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Role of Xenopus laevis integrin linked kinase (XILK) during early development.

Sharareh (Sherry) Savari
University of Windsor

Role of *Xenopus laevis* integrin linked kinase (*X*ILK) during early development

By
Sharareh (Sherry) Savari

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Abstract

Integrin linked kinase (ILK) is a serine/threonine protein kinase implicated in the phosphatidylinositol 3'kinase (PI3'K) pathway (Delcommenne et al, 1998). Integrin linked kinase has been investigated in different organisms such as mammalian systems (human, mice, rat), insects (*Drosophila*) and nematodes (*Cenorhabditis elegans*), however to date no data regarding ILK research on amphibians has been reported. This work is the first of its kind to investigate the role of ILK in *Xenopus laevis* development.

Xenopus is an ideal model system for developmental studies, because the fertilization process is carried out *in vitro* and embryos can be collected at different stages of development for ILK analyses. My project was mainly based on characterization of *Xenopus* ILK during early development.

In our study, we isolated and characterized a cDNA clone encoding ILK in *Xenopus laevis* (*X*-ILK). The experiments were executed in both embryos and adult tissues to compare the relationship between the ILK expression patterns. Sequence analysis of *X*-ILK revealed that it is 59% identical to human ILK (HILK) cDNA and 71% identical to human ILK (H-ILK) protein. The well-known domains of ILK (ankyrin domain, Pleckstrin homology domain, kinase domain and paxillin binding site) are preserved among human, mouse, rat and *Drosophila*. These domains are found in *Xenopus* ILK. Northern blot analysis showed that a 1.8 Kb transcript is present throughout early embryogenesis. However, there was a significant increase in *X*-ILK expression at the onset of neurulation. Interestingly, expression studies revealed the presence of only one transcript whereas Western blot analysis revealed the expression of two *X*-ILK proteins during early development. Expression analyses for various adult

tissues revealed that *Xenopus* heart expressed a lower level of *X*-ILK mRNA, while the protein level in heart was higher than the other tissues examined. We also measured *X*-ILK kinase activity in embryonic extracts using *in vitro* kinase assays. Our findings showed that *X*-ILK activity increased during early embryogenesis. As expected, we observed higher *X*-ILK activity in heart and muscle. Taken together, our data suggest that *X*-ILK may be an important signaling molecule during early *Xenopus* embryogenesis, and may have tissue specific functions in adult frogs.

For my father, my mother and my brothers with love and mercy.

