AMP-activated protein kinases (AMPKs) act as a metabolic sensor in mammalian cells. The kinases are activated by a relative increase in cellular AMP level, i.e. increase in the AMP to ATP ratio (1–5). AMPK activity can also be stimulated by 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), an adenosine analog. After entering into cells, AICAR is phosphorylated at its 5′ position, and the reaction product (identified as ZMP) mimics the stimulatory action of AMP on AMPK (6). Activation of AMPK either by physiological stimulation such as contraction of skeletal muscles or by pharmacological activator AICAR leads to a significant increase of glucose transport mediated by translocation of the GLUT4 transporter molecule from intracellular membranes to the plasma membrane (7–9). We have recently found that activation of AMPK by AICAR is also associated with the enhancement of GLUT1-mediated glucose transport in Clone 9 cells (10). However, signaling events upstream and downstream of AMPK in the regulation of glucose transport are unknown. Available evidence distinguishes the AMPK signaling pathway in glucose transport from the phosphatidylinositol 3-kinase (PI3K) cascade, a pathway involved in insulin-stimulated glucose transport, on the basis of the wortmannin-insensitive nature of the stimulation of glucose transport by AMPK (7).

The p38 mitogen-activated protein kinases (MAPKs) are activated in response to a variety of extracellular stimuli and have been found to be involved in the inflammatory process, cell growth, cell differentiation, cell cycle, and cell death (11–14). Available evidence from muscles and cultured cells indicates that p38 MAPK might also be involved in the regulation of glucose transport. Muscle contraction, which has been demonstrated to increase GLUT4-mediated glucose transport, activates p38 MAPK (15). Overexpression of constitutively active forms of mitogen-activated protein kinase kinase 3 (MKK3) and MKK6, the upstream regulatory kinases of the p38 MAPK pathway, stimulate translocation of GLUT1 and GLUT4 transporters in 3T3-L1 adipocytes (16). Increased glucose transport by ischemic preconditioning is also mediated by p38 MAPK (17). Moreover, it has been reported that a pathway involving p38 MAPK leads to an activation of the recruited glucose transporters in the plasma membrane in 3T3-L1 adipocytes and L6 myotubes (18).

A number of conditions like contraction, hypoxia, inhibition of oxidative phosphorylation, and hyperosmolarity, which evoke metabolic stress, are known to increase both glucose transport and AMPK activity in isolated skeletal muscles (19). Interestingly most of these physiological conditions also activate p38 MAPK (14, 20). Therefore, it is a logical question whether AMPK and p38 MAPK share the same or similar signaling pathway in the stimulation of glucose transport. In the present study, we investigated the possible involvement of p38 MAPK in the stimulation of glucose transport by AMPK in Clone 9 cells. Clone 9 is a rat liver-derived nontransformed cell line that expresses only the GLUT1 glucose transporters (21, 22). Our studies show that p38 MAPK is a downstream component of AMPK signaling pathway in AICAR-stimulated glucose transport.
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pcDNA3.1/Myc-His(+) and pcDNA3.1/His/ lacZ, the β-Gal Staining Kit, anti-Myc monoclonal antibody, and NOVEX Pre-Case gels were from Invitrogen (Carlsbad, CA). Anti-FLAG-M2 monoclonal antibody, anti-FLAG-M2 affinity gel, AICAR, 5-iodotubercidin, and other reagents were from Sigma.

Cell Culture—Clone 9 cells, obtained from the American Type Culture Collection (Manassas, VA), were cultured in Dulbecco’s modified Eagle’s medium containing 10% calf serum (21, 22). The cells were maintained in serum-free medium for 18 h prior to any treatment.

Glucose Transport Assay—Cytochalasin B-inhibitable 3-O-methyl-[3H]glucose uptake was carried out as described previously (23). Briefly, triplicate culture plates were incubated with [3H]glucose for 60 s in glucose-free medium consisting of 750 μCi of 3-O-methyl-[3H]glucose with a final concentration of 50 μCi cytochalasin B (CB) in Me2SO. An equal amount of Me2SO was added to the control set. Uptake of glucose was terminated by removal of the uptake medium followed by five rapid washes each with 5 ml of ice-cold 100 mM MeCl2 containing 0.1 mM phenol red. Cells were collected in 1 ml of distilled H2O, and the radioactivity was determined by scintillation counting. CB-inhibitable 3-O-methyl glucose uptake was calculated by the difference between uptake in the absence and presence of CB.

