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Insulin-stimulated GLUT4 Protein Translocation in Adipocytes Requires the Rab10 Guanine Nucleotide Exchange Factor Dennd4C*[S]

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Hiroyuki Sano[‡], Grantley R. Peck[‡], Arminja N. Kettenbach[§], Scott A. Gerber[§], and Gustav E. Lienhard[‡1]

From the [‡]Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03755 and [§]Department of Genetics and Norris Cotton Cancer Center, Dartmouth Medical School, Lebanon, New Hampshire 03756

Insulin-stimulated translocation of the glucose transporter GLUT4 to the cell surface in fat and muscle cells is the basis for insulin-stimulated glucose transport. Studies in adipocytes strongly support the following molecular mechanism for this process. Insulin-elicited phosphorylation of the GTPase-activating protein TBC1D4 (AS160) suppresses its activity toward Rab10 and thereby leads to an increase in the GTP-bound form of Rab10, which in turn triggers movement of vesicles containing GLUT4 to the plasma membrane and their fusion with the membrane. This process is expected to require the participation of a guanine nucleotide exchange factor (GEF) to generate the GTP-bound form of Rab10, but this GEF has not hitherto been identified. The present study identifies Dennd4C, a recently described GEF for Rab10, as the primary GEF required for GLUT4 translocation. Knockdown of Dennd4C markedly inhibited GLUT4 translocation, and ectopic expression of Dennd4C slightly stimulated it. Dennd4C was found in isolated GLUT4 vesicles. This study thus identifies another key component in the machinery of GLUT4 translocation. Moreover, it provides a potential explanation for the moderate association of a variant in the *Dennd4C* gene with type 2 diabetes.

Insulin rapidly stimulates glucose transport into fat and muscle cells. The basis for this stimulation is the insulin-stimulated movement of vesicles containing the glucose transporter GLUT4 to the plasma membrane and the insulin-stimulated fusion of these vesicles with the plasma membrane (1, 2). This process, which is known as GLUT4 translocation, leads to a marked increase in the amount of GLUT4 in the plasma membrane and a corresponding increase in glucose transport. The links between insulin signaling and GLUT4 translocation are not fully known. Considerable evidence supports the following scenario in adipocytes as one key connection (1–5). Insulin binds to its receptor and thereby initiates a well described signaling cascade that results in the activation of the protein kinase Akt; Akt then phosphorylates TBC1D4 (also known as AS160), a GTPase-activating protein (GAP)[2] for the small G protein Rab10. Phosphorylation by Akt suppresses the GAP activity of TBC1D4 and so leads to an elevation of the GTP-bound form of Rab10 on GLUT4-containing vesicles; the elevation of the Rab10-GTP triggers GLUT4 translocation.

A key component in the elevation of Rab10-GTP that hitherto has been unidentified is the guanine nucleotide exchange factor (GEF) for Rab10. The conversion of the GDP-bound form of a Rab to its GTP-bound form typically requires the action of a specific GEF (6). Recently the protein Dennd4C was discovered to be a GEF for Rab10 (6), and hence, Dennd4C became a candidate for the GEF that converts Rab10-GDP to the Rab10-GTP participating in GLUT4 translocation. In the present study, we provide strong evidence that Dennd4C acts in this role. This study thus identifies another important component of the GLUT4 translocation machinery. Previously it was found that a SNP in a noncoding region of the *Dennd4C* gene shows a moderate association with the disease type 2 diabetes (7). The participation of Dennd4C in GLUT4 translocation points to an explanation for this association.

