DOC2B: A Novel Syntaxin4 Binding Protein Mediating Insulin-Regulated GLUT4-Vesicle Fusion in Adipocytes

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ABSTRACT

Objective: Insulin stimulates glucose uptake in skeletal muscle and adipose tissues primarily by stimulating the translocation of vesicles containing a facilitative glucose transporter, GLUT4, from intracellular compartments to the plasma membrane. The formation of stable SNARE [soluble N-ethyl-maleimide-sensitive fusion protein (NSF) attachment protein receptor] complexes between VAMP-2 and syntaxin4 initiates GLUT4-vesicle docking and fusion processes. Additional factors such as munc18c and tomosyn were reported to be negative regulators of the SNARE complex assembly involved in GLUT4-vesicle fusion. However, despite numerous investigations, the positive regulators have not been adequately clarified.

Research design and Methods: We determined the intracellular localization of DOC2b by confocal immunoflorescent microscopy in 3T3-L1 adipocytes. Interaction between DOC2b and syntaxin4 was assessed by yeast two-hybrid screening system, immunoprecipitation and in vitro GST pull-down experiments. Cell-surface externalization of GLUT4 and glucose uptake were measured in the cells expressing DOC2b constructs or silencing DOC2b.

Results: Herein, we show that DOC2b, a SNARE related protein containing double C2 domains but lacking a transmembrane region, is translocated to the plasma membrane upon insulin stimulation and directly associates with syntaxin4 in an intracellular Ca\(^{2+}\) dependent manner. Furthermore, this process is essential for triggering GLUT4-vesicle fusion. Expression of DOC2b in cultured adipocytes enhanced, while expression of the Ca\(^{2+}\) interacting domain mutant DCO2b or knockdown of DOC2b inhibited insulin stimulated glucose uptake.

Conclusions: These findings indicate that DOC2b is a positive SNARE-regulator for GLUT4 vesicle fusion and mediates insulin stimulated glucose transport in adipocytes.

Abbreviations:
SNARE: soluble N-ethyl-maleimide-sensitive fusion protein (NSF) attachment protein receptor
BMAPT-AM: O,O'-Bis(2-aminophenyl)ethyleneglycol-\(N,N',N',N'\)-tetraaceticacid, tetraacetoxymethyl ester
eGFP: enhanced green fluorescent protein
MID: munc interacting domain
CIM: calcium interacting domain mutant
GLUT4: glucose transporter 4
Insulin stimulates glucose uptake in skeletal muscles and adipose tissues primarily by stimulating the translocation of vesicles containing a facilitative glucose transporter, GLUT4, from intracellular compartments to the plasma membrane (1, 2). In addition to this translocation step, membrane fusion processes are also controlled by insulin (3, 4). Like other regulated exocytotic processes in many cell types, the formation of stable SNARE complexes between VAMP-2 and syntaxin4 initiates GLUT4-vesicle docking and fusion processes (5). However, the precise mechanism by which insulin regulates SNARE complex assembly remains poorly understood.

In neurons, Ca\(^{2+}\) triggers exocytotic membrane fusion of synaptic vesicles to the plasma membrane and calcium sensor proteins such as synaptotagmins have critical roles in this process (6, 7). Similar mechanisms result in GLUT4-vesicle fusion in adipocytes and muscle cells. Whitehead et al. demonstrated and we confirmed that reduction of intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\text{in}) using the membrane permeable Ca\(^{2+}\) chelating agent BAPTA-AM diminished insulin-stimulated glucose transport, whereas this reagent did not inhibit GLUT4 translocation to the plasma membrane, i.e. GLUT4-vesicle trafficking was not impaired (8 and Fukuda N. and Emoto M. unpublished observation). These observations suggest that an appropriate intracellular Ca\(^{2+}\) level may be required for the final docking/fusion steps of GLUT4 vesicles in adipocytes.

The universal role of Ca\(^{2+}\) as a trigger for regulated exocytosis predicts the existence of conserved proteins capable of activating the fusion machinery upon binding Ca\(^{2+}\). Although many proteins have been suggested to play such a role, synaptotagmins have attracted the most attention as putative calcium sensor proteins functioning in regulated exocytosis (9). Synaptotagmin family proteins have tandem C2 domains at the C-terminus. These two domains, C2A and C2B, are conserved in all 13 synaptotagmins described to date, and constitute Ca\(^{2+}\) binding modules (10). Many proteins have been identified as being involved in the GLUT4 vesicle fusion machinery in adipocytes. However, neither synaptotagmins nor other calcium sensor proteins have as yet been reported to regulate GLUT4 vesicle fusion.

