TBC1D1 Regulates Insulin- and Contraction-Induced Glucose Transport in Mouse Skeletal Muscle

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OBJECTIVE—TBC1D1 is a member of the TBC1 Rab-GTPase family of proteins and is highly expressed in skeletal muscle. Insulin and contraction increase TBC1D1 phosphorylation on phospho-Akt substrate motifs (PASs), but the function of TBC1D1 in muscle is not known. Genetic linkage analyses show a TBC1D1 R125W missense variant confers risk for severe obesity in humans. The objective of this study was to determine whether TBC1D1 regulates glucose transport in skeletal muscle.

RESEARCH DESIGN AND METHODS—In vivo gene injection and electroporation were used to overexpress wild-type and several mutant TBC1D1 proteins in mouse tibialis anterior muscles, and glucose transport was measured in vivo.

RESULTS—Expression of the obesity-associated R125W mutant significantly decreased insulin-stimulated glucose transport in the absence of changes in TBC1D1 PAS phosphorylation. Simultaneous expression of an inactive Rab-GTPase (GAP) domain of TBC1D1 in the R125W mutant reversed this decrease in glucose transport caused by the R125W mutant. Surprisingly, expression of TBC1D1 mutated to Ala on four conserved Akt and/or AMP-activated protein kinase predicted phosphorylation sites (4P) had no effect on insulin-stimulated glucose transport. In contrast, expression of the TBC1D1 4P mutant decreased contraction-stimulated glucose transport, an effect prevented by concomitant disruption of TBC1D1 Rab-GAP activity. There was no effect of the R125W mutation on contraction-stimulated glucose transport.

CONCLUSIONS—TBC1D1 regulates both insulin- and contraction-stimulated glucose transport, and this occurs via distinct mechanisms. The R125W mutation of TBC1D1 impairs skeletal muscle glucose transport, which could be a mechanism for the obesity associated with this mutation. ***Diabetes*** **59:1358–1365, 2010**

Insulin and exercise are the most physiologically relevant stimulators of glucose transport in skeletal muscle, and in patients with type 2 diabetes, insulin- but not exercise-stimulated glucose transport can be severely impaired (1–4). GLUT4 translocation from an intracellular location to the sarcolemma and transverse tubules is the major mechanism through which both insulin and exercise increase glucose transport in muscle (1,2). Although it is well established that insulin works through a phosphatidylinositol 3 kinase (PI 3-kinase)–dependent mechanism and the exercise effect may involve multiple molecules including the AMP-activated protein kinase (AMPK) family of protein kinases, an important goal in this field has been to elucidate the mechanisms that connect the proximal insulin and exercise signals to GLUT4 translocation. Recent studies have identified two molecules, the Akt substrate of 160 kDa (AS160/TBC1D4) and its paralog, TBC1D1, as potential molecular links among multiple signaling pathways converging on GLUT4 translocation in skeletal muscle (5–12).

AS160 was first shown to modulate GLUT4 trafficking in insulin-sensitive 3T3-L1 adipocytes (13). A striking structural feature of AS160 is that the molecule harbors a Rab-GTPase (GAP) domain and its activity controls Rab-GTP loading and regulation of Rab function (11). AS160 contains at least six amino acids that can be phosphorylated in response to insulin by Akt (14), and these sites can be detected in aggregate with an antibody directed against a phospho-Akt substrate motif (PAS). Insulin, exercise, and the AMPK activator 5′-aminoimidazole-4-carboxymide-1-β-D-ribofuranoside (AICAR) all cause phosphorylation of AS160 on PAS sites in skeletal muscle (5,7,10), and there is good evidence that phosphorylation of these sites inhibits AS160 activity, leading to trafficking of GLUT4 vesicles to the cell surface and glucose transport (8,15). However, ablation of AS160 phosphorylation at PAS sites only partially inhibits insulin- and contraction-stimulated glucose transport (8,15). Furthermore, a preliminary report suggests that whole-body knockout of AS160 does not result in a significant increase in glucose transport in muscle (16). Taken together, these findings suggest that there may be one or more additional Rab-GAP proteins expressed in skeletal muscle that also function to regulate glucose transport.

TBC1D1 (tre-2/USP6, BUB2, cdc16 domain family member 1) is a member of the TBC1 Rab-GAP family of proteins. TBC1D1 and AS160 are 47% identical overall and have several comparable structural features. Both proteins contain two full or partial NH_2-terminal phosphotyrosine binding domains, a splice exon, and a putative calmodulin-binding domain. The COOH-terminal Rab-GAP domains of TBC1D1 and AS160 are 79% identical and exhibit similar Rab specificities in vitro (6). Both proteins are detected at a molecular weight of ~150–160 kDa upon SDS-PAGE, and both can be detected using the PAS antibody. Although there are several similarities between TBC1D1 and AS160, there are also clear differences in tissue distribution and protein phosphorylation. For example, whereas AS160 is expressed in multiple tissues at similar levels, TBC1D1 is several-fold higher in skeletal muscle compared with other tissues (12). Another key difference between TBC1D1 and AS160 relates to the phosphorylation status of the proteins. Insulin increases the phosphor-

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ylation of five of six Akt substrate motifs in AS160, whereas only one of these insulin-stimulated sites is conserved in TBC1D1 (6,12,13).

A recent report has shown that a mouse genetic model resulting in a truncation mutation of TBC1D1 that includes the Rab-GAP domain of the protein, as well as knockdown of TBC1D1 in C2C12 muscle cells, lead to increased fatty acid oxidation (17). Other studies have identified a genetic variation in TBC1D1 that confers risk for severe obesity in Caucasian U.S. and French pedigrees (18,19). This R125W missense variant occurs in a phosphotyrosine-binding domain of TBC1D1, but how this mutation leads to metabolic dysfunction and obesity is not known.