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List of abbreviations

ANK : Ankyrin

APC : Adenomatous Polyposis Coli

CH-ILKBP : Calponin homology-integrin linked kinase binding protein

CPI-17: Protein kinase C (PKC)-dependent phosphatase inhibitor of 17 KDa

Dsh : Dishevelled

ECM: Extra-cellular matrix

Ef1-α: Elongation factor 1-alpha

EGF : Epidermal growth factor

FA : Focal adhesions

FAK: Focal adhesion kinase

GSK-3: Glycogen synthase kinase 3

ILK : Integrin linked kinase

IRS : Insulin receptor substrate

Lef : Limphoid enhancer factor

MLCP: Myosin light chain phosphatase

PAT: Paralyzed and arrested elongation at the two fold stage

PBS : Paxillin binding sub-domain

PDGF-AB : Platelet derived growth factor AB

PH : Pleckstrin homology

PHI-1: Phosphatase holoenzyme inhibitor-1

PI3'K : Phosphatidylinositide 3'- kinase

PIP2 : Phosphatidylinositol diphosphate

PIP3 : Phosphatidylinositol triphosphate

PKB: Protein kinase B

PKC : Protein kinase C

PTEN : Phosphatidylinositol phosphatase

RTK : Receptor tyrosine kinase

Ser : Serine

Tcf : T cell factor

TNF : Tumor necrosis factor

Wnt/Wg: Wnt/Wingless

***X*fzd :** *Xenopus* frizzled

XLA: X-linked agammaglobulinemia

Introduction

1.1 Cell signaling

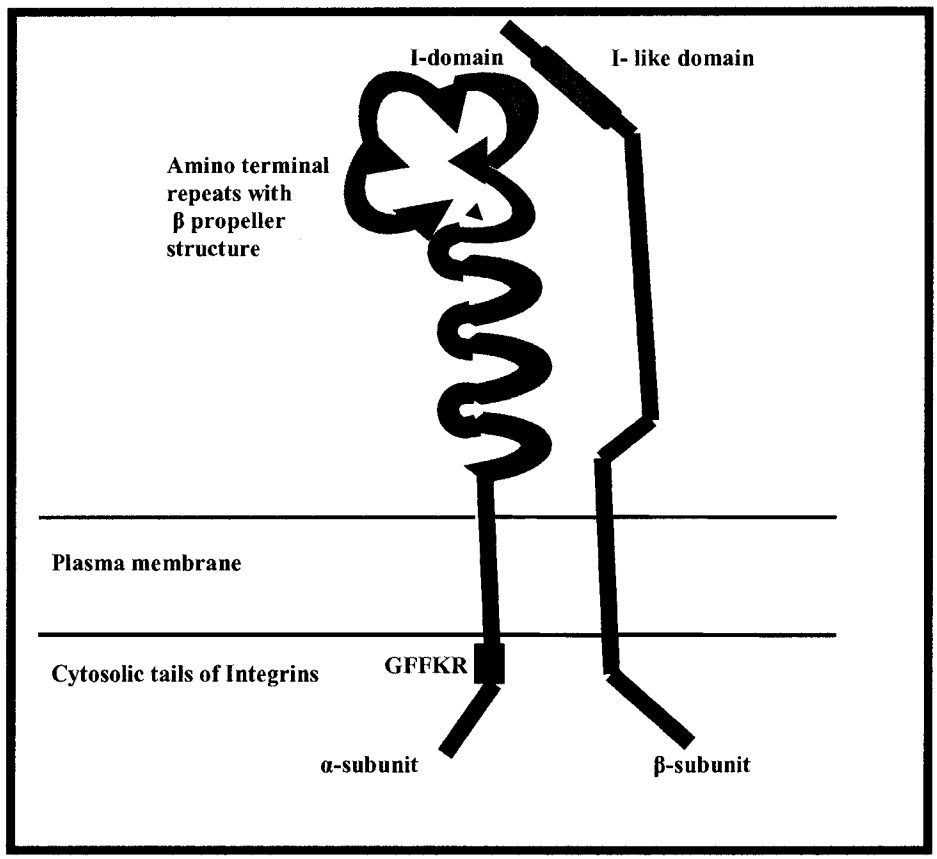
Cell signaling is the process by which cells communicate with their environment in order to survive, differentiate and migrate (Dedhar, 2000). A cell may communicate through an outside-in signaling mechanism, which involves external stimuli promoting the transfer of information from the extra-cellular matrix (ECM) into the cell (Cooper, 2000). These communications are mediated through cell's detection mechanisms such as receptors which bind or respond to ligands with high specificity. This kind of signaling results in a series of events that target a specific region or factor within the cell to bring about a particular change and modify cellular activities. One family of receptors that facilitate the interaction of cells with their extra-cellular matrix is integrins. Integrins are coupled with intracellular effectors such as integrin linked kinase (ILK), which mediate cell interactions, attachments and progression of signaling pathways (Hannigan et al, 1996).

1.2 Integrin receptors

Integrins are a family of trans-membrane receptors that mediate cell signaling events by transferring the signal from the outer surface of the cell to the interior. Integrins are the main receptors that link cells to their extracellular matrix (Cooper, 2000). They play a structural role in the formation of focal adhesions and hemidesmosomes, linking the cytoskeleton to the ECM (Cooper, 2000). These adhesive properties also allow them to be involved in signaling events by transferring the information from the outer surface of the cell to the inner surface (Cooper, 2000). Integrins are expressed in all multi-cellular animals but their diversity varies among species (Humphries, 2000). Integrins are hetero-

dimeric glycoproteins consisting of an α and a β subunit (Karp, 2002). The α and β subunits bind to the extracellular ligands at the surface of the cell and their cytosolic tails interact with the cytoplasmic components (Fig.1). The α-subunits of integrins are divided

Fig.1. Integrin structure (adopted from bme.virginia.edu/ley/integrin_activation)



into 2 groups based on the structure of their extracellular domain (Karp, 2002). The N-terminal extracellular portion of the α-subunit consists of seven repeating modules each comprised of 60 amino acids termed the seven bladed β-propeller. This site is the ligand binding site in the first group of integrins. In the second group, the α-subunits contain an I-domain structure (located on the top of the β-propeller), which is the portion that carries the ligand binding site. The β subunits of integrins lack the β-propeller structure, but have

an I-like domain containing the ligand binding site (Karp, 2002).

Integrins interact with the ECM components such as fibronectin (Cooper, 2000), and one of the early responses to such an interaction is protein tyrosine phosphorylation. However, the cytoplasmic tails of integrins lack enzymatic activity, which suggests that integrins are bound to non-receptor tyrosine kinases (Cooper, 2000). One major protein tyrosine kinase is focal adhesion kinase (FAK) which becomes autophosphorylated upon fibronectin attachment; however, the details of FAK activation remain unclear (Schlaepfer and Hunter, 1998).

1.3 Integrin linked kinase (ILK)

A downstream cytoplasmic effector of β-integrin protein is integrin linked kinase. This protein is a serine-threonine kinase which interacts with β_1, β_2 and β_3 cytoplasmic integrin domains (Hannigan et al, 1996; Zervas et al, 2001). Integrin linked kinase plays an important role in cell adhesion as well as Wnt/Wg, PI3'K and insulin signaling pathways; these pathways are involved in cell survival, growth and differentiation (Dedhar, 2000; Delcommenne et al, 1998; Novak et al, 1998). Integrin linked kinase also acts as a proto-oncogene when over-expressed in cultured cells (Dedhar, 2000). Integrin linked kinase is found to be conserved among various organisms ranging from nematode *Caenorhabditis.elegans* to humans. In *C. elegans*, the *pat-4* gene is reported to be the sole homologue of ILK (Mackinnon et al, 2002). It was found that mutant *pat-4* gene results in a failure to assemble integrin-mediated muscle attachments.

