The Role of Integrin-Linked Kinase (ILK) In Cellular Signalling

By

Sarah Attwell

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Abstract

Integrins are a family of heterodimeric transmembrane receptors that link the cytoskeleton to the extracellular matrix, and mediate cell adhesion and bidirectional signalling. The Integrin-linked kinase (ILK) was identified as a protein capable of interacting with β1 and β3 integrin subunits. ILK behaves as a potent oncogene, and is capable of transforming normal epithelial cells and forming tumours in nude mice. ILK is a serine/threonine kinase which phophorylates and activates PKB/Akt at serine-473, and phosphorylates and inhibits GSK-3α/β at serine 21/9. When normal epithelial cells detach from the extracellular matrix, they undergo suspension-induced apoptosis, or anoikis. Because ILK links integrins to the anti-apoptotic PKB/Akt pathway, we investigated whether ILK elicits its oncogenic effects by inhibiting anoikis. Here, we show that ILK inhibits anoikis in a PKB/Akt-dependent manner. Furthermore, inhibition of ILK activity in cancer cell lines induced anoikis. In prostate cancer cells which are lacking expression of the upstream regulator of ILK, PTEN, inhibition of ILK activity, as well as re-introduction of PTEN induces cell cycle arrest and apoptosis. We also examined the role of ILK, and its interacting partners CH-ILKBP and paxillin, in focal adhesion formation and inside out signalling. We found that proper recruitment of and activation of ILK is crucial for β integrin activation, cell attachment, and migration, as well as the recruitment of its binding partners to focal adhesion complexes. The data presented here underscores the importance of ILK as a central regulator of the PKB/Akt pathway and anoikis, as well as integrin-mediated functions and focal adhesion formation. They also identify ILK as a potential novel target in tumour therapy.

Table of contents

List of tables

List of figures

Abbreviations used in this thesis

ANK ankyrin repeat
AP-1 activator protein-1
APC adenomatous polyposis coli
CDK cyclin-dependent kinase
CH calponin homology
CH-ILKBP calponin homology domain-containing ILK binding protein
CKI CDK inhibitor
COX-2 cyclooxygenase-2
CREB cyclic AMP-response element binding protein
ECM extracellular matrix
EMT epithelial to mesenchymal transition
ERK extracellular signal-regulated kinase
FADD Fas-associated death domain
FAK focal adhesion kinase
FRNK FAK-related non-kinase
GAP GTPase activating protein
GEF guanine nucleotide exchange factor
GSK-3 glycogen synthase kinase-3
IAP integrin associated protein
IKK I-kappa B kinase
ILK Integrin-linked kinase
ILKAP ILK-associated phosphatase
IGF insulin-like growth factor
IRS insulin receptor substrate
JNK Jun-NH_2-terminal kinase
LEF1/TCF lymphoid enhancer/T cell factor
LPS lipopolysaccharide
MAPKAP-K2 mitogen-activated protein kinase associated protein kinase 2
MAP kinase mitogen-activated protein kinase
MBP myelin basic protein
MLCP myosin light chain phosphatase
MMP matrix metalloproteinase
MMTV mouse mammary tumour virus
mTOR mammalian target of rapamycin
NF-kappa B nuclear factor-kappa B
NGF nerve growth factor
NOS nitric oxide synthase
PAK p21 activated kinase
PCNA proliferating cell nuclear antigen
PDK-1 phosphoinositide-dependent kinase-1
PH pleckstrin homology
PIF PRK-2-interacting fragment
PINCH particularly interesting new Cys-His protein

PIP phosphatidylinositol-4-phosphate
PIP2 phosphatidylinositol-4,5-P2
PIP3 phosphatidylinositol-3,4,5-P3
PI3 kinase phosphatidylinositol-3 kinase
PKB/Akt protein kinase B
$P70^{S6K}$ 70 kDa ribosomal S6 kinase
$P90^{rsk}$ 90 kDa ribosomal S6 kinase
PTEN phosphatase and tensin homolog deleted on chromosome 10
Rb retinoblastoma
RTK receptor tyrosine kinase
SGK serum and glucocorticoid-induced protein kinase
SH src homology
TGF βtransforming growth factor β
TM4 tetraspanin
VCAM-1 vascular cell adhesion molecule-1

Preface

The majority of work presented in this thesis has been published in the following manuscripts:

Attwell, S., Roskelley, C., and Dedhar, S. (2000) The integrin-liked kinase (ILK) suppresses anoikis. Oncogene *19*: 3811-3815

Persad, S., Attwell, S., Gray, V., Delcommenne, M., Troussard, A., Sanghera, J., and Dedhar, S. (2000) Inhibition of integrin-linked kinase (ILK) suppresses activation of PKB/Akt and induces cell cycle arrest and apoptosis of PTEN-mutant prostate cancer cells. Proc. Natl. Acad. Sci. USA 97: 3207-3212

Persad, S., Attwell, S., Gray, V., Mawji, N., Deng, J.T., Leung, D., Yan, J., Sanghera, J., Walsh, M.P., and Dedhar, S. (2001a) Regulation of protein kinase B/Akt-Serine 473 phosphorylation by integrin-linked kinase. J. Biol. Chem. *276*: 27462-27469

Attwell, S., Mills, J., Troussard, A., Wu, C., and Dedhar, S. (2003) Integration of cell attachment, cytoskeletal localization, and signalling by integrin-linked kinase (ILK), CH-ILKBP, and the tumor suppressor PTEN. Mol Biol Cell. *14*:4813-25

All results presented in this thesis were solely the work of S. Attwell, except for those described in figure 3-1 and 3-2, which were jointly the work of A. Troussard and S. Attwell, and that described in figure 3-5, which was jointly the work of J. Mills and S. Attwell.

Chapter 1. Introduction

Cell interaction with the extracellular matrix (ECM) is crucial for the regulation of cell survival, growth, differentiation and migration. Virtually every cell in the human body requires interaction with the ECM at some point in development, and disregulation of this interaction can cause defects in tissue development, and contributes to many human diseases, including cancer. Many transmembrane receptors are responsible for interacting with the ECM, including heparan sulphate proteoglycans, syndecans, and the hyaluronan binding molecule layillin (Zamir and Geiger, 2001). The major ECM receptors which are present in virtually all cell types however, are the integrins.

Integrins

Integrins are a large family of integral plasma membrane glycoprotein receptors which are the major receptors responsible for cell adhesion to the ECM (Hynes, 1987). Integrins provide a physical link between the ECM and the actin cytoskeleton, thus serving as bi-directional signalling molecules capable of regulating both intracellular (outside-in) and extracellular (inside-out) signalling (Hynes, 1992). Defects in integrin expression and function contribute to many human diseases, including cancer, genetic and autoimmune diseases, and targeted integrin knockout studies in various organisms and in tissue culture underscore the essential role that integrins play in development (Hynes, 1996, Hynes 2002). Integrin expression may be both negatively (in the case of the $\alpha 5\beta 1$ integrin) and positively (in the case of the $\alpha v\beta 3$ integrin) associated with transformation and tumour formation (Giancotti and Ruoslahti, 1990, Felding-

Habermann *et al,* 1992). Many intracellular signalling pathways are activated upon integrin-mediated cell adhesion and integrin clustering, and these pathways are vital in regulating such diverse processes as embryonic development, cell survival, cell cycle progression, growth, differentiation, motility, and gene expression (Hynes, 1992, Giancotti and Maniero, 1994, Clark and Brugge, 1995, Dedhar and Hannigan, 1996, Boudreau and Bissel, 1998). Both receptor clustering and ligand occupancy are critical for the activation of intracellular integrin-mediated responses (Miyamoto *et al,* 1995). The diverse integrin-activated signalling pathways which regulate these processes include the activation of tyrosine and serine/threonine protein kinase cascades, induction of calcium and hydrogen transients via activation of calcium channels and the Na^+/H^+ antiporter, stimulation of lipid metabolism and generation of lipid second messengers, and organization of the actin cytoskeleton (Dedhar, 1999). Integrins may also be converted from an inert to an active state in response to intracellular signals, termed "inside-out" signalling. This regulation can then modulate the adhesive affinity of integrins to the extracellular matrix, and can activate ECM-degrading enzymes (Brakebusch *et al*, 2002). In addition to ECM and cytoplasmic proteins, integrins also associate with several membrane proteins, including IAP (Integrin Associated Protein), caveolin, and the tetraspanins (TM4) (Schwartz, 2001). The significance of these interactions is unclear, although evidence points to a possible adaptor function, as TM4 cytoplasmic domains can associate with signalling proteins such as protein kinase C, and caveolin links to the Shc-Src-Ras pathway (Schwartz, 2001).

Integrins are composed of an alpha and a beta subunit, each consisting of a long extracellular domain and a generally short intracellular domain, and are found in all metazoan species (Whittaker and Hynes, 2002). In mammals, there has been identified 8 beta and 18 alpha subunits, known to assemble into 24 distinct integrins. Integrins vary according to their substrate, such as the collagen, laminin or RGD receptors, or expression on certain cell types, such as the leukocyte specific receptors (Hynes, 2002). The most common integrins found in focal adhesions are $\alpha 5\beta 1$, the classical fibronectin (RGD) receptor, and $\alpha v\beta 3$, the vitronectin receptor (Geiger *et al,* 2001). Many integrins are not constitutively active, and can regulate the affinity by which they bind their ligand. This activation may be controlled by intracellular signals, and is very important in cell types such as platelets, which must bind to fibrinogen in response to injury (Brakebusch *et al,* 2002). It is thought that the high affinity conformation of integrins is induced by the binding of intracellular molecules to the cytoplasmic domains of the integrin alpha and beta subunits, and requires Mn^{2+} (Mastrangelo *et al,* 1999). The efficiency of cell attachment can also be regulated by the clustering of integrins, which leads to increased avidity. These clusters are found in points of cell contact termed focal contacts. Upon integrin engagement with the ECM, a large group of intracellular structural and signalling molecules is recruited to the integrin cytoplasmic domains, leading to the formation of actin stress fibers (Burridge and Chrzanowska-Wodnicka, 1996, Calderwood *et al,* 2000). These large complexes are termed focal adhesions. Stable and dynamic focal adhesions play a vital role in the regulation of actin organization, thus affecting cell spreading,

morphogenesis and migration, processes which play key roles in metastasis, angiogenesis, and development.

Over 20 structural and signalling focal adhesion proteins are known to interact directly with the cytoplasmic domains of integrins (Hemler, 1998). Structural cytoskeletal proteins such as talin, filamin, F-actin, paxillin, and α-actinin are capable of binding directly to integrins (Horwitz *et al,* 1986, Sharma *et al,* 1995, Kieffer *et al,* 1995, Tanaka *et al,* 1996, Otey *et al,* 1993). Talin and α-actinin then, in turn, associate with other focal adhesion proteins, such as vinculin, tensin and actin, through interactions with their protein-protein binding domains. Thus, a complex network of proteins is responsible for linking integrins to the actin cytoskeleton. Many structural proteins act as scaffolding proteins for signalling pathways, bringing signalling proteins such as phosphatidylinositol-3 kinase (PI3 kinase) and extracellular signal-regulated kinase (Erk1/2) to focal adhesions and thus mediating the initiation of cell attachment-induced signalling cascades (Brakebusch and Fassler, 2003). Many of these pathways, for example the Rho GTPase pathway, require the coordinated action of both integrin and growth factor mediated signalling pathways for full activation. In fact, integrin activation and clustering often involves co-clustering with growth factor receptors such as the insulin, platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) receptors (Giancotti and Ruoslahti, 1999). Two calcium binding proteins, CIB and calreticulin, have been shown to interact with integrin α subunits, and may mediate integrin-dependent calcium influxes (Naik *et al,* 1997, Coppolino *et al,* 1997). Catalytic proteins such as the tyrosine kinase Focal Adhesion Kinase (FAK) (Schaller *et al,* 1995)

are also associated with integrins although this interaction may be indirect. FAK, in turn, binds to proteins such as talin, paxillin, p130Cas, Src, and Guanine nucleotide exchange factors (GEFs) (Brakebusch and Fassler, 2003, Giancotti and Ruoslahti, 1999, Schwartz *et al,* 2001). In summary, integrin activation, due to cell attachment to an extracellular matrix substrate and integrin clustering, initiates the formation of focal adhesions and the reorganization of the actin cytoskeleton, and activates many diverse intracellular signalling processes.

Integrin-Linked Kinase (ILK)

Integrin-linked kinase (ILK) was identified by yeast-two hybrid screen as a protein capable of interacting with the cytoplasmic domain of β1 and β3 integrins (Hannigan *et al,* 1996). The ILK gene encodes a serine/threonine protein kinase located on human chromosome 11p15.5-p15.4 (Hannigan *et al,* 1997). ILK is highly evolutionarily conserved, with homologues identified in human, mouse, rat, *D. melanogaster,* and *C. elegans* (Hannigan *et al,* 1996, Li *et al,* 1997, Nikolopoulous and Turner, 2001, MacKinnon *et al,* 2002, Zervas *et al,* 2001). The mouse and human ILK genes are 99% identical, and both contain 451 amino acid residues and have an apparent molecular mass of 59 KDa (Hannigan *et al,* 1996, Li *et al,* 1997). Structurally, ILK is comprised of four ankyrin repeats, involved in protein-protein interactions at the extreme N terminus (see figure 1). This ANK domain appears to be similar in sequence to several recently solved protein structures, and is thus predicted to be a cupped hand structure consisting of an antiparallel β-sheet (fingers), and α-helix bundles (palm) (Wu, 1999, Sedgwick and Smerdon, 1999). Immediately following the ANK domain is a sequence

which bears resemblance to a phosphoinositide-binding motif normally found in pleckstrin homology (PH) domains, which are usually involved in phospholipid binding. Finally, a serine/threonine kinase catalytic domain is located at the C-terminus, which is predicted to fold into the bi-lobate structure that is characteristic of other kinase domains (Wu, 1999, Dedhar *et al,* 1999). Sequence analysis of ILK's kinase domain reveals divergence at three generally highly conserved subdomains. In the highly conserved GXGXXG sequence of subdomain I, ILK is missing 2 of the 3 critical glycine residues, resulting in NENHSG. ILK's sequence also shows variance in subdomains VIB (HRDL), and VII (DFG), raising questions about ILK's identity as a bona fide protein kinase. However, the ILK sequence does not show any variance in the crucial subdomain VIII APE sequence, or in the critical lysine residue between subdomain I and II which is necessary for the binding of ATP. Furthermore, other proteins lacking all three glycine residues in subdomain I have been identified as true protein kinases (Dedhar *et al,* 1999).

Co-immunoprecipitation and immunostaining confirm ILK's interaction with integrin β1 and β3 subunits, and the integrin interacting domain of ILK was found to be located at the extreme C-terminal end of ILK, within the last two subdomains of the kinase catalytic domain (Hannigan *et al,* 1996). ILK was found to co-localize with the β1 integrin at focal adhesion sites, but interestingly, not at cell-cell adhesion sites, which also contain high levels of the β1 integrin (Li *et al,* 1998). ILK is expressed in virtually all mammalian cell types, with the highest expression found in cardiac and skeletal muscle.

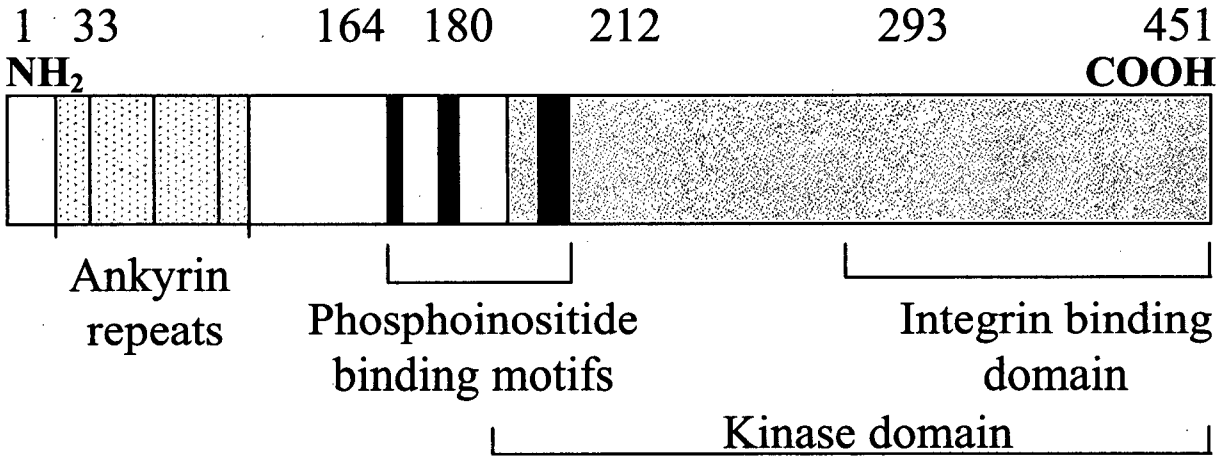


Figure 1-1. Structure of Integrin-Linked Kinase (ILK). The ILK protein is comprised of 451 amino acids, and contains 4 ankyrin repeats at the NH_2 terminus, followed by central phosphoinositide binding motifs, which partially overlap the COOH terminal serine/threonine kinase domain. The integrin binding domain of ILK is located at the extreme COOH terminus, within the kinase domain.

ILK kinase activity

Immunoprecipitated ILK is capable of serine/threonine phosphorylation of exogenous substrates such as myelin basic protein (MBP) *in vitro,* as well as phosphorylating a β1 integrin cytoplasmic domain peptide (Hannigan *et al,* 1996). Furthermore, a purified recombinant GST-ILK fusion protein can directly phosphorylate MBP, as well as ILK itself (autophosphorylation), protein kinase B (PKB/Akt), which ILK phosphorylates and activates at serine-473, and glycogen synthase kinase-3 (GSK-3), which ILK phosphorylates and inhibits at serine 21/9 (Hannigan *et al,* 1996, Delcommenne *et al,* 1998, Persad *et al,* 2001a). Other physiologically important substrates which recombinant ILK can phosphorylate *in vitro* are myosin light chain, and the adaptor protein affixin (Deng *et al,* 2001, Yamaji *et al,* 2001). Further evidence that ILK is a true kinase was the finding that substitution of Glu359, within the catalytic

subdomain VIII, with a lysine residue inhibits the kinase activity (Novak *et al,* 1998, Wu *et al,* 1998). ILK activity is PI3 kinase dependent, and can be temporarily activated both by integrin engagement and insulin stimulation. It is likely that the PH-like domain of ILK plays a role in its activation through PI3 kinase, as PH domains are known to bind PIP3, a product of PI3 kinase, and this phospholipid is able to stimulate ILK kinase activity *in vitro* (Delcommenne *et al,* 1998).

ILK function

Initial studies showed that ILK appears to function as a proto-oncogene, causing anchorage independent growth in soft agar (Hannigan *et al,* 1996), tumorigenicity in nude mice, cell cycle progression, and an epithelial to mesenchymal transition, characterized by enhanced fibronectin matrix assembly, increased invasion of extracellular matrices, loss of expression of keratins 14 and 18, and increased expression of vimentin (Radeva *et al,* 1997, Wu *et al,* 1998, Somasiri *et al*, 2001). These characteristics are likely due to the loss of E-cadherin expression, which is a hallmark of invasion and metastasis, as re-introduction of E-cadherin rescues the mesenchymal phenotype. Increased invasiveness of ILK-overexpressing cells is also due in part to the stimulation of invasion-related gene expression, such as the matrix metalloproteinase MMP-9 (Troussard *et al,* 2000).

In vitro studies performed to elucidate ILK function have employed 4 major methods: stimulation of ILK activity through overexpression of exogenous ILK and activation by growth factors and extracellular matrices; inhibition of ILK activity through dominant-negative ILK mutants; small molecule ILK inhibitors; and most recently, inhibition of ILK expression through small, interfering siRNA. The dominant-negative

form of ILK (ILK-E359K) contains a point mutation in the highly conserved subdomain VIII of the kinase domain, which abrogates much of the kinase activity and confers a dominant-negative phenotype (Wu *et al,* 1998, Persad *et al,* 2001a). Drug inhibition was achieved with the small molecule ATP-analog ILK inhibitor KP-392 (formerly KPSD1), which has previously been reported to inhibit ILK kinase activity in a highly selective, dose-dependent manner (Persad *et al,* 2000, Persad *et al,* 2001a, D'Amico *et al,* 2000, Troussard *et al,* 2000, Tan *et al,* 2002, Yoganathan *et al,* 2002, Mills *et al,* 2003, Cruet-Hennequart *et al,* 2003). KP-392 was identified in a high throughput kinase activity screen using active recombinant ILK (Persad *et al,* 2001a). These studies reveal critical roles for ILK in the downstream PKB/Akt and GSK-3 signalling pathways, as well as support the findings of *in vivo* studies that identify ILK as an important proto-oncogene (see figure 1-2 for a summary of ILK signalling).

ILK and Human Disease

ILK activity and expression levels have been found to be upregulated in several types of human cancer, including Ewing's sarcoma and primitive ectodermal tumours (Chung *et al,* 1998), and familial and sporadic colon carcinoma (Marotta *et al,* 2001, Marotta *et al* 2003). Ito and co-workers (2003) have recently reported high ILK mRNA expression in 63% of gastric carcinoma tumour samples, compared with 0% of control tissue. Furthermore, levels of ILK expression were found to correlate positively with invasion and metastasis, suggesting that ILK may serve as a novel marker for aggressive gastric cancer. ILK expression has also been demonstrated to correlate positively with

grade, and inversely with patient survival rates, in human prostate cancer samples (Graff *et al,* 2001). ILK upregulation has also been observed in response to ionizing radiation of lung cancer cells, hypoxia of hepatocellular carcinoma cells, and hyperthermia of prostate cancer cells (Cordes *et al,* 2002, Scandurro *et al,* 2001, Zhang *et al,* 2003) suggesting that ILK plays a role in tumour cell survival. In an asbestos-induced murine carcinogenesis model, ILK was one of several oncogenes found to be upregulated (Sandhu *et al,* 2000). The potent oncogene erbB-2, which constitutively activates growth factor signalling pathways, was found to also induce upregulation of ILK expression in the hyperplastic epidermis (Xie *et al,* 1998). ILK expression is correlated with melanoma progression and is inversely correlated with patient survival (Dai *et al,* 2003). Transforming growth factor-β (TGF-β)-induced transformation of human melanoma cells is accompanied by increased ILK expression, loss of E-cadherin, and nuclear translocation of beta-catenin, similar to the results seen when ILK is overexpressed in epithelial cells (Janji *et al,* 1999). Lastly, a role for ILK has been reported in ovarian cancer. αvβ3 and αvβ5 integrins, expressed on the surface of ovarian cancer cell lines, were shown to activate cell proliferation in an ILK, PKB/Akt, and $p27^{kip1}$ –dependent manner (Cruet-Hennequart *et al,* 2003). Both inhibitors of ILK and αv integrin neutralizing antibodies are able to block this proliferation. Furthermore, ILK expression has been reported to increase with ovarian tumour grade, and this expression appears to be sustained by peritoneal fluid (Ahmed, *et al,* 2003).

ILK may play a role in the progression of non-cancerous diseases as well. ILK expression and activity are upregulated in diabetic kidneys (Guo *et al,* 2001), and ILK

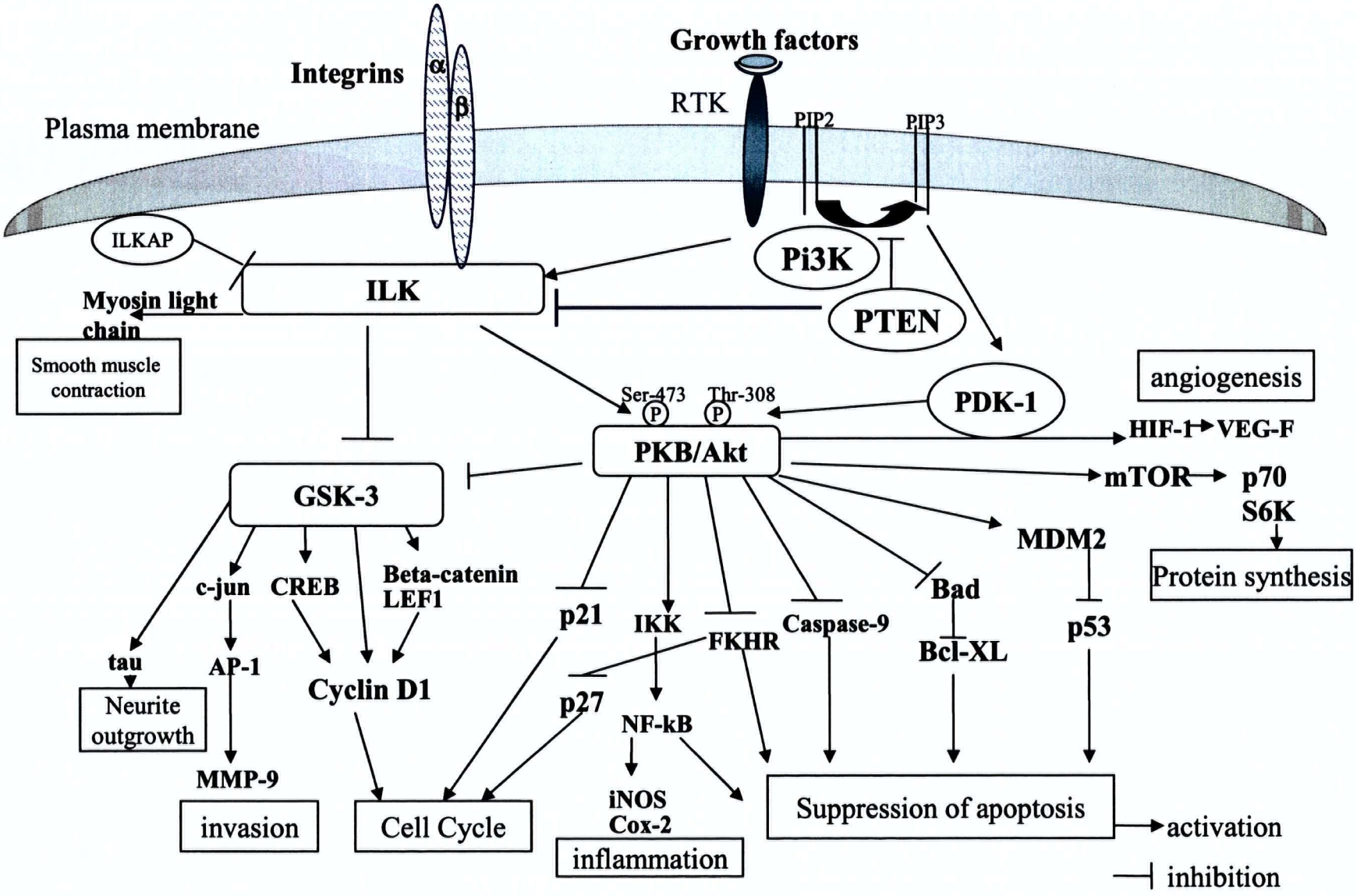


Figure 1-2. Summary of ILK signalling pathways.

was identified in an expression screen for glomerular filtration barrier disorder. It was then shown that ILK expression and activity are induced by murine kidney podocyte damage *in vitro* (Kretzler, 2001). ILK has also been demonstrated to play an important role in mediating tubular epithelial to mesenchymal transition induced by TGF-beta1 (Li *et al,* 2003).

In Vivo functional studies

Several *in vivo* studies have also been performed to elucidate the function of ILK. Overexpression of ILK in the mammary epithelium under control of the mouse mammary tumour virus promoter (MMTV/ILK) caused an initial development of benign hyperplasia, followed by the subsequent development of focal mammary tumours (White *et al,* 2001). Although the mammary tumours displayed a variety of phenotypes, ranging from papillary adenocarcinoma to spindle cell tumours, most exhibited some degree of epithelial to mesenchymal transformation, consistent with the *in vitro* studies. Further supporting the *in vitro* studies, these mice also displayed increased phosphrylation of PKB/Akt at serine 473 and GSK-3 at serine 21/9.

ILK mutant studies have been performed in both *D. melanogaster* and *C. elegans* (Mackinnon *et al,* 2002, Zervas *et al,* 2001). In *C. elegans,* pat-4 was identified as the sole homolog of ILK. In pat-4 null mutants, embryonic muscle cells form integrin foci, but fail to recruit the focal adhesion analog proteins vinculin, UNC-89, and actin and myosin filaments. In *D. melanogaster,* mutations in ILK cause embryonic lethality and defects in muscle attachment, and clones of cells lacking ILK in the adult wing fail to adhere, forming wing blisters. The ILK mutant phenotype is very similar to the integrin

mutant phenotype, although the muscle detachment in the ILK mutant is due to the detachment of actin filaments, while the integrin mutants display a primary defect in the attachment of the plasma membrane to the ECM. Interestingly, ILK mutants do not display the same defect in cuticle formation seen in PKB/Akt and beta-catenin mutants. The phenotypes of both of these mutants highlight the importance of ILK in integrin-mediated adhesion, while raising questions as to its importance as a kinase. ILK was shown to be crucial for integrin-mediated cell adhesion in muscles and epithelia, and its loss displayed a phenotype completely included within the set of defects found in the fly and worm integrin mutants. This suggests that the primary role of ILK is to contribute to integrin function. Surprisingly, mutations thought to be crucial to the kinase function of ILK had no phenotype in *C. elegans* or *Drosophila*, suggesting that the kinase activity of ILK is not important in its function. This result could mean that in *C. elegans* and *Drosophila,* ILK functions primarily as an adaptor protein, or that the kinase function of ILK in these species is more subtle. It also could be due to the nature of the ILK mutants tested. The ILK E359K mutant has been shown to retain some kinase activity (Persad *et al,* 2001), and is believed to function as a dominant-negative, affecting native ILK function when introduced to cultured cells *in vitro.* Therefore, in ILK-null *Drosophila* and *C. elegans* mutants, ILK E359K cannot elicit its dominant negative effects, and the small amount of ILK kinase activity left may be enough to rescue the null phenotype. The other mutants tested in these studies (ILK K220M and ILK P358S) have not been demonstrated to eliminate ILK kinase activity.

Recently, it has been reported that mice lacking ILK expression die at the peri-implantation stage because they fail to polarize their epiblast and to cavitate (Sakai *et al,* 2003). This failure to polarize appeared to be a result of abnormal F-actin accumulation at sites of integrin attachment to the basement membrane. Fibroblasts were obtained from the embryos, and these also displayed abnormal F-actin accumulation, impaired cell spreading, proliferation, and formation of focal adhesions. Interestingly, these impairments appeared to be PKB/Akt and GSK-3 phosphorylation-independent. Two groups recently reported the phenotype of a chondrocyte-specific ILK knockout in mice (Terpstra *et al,* 2003, Grashoff *et al,* 2003). Both reported that mice lacking ILK in their chondrocytes develop chondrodysplasia, characterized by a disorganized growth plate, dwarfism, and early death. ILK-deficient chondrocytes showed decreased proliferation, adhesion, spreading, and focal adhesion formation. However, the groups differed on the dependence of PKB/Akt and GSK-3 phosphorylation, and thus the importance of ILK's signalling role, versus its role as an adaptor protein, in chondrocytes. Reduced cyclin D1 expression was observed in the chondrocytes however, suggesting that signalling functions are disrupted.

The PI3 Kinase PKB/Akt pathway

The PI3 kinase- PKB/Akt signal transduction pathway has been studied extensively for its role in oncogenic transformation, cell survival, cell cycle progression, and regulation of transcription factors. Several components of this pathway are disregulated in many tumour types (Vivanco and Sawyers, 2002). The class 1A subgroup of PI3 kinases, the subgroup discussed here, are activated by growth factor receptor

tyrosine kinases. PI3 kinase is a phospholipid kinase comprised of a regulatory 85 kDa subunit, and a 110 kDa catalytic subunit. PI3 kinase can be activated by the binding of the p85 regulatory subunit directly to stimulated growth factor receptor tyrosine kinases, either through SH2 domain interactions, or via adaptor proteins such as insulin receptor substrate (IRS-1). This then allows the catalytic p110 subunit to phosphorylate its primary substrate, membrane-bound PtdIns(4,5)P2 (PIP2), converting it to PtdIns(3,4,5)P3 (PIP3). PtdIns-4-P (PIP) is also a minor substrate of PI3 kinase, although PIP2 is primarily produced by phosphatases such as SHIP. The production of PIP3 is very tightly controlled, and levels are virtually undetectable in unstimulated mammalian cells. However, within seconds to minutes after stimulation with growth/survival factors such as PDGF, nerve growth factor (NGF), insulin, or insulin-like growth factor (IGF), PI3 kinase is activated and phosphoinositides are produced (Cantley and Neel, 1999). This signalling is quickly downregulated, however, and this tight control is due in part to PTEN.

