

APPL1 Mediates Adiponectin-Induced LKB1 Cytosolic Localization Through the PP2A-PKC ζ Signaling Pathway

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We recently found that the adaptor protein containing pleckstrin homology domain, phosphotyrosine binding domain and leucine zipper motif (APPL)1 is essential for mediating adiponectin signal to induce liver kinase B (LKB)1 cytosolic translocation, an essential step for activation of AMP-activated protein kinase (AMPK) in cells. However, the underlying molecular mechanisms remain unknown. Here, we demonstrate that treating C2C12 myotubes with adiponectin promoted APPL1 interaction with protein phosphatase 2A (PP2A) and protein kinase C ζ (PKC ζ), leading to the activation of PP2A and subsequent dephosphorylation and inactivation of PKC ζ . The adiponectin-induced inactivation of PKC ζ results in dephosphorylation of LKB1 at Ser³⁰⁷ and its subsequent translocation to the cytosol, where it stimulates AMPK activity. Interestingly, we found that metformin also induces LKB1 cytosolic translocation, but the stimulation is independent of APPL1 and the PP2A-PKC ζ pathway. Together, our study uncovers a new mechanism underlying adiponectin-stimulated AMPK activation in muscle cells and shed light on potential targets for prevention and treatment of insulin resistance and its associated diseases. (*Molecular Endocrinology* 25: 1773–1785, 2011)

Adiponectin exerts its antidiabetic and antiinflammatory functions partly by binding to its membrane receptors adiponectin receptor 1 and adiponectin receptor 2 (1, 2). Recent evidence indicated that skeletal muscle tissue is one of the primary target sites for adiponectin action (3). Our previous study showed that the binding of adiponectin promotes the recruitment of adaptor protein containing pleckstrin homology domain, phosphotyrosine binding domain and leucine zipper motif (APPL)1 to the receptors, which leads to stimulate downstream targets including the AMP-activated protein kinase (AMPK) and various biological events, such as glucose uptake and fatty acid oxidation in muscle cells (4, 5). In

addition, we have found that adiponectin sensitizes insulin signaling by suppressing negative effect of p70 S6-kinase on insulin receptor substrate 1 serine phosphorylation (6), and APPL1 is essential for mediating the insulin sensitizer role of adiponectin (4). Accumulating evidence support the role of APPL1 in mediating adiponectin and insulin signaling in endothelial cells, adipocytes, HEK293 cells, zebrafish, as well as in mouse liver (7–12). Most recently, we showed that APPL1, together with its isoform APPL2, function as a “Yin-Yang” regulator of adiponectin signaling (13).

Several upstream kinases have been reported to activate AMPK in muscle cells, including liver kinase B

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Abbreviations: AMPK, AMP-activated protein kinase; APPL, adaptor protein containing pleckstrin homology domain, phosphotyrosine binding domain and leucine zipper motif; GST, glutathione S-transferase; LKB, liver kinase B; MO25 α/β , mouse protein 25; PKC ζ , protein kinase C ζ ; PP2A, protein phosphatase 2A; PS, pseudosubstrate; RNAi, RNA interference; shRNA, short hairpin RNA; STRAD α/β , Ste20-related adaptor protein.

(LKB)1 and Ca²⁺/calmodulin-dependent kinase kinase II (14–19). LKB1 is a constitutively active serine/threonine protein kinase that is predominately localized in the nucleus under normal physiological condition (20). By forming a heterotrimeric complex with Ste20-related adaptor protein (STRAD α/β) and mouse protein 25 (MO25 α/β) or associating with a LKB1 interacting protein, LKB1 is translocated to the cytosol, where it activates its substrates (20–26). It has been showed that LKB1 plays a critical role in adiponectin-induced activation of AMPK in muscle cells (22, 26). Our recent study revealed that adiponectin-stimulated AMPK activation in muscle cells is through two distinct mechanisms: APPL1-independent pathway stimulating Ca²⁺ release that activates Ca²⁺/calmodulin-dependent kinase kinase II and APPL1-dependent pathway that promotes LKB1 cytosolic translocation (26). APPL1 acts as an anchoring protein to tether LKB1 in cytosol in response to adiponectin stimulation, which leads to subsequent AMPK phosphorylation and activation (26). However, the underlying molecular mechanism by which APPL1 mediates adiponectin signal to stimulate LKB1 cytosolic translocation remains largely unknown.

Metformin is a widely used drug for the treatment of type 2 diabetes (27). Although studies have implicated AMPK activation as a mediator of metformin action, how metformin activates AMPK is poorly understood (28). One proposed mechanism is via inhibiting complex I activity of the respiratory chain and thereby increasing cellular AMP:ATP ratio and potentiating AMPK phosphorylation by the upstream kinase LKB1 (29, 30). Recent studies have shown that LKB1 is essential for metformin-stimulated AMPK activation *in vivo*, and LKB1 is translocated to cytosol in response to metformin stimulation (26, 31, 32). Despite its wide usage as an antidiabetic drug, the direct target of metformin is yet to be identified.

In the present study, we have demonstrated protein phosphatase 2A (PP2A) and protein kinase C ζ (PKC ζ) as two important regulators of adiponectin signaling in C2C12 cells. In addition, we found that metformin-stimulated AMPK activation is independent of APPL1 and the PP2A-PKC ζ pathway, suggesting a selective role of APPL1 in mediating adiponectin signaling. Our study reveals a novel molecular mechanism underlying APPL1-mediated adiponectin signaling in regulating LKB1-AMPK activation and function.

Results

Adiponectin stimulation leads to dephosphorylation of LKB1 at Ser³⁰⁷

We recently found that cytosolic localization of LKB1 is essential for adiponectin-stimulated AMPK

phosphorylation, and APPL1 plays an important role in this process (26). To elucidate the underlying molecular mechanism, we tested whether phosphorylation plays a role in LKB1 cellular trafficking in response to adiponectin stimulation. *In vivo* labeling experiments in C2C12 myoblasts revealed that LKB1 is phosphorylated under basal conditions and adiponectin treatment resulted in a decrease of this phosphorylation in a time-dependent manner (Fig. 1A).

By phosphopeptide mapping experiments, we found that LKB1 is phosphorylated exclusively on serine residue(s) in C2C12 myoblasts (Supplemental Fig. 1A, published on The Endocrine Society's Journals Online web site at <http://mend.endojournals.org>). In addition, adiponectin treatment diminished serine phosphorylation of LKB1 (Supplemental Fig. 1A, *right panel*). Sequence analysis revealed that LKB1 contains a putative PKC ζ phosphorylation site at Ser³⁰⁷ (Supplemental Fig. 1B), suggesting that PKC ζ is a putative upstream kinase of LKB1.

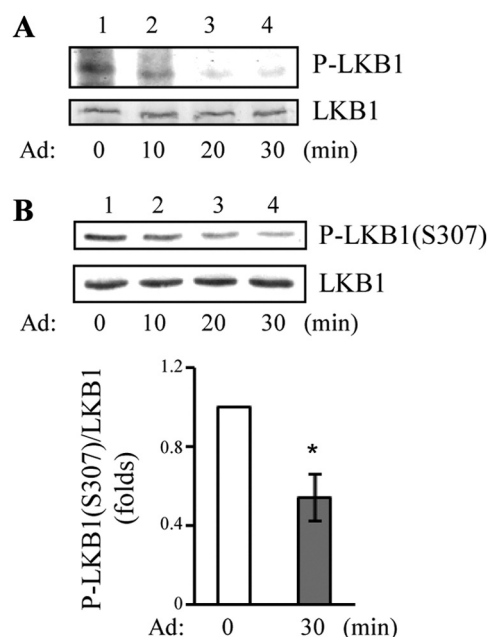


FIG. 1. Adiponectin (Ad) induces dephosphorylation of LKB1 at Ser³⁰⁷. A, LKB1 undergoes dephosphorylation in response to adiponectin stimulation. C2C12 myoblasts transiently expressing myc-tagged LKB1 were serum starved, incubated with Krebs-Ringer bicarbonate buffer containing 0.5 mCi of ³²P orthophosphate for 4 h, and then treated with or without adiponectin (1 μ g/ml) for indicated times. LKB1 was immunoprecipitated with anti-myc monoclonal antibody (*second panel*), and autoradiography was performed to detect LKB1 phosphorylation (P-LKB1, *top panel*). B, Adiponectin induces dephosphorylation of LKB1 at Ser³⁰⁷ in cells. After serum starvation, C2C12 myotubes were treated with adiponectin (1 μ g/ml) for indicated times. The phosphorylation of endogenous LKB1 at Ser³⁰⁷ and the protein levels were detected by Western blot analysis with antibodies specific to phospho-LKB1-Ser³⁰⁷ [P-LKB1 (S307), *top panel*] and LKB1 (*second panel*), respectively. The relative phosphorylation level of LKB1 was shown as graphic representation. The error bars represent mean \pm SEM from three independent experiments. *, $P < 0.05$.

