Mol Cell Biochem (2007) 306:239–245
DOI 10.1007/s11010-007-9575-6

AICAR induces phosphorylation of AMPK in an ATM-dependent, LKB1-independent manner

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Received: 11 May 2007 / Accepted: 10 August 2007 / Published online: 5 September 2007
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Abstract AMPK is an AMP-activated protein kinase that plays an important role in regulating cellular energy homeostasis. Metabolic stress, such as heat shock and glucose starvation, causes an energy deficiency in the cell and leads to elevated levels of intracellular AMP. This results in the phosphorylation and activation of AMPK. LKB1, a tumor suppressor, has been identified as an upstream kinase of AMPK. We found that in response to treatment with 5-aminoimidazole-4-carboxamide-1-β-4-ribofuranoside (AICAR), the LKB1 deficient cancer cell line, HeLa, exhibited AMPK-α phosphorylation. This indicates the existence of an LKB1-independent AMPK-α phosphorylation pathway. ATM is a protein that is deficient in the disease ataxia telangiectasia (A-T). We measured the activation of AMPK by AICAR in the normal mouse embryo fibroblast cell line, A29, and the mouse cell line lacking the ATM protein, A38. In A38 cells, the level of AICAR-induced AMPK-α phosphorylation was significantly lower than that found in A29 cells. Furthermore, phosphorylation of AMPK in HeLa and A29 cells was inhibited by an ATM specific inhibitor, KU-55933. Our results demonstrate that AICAR treatment could lead to phosphorylation of AMPK in an ATM-dependent and LKB1-independent manner. Thus, ATM may function as a potential AMPK kinase in response to AICAR treatment.

Keywords AMPK · ATM · AICAR · Protein phosphorylation

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Introduction

AMPK is an AMP-activated protein kinase that is an essential factor in maintaining energy homeostasis following multiple types of cellular stress including heat shock, metabolic poisoning, glucose starvation, oxygen deprivation, and disruption of blood supply [1]. AMPK is a heterotrimeric complex consisting of α, β, and γ subunits and is capable of phosphorylating various downstream targets, including acetyl-CoA carboxylase and HMG-CoA reductase [1, 2]. Through its acute phosphorylation of metabolic enzymes, as well as long-term effects on gene and protein expression, AMPK switches off ATP consumption pathways, such as fatty acid and cholesterol synthesis, and switches on ATP production pathways, such as fatty acid oxidation and glycolysis [1, 2].

AMPK can be activated through reversible phosphorylation at the Thr172 site within its α-subunit by upstream kinases [3]. For instance, AMPK is known to be phosphorylated in the presence of the adenosine analog, AICAR. AICAR is taken in by the cell and phosphorylated by the adenosine kinase to become ZMP, which then mimics the activating effect of AMP on AMPK without affecting the intracellular AMP:ATP ratio [2].

Recent studies have found that AMPK plays a role in the inhibition of cell proliferation in both tumor and non-malignant cells [4]. In particular, activation of AMPK by AICAR has been shown to cause the suppression of cell proliferation. In addition, activation of AMPK has also been shown to facilitate apoptosis of lung cancer cells, gastric cancer cells, pancreatic cancer cells, hepatic carcinoma cells, and prostate cancer cells [5]. Therefore, current studies are investigating whether AMPK can be used as a target in treating a number of cancers.

LKB1 protein kinase is a tumor suppressor that phosphorylates and activates AMPK in response to a decrease in energy stores. It can also phosphorylate multiple other kinases of the AMPK subfamily, thus functioning as a master upstream kinase [2, 6]. However, recent studies have shown that AICAR can also activate AMPK without direct activation of LKB1, indicating the existence of other upstream AMPK kinases (AMPKK) [5, 7].