PTEN

The lipid and protein phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10, also termed MMAC1 or TEP1) serves to decrease levels of PIP2 and PIP3 in the cell by dephosphorylating at the 3' position of the inositol ring, thus negatively regulating the PI3 kinase- PKB/Akt pathway (Stambolic *et al,* 1998). In addition to phospholipids, PTEN has been demonstrated to de-phosphorylate proteins such as FAK, although the *in vivo* significance of this finding requires further investigation (Tamura *et al,* 1998, Vivanco and Sawyers, 2002). PTEN is a potent

tumour suppressor, in fact, the knockout causes a cancer phenotype in mice, represented in humans by the inherited cancer syndrome Cowden's disease (Yamada and Araki, 2001). Furthermore, loss of PTEN occurs in the majority of glioblastomas, and in more than 60% of advanced prostate cancers (Tamura *et al,* 1999). These findings underscore the importance of the PI3 kinase- PKB/Akt pathway in cancer.

PKB/Akt

The production of PIP2 and PIP3 then results in the recruitment of PKB/Akt to the plasma membrane. The N-terminal domain of PKB/Akt acts as an autoinhibitory domain. This domain has a high affinity for membrane-bound PIP2 and PIP3, and binding to these phosphoinositides localizes PKB/Akt to the plasma membrane, inducing a conformational change which exposes the activation loop of PKB/Akt (which encompasses the threonine-308 site). Studies have shown that membrane targeting of PKB/Akt is sufficient for its activation (Andjelkovic *et al,* 1997).

PKB/Akt is a serine/threonine kinase with a wide variety of substrates involved in cell proliferation, survival, and growth. PKB/ Akt was originally identified as the cellular counterpart to the viral oncogene v-akt, and has now been shown to be overamplified in a variety of human cancers (Staal, 1987, Hill and Hemmings, 2002). PKB/Akt belongs to the family of structurally similar AGC kinases (Scheid and Woodgett, 2003), a super family of protein kinases which are often de-regulated in disease and oncogenesis. As with other AGC kinases, PKB/Akt is regulated by upstream second messengers and secondary, activating kinases. There are three isoforms of PKB/Akt (α, β, γ), and all are composed of an N-terminal PH domain, a central kinase domain, and a C-terminal

hydrophobic regulatory domain (Hill and Hemmings, 2002). For full activation, PKB/Aktα (the most abundant isoform) requires phosphorylation at threonine 308, in the activation loop of the kinase domain, and serine 473, in the C-terminal hydrophobic regulatory domain (Alessi *et al,* 1996).

PDK-1

The kinase which phosphorylates PKB/Akt at threonine 308 is 3-phosphoinositide-dependent kinase-1 (PDK-1), another PH domain-containing serine-threonine kinase (Alessi *et al,* 1997a) which binds strongly to PIP2 and PIP3 and thus is recruited to the plasma membrane in response to growth factor stimulation. In addition to PKB/Akt, PDK-1 also phosphorylates the activation loop of other AGC family kinases, including the 70 kDa ribosomal S6 kinase ($p70^{s6k}$), 90 kDa ribosomal S6 kinase ($p90^{RSK}$), serum and glucocorticoid-induced protein kinase (SGK), and protein kinase C isoforms (Alessi, 2001).

PDK-2

While the role of PDK-1 in phosphorylating PKB/Akt at threonine 308 has been well established, the identity of the kinase which phosphorylates serine 473, termed PDK-2, has been more controversial. Early candidates for PDK-2 included MAPKAP-K2 (mitogen-activated protein kinase associated protein kinase 2), and mTOR (mammalian target of rapamycin), although neither have been shown to regulate serine-473 phosphorylation *in vivo.* Although it has been widely shown that ILK is capable of stimulating serine 473 phosphorylation, it has been theorized that this effect may be indirect (Lynch *et al,* 1999). It has also been suggested that autophosphorylation, or

PDK-1, is responsible for serine 473 phosphorylation (Toker and Newton, 1999, Toker *et al,* 2000, Hill *et al,* 2002, Balendran *et al,* 1999, Zervas and Brown, 2002). However, this is unlikely due to the findings that serine 473 phosphorylation still occurs (and is indeed elevated) in PDK-1 null cells (Williams *et al,* 2000), and after treatment with staurosporine, which inhibits PKB/Akt activity (Hill *et al,* 2001). ILK has been demonstrated to induce serine 473, but not threonine 308 phosphorylation of PKB/Akt, in a PI3 kinase dependent manner, in response to both fibronectin and insulin stimulation (Delcommenne *et al,* 1998). Recent evidence of ILK's role in serine 473 phosphorylation has been confirmed by ILK knock-down with RNAi (Troussard *et al,* 2003). Further evidence that ILK is a major effector of serine 473 phosphorylation is demonstrated by ILK's effect on the many downstream targets of PKB/Akt.

ILK and the PKB/Akt pathway

PKB/Akt phosphorylates a diverse range of substrates, such as I-kappaB kinase (IKK), nitric oxide synthase (NOS) and cyclic AMP-response element binding protein (CREB), which are activated by PKB/Akt phosphorylation, and Raf, BAD, procaspase-9, the forkhead family of transcription factors (FKHR/AFX/FOX), GSK-3, and p21, which are inhibited by PKB/Akt phosphorylation (Hill and Hemmings, 2002). Each of these substrates has been shown to play a role in cancer progression, as discussed below.

Anti-apoptotic targets

PKB/Akt is a potent inhibitor of apoptosis, or controlled cell death, an observation that can be partially explained by its regulation of the targets BAD, caspase-9, CREB, IKK and the forkhead transcription factors. Because ILK acts upstream of PKB/Akt, it is

likely that ILK regulates these pathways as well, and recently, several reports have confirmed ILK's anti-apoptotic role.

BAD is a pro-death member of the Bcl-2 family that initiates an apoptotic cascade by binding to Bcl-XL on the mitochondrial membrane, thus opening a channel and causing the release of cytochrome c into the cytosol (Datta *et al,* 1997). PKB/Akt blocks this cascade by phosphorylating BAD at serine 136, which promotes the association of BAD with 14-3-3, thus sequestering it in the cytosol. PKB/Akt may also inhibit apoptosis at a later step in this cascade, by the phosphorylation (at serine 196) and inactivation of the cell death protease procaspase-9 (Fujita *et al,* 1999). Members of the forkhead family of transcription factors are involved in the upregulation of a subset of pro-apoptotic factors such as p27 and Fas. PKB/Akt can inactivate the forkhead family of transcription factors by phosphorylation at multiple serine/threonine residues (Burgering and Kops, 2002, Brunet *et al,* 1999). Another transcription factor, CREB, is activated by PKB/Akt, thus increasing the expression of the anti-apoptotic genes Bcl-2 and mcl-1 (Du and Montminy, 1998). The nuclear factor-kappaB (NF-kappaB) is a transcription factor that is translocated into the nucleus, driving the up-regulation of, among many other genes, prosurvival factors such as Bcl-XL, caspase inhibitors, and c-Myb (Barkett and Gilmore, 1999, Lauder *et al,* 2001). PKB/Akt regulates this pathway by phosphorylating and activating IKK-α, which then, in turn, phosphorylates I-kappaB, targeting it for degradation. NF-kappaB is then released, allowing its translocation to the nucleus and thus the upregulation of survival genes (Kane *et al,* 1999). ILK has also been shown to play a role in NF-kappaB activation in macrophages, as it is capable of

stimulating, in an NF-kappaB and PKB/Akt dependent manner, the upregulation of inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2), in response to lipopolysaccharide (LPS) stimulation. This implicates ILK in inflammatory processes; however, it is also possible that ILK may be involved in NF-kappaB's prosurvival functions. PKB/Akt also prevents apoptosis through the inhibition of the pro-apoptotic tumour suppressor p53. This is achieved through PKB/Akt's phosphorylation of MDM2, which binds to p53, and is thought to enhance its degradation (Mayo and Donner, 2001). PKB/Akt was recently shown to promote survival by transcriptional upregulation of PPARβ, and this control was found to be due, at least in part, to the transcriptional upregulation of ILK (Di-Poi *et al,* 2002).

Cell Growth

A protein that is emerging as a crucial regulator of cell growth is mTOR, a serine/threonine protein kinase that regulates the rate of protein synthesis according to the availability of nutrients. mTOR activates the p70 S6 kinase (RSK), which enhances translation of mRNAs. mTOR is directly phosphorylated by PKB/Akt, and its activity is PI3 kinase dependent, however, it is unclear how the phosphorylation of mTOR induces its activation (Nave *et al,* 1999).

Proliferation targets

Progression through the cell cycle is tightly coordinated by a number of proteins, including cyclins and cyclin –dependent kinases (Cdks). Cyclins are nuclear proteins that are transiently expressed in order to initiate progression into a phase of the cell cycle. Cdks are serine/threonine kinases that are activated by their respective cyclins. Cdks

then, in turn, phosphorylate key proteins such as Retinoblastoma (Rb), a necessary step in the transition of cells from the G1 to the S phase of the cell cycle. The activity of Cdks are inhibited by Cdk inhibitors (CKIs) (Chang *et al,* 2003). PKB/Akt regulates the activity of at least 2 CKIs: $p21^{Cip1/WAF1}$, and $p27^{kip1}$. $P21^{Cip1/WAF1}$ exists in a complex with a cyclin, a Cdk, and proliferating cell nuclear antigen (PCNA), and this binding directly prevents PCNA's activation of DNA polymerase activity (Waga *et al,* 1994). PKB/Akt phosphorylates $p21^{Cip1/WAF1}$ at threonine 145, causing the release of PCNA and Cdk2 and the induction of DNA synthesis. $P27^{kip1}$ is also regulated by PKB/Akt, in this case indirectly. PKB/Akt decreases transcription of $p27^{kip1}$ by phosphorylating and inactivating Forkhead family transcription factors (Medema *et al,* 2000).

PKB/Akt is also capable of activating the hypoxia-inducible factor HIF-1-alpha, which then, in turn, activates the vascular endothelial growth factor VEGF (Mazure *et al,* 1997). The production of VEGF in cancer cells can contribute to angiogenesis, or the growth of new blood vessels which supply the tumour with oxygen and nutrients. Without angiogenesis, the tumour is starved of oxygen and is unable to grow, and thus, anti-angiogenic drugs show much promise as cancer therapeutic agents. Recently, it has been shown that constitutive activation of ILK results in the expression and secretion of VEGF, and that inhibition of ILK activity downregulates this secretion (Tan *et al,* 2004).

Glycogen synthase kinase-3β (GSK-3β)

GSK-3β, and the minor isoform GSK-3α, were the first identified substrates of PKB/Akt (Cross *et al,* 1995). GSK-3 regulates both glucose metabolism and cell

proliferation. PKB/Akt phosphorylates GSK-3β on serine 9, (and GSK-3α on serine 21) which results in its inactivation. This then results in the activation of several proteins, including cyclin D1, beta-catenin, and the activator protein (AP-1) transcription factor. Interestingly, several studies have shown that ILK also affects these pathways, not only through its activation of PKB/Akt, but through its direct phosphorylation and inactivation of GSK-3 at serine 9 (Delcommenne *et al,* 1998, Persad *et al,* 2001b, Troussard *et al,* 1999). Overexpression of ILK leads to the increased expression of several key components of the cell cycle machinery, including cyclin A, cyclin D1, and CDK4 proteins, and reduced the inhibitory activity of p27 (Radeva *et al,* 1997).

Beta-catenin has been identified as both a cytoplasmic protein which interacts with E-cadherin at the membrane, and a nuclear protein which interacts with the T cell factor (TCF) family of transcription factors, leading to the activation of target genes in response to Wnt signalling (Nusse, 1998, Novak and Dedhar, 1999). GSK-3 phosphorylates beta-catenin at multiple sites, resulting in its ubiquitination and degradation. Therefore, when GSK-3 is inactivated (through Wnt signalling), this results in the stabilization and nuclear localization of beta-catenin, which then associates with T-cell/lymphoid enhancer factor (Lef1/TCF) transcription factor to increase the expression of cell cycle progression genes such as cyclin D1, c-myc and matrilysin (Moon, *et al,* 2002, Hill and Hemmings, 2002). Disregulation of the Wnt signalling pathway often contributes to oncogenesis. GSK-3 also affects cyclin D1 directly by phosphorylating it at threonine 286, which results in its ubiquitination and subsequent degradation. Inactivation of GSK-3 by PKB/Akt prevents this phosphorylation, thereby increasing

at threonine 286, which results in its ubiquitination and subsequent degradation. Inactivation of GSK-3 by PKB/Akt prevents this phosphorylation, thereby increasing cyclin D1 levels (Diehl *et al,* 1998). Cell attachment to the extracellular matrix induces beta-catenin translocation and cyclin D1 expression, and this was found to be ILK and GSK-3 dependent (Novak *et al,* 1998, Persad *et al,* 2001b). In addition, an up-regulation of LEF-1 expression was observed in ILK-overexpressing cells, resulting in beta-catenin/LEF1 complex formation and transcriptional activation. It is probable, however, that the ILK and GSK-3-dependent upregulation of cyclin D1 is primarily via GSK-3's phosphorylation of CREB, as activation of cyclin D1 by ILK overexpression was abolished by point mutation of the CREB binding domain in the cyclin D1 promoter (D'Amico *et al,* 2000). The finding that ILK is involved in the attachment-induced expression of cyclins was crucial to understanding the processes by which integrins and cell attachment are capable of regulating the cell cycle.

A component of the Wnt signalling pathway which negatively regulates beta-catenin-Lef1/TCF reporter activity, APC (Adenomatous Polyposis Coli), normally functions in binding beta-catenin and sequestering it in the cytosol. However, upon loss of APC (as seen in human colon carcinoma), beta-catenin is constitutively translocated into the nucleus. Interestingly, ILK activity is elevated in these APC-/- cells, and the inhibition of ILK activity resulted in a decrease in nuclear beta-catenin, an increase in GSK-3 activity, and a substantial inhibition of beta-catenin-Lef1/TCF-mediated transcriptional activity (Tan *et al,* 2001). This suggests a possible role for ILK downstream of APC, or perhaps a feedback loop mechanism of regulation.

GSK-3 also phosphorylates c-Jun, a component of the AP-1 transcription factor, rendering it inactive. Binding sites for AP-1 are found in the promoters of a wide variety of genes, including cell cycle regulators such as cyclin D1, and VEGF. ILK has been shown to contribute to this pathway; in response to cell adhesion, GSK-3 is phosphorylated in an ILK-dependent manner, thus activating AP-1 (Troussard *et al,* 1999). One of the consequences of ILK-induced upregulation of AP-1 activity appears to be the AP-1-mediated expression of the matrix metalloproteinase MMP-9 (Troussard *et al,* 2000). MMPs contribute to invasion and metastasis by degrading the extracellular matrix, and this finding may further explain ILK's oncogenic effects.

One of the consequences of ILK overexpression is the downregulation of the tumour suppressor E-cadherin (Novak *et al,* 1998, Somasiri *et al,* 2001), a transmembrane glycoprotein expressed on the surface of epithelial cells which is responsible for the formation of cell-cell adherin junctions (Thiery *et al,* 2002). Loss of E-cadherin expression is a critical step in the induction of an epithelial to mesenchymal transition (EMT), and is thought to be crucial in the process of invasion and metastasis. Loss of E-cadherin and EMT appears to be a consistent result of ILK overexpression in epithelial cells (Wu *et al,* 1998, Novak *et al,* 1998, Tan *et al,* 2001, Somasiri *et al,* 2001). Overexpression of ILK, but not dominant-negative ILK, was shown to result in the loss of E-cadherin expression, and the acquisition of a fibroblastic morphology (Novak *et al,* 1998). Furthermore, the inhibition of ILK activity in human cancer cell lines (in which E-cadherin levels are low), caused an induction of E-cadherin expression (Tan *et al,* 2001, Persad *et al,* 2001b). ILK overexpression also results in the increased production

of the ECM protein fibronectin (Wu *et al,* 1998), nuclear translocation of beta-catenin (Novak *et al,* 1998), downregulation of the epithelial markers cytokeratin18 and MUC1, and the upregulation of the mesenchymal markers LEF1 and vimentin, all hallmarks of EMT (Wu *et al,* 1998, Novak *et al,* 1998, Somasiri *et al,* 2001, Guaita *et al,* 2002). While it is unclear exactly how ILK represses E-cadherin, it appears to be at the level of transcription, as E-cadherin promoter activity is affected by ILK inhibition. Tan *et al* (2001) reported a concomitant increase of the E-cadherin transcriptional repressor snail upon ILK overexpression. It was however, unclear how ILK may control snail expression. Zeb-1 was recently identified as another transcriptional repressor of E-cadherin that is upregulated in cells overexpressing ILK, although this activation also appears to be indirect (Guaita *et al,* 2002).

Negative Regulation of ILK

PTEN

As mentioned previously, ILK activity is positively regulated by both PI3 kinase and PIP3. Therefore, the finding that the tumour suppressor PTEN antagonizes PI3 kinase by de-phosphorylating PiP3 (Stambolic *et al,* 1998) raised the possibility that ILK may be negatively regulated by PTEN. Indeed, it was found that prostate cancer cells which lack PTEN have constitutively high levels of ILK activity, resulting in the constitutive downstream activation of the PKB/Akt pathway (Persad *et al,* 2000). This relationship will be discussed in greater detail in chapter 2.

ILKAP

Recently, a protein phosphatase termed ILK associated phosphatase (ILKAP), was identified as a negative regulator of ILK activity (Leung-Hagesteijn *et al,* 2001). However, ILKAP appears to selectively inhibit ILK's phosphorylation of GSK-3 on serine 9, and has no effect on PKB/Akt serine 473 phosphorylation. This inhibition results in the repression of LEF-1/TCF reporter activity.

DOC-2 and SAP-1

Two other tumour suppressor genes, DOC-2 and SAP-1, have recently been identified as proteins which negatively regulate ILK activity (Wang *et al,* 2001, Takada *et al,* 2002). As is the case with PTEN, cells with inactivating mutations in DOC-2 and SAP-1 possess constitutively active ILK. SAP-1 (Stomach cancer Associated protein tyrosine phosphatase-1) a transmembrane phosphatase which is often deleted in stomach cancer, negatively regulates ILK and induces apoptosis in a PKB/Akt-PI3 kinase-dependent manner (Takada *et al,* 2002). DOC-2, which is often mutated in breast cancer, also negatively regulates ILK and induces anoikis, however, it is via a PKB/Akt-independent pathway (Wang *et al,* 2001).

Other Biological Roles of ILK

As mentioned previously, ILK is capable of phosphorylating a β1 integrin cytoplasmic peptide *in vitro* (Hannigan *et al,* 1996). While this finding has yet to be demonstrated *in vivo*, it is potentially an important role for ILK, as the β1 integrin requires threonine phosphorylation (at threonines 788-789) for its activation (Wennerberg *et al,* 1998), and this activation is essential for assembly of a fibronectin matrix, which ILK overexpression is known to induce (Wu *et al,* 1995, Wu *et al,* 1998).

ILK has recently been demonstrated to play a role in leukocyte recruitment: ILK activity in leukocytes is stimulated in response to the chemokine monocyte chemoattractant protein-1, and ILK overexpression in human monocytic cells diminishes firm adhesion to endothelial cells in a β1 integrin and vascular cell adhesion molecule-1 (VCAM-1)-dependent manner (Friedrich *et al,* 2002). These findings suggest that ILK plays a role in the signalling events involved in the modulation of integrin avidity of leukocytes in response to chemokines. This modulation is necessary to coordinate adhesion and transendothelial migration, a process important in immune system response. Another role for ILK in blood cell function has recently been identified in platelets. ILK has been demonstrated to regulate the avidity of integrin αIIbβ3, the major integrin involved in platelet aggregation, in a PI3 kinase-dependent manner (Pasquet *et al,* 2002). Chemical inhibition of PI3 kinase reduces binding to fibrinogen and platelet aggregation.

A role for ILK has also been described by Mills *et al* (2003) in NGF-stimulated neurite outgrowth. In rat pheochromocytoma cells and dorsal root ganglion neurons, both pharmacological and dominant negative inhibitors of ILK significantly reduced nerve growth factor-induced neurite outgrowth. This inhibition appears to be mediated through hyperphosphorylation of Tau, a substrate of GSK-3.

Perhaps most interestingly, a role for ILK has been identified in Ca2+-independent smooth muscle contraction (Deng *et al,* 2001). Microcystin-induced contraction of smooth muscle was found to correlate with phosphorylation of the myosin light chain at serine 19 and threonine 18 by a kinase distinct from myosin light chain kinase. This kinase was identified as ILK, and it was subsequently shown that ILK co-

localizes with myosin at the contractile machinery. These findings suggest a completely novel function for ILK, as well as confirming its identity as a true serine/threonine protein kinase. Further work by this group has determined that in addition to phosphorylating myosin, ILK is also capable of phosphorylating the myosin light chain phosphatase inhibitor protein CPI-17, and PHI-1, thus inhibiting myosin light chain phosphatase (MLCP) activity bound to myosin (Deng *et al,* 2002, Muranyi *et al,* 2002). This suggests that ILK may activate smooth muscle contraction by direct phosphorylation of myosin, or indirectly by phosphorylation and activation of CPI-17 and PHI-1, which leads to the inhibition of MLCP. Another phosphatase inhibitor target of ILK has since been identified: KEPI, a phosphorylation-dependent type-1 protein phosphatase inhibitor (Erdodi *et al,* 2003). ILK phosphorylates KEPI at threonine 73, dramatically increasing inhibition of the phosphatases PP1C and myosin phosphatase MPH. These results suggest a novel means by which ILK affects signalling pathways: by inhibiting protein phosphatases, as well as activating protein kinases. Huang *et al* (2000) also demonstrated a requirement for ILK in myogenic differentiation and myotube formation, with the finding that ILK activity and recruitment to focal adhesions is induced in response to myogenic differentiation, and that differentiation could be blocked by dominant negative mutants of ILK. These findings further underscore the importance of ILK in muscle cells.

ILK interactions

ILK was first identified based on the ability of its carboxy terminus to interact with the cytoplasmic domain of the β1 integrin (Hannigan *et al,* 1996), and has since

been demonstrated to localize with β1 integrins at focal adhesion plaques (Li *et al,* 1999). Since this discovery, ILK has been found to interact with several signalling and structural proteins, and is emerging as an important component of cell-ECM adhesion structures (see figure 1-3 for a summary of ILK interactions). Because genetic studies in *D. melanogaster* and *C. elegans* highlight the importance of ILK's role as an adaptor protein, the identification of ILK interacting proteins is currently an exciting area of research. It is possible that interactions with adaptor proteins are responsible for the regulation of ILK kinase activity, and thus downstream signalling.

The ILK-interacting protein that has been characterized in the most detail is the adaptor protein PINCH (Particularly Interesting New Cys-His protein). ILK complexes with PINCH through direct interaction of the ILK NH2-terminal ANK domain to the second zinc finger located within the LIM1 domain of PINCH (Li *et al,* 1999, Tu *et al,* 1999). PINCH is a LIM (cysteine-rich motif first identified in *C. elegans*, Lin-11, ISL-11, and Mec-3) domain only protein that provides a potential link between ILK and growth factor receptor tyrosine kinases (RTKs) and PI3 kinase. The PINCH LIM4 domain binds directly to the third SH3 (Src homology) domain of the NCK-2 adaptor protein, which in turn binds directly to phosphotyrosine residues on the cytoplasmic domain of ligand-activated RTKs. NCK-2 also binds the Insulin Receptor Substrate (IRS), which binds to PI3 kinase (Tu *et al,* 1998). These findings provide a possible explanation for the activation of ILK by both integrins and RTKs. It is known that integrin and RTK clustering occurs upon cell stimulation; ILK, PINCH and NCK-2

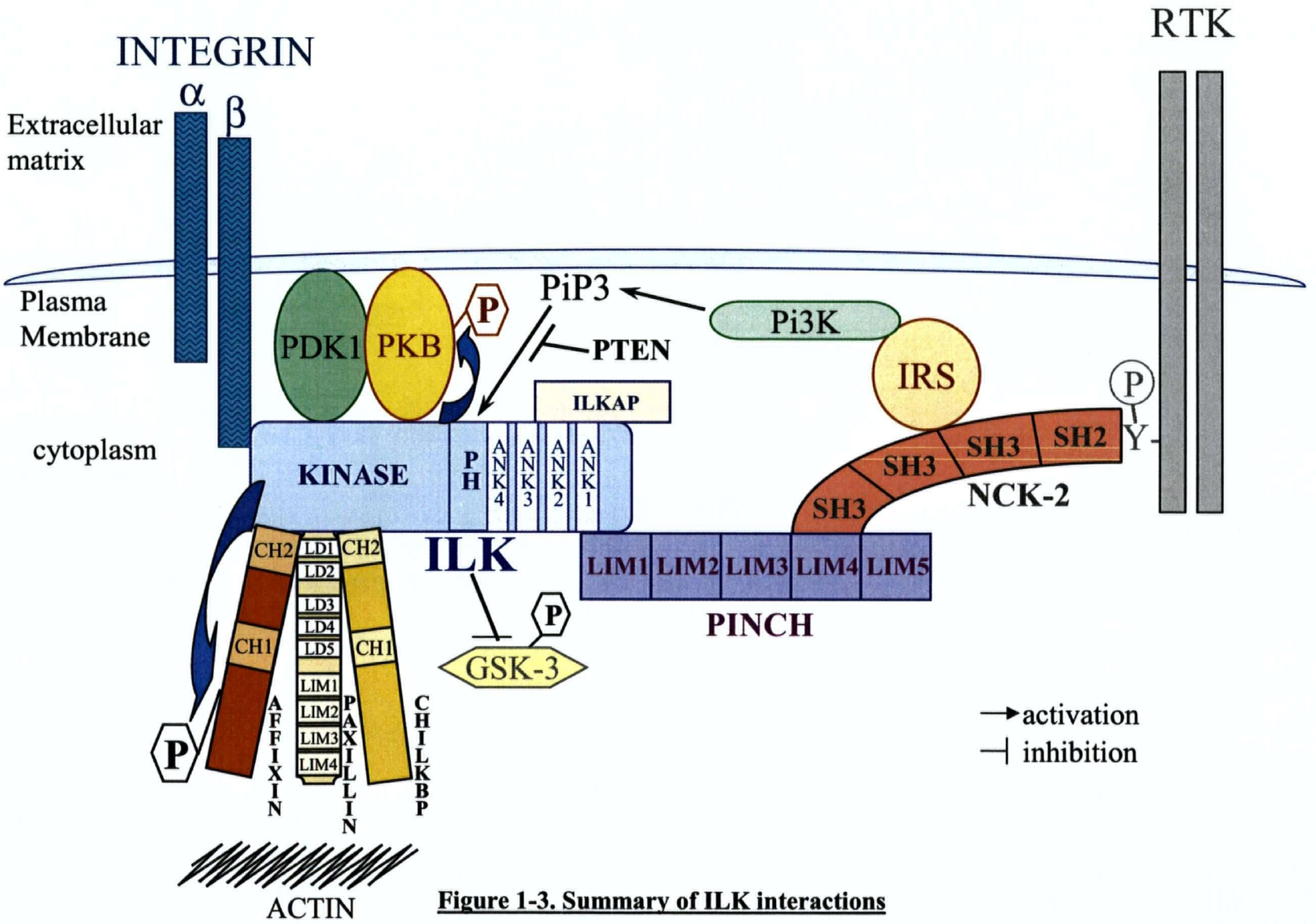


Figure 1-3. Summary of ILK interactions

may contribute to form a physical bridge to enable this occurrence. PINCH may also function in bringing ILK into close proximity with RTKs and PI3 kinase, thus allowing its activation by growth factor stimulation. PINCH is required for the localization of ILK to focal adhesion plaques, and exists in a ternary complex with ILK and another protein, CH-ILKBP (Calponin-Homology domain-containing ILK Binding Protein) (Tu *et al,* 1999, Tu *et al,* 2001). This complex is assembled and recruited to integrins upon cell adhesion, where it contributes to cell spreading. Zhang *et al* (2002) reported that this complex is formed prior to recruitment to integrins, and that each component is vital for the recruitment of the others. Interestingly, this complex formation was shown to be protein kinase C-dependent (Zhang *et al,* 2002), and disruption of this complex inhibits fibronectin matrix deposition, and cell proliferation, suggesting that the complex plays a role in integrin activation and "inside-out" signalling (Guo and Wu, 2002). Genetic studies have shown that *D. melanogaster* and *C. elegans* PINCH mutants display defects in muscle attachment similar to those seen in both integrin and ILK mutants, suggesting that ILK and PINCH are both important mediators of integrin function (Hobert *et al,* 1999, MacKinnon *et al,*). Another PINCH family member, termed PINCH-2, was recently identified, which also binds to the ANK repeats of ILK through its LIM-1 domain (Zhang *et al,* 2002, Braun *et al,* 2003). Because ILK-PINCH-2 binding is mutually exclusive to ILK-PINCH-1 binding, it was hypothesized that PINCH-2 regulates the binding of ILK to PINCH-1. Indeed, overexpression of PINCH-2 inhibits the PINCH-1/ILK interaction, and also inhibits cell spreading and migration.

Three adaptor proteins have recently been identified which bind directly to the C-terminal region of ILK: CH-ILKBP, affixin, and paxillin (Tu *et al,* 2001, Yamaji *et al,* 2001, Nikolopoulos and Turner, 2001). CH-ILKBP (also known as actopaxin and α-parvin, in rat and mouse, respectively,Yamaji *et al,* 2000, Olski *et al,* 2001) was identified in a yeast two-hybrid screen on the basis of its ability to interact with the C-terminal domain of ILK (Tu *et al,* 2001). The two calponin homology (CH) domains at the C-terminus of CH-ILKBP share sequence homology with those found in α-actinin, filamin, and other actin-binding proteins, suggesting that CH-ILKBP may play a role in actin binding. Indeed, another group discovered CH-ILKBP in rat cells (and termed it actopaxin) on the basis of its ability to bind F-actin, and the paxillin LD1 and LD4 motifs (Nikolopoulos and Turner, 2000). Both groups found that, like ILK, CH-ILKBP is recruited to focal adhesions in response to cell matrix, but not cell-cell adhesions. They also reported that CH-ILKBP is required during cell adhesion, motility, and spreading. However, these groups differed in the finding that CH-ILKBP is capable of binding paxillin. Curtis *et al* (2002) have recently identified an interesting novel function of CH-ILKBP in mitosis. It is known that prior to mitosis, cells adopt a round morphology that is involved changes in actin cytoskeleton structure and loss of focal adhesions. These changes are thought to be somehow initiated by cell cycle progression factors. It was found that CH-ILKBP contains six putative cdc2 phosphorylation sites at its N-terminus, and that this region is phosphorylated by cyclin B1/cdc2 kinase in vitro. It was also shown that CH-ILKBP is phosphorylated in this region during mitosis, and then de-phosphorylated during progression to the G1 phase. These findings suggest that CH-

ILKBP is involved in regulating actin cytoskeletal organization during mitosis, through an unknown mechanism.

Another CH domain-containing protein that is very closely related to CH-ILKBP was also recently shown to interact, through its CH2 domain, with the ILK C-terminal domain. This protein, termed affixin, was identified as an important regulator of early stage cell-substrate interaction (Yamaji *et al,* 2001). Affixin and ILK were found to co-localize at focal adhesions and at the tip of the leading edge of migrating cells, and also at sites of muscle attachment to the basal lamina. Interestingly, immunoprecipitated FLAG-tagged ILK was also capable of phosphorylating recombinant affixin. This was the first report of phosphorylation of an adaptor protein by ILK. Affixin is the human orthologue of mouse β parvin, which, like α-parvin, was identified as an actin-binding protein (Olski *et al,* 2001). Thus, affixin probably provides another means of linking integrins, ILK, and the actin cytoskeleton. This ILK-affixin interaction may play an important role in the integrin-cytoskeleton linkage during platelet aggregation, as stimulation by thrombin rapidly induces recruitment of the complex, and also stimulates ILK activity (Yamaji *et al,* 2002).