Using mass spectrometry, we have identified novel phosphorylation sites on TBC1D1 (12), and we and other laboratories have shown that one or more of these sites can be regulated by insulin, contraction, and/or AICAR in skeletal muscle (12,14,20,21). The function of these phosphorylation sites is not known. However, given that these stimuli are potent regulators of glucose transport, that TBC1D1 is highly expressed in skeletal muscle, and that R125W mutation of TBC1D1 is associated with obesity, we hypothesized TBC1D1 regulates glucose transport in skeletal muscle. We found distinct mutations of TBC1D1 differentially decrease insulin- and contraction-stimulated glucose transport in skeletal muscle, and this occurs in a Rab-GAP domain–dependent manner. Our findings demonstrate that TBC1D1 functions as a Rab-GAP protein, regulating both insulin- and contraction-stimulated glucose transport in skeletal muscle.

RESEARCH DESIGN AND METHODS

Animals. Female ICR mice 8–9 weeks old were purchased from Taconic (Hudson, NY) or Charles River Laboratories (Wilmington, MA). All of the mice were housed with a 12-h light/12-h dark cycle and fed standard laboratory chow and water ad libitum. The mice were fasted overnight (9:00 P.M. to 8:00 A.M.) prior to study. Protocols for animal use were reviewed and approved by the Institutional Animal Care and Use Committee of the Joslin Diabetes Center and were in accordance with National Institutes of Health guidelines.

Plasmid cDNA constructs. A mouse wild-type TBC1D1 cDNA construct was generated in our laboratory. Briefly, total RNA was isolated from mouse tibialis anterior muscle using the RNeasy Fibrous Tissue Mini Kit (74704; Qiagen). RNA was reverse transcribed to cDNA using the AccuScript High Fidelity 1st Strand cDNA Synthesis Kit (200820; Stratagene). The long form of TBC1D1 (AK122445) was amplified by PCR with the Phusion Hot Start High Fidelity Polymerase (New England Biolabs) using gene-specific primers: forward: 5′-ggaaaatagtagcacgttttcctg-3′; reverse: 5′-ctctgcctctgacttccatct ctcttg-3′. The PCR product was gel purified with the QIAquick Gel Extraction Kit (28706; Qiagen) and ligated to the Blunt II Topo cloning vector (K280020; Invitrogen). The ligation product was transformed into Top10 chemically competent *Escherichia coli* (C404003; Invitrogen). Transformants were plated and screened by selection with kanamycin. Single clones were cultured and plasmid DNA was extracted with the QIAprep Spin Miniprep Kit (27106; Qiagen) and sequenced in both the forward and reverse directions (Brigham and Women's Sequencing Center, Harvard Medical School). A hemagglutinin tag was added to the NH_2-terminus and *Xho*I sites to both termini of TBC1D1 by PCR cloning (Phusion) using the following composite primers: forward: 5′-tttctcgaggccaccatgtacccgtacgacgtgcccgactacgcg-3′; reverse: 5′-aaactcgag ctctgcctctgacttccatctctcttg-3′. The PCR product was gel purified, ligated to the Blunt II Topo cloning vector, and transformed into Top10 *E. coli*. True clones were identified by plating, kanamycin selection, and sequencing. The hemagglutinin-TBC1D1 open reading frame was cut from the Blunt II Topo vector with *Xho*I and ligated into the *Xho*I site of the PCAGGS mammalian expression vector. True clones were identified by ampicillin selection of transformed *E. coli* (Top10) and sequencing purified plasmid. This PCAGGS expression vector drives a target gene under the cytomegalovirus immediate-early enhancer chicken β-actin hybrid (CAG) promoter and has been demonstrated to have high activity in skeletal muscle (8). Five TBC1D1 mutant constructs were generated: *1*) TBC1D1 mutated to Ala at four phosphorylation motifs (Ser^{231}, Thr^{499}, Thr^{590}, and Ser^{621}), rendering these sites incapable of being phosphorylated (4P); *2*) TBC1D1 mutated to Trp at Arg^{125} (R125W); *3*) TBC1D1 mutated to Lys at Arg^{941} within its Rab-GAP domain to eliminate TBC1D1 GAP activity (R/K); *4*) TBC1D1 double mutant containing both the 4P and R/K mutations; and *5*) TBC1D1 double mutant containing both the R125W and R/K mutations. TBC1D1 cDNA constructs were sequenced to confirm accuracy, using the high-throughput DNA sequencing service at Brigham and Women's Hospital. Plasmid DNA was amplified in *E. coli* Top10 cells (Invitrogen), extracted using an endotoxin-free Plasmid Mega Kit (Qiagen), and suspended in saline at 4 μg/μl. TBC1D1 plasmids (100 μg) were injected into mouse tibialis anterior muscles using our protocol (22), modified to that originally described by Aihara and Miyazaki (23), and mice were studied 7 days later.

Contraction, glucose, and insulin treatment in the study of PAS phosphorylation. Mice were anesthetized with intraperitoneal administration of pentobarbital sodium (90 mg/kg of body wt). Peroneal nerves from both legs were surgically exposed for electrode placement. One leg was left unstimulated (basal/sham control) and the other leg was subjected to electrical stimulation using a Grass S88 pulse generator for 15 min of contractions (train rate 1/s; train duration 500 ms; pulse rate 100 Hz; duration 0.1 ms at 2–7 V), as we have previously described (5). To study insulin-activated PAS signaling, we injected mice with either a bolus of glucose that resulted in a physiological increase in plasma insulin concentrations (8) or an insulin bolus. For the glucose treatment, mice were anesthetized and administered a 20% glucose bolus (1.0 g of glucose/kg of body wt). For the insulin treatment, mice were anesthetized and injected with insulin (10 mU/g). Immediately after contraction, 15 min after glucose administration, or 6 min after insulin stimulation, tibialis anterior muscles were dissected and frozen in liquid nitrogen.

