THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 278, No. 1, Issue of January 3, pp. 48–55, 2003

Printed in U.S.A.

Identification of a Novel Protein Kinase Mediating Akt Survival Signaling to the ATM Protein*

Received for publication, June 17, 2002, and in revised form, October 28, 2002
Published, JBC Papers in Press, October 29, 2002, DOI 10.1074/jbc.M206025200

Atsushi Suzuki‡, Gen-ichi Kusakai‡§, Atsuhiro Kishimoto‡§, Jie Lu‡§, Tsutomu Ogura‡, Martin F. Lavin¶, and Hiroyasu Esumi‡‖

From the ‡Investigative Treatment Division, National Cancer Center Research Institute East, 6-5-1 Kashiwanoha, Kashiwa, Chiba 277-8577, Japan and the ¶Queensland Institute of Medical Research and the Department of Surgery, University of Queensland, Herston, Brisbane, Queensland 4029, Australia

We identified a novel human AMP-activated protein kinase (AMPK) family member, designated ARK5, encoding 661 amino acids with an estimated molecular mass of 74 kDa. The putative amino acid sequence reveals 47, 45.8, 42.4, and 55% homology to AMPK-α1, AMPK-α2, MELK, and SNARK, respectively, suggesting that it is a new member of the AMPK family. It has a putative Akt phosphorylation motif at amino acids 595–600, and Ser^{600} was found to be phosphorylated by active Akt resulting in the activation of kinase activity toward the SAMS peptide, a consensus AMPK substrate. During nutrient starvation, ARK5 supported the survival of cells in an Akt-dependent manner. In addition, we also demonstrated that ARK5, when activated by Akt, phosphorylated the ATM protein that is mutated in the human genetic disorder ataxia-telangiectasia and also induced the phosphorylation of p53. On the basis of our current findings, we propose that a novel AMPK family member, ARK5, is the tumor cell survival factor activated by Akt and acts as an ATM kinase under the conditions of nutrient starvation.

AMPK[1] is a mammalian homologue of sucrose non-fermenting protein kinase (SNF-1), which belongs to a serine/threonine protein kinase family, and its activation is well documented in cells under metabolic stress, hypoxia, heat shock, and ischemia (1, 2). The SNF-1/AMPK family is highly conserved in several species including mammals (3–8), and the α-subunit of AMPK has been shown to be the catalytic subunit (2). Four proteins, AMPK-α1 and AMPK-α2 (9–12), MELK (5), and SNARK (13), have been identified as catalytic subunits of the AMPK family to date. Although it has been reported that AMPK activation is initiated by phosphorylation at Thr^{172} (14), how the phosphorylation is initiated remains unclear.

AMPK is activated under various stress conditions where the cellular ATP concentration decreases and plays a key role in cellular adaptive responses to maintain energy balance. The well known targets of AMPK belong to those involved in energy metabolism including 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA), acyl-CoA decarboxylase, and glucose and amino acid transporters (15). However, the cellular mechanism for survival under stress conditions is complex and includes cell cycle regulation, repair of cellular components, remodeling tissue components, and regulation of the cell death pathway. Targets of AMPK are not fully described to date. Recently, we demonstrated that some tumor cells, such as the pancreatic cancer cell line PANC-1, showed high tolerance against glucose starvation, which is compatible with hypovascular findings on clinical angiography (16), although most cell lines, including a human hepatoma cell line and the human normal fibroblast cell line, underwent cell death under glucose starvation (16, 17). Similar tolerance to glucose starvation was induced by hypoxic conditions, and tolerance was found to be dependent on AMPK activity as well as Akt (16–18). During glucose starvation, a G_1 phase cell cycle delay occurs with a concomitant increase in p53 phosphorylation, and AMPK appeared to be involved in this process (19).

The tumor suppressor gene *ATM* has been identified as the gene defective in the human genetic disorder ataxia-telangiectasia, which is characterized by neurological degeneration and cancer predisposition (20–22). ATM is a member of the phosphatidylinositol 3-kinase family that activates the tumor suppressor p53 during the cellular response to DNA double strand break (23–25). Although several studies have reported a close involvement of the ATM/p53 pathway during the cellular response to DNA damage, the regulation of this pathway, particularly by non-DNA damage events, has not been well described, and it remains unclear how ATM is activated.

In the current study, we identified a novel AMPK family member through an investigation of human SNARK and designated it ARK5. ARK5, the activation of which was observed during glucose starvation, caused cells to survive in an Akt-dependent manner. Furthermore, Akt-activated ARK5 phosphorylated ATM both *in vivo* and *in vitro* and led to the phosphorylation of p53. We report here that a novel AMPK family member, ARK5, is a new target molecule of Akt and transduces a signal to activate ATM during nutrient starvation.

EXPERIMENTAL PROCEDURES

Preparation of FLAG-tagged ARK5—FLAG-tagged ARK5 was prepared from full-length KIAA0537-ligated pBluescript II SK^+ vector supplied from KAZUSA DNA Research Institute with LA PCR (Takara Biomedicals, Kyoto, Japan); the up-stream primer was 5′-AAGCTTAT-

* This work was partly supported by a second-term comprehensive 10-year Strategy of Cancer Control grant from the Ministry of Health, Welfare, and Labor and a Medical Frontier Program grant from the Ministry of Health, Welfare, and Labor. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Recipient of a research resident fellowship award from the Foundation for the Promotion of Cancer Research.

‖ To whom correspondence should be addressed. Tel.: 81-4-7134-6880; Fax: 81-4-7134-6859; E-mail: hesumi@east.ncc.go.jp.

[1] The abbreviations used are: AMPK, AMP-activated protein kinase; PI, propidium iodide; ATM, ataxia-telangiectasia mutated; H/ARK, HepG2 cells with transfected ARK5 expression vector; PBS, phosphate-buffered saline; GST, glutathione *S*-transferase; ARK5(AS), antisense RNA expression vector of ARK5; ARK5(S600A), ARK5 point mutant; PDK1, phosphoinositide-dependent kinase 1; DA-Akt, dominant active Akt; DN, dominant negative Akt; P/ATM, PANC-1 cell in which His-tagged ATM is stably expressed.