The *Drosophila* ILK (*D*ILK) homologue was first identified by sequence comparison from the Berkeley *Drosophila* genome project. Within the 5' end sequence of a cDNA clone LD02317, the encoded peptide was found to be 65% identical to

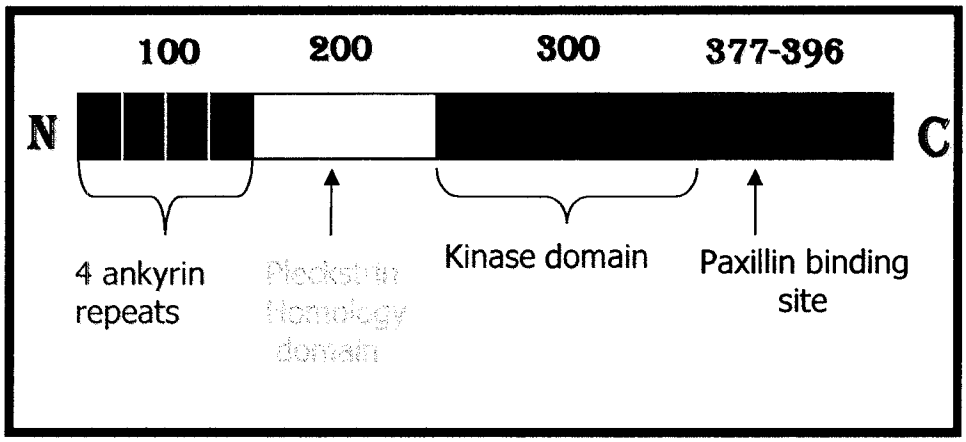
residues 1-45 of human ILK. This clone was used to screen an imaginal disc cDNA library and one full length clone of 1,813 bp was isolated that encoded a 448 amino acid protein that is similar to human ILK and ILK encoded in *C. elegans* genome.

Mammalian ILK was first identified using the yeast two-hybrid technique (Hannigan et al, 1996). This technique was used to identify genes encoding a protein that interacts with the β_1 integrin cytoplasmic domain. A new 59KDa serine-threonine kinase, known as ILK, was recognized to be involved in regulating integrin-mediated signal transduction. In order to investigate the role of ILK during development we have isolated and characterized *Xenopus* ILK in our present study.

1.4 Functional domains in ILK

Monomeric ILK (in mammals) weighs 59 KDa (Hannigan et al, 1996), and has four different domains. The well-known domains of ILK (ankyrin domain, Pleckstrin homology domain, kinase domain and paxillin binding site) are preserved among human, mouse, rat and *Drosophila*. The high conservation of the domains strongly suggests that ILK function is conserved during evolution (Zervas et al, 2001). In its N-terminus region it contains four ankyrin repeats that interact with the LIM1 domain, a cysteine rich domain, of protein PINCH. Downstream of the ANK domain is the pleckstrin homology domain (PH domain) which interacts with phosphoinositides. Proximal to the PH domain is a catalytic kinase domain which interacts with integrin β-subunits as well as its downstream effectors such as protein kinase B (PKB) and glycogen synthase kinase-3 (GSK-3) (Wu and Dedhar, 2001). Lastly, at the C-terminal domain ILK contains a paxillin binding site, which mediates its interaction with the paxillin proteins involved in focal adhesion assembly (Nikolopoulos and Turner, 2001) (Fig.2). ILK plays a structural

Fig.2. Structure of ILK protein domains (adopted from Zervas et al, 2001; Nikolopoulos and Turner, 2001)



role through interactions via its ANK domains and the paxillin binding domain, and a signaling role is carried out through the PH and kinase domains. However, there is evidence to indicate the significance of the ANK domain through interaction with PINCH in certain signaling pathways such as during insulin signaling (Tu et al, 1999). Integrin linked kinase interacts with several proteins including: integrins, PINCH, calponin homology-ILK binding protein (CH-ILKBP) (also named actopaxins and α-parvin), affixin (also named β-parvin) and paxillin (Nikolopoulos and Turner, 2000; Olski et al, 2001;Tu et al, 2001; Olski et al, 2001; Yamaji et al, 2001;Wu and Dedhar, 2001). ILK is involved in focal adhesions (Mulrooney et al, 2000) as well as fibrillar adhesions (Guo and Wu, 2002) through the interactions mentioned above (Wu and Dedhar, 2001). Integrin linked kinase interacts with α-parvin and β-parvin molecules to link cytoskeleton to the plasma membrane. It also mediates the interaction of PINCH with integrins (Li et al, 1999) and binds to paxillin to assemble focal adhesion structures (Nikolopoulos and Turner, 2001).

1.4.1 Ankyrin domain

The N-terminal domain of ILK, known as the ankyrin (ANK) repeat, is involved in focal adhesions and insulin signaling pathways (Li et al, 1999; Nikolopoulos and Turner, 2001; Tu et al, 1999). Mammalian ILK contains four ANK repeats at the N-terminus (Dedhar, 2000), whereas *D*ILK contains 3 ANK repeats (Zervas et al, 2001). The ANK repeat is a very common protein-protein interaction motif. ANK motifs are repeated modules of about 33 amino acids occurring in functionally diverse proteins, mainly in eukaryotes. The examples of ANK repeats within prokaryotes and viruses are a result of gene transfers (Bork, 1993). The conserved fold has been found in proteins with different functions such as transcriptional initiators, cell-cycle regulators, cytoskeletal factors, ion transporters and signal transducers. The ANK repeat is recognized within various proteins due to its structural similarity rather than its function. The repeat contains alpha helix loops and beta hairpin loops, each repeat folding into a helix-loop-helix structure with the beta-hairpin loops projecting out from the helices with a 90° angle. The repeats stack together to make an L-shape structure (Gornia and Pavletich, 1996). The ability of ANK repeats to bind to other proteins involves the beta hairpin tips and the surface of the helical bundle facing the ANK groove. To date, 1700 different proteins have been identified containing the ANK repeats. As mentioned earlier, the repeats are implicated in mediating protein-protein interactions, although no common theme among the known ANK domain protein targets has been established.

