Preferential Dependence of Breast Cancer Cells versus Normal Cells on Integrin-Linked Kinase for Protein Kinase B/Akt Activation and Cell Survival

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Abstract

The emerging paradigm of “oncogene addiction” has been called an Achilles’ heel of cancer that can be exploited therapeutically. Here, we show that integrin-linked kinase (ILK), which is either activated or overexpressed in many types of cancers, is a critical regulator of breast cancer cell survival through the protein kinase B (PKB)/Akt pathway but is largely dispensable for the survival of normal breast epithelial cells and mesenchymal cells. We show that inhibition of ILK activity with a pharmacologic ILK inhibitor, QLT-0267, results in the inhibition of PKB/Akt Ser473 phosphorylation, stimulation of apoptosis, and a decrease in mammalian target of rapamycin (mTOR) expression in human breast cancer cells. In contrast, QLT-0267 treatment has no effect on PKB/Akt Ser473 phosphorylation or apoptosis in normal human breast epithelial, mouse fibroblast, or vascular smooth muscle cells. The inhibition of PKB/Akt Ser473 phosphorylation by QLT-0267 in breast cancer cells was rescued by a kinase-active ILK mutant but not by a kinase-dead ILK mutant. Furthermore, a dominant-negative ILK mutant increased apoptosis in the MDA-MB-231 breast cancer cell line but not in normal human breast epithelial cells. The inhibitor was active against ILK isolated from all cell types but did not have any effect on cell attachment and spreading. Our data point to an “ILK addiction” of breast cancer cells whereby they become dependent on ILK for cell survival through the mTOR-PKB/Akt signaling pathway and show that ILK is a promising target for the treatment of breast cancer.

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Introduction

Tumor formation is currently viewed as a multistage process involving the acquisition of several mutations in growth-enhancing oncopogenes and growth-inhibiting tumor suppressor genes, leading to dysregulation of cell signaling pathways (1). Despite the myriad of genetic and epigenetic alterations necessary for the development and progression of human cancers, many tumors seem highly dependent on constitutive activity of a specific gene or gene subset (2). In other words, cancer cells become “addicted” to the continued expression and/or activity of a specific oncogene or tumor suppressor gene, creating a situation that can be exploited therapeutically to induce tumor regression (1, 3). Indeed, the concept of oncogene addiction has been considered an Achilles’ heel of cancer (1). Thus, by elucidating the molecular mechanisms that result in these aberrant changes, in particular the processes of cell survival and cell proliferation, we will be better able to predict the most appropriate targets for cancer therapy.

Protein kinase B (PKB)/Akt is a central player in many cellular processes, including cell proliferation, differentiation, and survival (4), and is activated by numerous extracellular stimuli (5). Constitutive activation of PKB/Akt is frequently described in many types of human cancers (6, 7). Full activation of PKB/Akt is phosphatidylinositol 3-kinase (PI3K) dependent and requires both recruitment to the plasma membrane and phosphorylation on two key residues, Thr308 and Ser473 (8). Whereas phosphoinositol-dependent kinase-1 (PDK-1) has been shown to phosphorylate PKB/Akt on Thr308, the mammalian target of rapamycin (mTOR) of the kinase(s) implicated in the phosphorylation of the Ser473 site remains controversial. PDK-1 was initially proposed as being responsible for Ser473 phosphorylation but this was subsequently disproved (9). Other proposed candidates include DNA-PKcs (10) and, more recently, the mammalian target of rapamycin (mTOR)/Rictor complex (11), as well as autophosphorylation by PKB/Akt itself (12).

Integrin-linked kinase (ILK) has been shown to play a role in PKB/Akt Ser473 phosphorylation (13–16) and there is compelling evidence for ILK as a PI3K-dependent kinase (reviewed in ref. 14). ILK is a β1-integrin cytoplasmic domain-interacting protein and functions as a scaffold in forming multiprotein complexes connecting integrins to the actin cytoskeleton and signaling pathways. ILK activity is stimulated by adhesion to the extracellular matrix and by growth factors in a PI3K-dependent manner, and the activation of ILK promotes cell survival and protects against apoptosis (14, 17–19). Overexpression of ILK in epithelial cells also leads to anchorage-independent cell growth and tumorigenicity in nude mice (14). This is important because a number of human malignancies, including melanoma, ovarian cancer, prostate cancer, and non–small cell lung cancer, show marked overexpression of ILK (20–23). ILK can phosphorylate PKB/Akt in vitro (13, 24, 25) and recent studies have reported that ILK-phospho-PKB/Akt signaling protected hepatocytes from apoptosis (26) and that formation of a functional thymosin β4-PINCH-ILK complex resulted in activation...
of PKB/Akt and promotion of cardiac cell survival (27). We have shown that dominant-negative inhibition of ILK in prostate cancer cells results in decreased PKB/Akt Ser473 levels (13). In addition, a small molecule inhibitor of ILK kinase activity decreases PKB/Akt phosphorylation on Ser473 in ovarian (28), prostate (29), and glioblastoma (30) cancer cells in vitro and a human orthotopic pancreatic cancer xenograft model in vivo (16). Knockdown of ILK protein expression with siRNA in HEK-293 (31) and PC3 (32) cells, or with Cre recombinase in ILK-floxed (ILK+/−/+) macrophages (31), also results in a significant decrease in PKB/Akt Ser473 phosphorylation levels. In contrast, genetic studies in Drosophila and Caeorhabditis elegans have shown that PKB/Akt phosphorylation on Ser473 was not affected in ILK mutants (33, 34). In addition, levels of Ser473 phosphorylation on PKB/Akt were equivalent in ILK-null and wild-type mouse fibroblasts (35) and in ILK-null chondrocytes (36) and cells of the cerebral cortex (37). These data suggest that the dependence of PKB/Akt Ser473 phosphorylation on ILK may be cell type and cell context dependent.