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting—Clone 9 cells were lysed in lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 20 mM pyrophosphate, 1 mM orthovanadate, 20 mM NaF, 0.1 mM each of phenylmethylsulfonyl fluoride, N-α-tosyl-l-lysine chloromethyl ketone, N-tosyl-l-phenylalanine chloromethyl ketone, and 1% Nonidet P-40), sonicated, and centrifuged (23). The resulting supernatant was fractionated by SDS-polyacrylamide gel electrophoresis (4–20% gradient gel) and transferred onto nitrocellulose paper. Membranes were blocked with 5% nonfat milk and incubated with primary antibodies. The secondary antibody was peroxidase-labeled goat anti-rabbit IgG or goat anti-mouse IgG. Immunocomplexes were detected using chemiluminescence.

Adenovirus Infection—Recombinant adenovirus containing a dominant negative form of p38α mutant was constructed as described previously (24). Clone 9 cells were infected with viruses containing mutant p38α cDNA or empty vector for 24 h in serum-free Dulbecco’s modified Eagle’s medium. The titers of viral stocks were ~5 x 10^11 plaque-forming units/ml.

Construction of Plasmids Expressing p38α MAPK or a Constitutively Active Form of AMPK and Co-transfection in Clone 9 Cells—p38α cDNA containing a FLAG epitope tag at its N-terminal region in pcDNA3 was prepared as described previously (25). cDNA fragments of the rat AMPKα1 subunit encoding its first 312 amino acids were generated by reverse transcription-polymerase chain reaction using the total RNA of Clone 9 cells as template. Sequences of upstream and downstream primers were 5’-ATGGCCAGAAGCAGAACACG and 5’-GTACAGGACGTCAGGACCC, respectively (26). The resulting polymerase chain reaction product was cloned into the EcoRI site of pCR2.1-TOPO and subcloned into the EcoRI site of pcDNA3.1-Myc/His(+) (27). Point mutation in the phosphorylation site (Thr-172 to Asp-172) within the AMPKα1 subunit was generated by in vitro mutagenesis using mutation primers 5’-GGTGAATTTATAAGAGATAGCTGTGGCTCGCCC and 5’-GGGCCAGCCACGAGTAATTAAATCCACC (27). The resulting AMPKα1 cDNA was verified by DNA sequencing. Clone 9 cells were co-transfected with either FLAG-p38 plus AMPK mutant or FLAG-p38 plus empty vector pcDNA3.1-Myc/His(+) (+). To evaluate the transfection efficiency, pcDNA3.1/His/lacZ was co-transfected with the above constructs, and the expression of β-galactosidase detected with the β-Gal Staining Kit. After 48 h, cells were collected in the above lysis buffer and used for the immunoprecipitation, AMPK activity assay, and Western blotting experiments. Cytochalasin B-inhibitable 3-O-methyl-[3H]glucose uptake was carried out as described above.

Immunoprecipitation and Western Blotting—Lysates of the transfected cells were subjected to immunoprecipitation and Western blotting as described previously (23). Anti-FLAG-M2 affinity gels were used for the immunoprecipitation of FLAG-p38α peptides, and anti-Myc antibodies were used for the immunoprecipitation of AMPK-Myc peptides. Phosphorylated active FLAG-p38α was detected by Western blotting using anti-phospho-p38 antibodies. The total amount of p38α MAPK was determined by Western blotting using anti-p38 antibodies. Expression of the AMPK mutant was determined by Western blotting using anti-Myc antibodies.

AMPK Activity Assay—The AMPK activity assay was carried out according to methods described previously (28, 29). Briefly, Clone 9 cells were collected in the lysis buffer, sonicated, and centrifuged to remove cell debris. Proteins in the supernatant were precipitated with a saturated ammonium sulfate solution. Assays were performed at 30 °C in 20-μl reaction mixtures containing 2 μg of total protein in reaction buffer (40 mM Hepes, pH 7.0, 80 mM NaCl, 5 mM magnesium acetate, 1 mM dithiothreitol, 200 μM AMP and ATP, and 2 μCi of [γ-32P]ATP) with or without 200 μM SAMS peptide. After the reaction, 15 μl of reaction mixture was spotted onto the phosphocellulose filter paper, and the filter paper washed extensively with phosphoric acid. The radioactivity on the filter paper was measured by scintillation counting, and AMPK activity was expressed as picomoles of [32P] incorporation into the peptide/minute/microgram of protein.

