Liver Kinase B1 Expression Promotes Phosphatase Activity and Abrogation of Receptor Tyrosine Kinase Phosphorylation in Human Cancer Cells

Background: Frequent loss of liver kinase B1 (LKB1) has been reported in human neoplasm. LKB1 depletion correlated with enhanced RTK phosphorylation in human lung cancer cell line. Ectopic LKB1 expression in deficient lung and cervical cancer cell lines abrogated a repertoire of phospho-RTKs associated with tumor development and progression.

Conclusion: LKB1 promotes protein tyrosine phosphatase activity and inhibition of RTK-activated pathways.

Significance: LKB1 protects against RTK-driven tumors.

Aberrant receptor tyrosine kinase phosphorylation (pRTK) has been associated with diverse pathological conditions, including human neoplasms. In lung cancer, frequent liver kinase B1 (LKB1) mutations correlate with tumor progression, but potential links with pRTK remain unknown. Heightened and sustained receptor activation was demonstrated by LKB1-deficient A549 (lung) and HeLaS3 (cervical) cancer cell lines. Depletion (siRNA) of endogenous LKB1 expression in H1792 lung cancer cells also correlated with increased pRTK. However, ectopic LKB1 expression in A549 and HeLaS3 cell lines, as well as H1975 activating-EGF receptor mutant lung cancer cell resulted in dephosphorylation of several tumor-enhancing RTKs, including EGF receptor, ErbB2, hepatocyte growth factor receptor (c-Met), EphA2, rearranged during transfection (RET), and insulin-like growth factor I receptor. Receptor abrogation correlated with attenuation of phospho-Akt and increased apoptosis. Global phosphatase inhibition by orthovanadate or depletion of protein tyrosine phosphatases (PTPs) resulted in the recovery of receptor phosphorylation. Specifically, the activity of SHP-2, PTP-1B, and PTP-PEST was enhanced by LKB1-expressing cells. Our findings provide novel insight on how LKB1 loss of expression or function promotes aberrant RTK signaling and rapid growth of cancer cells.

Although receptor tyrosine kinases (RTKs) belong to different families and/or sub-families, their activation as measured by phosphorylation of intracellular tyrosine residues is typically achieved through binding with respective growth factors. Receptor dimerization, auto-phosphorylation, and subsequent recruitment of adaptor molecules with varying anchoring domains (e.g. Shc, Grb2, and Src) result in the activation of specific downstream pathways. Aberrant RTK signaling has been implicated as mediators of several pathological conditions, including human neoplasms. Hence, modulation of dysregulated receptor activation may provide interventions against RTK-driven tumors and related disorders.

Among several RTKs linked with cancer, the epidermal growth factor receptor (EGFR) represents a well studied gene and perhaps one of the first receptors strongly associated with human malignancies (1, 2). In addition to intrinsic tyrosine kinase activity of the receptor, phosphatases also play a role in regulating the phosphorylation status of EGFR. More specifically, protein tyrosine phosphatases (PTPs) are described as a dynamic and reversible process required for regulated control of tyrosine phosphorylation (3, 4). EGFR has more than five major tyrosine phosphorylation sites and has been linked to various PTPs such as PTP1B and SHP (4). Therefore, the balance between kinases and phosphatases play an important role in regulating receptor activation.

Liver kinase B1 (LKB1) is another gene that is frequently associated with human neoplasms, including lung, breast, endometrial, and cervical cancers (5–9). For example, LKB1 mutations are thought to be present in about one-third of lung cancer cases and potentially contribute to the development or progression of the disease (10–12). The gene was initially identified in patients with Peutz-Jeghers syndrome, which is associated with gastrointestinal hamartomatous polyps and increased risk of malignant growths (8, 13, 14). LKB1 displays multiple functions, mainly via associations with several targets (13, 15–18). For example, complex formation with STRAD (STE20-related adaptor) and MO25 (mouse protein-25) promotes LKB1 activity, nuclear export, and cytokine cell distribution (19, 20). To date, LKB1 is reported to regulate 13 kinases, many of which lack clearly defined functions (21). One of the widely

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studied LKB1 substrate, AMP-activated protein kinase (AMPK) is regarded as a metabolic master switch (6, 22). Furthermore, the association of LKB1 with critical biological processes such as p53-mediated cell death support important roles in cell fate determination (7, 15, 17, 23). Together, these functions highlight why LKB1 alterations can severely impact malignant transformations.