Protein extraction, immunoprecipitation, and immunoblotting. Frozen muscle tissue was homogenized with a Polytron (Brinkman Instruments) in lysis buffer, as described previously (12). Homogenates were centrifuged at 14,000*g* for 10 min and supernatants were collected. Protein concentrations were determined by the Bradford assay. In some experiments, lysates were subjected to immunoprecipitation to remove AS160 before separation by SDS-PAGE. AS160 was immunoprecipitated with a Rabbit polyclonal antibody (07–741; Millipore, Billerica, MA). Bead-antibody-protein complexes were washed 1× with lysis buffer, 2× with lysis buffer + 500 mmol/l NaCl, and 1× with lysis buffer. Pellets were aspirated and spotted with 5–10 μl of 1 μg/μl BSA before elution. Proteins were eluted from protein G beads by adding Laemmli buffer and heated for 5 min at 95°C. For immunoblotting, lysates (50 μg protein) were separated by SDS-PAGE for Western blot analysis. Antibody-bound proteins were visualized, using enhanced chemiluminescence (Amersham Biosciences). The protein bands were scanned by ImageScanner (Amersham Biosciences) and quantitated by densitometry (Fluorchem 2.0; Alpha Innotech, San Leandro, CA).

Antibodies. Protein expression was assessed with antibodies to AS160 (07–741; Millipore), GLUT4 and GLUT1 (AB1346 and CBL242, respectively; Chemicon International), AMPK α2 (07–363; Upstate Biotechnology, Inc.), and Akt1/2, hexokinase II, and anti–α-tubulin (SC1619, SC6521, and SC5286, respectively; Santa Cruz Biotechnology). Serum-purified anti-TBC1D1 antibody was generated by Cell Signaling Technology by immunizing rabbits (12). Phosphorylation-specific antibodies included Akt Thr^{308} (9275), Akt Ser^{473} (4058), AMPK Thr^{172} (2535), and PAS (9611) from Cell Signaling Technology. Horseradish peroxidase–conjugated anti-rabbit, anti-mouse, and anti-goat antibodies (Amersham Biosciences) were used to bind and detect all primary antibodies.

In vivo skeletal muscle and glucose transport. Glucose transport was measured, as we previously described (5). Briefly, mice were fasted overnight and anesthetized with intraperitoneal administration of pentobarbital sodium (90 mg/kg of body wt). To examine contraction-stimulated glucose transport, muscle was contracted, as described above. To study insulin-stimulated glucose transport, mice were administered a saline or 20% glucose bolus (1.0 g of glucose/kg of body wt), along with [^{3}H]-2-deoxyglucose through the retro-orbital sinus. Our previous studies have shown that this dosage stimulates a physiologic insulin response (~75 μU/ml) without inducing significant hypoglycemia (8). Baseline blood samples were collected from the tail vein prior to retro-orbital injection. The blood samples were collected from the tail vein at 5, 10, 15, 25, 35, and 45 min after injection to determine blood glucose and [^{3}H]-2-deoxyglucose–specific activity. Subsequently, the animals were killed, and tibialis anterior muscles were removed and frozen in liquid nitrogen. Muscles were subsequently lysed and glucose transport ([^{3}H]-2-deoxyglucose-P) was determined via a precipitation protocol adapted from Ferré et al. (24).

Statistical analysis. Data are expressed as the means ± SE. Means were compared by one-way or two-way ANOVA. When ANOVA revealed significant differences, Tukey post hoc test for multiple comparisons was performed. *P* values less than 0.05 were considered statistically significant.

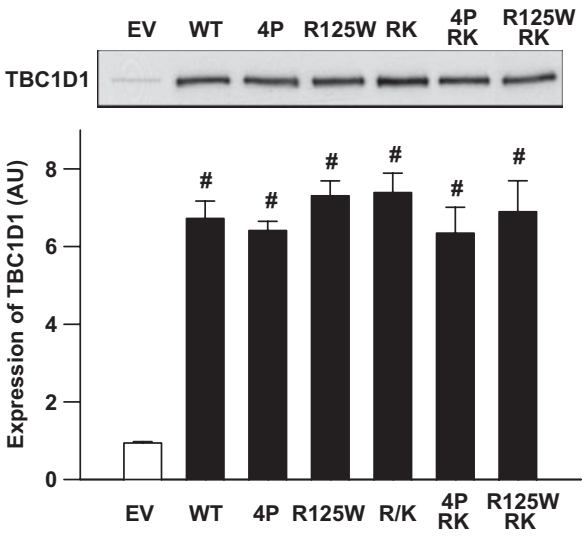

FIG. 1. TBC1D1 expression was significantly increased in mouse tibialis anterior muscles in response to in vivo cDNA injection and electroporation. Empty pCAGGS vector (EV) and TBC1D1 cDNA constructs (wild-type [WT] and 4P, R125W, R/K, 4P/RK, and R125W/RK mutants) were injected into the tibialis anterior muscles of anesthetized mice, followed by in vivo electroporation. The animals were allowed to recover, and protein expression was assessed 1 week after injection. Muscle proteins were separated by SDS-PAGE and immunoblotted with an anti-TBC1D1 antibody. The data are expressed as the means ± SE; n = 8–12/group. #$P < 0.05$ (vs. empty vector controls).

