polyclonal rabbit IgG, 1:100 dilution). Specimens were incubated for 90 min with avidin-biotinylated enzyme complex (ABC) (Oncogene Research Products) solution and rinsed three times in PBS for 5 min each. They were then exposed to nickel-free diaminobenzidine

tetrahydrochloride (DAB) (Sigma Chemical) solution for 1 min and 0.1% hematoxylin solution for 3 min. For the next steps, specimens were dehydrated in ethanol (50%, 60%, 70%, 80%, 90%, and 100%, respectively) for 30 s each and incubated in xylene twice for 10 s. Finally, slides were mounted with Canada balsam (Merck) and examined by light microscopy. Secondary antibody specificity was tested in a series of positive and negative control measurements.

Cell cycle analysis. Flow-cytometry examination of the cell cycle was performed to assay effects of insulin and transfection of recombinant caveolin-1. Cells cultured on 6-well plates were incubated in serum-free medium for 16–24 h and treated with or without 100 nM insulin for 24 h. After washing twice with PBS, cells were trypsinized, and cells (1×10^6 cells/ml) were fixed for 2 h in ice-cold citrate buffer (250 mM sucrose, 40 mM trisodium citrate, and 5% DMSO, pH 7.6) and centrifuged at 2000 rpm for 5 min. Pellets were incubated with a buffer containing 3.4 mM trisodium citrate, 0.1% NP-40, 1.5 mM spermine tetrahydrochloride, and 0.5 mM Tris, pH 7.6, for 10 min at room temperature (RT) and centrifuged at 2000 rpm for 5 min. They were then incubated in a buffer (20 μg/ml RNase A and trypsin inhibitor) containing 50 μg/ml propidium iodide (PI) for 5 min at RT. PI fluorescence of nuclei was determined and DNA content was analyzed by BECKMANCOULTER (Beckman Instruments, Palo Alto, CA).

Transfection with caveolin-1 cDNA. A full-length human caveolin-1 cDNA (kindly provided by Dr. S.H. Ryu at POSTEC) was subcloned into pcDNA3 and pEGFP-C2 vectors using *Eco*RI–*Bam*HI and *Eco*RI–*Xho*I (NEW ENGLAND BioLabs), respectively. These vectors (pcDNA3 alone, pcDNA3 + caveolin-1, pEGFP-C2 alone, and pEGFP-C2 + caveolin-1) were transiently transfected into cells using a modified calcium phosphate precipitation protocol. In brief, the cDNA constructs (30 μg of plasmid DNA/100-mm-diameter culture dish) were introduced into the serum-starved cells in the medium, which was replaced with 50 μl of 2.5 M $CaCl_2$ and 2× Hepes-buffered saline and incubated for 24 h at 37 °C. The medium was then removed, and the cells were treated with 20% DMSO for 3 min. After washing with PBS, fresh medium was added to the cells and incubated for another 24 h. The expression of caveolin-1 and GFP was then confirmed in cells by Western blots and immunofluorescence microscopy.

Immunoprecipitation. Serum-starved cells were incubated with or without 100 nM insulin for 10 min or 1 h, washed with ice-cold PBS, and lysed in a buffer containing 20 mM Tris–HCl, pH 7.4, 300 mM NaCl, 2 mM EDTA, 2 mM EGTA, pH 8.0, 0.4 mM sodium vanadate, 0.4 mM PMSF, and 2% Triton X-100. The cell lysates were centrifuged at 12,000 rpm for 20 min at 4 °C and the supernatants were subjected to immunoprecipitation with either anti-caveolin-2 or anti-ERK antibodies (1–5 μg/ml each). The immunocomplexes were absorbed to protein A–agarose (Calbiochem. OP25) and washed three times with lysis buffer. After extensive washing, samples were resolved by the addition of 2× sample buffer, separated by 12.5% acrylamide SDS-PAGE, and transferred to PVDF membranes. Blots were then probed by immunoblot analysis with anti-caveolin-2 (1:250 dilution), anti-caveolin-1 (1:1000 dilution), anti-ERK (1:5000 dilution), or anti-phosphospecific-ERK antibodies (1:2000 dilution).

Immunolabeling and immunofluorescence microscopy. Protein expression and cellular translocation of caveolin-2 and ERK upon insulin stimulation were investigated by immunofluorescence microscopy. Cells cultured on 12 mm glass coverslips were serum-starved overnight in the medium containing 0.2% BSA [24]. The coverslips were then incubated with 100 nM insulin for 10 min, 1 or 24 h, washed three times with PBS, and fixed with 2% paraformaldehyde and 0.05% glutaldehyde in PBS for 30 min at 4 °C. Fixed cells were rinsed with PBS and incubated for 5 min at RT with 0.1 mM glycine in 1% BSA-PBS and then with 0.5% Triton X-100 in PBS. Permeabilized cells were rinsed with PBS, incubated with 1% BSA-PBS for 30 min at RT and then with anti-caveolin-2 and anti-ERK antibodies diluted in 1% BSA-PBS for 90 min at RT. After three washes with 1% BSA-PBS, the primary antibodies were detected with fluorescein isothiocyanate (FITC)-conjugated anti-mouse (1:100 dilution) and tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-rabbit (1:100 dilution) IgG antibodies (Jackson ImmunoResearch Laboratories) for caveolin-2 and ERK, respectively. Cells were then washed three times with 1% BSA-PBS and slides were mounted using Canada balsam. Immunolabeled cells were visualized using appropriate filters on an Olympus BX51 microscope and imaged with an Olympus PM-20 exposure control unit. The barrier filter for the TRITC channel was 543 nm and for the FITC channel was 488 nm. Neither labeling in the absence of the primary antibody nor cross-reactivity between secondary and primary antibodies was observed.