Consistent with this, PKC ζ could phosphorylate LKB1 *in vitro*, and the phosphorylation was greatly suppressed in the S307A mutant of LKB1 (Supplemental Fig. 1C, *top panel*, lane 4 *vs.* lane 2), and two-dimensional phosphopeptide mapping showed that replacing Ser³⁰⁷ with Ala led to the loss of a major phosphopeptide in LKB1 (Supplemental Fig. 1D). During our study, Xie *et al.* (33) showed that PKC ζ phosphorylated LKB1 at Ser³⁰⁷ under metformin stimulation, further demonstrating that Ser³⁰⁷ of LKB1 is a PKC ζ -mediated phosphorylation site *in vitro*.

To determine whether phosphorylation of LKB1 at Ser³⁰⁷ is regulated by adiponectin, we examined LKB1 phosphorylation in C2C12 myotubes by Western blot analysis using a phospho-specific antibody to Ser³⁰⁷ of LKB1 (Supplemental Fig. 2A). Endogenous LKB1 is phosphorylated at Ser³⁰⁷ under basal conditions (Fig. 1B, *top panel*, lane 1). Adiponectin treatment attenuated LKB1 phosphorylation at Ser³⁰⁷ in a time-dependent manner (Fig. 1B, *top panel*, lanes 2–4 *vs.* lane 1), suggesting a

negative regulatory role of adiponectin on LKB1 phosphorylation in cells.

Dephosphorylation of LKB1 at Ser³⁰⁷ promotes LKB1 cytosolic translocation

To determine whether phosphorylation at Ser³⁰⁷ regulates LKB1 subcellular localization, we generated LKB1 mutant, in which Ser³⁰⁷ is mutated to Ala (S307A). Our previous study has shown that adiponectin treatment stimulated cytoplasmic translocation of LKB1 in C2C12 cells at both endogenous and overexpression level (26). Consistent with this finding, adiponectin treatment resulted in a significant increase of LKB1 cytosolic localization in C2C12 myoblasts (Fig. 2A). The LKB1^{S307A} mutant is largely localized in the cytosol at basal condition, and its subcellular localization pattern is very similar to that of the wild-type LKB1 in adiponectin-treated cells (Fig. 2A).

To further elucidate the role of LKB1 phosphorylation in adiponectin-regulated downstream signaling, we ex-

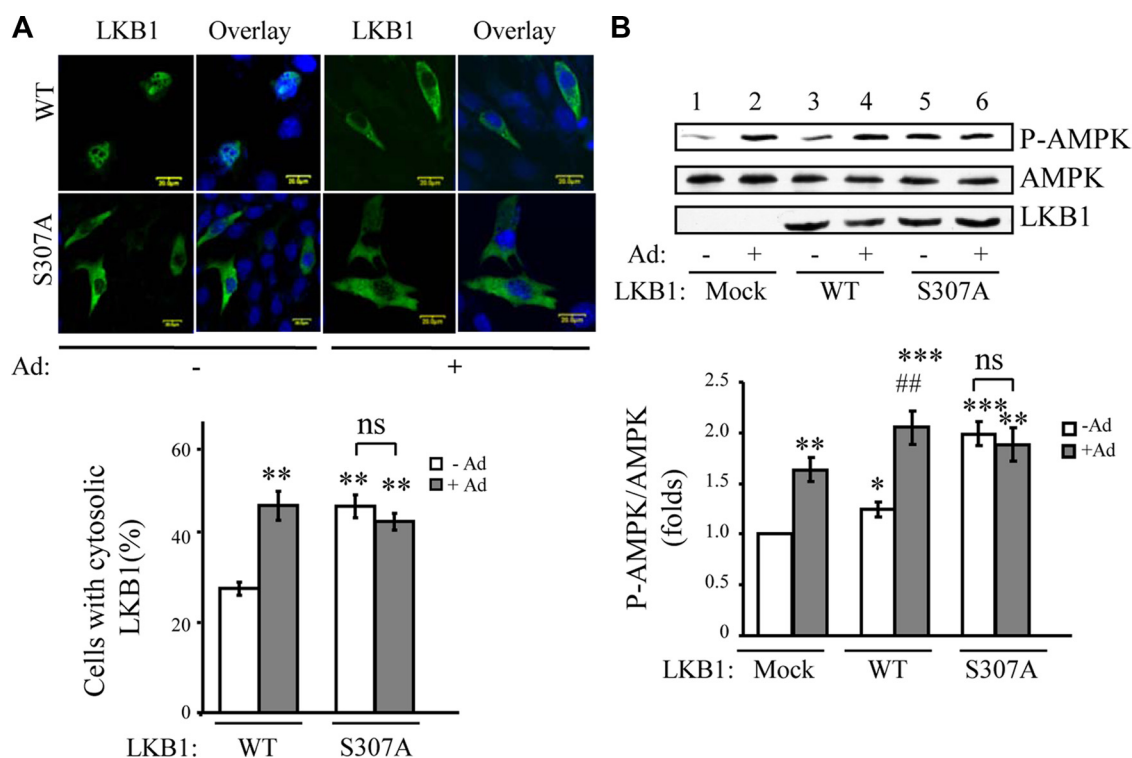


FIG. 2. Dephosphorylation at Ser³⁰⁷ stimulates cytosolic localization of LKB1. **A**, Localization of S307A mutant of LKB1 in C2C12 cells. Confocal microscopy images depict the localization of myc-tagged wild-type (WT) and S307A mutant form of LKB1 overexpressed in C2C12 myoblasts treated with or without adiponectin (Ad) (1 μ g/ml) for 20 min. The localization of LKB1 (green) was determined by an antibody to the myc-tag. The cell nuclei were stained with DAPI (blue). Scale bar, 20 μ m. The cells with cytosolic LKB1 were counted, analyzed, and shown as graphic representation. The error bars represent mean \pm SEM from three independent experiments. **, $P < 0.01$ *vs.* wild-type LKB1 without adiponectin treatment. ns, Nonstatistical significance. **B**, The effect of S307A mutant of LKB1 on adiponectin-stimulated AMPK activation. C2C12 myoblasts overexpressing myc-tagged wild-type or S307A mutant of LKB1 were treated with or without adiponectin (1 μ g/ml) for 20 min. The phosphorylation of AMPK (*top panel*), AMPK protein level (*second panel*), and myc-tagged LKB1 (*third panel*) were detected by Western blot analysis with specific antibodies as indicated. The relative phosphorylation level of AMPK was shown as graphic representation. The error bars represent mean \pm SEM from three independent experiments. *, **, ***, $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, *vs.* mock transfection without adiponectin treatment. ##, $P < 0.01$ *vs.* LKB1 (WT) without adiponectin stimulation. ns, Nonsignificant difference in statistics.

amined the effect of S307A mutant of LKB1 on AMPK phosphorylation in C2C12 myoblasts. Overexpression of LKB1 increased basal AMPK phosphorylation that was further enhanced by adiponectin treatment (Fig. 2B, *top panel*, lanes 3 and 4 *vs.* lanes 1 and 2). Interestingly, overexpression of LKB1^{S307A} mutant greatly enhanced the basal level of AMPK phosphorylation, which is not further stimulated by adiponectin (Fig. 2B, *top panel*, lanes 5 and 6 *vs.* lanes 1 and 2). Together, our data demonstrate that dephosphorylation at Ser³⁰⁷ plays a critical role in LKB1 cytosol translocation and adiponectin-stimulated AMPK activation.

