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Integrin-Linked Kinase (ILK): A "Hot" Therapeutic Target

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ABSTRACT. Integrin-mediated cell adhesion is known to regulate gene expression through the activation of transcription factors. We have recently revealed that these activations are mediated through integrin-linked kinase (ILK). ILK is an ankyrin repeat-containing serine–threonine protein kinase that can interact directly with the cytoplasmic domain of the β1 and β3 integrin subunits and whose kinase activity is modulated by cell–extracellular matrix interactions. We have shown that ILK overexpression results in the translocation of β-catenin to the nucleus, which then forms a complex formation with the lymphoid enhancer binding factor 1 (LEF-1) transcription factor, subsequently activating the transcriptional activity of promoters containing LEF-1 response elements. ILK phosphorylates the glycogen synthase kinase-3 (GSK-3), which inhibits GSK-3 activity. We have demonstrated that ILK stimulates activator protein-1 transcriptional activity through GSK-3 and the subsequent regulation of the c-Jun–DNA interaction. ILK also phosphorylates protein kinase B (PKB/Akt) and stimulates its activity. We have shown that ILK is an upstream effector of the phosphatidylinositol 3-kinase-dependent regulation of PKB/Akt. ILK has been shown to phosphorylate PKB/Akt on Ser-473 *in vitro* and *in vivo*. Our results clearly indicate that ILK is a key element in the regulation of integrin signaling as well as growth factor and Wnt signaling pathways. *PTEN* (phosphatase and tensin homolog detected on chromosome 10) is a tumor suppressor gene located on chromosome 10q23 that encodes a protein and phospholipid phosphatase. It is now estimated that inactivation mutants of *PTEN* exist in 60% of all forms of solid tumors. Loss of expression or mutational inactivation of *PTEN* leads to the constitutive activation of PKB/Akt via enhanced phosphorylation of Thr-308 and Ser-473. We have demonstrated that the activity of ILK is constitutively elevated in *PTEN* mutant cells. A small molecule ILK inhibitor suppresses the phosphorylation of PKB at the Ser-473 but not the Thr-308 site in the *PTEN* mutant cells. These results indicate that inhibition of ILK may be of significant value in solid tumor therapy. BIOCHEM PHARMACOL **60**;8:1115–1119, 2000. © 2000 Elsevier Science Inc.

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Signal transduction is the major communication network that links the outside of the cell with the inside. The signal flow goes in both directions, although the inward flow has been explored more than the outward flow. Part of this inward flow originates at cell surface transmembrane proteins, which exhibit the exquisite ability to deposit, upon ligand binding, a start signal on the inner side of the plasma membrane which sets off one, or more often, many cascades of intracellular signals.

Protein phosphorylation on serine and threonine or tyrosine residues has emerged as a critically important posttranslational modification at the heart of the regulatory mechanism controlling cell activities. Levels of protein phosphorylation are dictated by the coordinated activities of protein kinases and protein phosphatases, and the large number of genes encoding these enzymes testifies to their fundamental importance in the control of normal cell and body physiology. Mammalian protein kinases fall into two major families that phosphorylate either serine and/or threonine residues (serine–threonine kinases) or tyrosine residues (tyrosine kinase). One major role for cellular kinases is their participation in signal transduction pathways through which cells respond functionally to external messages or to extracellular stresses. Protein kinases play a key role in signal transduction pathways and cancer. The associations of aberrant kinase activity with pathological increases in cell proliferation and resistance to apoptosis make agents that modulate kinase pathways attractive potential therapeutic agents. Recent discoveries have also

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indicated a key role for protein kinases in angiogenesis, apoptosis, migration, and cell cycle controls at points downstream of initial events following growth factor stimulation. Part of the mechanism by which activated kinases may contribute to tumorigenesis is by decreasing apoptotic tendencies of target cells. Many human cancers, including colon cancer, breast cancer, lung cancer, and leukemias, have been shown to express dysregulated kinase activities. The kinase area is amenable to a system-based approach to drug discovery.