Due to the apparent differential roles of ILK-mediated PKB/Akt Ser473 phosphorylation in different cells, we investigated the importance of ILK as a molecular target in breast cancer. A small molecule ILK inhibitor was used to evaluate ILK-dependent phosphorylation of PKB/Akt on Ser473 in normal and cancer cells. We show that inhibition of ILK activity in breast cancer cells results in a decrease in PKB/Akt Ser473 phosphorylation, induction of apoptosis, and a decrease in mTOR expression. In contrast, inhibition of ILK kinase activity or genetic knockdown of ILK expression in normal epithelial and mesenchymal cells has no effect on PKB/Akt Ser473 phosphorylation, cell survival, or mTOR expression. We show that ILK promotes survival of breast cancer cells, likely through an mTOR-PKB/Akt pathway, validating ILK as an important therapeutic target.

Materials and Methods

Cells. Human breast cancer cells [MDA-MB-231, MDA-MB-435, MDA-MB-468, BT-549, MDA-MB-435-LCC6-wt, MDA-MB-435-LCC6-VCI9], and MDA-MB-435-LCC6 (mutant) were cultured in DMEM (Invitrogen, Burlington, ON, Canada) with 10% FBS (Invitrogen). Human colon carcinoma SW-480 and DLD1 cells (38) were cultured in DMEM containing 10% FBS. MDA-MB-435-LCC6-wt cells were transfected with empty, ILK wild-type, ILK-S343D (kinase-active), ILK-S343A (kinase-dead), and ILK-E399K (dominant-negative; ref. 13) pcDNA3-myc vector using LipofectAMINE (Invitrogen) or Silentfect (Bio-Rad Laboratories Ltd.) according to the instructions of the manufacturer. Thirty-six hours posttransfection, the cells were treated overnight with QLT-0267 as indicated. Cells were harvested 48 hours posttransfection.

Cell attachment assay. Cells were harvested in 5 mmol/L EDTA-PBS, resuspended in DMEM containing 1% BSA, incubated for 30 minutes with the indicated concentrations of QLT-0267, and plated for 1 hour on fibronectin, with or without inhibitor. Unattached cells were removed by three washes with PBS and attached cells were fixed in PBS containing 4% paraformaldehyde, stained with 1% toluidine blue, and lysed in NP40 lysis buffer. Absorbance at 570 nm was then measured. Results are expressed as percentage of cell attachment and represent the mean ± SE of three independent trials.

Immunocytochemistry. Immunocytochemical assays were done as previously described (28). Briefly, cells were grown on fibronectin-coated coverslips (Becton Dickinson Labware, Bedford, MA), washed in PBS, and fixed for 15 minutes in 4% paraformaldehyde. Cell monolayers were permeabilized in 0.1% Triton X-100, washed, and blocked in 10% normal goat serum (Sigma-Aldrich). Cells were incubated with the anti-ILK monoclonal antibody (1:200, Upstate Cell Signaling Solutions) for 18 hours at 4°C. Cells were then washed and incubated with fluorescein isothiocyanate (FITC)-conjugated antiserum to mouse IgG (Jackson Immunoresearch Laboratories Inc., West Grove, PA) for 1 hour at room temperature. Cells were washed and coverslips were mounted using Vectashield (Vector Laboratories, Burlingame, CA). Isotype-specific negative controls were included with each staining run.
Digital imaging. Digital images were captured using a Regita 1300i cooled single chip digital camera (QImaging, Burnaby, BC, Canada) attached to a Zeiss Axioplan microscope (Carl Zeiss Canada, North York, ON, Canada) equipped with epifluorescence optics.

