Characterization of an Alternative Splice Variant of LKB1*

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Fiona C. Denison†, Natalie J. Hiscock§, David Carling†‡, and Angela Woods†‡

From the †Cellular Stress Group, Medical Research Council Clinical Sciences Centre, Du Cane Road, London W12 0NN, United Kingdom and the ‡Unilever Discover, Personalised Vitality Platform, Colworth Science Park, Sharnbrook, Bedfordshire MK44 1LQ, United Kingdom

LKB1 is an upstream activating kinase for the AMP-activated protein kinase (AMPK) and at least 12 other AMPK-related kinases. LKB1 therefore acts as a master kinase regulating the activity of a wide range of downstream kinases, which themselves have diverse physiological roles. Here we identify a second form of LKB1 generated by alternative splicing of the LKB1 gene. The two LKB1 proteins have different C-terminal sequences generating a 50-kDa form (termed LKB1L) and a 48-kDa form (LKB1S). LKB1L is widely expressed in mouse tissues, whereas LKB1S has a restricted tissue distribution with predominant expression in the testis. LKB1S, like LKB1L, forms a complex with MO25 and STRAD, and phosphorylates and activates AMPK both in vitro and in intact cells. A phosphorylation site (serine 431 in mouse) and a farnesylation site (cysteine 433 in mouse) within LKB1S are not conserved in LKB1L, raising the possibility that these sites might be involved in differential regulation and/or localization of the two forms of LKB1. However, we show that phosphorylation of serine 431 has no effect on LKB1L activity and that both LKB1L and LKB1S have similar patterns of subcellular localization. These results indicate that the physiological significance of the different forms of LKB1 is not related directly to differences in the C-terminal sequences but may be due to their differential patterns of tissue distribution.

LKB1 was originally identified as a tumor suppressor because inactivating mutations in the LKB1 gene lead to a cancer predisposition disease in humans, termed Peutz-Jeghers syndrome. LKB1 is a serine/threonine protein kinase that forms a complex with two other proteins, STRAD (STE20-related adaptor) (1) and MO25 (mouse protein 25) (2). STRAD shares extensive sequence identity with protein kinases but lacks several residues required for catalytic activity and therefore has been termed a pseudokinase. MO25 binds to the C-terminal 3 amino acids of STRAD (1, 3), and binding of MO25 markedly increases the binding of STRAD to LKB1 (2). In addition to enhancing the catalytic activity of LKB1, it has been reported that binding of STRAD and MO25 causes LKB1 to relocalize from the nucleus to the cytoplasm (4). In addition, there has also been a report that claims that phosphorylation of serine 428 at the C terminus of human LKB1 (equivalent to Ser-431 in mouse) is necessary for LKB1 export from the nucleus (5). However, another study reported that phosphorylation at this site does not result in relocalization (6). The cytosolic localization of LKB1 appears to be important for its tumor suppressor functions, because mutants of LKB1 that are unable to enter the nucleus retain their full capability to suppress cell growth (7).

LKB1 phosphorylates AMP-activated protein kinase (AMPK)4 at a single site, Thr-172 within the T-loop segment of the catalytic α subunit (8–10). Phosphorylation of Thr-172 is essential for activation of AMPK (11). In addition to LKB1, Ca2+/calmodulin-dependent protein kinase kinase (12–14) and transforming growth factor-β-activated protein kinase 1 (15) have been shown to phosphorylate and activate AMPK. Although a complete understanding of the physiological relevance of the different upstream kinases in the AMPK cascade is some way off, it appears that LKB1 has a predominant role in activating AMPK in some tissues. For example, deletion of LKB1 in liver led to an almost complete loss of AMPK activity (16), and in skeletal muscle a lack of LKB1 abolished activation of the α2-containing AMPK complexes (17). More recently, LKB1 has been shown to phosphorylate 12 AMPK-related kinases within their T-loop regions, and this phosphorylation is essential for their activation (18).

LKB1 itself is phosphorylated on at least 8 serine/threonine residues. Four of these are autophosphorylation sites, whereas the remaining 4 sites are phosphorylated by other kinases (19).

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‡ To whom correspondence may be addressed: Cellular Stress Group, MRC Clinical Sciences Centre, Du Cane Road, London, W12 0NN, United Kingdom. Tel.: 44-20-8383-4313; Fax: 44-20-8383-8514; E-mail: dcarling@imperial.ac.uk.

§ To whom correspondence may be addressed: Cellular Stress Group, MRC Clinical Sciences Centre, Du Cane Road, London, W12 0NN, United Kingdom. Tel.: 44-20-8383-4313; Fax: 44-20-8383-8514; E-mail: angela. woods@imperial.ac.uk.

The abbreviations used are: AMPK, AMP-activated protein kinase; PKA, cAMP-dependent protein kinase; CREB, cAMP-response element binding protein; PBS, phosphate-buffered saline.

