Original Article

AMPK-Mediated AS160 Phosphorylation in Skeletal Muscle Is Dependent on AMPK Catalytic and Regulatory Subunits

Jonas T. Treebak,[1] Stephan Glund,[2] Atul Deshmukh,[2] Ditte K. Klein,[1] Yun Chau Long,[2] Thomas E. Jensen,[1] Sebastian B. Jørgensen,[1] Benoit Viollet,[3] Leif Andersson,[4] Dietbert Neumann,[5] Theo Wallimann,[5] Erik A. Richter,[1] Alexander V. Chibalin,[2] Juleen R. Zierath,[2] and Jørgen F.P. Wojtaszewski[1]

AMP-activated protein kinase (AMPK) is a heterotrimeric protein that regulates glucose transport mediated by cellular stress or pharmacological agonists such as 5-aminoimidazole-4-carboxamide 1 β-D-ribonucleoside (AICAR). AS160, a Rab GTPase-activating protein, provides a mechanism linking AMPK signaling to glucose uptake. We show that AICAR increases AMPK, acetyl-CoA carboxylase, and AS160 phosphorylation by insulin-independent mechanisms in isolated skeletal muscle. Recombinant AMPK heterotrimeric complexes (α1β1γ1 and α2β2γ1) phosphorylate AS160 in a cell-free assay. In mice deficient in AMPK signaling (α2 AMPK knockout [KO], α2 AMPK kinase dead [KD], and γ3 AMPK KO), AICAR effects on AS160 phosphorylation were severely blunted, highlighting that complexes containing α2 and γ3 are necessary for AICAR-stimulated AS160 phosphorylation in intact skeletal muscle. Contraction-mediated AS160 phosphorylation was also impaired in α2 AMPK KO and KD but not γ3 AMPK KO mice. Our results implicate AS160 as a downstream target of AMPK. *Diabetes* 55:2051–2058, 2006

From the [1]Department of Human Physiology, Institute of Exercise and Sport Sciences, Copenhagen Muscle Research Centre, University of Copenhagen, Copenhagen, Denmark; the [2]Department of Molecular Medicine and Surgery, Section Integrative Physiology, Karolinska Institute, Stockholm, Sweden; [3]René Descartes University, Institute Cochin, Paris, France; the [4]Department of Medical Biochemistry and Microbiology, Uppsala University, Uppsala Biomedical Center, Uppsala, Sweden; and the [5]Swiss Federal Institute of Technology, Zurich, Switzerland.

Address correspondence and reprint requests to Juleen R. Zierath, Department of Molecular Medicine and Surgery, Section for Integrative Physiology, Karolinska Institute, von Eulers väg 4, 4th Floor, S-171 77 Stockholm, Sweden. E-mail: juleen.zierath@ki.se.

Received for publication 7 February 2006 and accepted in revised form 23 March 2006.

J.T.T., S.G., and A.D. contributed equally to this work.

ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxamide 1 β-D-ribonucleoside; AMPK, AMP-activated protein kinase; CaMKK, calmodulin-dependent protein kinase kinase; EDL, extensor digitorum longus; GST, glutathione S-transferase; PAS, phospho-Akt substrate; TSC, tuberous sclerosis complex.

DOI: 10.2337/db06-0175

See related article by Kramer et al., p. 2067.

The 5′AMP-activated protein kinase (AMPK) is a serine/threonine protein kinase that is ubiquitously expressed and functions as an intracellular fuel sensor activated by depletion of high-energy phospho-compounds (1). Activation of AMPK initiates a complex series of signaling events that trigger increases in the uptake and oxidation of substrates important for ATP synthesis and decreases in ATP-consuming biosynthetic processes such as protein, lipid, and glycogen synthesis (2). Activation of AMPK has been linked to the regulation of glucose transport (3), but the substrates linking these events are elusive.

AMPK regulates GLUT4-dependent glucose transport across the sarcolemma in skeletal muscle in response to diverse forms of cellular stress including exercise, hypoxia, and agents that disrupt the intracellular ATP-to-AMP ratio (4–6). Evidence linking AMPK and glucose uptake has been provided by the use of different genetic approaches, including knockout (KO) and transgenic dominant-negative kinase mouse models (4–6). Expression of a dominant-negative α2 AMPK construct in skeletal muscle suppresses α2 and α1 isoform-specific AMPK activity and completely prevents 5-aminoimidazole-4-carboxamide 1 β-D-ribonucleoside (AICAR)-induced glucose transport (6). This observation was reinforced by recent reports showing that knockout of either the catalytic α2 (but not α1 AMPK isoform) or the regulatory γ3 AMPK subunit completely abolishes AICAR-induced glucose transport (4,5). Collectively, these data provide evidence to suggest that α2 and γ3 containing AMPK heterotrimeric complexes are involved in AICAR-induced glucose transport. Although AICAR-induced glucose transport in resting skeletal muscle is mediated by GLUT4 translocation to the plasma membrane (7,8), the link between AMPK signaling and GLUT4 translocation is presently unknown.

AS160 is a substrate for the protein kinase Akt that links insulin signaling and GLUT4 trafficking (9–11). AS160 contains a GTPase-activating protein homology domain that has been shown to regulate the GTPase activity of certain Rab proteins in vitro (12). Phosphorylation of AS160 by Akt is likely to inhibit its GTPase-activating protein activity, such that as a consequence, the GTP form of a Rab protein is elevated and this elevation in turn increases GLUT4 vesicle movement to, and/or fusion with,

the plasma membrane (9–11). In a cell-free assay, Akt phosphorylates AS160 (13). In 3T3-L1 adipocytes, insulin induces phosphorylation of at least six residues on AS160, of which five are placed in the Akt phosphorylation motif *RXRXXS*/T (10). Mutational analysis reveals that Ser-588 and Thr-642 on AS160 are of particular importance for the regulation of insulin-induced GLUT4 translocation (10). Insulin increases AS160 phosphorylation in a wortmannin-sensitive manner in 3T3-L1 adipocytes (13) and skeletal muscle (14). Thus, AS160 plays a pivotal role in insulin-mediated GLUT4 exocytosis at a step before the fusion of GLUT4-containing vesicles with the plasma membrane, such that when AS160 is dephosphorylated, GLUT4 vesicles remain in an intracellular compartment and upon phosphorylation by Akt, or other related kinases, GLUT4 is released for fusion with the plasma membrane (9,11).

AICAR treatment induces AS160 phosphorylation in isolated rat skeletal muscle (14). Insulin also increases AS160 phosphorylation in rat epitrochlearis (14) and human skeletal muscle (15–17). In type 2 diabetic patients (16), and in healthy humans infused with the cytokine tumor necrosis factor-α (17), AS160 phosphorylation and glucose transport are reduced in skeletal muscle. In contrast to effects of insulin, AICAR-induced AS160 phosphorylation occurs in the absence of Akt Ser-473 phosphorylation, a site necessary for full activation of the kinase, suggesting an Akt-independent regulation of AS160 (14). Since AICAR is a potent AMPK activator, we determined whether AS160 is an AMPK substrate in skeletal muscle. We hypothesized that AMPK mediates glucose transport in resting skeletal muscle via phosphorylation of AS160. This question is of clinical relevance since activation of AMPK bypasses insulin signaling defects, thereby increasing glucose transport and controlling glucose homeostasis (7). We also investigated whether contraction, a physiological stimulus for glucose uptake in skeletal muscle, increased AS160 phosphorylation by an AMPK-dependent mechanism. To this end, skeletal muscle from three genetically modified AMPK mouse models were studied: the whole-body α2 AMPK KO (18), γ3 AMPK KO (4,19), and the skeletal muscle–specific α2 AMPK kinase dead (KD) (6).