Inactivation of PKC ζ promotes LKB1 cytosolic localization and AMPK phosphorylation

Because S307A mutant of LKB1 is located predominantly in the cytosol (Fig. 2A), we investigated whether PKC ζ , which is responsible for LKB1 phosphorylation at Ser³⁰⁷ (Supplemental Fig. 1C), plays a role in regulating LKB1 cellular localization. Immunofluorescence studies revealed that under basal conditions, LKB1 is mainly localized in the nucleus in cells coexpressing wild-type PKC ζ (Fig. 3A). On the other hand, coexpression of the kinase-dead PKC ζ (PKC ζ -KD), which acts as a dominant negative mutant of this kinase (34), significantly increased the cytosolic localization of LKB1 under basal conditions. The PKC ζ (KD)-induced cytosol localization of LKB1 was not further stimulated by adiponectin treatment (Fig. 3A). These data indicate that the activity of PKC ζ positively regulates LKB1 nuclear localization. Consistent with this observation, overexpression of PKC ζ -KD led to increase of basal AMPK phosphorylation (Fig. 3B, *top panel*, lane 5 *vs.* lane 1). The PKC ζ (KD)-induced AMPK phosphorylation was not further enhanced by adiponectin treatment (Fig. 3B, *top panel*, lane 6 *vs.* lane 5).

To further confirm that the activity of PKC ζ negatively regulates LKB1 cytosolic localization, we treated C2C12 cells with a specific inhibitor for PKC ζ , PKC ζ -pseudosubstrate (PS) (35). Inhibition of PKC ζ activity with PKC ζ -PS promoted LKB1 translocation from the nucleus to cytosol to a similar extent to that induced by adiponectin treatment (Fig. 3C). Consistent with this observation, inhibition of PKC ζ activity by PKC ζ -PS decreased basal LKB1 phosphorylation at Ser³⁰⁷ (Fig. 3D, *top panel*, lane 4 *vs.* lane 1) and increased basal AMPK phosphorylation (Fig. 3D, *third panel*, lane 4 *vs.* lane 1). The inverse correlation of PKC ζ activity with LKB1 cytosolic localization was further proved by RNA interference (RNAi) approach. We found that suppression of PKC ζ expression by RNAi decreased basal LKB1S307 phosphorylation (Fig. 3E, *third panel*, lane 3 *vs.* lane 1) that was further diminished

by adiponectin treatment (Fig. 3E, *third panel*, lane 4 *vs.* lane 3). In addition, suppression of PKC ζ expression greatly enhanced AMPK phosphorylation in C2C12 myotubes (Fig. 3E, *top panel*, lane 3 *vs.* lane 1). Together, these results demonstrate that down-regulation of PKC ζ activity contributes to LKB1 cytosolic translocation and AMPK activation.

Adiponectin induces APPL1-dependent dephosphorylation of PKC ζ

To determine whether PKC ζ activity is regulated by adiponectin, we examined the effect of adiponectin on PKC ζ phosphorylation at Thr⁴¹⁰, an indicator of PKC ζ activation in cells (36). Treatment of C2C12 myotubes with adiponectin decreased phosphorylation of endogenous PKC ζ at Thr⁴¹⁰ in a time-dependent manner (Fig. 4A, *top panel*). A similar effect of adiponectin on PKC ζ phosphorylation was also observed when mouse primary skeletal muscle was treated with adiponectin *ex vivo* (Fig. 4B, *top panel*, lane 4 *vs.* lane 3). Because our previous study has shown that APPL1 plays an essential role in adiponectin-mediated AMPK activation (4, 26), we next tested the role of APPL1 in adiponectin-mediated PKC ζ dephosphorylation. As shown in Fig. 4C, the inhibitory effect of adiponectin on PKC ζ activity was greatly blocked in APPL1-suppressed C2C12 myotubes compared with the scrambled control cells (Fig. 4C, *top panel*, lanes 6–8 *vs.* lanes 2–4), suggesting a potential role of APPL1 in mediating adiponectin-induced down-regulation of PKC ζ activity.

To understand the mechanism underlying APPL1-mediated PKC ζ inactivation, we tested whether PKC ζ could interact with APPL1. Affinity binding experiments demonstrated that PKC ζ associates with APPL1 *in vitro*, and the C-terminal part of APPL1 is involved in this interaction (Supplemental Fig. 2B). The binding of PKC ζ to the bacterially purified C terminus of APPL1 *in vitro* was not affected by adiponectin stimulation (Fig. 4D, *top panel*, lanes 4–5 *vs.* lane 3). On the other hand, the interaction between APPL1 and PKC ζ in cells was stimulated by adiponectin (Fig. 4E, *top panel*, lane 4 *vs.* lane 3), suggesting that adiponectin-stimulated posttranslational modifications of APPL1 may regulate this interaction. It is also possible that another cellular component might be involved in regulating this interaction in cells.

PKC ζ activity is down-regulated by PP2A in response to adiponectin stimulation

To understand the mechanism underlying adiponectin-induced and APPL1-dependent dephosphorylation of PKC ζ , we asked whether PP2A, which has previously been shown

