Identification and characterization of four novel phosphorylation sites (Ser^{31}, Ser^{325}, Thr^{336} and Thr^{366}) on LKB1/STK11, the protein kinase mutated in Peutz–Jeghers cancer syndrome

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Peutz–Jeghers syndrome is an inherited cancer syndrome, which results in a greatly increased risk of developing tumours in those affected. The causative gene encodes a nuclear-localized protein kinase, termed LKB1, which is predicted to function as a tumour suppressor. The mechanism by which LKB1 is regulated in cells is not known, and nor have any of its physiological substrates been identified. Recent studies have demonstrated that LKB1 is phosphorylated in cells. As a first step towards identifying the roles that phosphorylation of LKB1 play, we have mapped the residues that are phosphorylated in human embryonic kidney (HEK)-293 cells, as well as the major *in vitro* autophosphorylation sites. We demonstrate that LKB1 expressed in HEK-293 cells, in addition to being phosphorylated at Ser^{431}, a previously characterized phosphorylation site, is also phosphorylated at Ser^{31}, Ser^{325} and Thr^{366}. Incubation of wild-type LKB1, but not a catalytically inactive mutant, with manganese-ATP *in vitro* resulted in the phosphorylation of LKB1 at Thr^{336} as well as at Thr^{366}. We were unable to detect autophosphorylation at Thr^{189}, a site previously claimed to be an LKB1 autophosphorylation site. A catalytically inactive mutant of LKB1 was phosphorylated at Ser^{31} and Ser^{325} in HEK-293 cells to the same extent as the wild-type enzyme, indicating that LKB1 does not phosphorylate itself at these residues. We show that phosphorylation of LKB1 does not directly affect its nuclear localization or its catalytic activity *in vitro*, but that its phosphorylation at Thr^{336}, and perhaps to a lesser extent at Thr^{366}, inhibits LKB1 from suppressing cell growth.

Key words: cell cycle, mass spectrometry, phosphopeptide mapping, signal transduction.

INTRODUCTION

Mutations in a widely expressed protein kinase, termed LKB1 (also known as STK11), cause Peutz–Jeghers Syndrome [1,2], an autosomal dominantly-inherited disorder that predisposes those affected to a wide spectrum of benign and malignant tumours [3,4]. The overexpression of LKB1 in a number of tumour cell lines that do not express this protein kinase has been shown to suppress cell growth by inducing a G_1 cell-cycle block [5]. Moreover, many of the mutations that have been mapped to LKB1 would be expected to impair its activity. These results suggest that LKB1 may function in cells as a tumour suppressor. Recent work indicates that LKB1 is required for cell death mediated by the tumour suppressor p53, and that the lack of LKB1 in Peutz–Jeghers syndrome patients results in a deficiency in apoptosis [6]. Another study found that LKB1 interacted with the brahma-related gene 1 protein (Brg1), an essential component of chromatin remodelling complexes, and this interaction was suggested to be required for Brg1 to suppress cell growth [7]. LKB1 also appears to play multiple roles in development, as mice that lack this enzyme die at day E9 of embryogenesis, with the embryos displaying numerous vascular abnormalities, as well as defects in the neural tube. Embryos lacking LKB1 also showed increased mesenchymal cell death and possessed markedly elevated levels of vascular endothelial growth factor [8].

LKB1 is a 436-amino-acid protein possessing a kinase domain (residues 50–319) that is only distantly related to other mammalian protein kinases. The N-terminal non-catalytic domain comprises a nuclear localization signal and, consistent with this, LKB1 has been reported to be a mainly nuclear protein [5,9]. A recent study, however, demonstrates that LKB1 can also be localized in the cytoplasm of cells through an interaction with a cytosolic protein called LKB1 interacting protein-1 ('LIP1') [10]. There are no yeast counterparts of LKB1, but there are counterparts in *Xenopus* (termed XEEK1; possesses 84% overall identity with human LKB1 [11]) and *Drosophila* [NCBI (National Center for Biotechnology Information) accession number AAF54972; possesses 44% overall identity with human LKB1]. It has been suggested that a *Caenorhabditis elegans* protein kinase, termed Par-4, may be a counterpart of LKB1 [12], however, in our opinion this is uncertain as *C. elegans* Par-4 possesses only 26% overall identity with human LKB1.

Recently, LKB1 has been found to be phosphorylated at a C-terminal serine residue (Ser^{431}) by the cAMP-dependent protein kinase (PKA) in response to agonists that elevate cAMP, as well as by the p90 ribosomal S6 kinase (p90RSK) in response to agonists that trigger the activation of the classical mitogen-activated protein (MAP) kinase pathway [13,14]. Mutation of Ser^{431} to alanine does not affect the ability of LKB1 to phosphorylate itself or the p53 protein *in vitro*, but prevents LKB1 from suppressing the growth of a G361 melanoma cell line that does not express LKB1 [14]. Ser^{431} is located two residues away from

Abbreviations used: AMPK, AMP-activated protein kinase; Brg1, brahma-related gene 1 protein; CHK1, checkpoint kinase 1; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase; HEK, human embryonic kidney; JNK, c-Jun N-terminal kinase; MALDI–TOF, matrix-assisted laser-desorption ionization–time-of-flight; MAP, mitogen-activated protein; MAPKAP-K2, MAP kinase-activated protein kinase-2; PKA, cAMP-dependent protein kinase; PKB, protein kinase B; S6K, p70 ribosomal S6 kinase; p90RSK, p90 ribosomal S6 kinase; T336-P antibody, phosphospecific antibody recognizing LKB1 phosphorylated at Thr^{336}; T366-P antibody, phosphospecific antibody recognizing LKB1 phosphorylated at Thr^{366}; YFP, yellow fluorescent protein.

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Cys[433], a residue on LKB1 that is farnesylated *in vivo* [13,14]. The function of farnesylation of LKB1 is unclear, as mutation of Cys[433] to alanine, to prevent farnesylation, has no effect on the ability of LKB1 to suppress cell growth and does not affect the phosphorylation of LKB1 at Ser[431] [14]. In the present study we identify and characterize four other phosphorylation sites on LKB1. We investigate the roles that these phosphorylation sites play in controlling LKB1 activity, cellular localization and its ability to suppress cell growth.

MATERIALS AND METHODS

Materials

Protease-inhibitor cocktail tablets, Fugene-6 transfection reagent and G418 were obtained from Roche, and Zwittergent 3-16 was purchased from Calbiochem. Microcystin-LR and tissue culture reagents were from Life Technologies. Precast SDS/4–12% polyacrylamide Bis-Tris gels were obtained from Invitrogen. All the peptides used in the present study were synthesized by Dr G. Bloomberg (University of Bristol, Bristol, U.K.). Mouse p53 expressed in bacteria was prepared as previously described [15]. Wild-type and mutant forms of glutathione S-transferase (GST)-tagged LKB1 were expressed in either human embryonic kidney (HEK)-293 cells or *Escherichia coli* and were purified on GSH–Sepharose as described previously [14].

Antibodies

The antibody recognizing LKB1 protein was raised in sheep against the GST–LKB1 protein expressed in *E. coli* [14]. The phosphospecific antibody recognizing LKB1 phosphorylated at Thr[336] (T336-P antibody) was raised in a rabbit against the peptide KDRWRSMTVVPYLED (corresponding to residues 329–343 of mouse LKB1) in which the underlined residue is phosphothreonine. The phosphospecific antibody recognizing LKB1 phosphorylated at Thr[366] (T366-P antibody) was raised in a rabbit against the peptide KIEDGIIYTQDFTVPGK (corresponding to residues 359–373 of mouse LKB1 with a lysine residue added to each end of the peptide to enhance its solubility) in which the underlined residue is phosphothreonine. All antibodies were affinity-purified on CH-Sepharose covalently coupled to the corresponding phosphorylated peptides, then passed through a column of CH-Sepharose coupled to the corresponding non-phosphorylated peptides. Antibody that did not bind to the latter column was selected. Secondary antibodies coupled to horseradish peroxidase (Pierce) were used for immunoblotting, and monoclonal antibodies recognizing GST and FLAG epitope (i.e. Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) tags were obtained from Sigma.

