LKB1, a novel serine/threonine protein kinase and potential tumour suppressor, is phosphorylated by cAMP-dependent protein kinase (PKA) and prenylated in vivo

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Peutz–Jeghers syndrome (PJS) is an autosomal dominant disease characterized by melanocytic macules, hamartomatous polyps and an increased risk for numerous cancers. The human LKB1 (hLKB1) gene encodes a serine/threonine protein kinase that is deficient in the majority of patients with PJS. The murine LKB1 (mLKB1) cDNA was isolated, sequenced and shown to produce a 2.4-kb transcript encoding a 436 amino acid protein with 90% identity with hLKB1. RNA blot and RNase-protection analysis revealed that mLKB1 mRNA is expressed in all tissues and cell lines examined. The widespread expression of LKB1 transcripts is consistent with the elevated risk of multiple cancer types in PJS patients. The predicted LKB1 protein sequence terminates with a conserved prenylation motif (Cys<sup>385</sup>-Lys-Gln-Gln<sup>388</sup>) directly downstream from a consensus cAMP-dependent protein kinase (PKA) phosphorylation site (Arg<sup>398</sup>-Arg-Leu-Ser<sup>401</sup>). The expression of enhanced green fluorescent protein (EGFP)–mLKB1 chimaeras demonstrated that LKB1 possesses a functional prenylation motif that is capable of targeting EGFP to cellular membranes. Mutation of Cys<sup>385</sup> to an alanine residue, but not phosphorylation by PKA, blocked membrane localization. These findings suggest that PKA does phosphorylate LKB1, although this phosphorylation does not alter the cellular localization of LKB1.

Key words: Peutz–Jeghers syndrome, membranes, green fluorescent protein.

INTRODUCTION

Peutz–Jeghers syndrome [PJS; OMIM (Online Mendelian Inheritance in Man) 175200] is an autosomal dominantly-inherited cancer syndrome. The symptoms include melanocytic macules on the lips, finger tips and oral mucosa, multiple gastro-intestinal hamartomatous polyps and an increased risk for various neoplasms [1,2]. The PJS gene has been mapped to chromosome 19p13.3 by linkage analysis [3], and LKB1/STK11, a widely expressed serine/threonine protein kinase, has been identified as the gene product [4,5]. Because PJS patients are at an increased risk for developing multiple types of cancer [6,7], it has been postulated that LKB1 may function as a tumour suppressor [4,5]. Most of the mutations in PJS families are point and truncation mutations within the kinase domain of LKB1, suggesting that the kinase activity of LKB1 is critical to its function [4,5]. To date, LKB1 mutations have been identified in only a small percentage of the sporadic tumour types examined [8–11]. Recently, four point mutations (G163D, D176N, W308C and L67P), which impair the ability of LKB1 to undergo autophosphorylation, have been found in PJS families [12,13].

Human LKB1 (hLKB1) encodes a protein that is highly homologous to the Xenopus kinase XEEK1 (Xenopus egg and embryo kinase 1) [14]. hLKB1 and XEEK1 share greater than 90% amino acid identity in their kinase domains, suggesting that LKB1 is the mammalian homologue of XEEK1 [4,5]. Both LKB1 and XEEK1 possess an N-terminal kinase domain followed by a C-terminal sequence of unknown function. XEEK1 is phosphorylated by cAMP-dependent protein kinase (PKA), probably in its C-terminal domain [14]. Like XEEK1, the hLKB1 protein contains a C-terminal consensus PKA phosphorylation site.

A single amino acid separates the consensus PKA phosphorylation site of hLKB1 from a C-terminal CAAX-box motif (CKQQ). Many proteins containing a CAAX-box motif undergo a stable post-translational modification in the form of prenylation. Prenylation of proteins has been shown to be important in targeting proteins to membranes and mediating protein–protein interactions [15]. In itself, prenylation is not sufficient to guarantee membrane localization [16,17]. Often, a number of positively charged amino acids are located directly preceding the CAAX-box motif. Such a basic region is essential for the membrane localization of Ki-Ras4B, and is believed to create an electrostatic interaction with acidic phospholipids in cell membranes [16]. Alternatively, Ha-Ras [18] possesses two palmitoylated cysteine residues upstream of the CAAX-box motif, which have been shown to be essential for membrane localization; hLKB1 is unique in that it possesses both a basic region and a potentially palmitoylated cysteine [19]. Phosphorylation of the PKA-site serine residue of hLKB1 could decrease the net positive charge or regulate reversible palmitoylation, and thus affect membrane localization. Thus the...
C-terminal of LKB1 may serve to localize LKB1 in the cell, and phosphorylation by PKA may affect the subcellular localization of LKB1.

