Characterization of the role of the AMP-activated protein kinase in the stimulation of glucose transport in skeletal muscle cells

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Stimulation of AMP-activated protein kinase (AMPK) in skeletal muscle has been correlated with an increase in glucose transport. Here, we demonstrate that adenoviral-mediated expression of a constitutively active mutant of AMPK α leads to activation of glucose transport in a skeletal-muscle cell line, similar to that seen following treatment with 5-amino-imidazolecarboxamide (AICA) riboside, hyperosmotic stress or insulin. In contrast, expression of a dominant-negative form of AMPK blocked the stimulation of glucose transport by both AICA riboside and hyperosmotic stress, but was without effect on either insulin or phorbol-ester-stimulated transport. These results demonstrate that activation of AMPK is sufficient for stimulation of glucose uptake into muscle cells, and is a necessary component of the AICA riboside- and hyperosmotic-stress-induced pathway leading to increased glucose uptake. On the other hand, AMPK is

not required in the insulin- or phorbol-ester-mediated pathways. Long-term (5 days) expression of the constitutively active AMPK mutant increased protein expression of GLUT1, GLUT4 and hexokinase II, consistent with previous reports on the chronic treatment of rats with AICA riboside. Expression of constitutively active AMPK had no detectable effect on p38 mitogen-activated protein kinase levels, although interestingly the level of protein kinase B was decreased. These results demonstrate that long-term activation of AMPK is sufficient to cause increased expression of specific proteins in muscle. Our results add further support to the hypothesis that long-term activation of AMPK is involved in the adaptive response of muscle to exercise training.

Key words: adenovirus, cellular stress, metabolic regulation, protein kinase cascade.

INTRODUCTION

AMP-activated protein kinase (AMPK) is a key regulatory enzyme in the control of cell-energy homeostasis [1–3]. It is activated by an increase in the AMP:ATP ratio following stresses that deplete ATP [1,2]. Activation is caused, in part, by the direct allosteric stimulation of AMPK through the binding of AMP and through the relieving of inhibition by ATP [4]. In addition, AMPK is activated by phosphorylation by an upstream kinase (AMPK kinase; 'AMPKK'), which is itself activated by AMP [5,6]. In certain tissues, such as skeletal muscle, AMPK also appears to be regulated by an increase in the creatine:phosphocreatine ratio [7], again reflecting the energy status of the cell. Once activated, AMPK responds to the decrease in cellular energy level by switching on energy-producing processes, while switching off energy-utilizing processes [2]. Stimulation of AMPK by a number of different mechanisms has been reported to result in an increase in glucose uptake into skeletal and cardiac muscle, as well as in cell culture [8–11]. The mechanism of this process appears to be distinct from insulin stimulation of transport, being both partially additive with insulin and wortmannin-insensitive [8,11].

AMPK can be activated pharmacologically by incubation with 5-amino-4-imidazolecarboxamide (AICA) riboside. This compound is taken up by cells and phosphorylated by adenosine kinase to the monophosphorylated form, ZMP [12]. ZMP acts as an AMP-mimetic, activating AMPK both allosterically and by promoting phosphorylation by the upstream kinase [12–14].

However, the efficacy and specificity of AICA riboside is dependent on its uptake by the cell and on the accumulation of ZMP [15]. In addition, ZMP has effects on other AMP-binding proteins [16], and the potential effect of accumulation of other metabolites derived from AICA riboside, e.g. ZDP and ZTP, on cellular metabolism is not fully understood [3]. Stresses that deplete ATP, e.g. hypoxia, metabolic poisons and mitochondrial uncouplers, have also been used to activate AMPK [9,11]. However, by their nature the effects of these stresses on the cell are severe, and are likely to have a number of other effects on different signalling pathways. Hyperosmotic stress has also been demonstrated to increase AMPK activity [9,11]; however, it increases the activity of a large number of other kinases within the cell, amongst them p38 mitogen-activated protein kinase (MAPK), stress-activated protein kinases ('SAPKs'), tyrosine kinases and phosphoinositide 3-kinase (PI-3K) [17–20].