Lastly, paxillin was reported to interact with the C-terminal domain of ILK through its LD1 motif (Nikolopoulos and Turner, 2001). Paxillin is an adaptor protein which consists of four LIM domains and five LD (leu-rich) domains, and is known to be recruited to focal adhesion sites upon integrin engagement (Schaller, 2001). The LD motifs of paxillin are known to bind to several focal adhesion proteins, including FAK, vinculin, and CH-ILKBP (Nikolopoulos and Turner, 2001). This region was also shown

to bind to the carboxy terminus of ILK (Nikolopoulos and Turner, 2001). Mutation of this region prevented ILK's binding to the paxillin LD-1 motif, and also prevented ILK's localization to focal adhesions upon cell attachment. These data suggest that the interaction between ILK and paxillin is physiologically relevant, and is required for ILK's correct localization. Because both CH-ILKBP and ILK have been shown to interact with the paxillin LD-1 motif, it was necessary to examine these interactions in more detail. Nikolopoulos and Turner (2002), report that a CH-2 domain mutant of CH-ILKBP (defective in paxillin binding), retains the capacity to bind ILK, indicating that paxillin and ILK bind CH-ILKBP at different sites. Paxillin was shown to be necessary for the proper localization of both ILK and CH-ILKBP to the focal adhesions. Interestingly, paxillin binding may play a role in ILK's previously identified regulation of the Wnt/beta-catenin pathway (Novak *et al,* 1998), as a ternary complex containing ILK, paxillin, and the Wnt pathway component dishevelled was precipitated in epithelial cells (Torres and Nelson, 2000).

Summary

In conclusion, the importance of ILK as both a signalling and adaptor protein has been well demonstrated through a combination of genetic, biochemical, and cell biological studies. ILK's role as a key mediator in the transduction of integrin and growth factor signals to the nucleus, via signalling pathways such as the PKB/Akt and GSK-3 pathways, has been established. Through these signalling pathways, ILK regulates processes such as cell survival, transformation, and proliferation. ILK also functions as a scaffolding protein, bridging the ECM and integrins to the actin

cytoskeleton, and regulating attachment and spreading. The relevance of these findings has been strongly supported by the disregulation of ILK signalling in many disease states: ILK overexpression or constitutive activation has been implicated in several types of cancer, and may also be involved in diabetes. The identification of ILK as a central regulator in oncogenesis and metastasis makes it an attractive target in the search for novel cancer and disease therapies, and underscores the need for understanding the complexities of ILK function. Due to the importance of ILK in oncogenic regulation, the major objective of this body of work was to further elucidate the pathways which ILK regulates.

In order to further understand the role of ILK in oncogenesis, we examined in greater detail ILK's role in the activation of the PKB/Akt pathway, and it's regulation of anoikis. We also investigated the role of ILK as an adaptor protein, in particular the role of ILK kinase activity in this function. The work presented in this thesis highlights the importance of ILK as a central regulator of the PKB/Akt pathway, and as a direct link between integrins and PKB/Akt. We also show that ILK inhibits anoikis and apoptosis, and induces cell cycle progression. We investigated the role of ILK in integrin function and focal adhesion formation, and found that both the adaptor and kinase properties of ILK are mutually required for proper focal adhesion formation, integrin activation, cell attachment, migration, and ILK downstream signalling. These findings significantly improve the understanding of how ILK elicits its oncogenic effects, as well as identify alternative targets for tumour therapy.

1.2 Materials and methods

Cell Culture.

SCP2 ILK(14) and SCP2 ILK(13) cells were cultured in DMEM-F12, containing 5% fetal calf serum (FCS) (Invitrogen), 5mg/mL insulin (Sigma), and 100 μg/mL G418 (Sigma). MDA 231 and MDA 453 cells were cultured in DMEM-F12 containing 5% FCS and 5 mg/mL insulin. PC3, Du145 and Hek-293 cells were cultured in DMEM (Sigma) containing 10% FCS. All cells were routinely grown on 10 cm diameter tissue culture plastic dishes. Cells were trypsinized at 90% confluence, using trypsin:PBS/EDTA diluted 1:5 in phosphate buffered saline (PBS), and were incubated for approximately 5 minutes, and then resuspended in the appropriate medium containing serum. Cells were re-plated at a 1: 10 ratio.

Coating Plates

Fibronectin-coated plates were prepared as follows: Fibronectin (FN) was diluted to 15 μg/mL in PBS from a stock solution of 1 mg/mL. 35 mm diameter tissue culture wells (in 6-well plates) were then coated overnight at 4 degrees. The plates were then washed 3X with PBS. Poly-HEMA-coated plates were prepared as follows: Poly-HEMA (Sigma) was solubilized in 95% ethanol, to a final concentration of 15 μg/ml. 35 mm tissue culture wells were then coated with 500 μL of solution, and allowed to dry at room temperature. Plates were then washed 3X with PBS.

Cell Lysis

Cell monolayers were washed three times with PBS, followed by lysis with NP-40 lysis buffer, or whole cell lysis buffer (for caspase-3 and PARP western blot) (volume

100 μL for 35 mm well). Cells were harvested by scraping, and after 30 minutes of incubation on ice, the cell lysates were centrifuged at 12, 000 x g for 10 minutes at 4 °C. The soluble fraction was then separated. Bradford Assay was performed using Biorad Protein Assay Reagent according to manufacturer's recommendations. Bovine Serum Albumin (BSA) was used for establishment of a protein concentration standard curve. Lysates were used immediately or stored at -80° C.

ILK Kinase Assays

After appropriate treatments, cells in 35mm wells were lysed as described above. 250 μg of protein were used in the kinase assay, and all samples were adjusted to 500 μL volume with NP-40 lysis buffer. 4 μg of either ILK antibody, or 4 μg of the appropriate IgG control, was added to each sample, and then each samples was incubated at 4 degrees overnight with rotation. 30 μL of Protein A/G plus Agarose beads (Santa Cruz) were washed once in NP-40, and added to the samples for 1 hour at 4 degrees with rotation. Beads were then washed 2x with NP-40 lysis buffer, followed by 2x in Kinase Last Wash buffer. All traces of Last Wash buffer were removed, and 25 μL of ATP kinase reaction buffer (containing either A: substrate Myelin Basic Protein (MBP) and γ-32P-ATP, or B: GSK-3 fusion protein and unlabelled ATP) was added to the samples. The samples were immediately incubated at 30 degrees for 25 minutes. The reaction was then stopped by addition of 10 uL of 4x sample buffer. The samples were then run on a 12% polyacrylamide gel, and visualized by A: autoradiography, or B: western blotting for GSK-3 serine 21/9.

Transfections

All plasmids were grown up in *E. coli.* cultures with the appropriate antibiotic resistance, and purified using Qiagen's Plasmid Maxi kit according to manufacturer's instructions.

SCP2 ILK(13) and SCP2 ILK(14) cells were plated in 35 mm wells at 50% confluence (5x 10^5 cells). 24 hours later, cells were transfected overnight with 2 μg of various constructs, using 3 μL of FUGENE 6 reagent (Boehringer Mannheim), according to manufacturer's instructions. Cells were then re-fed in fresh serum-containing media for 48 hours.

MDA 231 and MDA 453 cells were plated in 35 mm wells at 50% confluence (5x 10^5 cells). 24 hours later, cells were transfected overnight with 4 μg of various constructs, using 4 μL of Lipofectin (Invitrogen). Cells were re-fed in fresh serum-containing media, and the anoikis assay was performed after 48 hours.

PC3 cells were plated in 35 mm wells at 50% confluence (5x 10^5 cells). 16 hours later, cells were transfected for 3 hours with 4 ug various DNA constructs, using 6 μL of Lipofectin (Invitrogen) according to manufacturer's instrctions. After 3 hours, cells were re-fed with media containing serum.

Du145 cells were plated in 35 mm wells at 50 % confluence (5x 10^5 cells). 24 hours later, cells were transfected, in full serum, with 2 μg of various DNA constructs, using 6 μL of Fugene 6 reagent (Roche Molecular Biochemicals) according to manufacturer's instructions. After 16 hours, cells were re-fed with fresh media containing serum.

Construct	Tag
Empty GFP	GFP
Empty pcDNA3	His/V5
ILK WT	His/V5
ILK E359K	His/V5
ILK R211A	His/V5
ILK S343D	His/V5
ILK S343A	His/V5
ILK R211A S343D	His/V5
PKB AAA	HA
GSK-3	HA
FRNK	HA
PTEN	GFP
CH-ILKBP	FLAG
CH-ILKBP F271D	FLAG
TOP/FLASH	Luciferase reporter
FOP/FLASH	Luciferase reporter

Table 1. DNA Constructs used for transfection

Hek293 cells were plated in 35 mm wells at 50% confluence (5x 10^5 cells). 24 hours later, cells were transfected for 3 hours with 4 μg of various DNA constructs, using

4 μL of Lipofectamine (Invitrogen) according to manufacturer's instructions. After 3 hours, cells were re-fed in serum-containing media.

For transfections involving a dose response (ie. transfecting with different amounts of the same vector), the total amount of DNA was always kept constant, with the difference being made up with the appropriate empty vector. All experiments were coordinated for completion at 72 hours post-transfection.

Annexin-V Anoikis assay

Transfected SCP2 ILK(13) and SCP2 ILK(14) cells (1 x10^6) were plated on poly-HEMA or FN-coated plates for 10 hours in DMEM-F12 modified media containing 0.5% serum. Poly-HEMA plated cells were incubated under rotation to prevent cell aggregation. Cells plated on poly-HEMA were harvested and collected in 15 mL Falcon tubes, cells plated on FN were harvested by incubation with PBS+ 5mM EDTA. All cells were washed 3x in PBS, and then resuspended in 200 μL of annexin V binding buffer (Pharmingen). Cells were stained with annexin V:PE (Pharmingen) according to manufacturer's instructions. Cells were analyzed on a Coulter EXPO XL4 flow cytometer under FL1 (GFP) and FL2 (Annexin-PE) channels. The experiment was repeated three times and then graphed as the ratio of GFP-positive cells which are stained with annexin-PE.

Treatment with Inhibitors.

Cells were treated with 50 or 100 μM KP-392 (formerly KP-SD-1, Kinetek Pharmaceuticals), a highly selective inhibitor of ILK activity (Persad *et al,* 2001), for 16 hours, unless otherwise specified. Cells were also treated with 25 or 50 μM LY294002

(Sigma), 100 nM Wortmannin (Sigma), or 50 μM PD98059 (Cell Signalling) for 6 hours. An equivalent amount of vehicle control (DMSO) was added to all control reactions.

Caspase-3 Activity assay

SCP2 ILK(14) and SCP2 ILK(13) cells were grown in full serum, with or without 100 μM KP-392. 2x 10^6 cells were then plated on fibronectin or poly-HEMA coated 60 mm plates in serum free media for 8 hours. The ApoAlert CPP32/Caspase-3 Assay kit (Clontech) was then used according to manufacturer's instructions.

Trypan blue exclusion assay

Transfected MDA 231 and MDA 453 cells were plated on 35 mm poly-HEMA coated wells for 16 hours. Alternatively, untransfected cells were plated on 35 mm poly-HEMA coated wells in the presence of the inhibitor KP-392, for 16 hours. Cells were harvested and stained with 0.4% trypan blue (Sigma) in water, and counted on a VWR Levy Hemocytometer. At least 600 cells from each set were counted, and the experiment was repeated 3x.

Western Blots

Samples to be analyzed were boiled in the appropriate volume of 4x sample buffer for 5 minutes (typically 20 μg of sample). Samples were resolved by SDS-PAGE (typically 10% or 12% polyacrylamide) at 30 mA per gel for approximately 1 hour in SDS-PAGE running buffer. Samples were transferred to 0.2 μm nitrocellulose membrane (BioRad Laboratories) at 100 V for 1 hour in 1x transfer buffer. The membrane was then blocked for 1 hour at room temperature using 1x TBS-T + 5% nonfat dry milk. The membrane was then rotated overnight at 4° with the appropriate primary

antibody diluted (typically 1: 1000) in TBS-T +5% milk. The membrane was then washed 3x for 5 minutes with 1x TBS-T, and incubated with the appropriate secondary HRP-conjugated antibody (diluted 1: 10 000) in TBS-T +5% milk for 1 hour. Membranes were then washed 3x for 5 minutes in TBS-T, and visualized using ECL Western Blotting Detection Reagents (Amersham), or Supersignal (Pierce) according to manufacturer's instructions. Blots were stripped and re-probed a maximum of 1x, using Restore Western Blot Stripping Buffer (Pierce).

Antibody	**Source**	**Company**
ILK-592	Rb	UBI
ILK-575	Mo	UBI
ILK-550	Rb	UBI
ILK	Mo	Transduction Labs
V5 tag	Mo	Invitrogen
HA tag	Mo	Covance
PKB/Akt	Rb	CST
PKB/Akt phosphoserine-473	Rb	CST
GSK-3 serine 21/9	Rb	CST
Cleaved caspase-8	Rb	Calbiochem
Caspase-3	Rb	UBI
PARP	Rb	UBI

PDK-1	Mo	
Paxillin	Rb	Santa Cruz
Vinculin	Mo	Chemicon
Actin	Mo	Sigma
CH-ILKBP	Mo	Laboratory of Chuanyue Wu
FLAG tag	Mo	Sigma
α-pix	Goat	Santa Cruz
Rac	Mo	UBI
Rho	Rb	UBI

Table 2. Primary Antibodies used for microscopy and western blotting. Rb: Rabbit polyclonal antibody. Mo: Mouse monoclonal antibody

Antibody	**Dilution**	**Company**
Anti-rabbit:rhodamine	1:100	Santa Cruz
Anti-mouse:fluorescein	1:100	Santa Cruz
Anti-mouse:HRP	1:20 000	Jackson Immunoresearch
Anti-goat:HRP	1:20 000	Jackson Immunoresearch
Anti-rabbit:HRP	1: 20 000	Jackson Immunoresearch

Table 3. Secondary Antibodies used for microscopy and western blotting

PKB/Akt kinase assay

SCP2 cells were transfected and treated as in anoikis assay (above), but instead of staining, cells were lysed using cell lysis buffer (New England Biolabs), and the kinase assay was performed using the Akt kinase assay kit (New England Biolabs) according to manufacturer's instructions. Briefly, PKB/Akt was immunoprecipitated using antibody-conjugated agarose beads, and the kinase assay was performed on GSK-3 fusion protein.

Cell Cycle assay

PC3 cells were transfected as above with the following constructs: pcDNA3:His/V5/ empty GFP, ILK E359K:V5, or PTEN:GFP. Cells were grown in DMEM containing 10% FBS for 48 hours, serum-starved for 18 hours, and then either re-fed serum or starved for 3 hours. Cells (10^5) were harvested by trypsinization, rinsed in cold PBS, and fixed in 4% paraformaldehyde in PBS for 30 min. Samples were then rinsed in PBS and then stained with 50 μg/mL propidium iodide (Sigma) in PBS with 10 μg/mL RNAse (Sigma) and 1% Triton X-100 for 30 min. Samples were then analyzed by flow cytometry under the FL2 channel, and the percentage of cells in the G1, S and G2/M phases was determined. Results shown are representative of 4 independent trials.

Apoptosis Assay

PC3 cells were transfected as above with the following constructs: pcDNA3:His/V5/ empty GFP, ILK E359K:V5, or PTEN:GFP. Cells were re-fed with DMEM containing 10% FBS for 24 hours, then cells were serum starved for 48 hours for harvest at 72 hours post-transfection. Cells (10^5) were stained with ApoAlert Annexin

V:FITC (Clontech) according to manufacturer's instruction. Samples were analyzed by flow cytometry under the FL1 channel.

Co-immunoprecipitation

Cells were lysed as described above, and 250 μg of protein was rotated overnight at 4°C with either 4 μg of the appropriate antibody, or the equivalent IgG control. After 16 hours, 30 μL of washed protein A/G plus agarose beads (Santa Cruz) were added to the samples. After 1 hour, samples were centrifuged for 2 minutes at 10 000 rpm, and supernatant was removed. Beads were washed 5x, and then 15 μL of 4x sample buffer was added, and samples were boiled for 10 minutes. The supernatant was then analyzed by western blotting. The immunoprecipitation antibody and western blot antibody were never from the same source (eg mouse and mouse).

Adhesion Assay

Cells were serum starved for 18 hours (with or without various treatments), and washed 3x with PBS. Cells were harvested by scraping in the presence of PBS:EDTA, and were then centrifuged for 5 minutes at 800 rpm. Samples were then resuspended in media without serum, and plated for 1 hour on a fibronectin-coated 96 well plate (100 000 cells per 100 μL well). Medium was then removed, and attached cells are washed very gently with PBS. Attached cells were then fixed with 3.7% paraformaldehyde in PBS for 20 minutes, then washed 1x with PBS. Samples were stained with 1% toluidine blue in PBS for 10 minutes, then the plate was washed well under tap water. Wells (in triplicate) were read at 570 nm.

RNA inhibition.

A 21 base-pair double stranded small interfering RNA (siRNA) molecule targeting the PH-like domain of ILK (ILK-H), the kinase domain of ILK (ILK-FSF) or a control, non-specific 21 base-pair sequence, were made by Qiagen. PC3 cells were transfected with ILK-H, and Hek-293 cells were transfected with ILK-FSF. Cells were transfected with the siRNA molecules at concentrations of 10, 25, or 50nM, using lipofectin reagent (Invitrogen). Cells were transfected for 16 hours, and then allowed to recover for 72 hours.

ILK-H CCT GAC GAA GCT CAA CGA GAA

PH domain

ILK-FSF TGT CAA GTT CTC TTT CCA ATG

Kinase domain

Integrin activation studies

After transfection or treatment with KP-392, cells were harvested in PBS+ 5mM EDTA, resuspended in PBS containing 20 mM glucose and 1% BSA (PGB buffer). 4 μg of primary antibody were added to 5 X 10^4 cells. The following antibodies were used: mouse anti-β1 integrin, active conformation (MAB2079Z, Chemicon), and rat anti-CD29 (Integrin β1) (Pharmingen). Samples were incubated for 1 hour at room temperature. Cells were washed in PGB and incubated with FITC-conjugated secondary antibody (Jackson Lab) for 30 min at room temperature. After washes with PGB, samples were analysed by using a Coulter Expo XL-4 flow cytometer.

Wounding Assay

After transfection or treatment of PC3 cells with KP-392, wounding assays were performed as described in Carrieras *et al* (1999). Briefly, cells were grown on fibronectin-coated plates until confluence, and a "wound" was introduced into the monolayer by dragging a razorblade across the surface to detach and remove cells. The remaining cells were washed gently several times to remove any unattached cells, and were grown in serum free media. After 24 hours, cell migration was recorded using a Nikon Eclipse TE300 microscope, and cells which migrated into the wound were counted in 5 separate fields of vision.

Preparation of Soluble and Insoluble Fractions

Cells were plated on fibronectin or poly-HEMA-coated plates as described above, and treated with either 50 μm or 100 μm KP-392, or an equivalent concentration of vehicle control DMSO. Cells transfected with siRNA were plated on fibronectin-coated plates overnight in serum free DMEM. Cells plated on PH were collected by centrifugation, and cells plated on FN were left adherent. Cells were washed with Cell Solubilization Buffer (CSB) without Triton X-100. Cells were then washed for exactly 2 minutes in 37° C CSB containing 1% Triton X-100 (for western blots), or .5% Triton X-100 (for immunostaining). This soluble fraction was then removed, and the remaining cytoskeletal fraction was either fixed for immunostaining, or resuspended in cell extraction buffer. The cytoskeletal fraction was passed through a 25-gauge syringe 3X, and then the samples were sonicated prior to protein quantification and western blot analysis.

Immunofluorescence microscopy

Cells were grown on glass coverslips coated with 1% poly-L lysine, and serum starved overnight with or without KP-392. Cells were washed with PBS, and then either fixed directly with 3.7% paraformaldehyde in PBS for 10 minutes, or washed with warm Cell Solubilization Buffer containing 0.5 % Triton X-100 for 2 minutes and then fixed with 3.7% paraformaldehyde in PBS. After fixing, coverslips were removed from wells and placed on parafilm, and then washed 3x in PBS. Then, cells were permeabilized with 0.2% Triton in PBS for 20 minutes, followed by preblocking with 5% Normal Goat Serum (NGS) in Wash Buffer (0.1% BSA in TBS-TWEEN) for 20 minutes. Preblock was then removed, and the cells were incubated with 100μL/coverslip of primary antibody (diluted 1:50 in Antibody Dilution Buffer, containing 1% NGS in Wash Buffer) for 1 hour at 37°C. Coverslips were then washed 3x for 10 minutes in Wash Buffer, and the secondary antibody was incubated (1: 100 dilution in Antibody Dilution Buffer) for 45 minutes at 37°C. Coverslips were then washed again 3x for 10 minutes, and then mounted on slides with 15 μL of mounting media (Vector). For actin staining, Rhodamine-Phalloidin (Sigma) was diluted 1:1000 in PBS, and cells were stained for 30 minutes at room temperature.

Luciferase Assay

Hek-293 cells in 35 mm wells were transfected with 0.5 μg of either empty vector, CH-ILKBP, or CH-ILKBPF271D, 0.5 μg either TOP or FOP FLASH reporter constructs, and 0.01 μg of pRenilla as a reporter control. After 48 hours, the luciferase

assay was performed using Promega's dual luciferase assay reporter kit, according to manufacturer's instructions.

Rac and Rho activation assays

HEK-293 cells were transfected with siRNA, or treated with KP-392, as indicated. Rac and Rho activity assays were performed according to manufacturer's instructions using the Rac and Rho activity assay kits (UBI). Briefly, GTP-bound Rac is pulled down by a PAK-1 binding domain GST fusion protein bound to agarose beads. Because PAK-1 only binds the GTP-bound form of Rac, this form is specifically pulled down. Samples are then released from the agarose beads and western blotted with anti-Rac antibody. Similarly, activated Rho is specifically pulled down a Rhotekin Rho-binding domain-GST fusion protein. Samples are then western blotted with anti-Rho antibody.

Densitometric Analysis

Relevant blots were analysed densitometrically using Biorad's Quantity One program. Values are shown as a fraction of the first, or the most intense band.

1.3 Composition of Buffers and Solutions

NP-40 lysis buffer
1% NP-40
150 mM NaCl
50 mM Tris pH 7.6
1 mM EDTA
+ inhibitors (add fresh before each use)
2.5 µL/mL Aprotinin
1 µg/mL Leupeptin
1 mM PMSF
2 mM NaF
1 mM Na_3Vo4

Whole Cell lysis buffer
1x PBS
1% NP-40
0.1% SDS
0.5%sodium deoxycholate (DOC)
+inhibitors (as in NP-40 lysis buffer)

4x sample buffer (pH 6.8)
61 mM Tris/HCl
1.5 mM Tris Base
2.3% SDS
10% glycerol
mix together and then insert dry tip into bromophenol blue, and add to sample buffer

Tris Buffered Saline +Tween 20 (TBST)
10 mM Tris pH8
150 mM NaCl
0.005%Tween 20

1x Running Buffer
25 mM Tris Base
192 mM glycine
0.1% SDS

1x Transfer Buffer
25 mM Tris Base
192 mM glycine

10x Kinase Reaction Last Wash Buffer

500 mM HEPES
20 mM $MgCl_2$
20 mM $MnCl_2$
10 mM Na3V04

Kinase Reaction buffer A
1x kinase reaction last wash buffer
2mM NaF
5 μg MBP
200 mM γ-32P- ATP

Kinase Reaction buffer B
1x kinase reaction last wash buffer
2 mM NaF
2.5 μg GSK-3 fusion protein (CST)
20 mM ATP

Cell Solubilization buffer
0.01 M PIPES
0.05 M KCl
0.01 M EGTA
0.003 M $MgCl_2$
2 M glycerol
1% triton X-100 (.5% for immunostaining)
add before use:
1mM PMSF
1μg/mL leupeptin
2.5 μg/mL aprotinin

Extraction buffer
0.01 M Tris-HCL
0.01 M NaCl
0.003 M $MgCl_2$
1% triton X-100
0.5% SDS

Propidium Iodide
50 μg/mL propidium iodide
10 μg/mL Rnase
1x PBS

Chapter 2: Role of ILK in the regulation of anoikis and PI3 kinase-dependent regulation of apoptosis and cell cycle progression.

Introduction

Normal epithelial cells depend upon interactions between integrins and the extracellular matrix for survival, and the loss of this interaction induces apoptosis. This type of suspension-induced apoptosis is termed anoikis (Greek for "homelessness") (Frisch and Francis, 1994; Frisch and Ruoslahti, 1997). Anoikis plays an important role in the development of many normal tissues, for example, degradation of the ECM results in anoikis and thus the proper involution of both the mammary and prostate gland (Grossmann, 2002). Anoikis also appears to block the growth of detached cells at inappropriate locations, thus acting as an important safeguard against oncogenesis. Growth of cancer cells is generally independent of not only serum or growth factors, but also of adhesion to the ECM. In fact, reduced sensitivity to anoikis appears to be an important hallmark of oncogenesis, particularly in the process of metastasis, whereby cells detach from the primary tumour without undergoing apoptosis (Ruoslahti and Reed, 1994). Disregulation of anoikis is also observed in dermatological and glomerular diseases (Gniadecki *et al,* 1998, Makino *et al,* 2000).

Attached epithelial cells maintain growth and viability through the activation of survival signalling pathways, such as the mitogen-activated protein kinase (MAP kinase) family pathways and the protein kinase B (PKB)/Akt pathway (Kumar, 1998; Downward, 1998a). It is likely that in the absence of this signal (when cells become detached and

integrins are no longer ligated), these survival pathways are no longer activated, and "default" apoptotic pathways are initiated. Alternatively, pathways distinct from those activated during apoptosis may be induced to initiate anoikis. During oncogenic transformation, the activation of certain oncogenes and the loss of tumour suppressor genes can cause the constitutive activation of these pathways, resulting in anchorage-independent growth. Current evidence implicates several pathways in the regulation of anoikis, and these pathways may be cell and condition type specific (Grossmann, 2002).

Overexpression of oncogenes such as ras, raf, rac and src, as well as the deletion of tumour suppressor genes such as PTEN and p53 has been shown to render cell anoikis-resistant (Grossmann, 2002). Thus, the PKB/Akt, MAP kinase, ras-extracellular signal regulated kinase (ERK), and Jun-NH2-terminal kinase (JNK) pathways have all been implicated in the supression of anoikis (Khwaja *et al,* 1997, Frisch *et al,* 1996a). As of yet, there are only 2 candidates for integrin proximal factors which may regulate these pathways: Shc, which is known to activate MAP kinase pathways, and FAK, which activates the PKB/Akt, JNK, and ERK pathways (Wary, *et al,* 1998, Cary and Guan, 1999, Frisch *et al,* 1996b). Interestingly, death receptor signalling has also been implicated in the induction of anoikis, as expression of a dominant-negative Fas-associated death domain protein (FADD) prevented the induction of anoikis. The Fas pathway is normally activated by binding of the secreted Fas ligand (FasL) to the Fas receptor, resulting in the recruitment of FADD and the cleavage and activation of caspase-8 (Rytomaa *et al,* 1999, Frisch, 1999). It is unclear how detachment from the extracellular matrix could induce the Fas pathway, and whether this induction requires

FasL binding. Because PKB/Akt downregulates FasL via FKHR, it is possible that suppression of this PKB/Akt activity could have the secondary consequence of Fas pathway activation. It is also unlikely that the Fas pathway is the only pathway responsible for the induction of anoikis, as not all cell types possess Fas activity.

As discussed previously, PKB/Akt inhibits apoptosis by phosphorylating and inactivating apoptotic factors such as Bad and caspase-9 (Datta *et al,* 1997, Kops *et al,* 1999), as well as modulating the activity of the transcription factors NF-kappaB FKHRL1, thus preventing Fas ligand transcription (Romashkova and Makarov, 1999; Brunet *et al,* 1999). In the absence of a survival signal from PKB/Akt, Bad is translocated to the mitochondria thus altering the Bad/Bcl-2 ratio and causing pore formation in the mitochondrial outer membrane. The pores then allow release of cytochrome c into the cytoplasm, initiating an apoptotic cascade which involves the cleavage and activation of "executioner" pro-caspases such as caspase –8 and –3 (Grossmann, 2002). Active caspases then contribute to cell death by cleaving many structural and functional proteins in the cell, ultimately resulting in the dismantling of the cell architecture and DNA fragmentation.

PTEN (Phosphatase and tensin homolog deleted on chromosome TEN), also known as MMAC or TEP1, is a tumour suppressor gene located on chromosome 10q23, a region frequently deleted in a wide variety of human cancers, including melanoma, carcinomas of the breast, endometrium, lung, head and neck (Li and Sun, 1997, Yamada and Araki, 2002, Tamura *et al,* 1999, Cantley and Neel, 1999). Loss of PTEN expression also occurs in approximately 50% of human prostate tumours, and 80% of glioblastoma

multiforme (Whang *et al,* 1998, and Wang *et al,* 1997). The phenotype of mice heterozygous for a deletion in PTEN supports its role as a tumour suppressor gene, as these mice display hyperplastic changes in the prostate, colon, and skin similar to those seen in the dominantly inherited human equivalent, Cowden's disease (Di Cristofano *et al,* 1998, Stambolic *et al,* 1998). Mutations in the PTEN gene generally occur late in the tumour development, and thus serve as an important marker for tumour grade (Tamura *et al,* 1999).

Based on sequence homology, PTEN was initially thought to be a primarily a protein tyrosine phosphatase, but has since been proven capable of dephosphorylating phosphatidylinositol 3,4,5-triphosphate (PiP3), the product of PI3 kinase (Myers *et al,* 1997). While PTEN has been demonstrated to induce the dephosphorylation of both FAK and Shc (although possibly indirectly) and thus negatively regulate their activity, it has also clearly been demonstrated to antagonize PI3 kinase signalling (Gu *et al,* 1999, Li and Sun, 1997, Maehama and Dixon, 1999, Yamada and Araki, 2001). Indeed, a mutant of PTEN (G129E) which possessed no lipid phosphatase activity but still retains protein phosphatase activity, demonstrates that the lipid phosphatase activity of PTEN is required for the control of the cell cycle, growth, and apoptosis (Myers *et al,* 1998). Interestingly, this mutation is common in patients with Cowden's disease, suggesting that the lipid phosphatase activity of PTEN is crucial to its function. Reintroduction of PTEN into PTEN-negative cells results in the downregulation of PKB/Akt activity, and the induction of apoptosis (reversible by active PKB/Akt) in certain cell types(Li *et al,* 1998). Re-introduction of PTEN into PTEN-negative cells also inhibits cell cycle progression, and

induces the cell cycle inhibitor $p27^{kip1}$ (a downstream target of PKB/Akt) (Sun *et al,* 1999). ILK has been demonstrated to inhibit anoikis and induce cell cycle progression, and its activity is induced by PI3 kinase and PIP3 (Novak *et al,* 1998, Radeva *et al,* 1997, Delcommenne *et al,* 1998). Furthermore, ILK induces the phosphorylation of PKB/Akt on serine 473, which is required for its full activation. Thus, the dependence of ILK activity on PTEN was examined, and it was found that ILK and PKB/Akt are constitutively active in PTEN-negative prostate cancer (PC3) cells (Persad *et al,* 1999). Furthermore, transfection of dominant-negative ILK E359K into PC3 cells dramatically inhibits both serum and anchorage independent PKB/Akt serine 473 phosphorylation.