Apoptosis assay. Apoptosis was measured using the Cell Death Detection ELISA (Roche Applied Science, Mississauga, ON, Canada). Adherent or suspended cells were treated with increasing concentrations of the ILK inhibitor QLT-0267, with or without 20 μmol/L of the general caspase inhibitor ZVAD-fmk for 18 hours (BD Biosciences), pelleted at 1,200 rpm for 10 minutes, and resuspended in the supplied cell lysis buffer. After lysis, cell debris was pelleted and supernatants were used for the assay. The ELISA was then carried out as per the instructions of the manufacturer. Absorbance was read at 405 nm against the development solution as a blank with the secondary reference wavelength of 490 nm. Results are expressed as the mean fold change ± SD and the results shown are representative of three independent experiments.

Results

Inhibition of ILK kinase activity in vitro by a novel inhibitor. We have previously identified and characterized highly specific small molecule inhibitors of ILK (14, 16). In the current study, we have used a potent, second-generation ILK inhibitor, designated QLT-0267 (Fig. 1A), which inhibits the kinase activity of ILK at 26 nmol/L in a cell-free assay using highly purified recombinant ILK and has a selectivity profile when tested against 150 protein kinases under standard conditions similar to that reported for QLT-0254 (16). Furthermore, the inhibitor showed ~100-fold selectivity over PDK-1, DNA-PK, GSK-3, and all PKB isoforms.7 To show that QLT-0267 inhibits ILK kinase activity, we assessed its effect in an in vitro kinase assay in various nontumorigenic and tumorigenic cell lines. Specifically, we used human prostate cancer (PC3), breast cancer (MDA-MB-231 and BT-549) and mammary epithelial cells (MCF-10A), and murine embryonic fibroblasts and vascular smooth muscle cells. As ILK has been shown to phosphorylate phosphatase holoenzyme inhibitor-1 (PHI-1) on Thr57 (44), a PHI-T peptide was synthesized for use in kinase assays. Endogenous ILK was immunoprecipitated from cell lysates and in vitro phosphorylation of the PHI-T peptide substrate by ILK in presence of increasing concentrations of QLT-0267 was evaluated (Fig. 1B). We observed dose-dependent inhibition of PHI-T phosphorylation in non-tumorigenic and tumorigenic cells alike, demonstrating both the presence of kinase-active ILK in all cell types tested and efficient inhibition of ILK kinase activity by the QLT compound. Indeed, although cell-dependent differences in sensitivity to the inhibitor were noted, densitometric analyses showed that ILK kinase activity was decreased by at least 50% in all cell types upon treatment with 10 μmol/L of QLT-0267.

To verify the existence of ILK in the cell lines tested, we assessed ILK protein expression by Western blot. As shown in Fig. 1C, immunoreactive ILK protein was detected in all cell lines, albeit

Figure 1. In vitro inhibition of ILK kinase activity by QLT-0267. A, a generic structure of the K15792 class of ILK inhibitors, of which QLT-0267 is a derivative. B, ILK was immunoprecipitated (IP) from cancer (PC3, MDA-MB-231, BT-549) or normal (MCF-10A, fibroblast, vascular smooth muscle cell) cell lysates. A nonspecific control immunoprecipitation was done using an antihemagglutinin (HA) antibody. The immune complexes were isolated and kinase assays were done in the presence of QLT-0267 and using PHI-T peptide or PHI-A control peptide as substrates. Phosphorylated PHI-T was resolved by 15% SDS-PAGE and visualized by autoradiography. Gels were stained with Coomassie blue for PHI peptides to show equal amount of substrate. Numbers below each band, quantitative values from densitometric analyses. Representative of two independent experiments. C, ILK protein expression was assessed by Western blotting in several normal and cancer cell lines. FB, fibroblasts; VSMC, vascular smooth muscle cells.

7 D. Morrison, personal communication.
with variation among the lines tested. Importantly, human normal breast epithelial cells and breast cancer cell lines expressed ILK and there was no significant difference in expression levels between these cell types as has been proposed previously (18).

**Inhibition of ILK activity suppressed PKB/Akt Ser473 phosphorylation in all breast cancer cells but not in normal epithelial or mesenchymal cells.** We have previously shown that ILK can phosphorylate PKB/Akt on Ser473 in several cancer cell lines (14). Recent studies from our laboratory (32) and those of others (35, 36, 45) have provided apparently conflicting data on the role of PKB/Akt Ser473 phosphorylation in noncancerous cells. To address this issue, we have used genetic and pharmacologic strategies to evaluate the effect of inhibiting ILK kinase activity on the phosphorylation of Ser473 PKB/Akt in normal epithelial cells and breast cancer cell lines as well as in normal mesenchymal cells.

Robust knockdown of *ILK* gene expression was achieved through the introduction of Cre recombinase into *ILK* fl/fl murine embryonic fibroblasts (Fig. 2A) or vascular smooth muscle cells (Fig. 2B). Infected cells were treated with QLT-0267 and PDGF-induced PKB/Akt Ser473 phosphorylation was assessed by Western blot. Neither specific knockdown of ILK expression nor inhibition of ILK kinase activity, alone or in combination, produced a decrease in PKB/Akt Ser473 phosphorylation upon PDGF stimulation in these cell types. In addition to genetically modified mesenchymal cells, we examined the effect of ILK kinase activity inhibition in the widely used NIH3T3 fibroblast model. We compared QLT-0267 and its well characterized first-generation formulation, KP-392 (also called KP-SD1; ref. 46), on PDGF-induced PKB/Akt Ser473 phosphorylation (Fig. 2C). Neither inhibitor had an effect on PKB/Akt Ser473 phosphorylation in these cells.