In the present report, cDNAs encoding the murine homologue of LKB1 have been isolated and sequenced to determine which parts of the protein sequence are most highly conserved, and thus likely to be functionally important. Further, it was determined that the C-terminus of murine LKB1 (mLKB1) is phosphorylated by PKA and prenylated in vivo. Phosphorylation does not affect the membrane localization of enhanced green fluorescent protein (EGFP)–mLKB1 chimaeras. mLKB1 mRNA is widely expressed in murine tissues and cell lines suggesting that LKB1 is important to general cell function.

EXPERIMENTAL

Materials
Mevalonic acid (MVA) lactone (Sigma), mevastatin (Sigma) and [2,14C]mevalonolactone (55 mCi/mmol; American Radiolabeled Chemicals) were converted into their sodium salts before use [20]. [2,14C]Mevalonolactone was evaporated to dryness, incubated with 0.1 M NaOH for 1 h at 37 °C, neutralized with 0.5 M HCl and used immediately [21].

Isolation and sequencing of cDNA clones encoding mLKB1
A partial cDNA sequence coding for mLKB1 (I.M.A.G.E. Consortium clone 935323 5’/GenBank accession number AA542163) [22] was identified in a search of the expressed sequence-tag database for protein sequences similar to hLKB1, using the basic local alignment search tool (BLAST) algorithm [23]. This I.M.A.G.E. Consortium (LLNL) cDNA clone was obtained from Research Genetics. To obtain a cDNA clone possessing the entire mLKB1 open reading frame, cDNA library screening was performed essentially as described previously [24]. A 700 bp NcoI-SalI restriction fragment from clone 935323 was isolated and labelled by random primer extension with [32P]dATP (ICN Biomedicals). The resulting radioactively labelled DNA fragments were used to screen a mouse brain cDNA library. A single clone, mLKB1.1, was isolated and manually sequenced from both directions using Sequenase DNA polymerase (U.S. Biochemical Corp.). mLKB1.1 contains the entire open reading frame of mLKB1 and a full-length 3′-untranslated sequence with a putative poly(A) signal AATAAA (1537–1542) and a poly(A) tail. To determine the 5′-untranslated sequence of mLKB1, the reported sequences of two I.M.A.G.E. Consortium cDNA clones (I.M.A.G.E. Consortium clone 696173 5′/GenBank accession AA222428 and I.M.A.G.E. Consortium clone C0024E08 5′/GenBank accession AA408041) were aligned using DNASTAR software.

RNA-blot analysis
The template for the mLKB1 antisense probe was generated by PCR. PCR was performed with oligonucleotides 5′-GAGATCTGCTACGATCCATGGAGCTTGAGGACCCAGG-3′ and 5′-GGATCCCTCAGTGACTTGCTGTGGAGGACCCAGG-3′ with mLKB1.1 as the template. The resulting PCR fragment contained full-length mLKB1 flanked by BglII and NheI sites at the 5′ end and XhoI and BamHI sites at the 3′ end. A partial digest was performed on the fragment with BamHI and BglII and the digested fragment was ligated into the same sites in pGEM-T (Promega) to create pGEM-T–mLKB1, pGEM-T–mLKB1 was linearized by restriction digest with NheI and an antisense RNA radiolabelled probe was generated using T7 RNA polymerase. A mouse multiple-tissue Northern blot (Clontech) and a human RNA master blot (Clontech) were hybridized with the antisense probe at 60 °C for 16 h. Following hybridization, the blots were then washed at 70 °C for 2 h in 0.5 × SSC [5 mM Tris/HCl, pH 7.5, 5 mM EDTA, 0.5 %, (w/v) SDS] containing 0.1 % sodium pyrophosphate, dried and radiographed as described [25]. PhosphorImager quantitation was performed in a PhosphorImager apparatus and analysed with IMAGEQUANT software (Molecular Dynamics).

