LKB1 Catalytically Deficient Mutants Enhance Cyclin D1 Expression

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Abstract

Mutations in the serine-threonine tumor-suppressor kinase LKB1 are responsible for Peutz-Jeghers syndrome, characterized by hamartomatous proliferation and an increased risk of developing cancer. Mutations in lkb1 have also been identified in sporadic cancers, suggesting a wider role for LKB1 in cancer that is not limited to hamartomatous polyposis syndromes. Here, we show that LKB1 catalytically deficient mutants, when introduced into DLD1p21−/−p53−/− colorectal cancer cells, allowed for progression of cells through S phase of cell cycle and elicited the expression of Rb, cyclin E, and cyclin A2 whereas the introduction of LKB1 lead to G1 cell cycle arrest independent of p21WAF/CIP1 and/or p53 expression. Furthermore, we show that LKB1 catalytically deficient mutants activate the expression of cyclin D1 through recruitment to response elements within the promoter of the oncogene. In addition to compromising the tumor-suppressor function of LKB1, our findings highlight an emerging role for LKB1 catalytically deficient mutants, a gain of oncogenic properties. [Cancer Res 2007;67(12):5622–7]

Introduction

Mutations in the lkb1 gene are found in Peutz-Jeghers syndrome with loss of heterozygosity or somatic mutations at the lkb1 locus (1). Independent of Peutz-Jeghers syndrome, sporadic mutations in the lkb1 gene have been identified in breast, pancreatic, lung, prostate, and ovarian cancers (2, 3). As a multitasking tumor-suppressor kinase (4), LKB1 is involved in signaling pathways both nuclear and cytoplasmic; however, catalytically deficient mutants of LKB1 localize exclusively to the nucleus with no known mechanism for relocalization to the cytoplasm (5–7). The discovery that LKB1 catalytic activity is enhanced when bound to STRAD and MO25 proteins led to the discovery that LKB1 is the upstream kinase to AMP-activated protein kinase (AMPK) and AMPK family members and is linked to mammalian target of rapamycin signaling through the AMPK-TSC1/TSC2 cascade (3). For details regarding LKB1-AMPK signaling, readers are referred to the review by Alessi et al. (8).

LKB1 suppresses the growth of tumors by promoting Brg1-, p21WAF/CIP1 (herein called p21)−, and p53-mediated growth arrest (5–7). We previously showed that LKB1 binds to and regulates the helicase activity of the chromatin remodeling protein Brg1 enhancing Brg1-mediated cell growth arrest (6). Tiainen et al. (5) provide evidence for LKB1-mediated G1 cell cycle arrest by inducing the expression of p21 in a p53-dependent manner (7). More recently, LKB1-mediated activation of p21 was shown to result from p53-dependent association of LKB1 at the p21 promoter (9). LKB1-mediated up-regulation of p21 has also been observed in HeLaS3 cells (10) in which p53 is inactivated (11), supporting a p53-independent mode of p21 activation (10).

Here, we investigate the role of LKB1 catalytically deficient mutants (herein called LKB1 mutants) in regulating the expression of cyclin D1 in DLD1p21−/−p53−/− cells. Overexpression of the oncogene cyclin D1, CCND1, is one of the most common gene alterations in cancer (12). The primary function of cyclin D1 (herein called CD1) is to facilitate progression of cells from quiescence through to a proliferative state (12). We provide evidence that expression of the LKB1 catalytically deficient mutant D194A enhances the expression of proteins necessary for the transition of cells through the cell cycle, namely pRB, cyclin E, and cyclin A2. We show that LKB1 mediates G1 arrest independent of p21 and/or p53 expression. We show that LKB1 mutants are capable of enhancing CD1 expression through transactivation of the CD1 promoter. Finally, we discovered that LKB1 mutants are recruited to elements within the CD1 promoter, thereby providing a plausible explanation for LKB1 mutant–mediated enhanced expression of CD1. Our discovery provides a mechanism by which the loss of LKB1 tumor-suppressor function leads to cellular proliferation due to activation of CD1 by mutant LKB1.

