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AMPK-independent down-regulation of cFLIP and sensitization to TRAIL-induced apoptosis by AMPK activators

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ABSTRACT

The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a TNF superfamily member that is being considered as a new strategy in anticancer therapy because of its ability to induce apoptosis, alone or in combination with other stimuli, in many cancer cells. AMP-activated protein kinase (AMPK) is an evolutionarily conserved key regulator of cellular energy homeostasis that protects the cell from energy depletion and stress by activating several biochemical pathways that lead to the conservation, as well as generation, of ATP. Here we report that a number of AMPK activators, including the small molecule activator A-769662, markedly sensitize TRAIL-resistant breast cancer cells to TRAIL-induced apoptosis. However, silencing AMPKα1 expression with siRNA or over-expression of DN-AMPKα1 does not inhibit AICAR, glucose deprivation, phenformin or A-769662-induced sensitization to TRAIL. Furthermore, the expression of constitutively active AMPK subunits does not sensitize resistant breast cancer cells to TRAIL-induced apoptosis. The cellular FLICE-inhibitory proteins ($cFLIP_L$ and $cFLIP_S$) were significantly down-regulated following exposure to AMPK activators through an AMPK-independent mechanism. Furthermore, in cells over-expressing $cFLIP_L$, sensitization to TRAIL by AMPK activators was markedly reduced. In summary, our results indicate that AMPK activators facilitate the activation by TRAIL of an apoptotic cell death program through a mechanism independent of AMPK and dependent on the down-regulation of cFLIP levels.

1. Introduction

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL/Apo2L) is a cytokine of the TNF gene superfamily that selectively induces apoptosis in many tumor cells while leaving normal cells intact and thus is an attractive candidate for anti-tumor therapies [1]. However, despite the ubiquitous expression of TRAIL receptors, some tumor cells, including most breast cancer cells, show either partial or complete resistance to the apoptotic effects of TRAIL [2,3]. To overcome this resistance, TRAIL-induced signalling is being studied alone or in combination with different sensitizing agents, such as chemotherapeutic drugs, cytokines and inhibitors of survival pathways [4–6].

TRAIL induces apoptosis through its binding to membrane bound death domain (DD)-containing receptors TRAIL-R1/DR4 and TRAIL-R2/DR5. This interaction induces the recruitment of the intracellular adaptor molecule FADD (FAS-associated death domain protein), that concurrently engages procaspase-8 at the death-inducing signalling protein complex (DISC) [7]. Within the DISC, caspase-8 is activated by transcatalytic and autocatalytic cleavage and released into the cytoplasm, initiating the protease cascade that leads to the effector caspases activation, thereby triggering the execution of steps that induce apoptosis (extrinsic apoptotic pathway). In addition, activated caspase-8 is able to cleave Bid, a BH3-only pro-apoptotic member of the Bcl-2 family protein, releasing a truncated protein (tBid) that translocates to the mitochondrial external membrane and, in concert with other pro-apoptotic Bcl-2 family proteins, as Bax and Bak, induces the release

Abbreviations: cFLIP, cellular FLICE-inhibitory protein; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TNF, tumor necrosis factor; DISC, death-inducing signalling complex; FADD, Fas-associated death domain; Cyt c, cytochrome *c*; DD, death domain; DED, death effector domain; PARP, poly (ADP-ribose) polymerase; PFU, plaque-forming unit.

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of apoptogenic factors, thereby amplifying caspase activation [8]. The apoptotic signal from the DISC may be inhibited by the cellular FLICE-inhibitory protein (FLIP) [9]. In most cells, two alternatively spliced isoforms of cFLIP exist: a caspase-8 homologue $cFLIP_L$ that lacks the amino acids critical for proteolytic caspase activity; and $cFLIP_S$, which is comprised of the two death effector domains alone [9]. Although the role of cFLIP in apoptotic signalling remains controversial, there is strong evidence that it displays anti-apoptotic activity [10–12].

AMPK (AMP-dependent protein kinase) is a heterotrimeric protein kinase complex comprising of a catalytic subunit (α) and two regulatory subunits (β and γ). Isoforms of each subunit (α1, α2, β1, β2, γ1, γ2 and γ3) have been identified in mammals, allowing the generation of twelve different heterotrimeric complexes, whose expression is tissue-dependent [13]. AMPK acts as a sensor of the cellular energy status, responding to an increase in the AMP/ATP ratio by inducing ATP-producing pathways (catabolism activation) and reducing ATP-consuming pathways (anabolism inhibition), until cellular energy content reaches normal levels [14]. AMPK is activated by two mechanism. First, AMP allosterically activates AMPK by binding to the γ subunit. Secondly, AMPK is activated by phosphorylation of threonine 172 (Thr-172) within the catalytic αsubunit. Three different upstream AMPK kinases have been identified: LKB1, CaMKKβ (Ca(2+)/calmodulin-dependent protein kinase kinase beta) and TAK1 (transforming growth factor-beta-activated kinase 1) [15,16]. Importantly, AMP protects against the dephosphorylation of T172, thereby increasing the proportion of active AMPK [17]. Several metabolic stress (hypoxia, energy starvation) and certain external factors or drugs, such as AICAR (5-aminoimidazole-4-carboxamide riboside), phenformin (ATP mitochondrial sintase inhibitor) or metformin (antihyperglycemic agent used in the treatment of diabetes), are able to activate AMPK in vitro and in vivo. Recently, a novel small molecule has been proposed as a specific AMPK activator: A-769662 [18]. A-769662 activates AMPK both allosterically and by inhibiting dephosphorylation of AMPK on Thr-172, similar to the effects of AMP [19,20].

Although AMPK function in metabolism and maintenance of cellular energy homeostasis has been well studied, AMPK implication in cellular proliferation and survival remains unclear and controversial. Specifically, AMPK has been proposed to have a pro-apoptotic effect in different tumor cells. Moreover, its activation by AICAR has been shown to sensitize human colon adenocarcinoma cells to TRAIL and TNF-induced apoptosis [21]. Against these data, AMPK has been proposed as an anti-apoptotic molecule to protect cells from ischemia, hyperglycemia, glucose deprivation, fatty acids and ceramide [22–24].

Since most human breast tumor cells are resistant to TRAIL-induced apoptosis, the combination of TRAIL with other sensitizing stimuli is being studied to induce apoptosis in these cell types. In the present work, we have found that several AMPK activators, including the small molecule activator A-769662, markedly sensitize TRAIL-resistant breast cancer cells to TRAIL-induced apoptosis. However, silencing AMPKα1 expression with siRNA or over-expression of DN-AMPKα1 does not inhibit this sensitization to TRAIL. Furthermore, the expression of constitutively active AMPK subunits does not sensitize resistant breast cancer cells to TRAIL-induced apoptosis. In summary, our results indicate that a range of AMPK activators facilitate the activation by TRAIL of an apoptotic cell death program through a mechanism independent of AMPK. Interestingly, AMPK-independent down-regulation of cFLIP expression is observed upon treatment with AMPK activators and cFLIP over-expression markedly inhibits apoptosis induced by AMPK activators and TRAIL.

2. Materials and methods

2.1. Cell culture

The breast tumor cell lines were maintained in RPMI 1640 (MDA-MB231, MDA-MB435S, MCF7) or in DMEM (BT-474) medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 μg/mL streptomycin and 500 U penicillin (Sigma–Aldrich, St. Louis, MO, USA). Growth medium for MDA-MB435S cells was also supplemented with 10 μg/mL insulin. The cells were maintained at 37 °C in a humidified 5% CO_2, 95% air incubator. A stable cell line over-expressing $cFLIP_L$ was generated upon transfection of MDA-MB231 cells with pCR3.V64-Met-Flag-$FLIP_L$ (a kind donation of Dr. J. Tschopp, University of Lausanne) by electroporation. Mock-transfected cells and cells over-expressing $FLIP_L$ were selected in culture medium with 1 mg/mL G418 (Sigma–Aldrich, St. Louis, USA) and analyzed for the expression $cFLIP_L$ by Western blot.