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residue, but not to an alanine, prevented LKB1 from suppressing cell growth in G361 cells, suggesting that phosphorylation of this residue has an inhibitory effect on LKB1 activity (20). The physiological significance of this finding is unclear because phosphorylation of Thr-336 has only been reported to occur using manganese ATP as a substrate, rather than magnesium ATP, the physiologically relevant form of ATP (20). Ser-431 in mouse LKB1 (equivalent to Ser-428 in the human sequence) has been shown to be phosphorylated by a number of kinases, including cyclic AMP-dependent protein kinase (PKA), p90 ribosomal S6 protein kinase (21), and protein kinase Cζ (5). Mutation of Ser-431 to either alanine or glutamic acid prevented LKB1 from suppressing cell growth (21), suggesting that this residue is required for some aspect of LKB1 function. Recently, a role for the PKA-dependent phosphorylation of LKB1 has been proposed based on studies examining LKB1 in neuronal polarization (23, 24). Finally, it has been reported that phosphorylation of Ser-428 in human LKB1 is involved in relocalization of LKB1 from the nucleus to the cytoplasm and that this is required for the LKB1-mediated phosphorylation of AMPK (5).

Here we describe the identification of a previously uncharacterized form of LKB1, generated by alternative splicing, that has a different C-terminal sequence from the previously studied form and lacks the equivalent residue corresponding to Ser-428/431. We have called the new form of LKB1, LKB1S (for short form and lacks the equivalent residue corresponding to Ser-428/431). We have characterized this form of LKB1, generated by alternative splicing, that has been previously unrecognized. TV We show that phosphorylation of Ser-428/431 has no effect on LKB1 activity and is not involved in relocalization of the LKB1 complex between the nucleus and cytoplasm.

EXPERIMENTAL PROCEDURES

Materials—Forskolin, hydrogen peroxide, and oligonucleotide primers were purchased from Sigma-Aldrich. Mouse and human testis cDNA were obtained from Ambion. All of the microarray products were purchased from Agilent Technologies UK unless otherwise noted.

Tissue Harvesting and Preparation—Tissues from male mice (C57/B6), ~10 weeks old were harvested and immediately frozen in liquid nitrogen. Prior to analysis, the tissues were roughly homogenized in 50 μM Tris, pH 7.5, 5 mM sodium pyrophosphate, 50 mM NaF, 1 mM EDTA, 0.25 M sucrose, 1 mM dithiothreitol, 4 μg/ml trypsin inhibitor, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine) and then briefly homogenized with a rotor-stator homogenizer. Triton X-100 was added to the homogenates to 1% (v/v), and the homogenates were incubated on ice for 10 min before centrifugation at 16,000 × g for 15 min to remove insoluble material. Protein content in the supernatant was determined using the Bradford assay (25).

Antibodies—We raised a rabbit polyclonal antibody against full-length mouse LKB1, (expressed as a His-tagged fusion protein in Escherichia coli). A peptide (CMWKSQGAGLPGEE) corresponding to residues 390–403 of mouse LKB1s was used to raise anti-LKB1S antibodies (termed anti-sLKB1) in rabbit. Antibodies were affinity-purified from the serum using the immunizing-peptide coupled to thiol-activated Sepharose. Affinity-purified sheep antibody raised against residues 24–39 of human LKB1 was a kind gift from Prof. Dario Alessi (University of Dundee). Mouse monoclonal antibody recognizing both LKB1L and LKB1S (Ley37/D/G6) and anti-STRADα (STRAD N13) were from Santa Cruz Biotechnology. Mouse monoclonal antibody recognizing Ser(P)-428 in human LKB1L, monoclonal anti-MO25, and phospho-CREB (Ser-133, 87G3) were from Cell Signaling Technology. FLAG (M2) and anti-CREB were from Sigma, and anti-glyceroldehyde-3-phosphate dehydrogenase was from AbCam. A pan-β-specific AMPK antibody has been described elsewhere (26).

Western Blot Analysis—Primary antibodies were detected with the appropriate secondary antibody conjugated to either Alexa-Fluor 680 (Invitrogen) or IRDye800 (LI-COR) and scanned on the Li-COR Odyssey Infrared Imaging System. Quantification of results was performed using Odyssey software 2.0 (LI-COR Biotechnology).

LKB1 and AMPK Activity Assays—LKB1 activity was determined by a two-step assay that measures the ability of LKB1 to activate recombinant AMPK (10). For endogenous LKB1s activity, mouse tissue extracts were incubated with protein A-Sepharose beads for 1 h at 4 °C, and then LKB1s was immunoprecipitated by incubation with rabbit anti-sLKB1antibody bound to protein A-Sepharose for 2 h at 4 °C. LKB1 activity in transiently transfected cells was immunoprecipitated by incubation with anti-FLAG resin. In each case, the immune complexes were washed extensively with buffer A and then incubated with 0.2–1 μg of recombinant AMPK (α1β1y1) and 0.2 mM ATP, 5 mM MgCl2 in buffer A (20 μl of total volume) for 20 min at 37 °C. AMPK activation of was quantified by removing an aliquot (5 μl) of the reaction and measuring AMPK activity using the SAMS peptide assay (27). AMPK was immunoprecipitated from cell lysates (50 μg of total protein) using an anti-pan-β AMPK antibody, and the activity was measured by phosphorylation of the SAMS peptide.