**Figure 2.** Effect of the ILK inhibitor QLT-0267 and/or knockdown of *ILK* gene expression on PKB/Akt Ser473 phosphorylation in normal cells. Cre recombinase was introduced into murine embryonic fibroblasts (A) and murine vascular smooth muscle cells (B) containing the floxed *ILK* gene using adenoviral infection. Infected and noninfected control cells were subsequently treated overnight (16 hours) with or without QLT-0267 in serum-free medium. Cells were then stimulated with PDGF-BB for 5 minutes and expression levels of PKB/Akt Ser473, total PKB/Akt, ILK, and β-actin were assessed by Western blotting. Neither inhibition of ILK kinase activity by QLT-0267 nor knockdown of ILK gene expression, used separately or in combination, had an effect on PDGF-mediated phosphorylation of PKB/Akt. KP-392 (also called KP-SD1) is the predecessor compound to QLT-0267, and has been extensively characterized by us previously (13, 29, 46, 53). It has been shown to inhibit PKB/Akt phosphorylation as well as induce apoptosis in many cancer cell lines. D, normal human mammary epithelial cells were treated overnight with increasing amounts of QLT-0267 in medium containing 10% serum. Analyses of PKB/Akt Ser473 by Western blotting showed no effect of the ILK inhibitor on phosphorylation of PKB/Akt. Representative of three independent experiments.
To verify that the ILK-independent PKB/Akt Ser473 phosphorylation was not specific to murine mesenchymal cells, we investigated the effect of ILK inhibition on PKB/Akt Ser473 phosphorylation in several normal human mammary epithelial cell lines as well as in primary human mammary epithelial cells. In agreement with our observations in mesenchymal cells, the presence of QLT-0267 did not inhibit the phosphorylation of PKB/Akt on Ser473 in mammary epithelial cells, regardless of whether the cell line was immortalized or grown in primary culture (Fig. 2).

In striking contrast to the normal cells examined, treatment of several human breast cancer cells with QLT-0267 resulted in a significant, dose-dependent inhibition of PKB/Akt Ser473 phosphorylation (Fig. 3A). Indeed, in all four breast cancer cell lines studied (MDA-MB-231, MDA-MB-468, and MDA-435, BT-549), each harboring the loss of different tumor suppressors (MDA-MB-231 and MDA-MB-435 are negative for E-cadherin; MDA-MB-468 and BT-549 are negative for PTEN), PKB/Akt Ser473 phosphorylation was dramatically inhibited in the presence of 2 to 10 μmol/L QLT-0267.

Inhibition of PKB/Akt Ser473 phosphorylation by QLT0267 was also evident in the Her2-overexpressing MDA-MB-435-LCC6 cell line (Fig. 3A). Wild-type cells (LCC6-wt), vector control cells (LCC6-VC(P9)), and cells stably overexpressing Her2 (LCC6-Her2) were transiently transfected with kinase-active ILK (ILK-S343D), kinase-dead ILK (ILK-S343A), or empty myc-tagged vector. Cells were then treated overnight with the indicated amount of QLT-0267 and phosphorylation of PKB/Akt on Ser473 was assessed by Western blotting. Phosphorylation of PKB/Akt on Ser473 in cells expressing kinase-active ILK was protected from the inhibitory effect of 10 μmol/L QLT-0267 when compared with control cells. Representative of three independent experiments.

**Figure 3.** Effect of the ILK inhibitor QLT-0267 on PKB/Akt-Ser473 phosphorylation in cancer cells. A, breast cancer cell lines [MDA-MB-231, MDA-MB-468, MDA-MB-435-LCC6-wt, MDA-MB435-LCC6-VC(P9), and MDA-MB435-LCC6-Ser473] and colon carcinoma cell lines (SW-480, DLD-1) were treated overnight with increasing concentrations of QLT-0267 in medium containing 10% serum. Analyses of PKB/Akt-Ser473 by Western blotting showed a dose-dependent decrease in phosphorylation on Ser473 in presence of ILK inhibitor. The same blots were then reprobed to assess total PKB/Akt protein levels. B, protection of PKB/Akt-Ser473 phosphorylation level. MDA-MB-231 cells were transiently transfected with kinase-active ILK (ILK-S343D), kinase-dead ILK (ILK-S343A), or empty myc-tagged vector. Cells were then treated overnight with the indicated amount of QLT-0267 and phosphorylation of PKB/Akt on Ser473 was assessed by Western blotting. Representative of three independent experiments.
showed comparable, dose-dependent decreases in Ser\(^{473}\) phosphorylation in response to QLT0267 treatment. These results show that despite overexpression of Her2, a clinically important therapeutic target for breast cancer, the response to QLT0267 treatment is similar to that of other breast cancer cell lines.

