



# Vimentin binds IRAP and is involved in GLUT4 vesicle trafficking

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## ABSTRACT

Insulin-responsive aminopeptidase (IRAP) and GLUT4 are two major cargo proteins of GLUT4 storage vesicles (GSVs) that are translocated from a postendosomal storage compartment to the plasma membrane (PM) in response to insulin. The cytoplasmic region of IRAP is reportedly involved in retention of GSVs. In this study, vimentin was identified using the cytoplasmic domain of IRAP as bait. The validity of this interaction was confirmed by pull-down assays and immunoprecipitation in 3T3-L1 adipocytes. In addition, it was shown that GLUT4 translocation to the PM by insulin was decreased in vimentin-depleted adipocytes, presumably due to dispersing GSVs away from the cytoskeleton. These findings suggest that the IRAP binding protein, vimentin, plays an important role in retention of GSVs.

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## 1. Introduction

Insulin maintains glucose homeostasis by enhancing glucose transport into muscle and adipose tissues, a process mediated by recruitment of GLUT4 storage vesicles (GSVs) to the cell surface [1] and abnormalities in this machinery appears to be a cause of insulin resistance associated with type 2 diabetes [2,3]. Thus there have been many intensive explorations to characterize GSVs as well as the molecular mechanisms of insulin dependent signal transduction [1,4]. A large number of proteins have been identified as components of GSVs which can participate in and/or regulate some stage of their vesicular traffic [5,6].

Insulin-responsive aminopeptidase (IRAP) was also identified as an abundant cargo protein associated with GSVs that translocates in response to insulin in a manner seemingly identical to GLUT4 [7–11]. In fact, it is more abundantly expressed in the vesicles than the transporter [12]. A chimeric protein containing the intracellular domain of IRAP and the extracellular and transmembrane domains of the transferrin receptor displays IRAP- and GLUT4-like trafficking in 3T3-L1 adipocytes [13]. IRAP displays similar trafficking kinetics to GLUT4 in 3T3-L1 adipocytes [14], although there may be some differences in the internalization rate between these two proteins in rat adipocytes [15]. In any case, these citations

document considerable evidence that IRAP is a marker for insulin-dependent GLUT4/GSV trafficking.

IRAP shows increased intracellular sequestration upon differentiation of 3T3-L1 adipocytes in correlation with the development of an insulin responsive compartment whose formation precedes GLUT4 expression during the differentiation process [16,17]. When the cytoplasmic N-terminus of IRAP was microinjected into 3T3-L1 adipocytes, GLUT4 was localized on the plasma membrane even in the basal state [18]. Insulin-stimulated GLUT4 translocation was decreased after IRAP knockdown, but IRAP translocation was not changed in GLUT4 knockdown 3T3-L1 adipocytes [19,20]. These data suggested that IRAP can play a role in GSV movement/targeting. We reported that GLUT4 is not absolutely necessary for the formation of GSVs in engineered adipocytes, and that it is targeted to a preexisting vesicle, which can form independently of GLUT4 as adipocytes differentiate [21]. Similarly, IRAP fractionates and responds normally to insulin in denervated muscle under conditions where GLUT4 expression is decreased by 90% [22]. These findings strongly suggest that the involvement of IRAP with the retention and sorting machinery of GSVs, and that its potential association with the targeting and tethering proteins involved in this process.

IRAP has a single transmembrane domain with the N-terminal projecting into the cytoplasm [9], whereas GLUT4 has potential sorting and targeting motifs in three cytosolic domains corresponding to N- and C-termini as well as the central loop that connects helices 6 and 7 [23]. The N-terminus of IRAP has di-leucine motifs and an acidic cluster domain similar to that found in the c-terminus of GLUT4, which is thought to be important region for its trafficking [24–26]. In this study, we used the N-terminal

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cytoplasmic domain of IRAP, residues 1–109, conjugated to a 8 histidine tag with a chitin binding domain in order to find cytosolic proteins involved in GSV trafficking, and we identified vimentin as one such protein, in a manner similar to p115 [27] and AS160 [28,29]. When a vimentin-derived peptide, which interferes with the integrity of the filamentous network, is microinjected into 3T3-L1 adipocytes, GLUT4 peri-nuclear localization is disrupted [30]. The results of our study indicate that vimentin directly binds GLUT4 storage vesicles via IRAP and plays an important role in GSV retention.