Synthesis of cDNA Expression Constructs—cDNA encoding LKB1S was amplified from human or mouse testis cDNA with LKB1S-specific oligonucleotide primers using Phusion High-Fidelity DNA Polymerase (Finzymes). The primers used were: human, forward primer (including FLAG tag (underlined) and EcoRI restriction site in italics), AAGCTTGAATTCATCATGATTATAAAGATGATGACGA; reverse primer (including XhoI restriction site in italics), AAGCTTGAATTCGCGCCGTGAGAGGG; mouse, forward primer (including FLAG tag (underlined) and EcoRI restriction site in italics), AAGCTTGAATTCATGATTATAAAGATGATGACGA; reverse primer (including XhoI restriction site in italics), AAGCTTGAATTCATGATTATAAAGATGATGACGA; and reverse primer (including XhoI restriction site in italics), TCTAGACTGCTAGCTGCTGCGCCGAGGAGG; mouse forward primer (including FLAG tag (underlined) and EcoRI restriction site in italics), AAGCTTGAATTCATGATTATAAAGATGATGACGA; and reverse primer (including XhoI restriction site in italics), TCTAGACTGCTAGCTGCTGCGCCGAGGAGG; and reverse primer (including XhoI restriction site in italics), TCTAGAC-
with EcoRI and XhoI, and cloned into pCDNA3 mammalian expression vector (Invitrogen). The authenticity of the inserts were confirmed by DNA sequencing.

cDNA encoding human LKB1 was amplified from an IMAGE clone with an N-terminal FLAG tag sequence engineered at the 5’ end and cloned into pCDNA3 (a kind gift from Dr. Naveenan Navaratnam, Medical Research Council Clinical Sciences Centre). cDNAs encoding mouse LKB1, and mouse LKB1 harboring a mutation of aspartic acid 194 to alanine (LKB1D194A) were a kind gift from A. Ashworth. The cDNAs were amplified to include a FLAG tag at the N terminus and cloned into pCDNA3. The point mutations (S431A and S431E) were introduced into mouse LKB1 using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s protocol. The mutations were verified by DNA sequencing. cDNAs encoding human STRADα and MO25α (a kind gift from Prof. Dario Alessi, University of Dundee) were cloned into pCDNA3.

Mammalian Cell Culture—CCL13 and HEK293 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, N-glutamine (2 mM), and penicillin-streptomycin. The cells were transfected using the calcium phosphate precipitation method (CallPhos™ mammalian transfection kit; Clontech), following the standard manufacturers protocol. After ~18 h the cells were washed in PBS and shocked with 10% (v/v) Me2SO for 2 min. The cells were treated 36 h post-transfection.

Cell Transfection—Plasmid DNA was prepared using a Qia-gen maxiprep kit according to the manufacturer’s instructions. The cells were transfected using the calcium phosphate precipitation method (CallPhos™ mammalian transfection kit; Clontech), following the standard manufacturers protocol. After ~18 h the cells were washed in PBS and shocked with 10% (v/v) Me2SO for 2 min. The cells were treated 36 h post-transfection.

Immunofluorescence—CCL13 cells were plated on glass cover slips for transfection. 36 h post-transfection, the cells were fixed in 4% paraformaldehyde, permeabilized with PBS containing 0.1% (v/v) Triton X-100, and blocked with PBS containing 5% (v/v) donkey serum and 2% (w/v) bovine serum albumin before incubation with primary antibody. After thorough washing with PBS, the cells were incubated with the appropriate secondary antibody (conjugated with Alexafluor 488; Invitrogen). After further washing, the cells were mounted on coverslips with Vectashield and visualized on a Leica TCS SP1 confocal microscope. Image analysis was performed using Leica software.

Subcellular Fractionation—Mouse testis extract or HEK293 cell lysates were subjected to cell fractionation using a Qproteome Cell-Compartment kit (Qiagen) following the manufacturer’s protocol.

Microarray Analysis—RNA was extracted from whole mouse testis using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions, followed by purification on an RNeasy column (Qiagen) according to the standard manufacturer’s protocol. RNA quality and concentration was assessed using an Agilent bioanalyzer. 1 µg of RNA and 5 µl of (1:2500 dilution) Agilent One-Color RNA Spike-In RNA were labeled with reagents supplied in the Agilent low RNA input linear amplification kit PLUS, one-color according to the manufacturer’s instructions. The labeled cRNA was purified with the RNeasy mini kit (Qiagen) according to the manufacturer’s protocol.

Hybridization and Scanning—The Agilent hybridization kit was used in conjunction with Agilent Mouse whole genome Oligo Arrays. 2 µg of the labeled sample RNA were used for hybridization according to the Agilent one-color microarray-based gene expression analysis protocol. The hybridization was performed for 17 h at 65 °C with 10 rpm rotation. The slides were then washed as described in the manufacturer’s manual. The slides were then washed in acetonitrile for 1 min followed by 30 s in Agilent stabilization and drying solution. The slides were scanned with the Agilent G2565BA microarray scanner system.

Data Extraction and Deposition into Gene Expression Omnibus—For data extraction and quality control, the Agilent G2567AA feature extraction software (version 9.1) was used. The data files were deposited into the NCBI Gene Expression Omnibus to comply with Minimum Information About a Microarray Experiment requirements.