RNA-sequence analysis
RNA-sequence analysis procedures were essentially as described previously [26]. pGEM-T–mLKB1 was digested with BamHI and BglII. The 5′-coding fragment flanked by a BglII site and an internal BamHI site was isolated and ligated into BamHI and BglII-digested pSP73, creating pSP73–mLKB1. pSP73–mLKB1 was sequenced to verify the coding region sequence. This construct was linearized with either BglII or BamHI and used to synthesize antisense mLKB1 RNA probe or sense RNA standards respectively. Total RNA was isolated from mouse cell lines under denaturing conditions [27] using Trizol reagent (Gibco BRL). T7 RNA polymerase was used to generate [32P]UTP-labelled riboprobes from the linearized template, and sense RNA was generated with the use of SP6 polymerase. The radiolabelled probe was then incubated for 16 h at 50 °C with total cell-line RNA (50 μg). The radiolabelled probe was also incubated with various amounts of sense RNA samples (0, 0.3, 1, 3, 10 or 30 pg) brought up to 50 μg of RNA with yeast tRNA. The samples were then treated with RNase A (20 μg/ml) and RNase T1 (200 units/ml) (Sigma). The resulting protected fragments were isolated and electrophoresed through 6 % polyacrylamide sequencing gels. The gels were then dried and radiographed.

Construction of EGFP–mLKB1 and EGFP–mLKB1 mutant mammalian expression vectors
pEGFP–mLKB1 and pEGFP–mLKB1 mutant mammalian expression vectors were constructed by PCR. PCR fragments were generated using the following oligonucleotides as a reverse primer: 5′-GGATCCCTCAGTGACCTGCTGTGGAGGACCCAGG-3′ (WT), 5′-GGATCCCTCAGTGACTGCTGTGGAGGACCCAGG-3′ (S431A), 5′-GGATCCCTCAGTGACTGCTGTGGAGGACCCAGG-3′ (S431E), 5′-GGATCCCTCAGTGACTGCTGTGGAGGACCCAGG-3′ (C433A), 5′-GGATCCCTCAGTGACTGCTGTGGAGGACCCAGG-3′ (C433A). The oligonucleotide 5′-AGATCTGACTACAAGGACAGCATGACAAGGGTCAGAATGGACAGAGC-3′ was used as the forward primer and mLKB1.1 was used as the template in the PCR reactions to amplify 200 bp DNA fragments for the C-terminals of mLKB1 and LKB1 mutants. These DNA fragments possess an N-terminal FLAG epitope (DYKDDDDK) and were flanked by a BglII site and a BamHI site. These amplified fragments were digested with BglII and BamHI, isolated and ligated into the BglII/BamHI site of pEGFP.C1 (Clontech) to create pEGFP–mLKB1 and pEGFP–mLKB1 mutants. These plasmids were restriction mapped and the amplified regions were sequenced to verify the coding region sequence.

Transient transfection of CV-1 cells
CV-1 cells were grown on 10-cm plates to 30 % confluency and transfected using a standard calcium phosphate method [28]
with 10 μg of wild-type pEGFP–mLKB1 or mutant pEGFP–mlKBI and 5 μg of pMEV (mammalian expression vector for mevalonate transporter; A. T. C. C.) [21]. The total amount of plasmid DNA was brought to 25 μg with pCMV (cytomegalovirus)-Neo. After a 12 h incubation with DNA precipitates, cells were grown in Dulbecco’s modified Eagle’s Medium (DMEM) containing 10% (v/v) fetal-calf serum with or without 1 mM MVA for 24 h.

### Metabolic labelling of EGFP–mLKB1 chimaeras with [32P]P

Cells, 24 h post-transfection, were incubated for an additional 12 h in DMEM containing 1 mM MVA, but lacking fetal-calf serum. Serum-starved cells were washed twice with phosphate-free DMEM and then incubated for 2.5 h at 37°C in phosphate-free DMEM containing 1 mM MVA and 0.2 mCi/ml [32P]P (ICN). During the last 30 min of the incubation, cells were treated with or without 25 μM forskolin and 500 μM isobutylmethylxanthine (IBMX). Control cells were treated with an equivalent amount of DMSO alone.

### Metabolic labelling of EGFP–LKB1 chimaeras with [14C]MVA

Cells, 24 h post-transfection, were incubated in DMEM containing 25 μM mevastatin for 1 h at 37°C. This was followed by incubation for 11 h at 37°C with DMEM containing 10%, (v/v) dialysed fetal-bovine serum, 25 μM mevastatin and 2.5 μCi/ml [14C]MVA [21].