EXPERIMENTAL PROCEDURES

Reagents—siRNAs were Silencer Select ones purchased from Ambion, Inc. The sequences of the siRNAs are given in the supplemental material. The lentiviral plasmid for HA-GLUT4-GFP (8) and the plasmid for expression of FLAG-tagged Rab10 (9) were generous gifts from Drs. Cynthia Mastick and Mitsunori Fukuda, respectively. The cDNA encoding mouse Dennd4C (BC146602 encoding NP_908976) was purchased from Thermo Fisher Scientific. It was amplified by PCR without the codon for the start Met and then ligated into the NotI/SalI sites of the animal cell expression vector p3XFLAG-CMV-7.1 from Sigma-Aldrich. This yielded a plasmid that encodes Dennd4C with a 3XFLAG tag at its N terminus. Point mutations in the Dennd4C plasmid were introduced by use of the QuikChange II XL site-directed mutagenesis kit from Stratagene. Each mutation was verified by DNA sequencing of the entire Dennd4C sequence. The following antibodies were purchased (source, antigen, catalogue number): Sigma-Aldrich, Dennd4C, HPA015096; Cell Signaling Technology, phospho-Ser-473 Akt, 4051, and phospho-Akt substrate, 9614; Santa Cruz Biotechnology, Akt, 8312. The antibody against TBC1D4 was the one in Ref. 10. Antibodies against rabin8 (11) and the cytoplasmic domain of the insulin-regulated aminopeptidase (IRAP) (12) were generous gifts from Drs. Johan Peranen and Susanna Keller, respectively.

Rab10 GEF Assay—This assay was carried out as described in Ref. 5. In this assay, HEK293 cells were transfected with the

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[1] To whom correspondence should be addressed: Dept. of Biochemistry, Dartmouth Medical School, North College St., Hanover, NH 03755. Tel.: 603-650-1627; Fax: 603-650-1128; E-mail: gustav.e.lienhard@dartmouth.edu.

[2] The abbreviations used are: GAP, GTPase-activating protein; GEF, guanine nucleotide exchange protein; IRAP, insulin-regulated aminopeptidase.

plasmids for FLAG-tagged Rab10 and Dennd4C, labeled with $^{32}P_i$, and lysed with nonionic detergent. The Rab10 was immunoprecipitated from the lysate on anti-FLAG beads. The bound ^{32}P-labeled GTP and GDP were separated by TLC, and the radioactivity in each was measured by phosphorimaging.

Cell Culture and Assay for Cell Surface GLUT4—All experiments were performed with 3T3-L1 adipocytes that were carried as fibroblasts and differentiated into adipocytes. The relative amount of GLUT4 at the cell surface was measured through the use of the reporter form of GLUT4, HA-GLUT4-GFP, and flow cytometry, as described in detail in Ref. 13. In this assay, adipocytes expressed HA-GLUT4-GFP, which contains the HA tag in the first extracellular domain of GLUT4 and the GFP tag in the cytoplasmic C-terminal domain of GLUT4. Serum-deprived 3T3-L1 adipocytes were treated with 160 nM insulin for 30 min or left unstimulated and then chilled. The HA tag on the exterior of the cell surface was labeled with anti-HA and then with allophycocyanin-conjugated secondary antibody. The labeled adipocytes were analyzed by flow cytometry. The ratio of the allophycocyanin fluorescence to the GFP fluorescence for each cell, after correction for nonspecific binding of the secondary antibody, provides a measure of the relative amount of HA-GLUT4-GFP at the cell surface normalized for its expression level. The value for each assay was the average corrected ratio from at least 200 cells.

Two different protocols were used in the analysis of the cell surface GLUT4. For determination of the effect of an siRNA knockdown, 3T3-L1 fibroblasts were infected with sufficient lentivirus for HA-GLUT4-GFP that ~90% of the cells expressed HA-GLUT4-GFP (8). The fibroblasts were then differentiated into adipocytes. On day 4 of differentiation, cells from a 10-cm plate were electroporated with 5 nmol of siRNA in a 0.5-ml volume and then replated, as described (14). This procedure introduces siRNA into almost all the cells (14). After 72 h, the cells were analyzed for HA-GLUT4-GFP at the cell surface. Experiments in which the effect of control siRNA (Silencer Select negative control 1 from Ambion) was compared with that of electroporation without siRNA showed that the control siRNA had no effect on cell surface GLUT4 in the basal or insulin state. Consequently, electroporation without siRNA was used as the control condition in the knockdown experiments. For determination of the effect of ectopic expression of Dennd4C on GLUT4 translocation, cells at day 4 of differentiation from a 10-cm plate were electroporated with a combination of 75 μg of the HA-GLUT4-GFP plasmid and 100 μg of the 3XFLAG-Dennd4C plasmid in a 0.5-ml volume, as described previously (13). The cells were analyzed for cell surface GLUT4 after 24 h. In this procedure, 10–15% of the cells expressed HA-GLUT4-GFP. To assess whether cells expressing HA-GLUT4-GFP also expressed Dennd4C, we fixed cells with formaldehyde, permeabilized them with saponin, labeled them with anti-FLAG and Cy3-conjugated secondary antibody, and examined the cells for Cy3 and GFP by fluorescence microscopy, as described previously (13). In this experiment, 12% of the cells expressed Dennd4C, 11% expressed HA-GLUT4-GFP, and 90% of the cells expressing HA-GLUT4-GFP also expressed Dennd4C.

