

Calmodulin-Binding Domain of AS160 Regulates Contraction- but Not Insulin-Stimulated Glucose Uptake in Skeletal Muscle

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OBJECTIVE—Insulin and contraction increase skeletal muscle glucose uptake through distinct and additive mechanisms. However, recent reports have demonstrated that both signals converge on the Akt substrate of 160 kDa (AS160), a protein that regulates GLUT4 translocation. Although AS160 phosphorylation is believed to be the primary factor affecting its activity, AS160 also possesses a calmodulin-binding domain (CBD). This raises the possibility that contraction-stimulated increases in Ca²⁺/calmodulin could also modulate AS160 function.

RESEARCH DESIGN AND METHODS—To evaluate the AS160 CBD in skeletal muscle, empty-vector, wild-type, or CBD-mutant AS160 cDNAs were injected into mouse muscles followed by *in vivo* electroporation. One week later, AS160 was overexpressed by ~14-fold over endogenous protein.

RESULTS—Immunoprecipitates of wild-type and CBD-mutant AS160 were incubated with biotinylated calmodulin in the presence of Ca²⁺. Wild-type AS160, but not the CBD-mutant AS160, associated with calmodulin. Next, we measured insulin- and contraction-stimulated glucose uptake *in vivo*. Compared with empty-vector and wild-type AS160, insulin-stimulated glucose uptake was not altered in muscles expressing CBD-mutant AS160. In contrast, contraction-stimulated glucose uptake was significantly decreased in CBD-mutant-expressing muscles. This inhibitory effect on glucose uptake was not associated with aberrant contraction-stimulated AS160 phosphorylation. Interestingly, AS160 expressing both calmodulin-binding and Rab-GAP (GTPase-activating protein) domain point mutations (CBD + R/K) fully restored contraction-stimulated glucose uptake.

CONCLUSIONS—Our results suggest that the AS160 CBD directly regulates contraction-induced glucose uptake in mouse muscle and that calmodulin provides an additional means of modulating AS160 Rab-GAP function independent of phosphorylation. These findings define a novel AS160 signaling component, unique to contraction and not insulin, leading to glucose uptake in skeletal muscle. *Diabetes* 56:2854–2862, 2007

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AMPK, AMP-activated protein kinase; AS160, Akt substrate of 160 kDa; CaMK, Ca²⁺/calmodulin-activated protein kinase; CBD, calmodulin-binding domain; GAP, GTPase-activating protein; PAS, phospho-Akt substrate; PKC, protein kinase C.

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Physical exercise increases glucose uptake into contracting skeletal muscle and can improve systemic glucose homeostasis when consistently performed. This is particularly valuable for individuals with diabetes, who exhibit normal contraction-induced glucose uptake despite impaired insulin sensitivity in skeletal muscle. Although both insulin and contraction stimulate increases in glucose uptake through translocation of intracellular GLUT4 proteins to the sarcolemma and T-tubules (1,2), the upstream signaling mechanisms leading to GLUT4 translocation appear to be distinct. Insulin signaling involves sequential activation of the insulin receptor, insulin receptor substrates, and phosphoinositide 3-kinase (3,4). In contrast, muscle contraction activates multiple phosphoinositide 3-kinase-independent signaling pathways, including the Ca²⁺/calmodulin-activated protein kinase (CaMK) family (5–7), AMP-activated protein kinase (AMPK) (2,8), and atypical protein kinase C (PKC) (9), each of which has been proposed to enhance glucose uptake in skeletal muscle. For many years, the mutual but independent capacity to stimulate GLUT4 translocation by insulin and contraction has ignited speculation of an eventual point of signaling convergence just before GLUT4.

Our lab and others recently showed that these disparate insulin and contraction signals converge on and phosphorylate the downstream Rab-GAP (GTPase-activating protein) known as the Akt substrate of 160 kDa (AS160) in a distinct and additive manner (10,11). Studies in cells initially characterized AS160 as a molecular restraint of GLUT4 translocation through the activity of its Rab-GAP domain (12,13). Phosphorylation of AS160 on phospho-Akt substrate (PAS) motifs is thought to remove its inhibitory GAP activity and increases GLUT4 trafficking to the cell membrane (14,15). Multiple effectors of glucose uptake stimulate AS160 phosphorylation, including insulin (10,13–19), platelet-derived growth factor (14), 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside (10,11,14,17), 4-phorbol-12-myristate-13-acetate (a conventional/novel PKC activator) (14), K⁺-induced membrane depolarization (14), and contraction (10,11,16,17,20). Failure to achieve AS160 PAS phosphorylation inhibits GLUT4 translocation and glucose uptake in L6 and 3T3-L1 cells (14,15,21). In accordance with this, we determined that overexpression of a 4P-mutant form of AS160, incapable of PAS phosphorylation, significantly inhibited both insulin- and contraction-stimulated glucose uptake *in vivo* in adult mouse skeletal muscle (22).

Skeletal muscle contraction is initiated by a process

known as excitation-contraction coupling. Briefly, acetylcholine-induced depolarization of the sarcolemma and T-tubules liberates Ca^{2+} from the sarcoplasmic reticulum and enhances Ca^{2+} interaction with the contractile apparatus. The frequency and duration of stimulation determine the amplitude and duration of the Ca^{2+} transients and, as a result, the level of force output by the muscle (23). These intracellular Ca^{2+} spikes have also been proposed to stimulate GLUT4 translocation and glucose uptake during contraction (6,24). Several studies have shown that glucose uptake is increased in skeletal muscle when cytoplasmic Ca^{2+} concentrations are elevated (25–27) and that blockade of sarcoplasmic reticulum Ca^{2+} release by dantrolene effectively inhibits glucose uptake (27). In isolated rat epitrochlearis muscles, caffeine increases intracellular Ca^{2+} levels (28) and glucose uptake without inducing contractions or changes in high-energy phosphates (27). Similarly, K^{+} -induced depolarization of L6 cells increased intracellular Ca^{2+} levels and GLUT4-myc at the plasma membrane in a Ca^{2+} -dependent manner (21). Dantrolene, Ca^{2+} chelators (EDTA and EGTA), and PKC inhibitors (calphostin C), but not inhibitors of AMPK α 2 and CaMK, abolished the depolarization-associated net gain in surface GLUT4-myc. In a subsequent study using K^{+} -induced depolarization, transfection of L6 cells with 4P-mutant AS160 also inhibited GLUT4-myc trafficking in response to depolarization (14). Although depolarization in this model does not mimic contraction or contraction-induced Ca^{2+} release, the results collectively suggest that K^{+} depolarization enriches membrane GLUT4 through a signaling mechanism involving Ca^{2+} , PKC, and AS160 but not AMPK or CaMK.

