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LKB1 is a master kinase that activates 13 kinases of the AMPK subfamily, including MARK/PAR-1

Jose M Lizcano[1], Olga Göransson[1], Rachel Toth[1], Maria Deak[1], Nick A Morrice[1], Jérôme Boudeau[1], Simon A Hawley[2], Lina Udd[3], Tomi P Mäkelä[3], D Grahame Hardie[2] and Dario R Alessi[1,*]

[1]MRC Protein Phosphorylation Unit, School of Life Sciences, University of Dundee, Dundee, UK, [2]Division of Molecular Physiology, School of Life Sciences, University of Dundee, Dundee, UK and [3]Molecular Cancer Biology Program, Institute of Biomedicine and Helsinki University Central Hospital, Biomedicum Helsinki, University of Helsinki, Finland

We recently demonstrated that the LKB1 tumour suppressor kinase, in complex with the pseudokinase STRAD and the scaffolding protein MO25, phosphorylates and activates AMP-activated protein kinase (AMPK). A total of 12 human kinases (NUAK1, NUAK2, BRSK1, BRSK2, QIK, QSK, SIK, MARK1, MARK2, MARK3, MARK4 and MELK) are related to AMPK. Here we demonstrate that LKB1 can phosphorylate the T-loop of all the members of this subfamily, apart from MELK, increasing their activity >50-fold. LKB1 catalytic activity and the presence of MO25 and STRAD are required for activation. Mutation of the T-loop Thr phosphorylated by LKB1 to Ala prevented activation, while mutation to glutamate produced active forms of many of the AMPK-related kinases. Activities of endogenous NUAK2, QIK, QSK, SIK, MARK1, MARK2/3 and MARK4 were markedly reduced in LKB1-deficient cells. Neither LKB1 activity nor that of AMPK-related kinases was stimulated by phenformin or AICAR, which activate AMPK. Our results show that LKB1 functions as a master upstream protein kinase, regulating AMPK-related kinases as well as AMPK. Between them, these kinases may mediate the physiological effects of LKB1, including its tumour suppressor function.

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Introduction

Mutations in the widely expressed LKB1 protein kinase in humans result in a disorder termed Peutz–Jeghers syndrome (PJS), which predisposes to a wide spectrum of benign and malignant tumours (Hemminki *et al*, 1998; Jenne *et al*, 1998). $LKB1^{+/-}$ heterozygous mice develop tumours resembling those found in PJS in humans (reviewed in Boudeau *et al*, 2003c). The overexpression of LKB1 in LKB1-deficient cancer cells induced a G1 cell cycle arrest (Tiainen *et al*, 1999, 2002)l, and genetic studies in *Caenorhabditis elegans* (Watts *et al*, 2000), *Drosophila* (Martin and St Johnston, 2003) and *Xenopus* (Ossipova *et al*, 2003) indicated that the LKB1 homologue in these organisms plays a role in regulating cell polarity. Taken together, these findings support the notion that the LKB1 protein kinase functions as a tumour suppressor and that the benign and malignant tumours in PJS patients could result in defects in the ability of cells to regulate their proliferation and/or polarity.

Recently, we demonstrated that LKB1 is activated through its interaction with STRAD (Baas *et al*, 2003) and MO25 (Boudeau *et al*, 2003a). STRAD possesses a domain with high sequence homology to protein kinases but lacks the key catalytic residues required for catalysis and has therefore been classified a 'pseudokinase'. MO25 bears no sequence homology to other proteins in the database, but recent studies indicate that it is structurally related to the Armadillo repeat domain (Milburn *et al*, 2004). In addition to activating LKB1, STRAD and MO25 anchor it in the cell cytoplasm (Baas *et al*, 2003; Boudeau *et al*, 2003a; Brajenovic *et al*, 2003), where LKB1 appears to exert its cell cycle-arresting function (Tiainen *et al*, 2002).

AMP-activated protein kinase (AMPK) is a sensor of cellular energy charge that regulates physiological processes that consume or regenerate ATP to restore the energy charge in the cell (Hardie *et al*, 2003). Both catalytic subunit isoforms of AMPK (AMPKα1 and AMPKα2) are activated by ATP-depleting processes such as exercise and cellular stress, through a rise in cellular AMP that accompanies the fall of ATP levels due to the reaction catalysed by adenylate kinase. AMPK is also activated by metformin, the drug most commonly employed for the treatment of type II diabetes (Zhou *et al*, 2001). The mechanism by which metformin, or its closely related analogue phenformin, activates AMPK is unknown but is not thought to involve changes in intracellular levels of AMP or ATP (Zhou *et al*, 2001; Hawley *et al*, 2002). The activation of AMPK by both ATP depletion and phenformin requires phosphorylation of the AMPK catalytic (α) subunit at its T-loop residue (Thr172 in AMPKα1) by an upstream kinase. Studies performed in *Saccharomyces cerevisiae* indicated that the T-loop residue of the yeast orthologue of AMPK (SNF1) was phosphorylated by a group of three related protein kinases bearing homology to LKB1 (Hong *et al*, 2003; Nath *et al*, 2003; Sutherland *et al*, 2003). We and others have shown that LKB1 functions to phosphorylate and activate AMPK in mammalian cells (Hawley *et al*, 2003; Woods *et al*, 2003a). Studies in cell-free systems demonstrated that LKB1 complexed to STRAD and MO25 activated AMPK by phosphorylating Thr172, and that the STRAD and MO25 subunits enhanced phosphorylation of AMPK by over 100-fold (Hawley *et al*, 2003). Moreover, AMPK could not be

*Corresponding author. MRC Protein Phosphorylation Unit, School of Life Sciences, University of Dundee, Dow Street, Dundee DD1 5EH, UK. Tel.: +44 1382 344 241; Fax: +44 1382 223 778;
E-mail: d.r.alessi@dundee.ac.uk

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activated in mammalian cells that lacked LKB1 expression (Hawley *et al*, 2003) or in cells that were treated with Hsp90 inhibitors (Woods *et al*, 2003a), which decreases LKB1 expression (Boudeau *et al*, 2003b).

Inspection of the human kinome indicates that there are 12 protein kinases (BRSK1, BRSK2, NUAK1, NUAK2, QIK, QSK, SIK, MARK1, MARK2, MARK3, MARK4 and MELK) that are closely related to AMPKα1 and AMPKα2 (Figure 1A). The nomenclature we will employ for the 'AMPK-related kinases' is based upon that used by Manning *et al* (2002). MARK3 is also known as PAR1A or C-TAK1 (Peng *et al*, 1998; Spicer *et al*, 2003), NUAK1 as ARK5 (Suzuki *et al*, 2003b), NUAK2 as SNARK (Lefebvre *et al*, 2001) and QIK as SIK2 (Horike *et al*, 2003). The MARK kinases have been proposed to play key roles in controlling cell polarity and are known to be regulated by phosphorylation of their T-loop Thr residue (Drewes and Nurse, 2003; Spicer *et al*, 2003). Little is known about the function or mechanism of regulation of the remaining AMPK-related enzymes. The T-loop Thr residue of AMPKα1 and AMPKα2 that is phosphorylated by LKB1, and many of the surrounding residues, is conserved in the AMPK-related kinases (Figure 1A). This indicates that LKB1 might also phosphorylate the T-loop Thr residue and hence regulate the AMPK-related kinases in the same way that PDK1 regulates the activity of a group of related AGC kinases (Alessi, 2001). In this study, we sought to investigate the role that LKB1 plays in regulating the activity of the AMPK-related kinases.