RESULTS

Overexpression of TBC1D1 in mouse tibialis anterior muscle. To study TBC1D1 function in skeletal muscle, in vivo injection and electroporation were used to transfect mouse tibialis anterior muscles with recombinant wild-type TBC1D1 and five TBC1D1 mutants. The TBC1D1 mutants were *1*) TBC1D1 mutated to Trp at Arg^{125} (R125W); *2*) TBC1D1 mutated to Ala at four phosphorylation motifs (Ser^{231}, Thr^{499}, Thr^{590}, and Ser^{621}); *3*) TBC1D1 mutated to Lys at Arg^{941} within its Rab-GAP domain (R/K); *4*) TBC1D1 double mutant containing both the R125W and R/K mutations; and *5*) TBC1D1 double mutant containing both the 4P and R/K mutations. We selected the 4P mutant sites because these sites are highly conserved and are predicted to be phosphorylated by AMPK and/or Akt (i.e., Ser^{231} and Thr^{499} by AMPK, Thr^{590} by Akt, and Ser^{621} by both AMPK and Akt) (12). In vivo DNA injection and electroporation resulted in an approximate sevenfold increase in expression of TBC1D1 in mouse tibialis anterior muscle compared with endogenous TBC1D1 (Fig. 1). The magnitude of overexpression was comparable across all constructs, as each TBC1D1 variant was subcloned into the same pCAGGS vehicle.

TBC1D1 PAS phosphorylation. We first determined the effects of overexpressing wild-type and mutant TBC1D1 proteins on the phosphorylation state of TBC1D1, using the PAS antibody. Because the PAS antibody detects both AS160 and TBC1D1, AS160 was depleted from lysates by immunoprecipitation. After immunoprecipitation, AS160 was not detected in the muscle lysates and there was no effect of the AS160 immunoprecipitation on the TBC1D1 protein, as detected by immunoblotting (supplementary Fig. 1, available in an online appendix at http://diabetes.diabetesjournals.org/content/early/2010/03/10/db09-1266/suppl/DC1). The AS160-depleted lysates were used to study TBC1D1 PAS signaling. As expected, basal phosphorylation was significantly increased in muscles overexpressing wild-type, R/K, R125W, and R125W/RK TBC1D1 mutants compared with empty vector controls (Fig. 2*A* and *B*). Glucose injection resulting in physiological insulin concentrations (Fig. 2*A*), contraction (Fig. 2*B*), and maximal insulin (supplementary Fig. 2*A*) stimulated endogenous TBC1D1 PAS phosphorylation by 1.5- to 2-fold. PAS phosphorylation in response to the three stimuli was greater in muscles overexpressing wild-type, R/K, R125W, and R125W/RK TBC1D1 (Fig. 2*A* and *B*; supplementary Fig. 2*A*). PAS phosphorylation in the 4P and 4P/RK TBC1D1-expressing muscle was not increased in the basal state compared with empty vector controls.

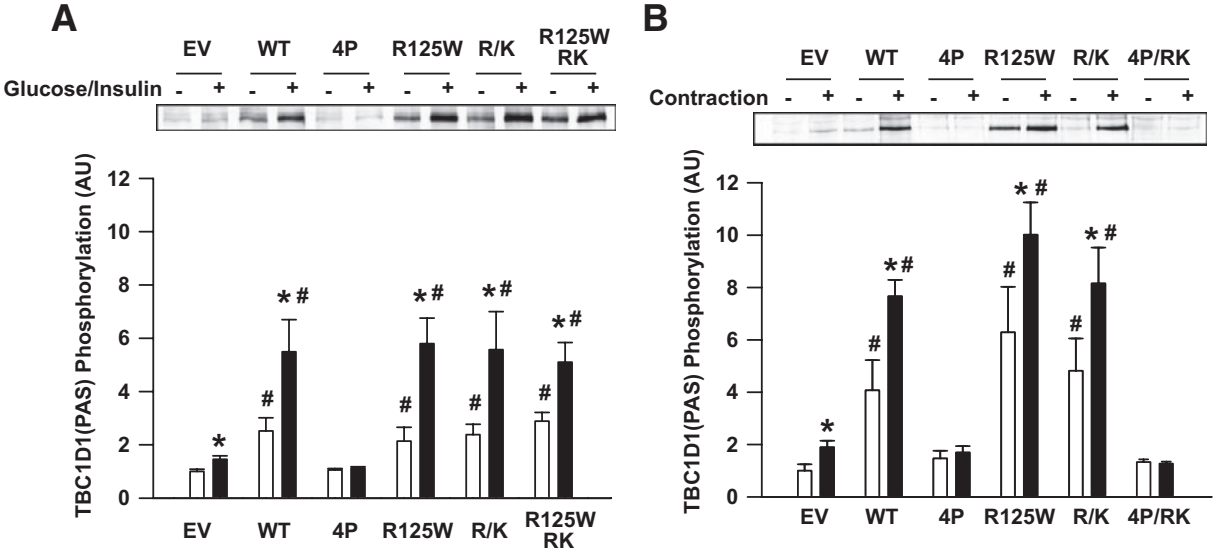

FIG. 2. Phosphorylation of TBC1D1 in transfected muscles. To determine whether expression of TBC1D1 altered TBC1D1 PAS phosphorylation, EV and TBC1D1 cDNA constructs were injected into tibialis anterior muscles followed by in vivo electroporation, and mice were studied 1 week later. *A*: Mice were anesthetized and injected intravenously with saline (−) or glucose (+; 1.0 g of glucose/kg of body wt), and tibialis anterior muscles were obtained 15 min later. *B*: Mice were anesthetized, one leg was sham treated (−) and the other leg was contracted in situ (+) for 15 min, and tibialis anterior muscles were dissected. Muscle lysates were immunoprecipitated to deplete AS160 and supernatants were separated by SDS-PAGE and immunoblotted with an anti-PAS antibody. White bars represent basal treatment, and black bars represent glucose/insulin (*A*) or contraction (*B*) treatment groups. Data are means ± SE, n = 8. *$P < 0.05$ (vs. basal); #$P < 0.05$ (vs. empty vector for respective treatment).