Preparation of Subcellular Fractions and GLUT4 Vesicles—Subcellular fractions from 3T3-L1 adipocytes were prepared by differential centrifugations, as described previously (5). Vesicles containing GLUT4 were isolated from cells in the basal state and ones treated with 160 nM insulin for 30 min, as described previously (5). In this procedure, the supernatant containing low density microsomes and cytosol was prepared from homogenized cells and then adsorbed onto protein A beads coupled to either anti-GLUT4 or, for the control, irrelevant rabbit immunoglobulin. The vesicle proteins were then released from the washed beads with nonionic detergent.

SDS Samples and Immunoblotting—In the experiments for analysis of cell surface, GLUT4 cells were plated in 35-mm wells. In each experiment, SDS samples were prepared by washing some 35-mm wells with PBS and dissolving the cells in 0.3 ml of SDS sample buffer with 10 mM dithiothreitol and a mixture of protease inhibitors (13). The concentration of protein in these SDS samples was measured by the precipitating Lowry assay (13). Immunoblotting was performed as in Ref. 13.

RESULTS

GEF Activity of Dennd4C for Rab10—Previously Yoshimura *et al.* (6) reported that recombinant Dennd4C acts as a GEF for recombinant Rab10 in an *in vitro* assay. To confirm that Dennd4C functions as a GEF for Rab10, we expressed Rab10 with and without Dennd4C in HEK293 cells, labeled the cellular nucleotides with $^{32}P_i$, isolated the Rab10 by immunoprecipitation, and determined the percentage of bound nucleotide that was GTP. The percentage of Rab10 in the GTP form was 7.0 ± 0.6 in the absence of ectopic Dennd4C and 13.1 ± 1.0 in its presence (mean ± S.E. from three experiments, supplemental Fig. S1). Thus, expression of Dennd4C with Rab10 led to a 1.9-fold increase in the percentage of Rab10 in the GTP-bound form. This finding supports the study demonstrating that Dennd4C is a GEF for Rab10 (6).

Inhibition of GLUT4 Translocation by Dennd4C Knockdown—To assess whether Dennd4C participates in the insulin-stimulated translocation of GLUT4, we measured the effect of Dennd4C knockdown on the relative amount of GLUT4 at the cell surface in unstimulated and insulin-stimulated 3T3-L1 adipocytes (Fig. 1*A*). The control showed the expected large increase in the amount of GLUT4 at the cell surface in response to insulin. Knockdown of Dennd4C had no significant effect on the low amount of GLUT4 at the cell surface in the basal state. In the insulin-stimulated state, knockdown of Dennd4C markedly lowered the amount of cell surface GLUT4 to 39% of that in the control. Immunoblotting showed that knockdown of Dennd4C reduced its level to less than 25% of the control level (Fig. 1*B*). To be certain of the specificity of the siRNA for Dennd4C, we also examined the effect of two other siRNAs for Dennd4C in a single experiment. These reduced the level of cell surface GLUT4 in the insulin-stimulated state to 43 and 54% of the value for the control (siRNAs s203347 and s203348, respectively), and immunoblotting showed effective knockdown of Dennd4C by these additional siRNAs (data not shown).

