ORIGINAL

Cysteine string protein 1 (CSP1) modulates insulin sensitivity by attenuating glucose transporter 4 (GLUT4) vesicle docking with the plasma membrane

Bayasgalan Jambaldorj[1], Eri Terada[1], Toshio Hosaka[1], Yuka Kishuku[1], Yukiko Tomioka[1], Kaori Iwashima[1], Yohko Hirata[1], Kiyoshi Teshigawara[1], Chung Thi Kim Le[1], Tadahiko Nakagawa[1], Nagakatsu Harada[1], Tohru Sakai[2], Hiroshi Sakaue[1], Toshio Matsumoto[3], Makoto Funaki[4], Akira Takahashi[5], and Yutaka Nakaya[1]

[1]*Department of Nutrition and Metabolism,* [2]*Department of Public Health and Applied Nutrition,* [3]*Department of Medicine and Bioregulatory Sciences and* [5]*Department of Preventive Environment and Nutrition, Institute of Health Biosciences, the University of Tokushima Graduate School, Tokushima, Japan and From* [4]*Clinical Research Center for Diabetes, Tokushima University Hospital, Tokushima, Japan*

Abstract : Insulin stimulates glucose transporter 4 (GLUT4) vesicle recruitment from its intracellular storage site to the plasma membrane. Cysteine string protein 1 (CSP1) is a SNARE-binding protein involved in the vesicular trafficking of neurotransmitters and other exocytic processes. In this study, we investigated the involvement of CSP1 in insulin-dependent GLUT4 recruitment in 3T3-L1 adipocytes. Over-expression of wild-type CSP1 led to attenuated insulin-stimulated glucose uptake without any change in GLUT4 content in the plasma membrane, rather it inhibits docking by blocking the association of VAMP2 with syntaxin 4. In contrast, knockdown of CSP1 enhanced insulin-stimulated glucose uptake. The mRNA and protein expression of CSP1 was elevated in 3T3-L1 adipocytes in insulin resistant states caused by high levels of palmitate and chronic insulin exposure. Taken together, the results of this study suggest that CSP1 is involved in insulin resistance by interrupting GLUT4 vesicle docking with the plasma membrane. J. Med. Invest. 60 : 197-204, August, 2013

Keywords **:** *GLUT4, cysteine string protein 1, SNARE, VAMP2*

INTRODUCTION

Insulin maintains glucose homeostasis by enhancing glucose transport into muscle and adipose tissues, a process mediated by recruitment of GLUT4 storage vesicles to the cell surface where these vesicles are first tethered, then docked and fused with the plasma membrane (1). One class of proteins

ABBREVIATIONS USED :
VAMP2 : vesicle associated membrane protein 2, SNARE : soluble NSF attachment receptor, SNAP : soluble NSF attachment protein, PKA : protein kinase A, FBS : fatal bovine serum, HRP : horse radish peroxidase, ECL : enhanced chemiluminescence, PBS : phosphate buffered saline.

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Address correspondence and reprint requests to Toshio Hosaka, MD, PhD, Department of Endocrinology and Diabetes, School of Medicine, Saitama Medical University, Morohongo 38, Moroyama, Iruma-gun, Saitama, 350-0495, Japan and Fax : +81-49-294-9752.

that function in this docking/fusion step are SNARE proteins (2), of which the vesicle SNARE VAMP2, and the t-SNAREs, syntaxin 4 and SNAP23 are the best characterized (3, 4). The SNARE core is extremely energetically stable; thus, the timing of its formation and subsequent dissociation is likely to be regulated by SNARE-associated proteins, such as Munc-18c, tomosyn, and synip (4).

Cysteine string protein 1 (CSP1) plays the role of a SNARE-binding protein or a chaperone and is expressed mainly in neuronal cells and also in cells such as adipocytes and islet cells (5-10). CSP1 is essential for presynaptic neurotransmission and also influences regulation of exocytosis in PC12 and pancreatic β-cells (10-13). Recent genetic and biochemical analyses have revealed a functional interaction of CSP with the syntaxin family or VAMP2 in neurons (11, 14-16). In addition, *in vitro* phosphorylation studies have revealed that Akt and PKA phosphorylate CSP1 (7, 17, 18). These findings suggest that CSP1 may be one of the proteins participating in insulin-stimulated GLUT4 trafficking at a step or steps close to the plasma membrane.

