Heat-shock protein 90 and Cdc37 interact with LKB1 and regulate its stability

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LKB1 is a widely expressed serine/threonine protein kinase that is mutated in the inherited Peutz–Jeghers cancer syndrome. Recent findings indicate that LKB1 functions as a tumour suppressor, but little is known regarding the detailed mechanism by which LKB1 regulates cell growth. In this study we have purified LKB1 from cells and establish that it is associated with the heat-shock protein 90 (Hsp90) chaperone and the Cdc37 kinase-specific targetting subunit for Hsp90. We demonstrate that Cdc37 and Hsp90 bind specifically to the kinase domain of LKB1. We also perform experiments using Hsp90 inhibitors, which indicate that the association of Hsp90 and Cdc37 with LKB1 regulates LKB1 stability and prevents its degradation by the proteasome. Hsp90 inhibitors are being considered as potential anti-cancer agents. However, our observations indicate that prolonged usage of these drugs could possibly lead to tumour development by decreasing cellular levels of LKB1.

Key words: chaperone, geldanamycin, Peutz–Jeghers syndrome, proteasome, radicicol, tumour suppressor.

INTRODUCTION

Peutz–Jeghers syndrome (PJS) is an autosomal dominant disorder predisposing those affected to a wide spectrum of benign and malignant tumours [1]. In most cases of PJS, the disease is caused by mutations in a gene encoding a widely expressed serine/threonine protein kinase, termed LKB1 [2] or STK11 [3] (reviewed in [4]). Mice lacking a single allele of the LKB1 gene also develop multiple tumours similar to those found in humans with PJS, confirming that mutations in LKB1 lead to the PJS phenotype [5–9]. The overexpression of LKB1 in a number of tumour cell lines that do not express this protein kinase suppresses cell growth by inducing a G_1 cell-cycle arrest, and this has led to the idea that LKB1 functions as a tumour suppressor [10,11]. LKB1 also appears to play multiple roles in normal embryo development, as mice that lack this enzyme die at day E9 of embryogenesis, displaying numerous vascular abnormalities, defects in the neural tube formation and increased mesenchymal cell death [12].

The serine/threonine protein kinase activity of LKB1 is likely to be essential in mediating the tumour-suppressor effects of this enzyme, as catalytically inactive LKB1 mutants are unable to suppress cell growth [10]. Moreover, the vast majority of LKB1 mutations identified in PJS patients result in the loss of LKB1 protein kinase activity [4]. Although LKB1 is predominantly localized in the nucleus, a significant fraction is also found in the cytoplasm of cells [10,13]. LKB1 cytoplasmic location may be important in enabling LKB1 to regulate tumour suppression, as it was shown that an LKB1 mutant which lacks a nuclear localization signal, and hence is solely localized in the cytoplasm, is still capable of suppressing cell growth [11]. Thus far no physiological substrates for LKB1 have been identified. There has also been a lot of interest in identifying LKB1-interacting proteins, which may regulate its function. To date, three proteins have been shown to bind LKB1, namely the p53 tumour suppressor protein [14], the brahma-related gene 1 protein (Brg1), which is a component of chromatin-remodelling complexes [15], and a novel protein termed LKB1 interacting protein 1 (LIP1) [16]. The binding of LKB1 to p53 was proposed to be essential for mediating p53-dependent cell death [14], whereas the binding of LKB1 to Brg1 was suggested to be required for Brg1-dependent cell growth arrest [15]. The interaction of LKB1 with LIP1 anchors LKB1 in the cytoplasm [16]. In this study we provide evidence that LKB1 physiologically interacts with the kinase-specific chaperone heterodimer consisting of Cdc37 and heat-shock protein 90 (Hsp90). We also establish that treatment of cells with Hsp90 inhibitors results in the proteasome-mediated degradation of LKB1. These findings indicate that Cdc37 and Hsp90 are likely to play a role in regulating the cellular stability of LKB1.

MATERIALS AND METHODS

Materials

Protease-inhibitor cocktail tablets were obtained from Roche. Geldanamycin, radicicol, the proteasome inhibitor benzyloxycarbonyl-Leu-Leu-Leu-aldehyde (MG132), the chymotrypsin-like serine protease inhibitor tosylphenylalanylchloromethane (Tos-Phe-CH_2Cl; 'TPCK'), the trypsin-like serine protease inhibitor tosyl-lysylchloromethane (Tos-Lys-CH_2Cl; 'TLCK'), the lysosome protease inhibitor *trans*-epoxysuccinyl-L-leucyl-amido-4-guanido butane (E64), the aspartate protease inhibitor pepstatin-A, dimethyl pimelimidate and the anti-FLAG M2–agarose affinity gel were purchased from Sigma. The proteasome inhibitors benzyloxycarbonyl-Ile-Glu(OBut)-Ala-Leu-aldehyde (PSI) and lactacystin, the caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-CH_2OCO-2,6-dichlorobenzene (ZVAD), the lysosome inhibitor leupeptin and Zwittergent 3-16 were from Calbiochem. Dialysed and tetracyclin-free foetal bovine sera (FBS) were from Perbio, and other tissue-culture reagents were from Biowhittaker unless otherwise stated. Precast SDS/4–12% polyacrylamide Bis-Tris gels were obtained from Invitrogen. The

Abbreviations used: Brg1, brahma-related gene 1; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated protein kinase; FBS, foetal bovine serum; GST, glutathione S-transferase; Hsp90, heat-shock protein 90; IKK, inhibitory κB kinase; LIP1, LKB1 interacting protein 1; PJS, Peutz–Jeghers syndrome; PSI, benzyloxycarbonyl-Ile-Glu(OBut)-Ala-Leu-aldehyde; TAPP, tandem pleckstrin-homology-domain-containing protein; Tos-Lys-CH_2Cl, tosyl-lysylchloromethane; Tos-Phe-CH_2Cl, tosylphenylalanylchloromethane; ZVAD, benzyloxycarbonyl-Val-Ala-Asp-CH_2OCO-2,6-dichlorobenzene.