GGATTATAAAGATGATGATGATAAAGAAGGGGCCGCCGCGCCTG-TGGCGGGG-3′ and the down-stream primer was 5′-TCTAGACTAGT-TGAGCTTGCTGCAGATCTCCAG-3′. The PCR product was ligated into pT7-Blue T vector for subcloning, and then insert cDNA digested by *Hin*dIII and *Xba*I was re-ligated into the pcDNA3.1(+) expression vector. Insert cDNA was also ligated into pcDNA3.1(−) expression vector for the preparation of antisense RNA expression vector.

The FLAG-ARK5 protein was prepared by immunoprecipitation technology using anti-FLAG-conjugated agarose. A HepG2 cell line overexpressing FLAG-ARK5 (H/ARK) was lysed with PBS containing 0.1% Nonidet P-40, and then immunoprecipitation was performed. Immunoprecipitates were washed eight times with PBS containing 0.1% Nonidet P-40, and purification was then examined with SDS-PAGE and silver staining.

Antibodies and Vectors—Monoclonal antibody against FLAG and His_6, anti-FLAG-conjugated agarose, recombinant active Akt1, and dominant negative and dominant active Akt1 expression vectors were purchased from Upstate Biotechnology. Polyclonal antibody against total Akt and phosphorylated Akt (Ser^{473}) were purchased from Cell Signaling Technology.

Cell Line and Culture—Cell lines used for current experiments were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Sigma). Cell viability was assessed by the Hoechst 33342/PI staining procedure. Hoechst 33342 and PI were purchased from Molecular Probes. After treatment, cells were collected and stained with Hoechst 33342 and PI and then observed by fluorescence microscopy. Cell induction ratio was indicated by the ratio of cells carrying PI-stained nuclei to total cells (about 1000 cells).

Akt1 Phosphorylation Assay—FLAG-tagged proteins were isolated from cell lysate by anti-FLAG-conjugated agarose and suspended in kinase buffer containing 25 mM Tris (pH 7.5), 2 mM dithiothreitol, and 10 mM $MgCl_2$. Subsequently, 100 μM [γ-^{32}P]ATP (20 μCi) and recombinant active Akt1 were added to a kinase buffer containing recombinant proteins and incubated for 30 min at 30 °C. After incubation, the agarose was washed six times with PBS containing 0.1% Nonidet P-40 and then re-suspended in PBS, and radioactivity was measured with a scintillation counter. Furthermore, measured samples were separated on 7.5% SDS-PAGE for autoradiography.

Preparation of Anti-human SNARK Antibody—Antibody to human SNARK was raised in rabbits by immunizing them against a peptide based on the predicted amino acid sequence of human SNARK (KKPRQRESGYYSSPEPS). The peptide was synthesized and coupled to keyhole limpet hemocyanin (KLH) at the C terminus. Japanese White rabbits were immunized with 0.5 mg of peptide conjugate by initially injecting it in 50% Freund's complete adjuvant (Sigma) and in 50% Freund's incomplete adjuvant (Sigma) for subsequent immunizations. One week after the booster injection of 0.5 mg of peptide conjugate, polyclonal anti-human SNARK antiserum was collected and used for immunoblotting analysis.

GST-SAMS Phosphorylation Assay—Crude cell extracts and immunoprecipitates were used to assay the phosphorylation of the SAMS peptide fused to glutathione *S*-transferase. An enzyme assay was performed at 30 °C in buffer containing 15 mM HEPES (pH 7.0), 200 mM 5′-AMP, 200 mM GST-SAMS, cell lysate, 0.01% Briji 35, 0.3 mM dithiothreitol, 15 mM $MgCl_2$, and 50 mM [γ-^{32}P]ATP (10 mCi). After incubation, the fusion protein was purified with glutathione-Sepharose (Amersham Biosciences) and counted in scintillation counter (Beckman Coulter). There was no significant difference in K_m value between SAMS peptide and GST-SAMS.

RESULTS

ARK5 Is the Novel AMPK Family Protein Kinase Leading Cells to Survival—Recently, a novel AMPK family member, SNARK, was identified from rat (13). On the basis of the reported amino acid sequence, BLAST search analysis revealed a human cDNA encoding SNARK (the amino acid sequence is listed in Fig. 1*B*), and we prepared a polyclonal antibody against the SNARK peptide. Using this antibody, the endogenous expression of SNARK in several human cell lines including lung carcinoma (PC-10 and A549), pancreatic cancer (PANC-1 and ASPC-1), hepatoma (HepG2 and HLE), and fibroblast (HF) was examined. As shown in Fig. 1*A*, Western blotting revealed the expression of an unknown cross-reacting protein at 74 kDa in some of cell lines tested (intense expression in PC-10, PANC-1, ASPC-1 and HLE; weak expression in A549 and HF; no expression in HepG2). Because the calculated molecular mass of human SNARK is 69.5 kDa, we tried to identify this 74-kDa unknown protein by BLAST search analysis using human SNARK amino acid sequence or the peptide sequence by which the anti-human SNARK antibody was prepared and a human protein data base, and we found a putative protein encoded by a KIAA0537 cDNA clone. As shown in Fig. 1*B*, the putative catalytic domain of SNARK and the KIAA0537 protein showed 84% similarity, and the peptide sequence used for the preparation of the anti-human SNARK antibody also showed strong similarity with the KIAA0537 protein. To investigate whether this KIAA0537 cDNA clone encodes the unknown 74-kDa protein detected by the SNARK antibody, we prepared a FLAG-tagged KIAA0537 cDNA. When the cDNA was expressed in HepG2 cells, Western blotting using monoclonal and polyclonal antibodies against FLAG and SNARK gave a common 74-kDa band, suggesting that KIAA0537 cDNA encodes the above mentioned unknown 74-kDa protein (Fig. 1*C*). Because four factors, AMPK-α1, AMPK-α2, MELK, and SNARK, have been identified as the catalytic subunits of the AMPK family (Fig. 1*D*), we tentatively designated KIAA0537 as ARK5.