Materials and Methods

Cell culture and transfection. The adenocarcinoma cell lines DLD1 p21−/−p53−/− and its isogenic counterpart DLDp21−/−p53−/− were provided by Dr. Vogelstein (Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University, Baltimore, MD). Cells were transfected with indicated plasmids using LipofectAMINE (Life Technologies). DEMEM and fetal bovine serum were from Life Technologies. Cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO2.

Cell lysis and Western blotting. Asynchronous cells were lysed as described previously (6). Protein concentration was determined by Bradford analysis. Proteins were resolved by SDS-PAGE then transferred to Immobilon-P membrane (Millipore). Membranes were blocked with 3% (w/v) powdered milk. Primary antibodies recognizing M2 (1:5,000; Sigma), LKB1 (Ley37D/G6; 1:500), Rb (1:1,000), p53 (1:1,000), CD1 (1:500), p21 (1:200), CDC2 (1:400), myc (1:500), GST (1:500), actin (1:5,000), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:5,000; Santa Cruz Biotechnologies) were used at the dilutions indicated in brackets. Secondary antibodies were horseradish peroxidase–conjugated anti-goat, anti-mouse, and anti-rabbit (Bio-Rad), used at 1:5,000 dilution. Protein expression was visualized by chemiluminescence (Pierce).

LKBtide assay. Human embryonic kidney (HEK293) cells were transfected with expression plasmids LKB1pEBG-2T, D194ApEBG-2T, or SL26pEBG-2T with Strado-FLAG-CMV and MO25-5xMYC-CMV (gifts from Drs. Clevers (Hubrecht Institute, Utrecht, Netherlands) and Alessi (Medical Research Council Protein Phosphorylation Unit, University of Dundee, Dundee, Scotland)) accordingly (13). Briefly, cells were harvested 36 h after transfection in Harvest buffer composed of 50 mmol/L...
Tris-HCl (pH 7.5), 0.27 mol/L sucrose, 0.1 mmol/L EGTA, and 0.1% (v/v) 2-mercaptoethanol; lysates were incubated with 30 μL of equilibrated glutathione-Sepharose slurry for 45 min at 4°C. GST-LKB1/Flag-STRAD/myc-MO complex was prepared as previously described (13). Purified complex was eluted from reduced glutathione (GSH) beads by incubating resin in 2 volume excess of Harvest buffer containing 30 mmol/L glutathione followed by filtration through a 0.44 μm filter (Spin-X, Costar). Elution was repeated twice; pooled eluents were divided into aliquots, snap frozen, and stored at −80°C. Phosphotransferase activity of LKB1 toward the LKBtide peptide (150 μmol/L; Upstate) was determined using 0.5 μg of protein complex and 0.1 mmol/L [γ-32P]ATP at 30°C for 15 min (13) and terminated by applying 40 μL of reaction mixture to P81 membranes and washed thrice with 3 mL of 1% cold phosphoric acid using a 12-well Millipore vacuum manifold. P81 membranes were dried at room temperature followed by measurement of incorporated radioactivity using a Beckman LS6000IC scintillation counter.

**Real-time PCR.** Real-time PCR was conducted according to the manufacturer's protocol (Stratagene). Primer sequences used for PCR can be found in Table 1. Data were analyzed by ΔΔCt normalized to GAPDH.

**Flow cytometry.** Cells were transfected with expression plasmids encoding p21WAF1/CIP1, CMV6 and p53-CMV and GFP-tagged LKB1 and mutants. Asynchronous cells were harvested and prepared for flow cytometry or were treated with 0.4 mmol/L mimosine 16 h posttransfection and harvested 20 h after treatment. Cells were fixed in ethanol and then stained (10 μg/mL propidium iodide/ Ribose A; Sigma). Cell cycle distribution was determined by flow cytometry using a Becton Dickinson FACSCalibur. Data were acquired using CellQuest software and analyzed using ModFitLT.