To assess potential involvement of LKB1 on receptor-mediated signaling in human tumors, we analyzed the phosphorylation profiles of RTKs in four human cancer cell lines, namely A549, H1792, H1975, and HeLaS3. We further measured the expression and activity of PTPs in the presence or absence of LKB1. Here, we report that LKB1 promotes the activity of PTPs, instigating rapid abrogation of a repertoire of phospho-RTKs in cancer cells.

EXPERIMENTAL PROCEDURES

Cell Lines—A549, H1792, and H1975 (lung) and HeLaS3 (cervical) cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA) and maintained at 37 °C in a humidified atmosphere of 5% CO2 in an open-air incubator with full medium consisting of DMEM or RPMI 1640 (Sigma) supplemented with 10% (v/v) heat-inactivated FBS, 2 mM glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin.

Cell Transfection—Wild-type or mutant LKB1 constructs (D194A and S428A) were generated as described previously (24). D194A is a kinase-dead LKB1 mutant, whereas S428A possesses a point mutation in the C-terminal phosphorylation domain. Plasmids encoding EGFP-fused SL-26 were kindly provided by Lily Dong (University of Texas Health Sciences Center, Madison, WI) or R&D Systems (Minneapolis, MN) respectively. For growth factor stimulation, cells were serum-starved overnight prior to stimulation at the indicated time intervals. For apoptosis, cells were fixed in 1% paraformaldehyde and Rh-EGF staining was detected by flow cytometry. For EGFR receptor recycling, monolayers of serum-starved LacZ- or LKB1-transfected cells were placed on ice for 10 min. Addition of Rh-EGF was added at indicated concentrations for 30 min. The reactions were stopped by removal of Rh-EGF and washing with cold PBS (2×). Cells were fixed with 1% paraformaldehyde, and Rh-EGF staining was detected by flow cytometry (LSRII analyzer).

Confocal Microscopy—Cells were fixed in 4% paraformaldehyde followed by permeabilization with either 0.1% Triton X-100 or 0.2% saponin. Immunofluorescence experiments were performed using the following antibodies: Alexa Fluor 555 or Alexa Fluor 647 conjugated anti-mouse IgG for LKB1 (1:100), Alexa Fluor 555 conjugated anti-rabbit IgG for EGFR or EGF (1:100), Alexa Fluor 488 conjugated anti-mouse IgG for pY1068-EGFR (1:100). All secondary Alexa Fluor® antibodies (Invitrogen) were used at a 1:400 dilution. Cells were mounted using SureMount® with DAPI. Imaging was carried out using a Zeiss LSM 510/710 confocal microscope with a 63× objective. pY1068 fluorescent intensity was measured using the LSM Zeiss 510 imaging software profile function. Briefly, a selected region of interest (profile) was determined in the cytoplasmic area defined by light field images and the exclusion of DAPI. The average peak fluorescence intensities were computed within the area of the cell. A total of 7–10 cells were counted for each group. Laser intensity, detector gain, and pinhole remained constant during data acquisition.

Statistical Analysis—Statistical analysis was done using Student’s t test, one-way analysis of variance, or two-way analysis of variance. p values of <0.05 were considered significant. Significance for each figure is noted as follows: #, p < 0.05; *, p < 0.01; **, p < 0.001, and ***, p < 0.0001. All analysis was done using GraphPad Prism software.

RESULTS

LKB1 Abrogates RTK Activation in Human Cancer Cell Lines—Tyrosine kinase phosphorylation serves as readout for receptor activation and mediates trans-membrane signal trans-
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Production pathways within cells. For measurement of phospho-RTK profiles, we selected LKB1-deficient A549 lung cancer cells due to the frequent inactivation of LKB1 in the disease. Empty-vector (LacZ) or LKB1-transfected cells were serum-starved and stimulated with a mixture of exogenous growth factors prior to incubation on phospho-RTK array membranes. Under basal, non-stimulatory conditions, RTK phosphorylation was undetected in both LacZ- and LKB1-expressing cells, as expected. However, upon growth factor stimulation, LKB1-expressing cells showed decreased phosphorylation of multiple RTKs compared with LacZ cells. C, LKB1-mediated phospho-RTK attenuation was observed in HeLaS3, another LKB1-deficient cancer cell line. D, in H1975 lung cancer cells that possess activating EGFR mutations, LKB1 overexpression blocked RTK activation compared with LacZ cells. E and F, confirmation of pY1234-HGFR and pY594-EphA2 dephosphorylation by LKB1 following stimulation with specific growth factor for the receptors. Cells were stimulated with 50 ng/ml of HGF or ephrin A1, respectively for 5 min.