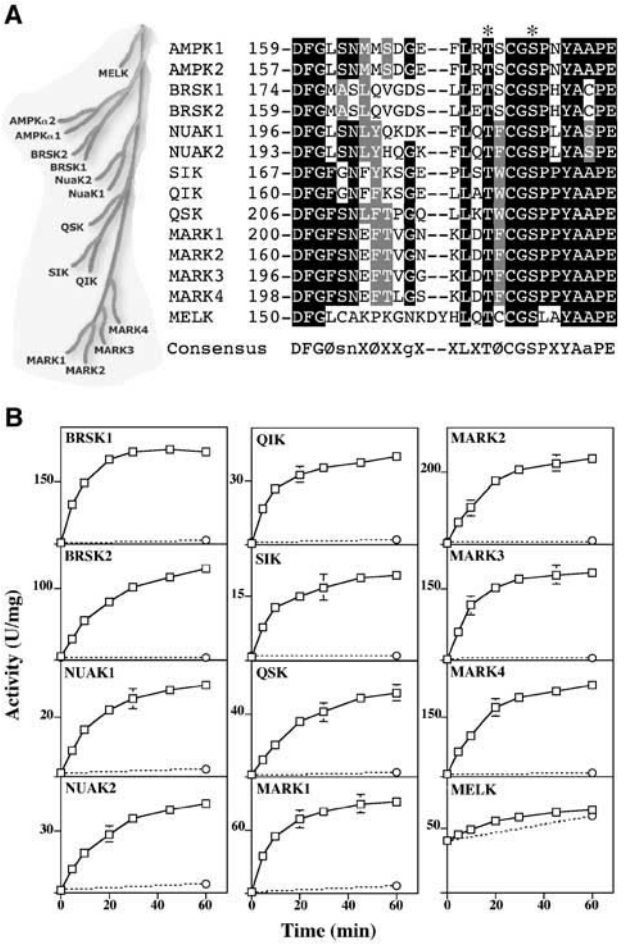

Figure 1 Activation of AMPK-related kinases by LKB1. (**A**) Dendrogram and T-loop sequences of AMPK subfamily of protein kinases (Manning *et al*, 2002). The identical residues are shaded black and the conserved residues in grey. The T-loop Thr and Ser are indicated with an asterisk. (**B**) The indicated AMPK-related kinases were incubated with wild-type LKB1:STRAD:MO25 (open squares) or catalytically inactive LKB1[D194A]:STRAD:MO25 (open circles) complexes in the presence of Mg^{2+} and ATP. At the indicated times, the activity of the AMPK-related kinases was assayed with the *AMARA* peptide, and the results are expressed as specific activity. Results shown are means ± s.d. of assays carried out in triplicate and representative of two independent experiments. The error bars are only shown when larger than the size of the open squares. The suggested consensus sequence for optimal LKB1 phosphorylation is indicated. Ø represents a large hydrophobic residue; X, any amino acid; s, n, g and a preferences for Ser, Asn, Gly and Ala, respectively.

Results

Activation of AMPK-related kinases by the LKB1 complex

We cloned and expressed in *Escherichia coli* the full-length versions of all of the 12 AMPK-related kinases and developed assays for these enzymes employing the *AMARA* peptide substrate for AMPK (Dale *et al*, 1995), which is not phosphorylated by the LKB1 complex (JM Lizcano, data not shown). We found that all of the AMPK-related kinases purified from *E. coli* phosphorylated this peptide but possessed low basal activities of <1 U/mg (1 U = 1 nmol peptide phosphorylated per minute), with the exception of MELK, which had an activity of $\sim$40 U/mg (Figure 1B). Following incubation of the AMPK-related kinases with the LKB1:STRAD:MO25 complex and MgATP, the activity of the AMPK-related kinases was increased 50- to 200-fold, except for MELK whose activity was hardly increased (Figure 1B). Catalytically inactive LKB1 complexed to STRADα and MO25α failed to increase the basal activity of the AMPK-related kinases, indicating that the kinase activity of LKB1 was required (Figure 1B).

Activation of AMPK-related kinases by LKB1 requires STRAD and MO25

To determine the importance of the STRAD and MO25 subunits in enabling LKB1 to activate the AMPK-related kinases, we expressed GST-tagged LKB1, FLAG-tagged STRADα/β and *myc*-tagged MO25α/β in various combinations in 293 cells, and affinity purified the complexes on glutathione–sepharose (Figure 2). The purified complexes were incubated in the presence of MgATP with the AMPK-related kinases and their activation was measured. LKB1 on its own did not activate any of the AMPK-related kinases significantly (Figure 2, compare lanes 1 and 14). The same result was obtained with LKB1 that had been coexpressed with MO25α or MO25β (Figure 2, lanes 4 and 5). This was as expected, as these proteins do not interact with LKB1 in the absence of STRADα/β (Boudeau *et al*, 2003a). An LKB1:STRADα complex gave a small activation (Figure 2, compare lanes 1 and 2), but a heterotrimeric complex containing LKB1, STRADα or STRADβ, and MO25α or MO25β was required to obtain a large activation (Figure 2, lanes 6–9). As previously reported for AMPKα1 (Figure 2; Hawley *et al*, 2003), all AMPK-related kinases were activated most efficiently by the LKB1:STRADα:MO25α complex, although the relative effectiveness of different heterotrimeric complexes varied from substrate to substrate (Figure 2). Heterotrimeric complexes containing a catalytically inactive mutant of LKB1 were

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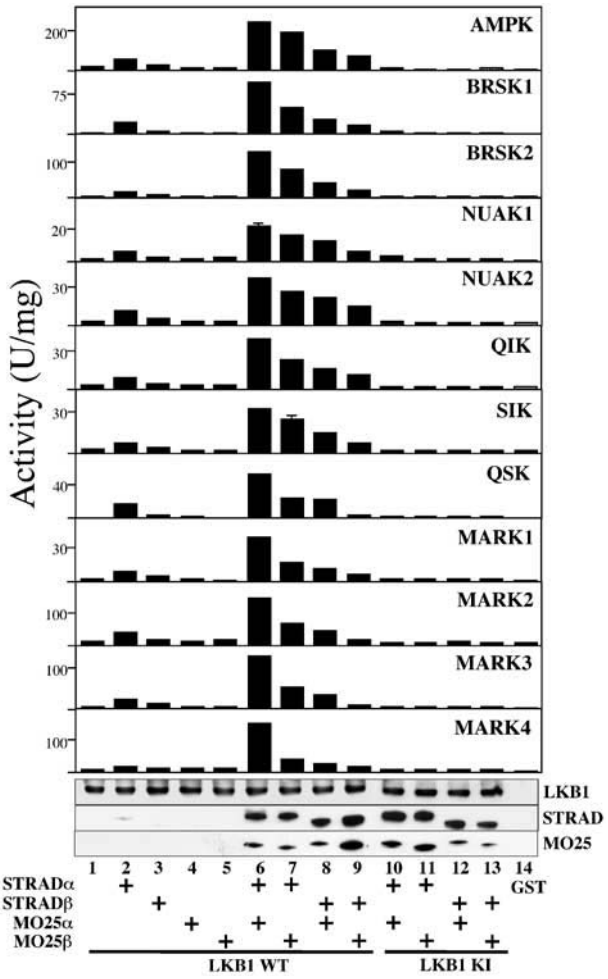

Figure 2 Efficient activation of AMPK-related kinases by LKB1 requires STRAD and MO25 subunits. The indicated combinations of GST-tagged wild-type LKB1 (WT, lanes 1–9), or catalytically inactive (KI, D194A, lanes 10–13) LKB1, or GST alone (lane 14), FLAG-tagged STRADα or STRADβ, and *myc*-tagged MO25α or MO25β were coexpressed in HEK-293T cells and purified on glutathione–sepharose. The complexes were tested for their ability to activate the catalytic domain of AMPKα1 or the indicated AMPK-related kinases. The results are expressed as specific activity employing the *AMARA* peptide as substrate. Results shown are means ± s.d. of assays carried out in triplicate and representative of two independent experiments. Samples from each incubation were also analysed by Western blotting and probed using the indicated antibodies (from top to bottom): anti-GST to detect LKB1; anti-FLAG to detect STRADα and STRADβ; and anti-*myc* to detect MO25α and MO25β. All proteins migrated with the expected mobility, taking into account the epitope tags.

unable to activate any enzyme (Figure 2, lanes 10–13). It should be noted that when STRAD isoforms were coexpressed with LKB1 in the absence of MO25, the amount of STRAD that co-precipitated with LKB1 was markedly reduced (Figure 2, compare lanes 2 and 3 with lanes 6 and 7), as MO25 is required to stabilise the LKB1:STRAD complex (Boudeau *et al*, 2003a).