RESEARCH DESIGN AND METHODS

All experiments were approved by the Danish Animal Experimental Inspectorate and the Regional Animal Ethical Committee (Stockholm, Sweden) and complied with the European Convention for the Protection of Vertebrate Animals Used for Experiments and Other Scientific Purposes (Council of Europe 123, Strasbourg, France). Male mice, 4–5 months of age, from four different strains were used: α2 AMPK KO (18), transgenic mice expressing a KD-dominant inhibitory mutant of α2 AMPK (kindly provided by Morris J. Birnbaum, Howard Hughes Medical Institute and University of Pennsylvania School of Medicine, Philadelphia, PA) (6), γ3 AMPK KO (4), and C57Bl/6 mice (Taconic, Ry, Denmark). Wild-type littermates were used as controls for each genetic model studied. Within each transgenic strain, mice of different genotypes were produced by intercross breeding (hetero-hetero for α2 and γ3 and hetero-wild type for KD). Mice were maintained on a 10 h/14 h light/dark cycle and received standard rodent diet.

Muscle incubation procedures. Extensor digitorum longus (EDL) muscles were quickly removed from anesthetized fed mice and incubated in Krebs-Henseleit prebuffer at 30°C oxygenated with a gas containing 95% O_2 and 5% CO_2. Mice were anesthetized with either Avertin (2,2,2-tribromo ethanol 99% and tertiary amyl alcohol, at 0.015–0.017 ml/g mouse body wt i.p. for γ3 AMPK KO) or pentobarbital (6 mg/100 g body wt i.p. for α2 AMPK KO and α2 AMPK KD mice). The content of the prebuffer has been described previously (4,18). Incubations were carried out using prebuffer, to which AICAR (2 mmol/l; Toronto Research Chemicals, Toronto, Canada), wortmannin (500 nmol/l; Sigma), insulin (60 nmol/l Actrapid; Novo Nordisk, Bagsværd, Denmark), and/or DMSO (0.01%; Bie & Berntsen, Rødovre, Denmark) was added. To test the time-dependent effect of AICAR on AS160 phosphorylation, EDL muscles from wild-type C57Bl/6 mice were preincubated for 30 min in prebuffer, followed by incubation with or without 2 mmol/l AICAR for 10, 20, 40, or 60 min. To evaluate whether wortmannin inhibits AICAR- or insulin-induced AS160 phosphorylation, EDL muscles from wild-type C57Bl/6 mice were preincubated for 30 min in prebuffer with or without wortmannin, after which muscles were incubated for 40 min in the absence or presence of either AICAR or insulin. To test whether AICAR increased AS160 phosphorylation in an AMPK-dependent manner, EDL muscles from α2 AMPK KO, α2 AMPK KD, and γ3 AMPK KO mice and corresponding wild-type littermates were preincubated for 10 min in prebuffer, followed by a 40-min exposure to AICAR. To assess the effects of contraction on AMPK signaling and AS160 phosphorylation, isolated EDL muscles from α2 AMPK KO, α2 AMPK KD, and γ3 AMPK KO mice and corresponding wild-type littermates were incubated for 40 min in prebuffer. Thereafter, contraction was induced by electrical stimulation with a 10-s train (100 Hz, 0.2-ms impulse, ~30 V) per minute for 10 min. Contralateral muscles were incubated for 10 min under resting condition. To assess the effect of insulin on AS160 phosphorylation in the α2 AMPK KO and wild-type animals, EDL muscles were preincubated for 10 min in prebuffer, followed by a 10-min exposure to insulin. After incubation, muscles were harvested, washed in ice-cold Krebs-Henseleit buffer, blotted on filter paper, quickly frozen with aluminum tongs precooled in liquid nitrogen, and stored at −80°C.

Muscle lysate preparation. Muscles were homogenized in ice-cold buffer (10% glycerol, 20 mmol/l sodium pyrophosphate, 150 mmol/l NaCl, 50 mmol/l HEPES [pH 7.5], 1% NP-40, 20 mmol/l β-glycerophosphate, 10 mmol/l sodium fluoride, 1 mmol/l EDTA, 1 mmol/l EGTA, 2 mmol/l phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mmol/l sodium orthovanadate, 3 mmol/l benzamidine [pH 7.4]) for 20 s using a homogenizer (PT 3100; Brinkman Instruments). Homogenates were rotated end-over-end for 1 h at 4°C and then subjected to centrifugation (17,000*g* for 20 min) at 4°C. Supernatants were quickly frozen in liquid nitrogen and stored at −80°C. Protein content in lysates was measured by the bicinchoninic acid method (Pierce, Rockford, IL).

Immunoblot analysis. Phosphorylation of AMPK on Thr-172 was measured using a phospho-specific antibody (Cell Signaling Technology, Danvers, MA). Acetyl-CoA carboxylase (ACC) β Ser-227 phosphorylation was determined using a phospho-specific antibody (Upstate Biotechnologies, Waltham, MA). Phosphorylation of AS160 was measured using a phospho (Ser/Thr)-Akt substrate (PAS) antibody (Cell Signaling Technology, Danvers, MA). Phosphorylation of Akt on Ser-473 was measured with a phospho-specific antibody (Cell Signaling Technology). Total AS160 protein level was determined using an affinity-purified rabbit antibody prepared against a glutathione S-transferase (GST) fusion protein with a portion of mouse AS160 corresponding to amino acids 584–833 of human AS160 (kindly provided by Gustav E. Lienhard, Dartmouth Medical School, Hanover, NH). Muscle lysates were adjusted to equal protein concentration and boiled in Laemmli buffer, loaded on either 7.5% Tris-HCl Criterion gels (Bio-Rad, Copenhagen, Denmark) or self-cast 6–12% or 6–15% gradient gels, and transferred to polyvinylidene difluoride membrane (Immobilon Transfer Membrane; Millipore, Copenhagen, Denmark). Membranes were blocked in washing buffer (10 mmol/l Tris-base, 150 mmol/l NaCl, 0.25% Tween 20) containing 7.5% low-fat milk protein for 1 h at room temperature. Membranes were then incubated with primary antibodies overnight at 4°C, followed by incubation with appropriate horseradish peroxidase–conjugated secondary antibody (Dako, Glostrup, Denmark) for 2 h at room temperature. Bands were visualized using a Kodak Image Station 2000MM and enhanced chemiluminescence. Immunoreactive proteins were quantified (Kodak 1D 3.6 software), and results were expressed in relative units in comparison with basal samples loaded on each gel.