Rabin8 is a GEF for Rab8 (11). In addition to Rab10, Rab8 is also a substrate for the insulin-regulated GAP TBC1D4 (5). Rab knockdown experiments show that in adipocytes, Rab10 partic-

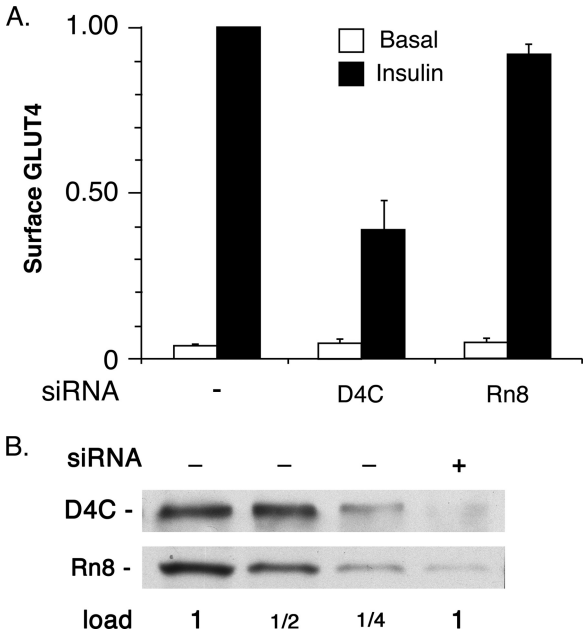

FIGURE 1. **Effect of Dennd4C knockdown on cell surface GLUT4.** *A*, 3T3-L1 adipocytes were treated with either no siRNA or siRNAs against Dennd4C (*D4C*) or Rabin8 (*Rn8*), and then GLUT4 at the cell surface in the basal and insulin-stimulated state was measured. Each experiment included the control without siRNA, and the amount of GLUT4 under each condition was normalized to the value for the control in the insulin-stimulated state, which was set at 100. The values are the mean ± S.E. for six separate experiments for the control, five for the Dennd4C siRNA, and two for the Rabin8 siRNA. *B*, samples of cells from the experiments in *panel A* were immunoblotted for Dennd4C and Rabin8. The 1× load was 50 μg of protein.

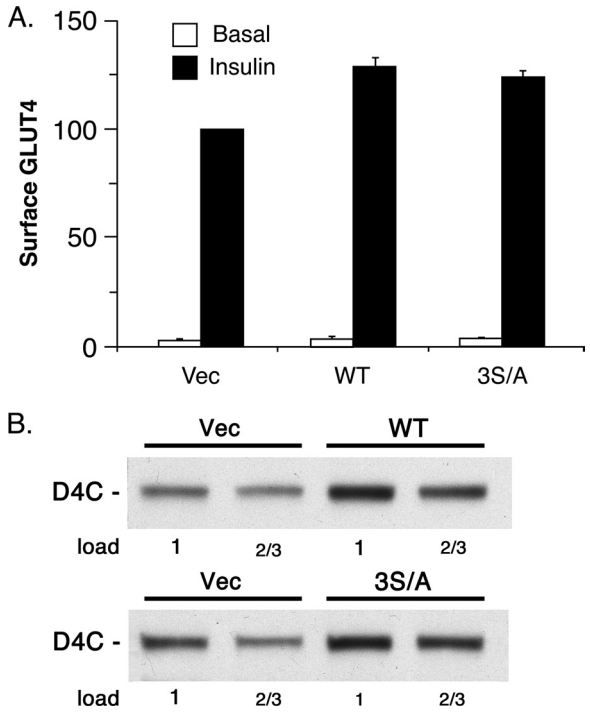

FIGURE 2. **Effect of Dennd4C overexpression on cell surface GLUT4.** *A*, 3T3-L1 adipocytes were transfected with the plasmids for HA-GLUT4-GFP and vector (*Vec*), wild-type Dennd4C (*WT*), or the Dennd4C S1043A/S1096A/S1321A mutant (*3S/A*), and cell surface GLUT4 was measured. The values are the mean ± S.E. from two separate experiments. *B*, samples from the experiments in *panel A* were immunoblotted for Dennd4C (*D4C*). The 1× load was 30 μg of protein. A replicate with the second set of samples gave similar results. Based upon quantification of the immunoblots, the increases in the total amount of Dennd4C averaged 1.9- and 2.2-fold upon transfection with wild-type and S1043A/S1096A/S1321A Dennd4C, respectively.

