“Yin-Yang” Regulation of Adiponectin Signaling by APPL Isoforms in Muscle Cells
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APPL1 is a newly identified adiponectin receptor binding protein that positively mediates adiponectin signaling in cells. Here we report that APPL2, an isoform of APPL1 that forms a dimer with APPL1, can interact with both AdipoR1 and AdipoR2 and acts as a negative regulator of adiponectin signaling in muscle cells. Overexpression of APPL2 inhibits the interaction between APPL1 and AdipoR1, leading to down-regulation of adiponectin signaling in C2C12 myotubes. In contrast, suppressing APPL2 expression by RNAi significantly enhances adiponectin-stimulated glucose uptake and fatty acid oxidation. In addition to targeting directly to and competing with APPL1 in binding with the adiponectin receptors, APPL2 also suppresses adiponectin and insulin signaling by sequestrating APPL1 from these two pathways. In addition to adiponectin, metformin also induces APPL1-APPL2 dissociation. Taken together, our results reveal that APPL isoforms function as an integrated Yin-Yang regulator of adiponectin signaling and mediate the crosstalk between adiponectin and insulin signaling pathways in muscle cells.

Adiponectin, an adipocyte-secreted hormone that regulates energy homeostasis and insulin sensitivity, has been shown to be a promising therapeutic drug target for the treatment of type 2 diabetes (1-3). Adiponectin binds to its membrane receptors (AdipoR1 and AdipoR2) and regulates lipid and glucose metabolism by activating downstream signaling molecules, such as AMPK, p38 MAP kinase (MAPK) and PPAR, in the muscle and liver (1,4). Activation of AMPK by adiponectin reduces S6 kinase-mediated IRS-1 serine phosphorylation and increases IRS-1 tyrosine phosphorylation thus sensitizes insulin signaling in C2C12 myotubes (5), suggesting a direct crosstalk between the adiponectin and insulin signaling pathways.

We have recently identified APPL1 (Adaptor protein containing PH domain, PTB domain and Leucine zipper motif) as a signaling protein immediately downstream of adiponectin receptors and positively mediates adiponectin signaling in muscle cells (6). This adaptor protein was previously shown to interact with the catalytic subunit of PI 3-kinase (p110) and Akt, which are two key kinases in the PI 3-kinase pathway downstream of the insulin receptor (7). The interaction between APPL1 and Akt is required for insulin-stimulated GLUT4 translocation (8) and for controlling Akt substrate selectivity (9). It has been shown that APPL1 potentiated Akt activity to suppress androgen receptor transactivation in prostate cancer cells (10). APPL1 has also been
suggested to function as an adaptor protein in regulating follicle-stimulated hormone (FSH)-mediated PI 3-kinase/Akt signaling pathway (11,12). Our results showed that APPL1 binds directly to the intracellular part of the adiponectin receptors and positively mediates adiponectin signaling to the AMPK and p38 MAPK pathways, leading to increased glucose uptake and fatty acid oxidation in muscle cells (6). In addition, we found that APPL1 plays a critical role in regulating the crosstalk between adiponectin and insulin signaling pathways (6). APPL1 has since been found to mediate adiponectin signaling in other types of cells such as endothelial cells to regulate nitric oxide production and endothelium-dependent vasodilatation (13) and to protect from IL-18 mediated cell death (14). A recent study also suggested that APPL1 has a potentiating effect on insulin-stimulated suppression of hepatic glucose production in mice (15). These evidence suggest that APPL1 is an essential mediator in both adiponectin and insulin signaling, which are the two major pathways regulating energy homeostasis.

APPL2 is an isoform of APPL1 and these two proteins display 54% identity in protein sequences (6,16). Similar to APPL1, APPL2 contains an N-terminal BAR domain, a central PH domain, and a C-terminal PTB domain. It has been shown that APPL2 is essential for cell proliferation and embryonic development (9,16). Recent studies indicated that APPL2 regulates FSH signaling by forming a dimer with APPL1 via the BAR domains of the isoforms, leading to the formation of a complex with the FSH receptor, APPL1 and Akt2. However, unlike APPL1, APPL2 does not directly interact with Akt2 (11,17). The role of APPL2 in adiponectin and insulin signaling remains largely unknown.

In the present study, we show that APPL2 functions as a negative regulator in adiponectin signaling. APPL2 competes with APPL1 in the binding to AdipoR1 and blocks adiponectin signaling in muscle cells. In addition, APPL2 also sequesters APPL1 from adiponectin signal pathway by forming a heretodimer with APPL1. Adiponectin as well as metformin induces a dissociation of this complex. These results provide a mechanism for negative regulation of adiponectin signaling and adiponectin-regulated glucose and lipid metabolism in muscle cells. Furthermore, APPL2 blocks APPL1-mediated insulin-sensitizing effect of adiponectin in muscle cells by heterodimerizing with APPL1 to inhibit insulin-stimulated Akt activation. Our data indicate that APPL isoforms function as an integrated “Yin-Yang” regulator in adiponectin signaling and reveals a novel molecular mechanism for adiponectin and insulin resistance.