Immunoprecipitation. EDL muscle lysate (350 μg protein) was immunoprecipitated with 3.5 μg COOH-terminal anti-AS160 antibody at 4°C with gentle rotation overnight. Samples were incubated with equal mixtures of protein A sepharose (Amersham, Uppsala, Sweden) and protein G agarose (Sigma-Aldrich, St. Louis, MO) for 3 h at 4°C and subsequently washed three times with homogenization buffer and four times with PBS. The immunocomplex was boiled in Laemmli buffer containing β-mercaptoethanol and subjected to SDS-PAGE.

Cell-free AMPK kinase assay. Recombinant functional AMPK heterotrimers (α1β1γ1 or α2β2γ1) were expressed in *Escherichia coli* (20) and purified from bacterial lysates by a single-step nickel-ion chromatography, utilizing a polyhistidine tag fused to the NH_2-terminus of the α subunit, as previously described (20). A GST fusion protein of calmodulin-dependent protein kinase kinase (CaMKK)β was expressed in *E. coli*, purified, and cleaved from GST as previously described (21). For activation of recombinant wild-type AMPK (α1β1γ1 or α2β2γ1), 0.5 mg enzyme (20) was incubated for 60 min at 30°C with 0.5 mg recombinant CaMKKβ (21) in activation buffer (10 mmol/l HEPES [pH 7.5], 5 mmol/l $MgCl_2$, 1 mmol/l AMP, 2 mmol/l ATP). EDL muscle lysates (100 mg) were incubated (30 min at 30°C) in assay buffer (10 mmol/l HEPES

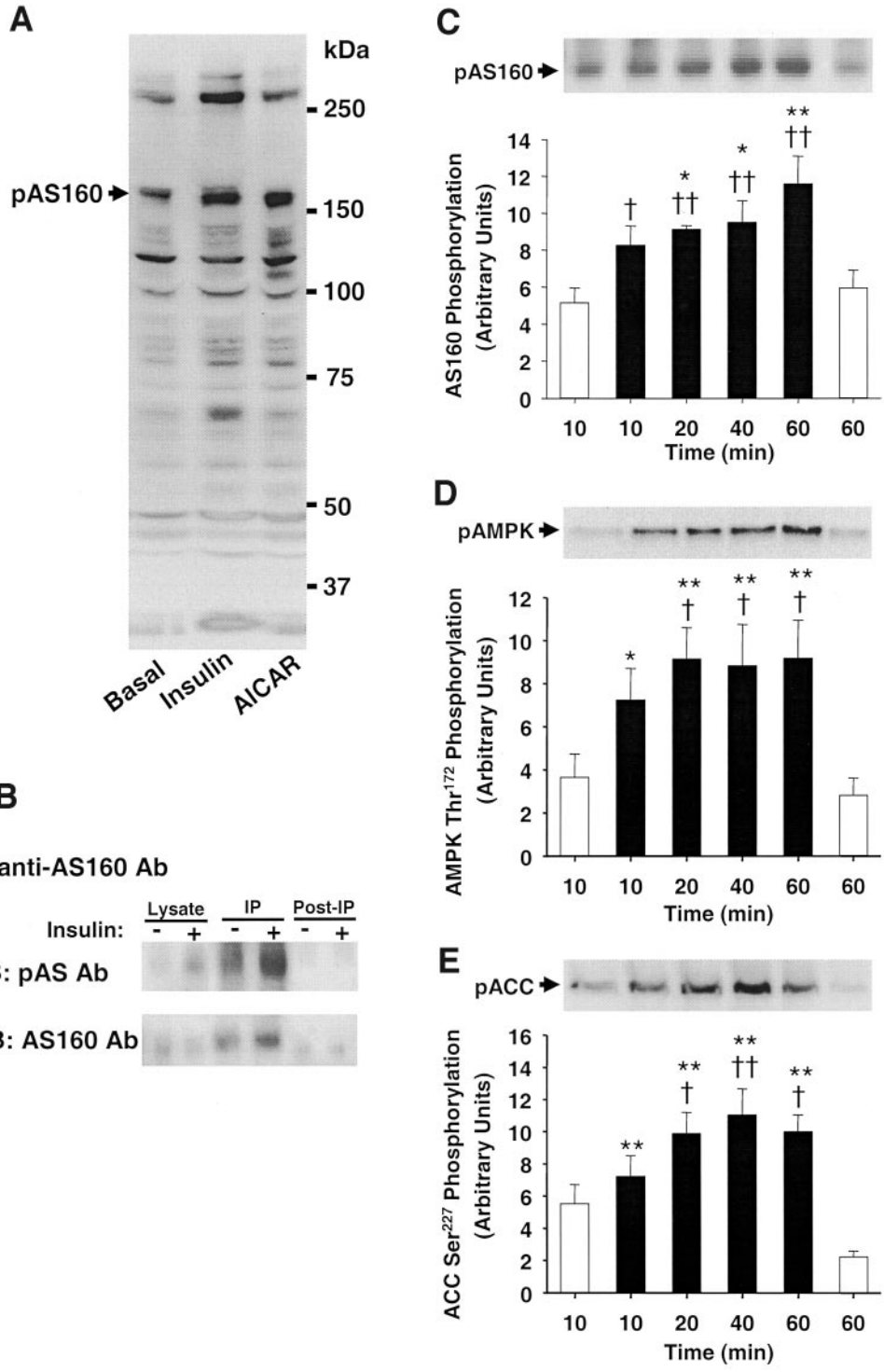

FIG. 1. Effect of AICAR on phosphorylation of AS160, AMPK, and ACC in EDL muscle from C57BL/6 mice. *A*: Representative PAS immunoblot of lysate prepared from EDL muscle incubated for 40 min in the absence (basal) or presence of either insulin or AICAR. *B*: Identification of AS160 by Western blot (WB) analysis of anti-AS160 immunoprecipitates with anti-PAS (*upper panel*) or anti-AS160 (*lower panel*). Time-dependent phosphorylation of AS160 (*C*), AMPK (Thr-172) (*D*), and ACC (Ser-227) (*E*) in EDL muscle incubated in the absence (□) or presence (■) of AICAR. Data shown in *C–E* are presented as means ± SE, $n = 6$. †/††$P <$ 0.05/0.01 vs. 10 min basal. */$P <$ 0.05/0.01 vs. 60 min basal.**

[pH 7.5], 5 mmol/l $MgCl_2$, 1 mmol/l EGTA, 0.5 mmol/l Na_3VO_4, 40 μmol/l P^1,P^5-di(adenosine-5′) pentaphosphate, 100 μmol/l AMP, 200 μmol/l ATP) with or without recombinant-activated wild-type AMPK. Assays were performed under six conditions: *1*) lysate and assay buffer; *2*) lysate, assay buffer, and AMP; *3*) lysate, assay buffer, AMP, and ATP; and *4-6*) lysate, assay buffer, AMP, ATP, and recombinant CaMKKβ or AMPK (α1β1γ1 or α2β2γ1). The reaction was stopped by addition of sample buffer (6×) and heating (96°C for 4 min). Samples were subjected to SDS-PAGE, followed by Western blot analysis with the PAS antibody.