The interaction of ILK with PINCH, a binding protein for ILK, has been observed through different methodologies such as: yeast two-hybrid assays, in solution, and in solid-phase-based binding assays (Li et al, 1999). In addition, ILK has been co-isolated

with PINCH through immunoaffinity chromatography in mammalian cells indicating their association *in vivo*. The ANK repeat in ILK interacts with the LIM domain, a cysteine rich domain, in protein PINCH (Li et al, 1999). The PINCH-ILK interaction is mediated through the N-terminal most LIM domain of PINCH (residues 1-70) with the N-terminal most ANK repeats within mammalian ILK (residues 1-66). Furthermore, ILK through its interaction with PINCH, is capable of forming a ternary complex with Nck-2, an SH2/SH3-containing adaptor protein implicated in growth factor and small GTPase signaling pathways (Tu et al, 1999).

1.4.2 Pleckstrin Homology Domain (PH Domain)

Downstream from the ANK repeat in ILK protein is the Pleckstrin homology (PH) domain implicated in PI3'K-mediated pathways through its binding to phospholipids (Shaw, 1996). First it was identified in 1993 as an internal repeat in Pleckstrin, a phosphoprotein in blood platelets. Since then, more than one hundred proteins have been identified that contain such a domain. This domain is common in vertebrates, *Drosophila*, *C.elegans* and yeast suggesting its early origin in eukaryotic biology (Gibson et al, 1994). The PH domain is also found in plant proteins, such as the dynamin-like protein, ADL3, isolated from *Arabidopsis thaliana* (Mikami et al, 2000). This domain has the capacity to exert the regulatory effects on the GTPase activity. The PH domain is approximately 120 amino acids long, although some domains are longer due to other inserted sequences (Rameh et al, 1997). The three-dimensional structure of PH domain was first determined from the N-terminal human pleckstrin protein and murine β-spectrin through NMR (Macias et al, 1994 and Yoon et al, 1994). The structure of a PH domain contains a seven-stranded β-sandwich capped at the C-terminus by an α-helix. The PH

domains occupy various positions in a protein. For example, in Pleckstrin they are located on the extreme N-terminus as well as the C-terminus, separated by a DEP domain (a domain which is important for membrane translocation of its protein). All PH domains at the C-terminus contain a tryptophan residue (Kraulis, 1991). Another common feature shared by PH domains which is seen in their three-dimensional structure is their electrostatic polarization. The positively charged end of the domain is the binding site for inositol phosphates observed by crystal structures (Ferguson et al, 1995). Nevertheless, the PH domain is rarely conserved and shows only 10-20% pair wise identity between different organisms; the only conserved residue is the nearly invariant tryptophan at the C-terminus of the domain which is part of the hydrophobic core (Kraulis, 1991). The low sequence identity of PH domains makes it difficult to find distant members of the family; thus more advanced and sophisticated searches are required.

The proteins carrying a PH domain are involved in signal transduction and cytoskeletal organizations. Unlike other domains of signaling proteins such as SH2 and SH3, the PH domains do not seem to be directly involved in signal transduction. Instead, they bind the inositol phospholipids and/or their soluble head derivatives, which are membrane bound ligands and in this way, are indirectly implicated in signaling pathways. The first evidence seen for PH interaction with phosphoinositides was derived from studies of the N-terminal PH domain of Pleckstrin (Harlan et al, 1994). The PH domain bound to vesicles containing phosphatidylinositol (4,5)-diphosphate (PtdIns (4,5)-P2) and this interaction was inhibited by excess of soluble inositol (1,4,5) triphosphate.

1.4.3 Kinase Domain

The eukaryotic protein kinases make up a large superfamily of homologous

proteins that share a kinase domain also known as the catalytic domain. A typical kinase consists of 250-300 amino acid residues containing 12 conserved sub-domains that fold into a catalytic core structure as revealed by the 3-dimensional structure of various protein serine kinases (Hank and Hunter, 1995). This superfamily is divided into two major subgroups: the serine-threonine protein kinases and the protein-tyrosine kinases (Hank and Hunter, 1995). Numerous studies have confirmed that ILK is a member of the serine-threonine subdivision (Hannigan et al, 1996).

The kinase domain of ILK has been observed among diverse organisms ranging from *C.elegans* and *Drosophila* to humans. Certain residues within the kinase domain of ILK vary from those of other typical serine-threonine kinases (Zervas et al, 2001). Notably these residues are not only different from other kinases, but also vary among different species, indicating that they are not conserved through evolution.

1.4.4 Paxillin Domain

At the C-terminus of ILK exists the paxillin-binding site (PBS), which binds to paxillin, a focal adhesion adaptor protein. This interaction aids in focal adhesion assembly. Microsequencing of a peptide derived from a 50-kDa paxillin LD-1 motif-binding protein (LD-1 motif consists of eight amino acids beginning with an invariant leucine-aspartate sequence, for which the LD repeats are named) revealed 100% identity with integrin-linked kinase-1 (Nikolopoulos and Turner, 2001). This domain was first discovered through sequence analysis of rat ILK and revealed a domain most similar to the PBS in other proteins such as focal adhesion kinase (FAK), vinculin and actopaxins. Cloning of rat ILK revealed 99.6% identity with the human ILK-1 homologue. A monoclonal antibody was produced against the C-terminus of rat and human ILK-1, which