To date there have been no reports of specific inhibitors for AMPK. A number of studies have used relatively non-specific methods for inhibiting AMPK, e.g. iodo-tubercidin, but the results of these experiments are obviously difficult to interpret. Because of the lack of specific inhibitors, and since the methods employed for activating AMPK are themselves not entirely specific, defining the physiologically relevant targets for AMPK has remained ambiguous. Recently, the role of AMPK in the stimulation of glucose uptake in skeletal muscle was addressed by studying the effect of expression of a dominant-negative inhibitor of the kinase in mouse muscle [21]. Expression of the mutant form of the kinase completely blocked the activation of

Abbreviations used: AICA, 5-amino-4-imidazolecarboxamide; AMPK, AMP-activated protein kinase; α CA, constitutively active mutant of AMPK α ; CCD, charge-coupled-device; α DN, dominant-negative mutant of AMPK α ; Bio-LC-ATB-BGPA, 4,4'-O-(2-(2-(2-(2-[6-(biotinylamino)hexanoyl]-amino)ethoxy)ethoxy)ethoxy)-4-(1-azido-2,2,2-trifluoroethyl)benzoyl)amino-1,3-propanediyl bis-D-glucose; MAPK, mitogen-activated protein kinase; PI-3K, phosphoinositide 3-kinase; p.f.u., plaque-forming units.

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glucose uptake in muscle by hypoxia or AICA riboside, while only partially reducing contraction-induced uptake. These results demonstrate that AMPK is involved in contraction-stimulated glucose uptake, but that other AMPK-independent pathways also have a role in the response.

In the present study, we have used adenovirus-mediated expression of a constitutively active mutant form (α CA) and a dominant-negative mutant form (α DN) of the catalytic (α) subunit of AMPK to study its role in the stimulation of glucose transport in muscle cells. Our results indicate that AMPK activity is sufficient for increasing glucose transport into skeletal-muscle cells, and is required for the stimulation of transport in response to AICA riboside and hyperosmotic stress. Furthermore, expression of the constitutively active AMPK construct leads to changes in protein expression that mirror some of the changes seen in muscle following exercise training.

EXPERIMENTAL

Materials

Cell-culture reagents were purchased from Life Technologies (Paisley, Renfrewshire, Scotland, U.K.). 2-Deoxy-D-[1-³H]-glucose was obtained from Amersham Biosciences (Little Chalfont, Bucks., U.K.) and [γ -³²P]ATP from NEN Life Sciences (Boston, MA, U.S.A.). Antibodies raised against protein kinase B, p38 MAPK and hexokinase II were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.), whereas those against creatine kinase were purchased from Biogenesis (Poole, Dorset, U.K.). An anti-Myc monoclonal antibody (clone 9E10) was used to detect expressed recombinant protein. Enhanced chemiluminescence reagent was obtained from Perbio Science UK Ltd (Tattenhall, Cheshire, U.K.).

Adenovirus constructs

Recombinant adenovirus constructs were prepared as described previously [22].

Cell culture and adenovirus infection

H-2K^b cells derived from heterozygous H-2K^b tsA58 transgenic mouse muscle were cultured at 33 °C under permissive conditions, as described previously [11]. For differentiation, the cells were densely plated (1×10^4 cells/cm²) and switched to non-permissive conditions (37 °C in the absence of interferon- γ). In experiments using adenovirus expression, cells were infected with 100 plaque-forming units (p.f.u.) adenovirus per cell following 2 days of growth in non-permissive conditions. After 4 h, the media was changed and cells were allowed to incubate for a further 5 days before treatments and harvesting. At 18 h before treatment, cells were transferred to media containing 0.5% fetal-calf serum and 1 mg/ml glucose. Treatment of the cells was performed in HEPES-buffered saline [20 mM HEPES/140 mM NaCl/5 mM KCl/2.5 mM MgSO₄/1 mM CaCl₂ (pH 7.4)] containing 5 mM glucose.