### Immunoprecipitation and Western-blot analysis

Cells were washed twice with ice-cold PBS, scraped into 1 ml of ice-cold Nonidet P-40 lysis buffer [10 mM Tris/HCl (pH 8.0), 150 mM NaCl, 1% (v/v) Nonidet P-40, 1 mM EDTA, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM PMSF, 1 μg/ml leupeptin and 1 μg/ml pepstatin A] and sonicated for 5 s at 5 W. Insoluble debris was removed by centrifugation at 1000 g for 15 min. Cell lysates were pre-cleared by mixing with Protein A/G plus agaro (Santa Cruz) for 1 h. An anti-FLAG epitope antibody (M2, 0.5 μg/ml; Eastman Kodak) was added to each supernatant and incubated for 1 h at 4°C. Protein A/G plus agaro (20 μl) was added to each supernatant and samples were mixed for an additional 1 h. The agarose beads were washed six times with Nonidet P-40 lysis buffer (1 ml). FLAG-tagged proteins were eluted from the agarose beads by the addition of 200 μM FLAG peptide (Eastman Kodak) to the final wash (100 μl, 12 h). Eluted proteins were resolved by SDS/PAGE (12% gels), transferred to nitrocellulose membranes and autoradiographed. The concentrations of eluted proteins were estimated by Western blotting. Membranes were blocked for 1 h in blocking buffer [50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.05% (v/v) Tween-20 and 5% (v/v) non-fat dried milk] and subsequently incubated with a 1:1000 dilution of an anti-FLAG epitope antibody (M2) in blocking buffer for 1 h. Filters were washed three times for 10 min with TBST (50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.05% (v/v) Tween-20) and incubated with a 1:1000 dilution of goat anti-mouse alkaline phosphatase (Gibco BRL), as the secondary antibody, in blocking buffer for 1 h. After the last of three washes for 10 min with TBST, the blots were developed with Nitro Blue Tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate system (Life Technologies Inc.)

### Fluorescence microscopy

CV-1 cells were grown in DMEM containing 10% (v/v) fetal-calf serum in 8-well tissue culture chambers on poly-d-lysine-coated glass slides (Lab-Tek) to 30% confluency. Cells were transiently transfectected using a standard calcium phosphate method [28] with 0.1 μg of pEGFP–C1, pEGFP–mLKB1 or pEGFP–mLKB1C433A and 0.05 μg of pMEV. Total plasmid concentration was maintained at 0.25 μg by addition of the parental vector, pCMV–Neo. After incubation for 12 h with DNA precipitates, cells were incubated with DMEM containing 10% (v/v) fetal-calf serum and 1 mM MVA for 24 h. Some cell preparations were stimulated with 25 μM forskolin and 500 μM IBMX in DMEM for 1 h at 37°C. After stimulation, cells were washed twice with ice-cold PBS, fixed with 4% formaldehyde in PBS for 10 min at room temperature and then in methanol/acetone (1:1, v/v) for 5 min. Before examination by fluorescence microscopy, the slides were incubated in PBS for 5 min.

### RESULTS

#### Isolation of mLKB1 cDNAs

To identify conserved features of the LKB1 protein sequence and to study LKB1 expression in murine tissues and cell lines, mLKB1 cDNAs were isolated. The amino acid sequence for hLKB1 [4,5] was used to search for orthologous murine sequences in the National Center for Biotechnology Information (NCBI) GenBank expressed-sequence-tag database using BLAST. This search identified a partial cDNA clone (I.M.A.G.E. Consortium clone 935323) possessing high predicted amino acid similarity to hLKB1. To obtain a cDNA encoding full-length mLKB1, cDNA library screening was performed. A radiolabelled probe generated from clone 935323 was used to screen a murine brain cDNA library. A single full-length cDNA clone, designated mLKB1.1, was isolated and fully sequenced. The mLKB1 cDNA sequence derived from overlapping cDNAs contained a short 21-bp 5'-untranslated sequence, an open reading frame of 1309 bp and a 3'-untranslated sequence with a putative poly(A) signal (AATAAA).

#### Analysis of mLKB1 protein sequence

The predicted mLKB1 protein is 436 amino acids with a calculated molecular mass of 49.5 kDa. Structurally, the mLKB1 protein contains a minimal kinase domain (262 residues) flanked by a short N-terminal (47 residues) and a longer C-terminal sequence (127 residues). The N-terminal sequence contains little sequence similarity to known proteins. The function of the N-terminal is unknown; however, small N-terminal sequences of other kinases have been shown to be important in properly positioning the glycine-rich loop and stabilizing the enzyme [29,30]. Whereas the mLKB1 kinase domain shows high amino acid identity to the kinase domains of hLKB1 and XEEK1 (Figure 1), it is only distantly related to the kinase domains of other protein kinase families. Overall, the C-terminal sequence of mLKB1 contains little similarity to known proteins; however, it does contain a consensus PKA phosphorylation site (RRLS) and a prenylation motif (CKQQ) (Figure 1).