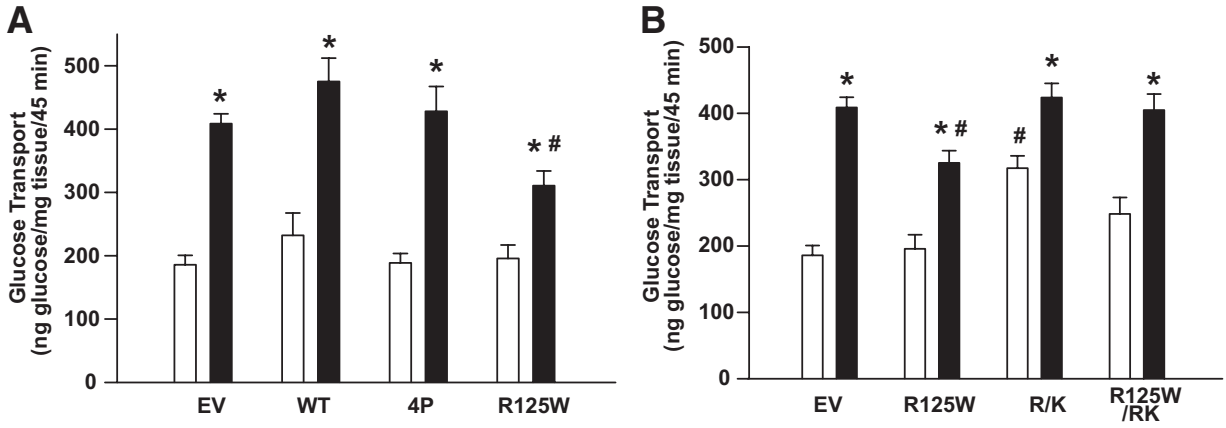

FIG. 3. Overexpression of TBC1D1 altered insulin-stimulated glucose transport in mouse skeletal muscles. To determine whether expression of wild-type TBC1D1 and TBC1D1 mutants altered insulin-stimulated glucose transport, EV or TBC1D1 cDNA constructs were injected into tibialis anterior muscles followed by in vivo electroporation. One week later, mice were anesthetized and administered a saline or 20% glucose bolus (1.0 g of glucose/kg of body wt) through the retro-orbital sinus to stimulate a physiologic insulin response. Insulin-stimulated glucose transport was measured in tibialis anterior muscles using [^{3}H]2-deoxyglucose. *A*: The effects of expressing the 4P and R125W mutants. *B*: The effects of the Rab-GAP (R/K) mutants. White bars represent the basal treatment, and black bars represent the glucose/insulin treatment group. Data are means ± SE, n = 8–16 mice/group. *P < 0.05 (vs. basal); #P < 0.05 (vs. empty vector for respective treatment).

Furthermore, glucose, contraction, and insulin did not significantly increase PAS phosphorylation in the 4P overexpressing muscles (Fig. 2*A* and *B*; supplementary Fig. 2*A*). These data demonstrate that the recombinant TBC1D1 is functionally modified in vivo, and that the integrity of the TBC1D1 point mutations (4P and 4P/RK) was preserved after gene expression. The R125W and R/K mutations did not interfere with the ability of insulin and contraction to increase PAS phosphorylation in the skeletal muscle.

TBC1D1 regulates insulin-stimulated glucose transport. Although there is minimal expression of TBC1D1 in adipocytes (12,16), a previous study has shown that overexpression of wild-type TBC1D1 dramatically reduces insulin-stimulated GLUT4 translocation in 3T3L1 adipocytes (6). In contrast to these findings, we found that overexpression of wild-type TBC1D1 did not decrease basal or submaximal insulin-stimulated glucose transport in skeletal muscle (Fig. 3*A*).

Insulin and contraction increase TBC1D1 and AS160 phosphorylation as detected in aggregate by the PAS antibody (5,12), and abolishing AS160 PAS phosphorylation impairs insulin- and contraction-induced glucose transport (8). Because of the structural similarity of TBC1D1 to AS160, we hypothesized that TBC1D1 phosphorylation sites detected by the PAS antibody would also be important for glucose transport. To address this question, we overexpressed the TBC1D1 4P construct that results in the loss of insulin- and contraction-stimulated PAS phosphorylation (Fig. 2) and measured basal and submaximal insulin-stimulated glucose transport in vivo. Surprisingly, expression of the TBC1D1 4P mutant did not alter insulin-stimulated glucose transport (Fig. 3*A*).

Genetic linkage analyses have shown a TBC1D1 R125W missense variant is linked to severe obesity in humans (18,19). Because skeletal muscle is the tissue with the highest level of expression of TBC1D1, we tested the hypothesis that the R125W mutation would alter glucose transport in this tissue. We found that overexpression of the TBC1D1 R125W mutant in skeletal muscle significantly impaired insulin-stimulated glucose transport (Fig. 3*A*). To explore whether disruption of the Rab-GAP domain reverses the decrease in glucose transport associated with the R125W mutation, we expressed TBC1D1 containing both the R125W and Rab-GAP mutations in the muscles. Figure 3*B* shows that glucose/insulin-stimulated glucose transport was normalized when the TBC1D1 R125W mutant was coexpressed with the mutated Rab-GAP domain. Interestingly, expression of TBC1D1 with a disrupted Rab-GAP domain (R/K) significantly increased basal glucose transport without altering insulin-stimulated glucose transport (Fig. 3*B*).