The LKB1 complex phosphorylates AMPK-related kinases at the activation loop

As the LKB1 complex was previously shown to phosphorylate AMPKα1 specifically at Thr172 in its activation loop, we first mutated the equivalent T-loop residue in the AMPK-related kinases to Ala, and tested how this affected phosphorylation of these enzymes by the LKB1 complex. We found that the LKB1 complex phosphorylated the wild-type AMPK-related kinases, and mutation of the T-loop Thr residue to Ala abolished or significantly reduced phosphorylation (Figure 3). We also performed detailed phosphorylation site analysis of catalytically inactive BRSK2 and NUAK2 mutants that had been phosphorylated *in vitro* by the LKB1 complex (Supplementary Figure 1). The ^{32}P-labelled BRSK2 and NUAK2 proteins were digested with trypsin and the resulting peptides were separated by chromatography on a C_{18} column. The major ^{32}P-labelled peptides were analysed by MALDI TOF–TOF mass spectrometry and solid-phase Edman sequencing, which demonstrated that these enzymes were phosphorylated at their T-loop Thr residue (Supplementary Figure 1).

T-loop phosphorylation activates AMPK-related kinases

To study the role of T-loop phosphorylation of BRSK1, BRSK2, NUAK1, NUAK2, QIK, QSK, SIK and MELK in their regulation, the T-loop Thr residues of these enzymes were mutated to

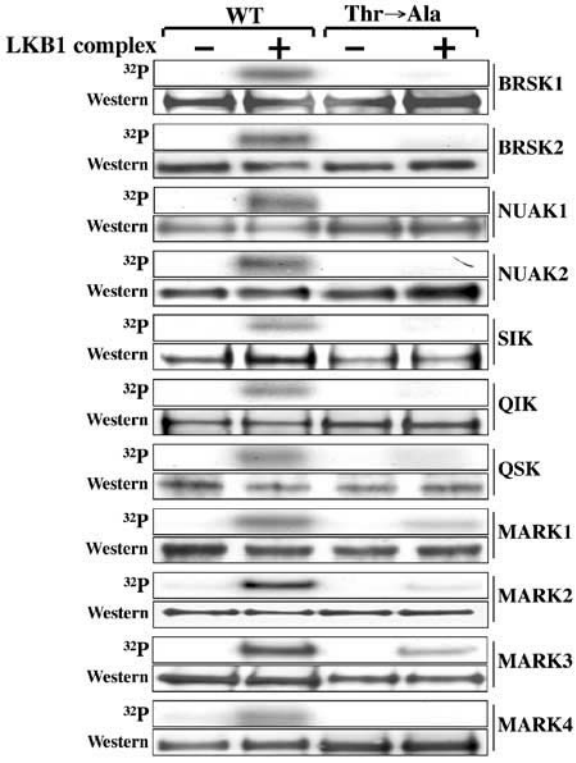

Figure 3 The T-loop Thr is the major site of LKB1 phosphorylation on the AMPK-related kinases. Wild-type (WT) and T-loop Thr to Ala (Thr→Ala) mutants of the indicated GST-AMPK-related kinases were incubated with the LKB1:STRAD:MO25 complex in the presence of Mg^{2+} and [γ^{32}P]ATP. Phosphorylation of protein substrates was determined by electrophoresis on a polyacrylamide gel and subsequent autoradiography of the Coomassie blue-stained bands corresponding to each substrate. An aliquot of each incubation was also analysed by Western blotting probing with an anti-HA antibody to ensure equal loading of wild-type and mutant AMPK-related kinases (which all possess an HA epitope tag). All proteins migrated with the expected mobility, taking into account the epitope tags. Similar results were obtained in three separate experiments.

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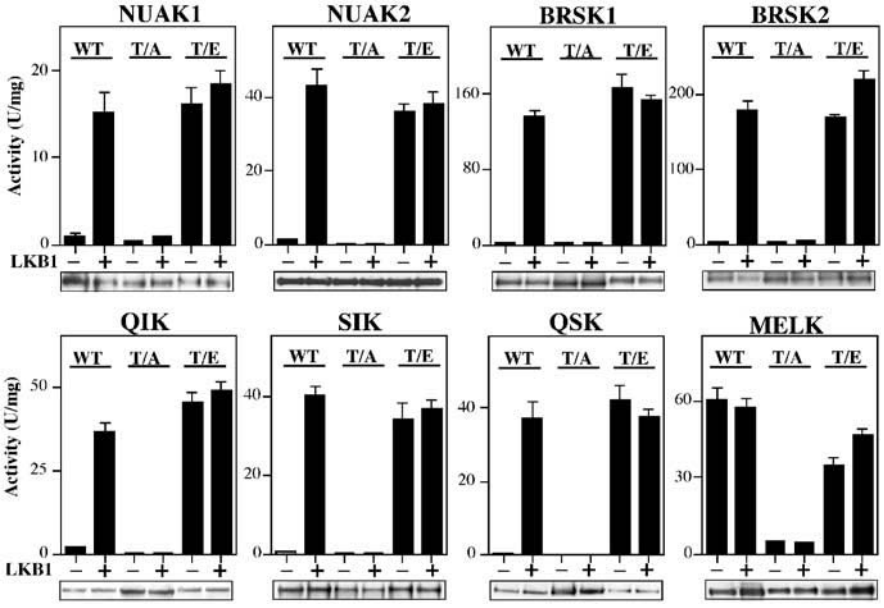

Figure 4 Effect of mutation of Thr in the T-loop on activation of AMPK-related kinases by LKB1. The indicated wild-type (WT) AMPK-related kinases or mutants of these enzymes in which the T-loop Thr was changed to either Ala (T/A) or Glu (T/E) were incubated in the absence (−) or presence (+) of wild-type LKB1:STRAD:MO25 in the presence of Mg^{2+} and ATP. After 30 min, the AMPK-related kinases were assayed with the *AMARA* peptide, and the results are expressed as specific activity. Results shown are means ± s.d. of assays carried out in triplicate and representative of two independent experiments. An aliquot of each incubation was also analysed by Western blotting probing with an anti-HA antibody to ensure equal loading of wild-type and mutant AMPK-related kinases (which all possess an HA tag).

either Ala to prevent phosphorylation or Glu to mimic phosphorylation. In all cases, the basal activity of the Ala mutants was low and their activity was not increased by incubation with the LKB1 complex (Figure 4). In contrast, the basal activity of the Glu mutants was similar to that of the wild-type enzyme phosphorylated by LKB1, and was not further increased following incubation with the LKB1 complex and MgATP (Figure 4). In the case of MELK, the Ala mutant possessed low activity and the Glu mutant was of similar activity to the wild-type enzyme, suggesting that wild-type MELK (like other AMPK-related kinases) required T-loop phosphorylation for activity. As wild-type MELK expressed in *E. coli* was active, MELK may be able to catalyse phosphorylation of its own T-loop Thr residue. Consistent with this, MALDI TOF–TOF analysis of wild-type MELK expressed in *E. coli* established that the T-loop was indeed phosphorylated (Supplementary Figure 1C).