We investigated, in detail, the mechanisms of Ca\(^{2+}\)-dependent GLUT4 vesicle-fusion in adipocytes. We searched for double C2 domain proteins as candidate Ca\(^{2+}\) sensor proteins suitable for the relatively slow (on the order of several minutes) SNARE complex formation, and we found that DOC2b bound syntaxin4 upon insulin stimulation in an intracellular Ca\(^{2+}\) dependent manner and mediated GLUT4 vesicle-fusion. DOC2b may be a downstream target of the insulin signal and a positive regulator of SNARE assembly involving regulated exocytosis in adipocytes.

**RESEARCH DESIGN AND METHODS**

**Materials.** Mouse DOC2a and DOC2b cDNA constructs were kindly provided by Dr. R.R. Duncan (University of Edinburgh, UK). Mouse Munc18c cDNA construct was kindly provided by Dr. T. Takuma (School of Dentistry, Health Sciences University of Hokkaido, Japan).

**Cell culture.** 3T3-L1 fibroblasts were grown in DMEM with 10% fetal bovine serum (FBS) at 37°C. The cells (3-5 days after confluence) differentiated into adipocytes with incubation in the same DMEM, containing 0.5 mM isobutylmethylxanthine, 0.25 μM dexamethasone and 4 μg/ml insulin, for 3 days and were then grown in DMEM with 10% FBS for an additional 5-8 days.
Plasmids and antibodies. Wild-type DOC2b was subcloned into pEGFP-C2, pDsRed2-C1 (Clontech, CA) and pGEX-6P-1 (GE Healthcare, UK) vectors. Calcium interacting domain mutants (CIM) of DOC2b (D157N, D163N, D297N, D303N) were subcloned into pEGFP-C2 and pGEX-6P-1 vectors. We also constructed syntaxin 4 and a series of deletion mutants of DOC2b corresponding to ΔMID (amino acids 36-413), ΔC2A (2-120, 252-413), and ΔC2B (2-264) in a pEGFP-C2 vector. Myc-tagged DOC2b WT and CIM, flag-tagged MID were subcloned into a pcDNA3 vector. All chemically synthesized and PCR-derived DNA sequences were verified by DNA sequencing.

Rabbit polyclonal DOC2b antibody was generated against the peptide sequence CGARDDDEDVDQL specific for DOC2b isoform. This antibody was found to cross-react minimally (Fig. S1). The following antibodies were used: monoclonal anti-GLUT4 (clone 1F8) (R&D Systems, MN), polyclonal anti-GLUT4, anti-GST (Santa Cruz Biotechnology, CA); anti-myc (clone 9E10) (Covance, NJ); polyclonal anti-syntaxin 4 (Synaptic Systems, Gottingen, Germany); anti-caveolin 1 (BD Bioscience, CA); and fluorescent-conjugated and horseradish peroxidase-conjugated secondary antibodies (Jackson Immuno Research, PA).

DOC2b-shRNA construct. Short hairpin-RNA (shRNA) specific for mouse DOC2b was designed to have a 5'-GCCAGATGTAGACAAAGAATC-3' sequence. Synthetic complementary single-stranded DNA of the target sequence was annealed, and the double-stranded DNA was inserted into a pcPUR+U6i cassette (11). This shRNA decreased DOC2b protein expression to 10-20% of the control level within 74h. A same cassette encoding non-specific scramble sequence was used as a negative control.

Preparation of recombinant adenovirus vectors. Adenovirus producing eGFP, myc-tagged DOC2b (wild type, CIM mutant), and shRNA (DOC2b, control) were prepared using an AdEasy adenovirus vector system according to the manufacturer's instructions (Stratagene, TX). All amplified viruses were purified by the cesium chloride centrifugation method and stored at -80 °C.

Live cell imaging of DOC2b. The pcEGF-DOC2b was electroporated into 3T3-L1 adipocytes, which were then re-seeded onto 0.1 mm glass bottom dishes (Matsunami, Tokyo Japan). At 24-48h after electroporation, cells were serum-starved for 3-4h in DMEM and then incubated at 37 °C for 2h in Krebs-Ringer Hepes (KRH) buffer [130 mM NaCl, 5 mM KCl, 1.3 mM CaCl2, 1.3 mM MgSO4, 25 mM Hepes (pH 7.4)]. The cells were treated with 100nM of insulin at 37 °C for the time indicated and observed by laser-confocal microscopy (LSM510 Pascal, Carl Zeiss, Germany). For the translocation analysis, fluorescent intensities at three distinct areas in plasma membrane, cytosol, and nucleus (9 areas/cell each) of three independent cells were analyzed by Photoshop software CS2.