AMPK activity in H-2K^b cells

Following treatment as described above, H-2K^b cells were lysed in a minimal volume of buffer A [50 mM Tris/HCl (pH 7.5)/1 mM EDTA/1 mM dithiothreitol/10% (v/v) glycerol/50 mM sodium fluoride/5 mM sodium pyrophosphate/1 mM benzamide/0.1 mM PMSF] containing 1% (v/v) Triton X-

100. Cell debris was removed by brief centrifugation (13000 *g*, 5 min). Endogenous AMPK was immunoprecipitated from cell lysates (100 μ g of protein) using a pan- β antibody [23], which cross-reacts with both β subunits, pre-bound to Protein A-Sepharose. Expressed recombinant proteins were immunoprecipitated using an anti-Myc (clone 9E10) antibody bound to Protein G-Sepharose. AMPK activity in the resulting immune complexes was measured by the incorporation of ³²P_i from [γ -³²P]ATP into the SAMS peptide substrate (a synthetic peptide of amino acid sequence HMRSAMSGHLVKRR) [24]. Under these conditions the amount of AMPK immunoprecipitated from each of the cell preparations is equivalent, as judged by Western blotting (results not shown). The activity measured within the immune complexes is therefore a reflection of the specific radioactivity of the kinase, rather than due to any change in enzyme content. In some cases, total AMPK activity present in the cell lysates was measured in a resuspended 0–12% (v/v) poly(ethylene glycol 6000) precipitate.

2-Deoxyglucose uptake in H-2K^b cells

Glucose uptake into H-2K^b cells was measured using radio-labelled 2-deoxy-D-[1-³H]glucose, as described previously [11].

Photolabelling of glucose transporters

H-2K^b cells (4 cm $\times$ 10 cm-diameter dishes) were transferred to differentiation medium and infected on day 4 with or without 50 p.f.u. of α CA. After 2 days, the cells were photolabelled with 500 μ M 4,4'-O-(2-(2-(2-[6-(biotinylamino)hexanoyl]amino)-ethoxy)ethoxy)ethoxy)-4-(1-azi-2,2,2,-trifluoroethyl)benzoyl-amino-1,3-propanediyl bis-D-glucose (Bio-LC-ATB-BGPA) ([25]; a gift from Professor G. D. Holman, University of Bath) by irradiation in open dishes twice for 1 min periods using a Rayonet RPR 100 photochemical reactor (RPR-3000 lamps) at 22 °C. After labelling, the cells were washed three times in ice-cold buffer A, lysed in 0.2 ml of buffer A containing 1% (w/v) Triton X-100, 2 μ M pepstatin A, 10 μ M leupeptin and 0.1 mM PMSF, and then centrifuged for 30 min at 100000 *g*. Biotinylated proteins present in the supernatant were precipitated by incubation with 75 μ l of a 50% (w/v) slurry of streptavidin-agarose overnight at 4 °C. The precipitates were washed three times in 0.1% (w/v) Triton X-100 in PBS, twice in PBS and then eluted by heating at 95 °C in 30 μ l of SDS-sample buffer. GLUT1 and GLUT4 present in the samples were detected by Western blotting using anti-GLUT1 or anti-GLUT4 antibodies, and visualized by enhanced chemiluminescence.

Quantitative analysis of protein expression

H-2K^b cells infected with adenovirus for 5 days were lysed into a minimal volume of buffer A containing 1% Triton X-100, cell debris was spun down at 13000 *g* for 5 min in a bench-top Microfuge, and the protein content of the supernatant was then determined. Equal amounts of protein (50 μ g) were separated by SDS/PAGE on 10% (w/v) polyacrylamide gels, and transferred on to PVDF membranes. The membranes were blocked in 5% (w/v) non-fat milk powder before probing with an appropriate antibody, followed by a secondary antibody linked to horseradish peroxidase. Proteins were visualized by enhanced chemiluminescence using a 16-bit charge-coupled-device (CCD) cooled camera (Gene Gnome; Syngene, Cambridge, U.K.). Quantification was achieved using GeneTools software (Syngene).

Statistical analysis

Results are expressed as means $\pm$ S.E.M. Analysis was carried out using a two-tailed unpaired Student's *t* test.

