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The Graduate School

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THE ROLE OF THE AKT SUBSTRATE OF 160 KDA (AS160) ON SKELETAL MUSCLE GLUCOSE UPTAKE

A Thesis in

Physiology

by

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ABSTRACT

Type 2 diabetes is characterized by a profound loss of insulin sensitivity. In humans and other mammals, skeletal muscle normally accounts for nearly 75% of whole body insulin-stimulated glucose transport. Impaired ability of the muscle to respond to insulin is therefore disruptive to systemic glucose homeostasis. Interestingly, skeletal muscle also possesses contractile properties that effectively restore glucose control in an insulin-independent manner, and this element is preserved in individuals with diabetes. While the precise mechanisms remain elusive, it is clear that both insulin and contraction signals converge on GLUT4 vesicles and promote their translocation to the cell membrane. Akt Substrate of 160 kDa (AS160) is a Rab-GTPase activating protein that was recently identified as the most distal regulatory molecule involved in insulin signaling to glucose uptake in adipocytes. However, the role of AS160 in skeletal muscle has not been explored. This dissertation examines AS160 phosphorylation and its putative regulation of glucose uptake in mouse skeletal muscle. It is demonstrated herein that: 1) insulin and contraction utilize distinct and additive upstream signaling mechanisms to stimulate AS160 phosphorylation in skeletal muscle *in vivo* and *in vitro*; 2) phosphorylation of AS160 by insulin and contraction is necessary for their full stimulation of skeletal muscle glucose uptake; and 3) the AS160 calmodulin-binding domain regulates contraction- but not insulin-stimulated glucose uptake in skeletal muscle. Collectively, our findings implicate AS160 as a point of convergence for both insulin and contraction signals leading to glucose uptake in skeletal muscle, although the mechanisms by which insulin and contraction mediate AS160 function are not identical. Future research should evaluate the efficacy of targeting AS160 for novel therapeutic diabetes interventions.

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ABBREVIATIONS

Abbreviations used extensively throughout this thesis include: *KDa*, kilodalton(s); *AS160*, Akt Substrate of 160 kilodaltons; *GAP*, GTPase activating protein; *PAS*, phospho-Akt substrate; *IRS*, insulin receptor substrate; *PI3-K*, phosphatidylinositol 3-kinase; *PKC*, protein kinase C; *AMPK*, AMP-activated protein kinase; *AICAR*, 5-aminoimidazole-4-carboxyamide ribonucleotide; *PCR*, polymerase chain reaction; *WT*, wild-type; *KO*, knockout; *TG*, transgenic; *4P-AS160*, AS160 mutated at four PAS motifs; *R/K-AS160*, mutation of arginine to lysine in AS160 GAP domain; *2M-AS160*, double mutant AS160 containing both 4P and R/K mutations; *CBD*, calmodulin binding domain; *E*, empty pCAGGS vector; *TA*, tibialis anterior; and *EDL*, extensor digitorum longus.

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CHAPTER 1

INTRODUCTION

Background and Significance

Type 2 diabetes was the fifth leading cause of death among U.S. adults in 2002 and accounted for an estimated $132 billion in annual U.S. health-care related costs (38). As a disease with multiple etiologies, diabetes is pervading virtually every demographic subset, with conspicuous and alarming proclivity toward younger individuals. It is projected that between 35 and 40 percent of all children born in the year 2000 will develop Type 2 diabetes in their lifetime (62), and experience one or more of its associated chronic complications including hypertension, stroke, blindness, nephropathy, and central and peripheral nervous system deterioration. These and other diabetic pathologies stem from the loss of insulin sensitivity in peripheral organs such as the liver, adipose tissue, and skeletal muscle. The molecular mechanisms triggering skeletal muscle insulin resistance are particularly destructive because skeletal muscle is the major glucose disposal site in humans by shear mass and storage capacity. Thus, in order to successfully isolate potential targets for therapeutic intervention, it is imperative that we first decipher the regulation of glucose transport into muscle.

Glucose Uptake in Skeletal Muscle

Insulin and physical exercise are potent stimulators of glucose uptake in skeletal muscle (29, 35). Importantly, in patients with type 2 diabetes, insulin- but not contraction-stimulated glucose uptake is impaired (49, 100). In addition to exercise, there are many other insulin-independent stimuli that can increase skeletal muscle

glucose uptake (33), although their physiological relevance is minimal in humans. GLUT4 is the predominant glucose transporter isoform expressed in skeletal muscle, and the translocation of GLUT4 from an intracellular location to the sarcolemma and T-tubules is the primary mechanism through which both insulin and exercise/muscle contraction increase skeletal muscle glucose uptake (29, 35). Some studies suggest there are different intracellular pools of GLUT4, one stimulated by insulin and one stimulated by exercise (15, 18, 76). Packaged in intracellular vesicles, GLUT4 proteins constantly migrate/recycle along cytoskeletal elements supporting molecular exocytosis and endocytosis in insulin-sensitive cells (83). Stimulation by insulin or contraction accelerates the rate of GLUT4 vesicular exocytosis and/or decreases endocytosis, such that GLUT4 is enriched at the cell surface and primed to enhance glucose uptake (83, 98).

Insulin Signaling to Glucose Transport

Insulin binds to its transmembrane insulin receptor (IR) on the exofacial leaflet of a target cell membrane, inducing rapid autophosphorylation of intracellular IR tyrosine residues, phosphorylation of insulin receptor substrate (IRS) proteins, and increased activity of phosphatidylinositol 3-kinase (PI3-K) (23, 28). PI3-K-generated phosphatidylinositol 3,4,5-triphosphates (PIP_3) engages the serine/threonine kinase Akt to the plasma membrane where it can be phosphorylated and activated by phosphoinositide-dependent kinase-1 (PDK-1) (16). In addition, both PI3-K and its lipid products directly activate atypical protein kinase C (aPKC) isoforms zeta, lambda, & iota (6, 21). The collective contribution of PI3-K-associated increases in activated Akt, particularly the Akt2 isozyme (89), as well as aPKC (21), have been proposed to be critical for insulin-

mediated GLUT4 translocation from endosomal storage sites to the sarcolemma (55, 57). However, the intermediate substrates connecting either Akt or aPKC to the GLUT4 machinery are only beginning to emerge.

Akt Substrate of 160 Kilodaltons

The Akt substrate of 160 kilodaltons (AS160) is a novel Rab GTPase activating protein (GAP) that modulates GLUT4 trafficking in insulin-sensitive 3T3-L1 adipocytes (20, 54, 79, 98) and L6 cells (40, 82, 83). AS160 was initially characterized as an Akt substrate in insulin-treated mouse 3T3-L1 adipocytes by the laboratory of Dr. Gustav Lienhard using an innovative two-step approach (46). First, phosphorylated Akt substrates, including AS160, were collectively immunoprecipitated with a newly generated phospho-Akt substrate (PAS) antibody that binds serine or threonine phosphorylated Akt recognition motifs (RXRXXS*/T*, where * denotes phosphorylation). Liquid chromatography mass spectrometry/mass spectrometry (LC-MS/MS) was then used to identify an immunoprecipitated 160 kDa Akt phospho-substrate as the mouse homologue of human protein gi7662198. This protein was renamed Akt Substrate of 160 kDa (AS160) (46).

LC-MS/MS targeted against putative AS160 phosphopeptides in 3T3-L1 adipocytes revealed as many as six insulin stimulated AS160 phosphorylation sites at serine 318, serine 341, serine 570, serine 588, threonine 642, and serine 751 (Figure 1) (46). Mutation of four of these residues (serine 318, serine 588, threonine 642, and serine 751) to alanine to create a 4P mutant form of AS160 inhibited insulin stimulated GLUT4 translocation by nearly 80% when transfected into adipocytes (79). These data

demonstrated that phosphorylation of AS160 on Akt recognition motifs is necessary for normal insulin stimulated GLUT4 translocation and glucose uptake. The discovery of AS160 was a major breakthrough because it extended the known insulin signaling pathway to a step beyond Akt and just prior to GLUT4. It is currently thought that AS160 regulates glucose uptake through its interaction with specific vesicular trafficking molecules called Rabs.

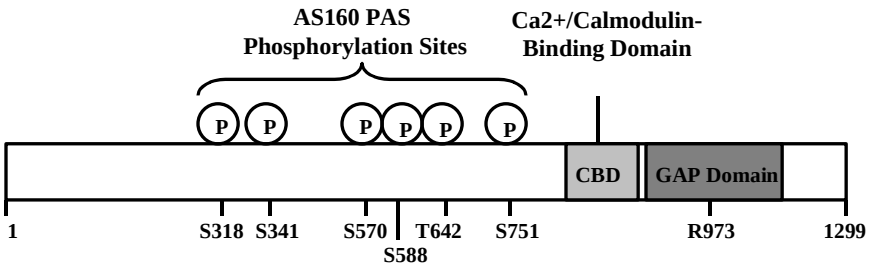


Figure 1. AS160 is structurally primed for phosphorylation events and protein-protein interactions that modulate the activity of its Rab-GAP domain. Unphosphorylated AS160 is thought to be a negative regulator of glucose transport. AS160 contains six characterized PAS motifs (P) sensitive to phosphorylation by Akt, AMPK, and potentially other kinases. AS160 also possesses a functional Rab-GAP domain, the activity of which is modulated by phosphorylation events and possibly protein-protein interactions. Calmodulin is a key associative molecule that interacts with AS160 via a calmodulin-binding domain (CBD), and may directly affect activity of the adjacent Rab-GAP domain.

Rabs are small GTP binding molecules (15-25 kDa) that regulate vesicle trafficking, including budding, delivery, tethering, and fusion of the vesicle membrane with that of the target location (Figure 2) (30).

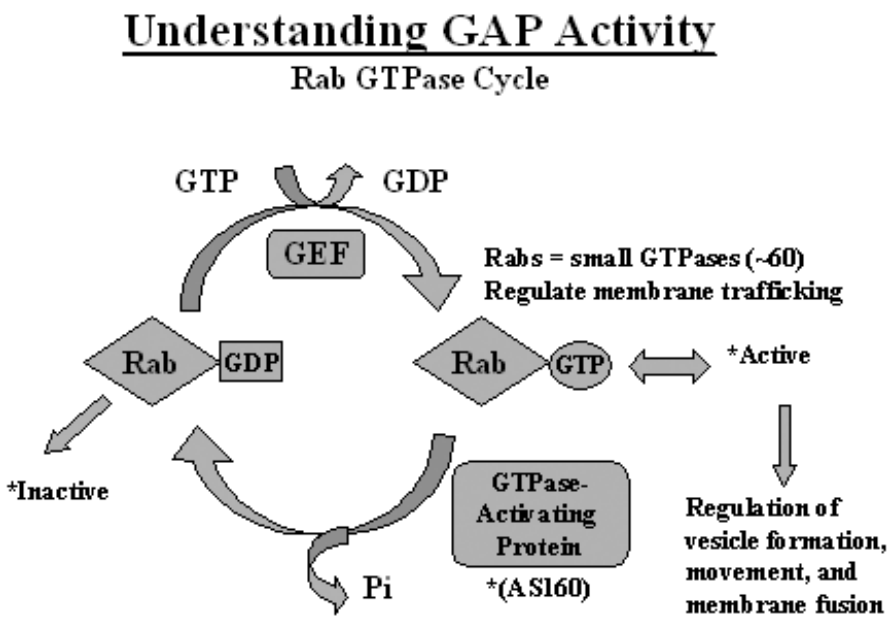


Figure 2. GTPase activating proteins such as AS160 modulate the activity of vesicle chaperones known as Rabs.

Rabs are considered active in their GTP bound state and inactive in their GDP bound state. AS160 contains a C-terminal Rab GTPase activating protein (GAP) domain (Figure 1), which has been demonstrated to be functional against specific Rab isoforms in vitro (61). Rab-GAPs such as AS160 catalyze the hydrolysis of Rab-GTP to inactive Rab-GDP (69). Hence, it is thought that the AS160 Rab-GAP domain tethers GLUT4 internally by keeping critical Rabs in their GDP-bound state (Figure 3) (88).

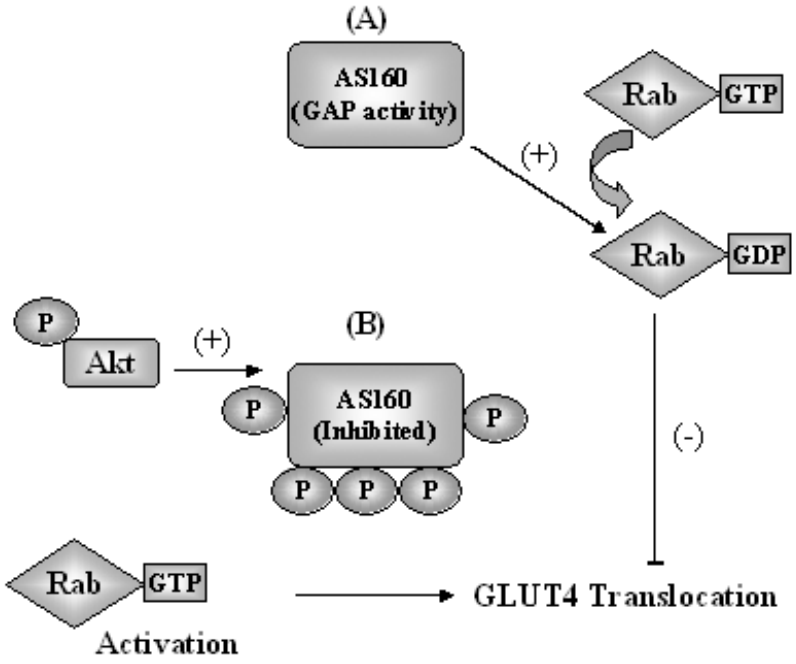


Figure 3. Unphosphorylated AS160 acts as a restraint on GLUT4 translocation by maintaining critical Rab proteins in their GDP-bound (inactive) state. However, phosphorylation of AS160 by upstream kinases such as Akt promotes inhibition of the AS160 Rab-GAP domain, and thereby permits GLUT4 translocation.

Experimental evidence supports this hypothesis. Rendering the AS160 Rab-Gap domain of 4P-mutant AS160 inactive by an arginine 973 to lysine point mutation (4P + R/K mutant) restores normal GLUT4 translocation in 3T3-L1 adipocytes (79) and L6 cells (82). Thus, when the AS160 Rab-GAP domain is disabled, phosphorylation of Akt recognition motifs is no longer necessary for insulin stimulated glucose uptake. Intriguingly, this suggests that phosphorylation of AS160 inhibits its Rab-GAP activity thereby allowing Rab-GTP(s) to stimulate GLUT4 translocation for increased glucose uptake (88). However, in 3T3-L1 adipocytes the mutation of the AS160 Rab-GAP domain alone does not result in increased GLUT4 translocation in the basal condition (79). Since mutation of the AS160 Rab-GAP domain by itself does not mimic insulin

signaling, AS160 is therefore necessary but not sufficient to stimulate increased GLUT4 translocation.

A yeast two-hybrid screen was recently used in conjunction with co-immunoprecipitation to identify an AS160 calmodulin-binding domain (CBD) positioned just amino terminal to the GAP domain (Figure 1) (45). Mutation of this region negated calmodulin-AS160 interaction, but did not interfere with the capacity of 4P-mutant AS160 to inhibit GLUT4 translocation in insulin-stimulated 3T3-L1 cells. It is meaningful to note that the association between calmodulin and AS160 was Ca^{2+}-specific (45). Therefore, while calmodulin binding does not appear to play a participatory role in AS160 mediation of insulin-stimulated GLUT4 translocation, it may instead function in GLUT4 translocation induced by muscle contractions.

Muscle Contraction Signaling to Glucose Transport

Muscle contractions and exercise also increase glucose uptake, but in a manner independent of and distinct from insulin (Figure 4). Whereas pharmacological inhibition of PI3-K by wortmannin abolishes insulin-stimulated GLUT4 translocation (57) and glucose uptake (55, 57, 96), wortmannin does not inhibit glucose uptake in isolated muscles incubated and contracted in vitro (55, 57, 96). Metabolic, chemical, and mechanical factors have all been implicated in contraction-mediated GLUT4 translocation, although the relative contribution of each of these mechanisms is likely dependent upon the duration, intensity, and frequency of the contractile stimulus. At the molecular level, multiple signaling cascades exhibit increased activity in response to muscle contraction. The AMP-activated protein kinase (AMPK), calmodulin-dependent

protein kinases (CaMK), and aPKC isoforms could all potentiate increases in glucose transport in response to exercise (6, 42, 94). In addition, data published by several laboratories indicates that Akt activity increases with muscle contractions (9, 26, 77, 78), but not through class I PI3-K (78). These findings are particularly compelling in light of an initial study that reported increased AS160 phosphorylation with in vitro contractions in rat epitrochlearis muscles (9). This raises the possibility that AS160 could operate as a common, downstream point of convergence mediating the effects of both insulin and contraction on GLUT4 translocation.

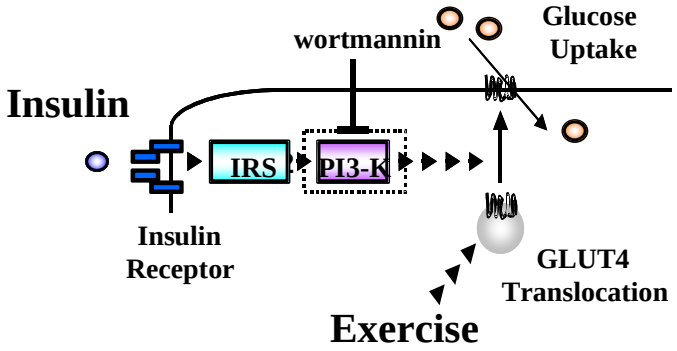


Figure 4. Insulin and exercise increase glucose uptake via GLUT4 translocation. The proximal signals leading to insulin- and exercise-stimulated glucose uptake in skeletal muscle are distinct. Whereas insulin initiates this metabolic effect through a signaling mechanism involving PI3-K and is susceptible to diabetes-induced impairment, exercise increases glucose uptake through PI3-K-independent signaling mechanisms that remain preserved in individuals with diabetes. IRS: insulin receptor substrate; PI3-K; phosphatidlyinositol 3-kinase; wortmannin: a pharmacological PI3-K inhibitor

Specific Aims of Doctoral Research

AS160 is currently recognized as the most distal regulatory molecule involved in insulin signaling to glucose uptake. AS160 phosphorylation is essential for full insulin-stimulated GLUT4 translocation in 3T3-L1 adipocytes (20, 54, 79, 98) and L6 cells (40,

82, 83). My preliminary studies in the Goodyear Laboratory have shown that both insulin and muscle contraction elicit AS160 phosphorylation events in mouse and rat skeletal muscle *in vivo*, and other laboratories have published comparable findings in vitro in rats. Despite these advances in our understanding of AS160 in muscle, major questions remain. My novel work suggests there are distinct upstream mediators of AS160 phosphorylation regulated by insulin and contraction; however, we have not identified the signaling proteins transducing this contraction-specific phenomenon. Furthermore, it is not known whether AS160 plays a regulatory role in insulin or muscle-contraction stimulated glucose uptake in adult skeletal muscle *in vivo*. Therefore, the major purposes of this dissertation are to understand the molecular kinases implicated in contraction-stimulated AS160 phosphorylation, and to then determine whether alterations in AS160 phosphorylation and/or activity ultimately affect glucose uptake into skeletal muscle.

Specific Aim #1: To characterize the signaling network(s) mediating increases in AS160 phosphorylation in response to muscle contraction in mouse skeletal muscle.

The upstream kinase(s) responsible for AS160 phosphorylation with muscle contraction have yet to be established. While Akt is known to phosphorylate AS160 at key regulatory phospho-motifs in cells in response to insulin (46, 79), it is possible that additional proteins participate in contraction-specific AS160 phosphorylation events as well. AMPK is a candidate kinase because it has been shown to phosphorylate substrates along consensus sequences similar to Akt (32) and is strongly activated by muscle contraction (31, 34). Furthermore, pharmacological activation of AMPK via AICAR

treatment increased AS160 phosphorylation in rat skeletal muscles probed with a phospho-Akt substrate antibody (9). For this aim, I employed genetic, pharmacological, and immunological strategies, alone and in combination, to systematically tease out the necessity of Akt and AMPK in contraction-stimulated AS160 phosphorylation (51).

Specific Aim #2: To determine the effects of expressing wild-type and mutant AS160 on basal, insulin- and contraction-stimulated glucose uptake in mouse skeletal muscle *in vivo*.