Homology search analysis of the ARK5 amino acid sequence revealed that it has 55.0% overall homology to human SNARK (data not shown). It also shows 47.0, 45.8, and 42.4% homology to AMPK-α1, AMP-α2, and MELK respectively (data not shown). These data strongly suggest that ARK5 is a serine/threonine protein kinase activated by AMP. This possibility was directly examined by transfecting an ARK5 expression vector into HepG2 cells (H/ARK), which do not otherwise show any cross-reactivity to anti-SNARK for the 74-kDa band (Figs. 1*A* and 2*A*). As shown in Fig. 2*A*, H/ARK showed a distinct band at 74 kDa. This was further confirmed by the immunoprecipitation method. Cellular extracts from parental HepG2 and H/ARK cell lines were immunoprecipitated with an anti-FLAG antibody first, and both the supernatant and the immunoprecipitates were subjected to Western blot analysis using anti-SNARK antiserum. Only immunoprecipitates from H/ARK cells gave strong reactivity (Fig. 2*A*). *In vitro* kinase activity showed a clear increase using GST-SAMS as a substrate after transfection. The activity was further stimulated in the presence of 200 μM AMP (Fig. 2*B*), indicating strongly that ARK5 is a novel member of the AMPK family. β- and γ-subunits of AMPK have been well known as the binding proteins of AMPK-α1 and AMPK-α2 (12, 26). In the previous report (13), it appeared that rat SNARK showed the enzyme activity without any binding protein. In the current study, immunoprecipitation and silver staining technologies showed that binding proteins were not required for the enzymatic activity of ARK5 (data not shown).

We have proposed that tolerance to nutrient starvation is one of the determinants of tumor malignancy (16, 18), and tolerance to glucose starvation can be induced by the activation of AMPK (18) or the hypoxic condition (17). In this context, the involvement of ARK5 in the mechanism for tolerance to glucose starvation was examined. When a human hepatoma cell line, HepG2, was subjected to glucose starvation under normoxic (21% O_2) conditions, more than 90% of the cells underwent necrotic cell death during 24 h treatment, but only 40% of H/ARK cells showed cell death during the same treatment (Fig. 2*C*). We prepared an antisense RNA expression vector of ARK5 (ARK5(AS)). When HepG2 or PANC-1 cells were transiently transfected with ARK5(AS), mRNA expression of ARK5 was specifically suppressed (Fig. 2*D*). When cells were subjected to glucose starvation at 1% oxygen tension, >90% of HepG2 cells survived, but ~50% cells died when ARK5(AS) was transfected