**EXPERIMENTAL PROCEDURE**

*Plasmids, adiponectin and antibodies.* The cDNAs of full-length and truncations of human APPL1 and mouse APPL2 were generated by PCR and subcloned into the mammalian expression vectors pcDNA3.1 (Myc-tagged), pBEX (HA-tagged), pFLAG-CMV2 (FLAG-tagged), or pCMV-3Tag-2 (Myc-tagged), respectively. The cDNA encoding full-length human AdipoR1 were subcloned into the pBEX as described previously (6). The recombinant globular adiponectin was produced as described previously (6). Antibodies specific to APPL1 and AdipoR1 were generated as described previously (6). Antibodies specific to APPL2 and to AdipoR2 were raised in rabbits using GST-APPL2c (amino acids 619-662) and GST-AdipoR2n (amino acids 78-219), respectively. The specificities of anti-APPL2 and anti-AdipoR2 antibodies were described in Supplementary Information (Figures S1A and S1B). The protein expression of AdipoR2 was detected with the AdipoR2 antibody in mouse tissues and the result indicated that the receptor was heavily expressed in the liver (Figure S1C).

*Cell culture:* C2C12 myoblasts (from the American Type Culture Collection (ATCC), Manassas, VA) were grown in DMEM (ATCC) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. Differentiation of C2C12 myoblasts into myotubes was induced by growing the cells in low-serum differentiation medium (99% DMEM, 0.1% FBS, 1% penicillin–streptomycin and 100 nM insulin). The medium was changed daily and multinucleated myotubes were normally observed 3-5 days later.

*APPL2-suppressed cells and Adeno-X APPL2/shRNA Virus:* The shRNA of APPL2 were chemically synthesized and ligated into the pSIREN–DNR (BDKnockout RNAi system, BD, San Jose, CA). The sense sequence corresponds to nucleotides 690-708 of mouse APPL2 (5'-
AGACATGGTTCAAGACATT-3’). Adeno-X APPL2/shRNA and scramble adenovirus were generated by recombination of shRNA into pLp-Adeno-X adenovirus backbone vector (BD knockout adenoviral RANi System 2, Clonetech, CA). To suppress APPL2 expression in C2C12 myotubes, the differentiated cells were changed into growth medium and treated with 25 of M.O.I. of pLp-Adeno-X APPL2/shRNA virus for 48 hours.

**Immunoprecipitation and Western blot:** The in vitro GST pull-down assay and co-immunoprecipitation experiments were performed as described previously (18). The western blot was carried out as described previously (6). Antibodies to APPL1, APPL2, AdipoR1 and AdipoR2 were obtained from rabbits using a GST–APPL1 (CT) (amino acids 455–709), GST-APPL2 (CT) (amino acids 619-662), GST-AdipoR1 (NT) (amino acids 4–142) or GST-AdipoR1 (NT) (amino acids 78-219), respectively. All other antibodies were obtained from Cell Signaling Technologies (Danvers, MA). Statistical analysis of the data was done using Student’s t-test.

**Immunofluorescence microscopy analysis:** C2C12 myoblasts were transfected with plasmids encoding Myc-tagged APPL1 or APPL2. 18 hours post transfection, cells were serum starved for 6 hours and then treated with 1 μg/ml globular adiponectin or 500 μM metformin for indicated time. The Immunofluorescence experiments were carried out as previously described (6). Briefly, the cells were fixed in 4% paraformaldehyde for 20 minutes. The proteins were detected with a monoclonal anti-Myc antibody. After three washes with PBS, cells were incubated with Alexa 488 conjugated secondary antibodies (green; Molecular Probes, Eugene, OR). Nuclear staining was performed with 4’,6-diamidino-2-phenylindole (DAPI; Sigma, St Louis, MO). Images were acquired on an Olympus FV-1000 laser-scanning confocal microscope. Statistical analysis of the data was done using Student’s t-test.

**Fatty acid oxidation assay:** After 2 hours serum-free starvation, C2C12 myotubes in 24-well plates were incubated with preincubation buffer (DMEM containing 12 mM glucose, 4 mM glutamine, 25 mM HEPES, 1% free fatty acid (FFA)-free BSA and 0.25 mM oleate) for 1 hour. 1 μCi/ml of 14C-oleic acid (American Radiolabeled Chemicals, St Louis, MO) was added into the medium and the wells were immediately covered with a piece of filter paper (Whatman paper #3; Whatman, Florham Park, NJ). The cells were incubated for another 1.5 hours at 37 °C in the presence or absence of globular adiponectin (1 μg/ml). The Whatman paper was wetted with 0.2 ml of 3 M NaOH and 0.1 ml of 70% perchloric acid (Sigma) was injected into the wells with a syringe. The cells were put at room temperature for 1 hour to collect CO2. The filter paper was removed from the wells and dried in the hood. The value of 14C-radioactivity was determined by a liquid scintillation counter. Statistical analysis of the data was done using Student’s t-test.