2.2. Reagents and antibodies

5-Aminoimidazole-4-carboxamide riboside (AICAR) was purchased from Toronto Research Chemicals (Ontario, Canada); phenformin, cycloheximide and 2-deoxyglucose (2DG) were from Sigma–Aldrich (St. Louis, MO, USA); glucose from MERCK (Darmstadt, Germany). Caspases inhibitor benzyloxy-carbonyl-Val-Ala-Asp-(OMe) fluoromethyl ketone (Z-VAD-fmk) was from Bachem, AG (Bachem, Bubendorf, Switzerland). The small molecule AMPK activator A-769662 was a gift from Dr. Anu Balendran (AstraZeneca, Molndal, Sweden). Soluble human His-tagged recombinant TRAIL was generated in our laboratory as described [25]. Human Factor X was obtained from Bioscience (Cambridge, UK). Anti-caspase 8 was a gift from Dr. Gerald Cohen (Leicester University, UK). Anti-bid was a gift from Dr. Wang (University of Texas Southwestern Medical Center at Dallas, USA). PARP and cytochrome *c* antibodies were from Pharmigen (San Diego, CA, USA). The antibodies to alpha-tubulin and to Flag-M2 were purchased from Sigma–Aldrich (St. Louis, MO, USA). Antibodies against Bax, GAPDH, actin (C-11) and c-Myc (9E10) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-cFLIP monoclonal antibody (NF6) was purchased from Alexis Corporation (Lausen, Switzerland). Anti-COX IV rabbit polyclonal antibody was from Abcam (Cambridge, UK). Antibodies against p-ACC, p-AMPK, AMPKα1, active caspase-9 and active caspase-3 were from Cell Signalling Technology, Inc. (Danvers, MA, USA). Horseradish peroxidase or FITC conjugated, goat anti-mouse, rabbit anti-goat and goat anti-rabbit secondary antibodies were obtained from DAKO (Cambridge, UK).

2.3. Determination of cell viability and apoptosis

Cell viability was determined by the crystal violet method as described [26]. Hypodiploid apoptotic cells were assessed by flow cytometry according to published procedures [27].

2.4. Immunoblot detection of proteins

Cells were lysed in ice-cold lysis buffer [50 mM Tris/acetate (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1% (w/v) Triton X-100, 1 mM sodium orthovanadate, 10 mM sodium glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate, 27 mM sucrose and protease inhibitor mixture (Roche Applied Science, Germany)]. The samples were frozen at −20 °C until analysis. Protein concentration was determined using the Bio-Rad Dc Protein Assay Reagent A, B and S (Hercules, CA, USA). Proteins were resolved on SDS-polyacrylamide minigels and detected as described previously [26].

2.5. Measurements of Bax translocation and cytochrome c release

Cells (3×10^5 well^{-1}) were treated in six-well plates as indicated in the figure legends. After treatment, cells were detached from the plate with RPMI/EDTA and trypsin, washed with PBS and lysed in 30 μL ice-cold lysis buffer (80 mM KCl, 250 mM sucrose, 500 μg/mL digitonin and protease inhibitors in PBS). For measurements of Bax translocation and cytochrome *c* release from mitochondria, cell lysates were centrifuged for 5 min at 10,000 × *g* to separate the supernatant (cytosolic fraction) and pellet (mitochondria-containing fraction). Amount of protein in each fraction was determined by the Bradford protein assay (BIO-RAD, Hertfordshire, UK). Proteins from the supernatant and pellet were mixed with Laemmli buffer and resolved on SDS-12% PAGE minigels. Bax and cytochrome *c* were determined by western blot analysis.

2.6. RNA interference

siRNAs against AMPKα1 (5′-UGCCUACCAUCUCAUAAUAdTdT) and nontargeting scrambled siRNAs were synthesized by Sigma-Proligo (St. Louis, MO, USA). Cells were transfected with the indicated siRNAs at 50 nM during 48 h using DharmaFECT transfection agent (Dharmacon Research, CO, USA) as described by the manufacturer.

2.7. Adenoviral infection

Cells were incubated with 100 PFU/cell of the indicated adenovirus overnight in a limiting serum-free medium volume containing 10.8 μg/mL of Factor X (Bioscience, Cambridge, UK) to facilitate adenoviral infection [28]. After the infection, complete medium was added and cells were incubated for 8–24 h before further treatments. Adenoviruses were produced by ViraQuest Inc (North Liberty, IA, USA).

2.8. Immunoprecipitation and AMPK assays

AMPK was immunoprecipitated from 100 μg of protein of cell lysate by overnight incubation at 4 °C with a sheep anti-alpha1 or alpha2 antibody [29] bound to protein G or A-Sepharose from Sigma–Aldrich (St. Louis, MO, USA). Immune complexes were collected by brief centrifugation and washed extensively in ice-cold buffer A (50 mM HEPES, pH 7.4, containing protease inhibitor mixture (Roche Applied Science, Germany), 1 mM EDTA, 50 mM NaF, 100 mM NaCl, 1 mM DTT, 10% glycerol (v/v) and 1% (v/v) Triton X-100). AMPK activity in the immune complex was determined by phosphorylation of the SAMS (full sequence: HMRSAMSGLHLVKRR) synthetic peptide substrate [30] during 20 min of incubation in the presence of 200 μM AMP.

2.9. Reverse transcription-PCR

Total RNA was isolated from cells with Trizol reagent (Life Technologies, Inc., Grand Island, NY, USA) as recommended by the supplier. cDNA was synthesized from 2 μg total RNA using a RNA PCR kit (Perkin-Elmer, Indianapolis, IN, USA) with the supplied random hexamers under conditions described by the manufacturer. PCRs were done using specific primers for $cFLIP_L$ and $cFLIP_S$ as described previously [31].

2.10. Statistical analysis

All data are presented as the mean ± SE of at least three independent experiments. The differences among different groups were determined by the Student's *t*-test. *P*-value <0.05 was considered significant.

3. Results

3.1. AMPK activators sensitize human breast cancer cell lines to TRAIL-induced apoptosis

To analyze whether AMPK activation sensitize breast tumor cells to the apoptotic ligand TRAIL, the breast tumor cell line MDA-MB231 was treated with different concentrations of 5-Aminoimidazole-4-carboxamide riboside (AICAR), a widely used AMPK activator, to determine the sub-toxic dose capable of sensitizing these cells to TRAIL-induced apoptosis. Results shown in Fig. 1A demonstrate that these cells were resistant to TRAIL-induced apoptosis and a marked sensitization to apoptosis by TRAIL can be observed at concentrations of 1–2 mM AICAR. To determine the AMPK-activating effect of these AICAR doses, we measured AMPK activity in cell extracts as described in Section 2. As shown in Fig. 1A in MDA-MB231 cells sensitization to TRAIL correlated well with AMPK activation by AICAR. In these cells AMPK activity was almost entirely associated with α1, with virtually no α2 activity detectable (data not shown). Similar association between AMPK activity and sensitization to TRAIL by AICAR was observed when AMPK activation was analyzed by determining the phosphorylation of AMPK and acetyl-CoA carboxylase (ACC), a well characterized substrate of AMPK. In subsequent experiments, we normally used 1 and 2 mM AICAR, because these concentrations produced a marked sensitization and AMPK activation (Fig. 1A) but were not toxic for the cells.

To further explore the sensitization to TRAIL-induced apoptosis by AMPK activators we used the small molecule A-769662, a recently identified direct activator of AMPK. As shown in Fig. 1B, A-769662 compound, as AICAR, was able to sensitize MDA-MB231 cells to TRAIL-induced apoptosis in a dose-dependent manner, with no toxic effect by itself. This sensitization correlated well with the activation state of AMPK, as measured by the *in vitro* assay and as p-ACC levels and p-AMPK by western blotting. Since A-769662 compound is considered a specific AMPK activator, this result suggested that AMPK activation could be responsible for the observed A-769662 sensitization to TRAIL-induced apoptosis in these breast cancer cells. In addition to the direct AMPK activators AICAR and A-769662, we examined if stimuli that change the intracellular AMP/ATP ratio, were also able to sensitize these cells to TRAIL-induced apoptosis. For this purpose, we tested glucose limitation in the culture medium and phenformin, a mitochondrial ATP synthase inhibitor. As shown in Fig. 1C and D, both stimuli activated AMPK and sensitized the cells to TRAIL-induced apoptosis in a dose-dependent manner.