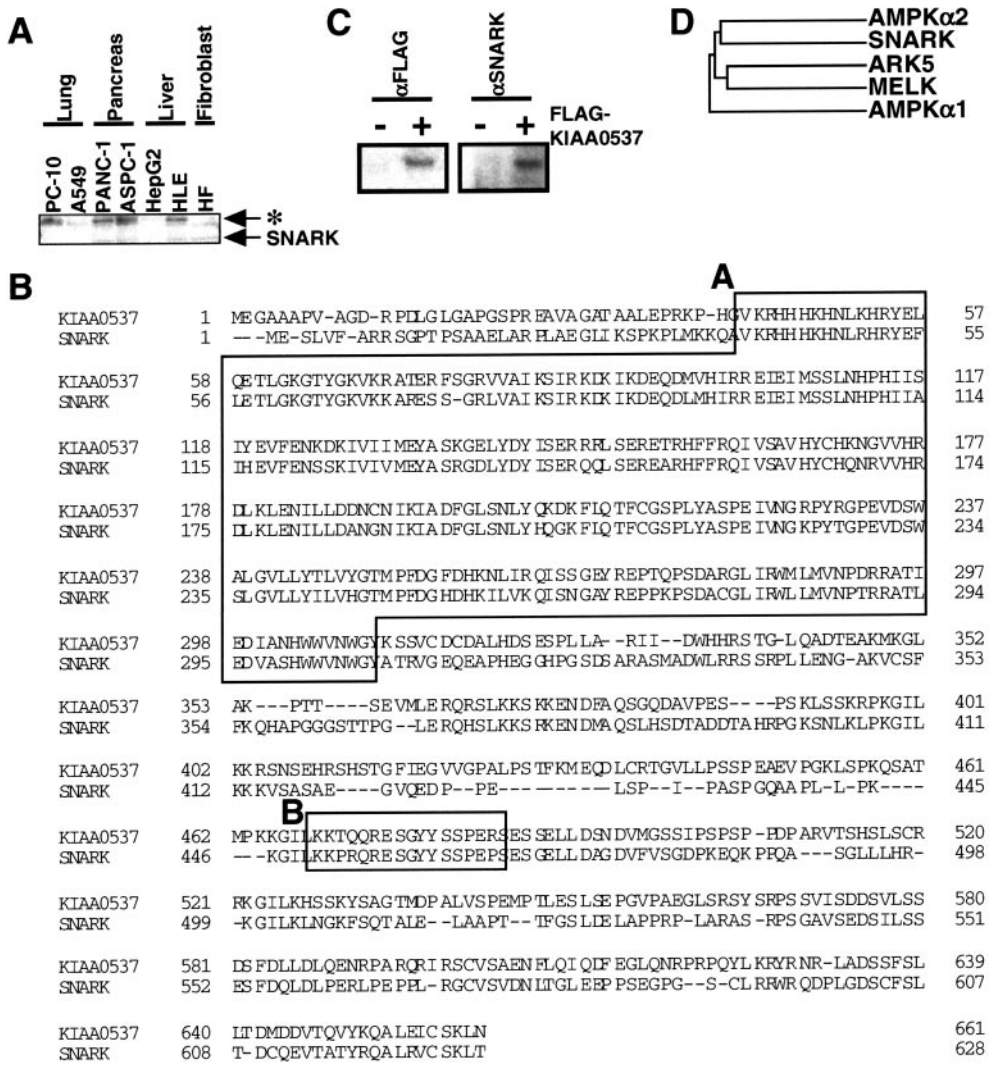

FIG. 1. **Identification of KIAA0537 as ARK5.** *A*, cell extracts from several lines were Western blotted with SNARK antibody. *Asterisk* shows the position of an unknown 74 kDa protein. *B*, putative amino acid sequence of KIAA0537 (*top lines*) and human SNARK (*bottom lines*). *Box A*, the region of putative catalytic domain. *Box B*, amino acid sequence used for the preparation of SNARK antibody. *C*, cell extracts from HepG2 transfected with (+) or without (−) an expression vector of FLAG-KIAA0537 were Western blotted with antibody against FLAG (*αFLAG*) or SNARK (*αSNARK*). *D*, evolutional tree of molecules belonging to AMPK family.

into HepG2 cells (Fig. 2*E*). These results clearly showed that ARK5 is involved in tolerance to glucose starvation.

ARK5 activity in whole cell extracts and anti-FLAG immunoprecipitates was measured as the GST-SAMS phosphorylation during glucose starvation. As shown in Fig. 2*F*, increased activity was observed in cell extracts and immunoprecipitates from H/ARK cells but not from HepG2 cells. In addition, more increased activity was detected when H/ARK cells were subjected to glucose-free medium for 1 h (Fig. 2*F*).

ARK5 Is Activated by Akt through Phosphorylation at Ser^{600}—We have previously found that another protein kinase, Akt, is activated immediately after nutrient starvation in HepG2 cells (16). As is evident in Fig 3*A*, Akt was phosphorylated rapidly. Amino acid sequence analysis of ARK5 revealed that there is a perfectly conserved putative Akt phosphorylation site in the C-terminal portion as shown in Fig. 3*B*. However, the putative Akt phosphorylation site was not detected in AMPK-α1, AMPK-α2, and SNARK (data not shown). These observations strongly indicate that ARK5 is activated by Akt through phosphorylation at Ser^{600}. This possibility was directly examined *in vitro*. A point mutant of ARK5 at Ser^{600} was constructed by substituting Ser with Ala, and an expression vector with a FLAG tag was constructed (ARK5(S600A)). Both wild-type and ARK5(S600A) were transfected into HepG2 cells, and FLAG-tagged ARK5 and ARK5(S600A) were purified by immunoprecipitation with anti-FLAG antibody-conjugated agarose. ARK5 phosphorylation was examined after incubation with or without PDK1-activated Akt1 *in vitro*. As shown in Fig. 3*C*, only ARK5 but not ARK5(S600A) was phosphorylated by Akt. In addition, an increased phosphorylation of GST-SAMS was induced by ARK5 but not by ARK5(S600A) in the presence of active Akt1 (Fig. 3*D*), suggesting that ARK5 phosphorylated by active Akt1 is enzymatically "active". To further examine the activation of ARK5 by Akt, physiological interaction of these components *in vivo* was examined by ARK5 and Akt co-immunoprecipitation. The results clearly showed that ARK5 and Akt were in association with each other, but they dissociated just after Akt activation either by glucose starvation or insulin treatment that activates Akt (Fig. 3*E*), which is consistent with the case of Mdm2 phosphorylation by Akt (27). No Akt was co-immunoprecipitated from HepG2 cells with anti-FLAG antibody (data not shown).

To determine whether enzymatic activation of ARK5 is really required for tolerance to glucose starvation, the effects of transfection of ARK5 mutants and the dominant active and dominant negative forms of Akt as well as the chemical inhibitor of

