LKB1 Associates with Brg1 and Is Necessary for Brg1-induced Growth Arrest*

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Inactivating mutations in the serine-threonine kinase LKB1 (STK11) are found in most patients with Peutz-Jeghers syndrome; however the function of LKB1 is unknown. We found that LKB1 binds to and regulates brahma-related gene 1 (Brγ1), an essential component of chromatin remodeling complexes. The association requires the N terminus of LKB1 and the helicase domain of Brγ1 and LKB1 stimulates the ATPase activity of Brγ1. Brγ1 expression in SW13 cells induces the formation of flat cells indicative of cell cycle arrest and senescence. Expression of a kinase-dead mutant of LKB1, SL26, in SW13 cells blocks the formation of Brγ1-induced flat cells, indicating that LKB1 is required for Brγ1-dependent growth arrest. The inability of mutants of LKB1 to mediate Brγ1-dependent growth arrest may explain the manifestations of Peutz-Jeghers syndrome.

Peutz-Jeghers syndrome (PJS) is an autosomal dominant disorder characterized by mucocutaneous pigmentation, hamartomatous polyps of the gastrointestinal tract, and an increased risk of developing certain types of cancer. The disease is caused by mutations in the serine-threonine kinase LKB1 (STK11), which is found in most patients with PJS. LKB1 is a tumor suppressor, and mutations in LKB1 have been found in a small number of sporadic cancers. Mutations in LKB1 are associated with loss of heterozygosity in tumors from patients with PJS, indicating that LKB1 is a tumor suppressor. The kinase-dead mutant of LKB1 is less able to inhibit cell growth and induce senescence than the wild-type LKB1.

LKB1 in G361 melanoma cells resulted in significant inhibition of cell growth. The SL26 mutant of LKB1 was isolated from a patient with PJS, and this mutant was found to be less effective in inhibiting cell growth than the wild-type LKB1.

Experimental Procedures

In Vitro Expression Cloning—A unidirectional HeLa cell-derived cDNA library in pcDNA3.1 (Invitrogen, Carlsbad, CA) was fractionated into 680 small pools containing ~100 different clones per pool, as described (19). Pools were transcribed and translated in vitro (coupled TNT system, Promega) in the presence of [35S]methionine. Each translated protein was resuspended in buffer 1 (20 mM Tris, pH 7.5, 150 mM NaCl, 10 mM EDTA, 200 mM sodium orthovanadate, 10 mM NaF, 10 mM tetrasodium pyrophosphate, 10% glycerol, 1% Nonidet P-40, 4 µg/ml of pepstatin, leupeptin, and 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF)), followed by pre-clearance with 40 pmol of GST bound to glutathione-agarose beads, for 2 h while rocking at 4 °C. Labeled proteins were eluted from the beads with Laemmli sample buffer, followed by ligation into pGEX4T1 at a ligation efficiency of ~100 different clones per pool, as described (20). LKB1 and SL26 were expressed in insect cells using GSH-agarose beads. The proteins were eluted from the beads with Laemmli sample buffer, digested with EcoRI and SmaI, and purified from insect cells using GSH-agarose beads.

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† The abbreviations used are: PJS, Peutz-Jeghers syndrome; GST, glutathione S-transferase; PAGE, polyacylamide gel electrophoresis; Rb, retinoblastoma.

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beads with GSH, concentrated, and then diluted to remove the GSH. Kinase assays were conducted at 30°C for 30 min in kinase buffer (20 mM Hapes, pH 7.5, 5 mM MgCl₂, 0.4 mM MnCl₂, 1 mM dithiothreitol, 75 μg/ml bovine serum albumin, 20 mM ATP, and 10 μCi of [γ-³²P]ATP). Samples were separated by SDS-PAGE, and radioactivity was visualized by autoradiography.

**FIG. 1.** LKB1 binds to Brg1. A, pools from a unidirectional HeLa cell-derived cDNA library were transcribed and translated in vitro in the presence of [³⁵S]methionine. Labeled proteins were incubated with GST-LKB1 (10 pmol) or GST (10 pmol) and washed, and bound proteins were then separated by SDS-PAGE and visualized using a molecular imager. The arrow indicates the 35-kDa protein that associates specifically with GST-LKB1, following isolation of the clone. ¹⁴C-MW, ¹⁴C-labeled molecular mass markers. B, LKB1 was immunoprecipitated from Saos-2 cells using LKB1 antibody (α-LKB1), and the immunoprecipitates were Western blotted for LKB1 and Brg1. Pre-immune serum was used as a control (PIS), and total cell lysates (TCL) were also blotted for LKB1 and Brg1.