**Reporter assays.** DLD1−/− cells were transfected with expression plasmids encoding LKB1, D194A, SL26 (100 or 200 ng), pRl-rlk (0.5 ng), and CD1 (−1745)–luc (100 ng) reporter (gift from Dr. Pestell, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA). Transcriptional activity was measured 24 h after transfection using the Dual-Luciferase Reporter Kit (Promega). Analysis was conducted using a Berthold AutoLumat Plus LB953 Luminesimeter.

**Chromatin immunoprecipitation assay.** DLD1−/− cells were used for chromatin immunoprecipitation assay (ChIP) analysis according to the manufacturer's protocol (Upstate Biotechnology). Precipitated DNA was analyzed by PCR. Primers directed toward human CD1 promoter elements can be found in Table 1.

**Results and Discussion.**

Mutations in the tumor suppressor *lkbi* leads to loss of LKB1 catalytic activity (herein called LKB1 activity) observed in Peutz-Jeghers syndrome (1) and some sporadic cancers (2, 3). A shift in the balance between the loss of tumor-suppressor function and the gain of oncogene activity is a reoccurring theme in malignancies. We investigated the role of LKB1 catalytically deficient mutants on expression of the prominent oncogene cyclin D1.

The catalytic activities of LKB1 mutants SL26 and SL8 have previously been described (5–7, 13); however, the catalytic profile of D194A, identified in malignant melanomas (14) and sporadic lung cancers (2), has not. We conducted LKBtide assays (13) using purified recombinant GST-LKB1, GST-D194A, or GST-SL26 in complex with STRADha/M0525a. Compared with LKB1, D194A was as catalytically deficient toward the LKBtide peptide as mutant SL26 (Fig. 1A). Based on these results, engineered D194A mutant was deficient for kinase activity. To determine whether the LKB1 mutants were deficient for G1 arrest, we conducted cell cycle analysis on HeLaS3 cells expressing ectopic LKB1 or mutants followed by treatment with mimosine to inhibit cell cycle in late G1 phase before the onset of DNA synthesis. We found that ectopic LKB1 lead to the G1 arrest of 45% of asynchronous cells compared with 22% to 27% of the cells expressing LKB1 mutants (Fig. 1B).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>LKB1</td>
<td>(ID 4507271a1)†</td>
</tr>
<tr>
<td>GAPDH</td>
<td>(ID 7669492a2)†</td>
</tr>
<tr>
<td>Bb</td>
<td>(ID 4506435a3)†</td>
</tr>
<tr>
<td>Actin</td>
<td>Fp: GTCCTCCTGAGCCAAAGTA Rp: TCACCTCCTGCTTGTGGACT</td>
</tr>
<tr>
<td>CyclinE</td>
<td>Fp: GGAGAGAGGGAAACCG    Rp: GCATAATCGAGGCTT</td>
</tr>
<tr>
<td>CyclinA2</td>
<td>Fp: TTCATTTGACACTCTACAGCAGCG Rp: TTGAGTGTGACTGGAGGTC</td>
</tr>
<tr>
<td>p21WAF1/CIP1</td>
<td>Fp: TGGAGACTCTGAGGTCGAAA Rp: GCGTTTGAGTGGAGAAATC</td>
</tr>
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Treatment with mimosine increased LKB1-mediated G1 arrest to 67% compared with 42% and 43 to 47% of the cells expressing vector or mutants, respectively (Fig. 1B). These results confirm that D194A, like SL26 and SL8 and unlike LKB1, was not able to arrest cells in G1.