Furthermore, similar results were obtained in two APC-negative colon carcinoma cell lines (Fig. 3A), indicating that this sensitivity of cancer cells is not limited to breast cancer cells. Time course experiments in MDA-MB-435 cells treated with 10 \(\mu\)mol/L of QLT-0267 showed complete inhibition of PKB/Akt Ser\(^{473}\) phosphorylation after a 3-hour incubation period (data not shown), demonstrating the rapid uptake and action of the inhibitor in susceptible cells.

A study published recently examining anaplastic thyroid cancer showed inhibition of ILK kinase activity, increased apoptosis, and decreased cell growth in response to QLT0267 in several thyroid cancer cell lines at concentrations similar to those used in the present study (47). In addition, QLT0267 inhibited PKB/Akt Ser\(^{473}\) phosphorylation in vivo (47). The difference in the inhibitory concentrations of QLT0267 in cell-free assays compared with cell-based assays is not unusual for small molecule inhibitors and has been noted for other kinase inhibitors, including epidermal growth factor receptor inhibitors and CDK2/cyclin D inhibitors (48, 49).

To confirm that the suppression of PKB/Akt Ser\(^{473}\) phosphorylation in response to QLT-0267 was ILK specific, we assessed the possibility of protecting, or rescuing, the decreased phosphorylation of PKB/Akt on Ser\(^{473}\) in cancer cells by overexpressing active ILK in these cells. MDA-MB-231 breast cancer cells were transiently transfected with a kinase-active (ILK-S343D) or kinase-dead (ILK-S343A) form of ILK or a control vector (13) and subsequently treated with 10 \(\mu\)mol/L of QLT-0267. The results of these experiments showed that exogenous expression of kinase-active but not kinase-dead ILK protected the inhibitory effect QLT-0267 on phosphorylation of PKB/Akt on Ser\(^{473}\) (Fig. 3B). Transfection of ILK-S343A kinase-dead ILK reduced PKB/Akt phosphorylation, as expected.

**Inhibition of ILK activity induces apoptosis in cancer cells but not in normal cells.** Given the differential response to PKB/Akt Ser\(^{473}\) phosphorylation upon treatment with the ILK inhibitor between normal and tumorigenic cells, we were interested in ascertaining whether such an effect would have downstream implications. We and others have shown previously that the inhibition of ILK in cancer cells using dominant-negative or kinase-dead ILK constructs, or a pharmacologic inhibitor of ILK (KP-392), results in apoptosis (13, 16, 29). In the present study, we assessed the ability of normal and cancer cells to undergo apoptosis, as detected by caspase-3 cleavage, in response to treatment with QLT-0267. Normal embryonic fibroblasts and vascular smooth muscle cells (Fig. 4A), primary and normal mammary epithelial cells (Fig. 4B), and breast cancer cells (Fig. 4C) were incubated for 20 hours with increasing concentrations of QLT-0267. Actinomycin D–treated cells served as a positive control for caspase-3 cleavage (Fig. 4A and B). As seen by Western blotting (Fig. 4), caspase-3 cleavage was virtually undetectable in fibroblasts, vascular smooth muscle cells, and normal epithelial cells at all inhibitor concentrations examined. In sharp contrast, QLT-0267 induced caspase-3 cleavage in all breast cancer cell lines at concentrations of \(\leq 10\) \(\mu\)mol/L QLT-0267.

To confirm our caspase-3 findings, we examined quantitatively the level of caspase-mediated apoptosis in normal mammary epithelial cells versus breast cancer cells. We observed a dose-dependent increase in apoptosis in the cancer cells to levels \(>3.5\)-fold higher than that seen in normal epithelial cells (Fig. 4D). Importantly, the general caspase inhibitor ZVAD-fmk eliminated the apoptotic effect of QLT-0267, suggesting the presence of caspase-mediated apoptosis (Fig. 4D). These data indicate that QLT-0267 induces apoptosis in a dose-dependent manner in cancer cells but not in normal cells.

To further confirm that the decrease in breast cancer cell survival in response to QLT0267 treatment was ILK specific, we blocked ILK function by transient transfection of a dominant-negative ILK construct (ILK-E359K) and examined apoptotic cell death in normal mammary epithelial cells and breast cancer cells (Fig. 4E). The ILK-E359K construct has previously been shown to function as dominant-negative (17, 29, 46), and earlier studies have shown the induction of anoikis by the dominant-negative construct in MDA-MB-231 cells (17). In the current studies, Htrt cells and MDA-MB-231 cells were transiently transfected with wild-type ILK or dominant-negative ILK and assessed for cell survival 48 hours post-transfection. The results of these experiments showed that breast cancer cells underwent increased cell death upon expression of the dominant-negative construct, whereas normal cells do not (Fig. 4E). The presence of myc-tagged ILK verified successful transfection of the various constructs.