Statistical Analysis—Results are expressed as means ± S.E. Student’s unpaired two-tailed t test was used, and p < 0.05 was considered significant (30).

RESULTS

Activation of p38 MAPK and MKK3 by AICAR—To study whether p38 MAPK was involved in AICAR-stimulated glucose transport, Clone 9 cells were treated with 1 mM AICAR for 1 h. Activation of p38 MAPK was determined by Western blotting using anti-phospho-p38 MAPK antibodies. These antibodies react specifically with the active dual-phosphorylated forms of p38 MAPK. As shown in Fig. 1, the activation was evident by Western blotting using anti-phospho-MKK3/6 antibodies. Total MKK3 was determined by using anti-MKK3 antibodies reacting with both inactive and active forms of MKK3. B, dose-dependent activation of p38 MAPK by AICAR. Clone 9 cells were treated with three different concentrations of AICAR for 1 h, and the relative contents of phosphorylated p38 MAPKs were determined by Western blotting using anti-phospho-p38 MAPK antibodies and laser scanning densitometry.

FIG. 1. Activation of p38 MAPK and MKK3 by AICAR. A, Clone 9 cells were treated with 0.25 or 1 mM AICAR for 1 h or pretreated with 10 μM SB203580 or 10 μM 5-iodotubercidin for 10 min prior to treatment with 1 mM AICAR. Phosphorylation and activation of p38 MAPK were determined by Western blotting using anti-phospho-p38 antibodies reacting specifically with the active dual-phosphorylated forms of p38 MAPK. Total p38 MAPK was determined by Western blotting using anti-p38 antibodies reacting with both inactive and active forms of p38 MAPK. Phosphorylation and activation of MKK3 were determined by Western blotting using anti-phospho-MKK3/6 antibodies. Total MKK3 was determined by using anti-MKK3 antibodies reacting with both inactive and active forms of MKK3. B, dose-dependent activation of p38 MAPK by AICAR. Clone 9 cells were treated with three different concentrations of AICAR for 1 h, and the relative contents of phosphorylated p38 MAPKs were determined by Western blotting using anti-phospho-p38 MAPK antibodies and laser scanning densitometry.
MKK3 is one of the upstream kinases of p38 MAPK (31). AICAR significantly activated MKK3, and 5-iodotubercidin completely blocked the activation (Fig. 1). However, SB203580 had no significant effect on the MKK3 activity, which is in good agreement with the fact that p38 MAPK is the target molecule of SB203580 (Fig. 1). These findings demonstrate that treatment of Clone 9 cells with AICAR stimulates the p38 MAPK cascade.

Inhibition of AICAR-stimulated Glucose Transport by SB203580—We have already reported that the activation of AMPK by AICAR is associated with the enhancement of GLUT1-mediated glucose transport in Clone 9 cells (10). To find out whether activation of p38 MAPK by AICAR was related to the observed increase of glucose transport, Clone 9 cells were treated in a manner similar to that in Fig. 1, and CB-inhibitable [3H]3-OMG uptake was measured. As shown in Fig. 2, pretreatment of the cells with 1 mM AICAR for 1 h led to a 5.4-fold increase in glucose transport. Pretreatment of the cells with 10 mM SB203580 for 10 min completely abolished AICAR-stimulated glucose transport, whereas treatment with 2 mM SB203580 also significantly inhibited glucose transport with a lesser effect than that noticed with 10 mM SB203580. As expected, pretreatment of the cells with 10 mM 5-iodotubercidin for 10 min completely blocked AICAR-stimulated glucose transport as well. However, neither SB203580 nor 5-iodotubercidin alone had a significant effect on glucose transport (Fig. 2). These results indicate that activation of p38 MAPK is required for AICAR-stimulated glucose transport.