TBC1D1 regulates contraction-stimulated glucose transport. We examined whether TBC1D1 regulates contraction-stimulated glucose transport. The contraction protocol used was maximal, and therefore the increase in glucose transport with contraction was greater than with glucose-induced physiological insulin (Figs. 3 and 4). Similar to the effects of insulin, expression of wild-type TBC1D1 did not alter contraction-stimulated glucose transport (Fig. 4*A*). However, in contrast to what was observed with glucose/insulin, muscles overexpressing the TBC1D1 4P mutant exhibited a significant decrease in contraction-stimulated glucose transport (Fig. 4*A*). Furthermore, although the expression of the TBC1D1 R125W mutation decreased insulin-induced glucose transport, it did not alter contraction-induced glucose transport (Fig. 4*A*).

TBC1D1 devoid of Rab-GAP activity significantly increased contraction-stimulated glucose transport by 60% (Fig. 4*B*). In addition, disruption of the Rab-GAP domain of TBC1D1 resulted in reversal of the decrease in contraction-stimulated glucose transport that occurred with expression of the 4P mutant (Fig. 4*B*). These results suggest that TBC1D1 plays an important role in contraction-stimulated glucose transport.

For the contraction experiments, all treatment groups had a higher basal rate of glucose transport compared with the basal glucose transport in the insulin experiment. This is likely the result of contraction of one leg altering glucose transport of the control sedentary leg due to changes in blood flow or concentration of blood hormones. Therefore, the higher basal rates of glucose transport in the contraction experiments may mask the more prominent effects of the Rab-GAP (R/K) mutant that are observed with the insulin experiments.

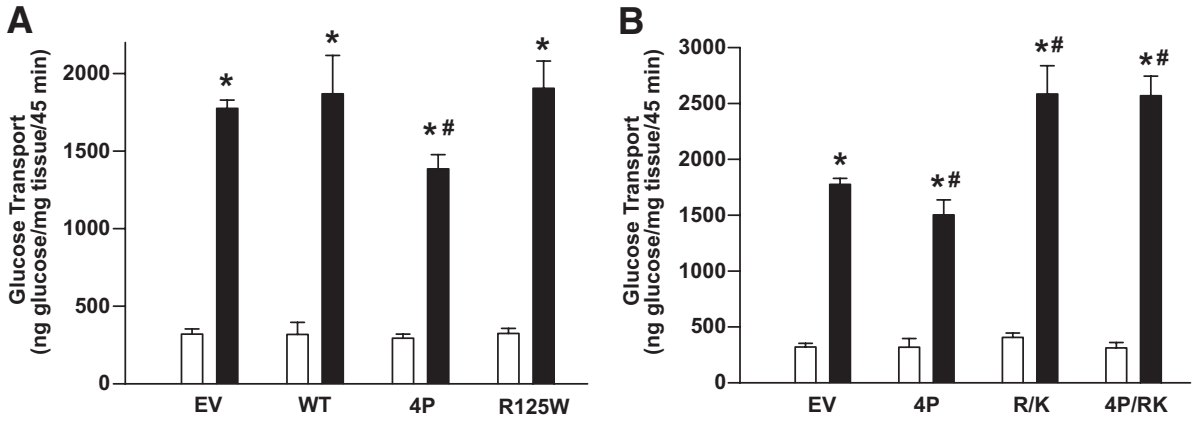

FIG. 4. Overexpression of TBC1D1 altered contraction-stimulated glucose transport in mouse skeletal muscles. To determine whether expression of wild-type TBC1D1 and TBC1D1 mutants altered contraction-stimulated glucose transport, EV or TBC1D1 cDNA constructs were injected into tibialis anterior muscles followed by in vivo electroporation. One week later, mice were anesthetized and contraction was performed by stimulation of the peroneal nerve. One leg was sham treated but unstimulated, and the other leg was contracted for 15 min. Contraction-stimulated glucose transport was measured in tibialis anterior muscles using [^{3}H]2-deoxyglucose. *A*: The effects of expressing the 4P and R125W mutants. *B*: The effects of the Rab-GAP (R/K) mutants. White bars represent the basal treatment, and black bars represent the contraction treatment group. Data are means ± SE, n = 8–16 mice/group. *P < 0.05 (vs. basal); #P < 0.05 (vs. empty vector for respective treatment).

Overexpression of TBC1D1 does not alter key signaling proteins and glucose transport. Akt plays a key role in mediating insulin-stimulated glucose transport, whereas the signaling mechanisms regulating contraction-induced glucose transport likely involve AMPK and AMPK-related kinases. To determine whether overexpression of the TBC1D1 constructs alter insulin- and contraction-stimulated Akt and AMPK signaling, Akt-Thr^{308}, Akt-Ser^{473}, and AMPK Thr^{172} phosphorylation were measured in muscles expressing the TBC1D1 constructs. Insulin-stimulated (supplementary Fig. 2*B* and *C*) or glucose-stimulated (supplementary Fig. *3*) Akt Thr^{308} and Akt Ser^{473} phosphorylation were not altered in muscles expressing wild-type and mutant TBC1D1. In addition, contraction-induced AMPK Thr^{172} phosphorylation (supplementary Fig. 4*A*) and Akt Thr^{308} and Akt Ser^{473} phosphorylation (supplementary Fig. 4*B* and *C*) were also normal in the muscles. These results indicate that TBC1D1 mutations alter glucose transport without interfering with Akt and AMPK signaling.