General methods and buffers

Phospho amino acid analysis of ^{32}P-labelled peptides, restriction enzyme digests, DNA ligations and other recombinant DNA procedures were performed using standard protocols. All DNA constructs were verified by DNA sequencing, which was performed by The Sequencing Service, School of Life Sciences, University of Dundee, Scotland, U.K., using DYEnamic ET terminator chemistry (Amersham Biosciences) on Applied Biosystems automated DNA sequencers. The ability of LKB1 to autophosphorylate, phosphorylate the p53 protein and to suppress G361 cell growth were measured as described previously [14]. Buffer A: 50 mM Tris/HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 1% (w/v) Triton-X 100, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 μM microcystin-LR, 0.1% (v/v) 2-mercaptoethanol and 'complete' proteinase inhibitor cocktail (one tablet/25 ml). Buffer B: 50 mM Tris/HCl (pH 7.5), 0.1 mM EGTA, 0.27 M sucrose and 0.1% (v/v) 2-mercaptoethanol. SDS sample buffer: 50 mM Tris/HCl (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol and 10 mM dithiothreitol.

DNA constructs

The FLAG- and GST-tagged mouse wild-type LKB1 and kinase-dead LKB1 (LKB1[D194A]) in the pCMV5 and pEBG2T vectors were described previously [14]. The Ser[31], Ser[325], Thr[336] and Thr[366] mutants of LKB1 that were employed in the present study were generated by performing site-directed mutagenesis using the QuickChange kit (Stratagene). The pEYFP-C1 vector was modified appropriately by inserting a linker that makes the existing *Eco*RI restriction site in this vector in-frame with the yellow fluorescent protein (YFP) in which the wild-type and mutant forms of FLAG–LKB1 could be subcloned as *Eco*RI fragments from the pCMV5 vector.

Cell culture and lysis

HEK-293 cells were cultured on 10-cm diameter dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, 10% (v/v) foetal bovine serum and 1× antibiotic/antimycotic solution (Sigma). The human G361 malignant melanoma cells were obtained from the European Collection of Cell Cultures. G361 cells were cultured on 10-cm diameter dishes in McCoy's 5A medium supplemented with 2 mM glutamine and 10% (v/v) foetal bovine serum. The cells were lysed in 1 ml of ice-cold buffer A and centrifuged at 4 °C for 5 min at 20000 ***g***. The supernatants were frozen in liquid nitrogen and stored at −80 °C. Protein concentrations were determined using the Bradford method and BSA was employed as the standard.

^{32}P-labelling of HEK-293 cells transfected with LKB1

HEK-293 cells were transfected with a pCMV5-encoded DNA construct expressing wild-type or kinase-dead FLAG–LKB1. The cells were washed with phosphate-free DMEM 36 h post-transfection, incubated for 3 h with [^{32}P]P_i (1 mCi/ml), and then lysed in 1 ml of buffer A. FLAG–LKB1 was immunoprecipitated from the cleared lysate with anti-FLAG antibodies (5 μg) conjugated to Protein G–Sepharose (5 μl). The immunoprecipitates were washed ten times with 1 ml of buffer A containing 0.5 M NaCl and once with buffer B, and then resuspended in a total volume of 40 μl of SDS sample buffer and heated at 100 °C for 1 min. After cooling, 4-vinylpyridine was added to a concentration of 0.5% (v/v) and the sample was left on a shaking platform for 30 min at room temperature to alkylate cysteine residues. The LKB1 was electrophoresed on an SDS/4–12% polyacrylamide Bis-Tris gel, which was then autoradiographed. The phosphorylated 55 kDa band corresponding to ^{32}P-labelled LKB1 was excised from the gel and cut into small pieces. These were washed sequentially for 15 min on a vibrating platform with 1 ml of the following: water; water/acetonitrile (1:1, v/v); 0.1 M ammonium bicarbonate; 0.2 M ammonium bicarbonate/acetonitrile (1:1, v/v); and finally acetonitrile. The gel pieces were dried by rotary evaporation and incubated in 0.3 ml of 50 mM ammonium bicarbonate/0.05% (w/v) Zwittergent 3-16 containing 2 μg of alkylated trypsin. After 16 h the supernatant was removed, and the gel pieces were washed for 10 min in 0.3 ml of 50 mM ammonium bicarbonate/0.05% (w/v) Zwittergent 3-

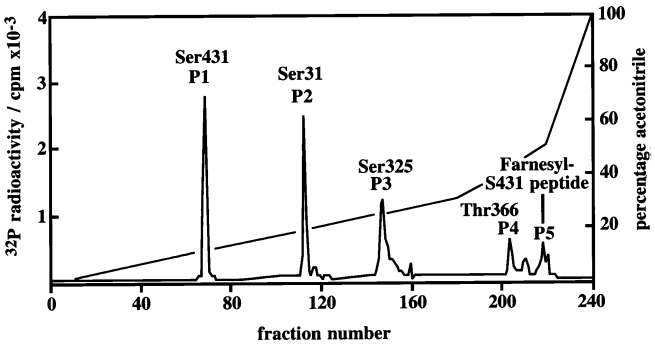

Figure 1 Tryptic peptide map of ^{32}P-labelled LKB1

HEK-293 cells transiently transfected with a DNA construct encoding wild-type FLAG–LKB1 were ^{32}P-labelled. The cells were lysed, and LKB1 was immunoprecipitated and electrophoresed on an SDS/polyacrylamide gel and then digested with trypsin as described in the Materials and methods section. The resulting ^{32}P-labelled peptides were chromatographed on a Vydac 218TP54 C_{18} column (Separations Group, Hesperia, CA, U.S.A.) equilibrated in 0.1% (v/v) trifluoroacetic acid in water. The column was developed with a linear acetonitrile gradient (diagonal line) at a flow rate of 0.8 ml/min, and fractions of 0.4 ml were collected; 80% of the radioactivity applied to the column was recovered in the fractions. Similar profiles were obtained in three separate experiments. The positions of the peptides, termed P_1, P_2, P_3, P_4 and P_5, are indicated.

16/0.1% (v/v) trifluoroacetic acid. The combined supernatants containing >90% of the ^{32}P-radioactivity were chromatographed on a Vydac C_{18} column as described in the legend to Figure 1.

Mapping the *in vitro* autophosphorylation sites on LKB1

GST–LKB1 or GST– LKB1[D194A] (2 μg) expressed in HEK-293 cells was incubated in 50 μl of reaction mixture containing 50 mM Tris/HCl (pH 7.5), 0.1% 2-mercaptoethanol, 0.1 mM EGTA, 10 mM manganese chloride, 0.5 μM microcystin-LR and 100 μM [γ-^{32}P]ATP (10000 c.p.m./pmol). The reactions were terminated by the addition of 1% (w/v) SDS and 10 mM dithiothreitol, and the samples were heated at 100 °C for 1 min. After cooling, 4-vinylpyridine was added to a concentration of 0.5% (v/v) and the samples were left on a shaking platform for 30 min at room temperature to alkylate cysteine residues. Each sample was subjected to electrophoresis and the 82 kDa ^{32}P-labelled band corresponding to GST–LKB1 was excised, digested with trypsin and analysed by chromatography on a C_{18} column as described above.