One particular study showed that in LKB1 knockout and LKB1 wild-type mouse embryo fibroblast cell lines, AICAR can induce phosphorylation of AMPK and inhibit cell proliferation to a similar degree in both cells. This result leads to the conclusion that AICAR is sufficient in causing activation of AMPK, which in turn suppresses cell proliferation, regardless of the LKB1 status of the cell [5]. This finding is important for treatment of cancers that are LKB1 independent. As a tumor suppressor, if LKB1 is absent or mutated in a cell, the result is uncontrolled cell growth. This is the case in Peutz-Jeghers syndrome where LKB1 is deficient [4]. Patients with this disease have a predisposition to various types of cancer. Thus, it is thought that AICAR may be used as a potential therapeutic drug against cancers that are independent of LKB1 [5]. However, the identity of other kinases that phosphorylate AMPK is unclear. One possible AMPKK candidate is the ATM protein kinase [8].

The *ATM* gene has been identified as the gene defective in ataxia telangiectasia, an autosomal recessive disorder characterized by cerebella ataxia, oculocutaneous telangiectasia, immunodeficiency, radiation sensitivity, growth retardation, premature aging, and cancer predisposition [9]. The ATM protein kinase is a member of the phosphatidylinositol 3-kinase (PI 3-Kinase) superfamily and controls cell cycle progression by phosphorylating and activating p53 [10, 11], Brca1 [12, 13], Chk2 [14], p95/nbs1 [15], and Smc1 [16] in response to ionizing radiation (IR) and DNA double strand breaks. Therefore, similar to LKB1, ATM also functions as a tumor suppressor.

There is evidence suggesting that in response to insulin-like growth factor-1 (IGF-1), ATM directly phosphorylates the AMPK-α subunit in vitro, independent of LKB1 [8]. In this study, we examined the effect of ATM on AICAR-mediated phosphorylation of AMPK. We conducted these experiments in two isogenic mouse embryonic fibroblast cell lines, A38 ($ATM^{-/-}$) and A29 ($ATM^{+/+}$), as well as a human cell line, HeLa, which lacks the expression of LKB1. Our results demonstrate that AICAR treatment can induce phosphorylation of AMPK in an ATM-dependent and LKB1-independent manner.

Materials and methods

Reagents

Genistein was purchased from Calbiochem. LY294002 and AICAR were purchased from Sigma-Aldrich. The ATM inhibitor, 2-morpholin-4-yl-6-thianthren-1-yl-pyran-4-one (KU-55933) was provided by Dr. Graeme Smith [17]. Rabbit polyclonal antibodies against total- and phospho-AMPK-α (Thr172) were purchased from Cell Signaling Technology. The LKB1 antibody was purchased from Santa Cruz Biotechnology. Antibodies against β-actin and β-tubulin were from Sigma. The anti-ATM monoclonal antibody, MAT3, was a generous gift from Dr. Yossi Shiloh [18].

Cell lines and cultures

A29 is a normal mouse embryonic fibroblast cell line, while A38 is an ATM-deficient mouse embryonic fibroblast cell line [19]. HeLa is a human cervical carcinoma cell line. All three cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics.

Treatment and lysis of the cell

After treating the cells, cellular extracts were prepared by lysing the cells for 1 h on ice with TGN lysis buffer containing 50 mM HEPES pH 7.4, 0.5% NP-40, 1.0% Tween 20, 150 mM NaCl, 1.0 mM PMSF, 1.0 mM NaF, 1.0 mM Na_3VO_4, and a protease inhibitor cocktail tablet (Roche). The supernatant was then collected by centrifugation at 14,000 rpm for 10 min, and the protein concentration was determined using a Bio-Rad D_C protein assay kit, with bovine serum albumin as the standard.

SDS-PAGE and immunoblotting

Protein samples were separated by SDS-PAGE and then transferred to a PVDF membrane using a Western blotting system. The membrane was blocked at room temperature for 2 h and then incubated overnight with a primary antibody. After washing with wash buffer, the membrane was incubated with a secondary antibody conjugated with horseradish peroxidase. The membrane was then washed again, and protein bands were visualized with the enhanced chemiluminescence system (Pierce).