were 100% identical and determined ILK in all cultured cells and tissues examined (rat tissues and cell lines). Binding experiments were carried-out and illustrated that ILK binds directly to paxillin LD-1 motif *in vitro.* Through co-immunoprecipitation of fibroblasts and cells in suspension, an *in vivo* interaction was found to occur between paxillin and ILK. Immunofluorescence microscopy of fibroblasts showed that endogenous ILK as well as transfected green fluorescence ILK co-localized with paxillin in focal adhesions. Analysis of amino acid sequence of ILK identified a paxillin-binding sub-domain in the carboxyl terminus of ILK. To further support the interaction of ILK with paxillin through the PBS domain (residues 377-396 of ILK protein), mutant PBS domains of ILK failed to bind paxillin LD1 motifs *in vitro* and were unable to localize to focal adhesions (Nikolopoulos and Turner, 2001).

1.5 Focal adhesions

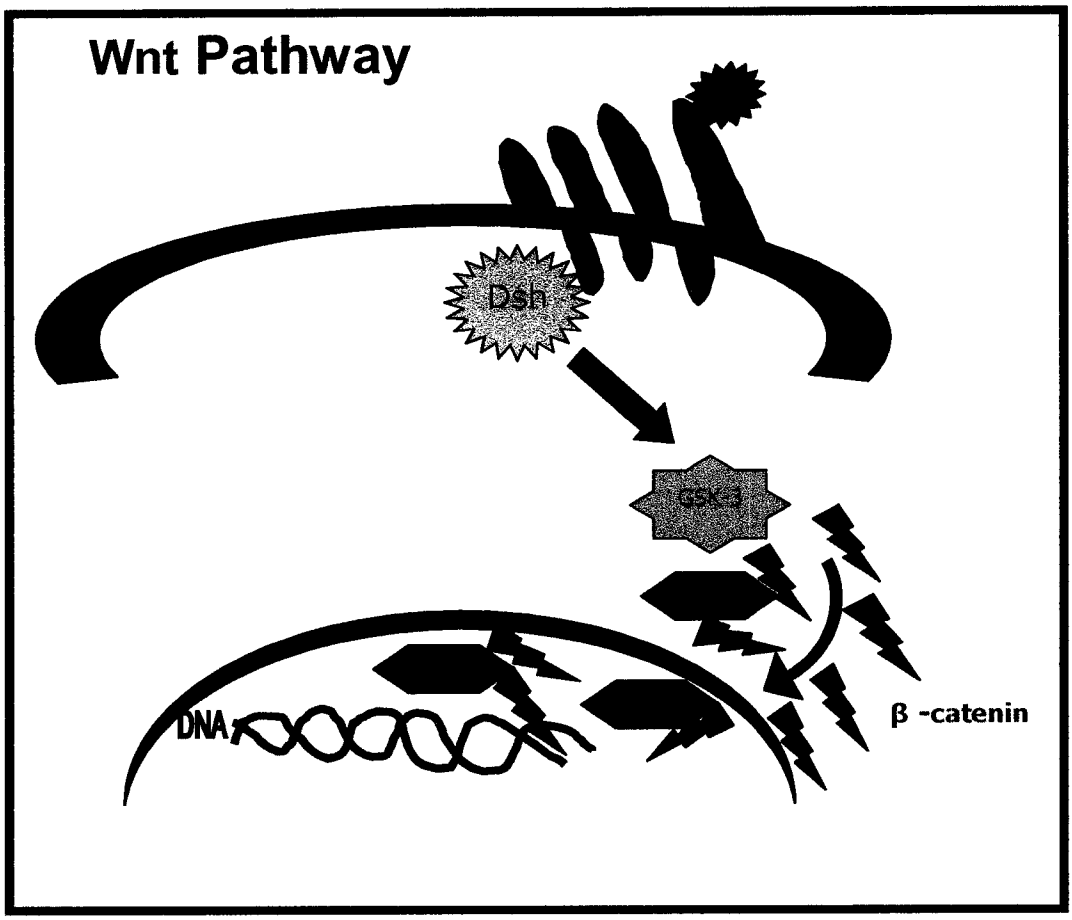
Integrins and other adaptor molecules are involved in cell adhesion through focal adhesion assemblies. As their name implies, adaptors supply the connection between two different components. A focal adhesion (FA) is a dynamic anchorage point of the plasma membrane of an adherent cell, which mediates a cell's attachment with its substratum. They are essential structures for cell adhesion, migration and morphogenesis. Focal adhesions disassemble when cells are stimulated to move or enter mitosis. Through integrins extra-cellular components link with components of the cell cytoskeleton such as actin filaments. At the focal adhesion points where integrins recruit, a number of cytoplasmic adaptor proteins are visible; among these proteins ILK as well as other proteins such as PINCH and paxillin can be specifically identified (Tu et al, 1999; Nikolopoulos and Turner, 2001). Mammalian PINCH, a binding protein for ILK, consists

of five LIM domains containing cysteine- rich sequences that are involved in protein-protein interactions (Tu et al, 1999). In focal adhesions, integrins bind to ILK and paxillin, a focal adhesion adaptor protein involved in integrin signaling (Nikolopoulos and Turner, 2001). Paxillins in turn bind with additional adaptor proteins such as vinculins (Tu et al, 1999) and actopaxins (Nikolopoulos and Turner, 2002), connecting integrins to actin cytoskeleton. However, the interaction of PINCH with ILK is necessary for the formation of focal adhesions (Li et al, 1999). From the information above it can be concluded that ILK is involved in cell adhesion as an adaptor protein.