Statistics. Data are expressed as means ± SE. Levene's test of equality of variance was used to test whether groups had equal variances, and data were log-transformed if Levene's test was significant. In all cases, this was sufficient to obtain equal variances among groups. Statistical evaluation was performed by two-way ANOVA with repeated measures or one-way ANOVA, using Fisher's least significant difference post hoc analysis to identify significant differences between groups. All statistical analyses were performed using SPSS 13.0. $P < 0.05$ was considered significant.

RESULTS

AICAR treatment in vitro leads to AS160 phosphorylation in skeletal muscle. Immunoblot analysis with the PAS antibody revealed that AICAR and insulin independently increased the phosphorylation of multiple proteins in EDL muscle from C57BL/6 mice (Fig. 1*A*). Previous studies provide evidence that exposure of isolated rat epitrochlearis muscle to AICAR increases AS160

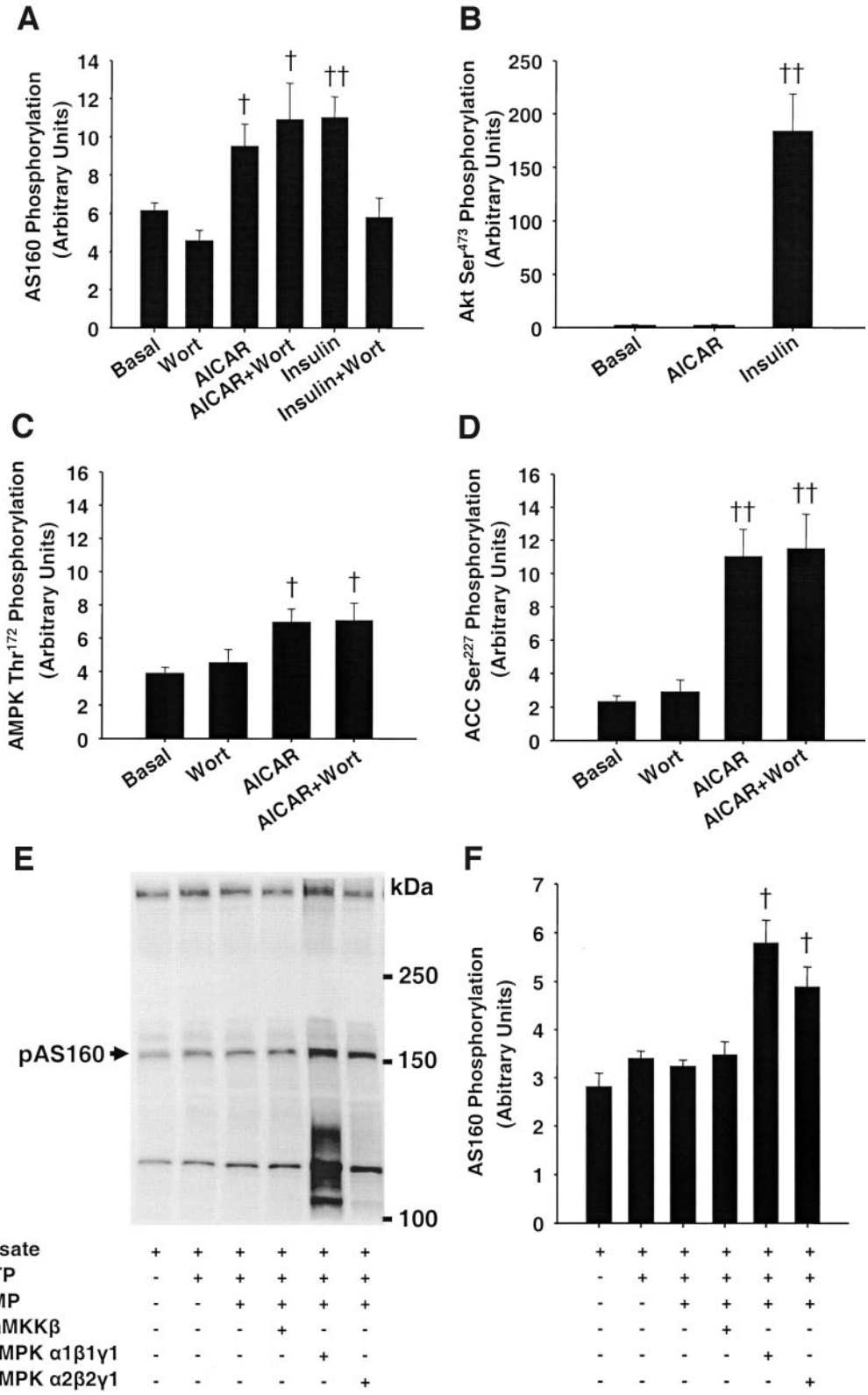

FIG. 2. Wortmannin-independent effects of AICAR on AS160 phosphorylation in EDL muscle and in vitro phosphorylation of AS160 by AMPK. EDL muscle was incubated for 40 min in the presence of either AICAR or insulin, and phosphorylation of AS160 (*A*), AKT (Ser-473) (*B*), AMPK (Thr-172) (*C*), or ACC (Ser-227) (*D*) was determined. In *A*, *C*, and *D*, EDL muscle was also incubated with or without wortmannin (Wort). Data are presented as means ± SE, $n = 4–6$. *E*: Effect of recombinant AMPK on AS160 phosphorylation in vitro was determined. Recombinant CaMKKβ or AMPK (α1β1γ1 or α2β2γ1) was added to EDL muscle lysates. Samples were incubated for 30 min, and AS160 phosphorylation was measured by immunoblot analysis using a PAS antibody. Representative PAS immunoblot (*E*) and quantification (means ± SE, $n = 3$) (*F*) is shown. †/††$P <$ 0.05/0.01 compared with basal/control.

phosphorylation (14). A phospho-protein of ~160 kDa, confirmed as AS160 in the immunoprecipitation experiments (Fig. 1*B*), was phosphorylated in response to insulin exposure. AICAR increased AS160 phosphorylation in a time-dependent manner (Fig. 1*C*) paralleled by increased phosphorylation of AMPK on Thr-172 and ACCβ, an AMPK substrate, on Ser-227 (Fig. 1*D* and *E*).

AICAR-induced AS160 phosphorylation is wortmannin independent. Insulin action on AS160 phosphorylation occurred by a wortmannin-sensitive mechanism (Fig. 2*A*), suggesting that this effect was mediated by signal transduction over a phosphatidylinositol-3 kinase– and Akt-dependent pathway. In contrast, AICAR increased AS160 phosphorylation in the presence of wortmannin

TABLE 1
Effect of AICAR on AMPK and ACC phosphorylation

Genotype	AMPK-P (T-172)		ACC-P (S-227)	
	Basal	AICAR	Basal	AICAR
α2 AMPK WT	100 ± 15	185 ± 22*	100 ± 17	218 ± 26*
α2 AMPK KO	15 ± 2†	16 ± 2†	15 ± 3†	45 ± 5*†
α2 AMPK WT	100 ± 13	203 ± 35*	100 ± 10	436 ± 49*
α2 AMPK KD	283 ± 32†	496 ± 75*†	28 ± 7†	131 ± 15*†
γ3 AMPK WT	100 ± 12	267 ± 36*	100 ± 29	507 ± 80*
γ3 AMPK KO	82 ± 10†	108 ± 16†	122 ± 20	167 ± 25

Data are means ± SE, n = 7–14. AMPK (Thr-172) and ACC (Ser-227) phosphorylation (AMPK-P and ACC-P, respectively) was determined in EDL muscle. Comparisons were made between each wild-type (WT) and genetically modified animal model. */†Effect ($P < 0.01$) of AICAR and genotype, respectively.