**The effect of ILK inhibition with QLT-0267 on cell attachment and spreading.** The finding that ILK inhibition has differential effects on normal and tumorigenic cells with respect to PKB/Akt Ser\(^{473}\) phosphorylation and apoptosis shows the existence of a differential sensitivity to targeted pharmacologic therapies. Thus, we were interested in elucidating the mechanism behind this observation. Given that ILK regulates integrin-mediated cell adhesion and that most tumorigenic cells are anoikis resistant, we reasoned that adhesion differences between cancerous and noncancerous cells may underlie the opposing responses to ILK inhibition. To determine what influence, if any, inhibition of ILK kinase activity may have on cell adhesion, normal and cancer cells were subjected to an adhesion assay. Suspended cells were pretreated for 30 minutes with the inhibitor and plated onto fibronectin in the presence of the inhibitor. The percentage of cells attached to fibronectin was then assessed. Regardless of the cell type tested, the presence of QLT-0267 at concentrations that significantly inhibited PKB/Akt phosphorylation had no significant effect on cell attachment to fibronectin (Fig. 5A). To confirm these findings, the MDA-MB-231 breast cancer cell line was plated onto fibronectin-coated coverslips in the presence or absence of QLT-0267 and stained for ILK expression. No differences in overall morphology, cell spreading, or ILK localization were noted after 4 hours of inhibitor treatment compared with controls (Fig. 5B). These data show that the effects of QLT-0267 on PKB/Akt phosphorylation and cell survival in the breast cancer cells are not a consequence of differences in cell attachment.

To further investigate the possibility that integrin-mediated adhesion of cells could be involved in the observed differences between normal cells and cancer cells, RGD peptide was used as an integrin-activating agent for suspended cells in the presence of QLT-0267. For this experiment, we focused on MCF-10A normal and MDA-MB-231 cancer cells as prototypic cell types. Cells were left adherent on plastic or placed in suspension in poly-HEMA-coated 96-well tissue culture plates in the presence of RGD and/or QLT-0267 and assayed for apoptosis. As expected, adherent MCF-10A cells were resistant to apoptosis in the presence of the ILK inhibitor (Fig. 5C). Suspended cells underwent substantial anoikis over the same time period but were significantly protected by the
presence of RGD. The effects of QLT could not be measured in suspended cells due to the high levels of anoikis but the protective effect of RGD was observed even with QLT-0267 present. However, ILK inhibitor-mediated apoptosis occurred in the MDA-MB-231 cells regardless of whether the cells were adherent, suspended, or treated with RGD (Fig. 5C). These results suggest that in normal epithelial cells, ILK activity is dispensable for the regulation of adhesion-dependent cell survival. However, in tumorigenic cells, ILK becomes a critical factor for adhesion-dependent or adhesion-independent cell survival.

Differential regulation of mTOR expression by ILK in normal epithelial cells and breast cancer cells. Both ILK and the mTOR/Rictor complex can promote phosphorylation of PKB/Akt on Ser473 (11). Inhibition of both ILK (siRNA; ref. 32) and mTOR (short hairpin RNA; ref. 11) expression results in the inhibition of PKB/Akt Ser473 phosphorylation in cancer cell lines. A decrease-dependent increase in apoptosis was observed in the breast cancer cells but not in the normal cells. Note that ZVAD-fmk abrogated the apoptotic effect of QLT-0267, indicating that the cell death observed in the cancer cells is caspase dependent. The mean basal absorbance values (i.e., 0 μmol/L QLT-0267) for each cell type are as follows: MCF-10A, 0.198 ± 0.004; MDA-MB-231, 0.060 ± 0.008; MDA-MB-468, 0.575 ± 0.074. *, P < 0.05. E, effect of dominant-negative ILK on epithelial cell survival. Human mammary epithelial cells (Htrt) and human breast cancer cells (MDA-MB-231) were transiently transfected with myc-tagged wild-type ILK, dominant-negative ILK, or empty vector control constructs. Cells were harvested 48 hours posttransfection and cell survival was examined using the cell death detection ELISA. Transfection levels were monitored by Western blotting for myc. Transfection with dominant-negative ILK resulted in a significant increase in apoptosis in the breast cancer cells but had no effect on the Htrt cell line. *, P = 0.004.
caspase inhibitor ZVAD-fmk. As seen previously in Fig. 4D, ZVAD-fmk treatment is sufficient to inhibit the induction of apoptosis by QLT-0267. We found that inhibition of apoptosis, however, did not reverse the decrease seen in mTOR expression levels in QLT0267-treated MDA-MB-231 breast cancer cells (Fig. 6B), indicating that this decrease is due to an inhibition of ILK activity. These data suggested that in cancer cells, ILK may acquire a novel function of regulating mTOR expression. Ongoing experiments are being done to elucidate the exact role of ILK in mTOR expression and whether ILK activity regulates the formation of the mTOR/Rictor complex.