The ability to screen compounds against a wide variety of kinases facilitates the search for selective kinase inhibitors. This approach can generate efficiencies in both resources as well as time in the drug discovery process for kinase inhibitors, which can lead to medicines in a larger number of therapeutic areas. Rapidly developing technologies such as combinatorial chemistry, structure-based drug design, and high-throughput screening are greatly impacting the drug discovery process. These approaches are being utilized to rapidly identify and advance new kinase inhibitors into the clinic.

At Kinetek Pharmaceuticals Inc., we are currently focusing on several of these kinases and using some of the most clinically relevant ones as a 'bait' in our screening program to identify specific small molecule inhibitors. More recently, we have added phosphatase targets to our portfolio. Over the last few years, we have been successful in finding potent and specific kinase inhibitors for several protein kinases. In this communication, we would like to highlight some of the scientific work carried out at Kinetek Pharmaceuticals Inc. and in the laboratory of our collaborators. In addition, we focus our attention herein on the ILK,* which regulates integrin-and growth factor-mediated signal transduction.

INTEGRIN SIGNALING

Integrins comprise a large family of cell surface receptors that are found in many eukaryotic cellular membranes [1]. Integrins recognize several extracellular matrix proteins including individual matrix proteins such as fibronectin (FN) collagens and vitronectin [1]. Integrin-mediated interactions of cells with components of the extracellular matrix regulate cell survival, cell proliferation, cell differentiation, and cell migration [1]. ILK was first identified using a yeast two-hybrid genetic screen, with the β1 integrin subunit being the bait [2]. ILK is a novel serine–threonine kinase, containing an ankyrin repeat domain, which also contains sequence similar to pleckstrin homology domains, capable of interacting with phosphoinositide lipids [3].

The kinase activity of ILK can be modulated by the interaction of cells with components of the extracellular matrix or by integrin clustering [4] and growth factors [3, 4]. It has been shown that overexpression of ILK in epithelial cells leads to the stimulation of anchorage-independent cell growth, cell cycle progression, and constitutive up-regulation of cyclin D and cyclin A expression [5]. Furthermore, overexpression of ILK in epithelial cells induces tumorigenicity in nude mice, indicating that ILK is behaving as a proto-oncogene [4]. Novak *et al.* reported that ILK overexpression results in the translocation of β-catenin to the nucleus, resulting in the formation of a complex with the LEF-1 transcription factor and activation of the transcription of promoters containing the LEF-1-binding site [6]. These findings suggested that ILK may activate the Wnt-1 signaling pathway, which could lead to the nuclear localization of β-catenin and the increased transcriptional activation of LEF-1 [6].

Integrin-mediated interactions also regulate the activity of transcription factors such as AP-1 [7]. We have recently shown that AP-1 activity is dependent upon the activation of ILK inhibiting the activity of GSK-3 [7]. The AP-1 activity could be regulated by both increased DNA binding of c-Jun or by phosphorylation of the N-terminal transactivation domain of c-jun [7]. We have recently demonstrated that adhesion to fibronectin activates ILK and consequently inhibits GSK-3, leading to higher AP-1 activity by modulating the c-Jun DNA-binding activity [7]. We have also shown that AP-1 activation is mediated through PI3K by using wortmannin, a specific PI3K inhibitor [7]. Our studies further supported the hypothesis that GSK-3 is an important regulator of AP-1 *in vivo*.

GSK-3 is involved in the regulation of several intracellular signaling pathways, of which the Wnt pathway is of particular interest. Wnt is a vertebrate matrix-associated protein homologous to Drosophila Wingless protein, which is involved in cell fate determination and activates a signaling pathway resulting in transient epithelial to mesenchymal transformation [8]. In mammalian cells, Wnt signaling increases the stability of β-catenin, resulting in transcriptional activation by LEF-1/TCF proteins in association with β-catenin. In the absence of Wnt signaling, LEF-1/TCF protein represses transcription in association with Groucho and CREB-binding proteins (CBP). The LEF-1/TCF transcription factors can also interact with other cofactors and play an architectural role in assembly of multiprotein enhancer complexes, which may allow for the integration of multiple signaling pathways [8, 9].