RESULTS AND DISCUSSION

In the present study we have used adenovirus-mediated expression of the α CA and α DN mutant forms of AMPK to investigate the role of the kinase in the activation of glucose transport and protein expression in a skeletal-muscle cell line. Previous studies exploring the potential role of AMPK in cellular pathways have been limited by the lack of specific inhibitors for

the kinase. Many studies have used activation of AMPK by treatment of cells with AICA riboside. However, due to the lack of a specific inhibitor of AMPK, it has not been possible to demonstrate unequivocally that the effects of AICA riboside are due to activation of the kinase. Furthermore, studies with AICA riboside have been frustrated by the finding that it does not lead to activation of AMPK in all cell types, e.g. cardiomyocytes and HEK-293 cells [26]. In an attempt to overcome these problems, we developed constitutively active and dominant-negative forms of the kinase as a means of specifically altering AMPK activity in cells. We used expression of these constructs in primary rat hepatocytes to investigate the role of AMPK in glucose-regulated

Figure 1 Adenovirus-mediated expression of α CA and α DN forms of AMPK in H-2K^b muscle cells

(A) Lysates from uninfected H-2K^b cells or cells infected with adenovirus (100 p.f.u./cell, 4 days) containing either a constitutively active mutant of AMPK (α CA) or a dominant-negative mutant (α DN) were analysed by Western blotting using an anti-Myc antibody. In each case, duplicate samples from separate infections were loaded. (B) AMPK activity in immune complexes prepared by immunoprecipitation using an anti-Myc antibody from uninfected cells, cells infected with α DN or those infected with α CA was measured by phosphorylation of the SAMS peptide. Cells were either untreated (control) or incubated in the presence of 100 nM insulin, 500 μ M AICA riboside (AICAR) or 0.6 M sorbitol for 30 min before harvesting. (C) Endogenous AMPK activity in cells, treated as in (B) and expressing either α DN or α CA, was measured in immune complexes prepared by immunoprecipitation with a pan- β antibody. Significant differences in AMPK activity in cells expressing α DN relative to uninfected cells are shown by asterisks (**P* < 0.05; ***P* < 0.005; and ****P* < 0.0005). (D) Total AMPK activity present in a 0–12% poly(ethylene glycol) fraction of H-2K^b cell lysates was determined. Cells were either left untreated (control), incubated in the presence of either 500 μ M AICA riboside (AICAR) or 0.6 M sorbitol for 30 min, or infected with α CA (100 p.f.u./cell, 4 days) before harvesting. In all cases, activities are the means $\pm$ S.E.M. for 4–6 independent experiments, and are expressed as nmol of 32 P₄ incorporated into SAMS peptide/min per mg of lysate protein.

gene expression [22]. The results presented here demonstrate that the constructs provide effective reagents for altering AMPK activity in a muscle cell line, indicating their usefulness for investigating diverse and tissue-specific roles of the kinase.

Expression of α CA and α DN in H-2K^b cells was monitored by Western blotting using an anti-Myc antibody, since both recombinant α subunits contain a Myc-epitope tag at their N-termini [22]. A representative blot is shown in Figure 1(A), demonstrating that adenoviral infection of H-2K^b cells results in readily detectable expression of both α CA and α DN. A weakly cross-reacting band migrating just ahead of α CA was also detectable, but this was present in all the lysates, including uninfected cells. Lysates from untreated (control) cells, or cells treated with either 500 μ M AICA riboside or 0.6 M sorbitol for 30 min before lysis, were immunoprecipitated with an anti-Myc antibody, and kinase activity in the immune complexes was measured using the SAMS peptide assay (Figure 1B). As expected, AMPK activity in anti-Myc immunoprecipitates was only detectable in cells expressing α CA. Truncation of the AMPK α 1 subunit at residue 312 yields a protein that no longer associates with the β and γ subunits, and is insensitive to activation by AMP [27]. Mutation of Thr¹⁷² within the truncated polypeptide to Asp¹⁷², which mimics the phosphorylated form of the enzyme, results in a constitutively active kinase (α CA) [22]. Additionally, in this study we find that the activity of α CA is not affected by either AICA riboside or hyperosmotic stress, indicating that this form of the kinase cannot be activated further by these treatments. Figure 1(C) shows that the activity of endogenous AMPK, measured from immunoprecipitates using a pan- β subunit antibody, was unaffected by expression of α CA. Expression of α DN, however, inhibited endogenous AMPK activity by approx. 40% in the basal state, and by up to 70% in the stimulated state (Figure 1C). α DN harbours a mutation of Asp¹⁵⁷ within the α subunit to Ala¹⁵⁷. This residue lies within the DFG (Asp-Phe-Gly) motif and has been shown to be essential for MgATP²⁻ binding in all protein kinases [28]. Correspondingly, mutation of this residue renders the kinase inactive, although it can still associate with the β and γ regulatory subunits [29]. The forced overexpression of the inactive α subunit acts as a dominant-negative inhibitor by displacing the native α subunit for binding of the regulatory subunits, leading to its degradation [22].