To demonstrate that the observed sensitization to TRAIL-induced apoptosis is not restricted to MDA-MB231 cells, dose–response experiments were performed in other breast tumor cell lines to determine whether AMPK activators also facilitated apoptosis induced by TRAIL. As shown in Fig. 2, treatment of BT474 (A and B), MDA-MB-435S (C and D) and MCF7 (E) cell lines with various AMPK activators sensitized these cells to TRAIL. All these data indicated that most AMPK activators were able to sensitize human breast cancer cells to TRAIL-induced apoptosis.

To further analyze the apoptotic mechanism induced by the combination of AMPK activator and TRAIL in breast tumor cells, we examined the caspase dependency of this cell death process in MDA-MB231 cells. We found that the generation of hypodiploid cells induced by the combination of AICAR and TRAIL was dependent on caspase activation as it was completely prevented by the general caspase inhibitor Z-VAD-fmk (Fig. 3A). During TRAIL-induced apoptosis, procaspase-8 is recruited and processed at the DISC in a FADD- and TRAIL receptor–dependent manner. Procaspase-8 is first cleaved to the p43/p41 intermediate fragments and then subsequently processed to generate the large

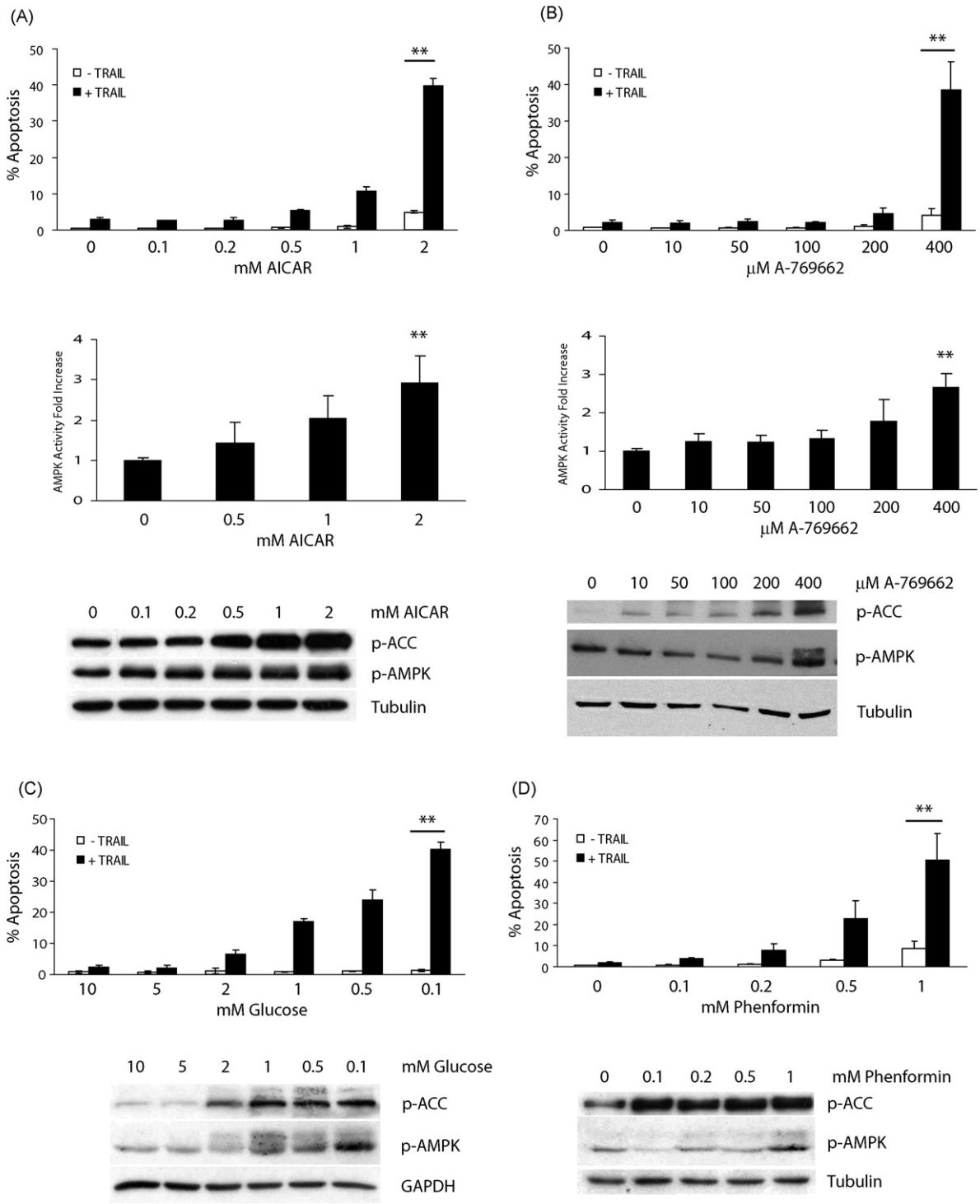

Fig. 1. AICAR, A-769662, glucose deprivation and phenformin sensitize MDA-MB231 human breast cancer cells to TRAIL-induced apoptosis. MDA-MB231 cells were incubated with the indicated concentrations of AICAR (A) or A-769662 (B) for 8 h, or glucose (C) or phenformin (D) for 16 h, before the addition of soluble recombinant TRAIL (1 μg/mL). Apoptosis was measured 16 h (A and B) or 8 h (C and D) after the addition of TRAIL as percentage of cells with subG1 DNA content as described in Section 2. AMPKα1 activity was measured at the end of the pre-treatment period as described in Section 2 and normalized to the activity detected in untreated cells. Error bars represent S.D. from three independent experiments. $^{**}P < 0.01$. p-ACC and p-AMPK levels were analyzed by immunoblotting. Tubulin was used as a protein loading control. In each case, results are representative of three independent experiments.

catalytically active p18 subunit. Accordingly, TRAIL-induced activation of caspase-8 was clearly enhanced by pre-treatment with AICAR (Fig. 3B). The same result was observed following pre-treatment of the cells with the protein synthesis inhibitor cycloheximide, used as a positive control of sensitization to TRAIL-induced apoptosis. Activation of caspase-8 leads to the processing of its substrate Bid generating a 15-kDa fragment, which translocates to mitochondria and induces the mitochondria-controlled apoptotic pathway. Thus, we examined the loss of intact Bid by western blotting in MDA-MB231 cells. Results shown in Fig. 3B indicate that the amount of intact Bid was clearly diminished in the cells treated with a combination of AICAR and TRAIL in comparison with the cultures treated only with TRAIL. Upon death receptor activation the cytoplasmic protein Bax