The information encoded in transient Ca^{2+} signals is deciphered by various intracellular Ca^{2+} -binding proteins that convert the signals into a wide variety of biochemical changes (29). Proteins such as calmodulin are intermediaries that couple the Ca^{2+} signals to biochemical and cellular changes. Calmodulin is a small (~17 kDa), evolutionarily conserved protein that serves as a ubiquitous intracellular receptor for Ca^{2+} . The Ca^{2+} /calmodulin complex is considered active and interacts with numerous substrates to regulate diverse cellular functions such as growth, proliferation, movement, and metabolism (23,30,31). PKC and CaMKII are two known substrates that bind to Ca^{2+} /calmodulin and are directly regulated in a Ca^{2+} -dependent manner.

Interestingly, human AS160 contains a region NH₂-terminal to the Rab-GAP domain (amino acids 834–857) that is ~58% identical to the calmodulin-binding domain (CBD) (amino acids 657–680) within the *Drosophila* protein Pollux (gi24644376) (32). Both of these human and *Drosophila* peptide sequences harbor key leucine and tryptophan residues (842/843 on AS160) shown to be essential for calmodulin association (32). Since contraction increases Ca^{2+} /calmodulin and AS160 possesses a functional CBD, the purpose of the present study was to determine whether the AS160 CBD regulates contraction-stimulated glucose uptake in mouse skeletal muscle. Our results indicate that the AS160 CBD is uniquely required for full contraction- but not insulin-stimulated muscle glucose uptake.

RESEARCH DESIGN AND METHODS

Antibodies. Total AS160 was detected using anti-AS160 (Upstate), while AS160 phosphorylation was detected with a PAS antibody (Cell Signaling Technology) (10,11,16,17,19,20,22). A polyclonal anti-myc antibody (CST) was

used to immunoprecipitate transfected AS160. Phosphorylation of AMPK-Thr¹⁷² (Biosource International) was used to evaluate muscle contraction efficacy. Horseradish peroxidase-conjugated anti-rabbit antibody (Amersham Biosciences) was used to bind and visually detect all primary antibodies.

Animals. Protocols for animal use were reviewed and approved by the Joslin Diabetes Center Institutional Animal Care and Use Committee and were in accordance with National Institutes of Health guidelines. Female ICR mice aged 8 weeks were purchased from Taconic (Hudson, NY). All mice were housed with a 12-h:12-h light:dark cycle and fed standard laboratory chow and water ad libitum. Mice were fasted overnight for 10 h before the morning of the experiment.

Plasmid cDNA constructs and mutagenesis. Human wild-type AS160 cDNA and four distinct mutant AS160 cDNA constructs have been characterized in 3T3-L1 cells as previously described (online appendix [available at <http://dx.doi.org/10.2337/db07-0681>]) (15,19). For in vivo gene transfer, AS160 cDNA was first subcloned into pCAGGS plasmids as previously described (online appendix) (22). Novel AS160 mutants were generated using the QuikChange XL kit from Stratagene (La Jolla, CA) and verified for accuracy using the Brigham and Women's Hospital DNA sequencing lab (Boston, MA).

In vivo gene transfer and glucose uptake in mouse skeletal muscle. Purified AS160 plasmids were directly injected into mouse tibialis anterior muscles by a protocol described by Aihara and Miyazaki (33) and modified in our laboratory (7,22,34). For glucose bolus studies, individual mice were transfected with different cDNA constructs in each tibialis anterior. Seven days later, mice were anesthetized with 90 mg/kg pentobarbital sodium and administered saline or 20% glucose (1.0 g glucose/kg body wt) through the retroorbital sinus (online appendix). For contraction studies, mice were transfected with identical cDNA constructs in each tibialis anterior. Seven days after DNA injection, mice were anesthetized with intraperitoneal administration of 90 mg/kg body wt pentobarbital sodium. Peroneal nerves from both legs were surgically exposed for electrode placement and remained intact throughout the experiment. While one leg was left unstimulated (basal/sham), the other leg was subjected to electrical stimulation for 15 min of contractions (22).

Baseline blood samples were collected from the tail vein before intravenous delivery of [³H]-2-deoxyglucose. This tracer was combined with unlabeled glucose or saline in glucose injection experiments and occurred simultaneously with the onset of in situ peroneal nerve stimulation (as a saline solution) in contraction studies. For all treatments, blood samples were taken from the tail vein at 5, 10, 15, 25, 35, and 45 min postinjection for the determination of blood glucose and [³H]-2-deoxyglucose-specific activity. After collection of the final blood sample, animals were killed and tibialis anterior muscles removed and frozen in liquid nitrogen. Accumulation of [³H]-2-deoxyglucose in pulverized tissue was determined via a precipitation protocol adapted from Ferre et al. (35) using barium-hydroxide/zinc-sulfate and perchloric acid (22). Small amounts of the muscle were set aside for immunoblots.

Modified tissue processing and immunoblotting protocol. Frozen muscle tissue was homogenized with a polytron (Brinkman Instruments) in chilled lysis buffer and processed for glucose uptake as previously described (22). Equal amounts of isolated skeletal muscle proteins (40–50 μg) were resolved by SDS-PAGE (36) for Western blot analysis (37). Antibody-bound proteins were visualized using enhanced chemiluminescence (Amersham Biosciences). Protein bands were scanned by ImageScanner (Amersham Biosciences) and quantitated by densitometry (Fluorchem 2.0; Alpha Innotech, San Leandro, CA).