Gene Expression Analysis—The data were analyzed using Genespring GX 7.3 Expression Analysis software (Agilent Technologies). Text tab delimited raw files were imported into Genespring. Standard data transformation, chip, and gene normalizations were applied, in addition to standard QC procedures. Differences in testis gene expression between the two groups were identified using Volcano Plot analysis. The data were corrected for multiple testing corrections with adjusted p values using the method of Hothberg and Benjamini with a user-defined false discovery rate of 5%.

RESULTS

We investigated the amount of LKB1 protein in mouse tissues following immunoprecipitation with a sheep polyclonal anti-LKB1 antibody. Western blot analysis of the resulting LKB1 immune complexes with an anti-LKB1 monoclonal antibody revealed a band migrating with a molecular mass of ~50 kDa in all of the tissues analyzed. In addition, a second cross-reacting band, migrating with a molecular mass of ~48 kDa, was detected in testis (Fig. 1A). The same pattern was observed using a different LKB1 antibody for immunoprecipitation (data not shown), suggesting that both bands are related to LKB1. We refer to the 50-kDa protein as LKB1L (for long form) and to the 48-kDa protein as LKB1S (for short form). Quantification of the blots showed that the 50-kDa protein is most highly expressed in brain and testis. Previous studies have shown that LKB1 requires binding of two other proteins, MO25 (2) and STRAD (1) for activity, and so we blotted the immune complexes with antibodies specific for the α isoforms of these two proteins. As can be seen from Fig. 1, both MO25α and STRADα co-immunoprecipitate with LKB1 in all of the tissues examined. In brain, a second slower migrating band was detected strongly by the STRADα antibody. However, this band was not detected when a different LKB1 antibody was used for immuno-
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precipitation (data not shown), and so we remain uncertain as to the nature of this cross-reacting protein.

We ruled out protein degradation as a likely explanation for the appearance of the 48-kDa band, because it was only detected significantly in the testis, and similar patterns of detection were observed on multiple occasions in the presence or absence of protease inhibitors in the buffers used for immunoprecipitation (data not shown). Next, we considered the possibility that alternative splicing of the LKB1 gene might give rise to LKB1s. Upon re-examination of the mouse LKB1 gene, we identified a potential exon within the intron between exons VIII and IX of the previously determined gene structure (28). This additional exon, which we termed IXa (exon IX becoming IXb), is conserved within the human and mouse LKB1 genes (Fig. 2A) and contains a termination codon. Alternative splicing of the LKB1 gene after exon VIII would lead to the different C-terminal amino acid sequences shown in Fig. 2B. Interestingly, the predicted amino acid sequence encoded by exon IXa lacks a phosphorylation site (Ser-428/431) and the farnesylation site (Cys-430/433) within the previously identified LKB1 sequence.

We raised an antibody (termed anti-sLKB1) against a predicted C-terminal peptide sequence (see “Experimental Procedures”) of mouse LKB1s and determined whether it could immunoprecipitate LKB1 from mouse tissues. Using this antibody, LKB1 activity, as determined by activation of recombinant AMPK, was readily detectable in immune complexes isolated from testis (Fig. 3A). Low, but detectable, activity was also observed in lung, heart, liver, and kidney, whereas no detectable activity was present in brain or muscle (Fig. 3A). Fig. 3B shows that the 48-kDa band is greatly depleted (greater than 85% of a control immunoprecipitation) from testis homogenates immunoprecipitated using the LKB1s antibody. This confirms that the 48-kDa band seen in Western blots corresponds to LKB1s. Immunoprecipitates of LKB1 isolated using an antibody raised to an N-terminal peptide sequence (which is present in both LKB1L and LKB1S) were analyzed by Western blotting using antibodies to total LKB1 or LKB1S (Fig. 3C). A doublet migrating at ~48 kDa, corresponding to the predicted molecular mass of the novel LKB1 protein, is recognized by both antibodies in testis but not in any other tissue tested. These results show that the 48-kDa protein highly expressed in testis is the alternative LKB1 splice variant and that this form of LKB1 is capable of phosphorylating and activating AMPK in vitro.

Based on the sequence of mouse and human LKB1s, we used specific oligonucleotide primers to amplify the cDNA from mouse or human testis. In both cases, single products of ~1.2 kb were obtained, and sequence analysis showed that they encoded the expected amino acid sequence of mouse or human LKB1s (data not shown). The cDNA products were cloned into a mammalian expression vector with a FLAG tag engineered at the N termini.

Fig. 4A shows overexpression of LKB1s, LKB1L, and a catalytically inactive mutant, LKB1D194A in CCL13 cells, which do not express endogenous LKB1. To investigate the ability of these expressed pro-
Expression of either LKB1L or LKB1S results in activation of endogenous AMPK immunoprecipitated from cells overexpressed MO25. The activities are plotted as units/mg lysate, where 1 unit is the activity of LKB1 required to activate recombinant AMPK by 1 nmol/min/mg. LKB1 activity in the immune complexes was measured by activation of recombinant AMPK. The activities are plotted as pmol/min/mg and are the means ± S.E. of three independent experiments.