We were unable to detect AMPK activity in crude lysates of H-2K^b cells and therefore, in order to assess the level of activity due to overexpression of α CA relative to endogenous AMPK, the total activity against the SAMS peptide was measured in a resuspended 0–12% poly(ethylene glycol) precipitate fraction (Figure 1D). The stimulation of AMPK by both AICA riboside and hyperosmotic stress measured under these conditions is somewhat lower than that determined in anti- β immune complexes (compare Figures 1C and 1D). A probable explanation for this is that the SAMS assay is not completely specific for AMPK [6]. Other kinases present in the poly(ethylene glycol) fraction capable of phosphorylating the peptide would therefore result in the apparently lower stimulation of AMPK relative to that observed in anti- β immune complex assays. Despite this, however, it is clear that overexpression of α CA increases kinase activity to at least the same extent as either AICA riboside or hyperosmotic stress.

A number of studies have implicated a role for AMPK in the stimulation of glucose uptake in skeletal muscle in response to a number of stimuli, including contraction [8,9,11,30,31]. Until recently, however, a definitive role for AMPK in the contraction-mediated stimulation of glucose uptake remained elusive [21]. Here, we show that expression of the constitutively active α subunit resulted in an approx. 2-fold increase in glucose uptake

into the H-2K^b cells, similar to that observed following stimulation of the cells with AICA riboside or following hyperosmotic stress (0.6 M sorbitol) (Figure 2A). No further increase in glucose uptake was seen when H-2K^b cells expressing α CA were treated with AICA riboside, and infection with a null adenovirus construct was without effect (results not shown). In order to determine whether the increase in uptake by expression of α CA was due to an increase in translocation of glucose transporters to the plasma membrane, we quantified cell-surface expression of GLUT1 and GLUT4 by photoaffinity labelling using a biotinylated bis-glucose photolabel [25]. As shown in Figure 2(B), there is a significant increase in surface expression of both GLUT1 and GLUT4. A previous study demonstrated that perfusion of rat hind-limb with AICA riboside increases glucose uptake and causes a concomitant increase in GLUT4 translocation to the plasma membrane [31]. Our results indicate that activation of AMPK alone is sufficient for stimulation of glucose uptake into muscle cells via an increase in cell-surface expression of glucose transporters. Expression of the α DN mutant almost completely blocked the increase in glucose uptake caused by either AICA riboside or 0.6 M sorbitol, but had no effect on basal glucose uptake or on the increase in uptake brought about by insulin or PMA (Figure 2C). These results show that both AICA riboside and hyperosmotic stress require activation of AMPK for their effects on glucose uptake.

During the preparation of this manuscript, Mu et al. [21] published data on the effects of the expression of a dominant-negative inhibitory mutant of AMPK on glucose transport in mouse skeletal muscle. In agreement with the results presented here, they determined that expression of the dominant-negative AMPK was sufficient to abolish AICA riboside-stimulated glucose transport. Furthermore, they showed that inhibition of AMPK also blocked completely the activation of glucose uptake in response to hypoxia. From our results, the response to hyperosmotic stress can also be included in the AMPK-dependent pathway for stimulation of glucose uptake in muscle. Mu and co-workers [21] also looked at the effect of inhibiting the AMPK signalling pathway on contraction-stimulated glucose transport. They found that expression of the dominant-negative mutant only partially reduced the increase in glucose uptake in response to electrical stimulation of contraction, indicating that muscle contraction activates glucose uptake via AMPK-dependent and -independent pathways [21].