Densitometry analysis. Autoradiograms were quantified with an imaging densitometer using the Molecular Analyst software (Bio-Rad Laboratories, model GS-700). Signal intensities of bands in immunoblots were determined by the scanning laser densitometry.

Results

Expression of insulin receptor and caveolin isoforms

To investigate insulin-induced modulation of the cell cycle, we have chosen Hirc-B cells, originally derived from Rat-1 fibroblasts, overexpressing the wild type human insulin receptor. To confirm the overexpression, the levels of IR-β protein were assessed in Hirc-B and Rat-1 cells by immunoblotting and immunohistochemistry (Fig. 1A). As expected, the expression of IR-β was much higher in Hirc-B cells as compared to Rat-1 cells. The results confirm that insulin receptors were overexpressed in Hirc-B cells and that Hirc-B cells can serve as a good model system to investigate insulin action on the cell cycle.

We then examined expression of caveolin-1 and -2 in Hirc-B and Rat-1 cells by Western blot analysis as shown in Fig. 1B. Caveolin-2 was expressed in both cells with a relatively lower expression in Hirc-B cells. Expression of caveolin-1, however, was greatly reduced in Hirc-B cells indicating caveolin-2 as a major isoform in Hirc-B cells as compared to caveolin-1 in Rat-1 cells. This observation was further confirmed by the Northern blot analysis with a caveolin-1 cDNA probe, showing no caveolin-1 gene expression was detected in Hirc-B cells (Fig. 1C). Thus, we chose Hirc-B cells to investigate the role of caveolin-2 in the regulation of cell cycle by insulin.

Transient expression of caveolin-1

Based on the recent reports [15–18] showing that up- or down-regulation of caveolin-1 affected the regulatory mechanism on cell growth and tumorigenicity, Hirc-B cells were subjected to cell cycle analysis in the absence or presence of recombinantly expressed caveolin-1 as described below. Accordingly, caveolin-1 was transiently expressed into Hirc-B cells that fail to express caveolin-1 but express caveolin-2 (Figs. 1B, C and 2) to assess the role of caveolin-1 and caveolin-2

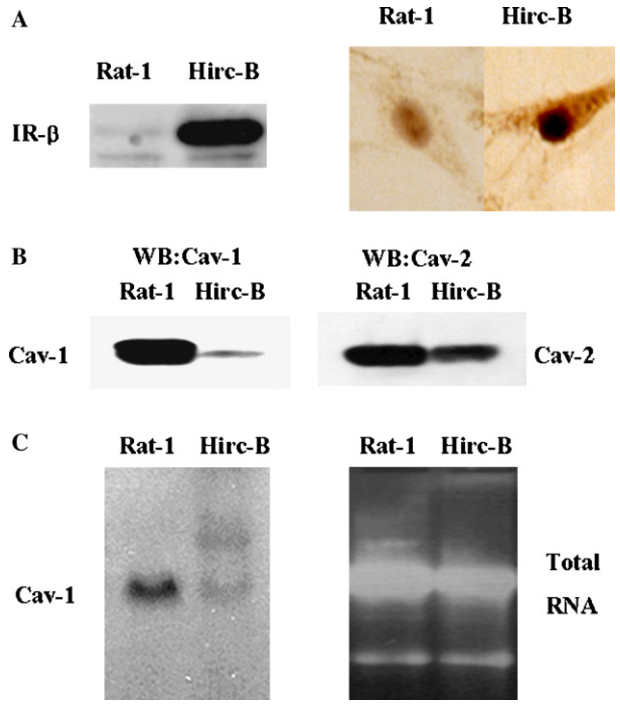

Fig. 1. Expression of insulin receptor and caveolin-1 and -2 in Rat-1 and Hirc-B cells. (A) Western blot and immunohistochemical analyses of insulin receptor expression. Protein samples (50 μg) from whole cell lysates were resolved by 12.5% acrylamide SDS–PAGE and immunoblotted with anti-insulin receptor β chain (IR-β) antibody. Expression level of IR-β was also directly confirmed in cells grown on glass coverslips in chamber slides by immunohistochemistry as described under Materials and methods. (B) Endogenous expression of caveolin-1 and -2. Whole cell protein extracts were separated by 12.5% acrylamide SDS–PAGE and transferred to PVDF membranes. Blots were then probed by immunoblot analysis with anti-caveolin-1 and anti-caveolin-2 antibodies. (C) Northern blot analysis of caveolin-1. Total RNA was isolated, denatured, subjected to 1% agarose/formaldehyde gel electrophoresis, transferred to nylon membranes, and then immobilized by UV cross-linking. Human caveolin-1 cDNA was labeled with [α-^{32}P]dATP in a random primed reaction. After UV cross-linking, membranes were hybridized and the final blots were analyzed for caveolin-1 mRNA as described under Materials and methods.

