

Analysis of the LKB1-STRAD-MO25 complex

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Summary

Mutations in the LKB1 tumour suppressor threonine kinase cause the inherited Peutz-Jeghers cancer syndrome and are also observed in some sporadic cancers. Recent work indicates that LKB1 exerts effects on metabolism, polarity and proliferation by phosphorylating and activating protein kinases belonging to the AMPK subfamily. In vivo, LKB1 forms a complex with STRAD, an inactive pseudokinase, and MO25, an armadillo repeat scaffolding-like protein. Binding of LKB1 to STRAD-MO25 activates LKB1 and re-localises it from the nucleus to the cytoplasm. To learn more about the inherent properties of the LKB1-STRAD-MO25 complex, we first investigated the activity of 34 point mutants of LKB1 found in human cancers and their ability to interact with STRAD and MO25. Interestingly, 12 of these mutants failed to interact with STRAD-MO25. Performing mutagenesis analysis, we defined two binding sites located on opposite

surfaces of MO25 α , which are required for the assembly of MO25 α into a complex with STRAD α and LKB1. In addition, we demonstrate that LKB1 does not require phosphorylation of its own T-loop to be activated by STRAD α -MO25 α , and discuss the possibility that this unusual mechanism of regulation arises from LKB1 functioning as an upstream kinase. Finally, we establish that STRAD α , despite being catalytically inactive, is still capable of binding ATP with high affinity, but that this is not required for activation of LKB1. Taken together, our findings reinforce the functional importance of the binding of LKB1 to STRAD, and provide a greater understanding of the mechanism by which LKB1 is regulated and activated through its interaction with STRAD and MO25.

Key words: Peutz-Jeghers syndrome, AMPK, Pseudokinase, Cancer and Cell polarity

Introduction

Mutations in the LKB1 threonine protein kinase gene lead to the inherited Peutz-Jeghers syndrome (PJS), in which subjects are predisposed to developing benign and malignant tumours (Hemminki et al., 1998; Jenne et al., 1998). Subsequent studies, including the finding that overexpression of LKB1 induced a G1 cell cycle arrest (Tiainen et al., 2002; Tiainen et al., 1999), have supported the notion that LKB1 functions as a tumour suppressor. A number of groups have demonstrated that knocking out one of the LKB1 alleles in mice is sufficient to induce a cancer syndrome similar to PJS in humans (reviewed by Boudeau et al., 2003c). Genetic analyses in *C. elegans* (Watts et al., 2000), *Drosophila* (Martin and St Johnston, 2003), *Xenopus* (Ossipova et al., 2003) and mammalian cells (Baas et al., 2004) also suggest that LKB1 is an important regulator of cell polarity. An increasing number of sporadic mutations in LKB1 are being reported in diverse cancers (Boudeau et al., 2003c), for example 30% of lung adenocarcinomas possess mutations in LKB1 (Sanchez-Cespedes et al., 2002).

Recent analysis has indicated that LKB1 phosphorylates and activates the AMP-activated protein kinase (AMPK) (Hawley et al., 2003; Shaw et al., 2004b; Woods et al., 2003), a regulator of cellular energy charge (Hardie et al., 2003). Recent studies have provided evidence that benign tumour formation in LKB1-deficient cells could result from deregulation of the

tuberous sclerosis complex/mTOR signalling pathway that is controlled by AMPK (Corradetti et al., 2004; Shaw et al., 2004a). A group of 11 kinases that belong to the AMPK subfamily, are also phosphorylated and activated by LKB1 (Lizcano et al., 2004). These enzymes comprise the MARK/PAR-1 kinases, which play roles in regulating cell polarity as indicated by genetic analysis (Biernat et al., 2002; Guo and Kempnes, 1995; Shulman et al., 2000). LKB1 activates AMPK and the AMPK-related kinases by phosphorylating a conserved Thr residue located in the T-loop of these enzymes.

In vivo, LKB1 forms a heterotrimeric complex with two proteins termed STE20-related adaptor (STRAD) and MO25 (Baas et al., 2003; Boudeau et al., 2003a). Although STRAD possesses a kinase-like domain that is related in sequence to STE20 kinases, it has been classified as a pseudokinase because it lacks several residues present in other kinases that are required for catalysis. Moreover, STRAD α does not autophosphorylate or phosphorylate a variety of exogenous kinase substrates that have been tested in vitro (Baas et al., 2003). Structural analysis revealed that MO25 α forms a curved rod-like structure made up of α -helical armadillo repeats (Milburn et al., 2004). A key function of MO25 α is to stabilise the binding of STRAD α to LKB1, which interact only weakly in the absence of MO25 α (Boudeau et al., 2003a). LKB1 expressed on its own is localised mainly in nuclei, but becomes

re-localised in the cytoplasm following its interaction with STRAD α and MO25 α (Baas et al., 2003; Boudeau et al., 2003a; Brajenovic et al., 2003). Most importantly, however, the binding of LKB1 to STRAD and MO25 activates LKB1 and vastly enhances the rate at which LKB1 phosphorylates AMPK subfamily members (Hawley et al., 2003; Lizcano et al., 2004; Shaw et al., 2004b). Binding site analysis suggests that the kinase domain of LKB1 binds to the pseudokinase domain of STRAD α (Baas et al., 2003), and that MO25 α binds to the STRAD α C-terminal Trp-Glu-Phe residues (Boudeau et al., 2003a). Analysis of the crystal structure of MO25 α complexed to a peptide encompassing the C-terminus of STRAD α revealed that the Trp-Glu-Phe residues bound to a deep hydrophobic pocket on the convex C-terminal surface of MO25 α (Milburn et al., 2004). In this study, we investigate the mechanism by which the LKB1 heterotrimeric complex is assembled and activated *in vivo*.

Materials and Methods

Protease-inhibitor cocktail tablets were obtained from Roche. Tissue culture reagents were from Biowhittaker. Precast 4-12% and 10% polyacrylamide Bis-Tris gels were obtained from Invitrogen. [γ - 32 P]ATP and glutathione-Sepharose were purchased from Amersham Biosciences. P81 phosphocellulose paper was from Whatman.

Antibodies

The anti-MO25 α antibody used for the immunolocalisation was raised in sheep against the human MO25 α protein expressed in *E. coli* and has been described previously (Boudeau et al., 2003a). The monoclonal antibody recognizing the STRAD α was described previously (Baas et al., 2003). Monoclonal antibodies recognizing the GST and Flag epitope tags were obtained from Sigma, the monoclonal antibody recognizing the Myc epitope tag was purchased from Roche, and secondary antibodies coupled to horseradish peroxidase used for immunoblotting were obtained from Pierce.