For this aim, I overexpressed wild type and mutant AS160 plasmid constructs by direct injection into the tibialis anterior (TA) muscle of mice followed by *in vivo* electroporation (52). This exciting gene transfer technique is described in detail in Chapter 2. The specific mutants subcloned and studied were: a) AS160 mutated at four PAS motifs, rendering these sites incapable of being phosphorylated (**4P mutant**); b) AS160 mutated at its Rab-GAP domain, effectively eliminating AS160 GAP activity (**R/K mutant**); c) AS160 double mutant carrying aforementioned 4P and R/K mutations (**2M mutant**); d) AS160 mutated within its calmodulin-binding domain (CBD), impeding the ability of the molecule to interact with calmodulin (**CBD mutant**); and e) another AS160 double mutant containing both the CBD and R/K mutations (**CBD + R/K mutant**). Subsequent measurement of glucose uptake *in vivo* using tracer methodology then allowed me to test the following hypotheses: a) AS160 phosphorylation at PAS motifs is required for full insulin and contraction-stimulated glucose uptake in mouse skeletal muscle. b) The GAP and calmodulin-binding domains within AS160 regulate insulin- and/or contraction-stimulated glucose uptake in mouse skeletal muscle.

CHAPTER 2

MATERIALS AND METHODS

Antibodies

Total AS160 was detected using an affinity-purified pan-AS160 antibody raised against the mouse C-terminal amino acid epitope (PTNDKAKAGNKP) (46, 79). AS160 phosphorylation was detected with a phospho-Akt substrate (PAS) antibody (Cell Signaling Technology; Danvers, MA), and a custom lot of anti-phospho-AS160 Thr^{642} antibody (#44-1071G; Biosource International; Camarillo, CA). This latter antibody was purified via epitope-specific chromatography and targets the peptide fragment RRRAH[pT]FSHPPS on AS160. Its specificity was validated using mutant AS160 incapable of phosphorylation at Thr^{642}. AMPK Thr^{172} phosphorylation (Biosource International) and phosphorylation of Akt Thr^{308} (Cell Signaling Technology) were also determined. Protein expression was assessed with anti-AMPK α2, anti-GLUT4, anti-GLUT1 (all Chemicon International), anti-Akt1/2 (Upstate), and anti-hexokinase II (Santa Cruz Biotechnology). A horseradish peroxidase-conjugated anti-rabbit antibody (Amersham Biosciences; Piscataway, NJ) was used to bind and visualize detections of all primary antibodies.

Animals

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. Female ICR mice 8-10 weeks old (25-30 grams) were purchased from Taconic (Hudson, NY). Male and female Akt2 knockout mice and

wild type littermates (C57BL/6N background; 10-14 weeks) were kindly provided by Dr. Morris Birnbaum (14) while α2-inactive (α2i) AMPK transgenic mice and wild type littermates (FVB background; 10-14 weeks) were generated as described previously (25). All mice were housed with a 12 hr:12 hr light:dark cycle and fed standard laboratory chow and water ad libitum. Mice were fasted overnight (10 pm to 8 am) prior to the morning of the experiment.

Methodology – Chapter 3

In vitro muscle incubations

Mice were sacrificed by cervical dislocation and extensor digitorum longus (EDL) muscles were rapidly dissected and preincubated in Krebs-Ringer bicarbonate (KRB) buffer + 2 mM pyruvate for 20 min, as described (78). Incubations were conducted in the absence or presence of maximal insulin (50 mU/mL) for 20 min, AICAR (2 mM) for 40 min, and/or contraction for 5 min (see below). For inhibition of PI3-kinase activity, muscles were co-incubated with 100 nM wortmannin dissolved in 0.05% DMSO. Muscles were immediately frozen in liquid N_2 following each treatment.

In vivo insulin administration

Fasted mice were assayed for basal blood glucose prior to intraperitoneal injections of 0.9% NaCl or maximal insulin (0.5U/mouse). After 10 min, blood glucose concentrations were measured to confirm an insulin response. Mice were cervically dislocated, and hindlimb muscles were immediately removed and frozen in liquid N_2.

Treadmill exercise

All mice were initially familiarized to the exercise protocol by running on a rodent treadmill over 3 separate days, during which they performed 10 min of exercise at 0.4 (day 1), 0.6 (day 2), and 0.8 (day 3) mph, with 0% grade. Three days later, mice were divided into basal or exercise groups. Exercised animals performed 30 min of running at 0.8 mph and 20% grade, or the equivalent of a moderate work pace, while basal animals remained sedentary. Mice were immediately sacrificed following the exercise or basal interval and gastrocnemius muscles were harvested and frozen in liquid N_2.

In situ and in vitro muscle contractions

Hindlimb muscles from anesthetized mice (pentobarbital 90 mg/kg) were contracted *in situ* by electrically stimulating sciatic or peroneal nerves, as described (15). While one leg was left unstimulated (basal/sham control), the other leg was subjected to contractions for 5 or 10 min. Mice were immediately sacrificed following the experiment and muscles were harvested and frozen in liquid N_2. *In vitro* tetanic contractions were induced for 5 min using established parameters (78). Force production was captured by IWORX 118 USB hardware (CB Sciences; Dover, NH) and analyzed with LabScribe software (CB Sciences). In experiments utilizing α2i transgenic mice, force production between transgenic and wild type littermates was normalized as done previously (25). Briefly, transgenic EDL was contracted using maximal *in vitro* stimulation settings (mean of 14 grams force/10 sec), while wild type muscles were only stimulated enough to match the force output generated by transgenic muscles (~50% of maximal force capacity). This precise voltage was determined using 2-3 brief contractions (pulse rate of 125 Hz for

180 ms) prior to the 5 min contraction protocol. Following contraction, muscles were immediately frozen in liquid N_2.

Tissue processing & immunoblotting

Frozen muscles were pulverized and homogenized as described previously (78), and protein concentrations were determined via Bradford assay. Equal amounts of skeletal muscle proteins (40-50 μg) were resolved by SDS-PAGE (53) for Western blot analysis (85). Antibody-bound proteins were visualized using enhanced chemiluminescence (Amersham Biosciences). Protein bands were scanned by ImageScanner (Amersham Biosciences) and quantitated by densitometry (Fluorchem2.0, Alpha Innotech; San Leandro, CA).

Methodology – Chapters 4 & 5

Plasmid DNA constructs

Human wild type (**WT**) AS160 DNA and four distinct mutant AS160 DNA constructs have been characterized previously in 3T3-L1 cells (46, 79). In addition, novel AS160 mutants were generated for critical experiments in chapter 5 (see DNA mutagenesis). The expressed mutant AS160 isoforms include: 1) AS160 mutated at four phospho-Akt substrate (PAS) motifs (Ser^{318}, Ser^{588}, Thr^{642}, and Ser^{751}), rendering these sites incapable of being phosphorylated (**4P**); 2) AS160 mutated to lysine at Arg^{973} within its Rab-GAP domain, effectively eliminating AS160 GAP activity (**R/K**); 3) AS160 double mutant containing both the 4P and R/K mutations (**2M**); 4) AS160 mutated to glycine at Leu^{842} and Trp^{843} within its calmodulin-binding domain (**CBD**), which

abolishes AS160 affinity for ca^{2+}/calmodulin; and 5) a novel AS160 mutant devoid of Rab-GAP activity and the ability to bind calmodulin (**CBD + R/K**). For the purposes of DNA injection and in vivo electroporation in adult mouse skeletal muscle, AS160 DNA was excised from the original CMV-10 vector, affixed to a myc-tag leader sequence via specific primers (see **Table 1**) and PCR amplification, and subcloned into pCAGGS plasmids. This expression vector drives a target gene under the CAG (cytomegalovirus immediate-early enhancer-chicken β-actin hydrid) promoter, and has been demonstrated to have high activity in skeletal muscle (24, 37). AS160 DNA constructs were confirmed for accuracy using the High-Throughput DNA Sequencing service at Brigham and Women's Hospital (Boston, MA). Plasmid DNA was then amplified in E. coli TOP10 cells (Invitrogen), extracted using an endotoxin-free Plasmid Mega Kit (Qiagen), and suspended in saline at [4 ug/ul].

Table 1. Primer pairs used for subcloning and sequencing human AS160 with N-terminal myc-tag

Composite Forward Primer (76mer):
5' AACTCGAGGCCACCATGGAGCAAAAGCTTATTTCTGAAGAGGACTT GATGGAGCCGCCCAGCTGCATTCAGGATGA **3'**
Composite Reverse Primer (*41mer*):
5' TTCTCGAGTTATGG CTTATTTCCTATCTTGGCTTTGTTGTT **3'**

Forward Sequencing Primer #1 (24mer): Coverage from 5' nucleotide 573 on human AS160
5' ATAATGAGGACGCCTTTTACAACT **3'**
Reverse Sequencing Primer #1 (23mer): Coverage from 3' nucleotide 646 on human AS160
5' AGCATAAAGACTGGGGCTGATTT **3'**

Forward Sequencing Primer #2 (26mer): Coverage from 5' nucleotide 1250 on human AS160
5' TTATGTATTCCAGTGTGCCAGCGAAT **3'**
Reverse Sequencing Primer #2 (24mer): Coverage from 3' nucleotide 1180 on human AS160
5' AATATCTTCCATATCACATCTGAT **3'**

Site-specific mutagenesis

Novel AS160 mutants were generated using the QuikChange XL Site-Directed Mutagenesis kit from Stratagene (La Jolla, CA). Briefly, myc-tagged mutant AS160 plasmids (CBD and 4P) served as parental templates and combined with specific mutagenic primers (see **Table 2**) to synthesize double mutants (CBD + R/K and CBD + 4P) via thermal cycling. Mutations were subsequently verified by DNA sequencing at Brigham and Women's Hospital.

Table 2. Primer pairs used for mutagenesis of myc-tagged CBD mutant AS160 and 4P mutant AS160 to create two additional double mutant forms of AS160

Double Mutant AS160 (CBD + R/K)
Start with CBD Mutant Template and Mutate GAP domain (R/K) with Following Primers:
Mutagenic Primer Sequences (33mer)
R973K Mutant Forward Primer:
5' CTCGTGGATTTAGGAAAGACGTTTCCTACTCAC **3'**
R973K Mutant Reverse Primer:
5' GTGAGTAGGAAACGTCTTTCCTAAATCCACGAG **3'**
Sequencing Primer Sequence (26mer)
R973K Mutant Sequencing Primer:
5' CAGAAAAGTTAAATTAGACTATGAAG **3'**

Double Mutant AS160 (4P + CBD)
Start with 4P Mutant Template and Mutate CBD w/Following Primers:
Mutagenic Primer Sequences (42mer)
CBD Mutant Forward Primer:
5' CAAAAGAACTGAGGAGCGGGGGGAGAAAAGCTATACACCAAC **3'**
CBD Mutant Reverse Primer:
5' GTTGGTGTATAGCTTTTCTCCCCCCGCTCCTCAGTTCTTTTG **3'**
Sequencing Primer Sequence (24mer)
CBD Mutant Sequencing Primer:
5' ACTGCCCCCTCTTTCCTGAAAAGC **3'**

In vivo gene transfer in mouse skeletal muscle

Enriched yields of AS160 plasmid clones were directly injected into mouse tibialis anterior (TA) muscles by a protocol first described by Aihara and Miyazaki (2) and modified in our laboratory (24, 37, 93). Mice were anesthetized with an

intraperitoneal injection of pentobarbital sodium (90 mg/kg), and 100 μg of DNA (empty pCAGGS or AS160 constructs) was injected longitudinally into the tibialis anterior muscle with a 29-gauge needle. Stainless steel electrode needles fixed 4 mm apart were then inserted into the transverse muscle belly and square-wave electrical pulses (200 V/cm) were applied eight times at a rate of one pulse per second (duration 20 ms) using a Grass S88 pulse generator (Grass Instruments, Quincy, MA). Our lab has demonstrated that transfection efficiency with the LacZ gene under this protocol is ~85%, and manifests relatively homogenously throughout the muscle (24).

In situ muscle contraction following gene transfer

Mice were transfected with identical DNA constructs in each tibialis anterior muscle. Seven days after DNA injection, mice were anesthetized with intraperitoneal administration of pentobarbital sodium (90 mg/kg BW). Peroneal nerves from both legs were surgically exposed for electrode placement. While one leg was left unstimulated (basal/sham control), 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).

Intravenous glucose injections following gene transfer

Mice were transfected with different DNA constructs in each tibialis anterior muscle. Seven days after DNA injection, mice were anesthetized with pentobarbital sodium (90 mg/kg) and administered a saline or 20% glucose bolus [1.0 grams glucose/kg BW] through the retroorbital sinus. This dosage stimulates a physiologic

insulin response (from ~0.82 to ~3.4 ng/ml spike within 5 min), followed by persistent but declining concentrations at 15-min post-injection, (see Figure 1, obtained from the DERC Specialized Assay Core Insulin ELISA) and does not induce significant hypoglycemia (19, 92).

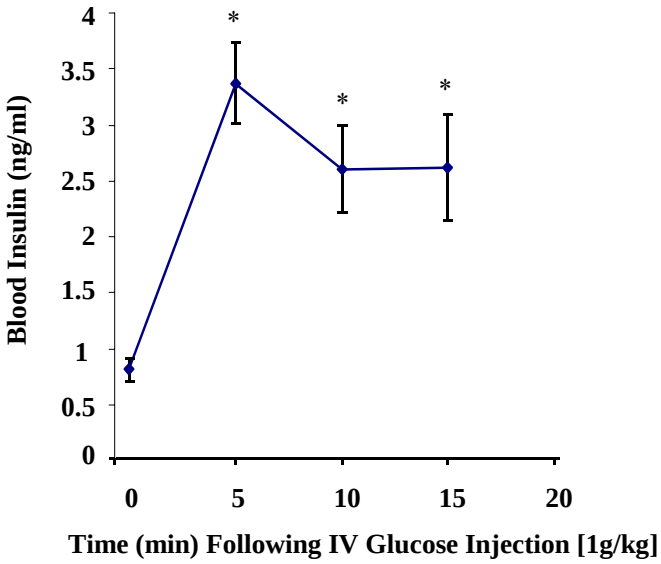


Figure 1. Plasma insulin is significantly increased following intravenous glucose injection in female ICR mice 8 weeks of age. Mice were anesthetized with pentobarbital sodium (90 mg/kg) and administered a saline or 20% glucose bolus [1 gram glucose/kg bodyweight] through the retroorbital plexus. Blood was collected from the tail vein prior to each injection (time 0) and at 5, 10, and 15 min post-injection. Plasma insulin concentrations were assayed via ELISA through the Joslin Diabetes Center DERC Specialized Assay Core. * $p<0.05$ versus 0 min (pre) insulin concentration.

In vivo skeletal muscle glucose uptake

Baseline blood samples were collected from the tail vein prior to intravenous delivery of [^{3}H]-2-deoxyglucose through the retroorbital vein. This tracer bolus was combined with the unlabelled glucose or saline solution in glucose injection experiments,

and occurred simultaneous with the onset of *in situ* peroneal nerve stimulation in contraction studies. For all treatments, blood samples were taken from the tail vein at 5, 10, 15, 25, 35, and 45 minutes post-injection for the determination of blood glucose and [^{3}H]-2-deoxyglucose specific activity. After collection of the final blood sample, animals were euthanized and tibialis anterior muscles were removed and frozen in liquid nitrogen. Accumulation of [^{3}H]-2-deoxyglucose in pulverized tissue was determined via a precipitation protocol adapted from Ferre et al. (22) using barium hydroxide/zinc sulfate and perchloric acid. Small amounts of the muscle were set aside and used for immunoblots.

Glycogen determination

Glycogen content of frozen, pulverized muscle was determined by HCl hydrolysis followed by NaOH neutralization (66). Resultant free glucosyl concentration was determined spectrophotometrically using a hexokinase-based assay kit (Sigma, St. Louis, MO).

Modified tissue processing and immunoblotting protocol

Frozen muscle tissue was homogenized with a Polytron (Brinkman Instruments) in chilled lysis buffer (20 mM Tris, pH 7.5, 5 mM EDTA, 10 mM $Na_4P_2O_7$, 100 mM NaF, 2 mM $NaVO_4$, 10 µM leupeptin, 3 mM benzamidine, 10 µg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Three-fourths of this homogenate was aliquoted for measurement of glucose uptake (see above). The remaining one-fourth was additionally processed with detergent (1% Nonidet P-40), rotated end over end at 4 ºC for 1 hr, and

then centrifuged at 14,000 x g for 15 min at 4 ºC. The supernatants were isolated and protein concentrations were determined via Bradford assay as described. Equal amounts of skeletal muscle proteins (40-50 µg) were resolved by SDS-PAGE (53) for Western blot analysis (85). Antibody-bound proteins were visualized using enhanced chemiluminescence (Amersham Biosciences). Protein bands were scanned by ImageScanner (Amersham Biosciences) and quantitated by densitometry (Fluorchem2.0, Alpha Innotech; San Leandro, CA).

Immunoprecipitation of myc-tagged AS160

Protein lysates (3-5 mg/ml) were placed in low-binding centrifuge tubes with 10 µl washed protein G beads. Tubes rotated end-over-end for 2 hours at 4º C as a pre-clearing measure to reduce IgG contamination. Samples were centrifuged for 15 seconds at 12,000 x g, and the supernatants were deposited in fresh low-binding centrifuge tubes containing 0.5-2.5 µg anti-myc antibody. Tubes then rotated end-over-end for 16 hours at 4º C, followed by addition of 20 ml washed protein G beads and another 2 hour incubation at 4º C. Beads were washed 3x in 500µl-1000 µl of the lysis buffer used to prepare the sample. After the final wash, the pellet was thoroughly aspirated and directly spotted with 5 µl of 1 µg/µl BSA, 25 µl 4x Laemmli's buffer, and heated at 80 Cº for 3 minutes. The purified anti-myc-derived precipitates were then 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 calmodulin binding assay (CBA) buffer (50 mM Tris–Cl, pH 7.5, 200 mM NaCl, and 50 mM $MgCl_2$, 4% BSA and 0.5 mM $CaCl_2$) for 2 hrs, and then treated with 100 ng/ml biotinylated calmodulin (Calbiochem, #208697) in CBA buffer for 2 hrs. After incubation with calmodulin, membranes were washed in CBA buffer containing 0.05% Tween 20 (5 x 15 min) followed by incubation with Streptavidin-HRP (1:5,000) in CBA buffer for 1 h. Membranes were washed and calmodulin association was detected using enhanced chemiluminescence. To determine total AS160 protein, membranes were subsequently stripped in CBA buffer containing the calcium chelator EGTA (5 mM) instead of $CaCl_2$ for 2 hrs and then incubated in the presence of anti-AS160 as described for standard Western analysis.

Statistical analysis

Data are expressed as means ± SE. For chapter 3, statistical analyses were performed using a paired Student's t test and one-way analysis of variance. For chapters 4 and 5, statistical analyses were performed using one- and two-way analysis of variance. When analysis of variance revealed significant differences, Tukey's post hoc test for multiple comparisons was performed. P-values less than 0.05 were considered statistically significant.

CHAPTER 3

DISTINCT SIGNALS REGULATE AS160 PHOSPHORYLATION IN RESPONSE TO INSULIN, AICAR, AND CONTRACTION IN MOUSE SKELETAL MUSCLE

Abstract

Insulin and contraction increase GLUT4 translocation in skeletal muscle via distinct signaling mechanisms. Akt substrate of 160 kDa (AS160) mediates insulin-stimulated GLUT4 translocation in L6 myotubes, presumably through activation of Akt. Using *in vivo*, *in vitro* and *in situ* methods, insulin, contraction, and the AMPK activator AICAR all increased AS160 phosphorylation in mouse skeletal muscle. Insulin-stimulated AS160 phosphorylation was fully blunted by wortmannin *in vitro*, and in Akt2 knockout mice *in vivo*. In contrast, contraction-stimulated AS160 phosphorylation was only partially decreased by wortmannin and unaffected in Akt2 knockout mice, suggesting additional regulatory mechanisms. To determine if AMPK mediates AS160 signaling, we used AMPKα2-inactive transgenic mice. AICAR-stimulated AS160 phosphorylation was fully inhibited, whereas contraction-stimulated AS160 phosphorylation was partially reduced in the AMPKα2-inactive transgenic mice. Combined AMPKα2 and Akt inhibition by wortmannin treatment of AMPKα2 transgenic mice did not fully ablate contraction-stimulated AS160 phosphorylation. Maximal insulin together with either AICAR or contraction increased AS160 phosphorylation in an additive manner. In conclusion, AS160 may be a point of convergence linking insulin, contraction, and AICAR signaling. While Akt and AMPKα2 activities are essential for AS160 phosphorylation by insulin and AICAR, respectively, neither kinase is indispensable for the entire effects of contraction on AS160 phosphorylation.