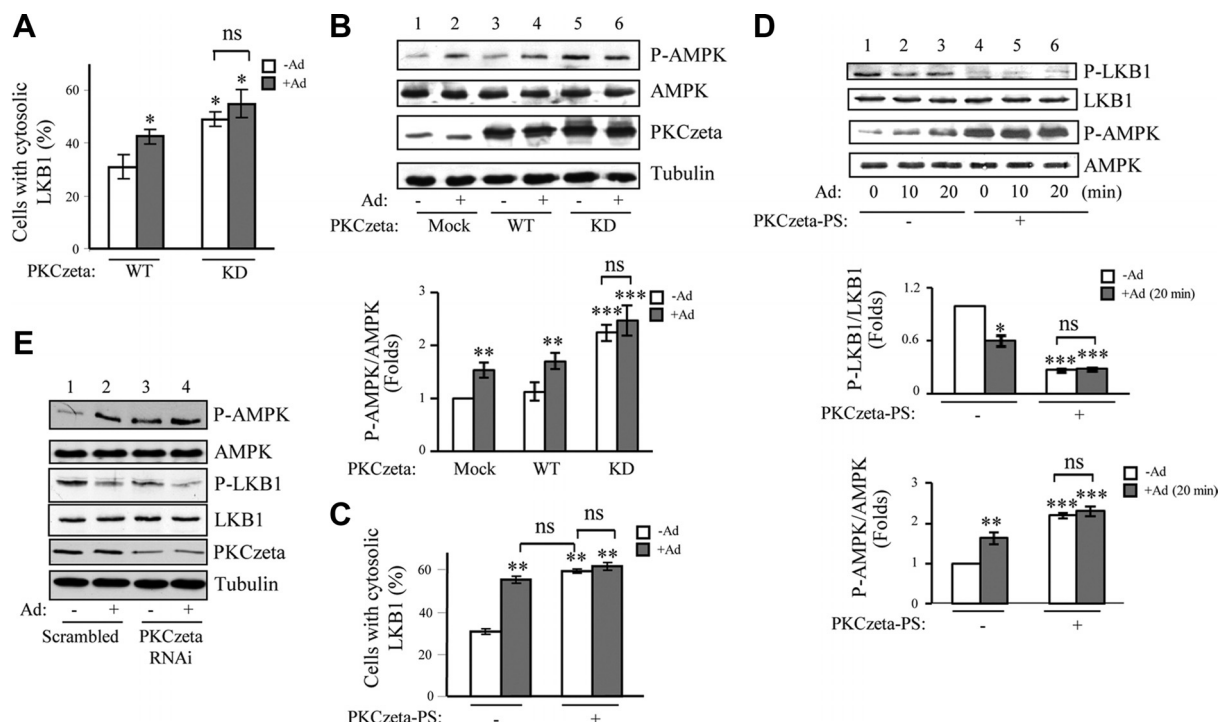


FIG. 3. Inactivation of PKC ζ induces cytosolic translocation of LKB1 and subsequent AMPK activation. **A**, The kinase activity of PKC ζ is inversely related to adiponectin (Ad)-induced LKB1 cytosolic translocation. Localization of hemagglutinin-tagged LKB1 coexpressed with myc-tagged wild type (WT) or kinase inactive mutant (KD) of PKC ζ in C2C12 myoblasts with or without adiponectin treatment (1 μ g/ml, 20 min) was determined by confocal microscopy images with specific antibodies to the tags as described in *Materials and Methods*. The cells with cytosolic LKB1 were counted, analyzed, and shown as graphic representation. The error bars represent mean \pm SEM from three independent experiments. *, $P < 0.05$ vs. wild-type LKB1 without adiponectin treatment. ns, Nonsignificant difference in statistics. **B**, The kinase inactive mutant of PKC ζ increases basal AMPK phosphorylation. C2C12 myoblasts were transiently transfected with myc-tagged wild-type or kinase inactive mutant (KD) of PKC ζ and treated with or without adiponectin (1 μ g/ml, 20 min). The phosphorylation of AMPK (*top panel*), the protein levels of AMPK (*second panel*) and PKC ζ (*third panel*), and β -tubulin loading control (*fourth panel*) were detected by Western blot analysis with specific antibodies as indicated. The relative phosphorylation level of AMPK was shown as graphic representation. The error bars represent mean \pm SEM from three independent experiments. ** and ***, $P < 0.01$ and $P < 0.001$, respectively, vs. mock transfection without adiponectin treatment. **C**, PS of PKC ζ blocked adiponectin-induced LKB1 translocation. Localization of myc-tagged LKB1 in C2C12 myoblasts with or without PKC ζ -PS pretreatment (10 μ M, 1 h) and with or without adiponectin (1 μ g/ml, 20 min) stimulation was determined by confocal microscopy images with specific antibodies to the tags as described in *Materials and Methods*. The cells with cytosolic LKB1 were counted, analyzed, and shown as graphic representation. The error bars represent mean \pm SEM from three independent experiments. **, $P < 0.01$ vs. PKC ζ -PS negative control without adiponectin treatment. **D**, Inhibition of PKC ζ using PKC ζ -PS increases basal AMPK phosphorylation. C2C12 myotubes were pretreated with or without PKC ζ -PS (10 μ M, 1 h) and treated with or without adiponectin (1 μ g/ml, 20 min). LKB1 phosphorylation at Ser³⁰⁷ (*first panel*), AMPK phosphorylation at Thr¹⁷² (*third panel*), and protein levels of LKB1 (*second panel*) and AMPK (*fourth panel*) were determined by Western blot analysis with specific antibodies as indicated. The relative phosphorylation levels of LKB1 and AMPK were shown as graphic representations. The error bars represent mean \pm SEM from three independent experiments. *, **, and ***, $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, vs. PKC ζ -PS negative control without adiponectin treatment. **E**, Suppression of endogenous PKC ζ expression enhances basal AMPK phosphorylation. shRNA PKC ζ -suppressed or the control C2C12 myotubes were treated with or without adiponectin (1 μ g/ml) for 20 min. Thr¹⁷² phosphorylation of AMPK (*first panel*), protein levels of AMPK (*second panel*), phosphorylation of LKB1 at Ser³⁰⁷ (*third panel*), protein levels of LKB1 (*fourth panel*) and PKC ζ (*fifth panel*), and tubulin loading control (*bottom panel*) were detected by Western blot analysis with specific antibodies as indicated.

to interact with PKC ζ in adult cardiac myocytes (37), could contribute to adiponectin-induced inactivation of PKC ζ . We found that PP2A can directly dephosphorylate PKC ζ , as demonstrated by *in vitro* dephosphorylation assay (Fig. 5A, *top panel*). The effect of adiponectin on PP2A activity was also assessed by PP2A substrate assay. As shown in Fig. 5B, adiponectin treatment significantly increased PP2A activity in C2C12 myotubes. Interestingly, adiponectin-induced activation of PP2A (Fig. 5B) is coincident with a decrease in PKC ζ phosphorylation in cells (Fig. 4, A and B). To investigate the direct involve-

ment of PP2A in adiponectin-induced PKC ζ inactivation, we tested the effect of PP2A activity on PKC ζ phosphorylation by treatment of the cells with cantharidin, a compound that can specifically inhibit PP2A at lower concentrations (38, 39). Inhibition of PP2A resulted in an increase of PKC ζ phosphorylation at basal level (Fig. 5C, *top panel*, lane 1 vs. lane 4). Adiponectin-induced down-regulation of PKC ζ phosphorylation was significantly blocked in the cells pretreated with cantharidin (Fig. 5C, *top panel*, lanes 5–6 vs. lanes 2–3). Interestingly, the effect of cantharidin on PKC ζ phosphorylation was corre-