General methods and buffers

Restriction enzyme digests, DNA ligations and other recombinant DNA procedures were performed using standard protocols. All mutagenesis was performed using the Quick-Change site-directed mutagenesis method (Stratagene). DNA constructs used for transfection were purified from *E. coli* DH5 α using Qiagen Plasmid Mega kit according to the manufacturer's protocol. All DNA constructs were verified by DNA sequencing, which was performed by the Sequencing Service, School of Life Sciences, University of Dundee, UK, using DYEnamic ET terminator chemistry (Amersham Biosciences) on Applied Biosystems automated DNA sequencers. Lysis buffer contained 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, 0.1% (v/v) 2-mercaptoethanol and 'complete' proteinase inhibitor cocktail (one tablet/50 ml). Buffer A contained 50 mM Tris/HCl pH 7.5, 0.27 M sucrose, 0.1 mM EGTA, and 0.1% (v/v) 2-mercaptoethanol. SDS sample buffer contained 50 mM Tris/HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.005% (w/v) bromophenol blue, and 1% (v/v) 2-mercaptoethanol.

DNA constructs

The DNA constructs encoding mouse wild-type GST-LKB1 or catalytically inactive GST-LKB1 [D194A] in the pEBG-2T vector (Sapkota et al., 2001), Flag-STRAD α , and myc-MO25 α in the

pCMV5 vector or pEBG-2T vector have been described previously (Boudeau et al., 2003a). The DNA constructs encoding human wild-type LKB1, in the pEBG-2T and pEGFP vectors have been described previously (Boudeau et al., 2003b). All the mutants of human LKB1 analysed in Fig. 1 have been reviewed previously (Boudeau et al., 2003c), and were generated by standard mutagenic procedures and subcloned into the pEBG-2T vector.

Cell culture conditions and cell lysis

Human embryonic kidney 293 (HEK293) and HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) FBS. For all experiments, cells were cultured on a 10 cm diameter dish and lysed in 0.5 to 1 ml of ice-cold lysis buffer. Lysates were clarified by centrifugation at 4°C for 10 minutes at 14,000 *g*.

Immunoblotting

The protein samples were subjected to SDS-PAGE and transferred to nitrocellulose. The membranes were blocked for 1 hour in 50 mM Tris/HCl (pH 7.5), 0.15 M NaCl, 0.5% (v/v) Tween (TBST buffer), containing 10% (w/v) skimmed milk powder for 1 hour. The membranes were then incubated in TBST buffer containing 5% (w/v) BSA and 0.5 μ g/ml antibody for 8 hours at 4°C. Detection was performed using the appropriate horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence reagent (Amersham Pharmacia Biotech).

Expression of GST-fusion proteins in HEK293 cells and affinity purification

10 cm diameter dishes of HEK293 cells were transiently transfected with 3-10 μ g of the pEBG-2T constructs together with the indicated pCMV5 constructs using a modified calcium phosphate method (Alessi et al., 1996). 36 hours post-transfection, the cells were lysed and the clarified lysates were incubated for 1 hour on a rotating platform with glutathione-Sepharose (25 μ l/dish of lysate) previously equilibrated in lysis buffer. The beads were washed four times with lysis buffer containing 150 mM NaCl and four times with Buffer A. The resin was incubated in a 3-volume excess of Buffer A containing 20 mM glutathione to elute the GST-fusion proteins. The beads were then removed by filtration through a 0.44 μ m filter and the eluate divided into aliquots, snap frozen in liquid nitrogen and stored at -80°C.

Localisation studies

HeLa cells were cultured to 50% confluence on 13-mm glass cover slips (no. 1.5) on 60 mm diameter dishes and transfected with a total of 0.4 μ g of a construct encoding wild-type EGFP-LKB1 or indicated mutants together with the indicated pCMV5 constructs using Effectene transfection reagent (Qiagen) according to the manufacturer's protocol. A duplicate set of dishes was used for each condition. The cells were washed with PBS 20 hours post-transfection, and were fixed for 10 minutes 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 then washed twice with PBS and permeabilised for 10 minutes with 1% (v/v) NP40 in PBS and blocked for 20 minutes with 5% skin gelatin. The cells were immunolabelled with both the sheep anti-MO25 α antibody and mouse anti-Flag antibody (to detect Flag-tagged STRAD α) for 1 hour, washed in PBS and counterstained with Texas Red anti-sheep IgG and Cy5 anti-mouse IgG antibodies for 1 hour. The cells were imaged using a Zeiss LSM 510 META confocal microscope. Each channel was scanned independently to avoid crosstalk (Multi-Tracking).

Assay of recombinant LKB1-STRAD α -MO25 α complexes using LKBtide substrate

All assays were performed by using 0.1–1 μ g of recombinant proteins expressed and purified from HEK293 cells as described above. Pilot studies were performed to ensure all assays were in the linear range. Phosphotransferase activity towards the LKBtide peptide [SNLYHQGKFLQTFCSPLYRRR (Lizcano et al., 2004)] was measured in a total assay volume of 50 μ l consisting of 50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 10 mM magnesium acetate, 0.1 mM [γ - 32 P]ATP (~200 cpm/pmol) and 200 μ M LKBtide peptide. The assays were carried out at 30°C and were terminated after 15 minutes by applying 40 μ l of the reaction mixture onto P81 membranes. The P81 membranes were washed in phosphoric acid, and the incorporated radioactivity was measured by scintillation counting as described previously for MAP kinase (Alessi et al., 1995).

Adenine nucleotide binding assays

Wild-type and the indicated mutant of GST-STRAD α were expressed in HEK293 cells and affinity purified as described above. The molar concentrations of the STRAD α fusion proteins were determined from their absorbance at 280 nm by using extinction coefficients calculated from the amino acid sequence. The proportion of full-length protein was estimated by densitometry of Coomassie-stained protein. Each protein (1 μ M) was incubated with the indicated concentrations of [γ - 32 P]ATP (5 MBq/ μ mol), in the presence or absence of 5 mM MgCl₂, for 30 minutes at 25°C in a 20 μ l total volume in HBS (50 mM HEPES; pH 7.4, 150 mM NaCl). 10 μ l of each mixture was spotted onto a Millipore MF filter membrane disc (2.5 cm), which was rapidly filtered under high vacuum (0.13 mbar), and washed with 1 ml of ice-cold HBS. Radioactivity associated with the membrane was determined by scintillation counting. Non-specific binding of radioactivity to the membrane was evaluated by control assays in which GST-STRAD α was replaced with the isolated GST fusion protein. The radioactivity associated with the control samples was typically 95% lower than that obtained with wild-type STRAD α and was subtracted as a blank. Data were fitted to binding models using GraphPad Prism as described in the figure legends.