1.6 Wnt/Wg pathway

Wnt/Wg pathway plays an important role during animal development (Wodarz and Nusse, 1998). Upon binding of a secreted protein, called wnt, to a seven trans-membrane receptor (frizzled (fz) receptor), disheveled (a docking protein for ILK) becomes hyper-phosphorylated and activated resulting in phosphorylation and inhibition of GSK-3 (Novak and Dedhar, 1999). The serine-threonine kinase GSK-3 makes a complex with adenomatous polyposis coli (APC), a tumor suppressor involved in colon carcinomas, and β-catenin, a structural molecule in cell adhesion. Inhibition of GSK-3 leads to inhibition of phosphorylation of β-catenin and APC leading to stabilization of β-catenin. Glycogen synthase kinase-3 acts as a negative regulator in this pathway. This inhibition raises the stability of β-catenin and its free cytoplasmic concentrations. In the cytoplasm, β-catenin binds to lymphoid enhancer factor/T-cell factor (Lef/Tcf transcription factor) and translocates to the nucleus stimulating gene expression (Novak and Dedhar, 1999; Ali et al, 2001) (Fig.3). Two particular genes that have been shown to be regulated through β-catenin and Lef/Tcf complex are *c-myc* and *cyclin D1* (Coyle-Rink et al, 2002). *In vitro*,

Fig.3. Wnt/Wg pathway (adopted from Ali et al, 2001)



ILK phosphorylates GSK-3β on ser-9 and leads to expression of *cyclin D1* gene (Novak and Dedhar, 1999). In addition, the experiments performed by Troussard et al (2003) show that ILK regulation by siRNA inhibited phosphorylation of GSK-3β on Ser-9, and decreased the expression of cyclin D. In conclusion, these data suggest that GSK-3 phosphorylation could be the result of ILK activity leading to inhibition of its activity.

Disheveled has been shown to interact with ILK at focal adhesion sites indicating its role in cell-ECM interactions (Torres and Nelson, 2000). Since ILK interacts with Dsh, and phosphorylates GSK-3 *in vitro*, one candidate for GSK-3 phosphorylation may be ILK

the specific interaction of ILK with Dsh.

Of β-catenins, one pool is involved in signaling pathways whereas, the other pool of β -catenin is involved in cell adhesion (Novak and Dedhar, 1999). β-catenin binds to cadherins at the intracellular surface of the membrane and functions as a structural protein. Cadherins are involved in the attachment of neighboring cells through their extracellular domain at the surface of the cell. Cadherins alter the signaling of β-catenin by recruiting cytoplasmic β-catenin towards the plasma membrane. Normally some β-catenin is found in the plasma membrane acting as a structural protein.

Over-expression of ILK leads to E-cadherin downregulation and increase in the cytoplasmic pool of β-catenin which is a hallmark of Wnt pathway. β-catenin forms a complex with Lef-1and is then translocated into the nucleus, however at this point ILK over-expression has led to an increase and stabilization in Lef-1 levels. ILK is shown to directly phosphorylate GSK-3 and inactivate it *in vitro* (Delcommenne et al, 1998). In ILK over-expressing cells, the levels of *cyclin D1* and *cyclin A*, two cell cycle regulators, are increased. Two explanations can be given for this effect. First, phosphorylation of *cyclin D1* by GSK-3 leads to its degradation, and since ILK inhibits GSK-3 activity, the increase in *cyclin D1* expression could be due to inhibition of GSK-3 activity via ILK. Second, because *cyclin D1* is a direct target of β-catenin-Lef/Tcf signaling and the *cyclin A* gene has a binding site for Lef/Tcf in its promoter, the increased expression of these genes resulting from ILK over-expression may be due to β-catenin-Lef/Tcf induced transcriptional activity of ILK.

1.6.1 Glycogen synthase kinase-3 (GSK-3)

Glycogen synthase kinase-3 (GSK-3) is a serine-threonine kinase that is

implicated in both PI3'K pathway and Wnt/Wg pathway where it can influence proliferation and cell survival (Ali et al, 2001; Delcommenne et al, 1998). *GSK-3* genes are highly conserved in eukaryotes and have been identified in all eukaryotes studied (Ali et al, 2001). *GSK-3* in mammals encodes two different isoforms known as GSK-3α and GSK-3β. GSK-3α has a molecular mass of 51 KDa and GSK-3β carries a mass of 47 KDa (Woodgett, 1991). These two genes exhibit 85% similarity overall in gene structure and 93% in the catalytic domain. These isoforms contain similar biochemical and substrate properties. GSK-3 was first noted to function in muscle energy storage and metabolism. However recently an advanced role for GSK-3 in cellular regulation has been observed, which is mediated by its interaction with downstream targets such as cytoplasmic proteins and transcription factors. GSK-3 plays a critical role in differentiation, cell fate determination, dorso-ventral axis determination and inducing bilateral embryonic symmetry. GSK-3 targets consist of proteins involved in Alzheimer's disease, neurological disorders and cancer; hence it plays a vital role in clinical aspects (Ali et al, 2001).