Glycogen determination. Glycogen content of frozen, pulverized muscle was determined by HCl hydrolysis followed by NaOH neutralization. Resultant free glucosyl concentration was determined spectrophotometrically using a hexokinase-based assay kit (Sigma, St. Louis, MO).

Immunoprecipitation of myc-tagged AS160. Protein lysates (3–5 mg/ml) were placed in low-binding centrifuge tubes containing 1 μg anti-myc antibody (CST) and rotated end over end for 16 h (4°C). Washed protein G beads (20 μl) were then added and incubated for 2 h (4°C). Beads were washed three times in 500-1,000 μl lysis buffer (22). The pellet was finally aspirated, and 5 μl of 1 μg/μl BSA was added with 30 μl 4× Laemmli's buffer and heated at 80°C for 3 min. Purified anti-myc-derived precipitates were used to determine calmodulin association with AS160 via immunoblotting.

Biotinylated calmodulin-binding assay. Immunoprecipitates of AS160 were separated via SDS-PAGE and transferred to nitrocellulose. Membranes were blocked in Buffer-A (32) with 4% BSA and 0.5 mmol/l CaCl₂ for 2 h and then treated with 100 ng/ml biotinylated calmodulin (no. 208697; Calbiochem) in Buffer-A for 2 h. After incubation with calmodulin, membranes were washed in Buffer-A containing 0.05% Tween-20 (5 × 15 min) followed by incubation with streptavidin horseradish peroxidase (1:5,000) in Buffer-A for 1 h. Membranes were washed, and calmodulin association was detected using enhanced chemiluminescence. To determine total AS160 protein, membranes

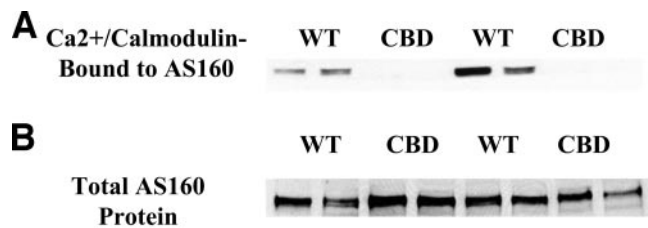


FIG. 1. Mutation of the AS160 CBD abolishes calmodulin binding to AS160. Myc-AS160 immunoprecipitates (wild type [WT] and CBD mutant) were probed with biotinylated calmodulin (100 ng/ml) in the presence of calcium (0.5 mmol/l CaCl₂) to determine whether overexpressed AS160 can bind calmodulin (*A*). The presence of AS160 protein was confirmed by immunoblotting after stripping membranes of Ca²⁺/calmodulin with the calcium chelator EGTA (5 mmol/l) (*B*). Data are representative blots; *n* = 4 per group.

were subsequently stripped in Buffer-A containing EGTA (5 mmol/l) for 2 h and then incubated in the presence of anti-AS160 as previously described (10,22).

Statistical analysis. Data are expressed as means \pm SE. Statistical analyses were performed using one- and two-way ANOVA. When ANOVA revealed significant differences, Tukey's post hoc test for multiple comparisons was performed. *P* values <0.05 were considered statistically significant.

RESULTS

Mutation of the AS160 CBD abolishes calmodulin binding to AS160. A previous study using recombinant AS160 and human AS160 immunoprecipitated from 3T3-L1 adipocytes showed that wild-type AS160, but not a CBD mutant, associates with calmodulin in the presence of Ca²⁺ (32). Here, we studied AS160 function by using an *in vivo* electroporation technique to express wild-type and CBD-mutant AS160 into the tibialis anterior muscle in mice. After 7 days, both wild-type and mutant AS160 expression was increased by over 14-fold compared with that in empty-vector controls (online appendix Fig. S1). Because of the differences in plasmid vehicle, transfection method, tissue type, and overall magnitude of expression between this system and the previous report in adipocytes (32), we first determined whether AS160 transfected and expressed in skeletal muscle could bind calmodulin.

Wild-type and CBD-mutant AS160 were immunoprecipitated from skeletal muscle lysates using the NH₂-terminal myc-epitope tag, separated via SDS-PAGE, and transferred to nitrocellulose. Membranes were then incubated with biotinylated calmodulin with 0.5 mmol/l CaCl₂. While wild-type AS160 strongly associated with biotinylated calmodulin, the CBD-mutant AS160 did not (Fig. 1*A*). Addition of EGTA fully prevented binding by wild-type AS160 (data not shown). Subsequently, membranes were stripped with EGTA and total AS160 expression assessed to account for differences in protein loading (Fig. 1*B*). These results demonstrate that wild-type AS160 derived from skeletal muscle transfections maintains the capacity to bind calmodulin. Furthermore, the point mutations in CBD-mutant AS160 cDNA were faithfully translated from gene to protein and prevented normal association with calmodulin.