Results

ATM mediates phosphorylation of the AMPK-α subunit in response to AICAR

ATM has been shown to be able to phosphorylate AMPK in response to IGF-1 [8]. To study the functional link between ATM and AMPK phosphorylation in response to AICAR, we investigated the role of ATM in the AICAR-mediated phosphorylation of the AMPK-α subunit in both normal mouse embryonic fibroblast A29 and ATM-deficient mouse embryonic fibroblast A38 cells. Although immunoblotting results revealed that AICAR induced phosphorylation of the AMPK-α subunit in both cell lines, A38 ($ATM^{-/-}$) cells exhibited a significantly lower level of AMPK phosphorylation (70% reduction) than did the A29 ($ATM^{+/+}$) cells (Fig. 1A). Interestingly, we also observed a slight up-shift of the AMPK protein in normal cells but not in the A-T cells. The results suggest the involvement of ATM in the phosphorylation of the AMPK-α subunit during AICAR treatment in mouse cells.

In order to elucidate why AMPK was phosphorylated, although at a significantly reduced level, following AICAR treatment in the ATM deficient A38 cells, we examined the expression of LKB1, a well-documented upstream kinase of AMPK [1, 2], in A29 and A38 cells. As seen in Fig. 1B, LKB1 was equally expressed in both A29 and A38 cell lines. Taken together, these results indicate that while the AICAR-induced AMPK phosphorylation in A38 cells may be caused by the LKB1 protein expressed in the cell, ATM, in addition to LKB1, also participates in the phosphorylation of AMPK-α subunit in response to AICAR treatment in A29 cells.

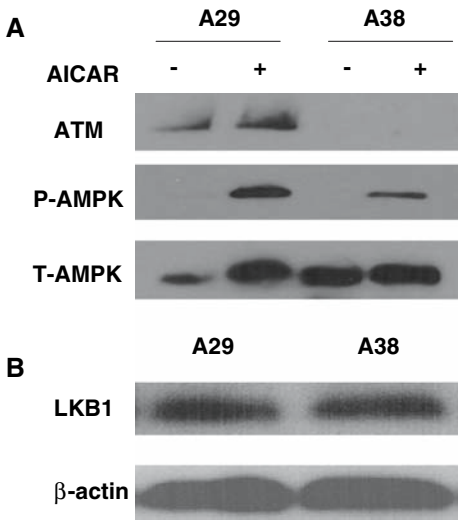

Fig. 1 Phosphorylation of AMPK-α subunit in normal (A29) and A-T (A38) mouse cells exposed to AICAR. (**A**) Subconfluent A29 or A38 cells were serum-starved overnight. The cells were then treated with AICAR (1.0 mM) for 1 h. Cells were then lysed, and equal amounts of protein in the cell lysate were subjected to SDS-PAGE as described in the Materials and Methods. Immunoblotting was then performed using antibodies against phosphorylated (P-AMPK, Thr172) or total AMPK-α subunit (T-AMPK), as well as an anti-ATM monoclonal antibody MAT3. (**B**) Subconfluent A29 or A38 cells were lysed and subjected to SDS-PAGE as described above. Immunoblotting was then preformed using antibodies against LKB1 or *β*-actin. The results in both A and B are representative of three individual experiments

Tyrosine phosphorylation within the ATM molecule is involved in the AMPK-α subunit

When activated, ATM is known to be phosphorylated at both threonine/serine and tyrosine residues [8, 20]. To examine how ATM is activated and mediates the phosphorylation of the AMPK-α subunit at the Thr172 site in response to AICAR treatment, we used two protein kinase inhibitors, LY294002 and genistein. LY294002 is a PI 3-kinase inhibitor and specifically suppresses threonine/serine phosphorylation of the downstream substrates of the PI 3-kinase while genistein is an inhibitor of tyrosine kinase and only inhibits tyrosine phosphorylation.