Background

Insulin and exercise share the capacity to increase glucose uptake into skeletal muscle and positively regulate glucose homeostasis in healthy individuals and people with type 2 diabetes. Each stimulus, alone and in combination, promotes migration of intracellular GLUT4 proteins to the cell membrane, thereby facilitating glucose uptake from blood into muscle for storage and/or utilization (29, 49, 83). Insulin exerts these partitioning effects through a well-characterized signaling network involving its transmembrane insulin receptor (IR), insulin receptor substrate (IRS) proteins, and downstream engagement of phosphatidylinositol 3-kinase (PI3-K) (23, 28). Briefly, activity of PI3-K catalyzes the formation of phosphatidylinositol 3,4,5-triphosphates (PIP_3), which in turn recruits the serine/threonine kinase Akt to the cell membrane where

it can be phosphorylated by phosphoinositide-dependent kinase-1 (PDK-1) (16, 83). In addition, both PI3-K and its lipid products directly activate atypical protein kinase C (aPKC) isoforms (21). The resultant pools of activated Akt (5), particularly the Akt2 isoform (89), as well as aPKC (21) have been proposed to be critical for insulin-mediated GLUT4 translocation from endosomal storage sites to the sarcolemma. However, the intermediate substrates connecting these signals with the GLUT4 machinery are only beginning to emerge.

Akt Substrate of 160 kDa (AS160) is a Rab GTPase activating protein (GAP) that modulates GLUT4 trafficking in insulin-sensitive 3T3-L1 adipocytes (79) and L6 myoblasts (83). The most striking structural features of AS160 are two phosphotyrosine binding domains, a C-terminal Rab-GAP domain, as well as six phosphorylation motifs (RXRXXS*/T*) targeted by, but not exclusive for, Akt (Ser^{318}, Ser^{341}, Ser^{570}, Ser^{588}, Thr^{642}, Ser^{751}) (46, 79). In addition, a yeast two-hybrid screen recently identified an AS160 calmodulin-binding region positioned just amino terminal to the GAP domain (45). Under basal conditions, AS160 exists primarily in an unphosphorylated state and retains GLUT4 vesicles intracellularly through the activity of its GAP domain (20, 61). When cells are treated with insulin, AS160 is rapidly phosphorylated at multiple Akt phospho-motifs (46). Both 3T3-L1 adipocytes (79) and L6 GLUT4-myc myoblasts (83) transfected with a constitutively active mutant AS160 (incapable of being phosphorylated at these regulatory sites) exhibit significantly reduced insulin-induced GLUT4 translocation. AS160 phosphorylation therefore appears to suppress and/or alter the intrinsic GAP activity of the protein such that exocytosis of GLUT4 vesicles is permitted.

Muscle contractions and exercise also potently stimulate glucose uptake, but in a manner independent of and distinct from insulin (29, 49). This insulin-independent mechanism is critical in the maintenance of whole body glucose homeostasis, especially in people with type 2 diabetes where there are defects in insulin action but normal or near normal effects on exercise on glucose disposal. Multiple metabolic, chemical, and mechanical factors have been implicated in contraction-mediated GLUT4 translocation, though pinpointing a primary mechanism has remained elusive. A growing body of literature suggests that increases in the activity of AMP-activated protein kinase (AMPK), calmodulin-dependent protein kinases (CaMK), and aPKC isoforms could all potentiate increases in glucose transport in response to exercise (6, 42, 94). In addition, Akt activity can increase with muscle contractions but not through class IA PI3-K (78). These findings are compelling in light of a recent study that reported increased AS160 phosphorylation with in vitro contractions in rat epitrochlearis muscles (9). This raises the possibility that AS160 operates as a common, downstream point of convergence mediating the effects of both insulin and contraction on GLUT4 translocation.

While Akt is known to phosphorylate AS160 at key regulatory phospho-motifs, it is possible that additional kinases phosphorylate AS160 at these sites and/or contraction-specific phosphorylation sites. For example, AMPK is a plausible candidate since it recognizes and phosphorylates substrates along consensus sequences similar to Akt (60, 72, 80), and AICAR treatment in rat muscle increases AS160 phosphorylation (9). In the current study, we first assessed the regulation of AS160 in response to insulin, contraction, and AICAR in mouse skeletal muscle. Once this was established, our

primary objective was to determine the signaling network(s) mediating skeletal muscle AS160 phosphorylation.

Results

Insulin-Stimulated AS160 Phosphorylation

We initially determined whether insulin regulates AS160 phosphorylation in isolated mouse skeletal muscle using two different antibodies. First, phosphorylation of AS160 at known insulin-responsive motifs was detected with the phospho-Akt substrate (PAS) antibody, which recognizes as many as six sites on AS160 bearing the amino acids RXRXXS*/T* (where "*" indicates residue of phosphorylation) (46, 79). Note that PAS immunoreactivity is not exclusive for Akt phosphorylation events, as other arginine sensitive kinases are able to phosphorylate substrates along similar PAS-detectable sequences (32, 60, 63, 80, 92). In addition, samples were also immunoblotted with a modification-state antibody, specific for phosphorylation at the AS160 Thr^{642} residue in mouse skeletal muscle. This latter motif is recognized by both antibodies and has been demonstrated to be the primary target of Akt in insulin-treated 3T3-L1 cells (73, 79). *In vitro* insulin treatment significantly increased AS160 phosphorylation in isolated mouse extensor digitorum longus (EDL) muscle using both anti-phospho-AS160 antibodies ($p<0.05$; Figure 1A & 1B). To understand whether the insulin-stimulated increase in AS160 phosphorylation *in vitro* is mediated through activation of Akt, we co-treated EDL muscles with insulin and the PI3-kinase inhibitor wortmannin (100 nM). Wortmannin completely inhibited insulin-stimulated Akt phosphorylation at Thr^{308} (Figure 1C) and concomitantly suppressed AS160 phosphorylation (Figure 1A & 1B).

These results demonstrate the involvement of Akt in insulin-induced AS160 phosphorylation *in vitro* in mouse skeletal muscle.

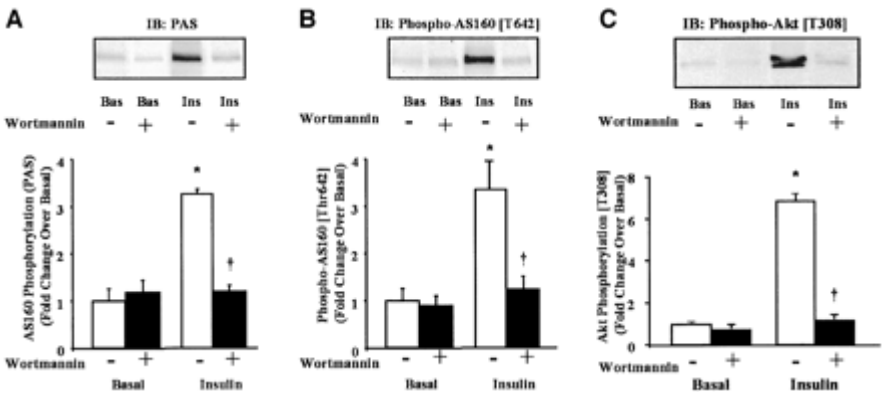


Figure 1. Effects of *in vitro* insulin on AS160 phosphorylation in EDL muscle. EDL muscles were incubated in the absence (-) or presence (+) of 100 nM wortmannin as described in Materials and Methods. Immunoblotting (IB) was with anti-PAS (*A*) anti-phospho-AS160 Thr^{642} (*B*), and anti-phospho-Akt Thr^{308} (*C*) antibodies. Data are means ± S.E.; $n = 6$/group.*, $p < 0.05$ (*versus* basal).

We next assessed whether insulin stimulates AS160 phosphorylation in skeletal muscle *in vivo* in normal ICR mice. Ten minutes following maximal insulin injection, there was a significant increase in AS160 phosphorylation detected with both antibodies ($p<0.05$; Figure 2A & 2B). In order to discern the upstream mechanism(s) underlying these phosphorylation events, we utilized wild type (WT) and Akt2 knockout (KO) littermates. Akt2 KO mice are characterized by specific whole-body deletion of Akt2, the isoform primarily responsible for insulin-stimulated glucose transport in skeletal muscle (5; 6). Insulin-stimulated AS160 phosphorylation was significantly blunted in Akt2 KO gastrocnemius muscle (Figure 2C & 2D) and reflected the phosphorylation status of Akt (data not shown). There were no alterations in pan-AS160 expression between wild type and Akt2 KO muscle (Figure 2C & 2D), nor differences in proximal

insulin signaling to IRS-1 and PI3-kinase (77). Taken together with the wortmannin data, these findings strongly support a model whereby Akt functions as the major upstream kinase regulating insulin-induced AS160 phosphorylation in mouse skeletal muscle.

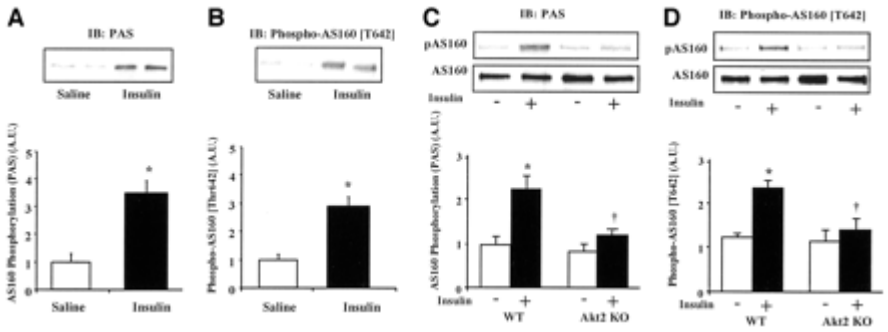


Figure 2. Effects of *in vivo* insulin on AS160 phosphorylation in gastrocnemius muscle. Fasted ICR mice (*A,B)*, and wild type and Akt2 knockout littermates (*C,D*) received saline or maximal intraperitoneal insulin injections and studied 10 min later. Immunoblotting (IB) was with anti-PAS (*A,C*), anti-phospho-AS160 Thr^{642} (*B,D*), and pan-AS160 (*C,D*) antibodies. Data are means ± S.E.; *n* = 6-8/group.*, $p < 0.05$ (*versus* basal);.†, $p < 0.05$ (*versus* wild type).

Contraction-Stimulated AS160 Phosphorylation

We next examined whether contraction alters AS160 phosphorylation in mouse skeletal muscle using treadmill exercise, *in situ* contraction, and *in vitro* contraction protocols (Figure 3A-C). Moderate intensity treadmill running (Figure 3A) and in situ muscle contractions (Figure 3B) increased AS160 phosphorylation detected by immunoblots with both antibodies, although the magnitude of PAS-detected increases was significantly more pronounced than that detected with the phospho-Thr^{642}-specific probe (~2.5-fold versus ~1.7-fold). High intensity tetanic contractions *in vitro* in isolated mouse EDL muscles (Figure 3C) produced greater increases in AS160 phosphorylation, ~3-fold over basal with both antibodies. Overall, each model of muscle

contraction/exercise increased AS160 Thr^{642} phosphorylation and potentially multiple PAS motifs that are also regulated by insulin stimulation.

In order to determine whether Akt mediates contraction-induced AS160 phosphorylation, we stimulated EDL muscles to contract *in vitro* in the absence or presence of wortmannin. Our laboratory and other groups have reported enhanced Akt phosphorylation with contraction in skeletal muscle (9, 26, 77, 78). As shown in Figure 3D, 5 min of tetanic contractions elicited significant increases in Akt Thr^{308} phosphorylation ($p<0.05$), which was abolished in the presence of wortmannin. In contrast, wortmannin only partially reduced contraction-stimulated AS160 phosphorylation detected with PAS (Figure 3E), and did not significantly decrease phosphorylation at Thr^{642} (Figure 3F). Thus, even in the absence of upstream Akt activity, AS160 is still phosphorylated in response to muscle contractions *in vitro*.

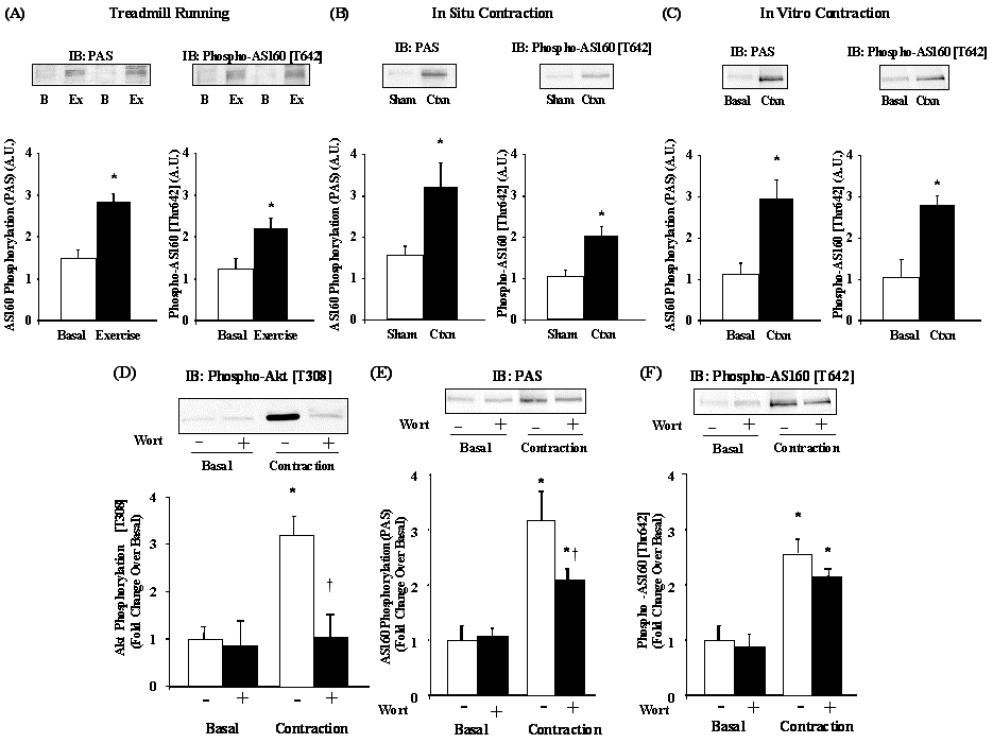


Figure 3. Effects of exercise/contractions on AS160 phosphorylation in mixed gastrocnemius and EDL muscles. (*A*): Mice performed moderate treadmill exercise for 30 min (ex) or remained sedentary (basal), after which gastrocnemius muscles were studied for signaling. *(B*): One gastrocnemius muscle from anesthetized mice contracted *in situ* (ctxn) for 10 min, while the contralateral leg served as a sham-operated control (sham). *(C,D,E,F*): EDL muscles were incubated (-) or (+) 100 nM wortmannin for 30 min, and thereafter rested (basal) or stimulated to contract for 5 min (ctxn). Immunoblotting (IB) was with anti-phospho-Akt Thr^{308} (*D*), anti-PAS (*A,B,C,E*), and anti-phospho-AS160 Thr^{642} *(A,B,C,F)* antibodies. Data are means ± S.E.; *n* = 5-8/group.*, $p < 0.05$ (exercise/contraction *versus* basal). †, $p < 0.05$ (*versus* contraction alone).

In addition to determining the effects of Akt inhibition *in vitro*, we also performed *in vivo* studies to examine whether contraction-mediated signaling to AS160 is preserved in the absence of Akt2 (Figure 4). Wild type and Akt2 KO mice were stimulated to generate hindlimb contractions *in situ*. As a control for contraction efficacy, phosphorylation of the contraction-sensitive kinase AMPK at Thr^{172} was significantly increased in both wild type and Akt2 KO mice (Figure 4A). Akt Thr^{308} phosphorylation was significantly increased in wild type but not Akt2 KO mice (Figure 4B). Despite the lack of Akt Thr^{308} phosphorylation in the Akt2 KO mice, both genotypes responded to contraction with comparable increases in AS160 phosphorylation ($p<0.05$) using both antibodies (Figure 4C & 4D). These data indicate that Akt activation cannot fully explain contraction-induced AS160 phosphorylation, in contrast to insulin. Another contraction-sensitive kinase(s) appears to converge upon and phosphorylate AS160 at key regulatory phospho-motifs.

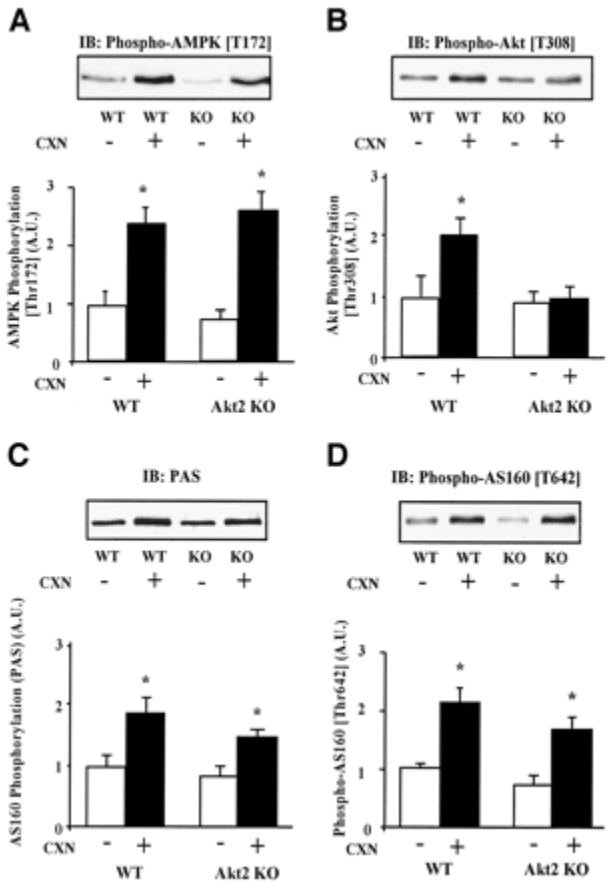


Figure 4. Effects of in situ contraction on AS160 phosphorylation in Akt2 KO muscle. Anesthetized wild-type and Akt2 KO tibialis anterior muscles were stimulated to contract *in situ*. Immunoblotting (IB) was with anti–phospho-Akt Thr^{308} (*A*), anti–phospho-AMPK Thr^{172} (*B*), anti-PAS (*C*), and anti–phospho-AS160 Thr^{642} (*D*) antibodies. Data are means ± SE; n = 5–6/group. *$P < 0.05$ vs. sham.

AICAR-Stimulated AS160 Phosphorylation

Our results, both *in vitro* and *in vivo*, provide evidence for the existence of an Akt-independent mechanism for contraction-stimulated AS160 phosphorylation. To determine whether AMPK regulates AS160 phosphorylation *in vitro*, we incubated isolated EDL muscle in the absence or presence of the AMPK activator AICAR (Figure 5). As expected, AICAR significantly increased AMPK Thr^{172} phosphorylation (Figure 5A) but had no effect on Akt phosphorylation (Figure 5B). AS160 phosphorylation

detected with the anti-PAS antibody revealed significant increases (Figure 5C), but there was not significant increase in phosphorylation when probing with the anti-phospho-AS160 Thr^{642} antibody (Figure 5D). Moreover, PAS-detectable increases in AS160 phosphorylation were not affected by wortmannin. These data suggest that AICAR-stimulated increases in AS160 phosphorylation apparently occur at serine-terminal PAS phospho-motifs and are wortmannin-insensitive.

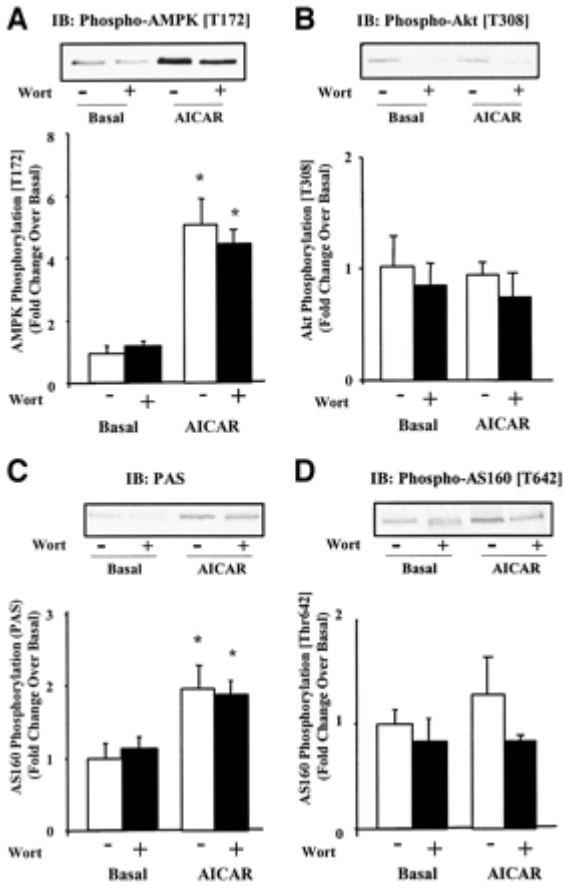


Figure 5. Effects of *in vitro* wortmannin (Wort) and AICAR on AS160 phosphorylation in EDL muscle. EDL muscles were incubated (-) or (+) 100 nmol/l wortmannin for 20 min and thereafter rested (Basal) or treated with 2 mmol/l AICAR for 40 min (AICAR). Immunoblotting (IB) was with anti–phospho-AMPK Thr^{172} (*A*), anti–phospho-Akt Thr^{308} (*B*), anti-PAS (*C*), and anti–phospho-AS160 Thr^{642} (*D*) antibodies. Data are expressed as means ± SE; n = 6–8/group. *$P < 0.05$ vs. basal.