Mutation of the AS160 CBD does not impair insulin-stimulated glucose uptake in transfected mouse skeletal muscles. We have previously shown that AS160 regulates insulin-stimulated glucose uptake in skeletal muscle tissue (22). Phosphorylation on key PAS motifs appears to be required to disengage the inhibitory effect of the Rab-GAP domain on glucose uptake, a mechanism also observed in various cell models (13–15). Although

the initial characterization of the AS160 CBD in adipocytes showed that mutation of this region had no effect on insulin-stimulated GLUT4 translocation (32), there are currently no reports examining a role for the AS160 CBD on insulin-stimulated glucose uptake in adult skeletal muscle. Therefore, we administered intravenous glucose into fasted mice to induce hyperglycemia and an associated physiologic insulin response (22), while glucose uptake into injected muscles was measured using a ³H-2-deoxyglucose tracer *in vivo*. Since there were no significant differences in blood glucose values between transfected animals, glucose curves represent data from all electroporated mice (Fig. 2*A*). In empty-vector control muscles, endogenous PAS phosphorylation at 160 kDa was significantly increased in response to the glucose bolus (Fig. 2*B*). Both wild-type- and CBD-mutant-expressing muscles exhibited twofold increases in basal AS160 phosphorylation, and these values were similarly increased by intravenous glucose. The effects of wild-type and CBD-mutant AS160 on skeletal muscle glucose uptake are shown in Fig. 2*C*. There were no differences between empty-vector controls or muscles expressing wild-type or CBD-mutant AS160 in either basal or insulin-stimulated glucose uptake. Collectively, these data indicate that expression of the CBD-mutant AS160 does not alter insulin-stimulated phosphorylation of AS160 at PAS motifs or glucose uptake *in vivo* in adult skeletal muscle.

Mutation of the AS160 CBD significantly inhibits contraction-stimulated glucose uptake in transfected mouse skeletal muscles. Increases in intracellular Ca²⁺ levels are a fundamental component of muscle contraction, and this rise in Ca²⁺ levels allows for the association of Ca²⁺ with calmodulin. Although AS160 has a functional CBD, it is not known whether this region regulates AS160 PAS phosphorylation events, AS160 Rab-GAP function, and/or contraction-stimulated glucose uptake.

To explore the effects of CBD-mutant AS160 on AS160 PAS phosphorylation and glucose uptake, mouse tibialis anterior muscles were transfected with wild-type and CBD-mutant AS160 and then stimulated to contract for 15 min (Fig. 3). Phosphorylation at PAS motifs was significantly increased by contraction in empty-vector control muscles and, despite their higher basal levels, further increased by contraction in wild-type and CBD-mutant AS160 transfected muscles (Fig. 3*A*). AMPK Thr¹⁷² phosphorylation was also increased similarly in control and transfected muscles in response to contraction (online appendix Fig. S2).

Despite a similar ability to increase AS160 PAS phosphorylation (Fig. 3*A*), muscles overexpressing CBD-mutant AS160 exhibited significant impairments in contraction-stimulated glucose uptake compared with that in empty-vector controls (~45%) and wild-type AS160 (~26%) (Fig. 3*B*). The reduction in contraction-stimulated glucose uptake due to wild-type AS160 overexpression is similar in magnitude to our previous findings in skeletal muscle (22). There were no differences observed between basal (sham) glucose uptake values among the various conditions. Blood glucose levels did not differ between mice injected with different constructs (online appendix Fig. S3). In addition, there were no alterations in glycogen metabolism pre- and postcontraction due to transfection with CBD-mutant AS160 (Fig. 3*C*). These results suggest that the AS160 CBD is essential for full contraction-stimulated glucose