Our results showed that the inhibitory effect of genistein on AMPK-α phosphorylation in A29 cells increased in a dose-dependent manner. The maximum inhibition of AMPK phosphorylation was achieved in the presence of 100 μM of genistein (Fig. 2A). Densitometry results revealed a nearly 90% decrease of the AICAR-induced phospho-AMPK signal after correcting for basal-level AMPK phosphorylation. However, treatment with LY294002 had no effect on the AICAR-promoted AMPK-α subunit phosphorylation (Fig. 2B), which shows that PI-3 kinase is not involved in the phosphorylation of the AMPK-α subunit during AICAR treatment. These results suggest the involvement of a tyrosine kinase, possibly upstream of the ATM molecule, in AICAR-stimulated AMPK phosphorylation (also see discussion).

AMPK phosphorylation in A29 cells is inhibited by a specific inhibitor of ATM

In order to further examine the role of ATM in AICAR-induced AMPK phosphorylation in A29 cells, we treated the cells with a specific ATM inhibitor, KU-55933 [17, 21]. KU-55933 has selectivity for ATM that is at least 100-fold greater than that for other related kinases, including the PI 3-kinase. It was found that at a concentration of 10 μM, KU-55933 does not inhibit kinases other than ATM [17].

After treatment with KU-55933, the AICAR-induced AMPK phosphorylation was almost reduced to the basal-level of AMPK phosphorylation seen in untreated control

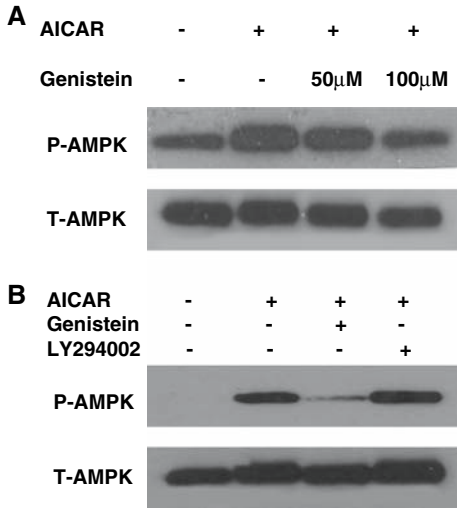

Fig. 2 AICAR mediated phosphorylation of AMPK-α subunit is inhibited by genistein but not LY294002. (**A**) Subconfluent A29 cells were serum-starved overnight. The cells were then pre-treated with genistein at different concentrations (50 μM or 100 μM) for 1 h and then incubated with AICAR (1.0 mM) for an additional hour. Cell lysis, SDS-PAGE, and immunoblotting were performed as described in Fig. 1. (**B**) Subconfluent A29 cells were serum-starved overnight. The cells were pre-treated with genistein (100 μM) or LY294002 (20 μM) for 1 h and then AICAR (1.0 mM) was added for another hour. Cell lysis, SDS-PAGE, and immunoblotting were then performed as described in Fig. 1. The results presented in both A and B are representative of three individual experiments

cells (Fig. 3). Since ATM is present in this cell line, these results further indicate that ATM mediates the phosphorylation of the AMPK-α subunit induced by AICAR in A29 cells.

AMPK phosphorylation in HeLa cells is LKB1-independent but ATM-dependent

HeLa cells lack the *LKB1* gene and do not express LKB1 mRNA and protein [8]. We therefore investigated whether AMPK can be phosphorylated in response to AICAR in HeLa cells. Immunoblotting results illustrate that AICAR treatment induced the phosphorylation of the AMPK-α subunit at the Thr172 site in the HeLa cell line (Fig. 4). This result is consistent with previous reports and confirms that the activation of AMPK by AICAR could be independent of LKB1 [7, 8].

We then used the specific ATM inhibitor, KU-55933, to examine whether or not the AICAR-promoted AMPK phosphorylation in HeLa cells is mediated through ATM. As seen in Fig. 4, AICAR-induced AMPK-α subunit phosphorylation was inhibited by both KU-55933 and genistein. In contrast, treatment with the PI 3-kinase inhibitor, LY294002, did not inhibit AICAR-induced AMPK phosphorylation. These data, in combination with