FIGURE 3. Tissue distribution of LKB1s. A, mouse tissue homogenates (200 μg of total protein) were immunoprecipitated using anti-LKB1S antibody coupled to protein A-Sepharose. LKB1 activity in the immune complexes was measured by activation of recombinant AMPK. The activities are plotted as units/mg lysate, where 1 unit is the activity of LKB1 required to activate recombinant AMPK by 1 nmol/min/mg. B, lysates were immunoprecipitated from mouse testis homogenate (50 μg of total protein) using anti-LKB1S antibody, and the resulting immunoprecipitate (IP) and depleted supernatant (LKB1s SN) were blotted with anti-LKB1S monoclonal antibody and secondary antibody conjugated to Alexa-Fluor 680 and visualized in the green channel (top panel). The same blot was reprobed with antibodies specific for MO25 and STRAD and visualized on the Li-COR System in the red channel (bottom panel). The migration of the 50-kDa molecular mass standard is indicated.

Proteins to associate with MO25α and STRADα, cell lysates were immunoprecipitated using an anti-FLAG antibody to isolate the expressed LKB1. The resulting immune complexes were probed with antibodies specific for MO25α and STRADα. All three forms of LKB1 co-immunoprecipitated with endogenously expressed MO25α and STRADα (Fig. 4B). The activity of endogenous AMPK immunoprecipitated from cells overexpressing LKB1L was measured using the SAMS peptide assay. Expression of either LKB1L or LKB1S results in activation of AMPK under basal conditions, compared with cells overexpressing LKB1D194A. Overexpression of LKB1D194A had no effect on AMPK activity compared with an untransfected control.

Previous reports have suggested that phosphorylation of Ser-428/431 may be responsible for regulation of LKB1 activity (6, 21, 23, 24). Because the Ser-428/431 phosphorylation site is not present in either human or mouse LKB1s, we wondered whether this site could be involved in differential regulation of the two splice forms. Therefore, to investigate this possibility we used the adenylylate cyclase activator forskolin to activate the PKA pathway and increase phosphorylation of Ser-428/431 in LKB1L. Forskolin treatment of CCL13 cells overexpressing LKB1L resulted in increased phosphorylation of Ser-428, as well as CREB at Ser-133, a known target of PKA activation (29) (Fig. 5A). LKB1 activity from cells overexpressing LKB1L, LKB1s, or LKB1D194A was determined. There was no change in the activity of either LKB1L or LKB1s following forskolin treatment, demonstrating that phosphorylation of Ser-428 in LKB1L does not have a direct effect on activity (Fig. 5B). Because it has been reported previously that phosphorylation of Ser-428 increases the association of LKB1 with AMPK (5), we looked at the effect of forskolin treatment on endogenous AMPK activity. Forskolin treatment led to a modest increase in AMPK activity, and this was detected in cells expressing LKB1L, LKB1s, or LKB1D194A (Fig. 5C). The finding that AMPK activity is increased with forskolin even in cells expressing catalytically inactive LKB1 indicates that this is not due to an effect on LKB1. Treatment with H₂O₂ resulted in a marked increase in the activity of endogenous AMPK in cells overexpressing either LKB1L, LKB1S, or LKB1D194A, demonstrating that AMPK is not fully activated by overexpression of LKB1.

It is possible that only a small proportion of the overexpressed LKB1 is phosphorylated by PKA in response to forskolin treatment so that the effect on LKB1 activity might be
underestimated. We therefore investigated the effect of mutating Ser-431 to either a negatively charged amino acid, glutamic acid (S431E) to mimic phosphorylation at this site, or an alanine (S431A), to prevent phosphorylation at this site. Fig. 6A shows expression of wild type and mutated LKBL protein in CCL13 cells. Mutation of Ser-431 to either alanine or glutamic acid had no effect on the activity of LKB1 as compared with wild type (Fig. 6B). In untransfected control cells, LKB1 was not detectable in this assay. Similarly, treatment with H2O2 had no effect on LKB1 activity, as we have previously reported (10). Furthermore, mutation of Ser-431 had no effect on activation of endogenous AMPK under basal or H2O2-treated conditions (Fig. 6C).

We then went on to look at cellular localization of LKB1L and LKB1S when overexpressed in CCL13 cells. When expressed alone, LKB1S and LKB1L are detected in both the nucleus and cytoplasm, as shown in Fig. 7A (left-hand panels). However, when co-expressed with MO25/H9251 and STRAD/H9251, there is a striking relocalization of LKB1S and LKB1L from the nucleus to the cytoplasm. Because overexpression of proteins may cause them to locate to compartments of the cell that are not representative of the endogenous protein, we looked at the localization of endogenous LKB1. We were unable to detect endogenous LKB1 by immunofluorescence in any cell types studied presumably because of limited sensitivity of the antibodies available. Therefore we used cell fraction-
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Western blot analysis of the different subcellular fractions demonstrates that both LKB1S and LKB1L are predominantly localized in the cytosol, with a small amount of LKB1S detected in the membrane fraction (Fig. 7C). However, there is no detectable LKB1S or LKB1L in the nuclear fraction.

To investigate the functional role of LKB1S, a microarray analysis was carried out to investigate differential gene expression in the testis of LKB1S knock-out mice compared with that of wild type. Table 1 lists all genes found to have significantly (p < 0.01) altered expression (>1.5-fold change relative to wild type).