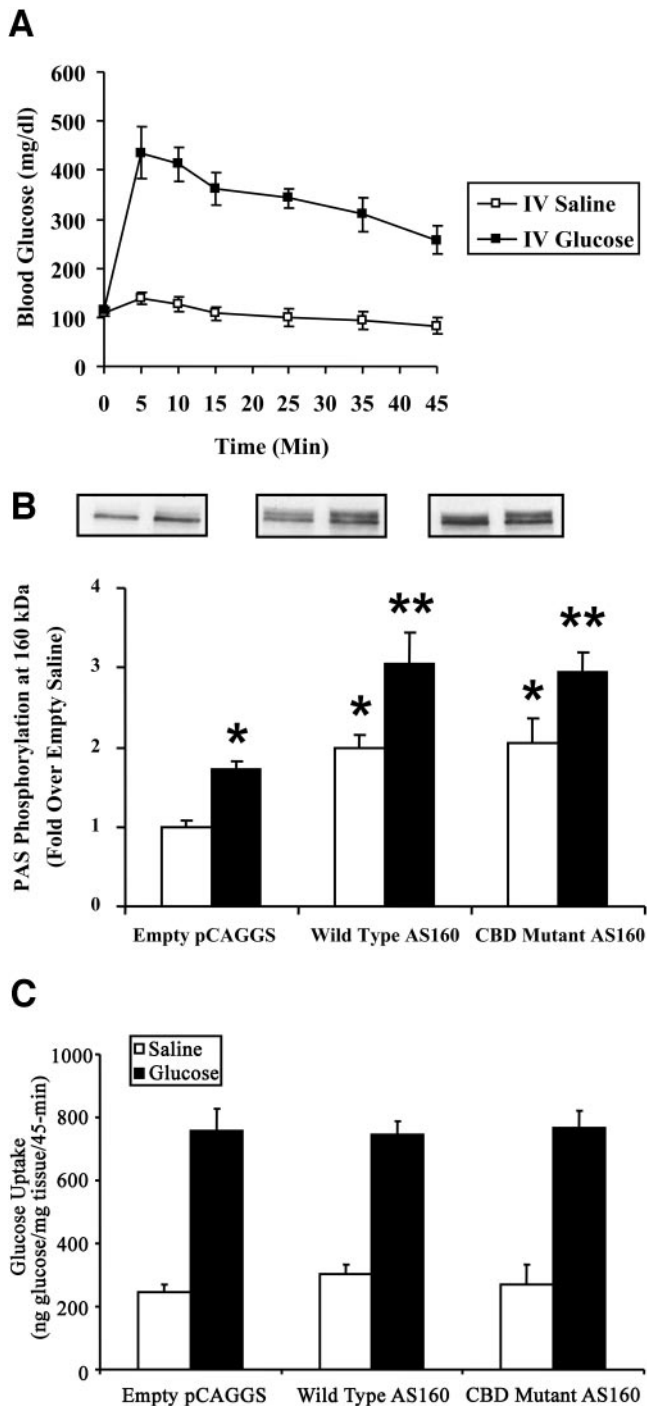


FIG. 2. Mutation of the AS160 CBD does not impair insulin-stimulated glucose uptake in transfected mouse skeletal muscles. Empty pCAGGS vector or myc-tagged AS160 cDNA constructs (wild type and CBD mutant) were injected into the tibialis anterior muscles of anesthetized mice, followed by *in vivo* electroporation. The animals were allowed to recover, and basal (saline) and insulin-stimulated intravenous glucose uptake was assessed *in vivo* 7 days postinjection. Transfected animals were administered intravenous [3 H]2-deoxyglucose combined with either saline (\square) or glucose (\blacksquare) bolus (1.0 g glucose/kg body wt), and blood was sampled at 0, 5, 10, 15, 25, 35, and 45 min postinjection. Blood glucose curves during the time course reflect aggregate data from all transfected animals (A). Muscles were harvested at 45 min and analyzed via immunoblot with the PAS antibody at 160 kDa (B), as well as processed for glucose uptake (C). B: * $P < 0.05$ vs. empty pCAGGS saline; ** $P < 0.05$ vs. empty pCAGGS glucose. Data are expressed as means \pm SE ($n = 4-6$ per group).

uptake in skeletal muscle and that this AS160-mediated inhibition of glucose uptake can occur independently of PAS phosphorylation status.

We have previously shown that a PAS (4P) mutant form of AS160 also inhibits contraction-stimulated glucose uptake in skeletal muscle (22). To determine whether expression of AS160 harboring both the CBD and 4P mutations inhibits contraction-stimulated glucose uptake in an additive manner, we generated a double-mutant (CBD + 4P) AS160 using site-directed mutagenesis. Mouse tibialis anterior muscles were then transfected with either 4P-mutant AS160, CBD-mutant AS160, or CBD + 4P-mutant AS160, and glucose uptake was subsequently evaluated following *in situ* contraction (Fig. 3D). Compared with empty-vector controls, each AS160 mutant clearly inhibited contraction-stimulated glucose uptake (47, 41, and 43% for 4P-mutant, CBD-mutant, and CBD + 4P-mutant AS160, respectively). No significant differences were observed between these groups, implying that glucose uptake is not further decreased by concomitant CBD + 4P mutations on AS160. Therefore, AS160 inhibitory action on glucose uptake may be maximally achieved by either the 4P or CBD mutations alone.

Contraction-stimulated glucose uptake is restored in muscles expressing CBD + R/K-mutant AS160. The AS160 CBD lies adjacent to the Rab-GAP domain. Therefore, it is possible that calmodulin association in this region physically hinders AS160 interaction with its target Rab(s) during contraction in skeletal muscle, which may then permit enhanced glucose uptake. To determine whether the inhibition in contraction-stimulated glucose uptake exerted by CBD-mutant AS160 was mediated by the Rab-GAP domain, we generated a double-mutant form of AS160 that contained point mutations in both the CBD and GAP regions of the protein. This resultant double-mutant CBD + R/K plasmid, as well as single CBD-mutant AS160 and empty pCAGGS control DNA, was then injected and electroporated into mouse skeletal muscle and examined following *in situ* contraction (Fig. 4). There were no significant differences in AS160 expression (online appendix Fig. S1) or PAS phosphorylation under basal or contraction conditions between CBD- and CBD + R/K-mutant AS160 (Fig. 4A).

Figure 4B shows that transfection with the double-mutant AS160 (CBD + R/K) reversed the CBD-mutant-induced decreases in glucose uptake. In fact, there was actually a significant increase (18%) in glucose uptake in muscles expressing the CBD + R/K-mutant AS160 compared with that in controls despite similar levels of AMPK phosphorylation (data not shown). Basal glucose uptake was also increased (61%) in muscles expressing CBD + R/K-mutant AS160 compared with that in control muscles and muscles expressing CBD-mutant AS160. These results are consistent with previous observations using R/K-mutant AS160 (22) and suggest that R/K mutants compete with endogenous AS160 at sites where the latter acts on Rabs and thus leads to Rab-GTP even in the absence of stimulus. Taken together, our findings indicate that the AS160 Rab-GAP domain acts as a restraint on basal and contraction-stimulated glucose uptake *in vivo*. Furthermore, an intact AS160 CBD appears to regulate AS160 GAP activity and is required for normal contraction-stimulated glucose uptake in mouse skeletal muscle.