Results

Characterisation of mutant forms of LKB1 found in cancer patients

Many mutations have been identified in the catalytic and C-terminal regions of LKB1 in PJS as well as sporadic cancers (Boudeau et al., 2003c). For most of these mutants, their activity and ability to interact with STRAD α and MO25 α have not been investigated. To study the properties of these mutants, we co-expressed 30 LKB1 catalytic domain point mutants reported in PJS and sporadic cancers (Boudeau et al., 2003c), as GST fusion proteins with STRAD α and MO25 α in HEK293 cells. Following glutathione-Sepharose affinity purification of the LKB1 mutants, LKB1 catalytic activity was assessed by using the LKBtide peptide substrate (Lizcano et al., 2004), and LKB1 association with STRAD α and MO25 α was analysed by immunoblotting. Strikingly, 12 of the LKB1 mutants were unable to interact with STRAD α and MO25 α and therefore possessed negligible catalytic activity, similarly to wild-type LKB1 expressed on its own (Fig. 1A). These LKB1 mutants comprised mutations of Leu67 and Phe157, as well as clusters of mutations located between residues 175–182, 239–242 and 297–308. When these mutations are evaluated in a structural model of the LKB1 catalytic domain (Fig. 1B), a number of

trends can be observed. With the exception of Leu67, all mutations that affect the interaction of LKB1 with STRAD α -MO25 α are found in the C-terminal lobe. The mutations that are on the surface of the fold (Leu67, Arg297, Arg304 and Trp308) are found on the ‘back’ of the kinase domain, away from the substrate-binding site. Surprisingly, however, the majority of the 12 mutations are found in the core of the C-terminal lobe, and are therefore unlikely to be involved in direct interactions with STRAD α -MO25 α . It is possible that these mutations lead to a destabilisation of the C-terminal lobe, thus indirectly affecting STRAD α -MO25 α binding.

The remaining 19 mutants of LKB1 bound STRAD α and MO25 α , similarly to wild-type LKB1. Seven of these were catalytically inactive, whereas the remainder possessed normal or reduced catalytic activity. We also analysed six C-terminal non-catalytic domain mutants of LKB1 that had not been studied previously and found that these were all able to bind STRAD α and MO25 α and possessed normal or slightly reduced catalytic activity (Fig. 1C). These findings will be considered further in the Discussion.

Role of the WEF-binding pocket on MO25 α

The MO25 α crystal structure revealed that MO25 α interacts with the C-terminal WEF sequence on STRAD α through a deep hydrophobic pocket lined with positively charged residues, on the convex surface of MO25 α (Milburn et al., 2004) (Fig. 2A). Consistent with the importance of this pocket in enabling MO25 α to bind to STRAD α (Milburn et al., 2004), mutation of several residues located in the WEF-binding pocket of MO25 α to Ala prevented the binding of STRAD α to MO25 α in the absence of LKB1, in a HEK293 cell co-expression based assay (Fig. 2B). Strikingly, however, in the presence of LKB1, the WEF-binding pocket mutants of MO25 α were still capable of forming heterotrimeric complexes with LKB1 and STRAD α (Fig. 2C). This suggests that MO25 α possesses an additional binding site at a separate location to the WEF-binding pocket, which interacts with either LKB1 and/or STRAD. The heterotrimeric LKB1 complexes containing WEF-binding pocket mutants of MO25 α possessed the same catalytic activity as the equivalent complexes formed with wild-type MO25 α , which indicates that occupancy of this pocket is not required for LKB1 activity in the complex (Fig. 2C).

Binding of wild-type MO25 α to STRAD α or to the LKB1-STRAD α complex in cells, can also be visualised by monitoring the nuclear exclusion of MO25 α (Boudeau et al., 2003a). Consistent with the inability of the WEF-binding pocket MO25 α [M260A] mutant to interact with STRAD α in the absence of LKB1, it remained localised in the nucleus of HeLa cells when co-expressed with STRAD α (Fig. 3, compare panels G and J). However, in the presence of LKB1 and STRAD α , the WEF-binding pocket MO25 α [M260A] mutant was re-localised to the cytoplasm (Fig. 3, compare panels M and P), which indicates that occupancy of the WEF-binding pocket of MO25 α is not required for cytoplasmic localisation of the complex.

Identification of a second STRAD α -LKB1-binding site on MO25 α

A striking feature of the MO25 α structure is the presence of a concave putative binding pocket, which is used by other