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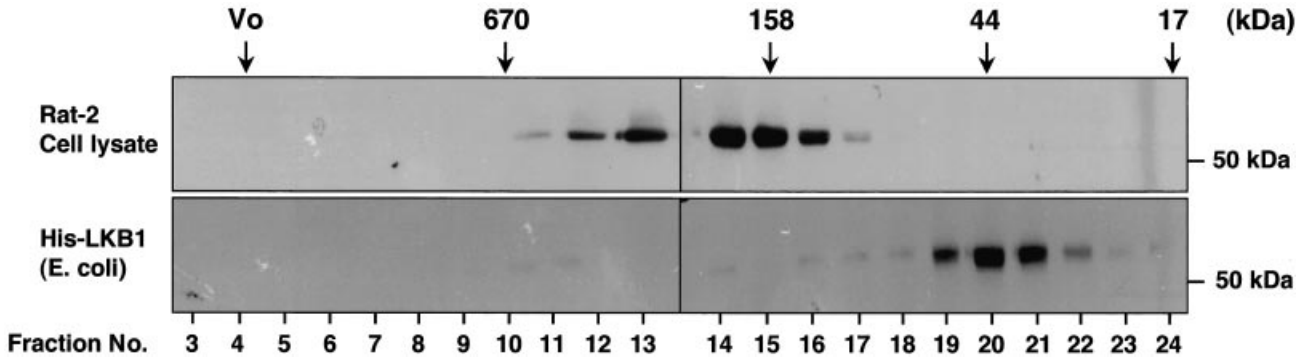


Figure 1 Evidence that endogenous LKB1 exists as a complex

Rat-2 cell lysates or bacterially expressed His-LKB1 were chromatographed on a Superdex 200 PC 3.2/30 gel-filtration column and fractions immunoblotted for LKB1 as described in the Materials and methods section. The elution positions of the peak fractions of the globular protein standards thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa) and equine myoglobin (17 kDa) are indicated. Similar results were obtained in three separate experiments.

FLAG peptide (DYKDDDDK) was synthesized by Graham Bloomberg at the University of Bristol (Bristol, U.K.). Hexahistidine-tagged LKB1 was expressed in *Escherichia coli* as described previously [17]. The gel-filtration standards thyroglobulin, bovine γ-globulin, chicken ovalbumin and equine myoglobin were from Bio-Rad.

Antibodies

The antibody recognizing LKB1 protein was raised in sheep against the glutathione S-transferase (GST)-LKB1 mouse protein expressed in *E. coli* [17]. Antibodies recognizing tandem pleckstrin-homology-domain-containing protein-1 (TAPP1) used in Figure 3 were raised against the TAPP1 protein as described before [18]. Antibodies recognizing Cdc37 and Hsp90 were obtained from Santa Cruz Biotechnology and antibodies recognizing extracellular signal-regulated protein kinase (ERK) 1 and ERK2 were purchased from Cell Signalling. Monoclonal antibodies recognizing the GST and FLAG epitope tags were obtained from Sigma, 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 DNA constructs were verified by DNA sequencing, which was performed by the Sequencing Service (School of Life Sciences, University of Dundee, Dundee, Scotland, U.K.), 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 NaF, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 μM microcystin-LR, 0.1 % (v/v) 2-mercaptoethanol and complete proteinase inhibitor cocktail (one tablet/50 ml). Buffer A contained 50 mM Tris/HCl, pH 7.5, and 0.1 mM EGTA. Buffer B contained 50 mM Tris/HCl, pH 7.4, 0.15 M NaCl and complete proteinase inhibitor cocktail (one tablet/50 ml). SDS sample buffer contained 50 mM Tris/HCl, pH 6.8, 2 % (w/v) SDS, 10 % (v/v) glycerol and 1 % (v/v) 2-mercaptoethanol.

DNA constructs

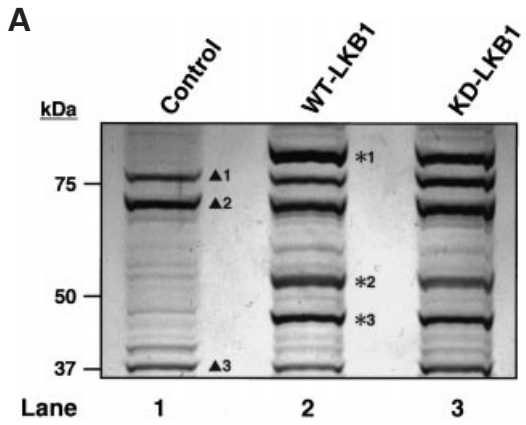
Constructs expressing the full-length wild-type and kinase-dead mouse LKB1 were as described previously [17]. In order to generate a cDNA encoding for the N-terminal 44 amino acids of LKB1 fused to GST, a stop codon was introduced at the 45th codon of wild-type LKB1 in the pEBG-2T vector [17]. The fragments of LKB1 cDNA encoding residues 44–319 and 343–436 of LKB1 were amplified by PCR and first subcloned into the pCR-Topo 2.1 vector (Invitrogen) and subsequently subcloned as an *Spe*I/*Spe*I fragment into pEBG-2T. The DNA constructs coding for expression in HEK-293 cells of N-terminal GST tagged protein kinase Bα ('PKBα') [19], 3-phosphoinositide-dependent protein kinase-1 (PDK1) [20], mitogen- and stress-activated protein kinase-1 (MSK1) [21], serum- and glucocorticoid-stimulated protein kinase-1 lacking the N-terminal 60 amino acids (ΔN-SGK1) [22], p70 ribosomal S6 protein kinase (p70S6K) [23], TAPP1 [24] and dual adaptor for phosphotyrosine and 3-phosphoinositide-1 (DAPP1) [25] in the pEBG-2T vector were as described previously.

Cell culture conditions

HeLa cells stably expressing wild-type or kinase-dead LKB1 have been described previously [26] and were cultured in Eagle's minimum essential medium supplemented with 10 % (v/v) tetracyclin-free FBS in the presence of 1 × non-essential amino acid solution, 1 × antibiotic/antimycotic solution (Sigma), 100 μg/ml zeocin and 5 μg/ml blasticidin (Invitrogen). HEK-293 and embryonic fibroblast Rat-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) FBS and 1 × antibiotic/antimycotic solution.

Immunoblotting

The amounts of proteins indicated in the Figure legends were subjected to SDS/PAGE and transferred to nitrocellulose membranes. The membranes were incubated in 50 mM Tris/HCl (pH 7.5)/0.15 M NaCl/0.5 % (v/v) Tween containing 5 % (w/v) BSA for 1 h, and then in the same buffer containing 1 μg/ml antibody (Cdc37, Hsp90 and ERK1/ERK2 antibodies) or 0.5 μg/ml (GST and FLAG antibodies) for 8 h at 4 °C. Immunoblots with the LKB1 antibody were carried out as above with 1 μg/ml antibody except that BSA was replaced with 10 % (w/v)



B

	Protein name	Peptide matches	% sequence coverage	NCBI gi number
▲1	eIF-4B	16/52	27%	4503533
▲2	Protein methyltransferase	24/74	39%	6164704
▲3	Hypothetical protein XP_052162	12/54	38%	14739415
*1	Hsp90	17/54	42%	13129150
*2	LKB1	28/64	48%	7106425
*3	Cdc37	20/39	51%	5901922