The consensus PKA site of mLKB1 is located adjacent to a C-terminal prenylation motif (CAAX-box motif). Directly upstream of the CAAX-box motif are a number of positively charge residues and a potentially palmitoylated cysteine (Figure 1). There are two potential PKA sites in mLKB1 compared with the four potential PKA sites found in the Xenopus homologue XEEK1. However, both the C-terminal PKA phosphorylation site and prenylation motif are conserved in hLKB1 and XEEK1 (Figure 1).
smaller-sized transcripts were observed in testis (Figure 2, top panel). In addition to the 2.4-kb transcript, multiple smaller transcripts observed in most tissues was smaller than the 3.1-kb transcript observed in all murine tissues examined (Figure 2, top panel). The 2.4-kb mLKB1 study in human [5], transcripts were observed in all murine brain regions.

Expression of LKB1 mRNA in adult tissues, fetal tissues and adult brain regions
To determine mLKB1 mRNA expression levels in tissues, a murine poly(A) + Northern blot was probed with an antisense RNA probe specific for mLKB1. Consistent with a previous study in human [5], transcripts were observed in all murine tissues examined (Figure 2, top panel). The 2.4-kb mLKB1 transcript observed in most tissues was smaller than the 3.1-kb hLKB1 transcript. In addition to the 2.4-kb transcript, multiple smaller-sized transcripts were observed in testis (Figure 2, top panel). The highest levels of expression were observed in the liver and skeletal muscle. The universal expression of LKB1 mRNA in all mammalian tissues is consistent with the elevated risk of multiple cancer types in PJS patients.

Figure 1 Alignment of mLKB1, hLKB1 and XEEK1
The predicted mLKB1 protein sequence was aligned, by the CLUSTAL method, with those of hLKB1 and Xenopus XEEK1 [4,14]. Amino acid residues, identical in any two of the three sequences, are boxed. The kinase domain is highly conserved in all three proteins. The highest divergence is seen in the N- and C-terminals. The catalytic aspartic acid (Asp176), conserved in all protein kinases, is marked with an asterisk above the alignment. The threonine residue, indicated by a plus sign above the alignment. The prenylation motif (CAAX-box motif) indicated by a broken line above the alignment. The potential PKA phosphorylation sites of MLKB1 are underlined residues, and the potentially phosphorylated threonine residue (Thr212) is indicated by a minus sign above the alignment. The activation loop is defined by doubly underlined residues, and the conserved switch regions of mLKB1 generated the expected 265-bp protected fragment in all cell lines tested (Figure 2, bottom panel). C

Phosphorylation of mLKB1 by PKA
mlKB1 has two potential PKA phosphorylation sites and numerous potential activation-loop phosphorylation sites (Figure 1). Fusion proteins consisting of the C-terminus of mLKB1 attached to glutathione S-transferase (GST) (GST–mLKB1) or EGFP (EGFP–mLKB1) via a FLAG-tag linker were constructed to selectively examine the role of the putative C-terminal PKA site of mLKB1. To determine if the C-terminal site was phosphorylated by PKA in vitro, GST and GST–mLKB1 were expressed in bacteria and purified to near homogeneity. Phosphorylation of GST–mLKB1 was apparent within 5 min and peaked within 30 min. No significant phosphorylation of GST alone was observed under similar conditions. Phosphorylation of mLKB1 by recombinant C subunit was blocked by inclusion of the LBK1 antisense probe. Hybridization was observed in all tissues and brain regions examined, with the highest levels in skeletal muscle, testis, small intestine and fetal liver (Figure 2, middle panel). Hybridization was specific, as no signal was observed with negative controls (yeast total RNA). Although fetal liver was the tissue showing the highest level of LKB1 expression, fetal tissues did not, in general, show higher levels of expression than their adult counterparts. For example, fetal lung and spleen showed lower levels of expression than adult lung and spleen (Figure 2, middle panel).

RNase-protection analysis of LKB1 expression in mouse-derived cell lines
RNA-blot analysis showed LKB1 mRNA widely expressed in all tissues examined. To determine if LKB1 mRNA was widely expressed in different cell types, the more sensitive RNase-protection assay was performed on various murine cell lines: L929 fibroblasts, NIH3T3 fibroblasts, C

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precipitated was estimated by Western-blot analysis. Western-blot analysis with the M2 anti-FLAG antibody detected a single band with an apparent molecular mass of 34 kDa, the size expected for the EGFP–mLKB1 chimaeras (Figure 3A). The protein expression levels of the EGFP–mLKB1 mutants were similar to those of the wild-type chimaera (Figure 3B). The level of wild-type EGFP–mLKB1 phosphorylation was low in serum-starved cells. Forskolin treatment increased wild-type EGFP–mLKB1 phosphorylation by 6-fold (Figure 3A).