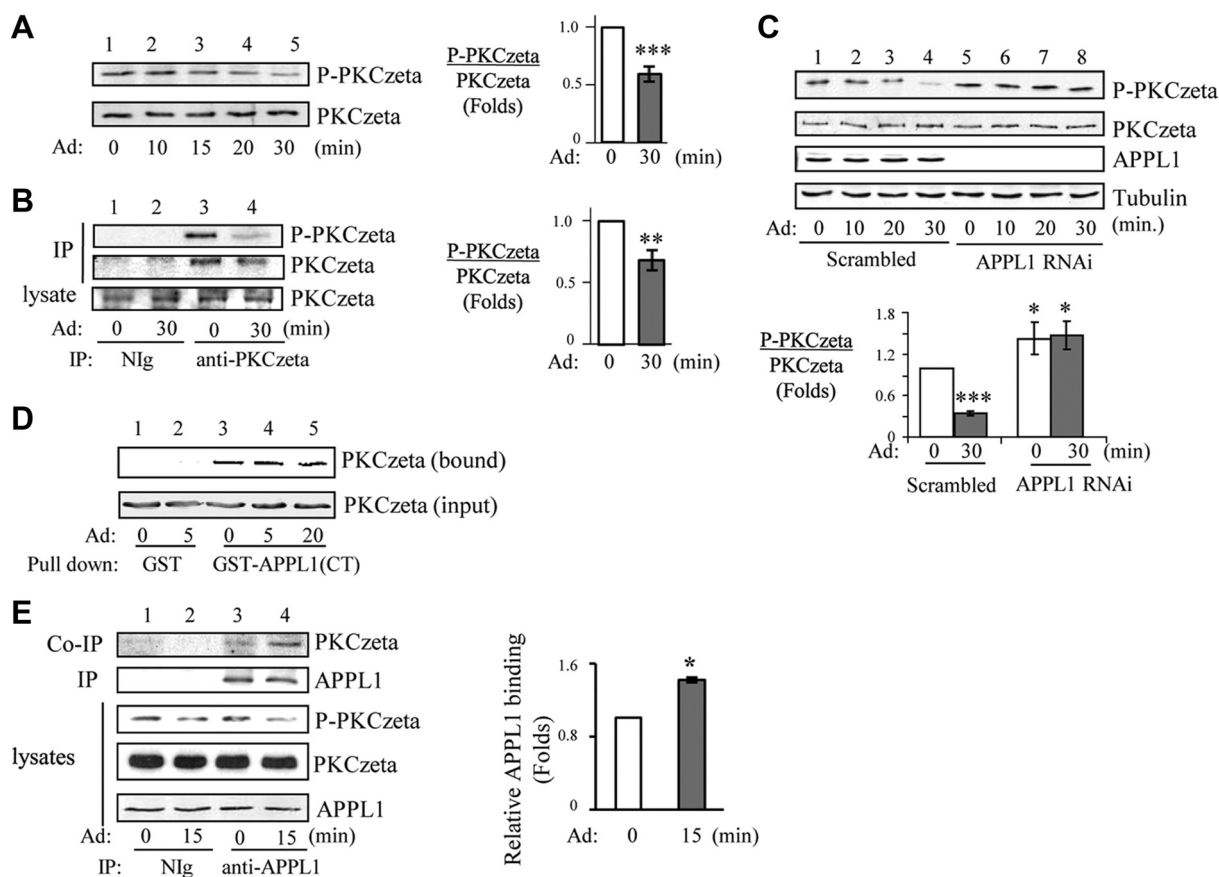


FIG. 4. Adiponectin (Ad) induces inactivation of PKC ζ with APPL1-dependent manner. **A**, The effect of adiponectin on PKC ζ activity. After serum starvation, C2C12 myotubes were treated with adiponectin (1 μ g/ml) for indicated times. Phosphorylation and protein levels of endogenous PKC ζ were detected by Western blot analysis with antibodies specific to phospho-PKC ζ -Thr⁴¹⁰ (top panel) and PKC ζ (second panel), respectively. The relative phosphorylation level of PKC ζ was shown as graphic representation. The error bars represent mean \pm SEM from three independent experiments. ***, $P < 0.001$. **B**, Adiponectin induces inactivation of PKC ζ in primary skeletal muscle tissue. Mouse skeletal muscle tissue was treated without or with adiponectin (2.5 μ g/ml) for 30 min, and endogenous PKC ζ was immunoprecipitated with PKC ζ antibody. Phosphorylation of PKC ζ at Thr⁴¹⁰ (top panel) and protein expression levels of PKC ζ (second and third panels) were determined by Western blot analysis with specific antibodies as indicated. The relative PKC ζ phosphorylation was shown as graphic representation. The error bars represent mean \pm SEM from three independent experiments. **, $P < 0.01$. **C**, Adiponectin-induced inactivation of PKC ζ is dependent on APPL1. C2C12 myotubes stably expressing APPL1 shRNA or the scrambled shRNA were serum starved and treated with adiponectin (1 μ g/ml) for indicated times. Phosphorylation of PKC ζ at Thr⁴¹⁰ (top panel), protein expression levels of PKC ζ (second panel) and APPL1 (third panel), and tubulin loading control (fourth panel) were determined by Western blot analysis with specific antibodies as indicated. The relative PKC ζ phosphorylation was shown as graphic representation. The error bars represent mean \pm SEM from four independent experiments. *** and *, $P < 0.001$ and $P < 0.05$, respectively, vs. the scramble control without adiponectin treatment. **D**, APPL1 interacts with PKC ζ via its C terminus *in vitro*. C2C12 myoblasts overexpressing myc-tagged PKC ζ were serum starved and treated with adiponectin (1 μ g/ml) as indicated. The myc-tagged PKC ζ was pulled down by GST or GST-APPL1(CT) fusion proteins. The bound PKC ζ (top panel) and PKC ζ expression control (input, second panel) were detected with anti-myc antibody. **E**, PKC ζ interacts with APPL1 in cells. C2C12 myotubes were serum starved and treated with adiponectin (1 μ g/ml) for 15 min. Endogenous APPL1 was immunoprecipitated (IP) with negative control immunoglobulin (NIg) or an anti-APPL1 antibody. Immunoprecipitated APPL1 (second panel), coimmunoprecipitated PKC ζ (top panel), and its Thr⁴¹⁰ phosphorylation (third panel) were detected with the antibodies as indicated. The endogenous protein levels of PKC ζ (fourth panel) and APPL1 (fifth panel) in cell lysates were determined with specific antibodies as indicated. The relative APPL1 binding with PKC ζ was shown as graphic representation. The error bars represent mean \pm SEM from three independent experiments. *, $P < 0.05$.

lated with LKB1 phosphorylation under both basal level and adiponectin treatment (Fig. 5C, top panel vs. third panel), demonstrating the involvement of PP2A-PKC ζ -LKB1 pathway in mediating adiponectin signaling in muscle cells. To determine the mechanism by which adiponectin stimulates PP2A activity, we examined whether PP2A could interact with APPL1. As shown in Fig. 5D, endogenous APPL1 interacts with endogenous PP2A in C2C12 myotubes, and this interaction was

stimulated by adiponectin (Fig. 5D, top panel, lane 4 vs. lane 3). The regulatory mechanism for adiponectin-mediated APPL1-PP2A interaction is currently unknown. It is possible that adiponectin-stimulated post-translational modifications in APPL1 or PP2A could play a role in this regulation. In conclusion, adiponectin induces PKC ζ dephosphorylation by promoting its interaction with PP2A, which is facilitated by adaptor protein APPL1.

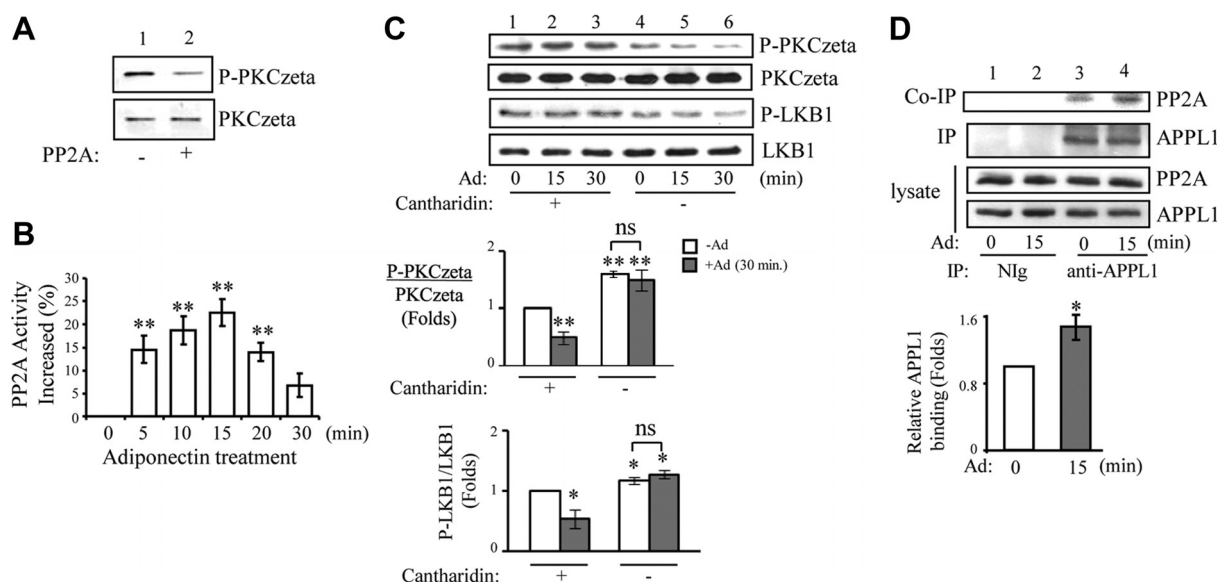


FIG. 5. PP2A inhibits PKC ζ activity in response to adiponectin (Ad) stimulation. **A**, *In vitro* dephosphorylation assay. Recombinant PKC ζ (50 ng) was incubated with 2 U of purified PP2A for 30 min at 30°C. After stopping the reaction, the phosphorylation at Thr⁴¹⁰ (top panel) and the protein (bottom panel) levels of PKC ζ were determined by Western blot analysis with specific antibodies as indicated. **B**, Adiponectin stimulates PP2A activity. C2C12 myotubes were treated with mock control or adiponectin (1 μ g/ml) for different times as indicated. PP2A activity was measured as described in *Materials and Methods*. The PP2A activity in the cells treated with mock control was set as 100% (activity change is 0% as shown); the activity changes with adiponectin treatments were compared with the control. **, $P < 0.01$. **C**, Adiponectin-mediated dephosphorylation of LKB1 and PKC ζ is inhibited by cantharidin. C2C12 myotubes were pretreated with or without cantharidin (1 μ M, 2 h) and treated with or without adiponectin (1 μ g/ml) for different times as indicated. PKC ζ phosphorylation at Thr⁴¹⁰ (first panel), LKB1 phosphorylation at Ser³⁰⁷ (third panel), and protein levels of PKC ζ (second panel) and LKB1 (fourth panel) were determined by Western blot analysis with specific antibodies as indicated. The relative phosphorylation levels of PKC ζ and LKB1 were shown as graphic representation. The error bars represent mean \pm SEM from three independent experiments. * and **, $P < 0.05$ and $P < 0.01$, respectively, vs. cantharidin treatment without adiponectin. ns, Nonsignificant difference in statistics. **D**, Adiponectin stimulates interaction of APPL1 with PP2A in cells. After serum starvation, C2C12 myotubes were treated with adiponectin (1 μ g/ml) for 15 min. Endogenous APPL1 was immunoprecipitated with an antibody specific to APPL1. Immunoprecipitated APPL1 (second panel) and coimmunoprecipitated endogenous PP2A (top panel), protein expression level of APPL1 (bottom panel) and PP2A (third panel) in the cell lysates were detected with specific antibodies as indicated. The relative APPL1 binding was shown as graphic representation. The error bars represent mean \pm SEM from three independent experiments. **, $P < 0.01$.