The molecular mechanism by which insulin stimulates the translocation, tethering, docking and fusion of GLUT4 to the cell surface has been extensively studied, but many details of insulin signaling linking specific molecules to these processes remain incompletely described, particularly those evens most proximal to the plasma membrane. The aim of the present study was to determine the role of CSP1 in processes regulating GLUT4-containing vesicles as they translocate to the plasma membrane. The results of this study indicate that CSP1 is a candidate insulin resistance protein by virtue of its ability to interrupt GLUT4 vesicle docking *via* blocking the binding of the v-SNARE, VAMP2 on GLUT4 vesicles to t-SNARE, syntaxin 4 on the plasma membrane.

MATERIALS AND METHODS

Materials

0.25% trypsin-EDTA, penicillin/streptomycin, and pcDNA 3.1 vector were purchased from Life Technologies (Carlsbad, CA). ECL detection kit and bicinchoninic acid (BCA) protein assay kits were purchased from Thermo Scientific (Rockford, IL). Dulbecco's modified Eagle's medium (DMEM), Dulbecco's PBS without Mg^{++} or Ca^{++} (D-PBS), dexamethasone, and 3-isobutyl-1-methylxanthine (IBMX) were obtained from Sigma-Aldrich (St. Louis, MO). Insulin was purchased from Wako (Osaka, Japan), and troglitazone was from Cayman Chemical (Ann Arbor, MI). Protein A-agarose, 2-deoxy-D-glucose and scintillation counting solution were from GE Healthcare (Pittsburgh, PA). TaKaRa PrimeScript™ RT reagent kits and TaKaRa SYBR Premix Ex Taq were from Takara Bio (Kyoto, Japan). pcDNA 3 myc bovine CSP1(CSP1-Myc) and pcDNA 3 myc bovine CSP1 whose serine 10 was mutated to alanine (S10ACSP-Myc) were a kind gift from Professor Alan Morgan at University of Liverpool (7). pcDNA3 myc GLUT4 construct (Myc-GLUT4) containing an myc epitope tag in the first exofacial loop of GLUT4 was kindly provided by Dr. Shuichi Okada (Gunma University, Japan). For small interference RNA (siRNA), constructs Mouse Dnajc5 (SMF27A-0991-2 or 3), siCSP1 and non-targeting siRNA control, siControl (S10CM-0600) were obtained from B-Bridge International (Sunnyvale, CA). All other chemicals were of analytical grade.

Antibodies

CSP1 antibody was obtained from Stress gen (Victoria, BC). VAMP2 antibody and syntaxin 4 antibody were purchased from Synaptic Systems (Gottingen, Germany). GLUT1 antibody was from Biogenesis, (Hackensack, NJ). Akt antibody and p-Akt (Ser473) antibody were from Cell Signaling Technology (Beverly, MA). GLUT4 antibody was raised and purified as described previously (19). HRP-conjugated IgG second antibodies were purchased from Life Technologies (Carlsbad, CA).

Cell culture

3T3-L1 fibroblasts were induced to differentiate as described previously with minor modification (19). Briefly, 3T3-L1 fibroblasts were cultured in 15 cm dishes for electroporation with DMEM containing 4.5 g/l glucose, L-glutamine and 10% calf serum. Two days after confluence, cells were induced to differentiate by changing media to DMEM supplemented with 10% FBS, 0.5 mM IBMX, 1 μM dexamethasone, 1.7 μM insulin, and 1 μM troglitazone. After 48 h of induction, the medium was changed to DMEM supplemented with 10% FBS, and cells were fed with fresh medium every other day thereafter. Cells were used for experiments 8 days after inducing differentiation, when > 90% of the cells expressed the adipocyte phenotype.