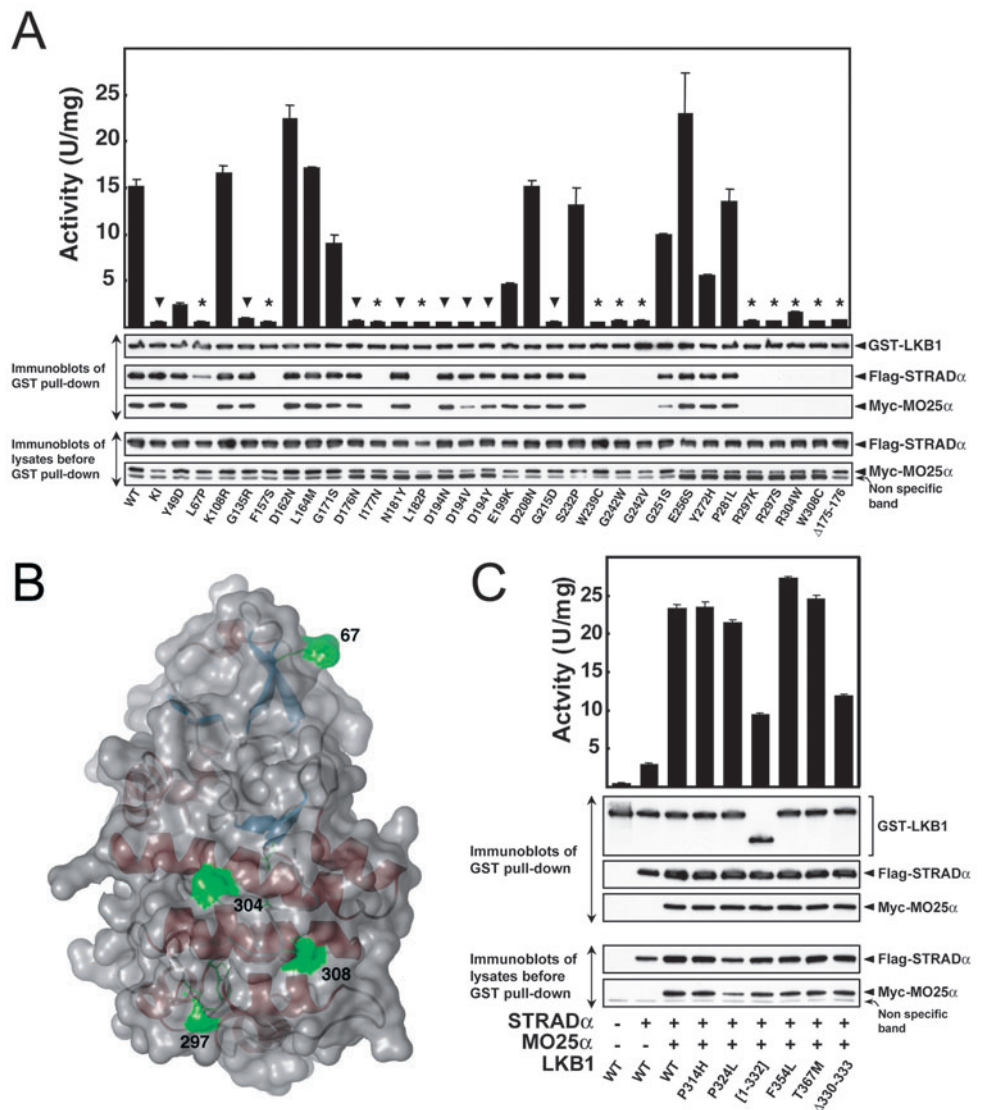
armadillo-repeat-containing proteins to bind their ligands. This concave surface has been shown to possess sequence motifs containing, for instance, basic residues in the Pumilio proteins that interact with an RNA-phosphate backbone (Wang et al., 2002). We have tried to identify such sequence motifs in MO25 α and found an Arg-His/Arg motif that is repeated at the same position in 4 out of the 6 α -helical repeats (Milburn et al., 2004). This forms a basic strip running along the length of the edge of the MO25 α concave surface, located on the opposite side of the WEF-binding pocket (Fig. 4A). To evaluate whether these surface-exposed residues could participate in the interaction with LKB1 and/or STRAD α , we tested how their mutation to Ala affected complex assembly in the HEK293 cell co-expression assay. We first explored whether the Arg-His/Arg motif MO25 α mutants could form

complexes with LKB1 and a mutant of STRAD α lacking the C-terminal WEF residues (STRAD α - Δ WEF). Interestingly, MO25 α mutants in which the fourth Arg-His/Arg motif was mutated (Arg240 and His241 changed to Ala), interacted poorly with the LKB1-STRAD α - Δ WEF complex (Fig. 4B), compared with the other Arg-His/Arg motif MO25 α mutants.

We next mutated Arg240 and His241 individually to Ala and found that mutation of Arg240, but not His241, was sufficient to impair binding of MO25 α to the LKB1-STRAD α - Δ WEF complex and hence LKB1 activation (Fig. 4B). Interestingly, the Arg240-His241-MO25 α mutant still interacts with wild-type STRAD α and LKB1, and forms a complex with normal catalytic activity (Fig. 4C), which indicates that complex assembly in this situation was mediated through the WEF-binding pocket. This was confirmed by the finding that MO25 α mutants in which both

Fig. 1. Characterisation of LKB1 mutants found in human cancers.

(A) HEK293 cells were transfected with 3 μ g of plasmids encoding wild-type or the indicated mutants of GST-LKB1 in the presence or absence of 3 μ g of plasmids encoding Flag-STRAD α and Myc-MO25 α . Thirty-six hours post-transfection, the GST-tagged proteins were affinity purified from the cell lysates using glutathione-Sepharose as described in Materials and Methods. Similar amounts of the purified GST fusion proteins were subjected to SDS-PAGE and immunoblotted with the anti-Flag and anti-Myc antibodies to detect copurified Flag-STRAD α and Myc-MO25 α , respectively, and with the anti-GST antibody to ensure that comparable amounts of the GST-tagged proteins were present in each lane (upper panels). 10 μ g of total cell lysates prior to affinity purification were also immunoblotted with the anti-Flag and anti-Myc antibodies to ensure that Flag-STRAD α and Myc-MO25 α were expressed at similar levels in each condition (lower panels). The purified LKB1 proteins were tested for their ability to phosphorylate the LKBtide peptide substrate as described in Materials and Methods. The results are expressed as the peptide kinase activity generated per mg of affinity purified protein added to the assay. Results shown are the mean \pm s.d. of two independent assays carried out in triplicate. Bars marked with an asterisk indicate LKB1 mutants that fail to bind STRAD α and MO25 α ; bars marked with an inverted triangle indicate LKB1 mutants that are catalytically inactive but still bind STRAD α and MO25 α . (B) Model of the LKB1 catalytic domain in which residues found to abolish binding of LKB1 to STRAD α are indicated. A sequence alignment of LKB1 with the structurally most related Aurora-related kinase-1 [30%, 1MUO (Cheatham et al., 2002)] was generated. The surface exposed residues that correspond to impaired LKB1 function/complex formation are shown in green patches on the grey surface representation of the kinase fold, and are mapped onto the structure of Aurora-related kinase-1, which is shown as a ribbon. (C) HEK293 cells were transfected with the indicated constructs and analysis performed as described in A. Results shown are the mean \pm s.d. of two independent assays carried out in triplicate.