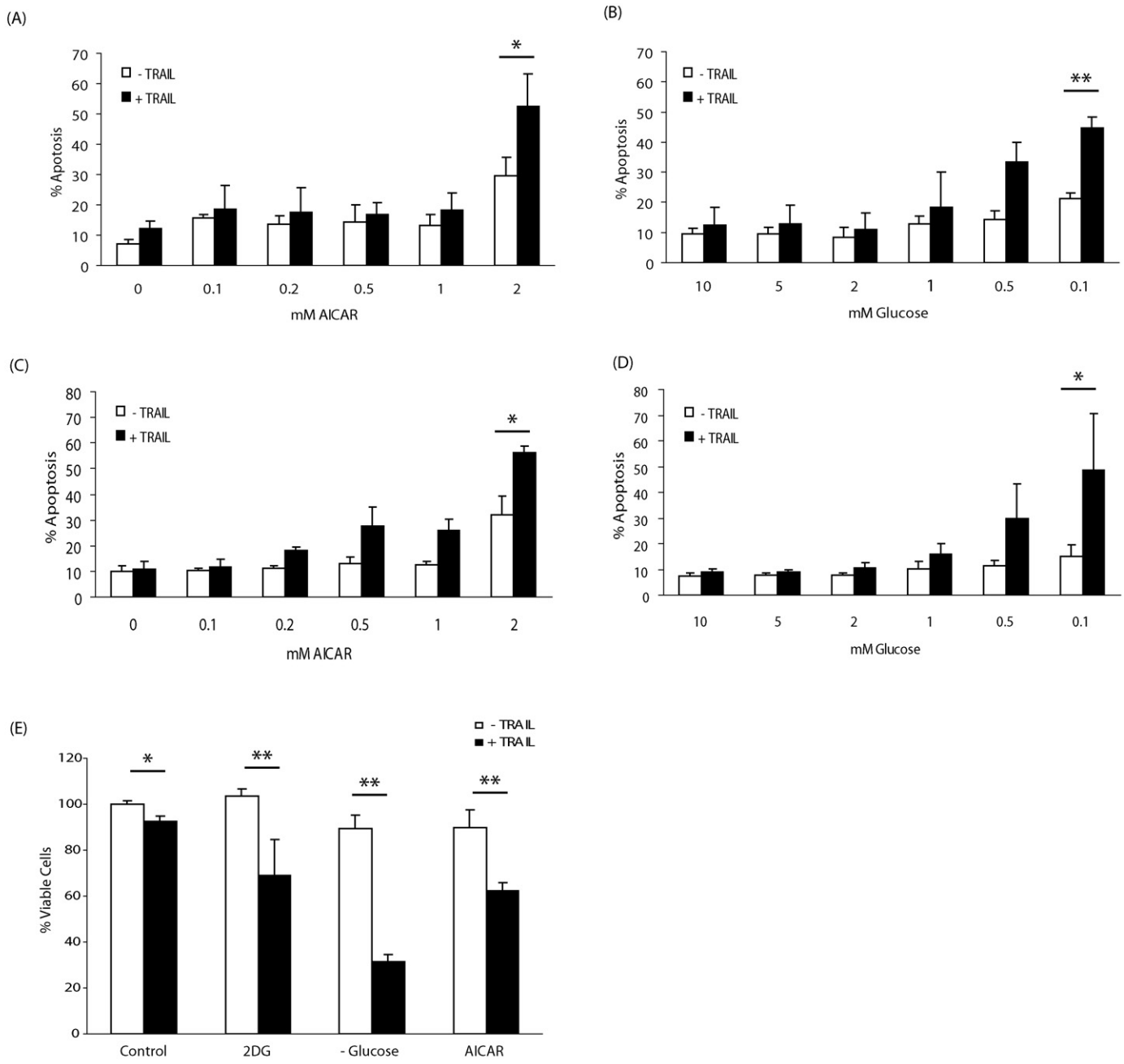

Fig. 2. AMPK activators sensitize different human breast tumor cell lines to TRAIL-induced apoptosis. BT-474 cells were incubated in medium with the indicated concentrations of AICAR (A) or glucose (B) for 20 h before the addition of soluble TRAIL (250 ng/mL). Apoptosis was measured 24 h after the addition of TRAIL as percentage of cells with subG1 DNA content as described in Section 2. Error bars represent S.D. from three independent experiments. MDA-MB435S cells were treated with the indicated concentrations of AICAR (C) or glucose (D) for 8 h before the addition of soluble TRAIL (25 ng/mL). Apoptosis was measured 16 h after the addition of TRAIL as percentage of cells with subG1 DNA content as described in Section 2. Error bars represent S.D. from three independent experiments. (E) MCF7 cells were incubated with 2-deoxyglucose (2DG) 5 mM, glucose-free medium or AICAR 1 mM with or without TRAIL (100 ng/mL). After 16 h of treatment, cell viability was measured by crystal violet staining, as described in Section 2. Error bars represent S.D. from three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$.

migrates to the mitochondria where it co-operates with truncated Bid in the release of cytochrome *c*. We next analyzed the levels of Bax and cytochrome *c* in cytosolic and mitochondrial fractions of cells treated with AICAR and TRAIL. Results shown in Fig. 3C demonstrate that the combination of AICAR and TRAIL clearly promotes Bax translocation to the mitochondria and the release of cytochrome *c* from this organelle. The activation of the mitochondrial apoptotic pathway leads to caspase-9 processing and activation in the apoptosome complex. Activated caspase-9 is able to cleave and activate caspase-3. Processing of both caspase-9 and caspase-3 were observed after treatment with AICAR and TRAIL in MDA-MB231 cells by western blotting (Fig. 3D). To confirm that the apoptosis cascade was fully active after this treatment, we analyzed the proteolytic degradation of the nuclear protein poly (ADP-ribose) polymerase (PARP), a substrate of effector caspases. As shown in Fig. 3D, PARP cleavage was clearly induced in cells pretreated with AICAR and subsequently treated with TRAIL, as well as those cells treated with cycloheximide and TRAIL. Similar results were obtained in the MCF-7 cell line (data not shown).

