Conditional Knock-out of Integrin-linked Kinase Demonstrates an Essential Role in Protein Kinase B/Akt Activation*

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Arlene A. Troussard‡§, Nasrin M. Mawji‡, Christopher Ong¶, Alice Mui¶, René St.-Arnaud**, and Shoukat Dedhar‡ ‡‡

From the British Columbia Cancer Agency and Department of Biochemistry, University of British Columbia, Jack Bell Research Centre, Vancouver, British Columbia V6H 3Z6, Canada, ¶The Prostate Centre, Vancouver Hospital Health Sciences Centre and Department of Surgery, University of British Columbia, Vancouver, ‡‡Supported by grants from the National Cancer Institute of Canada and Shriners Hospital Foundation and McGill University, Montréal, Québec H3G 1A6, Canada

Protein kinase B (PKB/Akt) plays a pivotal role in signaling pathways downstream of phosphatidylinositol 3-kinase, regulating fundamental processes such as cell survival, cell proliferation, differentiation, and metabolism. PKB/Akt activation is regulated by phosphoinositide phospholipid-mediated plasma membrane anchoring and by phosphorylation on Thr-308 and Ser-473. Whereas the Thr-308 site is phosphorylated by PDK-1, the identity of the Ser-473 kinase has remained unclear and controversial. The integrin-linked kinase (ILK) is a potential regulator of phosphorylation of PKB/Akt on Ser-473. Utilizing double-stranded RNA interference (siRNA) as well as conditional knock-out of ILK using the Cre-Lox system, we now demonstrate that ILK is essential for the regulation of PKB/Akt activity. ILK knock-out had no effect on phosphorylation of PKB/Akt on Thr-308 but resulted in almost complete inhibition of phosphorylation of Ser-473 and significant inhibition of PKB/Akt activity, accompanied by significant stimulation of apoptosis. The inhibition of PKB/Akt Ser-473 phosphorylation was rescued by kinase-active ILK but not by a kinase-deficient mutant of ILK, suggesting a role for the kinase activity of ILK in the stimulation of PKB/Akt phosphorylation. ILK knock-out also resulted in the suppression of phosphorylation of GSK-3β on Ser-9 and cyclin D1 expression. These data establish ILK as an essential upstream regulator of PKB/Akt activation.

PKB/Akt is activated in a PI 3-kinase-dependent manner and regulates cell survival, proliferation, differentiation, motility, and metabolism (1). The activity of PKB/Akt is constitutively activated under situations of chronic activation of PI 3-kinase, for example, by the mutational inactivation of the tumor suppressor PTEN. PKB/Akt regulates apoptosis and cell cycle progression by promoting the phosphorylation of pro-apoptotic proteins such as Bad, Forkhead transcription factors, and the cell cycle inhibitor p27(kip1) (2–5).

The full activation of PKB/Akt requires phosphorylation on Thr-308 and Ser-473 (6). Whereas PDK-1 has been demonstrated to phosphorylate PKB/Akt on Thr-308 (7), the kinase responsible for phosphorylation at the hydrophobic Ser-473 site has not been identified despite extensive efforts. PDK-1 was initially proposed as also being responsible for the stimulation of phosphorylation of Ser-473 (8). However, this site is inducibly phosphorylated in PDK-1 knock-out cells, pointing to the existence of a distinct Ser-473 kinase (9). Autophosphorylation is another proposed mechanism for the phosphorylation on Ser-473 (10). The integrin-linked kinase (ILK), an integrin- and growth factor-regulated PI 3-kinase-dependent kinase (11, 12), has also been shown to promote the phosphorylation of PKB/Akt on Ser-473 but not on Thr-308 (1, 11–15). Growth factor- and extracellular matrix-induced PKB/Akt Ser-473 phosphorylation is inhibited by kinase-deficient, dominant-negative ILK (1, 13, 14), as well as by small molecule ILK kinase inhibitors (14, 16). Inhibition of ILK activity also results in inhibition of constitutively activated PKB/Akt in PTEN-null cancer cells (16). In addition, ILK was identified as a PKB/Akt Ser-473 kinase by in-gel kinase analysis and protein purification (14). These data support a role of ILK in the regulation of phosphorylation of PKB/Akt on Ser-473. However, recently, a lipid-raft associated PKB Ser-473 kinase activity distinct from PDK-1, PKB, or ILK was detected (17), although its identity remains unknown. This finding and the reports showing that the PI 3-kinase pathway and PKB/Akt activation do not appear to be affected in Caenorhabditis elegans and Drosophila ILK mutants have prompted a debate over the physiological role of ILK in PKB/Akt activation (23, 24).