Phosphopeptide sequence analysis

^{32}P-labelled peptides were analysed by matrix-assisted laser-desorption ionization–time-of-flight (MALDI–TOF) MS on a PerSeptive Biosystems (Framingham, MA, U.S.A.) Elite STR mass spectrometer using α-cyanocinnamic acid as the matrix. Spectra were obtained in both the linear and reflector mode. The sequence identity of each peptide was confirmed by Edman sequencing. The site of phosphorylation was determined by solid-phase Edman degradation of the peptide coupled to Sequelon-AA membrane (Milligen) on an Applied Biosystems 476A sequenator as described previously [16].

Immunoblotting

For blots of total G361 cell lysates in Figure 7, 20 μg of protein was used. For purified wild-type and mutant GST–LKB1, 40 ng of protein was used. Samples were subjected to SDS/PAGE and transferred on to nitrocellulose. For the T336-P antibody, T366-P antibody and GST blots, membranes were incubated in 50 mM Tris/HCl (pH 7.5), 0.15 M NaCl, 0.5% (v/v) Tween ('TBS/Tween') and 5% (w/v) BSA for 7 h at 4 °C in the presence of 1 μg/ml antibody and, for the phosphospecific antibodies, the non-phosphorylated peptide (10 μg/ml) corresponding to the antigen used to raise the antibody was also included. The LKB1 immunoblot in Figure 7 was carried out as above except that the BSA was replaced with 10% (w/v) skimmed milk powder. Detection was performed using horseradish peroxidase-conjugated secondary antibodies and ECL® (Amersham Biosciences).

Cellular localization of YFP–LKB1

HeLa cells were cultured to 50% confluence on coverslips (no. 1.5) in 3.5-cm diameter dishes, in DMEM supplemented with 10% (v/v) foetal bovine serum. They were transfected with 0.4 μg of a cDNA construct encoding the wild-type or indicated mutants of pEYFP-LKB1 or empty pEYFP vector using Effectene (Qiagen) transfection reagent following the manufacturer's protocol. A triplicate set of dishes was used for each condition. The cells were washed with PBS 20 h post-transfection, and were fixed for 10 min in freshly prepared 4% (v/v) paraformaldehyde in PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA and 2 mM magnesium sulphate, pH 7.0). The cells were washed twice with PBS, mounted on to slides using MOWIOL and imaged on a Leica microscope (Improvision Ltd), and the data were analysed using the Openlab software.

RESULTS

Mapping the *in vivo* phosphorylation sites of LKB1

HEK-293 cells transiently transfected with a cDNA construct encoding the expression of FLAG epitope-tagged LKB1 were incubated with [^{32}P]P_i for 3 h, the cells were then lysed and the LKB1 was immunoprecipitated and electrophoresed on an SDS/polyacrylamide gel. Autoradiography of the gel revealed that LKB1 was heavily phosphorylated. To map the phosphorylation sites, the ^{32}P-labelled LKB1 was digested with trypsin and

Table 1 Identification of LKB1 tryptic peptides isolated from the ^{32}P-labelling of HEK-293 cells and *in vitro* autophosphorylation reactions

The peptides isolated from Figures 1 and 3 were analysed on a PerSeptive Biosystems Elite STR MALDI–TOF mass spectrometer in the linear and reflective mode, using 10 mg/ml α-cyanocinnamic acid as the matrix. Peptides P_4, P_A and P_B were not present in sufficient amounts for their masses to be detected by MALDI–TOF MS. The theoretical masses shown are for the monoisotopic masses. For peptides P_C and P_D the measured masses are closer to the average peptide masses because it is not possible to isotopically separate approx. 60-residue peptides. This is likely to account for the approx. 1 Da difference between the observed and theoretical masses of these peptides. Alkylated-Cys^{433} corresponds to pyridylethylated Cys^{433} due to alkylation of LKB1 with 4-vinylpyridine; and Met-SO corresponds to a methionine sulphone derivative.

			Mass	
Phosphopeptide	Residues	Modification	Measured	Theoretical
P_1	429–434	Alkylated-Cys^{433} + PO_4	862.40	862.40
P_2	29–39	PO_4	1400.31	1400.68
P_3	311–329	None	2038.22	2038.17
P_5	429–434	Farnesyl-Cys^{433}	961.53	961.53
P_C	334–391	None	6562.25	6561.07
P_D	334–391	PO_4 + Met-SO	6658.53	6657.08

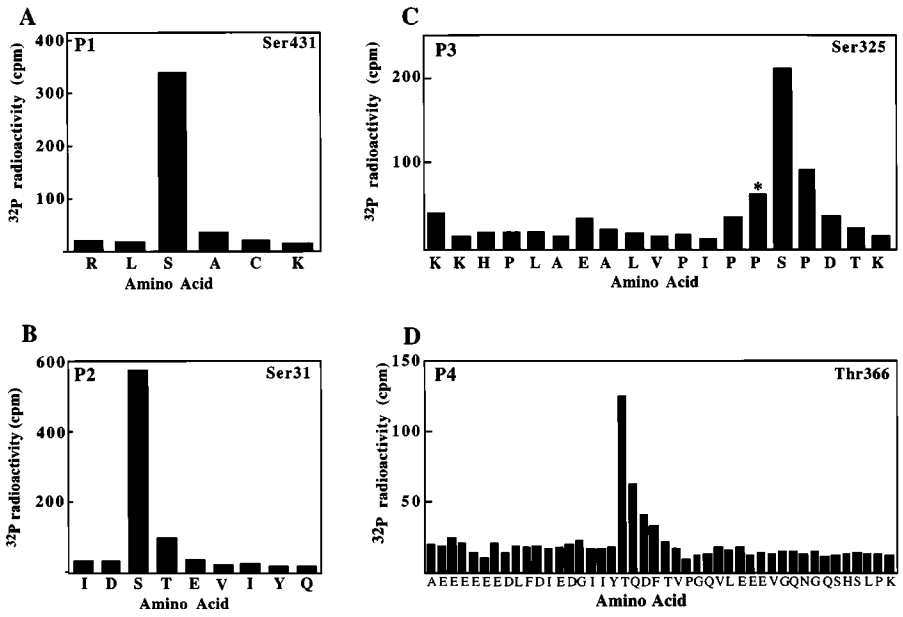

Figure 2 Identification of the phosphorylation sites in ^{32}P-labelled LKB1 peptides

Aliquots of the major ^{32}P-labelled peptides derived from ^{32}P-labelled LKB1 (Figure 1) were covalently coupled to a Sequelon arylamine membrane and analysed on an Applied Biosystems 476A sequenator. ^{32}P-radioactivity was measured after each cycle of Edman degradation. In combination with MALDI–TOF MS (Table 1) and phospho amino acid analysis (see the Results section), this enabled the identification of the sites of phosphorylation in each of the peptides. An asterisk in (**C**) indicates a minor release of ^{32}P-radioactivity at cycle 14, which, as discussed in the text, represents a minor form of the tryptic peptide that commences at residue 312.

the resulting peptides were separated by chromatography on a C_{18} column. Five major ^{32}P-labelled peptides were recovered and termed P_1, P_2, P_3, P_4 and P_5 (Figure 1). Phospho amino acid analysis revealed that P_1, P_2, P_3 and P_5 contained phosphoserine, whereas P_4 contained phosphothreonine (results not shown). MALDI–TOF MS analysis (Table 1) and solid-phase Edman sequencing (Figure 2A) established that the peptides P_1 and P_5 comprise the tryptic phosphopeptide encompassing residues 429–434 phosphorylated at Ser^{431} that has been previously characterized [14]. In peptide P_1, the Cys^{433} residue is not farnesylated, whereas in P_5, Cys^{433} is farnesylated, causing the peptide to elute late on the C_{18} chromatography (Figure 1).