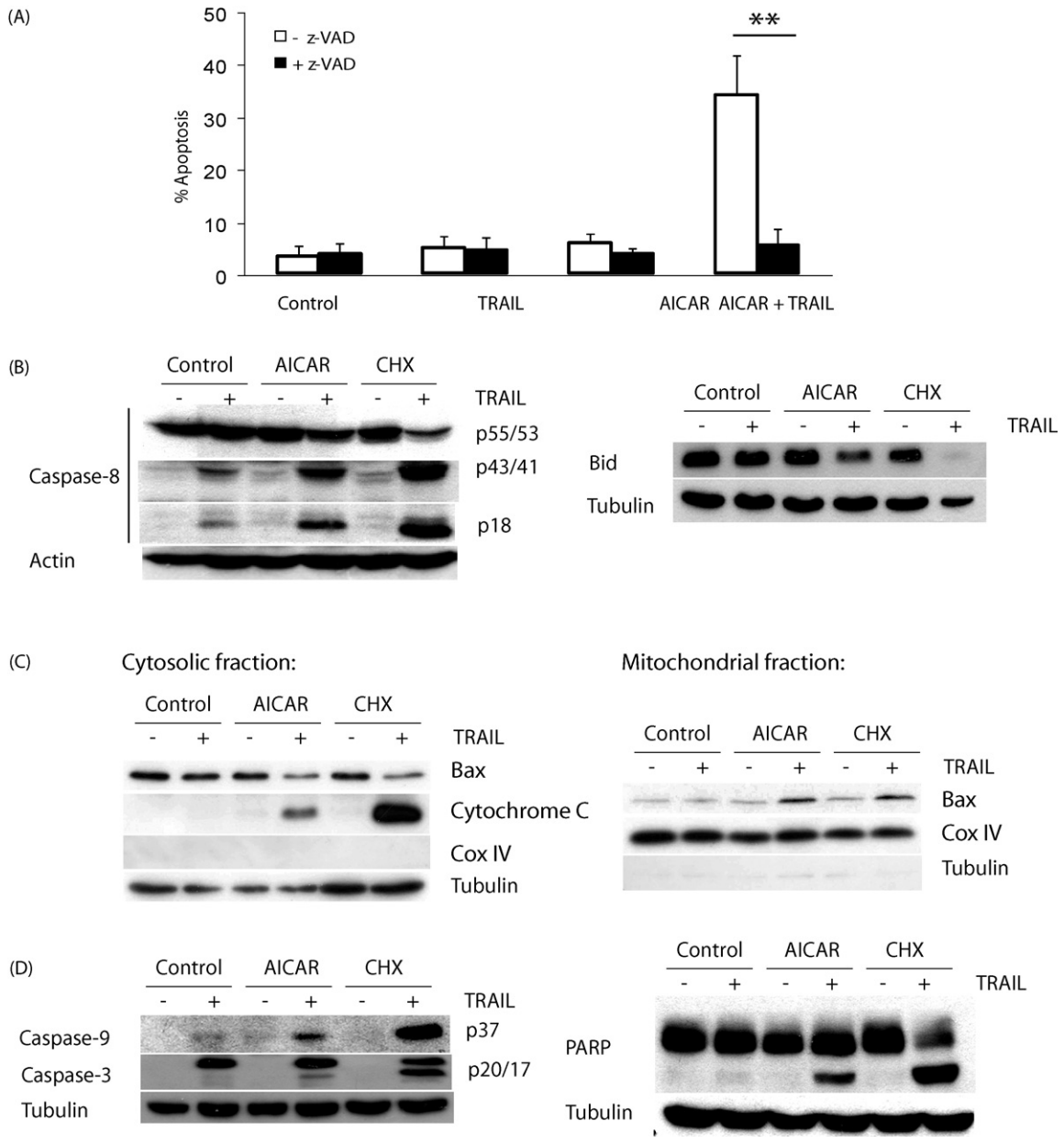

Fig. 3. Activation of a caspase-dependent mitochondria-operated apoptotic pathway by the combination of AICAR and TRAIL. (A) MDA-MB231 cells were incubated with AICAR (2 mM) for 8 h and then treated with soluble TRAIL (1 μg/mL) for 16 h; z-VAD-fmk (50 μM) was added 1 h before the addition of TRAIL. Apoptosis was measured as percentage of subG1 cells. Error bars represent S.D. from three independent experiments. $^{**}P < 0.01$. (B) MDA-MB231 cells were treated with AICAR (1 mM) for 16 h or 5 μg/mL cycloheximide (CHX) for 1 h before incubation with 1 μg/mL TRAIL for 4 h. Processing of caspase-8 and Bid were assessed by inmunoblotting as described in Section 2. Cells were treated as in (B) and levels of cytochrome *c* and bax in cytosolic and mitochondrial fractions (C) or processing of caspase-9, caspase-3 and PARP (D) were determined by Western blot as described in Section 2. Actin and Tubulin were used as protein loading controls. Results are representative of three different experiments.

3.2. AMPK activation is not required for the sensitization to TRAIL-induced apoptosis observed after treatment with AMPK activators

To investigate if the sensitization process required the activation of AMPK, we performed siRNA experiments to silence the expression of the catalytic AMPKα1 subunit in MDA-MB231 cells and analyzed the sensitivity of these cells to TRAIL under AMPK-activating conditions. As shown in Fig. 4A, the knockdown of AMPKα1 subunit did not inhibit AICAR sensitization to TRAIL-induced apoptosis. Similar results were also obtained in MCF-7 cells (data not shown). Next, we determined whether sensitization to TRAIL-induced apoptosis promoted by other AMPK activators was also observed in MDA-MB231 cells where AMPKα1 expression had been silenced by siRNA. As shown in Fig. 4B and C, silencing AMPKα1 expression in MDA-MB231 cells did not reduce apoptosis by TRAIL in cells subject to glucose deprivation or treated with phenformin. Although these results suggested that AMPK may not be required for the sensitization process, we could not exclude the possibility that the remaining AMPKα1 subunit may be sufficient to activate the signalling leading to enhanced TRAIL sensitivity in the presence of AMPK activators.

To further examine the role of AMPK in the sensitization to TRAIL induced by AMPK activators we over-expressed by adenoviral infection a Myc-tagged dominant negative isoform of AMPKα1 in MDA-MB231 cells, as described in Section 2. This isoform competes with the endogenous AMPKα1 in the formation of the trimeric active complex, rendering an inactive complex [32]. As shown in Fig. 5A and B, activation of AMPK in cells treated with

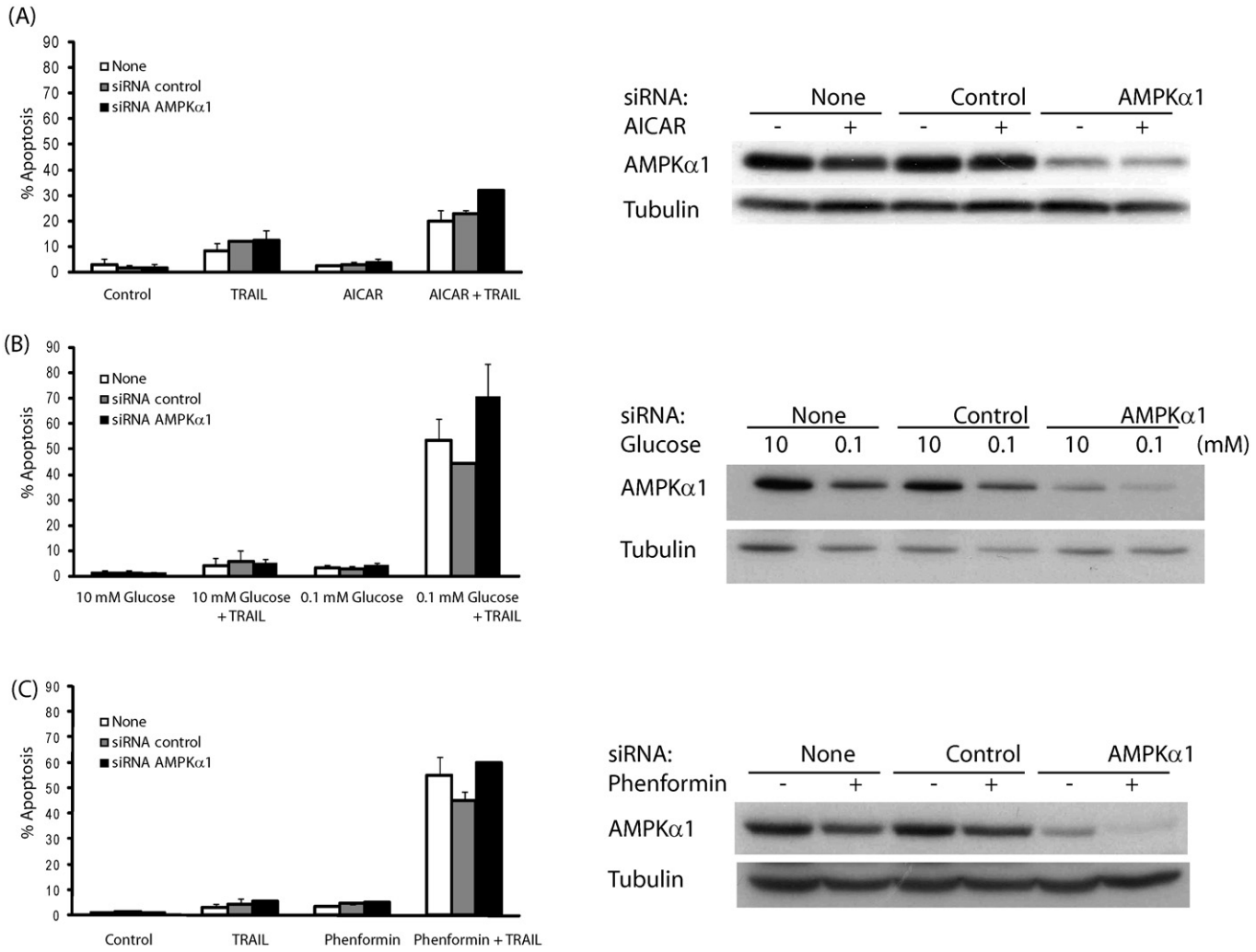

Fig. 4. Knockdown of endogenous AMPKα1 in MDA-MB231 cells does not prevent AMPK activators-induced sensitization to apoptosis by TRAIL. MDA-MB231 cells were transfected with either a siRNA oligonucleotide targeting AMPKα1 or a control siRNA oligonucleotide as described in Section 2. After 48 h cells were pre-treated with 1 mM AICAR (A), 0.1 mM glucose (B) or 1 mM phenformin (C) for 16 h before the addition of soluble recombinant TRAIL (1 μg/mL). Apoptosis was measured 4 h after the addition of TRAIL as percentage of subG1 cells. Error bars represent S.D. from three independent experiments. AMPKα1 and p-ACC levels were assessed by inmunoblotting. Tubulin was used as a protein loading control. Results are representative of two independent experiments.