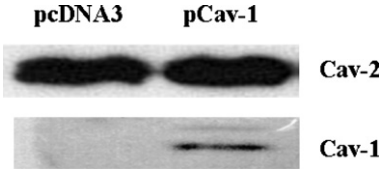

Fig. 2. Transient expression of caveolin-1 in Hirc-B cells. For expression of caveolin-1, Hirc-B cells were transfected with pcDNA3 alone (pcDNA3), pcDNA3 containing a caveolin-1 insert (pCav-1) using the calcium phosphate procedure. Cells were harvested by sonication, subjected to 12.5% acrylamide SDS–PAGE, and analyzed for caveolin-1 and -2 expressions by Western blot analysis.

on the cell cycle regulated by insulin. Fig. 2 shows the expression of the caveolin-1 in Hirc-B cells after the transfection.

Effect of insulin on cell cycle

Using flow-cytometry, modulation of the cell cycle by insulin was investigated. The data shown in Figs. 3A and B, revealed that insulin induced a 6-fold increase in the number of Hirc-B cells in the S phase of the cell cycle relative to untreated cells. This result indicates that the insulin-induced activation of the cell cycle in Hirc-B cell is mediated through activation of ERK, as previously reported [1,2,4,5]. We then examined the effect of the caveolin-1 transfection on the cell cycle of Hirc-B cells in response to insulin. In contrast to the non transfected cells which showed the 6-fold increased S phase (Fig. 3B), the transfected cells increased S phase only by approximately 1.7-fold in response to insulin (Fig. 3D). Thus, our results demonstrated that caveolin-1 acted as a negative regulator on the G1 to S phase transition, conceivably by inducing G1 phase arrest of the cell cycle in Hirc-B cells. Consistent with these results, caveolin-1 transfected cells showed retardation in cell growth as compared to non-transfected controls (Fig. 4). Our data, therefore, suggest that caveolin-2 is the only caveolin isoform that regulates the G1 to S phase transition positively in the insulin-induced cell cycle of Hirc-B cells.

Transcriptional control of caveolin-2 in response to insulin

To determine if insulin-mediated cell cycle regulation was accompanied by induction of the caveolin-2 gene in Hirc-B cells, transcriptional changes identified by expression profiling over time were examined by Northern blotting using cDNA probes specific for the caveolin-2 gene. As shown in Fig. 5, caveolin-2 was an early insulin response gene, showing insulin-induced maximum expression within 1 h of treatment. In contrast, Rat-1 cells in the presence of insulin exhibited relatively little change in the expression over the same (1 h) time period. Induction of an insulin-mediated genomic response of caveolin-2 facilitated positive action by caveolin-2 on the insulin-regulated cell cycle in Hirc-B cells, while negative action by caveolin-1 was not enhanced. Thus, our results again support that caveolin-2 is a positive regulator with a mitogenic effect on the cell cycle of Hirc-B cells in response to insulin.

Association of caveolin-2 with ERK-1/2

ERK-1/2 activates transcription factors and in turn stimulates activity of the cyclin D-cdk4 complex to regulate the cell cycle [2]. Recently, caveolin-1 was reported to interact directly with ERK-1/2 and inhibit ERK-1/2 activation as a negative regulator [18,26,27]. We investigated whether caveolin-2 can form a direct physical complex with ERK-1/2 in the cell cycle regulation of Hirc-B cells. Cell lysates were immunoprecipitated with anti-caveolin-2 (Fig. 6A) or anti-ERK antibodies