^{32}P-radioactivity was released from peptide P_2 after three cycles of Edman degradation (Figure 2B) and its identity was established by MALDI–TOF MS (Table 1) as the tryptic phosphopeptide comprising residues 29–39 phosphorylated at Ser^{31}. Solid-phase Edman degradation of P_3 resulted in a major release of ^{32}P-radioactivity at cycle 15 (Figure 2C). MALDI–TOF MS of peptide P_3 (Table 1) revealed the presence of the non-phosphorylated LKB1 tryptic peptide comprising residues 311–329 in this fraction, but the mass of the corresponding phosphorylated peptide could not be detected. This analysis indicates that peptide P_3 could comprise the 311–329 residue LKB1 peptide phosphorylated at Ser^{325} in which there is insufficient amounts present to detect by MALDI–TOF MS. As peptide P_3 eluted as a broad peak, a common feature of peptides containing multiple proline residues (this peptide possesses five), three separate fractions were pooled in order to obtain sufficient material for analysis. This could explain the presence of both the dephosphorylated and phosphorylated forms of this peptide in the same pool. Further evidence that we have correctly assigned the identity of peptide P_3 is that no other serine residues in a tryptic digest of LKB1 would be expected to be located 15 residues from the N-terminus. Furthermore, as both residues 311 and 312 of LKB1 are lysine, a trypsin digestion of LKB1 would be expected to generate two phosphopeptides encompassing Ser^{325}, commencing at either residue 311 or residue 312. Consistent with this, a minor release of ^{32}P-radioactivity at cycle 14 of the Edman sequencing of P_3 (Figure 2C) indicated the presence of a minor LKB1 tryptic peptide encompassing residues 312–329 and phosphorylated at Ser^{325}. ^{32}P-radioactivity was released from peptide P_4 after 19 cycles of Edman degradation (Figure 2D) and although we were unable to determine the mass of this peptide by MALDI–TOF MS, our analysis described below indicates that this peptide comprises residues 348–392 of LKB1 phosphorylated at Thr^{366}.

Identification of the major *in vitro* autophosphorylation sites on LKB1

LKB1 autophosphorylates when incubated *in vitro* with manganese-ATP but not magnesium-ATP [1,17]. To map the major residues on LKB1 that become phosphorylated under these conditions, we incubated wild-type GST–LKB1 that had been expressed in mammalian HEK-293 cells with manganese-[γ^{32}P]ATP for 1 h. This resulted in the phosphorylation of LKB1 to a stoichiometry of 0.2 mol of ^{32}P-radioactivity/mol of protein. In a parallel reaction, a catalytically inactive LKB1 mutant (LKB1[D194A]) was only phosphorylated to a trace level as expected from previous results [14]. The ^{32}P-labelled wild-type LKB1 was digested with trypsin and the resulting peptides were separated by chromatography on a C_{18} column. Four major ^{32}P-labelled peptides, termed P_A, P_B, P_C and P_D, were observed (Figure 3A). Phospho amino acid analysis revealed that these peptides contained only phosphothreonine (results not shown). Solid-phase Edman sequencing and MALDI–TOF MS (Table 1) indicated that peptide P_A corresponds to residues 334–347 phosphorylated at Thr^{336} (Figure 3B), peptide P_B corresponds to

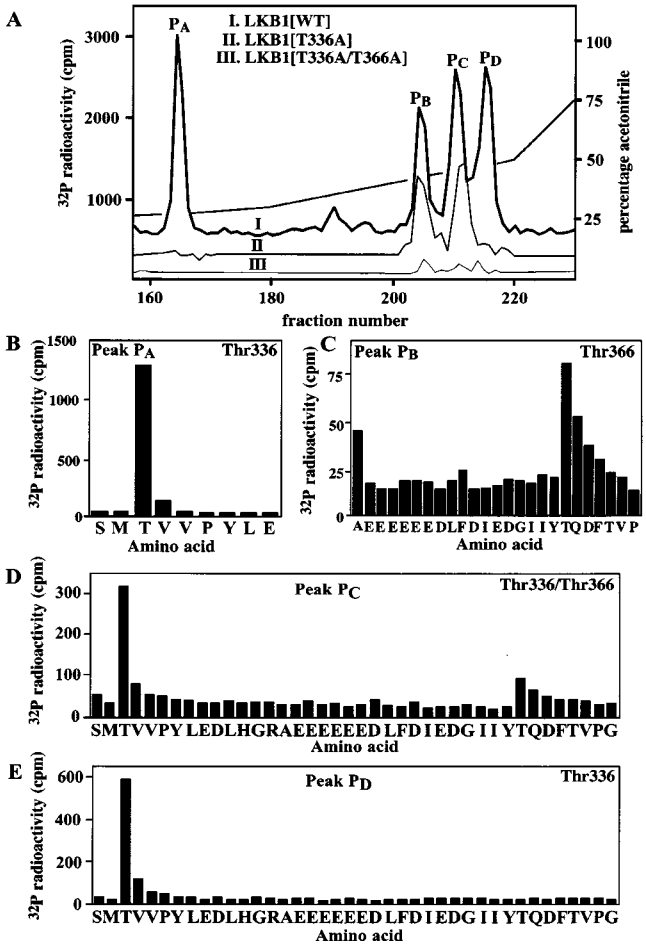

Figure 3 Identification of the *in vitro* autophosphorylation sites of LKB1

(**A**) Wild-type (WT) GST–LKB1, GST–LKB1[T336A] or GST–LKB1[T336A/T366A] expressed and purified from HEK-293 cells was incubated for 1 h with manganese-[γ^{32}P]ATP and electrophoresed on a 4–12% gradient polyacrylamide gel which was autoradiographed. The ^{32}P-labelled LKB1 was digested with trypsin and the resulting ^{32}P-labelled peptides were chromatographed on a C_{18} column as described in the legend to Figure 1. The positions of the peptides P_A, P_B, P_C and P_D are indicated. Fractions 160–230 from this chromatograph are shown; no other major ^{32}P-labelled peptides were observed to elute in any other fraction. (**B**–**E**) Each ^{32}P-labelled peptide was subjected to solid-phase sequencing as described in the legend to Figure 2. ^{32}P-radioactivity was measured after each cycle of Edman degradation and in combination with MALDI–TOF MS (Table 1) and phospho amino acid analysis (see the Results section) this enabled the identification of the phosphorylation sites in each of the peptides. The slight shift of peptide P_C derived from the LKB1[T336A] sample is due to this peptide only being phosphorylated at a single residue.

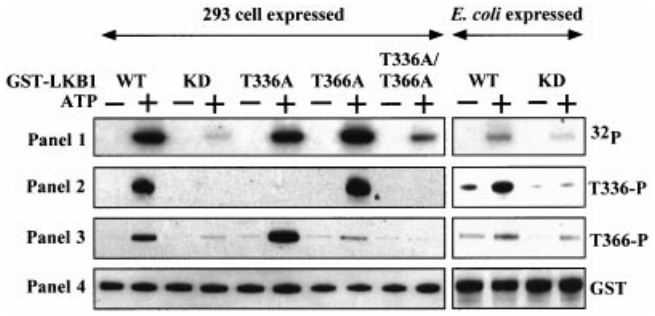

Figure 4 Generation of phosphospecific antibodies recognizing LKB1 phosphorylated at Thr^{336} and Thr^{366}

Wild-type (WT), kinase-dead (KD) or indicated mutants of LKB1 (2 μg), expressed either in mammalian HEK-293 cells or in *E. coli*, were incubated for 30 min in the absence (−) or presence (+) of [γ-^{32}P]ATP. Panel 1: a 1 μg aliquot of each sample was electrophoresed on a 4–12% gradient polyacrylamide gel which was subsequently autoradiographed. Panels 2, 3 and 4: 40 ng aliquots of each sample were electrophoresed on a 4–12% gradient polyacrylamide gel, transferred on to nitrocellulose and immunoblotted with either the T336-P antibody (panel 2) or the T366-P antibody (panel 3) in the presence of the dephosphorylated form of the peptide used to raise these antibodies, or with an anti-GST antibody (panel 4) to ensure similar loading of each form of LKB1. Similar results were obtained in at least three separate experiments.