Electroporation into 3T3-L1 adipocytes

This protocol was performed as described previously (20). Briefly, differentiated 3T3-L1 adipocytes were dislodged by incubation with trypsin-EDTA and centrifuged at 900 rpm for 3 min at room temperature (RT). Adipocytes were suspended again in D-PBS to an approximate concentration of 1.0×10^7 cells/500 μl and pipetted into a cuvette with a 0.4 cm electrode gap with 400 μg pcDNA3.1 plasmid, CSP1-Myc plasmid or S10ACSP1-Myc plasmid (and without or with 400 μg of Myc-GLUT4 for colorimetric assay, respectively). For RNA silencing, 2 nM siCSP1 or siControl was electroporated. This mixture was charged with 950 μF capacitance at 0.16 kV in a Gene Pulser II apparatus (Bio-Rad Laboratories, Hercules, CA), and then cells were immediately transferred to a tube containing warmed 10% FBS in DMEM and incubated at RT. After 10 min of incubation, the cells were plated for experiments. About 12 h after electroporation, cells were fed with new 10% FBS in DMEM, and until 48 h post-electroporation, cells were serum starved for at least 4 h before use in experiments.

Western blot analysis

After treatment of the cells as described in the figure legends, they were lysed in RIPA buffer. Cell lysates were obtained by centrifugation at 13,000 rpm for 20 min at 4°C, and protein content of each sample was determined by the BCA method. Equal amounts of proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were transferred to an Immobilon-P membrane (Millipore, Bedford, MA). Immunoblotting was performed with an ECL system according to the manufacturer's instructions. Densitometric analysis was performed using Image J 1.38× software (NIH, Bethesda, MD).

Glucose uptake assay

This protocol was carried out as described previously with minor alterations (19). Electroporated 3T3-L1 adipocytes were serum-starved for 4 h with serum-free DMEM and then cells were stimulated or not with 100 nM insulin for 15 min following preincubation for 30 min with Krebs Ringer phosphate buffer (KRP), pH 7.4. Glucose uptake was measured by incubation with 1 mM 2-deoxy D-glucose containing 1 μCi/ml 2-deoxy D-glucose for 4 min at 37°C. Cells were solubilized with PBS with 0.1% Triton X-100, and the radioactivity was detected by a liquid scintillation counter LSC-6100 (Aloka, Tokyo, Japan). Nonspecific 2-deoxy D-glucose uptake was measured in the presence of 1 μl of 120 mM phloretin/well and this value was subtracted from each determination to obtain specific uptake.

Immunoprecipitation (IP)

3T3-L1 adipocytes electroporated with siRNA were serum-starved for 4 h and treated with or without 100 nM insulin for 15 min. Cell lysates were obtained as described in '*Western Blot Analysis*'. VAMP2 antibody (1 μg) was added to 2 mg protein and mixed at 4°C overnight by rotation. Then 50 μl protein A plus sepharose beads were added and the mixture was rotated for 3 h at 4°C. Subsequently, the antibody-coupled beads were washed three times with RIPA buffer by centrifuging at 5,000 rpm for 2 min at 4°C. Finally, beads were mixed with SDS-PAGE sample buffer for Western blotting analysis. Before running gel electrophoresis, samples were boiled at 100°C for 5 min.

Colorimetric assay for detecting plasma membrane Myc-GLUT4 proteins

3T3-L1 adipocytes electroporated with 400 μg of CSP1-Myc or pcDNA3.1 plasmid plus 400 μg Myc-GLUT4 were reseeded onto a 24-well plate. After 48 h, wells were washed twice with serum-free DMEM at 37°C and incubated with DMEM with 0.2% BSA for 4 h. Then wells were washed twice with KRP buffer at RT and incubated with KRP buffer for 30 min at 37°C without or with 100 nM insulin. The dish was then locked on ice and fixed with 3.7% formaldehyde in PBS for 5 min. After washing several times with PBS, non-specific binding of the cells was blocked by 0.1 N glycine in PBS for 15 min at RT. Cells were incubated with myc antibody at a dilution of 1 : 1000 for 2 h at RT incubated and then with HRP-conjugated antibody in KRP with 0.1% BSA at a dilution of 1 : 1000 for 1 h, and finally the intensity value of ECL reaction was read by using Infinite F®500 (TECAN Männedorf, Switzerland).