ipates in GLUT4 translocation, whereas Rab8 does not (4, 5). However, in cultured muscle cells, similar knockdown experiments indicate that the reverse is true (15). Adipocytes express both Rab8 (5) and Rabin8 (Fig. 1*B*). Consequently, we also examined the effect of Rabin8 knockdown on cell surface GLUT4. Knockdown of Rabin8 had no effect on cell surface GLUT4 in the basal state and reduced cell surface GLUT4 in the insulin state by only 8% (Fig. 1*A*). The efficiency of the Rabin8 knockdown was greater than 75% as assessed by immunoblotting (Fig. 1*B*). This result is further evidence that in adipocytes, Rab8 does not participate significantly in GLUT4 translocation.

The molecular mechanism by which Dennd4C knockdown is inhibitory to GLUT4 translocation is most likely through a reduction in the amount of Rab10-GTP that results in the inhibition of the trafficking of GLUT4 vesicles. However, an alternative explanation is that Dennd4C knockdown in some way inhibits insulin signaling to GLUT4 translocation. To test this possibility, we examined by immunoblotting whether Dennd4C knockdown altered the amounts of Akt and TBC1D4 or the insulin-stimulated phosphorylation of these proteins. The knockdown had no effect on either (supplemental Fig. S2).

Stimulation of GLUT4 Translocation by Ectopic Expression of Dennd4C—Ectopic expression of Dennd4C resulted in a 29% increase in the amount of GLUT4 at the cell surface in the insulin-stimulated state without any effect on the amount in the basal state (Fig. 2*A*). Based upon immunoblotting for Dennd4C, ectopic expression led to a 1.9-fold increase in total Dennd4C (Fig. 2*B*). Because only 12% of the cells expressed the FLAG-tagged Dennd4C (see "Experimental Procedures"), the level of Dennd4C in the transfected cells was 9-fold that of endogenous Dennd4C.

Phosphorylation of Dennd4C—We have carried out a phosphoproteomic analysis of plasma membranes obtained from unstimulated and insulin-stimulated 3T3-L1 adipocytes.[3] The insulin-treated adipocytes were labeled with isotopically heavy Arg and Lys. This enabled the stable isotope labeling with amino acids in cell culture (SILAC) method of quantification (16), which yielded the relative amounts of each phosphopeptide in the basal and insulin-stimulated states. In this study, we detected three phosphopeptides from Dennd4C. The sites of phosphorylation were on Ser-1043, Ser-1096, and Ser-1321, and the amounts of the phosphopeptides increased by 1.49-, 1.25-, and 1.65-fold, respectively, in response to insulin treatment. Each of these sites is in the motif R*XX*(S/T)Φ, where *X* is any amino acid and Φ is a hydrophobic amino acid. This motif is a partial motif for the protein kinase Akt, which prefers R*X*R*XX*R(S/T)Φ (17). Because insulin treatment activates Akt, activated Akt may be the kinase that phosphorylates Dennd4C.

A priori, it seemed possible that the increased phosphorylation of Dennd4C on these sites might stimulate its GEF activity and thereby GLUT4 translocation. To test this possibility, we prepared the nonphosphorylatable mutant of Dennd4C in which all three phosphorylation sites were changed to Ala

[3] H. Sano, A. N. Kettenbach, S. A. Gerber, and G. E. Lienhard, unpublished results.