PKB/Akt activation is regulated in a complex manner, and requires the PI3 kinase-dependent phosphorylation of both threonine-308 and serine-473. Phosphorylation of serine 473 is rapidly inducible in resting cells, whereas threonine-308 phosphorylation is often constitutive (Downward, 1998b, Alessi *et al,* 1997b). It has therefore been hypothesized that PKB/Akt requires the initial phosphorylation at threonine-308 for basal levels of activity, and subsequent serine-473 phosphorylation leads to full activation. While the phosphorylation of threonine-308, in the activation loop, has been definitively attributed to PDK-1, some controversy remains as to the identity of the kinase which phosphorylates serine-473 in the hydrophobic motif. It has been suggested by PDK-1 may also be responsible for serine-473 phosphorylation, or that PDK-1 may regulate PKB/Akt autophosphorylation at this site (Toker and Newton, 2000).

Central to these theories is the regulatory C-terminal hydrophobic motif, which is present in many other AGC kinases (Scheid and Woodgett, 2003). The hydrophobic motif not only encompasses serine-473, but it also provides a docking site for PDK-1 which is crucial for threonine –308 phosphorylation (Balendran *et al,* 2000, Biondi *et al,* 2000). The hydrophobic motif also provides stability to the catalytic core by binding to a pocket within the kinase domain, and thus increasing kinase activity by several fold (Balendran *et al,* 1999). This pocket has been termed the "PRK-2-Interacting Fragment (PIF)-pocket" because it was initially determined to be the site of binding for the HM fragment (called PIF) of the kinase PRK2 (Balendran *et al,* 1999). Serine-473 phosphorylation within the hydrophobic motif greatly increases this affinity for the so-called PIF pocket. Therefore, one proposed mechanism for the activation of PKB/Akt involves the membrane recruitment of PKB/Akt via PH domain interactions, followed by a possible conformational change that allows binding and phosphorylation of threonine-308 by PDK-1. Because PDK-1 binds and masks the hydrophobic motif, another protein (the theoretical PIF fragment) must displace PDK-1 to allow for serine-473 auto-phosphorylation. Therefore, autophosphorylation of serine-473 would require the initial phosphorylation at threonine-308, and thus partial activity of PKB/Akt. However, a study on embryonic stem (ES) cells lacking PDK-1 seems to suggest that this autophosphorylation cannot occur, as these cells lacked threonine-308 phosphorylation, and thus lacked any activity, but still possessed serine-473 phosphorylation (Williams *et al,* 2000). In light of these results, it is likely that serine-473 is phosphorylated by a kinase distinct from PKB/Akt and PDK-1.

Because activation of the PKB/Akt pathway has been demonstrated to suppress apoptosis and anoikis in epithelial cells (both by PI3 kinase activation and PTEN inactivation) (Khwaja *et al,* 1997; Stambolic *et al,* 1998; Lu *et al,* 1999), and because ILK provides a potential direct link between integrins and the ECM and this pathway, we investigated the role of ILK in the suppression of anoikis in SCP2 mouse mammary epithelial cells. We also investigated the effect of the inhibition of ILK activity in breast cancer cell lines, and in PTEN-negative prostate cancer cells, on apoptosis, anoikis, and cell cycle regulation. Finally, we closely examined the mechanism by which ILK induces PKB/Akt serine 473 phosphorylation, and we investigated the possibility that ILK disrupts the PDK-1/PKB/Akt interaction to allow for serine-473 phosphorylation.

Results

ILK activity is cell substrate-dependent.

To determine if ILK kinase activity is fibronectin-dependent in mouse mammary SCP2 cells, we performed an ILK kinase assay. Serum-starved SCP2 cells were either harvested immediately (control), or plated on fibronectin or poly-HEMA (a hydrophobic substrate to which cells cannot adhere) for 1 hour. As seen in figure 2-1, immunoprecipitated ILK's ability to phosphorylate MBP is increased in cells plated on fibronectin, and is low in poly-HEMA plated cells.

ILK inhibits anoikis in a PKB/Akt and caspase-dependent manner.

To test whether constitutive ILK activity was protective against anoikis, we grew regular and ILK overexpressing cells in suspension (by plating on the substrate poly-HEMA), and measured anoikis by annexin-V staining, an early cell surface marker for apoptosis. SCP2 cell lines stably transfected with ILK in the sense (SCP2-ILK13) or antisense (SCP2-14) orientation were established previously (Novak *et al,* 1998), and it has been shown that the level of ILK protein in the SCP2-ILK13 cells is high, while that of the SCP2-14 cells is extremely low. As shown in figure 2-2, the control SCP2-14 cells are highly sensitive to anoikis when plated on poly-HEMA. However, anoikis is markedly inhibited in the ILK-overexpressing SCP2-ILK13 cells. Similar results were seen comparing SCP2 parental cells to the SCP2-ILK13 cells (data not shown). In order to ensure that this inhibition is ILK-dependent, we transiently transfected the SCP2 ILK13 cells with a dominant-negative form of ILK (ILK KD). As seen in figure 2-2, the

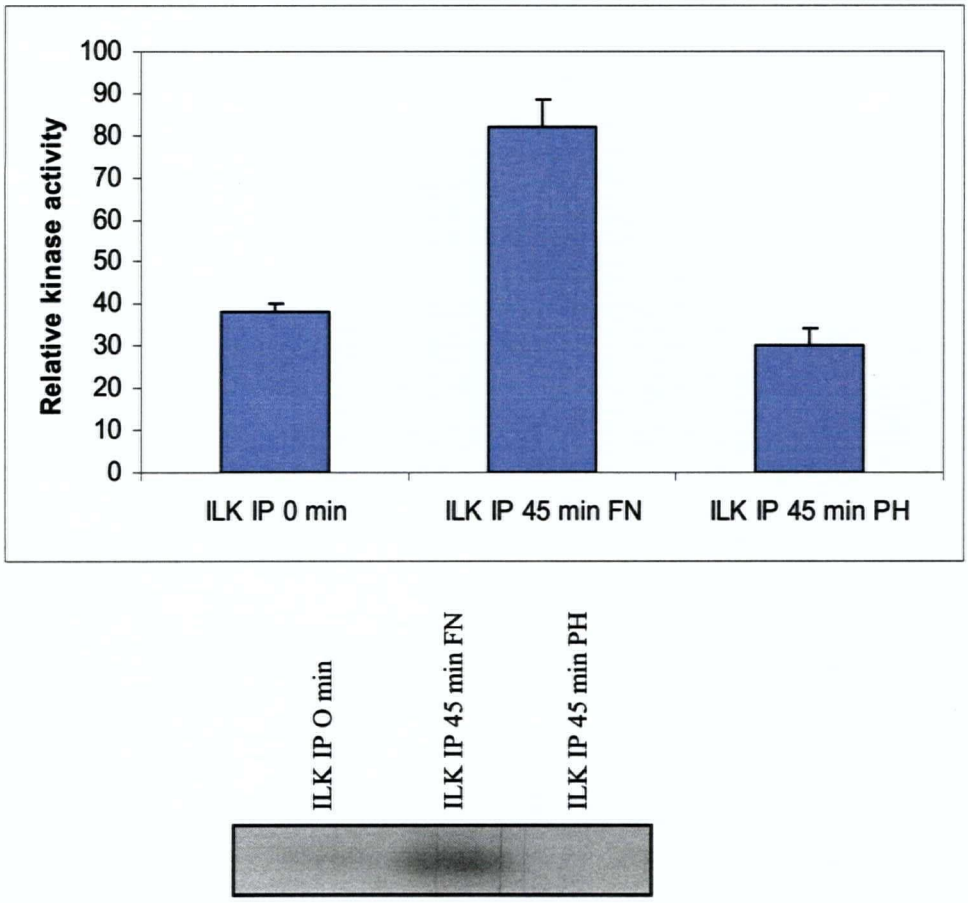
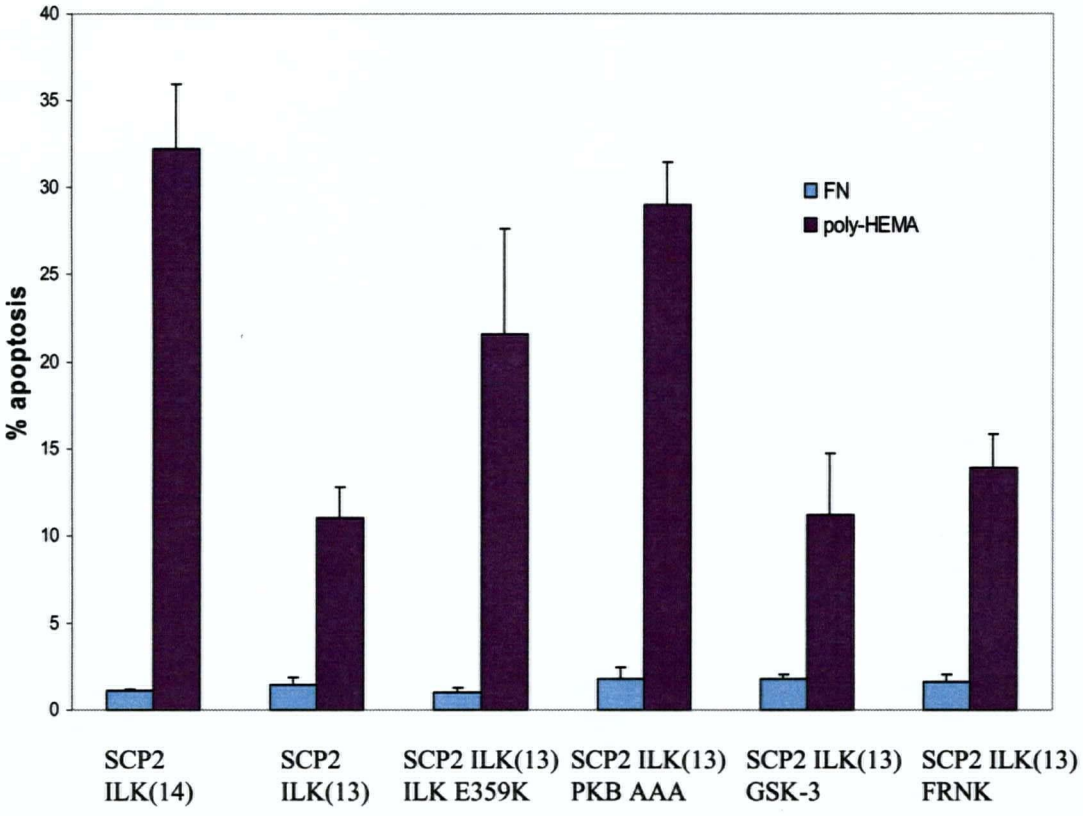


Figure 2-1. ILK activity is cell substrate dependent ILK kinase activity (on substrate myelin basic protein -MBP) of SCP2 cells is increased after 45 minutes on fibronectin (FN), but not on poly-HEMA (PH). SCP2 cells were serum-starved overnight, and then plated on either fibronectin or poly-HEMA coated plates, in serum-free media, for 45 minutes. Error bars are representative of 3 independent trials.



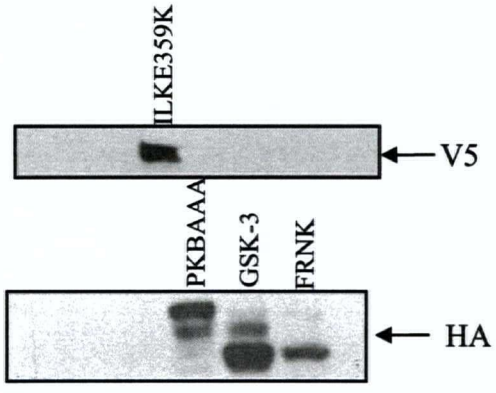


Figure 2-2. ILK suppresses anoikis in a PKB/Akt-dependent manner. **A)** SCP2 14 and SCP2 ILK13 cells were transfected with 2μg of either empty pcDNA3:His vector, ILK E359K:V5, PKB AAA:HA, GSK-3:HA or FRNK:HA and .2μg each of GFP vector. Cells were plated on either Fibronectin (FN) or poly-HEMA coated plates with .5% serum for 10 hours, and then stained with annexin-V PE. Cells were then analyzed by flow cytometry under FL1 and FL2 channels. Bar graph shown is representative of the percentage of GFP-positive cells which were stained with annexin-V PE. Error bars are representative of 3 independent trials. Expression of ILK-E359K:V5, PKB AAA:HA, GSK-3:HA, FRNK:HA is shown by western blot.

inhibition of anoikis by ILK is reversible by transient transfection of dominant negative ILK (ILK-E359K) and dominant negative PKB/Akt (PKB-AAA), but not by a naturally occuring, truncated dominant-negative FAK (FRNK-FAK-related non kinase) or by GSK-3. Because the transient transfection efficiency of SCP2 cells is only approximately 20%, the cells were co-transfected with green fluorescent protein (GFP) in a 1:10 ratio (1 GFP to 10 of experimental vector), to select for transfection. Thus, the percentage of GFP-positive cells which stained for annexin: PE was measured. The consistently low level of apoptosis of cells plated on Fibronectin (FN) indicates the lack of toxicity of any of the transfected constructs.

Because the inhibition of anoikis by ILK is reversible by dominant-negative PKB/Akt, we next determined if this correlates with PKB/Akt serine 473 phosphorylation and kinase activity, as shown in figure 2-3. The control SCP2 14 cells display low levels of serine 473 phosphorylation and activity, and these are both increased in the ILK-overexpressing SCP2 ILK13 cells. When the SCP2 ILK13 cells are transfected transiently with ILK E359K or PKB AAA, decreases in serine 473 phosphorylation and kinase activity are observed, consistent with the 20% transfection efficiency. As expected, GSK-3 transfection fails to reverse PKB/Akt serine 473 phosphorylation and activation in SCP2 ILK13 cells.

Next, we looked at the activation of downstream factors involved in apoptosis, specifically, the activity of caspases –8 and -3. We first examined the activation of the inducer caspase, caspase-8. As shown in Figure 2-4 a), significant processing of caspase-8 is observed in the control SCP2 14 cells, resulting in detection of the active 18 kDa

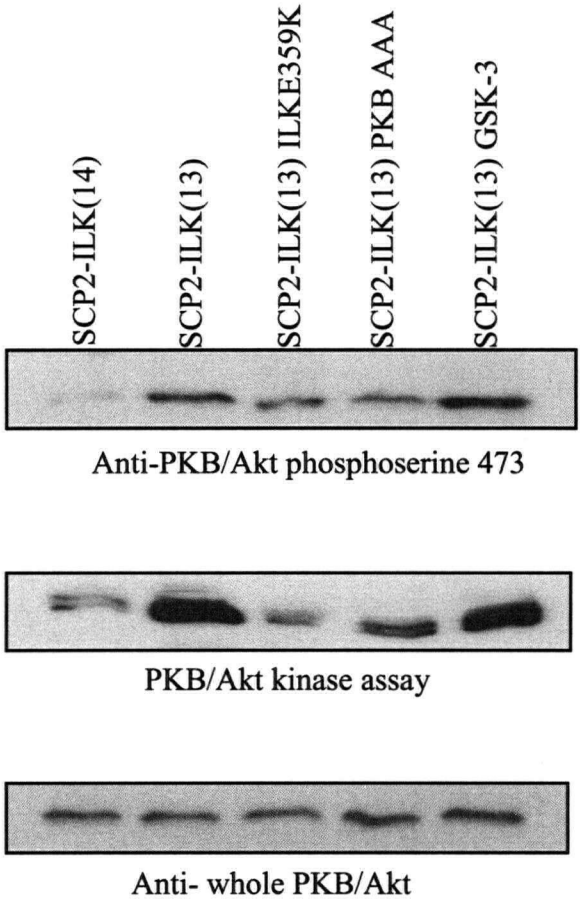


Figure 2-3. ILK induces PKB/Akt serine 473 phosphorylation and activity.
Transfected SCP2(14) and SCP2(13) cells were plated on poly-HEMA coated plates as described in figure 2-2, and lysed using cell lysis buffer (New England Biolabs) according to manufacturer's instructions. Western Blot analysis was performed using anti PKB/Akt Ser-473-P and anti PKB/Akt. PKB/Akt kinase assay was performed using the Akt kinase assay kit according to manufacturer's instructions. Results shown are representative of 3 independent trials.

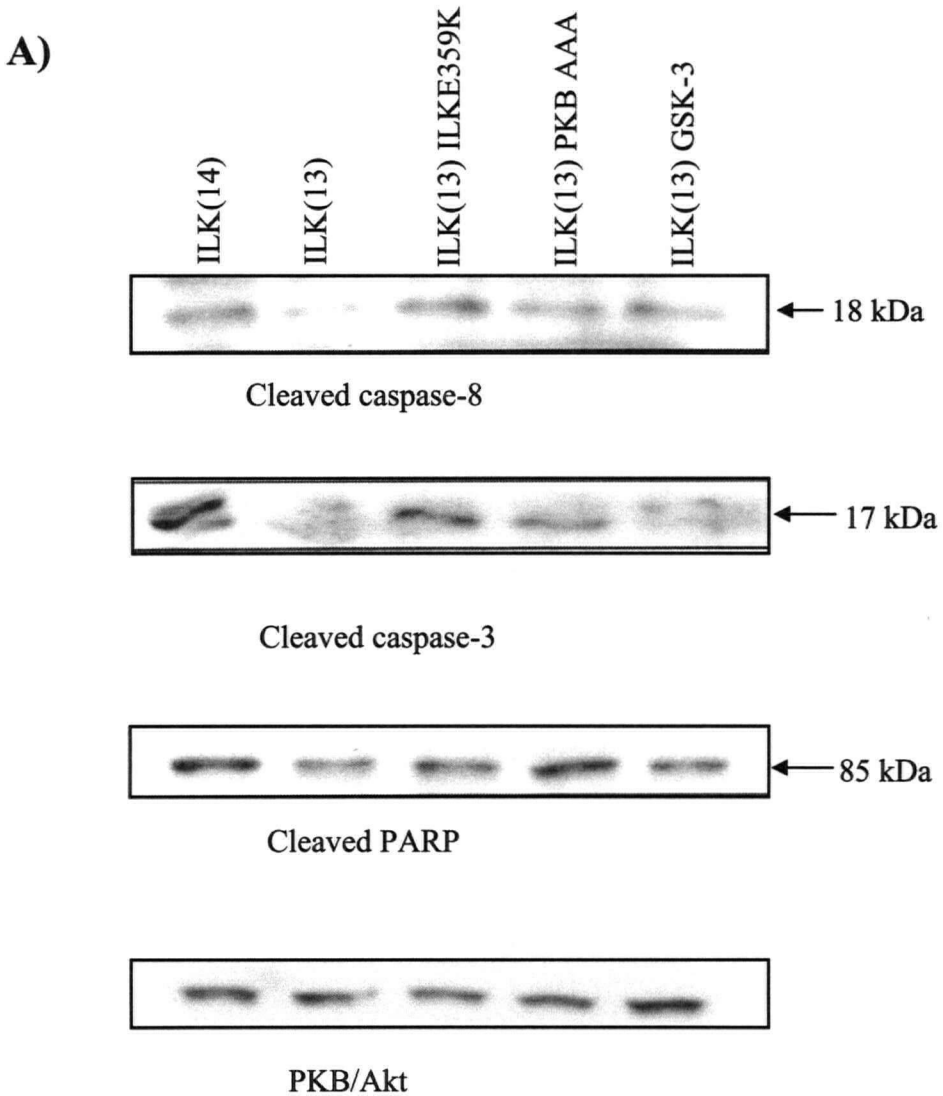
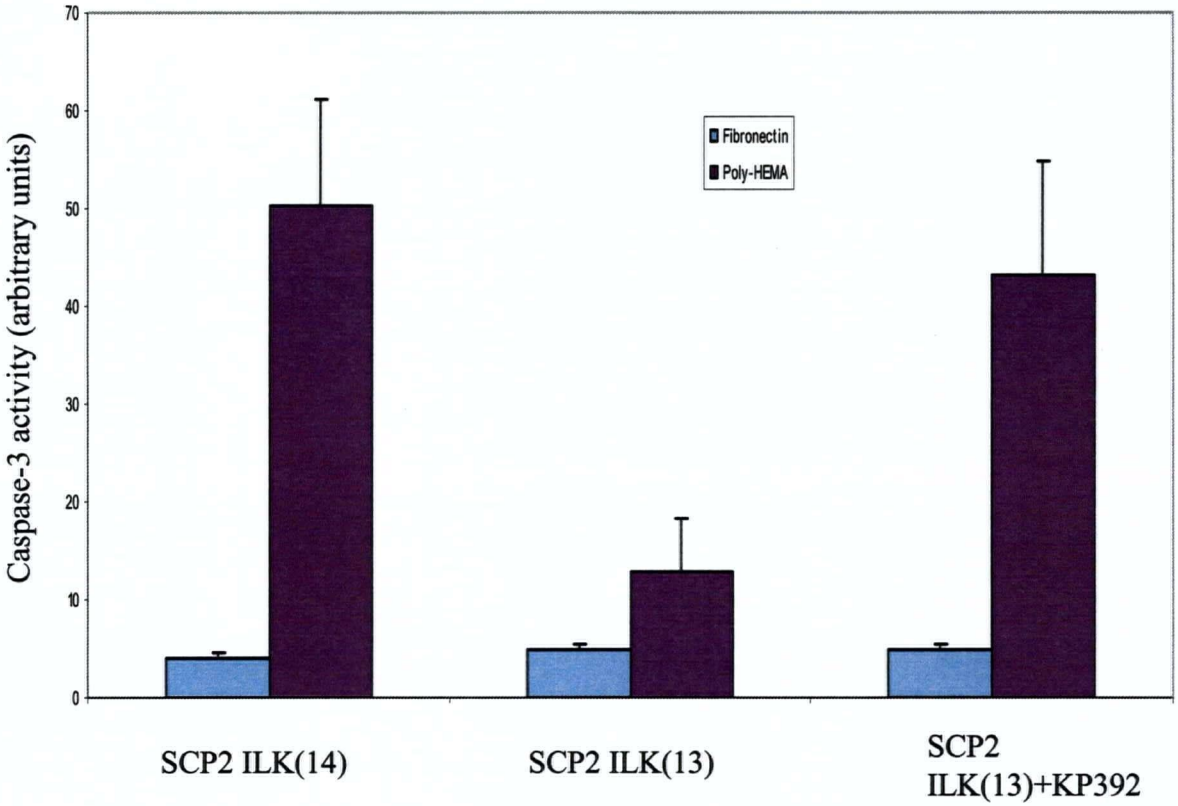


Figure 2-4. ILK inhibits the activation of Caspase-8 and Caspase-3. A) Transfected SCP2 ILK(14) and ILK(13) cells were plated on poly-HEMA as described in figure 2-2, and then lysed and western blotted for cleaved caspase-8, cleaved caspase-3 (Upstate Biotechnology), or cleaved PARP (UBI) **B)** SCP2 cells (2×10^6) were grown in DMEM containing 10% serum for 24 hours, with or without 100μm of KP-392, and then were plated on Fibronectin or Poly-HEMA, with no serum, for 8 hours. ApoAlert™ CPP32/Caspase-3 Assay Kit (Clontech) was used according to manufacturer's instructions. Error bars are representative of 3 independent trials.

Figure 2-4 B)


Caspase-3 activity (arbitrary units)
70
60
50
40
30
20
10
0
Fibronectin
Poly-HEMA
SCP2 ILK(14)
SCP2 ILK(13)
SCP2 ILK(13)+KP392

fragment. The extent of this processing is significantly lower in the ILK-overexpressing SCP2 ILK13 cells, indicating an inhibition of anoikis. The transfection of ILK E359K and PKB AAA, but not GSK-3, resulted in a slight increase in the amount of caspase-8 processing, again consistent with a 20% transfection efficiency. Because caspase-8 is capable of directly cleaving and activating the executioner caspase-3, resulting in a 17 kDa fragment (Dragovich *et al,* 1998), we examined this next. Like caspase-8, caspase-3 is cleaved in the SCP2 14 cells, but this cleavage is inhibited in the SCP2 ILK13 cells. Transfection of the ILK E359K and PKB AAA, but not GSK-3, results in the reversal of this inhibition. We also examined caspase-3 activity based on its ability to cleave a colourimetric substrate, DEVD-pNA (figure 2-4B). Caspase-3-like activity is dramatically inhibited in the ILK-overexpressing cells, but this inhibition is reversed upon the addition of a highly specific, small molecule ILK inhibitor, KP-392. The low level of caspase-3-like activity in the cells plated on fibronectin demonstrates that treatment with KP-392 does not induce apoptosis, but has a specific effect on anoikis.

Inhibition of ILK activity induces anoikis in breast cancer cell lines.

To examine the effect of ILK inhibition on an anoikis-resistant cell line, we employed 2 breast cancer cell lines, MDA 231 and MDA 453. As shown in figure 2-5 A), transfection and expression of ILK E359K induces a high level of cell death (measured by a trypan blue exclusion assay) in MDA 453 cells, and an intermediate level in MDA 231 cells, consistent with the relative transfection efficiencies of the two cell lines (60% for MDA 453, 40% for MDA 231). Exposure of the two cell lines to the inhibitor KP-392 also induced a significant level of anoikis. Different levels of

A)

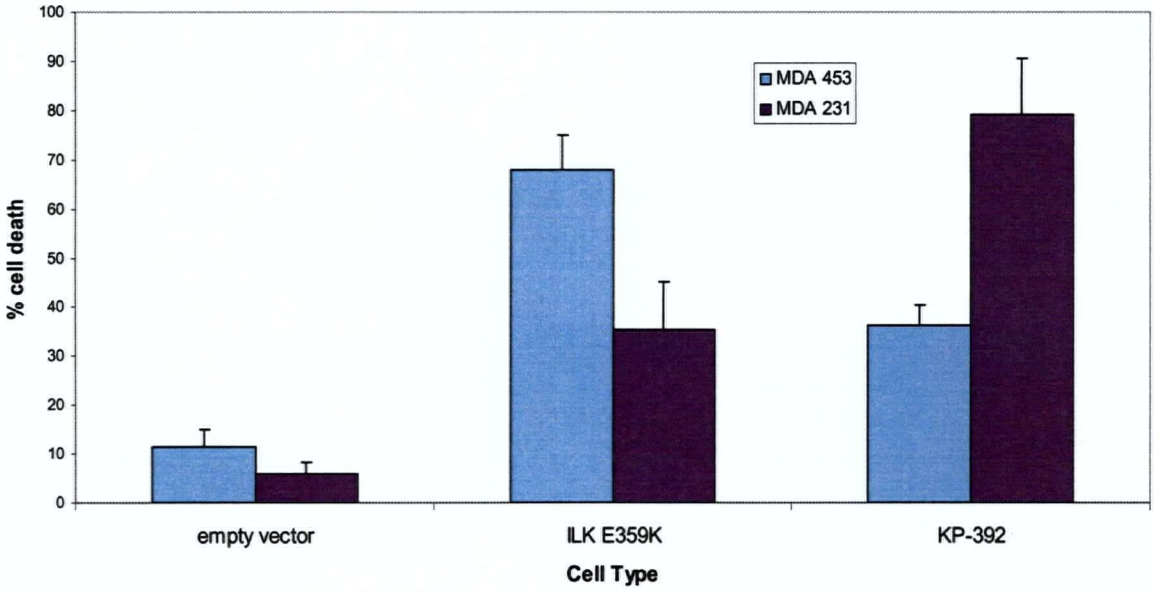
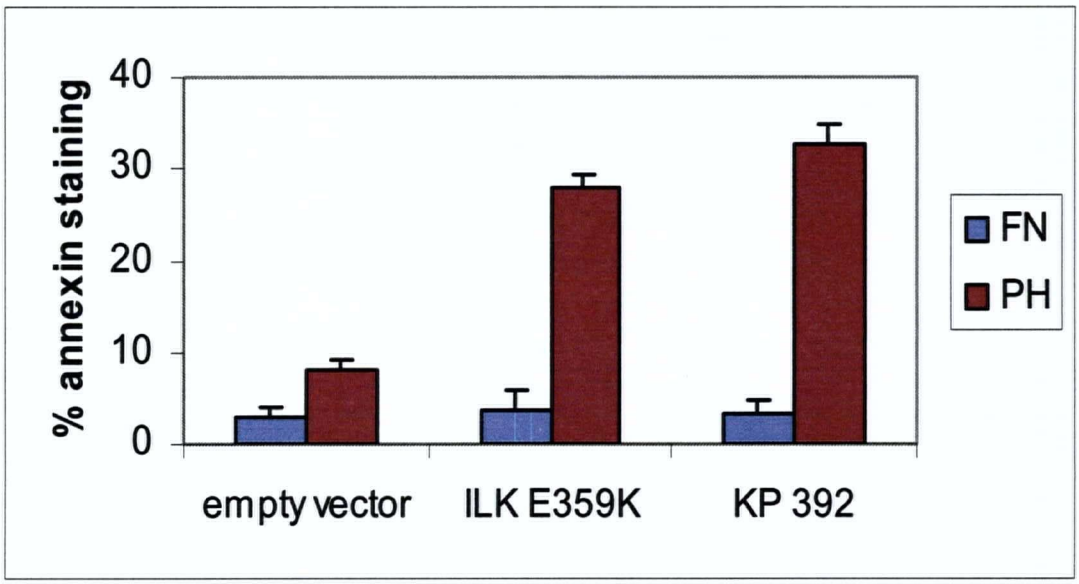


Figure 2-5. Dominant negative ILK (ILK E359K) and ILK inhibitor (KP-392) induce anoikis in two human mammary carcinoma cell lines (MDA 231 and MDA 453). **A)**Cells were transfected with either pcDNA3:His or ILK E359K, or, cells were treated with 100μm KP-392 for 24 hours. All cells were plated on poly-HEMA, in .5% serum, for 16 hours, 72 hours post-transfection. Cell were harvested and stained with trypan blue (Sigma), and counted on a VWR Levy hemocytometer. At least 600 cells from each set were counted. **B)** MDA 453 cells were treated as in figure 4a), and plated on either fibronectin or poly-HEMA in .5% serum for 16 hours. Cells were harvested and stained with annexin-V PE as in figure 2-2. Errors bars are representative of 3 independent trials.

Figure 2-5 B)


% annexin staining
40
30
20
10
0
empty vector
ILK E359K
KP 392
FN
PH

sensitivity in the two cell lines may be due to differing characteristics such as permeability, or the importance of ILK activity in each of the cell lines. These results are further supported by figure 2-5 B), which measures anoikis by annexin-V binding in the MDA 453 cells. This figure shows that when the cells are plated on fibronectin, neither the ILK E359K construct nor KP-392 had an effect on apoptosis. However, when the cells were plated on poly-HEMA, ILK E359K and KP-392 both significantly induce anoikis.

Dominant-negative ILK and PTEN induce cell cycle arrest and apoptosis in PC3 cells.

It is been shown previously by others that re-introduction of PTEN into PTEN-negative cells induces cell cycle arrest as well as apoptosis (Sun *et al,* 1999). Because PTEN-null cells possess constitutively elevated ILK activity, we wanted to determine if inhibition of endogenous ILK activity by dominant-negative ILK (ILK E359K) would also lead to cell cycle arrest and apoptosis. As shown in Figure 2-6 A), PTEN-transfected PC3 cells arrest in the G1 phase of the cell cycle compared with control empty vector-transfected PC3 cells, which continue to cycle in the absence or presence of serum. PC3 cells transfected with ILK E359K also arrest in G1 phase. We also noticed a sub-G1 peak in both PTEN and ILK E359K transfected cells, and wanted to determine if these cells were indeed undergoing apoptosis. As shown in Figure 2-6 B), compared with control (empty vector) transfected cells, both PTEN and ILK E359K transfected cells undergo enhanced apoptosis (measured by annexin-V:FITC staining) after 48 hours of serum starvation.