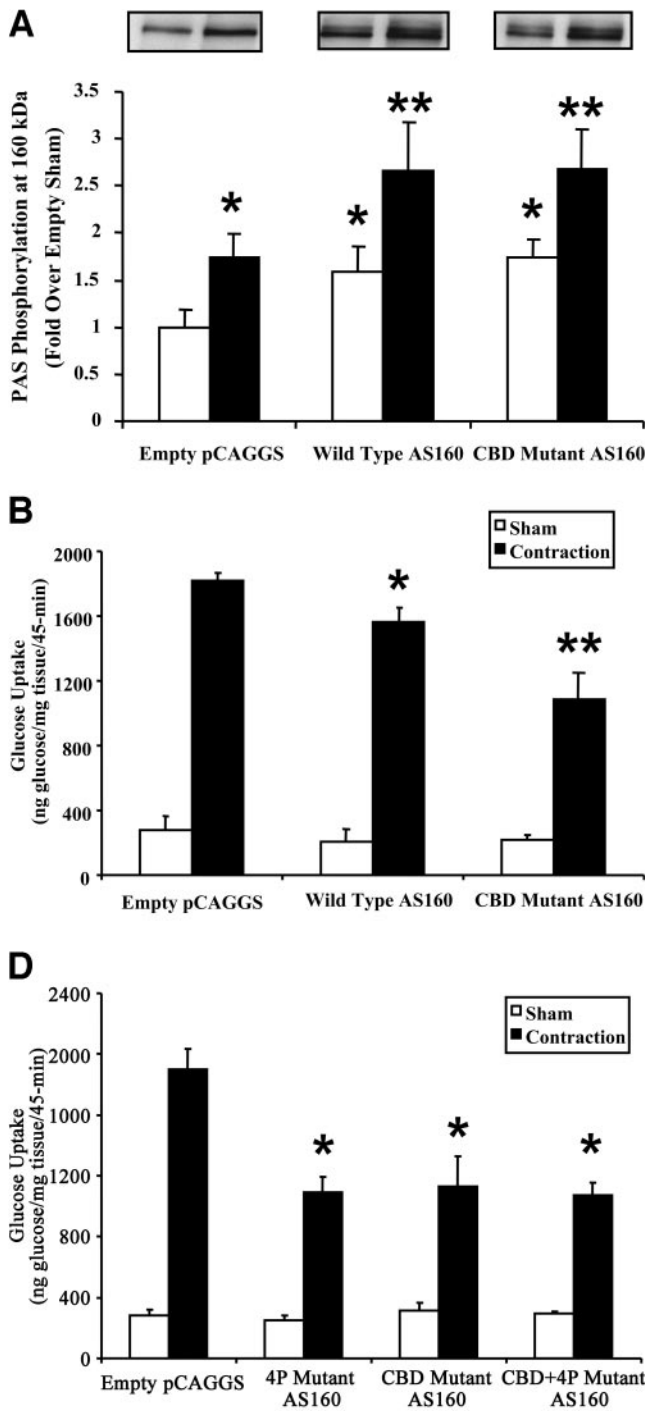


FIG. 3. Mutation of the AS160 CBD significantly inhibits contraction-stimulated glucose uptake in transfected mouse skeletal muscles. Empty pCAGGS vector or myc-tagged AS160 cDNA constructs (wild type, CBD mutant, 4P mutant, and CBD + 4P mutant) were injected into the tibialis anterior muscles of anesthetized mice, followed by in vivo electroporation. The animals were allowed to recover, and basal (sham [□]) and contraction-stimulated (■) glucose uptake were assessed in vivo 7 days postinjection. Transfected animals were administered intravenous [³H]2-deoxyglucose in a saline solution combined with either sham operations or 15 min in situ tibialis anterior muscle contractions. Muscles were harvested at 45 min and analyzed via immunoblot with the PAS antibody at 160 kDa (A). **P* < 0.05 vs. empty pCAGGS sham; ***P* < 0.05 vs. empty pCAGGS contraction. Muscles were also processed for absolute glucose uptake (B and C) and glycogen levels (D). B and D: **P* < 0.05 vs. empty pCAGGS contraction; ***P* < 0.05 vs. both wild-type AS160 and empty pCAGGS contraction conditions. Data are expressed as means ± SE (*n* = 4–6 per group).

DISCUSSION

Despite intensive research, the complete signal transduction networks mediating insulin- and contraction-stimulated glucose uptake in skeletal muscle have remained elusive. Both insulin and contraction clearly increase GLUT4 translocation; however, each stimulus utilizes independent proximal signaling cascades to achieve this effect. Our lab and others recently identified the Rab-GAP AS160 as a point of convergence for the distinct upstream signaling pathways activated by insulin and contraction (10,11,14,17). Furthermore, AS160 phosphorylation at critical PAS motifs appears to be necessary for full insulin- and contraction-stimulated glucose uptake in mouse skeletal muscle (22).

Here, we explored the functional significance of the AS160 CBD on insulin- and contraction-stimulated glucose uptake. Using an in vivo gene transfer technique (7,22,34,38), we overexpressed wild-type and CBD-mutant AS160 constructs in adult mouse skeletal muscle. Our results indicate the following: 1) CBD-mutant AS160 inhibits contraction- but not insulin-stimulated glucose uptake, 2) inhibited contraction-stimulated glucose uptake by the CBD-mutant AS160 is restored when the AS160 Rab-GAP domain is simultaneously mutated, and 3) regulation of glucose metabolism by AS160 can occur through PAS-independent signaling mechanisms in skeletal muscle. Intriguingly, this reveals that although AS160 is indeed a convergence molecule for insulin and contraction signals,

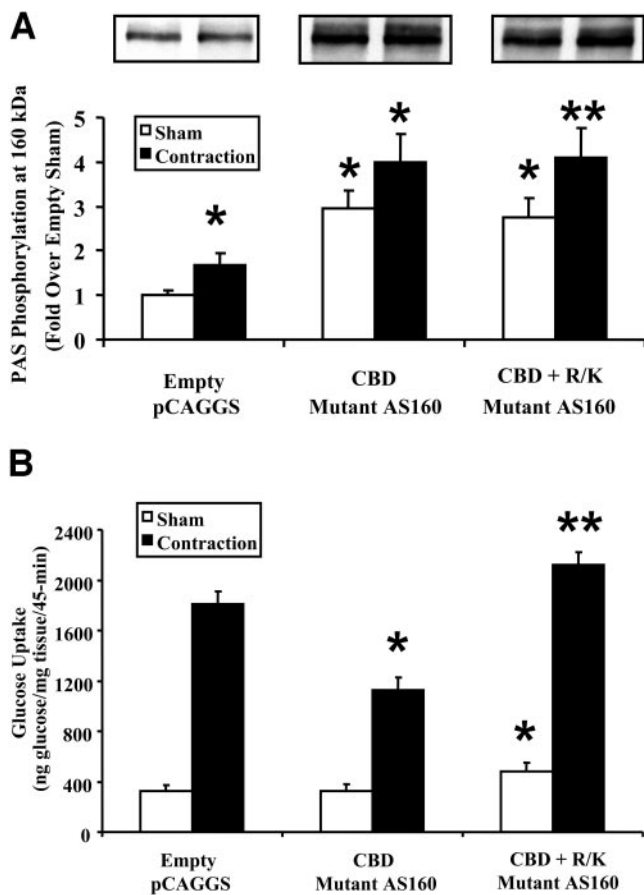


FIG. 4. Contraction-stimulated glucose uptake is restored in muscles expressing CBD + R/K-mutant AS160. Empty pCAGGS vector or myc-tagged AS160 DNA constructs (CBD and CBD + R/K mutants) were injected into the tibialis anterior muscles of anesthetized mice, followed by *in vivo* electroporation. The animals were allowed to recover, and basal (sham) and contraction-stimulated glucose uptake were assessed *in vivo* 7 days postinjection. Transfected animals were administered intravenous [3 H]2-deoxyglucose combined with either sham operations or 15 min *in situ* tibialis anterior muscle contractions. Muscles were harvested at 45 min and analyzed via immunoblot with the PAS antibody at 160 kDa (A). Muscles were also processed for absolute glucose uptake (B). * $P < 0.05$ vs. empty pCAGGS sham; ** $P < 0.05$ vs. empty pCAGGS contraction. Data are expressed as means \pm SE ($n = 5-6$ per group).