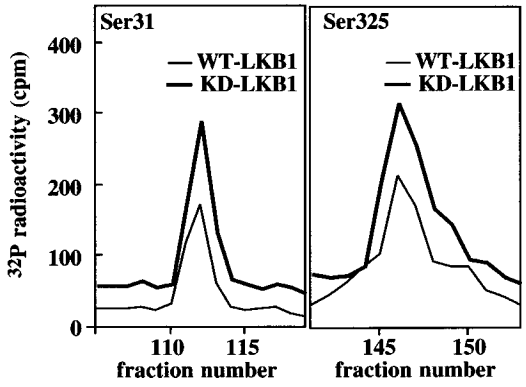

Figure 5 Kinase-dead LKB1 is labelled at Ser^{31} and Ser^{325} *in vivo*

HEK-293 cells transiently transfected with a DNA construct encoding either wild-type (WT) FLAG–LKB1 or kinase-dead (KD) LKB1 were ^{32}P-labelled. The cells were lysed, and LKB1 was immunoprecipitated and electrophoresed on an SDS/polyacrylamide gel and then digested with trypsin and chromatographed on a C_{18} column as described in the legend to Figure 1. The ^{32}P elution profile surrounding the fractions where peptides P_2 and P_3, corresponding to LKB1 peptides phosphorylated at Ser^{31} and Ser^{325}, are shown.

residues 348–392 phosphorylated at Thr^{366} (Figure 3C), peptide P_C corresponds to residues 334–392 phosphorylated at both Thr^{336} and Thr^{366} (Figure 3D), and peptide P_D corresponds to residues 334–392 phosphorylated at only Thr^{336} (Figure 3E). Peptide P_B was eluted in the same fraction on the C_{18} column as peptide P_4 derived from the *in vivo* labelling experiment of LKB1 and both peptides are phosphorylated at a single site 19 amino acids from the N-terminus (compare Figures 1 and 3). Consistent with this analysis, mutation of Thr^{336} to alanine in LKB1 resulted in the loss of peptides P_A and P_D. Moreover, the mutation of both Thr^{336} and Thr^{366} to alanine in LKB1 resulted in the loss of peptides P_A, P_B, P_C and P_D (Figure 3A). As expected, the single mutation of Thr^{366} to alanine resulted in the loss of peptides P_B and P_C (results not shown).

We subsequently generated phosphospecific antibodies against phosphopeptides corresponding to the sequences surrounding phosphorylated Thr^{336} and Thr^{366}, termed T336-P and T366-P antibodies respectively. These antibodies strongly recognized wild-type LKB1, but not a kinase-dead LKB1 mutant, following its incubation with manganese-ATP *in vitro* (Figure 4). The specificity of these antibodies was established by the findings that the T336-P antibody failed to recognize mutant GST–LKB1 [T336A] and the T366-P antibody failed to recognize mutant GST–LKB1[T366A] after incubation with manganese-ATP (Figure 4). Wild-type GST–LKB1 isolated from HEK-293 cells that had not been incubated with manganese-ATP was not recognized by either the T336-P antibody or the T366-P antibody, indicating that wild-type GST–LKB1 expressed in HEK-293 cells is phosphorylated at a low stoichiometry at Thr^{336} and

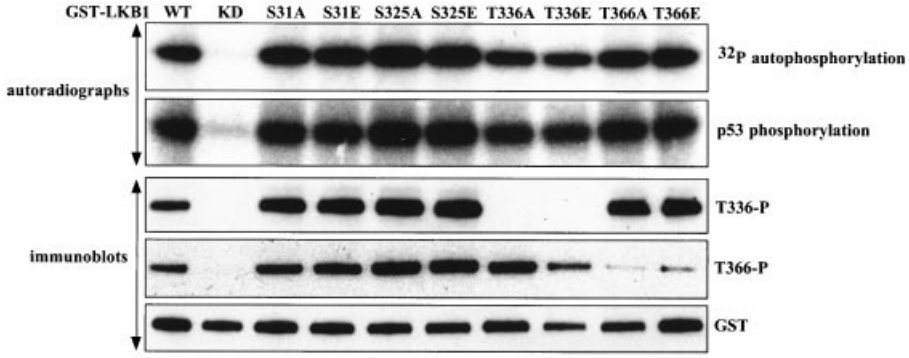

Figure 6 Evidence that phosphorylation of LKB1 does not affect its activity *in vitro*

Wild-type (WT) GST–LKB1, kinase dead (KD) GST–LKB1 or the indicated mutants of GST–LKB1, that had been expressed and purified from HEK-293 cells, were incubated for 30 min with manganese-[γ-^{32}P]ATP in the absence or presence of mouse p53 (2 μg) and electrophoresed on a 4–12% gradient polyacrylamide gel which was autoradiographed. The samples in which LKB1 had been incubated in the absence of p53 were also immunoblotted with T336-P and T366-P antibodies as well as an anti-GST antibody recognizing the GST tag to ensure that comparable amounts of wild-type and mutant forms of GST–LKB1 were used. Similar results were obtained in three separate experiments.

