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LKB1 Is the Upstream Kinase in the AMP-Activated Protein Kinase Cascade

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Summary

Inactivating mutations in the protein kinase LKB1 lead to a dominantly inherited cancer in humans termed Peutz-Jeghers syndrome [1, 2]. The role of LKB1 is unclear, and only one target for LKB1 has been identified in vivo [3]. AMP-activated protein kinase (AMPK) is the downstream component of a protein kinase cascade that plays a pivotal role in energy homeostasis [4]. AMPK may have a role in protecting the body from metabolic diseases including type 2 diabetes [5, 6], obesity [7], and cardiac hypertrophy [8]. We previously reported the identification of three protein kinases (Elm1, Pak1, and Tos3 [9]) that lie upstream of Snf1, the yeast homologue of AMPK. LKB1 shares sequence similarity with Elm1, Pak1, and Tos3, and we demonstrated that LKB1 phosphorylates AMPK on the activation loop threonine (Thr172) within the catalytic subunit and activates AMPK in vitro [9]. Here, we have investigated whether LKB1 corresponds to the major AMPKK activity present in cell extracts. AMPKK purified from rat liver corresponds to LKB1, and blocking LKB1 activity in cells abolishes AMPK activation in response to different stimuli. These results identify a link between two protein kinases, previously thought to lie in unrelated, distinct pathways, that are associated with human diseases.

Results and Discussion

LKB1 (also known as STK11) protein copurified with AMPKK activity during two successive chromatography steps of the purification procedure [10]. Western blot analysis of fractions from the Q-Sepharose ion exchange and Superdex-200 gel filtration chromatography steps showed that anti-LKB1 antibodies crossreact with polypeptides present only in the fractions containing AMPKK activity (Figures 1A and 1B). No AMPKK activity or LKB1 crossreactivity was detected in any other fractions from the columns (results not shown). Several closely migrating polypeptides were detected with a molecular mass of approximately 45 kDa, similar to the predicted mass of mouse and human LKB1 (49 kDa). Incubation with protein phosphatase 2C caused a shift in mobility of the polypeptides toward the faster-migrating band (Figure 1C), suggesting that the different polypeptides result from different states of phosphorylation.

AMPKK from the Q-Sepharose step was subjected to an immuoprecipitation reaction with anti-LKB1 antibodies. The resulting immune complex was able to activate bacterially expressed AMPK (Figure 1D); no significant activation was observed in a control reaction using an immune complex isolated by incubation with purified control sheep immunoglobulins. Immunoprecipitation with anti-LKB1 antibodies depleted AMPKK activity in the preparation by approximately 85% compared to modest depletion using a control antibody (Figure 1E). Similar results were observed using three different anti-LKB antibodies (results not shown). These results suggest that LKB1 accounts for the majority of AMPKK activity purified from rat liver. We have not found any evidence to suggest that AMPK and LKB1 activities coimmunoprecipitate (results not shown).

AMPK is heterotrimeric, consisting of an α catalytic subunit and two regulatory subunits, β and γ, and isoforms of all three subunits have been identified [4]. In rat, complexes containing the $\gamma 1$ subunit account for the majority of AMPK activity in most tissues [11]. We previously showed that recombinant LKB1 purified from mammalian cells phosphorylates and activates AMPK $\alpha 1\beta 1\gamma 1$ [9]. LKB1 also activates the other major forms of AMPK, and we did not detect any significant difference in the activation of these complexes by LKB1 (Figure 2A). LKB1 phosphorylated catalytically inactive mutants of AMPK on Thr172 within the α subunit (Figure 2B), and we have previously reported that phosphorylation of AMPK requires LKB1 catalytic activity [9]. Taken together, these results strongly suggest that LKB1 acts by directly phosphorylating AMPK rather than promoting autophosphorylation of AMPK. Catalytically inactive AMPK, which is unable to autophosphorylate, was phosphorylated to a stoichiometry of approximately 0.4 mol/mol by LKB1. Partially purified rat liver AMPKK was reported to be activated by AMP [12], but another study failed to detect this effect [13]. We did not detect any effect of AMP on the ability of LKB1 to phosphorylate and activate AMPK (Figures 2A and 2B). Using the same reagents, recombinant AMPK was activated allosterically by AMP (results not shown), consistent with an earlier study we have previously reported [14]. We do not know the reason for the difference between our results and those of Hawley et al. [12], although there are a number of explanations that could account for this