## 2. Materials and methods

### 2.1. Materials

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). The IMPACT system was obtained from New England Biolabs (Beverly MA). For vimentin knockdown (si-Vim) siGENOME SMARTpool Mouse VIM (MSS238655) was used and siGENOME non-Targeting siRNA Pool #2 (D-001206-13-20) as a control (si-Con) were obtained from Thermo Scientific (Rockford, IL). All other chemicals were of analytical grade.

### 2.2. Antibodies

Anti-vimentin antibody, anti-myc (9E10) antibody and anti-goat-GLUT4 antibody for immunofluorescence were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti- $\alpha$ -tubulin (T-6199) antibody was purchased from Sigma–Aldrich. Anti-IRAP and anti-GLUT4 antibodies for Western blotting were previously described [31]. Anti-phospho akt (Ser473), anti-akt and anti- $\beta$ -actin antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti-Syntaxin 4 antibody was purchased from Synaptic Systems (Goettingen, Germany). Anti-goat and anti-mouse Cy2 secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA).

### 2.3. Expression vectors

The pTYB4 IRAP (1–109), pGEX5X-1 GLUT4 c-terminal, pGEX-5T IRAP (55–82), delta LL-IRAP (55–82) construct and pcDNA3-myc-GLUT4 constructs (myc-GLUT4) were described before [27]. The pTYB4 IRAP (1–109)-His8, which contains 8 tandem histidines between IRAP and intein, was made by PCR.

### 2.4. Cell culture

3T3-L1 fibroblasts were induced to differentiate as described previously with minor modification [27]. Briefly, 3T3-L1 fibroblasts were cultured dishes with DMEM containing 4.5 g/l glucose, 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  $\mu$ M dexamethasone, 1.7  $\mu$ M insulin, and 1  $\mu$ M troglitazone. After 48 h of incubation, medium was changed to DMEM supplemented with 10% FBS, and cells were fed with fresh medium every other day thereafter. Electroporation was conducted 8 days after inducing differentiation, when >90% of cells expressed the adipocyte phenotype, and other experiments were conducted between 8 and 12 days post-differentiation.

### 2.5. Subcellular fractionation

Differentiated cells (15 cm dishes) were treated with insulin or not and then the cells were subjected to ice-cold HS buffer (2 mM

HEPES, pH 7.4, 250 mM Sucrose) for pull down by a Ni-column (or HS buffer with EDTA for Western blotting) and homogenized with a Teflon-glass homogenizer. Subcellular fractions [PM, LM (low density microsome) and cytosol] were obtained by differential centrifugation and resuspended in HS buffer as described before [27].

### 2.6. Bacterial expression and purification of IRAP(1–109)-His8 peptide

Media (LB) containing ampicillin (100 mg/L) was inoculated with bacteria harboring pTYB4 IRAP (1–109)-His8 at 37 °C until the OD<sub>600</sub> was 0.5–0.6. Isopropyl thiogalactoside was added to induce expression of the fusion protein, and then the culture was grown for overnight at 22 °C. The supernatant of bacterial extract which was sonicated and resuspended with 10% Triton X-100 was loaded onto a column of chitin beads. After the column was washed, 2%  $\beta$ -mercaptoethanol (BME) solution was added to the beads for intein cleavage. Peptides were concentrated by centrifugation, and then the removal BME was achieved by speed-vac. The IRAP (1–109)-His8 peptide was resuspended in PBS and it was loaded onto a column of Ni-Beads. The column was used for affinity chromatography as described below.

### 2.7. Affinity purification of IRAP-binding peptide with the IRAP-Ni-column

All the steps were conducted at 4 °C. The IRAP-Ni-column was equilibrated with purification buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.5 M NaCl, pH 8.0). Fractionated cell lysates (cytosol or LM) were loaded onto an IRAP-Ni-column. Then elution buffer was added to the column (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.5 M NaCl, 10 mM Imidazole, pH 8.0). The concentrated eluates were used for mass spectrometry analysis.