Phosphorylation and activation of the MARK kinases by LKB1

Previous studies have revealed that the MARK2 and MARK3 kinases, in addition to being phosphorylated at the T-loop Thr residue, are also phosphorylated at a nearby Ser residue (Figure 1A; Drewes *et al*, 1997; Timm *et al*, 2003). Peptide mapping of catalytically inactive MARK3 phosphorylated by the LKB1 complex revealed that both the Thr211 and Ser215 residues within the T-loop were phosphorylated (Figure 5A). Interestingly, catalytically inactive MARK3[T211A] mutant was not phosphorylated at Ser215 by LKB1, but the catalytically inactive MARK3[S215A] mutant was still phosphorylated at Thr211 (Figure 5A). We next investigated how mutation of the T-loop Thr or Ser residue affected activation of MARK3 by the LKB1 complex (Figure 5C). The MARK3[T211A] mutant could not be activated by the LKB1 complex, but the MARK3[S215A] mutant was activated to a small extent. The MARK3[T211E] mutant possessed only ~15% of the activity of the wild-type enzyme activated by the LKB1 complex and could not be activated further by LKB1. The MARK3[S215E] mutant was inactive and could not be activated by LKB1 (Figure 5C). As expected, we also found that mutation of the T-loop Thr to Ala in MARK1, MARK2 and MARK4 prevented their activation by LKB1 (Figure 5D–F). Mutation of the T-loop Thr residue to Glu in MARK1, MARK2 and MARK3 resulted in either no activation or only a small activation of these enzymes (Figure 5D–F). In contrast, the equivalent mutation in MARK4 vastly increased activity (Figure 5F). Peptide-mapping studies and MALDI TOF–TOF mass spectrometry demonstrated that LKB1 phosphorylated the MARK4 T-loop at only the Thr residue and not the Ser residue (Figure 5G and Supplementary Figure 1C). Consistent with these findings, it has recently been reported that LKB1 immunoprecipitated from cell lysates can phosphorylate and activate MARK4 (Brajenovic *et al*, 2003).

Evidence of differing substrate specificities of AMPK-related kinases

We investigated the substrate specificity of the AMPK-related kinases by comparing the rate at which these enzymes phosphorylated the AMARA peptide and two other peptides that are also efficiently phosphorylated by AMPK, namely the SAMS peptide (Davies *et al*, 1989) and the LNR peptide (Ross *et al*, 2002). While all AMPK-related kinases phosphorylated all three peptides, there were marked differences in the relative rates at which the enzymes phosphorylated them (Supplementary Figure 2). For example, the BRSK and NUAK enzymes had a marked preference for the LNR peptide,

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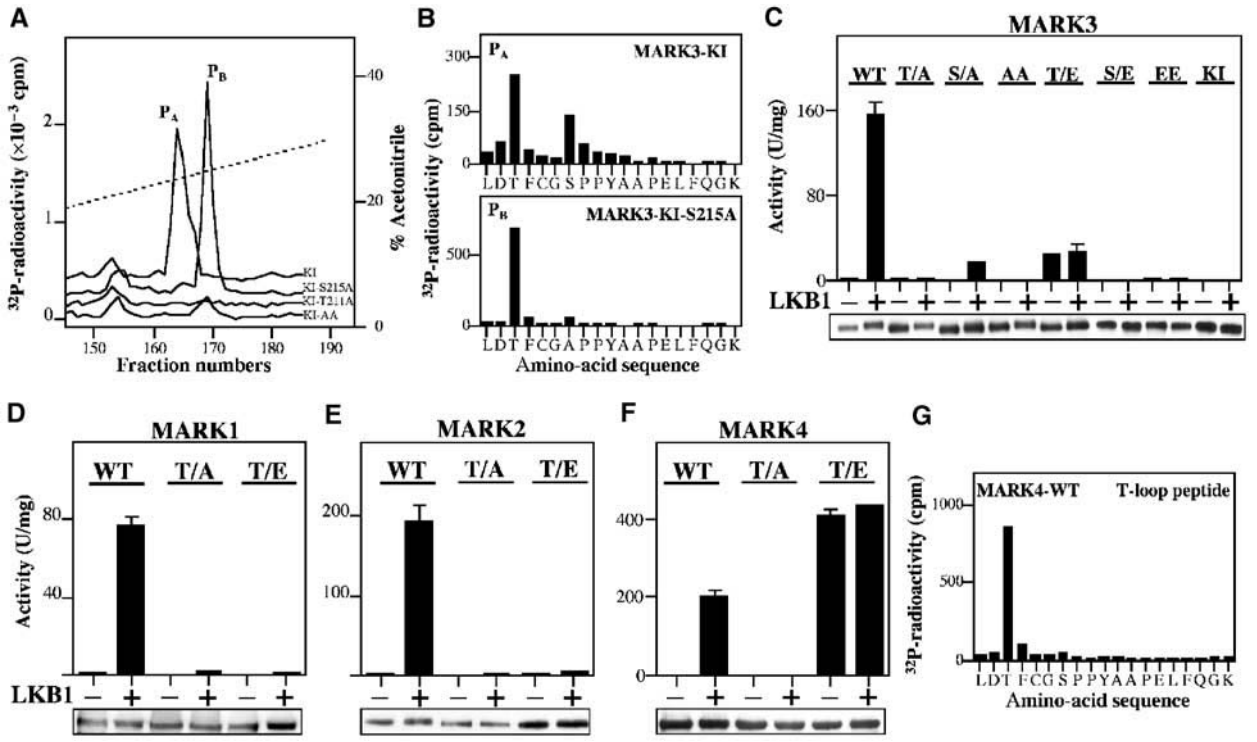

Figure 5 Analysis of phosphorylation and activation of MARK kinases. (**A**) Catalytically inactive MARK3[D196A] (KI), which cannot autophosphorylate, and the indicated mutants were incubated with the LKB1 complex for 30 min with Mg^{2+}-[γ^{32}P]ATP and separated by electrophoresis on a polyacrylamide gel, which was then autoradiographed. The ^{32}P-labelled MARK3 proteins were digested with trypsin and the resulting ^{32}P-labelled peptides were chromatographed on a C_{18} column. Fractions containing the ^{32}P-labelled T-loop tryptic peptide (peptides P_A and P_B) are shown. (**B**) Peptide P_A and P_B were subjected to solid-phase sequencing and ^{32}P-radioactivity was measured after each cycle of Edman degradation. In combination with MALDI TOF–TOF mass spectrometry, this enabled the identification of the sites phosphorylated in each peptide. Peptide P_A comprise the MARK3 T-loop peptide phosphorylated at Thr211 and Ser215, and Peptides P_B comprise the MARK3 T-loop peptide phosphorylated at Thr211. (**C–F**) The indicated wild-type (WT) or mutant forms of MARK kinases in which the the T-loop Thr or Ser was mutated to either Ala (T/A, S/A) or Glu to (T/E, S/E) were incubated in the absence (−) or presence (+) of wild-type LKB1:STRAD:MO25 in the presence of Mg^{2+} and ATP. After 30 min, the MARK kinases were assayed using the *AMARA* peptide, and the results are expressed as specific activity. An aliquot of each incubation was also analysed by Western blotting probing with an anti-HA antibody to ensure equal loading of wild-type and mutant MARK kinases (which all possessed an HA epitope tag). Results shown are average ± s.d. of a triplicate assay and are representative of at least two independent experiments. (**G**) MARK4 was phosphorylated with the LKB1 complex as in (A) and the major ^{32}P-labelled peptide was analysed by solid-phase Edman sequencing as in (B). In combination with MALDI TOF–TOF mass spectrometry (Supplementary Figure 1C), this peptide was shown to comprise the T-loop of MARK4 phosphorylated at only the Thr residue.

whereas QSK and QIK preferentially phosphorylated the AMARA peptide.