(Fig. 2*A*) and without effect on Akt (Ser-473) phosphorylation (Fig. 2*B*), implicating that this effect was mediated by a pathway independent of the canonical insulin signaling cascade. We hypothesized that the AICAR effect on AS160 occurs via the AMPK-signaling pathway. Consistent with this hypothesis, wortmannin did not alter AICAR-induced phosphorylation of AMPK (Thr-172) (Fig. 2*C*) or ACC (Ser-227) (Fig. 2*D*) in murine EDL muscle.

To test whether AMPK phosphorylates AS160, we performed a cell-free kinase assay adding either α1β1γ1 or α2β2γ1 recombinant AMPK heterotrimeric complexes to EDL muscle lysate (n = 3). Several phosphoproteins were identified by the PAS antibody in subsequent Western blot analysis (Fig. 2*E*). Phosphorylation of AS160 was enhanced in vitro by the addition of either α1β1γ1 or α2β2γ1 recombinant AMPK heterotrimeric complexes in all preparations tested, whereas the addition of recombinant CaMKKβ was without effect (Fig. 2*F*). This in vitro approach could indicate that AS160 is an AMPK substrate.

AS160 phosphorylation is regulated by AMPK in isolated EDL muscle. To establish the role of AS160 as an AMPK target, EDL muscles from α2 AMPK KO, α2 AMPK KD, and γ3 AMPK KO mice were isolated and incubated in the absence or presence of AICAR. In all experiments, genetically modified mice were compared with wild-type littermates to exclude any influence of genetic background on the measured phenotype. Differences in signaling responses were noted among wild-type mice of the three strains, further reinforcing the need to study each genetically modified animal against their own wild-type littermate. AICAR increased AMPK phosphorylation in wild-type mice from each genotype. This effect was blunted in α2 AMPK KO and γ3 AMPK KO but not in α2 AMPK KD mice (Table 1). The AICAR response on AMPK observed in the α2 AMPK KD mice was expected, since the dominant-negative construct can be phosphorylated but not activated (6). AICAR increased ACC phosphorylation in wild-type mice, with effects severely blunted in α2 AMPK KO, α2 AMPK KD, and γ3 AMPK KO mice (Table 1). In contrast to wild-type mice, the effect of AICAR on AS160 phosphorylation was significantly (P = 0.02) blunted (α2 AMPK KO) or ablated (α2 AMPK KD and γ3 AMPK KO) in EDL muscle from KO or transgenic mice (Fig. 3*A*). In EDL muscle from α2 AMPK KO and α2 AMPK KD mice, basal AS160 phosphorylation was also reduced compared with wild-type mice. This difference in basal AS160 phosphorylation may be related to a slight and nonsignificant reduction in AS160 protein expression in α2 AMPK KO and α2 AMPK KD mice (Fig. 3*B*). In contrast, basal AS160 phosphorylation and protein expression was similar between γ3 AMPK KO and wild-type mice.

To address whether physiological activation of AMPK by muscle contraction is associated with AS160 phosphorylation, isolated EDL muscle was subjected to intermittent electrical stimulation and forced to contract. Here, we provide evidence that AMPK phosphorylation after electrically induced muscle contraction is markedly increased in wild-type mice from all strains and blunted in α2 AMPK KO mice (Table 2). In contrast, contraction-induced AMPK phosphorylation was unaltered in α2 AMPK KD and γ3 AMPK KO mice. ACC phosphorylation, after electrically induced muscle contraction, was markedly increased in wild-type and γ3 AMPK KO mice and reduced in α2 AMPK KO and α2 AMPK KD mice. Similarly, contraction markedly increased AS160 phosphorylation in wild-type mice, with contraction-induced responses completely suppressed in α2 AMPK KO and α2 AMPK KD but not in γ3 AMPK KO mice (Fig. 3*C*).

Akt signaling is unaltered in α2 AMPK KO mice. Since AS160 phosphorylation was reduced under basal and AICAR-stimulated conditions in α2 AMPK KO and α2 AMPK KD mice, additional experiments were performed to determine whether insulin-mediated responses were impaired in these mice. Phosphorylation of Akt (Ser-473) and AS160 phosphorylation was increased in response to 60 nmol/l insulin, irrespective of genotype (Fig. 4*A* and *B*). Although basal and insulin-stimulated Akt phosphorylation was similar between α2 AMPK KO and wild-type mice, the absolute level of AS160 phosphorylation under basal and insulin-stimulated conditions was reduced. Nevertheless, the increase in AS160 phosphorylation in response to insulin was maintained, indicating an intact response to insulin. AMPK and ACC phosphorylation in α2 AMPK KO and wild-type mice was unaffected by insulin treatment (data not shown).

DISCUSSION

Using three different animal models in which the predominant catalytic and regulatory AMPK isoforms expressed in skeletal muscle have been genetically manipulated, we provide evidence that AS160 is a downstream target of AMPK in skeletal muscle. Exposure of isolated skeletal muscle to AICAR, an AMPK activator, increased AS160 phosphorylation in wild-type but only slightly or not at all in α2 AMPK KO, γ3 AMPK KO, or α2 AMPK KD mice. Taken together with previous observations that AICAR-induced glucose transport is totally abolished in these KO and transgenic mouse models (4–6), our results indicate that AICAR could increase glucose transport by a pathway involving AMPK and likely AS160. Moreover, heterotrimeric complexes containing the AMPK α2/γ3 subunits are likely to play a major role in the regulation of AS160 phosphorylation upon pharmacological activation by AICAR, since the AICAR effects on AS160 were ablated in α2 and γ3 AMPK KO mice. This is of physiological relevance, since the γ3 isoform is mainly found in complexes containing α2 and β2 subunits in skeletal muscle (22,23).