### 2.8. Mass spectrometry analysis; tandem mass spectrometer method (MS/MS)

The samples were subjected to SDS–PAGE, followed by Coomassie brilliant blue (CBB) staining. After visible bands were excised and digested, mass spectrometry was performed by MALDI-TOF-MS. The MSACOT peptide mass fingerprint online search engine (<http://www.matrixscience.com>) was used to identify the samples from NCBI non-redundant data-base.

### 2.9. Expression and purification of GST fusion proteins

For expression and purification of GST, GST-IRAP (55–82), GST-GLUT4 C-terminus, and GST-IRAP delta LL (55–82), each vector (Section 2.3) was expressed in BL21 and purified with Glutathione Sepharose 4B beads as described previously [27].

### 2.10. Western blotting

After treating the cells as described in the figure legends, proteins were separated by SDS–PAGE. After electrophoresis, the proteins were transferred to PVDF membranes. The membranes were then incubated with the primary antibodies described above. After HRP-conjugated secondary antibodies incubation, intensity was detected by ECL and exposure to autoradiographic film.

### 2.11. Immunoprecipitation

Cell lysates (200  $\mu$ g protein) were incubated with 1  $\mu$ g of IRAP antibody or control IgG at 4 °C for overnight, after which protein A-Sepharose beads were added and incubated at 4 °C for 2 h. The immune-complexes were subjected to SDS–PAGE and analyzed by Western blotting.

2.12. Electroporation into 3T3-L1 adipocytes

This protocol was performed as described before [27]. Briefly, differentiated 3T3-L1 adipocytes were suspended in D-PBS to an approximate concentration of  $1.0 \times 10^7$  cells/500  $\mu$ L and pipetted into electroporation cuvettes with 3 nM siVim or siCon. This mixture was charged with 950  $\mu$ F of capacitance at 0.16 kV in a Gene Pulser Xcell apparatus (Bio Rad, Richmond, CA). Following electroporation cells were plated for indicated experiments. Forty-eight hours after electroporation, cells were used for experiments.

2.13. Colorimetric assay detecting for plasma membrane myc-GLUT4 proteins

3T3-L1 adipocytes electroporated with 3 nM of si-Vim or si-Con plus 400  $\mu$ g of myc-GLUT4 were reseeded onto a 24-well plate. After 48 h, cells were treated with 100 nM insulin for 30 min. After fixation, cells were incubated with anti-myc antibody, followed by anti-mouse HRP-conjugated antibody. The intensity of the ECL reaction was determined using an Infinite F 500 reader (TECAN Marnedorf, Switherland).

2.14. Immunofluorescence

After treating cells as indicated in Fig. 4, cells were immunostained with antibody as indicated in Fig. 4. Images were taken on a Leica TSC-SP2 confocal microscope (Wetzlar, Germany).

2.15. Statistical analysis

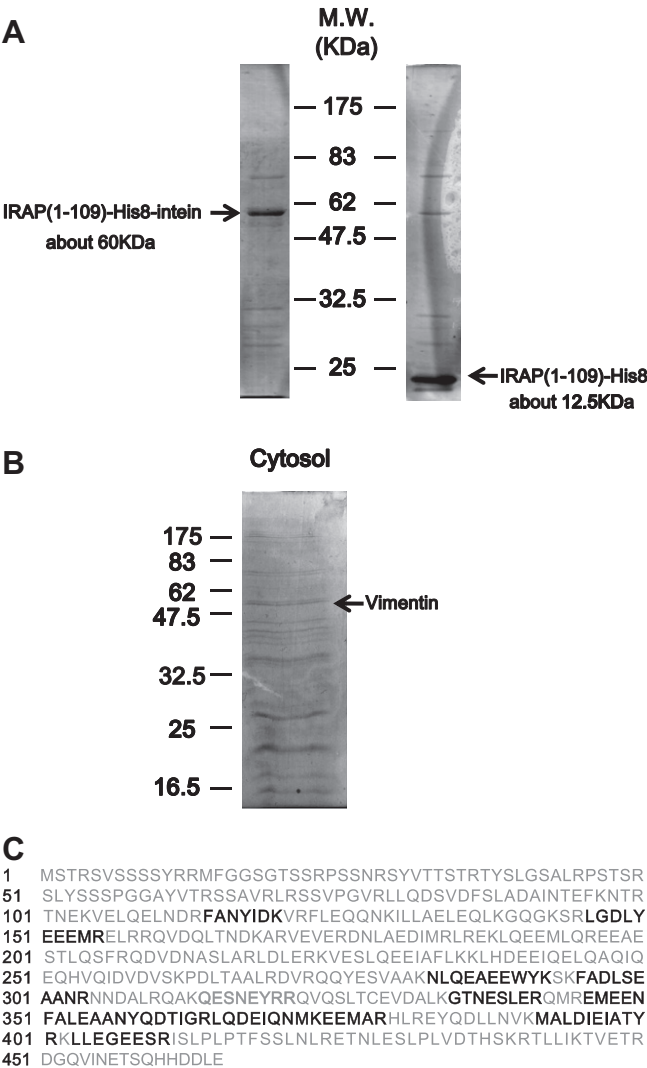
P values were calculated using unpaired Student's *t*-test and *p* values <0.05 were considered as significant differences.