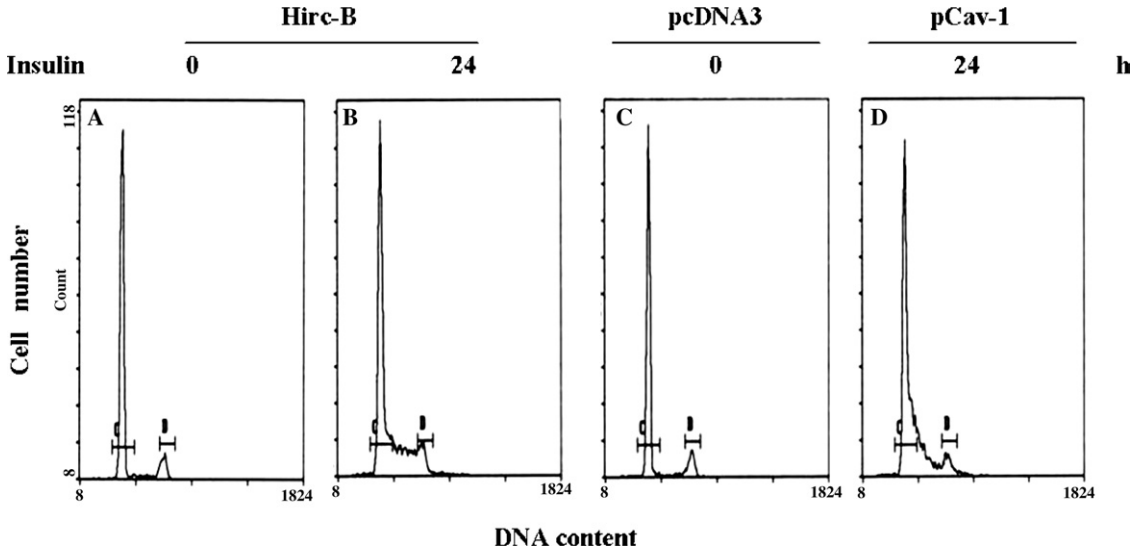

Fig. 3. Insulin simulation of cell cycle. Serum-starved cells (1×10^6 cells/ml), cultured on 6-well plates, were incubated in the absence or presence of 100 nM insulin for 24 h and analyzed by flow-cytometry as described under Materials and methods. To examine the effect of caveolin-1 transfection on the cell cycle regulated by insulin, Hirc-B cells were transiently transfected with pcDNA3 alone (pcDNA3) or pcDNA3 containing a caveolin-1 insert (pCav-1) as described in the legend to Fig. 2 and subjected to flow-cytometry analysis as were non-transfected control cells (Hirc-B). Peak area C indicates G0/G1 phase, peak area D G2/M phase, and the area between C and D S phase.

(Fig. 6B) and the immunopreciptates were analyzed by immunoblotting with anti-ERK and anti-caveolin-2 antibodies. As shown in Figs. 6A and B, the immunoblot analyses revealed the association of caveolin-2 and ERK-1/2 in Hirc-B cells. Interaction of ERK-1/2 with caveolin-1 was not detected (Fig. 6B). Thus, the results again confirmed that caveolin-2 in Hirc-B cells is the functional caveolin directly interacting with ERK-1/2 and the cells with no endogenous caveolin-1 (Figs. 1B, C and 2) rule out any possible regulatory action or effect of caveolin-1 in the interaction.

We further examined the activation of ERK-1/2 and the association of phosphorylated ERK-1/2 with caveolin-2 (Fig. 6C). Serum-starved cells were stimulated with insulin for the indicated time periods and immunoprecipitated with anti-ERK antibody. Phosphorylation of ERK-1/2 was analyzed with anti-phosphospecific-ERK (Thr 202 and Tyr 204) antibody. As expected, ERK-1/2 became phosphorylated maximally by approximately 2.4-fold with insulin stimulation in 10 min. The immunoblot with anti-caveolin-2 antibody revealed the strong interaction of ERK-1/2 with caveolin-2, 10-fold greater than control at 10 min of insulin treatment. Thus, phosphorylation of ERK-1/2 and molecular interaction between the resulting activated ERK-1/2 and caveolin-2 were sustained for 10 min after insulin stimulation. The interaction of caveolin-2 with phosphorylated ERK-1/2 returned to basal level at 1 h incubation with insulin (Fig. 6C). These results strongly suggest that caveolin-2 is a major ERK-1/2 interacting protein which is dependent on the phosphorylation of ERK-1/2 in response to insulin in Hirc-B cells.

Insulin-induced cellular localization of caveolin-2 and ERK

The insulin-stimulated interaction between caveolin-2 and ERK-1/2 in Hirc-B cells was further investigated by monitoring their cellular distribution over time by fluorescence microscopy. As presented in Fig. 7, fluorescence emitted for caveolin-2 (green) and ERK (red) indicated that both caveolin-2 and ERK are translocated and co-localized into a perinuclear region in the presence of insulin, demonstrated by the bright orange color from the superimposed images (Fig. 7, merge). Controls with no insulin treatment (Fig. 7A, 0 time) showed that both caveolin-2 and ERK were distributed throughout the cells with no co-localization, judged by the merge image (Fig. 7A, merge). However, treatment of these cells with 100 nM insulin resulted in distribution of the fluorescence of both to a region of the cell surrounding the nucleus, with a similar cellular distribution and extensive co-localization of caveolin-2 and ERK (Figs. 7B and C). The insulin-dependent co-localization appeared with maximum intensity as early as 10 min after insulin treatment at perinuclear region and increased in size over time (Fig. 7B). One hour after insulin treatment, the signal was still quite intense while the increased size concentrated in the area (Fig. 7C), and gradually returned to the basal level within 24 h as the co-localization became more diffuse (Fig. 7D). Thus, these observations are consistent with our immunoprecipitation analyses demonstrating an increase in the association of caveolin-2 with ERK-1/2 in response to insulin (Fig. 6C) and confirm the ability of insulin to

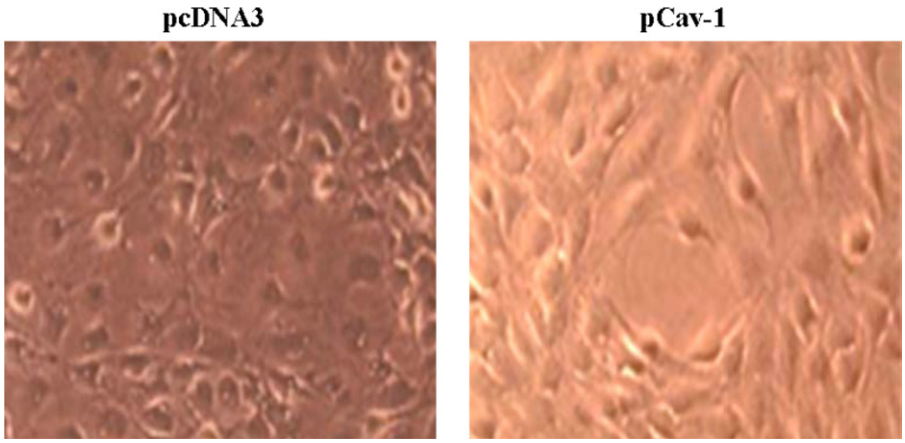

Fig. 4. Retardation of cell growth in caveolin-1 transfected Hirc-B cells. Cell morphology and growth of Hirc-B cells, transiently transfected with pcDNA3 alone (pcDNA3) or pcDNA3 containing a caveolin-1 insert (pCav-1) and cultured for 3 days after seeding (1×10^6 cells/ml) in chamber slides in DMEM, were observed by phase contrast microscopy (400×). Transfected cells (pCav-1) displayed an increase in cell size and grew to low cell densities relative to non-transfected cells. Representative images from the two cultures are shown.