**Glucose uptake assay:** The differentiated C2C12 myotubes were maintained in growth medium with Adeno-X APPL2/shRNA or Adeno-X APPL2/scramble for another 48 hours (M.O.I.=25). After 4 hours of serum-free starvation, the cells were washed with wash buffer for 2 times (20 mM HEPES (pH 7.4), 140 mM NaCl, 5 mM KCl, 2.5 mM MgSO4, 1 mM CaCl2), and then incubated in 1 ml of wash buffer in the presence or absence of globular adiponectin (1 μg/ml) for 1 hour. 0.5 μCi/ml of 2-deoxy-d-2-3H-glucose (American Radiolabeled Chemicals) and 10 μM 2-deoxyglucose (Sigma) were added to each well. 2-deoxyglucose uptake was allowed at 37 °C for 10 min. Glucose uptake was stopped by adding 2 ml of ice-cold 50 mM glucose in PBS. Cells were washed with 1 ml of wash buffer for 3 times and lysed in 0.5 ml of 0.1 M NaOH. The non-specific uptake was measured in the presence of 10 μM cytochalasin B (Sigma). Statistical analysis of the data was done using Student’s t-test.

**Tissue homogenization:** Mouse tissue samples were homogenized in ice-cold buffer containing 50 mM HEPES (pH 7.6), 150 mM sodium chloride, 20 mM sodium pyrophosphate, 20 mM beta-glycerophosphate, 10 mM sodium fluoride, 2 mM sodium orthovanadate, 2 mM EDTA, 1.0% Igepal (a nonionic, nondenaturing detergent), 10% glycerol, 2 mM phenylmethylsulfonyl fluoride, 1 mM magnesium chloride, 1 mM calcium chloride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Tissue homogenates were clarified by centrifugation, and...
protein concentrations in the supernatant were determined using the Bradford assay. Statistical analysis of the data was done using Student’s t-test.

RESULTS

APPL2 interacts with adiponectin receptors. To study the tissue distribution and the potential roles of APPL2, we have generated an APPL2-specific antibody by immunizing rabbits with GST-APPL2-CT fusion protein (Fig. S1A). By Western blot using this antibody, we found that APPL2 is expressed in insulin-target tissues including skeletal muscle, liver, fat, and brain (Figure 1A), which is comparable to APPL1 expression in these tissues. In contrast to APPL1, APPL2 is highly expressed in kidney and pancreas tissues (Figure 1A).

To determine if APPL2, like its isoform APPL1, is capable of interacting with the signaling molecules in the adiponectin pathway, we performed pull-down experiments. We found that endogenous AdipoR1 and AdipoR2 could be pulled down by GST-APPL2 fusion protein but not by the GST control (Figure 1B). Co-immunoprecipitation experiments confirmed the interaction between endogenous APPL2 and AdipoR1 in C2C12 myotubes (Figure 1C). Interestingly, treating C2C12 myotubes with adiponectin led to a significant dissociation between endogenous AdipoR1 and APPL2 (Figure 1C, lane 6 vs. lane 5, and Figure 1D), which is opposite to the effect of adiponectin on the interaction between AdipoR1 and APPL1 (Figure 1C, lane 4 vs. lane 3, and Figure 1D) (6).

Our previous study has shown that the PTB domain of APPL1 is essential for the interaction between AdipoR1 and APPL1 (6). To map the site in APPL2 interacting with AdipoR1, we performed truncation-mapping experiments. Deletion of the BAR domain in APPL2 resulted in a complete loss of the interaction between APPL2 and AdipoR1 (Figure 1E, lane 3 vs. lane 2, and Figure S2A), suggesting that the BAR domain of APPL2 is critical for its interaction with the receptor.

APPL2 negatively regulates adiponectin signaling in C2C12 myocytes. The findings that APPL1 and APPL2 interact with AdipoR1 via different mechanisms suggest that the two isoforms may exert distinct functions in mediating adiponectin signaling. To test this hypothesis, we examined the effects of APPL2 on adiponectin signaling in C2C12 cells. Similar to the results reported previously (6,13,14), overexpression of APPL1 potentiates adiponectin-stimulated AMPK and ACC phosphorylation (Figure 2A, lanes 3 and 4 vs. lanes 1 and 2). The stimulatory effect of adiponectin on AMPK and ACC phosphorylation, however, was greatly suppressed in cells overexpressing APPL2 (Figure 2A, lane 6 vs. lane 2) or the BAR domain of APPL2 (Figure 2A, lane 8 vs. lane 2), a domain critical for the interaction with AdipoR1 (Figure 1E) and APPL1 (Figure S2B). Overexpression of the full length or the BAR domain of APPL2 also inhibited adiponectin-stimulated p38 MAPK phosphorylation (Figure 2B, lanes 6 and 8 vs. lane 2). On the other hand, overexpression of the BAR domain truncated form of APPL2 had no effect on adiponectin signaling (Figures S3A and S3B). To further confirm the inhibitory role of APPL2 in adiponectin signaling, we suppressed the expression levels of endogenous APPL2 in C2C12 myotubes by infecting the cells with APPL2-RNAi adenovirus. We found that the activities of AMPK and p38 MAPK were significantly increased under both basal and adiponectin-stimulated conditions (Figures 2C and 2D).