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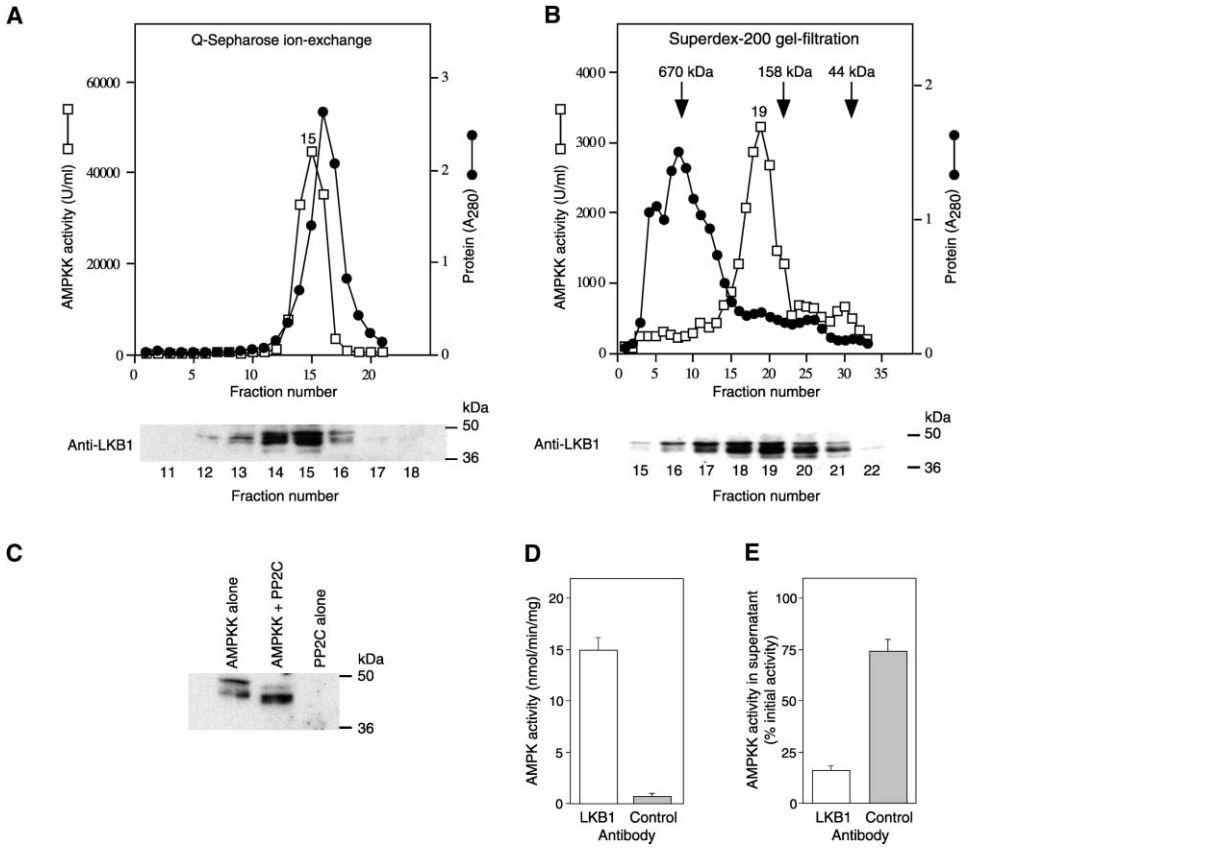