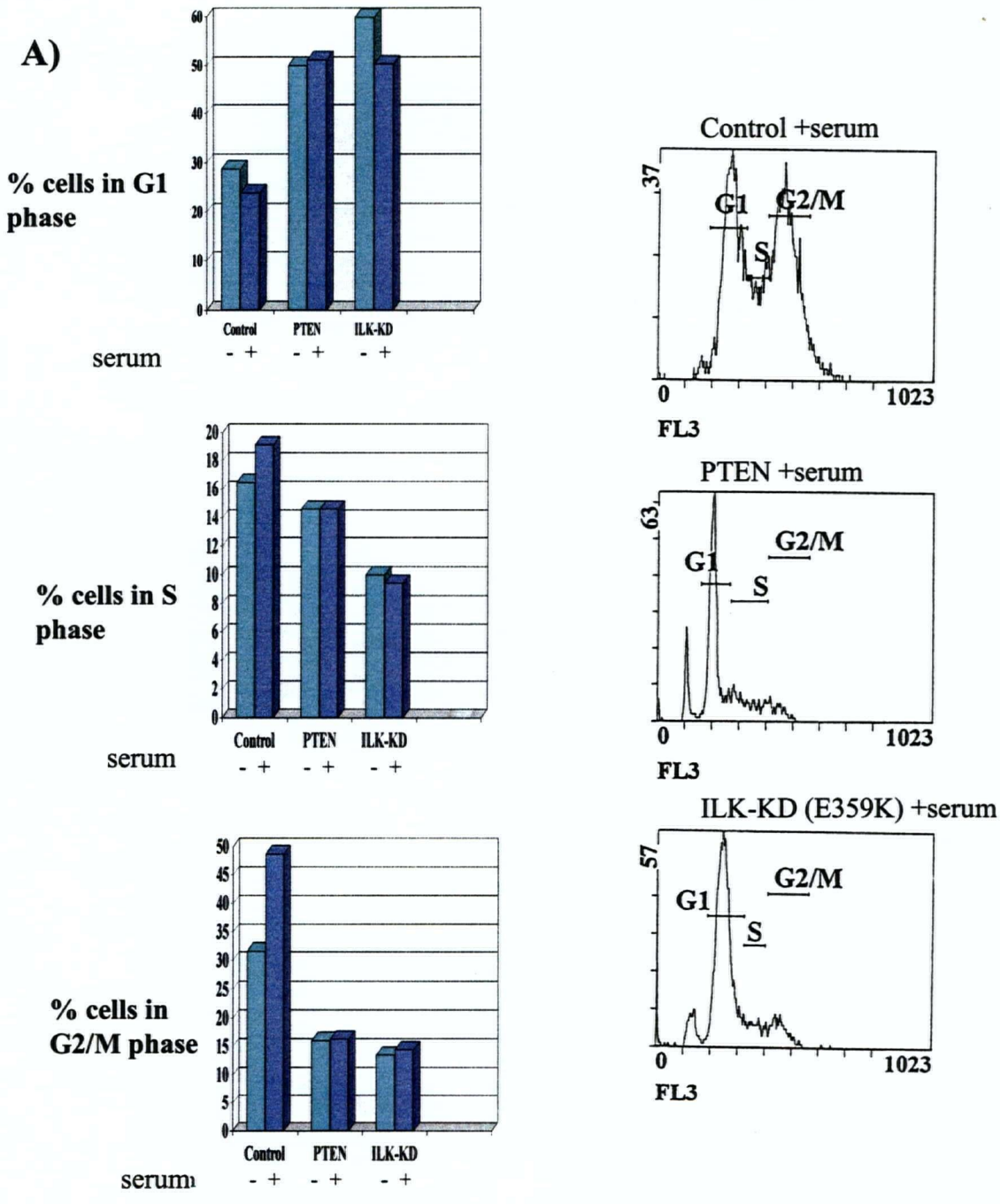
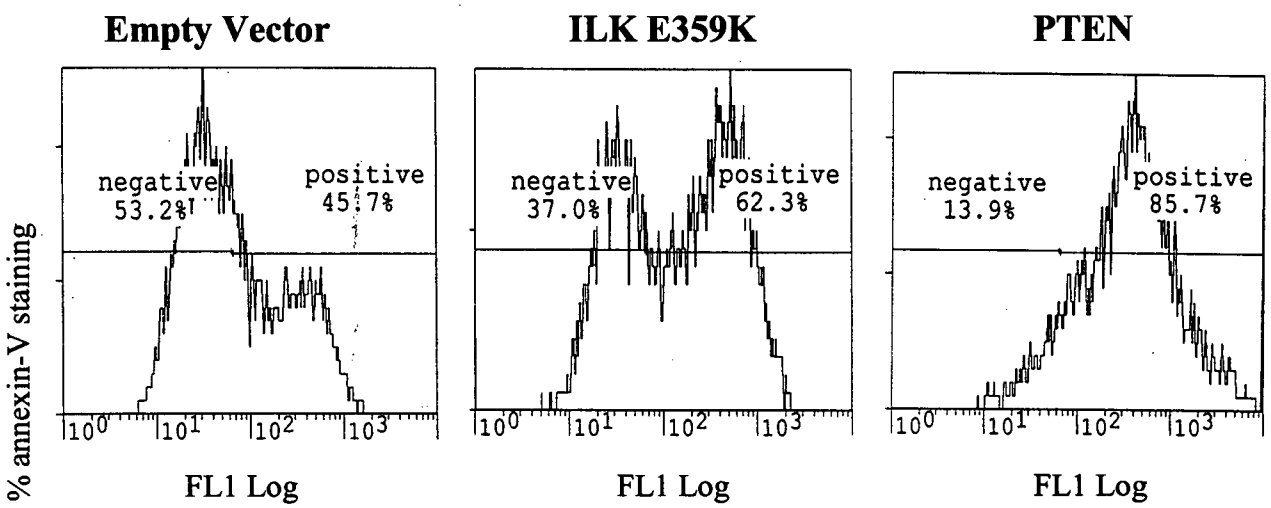


Figure 2-6. Dominant-negative ILK and PTEN-WT induce cell cycle arrest and apoptosis. **A)** Flow cytometric analysis of cell cycle inhibition in PC3 cells by PTEN-WT and ILK E359K. Propidium iodide staining of cells transfected with control (2 ug each of empty vector GFP or pcDNA3), PTEN-WT, or ILK E359K, and serum-starved for 18 hours with or without serum refeed for 3 hours. Bar chart shows cell cycle phase distribution percentages determined for each transfection with or without serum refeed for 3 hours. Example cell cycle profiles are shown on the right. **B)** Induction of apoptosis in PC3 cells by PTEN-WT and ILK E359K. Cells were transfected as above, serum refed for 24 hours, then serum starved for 48 hours. Cells were then stained with annexin-FITC, and analyzed by flow cytometry under the FL1 channel.

B)


Empty Vector
ILK E359K
PTEN
% annexin-V staining
negative
53.2%
positive
45.7%
negative
37.0%
positive
62.3%
negative
13.9%
positive
85.7%
10^0 10^1 10^2 10^3
FL1 Log
FL1 Log
FL1 Log

ILK interacts with PKB/Akt and PDK-1, and ILK's interaction with PKB/Akt depends on the activation loop of PKB/Akt.

In order to further understand the role of ILK in PKB/Akt activation, we utilized several point mutants of ILK and performed co-imunoprecipitation studies. As seen in figure 2-7, native ILK is capable of interacting with both PKB/Akt and PDK-1, and this interaction is moderately serum-inducible. Interestingly, of the several ILK mutants which are defective in activation, only the S343A mutant (mutation in the activation domain) lacks the ability to bind PKB/Akt (figure 2-8). Wortmannin also moderately inhibits the binding between PKB/Akt and ILK, indicating that this interaction is PI3 kinase-dependent.

ILK disrupts the PKB/Akt: PDK-1 interaction.

To determine if ILK is capable of disrupting the interaction between PKB/Akt and PDK-1, and thus unmasking the serine 473 site, we performed co-immunoprecipitation with wild-type PKB/Akt and PDK-1. As shown in figure 2-9, ILK-WT is capable of disrupting this interaction in a dose-dependent manner. Consistent with its inability to bind PKB/Akt, the ILK S343A mutant had no effect on this interaction.

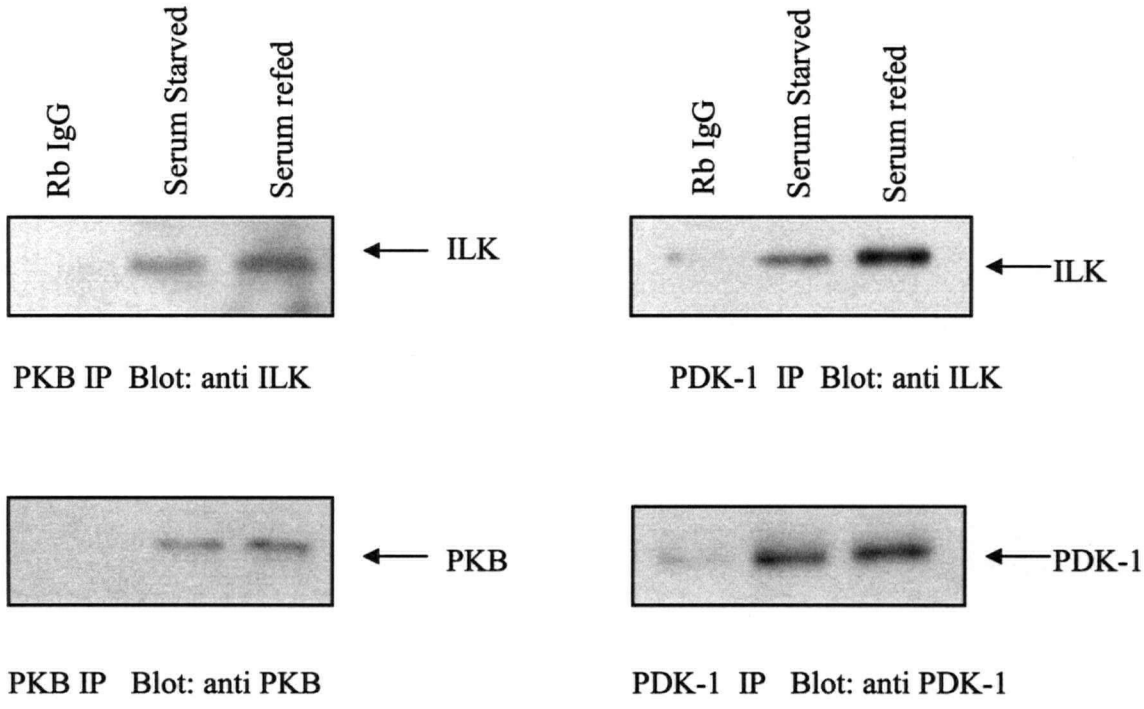


Figure 2-7. Endogenous ILK interacts with endogenous PKB/Akt and PDK-1 in a serum-dependent manner. Du145 cells were serum-starved overnight, and then either refed or starved for 1 hour. Lysates were immunoprecipitated with anti-PKB/Akt or anti-PDK-1 antibody, and then western blotted with anti-ILK antibody (mouse monoclonal UBI-575 for the PKB/Akt IP, rabbit polyclonal UBI-550 for the PDK-1 IP). Blots were stripped and reprobed to show equal loading. Data shown is representative of 3 independent trials.

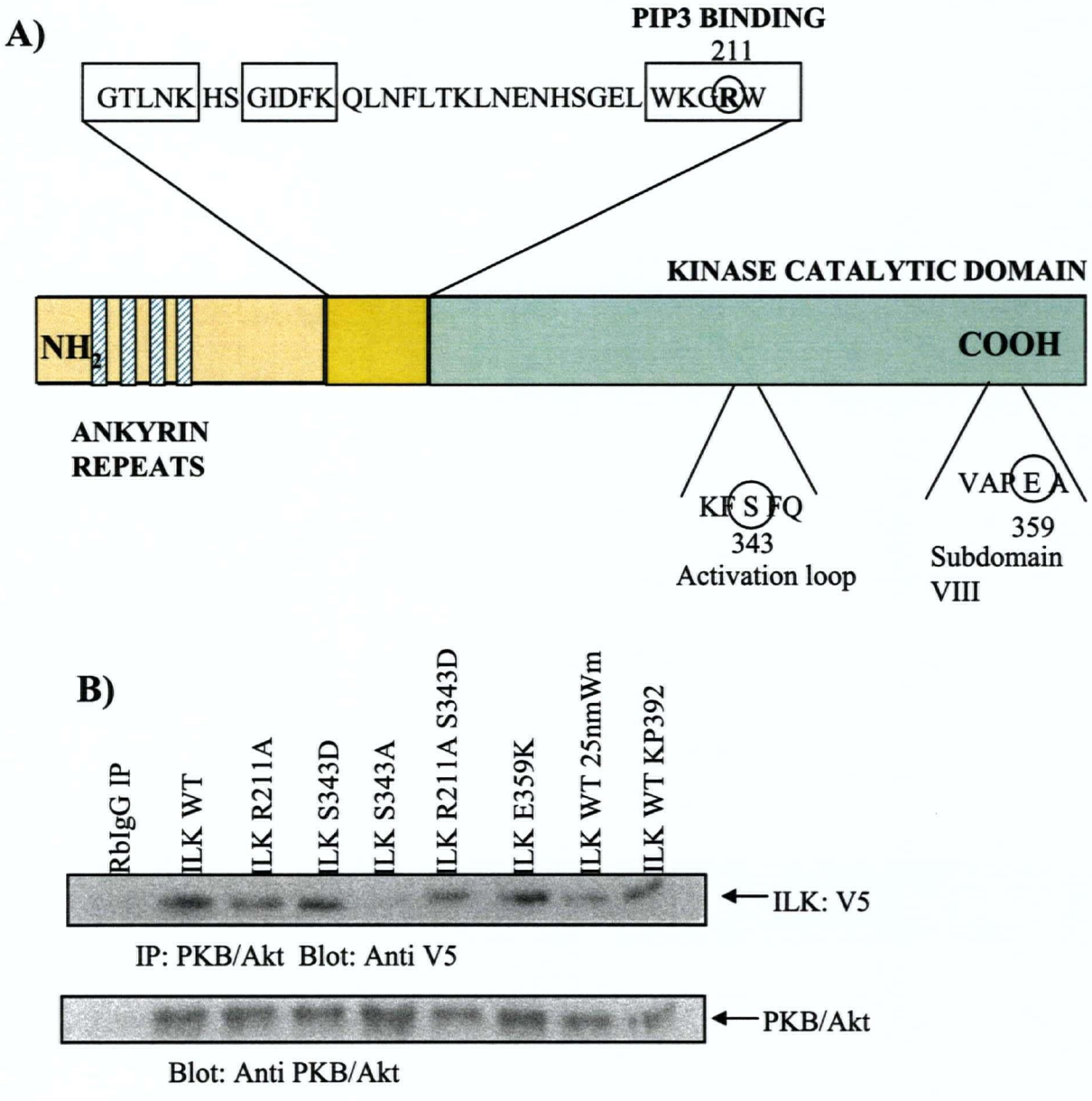


Figure 2-8. Mutation of the ILK activation loop domain (ILKS343A) disrupts ILK binding to PKB/Akt. A) Location of ILK point mutants (circled). B)Du 145 cells were transfected with various constructs, and cells were then grown in full serum for 72 hours. Drug treated cells were treated with 100 nM wortmannin (Wm) for 1hour, or 100 uM KP-392 overnight immediately prior to harvest. Cell lysates were immunoprecipitated with PKB/Akt antibody, and western blotted with V5 tag antibody. Data shown are representative of 3 independent trials.

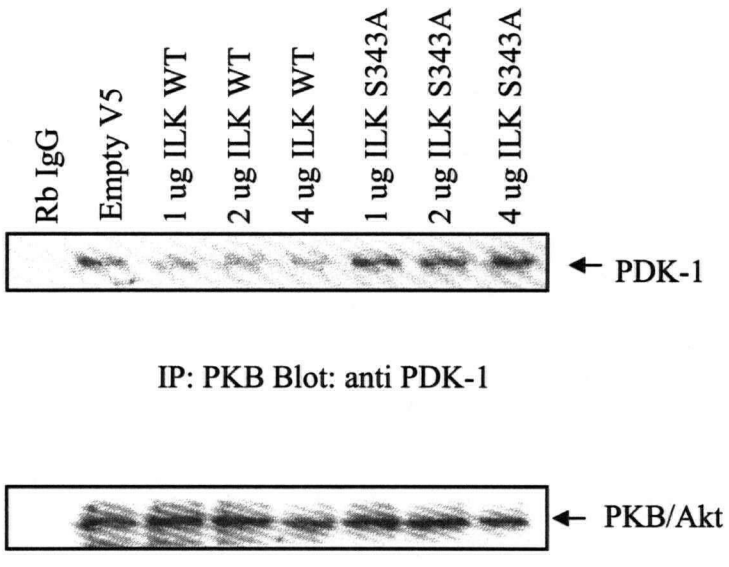


Figure 2-9. ILK disrupts the endogenous PKB:PDK-1 interaction. Du145 cells were transfected with increasing amounts of ILK-WT or ILK S343A. Lysates were immunoprecipitated with anti-PKB/Akt antibody, and western blotted with anti-PDK-1 antibody. While transfection of increasing amounts of ILK-WT resulted in a dose-dependent decrease in the interaction between PKB/Akt and PDK-1, transfection of the S343A mutant had no effect on the interaction.

Discussion

The role that ILK plays in oncogenic progression has been an area of great interest recently. Because the PKB/Akt pathway has been implicated in the inhibition of apoptosis, and ILK potentially links this pathway to integrins, we investigated the role of ILK in the inhibition of anoikis, or suspension-dependent apoptosis. Here, we show that in mouse mammary SCP2 cells, ILK activity is stimulated when cells are plated on fibronectin. Overexpression of ILK in these cells inhibits anoikis, as measured by annexin-V, caspase-8 and caspase-3. This inhibition is reversed by transfection and expression of a dominant-negative form of ILK (ILK E359K) or a dominant negative PKB (PKB AAA), but not by transfection of GSK-3. This indicates that ILK inhibits anoikis through its activation of the PKB pathway, and not through its inhibition of GSK-3. Focal adhesion kinase (FAK) has also been implicated as a possible regulator of anoikis, as its activity is also stimulated by integrin ligation and it has been reported to activate the PKB/Akt and Map kinase pathways (Frisch *et al,* 1996b; Khwaja *et al,* 1997). However, a naturally occuring, truncated form of FAK, FRNK, (FAK-related non kinase (Zhao *et al,* 1998) which functions as a dominant-negative, was unable to reverse the ability of ILK to inhibit anoikis, suggesting that FAK and ILK inhibit anoikis through two different pathways.

SCP2 cells are normal, untransformed mammary epithelial cells, and thus do not have high basal levels of ILK activity, and are sensitive to anoikis (Novak *et al,* 1998). However, many transformed epithelial cell lines are resistant to anoikis, possibly due to an increase in ILK activity. To test this, we examined the ability of a dominant negative

ILK, or the ILK inhibitor KP-392, to induce anoikis in the breast cancer cell lines MDA 231 and MDA 453, which have constitutively high ILK activity. Both ILK-E359K and KP-392 were capable of inducing anoikis in these cell lines, suggesting that one of the means by which breast cancer cells escape anoikis is through upregulation of ILK activity.

It has been reported that anoikis involves the activation of caspase-8 and the Fas-associated death domain (FADD) protein (Ryotomaa *et al,* 1999; Frisch, 1999). However, it has not been proven that death receptor signalling was necessary for the activation of this pathway, and thus it was unknown what factors initiated the caspase cascade. It is possible that integrin and death receptor signalling cooperate to regulate anoikis through an as yet unknown mechanism. It is however, highly likely that factors capable of communicating directly with integrins are crucial in anoikis regulation, as cell detachment is the initiating event in anoikis. Together, the data shown here suggest a model in which attached mammary cells signal through ILK to activate the PKB/Akt signalling cascade, resulting in the possible inhibition of downstream FasL expression and caspase activation. When normal cells are detached the ILK/PKB/Akt pathway is turned off, resulting in the activation of the caspase signalling cascade. However, if ILK becomes constitutively active due to oncogenic transformation, anoikis may be inhibited when cells are detached, potentially resulting in metastases. Thus, inhibition of ILK activity may be an effective means of breast cancer therapy, as it may prevent metastasis through anoikis induction. It is likely, however, that the role which ILK, and other signalling molecules such as FAK and FADD/caspase-8, play in the inhibition of anoikis

is complex, and maybe cell and tissue type specific. For example, Wang and co-workers (2001) have recently identified an ILK-dependent, but PKB/Akt-independent pathway by which the tumour suppressor gene DOC-2/hDab-2 induces anoikis in breast cancer cells. Epithelial cell survival and division are tightly regulated processes which ensure proper regulation of tissue development and homeostasis. Disregulation of the pathways which control these processes often leads to oncogenic transformation. One of the factors involved in the negative regulation of these pathways is the tumour suppressor PTEN. We have demonstrated the inverse dependency of ILK and PTEN signalling; in PTEN-negative prostate cancer cells, ILK activity is constitutive, and is both serum and anchorage-independent (Persad *et al,* 1999). Here, we show that the inhibition of ILK, as well as the re-introduction of PTEN, in these PTEN-negative cells induces G1 phase cell cycle arrest and apoptosis. Inhibition of ILK activity likely induces cell cycle arrest via decreased phosphorylation of GSK-3, and induces apoptosis via the PKB/Akt pathway.

The data presented here suggest that the disregulation of ILK activity in PTEN-null cells plays an important role in the suppression of apoptosis and cell cycle progression. Thus, the oncogenic properties of the PTEN-null cells can be at least partially attributed to the constitutive activation of ILK. They also present an interesting paradigm in which the inactivation of a tumour suppressor gene leads to the constitutive activation of an oncogene. In terms of cancer therapy, it may be more feasible to block the activity of an oncogene (through chemical means) than to re-introduce the activity of a tumour suppressor gene. Thus, the inhibition of ILK activity may be an attractive target in the treatment of PTEN-null cancers.

Here we have shown that wild type ILK interacts stably with both PKB/Akt and PDK-1, and that these interactions are moderately enhanced in the presence of serum. Through mutational analysis, we have identified serine 343, within the activation loop of ILK, as a critical residue involved in the interaction with PKB/Akt. This interaction probably involves serine 343 phosphorylation, as a mutant ILK in which serine was replaced with aspartate (which mimics phosphorylation) was able to bind to PKB/Akt, whereas a serine to alanine mutant (S343A) was not. Interestingly, both a PH-like domain mutant of ILK (R211A), and the subdomain VIII dominant-negative mutant (E359K) were both able to interact with PKB/Akt, although both are inhibited in the ability to promote phophorylation of PKB/Akt at serine 473 (Persad et al, 2001a). This suggests that the primary role of Arg-211 is in the regulation of ILK activity, not in the recruitment or co-localization of ILK to PKB/Akt. The E359K mutant may behave as a dominant-negative by binding PKB/Akt and competing with wild type ILK.

Because PDK-1 is thought to bind the HM and thus mask its phosphorylation, another factor must displace PDK-1 to allow serine-473 phosphorylation (Toker and Newton, 2000). While this has been attributed to a hypothetical PIF fragment, followed by PKB/Akt autophosphorylation, we wanted to determine if ILK was capable of displacing PDK-1 from PKB/Akt. Indeed, we found that ILK is capable of disrupting the PDK-1/PKB/Akt interaction in a dose-dependent manner, and that the PKB/Akt binding mutant (S343A) had no effect. See figure 2-10 for model of serine-473 phosphorylation. It is possible that ILK may interact directly with PDK-1 as well, as the critical serine 343

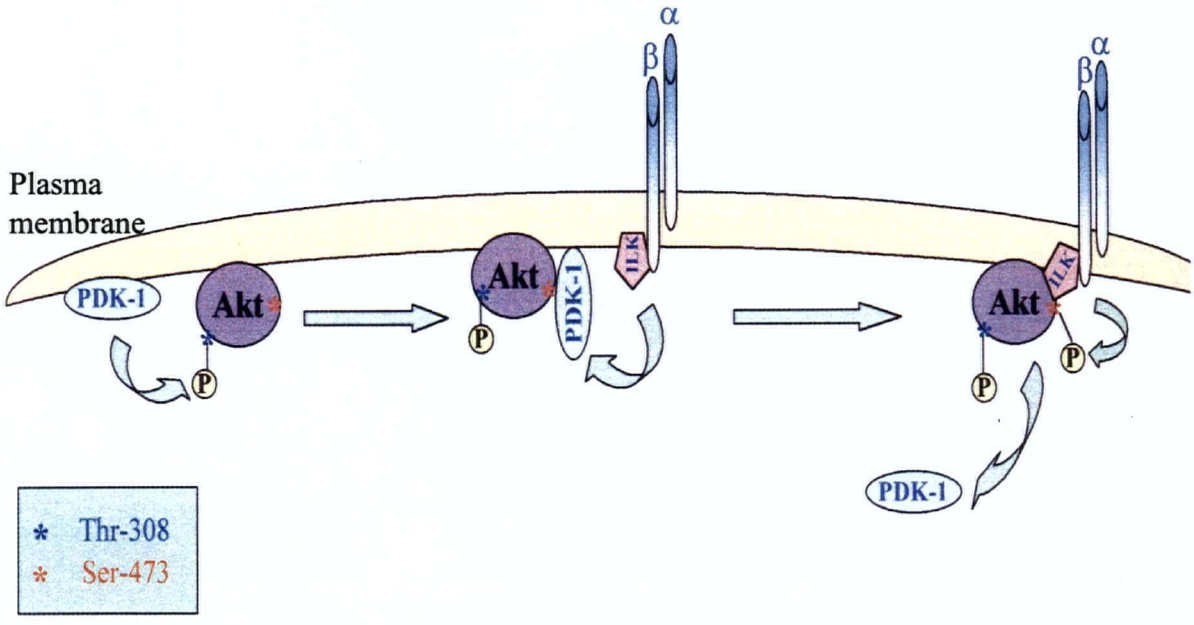


Figure 2-10. Model of PKB/Akt serine 473 phosphorylation by ILK. PKB/Akt is recruited to the plasma membrane by phosphoinositide species, causing a conformational change and allowing PDK-1 to phosphorylate threonine-308. PDK-1 then binds to the hydrophobic motif (HM) and masks the serine-473 site. ILK displaces PDK-1 and phosphorylates serine 473.

activation loop sequence is Val-Lys-Phe-Ser-Phe-Glu, which bears resemblance to the PIF consensus X-X-Phe-Asp-Tyr (Biondi *et al,* 2000).

Taken together, these data present a compelling argument for the central and interdependent roles of ILK, PTEN and PKB/Akt in cancer progression, metastasis, and the inhibition of apotosis and anoikis. They also provide strong evidence that ILK is a physiologically important PDK-2, and identify ILK as a target for cancer therapy.

Chapter 3: The role of ILK in cytoskeletal organization and integrin function

Introduction

It has been well established that ILK plays an important role in oncogenic transformation. ILK activity has been linked to several downstream signalling pathways which regulate the expression of several genes involved in cell division, growth, and apoptosis. The role of ILK in cytoskeletal organization and "inside-out" signalling however, has remained more elusive. It is known that upon integrin-mediated cell adhesion to the ECM, a massive reorganization of the actin cytoskeleton occurs, resulting in the formation of focal adhesion plaques (Petit and Thiery, 2000, Zamir *et al,* 1999). Many proteins, including catalytic proteins such as ILK (Li *et al,* 1999) FAK (Parsons *et al,* 2000), and structural proteins such as talin, vinculin and paxillin, are recruited to these focal adhesions in response to cell adhesion (Zamir and Geiger, 2001, Calderwood *et al,* 2000). This leads to morphological changes which contribute to cell spreading, migration and cell signalling.

ILK overexpression has been shown to cause an epithelial to mesenchymal transition, characterized by dramatic changes in cytoskeletal structure, a decrease in cell-cell contact, and loss of epithelial markers (Somasiri *et al,* 2001, Novak *et al,* 1998). While many of these changes have been attributed to changes in gene expression of factors such as E-cadherin, it is not known if ILK is capable of directly affecting cytoskeletal structure and integrin function.

As discussed in chapter 1, several structural focal adhesion components have been identified which interact with ILK directly. The calponin homology domain-containing ILK binding protein CH-ILKBP (also known as α-parvin and actopaxin), was identified as an interactor with the C-terminus of ILK (Tu *et al,* 2001). CH-ILKBP localizes to focal adhesions and the cytoskeleton, and has been shown to regulate cell adhesion and spreading, and the localization of ILK to focal adhesions (Zhang *et al,* 2002). It has also been demonstrated that ILK, CH-ILKBP, and the LIM protein PINCH form a ternary complex at fibrillar adhesions, and disruption of this complex reduces fibronectin deposition and cell proliferation in primary mesangial cells (Guo and Wu, 2002). A close homolog of CH-ILKBP, affixin (also known as β- parvin), also interacts with ILK and regulates cell spreading (Yamaji *et al,* 2001), as well as platelet aggregation (Yamaji *et al,* 2002). Also, the focal adhesion protein paxillin has been reported to interact with the C-terminal domain of ILK, through the paxillin LD1 motif (Nikolopoulos and Turner, 2001, Nikolopoulos and Turner, 2002). CH-ILKBP, affixin, and paxillin are all capable of interacting directly with actin, and thus provide a direct link between integrins, ILK, and the actin cytoskeleton.

The importance of ILK in regulating integrin-mediated function has been underscored in many recent studies. As shown in chapter 2, epithelial cells which overexpress ILK have increased resistance to anoikis, or the suspension-induced apoptosis which occurs when the integrin-extracellular matrix interaction is disrupted (Attwell *et al,* 2000, Wang *et al,* 2001). This suggests that constitutive ILK activation overrides the need for integrin engagement in cell survival. Recently, it has been

reported that the *C. elegans* pat-4/ILK null mutant shows serious defects at sites of integrin-mediated muscle cell attachments (Mackinnon *et al,* 2002). Similar findings in *Drosophila* ILK null mutants suggest that ILK functions as a crucial adaptor protein at sites of integrin muscle cell adhesion (Zervas *et al,* 2001). However, it was concluded from these studies that the kinase activity of ILK may be unimportant in the regulation of integrin adhesion, and that ILK functions mainly as an adaptor protein. This was due to the fact that an ILK "kinase-dead" mutant which has been shown to have partial loss of kinase activity was able to rescue the null mutant phenotype. Recently, it has been shown that mice lacking ILK expression die at the peri-implantation stage, and that ILK deficient fibroblasts display defects in cell adhesion, spreading, and formation of stress fibers (Sakai *et al,* 2003). Similarly, this study also questions the importance of ILK kinase activity, due to the fact that PKB/Akt Ser-473 levels remained unchanged, and a partial kinase dead mutant of ILK was able to rescue the phenotype.

The tumour suppressor PTEN has also been shown to play a role in the regulation of integrin-mediated function, by suppressing migration in a variety of cell types, regulating adhesion, invasion, and focal adhesion formation (Yamada and Araki, 2002, Tamura *et al,* 1998, Liliental *et al,* 2000). These observations have been attributed to PTEN's negative regulation of two pathways: the Shc/MAP kinase pathway, which regulates random migration, and the FAK p130Cas pathway, which is involved in actin cytoskeletal organization, focal adhesion formation, and directionally persistent cell motility (Tamura *et al,* 1999). Both of these targets rely on the protein tyrosine phosphatase activity of PTEN. There is however, some question as to whether PTEN is

actually capable of dephosphorylating these targets *in vivo*, due to the stoichiometrically high amounts of PTEN that were required to dephosphorylate FAK *in vitro* (Tamura *et al*, 1999, Cantley and Neel, 1999). Furthermore, the Cowden's disease mutation (PTEN G129E), which retains protein tyrosine phosphatase activity but is incapable of dephosphorylating phospholipids, underscores the physiological importance of PTEN as a lipid, rather than a protein phosphatase (Myers *et al,* 1998).

Members of the Rho GTPase family (such as Rho, Rac, and Cdc42) are key regulatory molecules that link cell surface receptors to the organization of the actin cytoskeleton. Rho GTPases are necessary for assembly of focal adhesion complexes, and processes such as polarized outgrowth and migration (Schwatz and Shattil, 2000). Rho GTPases are rapidly converted from an inactive (GDP-bound) form to an active (GTP-bound) form in response to external stimuli. This conversion is catalyzed by guanine nucleotide exchange factors (GEFs), and can be quickly reversed by GTPase Activating Proteins (GAPs) (Hall, 1998, Jaffe and Hall, 2002). αPIX (Cool-2) is a GEF specific for Rac1 and Cdc42, which signal to induce the formation of lamellopodia and filopodia, respectively, which are necessary for cell spreading. Mutations in αPIX were found in patients with X-linked non-specific mental retardation (MRX) (Kutsche *et al,* 2000). This finding supports a role for the Rho GTPases in neuronal morphogenesis, including migration, and axon growth and guidance (Luo *et al,* 2000). It appears that αPIX not only functions as a GEF for Rac and Cdc42 but also binds the Rho GTPase target, p21 activated kinase (PAK), bringing it within close proximity of GTP-bound Rac

and Cdc42 (Feng *et al,* 2002). PAK serves as an effector for Rac and Cdc42, promoting the motility of fibroblasts (Sells *et al,* 1997).