Discussion

Previous work indicates that STRAD α binds directly to the kinase domain of LKB1, as the isolated LKB1 kinase domain (residues 44 to 343) can bind STRAD α (Baas et al., 2003). We have also found that a shorter fragment of LKB1 encompassing the kinase domain (residues 44-309) binds

STRAD α -MO25 α , although binding is weaker than that observed with the LKB1[44-343] fragment (J.B., unpublished). The previous finding that a PJS LKB1 mutant lacking residues 303-306 of LKB1, termed SL26, failed to interact with STRAD α suggested that the C-terminal region of the LKB1 catalytic domain comprised a STRAD α binding site (Baas et al., 2003). Consistent with this notion, we found that four other mutations located between residues 297 and 308 of LKB1 also abolished binding to STRAD α and MO25 α (Fig. 1A). However, as mutations located in four other regions of the LKB1 catalytic domain (Leu67, Phe157, residues 175-182 and residues 239-242) also abolished binding of LKB1 to STRAD α , the STRAD α binding region on the LKB1 catalytic domain may comprise several sites. Our modelling of the LKB1 catalytic domain also indicates that many of the mutations may affect STRAD α -MO25 α binding by a general destabilisation of the C-terminal lobe of LKB1. Interestingly, an LKB1[D176Y] mutant (which has not been found in human cancer), has previously been used as a catalytically inactive LKB1 mutant for control experiments, and reported not to bind STRAD α (Baas et al., 2003). Although this finding was originally interpreted to mean that LKB1 needs to be catalytically active in order to bind STRAD α , our data indicate that Asp176 lies in one of the STRAD α -binding regions, which is likely to explain why this mutant failed to interact with STRAD α . Moreover, as our studies revealed that seven catalytically inactive LKB1 mutants still bound STRAD α (Fig. 1A), we conclude that catalytic activity of LKB1 is not required for LKB1 to bind to STRAD α . The inability of a significant number of PJS mutants of LKB1 found in human cancers to bind STRAD α -MO25 α further emphasises the importance that binding of STRAD α -MO25 α plays in controlling the physiological function of LKB1. It will be necessary to co-crystallise LKB1 and STRAD α in order to understand the molecular mechanism by which these proteins interact. We also attempted to investigate

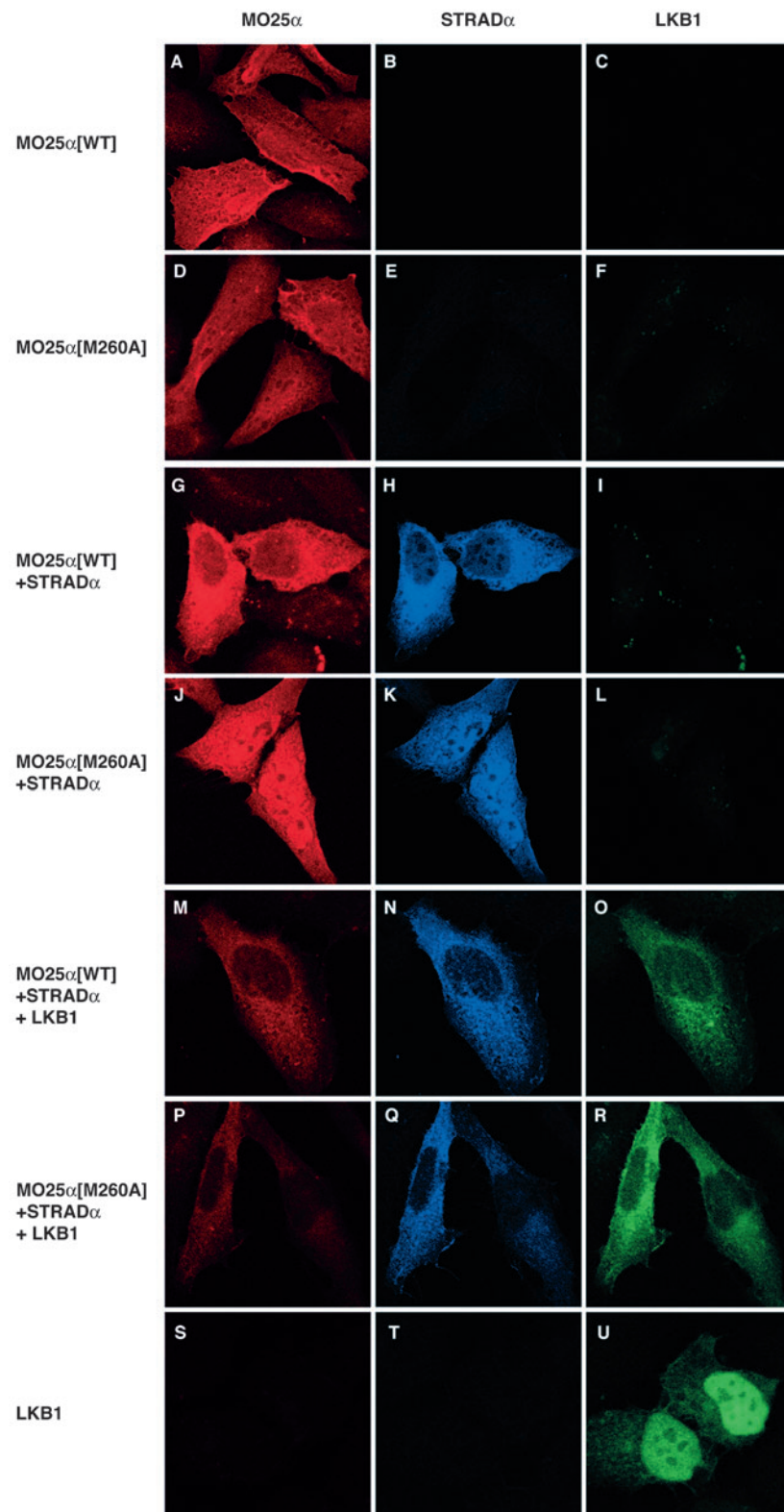


Fig. 3. Localisation of WEF-binding pocket MO25 α mutants in cells. HeLa cells were transfected with the construct encoding wild-type or indicated mutants of Myc-MO25 α in the absence or presence of GFP-LKB1 and Flag-STRAD α . Twenty-four hours post-transfection, the cells were fixed in 4% (v/v) paraformaldehyde and immunostained with the anti-MO25 α antibody to detect MO25 α (TR anti-sheep secondary antibody, red channel) and anti-Flag antibody to detect STRAD α (Cy5 anti-mouse secondary antibody, blue channel). GFP-LKB1 localization was visualized directly through the GFP fluorescence (green channel). The cells were imaged using a Zeiss LSM 510 META confocal microscope. The cells shown are representative images obtained in three separate experiments.

whether the 12 mutants of LKB1, as well as the SL26 mutant that failed to bind STRAD α , were still catalytically active. However, we were unable to detect any significant LKB1 activity in these mutants (J.B., unpublished).

We also found that SL26-LKB1 mutant was inactive and could not autophosphorylate itself, which is consistent with previous reports (Marignani et al., 2001; Ylikorkala et al., 1999). It should be noted that another group suggested that this mutant of LKB1 was still capable of autophosphorylation (Nezu et al., 1999). It is our opinion that LKB1 possesses negligible activity unless it is complexed to STRAD α . The low basal activity of wild-type LKB1 when expressed in mammalian cells is likely to result from low levels of endogenous STRAD-MO25 that interact with the overexpressed LKB1 enzyme.