In addition, it was recently demonstrated that ILK not only directly phosphorylates GSK-3 but can also phosphorylate PKB/Akt on serine-473, whereas a kinase–deficient form of ILK severely inhibits PKB serine-473 phosphorylation [3]. These data suggest that ILK is a receptor-proximal effector of PI3 signaling regulating the kinase activity of PKB.

The serine–threonine kinase PKB is activated by numerous growth factors and immune receptors through lipid

* *Abbreviations:* PTEN, phosphatase and tensin homolog deleted on chromosome 10; ILK, integrin-linked kinase; LEF-1, lymphoid enhancer-binding factor 1; AP-1, activator protein-1; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B/Akt; GSK-3, glycogen synthase kinase-3; TCF, T cell factor; NF-kappaB, nuclear factor-kappaB; and PP, protein phosphatase.

products of PI3K [10]. PKB is coupled to pathways that regulate cell survival and glucose metabolism [11]. PKB also regulates several transcription factors, including E2F, cyclic AMP (cAMP)- responsive factor (CREB), and the forkhead family member Daf-16 [12]. Recent studies indicated that PKB could regulate signaling pathways that lead to induction of the NF-κB family of transcription factors in the Jurkat T cell line [13]. This induction occurs, at least in part, at the level of degradation of the NF-κB inhibitor IκB and is specific for NF-κB [14]. These studies by Kane *et al.* uncovered a previously unknown interaction between PKB and NF-κB induction that could have significant implications for the control of T cell growth and survival [15]. Whether ILK also modulates NF-κB activity through PKB in this cell line remains to be established.

The above findings indicate that ILK signaling clearly affects gene expression and that it also induce changes in cell shape, cell–cell interaction, and cell migration. The end result of many cellular signaling processes is to alter the expression of genes at the level of transcription by acting on the DNA-binding transcription factors. In the last three years, we have witnessed an explosion of information describing the roles of ILK in signal transduction and gene expression. Recently, we have shown that ILK is regulated by the important receptor-proximal lipid kinase PI3K and lipid phosphatase *PTEN* [16].

Our findings that ILK regulates PKB serine-473 phosphorylation in a PI3K- and *PTEN*-dependent manner have been confirmed recently by Lynch *et al.* [17] and Morimoto *et al.* [18]. In the former, wild-type ILK was found to promote PKB Ser-473 phosphorylation in a PI3K-dependent manner, whereas a putative kinase dead, dominant negative mutant of ILK inhibited ErbB4-mediated PKB Ser-473 phosphorylation. Morimoto *et al.* showed that the tumor suppressor and lipid phosphatase, *PTEN*, inhibits PI3K mediated ILK activation, a finding that supports our observations that *PTEN* is a negative regulator of ILK activity in human prostate cancer cells [16].

Lynch *et al.*, however, suggest that ILK may regulate PKB Ser-473 phosphorylation indirectly rather than directly. This suggestion is based on their observation that the mutation of serine-343, a putative autophosphorylation site, to aspartic acid renders their supposed kinase active. They made a dominant negative mutant by mutating lysine-220 to alanine and mutating serine-343 to aspartic acid. They showed that this mutant could still phosphorylate PKB. However, they never measured ILK activity and assumed that this mutant would be kinase dead. Without such information, it is difficult to interpret these data.

On the other hand, we have previously reported a kinase-deficient, dominant negative form of ILK resulting from an E359K mutation [3, 6, 7]. We know by kinase assays that the activity of this mutant is severely decreased compared to that of wild-type ILK and that it inhibits PI3K-mediated phosphorylation of PKB Ser-473 in a dominant negative manner. We have now mutated Ser-343 on this background and find that this mutant remains inactive and continues to function as a dominant negative. Furthermore, an S343D mutant of wild-type ILK remains sensitive to PI3K, and its ability to phosphorylate PKB on Ser-473 is completely inhibited by ILK inhibitors*. These data strongly suggest that the kinase activity of ILK is required for PKB Ser-473 phosphorylation, supporting our previous observations that ILK can phosphorylate PKB Ser-473 directly *in vitro*.