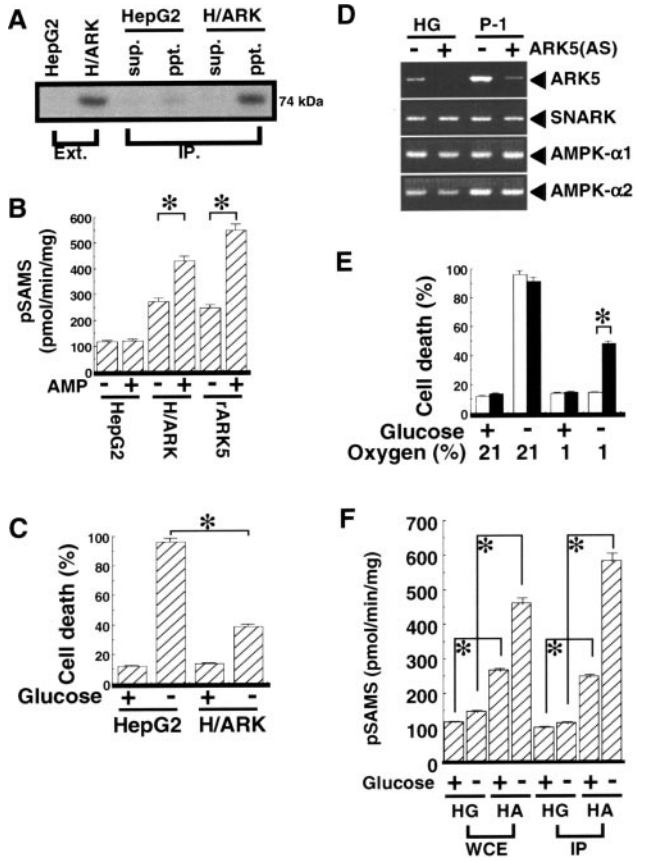

FIG. 2. **Characterization of ARK5 as an AMPK family member.** *A*, Western blotting analysis with SNARK antibody was performed on cell extracts (*Ext.*) or immunoprecipitates (*IP.*) with FLAG antibody (*sup.*, supernatant; *ppt.*, immunopellet) from HepG2 or H/ARK cells. *B*, SAMS phosphorylation by cell extracts (*HepG2* and *H/ARK*) or recombinant ARK5 (*rARK5*) was measured in the presence (+) or absence (−) of 200 μM AMP. Enzyme activity is shown as means of three experiments, and the *bars* represent S.E. value. *Asterisks* show the statistical significance $p < 0.01$ (*t* test). *C*, cell death induction was measured in HepG2 or H/ARK cells subjected to medium with (+) or without (−) glucose for 24 h. Ratio of cell death induction is shown as means of three experiments, and the *bars* represent S.E. value. *Asterisks* show the statistical significance $p < 0.01$ (*t* test). *D*, expressions of ARK5, SNARK, AMPK-α1, and AMPK-α2 were examined with the reverse transcription PCR method to total RNA (0.5 μg) from HepG2 (*HG*) or PANC-1 (*P-1*) cell lines transiently transfected with ARK5(AS). *E,* HepG2 cells transfected with (*filled bars*) or without (*open bars*) ARK5 antisense were subjected to medium with (+) or without (−) glucose under normoxic (21%) or hypoxic (1%) oxygen tension for 24 h. *F*, SAMS phosphorylation activity of cell extracts (*WCE*) or immunoprecipitates (*IP*) from HepG2 (*HG*) or H/ARK (*HA*) cells cultured in medium with (+) or without (−) glucose for 1 h was measured. Enzyme activity is shown as means of three experiments and the *bars* represent S.E. value. *Asterisks* show the statistical significance $p < 0.01$ (*t* test).

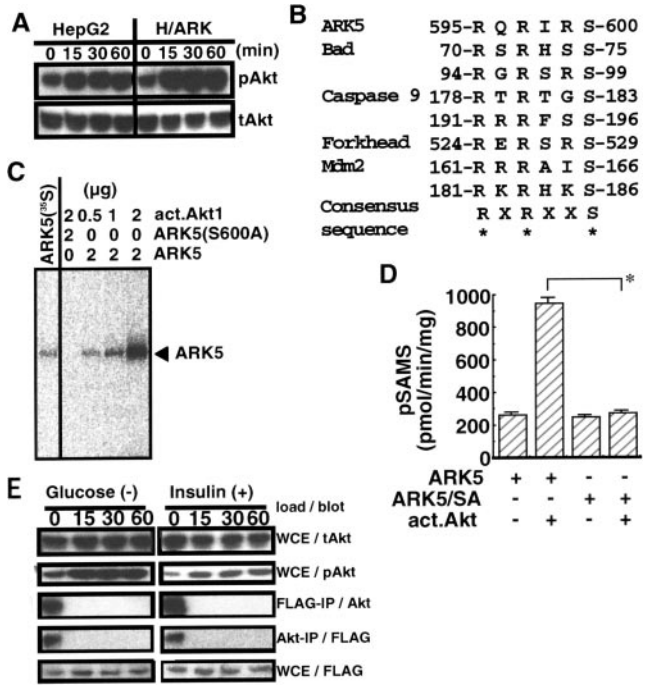

FIG. 3. **ARK5 is a novel substrate of Akt.** *A*, cell extracts from HepG2 or H/ARK cells subjected to glucose starvation for the indicated periods were Western blotted with polyclonal antibody against phospho Akt (*pAkt*) or total Akt (*tAkt*). *B*, comparison of Akt phosphorylation sites in ARK5 and other substrates. *C*, ARK5 or ARK5(S600A) was incubated with active Akt1 (*act.Akt1*) *in vitro*. After incubation, immunoprecipitates with anti-FLAG antibody were separated on SDS-PAGE. Immunoprecipitate with anti-FLAG antibody from H/ARK cells labeled with [^{35}S]methionine was also separated on SDS-PAGE. *D,* ARK5 and ARK5(S600A) was were incubated with (+) or without (−) active Akt1 (*act.Akt1*) *in vitro*. After incubation, SAMS phosphorylation was measured. ^{32}P incorporation is shown as means of three experiments, and the *bars* represent S.E. value. *Asterisks* show the statistical significance $p < 0.01$ (*t* test). *E*, H/ARK cells were subjected to glucose starvation or insulin stimulation for 0–60 min, and whole cell extracts (*WCE*) or immunoprecipitates with antibody against FLAG (*FLAG-IP*) or Akt (*Akt-IP*) were blotted with antibody for total Akt (*tAkt*), phospho Akt (*pAkt*) or FLAG.

Akt activation on HepG2 survival during glucose starvation were examined. As shown in Fig. 4*A*, >90% of HepG2 cells underwent necrotic cell death during 24 h of glucose starvation. Necrotic cell death was markedly suppressed to ~40% by transfecting wild-type ARK5 but not ARK5(S600A) or ARK5(ΔAPM) (ARK5 lacking 66 amino acids in the C-terminal region including the Akt phosphorylation site). When dominant active (DA-Akt), but not dominant negative, (DN-Akt) Akt was transfected into HepG2, cell death induction was also suppressed significantly. The combined expression of ARK5 and dominant active Akt suppressed cell death further, down to ~20%, but dominant negative Akt and/or mutants of ARK5 failed to do so (Fig. 4*A*). These results clearly indicated that ARK5 activated by active Akt is essential for tolerance to glucose starvation in HepG2 cells. These observations were further confirmed using H/ARK cells (Fig. 4*B*). When dominant active Akt is introduced into H/ARK cells, cell death was remarkably suppressed (Fig. 4*B*). However, when dominant negative Akt was used, induction of cell death occurred to a greater extent (Fig. 4*B*). When an inhibitor of phosphatidylinositol-3 kinase, LY294002 (28), was included, activation of Akt was clearly inhibited with a concomitant increase in cell death. These results are consistent with those for transient transfections.