In order to investigate whether AMPK α2 mediates AICAR-induced AS160 phosphorylation, we utilized wild-type mice and transgenic littermates overexpressing a skeletal muscle-specific α2-inactive (α2i) AMPK. These mice lack virtually all AMPK catalytic activity in skeletal muscle and exhibit no AICAR-stimulated glucose transport (25). AICAR incubation significantly increased AS160 phosphorylation detected by PAS immunoblotting wild type EDL muscles, but this effect was fully blunted in AMPK α2i transgenic muscles (Figure 6A). AICAR did not increase AS160 Thr^{642} phosphorylation in either wild type or α2i AMPK transgenic mice (Figure 6B). These data strongly suggest that AMPK activation directly or indirectly regulates AS160 serine-terminal PAS phosphorylation events associated with AICAR stimulation.

Contraction-Stimulated AS160 Phosphorylation in AMPK α2i Transgenic Mice

Based on our AICAR data, we next investigated whether contraction-stimulated AS160 phosphorylation is similarly abolished in α2i AMPK transgenic mice. We contracted tibialis anterior muscles from wild type and α2i AMPK transgenic mice *in situ* via peroneal nerve stimulation. This contraction protocol increases AMPK α2 activity by 1.5-fold in wild type mice, an effect completely abolished in α2i AMPK transgenic mice (25). Conversely, this protocol does not increase AMPK α1 activity in wild type (25, 87) or AMPK α2i transgenic mice (25). Contraction significantly increased AS160 phosphorylation in both wild type and transgenic mouse tibialis anterior muscle in anti-PAS immunoblots (Figure 6C). However, the increased AS160 phosphorylation in α2i AMPK transgenics was significantly reduced compared to wild type littermates ($p<0.05$). Phosphorylation at AS160 Thr^{642} exhibited a similar pattern, although transgenic mice

only showed a trend toward increases in AS160 phosphorylation (Figure 6D). Total AS160 protein expression was not different between wild type and transgenic mice (Figure 6C and D). Thus, AMPK α2 appears to be a primary regulator of contraction-specific AS160 phosphorylation events in mouse skeletal muscle, although the absence of AMPK α2 activity does not eliminate all contraction-stimulated increases in AS160 phosphorylation.

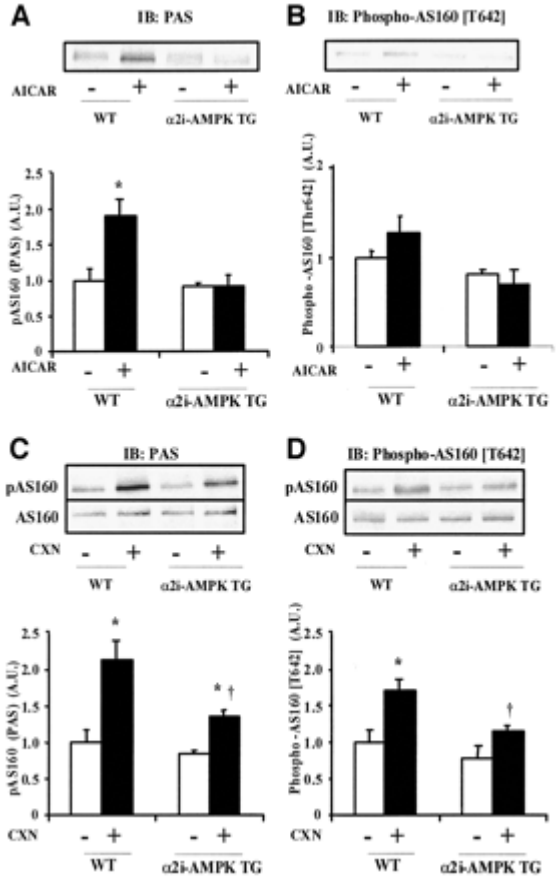


Figure 6. Effects of *in vitro* AICAR and *in situ* contraction on AS160 phosphorylation in α2i AMPK transgenic (α2i-AMPK TG) muscle. *A* and *B*: EDL muscles were incubated (-) or (+) 2 mmol/l AICAR for 40 min. *C* and *D*: Tibialis anterior muscles were stimulated to contract in situ. Immunoblotting (IB) was with anti-PAS (*A* and *C*), anti–phospho-AS160 Thr^{642} (*B* and *D*), and anti–pan-AS160 antibodies. Data are means ± SE; n = 4–8/group. *$P < 0.05$ vs. basal/sham; †$P < 0.05$ vs. wild type (WT).

Removal of AMPK α2 catalytic activity decreases contraction-stimulated AS160 phosphorylation by ~60% whereas abolished Akt activity reduces contraction-stimulated PAS-detectable phosphorylation by 10-30%. We next examined whether contraction-mediated signaling to AS160 is completely inhibited when Akt and AMPK α2 activities are simultaneously blocked. EDL muscles from wild type and α2i AMPK transgenic mice were stimulated to contract *in vitro* in the absence or presence of 100 nM wortmannin. Although the mean AS160 phosphorylation assessed by PAS (Figure 7A) or Thr^{642} immunoblotting was ~5-10% lower with combined Akt and AMPK α2 inhibition, these were not significantly different from the effects of AMPK α2 inhibition alone. Control immunoblots of Akt phosphorylation (Figure 7C) confirm both the efficacy of the inhibitor as well as the contraction stimulus. Collectively, these results suggest that AMPK α2 is largely responsible for contraction-mediated AS160 phosphorylation at regulatory PAS motifs, although other kinases may also account for some residual increases in AS160 phosphorylation following contraction.

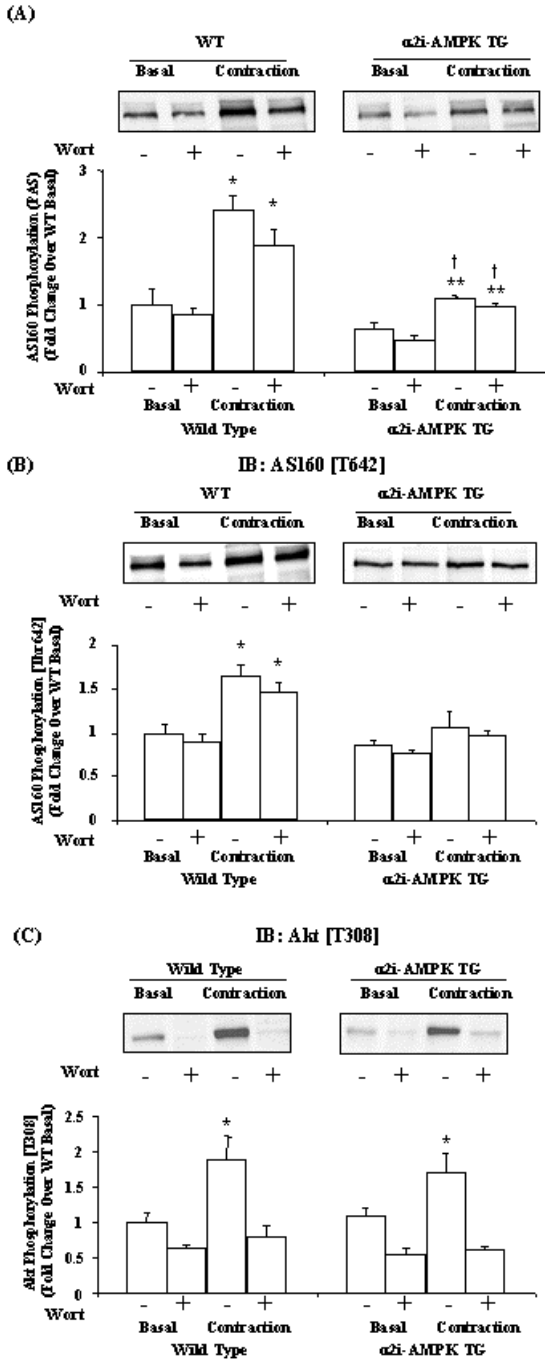


Figure 7. Effects of *in vitro* wortmannin on contraction-stimulated AS160 phosphorylation in α2i AMPK transgenic (α2i-AMPK TG) muscle. EDL muscles were incubated (-) or (+) 100 nmol/l wortmannin for 30 min and thereafter rested (Basal) or stimulated to contract for 5 min (Contraction). Immunoblotting (IB) was with anti-PAS (*A*), anti–phospho-AS160 Thr^{642} (*B*), and anti–phospho-Akt Thr^{308} (*C*) antibodies. Data are means ± SE; $n = 3$–4/group. *$P < 0.05$ vs. wild-type (WT) basal; **$P < 0.05$ vs. TG basal; †$P < 0.05$ vs. wild-type contraction.

Additive Effects of Insulin and AICAR or Contraction on AS160 Phosphorylation

AICAR-stimulated AS160 phosphorylation appears to occur at serine-terminal phospho-motifs detected with PAS, but not at Thr^{642}. Meanwhile, insulin and contraction treatments result in significant increases in Thr^{642} phosphorylation and possibly other PAS motifs. We therefore determined whether combined AICAR + insulin and contraction + insulin treatments generate greater increases in AS160 phosphorylation compared to either stimulus alone. Figures 8A and 8B show that AMPK Thr^{172} and Akt Thr^{308} phosphorylation were significantly increased, as expected, by AICAR + insulin, respectively ($p<0.05$). When samples were probed with anti-PAS (Figure 8C), the combined AICAR + insulin treatment resulted in the greatest magnitude of AS160 phosphorylation, with a significant increase above the independent effects of AICAR or insulin. In contrast, the increased AS160 phosphorylation at Thr^{642} observed with combined AICAR + insulin closely resembled the effect of insulin alone (Figure 8D). AS160 phosphorylation also increased in an additive manner during combined contraction + insulin treatment (Figures 8E and 8F). EDL muscles incubated in insulin and then stimulated to contract in vitro (5-min) exhibited significantly greater increases in AS160 phosphorylation compared to contraction or insulin treatment alone, as assessed with both antibodies. Thus, independent effectors of skeletal muscle glucose metabolism appear to converge upon AS160 PAS motifs.

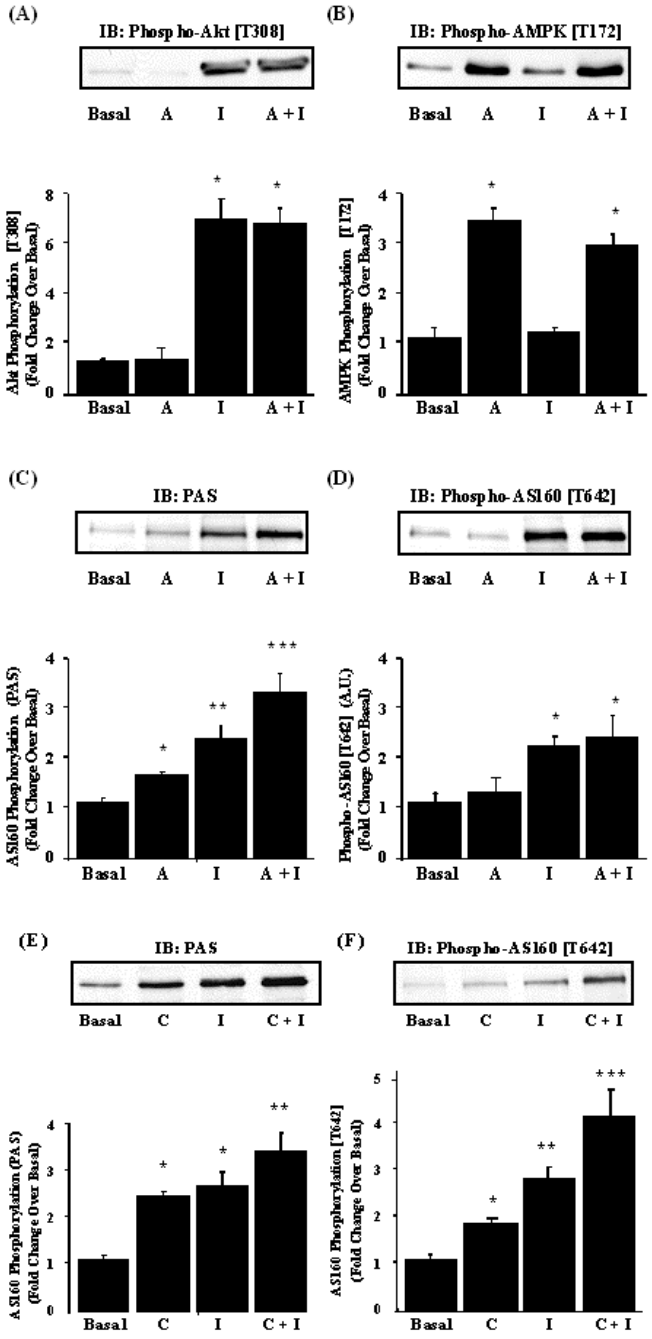


Figure 8. Effects of combined *in vitro* treatments involving AICAR, insulin, and contraction on AS160 phosphorylation. EDL muscles were incubated with nothing (Basal), AICAR (A), insulin (I), combined AICAR + insulin (*A*+ I) or stimulated to contract (C) ± insulin (I) treatment. Immunoblotting (IB) was with anti–phospho-Akt Thr^{308} (*A*), anti–phospho-AMPK Thr^{172} (*B*), anti-PAS (*C* and *E*), and anti–phospho-AS160 Thr^{642} (*D* and *F*) antibodies. Data are means ± SE; n = 6–11/group. *$P < 0.05$ vs. basal; **$P < 0.05$ vs. AICAR or contraction; ***$P < 0.05$ vs. all other conditions.

Discussion

The purpose of this study was to determine whether independent Akt and AMPK signaling pathways converge on the novel regulator of insulin-stimulated glucose transport, the Rab-GAP AS160. Our findings demonstrate that insulin, contraction, and AICAR stimulate AS160 phosphorylation through distinct upstream signaling mechanisms in mouse skeletal muscle. Using Akt2 knockout mice and α2i AMPK transgenic mice, we implicate convergent regulation of AS160 PAS phospho-motifs by both Akt and AMPK. Elucidation of the mechanisms regulating effectors of glucose transport common to both insulin and contraction signaling may yield information valuable for the treatment of type 2 diabetes.

Several important insights emerge from our data regarding the signaling mechanisms responsible for AS160 phosphorylation in mouse skeletal muscle. First, insulin increases AS160 phosphorylation at PAS motifs in an Akt-dependent manner both *in vivo* and *in vitro*. Second, exercise and muscle contractions also increase AS160 phosphorylation at PAS sites, principally through enhanced AMPK α2 activity. However, AMPK α2 alone does not account for all contraction-stimulated elevations. Third, AICAR increases AS160 phosphorylation in an AMPK α2-dependent manner and only at serine-terminal PAS motifs. Fourth, combined treatment of insulin + AICAR and insulin + contraction have additive effects on PAS-detectable AS160 phosphorylation.

A previous report in rat epitrochlearis muscle *in vitro* (9) has shown that wortmannin inhibits insulin-stimulated AS160 phosphorylation, and our findings using mouse muscle are consistent with these results. Wortmannin is a PI3-K inhibitor that blocks insulin-stimulated Akt phosphorylation, GLUT4 translocation, and ultimately

glucose transport in skeletal muscle (55). As a PI3-K inhibitor, wortmannin could potentially abolish the activity of other PI3-K-responsive proteins that may interact with AS160, in addition to Akt. Our use of Akt2 knockout mice provides strong evidence that Akt2, and not other wortmannin-sensitive kinases, is the primary regulator of insulin-stimulated AS160 phosphorylation in skeletal muscle. Interestingly, Birnbaum and colleagues have shown that Akt2 acts as the principle isoform regulating glucose transport in insulin-sensitive tissues (4, 14). Absence of Akt2 results in impaired skeletal muscle and hepatic insulin sensitivity, effects that cannot be compensated for by residual or overexpressed Akt1 and/or 3 activities (43). Our data suggest impairments in AS160 phosphorylation might be involved in the skeletal muscle insulin-resistant phenotype that characterizes Akt2 knockout mice at submaximal [insulin]. Consistent with this hypothesis, type 2 diabetes in humans is associated with reduced insulin-stimulated AS160 phosphorylation (48).

The molecular mediators governing contraction-stimulated AS160 phosphorylation are more obscure, although our data suggest the AMPK system is a major contributory mechanism. We found a modest effect of wortmannin on contraction-stimulated AS160 phosphorylation in mouse skeletal muscle. These data differ from the full inhibition of contraction-stimulated AS160 phosphorylation with wortmannin reported previously (9). Differences in species (rat vs. mouse), muscle fiber-type composition (epitrochlearis vs. EDL), and immunoblotting may account for the divergent results. It is important to note, however, that wortmannin has no effect on contraction-stimulated glucose uptake in muscle (42, 55). If phosphorylation of AS160 is a central event for contraction-induced glucose transport in skeletal muscle, then our data

indicating partial preservation of AS160 phosphorylation with concomitant contractions and wortmannin treatment appear reasonable. The dispensable nature of Akt2 for contraction-stimulated AS160 phosphorylation in vivo was confirmed in experiments employing Akt2 KO mice, since muscle contractions stimulated comparable increases in AS160 phosphorylation in both wild type and Akt2 KO mice. This Akt2-independent means of signaling to AS160 may reflect the ability of Akt2 KO mice to increase glucose transport normally in response to contraction (77).

AMPK is one of multiple proteins that potently respond to contraction in skeletal muscle (31, 34, 42), and both AMPK and Akt phosphorylate substrates along similar anti-PAS-detectable epitopes (32, 72, 80, 92). AICAR, a pharmacological activator of AMPK, increases AS160 phosphorylation at serine-terminal PAS motifs in both rat (9) and mouse skeletal muscle. Our use of α2-inactive AMPK transgenic mice demonstrated that these AICAR-induced AS160 phosphorylation events require AMPK α2 activity. Intriguingly, many established AMPK substrates are phosphorylated along PAS-like epitopes ending in the amino acid serine. In addition, PFK-2 (72) and Raf-1 (80) specifically contain PAS phosphorylation sites known to be targeted by both AMPK and Akt. AMPK may thus directly or indirectly phosphorylate AS160 at one or more serine-terminal PAS motifs.

Evidence from α2i AMPK transgenic mice also suggests an important role for AMPK in contraction-specific AS160 phosphorylation. α2i AMPK transgenic mice exhibit extreme diminutions in contraction-stimulated AS160 phosphorylation compared to wild type littermates. However, subtle elevations in PAS-detectable AS160 phosphorylation still occur in response to contraction, indicating other pathways may

account for the residual or compensatory phosphorylation. Although we cannot rule out AMPK α1 activity as a source for AS160 phosphorylation in α2i AMPK transgenic mice, we think it is unlikely because the contraction protocol used does not increase α1 activity (25, 87) and because AICAR-stimulated AS160 phosphorylation is fully inhibited in α2i AMPK transgenic mice despite normal AMPK α1 activation (25). Another putative upstream kinase family are atypical PKCs, which phosphorylate substrate sequences that are consistent with the PAS epitope (63) and are known to be activated by exercise in skeletal muscle (6). Thus, while downstream targets of PKC mediating glucose transport are not currently known, it is certainly plausible that AS160 is one such substrate. Scansite analysis of mouse AS160 reveals multiple domains and phospho-motifs distinct from PAS (65). Indeed, absence of an observable increase in AS160 phosphorylation in immunoblots with anti-PAS or anti-phospho-AS160 Thr^{642} does not preclude the possibility of alternative phosphorylation events on AS160 with contraction.

In conclusion, the mechanisms leading to insulin-, AICAR-, and contraction-stimulated AS160 phosphorylation in mouse skeletal muscle are distinct. Whereas insulin-stimulated AS160 phosphorylation is mediated by Akt, AICAR-stimulated AS160 phosphorylation occurs exclusively through the AMPK system. Contraction-stimulated AS160 phosphorylation events involve AMPK, possibly Akt, and other undefined kinase(s). Overall, all three stimuli increase AS160 phosphorylation at phospho-motifs recognized to be pivotal in L6 myotubes for insulin-stimulated GLUT4 translocation. That AS160 and its phosphorylation status could be a central point of convergence for both insulin and contraction makes it an attractive molecule for prospective pharmacological or genetic manipulation.