Adiponectin and metformin-induced subcellular localization changes of APPL isoforms. It is interesting to notice that the two APPL isoforms, though sharing high amino acid sequence homology and domain structure similarity, exert opposite effects on mediating adiponectin signaling in muscle cells. One of the potential mechanisms accounting for these differences could be their different subcellular localization. Under basal condition, the majority of APPL1 is in the cytosol (Figures 3A and 3C) while APPL2 is largely plasma membrane associated (Figures 3B and 3C). Adiponectin stimulation resulted in APPL2 translocation from the plasma membrane to cytosol (Figures 3B and 3C) and a simultaneous increase in APPL1 membrane translocation (Figures 3A and 3C). A similar stimulatory effect on APPL1/2 cellular localization was observed for the AMPK activator metformin (Figure 3), suggesting that altering the subcellular localization of APPL
isoforms could be a common mechanism for these insulin sensitizers to exert their function.

**APPL2 inhibits adiponectin signaling by blocking the interaction between AdipoR1 and APPL1.** APPL2 has been shown to undergo heterodimerization with APPL1 through its BAR domain (Figure S2B) (17). To determine whether APPL dimerization is regulated by adiponectin, we performed co-immunoprecipitation experiments. We found that APPL1 interacts with APPL2 in C2C12 myoblasts and the interaction was decreased by adiponectin treatment in a time-dependent manner (Figure 4A and Figure S4). Treating cells with metformin also inhibited APPL1/APPL2 interaction (Figure 4B), suggesting that regulation of APPL1-APPL2 heterodimer formation is a common mechanism for the insulin sensitization effects of adiponectin and metformin.

Based on the findings that adiponectin stimulates the dissociation of APPL2 from both AdipoR1 and APPL1, we attempted to determine whether APPL2 blocks the interaction between APPL1 and AdipoR1, an essential step for adiponectin signaling in muscle cells (6). Consistent with our earlier findings (6), APPL1 interacts with AdipoR1 in C2C12 myotubes and this interaction was stimulated by adiponectin (Figure 4C, lane 4 vs. lane 3). The interaction between APPL1 and AdipoR1 was greatly increased in APPL2-suppressed C2C12 myotubes even under basal conditions (Figure 4C, lane 7 vs. lane 3), suggesting that endogenous APPL2 could block adiponectin signaling by binding with AdipoR1 under un-stimulated condition and removal of APPL2 from the receptor is an essential step for adiponectin to activate downstream signaling events. Consistent with this view, overexpression of APPL2 competed with APPL1 in binding with AdipoR1 and inhibited adiponectin-stimulated binding of APPL1 to AdipoR1 (Figure 4D, lane 6 vs. lane 5). An increased APPL1 membrane localization was also observed in C2C12 myocytes with APPL2 expression suppressed compared with that in scrambled control cells (Figures 4E and 4F). Suppressing APPL1 expression in C2C12 myotubes led to an increase of APPL2-AdipoR1 interaction (Figure S5). Together, our data indicate that APPL2 negatively regulates adiponectin signaling by blocking the APPL1-AdipoR1 interaction and the relative expression levels of APPL isoforms are critical for the regulation of adiponectin signaling.

**APPL2 negatively regulates adiponectin-induced glucose uptake and fatty acid oxidation in C2C12 myotubes.** To determine the potential role of APPL2 in adiponectin action, we examined the effect of APPL2 on adiponectin-regulated glucose uptake and fatty acid oxidation. Suppression of APPL2 in C2C12 myotubes is sufficient to promote glucose uptake (Figure 5A) and fatty acid oxidation (Figure 5B) to an extent similar to that stimulated by adiponectin in the control cells. It is interesting to notice that adiponectin treatment further increased glucose uptake and fatty acid oxidation in the APPL2-suppressed C2C12 myotubes compared to un-stimulated cells (Figures 5A and 5B), suggesting that other mechanisms are involved in adiponectin-stimulated glucose uptake and fatty acid oxidation, in addition to the removal of the negative regulation due to APPL2 binding. Taken together, these data provide further evidence that APPL2 functions as a negative regulator in the adiponectin signal pathway in C2C12 myotubes.