We also observed that 12 of the 30 catalytic domain LKB1

mutants and all of the C-terminal LKB1 mutants analysed still interacted with STRAD α -MO25 α and were significantly activated (Fig. 1). It is not clear how these mutations would affect LKB1 function in cancer cells, which emphasises that there is still much to be learnt about the physiological regulation of LKB1 in vivo. The C-terminal non-catalytic region of LKB1 is phosphorylated in vivo at several sites [Ser325, Thr336, Thr366 and Ser431 (Sapkota et al., 2002; Sapkota et al., 2001)] and is farnesylated at its C-terminus (Collins et al., 2000; Sapkota et al., 2001). Mutation of some of these phosphorylation sites has been shown to suppress the ability of LKB1 to control cell polarisation in *Drosophila* (Martin and St Johnston, 2003) or to inhibit cell growth (Sapkota et al., 2002; Sapkota et al., 2001). Taken together, these observations indicate that the C-terminal region of LKB1 is likely to possess an important function in regulating LKB1 activity.

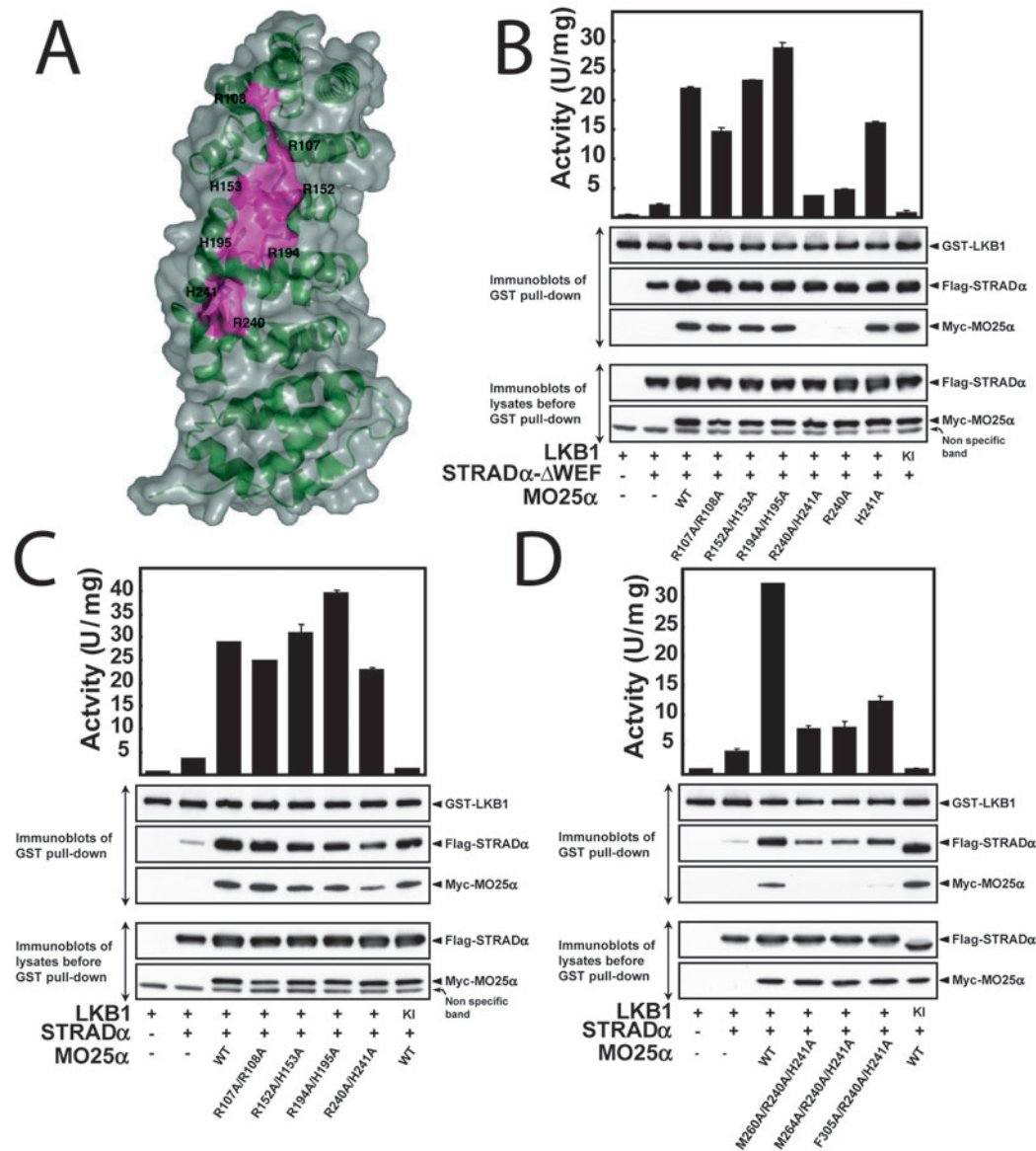


Fig. 4. Characterisation of the Arg240 binding site on MO25 α . (A) Structure showing the concave surface of MO25 α , in which the repeated Arg-Arg/His residues are labelled. (B-D) HEK293 cells were transfected with the indicated constructs and analysis performed as described in the legend to Fig. 1A. Results shown are the mean \pm s.d. of two independent assays carried out in triplicate.

Our results support the notion that MO25 α functions as a scaffolding component of the LKB1 heterotrimeric complex. We demonstrate that MO25 α possesses two binding sites, which we have termed the WEF-binding pocket and the Arg240 site, that are required for the assembly of an active LKB1-STRAD α -MO25 α complex. Sequence alignment indicates that the Arg240 and most of the residues located in the WEF-binding pocket that are required for binding to STRAD α are conserved in all species containing MO25 homologues, namely mammals, *Drosophila*, *C. elegans*, fission yeast, budding yeast and plants (data not shown). This suggests that the binding roles of this residue have been conserved in evolution. Interestingly, none of the other basic Arg-Arg/His motif residues on the concave surface of MO25 α , which are not required for interaction with STRAD and MO25, is conserved in all of these species. Our data

indicate that MO25 α mutants possessing only an intact Arg240 site or WEF-binding pocket can interact with LKB1-STRAD α and form a fully active complex that localises in the cytoplasm. This indicates that occupancy of either site is sufficient to enable MO25 α to interact with LKB1-STRAD α . At this stage we do not know which region of LKB1 and/or STRAD α that the Arg240 site on MO25 α interacts with. Our previous finding that wild-type MO25 α does not bind LKB1 directly (Boudeau et al., 2003a) suggests that the Arg240 site on MO25 α specifically recognises a site found only on the LKB1-STRAD α complex. It is possible that the interaction of STRAD α with LKB1 results in a conformational change that creates a novel binding-site for the Arg240 region on MO25 α .