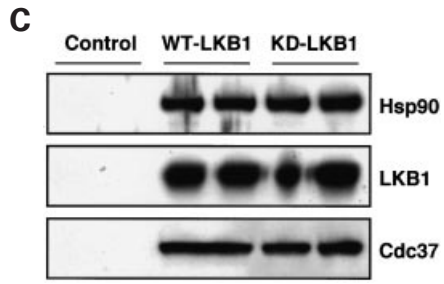


Figure 2 LKB1 is associated with Hsp90 and Cdc37

(**A**) Cell lysates derived from control parental HeLa cells or HeLa cells stably expressing FLAG-epitope-tagged wild-type (WT) or kinase-dead (KD) LKB1 were passed through an anti-FLAG M2-affinity agarose column, LKB1 eluted with the FLAG peptide and the samples concentrated as described in the Materials and methods section. The samples were electrophoresed on a polyacrylamide gel and the protein bands visualized with Colloidal Blue stain. (**B**) The Colloidal Blue-stained bands, labelled as indicated in (**A**), were excised from the gel and, following in-gel digestion with trypsin, their identities were determined by tryptic peptide mass-spectral fingerprint. The number of tryptic peptides, percentage of sequence coverage and NCBI gi numbers for each protein identified are indicated. (**C**) The samples purified in (**A**) were immunoblotted with the indicated antibodies. Identical results were obtained following two independent purifications of LKB1 from HeLa cells.

skimmed milk powder. Detection was performed using the appropriate horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence reagent (Amersham Biosciences).

Immunoprecipitation

The LKB1 antibody was covalently coupled to Protein G–Sepharose in a ratio of 1 mg of antibody to 1 ml of Sepharose using the dimethylpimelimidate cross-linking procedure [27]. Cleared lysates of Rat-2 cells containing 3 mg of total cell protein were incubated at 4 °C for 1 h on a vibrating platform with 10 μl of the LKB1 Protein G–Sepharose conjugate. Following centrifugation for 1 min at 14000 ***g***, the immunoprecipitates were washed twice with 1 ml of lysis buffer devoid of 2-mercaptoethanol containing 150 mM NaCl and twice with buffer A and resuspended in SDS sample buffer that did not contain 2-mercaptoethanol to give a final slurry volume of 25 μl. Immunoprecipitation of FLAG-LKB1 for the pulse-chase experiment shown in Figure 5(A) (see below), was carried out as above using 10 μl of anti-FLAG M2-affinity agarose gel except that four washes were performed with lysis buffer containing 0.5 M NaCl.

Expression of GST-fusion proteins in HEK-293 cells

Dishes of HEK-293 cells (10 cm diameter) were transiently transfected with 5–10 μg of the pEBG-2T construct using a modified calcium phosphate method [28] and the cells were lysed 36 h post-transfection in 0.5 ml of ice-cold lysis buffer. The lysates were centrifuged at 14000 ***g*** for 15 min at 4 °C, and the supernatants were incubated for 1 h on a rotating platform with 25 μl of glutathione–Sepharose equilibrated previously in lysis buffer. The suspension was centrifuged for 1 min at 14000 ***g***, the beads washed four times with lysis buffer containing 0.5 M NaCl, twice with buffer A containing 0.1 % (v/v) 2-mercaptoethanol, and resuspended in SDS sample buffer to give a final volume of slurry of 75 μl.

Gel-filtration analysis of LKB1

Rat-2 cells were cultured on 10 cm diameter dishes and lysed in 0.2 ml of lysis buffer, and then centrifuged at 14000 ***g*** for 15 min to remove insoluble material. The supernatant was filtered through a 0.2 μm filter and subjected to gel filtration on a Superdex 200 PC 3.2/30 column equilibrated in buffer B containing 0.1 mM EGTA and 0.1 % (v/v) 2-mercaptoethanol, on a SMART system maintained at 4 °C. Rat-2 cell extract (50 μl; 5–7 mg/ml) was loaded on to the column, which was run at 50 μl/min, and 50 μl fractions were collected. A 20 μl aliquot of each fraction was immunoblotted for LKB1. In the experiment shown in Figure 1, as a control, 50 μl of 10 μg/ml hexahistidine-tagged LKB1 expressed in *E. coli* was analysed in the same way.

Purification of FLAG-LKB1

A total of 40 15 cm-diameter dishes of HeLa cells stably expressing wild-type LKB1, kinase-dead LKB1 or control HeLa cells were cultured in the presence of 1 μg/ml tetracyclin for 24 h to induce LKB1 expression prior to lysis. The cells were washed once with PBS and lysed in 0.5 ml of lysis buffer containing 1 mM dithiothreitol rather than 2-mercaptoethanol. The lysates were pooled, centrifuged at 14000 ***g*** for 15 min, and the supernatants were filtered through a 0.44 μm filter. Cleared lysates (150 mg of protein) were then incubated for 60 min at 4 °C by end-over-end rotation with 1 ml of anti-FLAG M2-affinity agarose gel equilibrated previously in lysis buffer. The suspensions were loaded on to a 1 × 10 cm column and beads were washed four times with 25 ml of buffer B. Bound material was eluted from the anti-FLAG–agarose column by incubation for 60 min by end-over-end rotation with 5 ml of buffer B containing 100 μg/ml FLAG peptide. The eluate from the column was collected, the column was washed with 25 ml of the same elution buffer and 5 ml fractions were collected. These fractions were analysed for the presence of FLAG-LKB1 by immunoblotting 25 μl of each fraction with an anti-FLAG M2 monoclonal