Metformin-induced cytosolic translocation of LKB1 is independent of APPL1-PP2A-PKC ζ pathway

We and others have previously shown that metformin also induces LKB1 cytosolic translocation in C2C12 cells (26, 31). To determine whether metformin and adiponectin share a common mechanism to regulate LKB1 subcellular localization, we investigated metformin-induced LKB1 localization in APPL1-suppressed cells. We found that suppression of APPL1 expression had no significant effect on metformin-induced AMPK phosphorylation in C2C12 myotubes compared with scrambled control cells (Fig. 6A, top panel, lanes 6–8 vs. lanes 2–4), indicating that APPL1 is not required for metformin-stimulated AMPK activation. In addition, metformin stimulation led to a reduction of LKB1 phosphorylation at Ser³⁰⁷ (Fig. 6A, third panel, lanes 2–4), which is similar to adiponectin-induced dephosphorylation and cytosolic translocation of LKB1 (Figs. 1B and 2A). Interestingly, there is no significant change of metformin-induced dephosphorylation of LKB1 in APPL1 RNAi myotubes compared with that in C2C12 scrambled control (Fig. 6A, third panel, lanes 6–8 vs. lanes 2–4), suggesting that

metformin-regulated LKB1 dephosphorylation is independent of APPL1.

To further understand the difference of metformin and adiponectin actions on AMPK activation, we tested whether metformin has any effect on the activities of PP2A and PKC ζ . Our data indicated that metformin is unable to regulate phosphorylation of PP2A at Tyr³⁰⁷ (a marker of PP2A activity) (Fig. 6B, third panel) or PKC ζ at Thr⁴¹⁰ (Fig. 6B) (top panel) in either absence or presence of APPL1. In addition, PP2A substrate assays also indicated that metformin treatment has no effect on PP2A activity in C2C12 myotubes (Supplemental Fig. 3). Unlike adiponectin, metformin had no effect on the interaction between APPL1 and PP2A (Fig. 6C, top panel). Furthermore, we found that metformin did not stimulate PP2A and PKC ζ activities in skeletal muscle of db/db mice, although it significantly increased AMPK phosphorylation at Thr¹⁷² and reduced LKB1 phosphorylation at Ser³⁰⁷ (Fig. 6D), which is associated with increased insulin sensitivity in the db/db mice (Supplemental Fig. 4). These *in vivo* data further demonstrate that adiponectin-

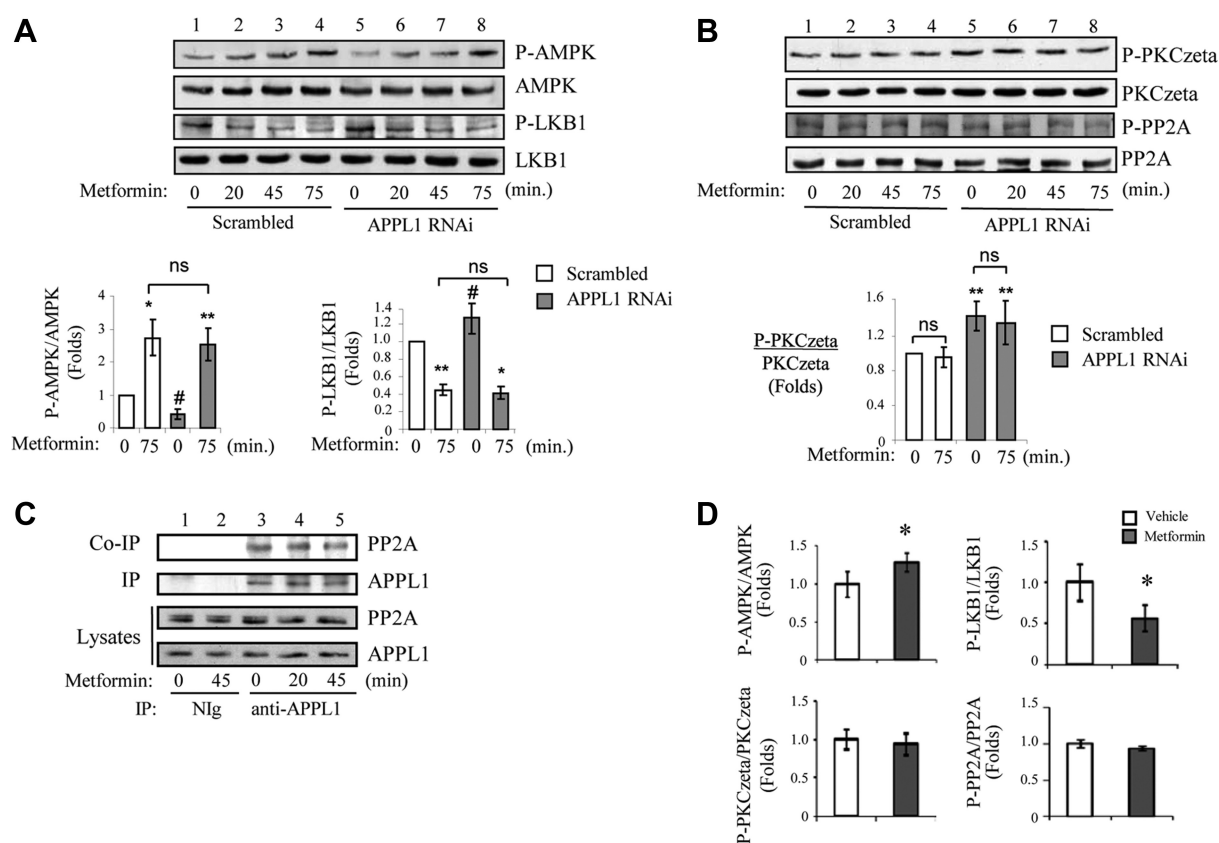


FIG. 6. Metformin-induced subcellular translocation of LKB1 is independent of APPL1-PP2A-PKC ζ pathway. **A**, Metformin-mediated AMPK phosphorylation and LKB1 dephosphorylation are independent of APPL1. C2C12 scrambled control and APPL1 RNAi myotubes were serum starved and treated with metformin (500 μ M) for indicated times. Phosphorylation levels of AMPK at Thr¹⁷² (top panel), LKB1 at Ser³⁰⁷ (third panel), and protein expression levels of AMPK (second panel) and LKB1 (fourth panel) were detected by Western blot analysis with specific antibodies as indicated. The relative phosphorylation levels of AMPK and LKB1 are shown as graphic representation. The error bars represent mean \pm SEM from three independent experiments. ** and *, $P < 0.01$ and $P < 0.05$, respectively, vs. the scramble control without metformin treatment. #, $P < 0.05$ vs. the scrambled control without metformin treatment. ns, Nonsignificant difference in statistics. **B**, Metformin treatment has no effect on the phosphorylation levels of PKC ζ and PP2A. C2C12 scrambled control and APPL1 RNAi myotubes were serum starved and treated with metformin (500 μ M) for indicated times. Phosphorylation levels of PKC ζ at Thr⁴¹⁰ (top panel), PP2A-C α / β at Tyr³⁰⁷ (third panel), and protein expression levels of PKC ζ (second panel) and PP2A (fourth panel) were detected by Western blot analysis with specific antibodies as indicated. The relative phosphorylation level of PKC ζ is shown as graphic representation. The error bars represent mean \pm SEM from three independent experiments. **, $P < 0.01$ vs. the scramble control without metformin treatment. **C**, Metformin has no effect on APPL1-PP2A interaction. C2C12 myotubes were serum starved and treated with metformin (500 μ M) for indicated times. Endogenous APPL1 was immunoprecipitated (IP) with negative control immunoglobulin (Nlg) or an antibody specific to APPL1. Immunoprecipitated APPL1 (second panel) and coimmunoprecipitated PP2A (first panel) and protein expression levels of APPL1 (bottom panel) and PP2A (third panel) in cell lysates were detected with specific antibodies as indicated. **D**, Metformin treatment in db/db mice increases AMPK and decreases LKB1 (Ser³⁰⁷) phosphorylation but has no effect on PKC ζ and PP2A phosphorylation in the skeletal muscle. Phosphorylation levels of AMPK at Thr¹⁷², LKB1 at Ser³⁰⁷, PKC ζ at Thr⁴¹⁰, PP2A-C α / β at Tyr³⁰⁷, and protein expression levels of AMPK, LKB1, PKC ζ , and PP2A in the skeletal muscle tissue homogenate from db/db mice administered with vehicle (n = 6) or metformin (150 mg/kg) (n = 6) were detected by Western blotting analysis. The relative phosphorylation levels of AMPK, LKB1, PKC ζ , and PP2A are shown as graphic representation. The error bars represent mean \pm SEM. *, $P < 0.05$.

tin and metformin activate LKB1-AMPK signaling via distinct mechanisms.