We also provide evidence that AMPK is the major contributor to the regulation of AS160 in response to muscle contraction. Despite our evidence supporting a role for AS160 as an AMPK-dependent target important for GLUT4-mediated glucose transport in resting muscle, the connection between AS160 and glucose transport is unre-

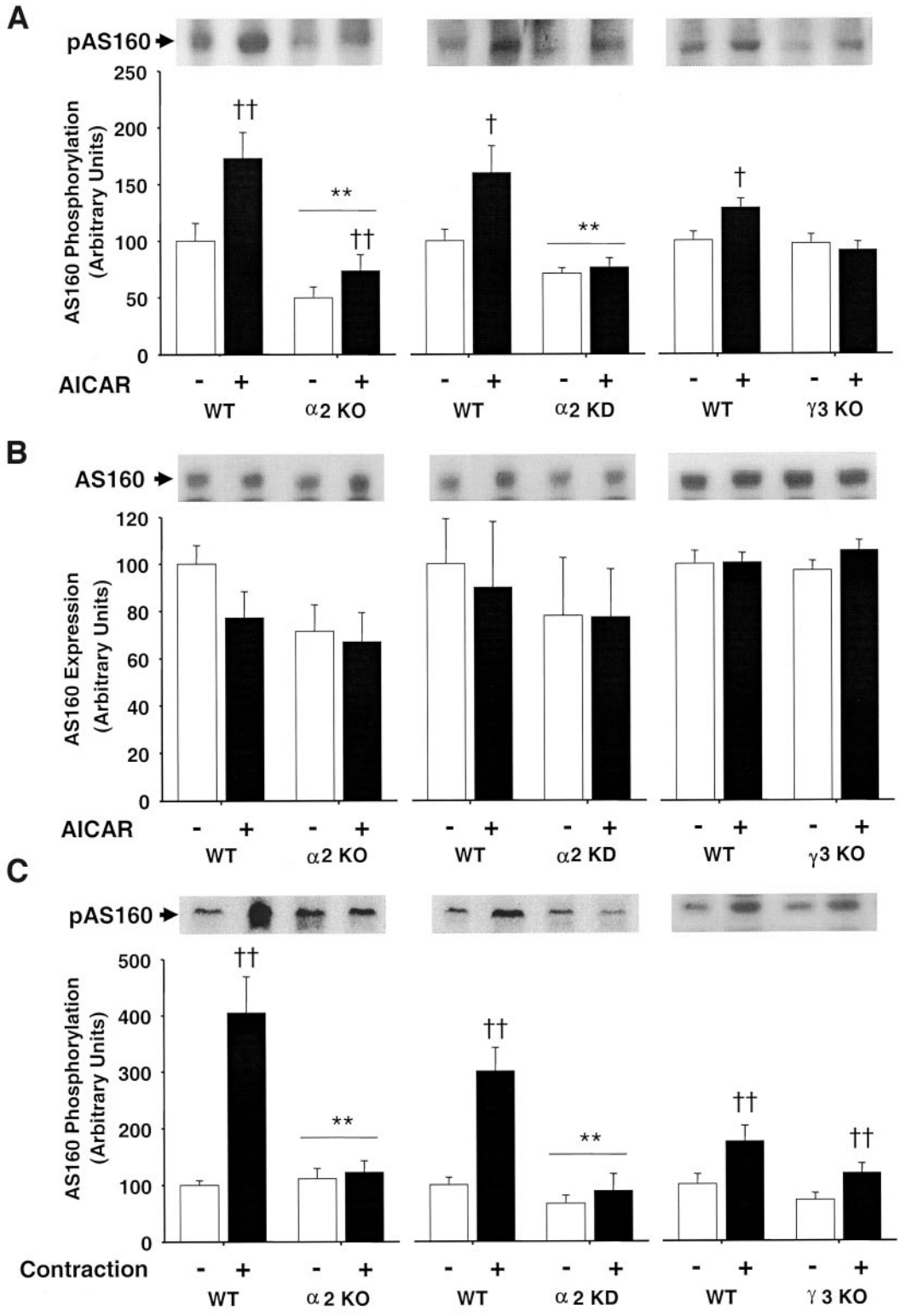

FIG. 3. Effect of AICAR or muscle contraction on AS160 phosphorylation. AS160 phosphorylation was determined in EDL muscle obtained from three genetically modified mouse models in which AMPK signaling has been disrupted and from corresponding wild-type (WT) littermates. *A*: Muscles were incubated in the absence (□) or presence (■) of AICAR, and AS160 phosphorylation was determined by immunoblot analysis using a PAS antibody. Results are means ± SE, n = 7–14. The increase with AICAR was significantly (P = 0.02) smaller in the α2 KO compared with WT mice. *B*: AS160 protein expression was determined in muscle lysate using an antibody recognizing mouse AS160 protein. Results are means ± SE, n = 5. *C*: Muscles were incubated under basal conditions (□) or induced to contract in vitro by electrical stimulation (■), and effects on AS160 phosphorylation were determined. Results are means ± SE, n = 9–14. †/††P < 0.05/0.01 vs. corresponding basal, **main effect (P < 0.01) of genotype.

solved in contracting skeletal muscle. Previous studies indicate that contraction-induced glucose uptake is unaltered (5) or only partly decreased (6) in the same AMPK-deficient mouse models in which contraction-induced AS160 phosphorylation was completely prevented. Thus, although contraction increases AS160 phosphorylation in skeletal muscle, AS160-independent mechanisms may elicit contraction-induced responses on glucose transport. In accordance, Ca^{2+}-mediated mechanisms have been proposed in the regulation of contraction-induced skeletal muscle glucose transport (24). Thus, AS160 is unlikely to be the sole mechanism involved in the release of GLUT4-containing vesicles. This hypothesis is supported by recent evidence showing that silencing of AS160 by short-hairpin RNA in 3T3-L1 adipocytes only partially released basal GLUT4 retention, thereby implicating AS160-dependent and -independent mechanisms in insulin signaling to GLUT4 exocytosis (25).

TABLE 2
Effect of contraction on AMPK and ACC phosphorylation

Genotype	AMPK-P (T-172)		ACC-P (S-227)	
	Basal	Contraction	Basal	Contraction
α2 AMPK WT	100 ± 21	1,479 ± 132*	100 ± 15	392 ± 25*
α2 AMPK KO	9 ± 4†	120 ± 14*†	11 ± 3†	181 ± 24*†
α2 AMPK WT	100 ± 16	1,266 ± 133*	100 ± 15	332 ± 42*
α2AMPK KD	460 ± 32	1,114 ± 141*	29 ± 8†	193 ± 25*†
γ3 AMPK WT	100 ± 7	1,475 ± 161*	100 ± 26	753 ± 232*
γ3 AMPK KO	69 ± 13	1,741 ± 199*	40 ± 7	778 ± 171*

Data are means ± SE, $n = 9$–14. AMPK (Thr-172) and ACC (Ser-227) phosphorylation (AMPK-P and ACC-P, respectively) was determined in EDL muscle. Comparisons were made between each wild-type (WT) and genetically modified animal model. */†Effect ($P < 0.01$) of contraction and genotype, respectively.

AMPK may phosphorylate AS160 directly or indirectly through unidentified substrates. Using an in vitro AMPK activity assay, where addition of activated recombinant α1β1γ1 or α2β2γ1 complexes to skeletal muscle lysate increased AS160 phosphorylation, we provided evidence to suggest that AMPK, per se, phosphorylates AS160. In contrast, recombinant CaMKKβ was without effect. Our results do not eliminate a possible indirect effect of AMPK on AS160. For example, the recombinant AMPK may have phosphorylated another kinase, which in turn may have directly phosphorylated AS160. However, if AMPK is an indirect regulator of AS160, wortmannin-sensitive components of the canonical insulin signaling cascade, including Akt, are unlikely candidates.