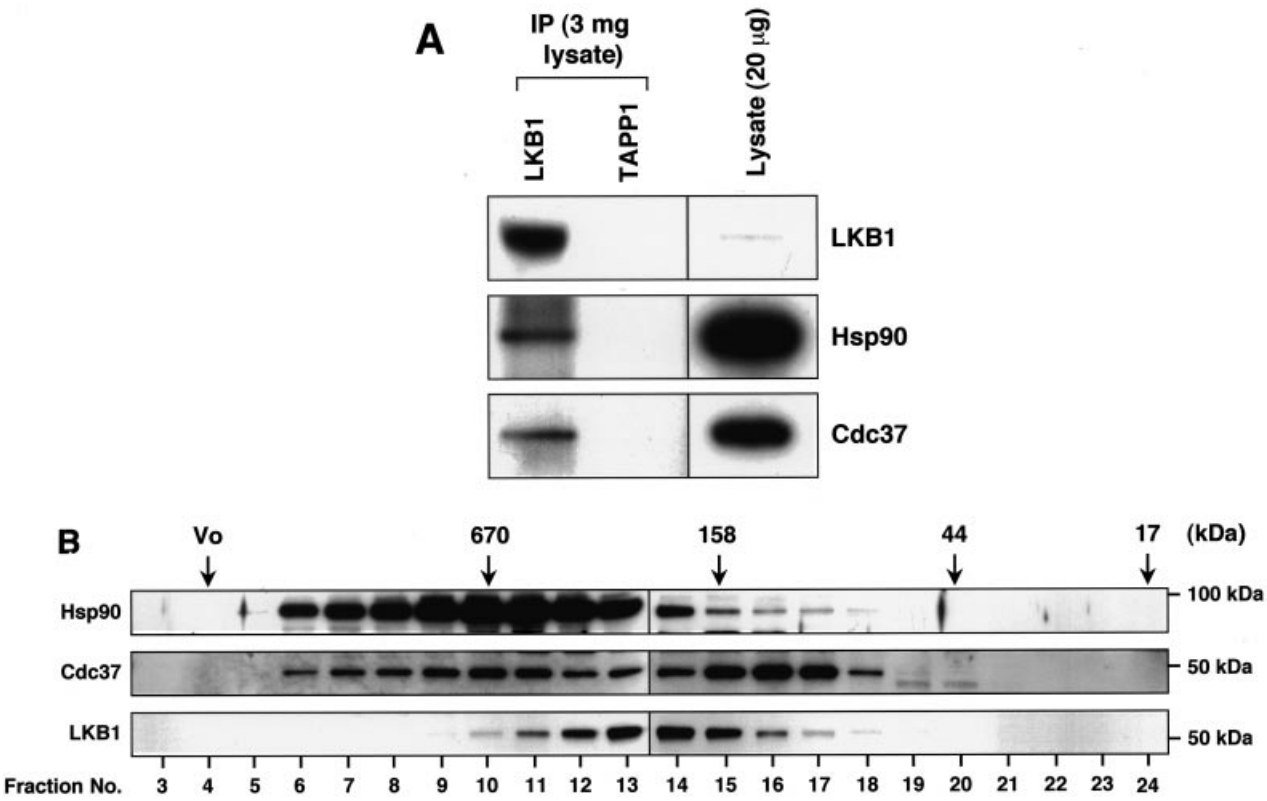


Figure 3 Endogenous LKB1 is associated with Hsp90 and Cdc37

(**A**) LKB1 was immunoprecipitated from 3 mg of Rat-2 cell lysate using LKB1 antibodies covalently coupled to Protein G–Sepharose, and the immunoprecipitates immunoblotted with the indicated antibodies. As a control, immunoprecipitations were also performed in parallel experiments with the non-specific TAPP1 antibody covalently coupled to Protein G–Sepharose. In each gel 20 μg of total Rat-2 cell lysate was also immunoblotted. Similar results were obtained in three separate experiments. (**B**) Rat-2 cell lysates were chromatographed on a Superdex 200 PC 3.2/30 gel-filtration column and fractions immunoblotted for Hsp90, Cdc37 and LKB1 as described in the Materials and methods section. The elution positions of the peak fractions of globular protein standards are shown (details are as in Figure 1).

antibody. The LKB1 was found to elute in the first two 5 ml fractions, which were pooled and concentrated to 0.1 ml using a 5 ml Vivaspin column (Vivascience, Lincoln, U.K.) and the samples were stored at -80 °C in aliquots.

MS analysis

The concentrated sample (20 μl) eluted from the anti-FLAG–agarose column was electrophoresed on a precast 4–12 % SDS/polyacrylamide gel, and the gel stained with Colloidal Blue and photographed. The bands indicated in Figure 2 were excised, washed and digested with trypsin as described previously [29]. Tryptic peptides were analysed by matrix-assisted laser-desorption ionization–time-of-flight ('MALDI-TOF') MS, using α-cyanocinnamic acid as the matrix, on a PerSeptive Biosystems Elite STR mass spectrometer. Spectra were acquired in the reflectron mode, internally calibrated and the tryptic peptide masses were scanned against NCBInr and SwissProt databases using the MS-FIT program of Protein Prospector (University of California at San Francisco, CA, U.S.A.) run on a local server.

Treatment of cells with Hsp90 and proteasome inhibitors

HeLa cells stably expressing wild-type LKB1 or HEK-293 cells were cultured on 6 cm-diameter dishes. For HeLa cells, expression of LKB1 was induced by culturing cells in the presence of 1 μg/ml tetracyclin for 16 h prior to treatment with the indicated inhibitors. Inhibitors were dissolved in DMSO (geldanamycin, radicicol, ZVAD, Tos-Lys-CH_2Cl, Tos-Phe-CH_2Cl, pepstatin, MG132, PSI and lactacystin) or water (leupeptin, E64 and NH_4Cl) at a 1000-fold higher concentration than that at which they were eventually used. Each protease inhibitor or the equivalent volume of vehicle DMSO as a control was added to the tissue-culture medium 3 h prior to treatment with geldanamycin or radicicol for the indicated times, and protease inhibitors were maintained throughout geldanamycin or radicicol treatments. The cells were then lysed in 0.3 ml of ice-cold lysis buffer, centrifuged at 14000 ***g*** for 15 min and the supernatants frozen in liquid nitrogen and stored at -80 °C. Protein concentrations were determined using the Bradford method, and BSA was employed as the standard.

Pulse–chase analysis

HeLa cells stably expressing wild-type LKB1 were cultured on 6 cm-diameter dishes and expression of FLAG-LKB1 was induced by culturing cells in the presence of 1 μg/ml tetracyclin for 16 h. The cells were washed once in PBS, and the culture medium was replaced with methionine/cysteine-free DMEM (Sigma) containing 10 % dialysed FBS and 1 μg/ml tetracycline. After 1 h, the cells were pulse-labelled with 100 μCi/ml [^{35}S]methionine/[^{35}S]cysteine in fresh methionine/cysteine-free DMEM containing 10 % dialysed FBS and 1 μg/ml tetracyclin for an additional 1 h. At this point ($t = 0$), the cells were washed

three times in DMEM containing 10% FBS and then either lysed immediately or incubated in complete medium in the presence or absence of 0.5 μM geldanamycin for the indicated times prior to cell lysis. FLAG-LKB1 was immunoprecipitated from the cleared lysates with anti-FLAG M2-affinity agarose beads as described above and subjected to SDS/PAGE. Gels were dried and the relative amounts of radiolabelled LKB1 were visualized and quantified by PhosphoImaging.