Subcellular membrane fractionation

Subcellular fractionations were performed as described in our previous report (19) with slight modification. Specifically, 3T3-L1 adipocytes were serum-starved for 4 hours at 37°C and then glucose-starved with KRP buffer, pH 7.4 for 30 min at 37°C. Subsequently, cells were treated with 100 nM insulin for 15 min at 37°C. Then the cells were scraped into HES (2 mM HEPES, pH 7.4, 5 mM EDTA and 250

mM sucrose) supplemented with protease inhibitor cocktail, and homogenized with 8 passages through a 29 gauge syringe. The homogenate was centrifuged at 19,000 g for 20 min at 4°C to precipitate cellular debris. The pellet was then suspended in HES buffer, layered onto a sucrose cushion (1.12 M sucrose), and subjected to ultracentrifuge at 100,000 g for 60 min at 4°C. The membrane fraction floating on the sucrose cushion was collected, resuspended in HES buffer and centrifuged at 40,000 g for 20 min at 4°C. The final pellet, designated as plasma membrane fraction, was resuspended in RIPA buffer and the same amounts of protein were used for Western blotting.

RNA extraction and reverse transcription PCR

Total RNA was extracted from 3T3-L1 adipocytes using TRIzol reagent. After RNA had been treated with DNase I, 0.5 μg DNA was reverse-transcribed using TaKaRa PrimeScript™ RT reagent kits according to the manufacturer's instructions.

Quantitative real-time PCR

Quantitative real-time reverse transcription polymerase chain reaction (quantitative real-time RT-PCR) was performed with a LightCycler system (Roche Diagnostics, Mannheim, Germany) using about 100 ng DNA of each reverse-transcribed reaction. The following oligonucleotide primers were used : CSP1 5'-GACGCCACGAAAAGAAACAT-3' (forward) and 5'-CAAACAGCAGCAGCAGTAGC-3' (reverse) ; 18S ribosomal RNA 5'-AAACGGCTACCACATCCAAG-3' (forward) and 5'-GGCCTCGAAAGAGTCCTGTA-3' (reverse). Samples were prepared by using TaKaRa SYBR Premix Ex Taq according to the manufacturer's instructions and reacted in the ligthCycler for PCR. After the reaction, each PCR product was verified for its single amplification by melting curve analysis. Expression levels were normalized to the expression of 18S ribosomal RNA.

Statistical analysis

P values were calculated using *unpaired Student's t-test*, and p values< 0.05 were considered as significant differences.

RESULTS

CSP1 contributes to insulin-stimulated glucose uptake at the step of GLUT4-vesicle docking to the plasma membrane.

First, we confirmed by immuno-fluorescence and subcellular fractionation that expression of CSP1 increased by about 4-folds after differentiation of adipocytes, that CSP1 existed both in the plasma membrane and intracellular membranes and its localization was not affected by insulin (data not shown).

To clarify the role of CSP1 for GLUT4 trafficking, CSP1 was over-expressed in 3T3-L1 adipocytes by electroporation (20). As shown in Figure 1, an approximately 2-fold over-expression of CSP1 protein did not influence protein expression of glucose transporter 1 (GLUT1), GLUT4, syntaxin 4 and VAMP2, which are important for GLUT4 vesicle trafficking, nor did it affect expression of Akt, which are required for insulin signaling (Figure 1A). Insulin-stimulated Akt phosphorylation also was not influenced by CSP1 over-expression (Figure 1B). On the other hand, over-expression of CSP1 significantly reduced basal and insulin-stimulated glucose uptake compared to that of control plasmid (Figure 1C).

In order to explain the decrease of insulin-stimulated glucose uptake by over-expressed CSP1 protein, GLUT4 recruitment to the plasma membrane was examined (Figure 2A). The amount of GLUT4 that fractionates with the plasma membrane after CSP1 over-expression was not different from that of control plasmid over-expression (Figure 2B). After movement of GLUT4 to the plasma membrane, the final steps by which GLUT4 vesicle facilitates glucose entry into the cell are tethering, docking and fusion (21). Thus, both of Myc-GLUT4 and CSP1 were co-expressed (Figure 2C) and the fusion of GLUT4 vesicles with the plasma membrane was monitored by Myc-GLUT4 (22). As shown in Figure 2D, surface incorporation of Myc-GLUT4 was significantly reduced after over-expression of CSP1 compared to that of the control.