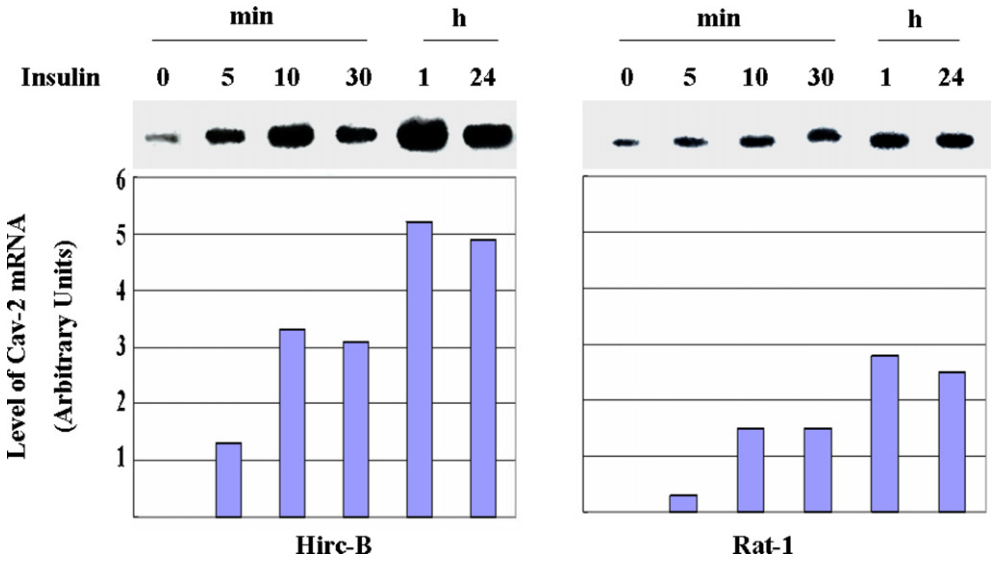

Fig. 5. Up-regulation of caveolin-2 gene expression in response to insulin. Serum-starved cells were treated with 100 nM insulin and incubated following a time course as indicated. Samples of total RNA (10 μg) were purified from cells at each time point, denatured, subjected to 1% agarose/formaldehyde gel electrophoresis, and transferred to nylon membranes following immobilization by UV cross-linking. For the caveolin-2 probe, caveolin-2 cDNA was labeled with [α-^{32}P]dATP in a random primed reaction. After UV cross-linking, membranes were hybridized and the final blots were analyzed for caveolin-2 mRNA. Signal intensities of bands in immunoblots were determined by scanning laser densitometry. The results presented are representative pictures of immunoblots showing phosphorimages of the ^{32}P-labeled caveolin-2 mRNA. The graphs are expressed relative to the immunoblot signals from control (i.e., 0 time incubation with insulin). The mean of duplicate determinations is shown.

induce translocation of ERK from cytoplasm into nucleus accompanied by caveolin-2.

Discussion

Caveolin-1 expression is reduced in a variety of tumor types, but increasing the level of caveolin-1 can suppress the transformed phenotype [17,18,26,27]. This is consistent with its antagonism of Ras-mediated cell transformation, in which caveolin-1 expression dramatically inhibited both Ras/MAPK-mediated and basal transcriptional activation of mitogen-sensitive promoter [18,19,27]. Thus, down-regulation of caveolin-1 expression is generally sufficient to drive oncogenic transformation and constitutively activate the MAPK cascade. Relevant to these findings, caveolin-1-overexpressed cells exhibited repression of the cyclin D1 promoter [15], needed by the cyclin D1 gene to encode the regulatory subunit of the holoenzyme that phosphorylates and inactivates the pRB protein [7], thereby promoting entry into the DNA synthetic S phase of the cell cycle.