The mechanism of activation of LKB1, which involves binding to a pseudokinase rather than being controlled by T-loop phosphorylation, is unusual. Most kinases require phosphorylation of their T-loop residue to induce a conformational change that stabilises these enzymes in an active conformation (Nolen et al., 2004). LKB1 may have evolved a distinct mechanism of activation to avoid the need for activation by another kinase, as LKB1 is itself an upstream kinase. Other upstream T-loop kinases such as PDK1 (Mora et al., 2004) and the cyclin activating kinase CDK7 (Harper and Elledge, 1998) have evolved distinctive mechanisms of activation. PDK1 activates many AGC kinases by phosphorylating their T-loop, and possesses a T-loop similar in sequence to those found on its substrates. However, PDK1, unlike LKB1, is capable of activating itself by trans-autophosphorylating its own T-loop residue (Casamayor et al., 1999; Wick et al., 2003). CDK7 phosphorylates the T-loop of CDK kinases and forms a complex with cyclin H and MAT1. Although CDK7 in complex with MAT1 can be partially active without T-loop phosphorylation, T-loop phosphorylation of CDK7 stabilises its interaction with cyclin H and MAT1 and is required for maximal activation of CDK7 (Larochelle et al., 2001). Interestingly, CDK7 cannot autophosphorylate its own T-loop, and one of the downstream kinases activated by CDK7, namely CDK2, has been reported to phosphorylate the T-loop of CDK7 (Garrett et al., 2001).

LKB1 has been shown to possess a strong intrinsic preference for phosphorylating peptides with a Leu located two residues N-terminal to a Thr (Shaw et al., 2004b). Interestingly, all AMPK subfamily kinases possess a Leu residue at the -2 position from the T-loop Thr phosphorylated by LKB1 (Fig. 1A). By contrast, mammalian and *Drosophila* LKB1 possess a Cys residue in this position of the T-loop (Fig. 5A), which might account for the inability of LKB1 to autophosphorylate its own T-loop residue.

The human genome comprises ~50 pseudokinases (10% of the total number of kinases) that lack one or more of the conserved catalytic residues (Manning et al., 2002). To our knowledge, the finding that STRAD α binds ATP (Fig. 6A) is the first report of a pseudokinase that can bind nucleotides. Our studies using an ATP-binding-defective mutant of STRAD α indicate that binding of ATP to STRAD α is not required for activation of

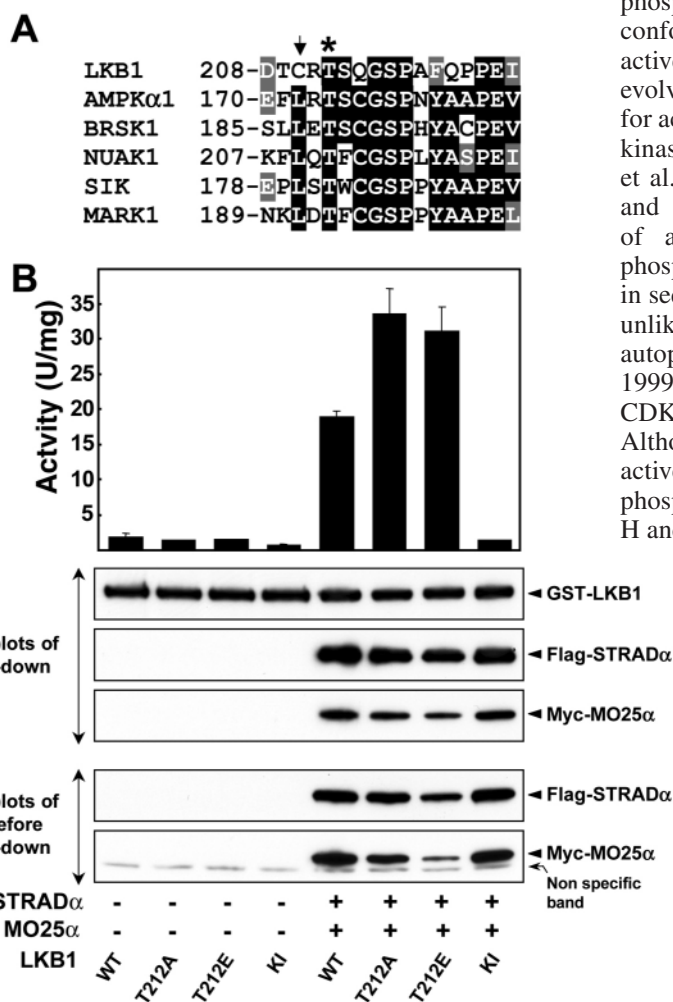


Fig. 5. Activation of LKB1 does not require T-loop phosphorylation. (A) Amino acid sequence alignment of the T-loop of LKB1 and protein kinases of the AMPK subfamily (Manning et al., 2002). The identical residues are boxed in black and the conserved residues are shaded in grey. The T-loop Thr is indicated with an asterisk. The conserved Leu residue found on AMPK subfamily kinases is marked with an arrow. (B) HEK293 cells were transfected with the indicated constructs and analysis performed as described in the legend to Fig. 1A. Results shown are the mean \pm s.d. of two independent assays carried out in triplicate.