Figure 1. LKB1 Is the Major AMPK Kinase Activity in Rat Liver

Partially purified AMPKK isolated from rat liver was fractionated by (A) ion-exchange chromatography on Q-Sepharose and (B) gel filtration on Superdex-200. Fractions (2 μl) were assayed for AMPKK by activation of recombinant AMPK (open squares) and protein (closed circles). The elution position of molecular mass standards from the gel-filtration column is indicated by arrows, and the peak fraction number of AMPKK activity in each case is labeled. In parallel, aliquots (25 μl) were analyzed by Western blotting using anti-LKB1 antibodies, and fractions across the peak of AMPKK activity are shown. No LKB1 crossreactivity was detected in any of the other fractions (results not shown). (C) The peak fraction of AMPKK activity from Superdex-200 was incubated in the presence or absence of protein phosphatase 2C (0.16 mg/ml) for 30 min at 37°C before Western blot analysis with anti-LKB1 antibody. The migration of molecular mass standards is shown. (D and E) AMPKK purified through the Q-Sepharose step was incubated with anti-LKB1 antibodies or sheep IgG immunoglobulins (Sigma) as a control. (D) AMPKK activity present in the immune complexes was determined by activation of recombinant AMPK. Results are expressed as AMPK activity and are the mean values ± SEM from three independent experiments. (E) AMPKK activity remaining in the supernatant after immunoprecipitation is plotted as a percentage of the initial activity before incubation with antibody. The values shown are the mean ± SEM from four independent experiments. We were unable to determine whether all of the AMPKK activity in the preparation could be accounted for by LKB1, since repeated incubation with control antibody led to nonspecific but substantial depletion of AMPKK activity (results not shown). One unit of AMPKK activity is the amount that increased the activity of recombinant AMPK by 1 nmol/min/mg.

discrepancy. For instance, it is possible that a factor required to mediate the stimulation of LKB1 by AMP was present in the partially purified AMPKK preparation, but it is either not present in the expression system or it is lost during the immunoprecipitation procedure used to isolate recombinant LKB1 in our study. Clearly, further work is required to investigate the effect of AMP on phosphorylation of AMPK by LKB1.

LKB1 is phosphorylated by a number of protein kinases at distinct sites, although the role of phosphorylation on LKB1 activity is unclear [15]. AMPKK activity is known to be unaffected by incubation with different protein phosphatases [10]. Consistent with this result, incubation of LKB1 with a mixture of protein phosphatases 1, 2A, and 2C had no effect on its ability to activate AMPK (results not shown).

In cells, AMPK is activated in response to ATP depletion, which leads to a concomitant increase in the AMP:ATP ratio [4]. AMPK is also activated in response to treatments that do not alter adenine nucleotide levels, e.g., incubation with the anti-diabetic agent metformin and hyperosmotic stress [16]. AMPK can be activated by pharmacological manipulation using the cell-permeable adenosine analog 5-amino-4-imidazolecarboxamide (AICA) riboside, which is phosphorylated to ZMP, an AMP mimetic [17]. To examine whether LKB1 activity is regulated, we immunoprecipitated LKB1 from lysates of H-2K^b muscle cells treated with stimuli known to alter AMPK activity [16]. LKB1 activity did not change significantly in response to any of the conditions tested (Figure 2C), even though AMPK activity varied substantially (Figure 2D). These results suggest that LKB1 is constitutively active and are consistent with a previous study that reported constitutive AMPKK activity in a pancreatic β