3. Results and discussion

3.1. Identification of vimentin as an IRAP binding protein

IRAP is a key protein in retaining GSVs at a peri-nuclear region and prior to sorting them to the PM upon insulin exposure, and proteins which associate with the N-terminal of IRAP have been shown to regulate GSV trafficking machinery [13–15,21,22]. Thus, we have looked for novel IRAP binding proteins which may regulate this process. In this study, we used a fusion protein, the N-terminus of IRAP (1–109) conjugated with both His and intein tags on its c-terminus to diminish the non-specific binding. Purifying fusion proteins, IRAP (1–109)-His8-intein (Fig. 1A left image), from bacteria by intein-chitin beads lowered non-specific binding of bacterial proteins. After cleavage of the intein by reducing reagents, fusion proteins, IRAP (1–109)-His8 (Fig. 1A right image), were purified by His-Ni-beads. Following affinity chromatography, we identified a number of proteins which were specifically bound to IRAP (1–109) from 3T3-L1 cytosol (Fig. 1B) and LM. In addition to those previously reported to be involved with GSV traffic but not to bind to them (see below), we identified vimentin (Fig. 1B and C) as an intermediate filament component (IF). We showed that vimentin binds specifically to the N-terminus of IRAP by pull down (Fig. 2A) and immunoprecipitation (Fig. 2B). As shown in Fig. 2C, the vimentin-binding activity of IRAP was not changed with insulin. Our results led us to think that vimentin might be involved in aspects of GSV recycling including endocytosis, and might not be limited to insulin-stimulated exocytosis of GSVs.

The di-leucine motif of IRAP and GLUT4 at the cytoplasmic terminus is an important site of GVS trafficking [24–26,32], and proteins that bind to the di-leucine motif may be involved in regulating GSVs. The 28-amino acid region of cytoplasmic domain of IRAP (55–82), particularly a di-leucine motif at position 76, 77, is