Yeast two-hybrid screening. The Matchmaker Yeast Two-hybrid System (Clontech, CA) was used for determination of DOC2b binding partners. Full-length DOC2b and munc18c cDNA were subcloned into a pLexA vector, and full-length syntaxin 4 and munc18c cDNA into a pB42AD vector. A standard lithium acetate/single stranded carrier DNA/polyethylene glycol method for transformation into yeast strain EGY48 (p8op-lacZ) was used and these proteins were expressed in this strain. Transcriptional activation of LacZ was determined by X-Gal assay. β-galactosidase activity was detected within 16 h of reaction at 30 °C.

In vitro GST pull-down assay. GST fusion proteins of wild type- and CIM- DOC2b, were purified according to the manufacturer's instructions. GST-syntaxin 4 was cleaved with PreScission protease (2 units/μl) (GE Healthcare, UK) in buffer containing 50 mM Tris-HCl (pH 7.0), 150 mM NaCl, 1mM
EDTA and 1mM DTT at 4 °C for 16 h. At the end of incubation, cleaved syntaxin4 protein was further purified using Amicon Ultra filter devices (Millipore, MA).

Recombinant individual GST-DOC2b’s or GST (1μg each) were incubated with 1 μg of recombinant syntaxin4 in 1 ml of Tris-buffered saline (20mM Tris, pH 7.4, 150mM NaCl) plus 0.5% Triton X-100 in the presence of 2mM EDTA or 1mM CaCl$_2$ for 4-6h. These mixture was immunoprecipitated by incubating with Glutathion Sepharose 4B (GE healthcare) for 1 h. The precipitates were washed four times and analyzed by SDS-PAGE and immunoblotting. Approximately 5% of the syntaxin4 was pulled down by the GST-DOC2b.

**Northern blotting.** Total RNAs were prepared from 3T3-L1 fibroblasts, 3T3-L1 adipocytes (days 3 and 9 of differentiation) or rat epididymal fat, and mouse brain using ISOGEN (Nippongene, Tokyo Japan), and denatured in formaldehyde/formamide, resolved by electrophoresis, and transferred to Hybond-N membranes (GH healthcare, UK). The membranes were hybridized with α-[32P]-labeled full-length DOC2a and DOC2b cDNAs as probes, and then washed three times with 1X SSC buffer [15 mM of NaCl, 15 mM of sodium citrate (pH 7.0), 0.1% SDS] at 65 °C. Radioisotopic measurements were conducted using Phosphorimagery FLA2000 (Fuji film, Tokyo Japan).

**Immunoprecipitation and immunoblotting.** A 10-cm plate of cells was lysed in 1 ml of lysis buffer [20 mM HEPES (pH 7.2), 100 mM NaCl, 25 mM NaF, 1 mM sodium vanadate, 1 mM benzamidine, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM PMSF, 1mM DTT, 0.5% NP-40] in the presence of 2mM EDTA or 1mM CaCl$_2$ and centrifuged for 15 min at 15,000 x g. The postnuclear lysates were used for the following experiments. The protein concentration was measured with BCA protein assay reagent (Pierce, IL). For immunoprecipitation, the cell lysates were pre-incubated with protein-G/A-Sepharose at 4 °C for 30 min to remove nonspecific bound proteins. Then, samples were incubated with primary antibodies at 4 °C for 8-12h followed by incubation with protein-G/A-Sepharose. Lysates and immunoprecipitates were resolved by SDS-PAGE and transferred to a PVDF membrane (GH healthcare, UK). The membranes were incubated with primary antibodies for 8-12h. Protein signals were visualized using horseradish peroxidase-conjugated secondary antibodies and an enhanced chemiluminescence substrate kit (GH healthcare, UK). The efficiencies of immunoprecipitation of the associated protein in Figure 3C, D, and E were approximately 1, 0.5, and 0.6%, respectively. All the images in figures are representative and we repeated the immunoblots at least 3 times and found similar results.