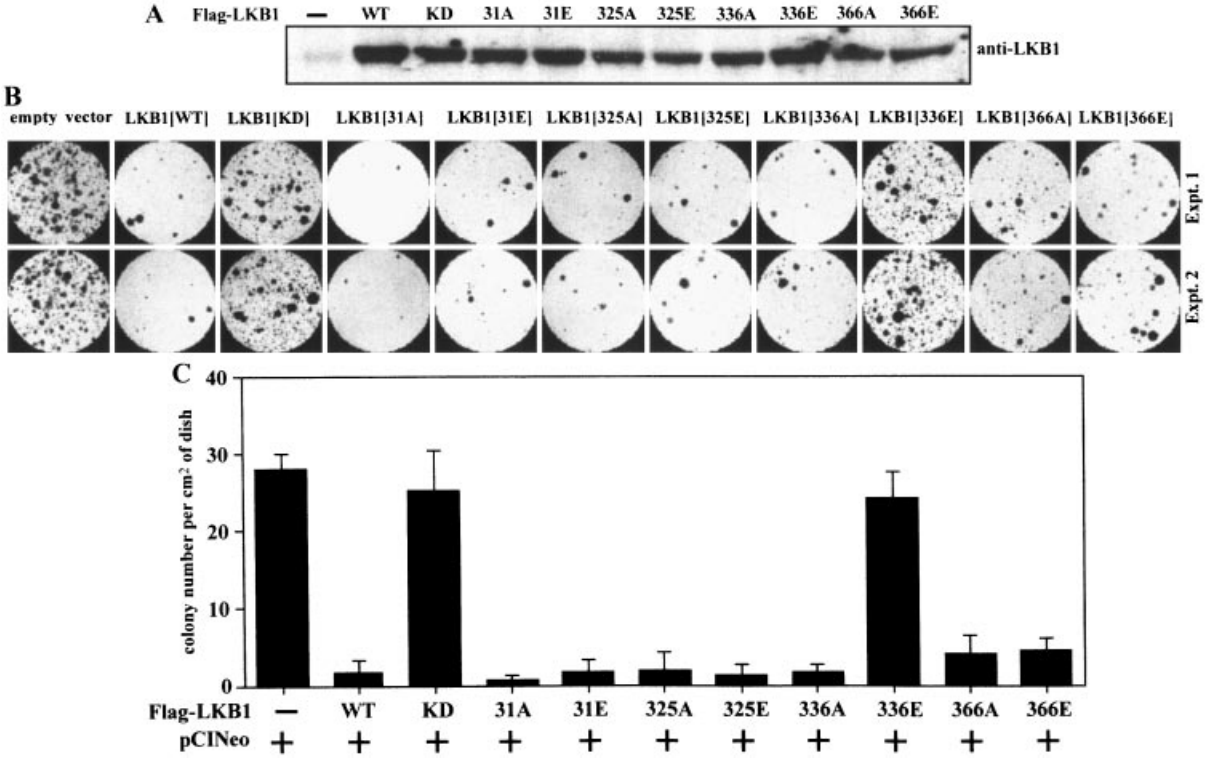

Figure 7 Evidence that phosphorylation of LKB1 at Thr^{336} prevents LKB1 from suppressing cell growth

G361 cells were transfected with pCMV5 constructs encoding the wild-type (WT), kinase dead (KD) and indicated mutant forms of FLAG–LKB1, together with the pCI-neo expression vector that encodes for G418 resistance. (**A**) After 2 days, a dish of each condition employed was lysed and the cell lysates were immunoblotted with the anti-LKB1 antibody to ensure that comparable amounts of wild-type and mutant forms of LKB1 were being expressed. (**B**) After 16 days of G418 selection Giemsa-stained colonies were photographed and (**C**) the number of colonies/cm^2 of the dish counted. Similar results were obtained in three separate experiments with each condition carried out in triplicate. The results of two separate experiments (Expt.) are shown in (**B**).

Thr^{366}. Further evidence that phosphorylation of LKB1 at Thr^{336} is mediated by autophosphorylation, rather than by a protein kinase that contaminates the HEK-293 cell GST–LKB1 preparation, stems from the observation that wild-type but not a kinase-dead LKB1 mutant expressed in *E. coli* is also phosphorylated at Thr^{336} following incubation with manganese-ATP *in vitro*, albeit to a lower stoichiometry than observed for GST–LKB1 expressed in HEK-293 cells (Figure 4). In contrast, wild-type LKB1 expressed in *E. coli* fails to phosphorylate itself significantly at Thr^{366}, and this finding is considered further in the Discussion section. As the [^{32}P]P_i incorporated into mutant GST–LKB1[T336A/T366A] following its incubation with manganese-ATP *in vitro* is significantly lower than wild-type GST–LKB1 (Figure 4), this indicates that Thr^{336} and Thr^{366} are the

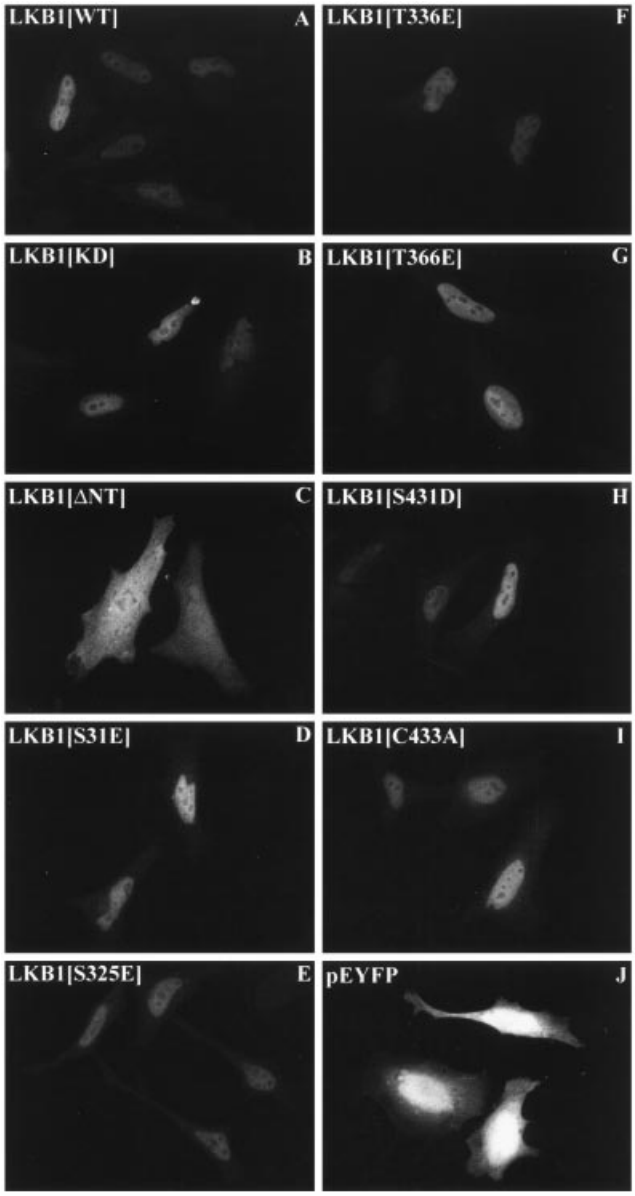

Figure 8 Localization of wild-type and mutant forms of LKB1

HeLa cells were transiently transfected with DNA constructs encoding for the expression of wild-type and indicated mutants of YFP–LKB1. The cells were fixed in 4% (v/v) paraformaldehyde 20 h post-transfection, and were imaged using a LEICA microscope as described in the Materials and methods section. The cells shown are representative images obtained in three separate experiments. KD, kinase-dead; LKB1[ΔNT], a mutant of LKB1 lacking the 44 N-terminal amino acids; WT, wild-type. Scale: 1 cm = 33 μm.

major sites that become phosphorylated in wild-type LKB1 under these conditions. As the autophosphorylation of the LKB1[T336A/T366A] mutant is not abolished this indicates that there is at least one other minor autophosphorylation site that we have been unable to identify.

Evidence that phosphorylation of LKB1 at Ser^{31} and Ser^{325} is not mediated by autophosphorylation

In order to investigate whether a catalytically inactive mutant of LKB1 is phosphorylated at Ser^{31} and Ser^{325} to the same extent as the wild-type enzyme in HEK-293 cells, we incubated HEK-293 cells expressing wild-type and kinase-dead LKB1 with [^{32}P]P_i. LKB1 was immunoprecipitated from the labelled cells, digested with trypsin and analysed by C_{18} chromatography. The ^{32}P-labelled peptides P_2 and P_3, corresponding to the Ser^{31} and Ser^{325} phosphorylation sites respectively, were present at a similar level in the chromatography derived from both the wild-type and kinase-dead LKB1 mutant (Figure 5).

Evidence that phosphorylation of LKB1 does not regulate its activity

The only assay available to gauge LKB1 activity *in vitro* is to measure either its autophosphorylation or the phosphorylation of the tumour suppressor p53 protein, which LKB1 phosphorylates very poorly [14]. In Figure 6, we demonstrate that mutation of Ser^{31}, Ser^{325}, Thr^{336} and Thr^{366} to either alanine, to prevent phosphorylation, or to glutamic acid, to mimic phosphorylation, does not significantly affect the extent to which LKB1 phosphorylates p53 or itself, as measured by ^{32}P-radioactivity incorporation or phosphorylation of Thr^{336} or Thr^{366} determined using the T336-P or T366-P antibodies.