**FIG. 2.** The amino terminus of LKB1 binds to the helicase domain of Brg1. A, [³⁵S]-Labeled Brg1HR, Brg1H, or Brg1ΔH was incubated with GST (10 pmol), GST-LKB1 (10 pmol), or GST-SL26 (10 pmol) bound to GSH beads. The beads were washed, and the associated proteins were separated by SDS-PAGE and visualized by autoradiography. To estimate the efficiency of binding, 10% of the [³⁵S]Brg1HR incubated with each fusion protein was also loaded onto the gel. B, [³⁵S]-labeled Brg1HR was incubated with GST-fusion proteins of LKB1 (10 pmol) or truncations, and association was detected as described above. To estimate the efficiency of binding, 10% of the [³⁵S]Brg1HR incubated with each fusion protein was also loaded onto the gel.

**FIG. 3.** Brg1 binds mutant SL26. A, Saos-2 cells were transiently transfected with vector alone (−), LKB1, or SL26. Cells were harvested after 48 h, immunoprecipitated with HA antibody, and Western blotted for Brg1. Total cell lysates (TCL) were also Western blotted for protein expression. B, autophosphorylation of recombinant GST alone, LKB1, or SL26 expressed in Sf9 insect cells were assayed as described (19).

**FIG. 4.** LKB1 and SL26 stimulate the ATPase activity of Brg1. A, Brg1 ATPase activity was assayed in the presence and absence of DNA and following the addition of GST-LKB1, GST-SL26, or GST proteins as described (20). The increase in activity because of LKB1 or SL26 was statistically significant (*, \(p < 0.0001\), and **, \(p < 0.005\), respectively, using Student's t test). B, Brg1 ATPase activity was assayed in the presence of GST-ΔC-LKB1.

ATPase Assays—ATPase assays were carried out as described using Flag-Brg1 purified from insect cells (20). The release of ³²PO₄ from [γ-³²P]ATP was determined using purified recombinant Flag-Brg1 (25 pmol) in the absence and presence of supercoiled DNA (pBluescript; 100 ng), purified recombinant GST-LKB1 (2.5 pmol), GST-SL26 (2.5 pmol), GST-ΔC-LKB1 (2.5 pmol), or GST (2.5 pmol).

Flat Cell Assay—2 × 10⁵ SW13, from primary small cell lung carcinoma from an adrenal metastasis (ATCC number CCL-105), were transiently transfected with Brg1, LKB1, SL26, pBJ5 vector alone, or Brg1 + LKB1 or SL26, pcDNA3.1 containing a neomycin resistance gene was included at a ratio of 5:1 for selection in neomycin. Beginning 48 h after transfection, cells were treated with neomycin (200 μg/ml) for an additional 14 days. Cells were washed three times in phosphate-buffered saline, fixed in 3% paraformaldehyde, washed, and then stained with 2% crystal violet. For each condition, the number of flat cells in 40 randomly selected fields were counted.

**RESULTS AND DISCUSSION**

To identify proteins that interact with LKB1, we adapted an in vitro expression cloning strategy (19) in which a HeLa cell cDNA library was divided into 680 pools of ~100 cDNAs per
TABLE I
Effect of LKB1 on Brg1-dependent flat cell formation

| Plasmids transfected | Number of flat cells/experiment | Mean flat cells | p Values |
|----------------------|---------------------------------|----------------|----------|
|                      | 1 | 2 | 3 | 4 | 5 | 6 | 7 | %   |
| Brg1                 | 194 (100) | 61 (100) | 193 (100) | 48 (100) | 53 (100) | 61 (100) | 84 (100) | 100 |
| Brg1 + LKB1          | 104 (54) | 63 (103) | 81 (42) | 28 (58) | 49 (92) | 81 (132) | 57 (68) | 78 ± 12 |
| Brg1 + SL26          | 61 (31) | 26 (43) | 58 (30) | 16 (33) | 15 (28) | 49 (80) | 21 (25) | 38 ± 7 <0.001* |
| LKB1                 | 41 (21) | 18 (29) | 85 (44) | 9 (18) | 9 (17) | 15 (24) | 10 (12) | 24 ± 4 <0.0001* |
| SL26                 | 38 (19) | 17 (28) | 48 (24) | 9 (18) | 5 (9) | 20 (32) | 19 (22) | 22 ± 3 <0.0001* |
| pBJ5                 | 11 (6) | 7 (14) | 10 (12) | 11 ± 2 <0.001* |

* Mean of seven experiments ± S.E.  
  * p value compared to Brg1 alone.  
  * p value compared to pBJ5 alone.