ATM Is a Candidate for Being the Target of ARK5—Tumor tolerance against nutrient starvation is found to be modulated by AMPK (18, 29), and AMPK activation induces a G_1 cell cycle delay as a result of p53 stabilization (19). Because ATM is well known as a p53 activator, we suspected that ATM might be a target of AMPKs. As shown in Fig. 5*A*, amino acid sequence analysis revealed that ATM contains some putative phosphorylation sites for Akt (two sites) and AMPK (three sites). Using the PANC-1 cell in which His-tagged ATM (30) is stably expressed (P/ATM), an *in vivo* ^{32}P-labeling study was performed. ^{32}P incorporation into His-ATM was detected in P/ATM cells cultured in ordinary medium, and an increase in ATM phosphorylation was detected when P/ATM cells were subjected to glucose starvation (Fig. 5*B*). Interestingly, ATM phosphorylation was completely suppressed by the transient expression of an antisense RNA expression vector of ARK5, and an increased phosphorylation of ATM was detected in cells transiently ex-

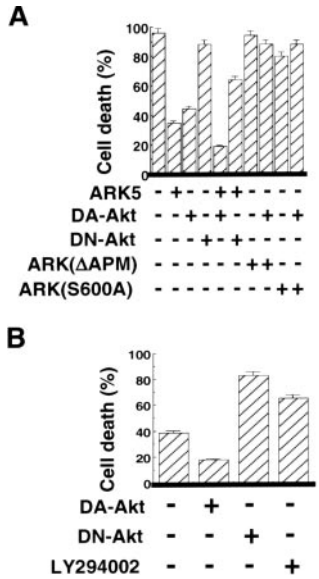

FIG. 4. **ARK5 mediates Akt-induced cell survival signaling.** *A*, HepG2 cells transiently transfected with (+) or without (−) ARK5, dominant active Akt (*DA-Akt*), dominant negative Akt (*DN-Akt*), ARK5-deletion mutant (*ARK(ΔAPM)*) and/or ARK5-point mutant (*ARK(S600A)*) were subjected to glucose starvation for 24 h. Ratio of cell death induction is shown as means of three experiments, and the *bars* represent S.E. value. *B*, H/ARK cells transiently transfected with or without DA-Akt or DN-Akt were subjected to glucose starvation in the presence or absence of LY294002 (20 μM) for 24 h.

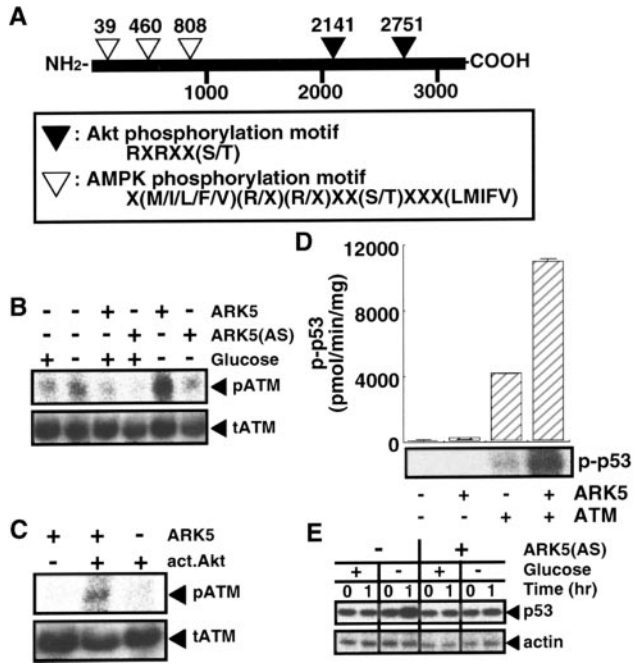

FIG. 5. **ARK5-induced ATM activation.** *A*, schematic model of phosphorylation site by AMPK or Akt was indicated by *arrowhead* on ATM sequence. *B*, P/ATM cells were transfected with (+) or without (−) ARK5 or ARK5(AS) for 24 h. After transfection, *in vivo* labeling of ^{32}P was performed, and cells were incubated in media with or without glucose for 1 h. His-ATM was collected with nickel-agarose and phosphorylated ATM (*pATM*; ^{32}P-autoradiography) and total ATM (*tATM*; Western blotting of His-ATM with anti-human ATM antibody) were detected. *C*, His-ATM (2 μg) was reacted with or without ARK5 (2 μg) in the presence or absence of active Akt1 (*act.Akt1;* 2 μg). *D*, ATM kinase assay was performed with full-length recombinant protein of human p53 in the presence or absence of His-ATM (2 μg) and/or ARK5 (2 μg). *E*, HepG2 cells transiently transfected with (+) or without (−) ARK5(AS). After 24 h, cells were subjected to medium with (+) or without (−) glucose for 1 h, and then Western blotting using antibodies for p53 and actin was performed.

pressing ARK5 (Fig. 5*B*), suggesting that ARK5 phosphorylates ATM during glucose starvation. To further confirm this possibility, *in vitro* phosphorylation was examined. As shown in Fig. 5*C*, ARK5- or PDK1-activated Akt1 alone did not phosphorylate ATM; however, ATM phosphorylation was detected in the presence of both ARK5 and active Akt1, indicating that ARK5 phosphorylates ATM directly. To determine that this phosphorylation of ATM was activating ATM kinase, we assayed for phosphorylation of p53, which has been shown to be a downstream effector of ATM (23–25). The results in Fig. 5*D* reveal that p53 is phosphorylated in the presence of both ATM and ARK5, suggesting that ARK5-phosphorylated ATM is functionally active. When HepG2 cells were subjected to glucose-free medium for 1 h, an accumulation of p53 protein was observed (Fig. 5*E*). This p53 accumulation induced by glucose starvation was completely suppressed by the transient transfection of ARK5(AS) (Fig. 5*E*). These results indicate that ARK5 activated during glucose starvation induces p53 accumulation through the functional phosphorylation of ATM in tumor cells.