its regulation of glucose uptake is differentially manipulated in response to each stimulus.

AS160 was initially shown to contain a CBD exhibiting sequence homology with the *Drosophila* protein Pollux, possessing strong affinity for Ca^{2+} /calmodulin (32). Expression of mutant AS160 incapable of binding calmodulin did not disrupt GLUT4 translocation in insulin-treated 3T3-L1 adipocytes. In the present study, we verified that wild-type AS160 strongly associated with calmodulin in the presence of Ca^{2+} when expressed in adult skeletal muscle. Conversely, this interaction was completely abolished by mutation of the AS160 CBD. It was not possible to capture and/or preserve evidence of calmodulin-AS160 interaction *in vivo* because of the fleeting nature of calcium transients in skeletal muscle. However, our biotinylated calmodulin binding experiments *ex vivo* coupled with the functional studies on glucose uptake *in vivo* coordinately show that these AS160-calmodulin interactions are likely to occur *in vivo* as well.

We found that expression of CBD-mutant AS160 did not alter insulin-stimulated AS160 PAS phosphorylation or

glucose uptake in skeletal muscle. Note that since glucose injections caused a three- to fourfold increase in plasma glucose, the glucose uptake values should be interpreted in the context of stimulation by hyperglycemia and hyperinsulinemia rather than exclusively as insulin-stimulated glucose uptake. These results are consistent with the only previous work on the AS160 CBD in adipocytes (32) and suggest that calmodulin association with AS160 is not required for insulin signaling to glucose uptake. Some studies have reported an important role for Ca^{2+} /calmodulin on insulin-stimulated glucose metabolism in adipocytes (39), L6 cells (21), and skeletal muscle tissue (40). However, these insulin-stimulated increases in Ca^{2+} are confined to a local microenvironment just below the plasma membrane (40) and likely would not regulate AS160 and/or GLUT4 translocation from deeper intracellular storage pools. Indeed, there are multiple nodes of insulin action on GLUT4 trafficking, including translocation, recruitment and/or docking, and fusion of GLUT4 to the plasma membrane, but its role in regulating fusion has not yet been directly determined (41,42). Therefore, local Ca^{2+} /calmodulin activity at or about the plasma membrane might regulate an insulin-stimulated fusion of docked GLUT4 vesicles that did not involve AS160 (42).

In contrast, we show that contraction-stimulated glucose uptake is significantly inhibited by expression of CBD-mutant AS160. This inhibitory effect is not associated with deficient AS160 PAS phosphorylation and therefore represents a novel means of contraction-regulated AS160 signaling to glucose uptake. Interestingly, contraction in this system induces robust increases in glucose uptake due to increases in blood flow (22), which may have diluted some of the inhibition observed. Ca^{2+} transients are intrinsic to the mechanism of contraction in skeletal muscle, and there is strong evidence that Ca^{2+} -encoded signals have metabolic implications as well (6,7,21). Here, we show that binding of calmodulin to AS160, in addition to PAS phosphorylation, is required for full contraction-stimulated glucose uptake in adult skeletal muscle. These two mechanisms of regulation have seemingly mutual effects, as concomitant mutation of AS160 at PAS sites and the CBD did not exert an additive inhibition on glucose uptake (Fig. 3C). It is noteworthy that AS160 mutated at PAS sites and/or the Rab-GAP domain strongly associates with calmodulin unless the CBD is mutated (online appendix Fig. S4), suggesting that these mutations do not impair the ability of AS160 to bind calmodulin.

AS160 is currently thought to be a restraint of GLUT4 translocation and glucose uptake in adipocytes, muscle cells, and skeletal muscle tissue (12,43). Multiple stimuli induce phosphorylation of AS160 (10,13-19), which removes the suppressive functions of the Rab-GAP domain and allows for normal glucose uptake (14,15,22). This study confirms that AS160 phosphorylation is necessary for full insulin-stimulated glucose uptake in mouse skeletal muscle. In contrast, based on these results and our previous study, contraction appears to require both phosphorylation and calmodulin-binding events for full stimulation of glucose uptake. It is unlikely that our results are artifactual or due to nonspecific effects because insulin-stimulated glucose uptake is normal in muscles expressing CBD-mutant AS160. Moreover, contraction-stimulated glucose uptake is completely restored when muscles express