Identification of peptide substrates for LKB1

We next tested whether the LKB1 complex was able to phosphorylate peptides encompassing the activation loop of AMPKα1, BRSK2, NUAK2, SIK, MARK3 and MELK. All of the peptides were phosphorylated, but with differing efficiencies (Figure 6A). Solid-phase Edman sequencing confirmed that every peptide was specifically phosphorylated by LKB1 at the T-loop Thr residue (Figure 6B). The optimal peptide substrate derived from the T-loop of NUAK2 was phosphorylated by LKB1 with a K_m value of 150 μM and was a better substrate than the peptide derived from the T-loop of AMPKα1 (K_m, 1.4 mM) (Figure 6A). The MELK peptide was the poorest substrate. We also assayed the different combinations of LKB1 STRADα/β and MO25α/β complexes used in Figure 2 with the T-loop peptides as substrates, and the pattern of LKB1 activity mirrored that observed using the full-length proteins (compare Figures 2 and 6C). These results indicate that the T-loop peptides can be employed as bona fide substrates to measure LKB1 activity in a facile single-step assay. The NUAK2 T-loop peptide was termed LKBtide. Based on sequence alignments of the T-loop of AMPK-related kinases, an optimal substrate phosphorylation motif for LKB1 phosphorylation is proposed in Figure 1A.

Role of LKB1 in regulating AMPK-related kinases in fibroblasts

In order to investigate the role of LKB1 in regulating AMPK-related kinases in intact cells, we generated peptide antibodies against specific sequences of the 12 AMPK-related kinases. The ability of these antibodies to immunoprecipitate the active forms of the AMPK-related kinases was assessed employing HEK-293 cell lysates in which the kinases had been overexpressed and found to be active (O Göransson, data not shown). Using this approach, we were able to develop antibodies that selectively immunoprecipitated all the AMPK-related kinases except for MARK2 and MARK3, whose activity was assessed using a commercial antibody that immunoprecipitated both of these kinases, but not MARK1 and MARK4 (O Göransson, data not shown). Using the specific antibodies, we were able to immunoprecipitate and assay endogenously expressed NUAK2, QIK, QSK, SIK

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	Peptide	K_m (mM)	V_{max} (U/mg)	V_{max}/K_m
NUAK2	LSNLYHQGKFLQ**T**FCGSPLYRRR	0.15 ± 0.02	86.8 ± 3.4	578
SIK	FGNFYKSGEPLS**T**WCGSPPYRRR	0.46 ± 0.05	63.9 ± 2.2	139
AMPK	LSNMMSDGEFLR**T**SCGSPNYRRR	1.40 ± 0.24	94.1 ± 8.3	67
BRSK2	MASLQVGDSLLE**T**SCGSPHYRRR	0.40 ± 0.06	10.6 ± 0.6	26
MARK3	FSNEFTVGGKLD**T**FCGSPPYRRR	0.62 ± 0.06	10.2 ± 0.4	16
MELK	AKPKGNKDYHLQ**T**CCGSLAYRRR	>3	>60	

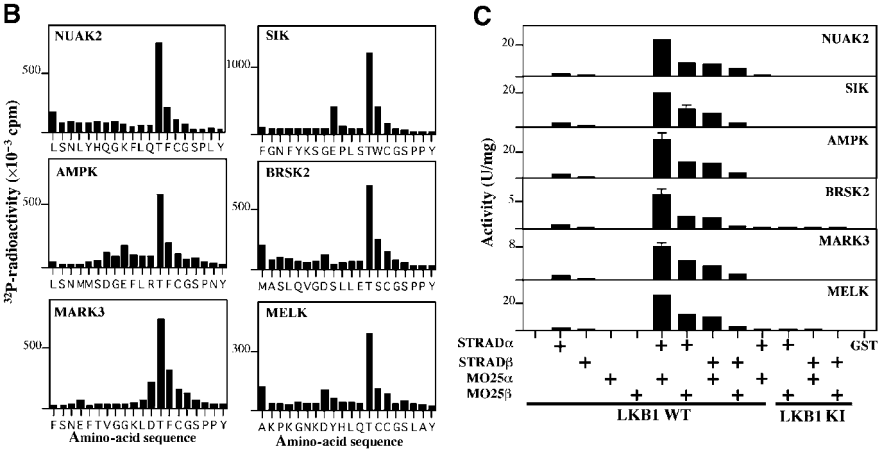

Figure 6 Identification of peptide substrates for LKB1. (**A**) Kinetic analysis of the phosphorylation of the indicated T-loop by the LKB1:STRAD:MO25 complex was performed. The T-loop Thr residue in each peptide is underlined and is in boldface type. Three Arg residues were added to the C-terminus of each T-loop peptide to enable their capture on phosphocellulose p81 paper. K_m and V_{max} values were determined from nonlinear regression. (**B**) An aliquot of each peptide phosphorylated by the LKB1 complex was subjected to solid-phase Edman sequencing and ^{32}P-radioactivity was measured after each cycle of Edman degradation. A small proportion of each peptide can become coupled to the Sequelon arylamine membrane through acidic internal Asp and Glu residues rather than their C-terminal carboxyl group. This accounts for the apparent small releases of ^{32}P-radiactivity that are observed at some Asp and Glu residues. (**C**) Same combinations of GST-tagged wild-type LKB1 (WT, lanes 1–9), or catalytically inactive (D194A, lanes 10–13) LKB1, or GST alone (lane 14), FLAG-tagged STRADα or STRADβ, and *myc*-tagged MO25α or MO25β employed in Figure 2 were tested for their ability to phosphorylate the indicated peptides (peptide concentration 200 μM). The results are expressed as the peptide kinase activity generated per mg of LKB1:STRAD:MO25 added to the assay. Results shown are average ± s.d. of three assays and are representative of at least two independent experiments.

MARK1, MARK2/3 and MARK4 in LKB1$^{+/+}$ mouse embryonic fibroblasts (MEFs) (Figure 7). We compared the activities of these kinases immunoprecipitated from LKB1$^{+/+}$ and LKB1$^{-/-}$ MEFs, and found that the activity of NUAK2, QIK, QSK, SIK, MARK1 and MARK4 was reduced from 7- to 35-fold in the LKB1$^{-/-}$ MEFs (Figure 7). The combined MARK2/3 activity was reduced ~3-fold in the LKB1$^{-/-}$ MEFs (Figure 7G). Immunoblotting demonstrated that the reduced activity of AMPK-related kinases in LKB1$^{-/-}$ cells was not caused by a decrease in the expression of the enzymes, which were either present at the same or slightly reduced levels in the knockout cells.

Interestingly, and in contrast to AMPKα1 (Figure 7A), the AMPK-related kinases assayed were not significantly stimulated by phenformin (Figure 7B–H). This suggested that phenformin was not triggering activation of AMPK by activating LKB1. Consistent with this, we demonstrated that increasing doses of phenformin that progressively activated AMPK (and increased the phosphorylation of Thr172) did not stimulate LKB1 activity (Figure 8A). The drug 5-aminoimidazole-4-carboxamide riboside (AICAR) activates AMPK in intact cells by being taken up and converted by adenosine kinase to AICAR monophosphate, which mimics the effect of AMP on the AMPK system (Corton *et al*, 1995). AICAR activated AMPK in LKB1$^{+/+}$ MEFs, but failed to activate markedly any of the AMPK-related kinases (Figure 8C).

Expression of LKB1 restores activation of AMPK-related kinases in HeLa cells

To obtain further genetic evidence that LKB1 acts as an upstream regulator of the AMPK-related kinases, we compared the activity of AMPK-related kinases in normal HeLa cells, which do not express LKB1, and in HeLa cells stably expressing either wild-type or catalytically inactive LKB1 (Sapkota *et al*, 2002; Hawley *et al*, 2003). In control HeLa cells not expressing LKB1, or cells expressing catalytically inactive LKB1, the activity of NUAK2, QIK, QSK and SIK was 20- to 40-fold lower than that observed in HeLa cells expressing wild-type LKB1 (Figure 9B–E). MARK1, combined MARK2/3 activity and MARK4 activity in control HeLa cells was about ~2- to 3-fold lower in normal HeLa cells than that in cells expressing LKB1 (Figure 9F–H). In contrast to AMPKα1 (Figure 9A), none of the AMPK-related kinases were significantly activated when HeLa cells were stimulated with phenformin (Figure 9B–H).