Discussion

We have recently shown that LKB1 undergoes adiponectin-stimulated and APPL1-dependent cytosolic translocation, a key step for adiponectin-induced activation of AMPK in muscle cells (26). However, the molecular mechanism underlying APPL1-dependent LKB1 cytosolic translocation remains unknown. In the present study, we

present evidence showing that PP2A-PKC ζ pathway is a missing link that mediates APPL1-dependent LKB1 cytosolic translocation in response to adiponectin stimulation. Under basal condition, PP2A activity is suppressed, which leads to enhancement of PKC ζ activity in cytosol and subsequent promotion of LKB1 nuclear localization by phosphorylating LKB1 at Ser³⁰⁷ (Fig. 7A). Adiponectin stimulation results in association of PP2A with APPL1, which induces activation of PP2A and deactivation of PKC ζ , a PP2A substrate that also associates with APPL1 in cells. As a consequence of adiponectin-induced

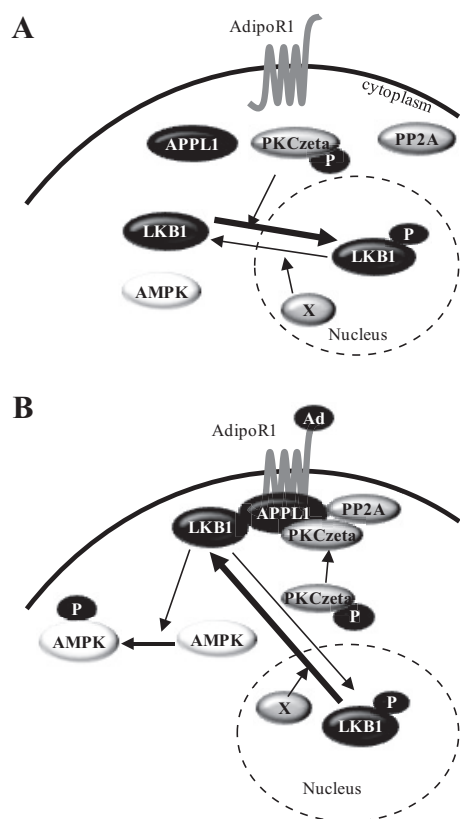


FIG. 7. A model of adiponectin-induced subcellular translocation of LKB1 in muscle cells. A, Under basal conditions, PP2A is inactive and dissociated from APPL1, leading to activation of PKC ζ in cytosol. The activated PKC ζ phosphorylates LKB1 at Ser³⁰⁷, which promotes LKB1 transporting into the nuclei. B, Adiponectin stimulation induces recruitment of both PP2A and PKC ζ onto APPL1, leading to activation of PP2A and subsequent dephosphorylation of PKC ζ by PP2A. The inactivated PKC ζ then results in less phosphorylation of LKB1 at Ser³⁰⁷ and an accumulation of LKB1 in the cytosol, which leads to the binding of LKB1 with APPL1 and activation of AMPK. A hypothetical nuclear phosphatase (X) may also contribute to LKB1 cytosolic translocation in response to adiponectin or metformin response. AdipoR, Adiponectin receptor.

inactivation of PKC ζ , LKB1 is accumulated in the cytosol due to less phosphorylation at Ser³⁰⁷ (Fig. 7B), which leads to more binding of LKB1 with APPL1 and activation of AMPK (26).

We and others have demonstrated that adiponectin-mediated LKB1 cytosolic translocation is a common mechanism employed by different cell types to mediate AMPK phosphorylation (26, 40, 41). Association of LKB1 with MO25 α and STRAD α is one of the mechanisms that anchors LKB1 in cytoplasm to mediate AMPK phosphorylation (21, 42). Our previous study has demonstrated that adiponectin has no effect on LKB1-STRAD-MO25 complex formation, and APPL1 do not interact with either STRAD or MO25 in the presence or absence of adiponectin, indicating the presence of an unidentified mechanism for LKB1 translocation (26). Here, we showed for the first time that PKC ζ -mediated phos-

phorylation of LKB1 at Ser³⁰⁷ plays a negative role in adiponectin-stimulated AMPK activation, probably by sequestering LKB1 in the nucleus. This finding is consistent with an early report that the *Xenopus* homolog of LKB1 (XEEK), which has an uncharged residue (Asn) corresponding to Ser³⁰⁷ in human and mouse LKB1 (Supplemental Fig. 1B), is primarily localized in the cytosol (43). Because AMPK is localized mainly in the cytosol (44), nuclear translocation of the AMPK upstream kinase LKB1 would thus provide a mechanism by which PKC ζ negatively regulates AMPK activity. Consistent with this, activation of PKC ζ by insulin has been shown to inhibit AMPK activity in heart and β -oxidation in L6 myotubes (45, 46). Recently, inhibitory role of PKC ζ on AMPK phosphorylation in L6 myotubes was reported that further supports our findings (47). On the other hand, our result is contradictory to a recent finding by Xie *et al.* (33), who showed that PKC ζ -mediated LKB1 phosphorylation at Ser³⁰⁷ is required for metformin-stimulated LKB1 cytosolic transfer in endothelial cells. The reason for this discrepancy remains unknown, but LKB1 cellular localization may be regulated via different mechanisms in different cells. Recently, another study showed that PKC ζ is not involved in metformin-induced AMPK activation in H9c2 myocytes and C2C12 myotubes (48). In addition, study by Hawley *et al.* (49) demonstrated that metformin-mediated activation of AMPK is through inhibition of mitochondrial respiration in HEK293 cells. Taken together, our data demonstrate that AMPK activation mediated by adiponectin and metformin are through two distinct pathways converging at LKB1.

In this study, we have identified PP2A as a critical regulator in adiponectin-induced LKB1 cytosolic localization and subsequent AMPK phosphorylation and activation. PP2A does not directly dephosphorylate LKB1 at Ser³⁰⁷, which is likely mediated by a distinct phosphatase located in the nucleus (Fig. 7B). Instead, PP2A promotes adiponectin signaling by dephosphorylation and inactivation of PKC ζ , which catalyzes LKB1 phosphorylation at Ser³⁰⁷. The mechanism of adiponectin-induced LKB1 dephosphorylation and its cytosolic transfer is not currently known and will be part of our future studies. One possibility is that APPL1/PP2A/PKC ζ complex will be translocated into the nucleus to dephosphorylate LKB1 in response to adiponectin stimulation. It is also possible that adiponectin could activate a nuclear phosphatase that specifically targets LKB1.

Interestingly, we found that metformin, which also stimulates LKB1 cytosolic translocation and AMPK activation (26), activates AMPK via an APPL1-PP2A-PKC ζ -independent mechanism. This conclusion is supported by the following observations. First, metformin and adi-

ponectin induce LKB1 cytosolic translocation with different time courses (45 min *vs.* 20 min) (26). In addition, metformin treatment had no effect on the activities of PKC ζ and PP2A in cells (Fig. 6B and Supplemental Fig. 3) and *in vivo* (Fig. 6D). Furthermore, suppressing APPL1 has little effect on metformin-induced dephosphorylation of LKB1 at Ser³⁰⁷ or activation of AMPK (Fig. 6A). Although how metformin promotes LKB1 cytosolic translocation remains unknown, one possibility may be that metformin may stimulate a nuclear phosphatase that directly dephosphorylates LKB1 at Ser³⁰⁷. Further studies will be needed to test this possibility.