PROTEIN PHOSPHATASE AND ILK

Protein phosphatases (PPs) also have an important role in regulating a variety of cellular processes, including metabolism, cell cycle, and intracellular signaling [19]. It has been speculated that PPs function as tumor suppressors by antagonizing protein kinases, many of which act as oncoproteins. In fact, recent studies have indicated that two PP genes are inactivated by somatic genetic alteration in human cancers. One is the *PTEN/MMAC1* gene encoding a dual-specificity PP, which was inactivated in a variety of human cancers including glioma, prostate carcinoma, melanoma, endometrial carcinoma, and lung carcinoma [20, 21]. *PTEN* is mutated or not present in 60% of all human cancers [16]. The other is the *PPP2RIB* gene encoding a regulatory subunit of PP2A which was stimulated in lung and colorectal carcinomas [22, 23]. Recent results also indicate that genetic variations of these two PP genes are involved in the susceptibility to tumor development [22]. Germline *PTEN* mutations have been reported to be responsible for several autosomal dominant cancer predisposition syndromes, including Cowden disease [24]. *PTEN* inactivation resulted in increased cell cycle progression through PKB-dependent phosphorylation and inactivation of GSK-3, which, in turn, leads to cyclin D1 expression [25]. In agreement with this notion, reduced levels of cyclin D1 are observed upon *PTEN* overexpression in C33A cells [26].

Recent studies have determined that ILK is part of the same pathway as *PTEN* [16]. We have demonstrated that ILK and PKB are constitutively activated in human prostate carcinoma cells lacking *PTEN* expression [16]. The transfection of kinase-deficient, dominant negative ILK into these cells inhibits both serum-and anchorage-independent PKB Ser-473 phosphorylation, as well as PKB activity [16]. In addition, we have shown that inhibition of ILK activity by a small molecule ILK antagonist inhibits serum-independent PKB phosphorylation [16]. These data suggested that ILK is important for the *PTEN*-sensitive regulation of PKB-dependent cell survival and is a downstream target in the *PTEN* signaling pathway. Hence, ILK represents an excellent therapeutic target for treating tumors associated with mutations in *PTEN*. Using high-throughput screening assays, we have identified several small molecules that specifically inhibit ILK activity *in vivo* and *in vitro* [16 and *]. These selective small molecule

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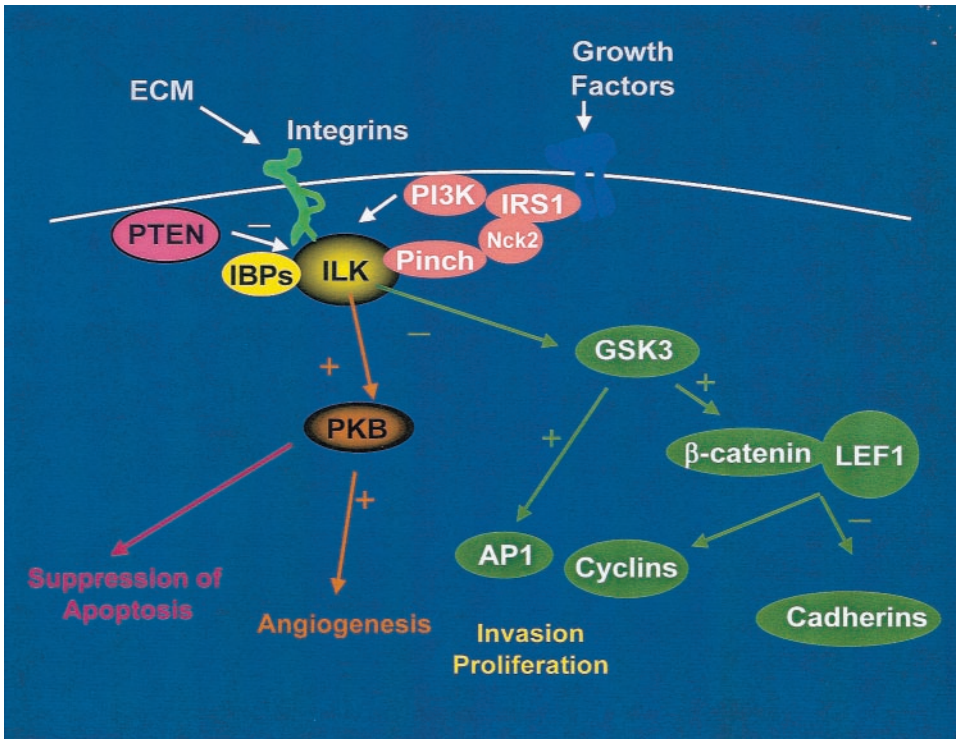