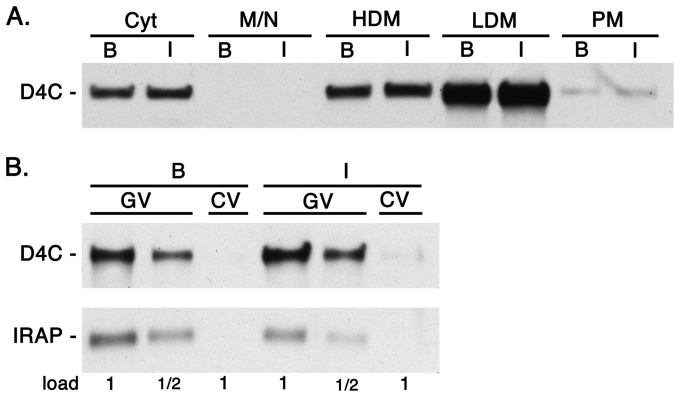

FIGURE 3. **Dennd4C in GLUT4 vesicles.** *A*, Immunoblot of subcellular fractions from basal (*B*) and insulin-treated (*I*) 3T3-L1 adipocytes for Dennd4C (*D4C*). Each lane contained 25 μg protein. *Cyt*, cytosol; *M/N*, mitochondria/nuclei; *HDM*, high density microsomes; *LDM*, low density microsomes; *PM*, plasma membrane. The relative mg amounts of protein in each fraction are: cytosol, 100; mitochondria/nuclei, 55; high density microsomes, 9.5; low density microsomes, 13; plasma membrane, 3.8 (5). *B*, immunoblot of GLUT4 vesicles from basal and insulin-treated adipocytes for Dennd4C and IRAP. Vesicles were adsorbed from the cytosol/low density microsome fraction with either anti-GLUT4 (designated *GV*) or control rabbit IgG (designated *CV*). The 1× loads for Dennd4C and IRAP were the vesicles derived from 40% and 0.2% of a 10-cm plate, respectively. This complete experiment, starting with the isolation of the GLUT4 vesicles from cells, was done three times, and each showed the same result.

(S1043A/S1096A/S1321A mutant) and used it in the assay described above. If phosphorylation enhanced the GEF activity, we would expect that this mutant of Dennd4C would be inhibitory to GLUT4 translocation. In fact, the S1043A/S1096A/S1321A mutant was as stimulatory as the wild-type Dennd4C (Fig. 2*A*). Immunoblotting showed that it was overexpressed to the same extent as wild-type Dennd4C (Fig. 2*B*). These results indicate that the insulin-stimulated phosphorylation of Dennd4C does not regulate its GEF activity.

Dennd4C in GLUT4 Vesicles—Immunoblotting of subcellular fractions from 3T3-L1 adipocytes revealed that Dennd4C was located primarily in the cytosol, high density microsomes, and low density microsomes and that insulin treatment caused no redistribution of the protein (Fig. 3*A*). The low density microsomes contain the exocytic GLUT4 vesicles as well as other small vesicles from the secretory and endosomal system. To determine whether the Dennd4C is present in GLUT4 vesicles, we isolated these vesicles from unstimulated and insulin-treated adipocytes and immunoblotted them for Dennd4C and for the IRAP, a well established marker of the vesicles (18). Dennd4C was present in the GLUT4 vesicles (Fig. 3*B*). The amount of Dennd4C in the vesicles did not change significantly in response to insulin. In contrast, the amount of IRAP in the vesicles decreased as the result of its translocation to the plasma membrane. In two previous proteomic analyses of GLUT4 vesicles, Dennd4C was not detected (19, 20). However, because each analysis found proteins not detected in the other, neither was entirely comprehensive.

DISCUSSION

Insulin-stimulated GLUT4 translocation requires the participation of Rab10 in its GTP-bound form (4, 5). Rabs oscillate between the inactive GDP-bound form and the active GTP-bound form (6). It has been known for several years that in adipocytes, TBC1D4 is the GAP that converts Rab10-GTP to Rab10-GDP in the regulation of GLUT4 translocation (1–5). However, until now, the identity of a Rab10 GEF that converts Rab10-GDP to Rab10-GTP in this process has been unknown. This study provides strong evidence that Dennd4C is the primary Rab10 GEF that generates the Rab10-GTP required for GLUT4 translocation. First, knockdown of Dennd4C, which should reduce the level of Rab10-GTP, markedly inhibited GLUT4 translocation. Second, overexpression of Dennd4C slightly increased GLUT4 translocation. This result indicates that although the GAP activity of TBC1D4 is suppressed in the presence of insulin, the balance between Rab10 GAP and GEF activities is such that the amount of Rab10-GTP is normally slightly limiting for GLUT translocation. Lastly, GLUT4 vesicles contained Dennd4C, and hence, Dennd4C is in a location where it can act upon Rab10, which is also present in the GLUT4 vesicles (5).