To identify the molecular mechanism by which LKB1 mutants mediate cellular proliferation in the absence of functional p21 and p53, we used the well-characterized DLD1p21+/+/p53−/− cell line and its isogenic counterpart DLD1p21−/−/p53−/− (15). We determined by PCR and Western blot analysis that asynchronous DLD1 and DLD1−/− cells express lkbi (Fig. 1C) and that expression of Rb and the G1 S transition cyclin E was 3-fold greater in DLD1−/− cells expressing D194A compared with vector control or DLD1 cells (Fig. 1D). The expression of cyclin A2, required for S phase transition from G1 (16), was reduced in DLD cells expressing LKB1 compared with D194A and vector control cells. Together, these results indicate that D194A enhances the expression of some genes that are necessary for cell cycle progression.

Because both p21 and p53 are implicated in LKB1-mediated G1 arrest (7, 10), we investigated the cell cycle profile of DLD1−/−. Briefly, cells were transfected with expression plasmids encoding LKB1 wild-type or mutants, p21, p53, or a combination of these plasmids. Asynchronous cells were harvested 24 h after transfection and prepared for flow cytometry. The introduction of LKB1, p21, or p53 into DLD1−/− cells resulted in 33%, 41%, and 45% of cells in G1, respectively (Fig. 2). The introduction of p21 or p53 when combined with LKB1 lead to 48% and 44% of the cells in G1, respectively, compared with LKB1 mutants that did not alter the cell cycle profile from that of vector control (Fig. 2). DLD1−/− transfected with a combination of LKB1, p21, and p53 lead to 56%...
of the cells in G1 compared with no observable difference in the cell cycle profiles from cells expressing LKB1 mutants combined with p21 and p53 or mutants alone (Fig. 2). These data suggest that in the absence of p21 or p53, LKB1 is able to arrest cells in G1 (Fig. 2), whereas the combination of LKB1, p21, and p53 modestly improved LKB1-mediated G1 arrest (Fig. 2). From these data, LKB1 arrests cells in G1 phase of the cell cycle independent of either p21 or p53. Furthermore, LKB1 activity is required for LKB1-mediated cell cycle arrest because LKB1 mutants did not arrest cells in the absence and/or presence of functional p21 or p53.

Figure 1. LKB1 expression in DLD1 and DLD1^+/^- cells. A, LKBtide assays were conducted to assess the catalytic activity of D194A toward LKBtide peptide. Expression of purified GST-LKB1, GST-D194A, or GST-SL26 plus Flag-STRADα/myc-MO25 was determined by Western blot analysis using the appropriate antibodies. Representative of three to four experiments in duplicate. Arrow, phosphorylated LKB1 (top band); PD, GSH pull down. B, LKB1-mediated G1 arrest was confirmed in HeLaS3 cells transfected with HA-LKB1pCMV, HA-D194A-pCMV, HA-SL26-pCMV, HA-SL8-pCMV, or vector for 16 h, left untreated, or treated with mimosine for an additional 20 h. Cell cycle distribution was determined by flow cytometry. C, endogenous expression of LKB1 was determined by reverse transcription-PCR (GAPDH control) and by Western blot analysis (actin control). D, quantitative PCR was used to determine the effect of Flag-LKB1 and Flag-D194A on gene expression of the indicated transcripts. Data were analyzed by the ΔΔCt method normalized to GAPDH and are expressed as fold change from control, set at a value of 1.0. Expression of Flag-LKB1 and Flag-D194A was determined by Western blot analysis. All data are representative of three to four experiments.
Because CD1 expression is elevated in adenocarcinomas and adenomatous polyps of the colon in Peutz-Jeghers syndrome–derived hamartomas (17) and Peutz-Jeghers syndrome–derived carcinomas (18), we assessed whether LKB1 mutants altered the expression of CD1 in DLD1−/− cells where p21-mediated regulation of CD1 is absent. To this end, we conducted transactivation reporter assays using a CD1-luciferase reporter (CD1-luc), −1,748 bp from the transcriptional start within the CD1 promoter (19). Briefly, DLD1−/− cells were transfected with expression plasmids containing LKB1, mutants, CD1-luc, and pRL-tk. Cells were prepared for reporter assays 24 h after transfection. LKB1 mutants significantly transactivated the CD1-luc promoter compared with LKB1 and vector (P < 0.01 and P < 0.005, respectively; Fig. 3A). LKB1 transactivation of CD1-luc was not significantly different from CD1-luc alone (Fig. 3A). Furthermore, we found that protein expression of CD1 in DLD1−/− was elevated in cells expressing LKB1 mutants compared with LKB1 alone (Fig. 3B). Expression of pRB and cdk2 were also elevated in cells transfected with mutants compared with LKB1 alone (Fig. 3B). We did not observe coimmunoprecipitation of CD1 with LKB1 or mutants; we also did not observe phosphorylation of CD1 by LKB1 (data not shown).