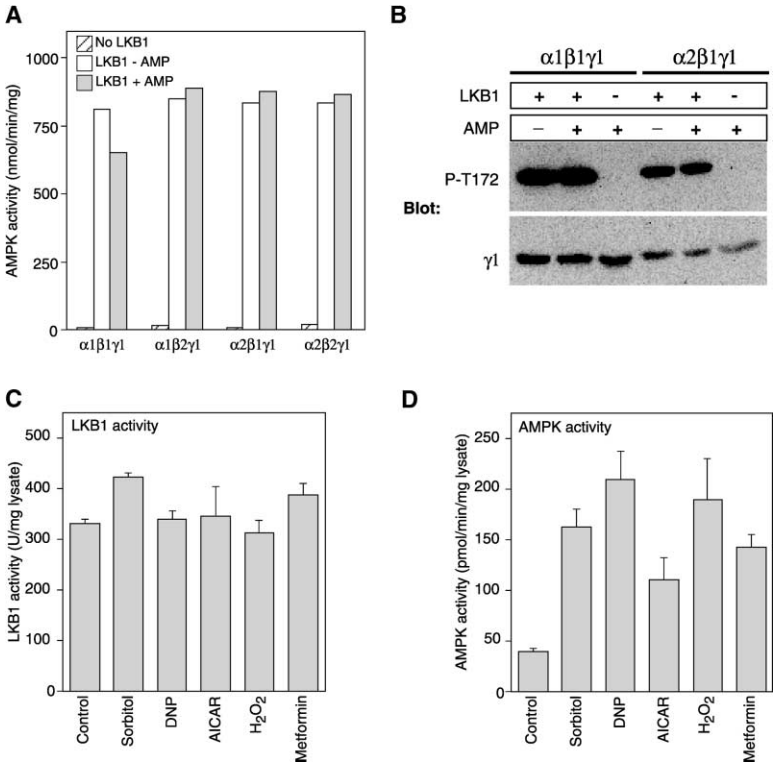

Figure 2. Regulation of LKB1

LKB1 expressed in COS7 cells was immunoprecipitated with anti-FLAG resin and used to phosphorylate recombinant AMPK complexes in vitro. (A) AMPK activity after incubation with recombinant LKB1 in the presence (shaded bars) or absence (open bars) of 0.2 mM AMP or in the absence of LKB1 (hatched bars) is shown. AMPK assays were carried out in the presence of 0.2 mM AMP, and the results shown are the average values from duplicate experiments that varied by less than 10%. (B) Catalytically inactive forms of AMPK ($\alpha1\beta1\gamma1$ or $\alpha2\beta1\gamma1$ complexes) were incubated in the presence or absence of recombinant LKB1 in the presence or absence of 0.2 mM AMP. Thr172 phosphorylation was determined by Western blot analysis using an anti-phosphothreonine 172 (P-T172)-specific antibody and total AMPK with an anti-$\gamma1$ specific antibody. (C and D) H-2K^b cells were incubated in the presence or absence of 0.5 M sorbitol, 0.5 mM dinitrophenol (DNP), 0.5 mM AICA riboside (AICAR), 0.5 mM hydrogen peroxide (H_2O_2), or 2 mM metformin for 30 min prior to harvesting and lysis. (C) LKB1 and (D) AMPK activities were determined in immune complexes isolated after immunoprecipitation from 100 μg total protein with anti-LKB1 or anti-AMPKβ antibodies. Results shown are the mean values $\pm$ SEM from four experiments.

cell line [13]. We cannot exclude, however, the possibility that the conditions used here cause phosphorylation or other modifications of LKB1 that are lost upon subsequent isolation, and hence no augmentation of activity or effect of phosphatases is observed.

To determine whether levels of LKB1 activity correlate with AMPK activity, we examined various cell lines including two tumor cell lines, HeLa S3 (cervical carcinoma) and G361 (melanoma), which have been reported to express very low or undetectable levels of LKB1 mRNA and protein [18] (Figures 3A and 3B). We detected LKB1 activity in all of the cell lines, and there was a remarkably close correlation between LKB1 activity and AMPK activity in all cases (Pearson correlation coefficient of 0.945, $p < 0.005$). In contrast, AMPK protein expression, as judged by Western blot analysis (Figure 3C), did not correlate with LKB1 or AMPK activity, indicating that the differences in AMPK activity are due to posttranslational effects.

LKB1 has been reported to associate with a number of proteins including heat-shock protein (Hsp) 90, which appears to be required for LKB1 stabilization [19]. Inhibitors of Hsp90, such as geldanamycin and radicicol, decrease LKB1 expression and activity in cells [19]. We tested the effect of radicicol on AMPK activity. Incubation of COS7 cells for 24 hr with 5 μM radicicol decreased LKB1 activity by 80%, from 60.1 $\pm$ 3.5 to 12.3 $\pm$ 1.1 U/mg ($n = 6$). AMPK activity was also reduced by as much as 70% after activation by hyperosmotic stress (0.5 M sorbitol), while AMPK protein expression was unchanged (Figures 3D and 3E). Thus, this effect of radicicol on AMPK activity is consistent with a role for LKB1 in activating AMPK in vivo, although it remains possible that inhibition of Hsp90 affects AMPK by some other mechanism.