**TABLE 1** Comparison of RTK phosphorylation profiles in LKB1 versus LacZ-transfected A549 lung cancer cell line

| Receptor family | Receptor tyrosine kinases attenuated by LKB1 |
|-----------------|-------------------------------------------|
| EGFR            | HGF, ErbB2 (HER2)                         |
| HGF             | HGF (c-Met)                               |
| EphR            | Epha2, Epha6, Epha7                       |
| Insulin receptor| IGF-1R, Insulin R                         |
| VEGFR           | VEGF R                                    |
| RET             | c-RET                                     |
| FGFR            | FGF R                                     |
| ROR             | ROR2                                      |
| Axl             | Axl, Dtk                                  |
| MuSK            | MuSK                                      |
| PDGFR           | Fkt-3, MCSF-R                             |
| Tie             | Tie-1                                     |

LKB1 Modulates Receptor Activation in a Dose- and Time-dependent Manner—Based on well established functions and links to lung cancer, the EGFR was selected for further investigations. LKB1 depletion (siRNA) in H1792 cells upon EGF stimulation correlated with increased phosphorylation at canonical Tyr-1068 and Tyr-1173 residues (Fig. 2A). Phosphorylation status of major EGFR tyrosine residues described previously by Sordella et al. (25) were subsequently assessed in A549 cells (Fig. 2B). Following 5 min of EGF stimulation, LKB1-expressing cells exhibited decreased phosphorylation across of RTKs within cancer cells and demonstrate a global, but differential attenuation by LKB1.
major EGFR tyrosine residues as compared with LKB1-null cells (Fig. 2C). Levels of total EGFR protein remained unchanged under basal or EGF stimulation, excluding receptor degradation or transcriptional regulation. Subsequent time-dependent stimulation demonstrated phosphorylation at Tyr-1068, and global phosphotyrosine-containing proteins (pTyr-20) within 5 min, which peaked at 15 min and declined by 30 min (Fig. 2D). Although similar activation patterns were exhibited across these time intervals, a stronger level was evident in LKB1-null cells. Dose-dependent stimulation revealed marked
receptor activation with 50 ng/ml EGF in LKB1-deficient cells (Fig. 2E). Receptor activation in this cell model has been achieved with lower EGF concentrations (~10 to 25 ng/ml EGF; data not shown). The dephosphorylation of Tyr-1068 by LKB1-expressing cells was further confirmed by confocal microscopy (Fig. 2, F and G).

LKB1-mediated Receptor Attenuation Impacts Downstream RTK-activated Targets and Enhances Apoptosis—Impact of negative receptor attenuation was investigated by assessing apoptosis and apoptosis-related targets. The activation of AKT was undetected under basal conditions in empty-vector or LKB1-positive cells; however, upon growth factor stimulation, decreased AKT phosphorylation was evident in LKB1 cells compared with LacZ cells (Fig. 2H).

Furthermore, LKB1 cells exhibited increased cleaved caspase-3 expression, which was further amplified in response to staurosporine treatment (Fig. 2D). Enhanced apoptosis by LKB1 was confirmed by TUNEL assay following treatment with receptor tyrosine kinase inhibitor, RPI-1 (Fig. 2, I and J). Together, these data support a role for LKB1 in phospho-RTK attenuation, thereby leading to inhibition of RTK-activated pathways of cancer cells.

Total Receptor Protein Is Not Impacted by LKB1 Expression—Despite changes in phosphorylation status, total receptor levels remained unaffected as demonstrated by Western blot data (Fig. 2). To further exclude differences in receptor availability, total EGFR expression and localization in the presence or absence of LKB1 was investigated. Confocal microscopy and flow cytometry data revealed comparable EGFR expression in
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LKB1-null or positive cells (Fig. 3, A–C). Under basal conditions, EGFR was predominately expressed at or near the cell surface in both LacZ and LKB1 transfected cells. However, upon EGF stimulation, the receptor was internalized to a similar degree across LKB1-expressing or null cells (Fig. 3A).