To definitively address the question of whether ILK is essential for the promotion of phosphorylation of PKB/Akt on Ser-473, we have taken two genetic approaches to down-regulate or eliminate the expression of ILK in mammalian cells and then to assess whether phosphorylation of PKB/Akt on Ser-473 is specifically affected.

We find that ILK expression is down-regulated in HEK-293 human kidney epithelial cells by double-stranded RNA interference (siRNA) and in immortalized mouse macrophages by conditional deletion of the ILK gene using the Cre-Lox system. ILK knock-out results in the inhibition of PKB/Akt activity and specific suppression of phosphorylation of PKB/Akt on Ser-473,
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without any effect on PKB/Akt expression or phosphorylation on Thr-308. Furthermore, the inhibition of PKB/Akt Ser-473 phosphorylation is rescued by transfection of a kinase-active ILK, but not by a kinase-inactive ILK. ILK knock-out also results in the inhibition of phosphorylation of GSK-3β and expression of cyclin D1, two known targets of PKB/Akt and ILK.

EXPERIMENTAL PROCEDURES

Cells and Plasmids—Human embryonic kidney HEK-293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% donor calf serum (Invitrogen).

Bone marrow cells were isolated from femurs and plated in tissue culture plates at 2 × 10⁶ cells/ml in 1% NFN at 37 °C in 100 ng/ml CSF-1 in 10% fetal calf serum/Isco's modified Dulbecco's media supplemented with 50% conditioned media from cells producing the PBS-Tex virus (18). The non-adherent cells were then removed and placed in fresh tissue culture plates and allowed to differentiate into macrophages. After several passages over weeks, 100% of the cells were Mac-1-positive.

Transfection of the following plasmids were carried out in macrophages using LipofectAMINE 2000: pcDNA-3-ILK-WT, pcDNA-3-ILK-S343A, pcDNA-3-ILK-S343D (14), and pEGFP.

siRNA—siRNA duplexes were synthesized by Xeragon, Inc., Germantown, MD. Twenty-one-base sequences of the human ILK gene (GenBank accession number gi 3150001) specifically targeting the kinase domain (ILK-FSP) and the p10 domain (ILK-H1) were chosen. A control-NS, non-silencing siRNA (from Xeragon's data base, 16-base overlap with Thermotoga maritima), a control-HC, inverted ILK-FSF siRNA, and a siRNA targeting calreticulin (GenBank accession number gi 16151096) were also designed. Each sequence was blasted to assess specificity. Transient transfections of HEK-293 cells were carried out by using 6 μl of Lipofectin reagent (Invitrogen), according to the manufacturer's guidelines.

Excision of the Floxed ILK Gene—Immortalized ILK fl/fl macrophages were infected with CRE recombinase adenoviruses. Cells were plated in a 6-well plate and used when they were 70% confluent. Cells were incubated with 1, 0.1, or 0.01 μl of Ads CRE (5.29 × 10⁹ viral particles/ml) in 500 μl of Dulbecco's modified Eagle's medium for 1 h at 37 °C. The volume was then increased to 2 ml, and the cells were incubated for an additional 3 h before replacement by complete media. By PCR, we assessed the presence of CRE recombinase (forward primer, 5'-CATT TCTGGGATTCTTTTATAACAC-3', reverse, 5'-TATTTGAAACTCCAG CGCGGGCC-3'), the presence of the floxed ILK gene (forward, 5'-AA GGTGCTGAAGGTGA-3', reverse, 5'-CAAGGAATAGGTGA GCCAAAGA-3'), and the presence of excision products (forward, 5'-C CAGGTTGCCAGTTAAGTTGA-3', reverse, 5'-CAAGGAATAGGTGA GCCTCAGA-3'). After infection, cells were maintained in 10% fetal bovine serum and 100 ng/ml CSF-1. ILK protein expression was then evaluated by Western blotting as described below. For the rescue experiments, macrophages were co-transfected with 1 μg of the indicated plasmids and 0.5 μg of pEGFP, prior to infection with Ads CRE. Transfection efficiencies were determined by counting GFP-positive cells. The transfection efficiencies varied from 20 to 40%. Because of this variability and the different amounts of GFP detected by Western blotting, we performed densitometric analyses and then normalized the phosphorylation status of PKB/Akt on Ser-473 relative to GFP expression. Ratios are shown under "Results."