RESULTS

Evidence that endogenous LKB1 exists as a complex

We chromatographed Rat-2 cell lysates, which express endogenous LKB1 [17], on a gel-filtration column and immunoblotted the eluted fractions for the presence of LKB1. In this analysis, endogenous LKB1 was consistently found to elute in the same fractions as globular $\approx$ 350–150 kDa proteins would be expected to elute (Figure 1). No LKB1 was detected in the fractions in which a 50 kDa globular protein would be expected to elute. In parallel experiments, bacterially expressed hexahistidine-tagged LKB1 was also gel-filtered and found to elute at markedly later fractions than the endogenous LKB1, immediately prior to the 44 kDa chicken ovalbumin marker (Figure 1). This analysis indicated that endogenous LKB1 expressed in Rat-2 cells is associated with another protein(s) and therefore migrates with a higher-than-expected apparent molecular mass on gel filtration.

Isolation of an LKB1 complex

To facilitate the isolation of potential LKB1-interacting proteins, we made use of stable HeLa cell lines expressing N-terminal FLAG-epitope-tagged wild-type or kinase-dead LKB1 that express about 10-fold higher levels of LKB1 than endogenous LKB1 expressed in Rat-2 cells [26]. It should be noted that the levels of LKB1 in these stable HeLa cell lines is $\approx$ 50–100-fold lower than those obtained following transient transfection of cells with LKB1 (results not shown). FLAG-LKB1 was immunopurified from 150 mg of HeLa cell lysate derived from the cells expressing wild-type or kinase-dead LKB1 (see the Materials and methods section). As a control the same purification was elaborated with a parental HeLa cell line that does not express LKB1. The preparations were subjected to electrophoresis on a polyacrylamide gel, which was subsequently stained with Colloidal Blue. Several bands were observed in the sample of purified wild-type FLAG-LKB1, which were not present in the control sample (compare lanes 1 and 2 on Figure 2A). The sample purified from cells expressing kinase-dead FLAG-LKB1 displayed the same composition as the wild-type FLAG-LKB1 sample (compare lanes 2 and 3 on Figure 2A). The identity of the major Colloidal Blue-stained bands labelled in Figure 2(A) was established by standard tryptic peptide mass-spectral fingerprinting procedures (Figure 2B). Three of the proteins present in the wild-type and kinase-dead FLAG-LKB1 preparations but not in the control sample were identified as FLAG-LKB1 itself, Hsp90 and Cdc37 (Figure 2B). The relative intensities of the Colloidal Blue-stained bands corresponding to Hsp90, LKB1 and Cdc37 indicate that these proteins are present in similar stoichiometric amounts (Figure 2A). Hsp90 and Cdc37 are known to form a 1:1 complex that has previously been documented to interact with a number of other protein kinases (reviewed in [30,31]). To validate the MS identification of LKB1-associated proteins, the purified samples were immunoblotted

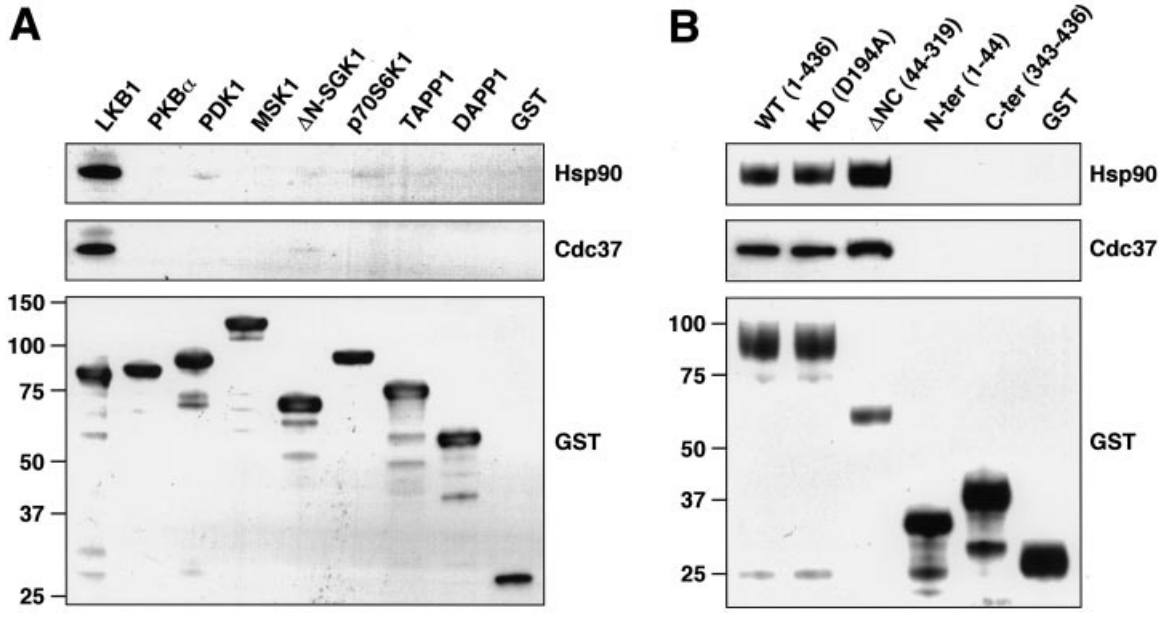


Figure 4 Specific association of Hsp90 and Cdc37 with the LKB1 catalytic core

(**A**) N-terminal GST-tagged LKB1 or the indicated proteins were expressed in HEK-293 cells and affinity-purified with glutathione–Sepharose. Similar amounts of the purified proteins were subjected to SDS/PAGE and immunoblotted with Hsp90 and Cdc37 antibodies to detect endogenous Hsp90 and Cdc37 associated with the GST fusion proteins. The membranes were also immunoblotted with a monoclonal GST antibody to ensure that comparable amounts of the GST fusion proteins were present in each lane. PKBα, protein kinase Bα; PDK1, 3-phosphoinositide dependent protein kinase-1; MSK1, mitogen- and stress-activated protein kinase-1; ΔN-SGK1, serum- and glucocorticoid-stimulated protein kinase-1 lacking the N-terminal 60 amino acids; p70S6K, p70 ribosomal S6 protein kinase; DAPP1, dual adaptor for phosphotyrosine and 3-phosphoinositide-1. (**B**) N-terminal GST tagged wild-type (WT) LKB1, kinase-dead (KD) LKB1 or the indicated fragments of wild-type LKB1 [the isolated catalytic domain, residues 44–319 (ΔNC), the N-terminal non-catalytic region, residues 1–44 (N-ter) and the C-terminal non-catalytic domain, residues 343–436 (C-ter)] were expressed in HEK-293 cells and analysed as in (**A**). Similar results were obtained in two separate experiments.