**The roles of APPL2 in the crosstalk between adiponectin signaling and insulin signaling pathways.** We previously showed that APPL1 plays an important role in the insulin sensitizing effect of adiponectin and regulates the crosstalk between adiponectin and insulin signaling (6). To investigate the potential role of APPL2 in the crosstalk between adiponectin and insulin signaling pathways, we examined the effect of APPL2 protein level on adiponectin-sensitized insulin signaling in C2C12 myotubes. As shown in Figure 6A, overexpression of APPL2 diminished the synergistic effect of adiponectin on insulin-stimulated Akt activation (lane 6 vs. lane 4). Overexpression of the BAR domain truncated form of APPL2, which is unable to bind to AdipoR1 (Figure 1E) and APPL1 (17), has no inhibitory effect on the insulin sensitizer role of adiponectin in the cross-talk between adiponectin and insulin pathways (Figure 6A, lane 8 vs. lane 6). On the other hand, suppressing APPL2 expression in C2C12 myotubes significantly increased the synergistic effect of adiponectin on insulin-induced Akt activation (Figure 6B, lane 8 vs. lane 4). Together, the results suggested that APPL2...
negatively regulates the insulin-sensitizer role of adiponectin in insulin signaling.

In contrast to APPL1, APPL2 is unable to interact with any insulin signaling molecules, such as Akt (11,17). It is interesting to notice that overexpression of APPL2 reduces the insulin-stimulated Akt phosphorylation (Figure 6A, lane 5 vs. lane 3). On the other hand, suppressing APPL2 expression in C2C12 myotubes significantly enhances insulin-stimulated activation of this kinase (Figure 6B, lane 7 vs. lane 3). Overexpression of the BAR domain truncated form of APPL2, APPL2(DBAR), led to an increase of insulin-stimulated Akt phosphorylation (Figure 6A, lane 7 vs. lane 3). These results suggest that APPL2 also plays a negative role in regulating insulin signaling in addition to blocking the insulin-sensitizer role of adiponectin.

**DISCUSSION**

We have recently identified APPL1 as a critical signaling molecule that positively mediates adiponectin signaling and action (6). However, the role of APPL2, a protein that shares a 54% identity in amino acid sequence with APPL1 (6,16), was unclear. It is interesting to notice that APPL1 but not APPL2 interacts directly with Akt (11). Thus, the two APPL isoforms may play distinct roles in adiponectin downstream signaling and action.

In the present study, we show that APPL2 negatively regulates adiponectin signaling in muscle cells (Figure 7A). Under the basal condition, APPL2 interacts with AdipoR1 via its BAR domain (Figure 1). The binding of APPL2 to AdipoR1 hinders the accessibility of APPL1 to AdipoR1 (Figures 4C and 4D) and thus inhibits the phosphorylation and activation of AMPK and p38 MAPK (Figure 2). Adiponectin treatment stimulates APPL2 dissociating from AdipoR1 (Figures 1C and S5), hence facilitates the recruitment of APPL1 to AdipoR1 (Figures 4C, S4, 4E and 4F), which is a critical step for adiponectin signaling transduction (6).

In addition to interacting with AdipoR1, APPL2 also undergoes heterodimerization with APPL1. The interaction between APPL2 and APPL1 could negatively regulate adiponectin signaling by sequestering APPL1 from the adiponectin receptor (Figure 7A). Treating cells with adiponectin or metformin promoted the dissociation between APPL1 and APPL2 (Figures 4A and 4B), suggesting that the heterodimer could be a central node in the regulation of adiponectin signaling. Taken together with the findings that APPL1 and APPL2 migrated to distinct cellular compartments under the adiponectin/metformin-stimulatory conditions (Figure 3), it is conceivable that a dynamic balance between association of APPL2 with APPL1 and with other molecules in the adiponectin signaling pathways could be a key mechanism regulating adiponectin signaling in cells. Interestingly, both APPL2 and AdipoR1 were simultaneously co-immunoprecipitated with APPL1 at endogenous level (Figure S4), suggesting that these three molecules could form a complex in cells. Further studies will be needed to elucidate these possibilities.