The SNARE complex consisting of syntaxin 4, VAMP2 and SNAP23, likely constitutes the molecular scaffold regulating GLUT4 vesicle docking and fusion to the PM (22). By an *in vitro* pull down assay from 3T3-L1 cell lysates, CSP1 was shown to interact with syntaxin 4 in 3T3-L1 adipocyetes (6). Therefore, to explore the mechanism of the attenuated GLUT4 insertion in CSP1-over-expressed cells, binding of VAMP2 to syntaxin 4 was examined by IP. VAMP2 antibody could not detect CSP1 by co-IP.

Also efficacy of IP by CSP1 or sytaxin 4 antibody was poor respectively. As shown in Figures 2E and F, in CSP1-over-expressed cells, the interaction of VAMP2 with syntaxin 4 by insulin was significantly reduced to less than half of control values, which might be responsible for the inhibition of GLUT4 insertion into the plasma membrane (Figure 2D). Previous studies reveal that Akt phosphorylates serine 10 site of CSP1 by Ca^{2+} stimulation in chromaffin cells and serine-phosphorylated CSP1 influences

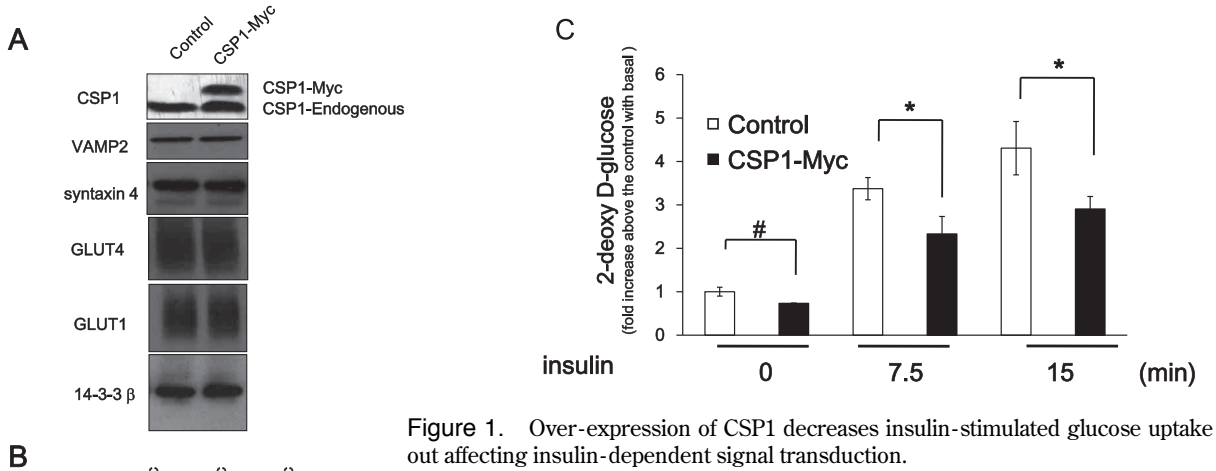

Figure 1. Over-expression of CSP1 decreases insulin-stimulated glucose uptake without affecting insulin-dependent signal transduction.
Cells were electroporated with either Control or CSP1-Myc plasmid. A. Total cell lysates were immunoblotted with CSP1, VAMP2, GLUT4, GLUT1, syntaxin 4 or 14-3-3 β antibody. B. Cells were serum-starved and were left without (-) or with insulin (+) for 15 min. Cell lysates were immunoblotted with either Akt or p-Akt antibody. Representative results of four (A) or three (B) independent experiments are presented. C. The electroporated cells were examined by glucose uptake after 0, 7.5, 15 min of insulin stimulation. The results of four independent experiments are presented as means± SEM (*$P< 0.01$ or #$P< 0.05$ compared with Control) and the fold-increase above control without insulin stimulation was shown.