FIGURE 7. Subcellular Localization of LKB1L and LKB1S. A, CCL13 cells were transiently transfected with FLAG-tagged human LKB1L or LKB1S alone (left panels) or co-transfected with STRADα and MO25α constructs (right panels). The cells were fixed in 4% paraformaldehyde and stained with a mouse monoclonal anti-LKB1 antibody (Ley37D/G6) that recognizes both LKB1L and LKB1S. Primary antibodies were detected with fluorescently linked secondary antibodies and visualized using a Leica TCS SP1 confocal microscope. B, HEK293 cells were treated with or without 20 μM forskolin for 30 min and fractionated into cytosolic (C), membrane (M), and nuclear (N) enriched fractions. An equal volume of each fraction was blotted with an anti-LKB1 antibody (Ley37D/G6). The same fractions were used to determine the expression of marker proteins for each of the fractions (Cytosolic, Na⁺/K⁺ ATPase; Membrane, Na⁺/K⁺ ATPase; Nuclear, CREB). C, cytosolic, membrane and nuclear fractions obtained from mouse testis were blotted with an anti-LKB1 antibody (Ley37D/G6). In each case, the blots shown are representative of blots obtained from three independent experiments. The migration of molecular mass markers is indicated.

DISCUSSION

Here we describe a previously uncharacterized splice variant of LKB1 (LKB1S), which has a different C-terminal amino acid sequence compared with that of the previously reported form of LKB1 (LKB1L) (30). The two proteins are products of the same gene, generated by alternative splicing of the final coding exon. LKB1S is the product of exons I–VIII and exon IXα, whereas LKB1L is product of exons I–VIII and exon IXb. In mouse, LKB1L is expressed predominantly in the testis. In a previous study, two pools of LKB1 activity were purified from rat liver and were shown to contain different molecular mass forms of LKB1, as judged by Western blotting (8). It is possible that these different forms correspond to the rat equivalent of LKB1L and LKB1S. Sakamoto et al. (17) described a faster migrating protein in testis detected in Western blots probed with an LKB1 antibody, which was absent from mice homozygous for a floxed allele of LKB1. Importantly, the method by which the floxed allele was generated in that study would result in the absence of LKB1S expression. This is because a cDNA cassette encoding exons V–VIII and IXb of LKB1L was used to replace part of the LKB1 gene, eliminating any possibility of alternate splicing. We used this animal model to investigate whether there is an absence of LKB1S in the testis of these mice. No activity was detected in immune complexes isolated from testis of these animals using anti-sLKB1 antibodies for immunoprecipitation (data not shown). The simplest interpretation of these results is that the lower molecular mass band seen by Sakamoto et al. (17) is LKB1S. Our finding that LKB1S is predominantly expressed in the testis leads us to speculate that the male sterility of the LKB1 floxed mice described in the earlier study is caused by a lack of LKB1S. In preliminary studies we have found that the absence of LKB1S leads to a defect in spermatogenesis.5 It will be interesting to investigate more fully the role of LKB1S in spermatogenesis and in particular its relationship with SNRK (Snf1-related kinase), a testis specific AMPK-related kinase.

In mouse tissues we show that LKB1 forms a complex with MO25α and STRADα. Two isoforms (α and β) of both MO25 and STRAD have been identified (1, 2). However, we have been unable to examine the association of LKB1 with the β isoforms of MO25 and STRAD because of the lack of available antibodies. Further studies are required to determine whether LKB1L and LKB1S interact with both the α and β isoforms of MO25 and STRAD or whether there is some selectivity in formation of the complex in vivo. Using a recombinant expression system, LKB1L was shown to form a complex with both the α and β isoforms of MO25 and

5 F. C. Denison and A. Woods, unpublished results.
**TABLE 1**
Differential gene expression in the testis of wild type versus LKB1<sub>S</sub> knock-out mice

Microarray analysis was carried out to determine changes in gene expression in the testis of LKB1<sub>S</sub> knock-out mice compared with that of wild type. Those genes with a fold change (FC) of greater than 1.5 compared with wild type and a p < 0.01 are listed. The accession numbers are shown in brackets. Negative values refer to decreased expression in the LKB1<sub>S</sub> knock-out relative to wild type.