Discussion

In this study, we provide evidence that LKB1 functions to regulate the activity of 11 of the 12 members of the AMPK-related family of protein kinases. In cell-free systems we established that all AMPK-related kinases tested, with the exception of MELK, are activated over 50-fold following the phosphorylation of their T-loop Thr residue, by the

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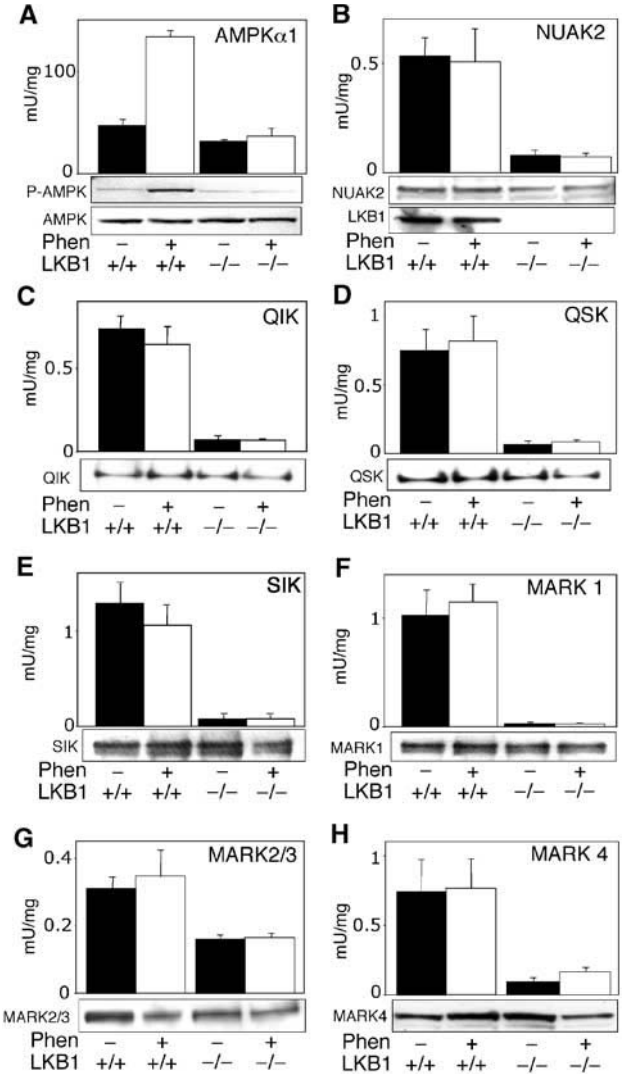

Figure 7 Activity of AMPK-related kinases in LKB1$^{+/+}$ and LKB1$^{-/-}$ MEFs. LKB1$^{+/+}$ or LKB1$^{-/-}$ MEFs were either left untreated (black bars) or stimulated with 10 mM phenformin (Phen, white bars) for 1 h. AMPKα1 and AMPK-related kinases were immunoprecipitated from the cell lysates, and *in vitro* kinase activity towards the *AMARA* peptide was measured as described in Materials and methods. To confirm equal expression of the kinases in each sample, cell lysates (AMPKα1, NUAK2, MARK2/3 and MARK4) or immunoprecipitated proteins (QIK, QSK, SIK and MARK1) were subjected to SDS–PAGE and Western blot analysis. All AMPK-related kinases migrated with the expected molecular mass. In the case of AMPKα1, cell lysates were immunoblotted with a phospho-Thr172 antibody (P-AMPK) that recognises the phosphorylated T-loop. A control immunoblot of LKB1 levels in cell lysates is also included in panel B. Results shown are average ± s.d. of 2–4 assays and are representative of at least two independent experiments.

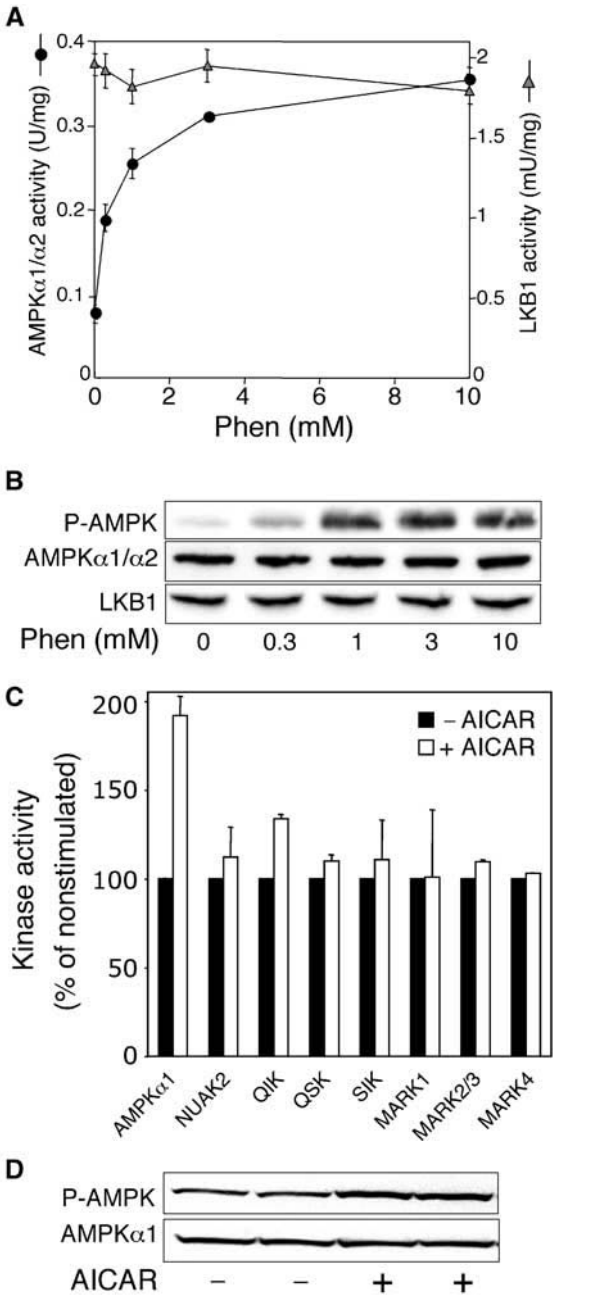

Figure 8 AICAR does not activate AMPK-related kinases. (**A**) LKB1$^{+/+}$ MEFs were either left untreated or stimulated with the indicated concentrations of phenformin (Phen) for 1 h. AMPKα1/α2 and LKB1 were immunoprecipitated from the cell lysates, and *in vitro* kinase activity towards the *AMARA* and LKBtide peptides, respectively, was measured as described in Materials and methods. Results shown are average ± s.d. of a triplicate assay and are representative of two independent experiments. (**B**) To confirm equal expression of the kinases in each sample, cell lysates were subjected to SDS–PAGE and Western blot analysis with the indicated antibodies. The phospho-Thr172 antibody (P-AMPK) recognises the phosphorylated T-loop of AMPKα1. (**C**) LKB1$^{+/+}$ MEFs were either left untreated (black bars) or stimulated with 2 mM AICAR (white bars) for 1 h. AMPKα1 and the indicated AMPK-related kinases were immunoprecipitated from the cell lysates, and *in vitro* kinase activity towards the *AMARA* peptide was measured as described in Materials and methods. Results are presented as % relative to the activity observed in nonstimulated cells, and are averages ± s.e.m. of two independent experiments. 100% corresponds to the following absolute activities: AMPKα1, 135 mU/mg; NUAK2, 0.25 mU/mg; QIK, 0.76 mU/mg; QSK, 1.9 mU/mg; SIK, 2.73 mU/mg; MARK1, 0.14 mU/mg; MARK2/3, 3.5 mU/mg; and MARK4, 1.6 mU/mg. (**D**) Immunoblotting of AMPK was performed as in (B).