It is known that integrins are capable of activating Rac and Cdc42, and that both proteins are involved in integrin-induced cell spreading (Price *et al,* 1998). It has recently been shown that the β integrin tail is sufficient to mediate signalling to Rac (Berrier *et al,* 2002). It is not known, however, the signalling events that link integrins to Rac and Cdc42. The recent finding that αPIX, a GEF for Rac and Cdc42, binds to β-parvin (also known as affixin) provides a potential explanation for this link. Affixin, and the closely related CH-ILKBP (α-parvin) are both adaptor proteins which bind ILK, which binds directly to integrin β subunits (Rosenberger *et al,* 2003). Furthermore, αPIX activity, like ILK activity, is PI3 kinase-dependent (Yoshii *et al,* 1999), and ILK has been identified at the leading edge of lamellipodia, suggesting that it may play a role in Rac activation(Yamaji *et al,* 2001).

We wanted to determine if ILK plays a role in inside-out signalling, by examining factors such as integrin activation, cell attachment, and migration. Because integrin function depends on proper focal adhesion formation, and because ILK-null mutants in *Drosophila* and *C. elegans* point to a role for ILK in focal adhesion formation, we examined this process in human cells. We also wanted to determine if ILK is capable of binding to αPIX and regulating the Rho GTPases, thus providing a potential link between β integrins and the Rho GTPases.

Here we show that inhibition of ILK kinase activity results in the inhibition of cell attachment to fibronectin and cell migration, as well as the localization of ILK binding

partners to the focal adhesions. We also show that ILK is preferentially active in the cytoskeletal fraction, and that the interaction of CH-ILKBP with ILK stimulates ILK-mediated signalling in DU145 prostate cancer cells. In PTEN-null prostate cancer cells (PC3), we show that the ILK: CH-ILKBP interaction is regulated by PTEN and the PI3 kinase pathway. These data suggest that upon integrin engagement, ILK and CH-ILKBP are recruited to focal adhesions in a PI3 kinase-dependent manner, resulting in ILK activation. Activated ILK is then involved in downstream outside-in signalling, and also in maintaining the β1 integrin in an activated state (inside-out signalling) by sustaining CH-ILKBP and paxillin localization to focal adhesions. We also show that ILK, CH-ILKBP and αPIX exist in a complex in PC3 cells, and that this complex is dependent on ILK kinase activity. We also show that Rac activity is dependent on ILK kinase activity. Together, these data demonstrate important co-operative roles for ILK, CH-ILKBP, PTEN, αPIX, and Rac in cytoskeletal organization, integrin-mediated cell attachment and signalling.

Results

Inhibition of ILK activity decreases cell attachment to FN and cell migration.

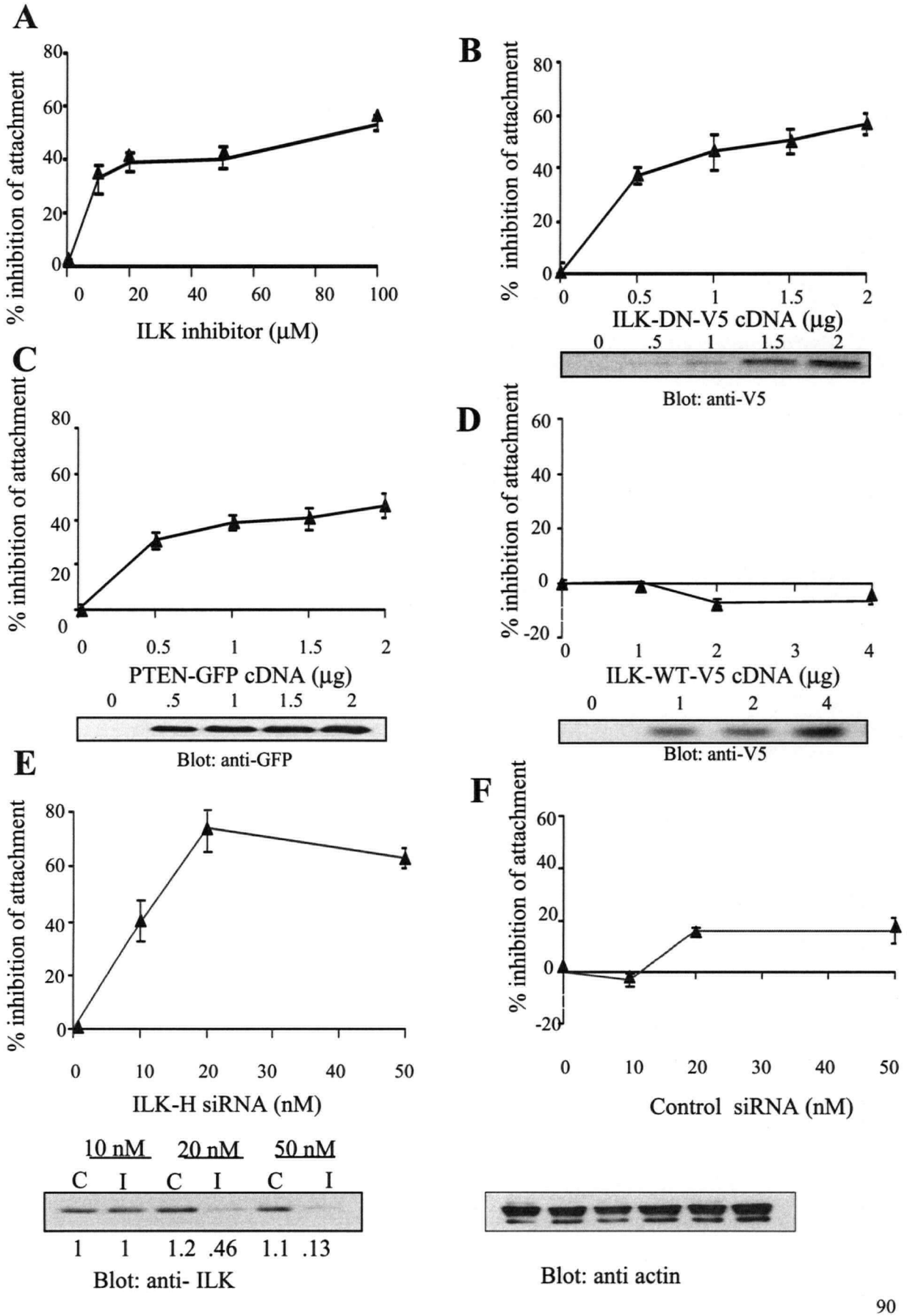
We have previously reported that ILK is constitutively active in the PTEN-null prostate cancer cell line PC3, and that re-introduction of PTEN inhibits this activity (Persad *et al,* 2000). To determine whether ILK kinase activity is crucial to the regulation of integrin function, we studied the effect of inhibition of ILK activity on integrin function in PC3 cells. This was done by treatment with the small molecule ATP-analog ILK inhibitor KP-392, transfection of a kinase deficient, dominant negative form of ILK (ILK-E359K) or re-introduction of PTEN, a negative regulator of ILK activity (Persad *et al,* 2000, Morimoto *et al,* 2000).

Inhibition of ILK activity with KP-392 showed a dose-dependent inhibition of cell attachment to fibronectin (figure 3-1A). Transient transfection of a dominant negative, kinase deficient form of ILK (ILK-DN, Fig 3-1 B), or transient transfection of PTEN-WT (figure 3-1 C) also decreased attachment of PC3 cells to FN in a dose-dependent manner. Transfection of wild-type ILK, on the other hand, (figure 3-1 D) did not affect attachment of PC3 cells to FN.

To test the effect of loss of total ILK protein, RNA inhibition experiments were also performed. Transfection of a small interfering RNA (siRNA) molecule targeted to the ILK sequence (I) inhibited cell attachment (figure 3-1 E), while a control, non-specific siRNA(C) (figure 3-1 F) had no effect.

Since ILK can interact with the cytoplasmic domain of the β1 integrin (Hannigan *et al,* 1996), we wanted to determine whether ILK regulated cell attachment to

Figure 3-1. Inhibition of ILK activity decreases cell attachment.
PC3 cells were treated with increasing amounts of inhibitor **(A)** for 24 hours, were transiently transfected with ILK-DN:V5**(B)**, PTEN:GFP**(C)**, or ILK-WT:V5**(D)** and left to recover for 48 hours, or were transfected with ILK-H siRNA specific to the PH-like domain of ILK (I) **(E)**, or control siRNA, using lipofectin **(F)**, and left to recover for 72 hours. Attachment assay was performed as described in methods. Each treatment was carried out in triplicate, as indicated by error bars, and the experiment was repeated 3 times. Data are plotted as % increase in the inhibition of attachment versus control.


A
% inhibition of attachment
ILK inhibitor (μM)
B
% inhibition of attachment
ILK-DN-V5 cDNA (μg)
Blot: anti-V5
C
% inhibition of attachment
PTEN-GFP cDNA (μg)
Blot: anti-GFP
D
% inhibition of attachment
ILK-WT-V5 cDNA (μg)
Blot: anti-V5
E
% inhibition of attachment
ILK-H siRNA (nM)
10 nM 20 nM 50 nM
C I C I C I
1 1 1.2 .46 1.1 .13
Blot: anti- ILK
F
% inhibition of attachment
Control siRNA (nM)
Blot: anti actin

fibronectin by regulating β1 integrin activation state. Using a specific antibody, we studied the activation state of β1 integrins on the cell surface after inhibition of ILK activity. Flow cytometric analysis showed that inhibition of ILK activity by KP-392 significantly decreased the amount of activated β1 on the cell surface, but did not affect the total amount of β1 integrin expressed on the cell surface (Fig 3-2 A). Transient transfection of ILK E359K (Fig 3-2 B) and wild type PTEN (Fig 3-2 C) also resulted in significant reduction of activated β1 on the cell surface, whereas transfection of wild-type ILK had no further effect on the activation state of β1 (Fig 3-2D).

Because integrin function is required for proper cell attachment and migration (Brakebusch *et al,* 2002), we next examined the effect of the inhibition of ILK activity on cell migration in a wounding assay. As seen in figure 3-3, KP-392 (A), ILK E359K (B), and wild-type PTEN(C) all decreased cell migration in a dose-dependent manner. Again, ILK-WT (D) did not affect migration in these cells. Together, these data demonstrate that ILK activity is required for cell attachment and migration.

Inhibition of ILK activity affects the localization of paxillin and CH-ILKBP, but not vinculin, to the focal adhesions.

We next examined the effect of inhibition of ILK activity on the proper localization of focal adhesion proteins. PC3 cells were plated on poly-HEMA (PH) or fibronectin (FN), and the localization of several proteins to either the soluble or cytoskeletal fraction was determined. As seen in figure 3-4 A), ILK localized to the cytoskeletal fraction when cells were plated on FN, and this localization was not affected when ILK activity was inhibited by KP-392. However, the cytoskeletal localization of

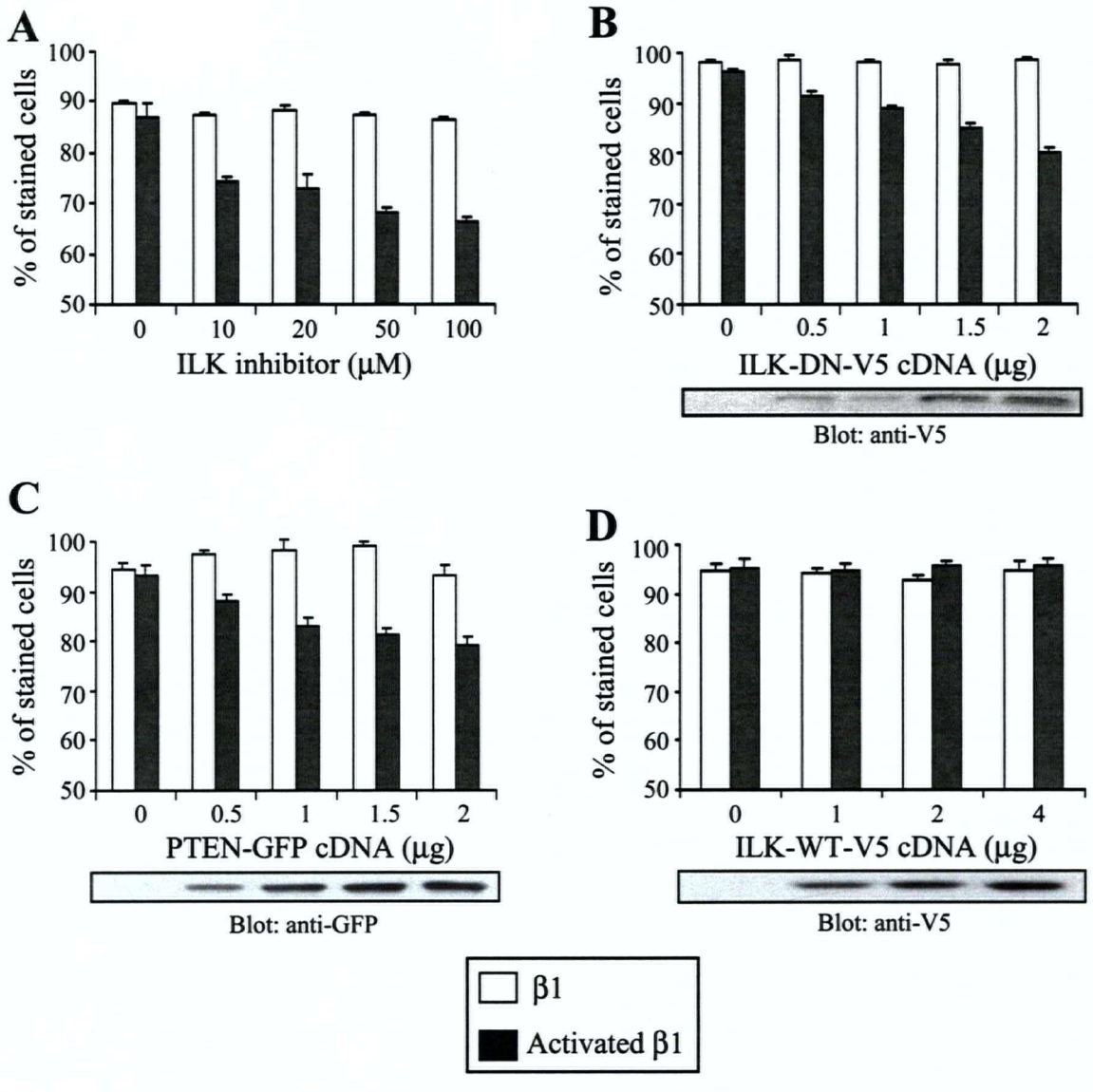
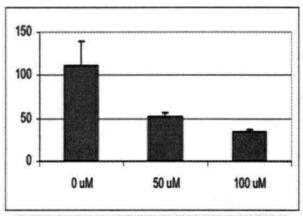
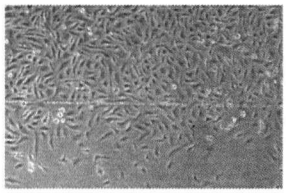


Figure 3-2. Inhibition of ILK activity decreases beta-1 integrin activation.
PC3 cells were treated with increasing amounts of inhibitor **(A)** for 24 hours, were transiently transfected with ILK-DN:V5**(B)**, PTEN:GFP**(C)**, or ILK-WT:V5**(D)** and left to recover for 48 hours. Cells were stained for activated or total beta-1 integrin, and then analyzed by flow cytometry. Each sample was repeated in triplicate, as indicated by error bars, and the experiment was repeated 3 times.

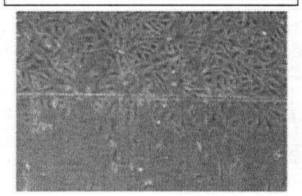
Figure 3-3. Inhibition of ILK activity disrupts cell migration.
(A) A wound was introduced to PC3 cells as described in Methods, and cells were then treated with increasing amounts of KP-392 for 24 hours. Migrated cells were photographed and counted in 5 separate fields. Alternatively, cells were transfected with PTEN:GFP**(B)**, ILK-DN:V5**(C)**, or ILK-WT:V5**(D)**. Wounding assay was then performed 24 hours post-transfection. Results are representative of 3 independent trials.

A)KP-392

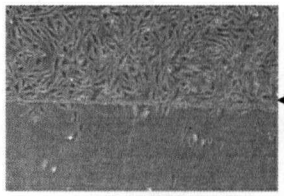




0 μM KP-392



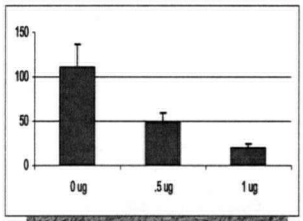
50 μM KP-392

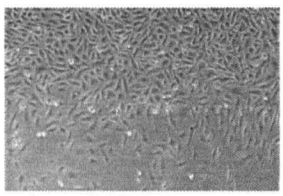


100 μM KP-392

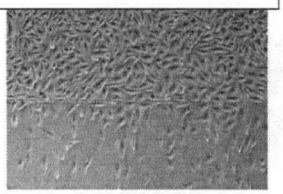
←wound

B)ILK-DN

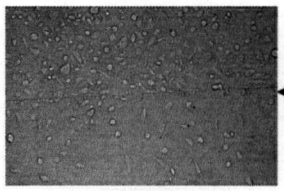




0 μg ILK-DN



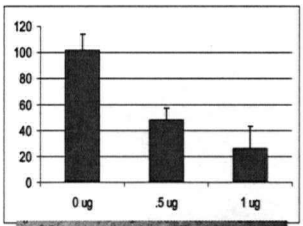
.5 μg ILK-DN

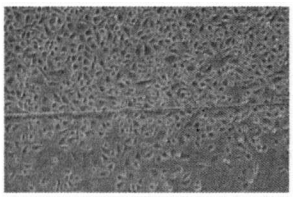


1 μg ILK-DN

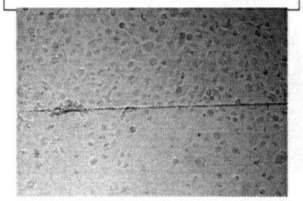
←wound

C)PTEN

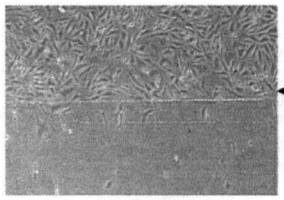




0 μg PTEN



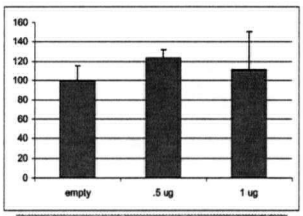
.5 μg PTEN

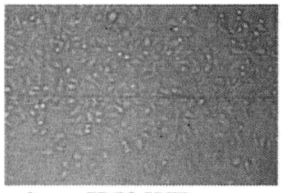


1 μg PTEN

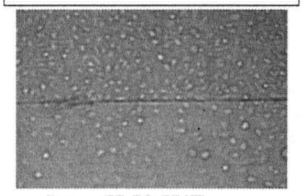
←wound

D)ILK-WT

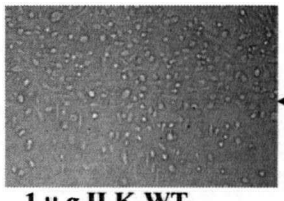




0 μ g ILK-WT



.5 μ g ILK-WT



1 μ g ILK-WT

←wound

paxillin, which has been shown to bind ILK directly (Nikolopoulos and Turner, 2001) was significantly inhibited by KP-392, whereas the localization of vinculin, which does not bind ILK directly, was not affected by KP-392. Because we have shown a dependence of ILK activity on the PI3 kinase pathway (Persad *et al,* 2000), we also examined the effect of PI3 kinase inhibition on the localization of paxillin and vinculin. As shown in figure 3-4A), inhibition of PI3 kinase by LY294002 also inhibited paxillin, but not vinculin, localization to the cytoskeletal fraction. The MEK 1 inhibitor compound PD98059 had no effect on the localization of paxillin and vinculin. We next examined the effect of the inhibition of ILK protein expression by RNA inhibition. As shown in figure 3-4 B), when cells were transiently transfected with ILK-H, an siRNA molecule specific to the PH-like domain of ILK (I), or a control siRNA (C), ILK-depleted cells displayed a dose-dependent loss of the localization of paxillin and CH-ILKBP, but not vinculin, to the cytoskeletal fraction. To further confirm the specific effect of ILK inhibition on the localization of paxillin, we also transfected cells with ILK E359K. As shown in figure 3-4 C), paxillin, but not vinculin, localization to the cytoskeletal fraction is inhibited in cells transiently transfected with ILK E359K. Inhibition of ILK activity also resulted in the inhibition of the interaction of ILK with its binding partner CH-ILKBP in the cytoskeletal fraction. As shown in figure 3-4 D), KP-392 inhibits the association of ILK with CH-ILKBP in the cytoskeletal fraction when cells are plated on fibronectin.

The localization of ILK, paxillin, CH-ILKBP, vinculin and actin were also examined by immunofluorescence microscopy. Paxillin, vinculin, and actin localization

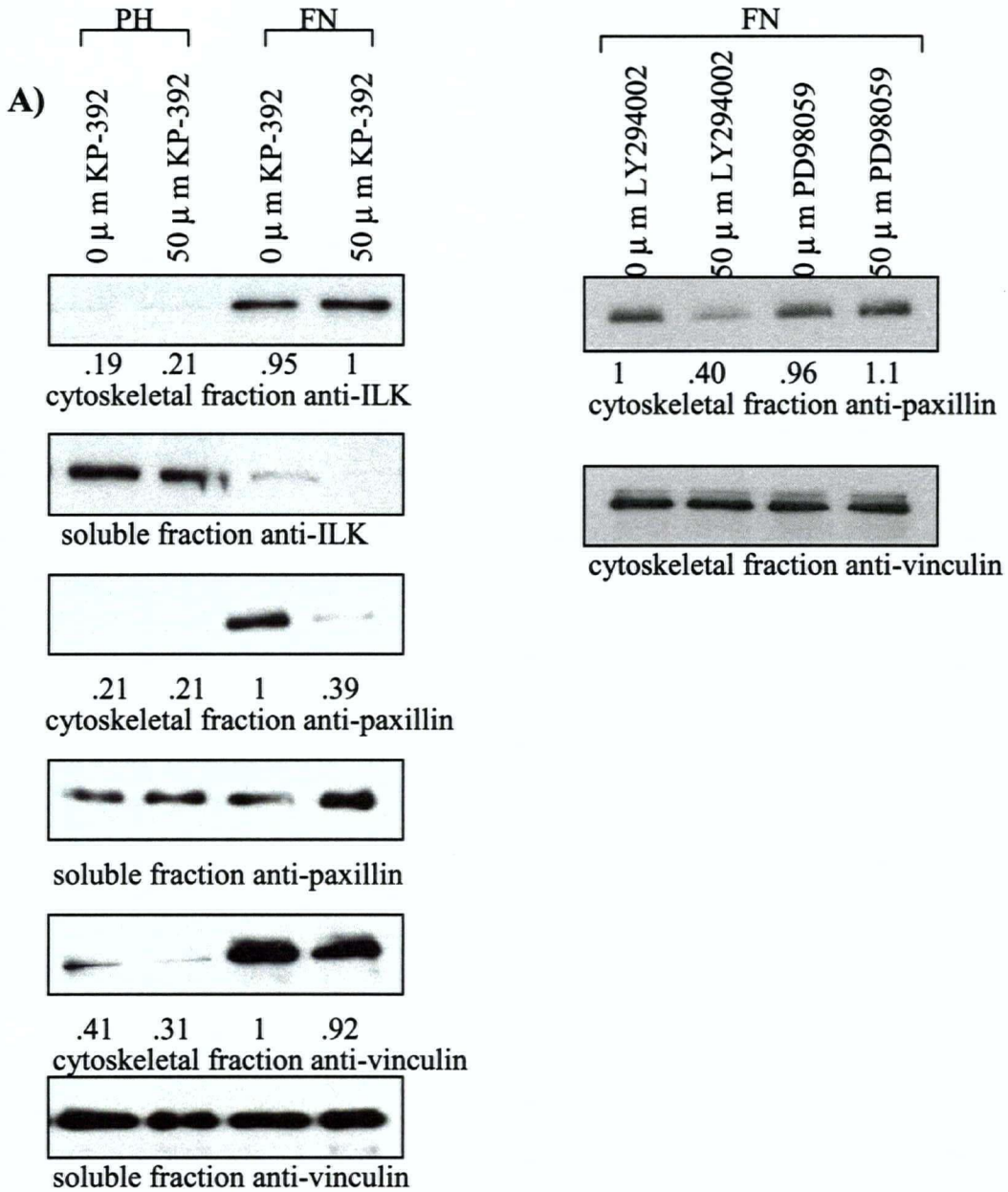


Figure 3-4. Inhibition of ILK activity disrupts the localization of paxillin to the Triton-insoluble fraction, and disrupts ILK:CH-ILKBP binding.

A) Inhibition by KP-392. Du145 cells were plated on either poly-HEMA (PH) or fibronectin (FN) coated plates, and treated with either DMSO or 50 uM KP-392 for 16 hours. Soluble and cytoskeletal fractions were then separated, and western blots performed as described in Methods.

B) PC3 cells were transiently transfected with ILK-H siRNA specific to the PH-like domain of ILK (I), or with control siRNA (C), at concentrations of either 10, 25, or 50 nM. After 72 hours, cells were then treated as in A). **C)** PC3 cells were transiently transfected with empty vector or ILK E359K, and after 48 hours, treated as in A). **D)** Du145 cells were plated on poly-HEMA or fibronectin and treated with increasing amounts of KP-392. The cytoskeletal fraction was isolated, and this fraction was immunoprecipitated with anti-ILK antibody. Western blots were then performed.

Figure 3-4 B)

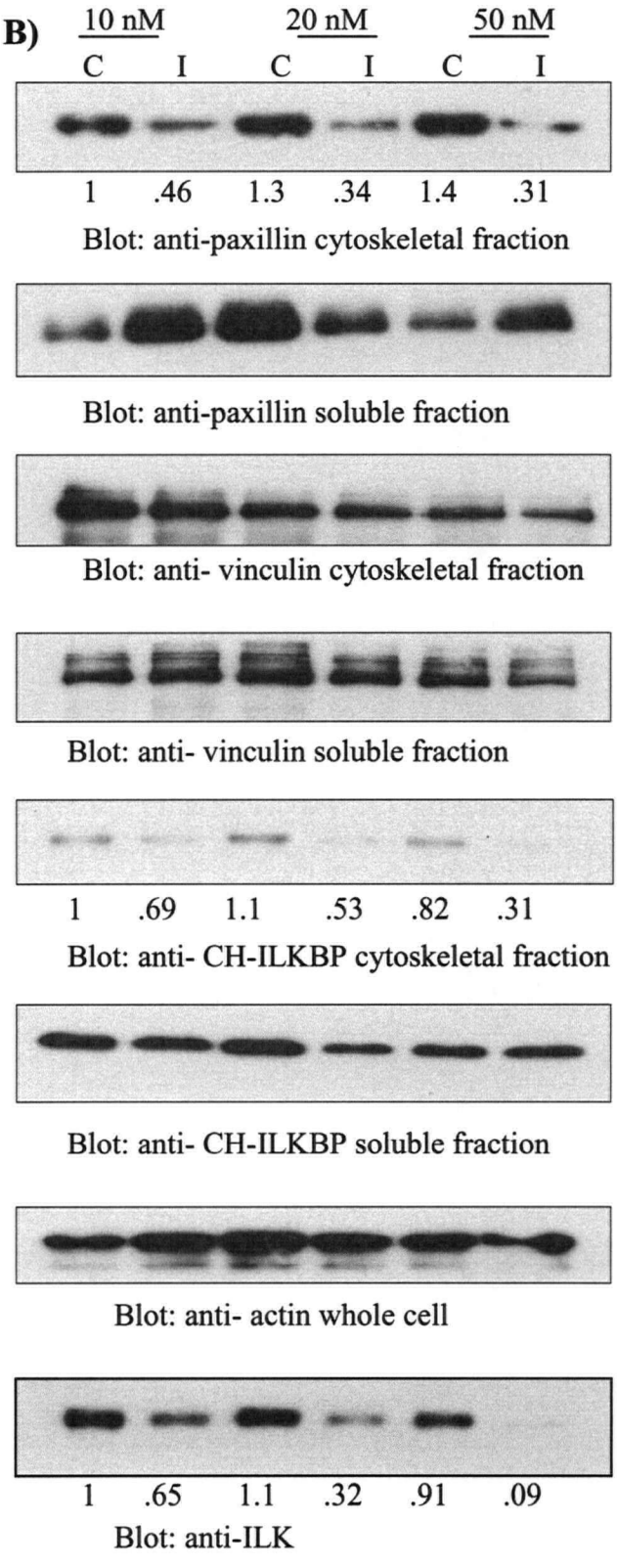

10 nM
20 nM
50 nM
C I C I C I
1 .46 1.3 .34 1.4 .31
Blot: anti-paxillin cytoskeletal fraction
Blot: anti-paxillin soluble fraction
Blot: anti- vinculin cytoskeletal fraction
Blot: anti- vinculin soluble fraction
1 .69 1.1 .53 .82 .31
Blot: anti- CH-ILKBP cytoskeletal fraction
Blot: anti- CH-ILKBP soluble fraction
Blot: anti- actin whole cell
1 .65 1.1 .32 .91 .09
Blot: anti-ILK

Figure 3-4 C)

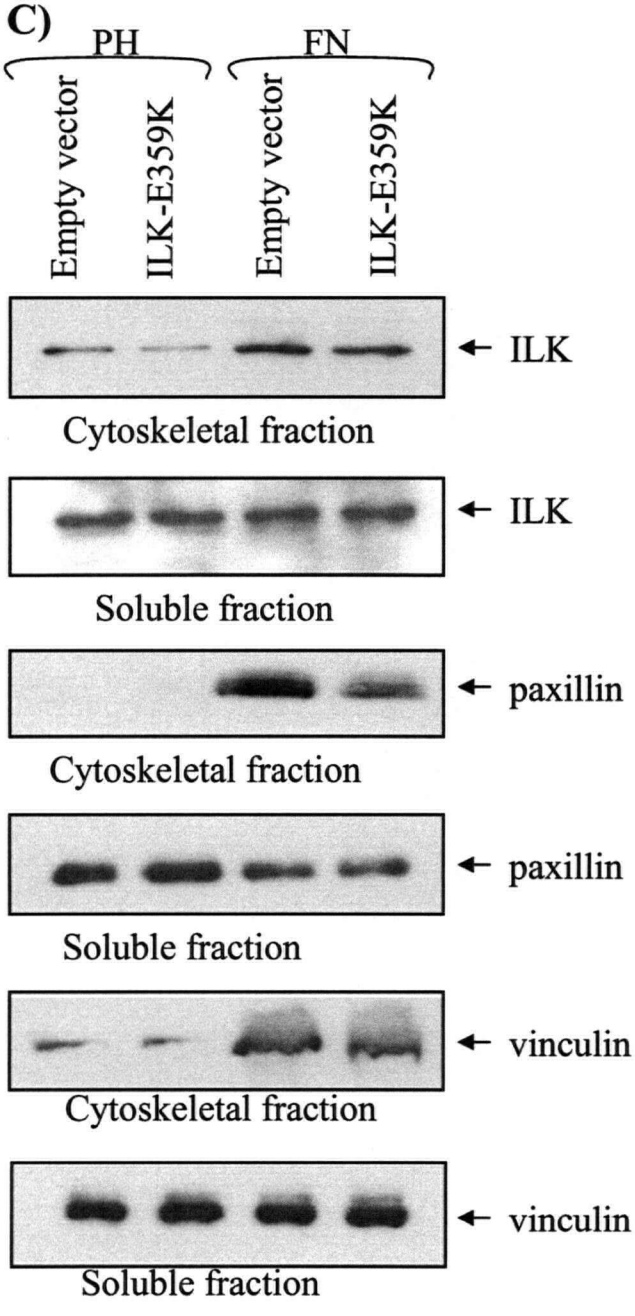
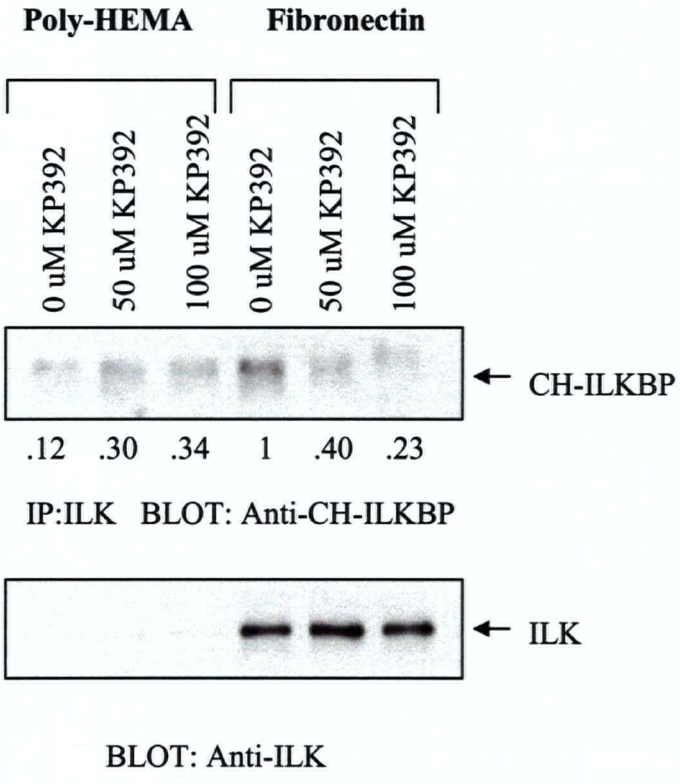

PH
FN
Empty vector
ILK-E359K
Empty vector
ILK-E359K
ILK
Cytoskeletal fraction
ILK
Soluble fraction
paxillin
Cytoskeletal fraction
paxillin
Soluble fraction
vinculin
Cytoskeletal fraction
vinculin
Soluble fraction

Figure 3-4 D)


Poly-HEMA
Fibronectin
0 uM KP392
50 uM KP392
100 uM KP392
0 uM KP392
50 uM KP392
100 uM KP392
CH-ILKBP
.12 .30 .34 1 .40 .23
IP:ILK BLOT: Anti-CH-ILKBP
ILK
BLOT: Anti-ILK

was examined in both whole cells and the cytoskeletal fraction. However, due to lower total levels of ILK and CH-ILKBP in Du145 cells, it was only possible to stain for these proteins in whole cells. As shown in figure 3-5 A), in the cytoskeletal fraction paxillin and vinculin co-localize to focal adhesion plaques. However, upon treatment with KP-392, paxillin is dramatically reduced from the focal adhesion plaques, while vinculin remains unchanged. Paxillin-vinculin costaining was also examined in whole cells (figure 3-5 B), however, due to much higher background staining levels of paxillin in the whole cells, only a slight change in focal adhesion staining is visible. However, the focal adhesions do appear reduced in size and number. Actin organization (shown by phalloidin staining) is also altered in the KP-392 treated cells, showing increased formation of stress fibers and accumulation of F-actin. This alteration in actin organization and accumulation is more obvious in the whole cell staining (figure 3-5B) where there is a clear increase in stress fibers, and loss of peripheral, cortical actin. There are also significant areas of F-actin accumulation (arrows). The whole cell staining also shows a selective loss of paxillin at the focal adhesions upon treatment with KP-392, as well as loss of CH-ILKBP at the focal adhesions. ILK staining, however, remains unchanged. It is important to note that cells were allowed to attach to fibronectin prior to treatment with KP-392. KP-392 does not cause detachment of attached cells, but will inhibit the rate of attachment with pre-incubation (as seen in figure 1).