DISCUSSION

We recently found that some tumor cells have a strong tolerance to nutrient starvation; tolerance to glucose starvation can be induced by hypoxia in normal human fibroblasts and the human hepatoma HepG2 cells, which are otherwise quite sensitive to glucose starvation. Akt and AMPK appear to be involved closely in the mechanism of tolerance (16–18). Taking all these findings into consideration, we suspected that there might be a connection between Akt and AMPK signaling pathways. The consensus sequence of the Akt phosphorylation is conserved in several species. ARK5, but not other members of AMPK family, contains this sequence at amino acids 595–600, and direct activation by Akt was demonstrated by phosphorylation *in vitro* in this study. This observation strongly indicates that ARK5 is a novel target of Akt. During glucose starvation, cell survival was induced by ARK5, and ARK5 mutants blocked DA-Akt1-induced cell survival, indicating that ARK5 acts as the tumor cell survival factor down-stream to Akt in HepG2 cells. In previous studies, we found that the antisense RNA expression constructs for AMPK-α1 and AMP-α2 subunits suppressed hypoxia-induced tolerance to glucose starvation in HepG2 cells (17) and constitutive tolerance in PANC-1 cells (29), indicating a close involvement of AMPK-α1 and AMP-α2 in tumor cell tolerance during glucose starvation. AMPK-α1 appeared to be more responsive to hypoxic conditions than AMPK-α2 (17). In our previous reports (16, 17), we also demonstrated an important role of Akt in tumor cell tolerance induced by hypoxia. The Akt phosphorylation was suppressed by the PI3K inhibitor LY294002 and wortmannin, but not by the AMPK inhibitor AraA (16, 17), suggesting an involvement of PI3K but not AMPK in the phosphorylation of Akt during glucose starvation and hypoxia. Because only ARK5 but not any other member of the AMPK family is responsive to Akt, it appears that ARK5 acts as the key mediator of Akt in tumor cell tolerance during glucose starvation. ARK5, the activation of which is induced by Akt during glucose starvation and hypoxia, may influence AMPK-α1 and AMPK-α2 to induce tumor cell tolerance to glucose starvation under the hypoxic condition.

Recently, we demonstrated that the AMPK family is involved in cell cycle arrest via p53 accumulation (19). ATM is a member of the phosphatidylinositol kinase family (20–22) and in response to radiation exposure activates the G_1/S checkpoint via p53 phosphorylation (23, 24). In response to DNA damage, activated ATM phosphorylates p53 directly on Ser^{15} and indirectly through Chk2 on Ser^{20}, causing cell cycle arrest or cell death (23–25). In addition to acting as a sensor of DNA dam-

age, it has been suggested that ATM is also a sensor of oxidative stress (31, 32). Evidence for increased oxidative stress in A-T cells and in ATM gene-disrupted mice supports this suggestion (33, 34). Nutrient starvation induces necrotic cell death in HepG2 cells, and a comet assay revealed that necrotic cell death is not due to DNA double strand break (data not shown). Recently, we found ATM-induced phosphorylation and accumulation of p53 following p21 up-regulation and cell cycle arrest at the G_1 phase in HepG2 cells during glucose starvation.[2] Our current observations that ARK5 phosphorylated ATM *in vitro* and *in vivo* in response to glucose starvation together with an involvement of the ATM/p53 pathway in maintaining cell survival during glucose deprivation provide additional support for a more general role for ATM in intracellular signaling. Amino acid sequence analysis revealed that ATM contained consensus sequences phosphorylated by AMPK and Akt. Other evidence of a role for ATM in non-damage signaling has been provided (35) where insulin activated ATM kinase to phosphorylate 4E-BPI (PHAS-1), a regulator of protein synthesis. The results described here are pertinent to that report (Ref. 35) in that we have demonstrated that insulin treatment activates Akt, which in turn activates ARK5, and this may lead to activation of ATM, which we also showed is a downstream effector of ARK5 under conditions of glucose starvation. The activation of ATM through this pathway would add an additional pathway to the scheme proposed after insulin treatment (21).

In the current study, we identified ARK5 as a novel member of the AMPK family. ARK5 activation is regulated by Akt, and activated ARK5, in turn, phosphorylated ATM during glucose starvation. Because phosphorylated ATM induces cell cycle arrest at the G_1 phase via p53 phosphorylation and accumulation (23–25), the present findings are consistent with our previous notion that the AMPK family is involved in p53 accumulation during glucose starvation (19). ARK5 mRNA expression in human normal tissue was observed in the heart, brain, skeletal muscle, kidney, and ovary, but not in the liver, pancreas, lung and intestine (official data of KIAA0537 on the KAZUSA DNA Research Institute homepage); however, protein and mRNA expression of ARK5 was observed in some tumor cell lines derived from hepatoma and pancreatic cancer, suggesting a possible involvement of ARK5 in tumorigenesis. On the basis of our current results, we propose here that ARK5 plays a key role in the tumor tolerance to nutrient starvation related to tumor malignancy. Therefore, ARK5 is a new target for cancer therapy.

Acknowledgment—We thank KAZUSA DNA Research Institute for KIAA0537 cDNA clone (GenBank™ accession number AB011109).

[2] G. Kusakai, A. Suzuki, A. Kishimoto, J. Lu, T. Ogura, M. Lavin, and H. Esumi, submitted for publication.