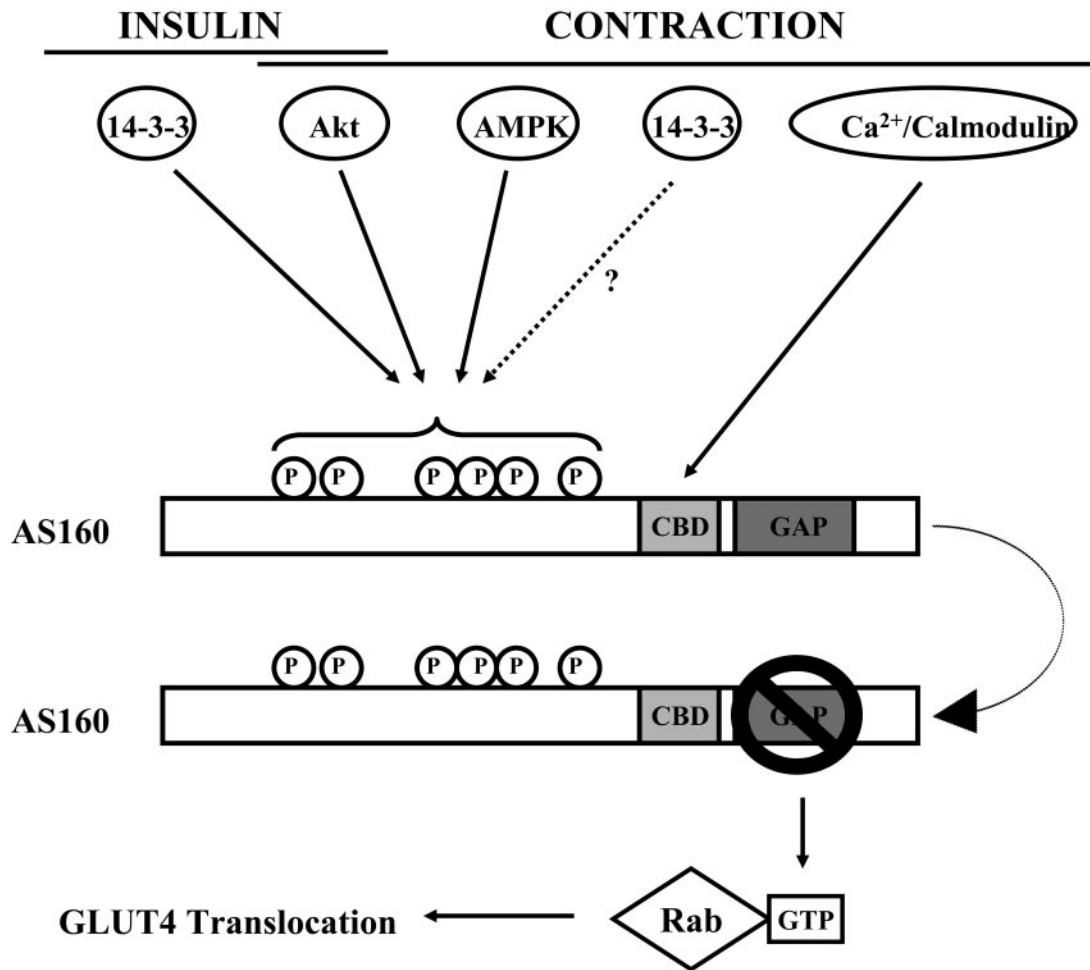


FIG. 5. A model for how insulin and contraction differentially regulate AS160 to stimulate GLUT4 translocation in skeletal muscle. The insulin signaling cascade leads to activation of Akt, which phosphorylates AS160 at known PAS motifs. In addition, 14-3-3 associates with AS160 in response to insulin. Both events are essential for full insulin-stimulated GLUT4 translocation and glucose uptake. Contraction activates multiple pathways including AMPK, Akt, and potentially other kinases, which phosphorylate AS160 at regulatory PAS motifs. It is not currently known whether 14-3-3 binds AS160 in response to contraction. Phosphorylation events are clearly necessary for full contraction-stimulated glucose uptake in skeletal muscle. But contraction also utilizes calmodulin as an additional regulatory input, which must associate with AS160 to permit full glucose uptake in contracting skeletal muscle.

a double mutant containing disrupted CBD and GAP domains (CBD + R/K).

The AS160 Rab-GAP domain exhibits activity toward multiple Rabs in adipocytes (44). Phosphorylation of AS160 may sufficiently hinder its GAP activity for specific Rabs involved in the insulin signaling pathway (45). However, to disable AS160 GAP activity toward specific contraction-regulated Rabs, both AS160 phosphorylation and association with calmodulin may be required. The AS160 CBD lies adjacent to the GAP domain (32), making it possible that binding of calmodulin physically interferes and/or competes with AS160-targeted Rabs. These Rabs would therefore predominantly exist in their GTP-bound form and be free to mobilize GLUT4 vesicles for translocation to the cell surface. Reports have described different intracellular pools of GLUT4 mobilized by insulin and contraction (46,47). If true, it is tempting to consider that these respective pools may be regulated by distinct Rabs, especially since AS160 interacts with different Rabs in muscle (45) and fat (48) cells. Whereas insulin-sensitive GLUT4 pools may be adequately liberated by AS160 phosphorylation alone, contraction-sensitive pools would require both phosphorylation and an intact CBD to facilitate GLUT4 translocation.

We must also consider the possibility that AS160 phosphorylation is necessary but not sufficient by itself for full insulin-stimulated glucose uptake. For example, AS160 association with phospho-14-3-3 was shown to be necessary for GLUT4 translocation triggered by insulin in adipocytes (49). Whereas calmodulin may function as the contraction-regulated molecular inhibitor of AS160 GAP activity, 14-3-3 proteins may exert similar functions on insulin-stimulated glucose uptake in skeletal muscle. In this scenario, both insulin and contraction require AS160 PAS phosphorylation and an additional signaling input to fully ablate AS160 Rab-GAP function (Fig. 5). Future studies are warranted to elucidate the potential role of 14-3-3 proteins on insulin- and contraction-stimulated glucose uptake.

In conclusion, we have identified the AS160 CBD as a novel regulator of contraction-stimulated glucose uptake in adult skeletal muscle. Expression of CBD-mutant AS160 inhibited contraction- but not insulin-stimulated glucose uptake, an effect reversed by AS160 coexpressing CBD and Rab-GAP domain mutations. From these data as well as previous reports, an emerging model for AS160 function and regulation of glucose uptake in skeletal muscle is presented in Fig. 5. Clearly, both insulin and contraction

signals converge on AS160 to regulate glucose uptake, but our findings reveal that contraction additionally requires the presence of the AS160 CBD for full stimulation of glucose uptake in skeletal muscle.

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