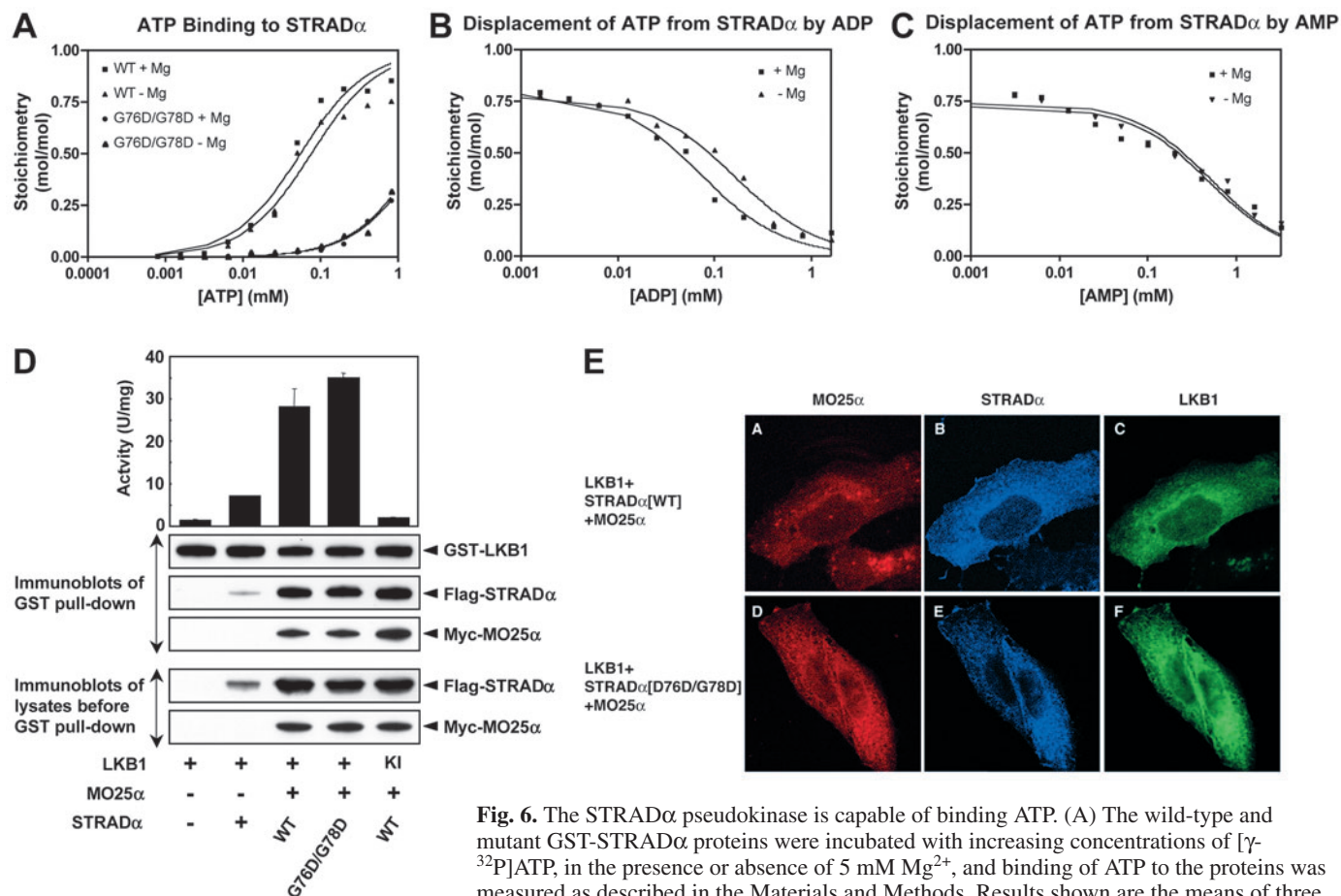


Fig. 6. The STRAD α pseudokinase is capable of binding ATP. (A) The wild-type and mutant GST-STRAD α proteins were incubated with increasing concentrations of [γ - 32 P]ATP, in the presence or absence of 5 mM Mg $^{2+}$, and binding of ATP to the proteins was measured as described in the Materials and Methods. Results shown are the means of three separate experiments carried out in duplicate. Data were fitted to a single-site binding

model: $\text{bound} = [\text{ATP}] / (K_d + [\text{ATP}])$. (B,C) Displacement of ATP from wild-type STRAD α by ADP (B) or AMP (C). A fixed concentration of [γ - 32 P]ATP (200 μ M) was incubated with the GST-STRAD α protein in the presence of increasing concentrations of either ADP or ATP, and in the presence or absence of 5 mM Mg $^{2+}$; binding of ATP to the proteins was measured as described in the Materials and Methods. Data were fitted to the binding models: $\text{bound} = [\text{ATP}] / ([\text{ATP}] + K_d \text{ATP} (1 + [\text{ADP}] / K_d \text{ADP}))$ or $\text{bound} = [\text{ATP}] / ([\text{ATP}] + K_d \text{ATP} (1 + [\text{AMP}] / K_d \text{AMP}))$. (D) HEK293 cells were transfected with the indicated constructs and analysis performed as described in the legend to Fig. 1A. Results shown are the mean \pm s.d. of two independent assays carried out in triplicate. (E) HeLa cells were transfected with the construct encoding wild-type or indicated mutant of Flag-STRAD α in the presence of GFP-LKB1 and Myc-MO25 α , and analysed as described in the legend to Fig. 3.

LKB1. One possibility is that STRAD α evolved from an active protein kinase and, despite losing its catalytic activity, has retained the ability to bind ATP. One might also speculate that, at one stage of evolution, STRAD α regulated LKB1 activity by phosphorylating LKB1 at its T-loop or other residue, and subsequently evolved into a protein that activated LKB1 by interacting with it instead. We have attempted to restore catalytic activity of STRAD α by mutating residues in subdomain VIb (Ser195 mutated to Asp) and subdomain VII (213Gly-Leu-Arg215 mutated to Asp-Phe-Gly), which are equivalent to those found in the STE20-family SPAK kinase (Johnston et al., 2000), STRAD α 's closest active kinase relative. However, the resulting STRAD α mutant was still judged to be catalytically inactive as it did not autophosphorylate or phosphorylate LKB1, histones H1, H2A, H2B, H3, H4 or myelin basic protein in vitro (J.B., unpublished).

Interestingly, other than STRAD α , the few mammalian pseudokinases that have been studied have also been found to interact with catalytically active kinases. For example, the

ErbB3 EGF receptor pseudokinase forms heterodimers with other catalytically active members of the ErbB tyrosine kinases, and binding of ErbB3 to these is required for their activation (Berger et al., 2004; Holbro et al., 2003). The KSR pseudokinase forms a scaffolding regulatory complex with Raf and regulates signal propagation through the ERK/MAPK pathway (Roy et al., 2002). The JAK tyrosine kinases possess a pseudokinase domain located next to the catalytically active tyrosine kinase domain. The JAK pseudokinase domain binds to and regulates the activity of the catalytically active domain (Luo et al., 1997; Saharinen et al., 2003). To our knowledge, there is no evidence that the ErbB3, KSR or Jak pseudokinases stimulate the autophosphorylation of the T-loop of their kinase-binding partners, and the mechanism by which these pseudokinases bind and regulate catalytically active kinases is poorly understood. The emerging picture is that pseudokinases function as key regulators of active protein kinases, and it is likely that much interesting information will be learnt from studying the physiological roles of this neglected class of proteins.

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