FIG. 1. Schematic representation of the regulation signal transduction from integrins and growth factor receptors through ILK to downstream targets regulating cell migration, cell cycle, and cell survival. ECM, extracellular matrix; IRS1, insulin receptor substrate; IBP, integrin-binding protein.

inhibitors of ILK are being developed for cancer therapy. In addition, these inhibitors will facilitate the identification of new ILK targets.

In summary, investigation of the integrin signaling pathway has provided valuable insights into oncogenesis. We have shown that ILK is a critical component in the integrin pathway and that it controls many downstream targets that are crucial for cell survival, cell migration, and the process of angiogenesis (Fig. 1). We have developed selective inhibitors of ILK that are showing promising results in cell and animal models of cancer.

References

1. Giancotti FG and Ruoslahti E, Integrin signaling. *Science* **285:** 1028–1032, 1999.
2. Hannigan GE, Leung-Hagesteijn EC, Fitz-Gibbon L, Coppolino MG, Radeva G, Filmus J, Bell JC and Dedhar S, Regulation of cell adhesion and anchorage-dependent growth by a new β1-integrin-linked protein kinase. *Nature* **379:** 91–96, 1996.
3. Delcommenne M, Tan C, Gray V, Rue L, Woodgett J and Dedhar S, Phosphoinositide-3-OH kinase-dependent regulation of glycogen synthase kinase 3 and protein kinase B/AKT by the integrin-linked kinase. *Proc Natl Acad Sci USA* **95:** 11211–11216, 1998.
4. Wu C, Keightley SY, Leung-Hagesteijn C, Radeva G, Coppolino M, Goicoechea S, McDonald JA and Dedhar S, Integrin-linked protein kinase regulates fibronectin matrix assembly, E-cadherin expression, and tumorigenicity. *J Biol Chem* **273:** 528–536, 1998.
5. Radeva G, Petrocelli T, Behrend E, Leung-Hagesteijn C, Filmus J, Slingerland J and Dedhar S, Overexpression of the integrin-linked kinase promotes anchorage-independent cell cycle progression. *J Biol Chem* **272:** 13937–13944, 1997.
6. Novak A, Hsu SC, Leung-Hagesteijn C, Radeva G, Papkoff J, Montesano R, Roskelley C, Grosschedl R, and Dedhar S, Cell adhesion and the integrin-linked kinase regulate the LEF-1 and beta-catenin signaling pathways. *Proc Natl Acad Sci USA* **95:** 4374–4379, 1998.
7. Troussard AA, Tan C, Yoganathan TN and Dedhar S, Cell–extracellular matrix interactions stimulate the AP-1 transcriptional factor in an integrin-linked kinase and glycogen synthase kinase 3-dependent manner. *Mol Cell Biol* **19:** 7420–7427, 1999.
8. Peifer M and Polakis P, Wnt signaling in oncogenesis and embryogenesis—a look outside the nucleus. *Science* **287:** 1606–1609, 2000.
9. Eastman Q and Grosschedl R, Regulation of LEF-1/TCF transcription factors by Wnt and other signals. *Curr Opin Cell Biol* **11:** 233–240, 1999.
10. Hass-Kogan D, Shalev N, Wong MM, Mills G, Yount G and Stokoe D, Protein kinase B (PKB/Akt) activity is elevated in glioblastoma cells due to mutation of the tumor suppressor *PTEN/MMAC*. *Curr Biol* **8:** 1195–1198, 1998.
11. Coffer P, Jin J and Woodgett JR, Protein kinase B (c-Akt): A multifunctional mediator of phosphatidylinositol 3-kinase activation. *Biochem J* **335:** 1–13, 1998.
12. Scheid MP and Woodgett JR, Protein kinases: Six degrees of separation? *Curr Biol* **10:** R191–194, 2000.
13. Sizemore N, Leung S and Stark GR, Activation of phosphatidylinositol 3-kinase in response to interleukin-1 leads to phosphorylation and activation of the NF-kappaB p65/relA subunit. *Mol Cell Biol* **19:** 4798–4805, 1999.
14. Romashkova JA and Makarov SS, NF-kappaB is a target of