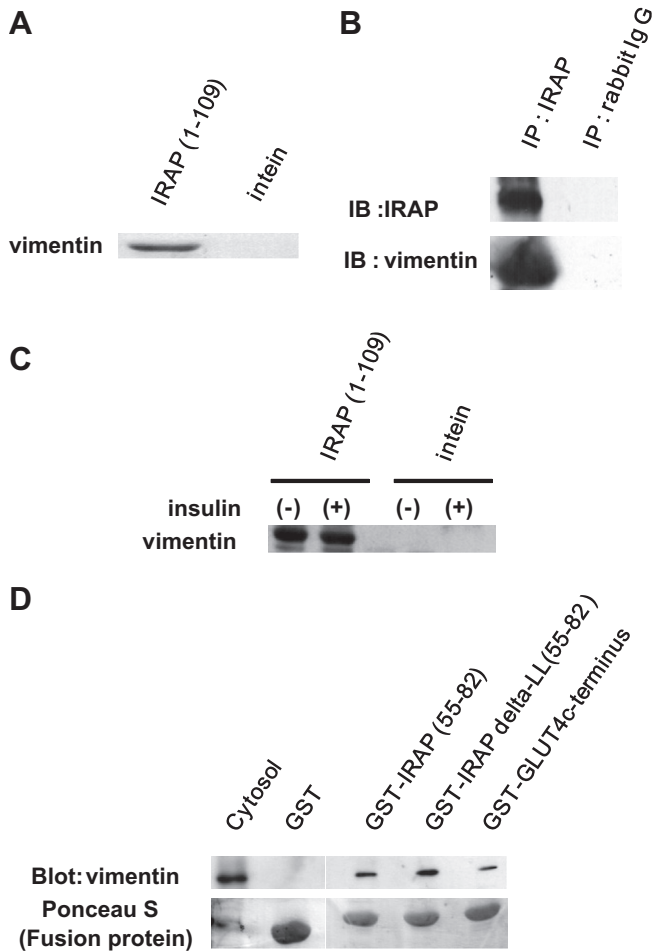


**Fig. 1.** Purification of IRAP (1–109)-His8 protein and identification of vimentin by affinity chromatography of IRAP (1–109)-His8-Ni column. (A) IPTG induced proteins from pTYB4 IRAP (1–109)-His8 transformed cultured bacteria were purified by chitin column affinity chromatography and the purified proteins subjected to SDS-PAGE and the gel (10%) was stained by CBB stain (left image). After cleavage of the intein domain, IRAP (1–109)-His8 was purified by Ni-column and the purified proteins subjected to SDS-PAGE and the gel (10%) was stained by CBB stain (right image). (B) The purified proteins from cytosol bound with IRAP (1–109)-His were subjected to SDS-PAGE, followed by CBB staining (10% gel). (C) After visible bands were excised, digested and analyzed by MS/MS method, the peptides of vimentin were identified. The identified peptides are shown in black bold letters in the amino acid sequence of vimentin.

required for GSV translocation to the plasma membrane [18]. In this study, we used a GST-IRAP delta-LL (55–82) whose di-leucine motif was changed to di-alanine. Our results showed that vimentin bound IRAP in a manner independent of its di-leucine motif, and also bound GLUT4, the binding of which was significantly less than IRAP (Fig. 2D). Tankyrase binds to another site of IRAP and plays a role in GSV exocytosis [33]. AS160, sortilin and p115 have also been shown to bind IRAP, their roles in GSV trafficking are still unclear [27–29,34].

3.2. Knockdown of vimentin expression by RNA silencing disperses GSVs to the periphery and impairs an insulin-stimulated translocation to PM

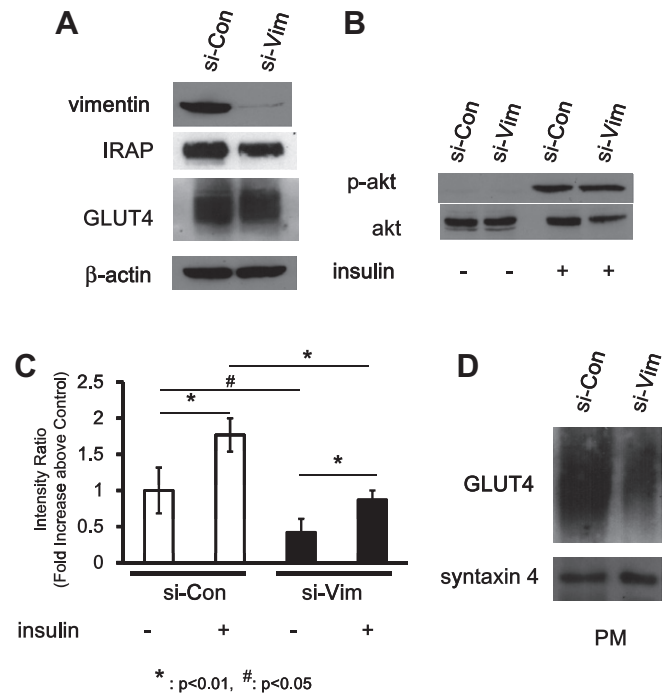
The integrity of microtubule networks and intermediate filaments is critical for correct peri-nuclear sequestration of GLUT4