AS160 is a negative regulator of basal GLUT4 exocytosis in 3T3-L1 adipocytes (9,25). In α2 AMPK KO and α2 AMPK KD mice, basal AS160 phosphorylation was impaired. Based on studies in 3T3-L1 adipocytes, in which AS160 expression is silenced (9,25), basal glucose uptake in α2 AMPK KO and α2 AMPK KD mice would be predicted to be decreased due to the reduction in AS160 phosphorylation. However, basal glucose transport is unaltered by modification of the AMPK α2 subunit (5,6). Thus, there may be a threshold effect, such that only a permissive level of AS160 phosphorylation may be necessary for basal glucose uptake. The reduced basal AS160 phosphorylation may be related to a slight but nonsignificant reduction in AS160 protein expression in α2 AMPK KO and α2 AMPK KD mice, although this alternative seems unlikely. In contrast, basal AS160 phosphorylation was preserved in the γ3 AMPK KO mice, an observation that may suggest that heterotrimeric complexes containing this isoform are dispensable for basal AS160 phosphorylation. The reduced basal AS160 phosphorylation in the α2 AMPK KO mice was not associated with impairments in insulin signaling, as insulin-induced phosphorylation of Akt and AS160, as well as glucose transport, are unaltered (18). Likewise, insulin-stimulated glucose uptake and Akt signaling is unaltered in α2 AMPK KD mice (6). Nevertheless, there may be excess signaling, such that partial phosphorylation of AS160 may be sufficient for full activation of insulin-mediated glucose transport.

The PAS antibody used to detect AS160 phosphorylation recognizes proteins with phosphorylated serine or threonine residues that follow arginine at positions P-3 and P-5 (R*X*R*XX*S/T). The recognition sequences of Akt and AMPK differ (13,26,27); consequently, AMPK might target other unidentified sites on AS160, which could regulate AS160 activity or localization. Interestingly, tuberous sclerosis complex (TSC)2 in the TSC1-TSC2 complex, which functions as a GTPase-activating protein for the small GTPase Ras homologue enriched in brain (Rheb) in the mammalian target of rapamycin pathway, is phosphorylated by Akt and AMPK but on different sites, leading to either inhibition or activation, respectively, of TSC2 (28,29).

The regulation of glucose transport is complex and requires multiple signaling inputs that converge on GLUT4 vesicle transport (30,31). The identification of AS160 as an AMPK substrate provides a mechanism to partly account for insulin-independent glucose uptake in resting skeletal muscle, whereas a role in contraction-mediated glucose uptake is unclear. Our results reveal that AMPK complexes containing α2 and γ3 are required for AS160 phosphorylation in response to the AMPK activator AICAR. Furthermore, AMPK increases AS160 phosphorylation in response to AICAR via a phosphatidylinositol-3 kinase/Akt-independent pathway. We also provide evidence that complexes containing the AMPK α2 subunit are

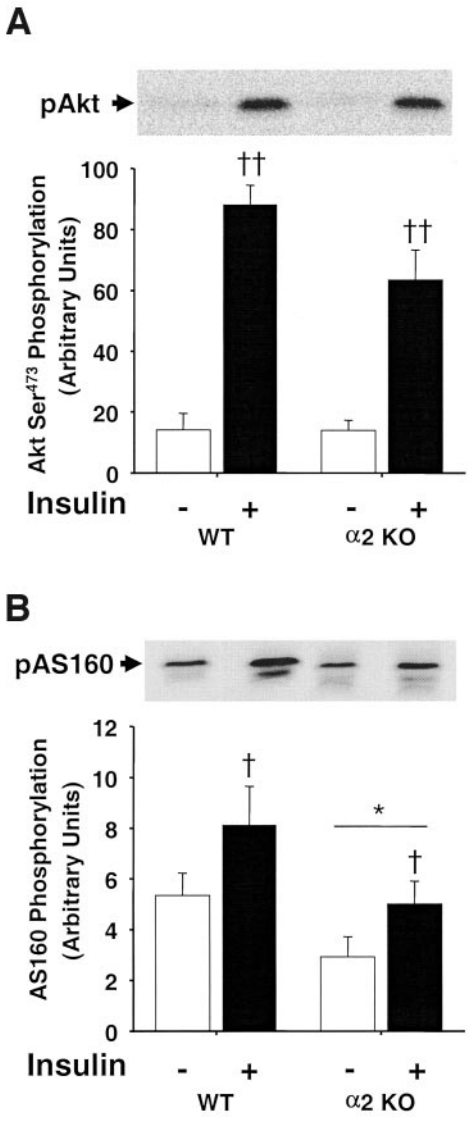

FIG. 4. Effect of insulin on AS160 phosphorylation in α2 AMPK KO mice. EDL muscle was obtained from α2 AMPK KO and wild-type (WT) mice and incubated in the absence (□) or presence (■) of insulin, and phosphorylation of Akt (Ser-473) (*A*) and AS160 (*B*) was determined. Results are means ± SE, $n = 7$–10. †/††$P < 0.05/0.01$ vs. basal, *genotype effect ($P < 0.05$).

required for AS160 phosphorylation in response to muscle contraction, whereas complexes containing the γ3 subunit are dispensable. Thus, AS160 may be viewed as a point of convergence for insulin-dependent and -independent signaling to glucose transport in resting skeletal muscle.

ACKNOWLEDGMENTS

This work was supported by the Swedish Research Council, the Swedish Diabetes Association, the Foundation for Scientific Studies of Diabetology, the Strategic Research Foundation, the Swedish Centre for Sports Research, the Commission of the European Communities (contract nos. LSHM-CT-2004-005272 EXGENESIS and LSHM-CT-2004-512013 EUGENE2), the Danish Medical Research Council, the Novo Nordisk Foundation, the Danish Diabetes Association, and the Copenhagen Muscle Research Centre.

J.F.P.W. was supported by a Hallas Møller fellowship from the Novo Nordisk Foundation.

We are especially grateful to Hiroshi Tokumitsu (Kagawa University, Kagawa, Japan), Professor Gustav E. Lienhard (Dartmouth Medical School, Hanover, NH), and Professor Morris J. Birnbaum (Howard Hughes Medical Institute and University of Pennsylvania School of Medicine, Philadelphia, PA) for providing valuable reagents.