We next sought to modulate LKB1 activity in a specific manner using a genetic approach. We expressed wild-type LKB1 or a catalytically inactive form of LKB1 harboring a mutation of aspartic acid residue 194 to alanine (D194A) in CCL13 cells. Expression of wild-type LKB1 caused a modest increase in AMPK activity relative to cells transfected with a control vector (expressing β-galactosidase) (Figure 4A). In contrast, expression of mutant LKB1 (D194A) completely abolished activation of AMPK in response to either hydrogen peroxide or hyperosmotic stress. Under these conditions, there was no detectable alteration in AMPK protein expression, as judged by Western blotting with an anti-AMPK β antibody (Figure 4B). These results indicate that catalytically inactive LKB1 acts in a dominant-negative manner, blocking AMPK activation and providing direct evidence that LKB1 functions to activate AMPK in cells. Furthermore, since AMPK activation was abolished after treatment of the cells with either hydrogen peroxide, which activates AMPK via an increase in the AMP:ATP ratio [20], or hyperosmotic stress, which activates AMPK by a nucleotide-independent pathway [16], these results demonstrate that LKB1 mediates both signaling pathways. Expression of catalytically inactive LKB1 did not result in a complete loss of AMPK activity (Figure 4A). One potential explanation for this observation is that expression of catalytically inactive LKB1 does not lead to total inhibition of endogenous LKB1. Alternatively, it is possible that another kinase, capable of activating AMPK under basal conditions, is present in the cells, and we are currently investigating this possibility.

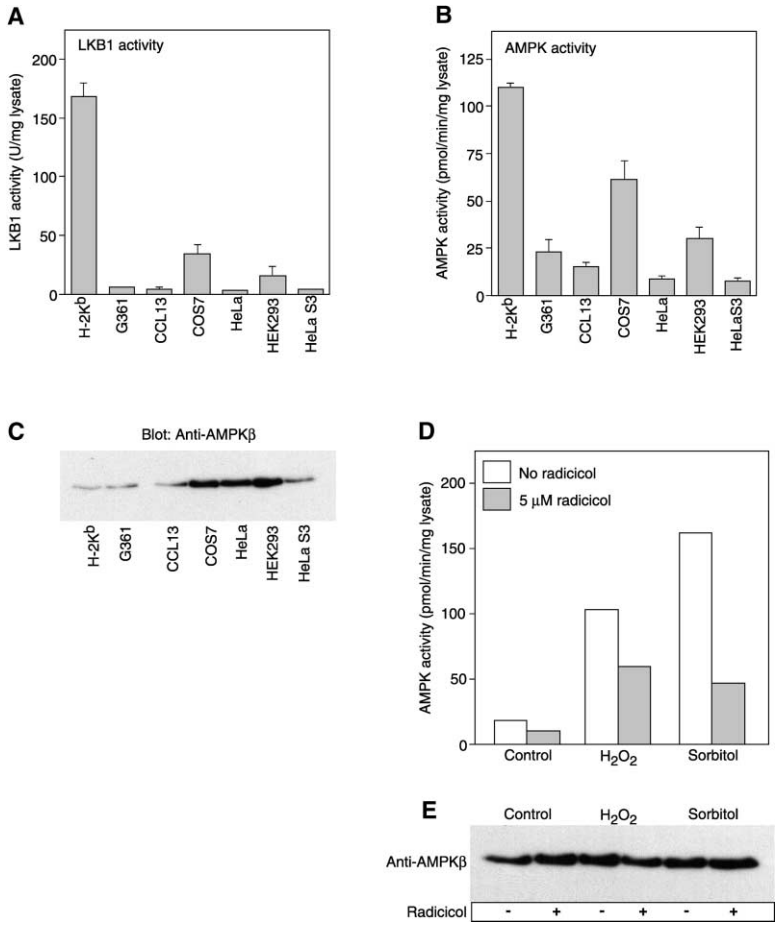

Figure 3. Reduced LKB1 Activity Is Associated with Decreased AMPK Activity

(A) Lysates (500 μg total protein) from the indicated cell lines were incubated with anti-LKB1 antibodies, and LKB1 activity in the immune complexes was determined by activation of recombinant AMPK.