The C-terminal PKA phosphorylation site of LKB1 is adjacent to its prenylation motif and a potentially palmitoylated cysteine (RKVCSSNKIRRLSA CKQQ) [19]. The proximity of these sequences suggests that prenylation or palmitoylation of LKB1 could block phosphorylation of Ser431 by PKA. However, PKA phosphorylated wild-type EGFP–mLKB1, EGFP–mLKB1C433A and EGFP–mLKB1C422A to similar extents,

RNA from various murine and human tissues was analysed by hybridizing with a radiolabelled antisense RNA probe generated from the full-length coding region of mLKB1. Top panel: Northern-blot analysis of mLKB1 expression in murine tissues. The sizes (kb) of RNA standards are indicated on the left of the Figure. H, heart; Br, brain; Sp, spleen; Lu, lung; Li, liver; SM, skeletal muscle; Ki, kidney; Te, testis. Middle panel: RNA-blot analysis of LKB1 expression in adult and fetal tissues. Poly(A)$^+$ RNA samples from adult and fetal human tissues were immobilized in separate dots. The signal intensity for each dot was quantified using a PhosphorImager system and normalized to the expression level in fetal liver (100%). Bottom panel: RNase protection analysis of LKB1 expression in mouse-derived cell lines. Total RNA (50 μg) or sense standard RNA (0, 0.3, 1, 3, 10 or 30 pg) was hybridized to a mLKB1-specific antisense RNA probe, treated with RNase and separated by denaturing gel electrophoresis. The positions of the RNA probe (330 bp) and the sense standards RNA-protected fragments (283 bp) are indicated on the right, and the position of the tissue RNA-protected fragments (265 bp) is indicated on the right.
with the wild-type EGFP–mLKB1 chimaera and labelled with the C-terminal of mLKB1 is palmitoylated, cells were transfected with the wild-type EGFP–mLKB1 chimaera or EGFP–mLKB1 chimaera mutants and labelled with [14C]palmitic acid (2.5 μCi/ml) for 4 h. The EGFP–mLKB1 chimaera was immunoprecipitated and the success of the immunoprecipitation was confirmed by immunoblotting. Although immunoblotting detected a single 34 kDa band, even with prolonged exposures, no [14C]palmitic acid incorporation was detected (results not shown). In general, prenylated proteins are palmitoylated on cysteine residues two to six residues N-terminal to their prenylated cysteine [18]. The finding that the EGFP–mLKB1 chimaera was not labelled with palmitate suggests that Cys433 was too distant from the prenyl-acceptor cysteine (Cys433) of mLKB1 to be palmitoylated efficiently.

Localisation of EGFP–mLKB1 chimaeras in mammalian cells

Prenylation of proteins has been shown to be important in targeting proteins to cell membranes [15]. To determine if the C-terminal of mLKB1 was capable of promoting membrane localization, CV-1 cells were transiently transfected with EGFP, EGFP–mLKB1 or EGFP–mLKB1C433A and examined by fluorescence microscopy 24 h after transfection. As expected, EGFP, normally a cytoplasmic protein, exhibited diffuse fluorescence in the nucleus and in the cytoplasm (Figure 5, EGFP). The wild-type EGFP–mLKB1 chimaera localized to both the plasma membrane and internal membranes (Figure 5, WT). Mutation of Cys433 to an alanine blocked membrane localization of the chimaera (Figure 5, C433A), suggesting that prenylation and not the C-terminal amino acid sequence was critical for membrane targeting. Interestingly, the C-terminal of mLKB1 prevented nuclear localization of EGFP. The fusion of the C-terminal of mLKB1 with a defective CAAX-box motif to EGFP was not sufficient alone to prevent nuclear localization, suggesting that nuclear restriction was not due solely to an increase in the size of the protein. This suggests that the membrane targeting ability of the CAAX-box motif is capable of blocking the nuclear localization of EGFP and that a large percentage of the expressed wild-type EGFP–mLKB1 chimaera is modified.