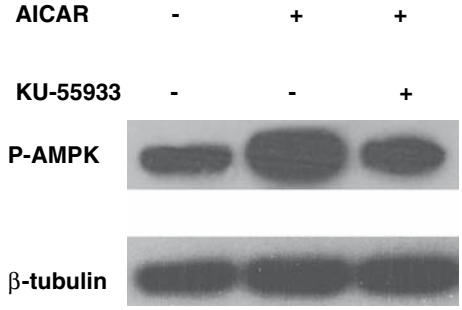

Fig. 3 AICAR-induced AMPK phosphorylation in A29 cells is inhibited by the specific ATM inhibitor KU-55933. Subconfluent A29 cells were serum-starved overnight and then pre-treated with KU-55933 (10 μM) for 1 h. Following treatment with KU-55933, cells were incubated with AICAR (1.0 mM) for an additional hour. Cell lysis and SDS-PAGE were then performed as described in Fig. 1. Immunoblotting was conducted using antibodies against P-AMPK at Thr172 or *β*-tubulin. The results presented are representative of three individual experiments

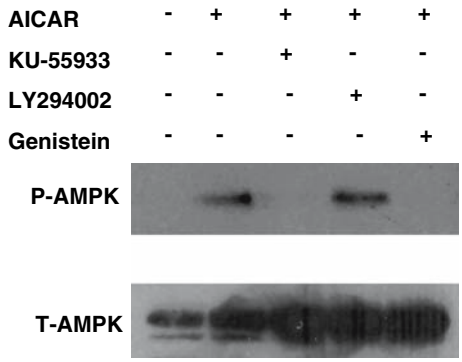

Fig. 4 AICAR-induced AMPK phosphorylation is LKB1-independent and ATM-dependent in HeLa cells. HeLa cells were pre-treated with KU-55933 (10 μM), LY294002 (20 μM), or genistein (100 μM) for 1 h and then incubated with AICAR (1.0 mM) for an additional hour. Cell lysis, SDS-PAGE, and immunoblotting were performed as described in Fig. 1. The results are representative of three individual experiments

the above results, strongly indicate that ATM participates in the phosphorylation of the AMPK-α subunit promoted by AICAR.

Discussion

In the present study, we showed that the phosphorylation of the AMPK-α subunit in response to AICAR treatment can be mediated in an ATM-dependent manner. This was demonstrated by the fact that although the LKB1 protein kinase is equally expressed in both A29 ($ATM^{+/+}$) and A38 ($ATM^{-/-}$) cells, phosphorylation of the AMPK-α subunit was observed to a much lesser degree in the ATM deficient A38 cells. These findings indicate the existence of an

ATM-dependent pathway in the AICAR-promoted phosphorylation of AMPK. Subsequently, the observed phosphorylation of the AMPK-α subunit at the Thr172 site in the LKB1-deficient HeLa cells demonstrates the presence of an LKB1-independent pathway for AMPK-α subunit phosphorylation in response to AICAR. The application of the specific ATM inhibitor, KU-55933, markedly inhibited the phosphorylation of AMPK induced by AICAR in both HeLa and A29 cells, further suggesting that ATM can function as an upstream AMPK kinase independent of LKB1.

As stated earlier, in response to IGF-1, ATM directly phosphorylates the AMPK-α subunit in vitro independent of LKB1 [8]. The findings from this study also indicate that ATM may be a candidate for a novel AMPK kinase in response to AICAR treatment. On the other hand, it should be noted that other studies have reported that IR induces the phosphorylation of LKB1 by ATM in vitro [22, 23], showing the involvement of ATM in regulating LKB1 activity following DNA damage. Although the functional link between ATM and LKB1 needs to be further confirmed in vivo, we cannot exclude the possibility that ATM may regulate AMPK phosphorylation indirectly by phosphorylating LKB1 or other potential upstream kinases of AMPK, such as CAMKK [24] or TAK1 [25].