A-769662, as determined either by measuring AMPK activity directly, or by monitoring ACC phosphorylation, was markedly reduced in cells expressing the dominant negative isoform of AMPKα1. Interestingly, abrogation of A-769662-induced AMPK activation in cells expressing DN-AMPKα1 was not sufficient to prevent sensitization to TRAIL-induced apoptosis in MDA-MB231 cells treated with A-769662 (Fig. 5C).

Based on these results, it seems very likely that AMPK activation is not involved in the sensitization to TRAIL-induced apoptosis observed in breast tumor cells incubated with the different AMPK activators used in our studies.

3.3. Constitutive AMPK activation is not sufficient to sensitize MDA-MB231 cells to TRAIL-induced apoptosis

To rule out AMPK activity involvement in the observed sensitization of breast tumor cells to TRAIL-induced apoptosis by AMPK activators, these cells were infected with adenovirus coding for constitutively active mutant AMPKγ1 [17] and γ2 isoforms [33] as described in Section 2. Despite their ability to activate AMPK independently of AMP (Fig. 6A and B), sensitization to TRAIL-induced apoptosis in cells infected with constitutively active isoforms of AMPKγ subunit was very small as compared to cells treated with the AMPK activator A-769662 (Fig. 6C). It is particularly relevant that although AMPK activity of cells expressing the mutant AMPKγ2RG isoform was significantly higher than the activity detected in cells incubated with the A-769662 compound, a clear sensitization to TRAIL-induced apoptosis was only observed in A-769662-treated cells. Therefore, AMPK activation is not sufficient to sensitize these cells to TRAIL-induced apoptosis.

3.4. AMPK-independent cFLIP down-regulation is required for the sensitization to TRAIL-induced apoptosis by AMPK activators in breast tumor cells

Several studies have suggested that a decrease in cFLIP, a short lived protein, sensitizes cells to death receptor-induced apoptosis. Although this is somewhat controversial [11,34], the anti-apoptotic activity of this protein is clearly supported by data obtained from cells stably over-expressing cFLIP, from mice deficient in cFLIP and from the selective knockdown of cFLIP [10,11,34]. In breast tumor cells, cFLIP appears to play an important role in preventing TRAIL-induced apoptosis [6]. Thus,

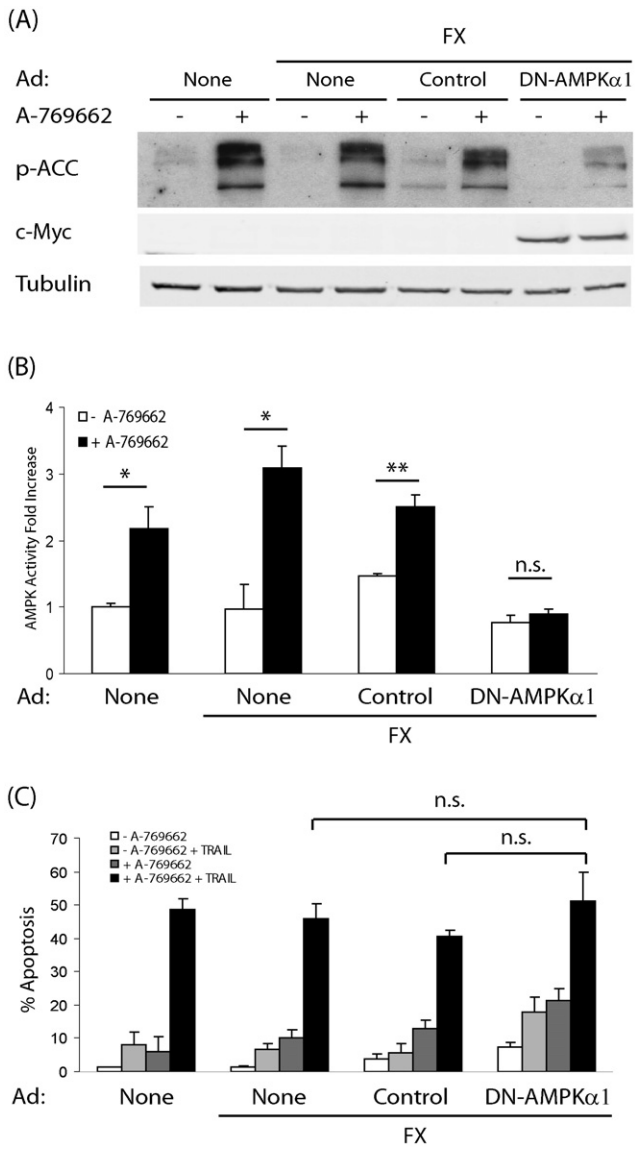

Fig. 5. Inhibition of AMPK activity by a dominant negative AMPKα1 mutant is not sufficient to abrogate A-769662-induced sensitization to TRAIL apoptosis in MDA-MB231 cells. (A) MDA-MB231 cells were infected for 24 h with 100 PFU of empty (control) or dominant negative AMPKα1 (DN-AMPKα1) adenovirus, as described in Section 2. Cells were then incubated with or without A-769662 (400 μM) for 16 h and p-ACC levels (A) or AMPKα1 activity (B) were determined as described in Section 2. Error bars represent S.D. from three independent experiments. p-ACC data are representative of three independent experiments. (C) Infected cells were treated with A-769662 as in (A) and (B) and then treated with TRAIL (1 μg/mL) for 24 h. Apoptosis was measured as percentage of subG1 cells. Error bars represent S.D. from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and n.s. = non-significant.