In addition to negatively regulating adiponectin signaling, APPL2 also plays an inhibitory role in mediating the insulin sensitizing effect of adiponectin in muscle cells (Figure 7B). Overexpression of APPL2 blocks the synergistic effect of adiponectin on insulin-stimulated Akt activation and the effects of insulin itself on this kinase (Figure 6A). Suppression of APPL2 expression levels, on the other hand, greatly enhanced both the sensitizer effect of adiponectin on insulin signaling and the direct insulin-simulated activation of Akt in C2C12 myotubes (Figure 6B). However, unlike APPL1, APPL2 does not interact with Akt (11) or PI 3-kinase (Data not shown), suggesting that APPL2 may negatively regulate insulin signaling through an indirect mechanism, probably via dimerization with APPL1. It is interesting to notice that adiponectin treatment inhibits APPL1/APPL2 dimerization (Figure 4A) and promotes APPL1 plasma membrane translocation (Figures 3A and 3C), suggesting that the adiponectin-induced dissociation of APPL1-APPL2 heterodimer may facilitate APPL1 translocation to the plasma membrane where APPL1 interacts with PI 3-kinase and Akt and promotes insulin signaling (Figure 7B). In addition to a direct interaction with signaling molecules in the insulin signaling pathway, membrane-association of APPL1 may also sensitize insulin signaling by promoting adiponectin-stimulated AMPK signaling pathway, which has been shown to suppress S6-kinase-mediated IRS-1 serine
phosphorylation, leading to increased IRS-1 tyrosine phosphorylation and Akt phosphorylation in response to insulin stimulation (5).

Adiponectin resistance has been shown to contribute significantly to insulin resistance and its associated metabolic diseases (1,19,20). Identification of APPL isoforms as a “Yin-Yang” regulator of adiponectin signaling and the adiponectin-insulin crosstalk in muscle cells provides insight not only to the molecular mechanism by which adiponectin signaling is regulated in cells but also on the identification of a potential therapeutic drug target for the treatment of insulin resistance and related disorders.

REFERENCES

FOOTNOTES
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The abbreviations used are: APPL, adaptor protein containing PH domain, PTB domain and leucine zipper motif; AdipoR, adiponectin receptor; IRS-1, insulin receptor substrate-1; AMPK, AMP-activated protein kinase; MAPK, mitogen-activated protein kinase.

FIGURE LEGENDS

Figure 1. APPL2 interacts with Adiponectin receptors. A. Protein expression of APPLs and AdipoR1 were detected in mouse brain (B), liver (Li), muscle (M), fat (F), heart (H), kidney (K), spleen (S) and pancreas (P) by western blot analysis with antibodies specific for APPL1, APPL2 and AdipoR1. Thirty microgram of protein in tissue homogenates was loaded in each lane. B. APPL2 interacts with adiponectin receptors in vitro. GST or GST-APPL2 fusion protein was incubated with cell lysates of C2C12 myotubes. Endogenous AdipoR1 and AdipoR2 associated with recombinant GST-APPL2 and their protein levels were detected by western blot analysis with antibodies specific for AdipoR1 and AdipoR2, respectively. The GST fusion proteins were visualized by Coomassie blue staining (right panel). C. APPL2 interacts with AdipoR1 in C2C12 myotubes. Serum starved C2C12 myotubes were treated with or without globular adiponectin (Ad, 1 μg/ml, 10 min). Endogenous APPL1 or APPL2 was immunoprecipitated. Co-immunoprecipitated endogenous AdipoR1 and the protein expression levels of AdipoR1, APPL1 and APPL2 in cell lysates were detected by western blot analysis with the antibodies specific to the proteins as indicated. D. Graphic presentation of effect of adiponectin on the interaction between APPLs and AdipoR1 shown in 1C. Error bars represents mean ± s.e.m (n=3). *, p<0.05. E. APPL2 interacts with AdipoR1 through its BAR domain. HA-tagged AdipoR1 and FLAG-tagged APPL2 or APPL2 (BAR) mutant were co-expressed in C2C12 myoblasts. APPL2 or APPL2 (BAR) mutants were immunoprecipitated by anti-FLAG-antibody. Co-immunoprecipitated AdipoR1 and the protein expression levels of AdipoR1 and APPL2 in cell lysates were detected by anti-HA or anti-FLAG-antibodies as indicated. The results shown in Figure 1 are representative of three independent experiments with similar findings.

Figure 2. APPL2 inhibits adiponectin signaling in C2C12 myocytes. A. Overexpression of APPL2 inhibits adiponectin-induced AMPK activation. C2C12 myoblasts overexpressing Myc-tagged APPL1, APPL2 or APPL2 (BAR) were serum starved for 4 hours and treated with or without 1 μg/ml globular adiponectin (Ad) for 20 minutes. Phosphorylation of AMPK at Thr172 and ACC at Ser79 as well as their protein levels were detected by western blot analysis using specific antibodies as indicated. B. Overexpression of APPL2 inhibits adiponectin-induced p38 MAPK activation. C2C12 myoblasts overexpressing Myc-tagged APPL1, APPL2 or APPL2 (BAR) were serum starved for 4 hours and treated with or without 1 μg/ml globular adiponectin (Ad) for 10 minutes. Phosphorylation of p38 MAPK at Thr180 and Tyr182 and the total p38 MAPK protein were detected by western blot analysis using specific antibodies as indicated. C. Suppression of APPL2 expression enhances AMPK activity. Scramble control or APPL2-suppressed C2C12 myotubes were serum starved for 6 hours and treated with or without 1 μg/ml globular adiponectin (Ad) for 20 minutes. Phosphorylation of AMPK at Thr172 as well as the total AMPK