ILK activity is stimulated in the cytoskeletal fraction.

To determine if ILK activity is dependent on its subcellular localization, an ILK kinase assay was performed on the soluble and cytoskeletal fractions of PC3 cells plated

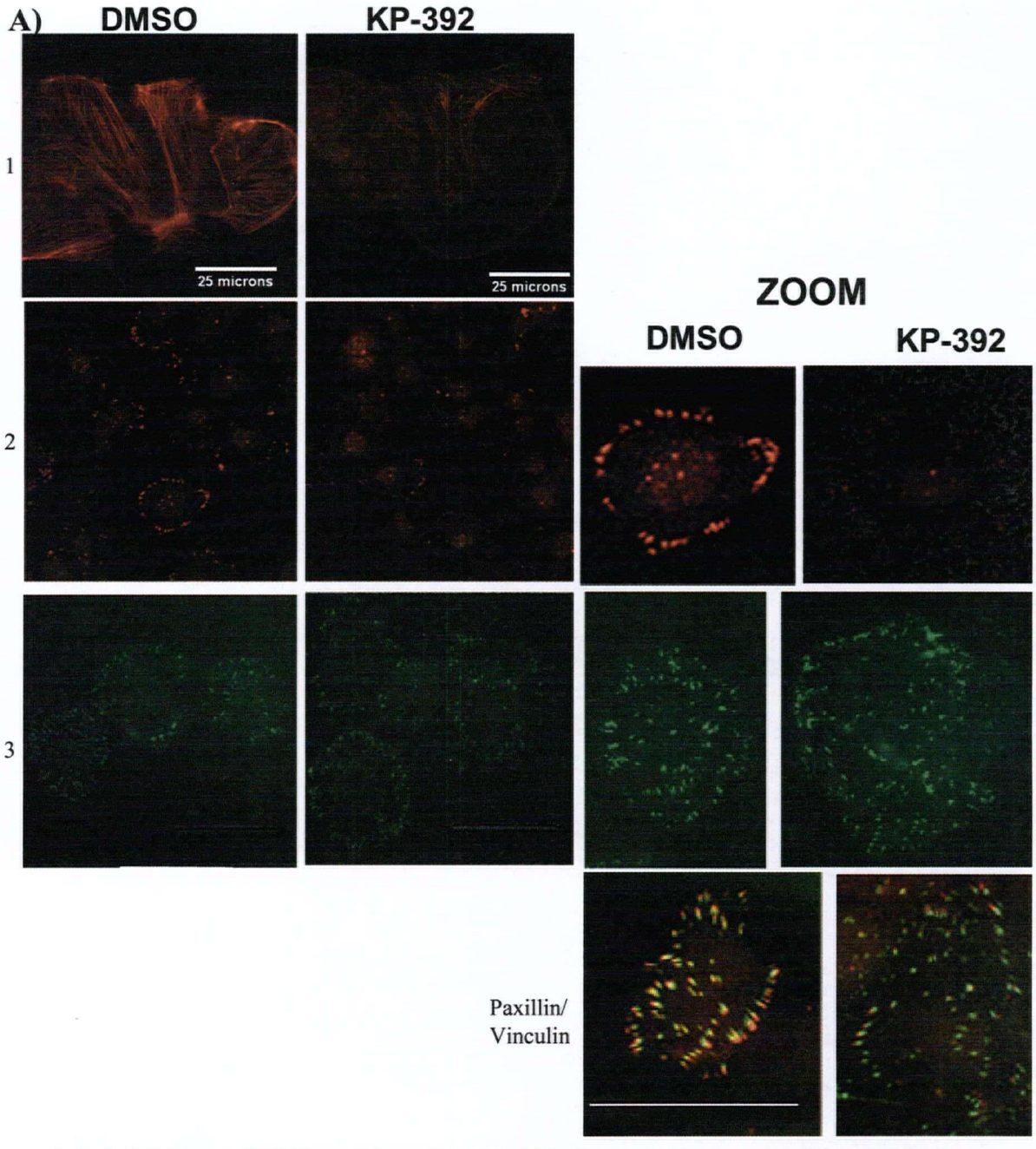
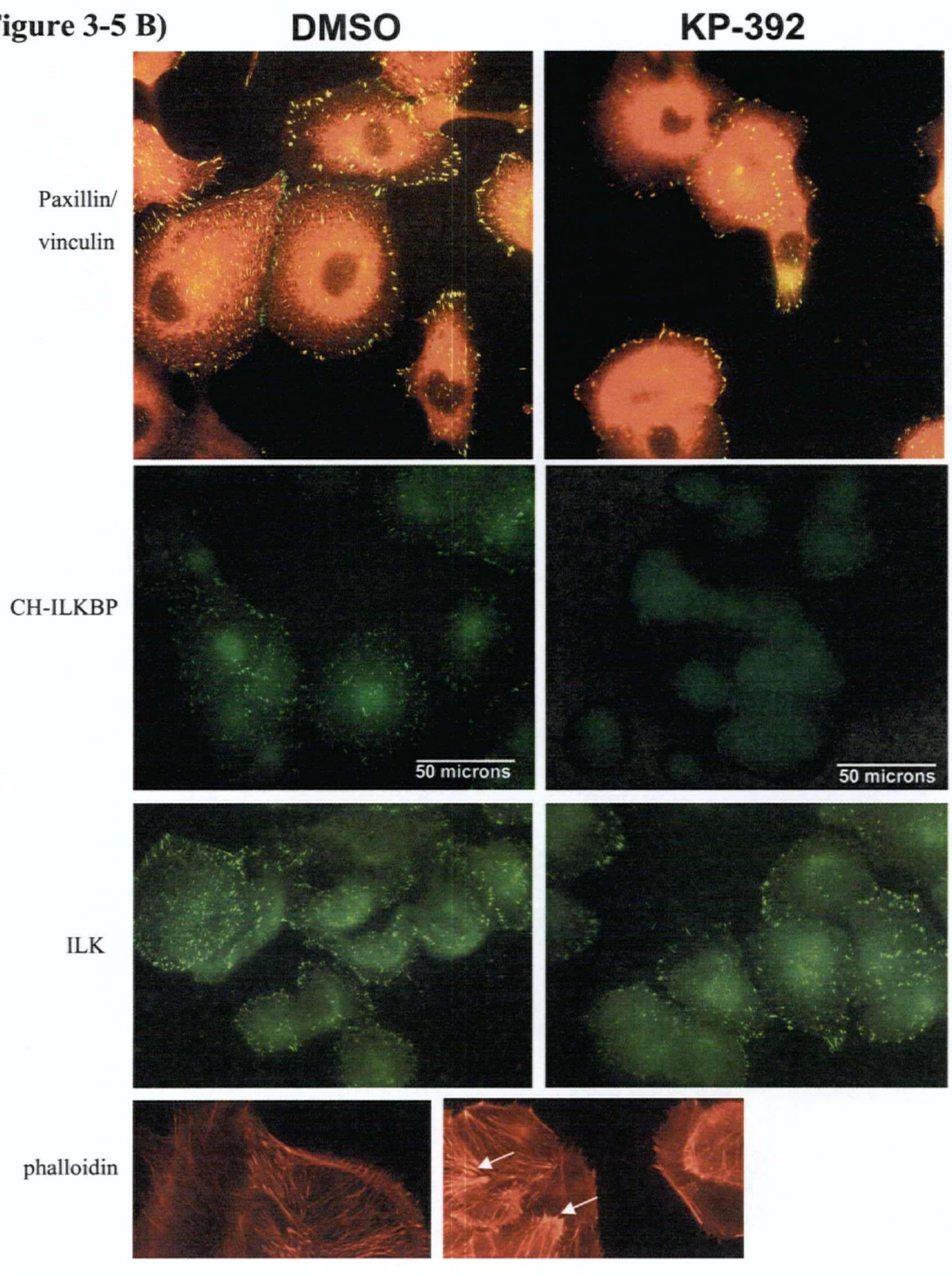


Figure 3-5. Inhibition of ILK activity with KP-392 disrupts the localization of paxillin and CH-ILKBP, but not ILK and vinculin, to focal adhesion plaques.

Du145 cells were plated on fibronectin-coated coverslips, with DMSO or 50 uM KP-392, for 16 hours. Cells were then either solubilized with a Triton wash **(A)**, or fixed as whole cells **(B)**. Samples were then stained with the appropriate antibodies: Phalloidin (1), paxillin (2), and vinculin (3) **A)** Solubilized staining. Rhodamine, bar represents 25 μm. Paxillin (rhodamine) and vinculin (FITC), bar represents 50 μm. Paxillin and vinculin zoom, Bar represents 39 μm. **B)** Whole cell staining. Paxillin (rhodamine) and vinculin (FITC) merge. CH-ILKBP (FITC), ILK (FITC), and phalloidin. Paxillin and CH-ILKBP are specifically dissociated from focal adhesion plaques upon inhibition of ILK activity. Results are representative of 3 independent trials. Bar represents 50 μm.

Figure 3-5 B)


DMSO
KP-392
Paxillin/
vinculin
CH-ILKBP
50 microns
50 microns
ILK
phalloidin

on the β1 integrin extracellular matrix substrate fibronectin, or on poly-HEMA, a control substrate to which cells cannot bind. As shown in figure 3-6 A), although roughly equal amounts of ILK are immunoprecipitated in 250 ug of protein in each of the samples, the ILK found in the cytoskeletal fraction of the fibronectin-plated cells plated is substantially more active than ILK present in the soluble fraction. This suggests that ILK is preferentially more active in the insoluble focal adhesions that are formed following integrin engagement.

Active ILK is bound to CH-ILKBP

As shown in figure 3-6 B), an immunoprecipitated CH-ILKBP complex is able to phosphorylate GSK-3 fusion protein on Serine 9, and this phosphorylation is blocked by the ILK inhibitor KP-392 (100nM KP-392, added to the reaction mixture). Stripping and reprobing this blot shows that ILK is present in this complex. When these depleted lysates are immunoprecipitated with anti-ILK antibody, there is very little ILK kinase activity left in the CH-ILKBP depleted lysate, showing that most active ILK is bound to CH-ILKBP.

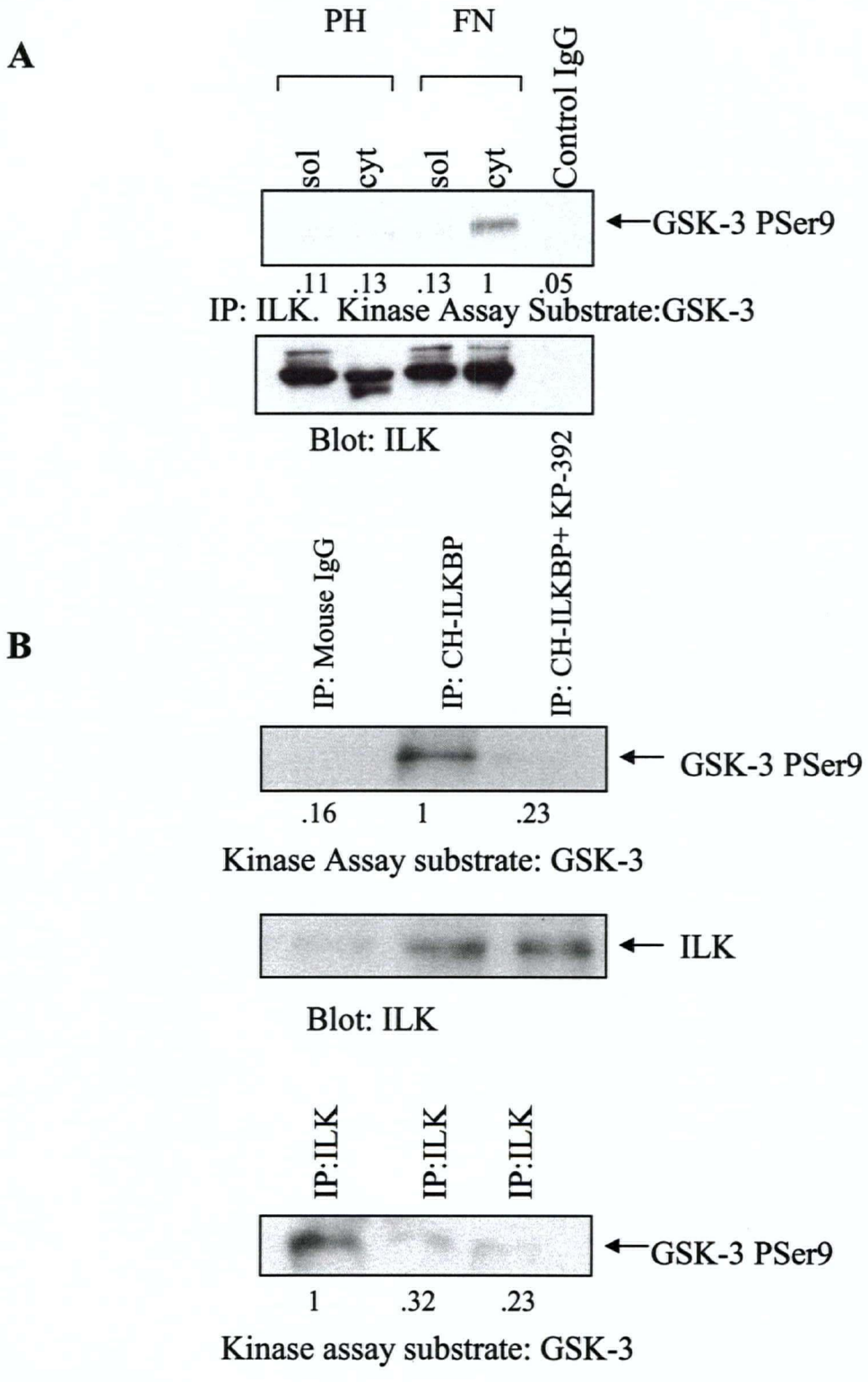
CH-ILKBP stimulates ILK activity and signalling.

Previously, it has been shown that CH-ILKBP is required for the recruitment of ILK to focal adhesions (Zhang *et al,* 2002). Because we have demonstrated that ILK is preferentially active in the cytoskeletal fraction, we examined the effect of CH-ILKBP on ILK signalling. We transfected either empty vector, CH-ILKBP, or an ILK-binding defective mutant form of CH-ILKBP (CH-ILKBP F271D) (Tu *et al,* 2001) into PTEN-

Figure 3-6. Active ILK is bound to CH-ILKBP, and is localized to the cytoskeletal fraction when cells are plated on fibronectin.

A)Serum-starved PC3 cells were plated on the substrates poly-HEMA (PH) or fibronectin (FN) for 1 hour, and the soluble and cytoskeletal fractions were separated. ILK (or control mouse IgG) was immunoprecipitated from the cytoskeletal fraction, and kinase assays were performed using GSK-3 fusion protein as a substrate. Samples were then western blotted with anti-phospho GSK-3α/β Ser 21/9, and anti-ILK.

B) PC3 cells lysates were immunoprecipitated with anti mouse IgG, or with anti CH-ILKBP, and kinase assays were performed using GSK-3 fusion protein as a substrate. 100 nM KP-392 was added to one of the anti-CH-ILKBP immunoprecipitates. The blot was stripped and re-probed with anti-ILK, to show immunoprecipitation. The leftover lysates (cleared with anti mouse IgG or anti CH-ILKBP) were then immunoprecipitated with anti-ILK antibody, and the ILK kinase assay was then performed on the GSK-3 fusion protein substrate. Results are representative of 3 independent trials.


A
PH
FN
sol
cyt
sol
cyt
Control IgG
GSK-3 PSer9
.11 .13 .13 1 .05
IP: ILK. Kinase Assay Substrate:GSK-3
Blot: ILK
B
IP: Mouse IgG
IP: CH-ILKBP
IP: CH-ILKBP+ KP-392
GSK-3 PSer9
.16 1 .23
Kinase Assay substrate: GSK-3
ILK
Blot: ILK
IP:ILK
IP:ILK
IP:ILK
GSK-3 PSer9
1 .32 .23
Kinase assay substrate: GSK-3

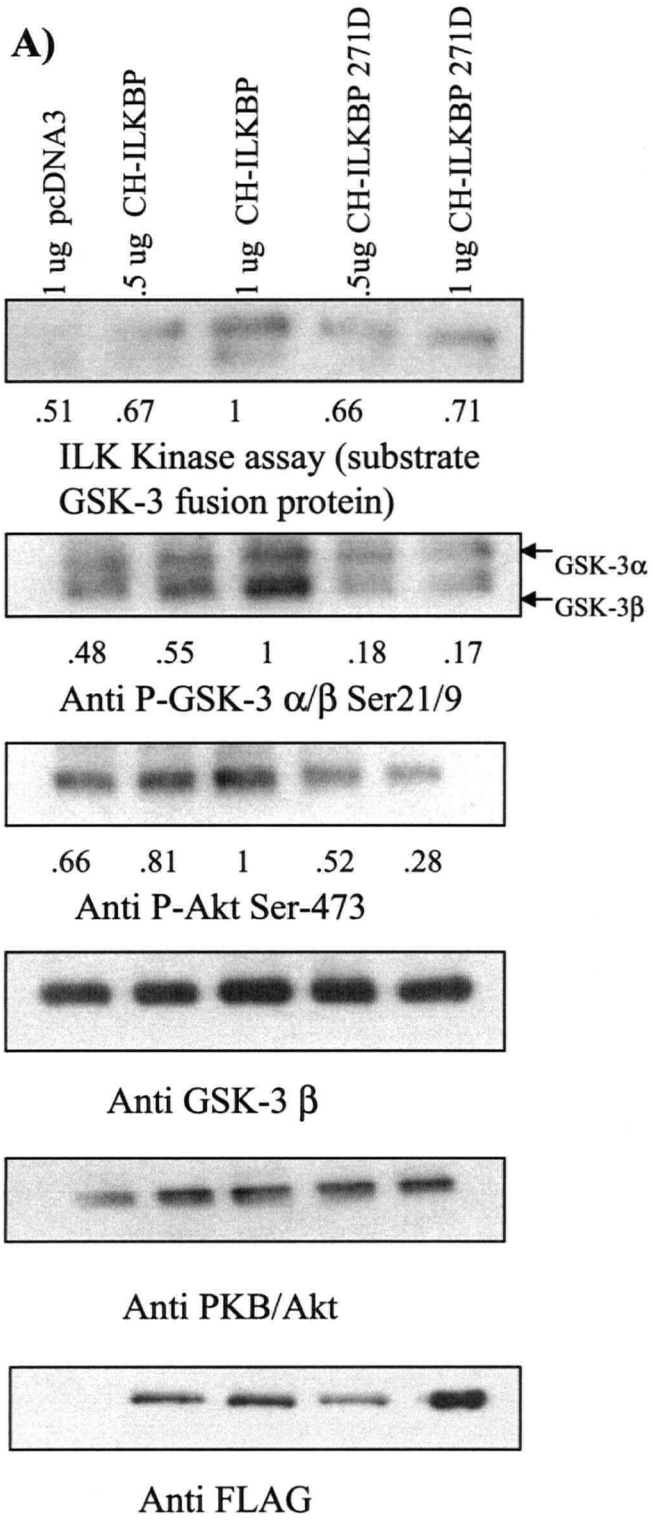
positive DU145 cells, in which ILK activity is inducible. When the cells were serum-starved and then re-fed for 1 hour, we observed that CH-ILKBP stimulated ILK kinase activity in a dose-dependent manner (figure 3-7 A). Furthermore, CH-ILKBP stimulated GSK-3 β phosphorylation on Ser 9, and phosphorylation of PKB/Akt on serine 473, both of which have been shown previously to be regulated by ILK (Delcommenne *et al,* 1998). CH-ILKBP also increased β catenin TCF/LEF reporter activity, as seen by the TOP/FOP FLASH reporter assay (figure 3-7 B). In contrast, the ILK-binding defective mutant form of CH-ILKBP (CH-ILKBPF271D), did not appear to stimulate ILK activity or signalling, and indeed, appeared to behave as a dominant-negative mutant, decreasing basal levels of GSK-3 and PKB/Akt phosphorylation, and dramatically decreasing β-catenin TCF/LEF reporter activity in these cells.

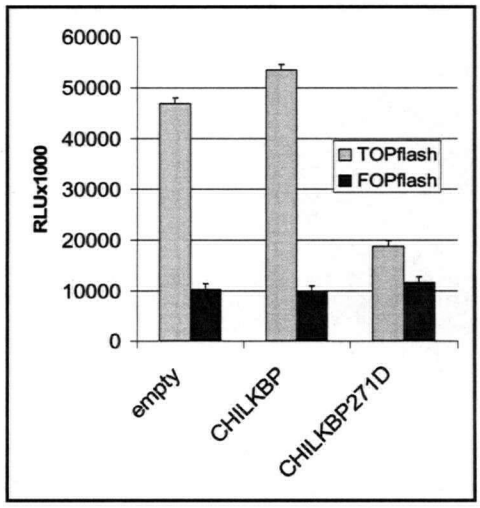
PTEN, and Inhibitors of PI3 Kinase Disrupt the ILK: CH-ILKBP interaction

Because ILK activity has been shown previously to be PI3 kinase dependent, we next tested the effect of disruption of the PI3 kinase pathway on the ILK: CH-ILKBP interaction in PC3 cells. As shown in figures 3-8 A) and B), both the pharmacological inhibition of PI3 kinase and re-introduction of PTEN disrupt the ILK: CH-ILKBP interaction. Trypan blue staining confirmed that PTEN, wortmannin and LY294002 had no effect on cell viability in the concentrations used (data not shown).

To further confirm the role of PI3 kinase and its product PiP3 in the regulation of the ILK:CH-ILKBP interaction, we utilized a PiP3 binding domain point mutant of ILK (ILK R211A), which disrupts the ability of ILK to promote PKB/Akt serine 473 phosphorylation (Persad *et al,* 2001). As shown in figure 3-8 C), this mutant is defective

Figure 3-7. CH-ILKBP, but not an ILK-binding mutant (CH-ILKBP F271D) stimulates ILK signalling in DU145 prostate cancer cells. **A)** Cells were transfected with empty vector, CH-ILKBP, or CH-ILKBP F271D. After 48 hours, cells were serum-starved overnight, then re-fed for 1 hour. Samples were western blotted with anti-phospho GSK-3α/β Ser 21/9, anti-phospho PKB/Akt Ser 473, anti-GSK-3 β, anti-PKB/Akt, and anti-FLAG. Kinase assay was performed using GSK-3 fusion protein as a substrate, and then western blotting with anti-phospho GSK-3α/β Ser 21/9. **B)** HEK-293 cells were transfected with empty vector, CH-ILKBP, or CH-ILKBP F271D, as well as either the TOP or FOP FLASH reporter constructs, and pRenilla. After 48 hours, a dual luciferase reporter assay was performed. Results are representative of 3 independent trials.


A)
1 ug pcDNA3
.5 ug CH-ILKBP
1 ug CH-ILKBP
.5ug CH-ILKBP 271D
1 ug CH-ILKBP 271D
.51 .67 1 .66 .71
ILK Kinase assay (substrate GSK-3 fusion protein)
GSK-3α
GSK-3β
.48 .55 1 .18 .17
Anti P-GSK-3 α/β Ser21/9
.66 .81 1 .52 .28
Anti P-Akt Ser-473
Anti GSK-3 β
Anti PKB/Akt
Anti FLAG


B)
60000
50000
40000
30000
20000
10000
0
RLUx1000
TOPflash
FOPflash
empty
CHILKBP
CHILKBP271D

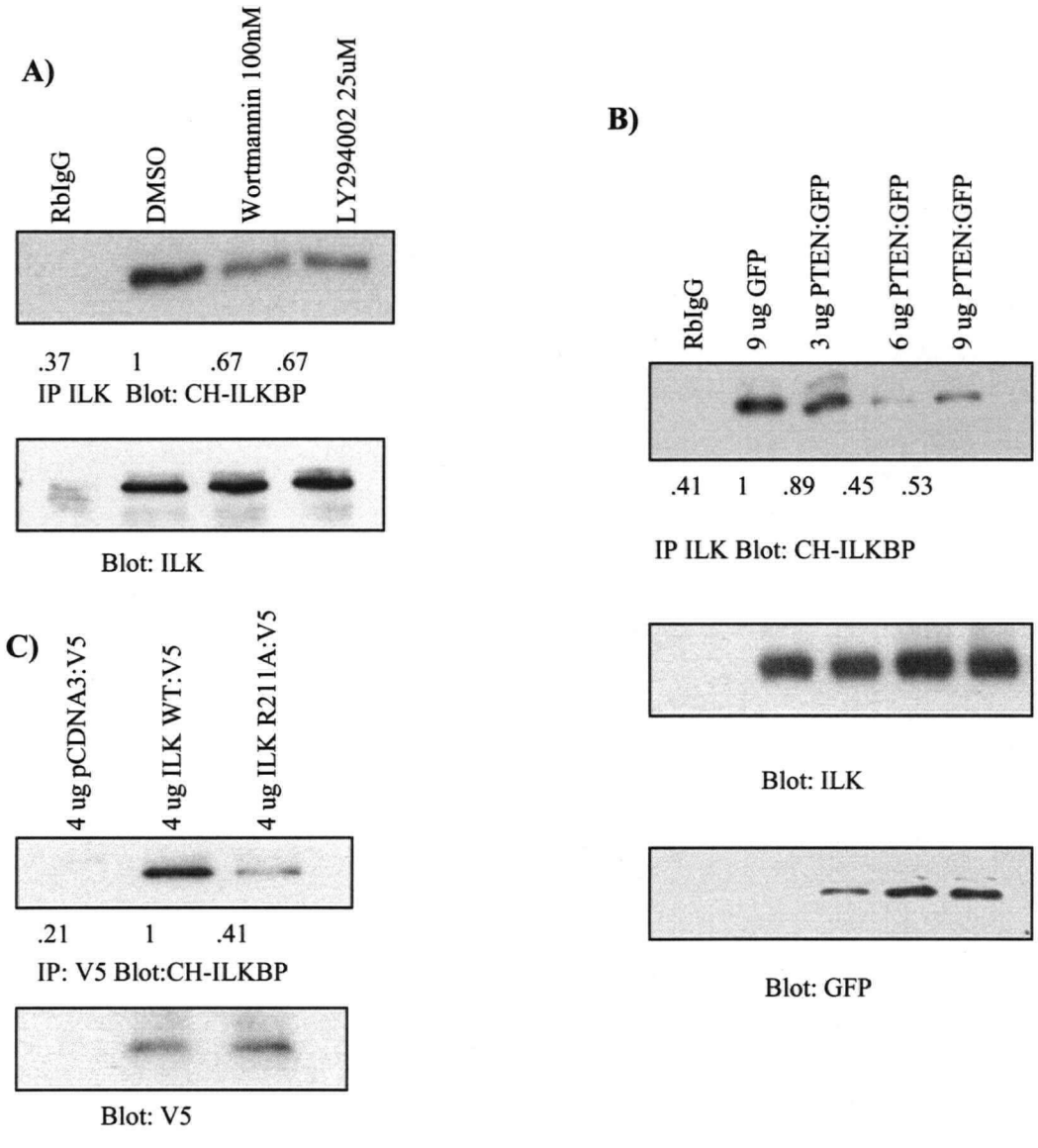


Figure 3-8. THE ILK:CH-ILKBP interaction is PI3 Kinase dependent. **A)** Chemical inhibitors disrupt the ILK:CH-ILKBP interaction. PC3 cells were treated with either DMSO, wortmannin, or LY294002 for 3 hours. Cells were then lysed with NP-40, and immunoprecipitated with anti-ILK. Samples were then western blotted with anti-CH-ILKBP, and stripped and re-probed with anti-ILK. **B)** PTEN re-introduction disrupts the ILK:CH-ILKBP interaction. PC3 cells were transfected with increasing amounts of PTEN. After 48 hours, cells were treated as in A), and samples were also stripped and re-probed with anti-GFP as a transfection control. **C)** The ILK:CH-ILKBP interaction is reduced when the proposed PiP3 binding domain of ILK is mutated. PC3 cells were transfected with pCDNA3:V5, ILKWT:V5, or ILK R211A:V5. After 48 hours, cells were lysed with NP-40 lysis buffer, immunoprecipitated with anti-V5 antibody, and then treated as in A). Results are representative of 3 independent trials.

in the ability to bind CH-ILKBP, providing further evidence that activation by PI3 kinase/ PiP3 pathway is required for proper ILK/ CH-ILKBP interaction and function.