Evidence that phosphorylation of Thr^{336} prevents LKB1 from suppressing cell growth

The expression of wild-type LKB1, but not kinase-dead LKB1, in G361 melanoma cells, which do not express LKB1, is known to potently suppress the ability of these cells to grow [5,14]. In order to determine whether mutation of Ser^{31}, Ser^{325}, Thr^{336} or Thr^{366} to alanine or glutamic acid affected the ability of LKB1 to suppress cell growth, G361 cells were transfected with an expression vector encoding either wild-type LKB1, the catalytically inactive LKB1, or the phosphorylation site mutants of LKB1, together with a plasmid encoding a neomycin/G418 resistance gene. After 16 days of selection with G418, the colonies that grew were visualized following staining with Giemsa stain solution. As reported previously [5,14], very few colonies of G361 cells were recovered when the cells were transfected with a plasmid encoding wild-type LKB1, compared with kinase-dead LKB1 (Figure 7). The mutation of Ser^{31} or Ser^{325} to either alanine or glutamic acid had no significant effect on the ability of LKB1 to inhibit G361 colony formation (Figure 7). Moreover, a mutant LKB1 in which Thr^{336} was changed to alanine still suppressed cell growth efficiently.

In contrast, when cells were transfected with a mutant of LKB1 in which Thr^{336} was changed to a glutamic residue, to mimic phosphorylation, > 10-fold more colonies were observed than when cells were transfected with wild-type LKB1 or LKB1[T336A] (Figure 7). Indeed, there was no significant difference in the number of colonies observed when cells were transfected with constructs expressing kinase-dead LKB1 or LKB1[T336E] (Figure 7). When G361 cells were transfected with constructs expressing LKB1[T366A] or LKB1[T366E], approx. 3-fold more colonies were observed compared with when the cells were transfected with wild-type LKB1 (Figure 7).

Phosphorylation of LKB1 does not alter its nuclear localization

To test whether phosphorylation of LKB1 could affect its cellular location, we transfected HeLa cells with an expression vector encoding YFP-tagged wild-type and mutant forms of LKB1 in which the Ser^{31}, Ser^{325}, Thr^{336}, Thr^{366} and Ser^{431} residues were individually mutated to a glutamic, aspartic or alanine residue.

Wild-type LKB1 and a catalytically inactive mutant were localized essentially in the nucleus (Figure 8A and 8B). In contrast, a mutant of LKB1 lacking the 44 N-terminal amino acids (LKB1[ΔNT]) that encompasses a putative nuclear localization signal (Figure 8C) as well as the YFP protein alone (Figure 8J) were distributed throughout the cell. All the mutants of LKB1 in which the phosphorylation sites were changed to glutamic acid (Figures 8D–8G), aspartic acid (Figure 8H) or alanine (results not shown) demonstrated the same nuclear localization as the wild-type LKB1. In addition, we demonstrated that a mutant of LKB1 in which the farnesylated Cys^{433} residue [14] is mutated to alanine is also still essentially localized in the nucleus (Figure 8I).

DISCUSSION

In the present study we demonstrate that LKB1, in addition to being phosphorylated at Ser^{431} [13,14], is also phosphorylated at three other residues *in vivo*, namely Ser^{31}, Ser^{325} and Thr^{366} (Figures 1 and 2). Furthermore, we show that, following incubation of LKB1 with manganese-ATP *in vitro*, it becomes phosphorylated at Thr^{336} as well as Thr^{366} (Figures 3 and 4). Ser^{31}, Thr^{336} and Thr^{366}, as well as the sequences surrounding these phosphorylation sites, are conserved in the human, mouse, *Xenopus* and *Drosophila* counterparts of LKB1 (Figure 9). Although Ser^{325} is not present in the *Drosophila* counterpart of LKB1, it is conserved in the human, mouse and *Xenopus* LKB1 (Figure 9). Individual mutations of Ser^{31}, Ser^{325}, Thr^{336} or Thr^{366} to alanine or glutamic acid did not significantly affect the rate at which LKB1 phosphorylated the p53 protein or itself (Figure 6).

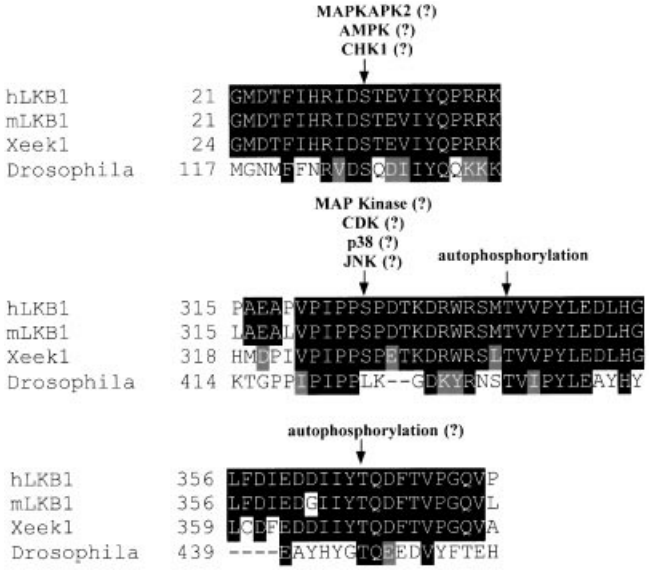

Figure 9 Alignment of the amino acid sequences surrounding the LKB1 phosphorylation sites, and potential kinases that could phosphorylate these residues

Residues that are identical with human LKB1 are shaded in black. The NCBI accession numbers for human, mouse, *Xenopus* and *Drosophila* LKB1 are U6333, AF151711, U24435 and AAF54972 respectively. The residues marked with an arrow correspond to the phosphorylation sites, and the possible identity of the upstream protein kinases that phosphorylate these residues is indicated. Although Thr^{366} may be catalysed by autophosphorylation, as *E. coli*-expressed LKB1 does not phosphorylate itself at this residue, we cannot exclude that the phosphorylation at this residue that we observe is mediated by a kinase that contaminates our preparation of wild-type LKB1 derived from HEK-293 cells.

These findings indicate that phosphorylation of these residues is not required for LKB1 activity and, consistent with this notion, incubation of wild-type LKB1 purified from HEK-293 cells with protein phosphatase-1 or protein phosphatase-2A did not inhibit LKB1 from phosphorylating itself or the p53 protein (G. P. Sapkota, unpublished work).

The *in vivo* peptide mapping analysis that was performed in the present study was carried out on LKB1 overexpressed in HEK-293 cells. We have also attempted to immunoprecipitate endogenous LKB1 from ^{32}P-labelled Rat-2 cells (i.e. rat embryonic fibroblast cells). Although we were able to confirm that endogenous LKB1 was phosphorylated, there was an insufficient amount present to enable us to map the phosphorylation sites (results not shown). It will be important in future studies to establish that endogenously expressed LKB1 is phosphorylated at Ser^{31}, Ser^{325}, Thr^{336} and Thr^{366}.

The mutation of Thr^{336} to a glutamic residue, to mimic phosphorylation, but not to an alanine, markedly prevented LKB1 from suppressing the growth of G361 cells (Figure 7). This indicates that phosphorylation of LKB1 at Thr^{336} may inhibit its function *in vivo*, without directly interfering with its intrinsic catalytic activity. Thr^{336} lies in a sequence Arg-Xaa-Arg-Xaa-Xaa-Thr-Hyd (where Xaa is any amino acid and Hyd is a large hydrophobic amino acid), which conforms to a consensus sequence for the AGC family of protein kinases [i.e. protein kinases similar to PKA, protein kinase G and protein kinase C] that includes protein kinase B (PKB), p70 ribosomal S6 kinase (S6K) and p90RSK [18–21]. However, despite Thr^{336} being a potential substrate for PKB, we have previously demonstrated that PKB does not phosphorylate LKB1 significantly *in vitro* [14]. We also found that other AGC kinase members tested, namely PKA, S6K1, p90RSK and mitogen- and stress-stimulated protein kinase 1 ('MSK1'), phosphorylated LKB1 only at Ser^{431} and not at Thr^{336} [14].