Tyrosine kinase is usually considered as part of the receptor of various growth factors including insulin and the IGF family [26]. We found that the tyrosine kinase inhibitor, genistein, blocked AMPK phosphorylation in response to AICAR treatment in A29 cells, but the PI 3-kinase inhibitor, LY294002, did not. Moreover, genistein also specifically inhibited AMPK phosphorylation following AICAR treatment in HeLa cells, which verifies that tyrosine phosphorylation of the ATM molecule is involved in the activation of the AMPK-α subunit. These results suggest that a tyrosine kinase is involved in the AICAR-promoted AMPK-α subunit phosphorylation and are consistent with the previous finding that a tyrosine kinase upstream of the ATM molecule may be required for ATM-dependent AMPK phosphorylation in response to IGF-1 stimulation [8].

It is not clear if any of the known upstream kinases of ATM can phosphorylate its tyrosine residues in response to AICAR stimulation. A recently identified AMPK family member, ARK5, phosphorylates ATM when activated by Akt in the event of nutrient starvation [20]. However, it is not likely that ARK5 acts as an upstream kinase of ATM in response to AICAR since it was shown that the phosphorylation of ATM at a threonine residue, which should be inhibited by LY-294002, is caused by the Akt/ARK5 pathway [20]. One study reported that the tyrosine kinase c-Src is associated with the phosphorylation of AMPK in bovine cells under hypoxia reoxygeneration [27]. More studies are needed to identify whether c-Src or other tyrosine kinases are potential upstream kinases of ATM in response to AICAR treatment.

The mechanism behind AMPK's ability to inhibit cellular proliferation is also controversial [4]. One possible explanation is that phosphorylated AMPK may inhibit mTOR, a key regulator of protein translation [28]. The mTOR pathway is normally activated by growth factors and functions to stimulate protein synthesis as well as cell growth and proliferation. It was found that when AMPK is activated, it phosphorylates TSC2 which in turn increases the activity of the TSC1-TSC2 complex. The TSC1-TSC2 complex is a tumor suppressor that when active inhibits mTOR and causes inhibition of protein synthesis and cell growth [4]. S6 is a p70S6 kinase that is a direct downstream target of mTOR. We tested the effect of AICAR treatment on the phosphorylation of S6 in HeLa cells. While AICAR dramatically increased the phosphorylation of AMPK, we did not observe any inhibition of S6 phosphorylation following AICAR treatment (data not shown), suggesting that AICAR-induced phosphorylation of AMPK may not affect the function of mTOR in HeLa cells.

Our findings place ATM in the AICAR-AMPK signaling pathway, suggesting that the absence or inactivation of ATM will cause a defect in the pathway as well as subsequent diseases. For instance, similar to LKB1 deficient patients, A-T patients are predisposed to cancer because of the lack of ATM. Although this can be readily explained by nuclear ATM's role as a central controller of cell cycle progression in response to DNA damage, findings from this study may provide new insights into the tumor-suppressor function of ATM.

In addition to AMPK's function in suppressing cancer cell growth, AMPK in skeletal muscle is phosphorylated in response to muscle contraction [29]. Previous studies demonstrate that AICAR-promoted AMPK phosphorylation, similar to the acute effect of exercise, also enhances insulin sensitivity and subsequently increases insulin-stimulated glucose transport in differentiated muscle cells [30, 31]. The effects of AMPK are not limited to skeletal muscles, as AICAR-mediated phosphorylation of AMPK also enhances insulin action in the liver of insulin resistant rats [32] and possibly controls hepatic glucose metabolism [33]. Interestingly, A-T patients also have a high incidence of type 2 diabetes. A-T patients who develop this disease show typical symptoms of insulin resistance and glucose intolerance [19]. The potential connection between ATM-mediated AMPK phosphorylation and insulin resistance symptoms observed in A-T requires further exploration.

In summary, our research has revealed novel information regarding the pathways through which the phosphorylation of AMPK is regulated in response to AICAR treatment. In addition to demonstrating ATM's

potential function as an AMPK kinase in response to AICAR treatment, findings from this study may also provide new insights into ATM's role in many of the symptoms observed in patients with A-T.

Acknowledgments We would like to thank Dr. Graeme Smith for providing us with the ATM inhibitor, KU-55933. This work was supported by The South Dakota Biomedical Research Infrastructure Network (BRIN) Program of the National Center for Research Resources of NIH (Grant Number: 2-P20-RR016479).

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