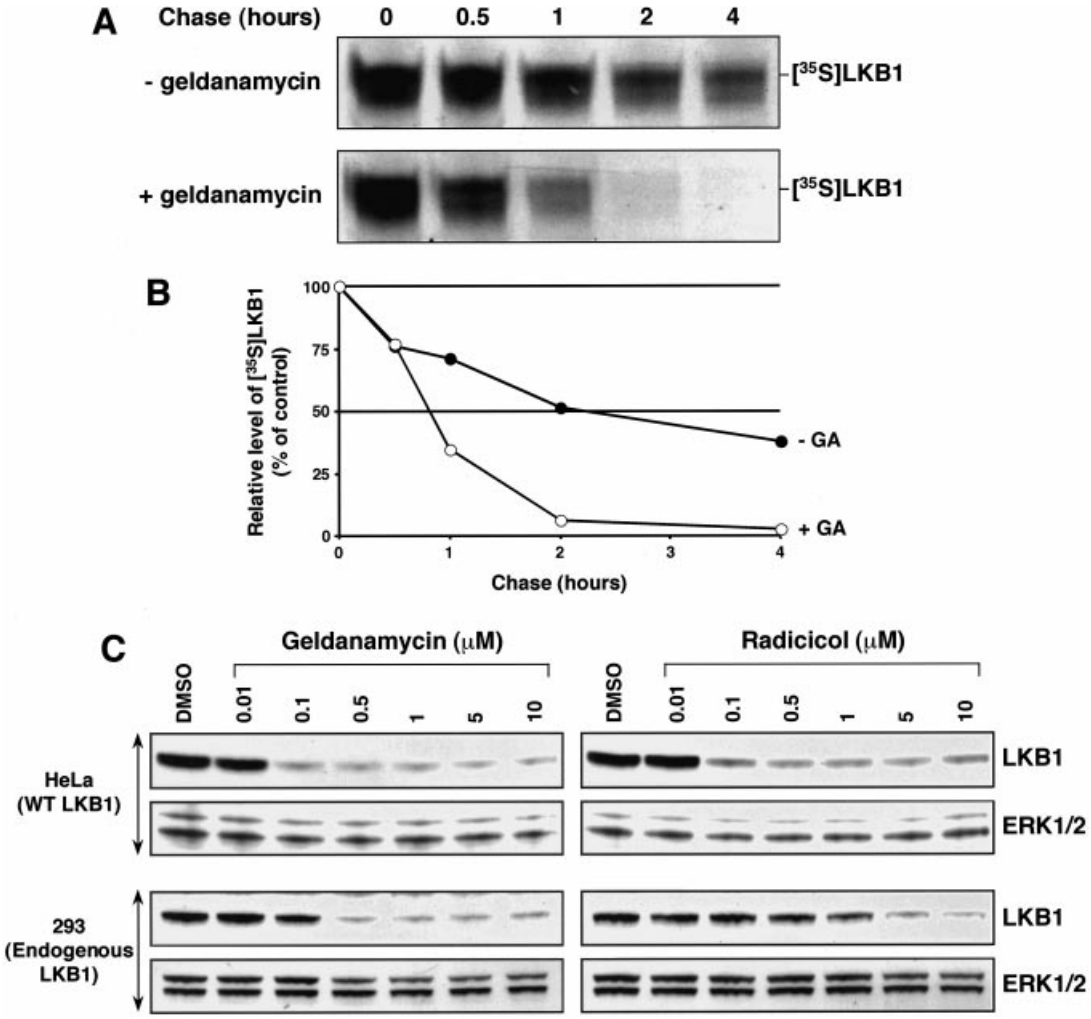


Figure 5 Evidence that Hsp90 regulates LKB1 intracellular stability

(**A**) HeLa cells stably expressing wild-type LKB1 were pulse-labelled with [^{35}S]methionine/[^{35}S]cysteine and chased in non-radioactive medium in the absence or presence of 0.5 μM geldanamycin as described in the Materials and methods section. At the indicated time periods following the chase, FLAG-LKB1 was immunoprecipitated, subjected to electrophoresis on a polyacrylamide gel and the dried gel autoradiographed. (**B**) The levels of radiolabelled LKB1 were quantified by PhosphoImaging. (**C**) HeLa cells stably expressing wild-type FLAG-LKB1 or non-transfected HEK-293 cells were treated with the indicated amounts of geldanamycin or radicicol for 4 or 24 h, respectively. Cells were lysed and lysates immunoblotted with LKB1 or ERK1/ERK2 antibodies to ensure that comparable amounts of proteins were present in each lane. Similar results were obtained in two separate experiments.

with antibodies recognizing Hsp90 and Cdc37 as well as LKB1 itself (Figure 2C). These results confirmed that Hsp90 and Cdc37 were specifically associated with wild-type and kinase-dead LKB1 but were not found in the control purification. Other proteins were also identified to be associated with wild-type and kinase-dead LKB1 and further investigation is underway to establish the physiological relevance of these interactions.

Endogenous LKB1 interacts with Hsp90 and Cdc37

We next immunoprecipitated endogenous LKB1 from 3 mg of Rat-2 cells lysates and demonstrated by immunoblotting that endogenous Hsp90 and Cdc37 were also coimmunoprecipitated (Figure 3A). To gain insight into the cellular fraction of Hsp90 and Cdc37 associated with LKB1, 20 μg of Rat-2 cell lysate was immunoblotted with these antibodies in parallel experiments. This demonstrated that only a very small proportion of the total cellular pool of Hsp90 and Cdc37 is associated with LKB1 (Figure 3A). Consistent with this observation, gel-filtration analysis of Rat-2 cell lysates revealed that Hsp90 and Cdc37 eluted across a wide range of molecular masses and that a large proportion of the cellular Hsp90 and Cdc37 complex was present in fractions not containing LKB1 (Figure 3B). These observations are not surprising as the abundant Hsp90 and Cdc37 chaperones are known to be associated with a large number of cellular proteins.