Overall, these data suggest that LKB1 mutants are capable of transactivating the CD1 promoter and increasing the expression of the oncogene.

Given that LKB1 mutants transactivate a synthetic CD1 promoter (Fig. 3A), we considered the possibility that transactivation occurs through recruitment of LKB1 mutants to the CD1 promoter. Briefly, endogenous LKB1 was immunoprecipitated from formaldehyde cross-linked DLD1−/− cells followed by the preparation of chromatin for ChIP analysis and PCR using primers designed for the cyclic AMP-responsive element (CRE)/Ets, AP1, SP1, and E2F elements within the CD1 promoter (Table 1). Endogenous LKB1 was recruited to the CD1 promoter at AP1, SP1, and CRE binding protein/Ets, whereas recruitment of LKB1 did not occur at E2F or at control region (Fig. 3C). To determine whether mutants were recruited to the CD1 promoter, DLD1−/− cells were transfected with LKB1, D194A, or SL26 expression plasmids, followed by ChIP analysis. Specifically, D194A was recruited to AP1, CRE/Ets, and SP1 elements, whereas SL26 was recruited to CRE/Ets and SP1 elements (Fig. 3C). Overall, recruitment of LKB1 mutants to promoter elements was consistently greater than recruitment of LKB1. Neither ectopic LKB1 nor mutants were recruited to the E2F element or the control region (data not shown). Introduction of p21, p53, or p21/p53 into DLD1−/− cells in combination with LKB1 or mutants did not improve recruitment of mutants or LKB1 to the CD1 promoter (data not shown), confirming that recruitment of LKB1 mutants to CD1 promoter elements is independent of p21 or p53. These data do not rule out...
the possibility of direct recruitment of LKB1 mutants to the CD1 promoter or indirect recruitment by tethering to proteins that in turn are recruited to the CD1 promoter.

Based on our findings, we postulate that in cancers where LKB1 is mutated, and therefore catalytically deficient, these mutants are recruited to the CD1 promoter (Fig. 3C), where they transactivate the oncogene (Fig. 3A and B) and enhance the expression of Rb and cyclins required for progression of cells through S and G2-M phases of cell cycle (Figs. 1D and 3A and B). Although it is not clear how LKB1 catalytically deficient mutants exert oncogenic activity, our findings are the first to identify a novel function for LKB1 mutants other than established loss of catalytic activity. Interestingly, p53 mutants that confer oncogenic status have been described as "gain of function" mutations (20). Because DLD1 cells express endogenous LKB1, LKB1 mutants may squelch the tumor-suppressor function of wild-type LKB1 thereby serving as dominant-negative proteins, a scenario similar to that described for p53 mutants (20). Because SL26 (6) and D194A (data not published) bind directly to Br1 and are involved in regulating Br1-mediated chromatin remodeling (6), it is not unreasonable to find LKB1 mutants influencing transcriptional events. Because LKB1 mutants localize exclusively to the nucleus, with no known mechanism for leaving this compartment, recruitment to the promoter of oncogenes, whether directly or indirectly, and the resulting transactivation may in essence be but one of many functions for LKB1 catalytic mutants. Our discovery described herein is significant in that it provides a plausible mechanism by which LKB1 catalytically deficient mutants contribute to the formation of malignancies when LKB1 tumor-suppressor function is lost.

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References

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