Prenylation is not sufficient to determine membrane localization. Stable membrane localization, in general, requires either a basic region or a palmitoylated cysteine upstream of the CAAX-box motif [16,17]. mLKB1 possesses a basic sequence N-terminal of its prenyl-acceptor cysteine (Figure 1). Prenylation of Ser433 could conceivably decrease the net positive charge and thus affect membrane localization. Experimentally, forskolin treatment elevated 32P incorporation into the wild-type EGFP–mLKB1 chimaera (Figures 3A and 3B); however, it did not discernibly affect its location (Figure 5, WT+F). Furthermore, no change in fluorescence was observed at other times following forskolin treatment (30 min and 2 h; results not shown). Likewise, mutation of Ser433 to a glutamate residue, to mimic

Figure 5 Fluorescence localization of EGFP, EGFP–mLKB1 and EGFP–mLKB1C433A in mammalian cells

Fluorescence-microscopy analysis of CV-1 cells transiently transfected with either pEGFP–C1 (EGFP), pEGFP–mLKB1 (WT) or pEGFP–mLKB1C433A (C433A). Forskolin (25 μM) and IBMX (500 μM) were added (+F) for 1 h, 24 h post-transfection. These reagents were omitted from the experiments shown in EGFP, WT and C433A. Cells were fixed with 4% paraformaldehyde and acetonemethanol (1:1, v/v). Scale bars, 20 μm.

Suggesting that putative prenylation or palmitoylation of mLKB1 does not affect PKA phosphorylation of Ser433 (Figure 3A).
phosphorylation, had no discernible effect on the localization of the chimera (results not shown).

To determine if phosphorylation of Ser431 may have more subtle effects on the membrane localization of EGFP-mLKB1, the partitioning of the EGFP-mLKB1 chimera was analysed by cell fractionation. CV-1 cells were transiently transfected with EGFP-mLKB1 or EGFP-mLKB1C433A and 24 h after transfection were fractionated into aqueous and detergent phases by Triton X-114 phase partitioning. Each phase was then analysed by immunoblotting with the anti-FLAG antibody. Whereas the EGFP-mLKB1 chimera was found in both the aqueous and detergent phases, the EGFP-mLKB1C433A mutant was found solely in the aqueous phase. Forskolin treatment did not affect the amount of the wild-type chimera in the detergent phase (results not shown). In conclusion, modification of the CAAX-box motif to prevent prenylation blocked membrane localization; however, manipulation of the PKA phosphorylation site adjacent to the CAAX-box motif had no observable effect.

DISCUSSION

The study of human cancer syndromes has greatly contributed to the present understanding of the origins of the more common sporadic cancers [34]. Mouse models of these syndromes have aided our understanding of the mechanisms by which these germline mutations cause cancers and the growth-control pathways in which these genes participate. In the present report, the cloning and characterization of the murine homologue of the novel serine/threonine protein kinase LKB1, which is mutated in PJS, is described. mLKB1 is highly conserved with hLKB1 (90% amino acid identity) and most likely functions in the same cellular pathway(s).

The kinase domain of LKB1 is reasonably similar to the kinase domains of other serine/threonine protein kinases [SNF1 kinases and AMP-activated protein kinases (AMPKs)] [35]; however, several LKB1 subdomain sequences differ significantly from these and other kinases [36]. Phosphorylation of residues located in the region spanning the highly conserved sequences DFG (subdomain VII) and APE (subdomain VIII) of the kinase activation loop is critical for activation of many kinases [37]. In LKB1, the DFG sequence is replaced by DLG and the APE sequence is replaced by PPE (Figure 1). The backbone structure of the APE motif is rigid in most kinase crystal structures, suggesting that the aspartate-to-proline change could significantly alter the structure of subdomain VIII in LKB1 [37].

Amino acids directly upstream of the APE motif are also highly conserved among different kinase families. This region has been shown to be important in substrate binding and contains the phosphorylated residues of the activation loop. AMPK kinase phosphorylates a threonine residue (Thr172) in this portion of the activation loop of AMPK, and causes a 20-fold activation [38]. Subdomain VIII of LKB1 exhibits amino acid sequence similarity with subdomain VIII of the AMPKs. Specifically, the position of the phosphorylated threonine residue in the z1 subunit of AMPK is identical with that found for Thr212 of LKB1 (Figure 1). The similarity in sequence between subdomain VIII of LKB1 and the AMPKs suggests that phosphorylation of Thr212 could play a critical role in LKB1 activation.

Additional evidence suggests that LKB1 may require phosphorylation for activation. All protein kinases possess an aspartic acid residue in subdomain Vlb which is believed to be important as a catalytic base. Kinases that are known to be activated by phosphorylation, in general, possess a basic residue immediately preceding the catalytic aspartate (arginine/aspartate kinases) [39]. It has been suggested that RD kinases require neutralization of this basic residue by the incorporated phosphate to obtain the active conformation. Although LKB1 possesses a basic residue directly upstream of its catalytic aspartate residue (Asp186), it is a lysine instead of an arginine residue (Figure 1). LKB1 and XEEK1 are the only serine/threonine protein kinases we know to possess a lysine in this position and its positive charge suggests that LKB1 may also require phosphorylation for activation.