Over the last few years, evidence has accumulated for a role of AMPK in the stimulation of glucose uptake, but because of the lack of specific reagents this has been largely circumstantial. In the current study, we provide the first definitive evidence that activation of AMPK is sufficient to cause an increase in glucose uptake in muscle cells. Our findings, together with the results reported recently by Birnbaum and colleagues on the dominant-negative AMPK mutant [21], show that activation of AMPK is necessary for the stimulation of glucose uptake in response to AICA riboside, hypoxia and hyperosmotic stress. Under most conditions, glucose uptake into skeletal muscle is the rate-limiting step in glucose metabolism [32], and perturbations in transport have been demonstrated in insulin-resistant states that may ultimately lead to type 2 diabetes [33]. Therefore the control of glucose uptake is of high importance in the maintenance of whole-body glucose homeostasis. It has been recognized for some time that, in muscle, there is an insulin-independent pathway that does not act through PI-3K to increase glucose uptake [8,34]. Muscle contraction is known to act through such a pathway, recruiting a separate pool of intracellular GLUT4 glucose transporters compared with those translocated in response to insulin [35]. In addition to contraction, a number of

Figure 2 Effect of α CA and α DN expression on glucose uptake in H-2K^b cells

(A) 2-[³H]Deoxyglucose uptake in cells infected with α CA (100 p.f.u./cell, 4 days) was measured as described in the Experimental section. Uptake in uninfected cells, either untreated (control) or incubated with 100 nM insulin or 500 μ M AICA riboside (AICAR) for 30 min, was also determined. (B) Cell-surface expression of GLUT1 and GLUT4 was measured in H-2K^b cells by photoaffinity labelling with Bio-LC-ATB-BGPA and Western blot analysis. Cells were infected with: lane 1, no adenovirus; lanes 2 and 3, α CA (50 p.f.u./cell, 2 days). Lanes 1 and 2 are samples from cells that had been labelled with Bio-LC-ATB-BGPA, whereas the sample in lane 3 was from unlabelled cells. Blots were probed with antibodies specific for either GLUT4 or GLUT1. (C) Cells infected with α DN (100 p.f.u./cell, 4 days) or left uninfected were either untreated (control) or incubated in the presence of 100 nM insulin, 500 μ M AICA riboside (AICAR), 0.6 M sorbitol or 1 μ M PMA for 30 min before measurement of 2-[³H]deoxyglucose uptake. Results are the means $\pm$ S.E.M. for 6–12 independent experiments, and are expressed

other stimuli have been shown to increase transport through a PI-3K-independent pathway, e.g. hypoxia [36], hyperosmotic stress [37] and mitochondrial uncoupling [38]. Recent reports have linked these varied stimuli to activation of AMPK, and the activation of AMPK to increasing glucose transport [9]. The results of our present study using constitutively active and dominant-negative mutants of AMPK have shown that increased AMPK activity is sufficient to stimulate glucose uptake, and is necessary for the increase in glucose uptake in response to AICA riboside and hyperosmotic stress. These data strengthen the hypothesis that AMPK is an important target for intervention in the control of glucose uptake and metabolism during conditions such as type 2 diabetes.

Previous studies have shown that long-term (5–28 days) injection of AICA riboside into rats caused an increase in the levels of several mitochondrial enzymes, as well as of GLUT4 [39,40]. In order to determine whether this process could be mediated directly via AMPK, we examined the effect of expression of constitutively active AMPK on protein expression of specific targets in muscle cells. Western blot analysis of lysates from cells infected with the α CA construct showed significantly increased expression of GLUT1 (1.3-fold; $P < 0.05$), GLUT4 (1.6-fold; $P < 0.0001$) and hexokinase II (1.7-fold; $P < 0.0005$) (Figures 3A and 3B) compared with uninfected cells. The expression of creatine kinase was also increased (1.3-fold), but this change did not reach statistical significance. The increases in protein expression do not appear to be non-specific effects, since the expression of p38 MAPK was unchanged, and the expression of protein kinase B actually showed a small decrease (approx. 20%). No significant change in expression of any of the proteins measured was seen in cells infected with the dominant-negative construct or with null adenovirus (results not shown). Our data provide strong evidence that activation of AMPK is sufficient to cause an increase in expression of specific proteins, including GLUT4, in muscle cells.