In conclusion, we have provided evidence showing that PP2A and PKC ζ are two novel signaling molecules involved in mediating adiponectin signaling from APPL1 to LKB1-AMPK pathway. In addition, we have demonstrated that the APPL1-PP2A-PKC ζ pathway mediates adiponectin-induced dephosphorylation of LKB1 at Ser³⁰⁷, which plays a key role in regulating LKB1 translocation from the nucleus to cytosol. We also show that metformin-induced LKB1 cytosolic translocation and subsequent AMPK activation is mediated by a mechanism independent of APPL1-PP2A-PKC ζ pathway. Our study reveals a novel molecular mechanism underlying adiponectin-induced AMPK activation and the role of APPL1 in this regulation. Because activation of AMPK is a key step in mediating the beneficial effects of adiponectin on fatty acid oxidation in muscle cells (27, 50–52), the intervention of the APPL1-PP2A-PKC ζ pathway in the current study may provide critical information for designing therapeutic drug for the treatment of insulin resistance and obesity-related metabolic disorders.

Materials and Methods

Plasmids, adiponectin, chemicals, and antibodies

Plasmids encoding full-length human APPL1, various glutathione S-transferase (GST)-APPL1 fusion constructs [GST-APPL1, GST-APPL1(COOH-terminus) (amino acids 455–693), and GST-APPL1(NH₂-terminus) (amino acids 1–270)], the globular form of adiponectin, and the anti-APPL1 antibody were described in our previous publications (4, 26, 53). Plasmids encoding mutant of mouse LKB1(S307A) or kinase inactive PKC ζ (PKC ζ -K273A) were generated by site-directed mutagenesis. Plasmids encoding amino acid 1–427 of LKB1 was cloned by PCR from a mouse cDNA library and subcloned into a mammalian expression vector pBEX1 (54), in-frame at its COOH terminus with a sequence encoding a hemagglutinin-tag, or the pcDNA 3.1 myc/His A plasmid (Invitrogen, Carlsbad, CA), in-frame at its COOH terminus with a sequence encoding a myc-tag. The phospho-specific antibody to Ser³⁰⁷ of LKB1 was raised in rabbits using phosphopeptide corresponding to mouse LKB1 299–314 (Ac-C-Ahx-IRQIRQH-pS³⁰⁷-WFRKKHP-amide) that was synthesized by 21st Century Biochemicals (Marlboro, MA). Monoclo-

nal antibodies to β -tubulin 2.1 (Sigma, St. Louis, MO), myc-tag (produced in house with the myc 1–9E10.2 cell clone from American Type Culture Collection, Manassas, VA), LKB1 (Upstate-Millipore, Bedford, MA) and polyclonal antibody to phospho-PP2A-Ca β (Tyr³⁰⁷) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used in the experiments. PKC ζ -PS was from Invitrogen and cantharidin was from Biomol International (Plymouth Meeting, PA). All other antibodies were obtained from Cell Signaling Technologies (Danvers, MA). Metformin was purchased from Sigma-Aldrich (St. Louis, MO).

Cell lines and cell culture

Conditions for culturing and differentiation of C2C12 cells, APPL1-suppressed C2C12 cells, and the scramble control C2C12 cells are described in our previous studies (4). PKC ζ short hairpin RNA (shRNA) construct or the control cloned into pLKO.1 vector (catalog no. RMM3981-9590281; Open Biosystems, Auburn, AL) was stably expressed in C2C12 myocytes and selected using puromycin as described previously (4).

In vitro and *ex vivo* binding assays and coimmunoprecipitation experiments

Experimental procedures for *in vitro* binding and coimmunoprecipitation are essentially the same as described previously (55). *Ex vivo* incubation of skeletal muscle with adiponectin was performed as described previously (26).

In vitro kinase assay and phosphopeptide mapping of LKB1

Myc-tagged wild-type LKB1 or LKB1(S307A) mutant was transiently expressed in C2C12 myoblasts and immunoprecipitated with an anti-myc antibody. *In vitro* phosphorylation was initiated with the addition of 30 μ l of kinase buffer [50 mM Tris-HCl (pH 7.5), 5 mM magnesium chloride, 1 mM sodium fluoride, 1 mM sodium orthovanadate, and 1 mM phenylmethanesulfonyl fluoride] containing 2 μ Ci of [γ -³²P]ATP and 0.2 μ g of recombinant human PKC ζ (Upstate, Temecula, CA). After incubation for 30 min at 30 C, the reaction mixture was washed with ice-cold buffer containing 50 mM HEPES (pH 7.4), 150 mM NaCl, and 0.1% Triton X-100. Phosphorylated LKB1 was separated by SDS-PAGE and visualized by autoradiography. ³²P-labeled LKB1 or LKB1(S307A) was trypsinized and examined by two-dimensional thin layer chromatography as described previously (56).

Western blot, immunofluorescence studies, and statistical analyses

Expression and phosphorylation levels of proteins were detected by Western blot analysis of cell lysates or immunoprecipitation with specific antibodies. Quantification of the relative increase in protein phosphorylation (expressed as percentage of basal phosphorylation) was performed using Scion Image Alpha 4.0.3.2 program (Scion Corp., Frederick, MD) and normalized for the amount of protein expression in each experiment. For all Western blot quantifications, “folds” represent the ratio of phosphorylated to nonphosphorylated protein. Percentage of cytosolic LKB1 is calculated as $P = C/(N + C)$, where N represents cells in which LKB1 is localized predominantly in the nucleus, and C represents cells with predominantly cytosolic LKB1. Sta-

tistical evaluation of the data was done using one-way ANOVA. * or #, $P < 0.05$; ** or ##, $P < 0.01$; ***, $P < 0.001$.

PP2A activity assays

The PP2A activity assay kit was purchased from Millipore (Bedford, MA), and the reaction was performed according to the manufacturer's instruction (Millipore). In brief, PP2A catalytic subunit was immunoprecipitated from C2C12 myotubes lysate (500 μ g of total protein) with 4 μ g of anti-PP2A C-subunit antibody. The immunoprecipitated PP2A C-subunit was incubated with 750 μ M phosphopeptide (K-R-pT-I-R-R) and Ser/Thr Assay buffer at 30°C for 10 min. The supernatant (25 μ l) was mixed with 100 μ l of Malachite Green phosphate detection solution provided by the manufactory and kept at room temperature for 10 min. The absorbance at 650 nm was measured. The same assay solution without PP2A incubation was used as a negative control.

In vitro dephosphorylation assays

Recombinant PKC ζ (0.05 μ g) was incubated in phosphatase buffer [50 mM Tris-HCl (pH 7.0), and 100 μ M CaCl₂] with indicated units of purified PP2A AC dimmer (subunit A and C mixed together and formed functional PP2A) (Upstate) for 30 min at 30°C. The reactions were terminated by adding sodium dodecyl sulfate gel loading buffer and run on 10% sodium dodecyl sulfate-polyacrylamide gels. The protein and phosphorylation levels of PKC ζ were detected by Western blot analysis with specific antibody as indicated.

In vivo metformin studies

Thirty db/db mice (11 wk old) were fed chow diet (diet 5008; Ralston Purina, St. Louis, MO) for 2 wk and then grouped to match blood glucose across treatments before dosing. The animals received either vehicle ($n = 10$), metformin150 mpk ($n = 10$), or metformin500 mpk ($n = 10$) by oral gavage once daily for three consecutive days. The mice were fasted overnight on the third day, and fasted baseline blood glucose was taken by tail vein blood glucometer readings next day morning. Animals then received a fourth dose of their respective treatment and blood glucose measured every hour thereafter for 4 h. After 4 h, animals were scarified by cervical dislocation, skeletal muscle was removed and frozen immediately in liquid nitrogen. From the frozen tissues, protein extraction was done as described previously (26). Animal protocol was approved by the Merck Research Laboratory Animal Care and Use Committee.

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