Hsp90 and Cdc37 specifically associate with the kinase domain of LKB1

To assess the specificity of the interaction of LKB1 with Hsp90 and Cdc37, we expressed in HEK-293 cells, as N-terminal GST fusion proteins LKB1, five distinct protein kinases, two adaptor proteins and GST itself. These proteins were purified by affinity chromatography on glutathione–Sepharose, and similar

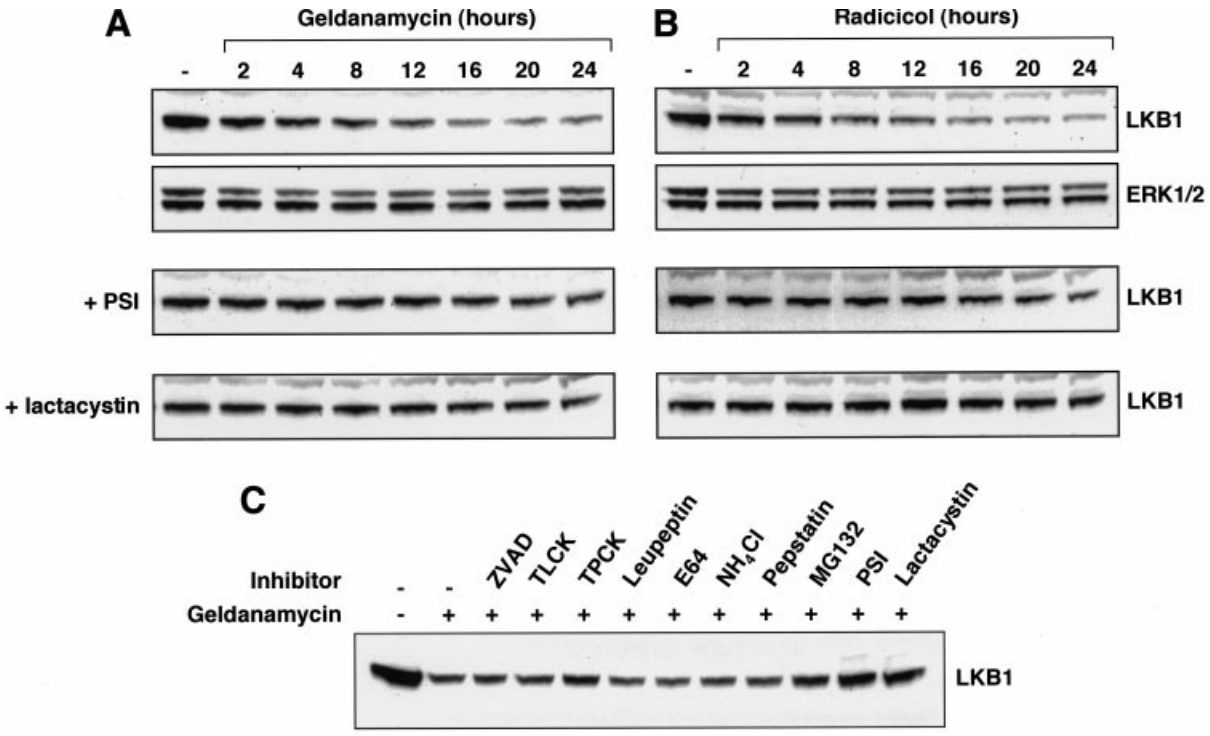


Figure 6 Evidence that LKB1 degradation is mediated by the proteasome

HEK-293 cells were treated for 3 h in the absence or presence of PSI (25 μM) or lactacystin (5 μM) and then incubated with 10 μM geldanamycin (**A**) or 10 μM radicicol (**B**). At the times indicated cells were lysed and lysates immunoblotted with LKB1 or ERK1/ERK2 antibodies, to ensure that comparable amounts of proteins were present in each lane. (**C**) HeLa cells stably expressing wild-type FLAG-LKB1 were treated for 3 h in the absence (−) or presence of 25 μM ZVAD, Tos-Lys-CH_2Cl (TLCK), Tos-Phe-CH_2Cl (TPCK), leupeptin, E64, NH_4Cl, pepstatin, MG132, PSI or 5 μM lactacystin. The cells were then incubated for 4 h in the absence (−) or presence (+) of 0.5 μM geldanamycin. Cells were lysed and lysates immunoblotted with the anti-LKB1 antibody. Similar results were obtained in two separate experiments.

amounts of the purified proteins were immunoblotted for the presence of associated endogenous Hsp90 and Cdc37. As shown in Figure 4(A), Hsp90 and Cdc37, were strongly associated with GST-LKB1, but not with any of the other proteins tested.

To establish the Hsp90- and Cdc37-binding regions within LKB1, wild-type LKB1, kinase-dead LKB1, the isolated catalytic domain of LKB1 (residues 44–319), the N-terminal non-catalytic region (residues 1–44) and the C-terminal non-catalytic domain (residues 343–436) were expressed in HEK-293 cells as N-terminal GST fusion proteins. These proteins were affinity-purified on glutathione–Sepharose and immunoblotted with Hsp90 and Cdc37 antibodies. The results shown in Figure 4(B) demonstrate that both endogenous Hsp90 and Cdc37 were equally associated with the isolated catalytic domain of LKB1 and full-length protein, but did not detectably interact with the non-catalytic regions of LKB1.

Hsp90 regulates LKB1 stability

To examine whether Hsp90 played a role in regulating LKB1 stability, we first tested whether geldanamycin, a derivative of ansamycin-type benzoquinoid, which inhibits the normal molecular chaperone function of Hsp90 [32], affected the stability of LKB1 in cells. The half-life of the LKB1 protein in HeLa cells stably expressing wild-type LKB1 was assessed in the presence or absence of geldanamycin. The cells were pulse-labelled with a mixture of [^{35}S]methionine and [^{35}S]cysteine for 1 h and the radioactivity then chased by placing the cells in non-radioactive medium in the presence or absence of 0.5 μM geldanamycin. At various times the cells were lysed, the LKB1 protein immunoprecipitated and the amount of radiolabelled LKB1 assessed by autoradiography and PhosphoImage analysis following electrophoresis. The half-life of the LKB1 protein was markedly shortened in HeLa cells treated with geldanamycin compared with control cells in two separate experiments (Figure 5A). Quantification of radiolabelled LKB1 revealed that the half-life of stably expressed LKB1 was ≈ 2 h in the absence of geldanamycin and ≈ 0.75 h in the presence of this drug (Figure 5B).

We next treated HeLa cells stably expressing wild-type LKB1 for 4 h with different doses of geldanamycin and another Hsp90 inhibitor, radicicol [33], and examined intracellular levels of LKB1 by immunoblotting. These experiments revealed that concentrations of geldanamycin and radicicol as low as 0.1 μM were sufficient to induce a $> 90\%$ decrease in the levels of the LKB1 protein without affecting the levels of the ERK1/ERK2 protein kinases that are not known to interact with Hsp90 (Figure 5C, upper panels). The levels of endogenously expressed LKB1 in HEK-293 cells (Figure 5C, lower panels) and Rat-2 cells (results not shown), were also markedly reduced by geldanamycin and radicicol treatments, although 0.5 μM geldanamycin and 5 μM radicicol was required to reduce the levels of endogenous LKB1 by over 90%. Moreover, it should be noted that in both HEK-293 cells (Figure 6A) and Rat-2 cells (results not shown) the endogenous LKB1 appears to have a longer half-life than in HeLa cells, as 12 h treatment with geldanamycin and radicicol is required to induce a substantial reduction in endogenous LKB1 levels.