AKT in anti-apoptotic PDGF signaling. *Nature* **401:** 86–90, 1999.
15. Kane LP, Shapiro VS, Stokoe D and Weiss A, Induction of NF-kappaB by the Akt/PKB kinase. *Curr Biol* **9:** 601–604, 1999.
16. Persad S, Attwell S, Gray V, Delcommenne M, Troussard A, Sanghera J and Dedhar S, Inhibition of integrin-linked kinase (ILK) suppresses activation of protein kinase B-Akt and induces cell cycle arrest and apoptosis of *PTEN*-mutant prostate cancer cells. *Proc Natl Acad Sci USA* **97:** 3207–3212, 2000.
17. Lynch DK, Ellis CA, Edwards PA and Hiles ID, Integrin-linked kinase regulates phosphorylation of serine 473 of protein kinase B by an indirect mechanism. *Oncogene* **18:** 8024–8032, 1999.
18. Morimoto AM, Tomlinson MG, Nakatani K, Bolen JB, Roth RA and Herbst R, the MMAC1 tumor suppressor phosphatase inhibits phospholipase C and integrin-linked kinase activity. *Oncogene* **19:** 200–209, 2000.
19. Tonks NK and Neel BG, Form and function: Signaling by protein tyrosine phosphatases. *Cell* **87:** 365–386, 1996.
20. Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, Puc J, Miliaresis C, Rodgers L, McCombie R, Bigner SH, Giovanella BC, Ittmann M, Tycko B, Hobshoosh H, Wigler MH and Parsons R, *PTEN*, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* **275:** 1943–1947, 1997.
21. Steck PA, Pershouse MA, Jasser SA, Yung WK, Lin H, Ligon AH, Langford LA, Baumgard ML, Hattier T, Davis T, Frye C, Hu R, Swedlund B, Teng DH and Tavtigian SV, Identification of a candidate tumour suppressor gene, *MMAC1*, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat Genet* **15:** 356–362, 1997.
22. Wang SS, Esplin ED, Li JL, Huang L, Gazdar A, Minna J and Evans GA, Alteration of *PPP2R1B* gene in human lung and colon cancer. *Science* **282:** 284–287, 1998.
23. Di Cristofano A and Pandolfi PP, The multiple roles of *PTEN* in tumor suppression. *Cell* **100:** 387–390, 2000.
24. Liaw D, Marsh DJ, Li J, Dahia PL, Wang SI, Zheng Z, Bose S, Call KM, Tsou HC, Peacocke M, Eng C and Parsons R, Germline mutation of the *PTEN* gene in Cowden disease, an inherited breast and thyroid cancer syndrome *Nat Genet* **16:** 64–67, 1997.
25. Sun H, Lesche R, Li DM, Liliental J, Zhang H, Gao J, Gavrilova N, Mueller B, Liu X and Wu H, *PTEN* modulates cell cycle progression and cell survival by regulating phosphatidylinositol 3,4,5-triphosphate and Akt/protein kinase B signaling pathway. *Proc Natl Acad Sci USA* **96:** 6199–6204, 1999.
26. Paramio JM, Navarro M, Segrelles C, Gomez-Casero E, and Jorcano JL, *PTEN* tumor suppressor is linked to the cell cycle control through the retinoblastoma protein. *Oncogene* **18:** 7462–7468, 1999.