CHAPTER 4

AS160 REGULATES INSULIN- AND CONTRACTION-STIMULATED GLUCOSE UPTAKE IN MOUSE SKELETAL MUSCLE

Abstract

Insulin and contraction are potent stimulators of GLUT4 translocation and increase skeletal muscle glucose uptake. We recently identified the Rab-GAP AS160 as a putative point of convergence linking distinct upstream signaling cascades induced by insulin and contraction in mouse skeletal muscle. Here, we studied the functional implications of these AS160 signaling events by using an *in vivo* electroporation technique to overexpress wild type (WT) and three AS160 mutants in mouse tibialis anterior muscles: 1) AS160 mutated to prevent phosphorylation on 4 regulatory phospho-Akt-substrate (PAS) sites (4P); 2) AS160 mutated to abolish Rab GTPase activity (R/K); and 3) double mutant AS160 containing both 4P and R/K mutations (2M). One week following gene injection, protein expression for all AS160 isoforms was elevated over 7-fold. To determine the effects of AS160 on insulin- and contraction-stimulated glucose uptake in transfected muscles, we measured ^{3}H-2-deoxyglucose uptake in vivo following intravenous glucose administration and *in situ* muscle contraction, respectively. Insulin-stimulated glucose uptake was significantly inhibited in muscles overexpressing 4P mutant AS160. However, this inhibition was completely prevented by concomitant disruption of AS160 Rab-GAP activity. Transfection with 4P mutant AS160 also significantly impaired contraction-stimulated glucose uptake, as did overexpression of wild type AS160. In contrast, overexpressing mutant AS160 lacking Rab-GAP activity resulted in increases in both sham and contraction-stimulated muscles. These data suggest that AS160 regulates both insulin- and contraction-stimulated glucose metabolism in mouse skeletal muscle *in vivo*, and that the effects of mutant AS160 on the actions of insulin and contraction are not identical. Our findings directly implicate AS160 as a critical convergence factor for independent stimulators of skeletal muscle glucose uptake.

Background

Skeletal muscle insulin resistance is a salient feature of type 2 diabetes. In humans and other mammals, skeletal muscle normally accounts for approximately 75% of whole body insulin-stimulated glucose transport (7, 17, 99). Impaired ability of the muscle to respond to insulin is therefore disruptive to systemic glucose homeostasis. Skeletal muscle also possesses contractile properties that can effectively restore glucose control in an insulin-independent manner, and this element is preserved in individuals with type 2 diabetes (29, 49). While the precise mechanisms remain elusive, it is clear that both insulin and contraction signals converge upon GLUT4 vesicles and promote their appearance at the cell membrane (42, 50).

Akt substrate of 160 kDa (AS160) is a Rab GTPase activating protein (GAP) shown to regulate GLUT4 translocation in insulin-sensitive 3T3-L1 adipocytes (79) and L6 myoblasts (83). In addition to its Rab-GAP domain, AS160 also contains two phosphotyrosine binding domains and multiple putative phosphorylation sites, including six phospho-Akt substrate (PAS) motifs (RXRXXS*/T*) targeted by Akt, AMPK and potentially other upstream kinases (51). Under basal conditions, AS160 has been shown to retain GLUT4 vesicles intracellularly through the activity of its GAP domain in 3T3-L1 cells (20, 54). GLUT4 vesicles are dynamic complexes that constantly migrate/recycle along cytoskeletal elements, and are directed by GTP-bound Rab proteins and other molecular chaperones (83, 88). Thus, AS160 GAP activity could inactivate a critical, still unidentified Rab protein as part of the mechanism for controlling basal GLUT4 trafficking (88). When cells are treated with insulin, however, AS160 is rapidly phosphorylated at PAS motifs (46) and dissociates from GLUT4 vesicles (54). This is associated with accelerated rates of GLUT4 vesicular exocytosis, such that GLUT4 manifests predominantly at the cell surface and enhances glucose transport (98).

Both 3T3-L1 adipocytes and L6 GLUT4-myc myoblasts transfected with a constitutively active AS160 incapable of being phosphorylated at four PAS regulatory motifs (4P mutant) exhibit significantly reduced insulin-induced GLUT4 translocation (79, 83). AS160 phosphorylation therefore appears to function in a permissive role by decreasing the GAP activity of the protein such that exocytosis of GLUT4 vesicles is allowed. Supporting this hypothesis, inactivation of the Rab-GAP domain via point mutation of Arginine^{973} to lysine appears to restore insulin-stimulated GLUT4

translocation in adipocytes co-expressing phosphorylation site-specific mutations in AS160 (2M) (79).

There are currently no reports establishing a regulatory role for AS160 on skeletal muscle glucose metabolism. Data published by our lab (51) and others (9) indicates that AS160 phosphorylation at PAS motifs occurs following both insulin and contraction in skeletal muscle. Furthermore, these phosphorylation events are regulated in a distinct and potentially additive manner by insulin-stimulated Akt2 and contraction-stimulated AMPKα2 activities (51). It is plausible, then, that AS160 is a common, downstream point of convergence mediating the effects of both insulin and contraction on skeletal muscle glucose uptake. The purpose of this study was to determine the effects of wild type and mutant AS160 on basal, insulin- and contraction-stimulated glucose uptake in mouse skeletal muscle *in vivo*. Specifically, we overexpressed wild type and three different mutant AS160 DNA constructs by direct injection into the tibialis anterior (TA) muscle of mice followed by *in vivo* electroporation. Mutant constructs included: 1) AS160 mutated to prevent phosphorylation on 4 regulatory phospho-Akt-substrate (PAS) sites (4P); 2) AS160 mutated to inhibit Rab GTPase activity (R/K), and 3) a double mutant AS160 containing both 4P and R/K mutations (2M). Measurements of glucose uptake *in vivo* using tracer methodology suggest that AS160 phosphorylation at PAS motifs is required for full insulin- and contraction-stimulated glucose uptake in mouse skeletal muscle. The observed inhibition with 4P mutant overexpression was dependent upon the Rab-GAP activity of AS160, since muscles transfected with mutant AS160 coexpressing 4P and Rab-GAP mutations (2M) exhibited normal insulin-induced glucose uptake and increased glucose uptake following contraction. Basal skeletal muscle

glucose uptake in sham-operated transfected muscles was also significantly increased by Rab-GAP mutant overexpression (R/K and 2M) compared to empty vector and wild-type AS160 controls. Thus, AS160 directly regulates insulin- and contraction-stimulated uptake in mouse skeletal muscle. Furthermore, the effects of wild-type and mutant AS160 on the actions of insulin and contraction are not identical and suggest distinct modes of regulation.

Results

Overexpression of AS160 in Transfected Mouse Skeletal Muscle

We initially determined the expression of recombinant wild type (WT) AS160 and 4P, R/K, and 2M mutant AS160 isoforms using our gene transfer approach. AS160 DNA injection and in vivo electroporation induced consistent 6- to 8-fold increases in AS160 protein after seven days in mouse tibialis anterior muscles (Figure 1). As expected, the magnitude of overexpression was comparable across all constructs, since each AS160 variant was subcloned into the same pCAGGS vehicle. Note that the pan-AS160 antibody detected both the recombinant myc-tagged AS160 (upper band) and endogenous mouse AS160 (lower band). Both AS160 protein bands were used in densitometry quantifications. Overexpressed AS160 did not silence or enhance expression of endogenous AS160 in experimental muscles. Furthermore, AS160 overexpression was only detected in tibialis anterior muscles, as the adjacent extensor digitorum longus muscles exhibited no increases in AS160 (data not shown). These results validate the efficacy of localized DNA injections as a means of overexpressing wild type or altered gene products into skeletal muscle tissue.

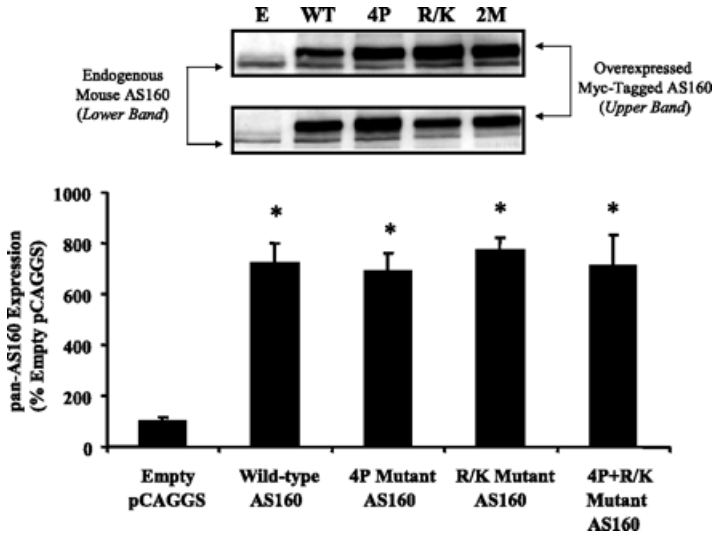


Figure 1. **Gene injection and *in vivo* electroporation of AS160 in mouse tibialis anterior muscles results in significant overexpression after 7 days.** Empty pCAGGS vector (*E*) or Myc-tagged AS160 DNA constructs (wild type and 4P, R/K, and 2M mutants) were injected into the tibialis anterior muscles of anesthetized mice, followed by *in vivo* electroporation. The animals were allowed to recover, and recombinant protein expression was assessed 7 days post-injection. The muscle proteins were resolved by SDS-PAGE and immunoblotted with anti-pan-AS160 antibody. The data are expressed as the means ± S.E.; n = 12–16/group. *, $p < 0.05$ (*versus* empty pCAGGS controls).

Phosphorylation of Overexpressed AS160

We next examined the phosphorylation of overexpressed AS160 at Thr^{642}, a critical PAS motif targeted by Akt and other kinases (Figure 2A), as well as collective phosphorylation events at all PAS sites via the phospho-Akt substrate antibody (Figure 2B). Basal phosphorylation of overexpressed WT and R/K AS160 at Thr^{642} was significantly increased compared to empty vector (E) controls. However, 4P and 2M mutant AS160, which express point mutations at Thr^{642} and three other PAS motifs, exhibited no discernible increases in basal Thr^{642} phosphorylation compared to empty vector controls. Both contraction and insulin stimulated endogenous AS160 Thr^{642}

phosphorylation, and further increased Thr^{642} phosphorylation of overexpressed WT and R/K AS160. These trends were similarly observed in immunoblots with the PAS antibody (2B). Here, electrophoretic optimization (8% gel; 50:1 ratio acrylamide:bisacrylamide) enabled clear discernment of upper (exogenous) and lower (endogenous) AS160 protein phosphorylation. This demonstrates that recombinant myc-tagged AS160 is phosphorylated *in vivo*, and that the integrity of the AS160 point mutations (4P and 2M) is preserved following gene expression.

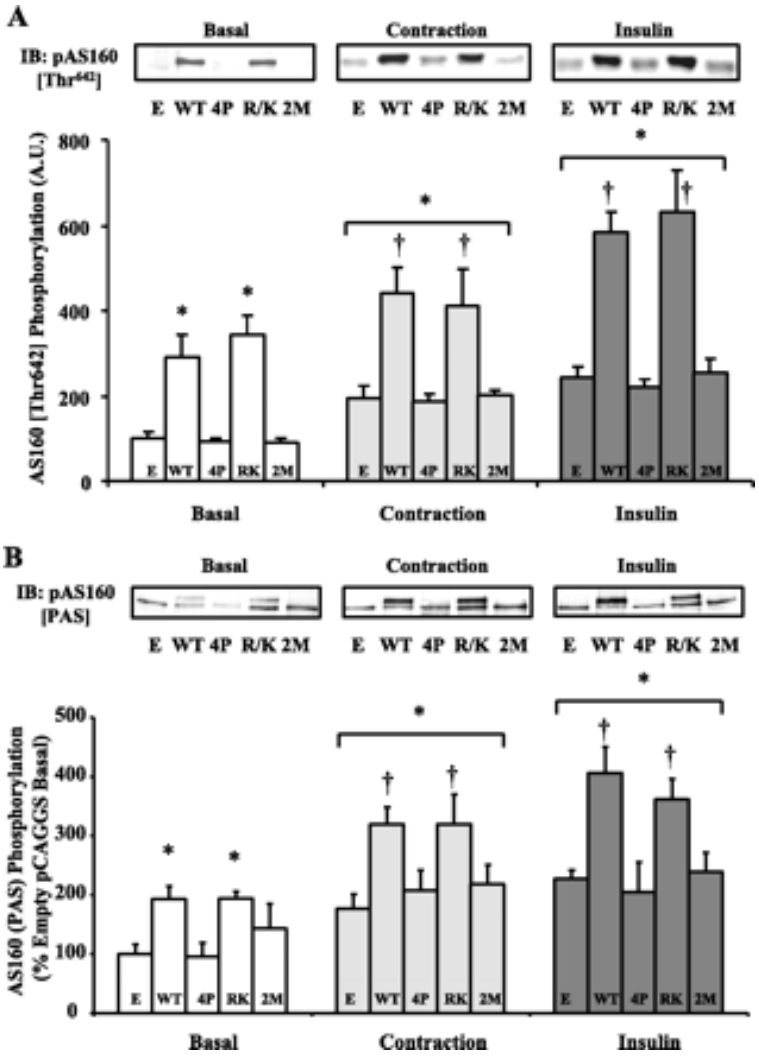
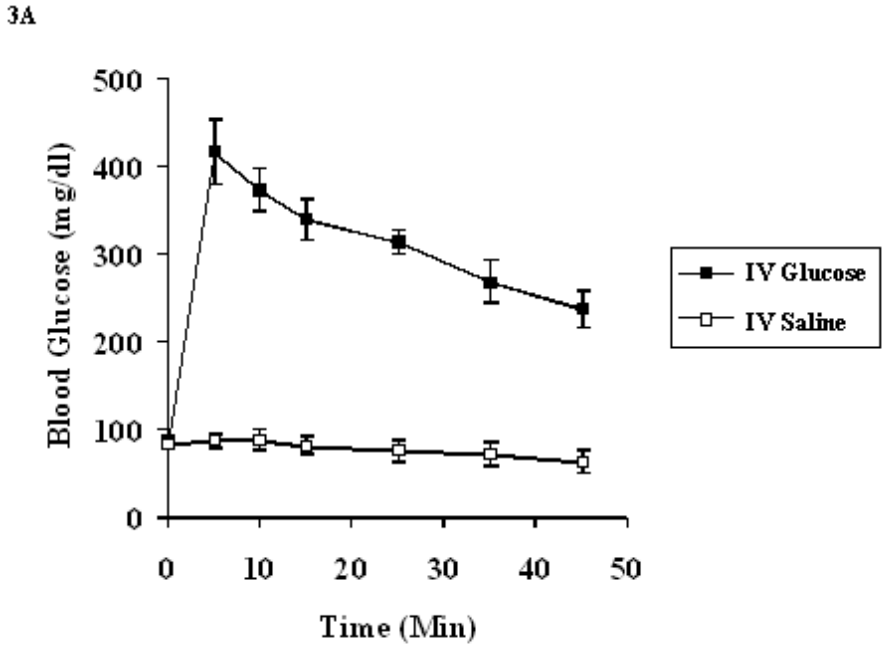


Figure 2. Phosphorylation of overexpressed AS160 in transfected muscles following basal or contraction- or insulin-stimulated conditions. Empty pCAGGS vector (*E*) or Myc-tagged AS160 DNA constructs (wild type and 4P, R/K, and 2M mutants) were injected into the tibialis anterior muscles of anesthetized mice, followed by *in vivo* electroporation. The animals recovered for 1 week and then were stimulated to contract *in situ* (15 min) or received intravenous glucose (1 g/kg). The mice were immediately sacrificed following the experimental protocol, and tibialis anterior muscles were harvested and processed for signaling. *A*, proteins were resolved by 10% SDS-PAGE (37.5:1 acrylamide:bisacrylamide) and immunoblotted (*IB*) with an anti-phospho-AS160 Thr^{642} antibody. *B*, proteins were resolved by 8% SDS-PAGE (50:1 acrylamide:bisacrylamide) to increase electrophoretic separation of high molecular weights, and immunoblotted with an anti-PAS antibody. This strategy enabled crisp discernment of upper (exogenous) and lower (endogenous) AS160 protein bands. Basal and contraction- and insulin-stimulated samples were run together in the same gels; however, because of the 15 different treatment conditions, it was not possible to load all of the treatments in a single gel. Therefore, internal loading controls were placed in all of the gels to enable comparisons from blot-to-blot. The images are cropped to elicit clarity, and the quantitations represent the normalized aggregate of six pairs of gels. The data are expressed as the means ± S.E. (n = 6–12/group). *, $p < 0.05$ (*versus* empty pCAGGS basal). †, $p < 0.05$ (*versus* empty pCAGGS for respective condition).

AS160 Regulates Insulin-Stimulated Glucose Uptake

Although AS160 appears to be involved in insulin-mediated GLUT4 translocation in cellular models (20, 54, 79, 83), there is currently no data concerning the role of AS160 on glucose metabolism in adult skeletal muscle tissue. Intravenous glucose injections were conducted in fasted mice to induce hyperglycemia (Figure 3A) and an associated physiologic insulin response (~4-fold increase in circulating concentrations within 5-min) (supplementary data, (19, 92)) while glucose uptake into injected muscles was measured *in vivo* using radio-labeled tracer. Blood glucose curves reflect aggregate data from all electroporated mice, and are graphed as a single line because there were no significant differences between transfected animals. The regulatory effects of overexpressed WT and mutant AS160 on insulin-stimulated glucose uptake are shown in Figure 3B. Muscles overexpressing 4P mutant AS160 exhibited significant decreases

(~33%) in insulin-stimulated glucose uptake compared to both empty vector and overexpressed WT controls. Interestingly, AS160 co-expressing a mutated Rab-GAP domain (2M) prevented the inhibitory effects of the 4P mutations alone and fully restored insulin-stimulated glucose uptake. These changes occurred concomitantly with normal insulin-stimulated signaling to Akt Thr^{308} across all muscles (Figure 3C). No significant differences in insulin-stimulated glucose uptake were observed between empty vector controls and overexpressed WT, R/K, and 2M AS160. In addition, there were no significant alterations in glucose uptake between injected muscles in mice administered saline as a control. These data suggest that phosphorylation of AS160 at PAS motifs is required for full insulin-stimulated glucose uptake in vivo in adult skeletal muscle.



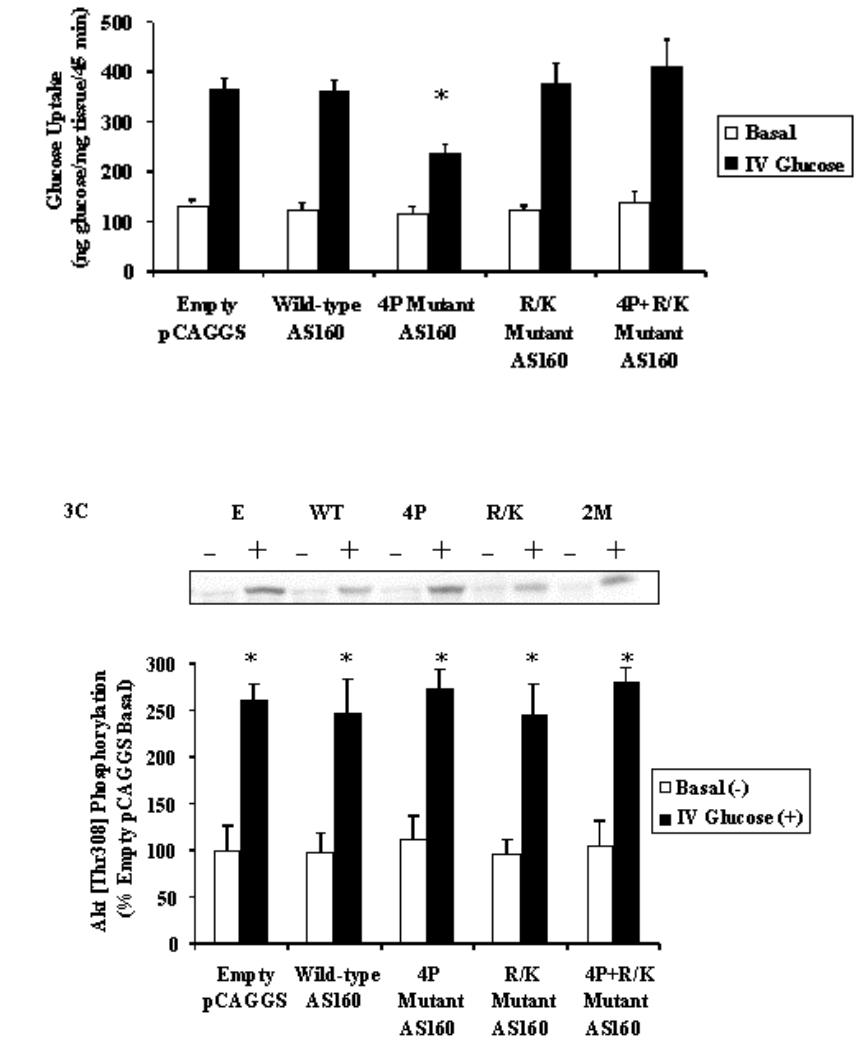


Figure 3. AS160 overexpression regulates insulin-stimulated glucose uptake without affecting Akt Thr^{308} phosphorylation in transfected mouse skeletal muscles. Empty pCAGGS vector (*E*) or Myc-tagged AS160 DNA constructs (wild type and 4P, R/K, and 2M mutants) were injected into the tibialis anterior muscles of anesthetized mice, followed by *in vivo* electroporation. The animals were allowed to recover, and basal and insulin-stimulated glucose uptake were assessed *in vivo* 7 days post-injection. The blood glucoses were evaluated in all electroporated mice at 0, 5, 10, 15, 25, 35, and 45 min following [^{3}H]2-deoxyglucose injection combined with either saline or glucose bolus (1.0 grams glucose/kg of body weight) (*A*). Absolute skeletal muscle glucose uptake was determined 45 min after saline/glucose injections (*B*). The data are expressed as the means ± S.E. (n = 6–24/group). *, $p < 0.05$ (*versus* insulin-stimulated empty pCAGGS controls). In addition, muscles from basal (–) and insulin-stimulated conditions (+) were immunoblotted for antiphospho-Akt Thr^{308} (*C*). The data are expressed as the means ± S.E. (n = 5–12/group). *, $p < 0.05$ (*versus* basal).