**Immunofluorescence microscopy and digital image analysis.** Differentiated 3T3-L1 adipocytes were left untreated or electroporated by eGFP-DOC2b (wild type, CIM, ΔMID, ΔC2A, ΔC2B), eGFP alone, DsRed2-DOC2b (wild type, CIM), shRNAs(control, DOC2b), or myc-GLUT4-eGFP. The cells were then re-plated onto coverslips and allowed to recover for 48h. Cells were pre-incubated in the presence or absence of 50 μM of BAPTA-AM for 10 min, followed by incubation with or without insulin for 20 min at 37 °C. Next, the cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) and permeabilized with buffer A (0.5% Triton X-100, 1% FBS in PBS) for 15min. For the detection of endogenous proteins, the coverslips were incubated for 2h with primary antibodies at room temperature. The cells were washed and incubated with an appropriate secondary antibody for 30 min. The coverslips were washed thoroughly again and mounted on glass slides. Immunostained cells were observed at room temperature with a LSM 5 PASCAL laser scanning confocal
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microscope and its two channel-scanning module (Carl Zeiss) equipped with an inverted Zeiss Axiovert 200M using the 63x oil objective lens (numerical aperture 1.4) run by LSM 5 processing software and Adobe Photoshop CS2. At least 5 cells were observed in a condition. The experiments were repeated at least three times, unless stated otherwise. All the images in figures are representative, and the conclusions are based upon qualitative visual impression. The cell surface myc-GLUT4-eGFP was measured by subtracting the internal myc signal from total myc signal of electroporated cells. The plasma membrane eGFP content was also measured. The cell surface GLUT4 was calculated as: (total myc - internal myc) / (total eGFP - internal eGFP) (22).

2-deoxy-glucose uptake. Differentiated adipocytes were prepared in 24-well plates. Cells were infected with the recombinant adenoviruses. Two days thereafter, the cells were serum-starved for 2 h at 37 °C in KRH buffer. Then, the cells were stimulated with or without 100 nM of insulin for 10 min, and 2-deoxy glucose uptake was determined by 2-deoxy-D-[2,6 3H] glucose incorporation. Nonspecific glucose uptake was measured in the presence of 20 μM cytochalasinB and subtracted from each determination to obtain specific uptake. The results were normalized by the protein amount.

Statistical analysis. Multiple comparisons among groups were performed using One-way ANOVA (post-hoc test: Tukey-Kramer). Results are presented as means ± SD. Values of P < 0.05 were considered statistically significant.

RESULTS

DOC2b translocates to the plasma membrane in response to insulin. Type C tandem C2 domain proteins are classified into three groups, i.e. synaptotagmin-, synaptotagmin like protein-, and DOC2-family proteins. The first two groups of proteins regulate relatively fast membrane fusion (on the order of millisecond to a few seconds) (12, 13). This time scale is not suitable for GLUT4 vesicle fusion. Therefore, we focused on DOC2 family proteins as candidate regulators of GLUT4 vesicle fusion. First, we determined the expression profile of DOC2 mRNA in adipocytes. As shown in Fig. 1A, DOC2a was not expressed in 3T3-L1 adipocytes. According to a previous study, DOC2γ is localized to the nucleus and has no Ca2+-binding activity because of amino acid substitutions at the Ca2+ binding sites. (14). Thus, we investigated the function of the DOC2b isoform involved in GLUT4 membrane fusion. Next, we examined the intracellular localization of DOC2b in differentiated 3T3-L1 adipocytes using anti-DOC2b antibody (Fig. 1B) or by expressing an eGFP (enhanced green fluorescent protein) fused to DOC2b (Fig.2A). As shown in Fig. 1B, DOC2b results in a fine punctate or granular appearance throughout the cytoplasm under basal conditions. In contrast, the addition of insulin yields relatively slow (~5 min) translocation of DOC2b to the cell periphery (Fig. 2A). This time-scale of translocation was very similar to that of GLUT4 vesicles. It is noteworthy that deletion of the munc13 interaction domain (MID) enhanced plasma membrane localization even in the absence of insulin, and that the C2B domain is necessary for membrane targeting (Fig. 2B). These observations are interesting in considering the distinct roles of the C2A and C2B domains and the negative regulatory function of MID.

We performed additional experiments to determine the role of calcium in the translocation of DOC2b. As shown in Fig. 2C, the cell membrane permeable Ca2+ chelating agent BAPTA-AM inhibited insulin-dependent translocation of DOC2b.

DOC2b binds syntaxin4 upon stimulation with insulin. Since DOC2b is thought to be a soluble calcium sensing protein,
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compartment-specific targeting must be achieved through interaction with membrane-bound proteins such as SNARE and SNARE-related proteins. Therefore, we attempted to identify DOC2b binding partners among these proteins using a yeast two-hybrid system. We found a very strong interaction between DOC2b and syntaxin4 as compared with the already known binding between munc18c and syntaxin4 (Fig. 3A). Interestingly, this t-SNARE protein is reportedly a key molecule for GLUT4-vesicle fusion in response to insulin (5, 15). Although this interaction was very strong, SNARE proteins are quite ‘sticky’ and can on occasion bind with many proteins non-specifically. Therefore, we performed the following three additional experiments. First, we determined the direct interaction in vitro using recombinant proteins, GST-tagged DOC2b and synthesized syntaxin4. As shown in Fig. 3B, the interaction was easily detected by immunoblotting in the presence of calcium. Second, we examined endogenous protein-protein interactions by immunoprecipitation experiments using polyclonal anti-DOC2b. As shown in Fig. 3C, insulin treatment increased DOC2b-syntaxin4 binding and BAPTA-AM abolished this interaction. Since the molecular weights of DOC2b and the IgG heavy chain are quite similar (computed molecular weight of DOC2b and syntaxin4 are 46 and 34 kDa, respectively), it was difficult to perform a reverse immunoprecipitation experiment. Third, our results were confirmed by immunoprecipitation experiments using adipocytes expressing myc-tagged DOC2b (Fig. 3D, E). It is important to note that we could not detect the interaction between DOC2b and syntaxin4 in the buffer containing EDTA (data not shown). Furthermore, we could not find interaction between DOC2b and syntaxin6 in 3T3-L1 adipocytes (Fig. S2).