(B and C) In parallel, (B) AMPK activity in anti-AMPKβ immunoprecipitates (from 100 μg total protein) was measured and (C) AMPK expression determined by Western blot analysis using anti-AMPKβ antiserum.

(D) COS7 cells were incubated in the presence or absence of 5 μM radicicol for 24 hr. Cells were incubated with no added reagent (control), 1 mM H_2O_2, or 0.5 M sorbitol for 15 min prior to harvesting. AMPK activity in immune complexes isolated from 100 μg total protein was determined. Results shown are the average values of duplicate experiments that varied by less than 10%.

(E) A representative Western blot showing AMPKβ expression.

Conclusions

Our findings show that LKB1 is the major upstream kinase in the AMPK cascade in mammalian cells. We do not exclude, however, the possibility of additional upstream kinases that activate AMPK in response to stimuli other than those tested in our current study. The presence of other upstream kinases might explain our finding that dominant-negative LKB1 does not lead to complete loss of basal AMPK activity. The recent demonstration that in yeast there are three upstream kinases that activate SNF1 [9, 21] also suggests that there may be more than one AMPK kinase in mammals. Clearly it will be important to determine whether there are other kinases acting upstream of AMPK in addition to LKB1.

The identification of LKB1 as an AMPK kinase opens many new avenues of investigation regarding both the regulation of AMPK and the physiological role of LKB1. To our knowledge, there is no direct evidence linking Peutz-Jeghers syndrome to metabolic diseases. However, since LKB1 is directly upstream of AMPK, a predicted consequence of loss of LKB1 function would be reduced, or perhaps abolished, AMPK activity, which would in turn lead to altered metabolic regulation. Whether this has a direct role in the development of tumors is unclear, but it is likely to affect cell growth. A recent report identified AMPK β1 as a p53-independent stress-responsive protein that inhibits tumor growth [22], and AMPK has been linked to the progression of cellular senescence [23]. It is intriguing that LKB1 has been implicated in both of these processes [24], and in the light of our recent findings, it will be important to investigate the role of AMPK in cancer.

Supplemental Data

Supplemental Data including experimental procedures are available at http://www.current-biology.com/cgi/content/full/13/22/2004/DC1/.

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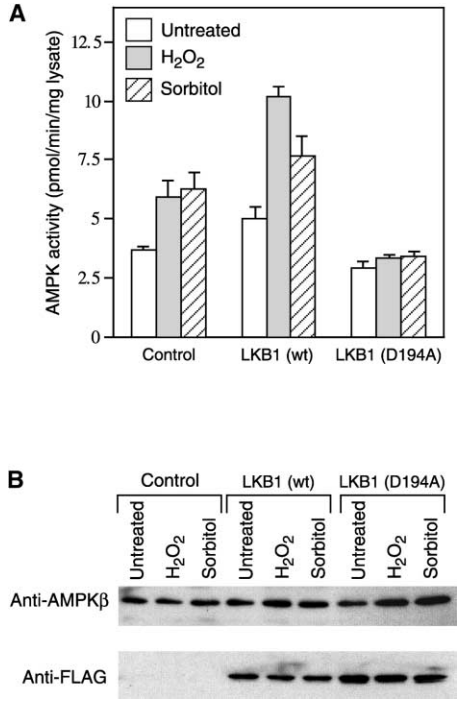

Figure 4. Expression of Dominant-Negative LKB1 Blocks AMPK Activation

CCL13 cells were transiently transfected with either wild-type LKB1 (wt), a catalytically inactive mutant LKB1 (D194A), or as a control, β-galactosidase. Cells were incubated in the absence (untreated) or presence of 1 mM H_2O_2 or 0.5 M sorbitol for 15 min prior to harvesting. (A) AMPK was immunoprecipitated from 100 μg total protein using anti-AMPKβ antibodies, and activity in the immune complexes was measured. Results are the mean ± SEM of six independent experiments. (B) The levels of AMPK and recombinant LKB1 were determined by Western blot analysis of total cell lysates (20 μg) using either anti-AMPKβ or anti-FLAG antibodies, respectively. A blot representative of results obtained in three independent experiments is shown in each case.

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