Previously we found that only 10% of the total Rab10 in 3T3-L1 adipocytes was in the GTP-bound form and that the percentage in this form did not detectably increase in response to insulin treatment (5). The amount of Rab10 located in GLUT4 vesicles was only 5% of the total amount in the cell (5). One possible explanation for the fact that insulin treatment did not increase the GTP-bound form of Rab10 is that the stimulatory effect of insulin is limited to the small percentage of Rab10 in GLUT4 vesicles (5). Because of this situation, as well as the low transfection efficiency with the plasmid for Dennd4C, we did not directly examine the effects of Dennd4C knockdown and overexpression on the level of Rab10-GTP in adipocytes.

Our finding in a separate study that insulin treatment slightly increased the phosphorylation of Dennd4C on three putative Akt sites suggested the possibility that this phosphorylation might increase its Rab10 GEF activity. However, because the ectopic nonphosphorylatable mutant of Dennd4C affected cell surface GLUT4 in the same way as ectopic wild-type Dennd4C, this regulation is unlikely to occur. In this regard, it should be noted that our analysis of phosphorylation did not yield values for the stoichiometry of phosphorylation; it is possible that even in the presence of insulin, the stoichiometry of phosphorylation on these sites is low.

The mouse Dennd4C used in this study (NP_ 908976) is a protein of 1906 amino acids. In addition, shorter and longer Dennd4C splice variants of 1615 (NP_001074483) and 1955 (CAM27931) amino acids may occur. The Denn domain, which is the Rab GEF domain (6), occupies ~500 amino acids from about positions 150–650 in all three forms (6). The remainder of the protein does not have known domains. In the future, it will be of interest to determine the functions of these other regions, as well as to elucidate the basis for the binding of Dennd4C to GLUT4 vesicles.

Dennd4A (NP_001156389) and Dennd4B (NP_958809) are two paralogs of Dennd4C. They are similar to Dennd4C in the N-terminal region through the Denn domain but are less similar in the C-terminal half. Dennd4A and -B have also been reported to be specific GEFs for Rab10 *in vitro* (6). Hence, it is possible that one or both of these other Dennd4s also participate in GLUT4 translocation. We have conducted initial experiments with two different siRNAs against both Dennd4A and

Dennd4B (listed in the supplemental material), as described under "Experimental Procedures" and in the legend for Fig. 1. In the case of Dennd4A, in single experiments, one siRNA reduced the amount of GLUT4 at the cell surface in the insulin state by 24%, and the second reduced it by 23%. In the case of Dennd4B, one siRNA reduced cell surface GLUT4 in the insulin state by 31 ± 4% (four experiments), and the second siRNA did so by 20% (one experiment). These results indicate that Dennd4A and -B may also each participate slightly in GLUT4 translocation, and their participation may account for the incomplete inhibition by the knockdown of Dennd4C. However, the 61% reduction in cell surface GLUT4 in the insulin state found with the knockdown of Dennd4C shows that Dennd4C is the primary Rab10 GEF that acts in GLUT4 translocation. Specific antibodies for Dennd4A and -B are not yet available. When they are, it will be possible to examine the roles of these two Dennds in a similar way to that done here for Dennd4C.

A large human genome-wide association study found that an SNP (rs17818670) in a noncoding region of *Dennd4C* showed moderate association with type 2 diabetes (see Ref. 7, additional file 2). Our study suggests that a variation in the *Dennd4C* gene that leads to decreased expression of Dennd4C will reduce GLUT4 translocation in adipocytes. A reduction in the insulin stimulation of glucose transport in adipocytes could contribute to the development of diabetes. In mice, fat-specific down-regulation of GLUT4 is known to cause insulin resistance (21).

A remaining challenge in this area is to identify the effectors for Rab10-GTP that function in GLUT4 translocation. Rab10-GTP is known to bind to myosin V, a microfilament-based motor, and to the exocyst, a complex that docks exocytic vesicles to the plasma membrane (22, 23). Previous studies have provided evidence that both myosin V and the exocyst complex are required for GLUT4 translocation (24, 25). Thus, the elevation of Rab10-GTP may allow GLUT4 vesicles to move to the plasma membrane via myosin V movement along microfilaments and to dock there via the exocyst.

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