In order to better assess the calcium dependency of DOC2b translocation and interaction with syntaxin4 in adipocytes, we conducted the following additional experiments using a Ca\(^{2+}\)-unbound mutant. Based on the information from crystallographic analysis of synaptotagmin1 (16-18), we created mutations in the putative Ca\(^{2+}\) binding sites of DOC2b, i.e. C2A (D157N, D163N) and C2B (D297N, D303N), and designated the product obtained the Ca\(^{2+}\)-interacting domain mutant (CIM) (Fig. 4A). This type of mutant reportedly loses its calcium-dependent phospholipid targeting capacity (19, 20). As shown in Fig. 4B, the CIM mutation markedly inhibited insulin-induced DOC2b translocation. Furthermore, CIM-DOC2b also failed to interact with syntaxin4 in both the in vivo and the in vitro setting (Fig. 4C-E). These results raise the possibility that Ca\(^{2+}\) binding is essential for insulin-stimulated DOC2b translocation as well as the interaction with syntaxin4. DOC2b regulates the step of GLUT4-vesicle fusion in response to insulin. One interpretation of the above findings is that DOC2b may act as a Ca\(^{2+}\) sensor protein for SNARE complexes triggering GLUT4-vesicle fusion. To assess the role of DOC2b in GLUT4-vesicle fusion to the plasma membrane, we employed a GLUT4 construct containing four-myc epitopes in the first exofacial loop of GLUT4 and an eGFP fusion at its cytoplasmic COOH terminus. These exofacial tags are easily detected by anti-myc antibody, but only when GLUT4 vesicles are fused to the plasma membrane (21). Adipocytes expressing this GLUT4 construct and DsRed2-DOC2b (wild type or CIM), or short hairpin RNAs were observed by confocal microscopy. As shown in Fig. 5A-B, the externalized GLUT4 was increased in the cells co-expressing wild type DOC2b, while being decreased in those co-expressing CIM-DOC2b or shRNA (shRNA\(_{DOC2b}\)), as compared to the control cells. Since above conclusions were based upon qualitative visual impression, we next estimated the
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fluorescent signals of the cellular rims in Figure 5A-B in two ways. First, we counted the number of the cells with eGFP rims (50 cells in each condition) in the cells expressing myc-GLUT4-eGFP. As shown in Fig. 5C, just translocated- or docked- GLUT4 detected by eGFP fluorescence did not change under either condition. Second, we quantified the ratio of cell surface GLUT4 (myc signal) to GLUT4 translocated to plasma membrane (eGFP signal) as previously described (22). As shown in Fig. 5D-E, cell surface GLUT4 was increased in the cells expressing wild type DOC2b, but decreased in those expressing CIM-DOC2b and silenced DOC2b (Fig. 5D-E). These results, taken together with the data shown in Fig. 5A, are consistent with the idea that DOC2b is a calcium sensing protein and regulates the GLUT4 vesicle fusion step in response to insulin.

Role of DOC2b in glucose transport in 3T3-L1 adipocytes. We next focused on the role of DOC2b in insulin dependent-glucose uptake in adipocytes expressing wild type DOC2b and CIM-DOC2b. As shown in Fig. 6A, overexpression of wild-type DOC2b increased insulin stimulated-glucose uptake to 122% of the control level. In contrast, overexpression of CIM-DOC2b decreased to 78% of the control level. Furthermore, we introduced short hairpin RNAs (shRNA_{DOCAb, control}) by adenovirus vectors into cultured adipocytes to induce specific degradation of the DOC2b mRNA. DOC2b protein expression was decreased to 50-10% of the control level, in the cells infected viruses at m.o.i. of 20-50, respectively (Fig. 6B). As expected, glucose uptake was decreased to 87-60% in adipocytes infected the adenovirus vectors at m.o.i of 20-50 (Fig. 6B). To confirm the specificity of silencing, we conducted add-back style rescue experiment using adenovirus vector containing wild type DOC2b. As shown in Fig. S3, overexpression of DOC2b rescued the inhibitory effect on glucose uptake in DOC2b silenced cells. Under these conditions, DOC2b overexpression and silencing had no effects on serine phosphorylation of Akt (Fig. 6A-B, S3). These results, taken together with the data presented in Fig. 2-5, suggest that DOC2b regulates glucose transport through modulating vesicle fusion processes, but not insulin signaling.