Immunoblots—Cells, grown in the conditions described above, were lysed in 1% Nonidet P-40, 150 mM NaCl, 50 mM Tris, pH 7.6, 1 mM EDTA, proteases mixture inhibitor (Roche), 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 0.1 mM sodium fluoride. Protein extracts were then resolved by SDS-PAGE and either transferred onto Immobilon-P membranes (Millipore). Membranes were probed with one of the following primary antibodies: anti-ILK (Upstate Biotechnology Inc., catalog no. 06-550), anti-PKB/Akt-Pser473 (Cell Signaling Technology), anti-PKB/Akt-Pthr308 (Cell Signaling Technology), anti-PKB/Akt (Transduction Laboratories), anti-GSK-3-Pser9 (New England Biosciences Inc.), anti-GSK-3 (Transduction Laboratories), anti-akt (Sigma), anti-calreticulin (Santa Cruz Biotechnology), anti-cyclin D1 (UBI), or anti-GFP (Roche Molecular Biochemicals). Detection was carried out with the appropriate horseradish peroxidase-conjugated secondary antibody (Jackson Laboratories, Inc.) and an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech), as described above. Densitometric analyses were performed using Quantity One (software from Bio-Rad).

AKT Kinase Assay—Non-radioactive AKT kinase assays were performed following the manufacturer's guidelines (Cell Signaling Technology, kit no. 9840). Briefly, 150 μg of protein were immunoprecipitated using an anti-AKT antibody, and immunoprecipitates were incubated with a GSK-3 fusion protein as a substrate. Phosphorylation of Ser-921 of GSK-3β, as a measure of AKT kinase activity, was then detected by Western blotting.

Apoptosis Assay—Cells were stained with annexin V-fluorescein isothiocyanate and propidium iodide according to the manufacturer's instructions (BD Biosciences). Annexin V-positive cells undergoing apoptosis were then detected by flow cytometry analysis.

RESULTS AND DISCUSSION

To determine whether ILK is essential for the regulation of PKB/Akt phosphorylation on Ser-473, we have taken two approaches to knock down the expression of ILK and then assess PKB/Akt Ser-473 phosphorylation. The first approach was the use of double-stranded RNA (siRNA) to knock down ILK expression. We identified and synthesized two specific ILK siRNAs, as well as three control RNAs (Fig. 1A). As shown in Fig. 1B, the ILK siRNA but not a control RNA (Fig. 1A) inhibited, in a dose-dependent manner, the expression of V5-epitope-tagged ILK transfected into HEK-293 cells. We next determined the ability of the ILK siRNAs, a control non-silencing siRNA and a siRNA directed against a sequence from calreticulin, to down-regulate the expression of endogenous ILK. As shown in Fig. 1C, transfection of one of the ILK-specific siRNAs but not the non-silencing control siRNA or the calreticulin siRNA resulted specifically in substantial knockdown of ILK expression in HEK-293 cells as determined by Western blot analysis. The expression of calreticulin protein was only down-regulated by the calreticulin siRNA, but this siRNA had no effect on ILK expression.