**Fig. 2.** Vimentin interacts with N-terminus of IRAP independent of the di-leucine motif. (A) The cytosolic proteins prepared from 3T3-L1 adipocytes were passed over the chitin-column conjugated with IRAP (1–109) or intein. Adherent proteins were eluted and blotted with anti-vimentin antibody, details are in Section 2. (B) Total cell lysates from 3T3-L1 adipocytes were immunoprecipitated with anti-IRAP or non-specific IgG, and immunoprecipitates were blotted by anti-IRAP or anti-vimentin antibody. (C) The cytosolic proteins prepared from 3T3-L1 adipocytes with or without 15 min. of insulin stimulation were pull-downed and blotted with anti-vimentin antibody using the same method as (A). (D) Cytosolic proteins prepared from 3T3-L1 adipocytes were pulled down by GST, GST-IRAP (55–82), GST-IRAP delta-LL (55–82) or GST-GLUT4 c-terminus conjugated GS4B beads column and blotted with anti-vimentin antibody (upper lane). Equal amounts of beads from each column were suspended in Laemli sample buffer. These samples were subjected to SDS-PAGE and the gel was stained by Ponceau S (lower lane). Representative results of three (A, B, and D) or four independent (C) experiments are presented.

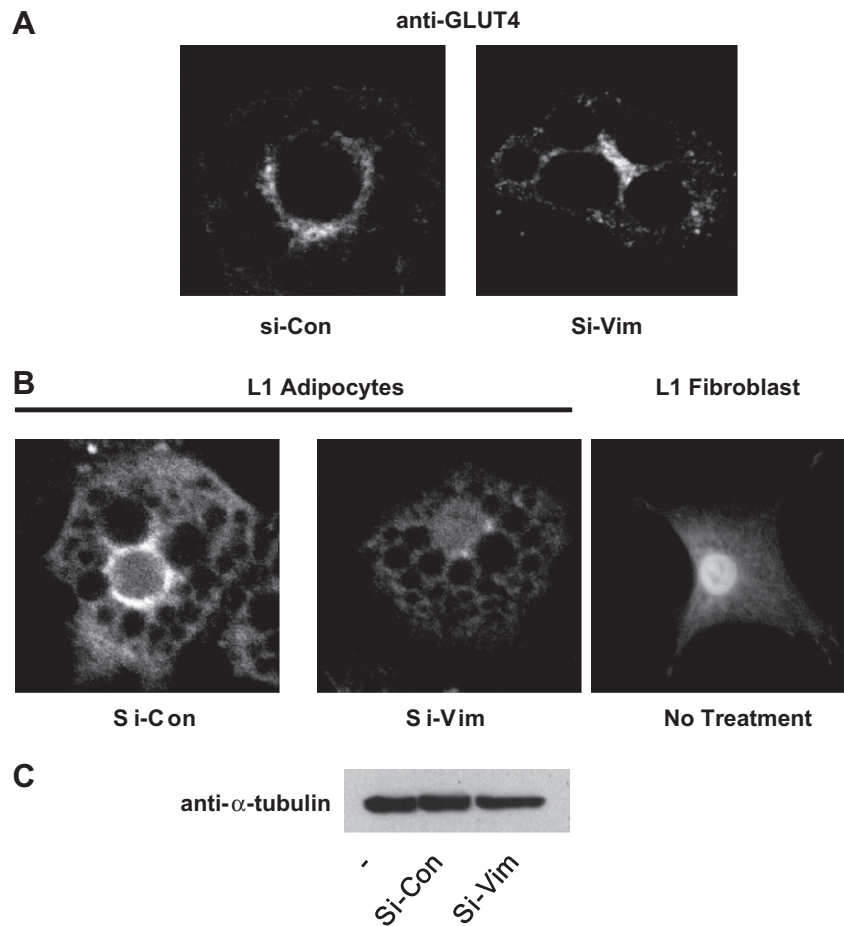
[30]. In this study, we sought specifically to knockdown the expression of vimentin in 3T3-L1 adipocytes (as shown in Fig. 3A) to clarify the role of vimentin in GSV trafficking. An almost complete knockdown of vimentin protein did not influence IRAP and GLUT4 expression (Fig. 3A), or insulin-stimulated phosphorylation of akt (Fig. 3B) compared to control siRNA. Insulin-stimulated GLUT4 insertion into PM in si-Vim groups was completely disrupted compared to the si-Con groups (Fig. 3C). This effect was verified by showing that translocation of GLUT4 to the PM was impaired in vimentin knockdown cells (Fig. 3D). In fibroblasts, vimentin's contribution to vesicle fusion machinery is to reserve and dissociate the SNAP23 [35]. The extent of decrease by a colorimetric assay method, about half, for si-Vim compared to si-Con groups was similar to the decrease in GLUT4 traffic as shown by subcellular fractionation (Fig. 3C and D).