1.7 Wnt/Wg pathway in development

Role of Wnt signaling during embryonic development has been shown in several studies (Wodarz and Nusse, 1998; Ali et al, 2001). Early *Xenopus* development is an ideal model to investigate the Wnt/Wg signaling response especially at the onset of zygotic transcription. Glycogen synthase kinase-3β plays a central role in the development of invertebrates and vertebrates (Woodgett 1991; Plyte et al, 1992; Hedgepeth et al, 1997; Ali et al, 2001). Wnt signaling components encoded by maternal mRNA are able to establish the dorsal embryonic axis (Heasman et al, 1994; Schneider et al, 1996; Larabell

et al, 1997). Loss of function and dominant negative mutations in GSK-3ß lead to activation of the Wnt/Wg pathway in *Drosophila* and *Xenopus* (Hedgepeth et al, 1997). *X*GSK-3 and the intracellular protein β-catenin are necessary for the establishment of the dorsal-ventral axis in *Xenopus* (Yost et al, 1996). A mutation in *X*GSK-3 mimics Wnt pathway just as mutation on shaggy, the homolog of GSK-3 in *Drosophila*, mimics the effects of wingless signaling (Pierce and Kimelman, 1995; Dominguez et al, 1995). The *X*GSK-3 mutant, like Wnt, induces dorsal axis formation when expressed in the deep vegetal cells that usually do not contribute to the axis. Dorsal fate is actively repressed by *X*GSK-3, which must be inactivated for dorsal axis formation to occur.

Different transcription factors of the Lef/Tcf family function in different tissues to bring about tissue-specific responses downstream of Wnt signaling. The function of the transcription factor *X*Tcf-3 is required for early Wnt signaling to establish the dorsal embryonic axis, while closely related *X*Lef-1 is required for Wnt signaling to pattern the mesoderm after the onset of zygotic transcription. Zygotic expressed *X*wnt-8 causes almost the opposite effect of other components of Wnt/Wg pathway by promoting ventral and lateral axis and restricting dorsal mesodermal development (Christian et al, 1991).

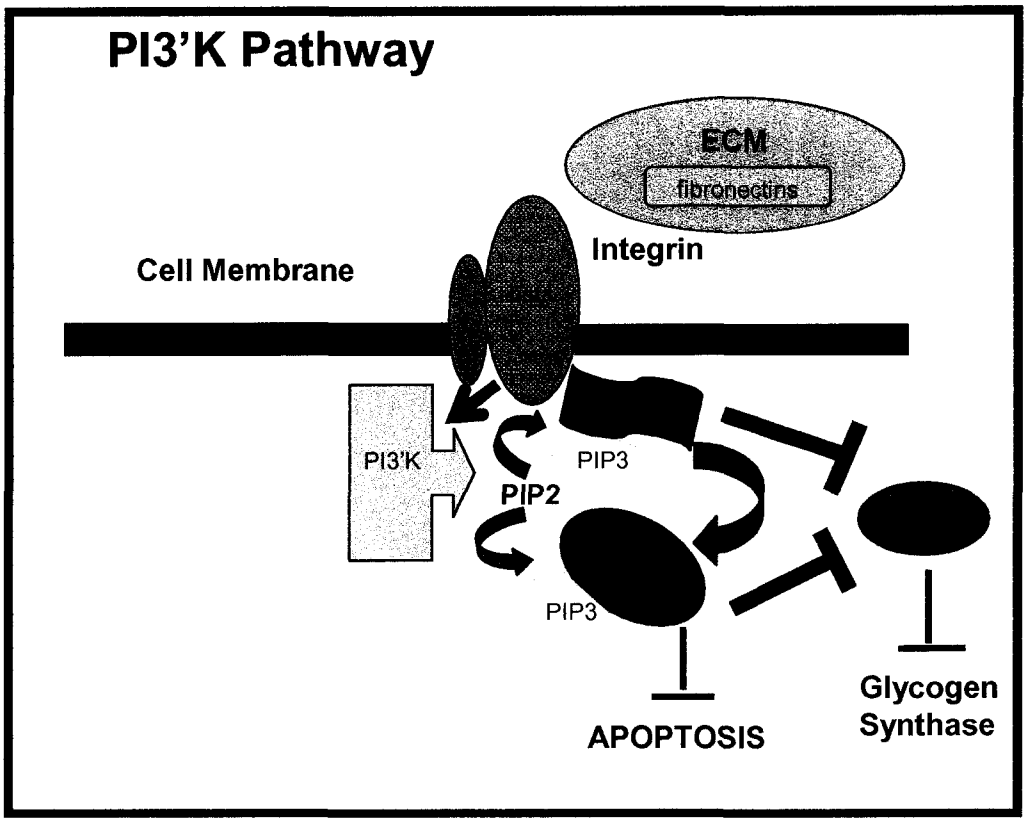
Many components of Wnt/Wg signaling pathway are involved in the development of the nervous system (Patapoutian and Reichardt, 2000). Recent work has shown that Wnt signaling controls initial formation of the neural plate as well as other subsequent patterning events such as formation of the neural crest. Wnt signaling continues to process at later stages of development. Wnt proteins have been shown to regulate the anatomy of the neuronal cytoskeleton and the differentiation of synapses in the cerebellum. Wnt signaling cascade has also been observed to regulate apoptosis and may participate in degenerative processes leading to cell death in the aging brain.