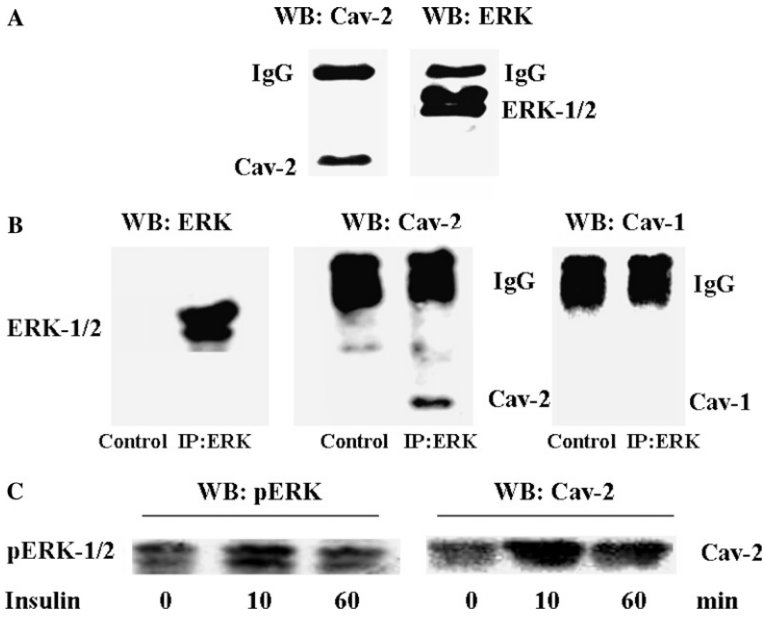

Fig. 6. Increased interaction of caveolin-2 and phosphorylated ERK-1/2 by insulin in Hirc-B cells. (A) Total cell lysates were immunoprecipitated with anti-caveolin-2 antibody. Immune complexes were immobilized on protein A–agarose, washed three times with lysis buffer, separated by 12.5% acrylamide SDS–PAGE, and immunoblotted with anti-caveolin-2 and anti-ERK antibodies. Molecular weights of ERK-1, ERK-2, and caveolin-2 are 44, 42, and 22 kDa, respectively. (B) The interaction shown in (A) was reconfirmed by reverse immunoprecipitation using anti-ERK antibody followed by immunoblot with anti-ERK, anti-caveolin-2, and anti-caveolin-1 antibodies. (C) Serum-starved cells were stimulated with 100 nM insulin for the indicated time periods. Total cell lysates were immunoprecipitated with anti-ERK antibody. The immune complexes were analyzed for the phosphorylation of ERK-1/2 and the association of the activated ERK-1/2 with caveolin-2 by Western blot using anti-phosphospecific-ERK and anti-caveolin-2 antibodies, respectively.

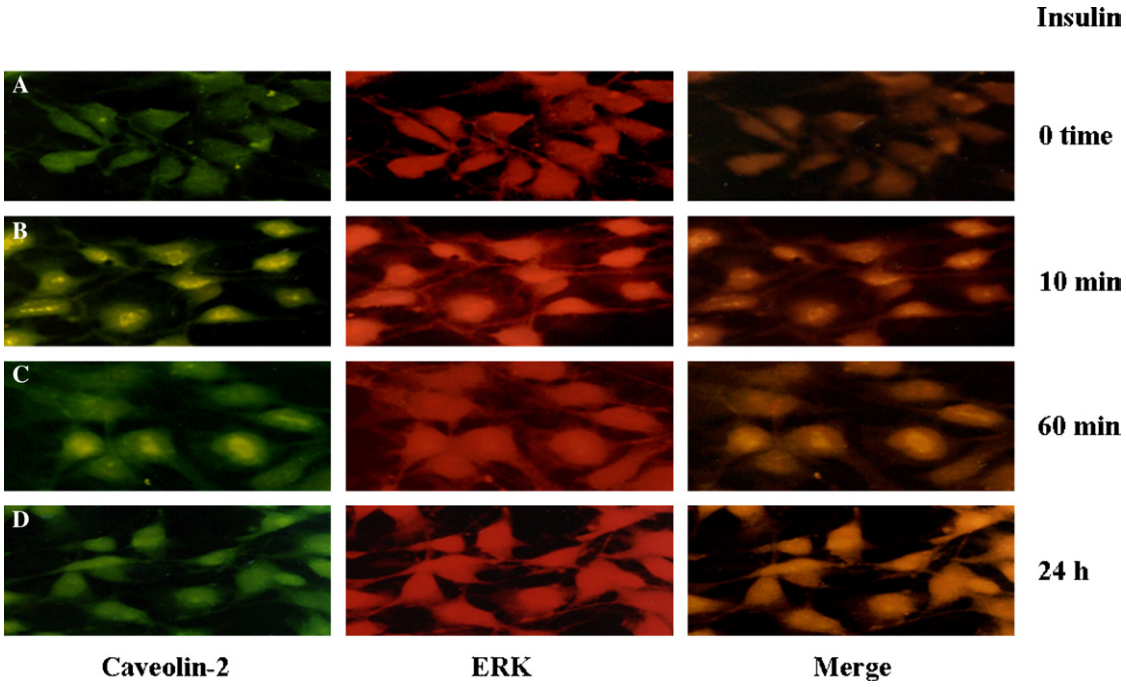

Fig. 7. Co-localization of caveolin-2 and ERK in the perinucleus of insulin-stimulated Hirc-B cells. Representative images of cells before and at various times after 100 nM insulin treatment are presented. Caveolin-2 fluorescence appears green, ERK fluorescence appears red, and regions of co-localization (merge) appear orange/yellow. Images were collected prior to insulin treatment, 0 time (A) and following a 10 min (B), 1 h (C), and 24 h (D) stimulation with 100 nM insulin.