LKB1:STRAD:MO25 complex. In the case of MELK, our results indicate that this enzyme requires T-loop phosphorylation for activity, but that it is able to phosphorylate its own T-loop residue. To our knowledge this is the first demonstration that the BRSK1, BRSK2, NUAK1, NUAK2, QIK, QSK, SIK as well as MELK enzymes can be activated by phosphorylation. Mutation of the T-loop Thr residue to Ala prevented the activation of all of these enzymes by the LKB1 complex, whereas its mutation to Glu was sufficient to activate BRSK1,

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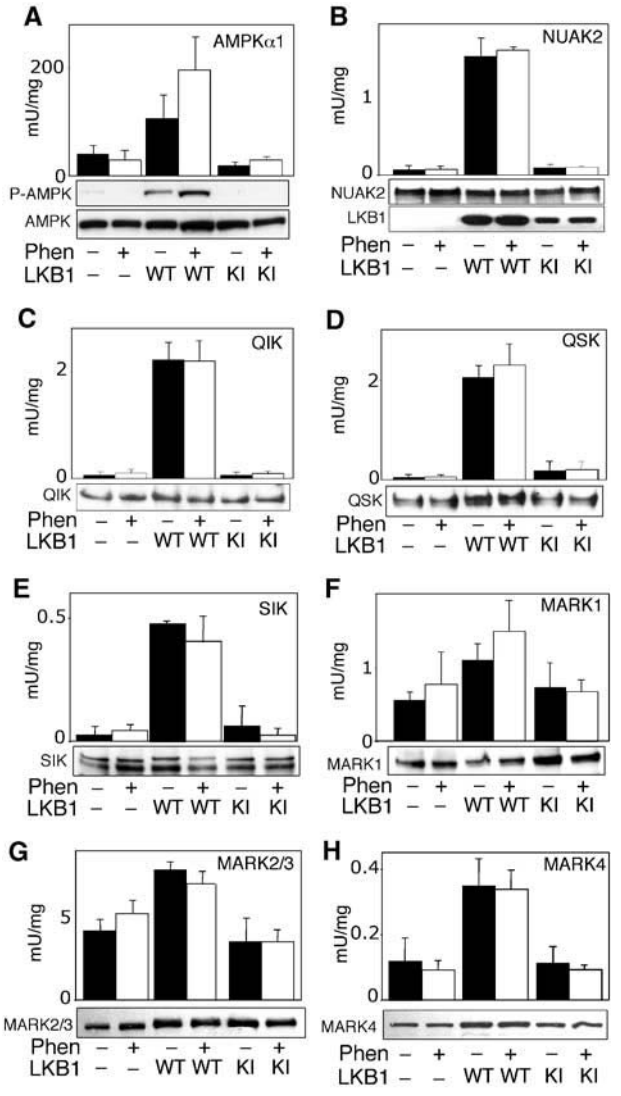

Figure 9 Activity of AMPK-related kinases in HeLa cells. Control HeLa cells lacking LKB1 expression (−), or HeLa cells stably expressing wild-type LKB1 (WT) or kinase inactive LKB1 (KI), were either left untreated (black bars) or stimulated with 10 mM phenformin (Phen, white bars) for 1 h. AMPKα1 and AMPK-related kinases were immunoprecipitated from the cell lysates, and *in vitro* kinase activity towards the *AMARA* peptide was measured as described in Materials and methods. To confirm equal expression of the kinases in each sample, cell lysates (AMPKα1, MARK2/3 and MARK4) or immunoprecipitated proteins (NUAK2, QIK, QSK, SIK and MARK1) were subjected to SDS–PAGE and Western immunoblot analysis. All AMPK-related kinases migrated with the expected molecular mass. In the case of AMPKα1, cell lysates were immunoblotted with a phospho-Thr172 antibody (P-AMPK) that recognises the phosphorylated T-loop. A control immunoblot of LKB1 levels in cell lysates is also included in panel B. Results shown are average ± s.d. of 2–4 assays and are representative of at least two independent experiments.

BRSK2, NUAK1, NUAK2, SIK, QIK, QSK and MARK4 to specific activities similar to that observed for the LKB1-phosphorylated forms of these enzymes (Figure 4). This latter finding could be exploited in future overexpression or knock-in studies to introduce constitutively active forms of these enzymes in cells to examine their cellular roles.

Previous work has indicated that MARK2 and MARK3 were phosphorylated at the T-loop Thr residue as well as at a nearby Ser residue (Drewes *et al*, 1997). The T-loop Ser residue phosphorylated in MARK2/MARK3 is conserved in AMPKα1, AMPKα2 and all AMPK-related kinases (Figure 1A and B), but at least for AMPKα1 there is no evidence that this residue is phosphorylated *in vivo* (Woods *et al*, 2003b). Our analysis (Figure 5A and B), as well as that of a recent study (Timm *et al*, 2003), showed that phosphorylation of the T-loop Thr residue of the MARK kinases played the most crucial role in regulating the activity of this class of kinase. The LKB1 complex phosphorylated a catalytically inactive MARK3 mutant at both the Thr and Ser T-loop residues (Figure 5A). However, the isolated MARK3 T-loop peptide was only phosphorylated by LKB1 at the Thr residue (Figure 6), signifying that the primary sequence of the peptide was not sufficient to enable LKB1 to phosphorylate the Ser residue. The finding that mutation of the T-loop Thr211 to Ala on MARK3 prevented phosphorylation of Ser215 by the LKB1 complex suggests that phosphorylation of Thr211 is required for Ser215 phosphorylation. MARK4 may be regulated differently, as peptide-mapping studies indicated that MARK4 was only phosphorylated at the T-loop Thr by LKB1 (Figure 5G), and that mutation of the T-loop Thr to Glu increased activity to a much greater level than observed for other MARK isoforms (Figure 5F). It was previously reported for MARK2 that mutation of the T-loop to Glu increased its specific activity three-fold (Timm *et al*, 2003), and we have made similar findings with this mutation increasing MARK2 activity from 2.2 to 6.7 U/mg (Figure 5D). However, in contrast to TAO1, which only activated MARK2 10-fold (Timm *et al*, 2003), the LKB1 complex increased MARK2 activation 200-fold, showing that in this case the Thr to Glu mutation only modestly activated MARK2 compared to phosphorylation by LKB1.

As observed for the activation of AMPKα1 and AMPKα2 (Hawley *et al*, 2003), the presence of STRAD and MO25 subunits was essential for LKB1 to activate all of the AMPK-related kinases in cell-free assays. There is considerable evidence that many protein kinases rely on sequences, termed 'docking sites', lying outside of the catalytic core, which stabilise the interaction between the kinase and its substrate. It was possible that the STRAD and MO25 subunits played a similar role in docking the substrate to the kinase complex. However, the finding that STRAD and MO25 subunits stimulated phosphorylation of the short T-loop peptides and the whole kinase subunits in a very similar manner suggests that they activate LKB1 directly rather than just acting as docking sites. Nevertheless, these results do not rule out additional roles of the STRAD and MO25 subunits in targeting the complex or in substrate recognition.