As wild-type LKB1 phosphorylates itself efficiently at Thr^{336} *in vitro*, it is perhaps surprising that LKB1 isolated from HEK-293 cells is not detectably phosphorylated at Thr^{336} (Figures 1 and 4). We have stimulated HEK-293 cells expressing GST–LKB1 with agonists known to activate the classical extracellular signal-regulated kinase (ERK)1/ERK2 MAP kinase pathway (serum, phorbol ester and epidermal growth factor), the phosphoinositide 3-kinase pathway (insulin-like growth factor 1 and epidermal growth factor) and the stress-activated p38/c-Jun N-terminal kinase (JNK) pathways (H_2O_2, sorbitol and anisomycin) and have not been able to demonstrate that any of these agonists induce the phosphorylation of LKB1 at Thr^{336} *in vivo* (G. P. Sapkota, unpublished work). It is possible that LKB1 may be associated with an inhibitory factor in cells that prevents it from phosphorylating itself at Thr^{336}, which is lost when LKB1 is isolated from cells, or that a stimulus which we have not tested promotes phosphorylation of LKB1 at Thr^{336} *in vivo*. We [14] and others [5,17] have shown that LKB1 only phosphorylates itself *in vitro* in the presence of manganese-ATP, but not magnesium-ATP, which is the physiological form of ATP in cells. It is therefore possible that it is the lack of the manganese-ATP complex *in vivo* that accounts for the lack of phosphorylation of LKB1 at Thr^{336} in cells. Thomas and co-workers [22] have recently shown that mammalian target of rapamycin ('mTOR'), another kinase that utilizes manganese-ATP in preference to magnesium-ATP, has a very high K_m for ATP (approx. 1 mM). We have tested whether LKB1 is capable of phosphorylating itself in the presence of magnesium and either 1 or 2 mM ATP, but found that this is not the case. The extent of autophosphorylation of LKB1 is the same in the presence of manganese and either 0.1 or 1 mM ATP, and only trace

levels of autophosphorylation are observed in the presence of 10 mM magnesium and 1 mM ATP.

Mutation of Thr^{366} to either alanine or glutamic acid also moderately reduced the ability of LKB1 to suppress cell growth, indicating that phosphorylation or dephosphorylation of this residue could play a role in regulating LKB1 (Figure 7). We found that LKB1 expressed in ^{32}P-labelled HEK-293 cells was detectably phosphorylated at Thr^{366} (Figures 1 and 2). Employing a T366-P antibody, we showed that phosphorylation of this site can be increased vastly by incubating LKB1 with manganese-ATP *in vitro* (Figure 4). As catalytically inactive LKB1 does not become phosphorylated at Thr^{366} following incubation with manganese-ATP *in vitro*, it is reasonable to conclude that LKB1 phosphorylates itself at this site. However, as wild-type LKB1 expressed in *E. coli* does not autophosphorylate itself at Thr^{366}, despite being able to phosphorylate itself at Thr^{336}, it cannot formally be ruled out that there is a distinct Thr^{366} protein kinase that binds only to wild-type LKB1 and not to the catalytically inactive LKB1, which is phosphorylating LKB1 at Thr^{366} in our experiments. It is also possible that the lack of phosphorylation of *E. coli*-expressed LKB1 at Thr^{366} is due to a mis-folding of the enzyme as this form of LKB1 phosphorylates itself poorly and does not phosphorylate the p53 protein [14].

Maller and co-workers [11] found that the *Xenopus* LKB1 counterpart autophosphorylated itself on threonine residue(s) and also demonstrated that mutation of a conserved threonine residue (Thr^{192} in *Xenopus* LKB1; equivalent to Thr^{189} in mammalian LKB1) to alanine, which is located in the kinase domain of LKB1, inhibited autophosphorylation. More recently, mutation of Thr^{189} to alanine in mammalian LKB1 has also been shown to inhibit LKB1 autophosphorylation [6]. These findings were interpreted to indicate that Thr^{189} was the major autophosphorylation site in LKB1. However, as peptide mapping studies to establish that LKB1 was indeed phosphorylated at Thr^{189} were not performed, an alternative explanation for these results is that mutation of Thr^{189} to alanine actually inactivates LKB1, thereby inhibiting autophosphorylation at other residues. Our peptide mapping results presented in Figures 3 and 4 suggest that Thr^{336} and Thr^{366} are the major sites of autophosphorylation of LKB1 and we have thus far been unable to identify any ^{32}P-labelled peptides present in this digest that could correspond to the LKB1 tryptic peptide phosphorylated at Thr^{189}. To obtain further evidence that LKB1 does not autophosphorylate at Thr^{189} we took advantage of the finding that LKB1 only phosphorylates itself on threonine residues. We mutated Thr^{189} to a serine residue and incubated this mutant with [γ-^{32}P]ATP. The LKB1[T189S] mutant autophosphorylated to the same extent as wild-type LKB1 and phospho amino acid analysis revealed phosphorylation of wild-type and mutant LKB1 occurred detectably solely on threonine and not serine (results not shown). We also demonstrated that phosphorylation of LKB1-[T189S] at Thr^{336} and Thr^{366} occurred to a similar extent as wild-type LKB1 in these experiments (results not shown).

LKB1 does not phosphorylate itself at Ser^{31} or Ser^{325} *in vitro*, and a catalytically inactive mutant of LKB1 when expressed in HEK-293 cells is phosphorylated at these sites (Figure 5). These observations indicate that these sites on LKB1 are phosphorylated by upstream protein kinase(s) that are distinct from LKB1. Ser^{31} lies in a consensus sequence (Hyd-Xaa-Arg-Xaa-Xaa-Ser) which matches that for the optimal phosphorylation motif for MAP kinase-activated protein kinase-2 (MAPKAP-K2) [23] and the AMP-activated protein kinase (AMPK) [24], as well as the cell cycle checkpoint kinase 1 (CHK1) [25]. Ser^{325} is located in a proline-rich region of LKB1 and is followed by a proline residue. Therefore Ser^{325} could be phosphorylated by one of the many proline-directed protein kinases that exist in cells. As mutation of Ser^{31} or Ser^{325} to alanine or glutamic acid does not affect LKB1 activity (Figure 6), the ability to inhibit growth of cells (Figure 7) or the nuclear localization of LKB1 (Figure 8), it is unclear at the moment what role phosphorylation of these conserved residues plays in regulating LKB1 activity. It will be important in future experiments to test whether stimuli that activate MAPKAP-K2, AMPK or CHK1 stimulate phosphorylation of LKB1 at Ser^{31}, or whether agonists that activate proline-directed kinases, including ERK1, p38, JNK or cyclin-dependent kinase family members, promote phosphorylation of LKB1 at Ser^{325}.

A major challenge for future work will be to establish that endogenously expressed LKB1 is phosphorylated at Ser^{31}, Ser^{325}, Thr^{336} and Thr^{366}, as well as identifying the specific molecular roles that these phosphorylations play in regulating its physiological functions. The work that we have performed in the present study indicates that phosphorylation of LKB1 at any of these residues is not regulating its intrinsic activity (Figure 6) or nuclear location (Figure 8). It is possible that phosphorylation of LKB1 could regulate its interaction with regulatory proteins or protein substrates. Identification of LKB1-binding proteins and how phosphorylation of LKB1 regulates its interaction with these proteins is likely to provide important insights into the molecular mechanism by which LKB1 might function as a tumour suppressor in cells.

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