EGFR Availability and Recycling Not Impacted by LKB1—We next assessed cell surface availability of the receptor under basal conditions via rhodamine-EGF (Rh-EGF) binding assay. Similar binding curves and biochemical parameters ($B_{\text{max}}$, 1700 and 1615; $K_d$, 0.0040 and 0.0045 nM for LacZ and LKB1, respectively) were observed, indicating that LKB1 has minimal effects on EGF receptor availability (Fig. 3D). Next, we examined whether LKB1 altered the recycling of EGFR, thereby attenuating receptor availability at the cell surface. Non-labeled exogenous EGF was initially applied to achieve internalization of the receptor. Subsequent Rh-EGF binding at the cell surface of LKB1-null or positive cells resulted in similar mean fluorescent intensities for each time point, with peak recycling occurring within 30–60 min (Figs. 3E). This indicates that the rate of EGFR recycling is unaffected by LKB1. Together, these data demonstrate that total receptor levels and availability remain unaffected in the presence of LKB1.

Full-length, Functional LKB1 Protein Is Critical to Phospho-RTK Attenuation—To define specific domain(s) within the LKB1 gene critical for receptor abrogation, we transfected A549 cells using wild-type LKB1 vector, in parallel with various LKB1 mutants. Our data suggest that a fully functional LKB1 protein is required for phospho-RTK abrogation, as LKB1 mutations that sequester LKB1 to the nucleus (SL-26), kinase-dead (D194A), or serine 428 phosphorylation (S428A) resulted in the recovery of EGFR activation (Fig. 4A).

Phosphatase Activity Is Involved with Rapid RTK Dephosphorylation Instigated by LKB1—Regulatory balance between kinase and phosphatase activity contributes to homeostasis of RTK activation (3, 4) and prompted investigation of phosphatase activity. Enhanced phosphatase activity was demonstrated by LKB1-expressing cells compared with null cells (Fig. 4B). As would be expected, treatment with pervanadate, a phosphatase inhibitor, resulted in the loss of phosphatase activity in the presence or absence of LKB1 (Fig. 4B). Biochemical analysis confirmed recovery of EGFR phosphorylation in LKB1-expressing cells following pervanadate treatment (Fig. 4C). Rescue of additional phospho-RTKs (HGFR and EphA2) upon pervanadate treatment was further demonstrated (Fig. 4, D and E). The data indicate that LKB1 expression confers significant enhancement of phosphatase activity, promoting rapid abrogation of RTK phosphorylation.

LKB1 Promotes SHP-2, PTP-1β, and PTP-PEST activity—To identify specific PTPs modulated by LKB1, we tested expression of cytosolic phosphotyrosine PTPs, namely SHP-1, SHP-2, PTP-1β, and PTP-PEST (3, 4). Expression of the selected PTPs was not affected by LKB1 (Fig. 5A). We next pulled down and
FIGURE 5. LKB1 promotes activity of specific PTPs. A, protein expression of intracellular PTPs involved in regulation of tyrosine phosphorylation was not affected by LKB1 in A549 cells. B, the activities of SHP-2, PTP-1β, and PTP-PEST were strongly up-regulated by LKB1 but not SHP-1. With the exception of SHP-1 activity, PTPs activity in LKB1 cells was significantly enhanced compared with LacZ cells; **, p < 0.001. C and D, knockdown of respective PTPs in LKB1-transfected A549 or HeLaS3 cells confirm differential recovery of phosphotyrosine residues. E, in A549 cells, LKB1-mediated phospho-RTK attenuation is further enhanced by AMPK. F, proposed mechanism of rapid phospho-RTK abrogation instigated by LKB1 via enhanced PTP activity. GF, growth factor.
measured activity of respective PTPs from LKB1-null or positive cells. Specific up-regulation of SHP-2, PTP-1β, and PTP-PEST activity (but not SHP-1) was evident in LKB1-cells (Fig. 5B). Finally, confirmation of PTP-mediated receptor attenuation was confirmed following depletion (siRNA) of specific PTPs in the presence of LKB1. In both A549 and HeLaS3 cell lines, variable recovery of phosphorytrosine residues was evident upon knockdown of specific PTPs (Fig. 5, C and D), and in agreement with distinct or combinatorial receptor modulation by PTPs (3, 4). Subsequent investigations evaluated whether the major LKB1 substrate, AMPK, played a role in phospho-RTK attenuation (22, 26). We silenced predominant major LKB1 substrate, AMPK, expressed a role in phospho-RTK attenuation (22, 26). We silenced predominant AMPK sub-units in LKB1-null or positive cells, and in the LKB1-deficient cells, AMPK expression failed to abrogate receptor tyrosine activation (Fig. 5E). Importantly, phospho-RTK inhibition mediated by LKB1 was enhanced in the presence of AMPK (Fig. 5E).