AS160 mutated at four PAS phosphorylation sites significantly decreases insulin- and contraction-stimulated glucose transport in skeletal muscle (8). Therefore, we determined whether overexpression of wild-type or mutant TBC1D1 would alter AS160 protein expression and/or insulin- and contraction-stimulated AS160 PAS phosphorylation. AS160 was immunoprecipitated from tibialis anterior muscle lysates expressing wild-type and mutant TBC1D1 and immunoblotted with the PAS antibody. Stimulation of AS160 phosphorylation by insulin (supplementary Fig. 5*A*) and contraction (supplementary Fig. 5*B*) was not affected by overexpression of wild-type or mutant TBC1D1. Overexpression of TBC1D1 had no effect on AS160 protein expression (supplementary Fig. 6). These results suggest that TBC1D1 alters glucose transport without altering AS160 PAS phosphorylation.

GLUT proteins and glycolytic enzymes can be critical regulators of glucose metabolism in skeletal muscle. Therefore, we determined whether TBC1D1 overexpression alters the protein expression of GLUT1, GLUT4, or hexokinase II in the transfected muscles. As shown in supplementary Fig. 6, there was no difference in expression among muscles injected with empty vector or any of the TBC1D1 constructs. Therefore, TBC1D1 overexpression does not alter expression of these key regulatory proteins of glucose metabolism in skeletal muscle.

DISCUSSION

Under normal physiological conditions, skeletal muscle glucose transport is the rate-limiting step in glucose utilization, and impairment in insulin-stimulated glucose transport in this tissue is a major factor in the development of type 2 diabetes. Despite the importance of skeletal muscle in metabolic health, the intracellular signaling mechanisms regulating glucose transport in this tissue are not completely understood. TBC1D1 is a Rab-GAP protein and a paralog of AS160, the latter protein having recently been recognized as a critical regulator of glucose transport in both adipocytes and skeletal muscle (8,13,15,25). Although TBC1D1 is known to have the highest levels of expression in skeletal muscle and is phosphorylated in response to multiple factors that stimulate glucose transport in skeletal muscle (12), whether TBC1D1 functions in the regulation of glucose transport in adult skeletal muscle was not clear. A recent study using a mouse model with a TBC1D1 truncation mutation (SJL) showed that depletion of TBC1D1 in skeletal muscle increased fatty acid oxidation but decreased glucose utilization (17). Because muscle from the SJL mouse has decreased GLUT4 expression, it is not known whether alterations in TBC1D1 function contributed to the inhibition of glucose uptake observed in the extensor digitorum longus muscle (17). In the current study, there was no change in GLUT4 expression, and therefore, TBC1D1 was established as a regulator of both insulin- and contraction-stimulated glucose transport in skeletal muscle.

Numerous phosphorylation sites have been identified on TBC1D1 (6,12,22), but the potential role of these sites in the regulation of glucose transport in skeletal muscle is not known. To determine whether TBC1D1 phosphorylation regulates glucose transport, we mutated Ser^{231}, Thr^{499}, Thr^{590}, and Ser^{621} to Ala to inhibit their phosphorylation (4P). We chose these sites because they *1*) are highly conserved phosphorylation sites on TBC1D1; *2*) are not subject to splice variation; and *3*) represent predicted

consensus sequences for both Akt and AMPK. Of these sites, Thr^{590}, a predicted Akt motif, is phosphorylated in response to insulin, and corresponds to Thr^{649} in AS160 (12). TBC1D1 Ser^{621} is a partial match for both the Akt and AMPK recognition motifs (12). Given the importance of the Akt sites on AS160 in the regulation of glucose transport (8,13), we were surprised to find that expression of this TBC1D1 4P mutant had no effect on insulin-stimulated glucose transport. Thus, our findings suggest that either TBC1D1 phosphorylation is not required for insulin-stimulated glucose transport due to the existence of another Rab-GAP protein or there are other Akt sites on TBC1D1 that are critical to mediate insulin-induced glucose transport. In contrast, expression of the TBC1D1 4P mutant significantly decreased contraction-stimulated glucose transport and this decrease was completely reversed by concomitant disruption of TBC1D1 Rab-GAP activity. It is possible that Ser^{231} and/or Thr^{499} mediate this inhibition because these sites are predicted to be phosphorylated by AMPK, and muscle contraction is a potent stimulus for AMPK activation. Taken together, our data suggest that phosphorylation of the putative AMPK sites Ser^{231} and/or Thr^{499}, but not Thr^{590} and Ser^{621}, may be critical in regulating TBC1D1 Rab-GAP activity and glucose transport. These findings also raise the possibility that TBC1D1 phosphorylation on Ser^{231} and/or Thr^{499} function predominantly in the regulation of contraction effects on glucose transport, whereas the phosphorylation of AS160 may be key in regulating insulin-stimulated glucose transport.

A TBC1D1 R125W missense variant causes familial obesity predisposition in humans (18,19); however, the mechanism by which this mutation leads to obesity is not known. Our finding that expression of the R125W mutation in skeletal muscle impairs insulin-stimulated glucose transport and our previous work showing that TBC1D1 is abundant only in skeletal muscle (12) suggest that metabolic dysfunction in skeletal muscle caused by alterations in TBC1D1 function could be a primary phenotypic manifestation of the TBC1D1 R125W missense variant. Impairment of glucose transport in muscle could lead to increased fat accumulation in adipose tissue, and subsequent obesity. Although the precise molecular mechanism by which the R125W mutation in TBC1D1 impairs insulin-stimulated glucose transport is not known, it is interesting to note that this mutation does not result in defects in upstream insulin signaling, as Akt phosphorylation was normal.