REFERENCES

1. Kahn BB, Alquier T, Carling D, Hardie DG: AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metab* 1:15–25, 2005
2. Hardie DG: The AMP-activated protein kinase pathway: new players upstream and downstream. *J Cell Sci* 117:5479–5487, 2004
3. Merrill GF, Kurth EJ, Hardie DG, Winder WW: AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle. *Am J Physiol* 273:E1107–E1112, 1997
4. Barnes BR, Marklund S, Steiler TL, Walter M, Hjalm G, Amarger V, Mahlapuu M, Leng Y, Johansson C, Galuska D, Lindgren K, Abrink M, Stapleton D, Zierath JR, Andersson L: The 5′-AMP-activated protein kinase gamma3 isoform has a key role in carbohydrate and lipid metabolism in glycolytic skeletal muscle. *J Biol Chem* 279:38441–38447, 2004
5. Jørgensen SB, Viollet B, Andreelli F, Frøsig C, Birk JB, Schjerling P, Vaulont S, Richter EA, Wojtaszewski JF: Knockout of the alpha2 but not alpha1 5′-AMP-activated protein kinase isoform abolishes 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranosidebut not contraction-induced glucose uptake in skeletal muscle. *J Biol Chem* 279:1070–1079, 2004
6. Mu J, Brozinick JT Jr, Valladares O, Bucan M, Birnbaum MJ: A role for AMP-activated protein kinase in contraction- and hypoxia-regulated glucose transport in skeletal muscle. *Mol Cell* 7:1085–1094, 2001
7. Koistinen HA, Galuska D, Chibalin AV, Yang J, Zierath JR, Holman GD, Wallberg-Henriksson H: 5-amino-imidazole carboxamide riboside increases glucose transport and cell-surface GLUT4 content in skeletal muscle from subjects with type 2 diabetes. *Diabetes* 52:1066–1072, 2003
8. Lemieux K, Konrad D, Klip A, Marette A: The AMP-activated protein kinase activator AICAR does not induce GLUT4 translocation to transverse tubules but stimulates glucose uptake and p38 mitogen-activated protein kinases alpha and beta in skeletal muscle. *FASEB J* 17:1658–1665, 2003
9. Larance M, Ramm G, Stockli J, van Dam EM, Winata S, Wasinger V, Simpson F, Graham M, Junutula JR, Guilhaus M, James DE: Characterisation of the role of the RabGAP AS160 in insulin-regulated GLUT4 trafficking. *J Biol Chem* 280:37803–37813, 2005
10. Sano H, Kane S, Sano E, Miinea CP, Asara JM, Lane WS, Garner CW, Lienhard GE: Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. *J Biol Chem* 278:14599–14602, 2003
11. Zeigerer A, McBrayer MK, McGraw TE: Insulin stimulation of GLUT4 exocytosis, but not its inhibition of endocytosis, is dependent on RabGAP AS160. *Mol Biol Cell* 15:4406–4415, 2004
12. Miinea CP, Sano H, Kane S, Sano E, Fukuda M, Peranen J, Lane WS, Lienhard GE: AS160, the Akt substrate regulating GLUT4 translocation, has a functional Rab GTPase-activating protein domain. *Biochem J* 391: 87–93, 2005
13. Kane S, Sano H, Liu SC, Asara JM, Lane WS, Garner CC, Lienhard GE: A method to identify serine kinase substrates: Akt phosphorylates a novel adipocyte protein with a Rab GTPase-activating protein (GAP) domain. *J Biol Chem* 277:22115–22118, 2002
14. Bruss MD, Arias EB, Lienhard GE, Cartee GD: Increased phosphorylation of Akt substrate of 160 kDa (AS160) in rat skeletal muscle in response to insulin or contractile activity. *Diabetes* 54:41–50, 2005
15. Karlsson HK, Hällsten K, Björnholm M, Tsuchida H, Chibalin AV, Virtanen KA, Heinonen OJ, Lönnqvist F, Nuutila P, Zierath JR: Effects of metformin and rosiglitazone treatment on insulin signaling and glucose uptake in patients with newly diagnosed type 2 diabetes: a randomized controlled study. *Diabetes* 54:1459–1467, 2005
16. Karlsson HK, Zierath JR, Kane S, Krook A, Lienhard GE, Wallberg-Henriksson H: Insulin-stimulated phosphorylation of the Akt substrate AS160 is impaired in skeletal muscle of type 2 diabetic subjects. *Diabetes* 54:1692–1697, 2005
17. Plomgaard P, Bouzakri K, Krogh-Madsen R, Mittendorfer B, Zierath JR, Pedersen BK: Tumor necrosis factor-α induces skeletal muscle insulin resistance in healthy human subjects via inhibition of Akt substrate 160 phosphorylation. *Diabetes* 54:2939–2945, 2005
18. Viollet B, Andreelli F, Jorgensen SB, Perrin C, Geloen A, Flamez D, Mu J, Lenzner C, Baud O, Bennoun M, Gomas E, Nicolas G, Wojtaszewski JF, Kahn A, Carling D, Schuit FC, Birnbaum MJ, Richter EA, Burcelin R, Vaulont S: The AMP-activated protein kinase alpha2 catalytic subunit controls whole-body insulin sensitivity. *J Clin Invest* 111:91–98, 2003
19. Barnes BR, Glund S, Long YC, Hjalm G, Andersson L, Zierath JR: 5′-AMP-activated protein kinase regulates skeletal muscle glycogen content and ergogenics. *FASEB J* 19:773–779, 2005
20. Neumann D, Woods A, Carling D, Wallimann T, Schlattner U: Mammalian AMP-activated protein kinase: functional, heterotrimeric complexes by co-expression of subunits in Escherichia coli. *Protein Expr Purif* 30:230–237, 2003
21. Tokumitsu H, Iwabu M, Ishikawa Y, Kobayashi R: Differential regulatory mechanism of Ca2+/calmodulin-dependent protein kinase kinase isoforms. *Biochemistry* 40:13925–13932, 2001
22. Mahlapuu M, Johansson C, Lindgren K, Hjalm G, Barnes BR, Krook A, Zierath JR, Andersson L, Marklund S: Expression profiling of the gamma-subunit isoforms of AMP-activated protein kinase suggests a major role for gamma3 in white skeletal muscle. *Am J Physiol Endocrinol Metab* 286:E194–E200, 2004
23. Wojtaszewski JF, Birk JB, Frosig C, Holten M, Pilegaard H, Dela F: 5′AMP activated protein kinase expression in human skeletal muscle: effects of strength training and type 2 diabetes. *J Physiol* 564:563–573, 2005
24. Holloszy JO: A forty-year memoir of research on the regulation of glucose transport into muscle. *Am J Physiol Endocrinol Metab* 284:E453–E467, 2003
25. Eguez L, Lee A, Chavez JA, Miinea CP, Kane S, Lienhard GE, McGraw TE: Full intracellular retention of GLUT4 requires AS160 Rab GTPase activating protein. *Cell Metab* 2:263–272, 2005
26. Hardie DG, Carling D, Carlson M: The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? *Annu Rev Biochem* 67:821–855, 1998
27. Weekes J, Ball KL, Caudwell FB, Hardie DG: Specificity determinants for the AMP-activated protein kinase and its plant homologue analysed using synthetic peptides. *FEBS Lett* 334:335–339, 1993
28. Inoki K, Li Y, Zhu T, Wu J, Guan KL: TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat Cell Biol* 4:648–657, 2002
29. Inoki K, Zhu T, Guan KL: TSC2 mediates cellular energy response to control cell growth and survival. *Cell* 115:577–590, 2003
30. Ramm G, James DE: GLUT4 trafficking in a test tube (Letter). *Cell Metab* 2:150–152, 2005
31. Thong FS, Dugani CB, Klip A: Turning signals on and off: GLUT4 traffic in the insulin-signaling highway. *Physiology (Bethesda)* 20:271–284, 2005