**DISCUSSION**

A plethora of cellular processes and downstream signal transduction pathways are modulated by RTKs. However, dysregulated RTK activation has been implicated in several pathological conditions, including human neoplasms. The regulatory mechanisms governing RTK activation are complex and include autocrine, paracrine, or juxtacrine events, ligand specificity, as well as duration or amplitude of stimulation (1, 27). In addition, signaling outcomes are variable and cell-type/context-dependent (1, 27). Here, we identify novel regulation of a repertoire of phospho-RTKs by the LKB1 tumor suppressor in lung and cervical cancer cell lines. The consequences of frequent LKB1 mutations and loss-of-function in human tumors have been widely described (5–9, 28). We report that the loss of LKB1 expression in cancer cells contributes to aberrant receptor activation, RTK-activated pathways and tumor growth (Fig. 5F). Proliferation profile of LKB1-null or positive cells in the presence or absence of growth factor stimulation was measured in real time using electrode-coated plates and was consistent with growth inhibition by LKB1 under all conditions (data not shown).

As readout for receptor activation, we assessed phosphorylation profiles of RTKs in the presence or absence of LKB1. Re-expression of LKB1 into the LKB1-deficient A549 cells demonstrated dramatic inhibition against several tumor-enhancing RTKs, including EGFR, ErbB2, HGF, and EphA2. The activation of additional RTKs such as, Axl, RET, insulin-like growth factor I receptor, ROR, and MusK was also blocked. Attenuation of selected RTKs was subsequently validated in A549, H1792, H1975 (lung adenocarcinoma), and HeLaS3 (cervical) cancer cell lines. Activating EGFR mutations significantly contribute to aberrant signaling by the receptor. However, enhanced LKB1 expression in a H1975 cell line that possesses a primary EGFR mutation (exon 21) was sufficient to block its activation. Importantly, phospho-RTK attenuation was further enhanced by AMPK, suggesting that AMPK may play a role in receptor modulation.

Mechanistic insight of RTK attenuation revealed that LKB1 expression correlated with increased PTP activity. The modulation of PTP is a dynamic and reversible process and subject to regulatory phosphorylation, which can result in the activation or inhibition of PTPs (3, 4, 29). It can also be regulated by reversible oxidation of cysteine in the active site of PTPs (30). Cellular oxidation states impacted by reactive oxygen or nitrogen species production result in the modulation of PTPs. Reactive oxygen or nitrogen species production can be influenced by several factors, including natural redox states or growth factor stimulation (29, 30). Interestingly, LKB1 has been described as a master kinase associated with the activation of 13 kinases of the AMPK subfamily (21). AMPK is at the cross-roads of two important processes, namely metabolic (bioenergetics) and redox regulation (31). Similar to the activation of LKB1 enzyme, AMPK responds to stress conditions such as serum deprivation or hypoxia (1, 26). AMPK-based redox signaling has been linked with redox-related processes, including autophagy and apoptosis (31). It is therefore conceivable that redox or metabolic alterations that frequently occur in cancer cells has impact upon reactive oxygen species or reactive nitrogen species production as well as AMPK activity (31–36). In turn, reactive oxygen or nitrogen species production play critical roles in PTP regulation, providing a link for phospho-RTK modulation by LKB1. The regulatory mechanisms of PTPs via AMPK and/or other kinases of the AMPK subfamily are a current area of research interests.

Aberrant expression of several RTKs such as EGFR, ErbB2, HGF, EphA2, and RET have been associated with tumor development, progression, or advanced disease, and often correlate with adverse prognosis (37–41). Persistent problems with drug resistance against tyrosine kinase inhibitors in addition to cross-activation of multiple RTKs by different growth factors represent a major challenge for effective cancer treatment. Therefore, inhibition of a repertoire of RTKs by a single gene, LKB1, may provide an attractive option for effective targeting of aberrant RTK activity and improve drug sensitivity in a subset of RTK-driven tumors. Our preliminary data has demonstrated modest sensitivity at low gefitinib concentrations (0.02, 0.05, and 0.1 μM) in the presence of LKB1 but requires further characterization (data not shown). Beyond cancer, these findings merit further investigation in other RTK-driven pathological conditions, especially the receptors identified in this study. For instance, muscle-specific tyrosine kinase has been implicated in neuromuscular junction defects through reciprocal tyrosine phosphorylation with Abl (42), whereas insulin-like growth factor I receptors and insulin receptor are intimately linked with diabetes (43–45). Finally, PTPs such as PTP-1β and SHP-2 have been associated with cancer, diabetes, restenosis, and cardiac hypertrophy (29, 30), providing potential therapeutic interventions by LKB1.

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