Despite numerous findings of caveolin regulation in signaling, including the Ras/MAPK-mediated signal cascade of the cell cycle, most of the studies reported have been concentrated exclusively on the role of caveolin-1. Caveolin-2, an additional isoform, is widely present in many cell types and generally known for its structural role in caveolae formation in many cell systems [21–23]. Although recent reports show that caveolin-2 can be tyrosine-phosphorylated by c-Src and serine-phosphorylated likely by casein kinase 2 [28,29], and that caveolin-2-deficient transgenic mice have pulmonary dysfunction [30], there have been no data demonstrating that caveolin-2, per se, can modulate signaling in a manner similar to caveolin-1. Therefore, we investigated the modulation of caveolin isoforms in mitogenic signaling efficiency and cell cycle progression mediated by ERK by taking advantage of the Hirc-B cells which express caveolin-2 as a major caveolin isoform.

Our results demonstrated that insulin treatment increased the S phase of the cell cycle 6-fold in Hirc-B cells (Fig. 3). This increased S phase was markedly reduced when caveolin-1 was transiently expressed, which conceivably resulted from G0/G1 phase cell cycle arrest (Fig. 3). This observation is in good agreement with previous reports demonstrating caveolin-1 as a negative regulator of mitogenic MAPK signaling and the cell cycle [17–19,26].

When we examined the level of caveolin-2 upon insulin treatment, the expression was increased in a time-dependent manner (Fig. 5). Up-regulation of caveolin-2 increased the insulin responses and modulated the subsequent downstream signal cascades, such as the increased phosphorylation of ERK-1/2 (Fig. 6), the nuclear translocation of phospho-ERK (Fig. 7), and subsequent activation of G0/G1 to S phase transition of the cell cycle upon insulin stimulation in Hirc-B cells (Fig. 3). More precisely, our results, demonstrated in Figs. 6 and 7, indicate that upon insulin stimulation, caveolin-2 directly interacts with activated phospho-ERK-1/2 (Fig. 6C) and facilitates its translocation from cytosol to nucleus (Figs. 7B and C). Then the activated ERK-1/2 rapidly induces a transition from G0/G1 into S phase in the cell cycle of Hirc-B cells. It is well documented that growth factors induce ERK-1/2 activation by phosphorylation and promote nuclear translocation [4,5]. Thus, DNA synthesis and activation of cell cycle are achieved in response to insulin concomitantly with an increase in the interaction between caveolin-2 and the phospho-ERK (Fig. 6C), and the co-localization and nuclear translocation of the complex in Hirc-B cells (Fig. 7).

In summary, we found that caveolin-2 acts as a positive regulator in mitogenic signaling in response to insulin in Hirc-B cells. It is tempting to speculate that cdc and cdk families regulating the G0/G1 to S phase transition of the cell cycle might be modulated by caveolin-1 and -2 as a negative and positive regulator, respectively. Our finding of the regulatory role of caveolin-2 in addition to its generally known role as a structural component of caveolae also opens up many exciting future investigations regarding caveolin-2 function in mitogenic MAPK signaling and the cell cycle as well as in interaction with other signaling molecules yet to be revealed.

Acknowledgments

We thank all of the members of our laboratory for their help during the course of these studies. This work was supported in part by KOSEF R01-1999-00091 and Brain Korea 21 to Y.P.

References

[1] J.P. Whitehead, S.F. Clark, B. Urso, D.E. James, Signaling through the insulin receptor, Curr. Opin. Cell Biol. 12 (2000) 222–228.
[2] M.J. Robinson, M.H. Cobb, Mitogen-activated protein kinase pathways, Curr. Opin. Cell Biol. 9 (1997) 180–186.
[3] S. Zimmermann, C. Rommel, A. Ziogas, J. Lovric, K. Moelling, G. Radziwill, MEK1 mediates a positive feedback on Raf-1 activity independently of Ras and Src, Oncogene 15 (1997) 1503–1511.
[4] P. Lenormand, J.M. Brondello, A. Brunet, J. Pouyssegur, Growth factor-induced p42/p44 MAPK nuclear translocation and retention requires both MAPK activation and neosynthesis of nuclear anchoring proteins, J. Cell Biol. 142 (1998) 625–633.
[5] A.V. Khokhatchev, B. Canagarajah, J. Wilsbacher, M. Robinson, M. Atkinson, E. Goldsmith, M.H. Cobb, Phosphorylation of the MAP kinase ERK2 promotes its homodimerization and nuclear translocation, Cell 93 (1998) 605–615.
[6] R.A. Weinberg, The retinoblastoma protein and cell cycle control, Cell 81 (1995) 323–330.
[7] N. Dyson, The regulation of E2F by pRB-family proteins, Genes Dev. 12 (1998) 2245–2262.
[8] S.J. Elledge, Cell cycle checkpoints: preventing an identity crisis, Science 274 (1996) 1664–1672.
[9] D.O. Morgan, Principles of CDK regulation, Nature 374 (1995) 131–134.
[10] C.J. Sherr, Cancer cell cycles, Science 274 (1996) 1672–1677.
[11] B. Razani, S.E. Woodman, M.P. Lisanti, Caveolae: from cell biology to animal physiology, Pharmacol. Rev. 54 (2002) 431–467.
[12] P. Liu, M. Rudick, R.G.W. Anderson, Multiple functions of caveolin-1, J. Biol. Chem. 277 (2002) 41295–41298.
[13] J-P. Gratton, P. Bernatchez, W.C. Sessa, Caveolae and caveolins in the cardiovascular system, Circ. Res. 94 (2004) 1408–1417.
[14] A.W. Cohen, T.P. Combs, P.E. Scherer, M.P. Lisanti, Role of caveolin and caveolae in insulin signaling and diabetes, Am. J. Physiol. Endocrinol. Metab. 285 (2003) E1151–E1160.
[15] J. Hulit, T. Bash, M. Fu, F. Galbiati, C. Albanese, D.R. Sage, A. Schlegel, J. Zhurinsky, M. Shtutman, A. Ben-ze'ev, M.P. Lisanti, R.G. Pestell, The cyclin D1 gene is transcriptionally repressed by caveolin-1, J. Biol. Chem. 275 (2000) 21203–21209.
[16] J.A. Engelman, R.J. Lee, A. Karnezis, D.J. Bearss, M. Webster, P. Siegel, W.J. Muller, J.J. Windle, R.G. Pestell, M.P. Lisanti, Reciprocal regulation of neu tyrosine kinase activity and caveolin-