The effect of the constitutively active AMPK on the expression of specific proteins led us to consider whether these effects may be linked to a pro-differentiating effect of the kinase on muscle cells. We therefore examined the levels of myosin, a muscle-cell-specific differentiation marker, and α -tubulin, which has been shown previously not to change with muscle cell differentiation [41]. As expected, myosin could not be detected in undifferentiated cells, and expression of α CA did not alter this (Figures 3C and 3D). However, in differentiated cells myosin was readily detectable. Furthermore, there was a significant increase in myosin levels following expression of α CA. The levels of tubulin were similar in undifferentiated and differentiated cells and, in both cases, expression of α CA caused a small decrease in tubulin content. Since expression of α CA does not lead to expression of myosin in undifferentiated H-2K^b cells, it seems unlikely that activation of AMPK in itself is sufficient to induce muscle cell differentiation. However, the increase in myosin levels seen following expression of α CA raises the interesting possibility that AMPK might be involved in some process during muscle cell development. Further investigation is required in order to substantiate the potential role of AMPK in skeletal muscle development.

An increase in GLUT4 expression in skeletal muscle has been shown to occur following exercise training in both rats and

as the percentage uptake relative to uninfected control cells (indicated by the dashed line). Significant differences in uptake in treated cells relative to control cells are denoted by asterisks (* $P < 0.05$; *** $P < 0.0005$).

Figure 3 Effect of α CA expression on protein levels in H-2K^b cells

(A) Representative Western blots of lysates prepared from two separate sets of uninfected H-2K^b cells (control) or cells infected with α CA (100 p.f.u./cell, 4 days) probed with antibodies against GLUT1, GLUT4, hexokinase II (HXT II), creatine kinase (CK), p38 MAPK and protein kinase B (PKB) are shown. (B) In each case, the level of expression was quantified by direct measurement of chemiluminescence using a 16-bit CCD cooled camera. Results are the means $\pm$ S.E.M. for 8–12 independent experiments, and are plotted as a percentage of the expression in uninfected cells. Significant differences in protein levels in cells expressing α CA relative to uninfected cells are denoted by * and ** ($P < 0.05$ and $P < 0.0001$ respectively). (C) Representative Western blots of lysates prepared from either undifferentiated (Undiff) or differentiated (Diff) H-2K^b cells infected with or without α CA (100 p.f.u./cell, 4 days) and probed with antibodies against either myosin or α -tubulin are shown. (D) Protein levels were quantified as described for (B). Results are the means $\pm$ S.E.M. for 8–12 independent experiments, and are plotted as a percentage of the expression in uninfected cells. Significant differences in protein levels in cells expressing α CA relative to uninfected cells are denoted by * and ** ($P < 0.01$ and $P < 0.0005$ respectively).

humans [42,43]. Previous studies have shown that AMPK is activated in muscle following contraction [44,45], and evidence has accumulated over the last few years demonstrating a role for AMPK in the regulation of gene expression [22,46,47]. Together, these findings suggest a potential link between AMPK and the changes in protein expression that result from exercise training. The increased protein expression observed in muscle from rats treated chronically with AICA riboside supported this hypothesis, although direct evidence was still lacking [39,40]. Our results in the current study demonstrate that increased AMPK activity can lead directly to increased protein expression in muscle cells. Taken together with the data obtained from the administration of AICA riboside *in vivo*, these findings offer an attractive hypothesis that AMPK is involved, at least in part, in the mechanism leading to a specific increase in protein expression in muscle in response to exercise training.

The mechanism by which AMPK increases protein levels within skeletal muscle has not yet been addressed. Since AMPK has been shown to be involved in the regulation of gene expression [22,46,47], it is possible that this may have a role in alteration of protein content. Indeed, the levels of GLUT4 mRNA have been shown to increase following exercise [43,48]. At present, however, activation of AMPK has only been shown to decrease the transcription of specific genes [22,46,47,49], and there is no information concerning the effect of AMPK on exercise-induced genes, such as GLUT4. Further studies are required to elucidate the mechanisms involved in the AMPK-mediated induction of protein expression in muscle.

In summary, the results of our present study show that activation of AMPK is sufficient to cause increased glucose uptake into muscle cells, and is required for the increased uptake in response to hyperosmotic stress. Chronic activation of AMPK

leads to increased expression of specific proteins, including GLUT4. These consequences of AMPK activation in muscle lead to increased glucose uptake, and are therefore beneficial in conditions where blood glucose is high, e.g. in patients with type 2 diabetes. Compounds that would cause activation of AMPK in skeletal muscle promise to be attractive agents for therapeutic intervention.

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