In contrast to insulin, expression of the TBC1D1 R125W mutant had no effect on contraction-stimulated glucose transport in mouse skeletal muscle, and this difference may provide important clues as to the mechanism by which the R125W mutation causes defects in glucose transport. This difference in R125 function for insulin- and contraction-stimulated glucose transport is likely due to distinct proximal signals for insulin and contraction. Insulin-stimulated glucose transport involves insulin binding to its receptor and subsequent activation of insulin receptor substrate, PI 3-kinase, and Akt (26,27). In contrast, contraction-stimulated glucose transport cannot be inhibited by the PI 3-kinase inhibitor wortmannin, and although the exact mechanism is still not fully understood, it may be mediated through AMPK or other AMPK-related kinases (1,28,29). Therefore, insulin and contraction may phosphorylate TBC1D1 on distinct sites, which may lead to different effects on its Rab-GAP activity or result in different affinities of TBC1D1 for other proteins. The R125 site is located in a tyrosine-binding domain in TBC1D1. Given that this domain mediates binding of AS160 with other proteins (25,30–32), the R125W mutation may alter the affinity of TBC1D1 for proteins involved in insulin but not contraction signaling, resulting in impaired insulin-stimulated glucose transport. The mechanism by which the R125W mutation impairs glucose transport will be an important topic for future investigation. Our finding that the impairment of insulin-stimulated glucose transport could be reversed by simultaneous disruption of Rab-GAP activity in TBC1D1 suggests a possible therapeutic approach to treat patients with this condition in the future.

Our results show that overexpression of wild-type TBC1D1 had no effect on both insulin- and contraction-stimulated glucose transport, in contrast to previous studies using adipocytes (6,16). The discrepancy is likely because there are very different expression levels of endogenous TBC1D1 in skeletal muscle and adipocytes. We find little to no expression of TBC1D1 in adipose tissue (12), and it has been reported that adipocytes have 20 times more AS160 compared with TBC1D1 (16). Thus, overexpression of a large amount of TBC1D1 in adipocytes may interfere with AS160 phosphorylation, suppressing GLUT4 translocation, resulting in inhibition of glucose transport. If TBC1D1 functions as a brake to restrain GLUT4 translocation and adipocytes lack this protein, overexpressing this foreign protein may overwhelm the system, preventing GLUT4 translocation. In contrast, in mouse skeletal muscle, expression of endogenous TBC1D1 is so abundant that it is sufficient to inhibit GLUT4 translocation. Therefore, overexpression of wild-type TBC1D1 in skeletal muscle did not inhibit glucose transport as it did in adipocytes. In addition, in 3T3-L1 adipocytes, mutation of R125W had no further effect on insulin-stimulated glucose transport compared with wild-type TBC1D1 (33). It is likely that in adipocytes, overexpression of wild-type TBC1D1 inhibits glucose transport enough to mask the effect of the R125W mutation on glucose transport. Indeed, in our studies in skeletal muscle, expressing the TBC1D1 R125W mutant significantly decreased insulin-stimulated glucose transport, under conditions where there was no effect of wild-type TBC1D1. Thus, the studies using adipocytes should be interpreted with caution and not extrapolated to skeletal muscle because TBC1D1 is minimally expressed in adipocytes and because the TBC1D1 splice variant expressed in the adipocyte study is not the variant expressed in skeletal muscle (6).

The current work suggests that in addition to AS160, TBC1D1 functions as a Rab-GAP protein regulating glucose transport in mouse skeletal muscle. In comparing TBC1D1 with AS160, mutation of four Akt phosphorylation sites on AS160 inhibited both insulin- and contraction-stimulated glucose transport, and both of these effects were reversed by disruption of the Rab-GAP domain (8). In contrast, in the current study, mutations of four phosphorylation sites on TBC1D1, which consisted of both predicted Akt and AMPK sites, impaired only contraction-stimulated glucose transport. One explanation for this finding is that TBC1D1 phosphorylation may not mediate insulin-stimulated glucose transport in mouse skeletal muscle. The second possibility is that the mutation of the two putative Akt sites (Thr^{590} and Ser^{621}) is insufficient to inhibit Rab-GAP activity of this protein, and that there must be one or more additional putative Akt sites in TBC1D1 that also regulate its Rab-GAP activity after

insulin stimulation. In future studies, it will be important to investigate the regulation and function of additional phosphorylation sites on TBC1D1. In addition, because different muscles have different relative levels of expression of AS160 and TBC1D1, these two Rab-GAP domains may contribute differently in terms of glucose transport in various muscle types.

In summary, we used in vivo gene injection and electroporation to overexpress wild-type and mutant TBC1D1 in mouse tibialis anterior muscles and evaluated the effect of TBC1D1 on glucose transport. Our studies show that the R125W mutation in TBC1D1 impairs insulin-stimulated glucose transport and has no effect on contraction-stimulated glucose transport, which may be a major mechanism for the obesity associated with this mutation. In contrast, mutation of four conserved phosphorylation sites compromised only contraction-stimulated glucose transport. Furthermore, we find that the Rab-GAP domain of TBC1D1 is critical in the regulation of glucose transport in muscle. TBC1D1 mediates stimuli-specific upstream signals, leading to regulation of glucose transport. Taken together with the reported role of this protein in fatty acid metabolism, TBC1D1 is a central regulator of metabolic function in skeletal muscle.

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