REFERENCES

1. Carlson, M. (1999) *Curr. Opin. Microbiol.* **2,** 202–207
2. Hardie, G. D., Carling, D., and Carlson, M. (1998) *Annu. Rev. Biochem.* **67,** 821–855
3. Becker, W., Heukelbach, J., Heiner, K., and Joost, H.-G. (1996) *Eur. J. Biochem.* **235,** 736–743
4. Gardner, H. P., Wertheim, G. B., Ha, S. I., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Marquis, S. T., and Chodosh, L. A. (2000) *Genomics* **63,** 46–59
5. Heyer, B. S., Warsowe, J., Solter, D., Knowles, B. B., and Ackerman, S. L. (1997) *Mol. Reprod. Dev.* **47,** 148–156
6. Inglis, J. D., Lee, M., and Hill, R. E. (1993) *Mamm. Genome* **4,** 401–403
7. Ruiz, J. C., Conlon, F. L., and Robertson, E. J. (1994) *Mech. Devel.* **48,** 153–164
8. Wang, Z.-N., Takemori, H., Halder, S. K., Nonaka, Y., and Okamoto, M. (1999) *FEBS Lett.* **453,** 135–139
9. Aguan, K., Scott, J., See, C. G., and Sarkar, N. H. (1994) *Gene* **149,** 345–350
10. Carling, D., Clarke, P. R., Zammit, V. A., and Hardie, D. G. (1989) *Eur. J. Biochem.* **186,** 129–136
11. Davies, S. P., Sim, A. T., and Hardie, D. G. (1989) *Eur. J. Biochem.* **186,** 123–128
12. Stapleton, D., Mitchelhill, K. I., Gao, G., Widmer, J., Michell, B. J., Teh, T., House, C. M., Fernandez, C. S., Cox, T., Witters, L. A., and Kemp, B. E. (1996) *J. Biol. Chem.* **271**, 611–614
13. Lefebvre, D. L., Bai, Y., Shahmolky, N., Poon, R., Drucker, D. J., and Rosen, C. F. (2001) *Biochem. J.* **355,** 297–305
14. Hawley, S. A., Davidson, M., Woods, A., Davies, S. P., Beri, R. K., Carling, D., and Hardie, D. G. (1996) *J. Biol. Chem.* **271,** 27879–27887
15. Sullivan, J. E., Carey, F., Carling, D., and Beri, R. K. (1994) *Biochem. Biophys. Res. Commun.* **200,** 1551–1556
16. Izuishi, K., Kato, K., Ogura, T., Kinoshita, T., and Esumi, H. (2000) *Cancer Res.* **60,** 6201–6207
17. Esumi, H., Izuishi, K., Kato, K., Hashimoto, K., Kurashima, Y., Kishimoto, A., Ogura, T., and Ozawa, T. (2002) *J. Biol. Chem.* **277**, 32791–32798
18. Hashimoto, K., Kato, K., Imamura, K., Kishimoto, A., Yoshikawa, H., Taketani, Y., and Esumi, H. (2002) *Biochem. Biophys. Res. Commun.* **290,** 263–267
19. Imamura, K., Ogura, T., Kishimoto, A., Kaminishi, M., and Esumi, H. (2001) *Biochem. Biophys. Res. Commun.* **287,** 562–567
20. Lavin, M. F., and Shiloh, Y. (1997) *Annu. Rev. Immunol.* **15,** 177–202
21. Lavin, M. F. (2000) *Nat. Cell Biol.* **2,** 215–217
22. Savitsky, K., Bar-Shira, A., Gilad, S., Rotman, G., Ziv, Y., Vanagaite, L., Tagle, D. A., Smith, S., Uziel, T., and Sfez, S., Ashkenazi, M., Pecker, I., Frydman, M., Harnik, R., Patanjali, S. R., Simmons, A., Clines, G. A., Sartiel, A., Gatti, R. A., Chessa, L., Sanal, O., Lavin, M. F., Jaspers, N. G. J., Malcolm, A., Taylor, R., Arlett, C. F., Miki, T., Weissman, S. M., Lovett, M., Collins, F. S., and Shiloh, Y. (1995) *Science* **268,** 1749–1753
23. Banin, S., Moyal, L., Shieh, S., Taya, Y., Anderson, C. W., Chessa, L., Smorodinsky, N. I., Prives, C., Reiss, Y., Shiloh, Y., and Ziv, Y. (1998) *Science* **281,** 1674–1677
24. Canman, C. E., Lim, D. S., Cimprich, K. A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M. B., and Siliciano, J. D. (1998) *Science* **281,** 1677–1679
25. Sheih, S. Y., Ikeda, M., Taya, Y., and Prives, C. (1997) *Cell* **91,** 325–334
26. Hardie, D. G., and Carling, D. (1997) *Eur. J. Biochem.* **246,** 259–273
27. Mayo, L. D., and Donner, D. B. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 11598–11603
28. Vlahos, C. J., Matter, W. F., Hui, K. Y., and Brown, R. F. (1994) *J. Biol. Chem.* **269,** 5241–5248
29. Kato, K., Ogura, T., Kishimoto, A., Minegishi, Y., Nakajima, N., Miyazaki, M., and Esumi, H. (2002) *Oncogene* **21,** 6082–6090
30. Zhang, N., Chen, P., Khanna, K. K., Scott, S., Gatei, M., Kozlov, S., Watters, D., Spring, K., Yen, T., and Lavin, M. F. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 8021–8026
31. Rotman, G., and Shiloh, Y. (1997) *Cancer Surv.* **29,** 285–304
32. Pearce, A. K., and Humphrey, T. C. (2001) *Trends Cell Biol.* **11,** 426–433
33. Barlow, C., Brown, K. D., Deng, C. X., Tagle, D. A., and Wynshaw-Boris, A. (1997) *Nat. Genet.* **17,** 453–456
34. Gatei, M., Shkedy, D., Khanna, K. K., Uziel, T., Shiloh, Y., Pandita, T. K., Lavin, M. F., and Rotman, G. (2001) *Oncogene* **20,** 289–294
35. Yang, D. Q., and Kastan, M. B. (2000) *Nat. Cell Biol.* **2,** 893–898

Identification of a Novel Protein Kinase Mediating Akt Survival Signaling to the ATM Protein

Atsushi Suzuki, Gen-ichi Kusakai, Atsuhiro Kishimoto, Jie Lu, Tsutomu Ogura, Martin F. Lavin and Hiroyasu Esumi

J. Biol. Chem. 2003, 278:48-53.
doi: 10.1074/jbc.M206025200 originally published online October 29, 2002