Transfection of the ILK-specific siRNA, which suppressed ILK expression, was the only siRNA that also suppressed phosphorylation of PKB/Akt on Ser-473 (Fig. 1C). To determine the specificity of the effect of down-regulating ILK expression on PKB/Akt phosphorylation, the extent of PKB/Akt phosphorylation of Thr-308 (in addition to phosphorylation of Ser-473) was determined by Western blotting using phosphospecific antibodies. As shown in Fig. 2A, whereas expression levels of actin and PKB/Akt, as well as the phosphorylation of PKB/Akt on Thr-308 were unchanged upon ILK knockdown, the phosphorylation of PKB/Akt on Ser-473 was dramatically and specifically suppressed concomitantly with ILK down-regulation. This effect was seen independently with two different ILK-specific siRNAs (Fig. 2A).

ILK has previously been shown to regulate the phosphorylation of Ser-9 of GSK-3β, as well as expression of cyclin D1 (19, 20). In addition, activated PKB/Akt also phosphorylates GSK-3β on Ser-9 (21). We therefore wanted to determine whether the siRNA-mediated down-regulation of ILK also inhibited the phosphorylation and expression of GSK-3β and cyclin D1, respectively. As shown in Fig. 2B, the phosphorylation of GSK-3β is also inhibited upon the knockdown of ILK expression, whereas the levels of GSK-3β protein remained unchanged. In addition, the expression levels of cyclin D1 are also significantly suppressed by the ILK siRNAs, compared with the control. At the time point at which phosphorylation of PKB/Akt and GSK-3β were analyzed, there was a clear effect on cell growth but no significant effect on cell adhesion as the cells remained attached and spread (data not shown). Inhibition of ILK activity has previously been shown to induce apoptosis and cell cycle arrest (12, 13, 15, 16).

Collectively, these data provide strong genetic support for previous findings that inhibition of ILK activity by dominant-negative ILK or highly selective small molecule ILK inhibitors inhibit PKB/Akt Ser-473 phosphorylation.

In addition to siRNA, we have used another approach for knocking out expression of ILK in mammalian cells. We have engineered pLox-ILK mice in order to knock out ILK expres-
Fig. 1. siRNA design and specificity. A, 21-base sequences of the human ILK gene specifically targeting the kinase domain (ILK-FSF) and the pH domain (ILK-H) were chosen. A non-silencing siRNA (control-NS), an inverted ILK-H siRNA (control-HC), and a siRNA targeting calreticulin (CRT) were used as controls. B, HEK-293 cells were co-transfected with wild-type ILK-V5 and the indicated siRNA. ILK-H siRNA prevented the expression of exogenous ILK-V5, as shown by Western blot analysis. C, HEK-293 cells were transfected with the indicated siRNA. Four days post-transfection, the specificity of each siRNA was assessed by Western blot analysis of the corresponding protein. ILK-H siRNA specifically inhibited endogenous ILK expression, and calreticulin siRNA only decreased calreticulin expression.

Fig. 2. Effects of ILK knockdown by siRNA on downstream ILK effectors. A, 4 days post-transfection of the indicated siRNA in HEK-293 cells, the phosphorylation status of PKB/Akt on both Ser-473 and Thr-308 was assessed. Knocking down ILK expression by siRNA significantly decreased phosphorylation on Ser-473, but Thr-308 phosphorylation and PKB/Akt protein expression were not affected. Numbers indicated below each band are the quantitative values from densitometric analyses. Data shown are representative of six independent experiments. B, in the same experimental conditions as above, suppression of ILK expression by siRNA inhibited phosphorylation of GSK-3β on Ser-9, but did not affect GSK-3β protein expression. Cyclin D1 expression was also decreased. Numbers indicated below each band are the quantitative values from densitometric analyses. Data shown are representative of four independent experiments.