Discussion

The theory of oncogene addiction in cancer cells represents an interesting and pharmacologically manipulative aspect of genetic mutation in tumor progression. Perhaps the best human example of this is the importance of the BCR-Abl mutation in myeloid leukemia and the manipulation of this addiction with respect to current treatments of chronic (early) stages of the disease (1). In this paper, we have shown the preferential dependence of breast cancer cells versus normal mammary epithelial cells on ILK for PKB/Akt activation and cell survival. Our results suggest that breast cancer cells become “addicted” to ILK for survival.

We have shown that the ability of normal mammary epithelial cells to regulate PKB/Akt activity in an ILK-independent manner allows them to maintain growth and inhibit apoptosis in the presence of pharmacologic inhibition of ILK activity. In striking contrast, inhibition of ILK activity in human breast cancer cells results in dramatic inhibition of Ser473 phosphorylation on PKB/Akt. The decrease in this phosphorylation in cancer cells is associated with a dysregulation of downstream cell survival pathways.

Figure 5. Effect of QLT-0267 on cell attachment and ILK localization to focal adhesions. A, normal and cancer cells maintained in suspension in DMEM containing 1% BSA were treated for 30 minutes with the indicated concentrations of QLT-0267 and plated on fibronectin for 2 hours in the presence of inhibitor. ILK inhibitor did not alter cell attachment to fibronectin. B, MDA-MB-231 cells were detached and replated on fibronectin-coated coverslips in serum-containing medium in the absence or presence of increasing concentrations of ILK inhibitor. Cells were incubated for 4 hours, fixed, and stained for ILK. Note that the cells are spread and ILK localizes to focal adhesions (arrows) at all inhibitor concentrations. Original magnification, ×100. C, human normal mammary epithelial cells (MCF-10A) and breast cancer cells (MDA-MB-231) were placed in suspension under serum-free conditions and treated with 10 μmol/L QLT-0267 in the presence or absence of 250 μg/mL RGD peptide for 24 hours (MCF-10A) or 30 hours (MDA-MB-231). Cell lysates were analyzed for the extent of apoptosis. The significant level of anoikis induced in the MCF-10A cells was fully protected in the presence of RGD peptide, regardless of whether QLT-0267 was also present. In the cancer cells, however, the ILK inhibitor induced apoptosis even in the presence of RGD peptide. Columns, mean fold change of triplicate samples; bars, SD. Representative of three independent experiments. The mean absorbance values for the control condition for each cell type (±SD) are as follows: MCF-10A, 0.198 ± 0.004; MDA-MB-231, 0.195 ± 0.009. *P ≤ 0.05 versus DMSO control-treated cells.
pathways, as indicated by an increase in caspase-mediated apoptosis and a concomitant decrease in cyclin D1 expression and cell proliferation. Similar results have been reported recently for SL0101, a novel, small molecule inhibitor of p90 ribosomal S6 kinase (RSK). SL0101 was found to inhibit the proliferation of the human breast cancer cell line MCF-7 but did not affect the growth of MCF-10A cells, a normal human breast cell line (50). The selective inhibition of proliferation in the cancer cells occurred despite the ability of SL0101 to inhibit RSK activity in both cell types (50). Together with these studies, our data suggest an increased reliance of cancer cells on particular pathways. Thus, breast cancer cells become dependent on the ILK pathway, resulting in an increased susceptibility to inhibition by QLT-0267. Normal cells, however, retain redundant mechanisms capable of circumventing ILK inhibition. The apparent ILK addiction of cancer cells relative to their normal counterparts provides an attractive therapeutic avenue that can be exploited clinically.

We have also shown here that although normal breast epithelial and breast cancer cells show differences in sensitivity to the effects of ILK inhibition, they do not show differences in attachment to or spreading on fibronectin or the distribution or expression levels of ILK when treated with the inhibitor. Interestingly, differences in response to the effects of ILK inhibition were seen in cells placed in suspension. Normal mammary epithelial cells in suspension underwent anoikis and this cell death was blocked by the addition of RGD peptide, regardless of ILK activity. In contrast, breast cancer cells were resistant to anoikis in suspension but remained sensitive to ILK inhibition even in the presence of RGD-mediated integrin activation. Recent studies in normal rat hepatocytes have also shown a protective effect of RGD peptide against apoptosis, with integrin activation leading to increased ILK activity and PKB/Akt phosphorylation (22). However, although our data support the idea that integrin activation is sufficient to protect normal cells against anoikis, this simple induction of ILK activity does not seem to be sufficient to produce sensitivity to ILK in these cells, given that breast cancer cells but not normal breast cells are sensitive to the effects of the inhibitor in the presence or absence of RGD. More recently, a study has been published demonstrating a role for ILK in regulating PKB/Akt phosphorylation and survival in human lung fibroblasts through a β1-integrin-mediated pathway (43). Although these data initially seem to conflict with our data in mouse fibroblasts and vascular smooth muscle cells, we have not, to date, tested our cells in three-dimensional matrix.