1 protein expression in vitro and in vivo: implications for human breast cancer, J. Biol. Chem. 273 (1998) 20448–20455.
[17] S.W. Lee, C.L. Reimer, P. Oh, D.B. Campbell, J.E. Schnitzer, Tumor cell growth inhibition by caveolin re-expression in human breast cancer cells, Oncogene 16 (1998) 1391–1397.
[18] F. Galbiati, D. Volonte, J.A. Engleman, G. Watanabe, R Burk, R.G. Pestell, M.P. Lisanti, Targeted downregulation of caveolin-1 is sufficient to drive cell transformation and hyperactivate the p42/p44 MAP kinase cascade, EMBO J. 17 (1998) 6633–6648.
[19] K.A. Cho, S.J. Ryu, J.S. Park, I.S. Jang, J.S. Ahn, K.T. Kim, S.C. Park, Senescent phenotype can be reversed by reduction of caveolin status, J. Biol. Chem. 278 (2003) 27789–27795.
[20] P.E. Scherer, T. Okamoto, M. Chun, I. Nishimoto, H.F. Lodish, M.P. Lisanti, Identification, sequence, and expression of caveolin-2 defines a caveolin gene family, Proc. Natl. Acad. Sci. USA 93 (1996) 131–135.
[21] R. Mora, V. Bonilha, A. Marmostein, D Brown, P.E. Scherer, M.P. Lisanti, E. Rodriguez-Boulan, Caveolin-2 localizes to the golgi complex but redistributes to plasma membrane, caveolae, and rafts when co-expressed with caveolin-1, J. Biol. Chem. 274 (1999) 25708–25717.
[22] P.E. Scherer, R.Y. Lewis, D. Volonte, J.A. Engelman, F. Galbiati, J. Couet, D.S. Kohtz, E. van Donselaar, P. Peters, M.P. Lisanti, Cell-type and tissue-specific expression of caveolin-2: caveolin 1 and 2 co-localize and form a stable hetero-oligomeric complex in vivo, J. Biol. Chem. 272 (1997) 29337–29346.
[23] U. Lahtinen, M. Honsho, R.G. Parton, K. Simons, P. Verkade, Involvement of caveolin-2 in caveolar biogenesis in MDCK cells, FEBS Lett. 538 (2003) 85–88.
[24] Y. Pak, C.R. Paule, Y.-D. Bao, L.C. Huang, J. Larner, Insulin stimulates the biosynthesis of *chiro*-inositol-containing phospholipids in a rat fibroblast line expressing the human insulin receptor, Proc. Natl. Acad. Sci. USA 90 (1993) 7759–7763.
[25] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[26] J.A. Engleman, C. Chu, A. Lin, H. Jo, T. Ikezu, T. Okamoto, D.S. Kohtz, M.P. Lisanti, Caveolin-1-mediated regulation of signaling along the p42/p44 MAP kinase cascade in vivo: a role for the caveolin-scaffolding domain, FEBS Lett. 428 (1998) 205–211.
[27] J.A. Engelman, X.L. Zhang, B. Razani, R.G. Pestell, M.P. Lisanti, p42/p44 Map kinase-dependent and -independent signaling pathways regulate caveolin-1 gene expression: activation of Ras-MAP kinase and protein kinase A signaling cascades transcriptionally down-regulates caveolin-1 promoter activity, J. Biol. Chem. 274 (1999) 32333–32341.
[28] G. Sowa, M. Pypaert, D. Fulton, W.C. Sessa, The phosphorylation of caveolin-2 on serines 23 and 36 modulates caveolin-1-dependent caveolae formation, Proc. Natl. Acad. Sci. USA 100 (2003) 6511–6516.
[29] H. Lee, D.S. Park, X.B. Wang, P.E. Scherer, P.E. Schwartz, M.P. Lisanti, Src-induced phosphorylation of caveolin-2 on tyrosine 19: phosphor-caveolin-2 ($Tyr(P)^{19}$) is localized near focal adhesions, remains associated with lipid rafts/caveolae, but no longer forms a high molecular mass hetero-oligomer with caveolin-1, J. Biol. Chem. 277 (2002) 34556–34567.
[30] B. Razani, X.B. Wang, J.A. Engelman, M. Battista, G. Lagaud, X.L. Zhang, B. Kneitz, H. Hou Jr., G.J. Christ, W. Edelmann, M.P. Lisanti, Caveolin-2-deficient mice show evidence of severe pulmonary dysfunction without disruption of caveolae, Mol. Cell. Biol. 22 (2002) 2329–2344.