**DISCUSSION**

Regulation of glucose uptake in muscle and adipose tissues by insulin is of fundamental importance for proper maintenance of postprandial hyperglycemia. This hormone stimulates translocation of the GLUT4 glucose transporter from the intracellular membrane to the cell surface (1, 2). In addition to this movement of intracellular vesicles containing GLUT4, it has been suggested that the docking and fusion step of GLUT4-vesicles is also critically regulated by insulin (3, 4, 23). However, the precise mechanism by which insulin regulates vesicle fusion is still largely unknown.

A key finding of this study is identification of the double C2 domain protein DOC2b, which mediates insulin regulated GLUT4 vesicle fusion. Like other membrane fusion processes, GLUT4-vesicle fusion occurs essentially through the formation of a “core complex” consisting of syntaxin4 and VAMP2 (5). In general, however, a number of additional factors are required to bring about SNARE-mediated membrane fusion in vivo. Many of these factors, which can collectively be called SNARE regulators (e.g. Munc18, synaptotagmin, Munc13, GATE-16/Apg8, LM1, synaptophysin, tomosyn and Vsm1/Ddi1), bind directly to SNARE proteins and are involved in membrane trafficking and fusion events (24). Among these SNARE regulators, munc18c and tomosyn were reported to be negative regulators of the SNARE complex assembly.
involved in GLUT4 vesicle fusion (25-27). Despite numerous investigations, the positive SNARE regulators for GLUT4-vesicle fusion have not been adequately clarified. In this report, we have shown that DOC2b mediates insulin stimulated GLUT4-membrane fusion in adipocytes, while having no effect on the GLUT4-vesicle translocation step. These data are consistent with the hypothesis that DOC2b regulates insulin stimulated GLUT4-vesicle fusion. DOC2b may be a positive SNARE regulator for vesicle fusion processes in adipocytes.

A second significant finding reported herein is the identification of a DOC2b binding partner. DOC2b interacts with t-SNARE syntaxin4 upon stimulation with insulin in the presence of calcium. Syntaxin4 is thought to be a SNARE protein on the target membrane for GLUT4-vesicle fusion (28, 29). As shown in Fig. 3A, this interaction appears to be very strong compared to that between munc18c and syntaxin4 demonstrated by the yeast two-hybrid method. Although this interaction appeared to be very strong, SNARE proteins are quite sticky and can on occasion bind with many proteins non-specifically. Therefore, we performed three additional experiments. As shown in Fig. 3B-E, we confirmed the interaction between DOC2b and syntaxin4 in both the in vivo and the in vitro setting. Furthermore, changes in the intracellular localization of DOC2b also supported the functional interaction. As shown in Fig. 2A, DOC2b translocates to the plasma membrane in response to insulin stimulation. Importantly, the time scale of DOC2b translocation coincides with relatively slow externalization of GLUT4 vesicles. Taken together, our data are consistent with the aforementioned hypothesis that DOC2b regulates GLUT4 vesicle fusion by triggering SNARE complex assembly.

Another interesting observation made in this study is the essential role of [Ca\(^{2+}\)]\(_i\) in insulin stimulated GLUT4-vesicle fusion. In 2001, Whitehead et al. first reported that a calcium chelator, BAPTA-AM, inhibited GLUT4 externalization and glucose uptake (8). However, the precise mechanism underlying calcium dependent GLUT4-vesicle fusion remains unknown, because no studies have as yet focused on Ca\(^{2+}\) sensing proteins in adipocytes. DOC2b is structurally similar to the well-known calcium sensing SNARE regulator synaptotagmins (Fig. 4A). Taking these observations together, we hypothesized that DOC2b is a Ca\(^{2+}\) sensing protein which regulates GLUT4-vasicle fusion in adipocytes. In order to better assess the role of Ca\(^{2+}\) in GLUT4-vesicle fusion, we confirmed the following. First, DOC2b translocation and its binding to syntaxin4 were shown to be [Ca\(^{2+}\)]\(_i\) dependent. Second, mutations in calcium binding sites on C2 domains of DOC2b resulted in loss of syntaxin4 binding, GLUT4 externalization, and glucose uptake. In contrast, we also observed DOC2b-syntaxin4 binding, without insulin action, in GST pull down assay suggesting that [Ca\(^{2+}\)]\(_i\) is necessary for this interaction in vitro (Fig.3B). In parallel, we also found that insulin initiates the DOC2b translocation to plasma membrane and promotes interaction between DOC2b and syntaxin4 in the presence of Ca\(^{2+}\) ions. Based on the above observations, we propose a simple model whereby insulin regulates SNARE regulator DOC2b and basal level of [Ca\(^{2+}\)]\(_i\) may act in a constitutive manner to promote DOC2b-syntaxin4 interaction involved in insulin-stimulated GLUT4 vesicle fusion.