Next, we examined the peri-nuclear localization of GLUT4 by immunofluorescence. As shown in Fig. 4A, the reduction of



**Fig. 3.** Knockdown of the expression of vimentin decreases GLUT4 translocation to the PM. Cells were electroporated with either si-Con or si-Vim as described in Section 2. (A) Total cell lysates were blotted with anti-vimentin, anti-IRAP, anti-GLUT4, or anti- $\beta$ -actin antibody. (B) Serum-starved cells were left untreated (-) or treated with 100 nM insulin (+) for 15 min. Then, total cell lysates were blotted with either anti-akt or anti-phospho-serine akt (p-akt) antibodies. (C) The amount of myc-tag exposure to the extracellular surface treated with or without insulin was quantified and the fold-increase from si-Con without insulin is shown. The data are shown means  $\pm$  SEM. (D) PM fractions were prepared from insulin-stimulated L1 adipocytes as described in Section 2 and blotted with GLUT4 or syntaxin4 antibody. Figures (A, B, or C) are presented as a representative result of three independent experiments.

vimentin expression increased the peripheral dispersion of GLUT4 in the basal state. This result was consistent with the effect of filamentous network disassembly as seen before [30]. These findings indicated that vimentin contributes to the GSVs peri-nuclear localization through the interaction with IRAP before insulin-stimulated GSV translocation. The IF network cooperates with the microtubule network and disassembly of the IF network destabilizes the microtubule network [36]. The microtubule network is involved not only in GSV trafficking [37–40], but also peri-nuclear retention of GSVs [41–43]. It was hard to detect filamentous images in L1 adipocytes by confocal microscopy as compared to L1 fibroblast because of the lipid droplets (Fig. 4B). In si-Vim cells, staining of  $\alpha$ -tubulin in the peri-nuclear region and cytoplasm became faint (Fig. 4B) and total expression of  $\alpha$ -tubulin, however, was not changed compared to si-Con (Fig. 4C). Our results might indicate that the microtubule network was disrupted and individual  $\alpha$ -tubulin might be seen as a faint image, but concentrated bundles of  $\alpha$ -tubulin should be more highly visible. On the other hand, treatment of cells with nocodazole, which disrupts the microtubular network, has little effect on the IF network in L1 adipocytes [38]. Together with those reports as well as our data, we concluded that microtubule network could contribute to insulin-sensitive peri-nuclear storage of GSVs through the interaction of IRAP with IFs. However, the extent of the increase from insulin minus to plus between si-Con and si-Vim in Fig. 3C was similar, suggesting that IFs do not influence insulin-stimulated GSV translocation. This translocation might be explained by the remaining intact parts of microtubule network which were not completely disrupted (Fig. 4B).



**Fig. 4.** Knockdown of vimentin expression dispersed GLUT4 to the cytoplasm. (A) 3T3-L1 adipocytes were electroporated with either si-Con or si-Vim as in Fig. 3. Cells were immunostained for GLUT4, for details see references cited in Section 2. Electroporated 3T3-L1 adipocytes or 3T3-L1 fibroblasts were immunostained (B) or immunoblotted (C) for  $\alpha$ -tubulin – represents non-electropolated 3T3-L1 adipocytes. Representative images from four independent experiments are shown.

In this study, we found that the IRAP interacts with vimentin and that their interaction plays an important role in GSV retention. We are concentrating on exploring the precise function of the surrounding IF network, which would be an important aspect of insulin-sensitive cytoskeletal functions.

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