| Gene Name      | Description                                                                 | FC      | p value     |
|----------------|------------------------------------------------------------------------------|---------|-------------|
| Usp4          | Ubiquitin specific peptidase 44, [NM_183199]                                | 25.66   | 7.37E-05    |
| Ntn4          | Netrin 4, [NM_021320]                                                       | 13.59   | 3.04E-04    |
| Rkn2          | Rhoetkin 2, [AK045134]                                                      | 6.70    | 1.47E-07    |
| Eva1          | Epithelial V-like antigen 1, [NM_007962]                                    | 6.27    | 7.56E-04    |
| Robo4         | Roundabout homolog 4 (Drosophila), [NM_028783]                              | 4.90    | 6.48E-03    |
| Hail          | Histidine ammonia lyase, [NM_010401]                                        | 4.34    | 2.90E-03    |
| Klrb1a        | Killer cell lectin-like receptor subfamily B member 1A, [NM_010737]         | 3.81    | 3.08E-03    |
| Cadps         | Ca<sup>2+</sup> dependent activator protein for secretion, [NM_012061]       | 3.59    | 6.47E-04    |
| Atf3          | Activating transcription factor 3, [NM_007498]                              | 3.51    | 5.80E-03    |
| Padi2         | Peptidyl arginine deiminase, type II, [NM_008812]                           | 3.41    | 1.84E-03    |
| Tcor          | T-cell receptor alpha chain precursor V-J region, [AK037357]                | 3.23    | 1.55E-03    |
| Lcp1          | Lymphocyte cytosolic protein 1, [NM_008879]                                 | 2.99    | 6.67E-03    |
| Sik6a8        | Solute carrier family 6, member 8, [NM_133987]                              | 2.84    | 6.51E-03    |
| Rab3b         | RAB3B, member RAS oncogene family, [NM_023537]                              | 2.75    | 2.37E-03    |
| Cfi           | Complement component factor I, [NM_007686]                                  | 2.70    | 2.37E-03    |
| Prss12        | Protease, serine, 12 neurotrophin (motopsin), [NM_008939]                   | 2.31    | 5.80E-03    |
| Timp3         | Tissue inhibitor of metalloproteinase 3, [NM_011595]                        | 2.20    | 2.07E-03    |
| P2ry2         | Purinergic receptor P2Y, G-protein coupled 2, [NM_008773]                    | 2.15    | 5.80E-03    |
| Phlda1        | Pleckstrin homology-like domain family A, member 1, [NM_009344]             | 2.09    | 5.80E-03    |
| Insc          | Insusubtact homolog (Drosophila), [NM_173767]                               | 2.05    | 7.72E-03    |
| Gpe4          | Glypican 4, [NM_008150]                                                     | 2.02    | 2.90E-03    |
| U90926        | Putative TNF-resistance related protein, [U90926]                           | 2.00    | 1.55E-03    |
| Rab3b         | RAB3B, member RAS oncogene family, [NM_023537]                              | 1.98    | 2.90E-03    |
| Trip6         | Thyroid hormone receptor interactor 6, [NM_011639]                          | 1.81    | 6.48E-03    |
| Scarb2        | Scavenger receptor class B, member 2, [NM_007644]                           | 1.81    | 6.48E-03    |
| 1700025G04Rik | RIKEN cDNA 1700025G04 gene, [NM_197990]                                     | 1.77    | 5.80E-03    |
| Nkb2          | Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, [NM_019408] | 1.69    | 5.80E-03    |
| Ubasb3b       | Ubiquitin associated SH3 domain containing B, [NM_176860]                   | 1.67    | 5.80E-03    |
| Cnr3          | Cysteine rich transmembrane BMP regulator 1, [NM_015800]                    | 1.59    | 8.83E-03    |
| Rnd3          | Rho family GTPase 3, [NM_028810]                                           | 1.57    | 5.13E-03    |
| Sema7a        | Sema domain, immunoglobulin domain (lg), and GPI membrane anchor, [NM_011352] | 1.56    | 5.13E-03    |
| Pibf1         | Progesterone immunomodulatory binding factor 1, [NM_029454]                 | -1.53   | 7.72E-03    |
| Prtg          | Progesterin homolog (Gallus gallus), [AK039115]                             | -1.64   | 8.78E-03    |
| Hcf1c2        | Host cell factor C2, [AK040565]                                            | -1.67   | 6.56E-03    |
| Cln3          | Calcytenin 3, [NM_153508]                                                  | -1.79   | 8.16E-03    |
| Glb3d2        | Glycosyltransferase 8 domain containing 2, [NM_029102]                      | -1.81   | 7.18E-03    |
| Txnrd1        | Thioredoxin reductase 1, [NM_015762]                                        | -1.83   | 6.47E-04    |
| 4930547N16Rik | RIKEN cDNA 4930547N16 gene, [BC070476]                                      | -1.93   | 9.21E-03    |
| Pflag1        | Pleiomorphic adenoma gene-like 1, [BC065150]                                | -1.97   | 6.13E-03    |
| 1810014B01Rik | RIKEN cDNA 1810014B01 gene, [AK004515]                                      | -2.38   | 7.56E-04    |
| Pappa2        | Pappalysin 2, [NM_001085376]                                               | -2.82   | 6.08E-03    |
STRAD (2). In our current study, we show that LKB1<sub>L</sub> expressed in CCL13 cells (lacking endogenous LKB1) forms an active complex with endogenous MO25α and STRADα. Overexpression of either LKB1<sub>L</sub> or LKB1<sub>S</sub> in cells that do not express endogenous LKB1 showed that both forms were able to activate endogenous AMPK to a similar extent, showing that both forms are functionally equivalent, at least under these conditions.

At present, there is some controversy regarding the regulation of LKB1 by post-translational modification. There have been several reports in the literature specifying a role for phosphorylation of Ser-428/431 by upstream kinases. Two recent studies have suggested an important role for phosphorylation of Ser-428/431 in axon differentiation (24) and neuronal polarization via activation of SAD-A and SAD-B (also known as brain-specific kinase 1 and 2) by LKB1 (23). However, we recently reported that phosphorylation of LKB1 by PKA had no effect on brain-specific kinase 1 or 2 (31). Another study reported that phosphorylation of LKB1 at Ser-431 did not alter its ability to phosphorylate p53, but S431A mutation abolished the ability of LKB1 to arrest cell growth (21). In our study, activation of the PKA pathway by forskolin increased Ser-431 phosphorylation of LKB1; however, we did observe a small increase in AMPK activity upon forskolin treatment, but this appears to be independent of LKB1, because the effect is observed following expression of a catalytically inactive form of LKB1. These results show that phosphorylation of LKB1 on Ser-428/431 does not alter its activity and therefore does not support the notion that this site is involved in differential regulation of LKB1<sub>L</sub> relative to LKB1<sub>S</sub>.