A recent report suggests that DOC2 proteins remove munc18 from syntaxin thereby regulating core SNARE complex formation (30). This interpretation is very attractive because munc18c is thought to be a negative regulator of GLUT4 vesicle fusion. Although we have no direct data pertaining to munc18c, it is possible that munc18c and DOC2b may modulate SNARE assembly in a
counter-regulatory manner. Very recently, Ke et al reported that DOC2b bound munc18c, but not syntaxin4, in pancreatic beta cells (31). This apparent discrepancy on syntaxin4-binding may be attributable to the different experimental conditions. They performed most of their experiments using a buffer without calcium, i.e. “Nonidet P-40 lysis buffer”. Since DOC2b has conserved Ca$^{2+}$ binding sites and thought to be a Ca$^{2+}$ sensor protein in other cell types such as neuron (32), chromaffin cells (33), Ca$^{2+}$ might be important for the physiological properties of DOC2b in beta cells. Further work is required to uncover the underling mechanisms by which DOC2b regulates SNARE assembly in response to insulin.

In summary, we have identified DOC2b as a syntaxin4-binding protein in adipocytes. This protein regulates GLUT4-vesicle fusion as well as glucose uptake in response to insulin stimulation. We have further revealed that DOC2b requires [Ca$^{2+}$]i and positively regulates the step of GLUT4-vesicle fusion.

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Figure 1. The expression profiles and intracellular localization of DOC2b in 3T3-L1 adipocytes (A) The expressions of DOC2a and DOC2b in 3T3-L1 fibroblasts, 3T3-L1 adipocytes (days 3 and 9 of differentiation), rat epididymal fat, and mouse brain were analyzed by mRNA blot. (B) Differentiated 3T3-L1 adipocytes were serum-starved for 3-4h and then stimulated with 100 nM of insulin for 20min, fixed and immunostained. The endogenous DOC2b and GLUT4 were visualized using anti-DOC2b and anti-GLUT4 antibodies and observed by confocal microscopy.

Figure 2. Insulin augments DOC2b translocation to the plasma membrane (A) 3T3-L1 adipocytes were electroporated with eGFP-DOC2b, and then treated with 100 nM of insulin for the times indicated. Live fluorescent images of the cells were captured by confocal microscopy. Relative fluorescences of the plasma membrane, nucleus and cytosol were each measured in three distinct areas. At least 3 cells were analyzed for each condition. Results are means ± SD from three independent experiments. (B) 3T3-L1 adipocytes expressing eGFP-DOC2b (WT, ΔMID, ΔC2A or ΔC2B) were treated with or without 100 nM of insulin and observed by
confocal microscopy. (C) 3T3-L1 adipocytes were electroporated with eGFP-DOC2b and then pre-treated with 50 μM of BAPTA-AM for 10min, before treatment with insulin and observed under a confocal microscope.
DOC2b regulates GLUT4 vesicle fusion

Figure 3. Insulin promotes Ca\(^{2+}\) dependent interaction between DOC2b and syntaxin4

(A) pLexA containing DOC2b or munc18c and pB42AD containing munc18c, syntaxin4 (synt.4) or empty vectors were co-expressed in the yeast strain of EGY48 (p8op-lacZ), followed by incubation at 30ºC for 16h. Transcriptional activation of LacZ was determined by β-galactosidase assay. (B) GST-tagged DOC2b and syntaxin4 were bacterially expressed. Syntaxin4 protein was cleaved by PreScession protease and further purified with Amicon Ultra filter devices. Both proteins (1μg each) were mixed in Tris buffered saline in the presence of 2mM EDTA or 1mM CaCl\(_2\) and pulled down by Glutathione Sepharose. The precipitates were analyzed by Western blotting with anti-syntaxin4 and anti-GST antibodies. (C) 3T3-L1 adipocytes were serum-starved for 3-4h and pre-treated with or without 50 μM of BAPTA-AM before insulin treatment. The DOC2b-syntaxin4 interaction was determined by immunoprecipitation using anti-DOC2b antibody. The immunoprecipitated proteins were immunoblotted with anti-syntaxin4 and anti-DOC2b antibodies. (D and E) Myc-tagged DOC2b was expressed by adenovirus vector in 3T3-L1 adipocytes. After serum-starvation and pre-treatment with or without 50 μM of BAPTA-AM, prior to insulin treatment, the cells were stimulated with or without 100 nM of insulin for 20 min and then immunoprecipitated with anti-myc or anti-syntaxin4 antibodies. The immunoprecipitated proteins were immunoblotted with anti-myc and anti-syntaxin4 antibodies.
Figure 4. Calcium binding to DOC2b is essential for its translocation and interaction with Syntaxin4