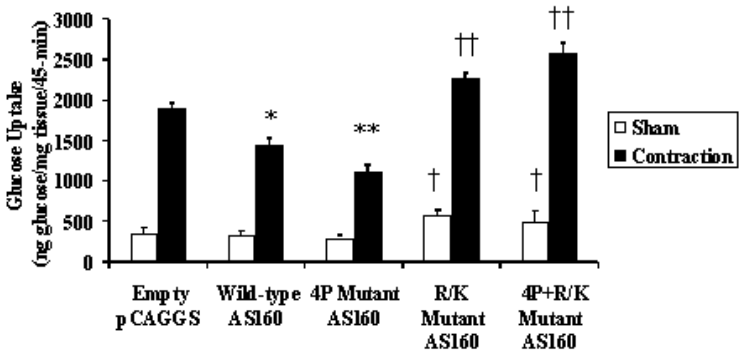
AS160 Regulates Contraction-Stimulated Glucose Uptake

Exercise and muscle contractions stimulate AS160 phosphorylation at PAS motifs through a wortmannin-insensitive pathway involving AMPK (51). However, it is not known whether these AS160 phosphorylation events, in turn, regulate contraction-stimulated glucose uptake. Therefore, we explored the effects of overexpressed WT AS160 and mutant AS160 on glucose uptake following 15-min of *in situ* contractions in transfected mouse tibialis anterior muscles (Figure 4A). Muscles overexpressing 4P mutant AS160 exhibited significant impairments (~44%) in contraction-stimulated glucose uptake compared to empty vector controls. Contraction-stimulated glucose uptake *in vivo* was also inhibited (~24%) in muscles overexpressing wild type AS160, although it was still significantly higher (20%) than in 4P-overexpressing muscles. In contrast, in vivo glucose uptake after contraction was significantly increased in muscle overexpressing mutant AS160 devoid of Rab-GAP activity (21% and 40% for R/K and 2M, respectively). It is important to note that contraction-stimulated phosphorylation of Akt Thr^{308} (Figure 4B) and AMPK Thr^{172} (Figure 4C) was normal and not significantly different between muscles. These results suggest that AS160 phosphorylation at PAS motifs is essential for full contraction-stimulated glucose uptake in skeletal muscle. Overexpression of constitutively active (4P) AS160 exerted a dominant-inhibitory effect on glucose uptake following muscle contractions *in vivo*, while overexpression of AS160 lacking Rab-GAP activity surprisingly enhanced skeletal muscle glucose uptake.

Significant differences in basal glucose uptake were observed between sham-operated muscles. Under these conditions, muscles overexpressing Rab-GAP mutant AS160 exhibited significant increases in basal glucose uptake compared to empty vector

controls (71% and 49% for R/K and 2M AS160, respectively), without detectable changes in fasting glycogen content (data not shown). There were no differences between empty vector, WT, and 4P mutant injected muscles on basal glucose uptake. Taken together, overexpression of mutant AS160 devoid of Rab-GAP activity appears to regulate both basal and contraction-stimulated glucose uptake *in vivo*. The combined regulatory effects that AS160 exerts on both insulin- and contraction-stimulated glucose uptake with in vivo transfections are consistent with a role for AS160 on molecular localization or trafficking.

4A



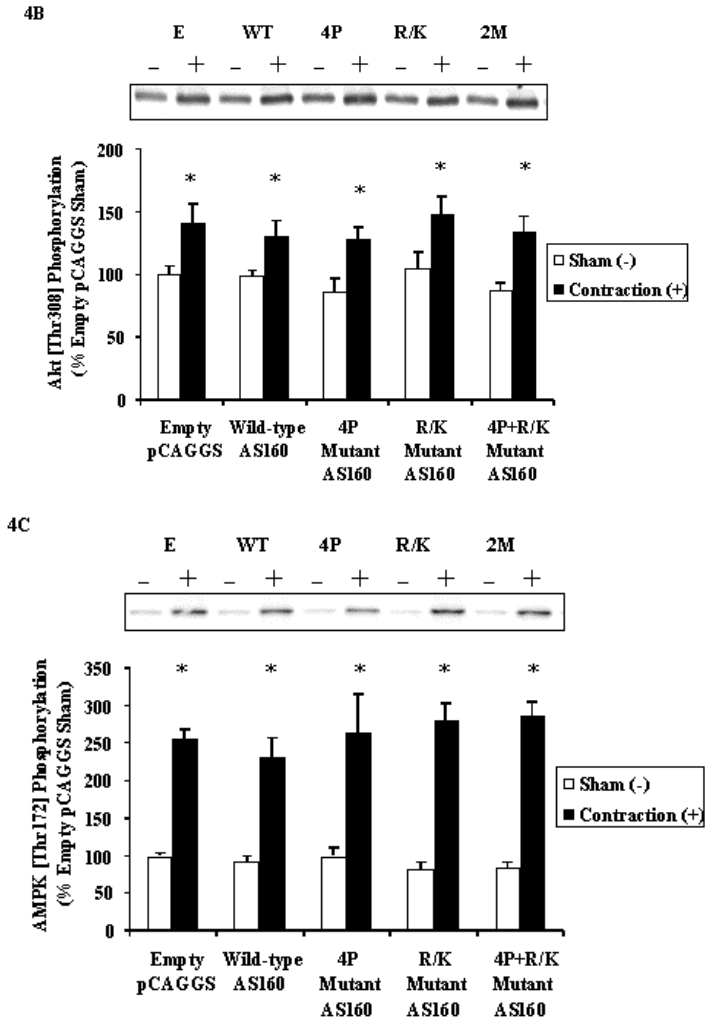


Figure 4. AS160 overexpression regulates basal (sham) and *in situ* contraction-stimulated glucose uptake without alterations in Akt Thr^{308} and AMPK Thr^{172} phosphorylation in transfected mouse skeletal muscles. Empty pCAGGS vector (*E*) or Myc-tagged AS160 DNA constructs (wild type and 4P, R/K, and 2M mutants) were injected into the tibialis anterior muscles of anesthetized mice, followed by *in vivo* electroporation. The animals were allowed to recover, and basal and contraction-stimulated glucose uptake were assessed *in vivo* 7 days post-injection. Absolute skeletal muscle glucose uptake was determined 45 min after [^{3}H]2-deoxyglucose injection combined with either sham operations or 15 min *in situ* tibialis anterior muscle contractions (*A*). The data are expressed as the means ± S.E. (n = 6–24/group). *, $p < 0.05$ (*versus* contraction-stimulated empty pCAGGS controls); **, $p < 0.05$ (*versus* contraction-stimulated WT AS160 and empty vector controls); †, $p < 0.05$ (*versus* basal empty pCAGGS controls); ††, $p < 0.05$ (*versus* contraction-stimulated empty pCAGGS, wild type AS160, and 4P-AS160). In addition, muscles from basal/sham (–) and contraction-stimulated conditions (+) were immunoblotted with anti-phospho-Akt Thr^{308} (*B*) and anti-phospho-AMPK Thr^{172} antibodies (*C*). The data are expressed as the means ± S.E. (n = 5–12/group). *, $p < 0.05$ (*versus* sham).

AS160 Overexpression Does Not Alter Expression of Proteins Involved in Skeletal Muscle Glucose Uptake

To determine whether changes in skeletal muscle glucose uptake observed with AS160 overexpression might be due to adaptive effects on regulatory upstream and downstream proteins, we performed immunoblots for AMPKα2, Akt1/2, GLUT1, GLUT4, and hexokinase II (Table 1). There were no significant differences between muscles injected with empty vector or any of the AS160 gene constructs. Thus, gene delivery by localized AS160 plasmid injections does not cause genomic disruption or alterations in the expression of key signaling proteins that regulate glucose metabolism in skeletal muscle.

Table 1. Immunoblot quantitations of AMPKα2, Akt1/2, GLUT1, GLUT4 and hexokinase II expression and AS160 expression and phosphorylation [T642] in tibialis anterior muscles one-week following intramuscular injection of empty vector control (E), wild-type AS160 (WT), 4P-mutant AS160, R/K mutant AS160, or double mutant (2M) AS160 DNA constructs. [* = Significant ($p<0.05$) differences compared to E controls]

IB	E	WT	4P	R/K	2M
AMPKa2	100±12	119±21	94±20	111±16	107±18
Akt1/2	100±9	117±17	96±15	92±21	112±16
GLUT1	100±5	103±8	101±6	107±6	107±3
GLUT4	100±10	108±20	98±22	98±12	109±14
Hexokinase II	100±11	105±6	107±7	105±15	104±8

Discussion

Skeletal muscle is unique in its ability to promote glucose homeostasis through both insulin-dependent and insulin-independent pathways leading to GLUT4 translocation. Successful isolation of a point of signaling convergence just prior to GLUT4 would provide a promising target for therapeutic diabetes intervention. Our lab previously determined that insulin- and contraction-stimulated signaling cascades phosphorylate AS160 phospho-Akt-substrate (PAS) motifs in a distinct and additive manner in mouse skeletal muscle (51). The purpose of this study was to characterize a functional role for AS160 on skeletal muscle glucose uptake. Using a local model of protein overexpression, we provide direct evidence that AS160 regulates insulin- and contraction-stimulated glucose uptake *in vivo*. Phosphorylation of AS160 at PAS motifs is essential for full insulin- and contraction-stimulated glucose uptake in adult skeletal muscle. To our knowledge, this is the first report of a single protein upstream of GLUT4 capable of orchestrating independent insulin and contraction effects on skeletal muscle glucose uptake.

We investigated AS160 function on glucose metabolism by transfecting mouse tibialis anterior muscles with empty vector, wild-type AS160, and three mutant AS160 DNA constructs. Utilization of direct intramuscular DNA injection in combination with *in vivo* electroporation allows for local delivery and expression of the target gene without an immune response. Furthermore, this approach temporarily bypasses developmental compensations inherent to other genetic manipulations such as transgenic or knock-out models (58). Because it is limited to the superficial tibialis anterior muscle, isolated skeletal muscle preparations cannot be performed in a complementary manner.

Nonetheless, the prevailing advantage of *in vivo* electroporation lies in its physiologic nature. The efficacy of this strategy has been validated previously by our lab (24, 37) and others (2), and very clearly allowed for consistent overexpression of wild-type and all mutant AS160 isoforms in the current study. There were no changes in the expression or activity of known signaling proteins upstream of AS160, nor downstream effects on expression of GLUT1, GLUT4, and hexokinase II, which might otherwise confound interpretation of the results of AS160 on skeletal muscle glucose uptake.

AS160 regulation of insulin-stimulated glucose uptake in mouse skeletal muscles was determined following intravenous administration of glucose. Thus, instead of a supramaximal insulin stimulus, our experiments utilizing a glucose bolus were designed to induce a physiological insulin response (Methods Figure 1) (19, 92). We found that 4P mutant AS160 acted in a dominant-interfering capacity, inhibiting insulin-stimulated glucose uptake by over 33% compared to empty vector and WT AS160 controls. This result is consistent with impaired insulin-stimulated exocytosis of GLUT4 in 4P-transfected adipocytes (98). Furthermore, overexpression of the double mutant (2M) AS160, which coexpresses the 4P and Rab-GAP domain mutations, effectively restored full insulin-stimulated glucose uptake relative to empty vector and WT-AS160 transfected skeletal muscle. These data suggest that activity of the AS160 Rab-GAP domain acts to restrain or inhibit glucose uptake, and is reflected in initial studies by Sano et al., which explore the effects of each mutant on GLUT4 translocation in 3T3-L1 cells (79). However, we did not observe uncontrolled basal glucose transport following overexpression of Rab-GAP mutant AS160, which is consistent with studies reporting AS160-independent mechanisms for basal GLUT4 retention (20). Residual activity of

endogenous AS160 protein may also be sufficient to ameliorate basal dysregulations in glucose transport. Collectively, our findings implicate a requirement for AS160 phosphorylation to suppress intrinsic Rab-GAP activity, and thereby facilitate the full effects of insulin on glucose uptake. These are the first data directly determining a functional role for AS160 on insulin-stimulated glucose metabolism in adult skeletal muscle tissue.

The observed effects of AS160 on contraction-stimulated glucose uptake are also entirely novel, albeit more complex. Phosphorylation of AS160 on phospho-Akt substrate (PAS) motifs is regulated by Akt2 in insulin-dependent signaling to glucose uptake, however, these same motifs are also regulated by AMPKα2 and potentially other kinases involved in insulin-independent signaling to glucose uptake (51). In transgenic mice lacking AMPKα2 activity, both AICAR-stimulated AS160 phosphorylation (51) and glucose transport (25) are abolished, and therefore suggests that AMPKα2-associated AS160 phosphorylation is necessary for AICAR-stimulated glucose uptake. However, these mice also exhibit significantly blunted contraction-stimulated AS160 phosphorylation (although they do produce significant contraction-stimulated increases compared to basal) (51) while glucose uptake following contraction appears normal after controlling for intrinsic differences in maximum contraction force (25). Developmental compensation could account for this apparent "mismatch" in transgenic animals (58), so we directly tested the function of contraction-stimulated AS160 phosphorylation at PAS motifs using our protein overexpression model in this study. Our data indicate AS160 phosphorylation at four PAS motifs is necessary for the full effects of contraction on skeletal muscle glucose uptake *in vivo*. As in the insulin-stimulated condition,

overexpression of 4P mutant AS160 exerted a significant dominant-inhibitory effect, reducing contraction-stimulated glucose uptake by over 44% compared to empty vector control muscles, and by 20% compared to muscles overexpressing WT AS160. These data provide evidence that contraction-mediated phosphorylation events on AS160 are functionally permissive for full stimulation of glucose uptake.

We were surprised to find that overexpression of wild-type AS160 also decreased glucose uptake (24%) following contraction, although this value was still significantly higher than 4P-injected muscles. There are at least two possible interpretations of these data. The magnitude of overexpressed wild-type and endogenous AS160 was too great for normal contraction-stimulated AS160 phosphorylation, and thus the remaining pool of unphosphorylated or partially phosphorylated AS160 exerted a dominant inhibitory effect. Our findings indirectly support this as we found substantial differences between AS160 overexpression (8-fold vs. empty vector controls) and enhanced contraction-stimulated AS160 phosphorylation (~2-fold vs. empty vector controls). These differences suggest that contraction-mediated kinase activity does not phosphorylate the exogenous AS160 in proportion to its overexpression, which may therefore account for the partial inhibition of contraction-stimulated glucose uptake by wild-type AS160 overexpression. Alternatively, overexpression of wild-type AS160 might suppress a molecule specifically activated by contraction but not insulin, or perturb native stoichiometries that disrupt contraction-induced glucose uptake.

Overexpression of mutant AS160 devoid of Rab-GAP activity (both R/K and 2M isoforms) generated increases in both unstimulated sham and contraction-stimulated glucose uptake compared to controls. These elevations in basal glucose uptake in

transfected muscles are intriguing because utilization of siRNA to specifically knock-down endogenous AS160 in 3T3-L1 adipocytes also resulted in increased basal glucose transport (20, 54). Re-expression of human wild-type AS160 restored normal basal glucose transport in this model, whereas expression of R/K mutant AS160 did not. The interpretation of these data concluded that AS160 GAP activity is required for full intracellular retention of GLUT4 in the basal state (20). Overexpression of GAP-inactive AS160 in our skeletal muscle transfections resulted in similar dysregulation of basal glucose uptake, perhaps mediated by the dominant-interfering capacity of these mutants on normal GLUT4 retention. To clarify, the basal glucose uptake values recorded in these experiments were from sham-operated muscles in the opposing leg of muscles stimulated to contract *in situ*. We did not detect significant changes in basal glucose uptake in animals administered saline as a control during IV glucose protocols. These latter saline basal glucose uptake values were ~2/3 lower than the sham basal uptake values obtained in contraction experiments. We have previously determined that sham operation by itself does not significantly increase AS160 phosphorylation above untreated or saline-injected control animals *in vivo* (51). Muscle contractions are well known to stimulate increases in cardiac output while reducing resistance of the peripheral vasculature. Thus, differences in blood flow and/or secretion of humoral factors likely account for the variation between saline and sham controls in our study, as has been shown in previous investigations (1, 59). However, the relative magnitude of sham basal uptake values afforded an increased sensitivity to detect significant differences in basal glucose uptake between the various AS160 isoforms.

Transfection of GAP-inactive AS160 also resulted in supraphysiological increases in contraction-stimulated glucose uptake in mouse skeletal muscle. In this scenario, we overexpressed a considerable pool of AS160 lacking normal braking (GAP) function on GLUT4 trafficking. AS160 is an extremely large protein with multiple domains, phosphorylation motifs, and docking sites conceivably designed for protein-protein interactions (65). It is possible that abundant AS160 without its inhibitory activity could act as a scaffold encouraging contraction-specific interactions that enhance glucose uptake. The role of AS160 as a scaffold protein will be pursued in future experiments.

To summarize, we used an *in vivo* electroporation technique to overexpress wild-type and mutant AS160 in adult skeletal muscle and subsequently evaluated AS160 function on glucose uptake. Our findings reveal a regulatory role for AS160 on insulin-, and contraction-stimulated glucose uptake. Overexpression of 4P mutant AS160, incapable of being phosphorylated at key phospho-Akt-substrate (PAS) motifs, significantly inhibited glucose uptake following insulin or contractile stimuli in transfected muscles. Wild-type AS160 overexpression also decreased contraction-induced uptake, although the mechanisms for this remain unclear. In contrast, both sham and contraction-stimulated glucose uptake were significantly increased by overexpressing mutant AS160 lacking normal Rab-GAP activity. These collective changes occurred in the absence of significant alterations in the activity or expression of putative upstream AS160 kinases or downstream expression of GLUT4. Therefore, AS160 directly regulates insulin- and contraction-stimulated glucose transport in mouse skeletal muscle.

CHAPTER 5

THE CALMODULIN-BINDING DOMAIN OF AS160 REGULATES CONTRACTION- BUT NOT INSULIN-STIMULATED GLUCOSE UPTAKE

Abstract

Insulin and contraction increase skeletal muscle glucose uptake through distinct and additive mechanisms. However, recent reports have demonstrated that insulin and contraction signals converge on AS160, a protein that regulates GLUT4 translocation. Although AS160 phosphorylation on phospho-Akt substrate (PAS) sites 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 Ca^{2+}/calmodulin could also modulate AS160 function. To evaluate the role of the AS160 CBD in skeletal muscle, empty vector, wild type or CBD mutant AS160 DNAs were injected into mouse tibialis anterior muscles followed by *in vivo* electroporation. This resulted in consistent AS160 protein overexpression (10-fold) 1-week later. To verify that the transfected CBD mutant AS160 could not bind calmodulin in a cell free system, immunoprecipitated wild-type and CBD mutant AS160 were incubated with biotinylated calmodulin in the presence of ~0.5 mM Ca^{2+}. Wild type AS160, but not the CBD mutant AS160, associated with calmodulin. Next, we measured basal, insulin-, or contraction-stimulated glucose uptake *in vivo*. Compared to empty vector and wild-type AS160, basal and insulin-stimulated glucose uptake were not altered in muscles expressing the CBD-mutant AS160. In contrast, contraction-stimulated glucose uptake was significantly decreased (45%) in CBD mutant expressing muscles. This strong inhibitory effect on glucose uptake was not associated with aberrant contraction-stimulated AS160 PAS phosphorylation. Interestingly, expression of a double mutant AS160 containing both calmodulin-binding and Rab-GAP domain point mutations (CBD + R/K) fully restored contraction-stimulated glucose uptake. Our results suggest that the calmodulin-binding domain of AS160 directly regulates contraction-induced glucose uptake in mouse skeletal muscle, and that calmodulin provides an additional means of modulating AS160 Rab-GAP function independent of PAS phosphorylation status. These findings define a novel AS160 signaling component leading to glucose uptake in contracting skeletal muscle.