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A.

| siRNA sequences | DNA target |
|-----------------|------------------|
| Sense UGU CAA GUU CUC UUC CAA AUG dTT | ILK kinase domain 1176-1194 |
| Sense CCC GAC GAA GCU CAA CGA GAA dTT | ILK pH domain 741-759 |
| Sense UUC UCG UUG AGC UUC GUC AGG dTT | Control-NS |
| Sense AAG AGC AAC UCG AAG CAG UCC dTT | Control-HC |
| Sense ACU GAG UGU GCC GGG ACA CAA dTT | Calreticulin (CRT) 590-610 |

B.

| siRNA (nM) | PKB/Akt | GSK-3 | Cyclin D1 | Actin |
|------------|---------|-------|------------|-------|
| 0          | 0.47    | 0.52  | 0.99       | 1.13  |
| 1          | 0.47    | 0.52  | 0.99       | 1.13  |
| 10         | 0.47    | 0.52  | 0.99       | 1.13  |
| 100        | 0.47    | 0.52  | 0.99       | 1.13  |

C.

| siRNA (nM) | PKB/Akt | GSK-3 | Cyclin D1 | Actin |
|------------|---------|-------|------------|-------|
| 0          | 0.47    | 0.52  | 0.99       | 1.13  |
| 1          | 0.47    | 0.52  | 0.99       | 1.13  |
| 10         | 0.47    | 0.52  | 0.99       | 1.13  |
| 100        | 0.47    | 0.52  | 0.99       | 1.13  |

Derived from the pLox-ILK mice, we isolated and immortalized macrophages from these mice as described under “Experimental Procedures.” The cells were then infected with adenoviral Cre-recombinase particles (22) to excise the ILK gene and eliminate its expression. PCR showed the expression of Cre-recombinase, as well as the excision of the Lox-P site from the floxed ILK gene (Fig. 3A). The expression of ILK protein was substantially inhibited in a dose-dependent manner 5 days post-infection of the Cre-recombinase (Fig. 3B). As shown in Fig. 3B, phosphorylation of PKB/Akt on Ser-473 was significantly inhibited concomitant with the knockdown of ILK expression in cells grown in the presence of 10% fetal bovine serum and 100 ng/ml CSF-1 (conditions which lead to PI3-kinase-dependent activation of PKB/Akt). Phosphorylation of PKB/Akt on Thr-308 was unaffected as were the expression levels of PKB/Akt and actin. No detectable phosphorylation of PKB/Akt on Thr-308 was detected in the absence of serum and CSF-1 in cells not infected with AdCre (data not shown). ILK expression was also unaffected under these conditions (data not shown). As shown in Fig. 3B, ILK knockdown also resulted in the inhibition of phosphorylation of GSK-3β on Ser-9 as well as cyclin D1 expression, whereas GSK-3β protein levels remained unchanged. A similar analysis in mammary epithelial cells derived from the pLox-ILK mice also resulted in inhibition of PKB/Akt Ser-473 phosphorylation after ILK deletion by CRE infection. Furthermore, as shown in Fig. 3C, both phosphorylation of PKB/Akt on Ser-473 and PKB/Akt kinase activity were substantially inhibited upon knockdown of ILK expression.

These data support results from the siRNA experiments and further demonstrate that ILK expression is essential for the specific phosphorylation of PKB/Akt on Ser-473 and for the regulation of PKB/Akt activity.

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Ser-473 inhibition was 83% of inhibition of ILK expression was 87.4% in four independent experiments (numbers staining in the same macrophages used in independent experiments). ILK resulted in a decrease of both PKB/Akt Ser-473 phosphorylation in ILK fl/fl macrophages, 5 days post-infection with AdCre. Excision of ILK does not have catalytic activity as determined by in vitro kinase assay of recombinant proteins (14). As shown in Fig. 4A, analysis of the phosphorylation of PKB/Akt Ser-473 relative to the amount of GFP expression demonstrates that ILK-WT was more efficient at rescuing the inhibition of phosphorylation of PKB/Akt than the kinase-inactive ILK-S343A mutant. Because the transfection efficiencies for each mutant varied (see “Experimental Procedures”), we quantified the ratio of Ser-473-PKB/Akt phosphorylation to GFP for each mutant transfected are shown. Data show that ILK-WT rescued Ser-473 phosphorylation by an average factor of 2.303 ± 0.315 compared with the ILK-S343A mutant. This difference is statistically significant (p < 0.05) (Fig. 4B). C, in an independent experiment, transfection of both ILK-WT and kinase-active S343D constructs rescued PKB/Akt Ser-473 phosphorylation, compared with kinase-inactive S343A. Numbers indicated below each band represent quantification by densitometric analysis. Data shown in Fig. 4 are representative of three separate experiments.