(A) ClustalW sequence alignment of C2A and C2B domains from synaptotagmin1, 2, 3, 5, 9, DOC2a, and DOC2b. Two aspartic acid residues in each C2 domain (shown on a black background) are well conserved putative Ca\(^{2+}\) binding sites. These four aspartic acid residues were mutated into asparagine for CIM-DOC2b (D157N, D163N, D297N, D303N) construction.

(B) Wild type (WT) or calcium-interacting domain mutants (CIM) of eGFP-DOC2b were expressed in 3T3-L1 adipocytes, which were then treated with insulin and observed under a confocal microscope. Figures show representative images of 3 independent experiments.

(C) Purified GST-DOC2b (WT or CIM) and syntaxin4 were mixed in the presence of 2mM EDTA or 1 mM of CaCl\(_2\) and pulled down with Glutathione Sepharose. The precipitates were analyzed by Western blotting with anti-syntaxin4 and anti-GST antibodies.

(D and E) Myc-tagged DOC2b (WT or CIM) was expressed in 3T3-L1 adipocytes. After serum-starvation, the cells were stimulated with or without 100 nM of insulin for 20 min and then immunoprecipitated with anti-myc or anti-syntaxin4 antibodies. The immunoprecipitated proteins were immunoblotted with anti-myc and anti-syntaxin4 antibodies.
Figure 5. DOC2b enhances GLUT4 vesicle fusion in 3T3-L1 adipocytes (A-B) Exofacial myc-tagged GLUT4-eGFP and DsRed2-DOC2b or pcPUR-U6i-shRNA_{DOC2b} Control were co-electroporated into 3T3-L1 adipocytes. The cells were serum starved for 2-4h and either untreated or treated with 100 nM of insulin for 20min. The cells were then fixed and stained with anti-myc antibody and Cy5-labeled secondary antibody without detergents. In the cells electroporated with shRNAs, endogenous DOC2b were visualized with anti-DOC2b antibody, followed by Cy3-labeled secondary antibody. Stained cells were observed by confocal microscopy. This shRNA system decreased DOC2b protein expression to 10-20% of control level. Images are representative of 3 independent experiments. (C-E) Percents of cells with GLUT4-eGFP rims and the cell surface myc-GLUT4 contents (the ratio of myc signal / eGFP signal at plasma membrane rims) in Figure 5A was calculated as described under “Research design and Methods”. The graphs represent values from at least 3-5 independent experiments, and error bars show standard deviation.
DOC2b regulates GLUT4 vesicle fusion

A

GLUT4

Exofacial c-term DOC2b- myc eGFP DsRed2 Merged

control

- +

WT

- +

DOC2b

- +

CIM

- +

Insulin

Bars, 10μm

C

% cells with GLUT4 eGFP rims

- insulin + insulin

cont. wt. CIM cont. DOC2b DOC2b shRNA

D

Cell surface GLUT4

(ratio of myc signal / total eGFP)

- insulin + insulin

Control WT CIM p<0.05

E

Cell surface GLUT4

(ratio of myc signal / total eGFP)

- insulin + insulin

Control DOC2b p<0.05
Figure 6. DOC2b regulates insulin-stimulated glucose uptake in 3T3-L1 adipocytes

3T3-L1 adipocytes were infected with recombinant adenovirus vectors encoding eGFP, myc-tagged DOC2b (WT; CIM) at m.o.i. of 50 (A, B) or adenovirus vectors encoding shRNA specific for DOC2b or non targeting control at m.o.i of 0, 20 and 50 (C, D). After serum-starvation, the cells were treated with or without 100 nM of insulin for 10min. (A, C) 2-deoxy-glucose uptake was measured under each condition. Results are presented as mean ± S.D. of at least five independent experiments. (B, D) The cell lysates were also immunoblotted with anti-myc, anti-GFP, anti-DOC2b, anti-Akt, anti-phosphoserine-Akt and anti-GAPDH antibodies. Immunoblots were representative of at least 3 independent experiments.