Background

Physical exercise increases glucose uptake into contracting skeletal muscle, and can improve systemic glucose homeostasis when performed consistently. 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 (29, 35), the upstream signaling mechanisms leading to GLUT4 translocation appear to be distinct. Insulin signaling involves sequential activation of the insulin receptor, insulin receptor substrates

(IRS), and phosphoinositide 3-kinase (PI3-K) (23, 28). In contrast, muscle contraction activates multiple PI3-K-independent signaling pathways, including the Ca^{2+}/calmodulin-activated protein kinase (CaMK) family (74, 75, 94), AMPK (31, 34, 35), and atypical PKC (6), each of which may enhance glucose uptake in skeletal muscle.

We previously showed that these disparate insulin and contraction signals converge upon and phosphorylate the downstream Rab-GAP known as Akt Substrate of 160 kDa (AS160) in a distinct and additive manner (51). Studies in cells initially characterized AS160 as a molecular restraint of GLUT4 translocation through the activity of its Rab-GAP domain (20, 54). Phosphorylation of AS160 on phospho-Akt substrate (PAS) motifs removes its inhibitory GAP activity and increases GLUT4 trafficking to the cell membrane (79, 82). Multiple effectors of glucose uptake stimulate AS160 phosphorylation, including insulin (3, 9, 46, 48, 51, 54, 79, 82), platelet-derived growth factor (PDGF) (82), AICAR (9, 51, 82, 86), 4-phorbol-12-myristate-13-acetate (a conventional/novel PKC inhibitor) (82), potassium (K^+) depolarization (82), and contraction (3, 9, 51, 84, 86). Failure to achieve AS160 phosphorylation inhibits GLUT4 translocation and glucose uptake in cells (79, 82, 91). 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 (52).

Skeletal muscle contraction is initiated by a process known as excitation-contraction coupling. Briefly, acetylcholine-induced depolarization of the sarcolemma and t-tubules liberates calcium (Ca^{2+}) from the sarcoplasmic reticulum (SR) 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 (95). These intracellular Ca^{2+} spikes have also been proposed to stimulate GLUT4 translocation and glucose uptake following contraction (39, 94). Several studies have shown that glucose uptake is increased in skeletal muscle when cytoplasmic Ca^{2+} concentrations are elevated (36, 41, 97), and blockade of SR Ca^{2+} release by dantrolene effectively inhibits glucose uptake (64, 97). In isolated rat epitrochlearis muscles, caffeine was demonstrated to increase intracellular Ca^{2+} levels (81) and glucose uptake without inducing contractions or changes in high-energy phosphates (97). Similarly, K^+-induced depolarization of L6 cells increased intracellular Ca2+ levels and GLUT4-myc at the plasma membrane in a Ca2+-dependent manner (91). Dantrolene, calcium chelators (EDTA and EGTA), and PKC inhibitors (Calphostin C), but not inhibitors of AMPKα2 and CaMK, abolished the depolarization-associated net gain in surface GLUT4-myc. In a subsequent study using the identical model, transfection of L6 cells with 4P mutant AS160 also inhibited GLUT4-myc trafficking in response to depolarization (82). These 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 (11). Proteins such as protein kinase C and CaMKII, bind to Ca^{2+} and are directly regulated in a Ca^{2+}-dependent manner. Other Ca^{2+}-binding 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 (11, 12, 44, 68, 95).

Interestingly, human AS160 contains a region N-terminal to the Rab-GAP domain (amino acids 834-857) that is ~58% identical to the calmodulin-binding domain (amino acids 657-680) within the *Drosophila* protein Pollux (gi24644376) (45). Both of these human and *Drosophila* peptide sequences harbor key hydrophobic leucine and tryptophan residues (amino acids 842 and 843 of AS160, respectively) that have been shown to be essential for calmodulin association (45). Since contraction increases Ca^{2+}/calmodulin and AS160 possesses a functional calmodulin-binding domain (CBD), the purpose of the present study was to determine if the AS160 CBD regulates insulin and contraction-stimulated glucose uptake in mouse skeletal muscle tissue.

Results

CBD Mutant AS160 Does Not Associate with Calmodulin in a Cell-Free Assay

Initial evidence using recombinant AS160 and human AS160 immunoprecipitated from transfected 3T3-L1 adipocytes suggests that wild type AS160, but not a calmodulin-binding domain (CBD) mutant, associates with calmodulin in the presence of calcium. 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 seven days, both wild type and mutant AS160 expression was increased by over 10-fold compared to empty vector controls (Supplementary Figure S1). Due to the differences in plasmid vehicle, transfection method, tissue type, and overall magnitude of expression

between this system and the previous report in adipocytes (45), 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 N-terminal myc epitope tag, denatured and separated via SDS-PAGE, and transferred to nitrocellulose. Membranes were then incubated with biotinylated calmodulin in the presence of 0.5 mM $CaCl_2$. While wild type AS160 strongly associated with biotinylated calmodulin, the CBD mutant AS160 did not (Figure 1A). Addition of the calcium chelator EGTA (5 mM) in combination with $CaCl_2$ and calmodulin fully prevented binding by wild type AS160 (data not shown). Subsequently, membranes were stripped with 5 mM EGTA and total AS160 expression was assessed to account for differences in protein loading (Figure 1B). These results demonstrate that wild type AS160 derived from skeletal muscle transfections maintains the capacity to bind calmodulin, at least in the partially denatured state. Furthermore, the point mutations in CBD mutant AS160 DNA were faithfully translated from gene to protein and prevented normal association with calmodulin.

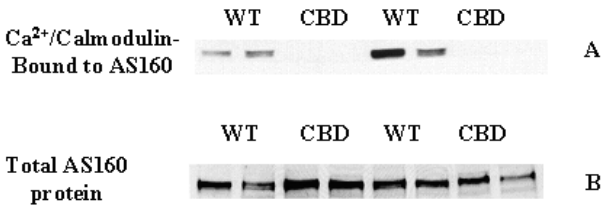


Figure 1. Although wild type AS160 binds calmodulin in the presence of calcium, this interaction is inhibited by mutation of the AS160 calmodulin-binding domain (CBD). Myc-AS160 immunoprecipitates (wild type and CBD mutant) were probed with biotinylated calmodulin (100 ng/ml) in the presence of calcium (0.5 mM $CaCl_2$) to determine whether overexpressed AS160 can bind calmodulin (A). Subsequently, total AS160 protein was assessed after stripping membranes with the calcium chelator EGTA (5 mM) (B). Data are representative blots; n = 4/group.

Insulin-Stimulated AS160 Phosphorylation and Glucose Uptake are Normal in Muscles Expressing CBD Mutant AS160

We have previously shown that AS160 regulates insulin-stimulated glucose uptake in skeletal muscle tissue. Phosphorylation on key phospho-Akt substrate (PAS) motifs appears to be required to disengage the inhibitory effect of the Rab-GAP domain on glucose uptake, a mechanism observed in various cell models as well. Although the initial characterization of the AS160 CBD in adipocytes showed that mutation of this region had no effect on GLUT4 translocation, 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 (21,22) while glucose uptake into injected muscles was measured using a ^{3}H-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 (Supplementary Figure S2). In empty vector control muscles, endogenous AS160 PAS phosphorylation was significantly increased in response to the glucose bolus (Figure 2A). Both wild type- and CBD mutant-expressing muscles exhibited two-fold increases in basal AS160 phosphorylation, and these values were similarly increased by IV glucose. The effects of wild type and CBD mutant AS160 on skeletal muscle glucose uptake are shown in Figure 2B. There were no differences between empty vector controls or muscles expressing wild type or CBD mutant AS160 on either basal (saline) 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 and glucose uptake in vivo in adult skeletal muscle.

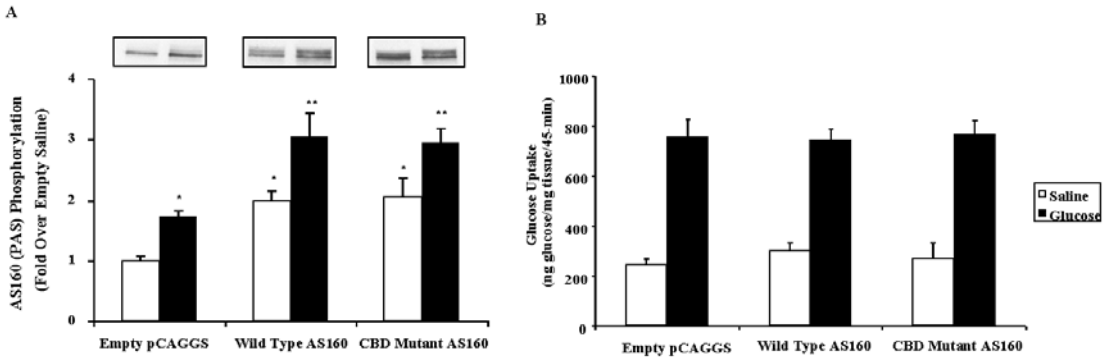


Figure 2. Insulin-stimulated AS160 phosphorylation and glucose uptake are normal in transfected mouse skeletal muscles expressing CBD mutant AS160. Empty pCAGGS vector or myc-tagged AS160 DNA constructs (wild type and CBD mutant) were injected into the tibialis anterior muscles of anesthetized mice, followed by *in vivo* electroporation. The animals were allowed to recover, and basal (saline) and insulin-stimulated glucose uptake were assessed *in vivo* 7 days post-injection. Transfected animals were administered intravenous [^{3}H]2-deoxyglucose combined with either saline or glucose bolus (1.0 grams glucose/kg of body weight), and blood was sampled at 0, 5, 10, 15, 25, 35, and 45 minutes post-injection. Muscles were harvested at 45 minutes and analyzed via immunoblot for phospho-AS160 with the PAS antibody (*A*) as well as processed for glucose uptake (*B*). The data are expressed as the means ± S.E. (n = 4–6/group). *, $p < 0.05$ (*versus* empty pCAGGS saline); **, $p < 0.05$ (*versus* empty pCAGGS glucose).

Contraction-Stimulated Glucose Uptake is Inhibited in Muscles Expressing CBD Mutant AS160 Despite Normal AS160 Phosphorylation

Muscle contractions stimulate rapid increases in calcium-mediated calcium release by the sarcoplasmic reticulum. These calcium transients activate Ca^{2+}/calmodulin, which in turn may mediate a number of physiological effects in contracting skeletal muscle. Although AS160 has a functional Ca^{2+}/calmodulin-binding domain, it is not known whether this region regulates AS160 PAS phosphorylation events, AS160 Rab-GAP function, and/or contraction-stimulated glucose uptake. Therefore, we explored the effects of wild type and CBD mutant AS160 on AS160 PAS phosphorylation and glucose

uptake following 15-min of *in situ* contractions in transfected mouse tibialis anterior muscles (Figure 3). AS160 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 (Figure 3A). As a control for contraction efficacy, we also evaluated AMPK Thr^{172} phosphorylation and found similar increases in control and transfected muscles in response to contraction (Supplementary Figure S3).

Despite similar ability to increase AS160 PAS phosphorylation (Figure 3A), muscles overexpressing CBD mutant AS160 exhibited significant impairments (~45%) in contraction-stimulated glucose uptake compared to empty vector controls (Figure 3B). Contraction-stimulated glucose uptake *in vivo* was also subtly inhibited (~19%) in muscles overexpressing wild type AS160, although it was still significantly higher (26%) than in CBD mutant-expressing muscles. This reduction due to wild type AS160 overexpression is similar in magnitude to our previous findings in skeletal muscle (Kramer et al. 2006). There were no differences observed between basal (sham) glucose uptake values among the various conditions. These results suggest that the calmodulin-binding domain of AS160 is essential for full contraction-stimulated glucose uptake in skeletal muscle, and that AS160-mediated inhibition of glucose uptake can occur independently of PAS phosphorylation status.

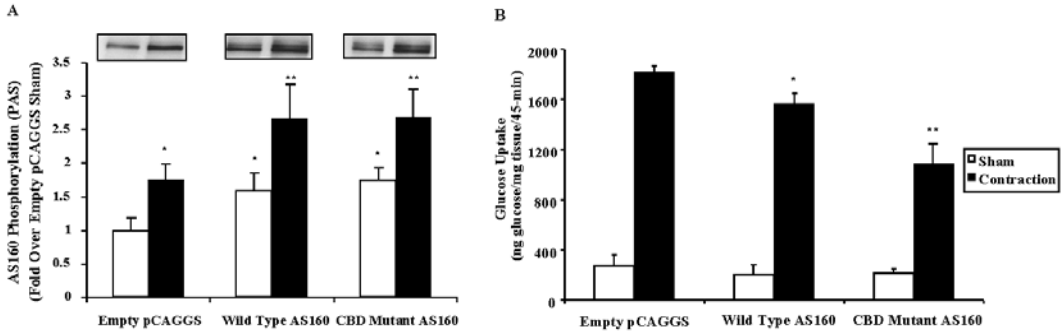


Figure 3. Contraction-stimulated glucose uptake is significantly inhibited in muscles expressing CBD mutant AS160 despite normal AS160 phosphorylation. Empty pCAGGS vector or myc-tagged AS160 DNA constructs (wild type and CBD mutant) were injected into the tibialis anterior muscles of anesthetized mice, followed by *in vivo* electroporation. The animals were allowed to recover, and basal (sham) and contraction-stimulated glucose uptake were assessed *in vivo* 7 days post-injection. 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 minutes and analyzed via immunoblot for phospho-AS160 with the PAS antibody (*A*). The data are expressed as the means ± S.E. (n = 4–6/group). *, $p < 0.05$ (*versus* empty pCAGGS sham); **, $p < 0.05$ (*versus* empty pCAGGS contraction). Muscles were also processed for absolute glucose uptake (*B*). The data are expressed as the means ± S.E. (n = 4–6/group). *, $p < 0.05$ (*versus* empty pCAGGS contraction); **, $p < 0.05$ (*versus* both wild type AS160 and empty pCAGGS contraction conditions).

Contraction-Stimulated Glucose Uptake is Restored in Muscles Expressing CBD + R/K Mutant AS160

The AS160 calmodulin-binding domain lies adjacent to the Rab-GAP domain. Therefore, it is possible that Ca^{2+}/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 were then injected and electroporated into mouse skeletal muscle, and examined in the context of contraction (Figure 4) as previously described. There were no significant differences in AS160 expression (Supplementary Figure S1) or AS160 PAS phosphorylation under basal or contraction conditions (Figure 4A) between CBD mutant AS160 and CBD + R/K mutant AS160.

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 to controls. Basal (sham) glucose uptake was also increased (61%) in muscles expressing CBD + R/K mutant AS160 compared to control muscles and muscles expressing CBD mutant AS160. Taken together, these findings suggest that the AS160 Rab-GAP domain acts as a restraint on basal and contraction-stimulated glucose uptake *in vivo*. Furthermore, an intact AS160 calmodulin-binding domain appears to regulate AS160 GAP activity and is required for normal contraction-stimulated glucose uptake in mouse skeletal muscle.

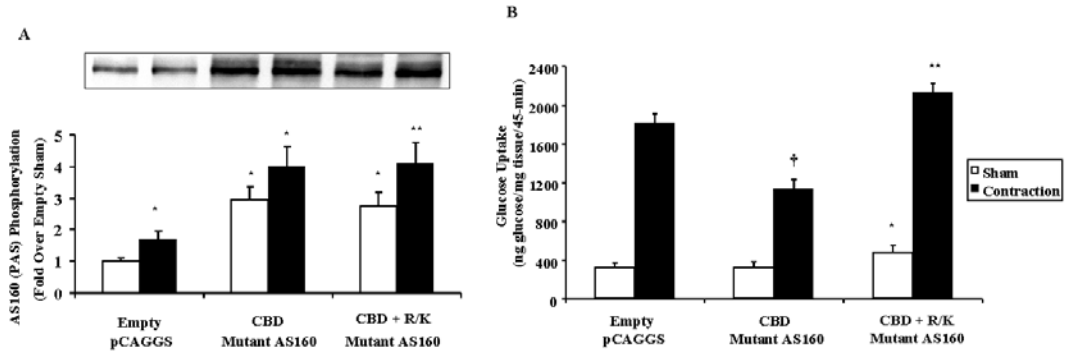


Figure 4. Contraction-stimulated glucose uptake is restored in muscles expressing CBD + R/K mutant AS160. Empty pCAGGS vector or myc-tagged AS160 DNA constructs (CBD and CBD + R/K mutants) were injected into the tibialis anterior muscles of anesthetized mice, followed by *in vivo* electroporation. The animals were allowed to recover, and basal (sham) and contraction-stimulated glucose uptake were assessed *in vivo* 7 days post-injection. 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 minutes and analyzed via immunoblot for phospho-AS160 with the PAS antibody (*A*). The data are expressed as the means ± S.E. (n = 5–6/group). *, $p < 0.05$ (*versus* empty pCAGGS sham); **, $p < 0.05$ (*versus* empty pCAGGS contraction). Muscles were also processed for absolute glucose uptake (*B*). The data are expressed as the means ± S.E. (n = 5–6/group). *, $p < 0.05$ (*versus* empty pCAGGS sham); **, $p < 0.05$ (*versus* empty pCAGGS contraction); †, $p < 0.05$ (*versus* empty pCAGGS contraction).

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. We recently identified the Rab-GAP AS160 as a point of convergence for the distinct upstream signaling pathways activated by insulin and contraction (51). Furthermore, AS160 phosphorylation at critical phospho-Akt substrate (PAS) motifs appears to be necessary for full insulin- and contraction-stimulated glucose uptake in skeletal muscle (52).

Here, we explored the functional significance of the AS160 calmodulin-binding domain (CBD) on insulin- and contraction-stimulated glucose uptake. Using an *in vivo* gene transfer technique (24, 37, 52, 93), we overexpressed wild type AS160 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.

AS160 was initially shown to contain a CBD exhibiting sequence homology with the *Drosophila* protein Pollux, and strong affinity for Ca2+/calmodulin (45). Expression of mutant AS160 (LW/GG) incapable of binding calmodulin did not disrupt GLUT4 translocation in insulin-treated 3T3-L1 adipocytes. In the present study, we verified that overexpressed wild type AS160 strongly associated with calmodulin in the presence of Ca^{2+}. However, this interaction was completely abolished by mutation of the AS160 CBD. In addition, we found that expression of the CBD mutant AS160 did not alter insulin-stimulated AS160 PAS phosphorylation or glucose uptake in skeletal muscle. These results are consistent with the only previous work on the AS160 CBD in adipocytes (45), and suggest that calmodulin association with AS160 is not required for insulin signaling to glucose uptake. It is noteworthy that some studies report an important role for Ca^{2+}/calmodulin on insulin-stimulated glucose metabolism in adipocytes (90), L6 cells (91), and skeletal muscle tissue (10). However, these insulin-stimulated increases in Ca^{2+} are confined to a local microenvironment just below the

plasma membrane (10), 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 (5, 27). AS160 has been shown to regulate the initial translocation phase of GLUT4 trafficking in response to insulin, but not the latter prefusion and fusion phases (27). Therefore, local Ca^{2+}/calmodulin activity at or about the plasma membrane likely regulates insulin-stimulated GLUT4 prefusion and/or fusion events, and would not be affected by expression of CBD mutant AS160.

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 an exciting and novel means of contraction-regulated AS160 signaling to glucose uptake.

Ca^{2+} transients are fundamental for contraction in skeletal muscle. Under resting conditions, the intracellular Ca^{2+} concentration in isolated single muscle fibers is ~30–50 nM (95). Neural activation causing contraction elicits Ca^{2+} increases in the range of ~100–300 nM (13) in slow-twitch (type I) fibers and even higher spikes of ~1–2 μM (95) in fast-twitch fibers (type IIA and IIB). Both the amplitude and duration of the Ca^{2+} transient in skeletal muscle are determined by the motor unit firing frequency (12). The frequency component, in turn, determines the amplitude of the Ca^{2+} signal. Ultimately, elevations in intracellular Ca^{2+} must be decoded by intermediate binding proteins such as calmodulin (11, 12). Activation of the Ca^{2+}/calmodulin complex is directly related to the frequency and amplitude of Ca^{2+} oscillations, and has been implicated in the metabolic alterations and changes in gene expression accompanying physical exercise (12).

Agonists of intracellular Ca^{2+} release directly increase glucose uptake in cells and skeletal muscle tissue through insulin-independent signaling mechanisms. Caffeine, for example, was shown to enhance glucose uptake via activation of CaMK in skeletal muscle. K^{+}-induced depolarization of L6 cells increased intracellular Ca^{2+} levels and resulted in increased GLUT4 at the cell membrane in a Ca^{2+}-sensitive manner, although not through activation of CaMK (91). Interestingly, this latter depolarization model showed a partial dependency on AS160 PAS phosphorylation for gains in surface GLUT4 (82). Our results further suggest that binding of Ca2+/calmodulin to AS160, in addition to PAS phosphorylation, is required for full contraction-stimulated glucose uptake in adult skeletal muscle.