protein was detected by western blot analysis using specific antibodies as indicated. Equal loading of protein was determined by western blot analysis using anti-β-tubulin antibody. D. Suppression of APPL2 expression enhances p38 MAPK activity. Scramble control or APPL2 suppressed C2C12 myotubes were serum starved for 6 hours and treated with or without 1 μg/ml globular adiponectin (Ad) for 10 min. Phosphorylation of p38 MAPK at Thr180 and Tyr182 as well as the total p38 MAPK protein were detected by western blot analysis using specific antibodies as indicated. Equal loading of protein was determined by western blot analysis using anti-β-tubulin antibody. The results shown in Figure 2 are representative of three independent experiments with similar findings.

Figure 3. Adiponectin and metformin regulate the subcellular translocation of APPLs. A. Translocation of APPL1 in response to adiponectin and metformin stimulation. C2C12 myocytes overexpressing Myc-tagged APPL1 were serum starved for 6 hours and treated with or without globular adiponectin (Ad, 1 μg/ml) for 10 minutes or metformin (500 μM) for 45 minutes. The localization of APPL1 was detected by immunofluorescence staining with Myc-antibody (Green). The cell nuclei were stained with DAPI (Blue). The scale bars represent 50 μm. B. Translocation of APPL2 in response to adiponectin and metformin stimulation. C2C12 myocytes overexpressing Myc-tagged APPL2 were serum starved for 6 hours and treated with or without globular adiponectin (Ad, 1 μg/ml) for 10 minutes or metformin (500 μM) for 45 min. The localization of APPL2 was detected by immunofluorescence staining with Myc-antibody (Green). The cell nuclei were stained with DAPI (Blue). The scale bars represent 50 μm. C. Graphic representation of percentage of cells with APPLs localizing on membrane as shown in 3A and 3B. Error bars represent mean ± s.e.m (n=3). * indicates p<0.05.

Figure 4. APPL2 forms heterodimer with APPL1 and interrupts the APPL1-AdipoR1 interaction. A. Adiponectin induces dissociation of APPL1-APPL2 heterodimer. C2C12 myoblast overexpressing Myc-tagged APPL1 and HA-tagged APPL2 were serum starved for 6 hours and treated with or without globular adiponectin (Ad, 1 μg/ml) for the indicated time. APPL2 was immunoprecipitated with anti-HA antibody. Co-immunoprecipitated APPL1 as well as the protein expression levels of APPL2 and APPL1 in cell lysates were detected by western blot analysis with anti-HA or anti-Myc antibody as indicated. B. Metformin treatment leads to dissociation of APPL1-APPL2 heterodimer. C2C12 myoblast overexpressing Myc-tagged APPL1 and HA-tagged APPL2 were serum starved for 6 hours and treated with or without metformin (500 μM) for the indicated times. APPL2 was immunoprecipitated with anti-HA antibody. Co-immunoprecipitated APPL1 as well as the protein expression levels of APPL2 and APPL1 in cell lysates were detected by western blot analysis with anti-HA or anti-Myc antibody as indicated. C. Suppression of APPL2 expression enhances APPL1-AdipoR1 interaction. Scrambled control or APPL2-suppressed C2C12 myotubes were serum starved for 6 hours and treated with or without globular adiponectin (Ad, 1 μg/ml) for 20 minutes. Endogenous APPL1 was immunoprecipitated with anti-APPL1 antibody. Co-immunoprecipitated AdipoR1 as well as the protein expression levels of AdipoR1, APPL1 and APPL2 in cells were detected by western blot analysis with the antibodies specific to these proteins as indicated. D. Overexpression of APPL2 interrupts APPL1-AdipoR1 interaction. C2C12 myocytes overexpressing HA-tagged AdipoR1, Myc-tagged APPL1 and different doses of HA-tagged APPL2 were serum starved for 6 hours and treated with or without globular adiponectin (Ad, 1 μg/ml) for 10 minutes. APPL1 was immunoprecipitated with anti-Myc antibody. Co-immunoprecipitated AdipoR1 as well as the protein expression levels of AdipoR1, APPL1 and APPL2 in cell lysates were detected by western blot analysis with anti-Myc or anti-HA antibody as indicated. E. Effect of APPL2 expression suppression on the translocation of APPL1 in response to adiponectin stimulation. Scrambled control or APPL2-suppressed C2C12 myocytes overexpressing Myc-tagged APPL1 were serum starved for 6 hours and treated with or without globular adiponectin (Ad, 1 μg/ml) for 10 minutes. The localization of APPL1 was detected by immunofluorescence staining with Myc-antibody (Green). The cell nuclei were stained with DAPI (Blue). The scale bars represent 20 μm. F. Graphic representation of percentage of cells with APPL1 localizing on
membrane as shown in 4E. Error bars represent mean ± s.e.m (n=3). * indicates p<0.05. The results shown in Figure 4 are representative of three independent experiments with similar findings.