Effect of Overexpression of Dominant Negative p38 MAPK Mutant on AICAR-stimulated Glucose Transport—Data shown in Figs. 1 and 2 demonstrated that AICAR-stimulated glucose transport was mediated by the p38 MAPK cascade. To further strengthen the inferences drawn from previous results, Clone 9 cells were infected with adenoviruses carrying the dominant negative p38 MAPK mutant for 24 h. Cells were then treated with either 1 mM AICAR or diluent for 1 h prior to glucose uptake measurement. Expression levels of mutant p38 MAPK protein were determined by Western blotting using anti-p38 MAPK antibodies reacting with both inactive and active forms of p38 MAPK. As shown in Fig. 3, overexpression of p38 MAPK mutant caused a significant inhibition (68%) in AICAR-stimulated glucose transport compared with that in control cells infected with the virus containing empty vector. These results strengthen the notion that the p38 MAPK cascade mediates AICAR-stimulated glucose transport in Clone 9 cells.
peptide of AMPK contains a Myc epitope at its C-terminal region. 48 h later, FLAG-p38α peptides were immunoprecipitated with anti-FLAG-M2 affinity gel, and the activation of p38α was determined by Western blotting with anti-phospho-p38 antibodies. As shown in Fig. 5, overexpression of the constitutively active form of AMPK mutant resulted in a marked activation of p38α MAPK compared with that in cells transfected with the empty vector. Glucose transport measurement and the AMPK activity assay were carried out as described in Figs. 2 and 4. A 62 ± 10% increase in AMPK activity and a 78 ± 10% increase in CB-inhibitable glucose transport were observed in cells co-transfected with p38α plus AMPK mutant compared with that in cells transfected with p38α plus empty vector. These results strongly suggest that the effect of AICAR on activation of p38 MAPK is indeed mediated by AMPK in Clone 9 cells.

DISCUSSION
Facilitative, sodium-independent glucose transport is mediated by a glycoprotein family of glucose transporters, GLUT1 to GLUT5 (34, 35). Among them, GLUT4 is responsible for the insulin-stimulated glucose transport in muscle and fat cells. Insulin increases glucose transport in these cells by translocation of GLUT4 molecules to the plasma membrane from their intracellular pools, and the PI3K cascade has been demonstrated to be involved in this action (36–38). In contrast, GLUT1, which is mainly responsible for the basal level of glucose transport, is present in almost all tissues and cultured cells. The signaling pathway for the GLUT1-mediated glucose transport is poorly understood (34). Clone 9, a rat liver-derived nontransformed cell line, expresses only the GLUT1 isoform of transporters in their predifferentiation states (10). Whether the AICAR-stimulated glucose transport in these cells and others is also mediated by the p38 MAPK cascade needs to be examined.

It has been demonstrated that the activation of AMPK by AICAR or contraction is associated with an increased glucose transport in skeletal and heart muscles (7–9). Recently, Mu et al. (43) reported that expression of the dominant negative mutant of AMPK in mouse muscle completely abolished AICAR-stimulated glucose transport. This finding showed that AICAR-stimulated glucose transport, at least in skeletal muscle, was indeed mediated by AMPK (43). However, signaling events upstream and downstream of AMPK in the up-regulation of glucose transport is unknown. The available evidence distinguishes the AMPK pathway from the PI3K cascade on the basis of the wortmannin-insensitive nature of AICAR-stimulated glucose transport and a partially additive effect of insulin and AICAR on the stimulation of glucose transport (7). It has been hypothesized that there are at least two independent signaling pathways involved in glucose transport in muscle: the PI3K cascade is responsible for the insulin-stimulated glu-
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FIG. 6. A schematic representation showing that p38 MAPK is a downstream component of the AMPK signaling pathway in AICAR-stimulated glucose transport.

cose transport, whereas AMPK mediates contraction-induced glucose transport. In 3T3-L1 adipocytes, a well known model for the study of insulin-stimulated glucose transport, AICAR has been found to inhibit insulin-stimulated glucose transport (44). By the overexpression of the dominant negative form of AMPK mutant in transgenic mice Mu et al. (43) found that AMPK was only partially responsible for the contraction-stimulated glucose transport in skeletal muscle, indicating that AMPK-independent pathways also contributed to the response (43). Derave et al. (45) reported a contradictory effect of AMPK on glucose transport in different kinds of muscles. They found a significant positive correlation between AMPK activity and glucose transport in contracting fast-twitch muscle but not a significant correlation between them in slow-twitch muscle (45). These results suggest that there are multiple signaling pathways in cells in response to the stimulation. Interaction between AMPK and another signaling pathway(s) in the regulation of glucose transport needs further investigation.