Western blotted for Brg1. The SL26 mutant associated with Brg1, as well as wild type LKB1, indicating that the lack of association of LKB1 with Brg1 is not responsible for PJS (Fig. 3A). We also determined whether the kinase activity of LKB1 is necessary for association with Brg1. The SL26 mutant has been reported to lack kinase activity; however this finding is controversial (15, 16, 18), so we assayed the activity of the SL26 mutant. We did not detect autophosphorylation in either immunoprecipitates from transiently transfected cells (data not shown) or from recombinant GST-SL26 produced in insect cells (Fig. 3B). Furthermore, K252a, a kinase inhibitor that blocks autophosphorylation of LKB1, had no effect on the association of [35S]Brg1HR made in reticulocyte lysate with LKB1 produced in insect cells (data not shown). Thus, the kinase activity of LKB1 is not necessary for association with Brg1.

Because the activity of Brg1 is regulated by phosphorylation (23, 40) we used Brg1 complexes purified from mammalian cells or recombinant Brg1 expressed in insect cells as substrates for LKB1. We detected no increase in phosphorylation above background, with the exception of LKB1 autophosphorylation, in samples to which LKB1 was added (data not shown). These results indicate that the kinase activity of LKB1 is not likely to regulate Brg1-containing SWI/SNF complexes directly.

Because LKB1 did not phosphorylate Brg1, we investigated whether its association with LKB1 might regulate Brg1 by measuring the effect of LKB1 and SL26 on the ATPase activity of recombinant Brg1. DNA alone stimulated Brg1 ATPase activity 3-fold, as expected (Fig. 4A). In the absence of DNA, LKB1 or SL26 also stimulated Brg1 ATPase activity 3-fold. In the presence of both DNA and LKB1 or SL26, Brg1 ATPase activity was stimulated 6-fold, compared with Brg1 alone (Fig. 4A). LKB1, SL26, or GST alone did not hydrolyze ATP in the absence of Brg1 (data not shown). These results indicate that LKB1 binding stimulates Brg1 function. We also tested whether the GST fusion protein containing only the N terminus of LKB1 activated the ATPase activity of Brg1 (Fig. 4B). Even though this protein binds to Brg1, it did not enhance Brg1-ATPase activity, indicating that the C terminus of LKB1 is required to stimulate the ATPase activity of Brg1.

Both LKB1 and the SL26 mutant stimulated the ATPase activity of Brg1, raising the question of whether loss of this function could account for PJS. It is not known whether mutant LKB1 proteins are expressed in patients with PJS; if the mutant proteins are not stable, lack of binding to Brg1 could explain the manifestations of PJS. However, the SL26 mutant is stable when ectopically expressed and therefore may be present in the cells of patients with PJS. Furthermore, several mutations in LKB1 found in patients with PJS are point mu-
What are the key mechanisms by which LKB1 regulates Brg1 function?

- LKB1 binds to Brg1 and stimulates its ATPase activity, suggesting a role in cell cycle regulation.
- Brg1 heterozygous mice develop epithelial tumors, indicating a potential role in carcinogenesis.
- The lack of LKB1 kinase activity contributes to or causes PJS.

How does LKB1 affect cell cycle progression?

- LKB1's kinase activity is required for Rb-induced cell cycle arrest in both G1 and S phases.
- LKB1 or SL26 alone induce a 2-fold increase in flat cell formation compared with expression of vector alone.
- Co-expression of SL26 and Brg1 reduces the number of flat cells, indicating a dominant-negative effect.
- SW13 cells, which do not express LKB1 or SL26, show a 2-fold increase in flat cell formation when co-transfected with Brg1 and LKB1 or SL26. This suggests an endogenous LKB1 function in these cells.

What is the significance of LKB1 in PJS and cancer?

- LKB1 mutations are associated with a subset of PJS cases, indicating a role in the predisposition to cancer.
- LKB1 appears to have both kinase-dependent and -independent functions, affecting cell cycle progression and tumor suppression.

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