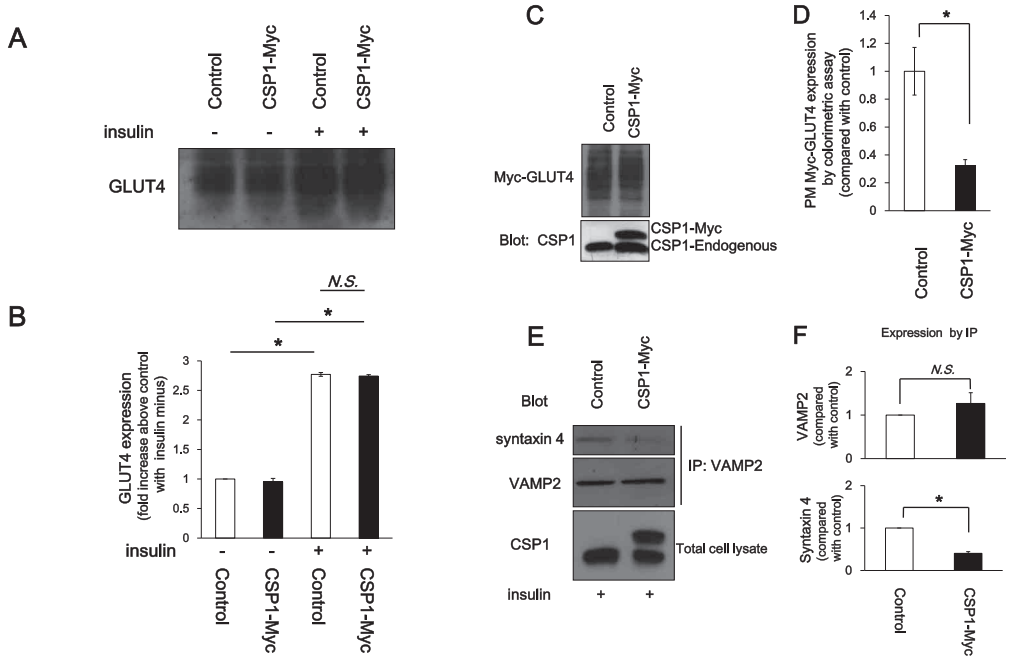

Figure 2. Over-expression of CSP1 inhibits SNARE complex formation concomitant with exposure of the cell surface to GLUT4 but not translocation of GLUT4.
Cells were electroporated with either Control or CSP1-Myc plasmid (A-F) together with Myc-GLUT4 (C, D). Cells were serum-starved and were either left without (-) or with insulin (+) for 15 min (E, F) or 30 min (A-D). A. Plasma membrane fraction was obtained and immunoblotted with GLUT4 antibody. Representative results of three independent experiments are presented. B. Results from all three experiments of A were also quantified, and the fold-increase from Control without insulin is shown. C. Cell lysates were immunoblotted with either GLUT4 or CSP1 antibody. D. Surface incorporation of Myc-GLUT4 with insulin was quantified as described in the Methods section. Results from three independent experiments were quantified, and the fold-increase from Control with insulin is shown. E. Insulin-stimulated cell lysates were immunoprecipitated with VAMP2 antibody and immunoblotted with either syntaxin 4 or VAMP2 antibody. F. Results from three independent experiments of E were quantified and the fold-increase from Control with insulin is shown. Data shown are means± SEM (*N.S. : not significant*, *$P< 0.01$* compared with Control).

the binding of syntaxin family members (7). We checked whether this insulin-stimulated phosphorylation of CSP1 involved GLUT4 trafficking. Over-expression of S10ACSP1 mutant protein decreased insulin stimulated glucose uptake to a similar extent as did over-expression of wild type CSP1 (Figure 3A).

To confirm the function of CSP1, it was knocked-down in 3T3-L1 adipocytes (Figure 3B). Gene silencing of CSP1 led to enhanced insulin-stimulated glucose uptake (Figure 3C). Knockdown of CSP1 protein did not influence the expression of proteins involved in insulin-signaling such as Akt and its phosphorylation by insulin stimulation, or GLUT4 content in the plasma membrane (not shown).