ILK, αPIX and CH-ILKBP exist in a complex which is dependent on ILK kinase activity.

Because αPIX has been shown to bind β-parvin, we wanted to determine if it could also interact with the closely related CH-ILKBP (also known as α-parvin), and the CH-ILKBP binding protein ILK. Here, we show that CH-ILKBP, αPIX, and ILK exist in a complex which is somewhat dependent on ILK activity (figure 3-9).

Rac activity is dependent on ILK kinase activity.

To determine if Rac activity is dependent on ILK activity in HEK-293 cells, we performed Rac activity assays after treatment with increasing amounts of KP-392 or ILK siRNA. As shown in figure 3-10, Rac activity decreases with downregulation of both ILK activity and total protein levels.

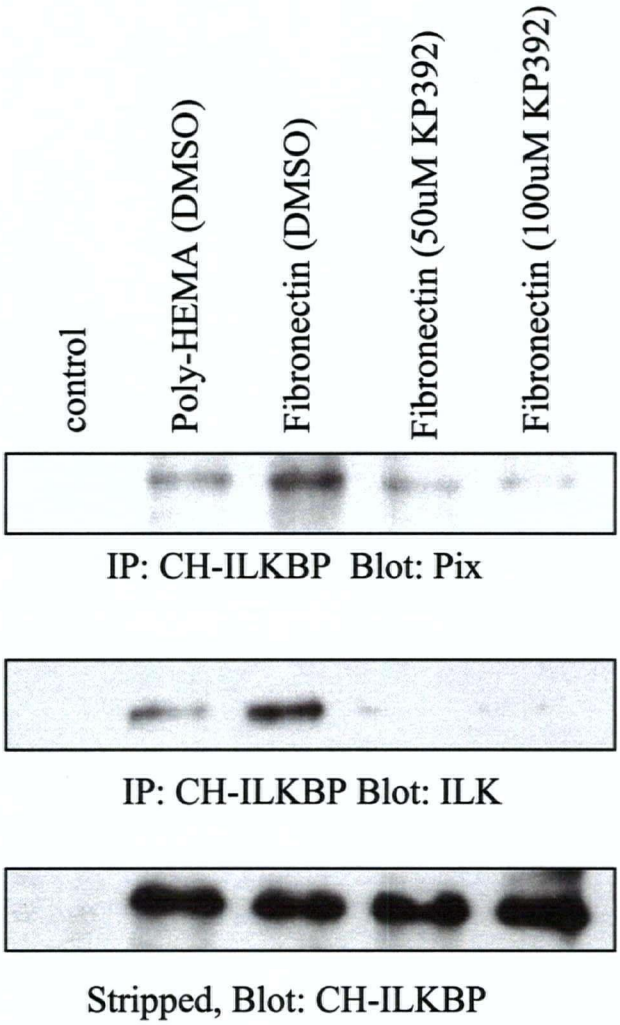


Figure 3-9. ILK, CH-ILKBP and αPIX exist in a complex that is dependent on ILK kinase activity. HEK-293 cells were treated with either DMSO or KP-392 overnight, and then plated on PH or FN for 1 hour. Lysates were immunoprecipitated with anti-CH-ILKBP or anti-mouse IgG (control), and then western blotted with anti-αPIX or anti-ILK. Results are representative of 3 independent trials.

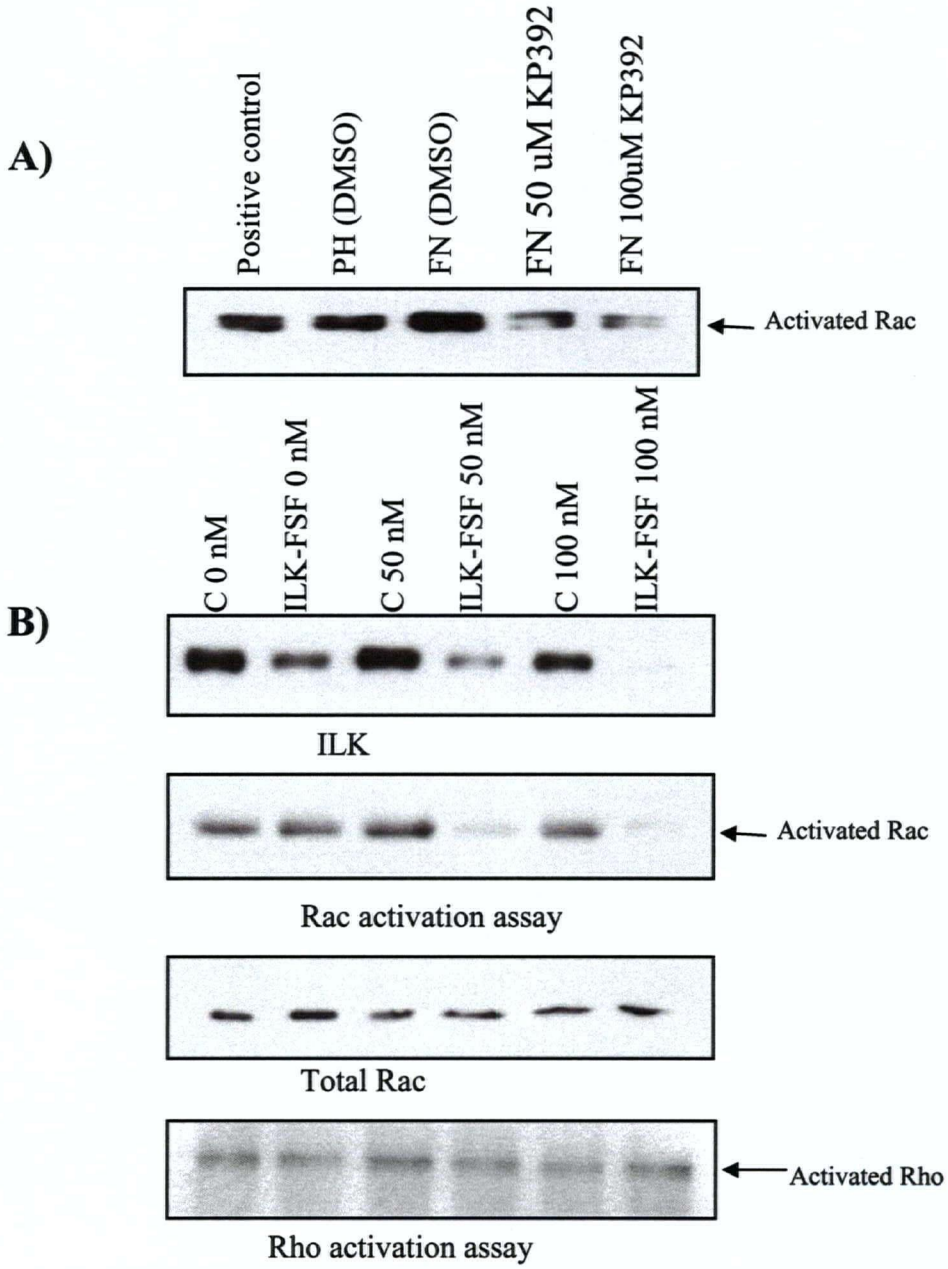


Figure 3-10. Rac, but not Rho activity is dependent on ILK. **A)**HEK-293 cells were treated with DMSO or KP-392 overnight, and plated on PH or FN for 1 hour. Rac assay was performed as described in materials and methods. **B)**HEK-293 cells were transfected with siRNA, and after 72 hours, cells were plated on FN for 1 hour. Rac and Rho assays were performed as described in materials and methods. Samples were also western blotted with anti-ILK (Transduction Labs) and anti-Rac.

Discussion

Recent studies in *Drosophila, C. elegans* and mouse have demonstrated that ILK null mutants display significant inhibition of integrin-related cell adhesion and cytoskeletal organization, (Mackinnon *et al,* 2002, Zervas *et al,* 2001, Sakai *et al,* 2003), supporting a crucial role for ILK in regulating cell adhesive functions. Here, we have employed several different methods to inhibit ILK activity: KP-392, a small molecule ILK inhibitor which has previously been shown to inhibit ILK kinase activity in a highly selective manner (Persad *et al,* 2001), ILK-DN, a kinase-deficient point mutant of ILK which behaves as a dominant negative (Wu *et al,* 1998), the tumor suppressor PTEN, which when re-introduced into PTEN-deficient PC3 cells, decreases the kinase activity of ILK (Persad *et al,* 2000, Morimoto *et al,* 2000), and finally, small-interfering RNA (siRNA) targeting the ILK protein. Here, we show that reducing ILK kinase activity or downregulating ILK expression by siRNA inhibits cell attachment, β1 integrin activation, and cell migration. These results agree with the effects of knocking out ILK in embryonic stem cells and chondrocytes (Sakai *et al,* 2003, Grashoff *et al,* 2003, Terpstra *et al,* 2003), which have shown that ILK knockout results in embryonic lethality, and severe defects in cell attachment, migration, proliferation, and F-actin accumulation. However, some of these studies raised questions about the importance of the kinase activity of ILK in regulating attachment and migration, due to a rescue of these phenotypes by a partial kinase deficient mutant of ILK (Sakai *et al,* 2003). Therefore, the mechanism for the inhibition of cell attachment and spreading in ILK-null cells remains unclear. We hypothesized that the inhibition of cell attachment, integrin activation, and migration

upon inhibition of ILK activity could be due to a role of ILK in focal adhesion formation and actin organization. To investigate this possibility, we studied the effect of inhibiting ILK activity, and ILK protein expression, on the localization of several focal adhesion proteins to the actin cytoskeleton. Here, we have shown that both inhibition of ILK activity and ILK protein expression decreased the localization of the ILK binding partners, paxillin (Nikolopoulos and Turner, 2001), and CH-ILKBP to focal adhesions, and also decreased the association of ILK with CH-ILKBP, in response to cell adhesion on fibronectin. Interestingly, another component of focal adhesion plaques, vinculin, which does not bind ILK directly, was unaffected by inhibition of ILK activity. In addition, we have found that the inhibition of ILK activity also results in altered F-actin accumulation similar to that observed in ILK knockout fibroblasts (Sakai *et al,* 2003). This suggests that in the absence of ILK activity, currently unidentified substrates are not phosphorylated, thus preventing proper focal adhesion formation and F-actin organization. This, in turn could lead to defective integrin function, and changes in actin cytoskeletal formation. Alternatively, ILK in the inactive conformation may be unable to bind paxillin and CH-ILKBP, thus preventing their recruitment to focal adhesion plaques. To substantiate the evidence that both the kinase activity and adaptor functions of ILK are crucial in regulating integrin function, we have shown three different methods of inhibiting ILK. Although complete loss of ILK protein is generally lethal to most cell types, we have found here that largely downregulating ILK protein levels and activity causes dramatic, dose-dependent changes to integrin and focal adhesion function. We believe that taken together, these data provide strong evidence that ILK plays an integral

role, as a kinase as well as an adaptor protein, in regulating cell adhesion and focal adhesion formation.

The kinase activity of ILK is transiently stimulated upon integrin engagement with the extracellular matrix (Dedhar, 2000, Wu *et al,* 1998). It has also been reported that ILK is recruited to focal adhesions upon cell attachment (Li *et al,* 1999), and that the ILK-interacting proteins CH-ILKBP (Tu *et al,* 2001, Zhang *et al,* 2002) and affixin (Yamaji *et al,* 2001) play crucial roles in this process. Here, we show that the fraction of ILK which is recruited to focal adhesions in response to cell adhesion has higher enzymatic activity, and that active ILK is specifically bound to CH-ILKBP. This suggests that ILK is activated once recruited to the focal adhesions, or, alternatively, that ILK is recruited specifically in its active conformation.

It has recently been reported that CH-ILKBP is necessary for the proper recruitment of ILK to focal adhesions following cell attachment (Zhang *et al*, 2002). Given that the activity of ILK depends on its localization, we wanted to determine if CH-ILKBP affected ILK signalling. It was observed that CH-ILKBP transiently increased ILK activity, and stimulated its downstream targets, in a dose-dependent manner. A mutant form of CH-ILKBP (CH-ILKBPF271D) which does not bind ILK, however, displayed a dominant-negative effect in down-regulating ILK signalling. From these results, we propose that CH-ILKBP is responsible for recruiting ILK, in its active conformation, to focal adhesion complexes, where it then participates in downstream signalling events such as the stimulation of PKB/Akt and GSK-3 phosphorylation. Since the ILK-binding mutant of CH-ILKBP inhibited ILK's downstream signalling, it is

possible that this mutant is competing with wild-type CH-ILKBP, and thus preventing ILK recruitment and having a negative effect on ILK signalling. Interestingly, knock-down of CH-ILKBP results in the inhibition of PKB/Akt activation and stimulation of apoptosis (Fukuda *et al,* 2003). The apoptosis was rescued by membrane targeted PKB/Akt, implicating a direct role for CH-ILKBP in the activation of PKB/Akt. However, in light of the data presented in this paper, another explanation could be that in the absence of CH-ILKBP, ILK activity is reduced, resulting in decreased PKB/Akt phosphorylation on Serine-473 and decreased kinase activity. We have recently shown that ILK knockout results in complete inhibition of PKB/Akt phosphorylation on Serine-473, and that ILK is essential for PKB/Akt activation (Troussard *et al,* 2003).

The tumor suppressor PTEN has previously been shown to inhibit cell migration, and proper focal adhesion formation, although this was thought to be due to its effects on the inhibition of FAK and Shc (Tamura *et al,* 1998, Yamada *et al,* 2002). As mentioned previously, PTEN also has a negative effect on ILK kinase activity (Persad *et al,* 2000, Morimoto *et al,* 2000), due to its ability to de-phosphorylate PiP3, a product of PI3 kinase which activates ILK (Delcommenne *et al,* 1998). Thus, in PTEN negative cells, ILK is constitutively active (Persad *et al,* 2000). Here, we have shown that in PTEN-negative PC3 cells, the interaction of ILK and CH-ILKBP is constitutive, even under serum-starved conditions, and that re-introduction of PTEN disrupts the interaction. Pharmacological inhibition of PI3 kinase also has the same effect. Thus, the observed effect of PTEN on cell migration and focal adhesion formation may be partially due to the disruption of the ILK:CH-ILKBP interaction. It is unclear exactly how PTEN

disrupts the ILK:CH-ILKBP interaction; it is possible that partial activation of ILK by the PI3 kinase pathway is required for binding and subsequent recruitment by CH-ILKBP. It is also possible that the effect may be mediated by other molecules that are regulated by PTEN and the PI3 kinase inhibitors.. Further evidence that PI3 kinase activation is required for proper ILK:CH-ILKBP interaction is provided by experiments involving the PiP3-binding mutant of ILK (ILKR211A) (figure 3-8 C). This mutant, which is unable to stimulate PKB/Akt serine 473 phosphorylation, presumably due to its inability to bind PiP3, is also defective in CH-ILKBP binding. It is important to note that CH-ILKBP has been reported to interact with the C-terminal end of ILK (Tu *et al,* 2001), so it is unlikely that a point mutation within the PH-like domain of ILK would disrupt binding to CH-ILKBP.

PI3 kinase is transiently activated upon integrin engagement, probably via integrin aggregation with growth factor receptors (Downward, 1998, Wu, 1999), and activation by Focal Adhesion Kinase (FAK) (Parsons *et al,* 2000). Stimulation of ILK activity is dependent on PI3 kinase (Wang *et al,* 2001, Lynch *et al,* 1999, Yamaji *et al,* 2002), and ILK activity is inhibited by PTEN (Persad *et al,* 2000, Morimoto *et al,* 2000). Here, we have shown that the ILK:CH-ILKBP interaction is also dependent on PI3 kinase, and that PTEN disrupts this interaction. We propose a model (see figure 3-11 for summary) where upon integrin engagement with the extracellular matrix, PI3 kinase is activated, resulting in the stimulation of ILK activity and inducing the ILK:CH-ILKBP interaction, causing translocation to focal adhesions. In focal adhesions, ILK is crucial for both proper focal adhesion formation and activation of the β1 integrin, and

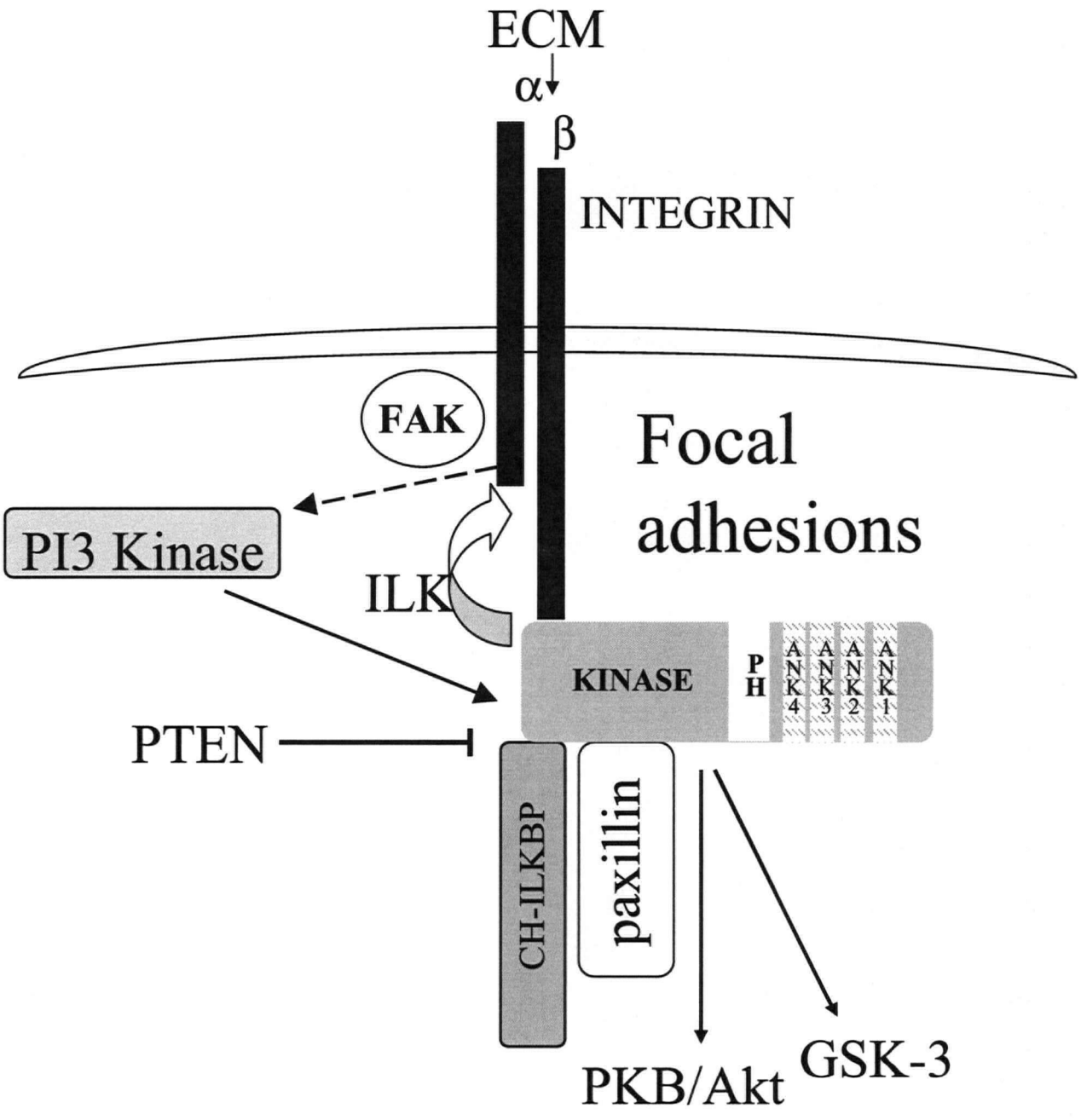


Figure 3-11. Summary of ILK recruitment and activity at focal adhesions.
Upon integrin engagement with the extracellular matrix (ECM), PI3 Kinase is activated, through FAK, and clustering and co-activation of growth factor receptor tyrosine kinases. ILK is then activated through Pi3 Kinase, allowing binding with CH-ILKBP and paxillin, and recruitment to focal adhesion plaques. At the focal adhesion plaques, ILK activity is crucial for maintaining upstream signaling to β1 integrins, and downstream signaling to PKB/Akt and GSK-3.

downstream signalling to both PKB/Akt and GSK-3. In the absence of ILK activation, paxillin and CH-ILKBP are not properly localized to focal adhesion plaques, resulting in alterations in actin organization and accumulation and inhibition of β1 integrin function.

It is known that cell adhesion activates Rho family GTPases in an integrin-dependent manner, although the mechanism by which this occurs is unclear. In light of ILK's effects on cell migration and actin cytoskeletal orgainization, we wanted to investigate the role of ILK in this activation. The data presented here shows that Rac activity is dependent on upstream ILK activity. Furthermore, ILK and CH-ILKBP interact, in an ILK activity-dependent manner, with αPIX, a GEF which activates Rac and Cdc42. This provides a potential link between integrins and Rac and Cdc42, and also sheds light on the upstream activation of αPIX, a protein implicated in X-linked mental retardation. Thus, defects in αPIX may lead to X-linked mental retardation through faulty relaying of cell adhesion and integrin signalling. These findings may provide further explanation for ILK's effect on actin cytoskeletal organization and cell migration.

Taken together, the results underscore the importance of ILK, CH-ILKBP and PTEN in cell migration, cytoskeletal organization and focal adhesion formation, as well as identifying a new role for ILK in the activation of the Rho family GTPase Rac.

Chapter 4. Summary

Conclusions

The data presented here provide further insight into the molecular mechanisms by which ILK elicits its function in normal and cancer cells. The finding that ILK is capable of inhibiting anoikis via the PKB/Akt pathway is crucial to the understanding of how apoptotic signals are transduced following integrin detachment. ILK potentially provides a direct link between integrins and anti-apoptotic signalling pathways, suggesting that it may play a crucial physiological role in the control of anoikis during development. Likewise, targeting ILK in tumours may be beneficial in the prevention of metastases, so that when cell detach from the primary tumour they will undergo anoikis.

Furthermore, these data highlight the importance of ILK regulation in PTEN-null tumours; in these cell lines, ILK activity is constitutively high, further supporting a dependence of ILK kinase activity on PI3 kinase and PiP3. Re-introduction of PTEN into PTEN-negative cells induces cell cycle arrest and apoptosis. Interestingly, introduction of a dominant-negative ILK has the same effect. These results suggest that at least some of the oncogenic properties of PTEN-null cancer cells are due to the constitutive elevation of ILK activity in these cells. This finding bears clinical relevance, as it is usually more feasible to inhibit an overactive enzyme (through drug therapeutics), than to replace an activity that is missing. Thus, inhibiting ILK in PTEN-negative tumour cancer patients may prove to be easier than replacing PTEN activity. This finding is particularly interesting because PTEN mutations are very commonly seen in

certain types of cancers, including glioma and prostate cancers, and loss of PTEN is often associated with aggressivity of the tumour.

The work in this thesis relies on the model that ILK is a PDK-2, an active kinase which is capable of phosphorylating PKB/Akt at serine 473. However, there has been some controversy over whether ILK is able to induce this phosphorylation directly, and indeed, whether ILK is a bona fide serine/threonine kinase. The data presented here show that ILK is capable of forming a complex, in a serum-dependent manner, with both PKB/Akt and PDK-1, and identify the activation loop of ILK as crucial in this interaction. Furthermore, ILK is capable of disrupting the interaction between PDK-1 and PKB/Akt. This supports the model that PDK-1 binds to the serine 473 site of PKB/Akt and prevents its phosphorylation, another molecule must displace PDK-1 from this site, thus allowing its phosphorylation. From the data presented here, we suggest that this molecule is ILK. ILK would disrupt the PDK-1: PKB/Akt interaction, and subsequently phosphorylate PKB/Akt on serine 473. It is also possible that ILK exposes the serine 473 site and allows a distinct PDK-2 to phosphorylate this site. However, in light of recent data that strongly suggests that ILK is a true kinase and is indeed a PDK-2, such as unbiased in-gel kinase assays and siRNA, the theory that ILK both displaces PKD-1 and phosphorylates serine 473 seems most likely. The degree by which ILK acts as the primary PDK-2 may vary according to cell and tissue types, and this may account for some of the discrepancies reported in the field.

It is however, highly unlikely that all of ILK's properties can be attributed to its kinase activity. Loss of ILK protein seems to be more detrimental to cells than specific

loss of ILK kinase activity. Furthermore, ILK is capable of binding not only β integrins, but also several cytoskeletal proteins which possess no enzymatic activities. Thus, it is likely that ILK also behaves as an adaptor protein, linking these proteins to integrins and the ECM. The phenomenon of inside-out signalling through integrins is poorly understood; it is known that upon cell adhesion to the ECM, several cytoskeletal proteins are recruited to sites of integrin adhesion, forming focal adhesions. The formation of these focal adhesions is crucial for the conversion of integrin subunits to the active conformation, which alters their affinity to the ECM, and regulates such processes as cell adhesion, spreading, and migration. The data presented here support a role for ILK kinase activity in inside-out signalling, as the inhibition of ILK activity decreases β1 integrin activation, cell attachment and migration. Interestingly, ILK is also required for the recruitment of select proteins to focal adhesions, as inhibition of ILK activity, or total ILK protein, prevents the localization of the ILK binding partners paxillin and CH-ILKBP, but not vinculin, to focal adhesions, as well as causing dramatic changes in actin cytoskeletal organization. This suggests that active ILK specifically binds and recruits CH-ILKBP and paxillin to focal adhesions. Indeed, we found that active ILK is specifically localized at focal adhesions, and that active ILK is specifically bound to CH-ILKBP. We also show here that CH-ILKBP is necessary for downstream ILK signalling to PKB/Akt, GSK-3, and the LEF-1/TCF transcription factor complex. Because CH-ILKBP has been shown to be required for the localization of ILK to focal adhesions, we suggest a model where active ILK and CH-ILKBP interact, and require each other for recruitment to focal adhesions, where ILK can participate in downstream signalling to

PKB/Akt and GSK-3. ILK is required for the recruitment of paxillin and CH-ILKBP to focal adhesions, and this proper focal adhesion formation is crucial for β1 integrin activation, cell attachment and migration. It is unclear why ILK kinase activity is required for this recruitment and activation; it is possible that ILK phosphorylates paxillin and CH-ILKBP, or it is also possible that ILK, in its active conformation, is capable of binding and recruiting these proteins. The work presented here show that the adaptor functions of ILK are dependent on ILK kinase activity. Interestingly, ILK downstream signalling is also dependent on the adaptor functions of ILK, as CH-ILKBP binding is required for downstream ILK signalling. This suggests that both the adaptor and kinase functions of ILK are equally important and interdependent for ILK function.

Another way by which ILK may regulate actin cytoskeletal organization, attachment and migration is by activation of the Rho family GTPases. It is known that upon integrin-mediated cell adhesion, Rho family GTPases are transiently activated, thus inducing such processes as actin reorganization and the formation of lamellopodia and filopodia. In fact, β1 integrins have been shown to be necessary and sufficient to activate Rac and Cdc42. It is however, unclear which events take place proximal to the integrin receptor to activate these pathways. Here, we show data strongly supporting a role for ILK in this process. CH-ILKBP was shown to interact with the GNEF αPIX, and ILK in a matrix inducible manner, and inhibition of ILK activity and protein expression was found to downregulate Rac, but not Rho, activity. The finding that CH-ILKBP interacts with αPIX was not surprising, given it's high sequence homology to another αPIX-interacting protein, β-parvin. This suggests that ILK may be involved in bridging CH-

ILKBP and αPIX to Rac, where αPIX then catalyzes its activation. This finding presents a completely novel role for ILK in coupling integrins to Rho GTPase signalling pathways.

The results presented here underscore ILK's role as a central regulator of β integrin signalling, as both a kinase and adaptor molecule. They also explain some of ILK's many oncogenic effects, as they identify ILK as an inhibitor of anoikis, and a promoter of apoptosis, cell cycle progression, actin cytoskeletal re-organization, Rac activation, β integrin activation, cell attachment and migration. Furthermore, ILK antagonizes the effects of the tumour suppressor PTEN, and may be very useful in the treatment of PTEN-null tumours. We have identified ILK as an attractive potential target in tumour therapy.

Future directions

With the availability of new tools to downregulate ILK activity, such as siRNA, the Cre-Lox system, and superior caspase antibodies, it is now possible to further examine the role of ILK in the inhibition of anoikis. Some of the questions that may be asked include:

1. What are the events which take place immediately downstream of PKB/Akt in ILK's inhibition of anoikis? For example, does ILK upregulate FasL expression in a PKB/Akt-dependent manner? Or does ILK induce PKB/Akt's phosphorlyation of Bad, caspase-9 or FKHR? The ILK-overexpressing cell lines, and targeting of ILK by siRNA, can be used to answer these questions. Western Blot analysis will be

performed with phospho-specific antibodies against Bad, caspase-9 and FKHR, and supernatant will be western blotted to detect FasL.

2. Does ILK prevent apoptosis and anoikis in vivo? Preliminary results indicate that the ILK inhibitors prevent metastasis of transplanted tumours in vivo, suggesting that induction of anoikis is occuring. By use of various pLox ILK/Cre recombinase mice we can test the effects of loss of ILK in vivo. An ILK pLox mouse has been established, and it has thus far been crossed with chondrocyte and glial cell specific Cre recombinase mice. ILK-negative tissues obtained from these mice will be stained for activated caspase antibodies to determine if cells are undergoing apoptosis.

SiRNA has proven to be an effective means of dramatically downregulating the levels of ILK protein expression. With this novel tool, several questions may be answered, such as:

1. What happens to the transcriptional profile when ILK protein expression is downregulated? To answer this question, microarray analysis will be performed. PC3 or HEK-293 cells will be treated with ILK siRNA vs. Control siRNA, and RNA from treated cells will be collected over several time points. A transcriptional profile will then be obtained by probing 19K human microarray chips with the labeled mRNA. Transcripts which display a variance between control and ILK-downregulated will be confirmed by Northern and Western blot analysis.

2. What happens to the phosphorylation profile of proteins in the cytoplasm when ILK protein expression is downregulated? With commercially available arrays which detect the phosphorylation state of many cytoplasmic proteins, it may be possible to identify novel proteins which are differentially phosphorylated upon loss of ILK expression. These proteins may be previously unidentified downstream or direct targets of ILK.

We have shown here that ILK kinase activity is required for the localization of paxillin and CH-ILKBP to the focal adhesions. This finding raises the question: what, if any, are the targets of ILK phosphorylation necessary for this event? Preliminary data shows that paxillin tyrosine (31 and 118) phosphorylation decreases upon treatment with ILK inhibitors. Since ILK is incapable of tyrosine phosphorylation, this suggests an intermediate kinase, such as FAK, is being inhibited (data shows that the ILK inhibitor has no direct effect on FAK kinase activity). Alternatively, tyrosine phosphorylation of paxillin may be decreased because of failure to localize to focal adhesions and thus be in close proximity of tyrosine kinases. To answer these questions, we will look at FAK activity in ILK inhibitor-treated cells. We will also examine immunoprecipitated ILK's ability to phosphorylate CH-ILKBP and paxillin *in vitro*.

We have also presented here interesting data implicating ILK in Rac activation. Several questions may be asked to verify and further investigate these findings, such as:

1. Does downregulation of ILK by siRNA prevent CH-ILKBP and αPIX from interacting? Hek-293 cells will be transfected with siRNA, and co-immunoprecipitation experiments will be performed as in figure 3-9.
2. Does inhibition of ILK prevent formation of lamellopodia and filopodia? Hek-293 cells treated with either siRNA or KP-392 will be plated onto fibronectin-coated coverslips, and fixed and stained with phalloidin. The leading edges of treated versus control cell will be examined by immunofluorescence microscopy.
3. Does inhibition of ILK prevent Cdc42 activation? Because we have shown that ILK is involved in Rac activation, we will next examine the closely related Rho GTPase Cdc42. Cdc42 activation assays (CST) will be performed as in figure 3-10.
4. Does ILK inhibition prevent downstream Rac and Cdc42 signalling to PAK? Activated PAK antibodies will be utilized to examine downstream signalling to PAK in siRNA and inhibitor-treated cells.

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