It is well established that the full activation of PKB/Akt requires the phosphorylation of both Thr<sup>308</sup> and Ser<sup>473</sup>. The phosphorylation of Thr<sup>308</sup> is carried out by PDK-1 but the kinase(s) responsible for phosphorylation at the Ser<sup>473</sup> site, the so-called PDK-2, has been attributed to several candidates. Together with DNA-PK and PKCα, ILK represents a compelling candidate for physiologically significant Ser<sup>473</sup> kinase activity in the mammalian system (4). It has been suggested that the function of this kinase is at least partially redundant, possibly with context- or agonist-dependent specificity (4). Our data demonstrating ILK-independent Ser<sup>473</sup> phosphorylation in normal cells and ILK-dependent phosphorylation in cancer cells are congruent with this view. One of the more recent candidates put forward as a PKB Ser<sup>473</sup> kinase is mTOR. Recent data have shown a role for the rapamycin-insensitive mTOR/Rictor complex in regulation of the actin cytoskeleton (including actin polymerization, cell spreading, and Rac1 activation) and PKB/Akt Ser<sup>473</sup> phosphorylation (11, 51). Although the mechanism involved in the differential regulation of PKB/Akt Ser<sup>473</sup> phosphorylation by normal and cancer cells remains unclear, our preliminary data suggest that ILK may play an important and novel role in mTOR/Rictor-mediated PKB/Akt activation. Based on these early findings, we speculate that ILK lies upstream of the mTOR/Rictor complex and the stabilization of this complex is dependent on ILK activity. Inhibition of ILK activity in cancer cells may lead to destabilization and subsequent degradation of the mTOR protein. Further studies are under way looking at the specific interaction of mTOR/Rictor with ILK to determine the exact role of ILK in mTOR/Rictor signaling to PKB/Akt. The differential dependence of normal versus cancer cells on ILK-mediated phosphorylation of PKB/Akt does not seem to depend on the presence of a single oncogene or absence of a tumor suppressor gene, as the cell lines used here represent a variety of genetic mutations. This suggests that ILK is a central “hub” in relaying signals promoting cell survival and proliferation. Thus, the inhibition of ILK activity represents a powerful mechanism in tumor growth reduction.

Importantly, we have shown that ILK protein is expressed and is active in both human epithelial cells and human breast cancer cell lines. These data differ from those reported by Chen et al. (18), where ILK levels were suggested to be reduced in breast cancer compared with normal mammary epithelial cells. There may be several reasons for the apparent differences in the data sets, the most significant of which may be that the data reported by Chen et al. rely heavily on mRNA expression and offer little evidence of protein expression or kinase activity, particularly in the mammary epithelial cell lines and the breast cancer cell lines. This report remains unique amid countless other reports of ILK expression in many types of cancers. Indeed, ILK
overexpression is a hallmark of several solid tumors (reviewed in ref. 14) and, in many cases, the increased expression levels of ILK correlate with poor patient survival and drug resistance (20, 22, 52).

In agreement with the results presented in this article, recent studies have shown the potent effect of inhibiting ILK activity on tumor progression in vivo. In a xenograft model of PTEN mutant human glioblastoma, suppression of ILK activity using antisense oligonucleotides caused a delay in growth when compared with control tumors (30). Furthermore, staining of the treated tumors revealed a significant loss of Ser473-PKB/Akt expression in vivo (30). Similar results have been reported in human orthotopic primary pancreatic xenografts, where treatment with a well-tolerated ILK inhibitor (QLT-0254, a QLT-0267 derivative) led to a significant reduction in tumor growth (16). Moreover, in combination with gemcitabine, a single dose of QLT-0254 substantially enhanced acute apoptosis in tumors (16). Recent data have also shown that knockdown of ILK expression by siRNA in chemoresistant-pancreatic adenocarcinomas induced chemosensitization to gemcitabine via increased caspase-mediated apoptosis (52). This suggests that directed therapies against ILK may lead to enhanced efficacy of gemcitabine-based chemotherapy. Most importantly, studies in anaplastic thyroid cancer have shown that treatment with QLT-0267 led to a reduction in tumor size and a suppression of Ser73 phosphorylation in mice (47). Thus, these studies, together with the data presented here, clearly show ILK as an important therapeutic target. In particular, the dichotomy in the sensitivity of normal and cancer cells to small molecule inhibitors of ILK, in tandem with the data suggesting synergy between ILK inhibition and conventional chemotherapy, make the use of ILK kinase inhibitors an attractive option in the development of novel therapeutic strategies for the treatment of cancer.

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References

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