The macrophages in which ILK expression was depleted were observed to grow poorly compared with the control cells. Closer examination demonstrated that these cells were undergoing apoptosis as determined by annexin-V staining (Fig. 3D) as would be expected because of the inhibition of PKB/Akt activity.

The role of the kinase activity of ILK in its function has been questioned based on data showing that ILK kinase activity is not required for certain functions in C. elegans and Drosophila (23, 24). In addition, it has previously been suggested that ILK may regulate PKB/Akt phosphorylation indirectly (13). We therefore carried out rescue experiments to assess the role of the ILK kinase activity in the regulation of PKB/Akt Ser-473 phosphorylation. We co-transfected the macrophage cells from the pLox-ILK mice with expression constructs encoding wild-type ILK (ILK-WT) or kinase-inactive ILK-S343A mutant (14) and a GFP construct and then infected the cells with AdCre. We have previously demonstrated that the S343A mutant of ILK is required for PKB/Akt Ser-473 phosphorylation.
These results demonstrate that the regulation of the phosphorylation of PKB/Akt on Ser-473 by ILK is dependent on its kinase activity and suggest that ILK may phosphorylate this site directly. These data are in agreement with our previous biochemical data showing that ILK phosphorylates PKB/Akt in an in-gel kinase assay (14) and that highly specific small molecule inhibitors of ILK kinase activity inhibit PKB/Akt phosphorylation on Ser-473 (14, 16). It should be noted that although the kinase catalytic domain of ILK differs from other kinase domains, ILK has been demonstrated to behave as a bona fide serine-threonine kinase in the context of phosphorylation of other substrates besides PKB/Akt, such as myosin light chain, myosin phosphatase target subunit, and myosin phosphatase inhibitors, PH1-1 and CPI-17 (25–27).

Based on the extensive level of inhibition of phosphorylation of Ser-473 upon knockdown of ILK expression in the cell types analyzed, ILK either functions as a direct PKB/Akt Ser-473 kinase (or PDK-2), or it is a major cellular regulator of the putative PKB/Akt Ser-473 kinase activity identified recently in a high molecular weight plasma membrane lipid-raft complex (17). Thus, an alternative explanation of our results could be that Ser-343 of ILK is a phosphorylation site important for the recruitment of PKB/Akt. Although the phosphorylation of Ser-343 has not been demonstrated directly, we have previously shown that ILK and PKB interact and that substitution of ILK-Ser-343 with alanine results in the inhibition of interaction with PKB/Akt, whereas substitution with an aspartic acid (mimicking phosphorylation) maintains the interaction (14). Therefore, both ILK-WT and the ILK-S343D mutant can rescue PKB/Akt Ser-473 phosphorylation because they may promote both interaction and phosphorylation (either by ILK itself or by another kinase), whereas the ILK-S343A mutant can do neither.

A recent report further solidifies a role of ILK in the regulation of PKB/Akt-mediated suppression of apoptosis. It has been demonstrated (28) that peroxisome proliferator-activated receptor-β (PPARβ) modulates PKB/Akt activation via the specific transcriptional up-regulation of ILK and PDK-1, revealing a novel mechanism for the control of PKB/Akt signaling.

Collectively these data demonstrate an essential role for ILK in the regulation of phosphorylation of PKB/Akt on Ser-473 and GSK-3β on Ser-9, as well as in the regulation of expression of cyclin D1. In further support for this, the targeted knock-out of ILK in chondrocytes, by crossing the pLox-ILK mice with type-2 collagen-Cre mice, also results in the inhibition of PKB/Akt Ser-473 phosphorylation and suppression of expression of cyclin D1 in vivo in the chondrocytes of long bones.2

The data presented in this paper establish ILK as an important and essential physiological regulator of the phosphorylation of PKB/Akt on Ser-473 and PKB/Akt activation.

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Armelle A. Troussard, Nasrin M. Mawji, Christopher Ong, Alice Mui, René St.-Arnaud and Shoukat Dedhar

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