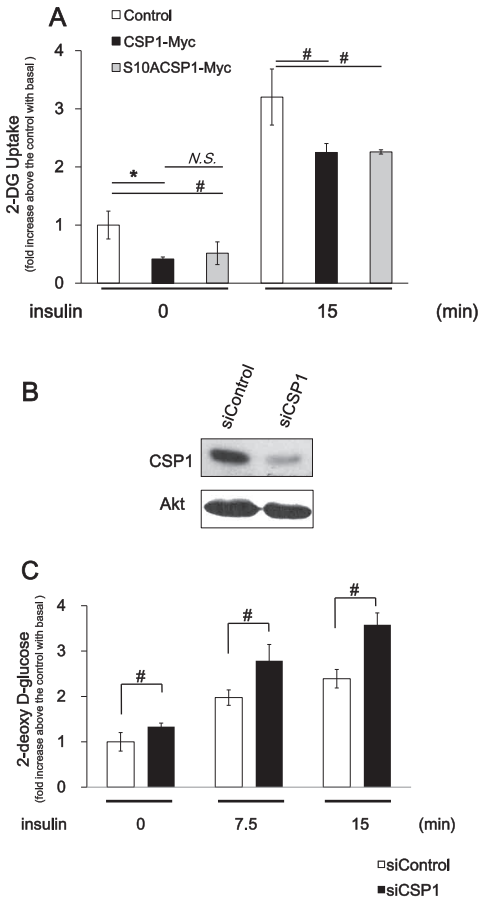

Figure 3. The effect of gene-manipulated CSP1 on insulin-stimulated glucose uptake.
A. Cells were electroporated with either Control CSP1-Myc or S10ACSP1-Myc plasmid. The electroporated cells were examined by glucose uptake after 0, 15 min of insulin stimulation. The results of three independent experiments are presented as means± SEM (*N.S. : not significant*, *#P< 0.05*, **P< 0.01* compared with Control) and the fold increase above Control without insulin stimulation was shown. B. Cells were electroporated with either siControl or siCSP1 and cell lysates were immunoblotted with either CSP1 or Akt antibody. C. The electroporated cells were examined by glucose uptake after 0, 7.5, 15 min of insulin stimulation. The results of four independent experiments are presented as means± SEM (*#P< 0.05* compared with siControl) and the fold increase above siControl without insulin stimulation was shown.

The mRNA and protein expression of CSP1 in 3T3-L1 adipocytes is increased under insulin resistance states.

To assess the possible pathological aspects of CSP1 expression, its mRNA was examined in an insulin resistance state. Exposure to a high level of insulin (100 nM) significantly increased mRNA expression of CSP1 (Figure 4A) under these conditions. Elevated free fatty acid also causes insulin resistance in a diabetic state or obesity (23). As shown in Figure 4B, incubation with 250 mM palmitate for 24 hours dramatically increased the mRNA expression of CSP1. Moreover, incubation with both 100 nM insulin and 250 mM palmitate for 48 hours increased the protein expression of CSP1 (Figure 4C).