Evidence that the proteasome is involved in degrading LKB1

Protein degradation can be achieved by several protease systems in cells; however, most proteins whose stability is regulated by Hsp90 appear to be degraded by the proteasome [34,35]. We therefore examined the effects of the proteasome inhibitors PSI [36] and lactacystin [37] in preventing the geldanamycin- and radicicol-induced degradation of endogenous LKB1 in HEK-293 cells (Figures 6A and 6B) or stably expressed LKB1 in HeLa cells (Figure 6C). These proteasome inhibitors substantially prevented the degradation of LKB1 over a 24 h time course of treatment with geldanamycin or radicicol. Consistent with proteasome mediating the degradation of LKB1, inhibitors of the trypsin-like serine protease inhibitor Tos-Lys-CH_2Cl, the lysosome protease inhibitors E64 and leupeptin, the lysosome acidification inhibitor NH_4Cl, the aspartate protease inhibitor pepstatin-A or the caspase inhibitor ZVAD could not significantly prevent geldanamycin-induced degradation of LKB1 in HeLa cells (Figure 6C). The chymotrypsin-like serine protease inhibitor Tos-Phe-CH_2Cl, which also inhibits the proteasome machinery, substantially reduced Hsp90-inhibitor-induced degradation of LKB1 (Figure 6C).

DISCUSSION

The initial aim of this study was to purify LKB1 from a mammalian cell lysate and determine the identity of the major LKB1-associated proteins. This analysis revealed that Hsp90 and Cdc37 proteins were stably associated with endogenously expressed LKB1. The Hsp90 and Cdc37 proteins were estimated to be present at similar molar ratios to LKB1 in the complex purified from HeLa cells stably expressing LKB1. Hsp90 and Cdc37 interacted with both wild-type and kinase-dead LKB1, indicating that the catalytic activity of LKB1 is not required for its association with these proteins. It is likely that LKB1 associates with cellular proteins other than those we have identified in this study. In the experiment shown in Figure 2(A) there are other bands present in the LKB1 preparation which we have identified, none of which have been previously reported to bind to LKB1. We are currently characterizing the physiological relevance, if any, of these interactions. There were also a number of bands that were too low in abundance to enable us to identify them by MS. These could also represent other LKB1-interacting proteins, namely p53 [14], LIP1 [16] and Brg1 [15], which were previously shown in genetic/co-expression-based screens to bind LKB1.

Hsp90 is an abundant cytosolic protein that functions as a chaperone and plays an essential role in numerous cellular processes. In contrast to Hsp60 and Hsp70, it is thought that Hsp90 has a more limited number of target proteins, many of which appear to be protein kinases and hormone receptors [38,39]. Hsp90 requires the presence of co-chaperone proteins to enable it to interact specifically with its client proteins. One of these co-chaperone proteins is Cdc37, which appears to specifically target Hsp90 to a variety of protein kinases, including the cyclin-dependent kinase CDK4 [40], the inhibitory κB kinase (IKK) IKKα/IKKβ complex [41], Raf1 [42,43] and the mitogen-activated protein kinase (MAPK) family member MAPK-overlapping kinase (MOK) [44]. To our knowledge, in all cases examined, the Hsp90/Cdc37 heterodimeric complex binds directly to the catalytic domain of its target protein kinases. As expected, we have made a similar observation with LKB1 (Figure 4B). Not surprisingly, as Hsp90/Cdc37 interacts with the catalytic domains of kinases, it influences the activity of some of the protein kinases to which it binds. For example, the interaction of Hsp90/Cdc37 with the IKK complex is required for its activation by tumour necrosis factor [41], and the interaction of CDK4 with Hsp90/Cdc37 is required for its proper assembly with cyclin D.

Another common role of Hsp90 is to stabilize its target proteins and prevent their degradation by the proteasome complex. A significant number of oncogenes, such as the ErbB receptor [45], $p210^{bcr-abl}$ and v-Src [46], as well as the mitogen-regulated MOK kinase [44], are rapidly degraded following treatment of cells with Hsp90 inhibitors such as geldanamycin. Consistent with the key role of Hsp90 in regulating the stability of LKB1, treatment of cells with Hsp90 inhibitors markedly lowered LKB1 protein levels (Figures 5 and 6). Pulse–chase experiments established that geldanamycin shortened the half-life of the stably expressed LKB1 protein in HeLa cells from ≈ 2 to ≈ 0.75 h. It is known that Hsp90 inhibitors function by inducing the dissociation of Hsp90 from its client proteins, thereby exposing them for ubiquitination/SUMOlation and subsequent attack by the proteasome complex. This is supported by the finding that the Hsp90-inhibitor-induced degradation of protein kinases is invariably prevented by proteasome inhibitors. As two distinct proteasome inhibitors (PSI and lactacystin) significantly prevented Hsp90-inhibitor-induced LKB1 degradation, it is likely that LKB1 degradation under these conditions is also mediated by the proteasome. Further investigation is required to establish the mechanism by which LKB1 becomes targeted by the proteasome following inhibition of Hsp90 and whether this may involve ubiquitination or SUMOlation.

In order to test whether binding of Hsp90/Cdc37 to LKB1 affects its activity we have added geldanamycin to GST-LKB1 purified from HEK-293 cells and found that this did not affect its ability to autophosphorylate or phosphorylate the exogenous substrate MBP *in vitro* (J. Boudeau, unpublished work). We have also immunoprecipitated FLAG-LKB1 from stable HeLa cells following treatment with geldanamycin in the presence of proteasome inhibitors to prevent LKB1 degradation and found that this did not affect immunoprecipitated LKB1 activity (J. Boudeau, unpublished work). These observations indicate that Hsp90/Cdc37 does not directly influence the intrinsic LKB1 catalytic activity.

There is significant interest in developing anti-cancer drugs that inhibit Hsp90 as these may induce the inactivation/degradation of a number of oncogenes [32]. However, our discovery that Hsp90 inhibitors reduce the intracellular levels of LKB1 suggests that prolonged use of these drugs could, instead of preventing cancer, lead to the formation of both benign and malignant tumours similar to those observed in patients with PJS. The finding that virtually all heterozygous LKB1 mice, lacking a single allele of this gene, develop gastrointestinal tumours by 20 weeks of age, perhaps due to a haploinsufficiency of LKB1 [6,7], indicates that even a moderate reduction of intracellular levels of LKB1 is sufficient to lead to tumour development.

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