The p38 MAPK signaling cascade activated by diverse stimuli is involved in a variety of biological processes (14). An increasing body of evidence indicates that the p38 MAPK pathway may also be involved in the regulation of glucose transport. In muscles, contraction known to increase GLUT4-mediated glucose transport activates p38 MAPK (15, 46). Enhanced glucose transport by ischemic preconditioning in heart is also mediated by p38 MAPK in a PI3K-independent manner (17). Contradictory results have been reported in differentiated 3T3-L1 adipocytes. Kayali et al. (47) found that although insulin markedly increased glucose transport in differentiated 3T3-L1 adipocytes, there was no significant increase in p38 MAPK activity. Fijishimo et al. (48) also found that suppression of p38 MAPK activation by overexpression of a dominant negative p38 MAPK or MKK6 mutant did not affect insulin-induced glucose transport in differentiated 3T3-L1 adipocytes. However, Sweeney et al. (18) reported that inhibition of p38 MAPK activity prevented insulin-stimulated glucose transport but did not affect translocation of glucose transporters in differentiated 3T3-L1 adipocytes and L6 myotubes. Borros et al. (39) also reported that treatment of differentiated 3T3-L1 adipocytes and Clone 9 cells with anisomycin, a potent activator of p38 MAPK, resulted in a significant increase in glucose transport as well as a marked activation of p38 MAPK. It was unclear whether different differentiation states of 3T3-L1 adipocytes contributed to the above contradictory effects. However, it has been known that 3T3-L1 preadipocytes express only the GLUT1 isoform, whereas differentiated 3T3-L1 adipocytes express both the GLUT1 and GLUT4 isoforms. It is also known that p38 MAPK activity is modulated during differentiation in 3T3-L1 adipocytes and drops dramatically during the later stages of differentiation (47, 49). In the present study, we have demonstrated that in Clone 9 cells, which express only the GLUT1 isoform of glucose transporters, p38 MAPK mediates AICAR-stimulated glucose transport.

Unlike the GLUT4 transporter, most of the GLUT1 molecules are present in the plasma membrane under basal conditions, and their activation by a variety of stimuli increases glucose transport (40–42). We have previously demonstrated that the enhancement of GLUT1-mediated glucose transport by AICAR in Clone 9 cells occurs without any significant increase in the number of transporter molecules in the plasma membrane, reflecting an activation of GLUT1 transporters already present in the plasma membrane (10). Interestingly available evidence shows that an activation of GLUT4, following its translocation to the plasma membrane, appears to be a necessary step for the stimulatory action of insulin on glucose transport without the involvement of the PI3K cascade. It has been reported that GLUT4 translocation is dissociated with insulin-stimulated glucose transport in transgenic mice overexpressing GLUT1 in skeletal muscle, suggesting that the intrinsic activity of GLUT4 is subject to regulation (50). In addition, it was found that inhibition of p38 MAPK activity did not affect insulin-induced translocation of GLUT4 transporters in 3T3-L1 adipocytes and L6 myotubes; rather it largely reduced the activation of relocated glucose transporters in the plasma membrane (18). It has been proposed that insulin-stimulated glucose transport is likely to be regulated by at least two independent signaling pathways, i.e. 1) the PI3K pathway facilitates the translocation of glucose transporters to the plasma membrane, and 2) a wortmannin-insensitive pathway activates translocated glucose transporters (18). Although GLUT1 and GLUT4 are predominantly present in different tissues or different differentiation states, our data and that of others support the notion that these transporters may share the same or similar signaling pathway in activation, and the p38 MAPK cascade appears to be involved in this action.

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Stimulation of Glucose Transport by AMP-activated Protein Kinase via Activation of p38 Mitogen-activated Protein Kinase
Xia Xi, Jiahuai Han and Jin-Zhong Zhang

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