**Figure 5. Biological functions of APPL2 in muscle cells.**

* A. Suppression of APPL2 expression enhances adiponectin-stimulated glucose uptake in C2C12 myotubes. Scrambled control or APPL2-suppressed C2C12 myotubes were serum starved for 4 hours and treated with or without globular adiponectin (Ad, 1 µg/ml) for 1 hour. Glucose uptake was measured as described in Experimental Procedures. The graph represents the folds of glucose uptake increase. * indicates p<0.05 (n=6). B. Suppression of APPL2 expression enhances adiponectin-stimulated fatty acid oxidation in C2C12 myotubes. Scrambled control or APPL2-suppressed C2C12 myotubes were serum starved for 2 hours and treated with or without globular adiponectin (Ad, 1 µg/ml) for 1.5 hour. Fatty acid oxidation was measured as described in Experimental Procedures. The graph represents the folds of increased fatty oxidation. * indicates p<0.05 (n=6).

**Figure 6. APPL2 blocks the insulin sensitization effects of adiponectin.**

* A. Overexpression of APPL2 inhibits the insulin sensitization effect of adiponectin. C2C12 myoblasts overexpressing FLAG-tagged full length or BAR domain truncated form of APPL2 were serum starved for 6 hours and pretreated with or without globular adiponectin (Ad, 1 µg/ml) for 10 minutes followed with insulin (Ins, 100 nM) for 5 minutes. Phosphorylation of Akt (T308) as well as the protein levels of Akt and FLAG-tagged APPL2 was detected by western blot analysis with specific antibodies as indicated. B. Suppression of APPL2 expression enhances the insulin sensitization effect of adiponectin. Scrambled control and APPL2-suppressed C2C12 myotubes were serum starved for 16 hours and pretreated with or without globular adiponectin (Ad, 1 µg/ml) for 15 minutes followed with insulin (Ins, 10 nM) for 5 minutes. Phosphorylation of Akt (Thr308) as well as the protein levels of Akt, APPL2 and β-tubulin were detected by western blot analysis with specific antibodies as indicated. The results shown in Figure 6 are representative of three independent experiments with similar findings.

**Figure 7. APPL isoforms play “Yin-Yang” regulatory roles in adiponectin signaling and adiponectin-insulin crosstalk.**

* A. The roles of APPL isoforms in adiponectin signaling. Under the basal condition, APPL2 occupies the N-terminus of AdipoR1 and sequesters APPL1 in cytosol by forming an APPL1-APPL2 heterodimer, thus inhibits adiponectin signaling. Upon adiponectin stimulation, APPL2 dissociates from AdipoR1 and releases APPL1, facilitating the recruitment of APPL1 onto AdipoR1, which is a critical step for adiponectin signaling. B. The role of APPL isoforms in the cross-talk between adiponectin and insulin pathways. Under the basal condition, APPL2 sequesters APPL1 and prevents the interaction of APPL1 with the components in insulin signaling. Upon adiponectin stimulation, APPL2 releases APPL1, facilitating the interaction of APPL1 with components in insulin signaling. Meanwhile, AMPK is activated, which in turn prevents the inhibitory effect of S6K on insulin signaling.
Figure 1

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Figure 2

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IgG H.C.
APPL1 (Myc)
APPL2 (HA)

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AdipoR1
APPL1
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APPL1

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AdipoR1 (HA)
APPL1 (Myc)
AdipoR1 (HA)
APPL1 (Myc)
APPL2 (HA)
APPL2 (HA)

E

Treatments

Control
Adiponectin
Scramble APPL2/shRNA

F

% membrane localization

*
Figure 5

A. Glucose Uptake (Folds)

B. Fatty Acid Oxidation (Folds)
Figure 6

A

1 2 3 4 5 6 7 8

Ad: - + - + - + + +
Ins: - - + + + + + +

p-Akt (T308)
Akt
APPL2 (FLAG)
APPL2 ([B]AR)(FLAG)

B

1 2 3 4 5 6 7 8

Ad: - + - + - + + +
Ins: - - + + - - + +

p-Akt (T308)
Akt
APPL2
[tubulin]

Scrambled
APPL2 shRNA
"Yin-Yang" regulation of adiponectin signaling by APPL isoforms in muscle cells
Chanhua Wang, Xiaoban Xin, Ruihua Xiang, Fresnida J. Ramos, Meilian Liu, Hak Joo Lee, Hongzhi Chen, Xuming Mao, Chintan K. Kikani, Feng Liu and Lily Q. Dong

J. Biol. Chem. published online August 6, 2009

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