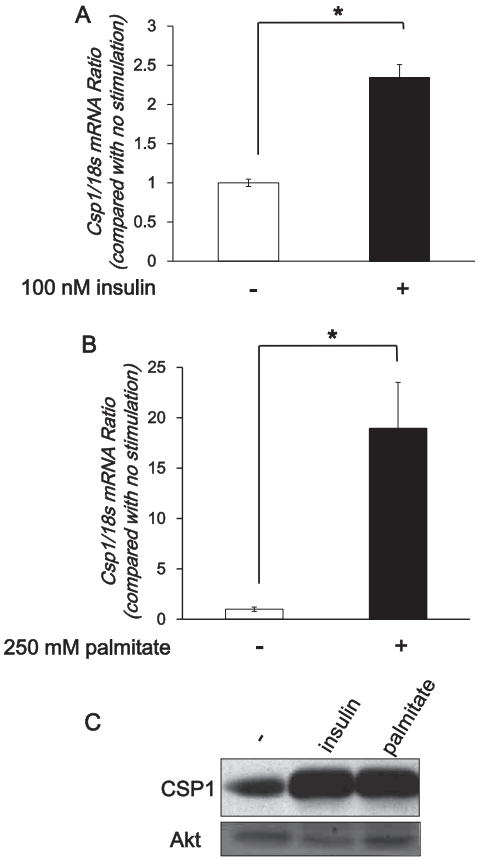

Figure 4. mRNA and protein expression of CSP1 is increased in an insulin resistant state.
Differentiated 3T3-L1 adipocytes were serum-starved for 4 hours. A. Cells were treated with or without insulin for 24 hours at 37°C. B. The adipocytes were treated with or without palmitate for 24 hours. Total mRNA that had been treated as described in A, B was extracted from cultured cells and subjected to quantitative real-time PCR with specific primers for CSP1. Results are means± SEM from three different experiments (**P< 0.01* compared with untreated condition). C. Total cell lysates of 3T3-L1 adipocyte treated with 100 nM insulin or 250 mM palmitate or non-treated were immunoblotted with either CSP1 or Akt antibody. Representative results of three independent experiments are presented.

DISCUSSION

Recent data from a number of laboratories has established that a key step in the insulin-dependent-regulation of GLUT4 translocation requires Akt and takes place at or very near to the plasma membrane (24, 25). The individual steps might involve tethering, docking and fusion and the latter two steps likely involve SNARE proteins. In the current study of 3T3-L1 adipocytes, it was found that a SNARE-binding protein, CSP1, participates in GLUT4 vesicle trafficking to the plasma membrane. Interestingly, the mRNA and protein expression of CSP1 in insulin resistant 3T3-L1 adipocytes were elevated, suggesting that CSP1 is closely related to the development of insulin resistance.

CSP has four isoforms. CSP2 is a splicing isoform of CSP1. CSPβ and γ are expressed only in the testis, and the physiological roles of these isoforms are still not well-understood (5). On the other hand, the function of CSP1 as a chaperone or a SNARE-binding protein in neurons has been investigated. It has been shown that CSP1 binds either to VAMP2 in neurons (6, 15) or syntaxin 4 in adipocytes (6, 15) as determined by pull-down protocols. CSP1 might directly bind either to VAMP2 or syntaxin 4 *in vivo*, but this finding was not confirmed in the present study, because syntaxin 4 or CSP1 antibodies were not available for IP (not shown). Also, VAMP2 antibody could not detect CSP1 by co-IP. It was speculated that a small fraction of CSP1 might bind to VAMP2.

Studies in which the effects of suppression or over-expression of CSP1 in β-cells and PC12 cells was investigated have shown both positive and negative results regarding the role of CSP1 in vesicle trafficking (9, 10, 12, 13, 16). The differences observed in their effects even in the same cell line might be explained by the differences in methods, i.e., stable or transient CSP1-over expression, or the difference in expression levels of CSP1. Moreover, the differences between β-cells and PC12 cells might be explained by the different organization of exocytosis in neuroendocrine cells from that in endocrine cells (12). As already mentioned, in 3T3-L1 adipocytes, CSP1 prevented insulin-stimulated GLUT4 vesicle docking on the plasma membrane when the expression of CSP1 was over-expressed, that could explain, in part, the decrease of GLUT4 trafficking in an insulin resistant state because CSP1 expression was increased after exposure of high insulin and palmitate.

Serine-phosphorylated CSP1 influences the binding of syntaxin family members. In 3T3-L1 adipocytes, over-expression of mutated S10ACSP1 decreased insulin-stimulated glucose uptake in the case of over-expression of wild type CSP1. This experiment suggested that serine phosphorylation of CSP1 is not involved in GLUT4 vesicle docking.

From of our studies, the precise mechanism of CSP1 to modulate the interaction of VAMP2 with syntaxin 4 was still not cleared. To clarify the precise function of CSP1, we have been trying to clone a novel protein that binds to CSP1.

FOOTNOTE

The first three authors contributed equally to this work.

DISCLOSURES

The authors declare they have no conflict of interest or financial ties to disclose

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