Employing $LKB1^{-/-}$ MEFs and/or HeLa cells that lack LKB1, we were able to demonstrate that the activities of endogenously expressed NUAK1, NUAK2, QIK, QSK and SIK were 7- to 40-fold lower than in $LKB^{+/+}$ MEFs or HeLa cells that stably express wild-type LKB1. This provides genetic evidence that LKB1 is rate limiting in the activation of these enzymes in intact cells. However, our data do not rule out the possibility that other kinases can regulate the activity of the AMPK-related kinases *in vivo* in addition to LKB1. Recently, the TAO1 kinase was purified from pig brain as the major activity that phosphorylated the T-loop Thr residue of MARK2, resulting in its activation (Timm *et al*, 2003). Although TAO1 also activated MARK2 in overexpression

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studies (Timm *et al*, 2003), so far no genetic evidence indicating how the lack of TAO1 affected the activity of MARK family kinases *in vivo* has been reported. Such evidence may be hard to acquire, as mammalian cells possess three closely related TAO isoforms (Manning *et al*, 2002). Lack of specific immunoprecipitating antibodies meant that we were unable to establish how a deficiency of LKB1 affected the individual activities of MARK2 or MARK3. However, the finding that the combined activity of MARK2 and MARK3 in LKB1$^{-/-}$ and control HeLa cells was substantial implies that other kinases, such as TAO1, may regulate the activities of these enzymes. It should be noted that LKB1 and TAO1 share no obvious amino-acid sequence homology and lie in distinct regions of the human kinase dendrogram (Manning *et al*, 2002). TAO1 belongs to the STE20 group of kinases and is therefore related to STRADα and STRADβ, but whether this has any significance is not clear.

The MARK (MAP/microtubule affinity regulating kinase) family of kinases are the most studied AMPK-related kinase family members and are thought to play key roles in establishing cell polarity. For example, genetic studies indicate that MARK homologues control partitioning of the *C. elegans* zygote (Guo and Kemphues, 1995) and embryonic axis formation in *Drosophila* (Shulman *et al*, 2000). In neuronal cells, MARK kinases phosphorylate the neuronal microtubule-associated protein Tau, resulting in destabilisation of microtubules (Drewes *et al*, 1997). Interestingly, the counterpart of mammalian LKB1 in *C. elegans* (Watts *et al*, 2000), termed PAR4, was originally identified as a member of the maternally expressed *PAR* (partitioning defective) gene family, required for establishing cell polarity during the first cycle of *C. elegans* embryogenesis (Kemphues *et al*, 1988). Maternal lethal mutations in the gene encoding *C. elegans* PAR4 have been shown to affect several aspects of cell polarity (Morton *et al*, 1992). These lead to phenotypes similar to those observed in *C. elegans* mutated in the PAR1 gene, which encodes the homologue of MARK3 (Guo and Kemphues, 1995). More recently, both the *Drosophila* (Martin and St Johnston, 2003) and *Xenopus* (Ossipova *et al*, 2003) homologues of human LKB1 were shown to play important roles in regulating cell polarity. Originally, it was suggested that *Drosophila* LKB1 functioned downstream of PAR1/MARK3, as overexpression of *Drosophila* LKB1 suppressed the polarity phenotype of PAR1/MARK3 mutants (Martin and St Johnston, 2003). However, in HeLa cells that do not express LKB1, MARK3 was found to exist in a dephosphorylated state and reintroduction of LKB1 promoted phosphorylation of MARK3 at its T-loop (Spicer *et al*, 2003), signifying that LKB1 was upstream of MARK3. One explanation that would reconcile the apparent discrepancy between the *Drosophila* and mammalian studies would be whether overexpression of *Drosophila* LKB1 suppressed the polarity phenotype of PAR1/MARK3 mutants, through the activation of other AMPK-related kinases, which might be able to compensate for the loss of PAR1/MARK3 function.

Much less is known regarding the function of other AMPK-related kinases, that is, BRSK1, BRSK2, NUAK1, NUAK2, QIK, QSK and SIK. Previous Northern blotting of NUAK1, NUAK2, QIK and SIK performed by other groups (see below) and analysis of EST clones (Supplementary Table I) indicated that the AMPK-related kinases with the exception of BRSK1, BRSK2 and MELK are likely to be expressed in many tissues. BRSK1 and BRSK2 EST clones were mainly derived from neuronal tissues and, after immunoblot analysis of a variety of rat tissues, these enzymes were only detected in the brain and at low levels in the testis (K Sakamoto, unpublished results). To date, MELK (maternal embryonic leucine zipper kinase) has only been reported to be expressed during mammalian embryogenesis, with the strongest expression detected during maturation of oocytes and preimplantation development (Heyer *et al*, 1999). A recent study indicates that MELK can inhibit spliceosome assembly by interacting with the splicing factor NIPP1 (Vulsteke *et al*, 2003). Although our antibodies raised against BRSK1, BRSK2 and MELK readily immunoprecipitated the recombinant enzymes, we were unable to detect their activity in MEFs or HeLa cells (O Göransson, data not shown). SIK (Salt-inducible kinase) was first cloned from the adrenal glands of rats fed a high-salt diet (Wang *et al*, 1999). SIK mRNA was also induced by membrane depolarisation in the brain (Feldman *et al*, 2000), and recent studies have indicated that when SIK is overexpressed in cells, it might play a role in steroidogenesis (Takemori *et al*, 2003). The mRNA expressing QIK (also termed SIK2) was highest in adipose tissue and, in overexpression studies, QIK was reported to phosphorylate human IRS1 at Ser794 (Horike *et al*, 2003), the residue equivalent to Ser789 in rat IRS1, shown to be phosphorylated by AMPK (Jakobsen *et al*, 2001). In other overexpression studies, NUAK1 (ARK5) was shown to suppress apoptosis induced by some stimuli, including nutrient starvation (Suzuki *et al*, 2003a). Furthermore, it has been claimed that Akt/PKB phosphorylated NUAK1 at a C-terminal site outside of the catalytic domain, leading to a three-fold activation of the enzyme (Suzuki *et al*, 2003b). NUAK2 (SNARK) was most highly expressed in the kidney, and its activity was reportedly stimulated by glucose starvation of cells (Lefebvre *et al*, 2001; Suzuki *et al*, 2003b). To our knowledge, no previous studies have addressed the roles of BRSK1, BRSK2 and QSK.

Clearly, further work needs to be carried out to characterise the mechanism of regulation and function of the AMPK-related kinases. Our studies indicate that, at least in MEFs and HeLa cells that stably express LKB1, the AMPK-related kinases were not activated in response to the drugs phenformin or AICAR in contrast to AMPK. This suggests that the beneficial antidiabetic effects of phenformin and metformin may be mediated through the activation of AMPK rather than the AMPK-related kinases. The finding that AMPK-related kinases are not activated by phenformin or AICAR is consistent with findings that these treatments do not activate LKB1 directly in LKB1$^{+/+}$ MEFs (Figure 8A) or in COS7 cells (Woods *et al*, 2003a). Phenformin stimulates AMPK without affecting the AMP or the ADP/ATP ratio in cells (Fryer *et al*, 2002; Hawley *et al*, 2002). It is therefore currently unclear how phenformin activates AMPK. Possibilities include phenformin inhibiting an AMPK-specific phosphatase that does not target the AMPK-related kinases, although the related drug metformin did not affect dephosphorylation of AMPK by protein phosphatase-2C *in vitro* (Hawley *et al*, 2002). Alternatively, phenformin may somehow generate a metabolite or another molecule that inside the cell binds to AMPK and promotes its activation by the LKB1 complex. In future studies it will be important to define the physiological stimuli that do cause activation of the AMPK-related kinases, and establish whether these enzymes, like AMPK, possess

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regulatory subunits that control their activation. The finding that AMPK-related kinases phosphorylate peptide substrates at different relative rates (Supplementary Figure 2) indicates that these enzymes have distinct substrate specificity preferences and may thus phosphorylate different substrates *in vivo*.

A significant number of inherited forms of PJS found in certain families do not display mutations in the LKB1 gene (Buchet-Poyau *et al*, 2002), indicating that there is a second causative locus for PJS. It will clearly now be critical to find out whether PJS families that express the normal LKB1 protein have mutations in other AMPK-related kinases. In conclusion, we have shown that LKB1 functions as a master upstream protein kinase activating 11 AMPK-related kinases in addition to AMPKα1 and AMPKα2. It is possible that these enzymes mediate some of the physiological effects previously ascribed to LKB1 and that one or more of these kinases may themselves function as tumour suppressors.

Materials and methods

A detailed Materials and methods section is provided in the supplementary section.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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