Commonly, patients with germline mutations in cancer-susceptibility genes have a large increased risk of a small number of cancer types even though many cancer-susceptibility genes are widely expressed [34]. This phenomenon has been attributed to the presence of cell-type specific redundancy in growth-control pathways. PJS is a unique human cancer syndrome in that patients have a small increased risk for a number of cancer types. As shown previously for hLKB1 [5], mLKB1 transcripts were expressed in all fetal and adult tissues examined. Expression of mRNA in all tissues is consistent with the increased cancer risk of PJS patients. In this report, mLKB1 transcripts were shown to be widely expressed in mouse-derived cell lines, suggesting that LKB1 participates in a growth-control pathway common to all cells. These cell lines should be useful for examining the mechanism(s) by which defects in the LKB1 gene contribute to PJS.

The Xenopus LKB1 homologue, XEEK1 is phosphorylated by PKA at multiple sites, most likely in its C-terminal [14]. Elevated PKA activity has been shown to be important in maintaining Xenopus oocyte arrested at the G2 phase in the cell cycle [40]. XEEK1 is a PKA substrate which is potentially important in the regulation of the Xenopus cell cycle. Like XEEK1, LKB1 protein contains potential sites for phosphorylation by PKA (Figure 1). The results of the present work suggest that Ser431 is indeed a PKA phosphorylation site in vivo. Prenylation of an adjacent cysteine residue and the resulting membrane localization did not affect phosphorylation at this site. Endogenous PKA is either localized diffusely in the cell or localized to specific cellular compartments by PKA anchoring proteins [41]. These results suggest that a significant percentage of the endogenous PKA in CV-1 cells must have access to membrane-bound proteins.

Phosphorylation of the EGFP-mLKB1 chimera at Ser431 by PKA did not discernibly affect the membrane localization of the chimera (Figure 5). However, this finding does not mean that phosphorylation at this site by PKA is not regulatory in terms of LKB1 function. Phosphorylation at Ser431 may regulate the kinase activity of LKB1 or the binding of regulatory subunits, and it is possible that LKB1 phosphorylation by PKA could be important in regulation of the cell cycle by PKA.

Recombinant XEEK1 autophosphorylates in vitro in a reaction in which Mn2+ is preferential to Mg2+. The preference for Mn2+ by a serine/threonine protein kinase suggests that the enzyme may require additional regulatory subunits [14]. In addition, immunoprecipitation of Xenopus oocyte extracts with anti-XEEK1 sera results in the precipitation of a 155 kDa protein that is likely to be a regulatory subunit or substrate of XEEK1 [14]. To determine if mLKB1 possesses similar regulatory subunits, [35S]-labelled mLKB1 and a catalytically inactive mutant (mLKB1K78R) from HEK293 cells stably expressing these proteins were immunoprecipitated. This revealed a prominent 90 kDa protein that associated with both mLKB1 and the mLKB1K78R mutant (results not shown). This band was specific, as it was not detected in precipitates from [35S]-labelled wild-type HEK293 cells. It will be of interest to determine if the binding of p90 is regulated by PKA phosphorylation.
The substrate specificity of a protein kinase in vitro is determined partly by its subcellular localization. Modification of protein kinases by covalent addition of lipids has been shown to be important in directing these proteins to specific membrane locations [43]. Rhodopsin kinase (G-protein-coupled receptor kinase 1; GRK1) is an example of a protein kinase that requires prenylation for proper localization and function. Farnesylation is essential to the light-dependent membrane localization of GRK1 and efficient phosphorylation of rhodopsin [44]. In the present study we have shown that LKB1 contains a functional CAAX-box motif that is capable of directing EGFP to membranes. To date, no LKB1 substrates have been identified which will allow examination of the possible role of this motif in the substrate specificity of LKB1 in vitro.

The Ras GTPases are a well characterized family of oncogenic proteins that contain CAAX-box motifs. The frequent association of mutated Ras proteins with human malignancies has brought about investigations into chemotherapies targeted at Ras. Anti-prenylation drugs have been examined as potential chemotherapeutic agents [45]. Of particular concern is the non-specific complications with other proteins that require prenylation for proper function (e.g. lamin B, GRK1 and phosphodiesterases). Our findings suggest that LKB1, a postulated tumour suppressor, may require prenylation for proper cellular function. Therefore anti-cancer therapies targeting Ras prenylation may compromise LKB1 function and this should be considered in the application of such therapies.

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