AS160 is currently thought to be a restraint of GLUT4 translocation and glucose uptake in adipocytes, muscle cells, and skeletal muscle tissue (20, 83, 88). Multiple stimuli induce phosphorylation of AS160 (3, 9, 46, 48, 51, 54, 79, 82), which removes the suppressive functions of the Rab-GAP domain and allows for normal glucose uptake (52, 79, 82). This study confirms that AS160 phosphorylation is necessary and sufficient for full insulin-stimulated glucose uptake in 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 artifact or due to non-specific 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 a double mutant containing disrupted CBD and GAP domains (CBD + R/K). There are several plausible, albeit speculative, interpretations of the data.

The AS160 Rab-GAP domain exhibits activity toward multiple Rabs in adipocytes (61). Phosphorylation of AS160 may sufficiently hinder its GAP activity for specific Rabs involved in the insulin signaling pathway (40). However, in order 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 (45), making it likely 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 (56, 71). If true, it is tempting to consider that these respective pools may be regulated by distinct Rabs. 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 (73). 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. 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 that was reversed by AS160 coexpressing CBD and Rab-GAP domain mutations. Both insulin- and contraction-induced AS160 PAS phosphorylation were unaltered in mutant-expressing muscles. Taken together, both insulin and contraction signals converge on AS160 to regulate glucose uptake, but contraction additionally requires the presence of the AS160 calmodulin-binding domain for full stimulation of glucose uptake in skeletal muscle.

Supplementary Data

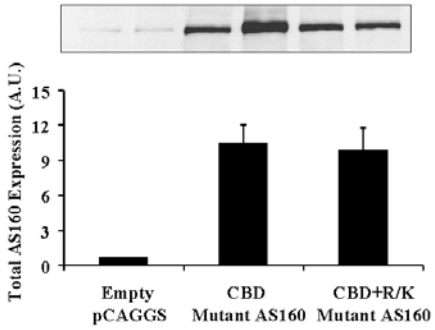


Figure S1. AS160 expression in transfected skeletal muscles. Empty pCAGGS vector or myc-tagged AS160 DNA constructs (CBD and CBD + R/K mutants) were injected into the tibialis anterior muscles of anesthetized mice, followed by *in vivo* electroporation. Animals were sacrificed 7 days post-injection, and muscles were processed for analysis of total AS160 expression via immunoblot. The data are expressed as means ± S.E. (n = 5–6/group).

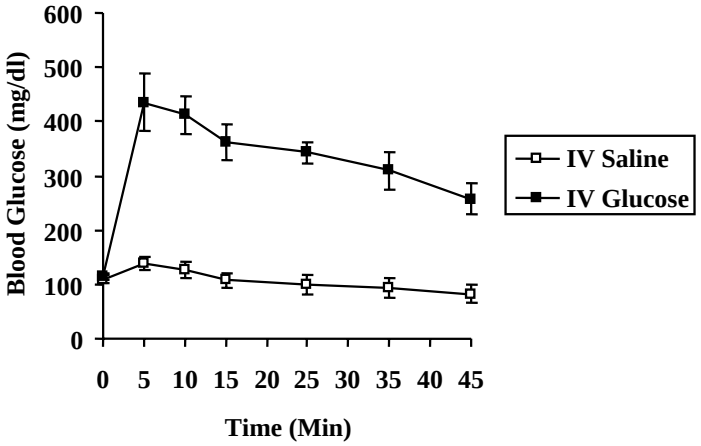


Figure S2. Blood glucose response to intravenous glucose administration in transfected mice. Empty pCAGGS vector, wild type AS160, or CBD mutant AS160 were injected into the tibialis anterior muscles of anesthetized mice, followed by *in vivo* electroporation. Seven days later, blood glucose values were evaluated in all electroporated mice at 0, 5, 10, 15, 25, 35, and 45 min following [^{3}H]2-DG injection combined with either saline or glucose bolus (1.0 g glucose/kg of BW). Data are expressed as means ± S.E. (n = 4–7/group).

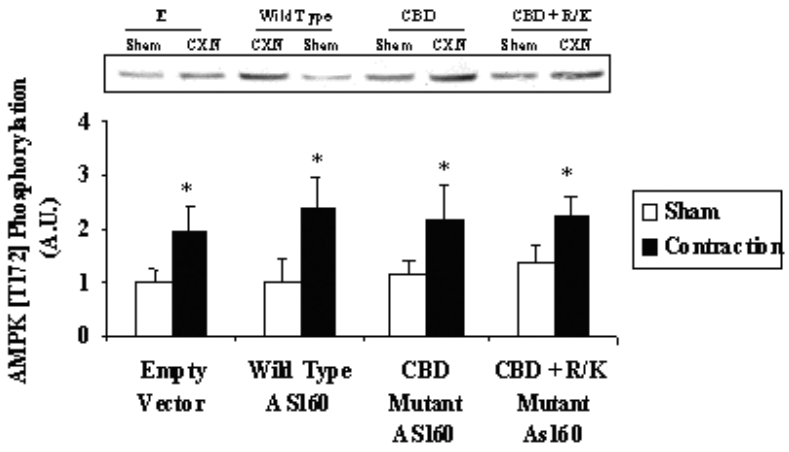


Figure S3. Contraction-stimulated AMPK Thr^{172} phosphorylation is normal in skeletal muscle transfected with wild type and CBD mutant AS160. Empty pCAGGS vector or myc-tagged AS160 DNA constructs (wild type and CBD mutant) were injected into the tibialis anterior muscles of anesthetized mice, followed by *in vivo* electroporation. Transfected muscles were harvested 7 days later following basal treatment (sham operation) or 15 min *in situ* muscle contractions, and subsequently analyzed via immunoblot for phospho-AMPK Thr^{172}. The data are expressed as the means ± S.E. (n = 4/group).

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

Summary of Dissertation Research

Distinct signals regulate AS160 phosphorylation in response to insulin, AICAR, and contraction in mouse skeletal muscle (51)

The purpose of the study presented in Chapter 3 was to characterize the upstream signals that mediate AS160 phosphorylation by insulin, contraction and AICAR. Insulin-stimulated AS160 phosphorylation detected using the phospho-Akt substrate (PAS) antibody was fully blunted by the PI3-K inhibitor wortmannin *in vitro*, and in Akt2 knockout mice *in vivo*. In contrast, contraction-stimulated AS160 phosphorylation on PAS sites was only partially decreased by wortmannin and unaffected in Akt2 knockout mice, suggesting regulatory mechanisms other than Akt may participate in AS160 phosphorylation with contraction. To determine if AMPK mediates AS160 signaling, we utilized muscle-specific AMPKα2 inactive transgenic mice. AICAR-stimulated AS160 phosphorylation was fully inhibited, whereas contraction-stimulated AS160 phosphorylation was partially reduced in the AMPKα2 inactive mice. Combined AMPKα2 and Akt inhibition by wortmannin treatment of AMPKα2i mice did not fully ablate contraction-stimulated AS160 phosphorylation. These data demonstrate that there must be additional contraction-mediated signals regulating AS160 phosphorylation, and likely reflect the multiplicity of molecular inputs involved in contraction-stimulated glucose uptake. In addition to looking at specific signaling pathways, we also showed that maximal insulin together with either AICAR or contraction increased AS160

phosphorylation in an additive manner. The collective results of this study provide evidence that multiple signals converge at the level of AS160, further implicating this molecule as a critical mediator of cell metabolism.

AS160 regulates insulin- and contraction-stimulated glucose uptake in mouse skeletal muscle (52)

The purpose of the study presented in Chapter 4 was to explore the functional consequences of AS160 phosphorylation in skeletal muscle. For these experiments we used an *in vivo* electroporation technique to overexpress wild-type and mutant AS160 in adult skeletal muscle and subsequently evaluated AS160 function on glucose uptake. Our findings reveal a regulatory role for AS160 on basal, insulin-, and contraction-stimulated glucose uptake. Overexpression of 4P mutant AS160, incapable of being phosphorylated at key PAS motifs, significantly inhibited glucose uptake following insulin or contractile stimuli in transfected muscles. Wild-type AS160 also decreased contraction-induced uptake, although the mechanisms for this remain unclear. In contrast, both basal and contraction-stimulated glucose uptake were significantly increased by overexpressing mutant AS160 lacking normal Rab-GAP activity. These changes occurred in the absence of significant alterations in the activity or expression of putative upstream AS160 kinases or downstream expression of GLUT4. Therefore, AS160 phosphorylation by Akt, AMPK, and/or other kinases appears to be necessary for full insulin- and contraction-stimulated glucose transport in mouse skeletal muscle.

The calmodulin-binding domain of AS160 regulates contraction- but not insulin-stimulated glucose uptake in skeletal muscle

The purpose of the study presented in Chapter 5 was to evaluate the functional significance of the AS160 calmodulin-binding domain (CBD) on insulin- and contraction-stimulated glucose uptake in skeletal muscle. Although AS160 phosphorylation on phospho-Akt substrate (PAS) sites is believed to be the primary factor affecting its Rab-GAP activity, it is plausible that contraction-stimulated increases in Ca^{2+}/calmodulin may alter AS160 function through interaction with the AS160 CBD. Therefore, empty vector, wild type or CBD mutant AS160 DNAs were injected into mouse tibialis anterior muscles followed by *in vivo* electroporation, which resulted in robust protein overexpression one-week later. To confirm that the transfected CBD mutant AS160 could not bind calmodulin in a cell free system, immunoprecipitated wild-type and CBD mutant AS160 were incubated with biotinylated calmodulin in the presence of excess (~0.5 mM) Ca^{2+}. We found that wild type AS160, but not the CBD mutant AS160, associated with calmodulin. Next, we measured basal, insulin-, or contraction-stimulated glucose uptake *in vivo*. Compared to empty vector and wild-type AS160, basal and insulin-stimulated glucose uptake was not altered in muscles expressing the CBD-mutant AS160. In contrast, contraction-stimulated glucose uptake was significantly impaired in muscles transfected with CBD mutant AS160. This strong inhibitory effect on glucose uptake was not associated with altered contraction-stimulated AS160 PAS phosphorylation. Interestingly, expression of a double mutant AS160 containing both calmodulin-binding and Rab-GAP domain point mutations (CBD + R/K)

fully restored contraction-stimulated glucose uptake. Our results suggest that the AS160 calmodulin-binding domain is important for the full effects of contraction on glucose transport in adult skeletal muscle, and that regulation of glucose metabolism by AS160 can occur through PAS-independent signaling mechanisms.

Review of Most Recent AS160 Findings

Altered regulation of AS160 in patients with diabetes and reversal by exercise training

Dysregulation in AS160 function is clearly associated with diabetes and insulin resistance. For example, AS160 phosphorylation was found to be impaired in skeletal muscle from individuals with type 2 diabetes (48), as well as in muscles of first-degree relatives of patients with diabetes (47). TNF-α, a prominent cytokine implicated in the pathogenesis of diabetes, was shown to induce skeletal muscle insulin resistance by interfering with insulin-induced AS160 phosphorylation and GLUT4 translocation (70). Interestingly, gene silencing of mitogen activated protein kinase kinase kinase kinase isoform 4 (MAP4K4) ameliorated the desensitizing effect of TNF-α on insulin-stimulated AS160 phosphorylation (8).

As discussed in Chapter 1, exercise-stimulated glucose uptake occurs independently of insulin signaling and constitutes a valuable mechanism for improved glucose homeostasis in individuals with diabetes. Exercise is also a potent physiological agonist for subsequent insulin action. The role of AS160 on post-exercise insulin sensitivity has not been thoroughly evaluated; however, AS160 phosphorylation was increased four hours after swimming exercise in rodents and correlated strongly with increased insulin-stimulated glucose uptake in skeletal muscle (3). Furthermore, acute

contraction of obese, insulin resistant rodent muscles partially restored AS160 phosphorylation despite severely impaired insulin-stimulated tyrosine phosphorylation of the insulin receptor and insulin receptor substrate-1 (84). These data suggest that AS160 may be a putative regulator of post-exercise increases in insulin action.

Insulin regulates multiple steps of GLUT4 trafficking

Insulin stimulates several discrete GLUT4 exocytotic trafficking steps in adipocytes, including an initial translocation phase, prefusion (defined by recruitment and/or docking within a perimembrane region <250 nM from the plasma membrane), and fusion with the membrane (27). While AS160 mediates GLUT4 translocation, it does not appear to be necessary for fusion with the adipocyte plasma membrane (5, 27). These data underscore the existence of multiple differentially regulated nodes of insulin action in fat cells, and suggest that both AS160-dependent and AS160-independent signaling is necessary to achieve full insulin-stimulated GLUT4 exocytosis. For the purposes of therapeutic interventions designed to circumvent defective insulin signaling, sole manipulation of AS160 may not be as successful as a comprehensive approach targeting AS160 and other regulatory molecules involved in GLUT4 prefusion and fusion phases.

AS160 interactions with downstream proteins involved in insulin-stimulated GLUT4 translocation

In addition to AS160 PAS sites and Rab-GAP domain, structural analysis by Scansite 2.0 software also predicts an abundance of other phosphorylation events and protein interactions on AS160. Recently, the insulin-regulated aminopeptidase (IRAP)

(54, 67) and multiple 14-3-3 isoforms (73) were identified as AS160-interacting proteins in adipocytes. The colocalization of AS160 and IRAP occurred independently of PAS phosphorylation status (67). In contrast, 14-3-3 proteins were found to bind AS160 in an insulin- and Akt-dependent manner via phosphorylation of the Thr^{642} PAS site (73). This association was blocked by expression of the dominant inhibitory 4P-mutant AS160. However, introduction of a constitutive 14-3-3 binding site into the 4P-mutant AS160 fully restored 14-3-3 binding without disrupting AS160-IRAP interaction and reversed the inhibitory effect of 4P-mutant AS160 on GLUT4 translocation. These data indicate that the insulin-dependent association of 14-3-3 with AS160 plays an important role in GLUT4 trafficking in adipocytes.

Another emerging niche of AS160 research involves the identification of Rab substrates directly regulated by AS160. In adipocytes, the AS160 Rab-GAP domain was demonstrated to have specific activity against Rabs 2A, 8A, 10 and 14 in an assay utilizing a partial fragment of AS160 (61). Of these candidates, only Rabs 10 and 14 appear to be colocalized with GLUT4-containing vesicles (54). Recently, Rabs 8A and 14 were found to be targets of AS160 regulating GLUT4 traffic in muscle cells (40). Future studies should directly test the effects of these Rabs (or mutant derivatives) on insulin- and/or contraction-stimulated glucose uptake. It is tempting to speculate that insulin and contraction might mobilize distinct intracellular GLUT4 pools through different AS160-Rab interactions.

Prospective Analysis of AS160 in Skeletal Muscle

Multiple AS160 splice variants are expressed in mouse skeletal muscle

The Goodyear Lab has conducted preliminary experiments revealing that skeletal muscle expresses at least two AS160 splice variants (Figure 1). This finding was confirmed by PCR with splice-exon flanking primers, immunoprecipitation with a splice-exon-specific antibody, and optimizing electrophoresis for maximal AS160 separation. The updated AS160 annotation map (Figure 2) is rapidly evolving. Future experiments will determine if AS160 splice variants are differentially regulated by insulin and contraction, and whether one or both of these variants regulate insulin- and contraction-stimulated glucose uptake.

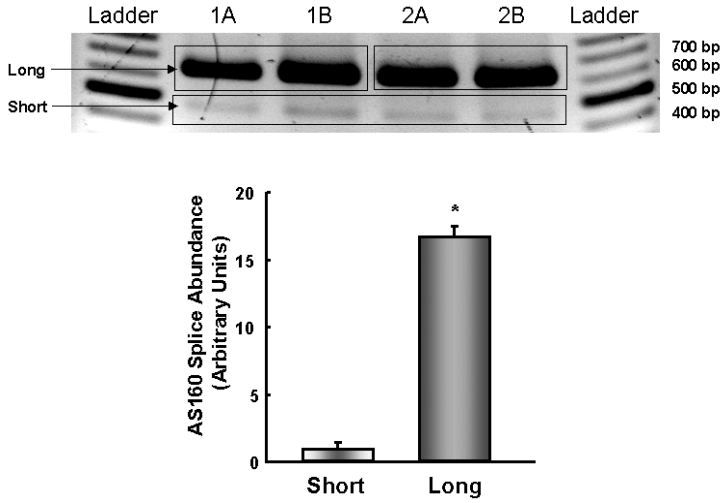


Figure 1. Two AS160 splice variants are expressed in mouse skeletal muscles. Total RNA was isolated from mouse quadriceps muscles and reverse transcribed to cDNA. PCR was conducted using primers flanking the putative AS160 splice exon sequence reported in GenBank. Relative splice abundance is expressed as means ± S.E. (n = 4 muscles). Results obtained in collaboration with Dr. Taylor of the Goodyear Lab.

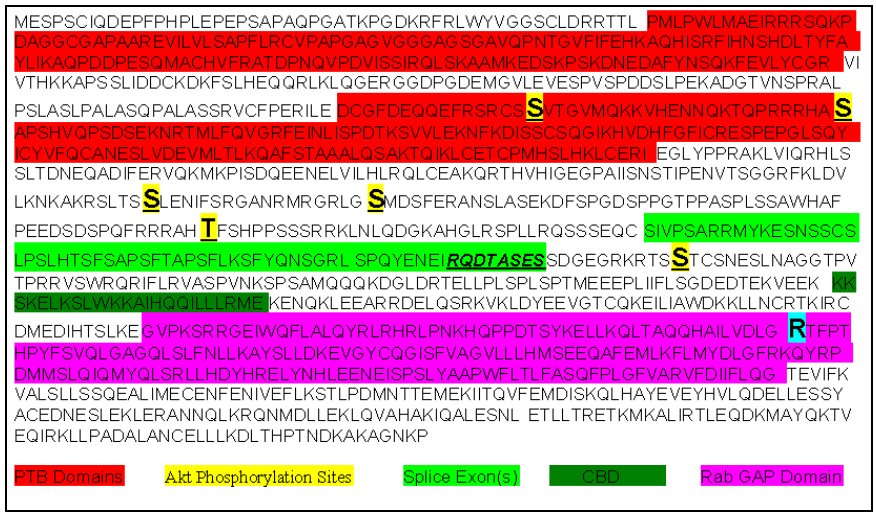


Figure 2. AS160 annotation map reflects updated structural landmarks

AS160 expression and phosphorylation are regulated in a fiber-type dependent manner in mouse skeletal muscle

In rodents and humans, individual skeletal muscles vary in their fiber-type composition. Furthermore, the relative distribution of fiber types can profoundly alter the gross metabolic characteristics of a given muscle. The Goodyear Lab has recently determined that the expression of AS160 varies across muscle fiber types, with the soleus (a muscle rich in slow Type I fibers) expressing significantly more AS160 than the TA or EDL (muscles rich in fast Type II fibers). In contrast to expression, however, AS160 phosphorylation in response to insulin or AICAR was highest in the TA and EDL, and blunted in the soleus. These results suggest that AS160 may be differentially regulated in muscles dominated by oxidative or glycolytic energy metabolism. Future experiments will further elucidate whether the fiber-type specificity of AS160 expression and phosphorylation is correlated with GLUT4, expression of mitochondrial markers, and/or activity of cytochrome C.

AS160 is phosphorylated at novel sites in response to contraction in skeletal muscle

The Lienhard laboratory originally identified AS160 PAS sites using the Scansite 2.0 program. These were confirmed as in vivo Akt phosphorylation sites in 3T3-L1 adipocytes by immunoprecipitation and mass spectrometry (69). Although these sites were identified within consensus Akt-substrate motifs, it is now known that kinases other than Akt can result in AS160 phosphorylation. Therefore, future experiments will adapt this highly successful approach to find novel contraction-regulated AS160 sites in skeletal muscle. In preliminary work, examining AS160 for additional phosphorylation motifs with the Scansite program at medium stringency reveals a multitude of putative phosphorylation sites for seventeen different kinases. Included among these are multiple kinases that are known or hypothesized to be regulated by muscle contraction (CaMKII, several PKC isoforms, glycogen synthase kinase-3, casein kinase II, ERK-1, and protein kinase A). In addition, there are near consensus matches for AMPK and CaMKI.

After successful identification of novel AS160 phosphorylation sites, the next step will be to characterize the effects of muscle contraction on these new sites. While mass spectrometry is an excellent technique for identifying phosphorylation sites, it is only semi-quantitative and therefore not suitable for characterizing the degree of phosphorylation. Therefore, development of phospho-specific antibodies engineered against novel AS160 phosphorylation sites and used in combination with immunoblotting will enable valuable quantitative calculation of phosphorylation events. Finally, the role of newly identified AS160 phosphorylation sites in contraction-stimulated glucose uptake can be assessed by transfecting mouse skeletal muscle with AS160 cDNA mutated at these sites to alanine (see Chapter 2 for mutagenesis methodology). It is likely that

multiple phosphorylation events govern AS160 Rab-GAP activity following contraction, and that mutating contraction regulated AS160 phosphorylation sites identified by mass spectrometry will blunt or abolish contraction-stimulated glucose uptake. In addition to enhancing our understanding of contraction-stimulated glucose transport in skeletal muscle, these studies will pinpoint specific targets on AS160 that may ultimately be utilized for future therapeutic diabetes interventions.

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