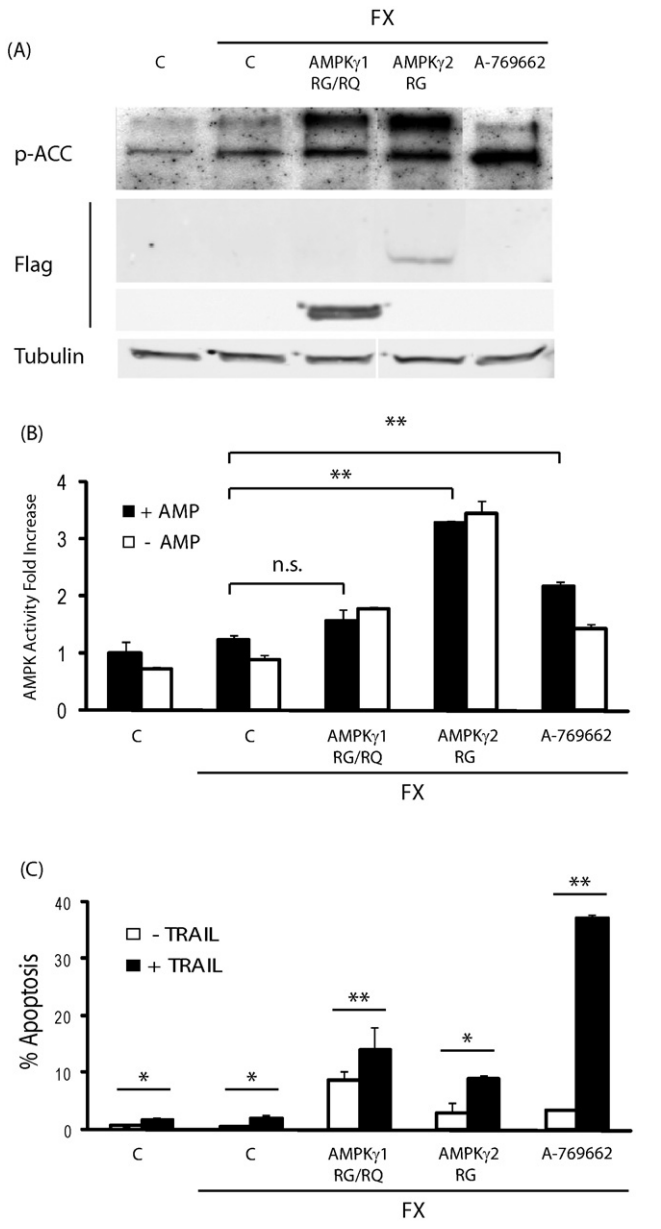

Fig. 6. Over-expression of constitutively active AMPK isoforms does not sensitize MDA-MB231 cells to TRAIL-induced apoptosis. MDA-MB231 cells were infected for 40 h with 100 PFU of control, RG/RQ-AMPKγ1, or RG-AMPKγ2 adenovirus as described in Section 2 or treated with A-769662 (400 μM) for 16 h before the addition of TRAIL (1 μg/mL). (A) p-ACC levels and constitutively active AMPK expression were assessed by immunoblotting. Tubulin was used as a protein loading control. Results are representative of three independent experiments. (B) AMPKα1 activity was measured in the absence or in the presence of AMP as described in Section 2 and normalized to the activity detected in control untreated cells. Error bars represent S.D. from three independent experiments. (C) Apoptosis was measured 24 h after the addition of TRAIL as percentage of subG1 cells. Error bars represent S.D. from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and n.s. = non-significant.

we investigated whether treatment with AMPK activators affected the levels of $cFLIP_L$ and $cFLIP_S$ proteins in MDA-MB231 cells. As shown in Fig. 7A and B the protein levels of both $cFLIP_L$ and $cFLIP_S$ decreased in these cells following exposure to AICAR, A-769662, low glucose or phenformin. Similar results were obtained in MCF-7 cells treated with AICAR or glucose deprivation (Fig. 7C). In contrast, TRAIL treatment did not induce a reduction in cFLIP levels in MDA-MB231 cells (Fig. 7A).

We next examined the role of AMPK in the observed down-regulation of cFLIP levels by AMPK activators. Silencing of AMPK expression by siRNA did not change the basal levels of $cFLIP_L$ and $cFLIP_S$ proteins in MDA-MB231 cells (Fig. 7D). Interestingly, knockdown of AMPK did not abrogate the decrease in $cFLIP_L$ and $cFLIP_S$ levels observed in cells treated with AMPK activators (Fig. 7D). These results suggest that, as is the case with sensitization to TRAIL-induced apoptosis, AMPK activation is most likely not involved in the down-regulation of cFLIP levels observed in breast tumor cells following incubation with AMPK activators. To test whether modulation of cFLIP expression by AMPK activators in MDA-MB231 cells occurred at the mRNA level, we performed RT-PCR analysis of $cFLIP_L$ and $cFLIP_S$ mRNA in cells treated with AMPK activators. Results shown in Fig. 7E indicate

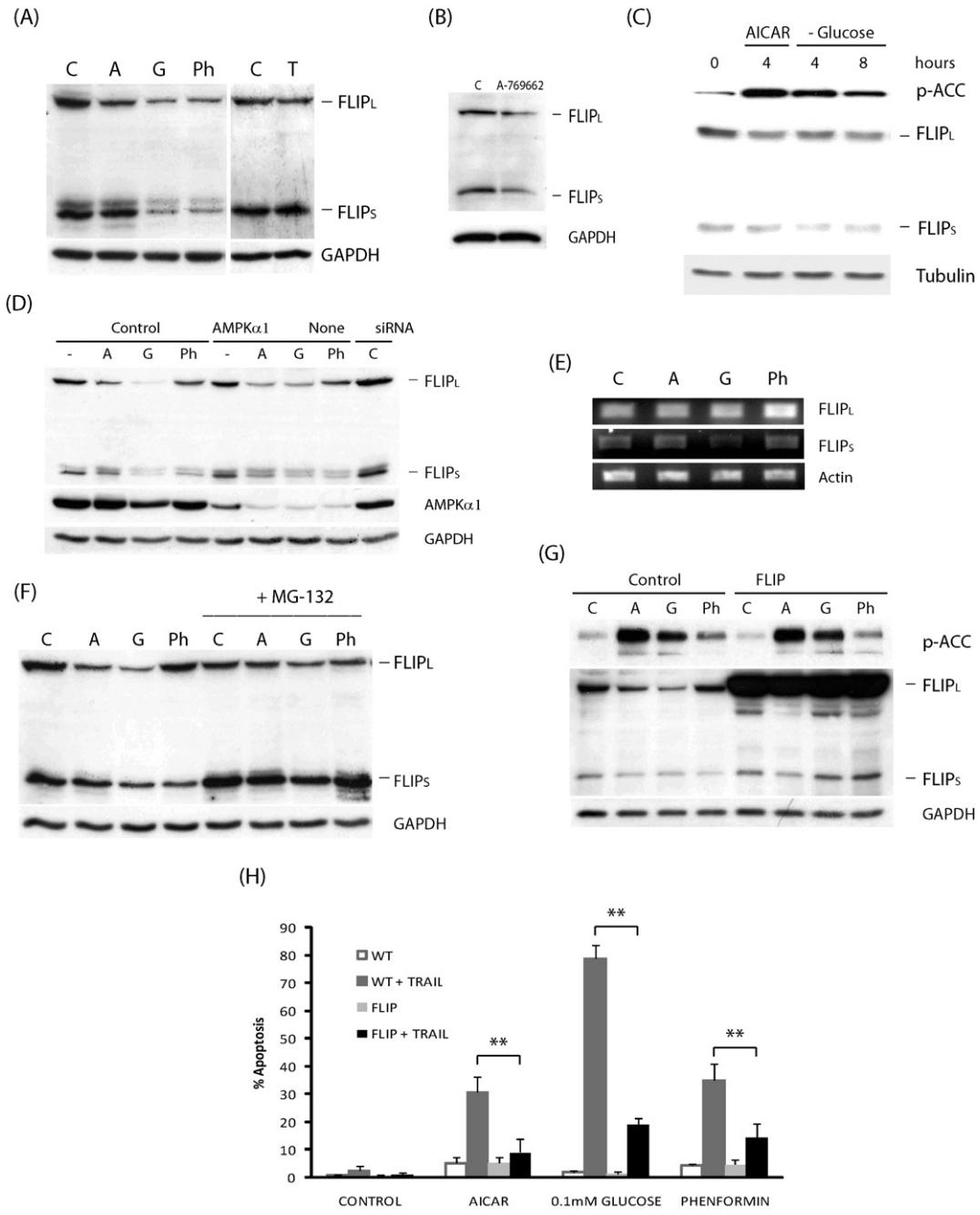

Fig. 7. Down-regulation of cFLIP levels by AMPK activators is involved in the sensitization of breast tumor cells to TRAIL-induced apoptosis. MDA-MB231 cells were incubated with AICAR (A, 2 mM), low glucose (G, 0.1 mM), phenformin (P, 1 mM) or TRAIL (T, 1 μg/mL) for 16 h (A) or incubated with A-769662 (400 μM) for 16 h (B). p-ACC and FLIP levels were analyzed by immunoblotting. Tubulin or GAPDH were used as protein loading controls. Results are representative of three independent experiments. (C) MCF-7 cells were incubated with AICAR 1 mM or glucose-free medium for the times indicated. p-ACC and cFLIP levels were analyzed by immunoblotting. Tubulin was used as protein loading control. Results are representative of three independent experiments. (D) MDA-MB231 cells were transfected either with a siRNA oligonucleotide targeting AMPKα1 or a siRNA oligonucleotide control as described in Section 2. After 48 h cells were treated with AICAR (1 mM), low glucose (0.1 mM) or phenformin (1 mM) for 16 h. cFLIP and AMPKα1 levels were assessed by immunoblotting. GAPDH was used as a protein loading control. Results are representative of two independent experiments. (E) Cells were treated with AMPK activators as described in (A) and $cFLIP_L$ and $cFLIP_S$ mRNA levels were determined by RT-PCR as described in Section 2. Results are representative of three independent experiments. (F) MDA-MB231 cells were incubated with AICAR (A, 2 mM), glucose (G, 0.1 mM), or phenformin (P, 1 mM) for 16 h with or without MG-132 (MG, 500 nM). cFLIP levels were assessed by immunoblotting. GAPDH was used as a protein loading control. Results are representative of three independent experiments. Wild type (WT) and FLIP-over-expressing (FLIP) MDA-MB231 cells were treated with AICAR (2 mM), low glucose (0.1 mM) or phenformin (1 mM) for 8 h (G and H) prior to the incubation with TRAIL (1 μg/mL) (H). FLIP and p-ACC levels (G) were assessed by immunoblotting. GAPDH was used as a protein loading control. Results are representative of three independent experiments. (H) Apoptosis was measured 16 h after the addition of TRAIL as percentage of subG1 cells. Error bars represent S.D. from three independent experiments. $^{**}P < 0.01$.