There have been a number of studies investigating the subcellular localization of LKB1. LKB1<sub>L</sub> overexpressed in COS cells was localized mainly to the nucleus, but co-expression with STRAD caused some relocalization of LKB1<sub>L</sub> to the cytoplasm (1), and co-expression with STRAD and MO25 resulted in predominantly cytoplasmic localization of LKB1<sub>L</sub> (2). In our present study, LKB1<sub>L</sub> or LKB1<sub>S</sub> expressed alone were distributed in both the cytoplasm and the nucleus. Co-expression with MO25α and STRADα resulted in a striking redistribution of both LKB1<sub>L</sub> and LKB1<sub>S</sub> to a largely cytoplasmic localization (Fig. 7). Our interpretation of these results is that when LKB1 is expressed alone a proportion of it forms a complex with endogenous MO25 and STRAD and is localized in the cytoplasm, whereas some remains unassociated and localizes to the nucleus. In these heterologous expression systems, the amount of LKB1 that is unassociated and localized in the nucleus will depend on the relative levels of expressed LKB1 with endogenous MO25 and STRAD. When all three proteins are co-expressed, the amount of unassociated LKB1 is relatively low, and therefore LKB1 localizes predominantly to the cytoplasm.

A recent study reported that STRADα prevents shuttling of LKB1 from the cytoplasm to the nucleus by competing with importin α for binding of LKB1 (32), which could explain the fact that if STRADα is limiting, LKB1 localizes to the nucleus.

We found that endogenous LKB1<sub>L</sub> in HEK 293 cells was undetectable in the nucleus but was readily detected in the cytosolic and membrane fractions. Interestingly, treatment with forskolin appeared to cause a decrease in the amount of LKB1<sub>L</sub> in the membrane fraction. Two previous studies, however, reported that phosphorylation of Ser-431 did not alter the amount of LKB1 associated with the membrane fraction (6, 21). Given these conflicting results it will be important to investigate further whether phosphorylation of Ser-428/431 could play some role in membrane localization of LKB1<sub>L</sub>. Other studies have implicated a role for phosphorylation of Ser-428/431 in regulating the nuclear export of LKB1<sub>L</sub>. Phosphorylation of Ser-428 by PKC-ζ has been reported to lead to the nuclear export of LKB1<sub>L</sub> in endothelial cells (5, 33, 34). However, our data and that of others (1, 2) shows that LKB1 in complex with MO25 and STRAD is not detected in the nucleus. Moreover, in HEK293 cells we have been unable to detect endogenous LKB1<sub>L</sub> in the nuclear fraction irrespective of the conditions used to treat the cells. This finding suggests that LKB1<sub>L</sub> is excluded from the nucleus independently of the level of Ser-428/431 phosphorylation. We cannot rule out the possibility that LKB1 might be present in the nucleus in certain cell types. However, using mouse testis, the one tissue in which both LKB1<sub>L</sub> and LKB1<sub>S</sub> are highly expressed, we found no evidence to suggest that either form is present in the nucleus in vivo.

One of the obvious differences between LKB1<sub>L</sub> and LKB1<sub>S</sub> is the presence of a farnesylation site (Cys-430/433) in LKB1<sub>L</sub> that is not conserved in LKB1<sub>S</sub>. Farnesylation of Cys-430/433 has been shown previously to be involved in the membrane localization of LKB1<sub>L</sub> (6). To examine the subcellular localization of LKB1<sub>L</sub>, we used mouse testis, because this is the only tissue with readily detectable expression of LKB1<sub>L</sub> and LKB1<sub>S</sub>. Both forms were detected primarily in the cytosolic fraction, although we did detect some LKB1<sub>L</sub> in the membrane fraction. It is possible, therefore, that LKB1<sub>S</sub> may be able to associate with the membrane fraction even though it lacks a farnesylation motif. Whatever the role of the farnesylation modification in LKB1<sub>L</sub>, it is clearly not required for a functional LKB1 complex, because LKB1<sub>L</sub> in complex with MO25, and STRAD activates AMPK with a similar efficiency to the LKB1<sub>L</sub> complex.

In this study we have identified a new form of LKB1 generated by alternative splicing. The finding that this form is predominantly expressed in testis suggests that it may play a specific role in this tissue. As a first step to explore the physiological role of LKB1<sub>S</sub>, we conducted a microarray analysis of gene expression in testis. Our results show that deletion of LKB1<sub>S</sub> results in altered expression of many genes (Table 1), demonstrating that LKB1<sub>S</sub> has a specific and nonredundant role in testis. This is consistent with the observation that the only detectable phenotype in mice lacking LKB1<sub>S</sub> is male infertility, which may be caused by a defect in spermatogenesis (17, 35). In future studies it will be interesting to explore the consequences of this altered gene expression in fertility, which may lead to new insights into causes of male sterility.

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6 F. C. Denison, N. J. Hiscock, D. Carling, and A. Woods, unpublished results.
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