that in contrast to the observed down-regulation of cFLIP protein levels, incubation of cells in the presence of the different AMPK activators did not change significantly the levels of cFLIP mRNA. It has been previously described that cFLIP levels can be modulated through an ubiquitin-proteasome pathway [35]. To assess the role of the proteasome in the down-regulation of cFLIP protein levels upon treatment with AMPK activators cells were treated with the proteasome inhibitor MG-132 before the addition of activators. As shown in Fig. 7F, treatment with MG-132 blocked down-regulation of both cFLIP isoforms induced by AMPK activators.

To demonstrate the importance of the reduction in cFLIP levels caused by treatment with AMPK activators in the sensitization observed to TRAIL-induced apoptosis, we performed some experiments with MDA-MB231 cells over-expressing $cFLIP_L$ (Fig. 7G and H). In these cells, $cFLIP_L$ levels were maintained in cells treated with the different AMPK activators (Fig. 7G). Interestingly, as shown in Fig. 7H cells over-expressing $cFLIP_L$ were clearly more resistant to AMPK activators-induced sensitization to TRAIL apoptosis than cells expressing normal cFLIP levels. Taken together, these results indicate that a key step in the sensitization to TRAIL apoptosis by AMPK activators is the down-regulation of cellular FLIP levels.

4. Discussion

It is well established that the key function of AMPK is to regulate the energy balance within the cell [13]. Once activated, AMPK phosphorylates downstream substrates, the overall effect of which is to switch off ATP-consuming pathways and to switch on catabolic pathways that generate ATP. Activation of AMPK by energy stress also causes a metabolic checkpoint, in which cells with intact AMPK signalling undergo cell-cycle arrest, while those cells defective for AMPK activation, or key components of the AMPK pathway, continue cycling and subsequently undergo apoptosis [36]. Activation of AMPK induces p53-dependent apoptotic cell death in response to energetic stress [37]. Moreover, AMPK activation with AICAR further enhances E2F1-mediated apoptosis [38]. All these studies suggested that AMPK activation could play a pro-apoptotic role in different types of cells.

Anti-tumor therapy based on the apoptosis-inducing properties of TRAIL and agonistic TRAIL receptor antibodies is currently under consideration [39]. However, despite the fact that TRAIL induces selective cell death in human tumor cells, sparing most untransformed cells, resistance to TRAIL is not uncommon in certain tumor cell lines. Furthermore, TRAIL might also promote cell migration and invasion in some apoptosis-resistant cells [40]. Hence, sensitization of cells to TRAIL-induced apoptosis through different strategies would augment the therapeutic potential of TRAIL against its capacity to stimulate invasion, resolving the potential risk to patients with TRAIL-resistant cancers. Combination strategies have been implemented to facilitate TRAIL apoptotic signalling [41], and we have been investigating the resistance of human breast tumor cells to TRAIL and the how to overcome it. We previously reported that the formation of the death-inducing signalling complex (DISC) is a common target for different sensitizing regimes to death receptor-induced apoptosis in breast tumor cells [6,42].

Glucose deprivation, a potent AMPK stimulus, enhances death receptor-triggered formation of death-inducing signalling complex and markedly sensitizes tumor cells to death receptor-mediated apoptosis [27]. Furthermore, AICAR treatment of a colon cancer cell line has been reported to sensitize these cells to TNF-alpha or TRAIL-induced apoptosis [21]. In our study, we demonstrate that pre-treatment of human breast cancer cells with AMPK activators promotes the activation by TRAIL of a caspase-dependent mechanism of apoptosis in these cells. We show that sensitization to TRAIL-induced cell death by AMPK activators is a general phenomenon occurring in all human breast cancer cell lines examined. However, several evidences indicate that AMPK is not involved in the sensitization process. First, there is no correlation between the doses of phenformin that activate the AMPK pathway and those sensitizing the cells to TRAIL-induced apoptosis. Second, silencing AMPK expression by siRNA does not abrogate sensitization to TRAIL apoptosis by AMPK activators. Third, expression of a dominant negative AMPKα1 mutant isoform which inhibits AMPK activation does not prevent sensitization to TRAIL-induced apoptosis. Finally, over-expression of a constitutively active form of AMPK does not sensitize the cells to TRAIL. On the basis of these findings, we propose that AMPK-independent mechanism(s) must be responsible for the observed sensitization of breast tumor cells to TRAIL.

AMPK activators may have other targets than AMPK [43,44]. In this respect, a recent study has demonstrated that AICAR and phenformin cause dephosphorylation of Akt and glycogen synthase kinase-3 (GSK-3) independently of their effects on AMPK activity [45]. Moreover, metformin, an anti-diabetic drug targeting AMPK has been demonstrated to down-regulate HER2 protein levels and to inhibit the development of mammary carcinomas through an AMPK-independent mechanism [46]. Furthermore, the apoptosis-inducing effect of AICAR could be observed in the absence of concomitant activation of AMPK in some human leukemic cells [47].

Although we have not identified the molecular target(s) of AMPK-activating agents that cause sensitization to TRAIL-induced apoptosis in breast tumor cells, our data indicate that down-regulation of the apoptosis inhibitor cFLIP is a common event to all treatments. How cFLIP levels are lowered by AMPK activators in breast tumor cells is not known. Our siRNA data indicate that cFLIP down-regulation does not require AMPK. It has been reported that cellular levels of FLIP can be regulated by the PI3K/Akt/Foxo3a pathway [48]. AMPK-independent inhibition of Akt by AICAR and phenformin has been recently demonstrated [45] and glucose deprivation has been shown to down-regulate cFLIP expression through the elevation of ceramide levels and inhibition of Akt [49]. However, results from our laboratory indicate that the basal levels of cFLIP are not affected by Akt inhibition in breast tumor cells (Yerbes et al., unpublished results).

The importance of cFLIP down-regulation by AMPK activators in the sensitization process is supported by the finding that siRNA silencing of cFLIP is sufficient to sensitize breast tumor cells to TRAIL-induced apoptosis [6]. The decrease in cFLIP levels upon treatment with AMPK activators can result in an elevation of the caspase-8/cFLIP ratio in the DISC of treated cells, which should facilitate caspase-8 activation and promote apoptosis [50]. On the other hand, competition between FADD and $cFLIP_L$ for the death domain of TRAIL receptor DR5 [51] may also modulate DISC formation. The decrease in $cFLIP_L$ levels upon treatment with AMPK-activating regimes observed in our work could favour binding of FADD and procaspase-8 to the TRAIL DISC and enhance the processing and activation of caspase-8. Our results demonstrate that facilitation of TRAIL-induced apoptosis by AMPK activators is markedly inhibited in breast tumor cells over-expressing $cFLIP_L$, further supporting the role of cFLIP down-regulation in the sensitization process.

In conclusion, our results demonstrate that treatments frequently used to activate AMPK and to examine the role of this kinase in apoptosis regulation, trigger the down-regulation of an important inhibitor of death receptor-mediated apoptosis through an AMPK-independent mechanism. Consequently, to determine the importance of AMPK in the resistance of tumor cells to TRAIL and other therapeutic strategies it will be necessary to use more specific approaches such as RNA interference or the expression of dominant inhibitory forms of AMPK.

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