1.8 PI3'K pathway

Integrin linked kinase is implicated in another crucial signaling pathway in the cell, the PI3'K pathway. Phosphatidylinositide 3-kinase (PI3'K) activation is initiated by the binding of extracellular ligands, such as fibronectins, to the extracellular α and β subunits of integrins. PI3'K phosphorylates phosphatidylinositol diphosphate (PIP2) (a phospholipid) on the 3'-position of inositol. Phosphorylation of PIP2 yields phosphatidylinositol 3,4,5-triphosphate (PIP3), a phospholipid that acts as a second messenger (Rameh and Cantley, 1999). One of PIP3's targets is protein kinase B (PKB) to which PIP3 binds through PKB's pleckstrin homology domain (PH domain). Through this interaction, PKB recruits to the inner surface of the plasma membrane (Scheid et al, 2002) where it can interact with other molecules such as ILK. Likewise, ILK recruits to the plasma membrane through interaction with PIP3 after which it phosphorylates PKB. PKB becomes fully activated once it is phosphorylated by phosphoinositide-dependent kinases 1 and 2 (PDK-1 and PDK-2), serine-threonine kinases which also contain PH domains and interact with PIP3. PDK-1 phosphorylates PKB on threonine 308 and PDK-2 phosphorylates PKB on Ser-473. Integrin linked kinase phosphorylates PKB on Ser-473 (Downward 1998), thus indicating that ILK may be a potential PDK-2 molecule (Persad et al, 2001a). Active PKB will regulate a series of pathways which lead to cell survival and cell metabolism (Fig.4). Primarily, cell survival through PKB is regulated by inhibition of apoptosis (Downward, 1998; Cardone et al, 1998; Brunet et al, 1999). PKB can lead to inhibition of apoptosis through: phosphorylation of protein BAD, a member of the Bcl-2 family; inactivation of caspase 9;and repression of forkhead transcription factor (FKHRL-1).

family; inactivation of caspase 9;and repression of forkhead transcription factor (FKHRL-1).

Fig.4. PI3'K pathway (adapted from Dedhar, 2000)



Regulation of apoptosis by PKB is initiated through a survival factor which binds to receptor protein tyrosine kinases. Survival factors, such as nerve growth factor (NGF) in neurons, trigger receptor phosphorylation and PI3'K activity leading to phosphorylation of PKB through PDKs (Talapatra and Thompson, 2001) as well as inactivation of Bad, caspase-9 and transcription factor forkhead. In contrast, once Bad is activated it translocates to the mitochondria stimulating the release of cytochrome-C. Cytochrome-C binds to Apaf-1 adaptor protein which in turn binds to caspase-9. Binding of cytochrome-C to apaf-1 and caspase-9 is necessary for regulation of cell death. This complex leads to

downstream caspases to induce cell death. Phosphorylation of Bad and caspase-9 by PKB leads to their inactivation and inhibition of apoptosis. Phosphorylation of Bad by PKB creates a binding site for other cytosolic proteins thus preventing its mitochondrial translocation and inhibiting the cytochrome-C release.

PKB also phosphorylates transcription factor forkhead (FKHRL-1); this interaction leads to the retention of FKHRL-1 in the cytoplasm and inhibition of its dependent transcription (Brunet et al, 1999). Survival factor withdrawal induces FKHRL-1 de-phosphorylation and nuclear translocation, initiating transcription of *Fas ligand* gene (a cell death gene), thus triggering apoptosis. In conclusion, phosphorylation of FKHRL-1 by PKB suppresses transcription of death genes leading to inhibition of apoptosis and cell survival.

As mentioned previously integrin linked kinase regulates GSK-3 leading to transcriptional activity of *cyclin D1* and *cyclin A*, two key targets for the transition of G1 to S phase during cell cycle (Assoian, 1997). Although the mechanism of this regulation through integrins is not obvious, integrin dependent cell adhesion is necessary for the regulation of *cyclin D1* and *cyclin A* expression. ILK has been suggested to be important in regulating such an interaction given that its over-expression results in anchorage independent cell progression induced by elevated *cyclin D1* levels (Dedhar, 2000).

An inhibitor of PI3'K pathway, phosphatidylinositol phosphatase PTEN [phosphatase and tensin homolog (mutated in multiple advanced cancers 1)], negatively regulates this cascade. PTEN maintains the PIP2/PIP3 balance by de-phosphorylation of PIP3 producing PIP2 and preventing activation of this pathway. PTEN primarily regulates cell cycle progression and cell death. Inhibition of PTEN leads to an increase in cell proliferation signifying its role in cell cycle control (Schmidt-Weber et al, 2002). From the

above mentioned data it can be concluded that ILK can regulate cell survival through cell cycle progression and inhibition of apoptosis.

The PI3'K and Wnt/Wg pathways are not mutually exclusive. In fact ILK is proposed to be the connection between these two pathways. Protein kinase ILK has the ability to phosphorylate GSK-3 in the PI3'K-dependant manner (Delcommenne et al, 1998). Through PI3'K activation it leads to metabolic effects as well as cell survival. It also has the ability to phosphorylate GSK-3 leading to *cyclin D1* gene expression in the Wnt/Wg pathway. In addition, Novak et al (1998) have shown that Lef-1 expression is modulated through cell detachment from the ECM. Integrin linked kinase over-expression leads to up-regulation of Lef 1 protein expression. These results are specific to ILK, indicating that the oncogenic properties of ILK involve Lef/β-catenin regulation and that ILK may be an intermediate molecule between the cell adhesion and cell matrix interactions as well as the Wnt signaling pathways. In another study, Dsh has been demonstrated to localize at focal adhesion plaques through interaction with ILK and paxillin (Torres and Nelson, 2000) supporting the idea that ILK is involved in both cell adhesion and Wnt/Wg pathways.

1.8.1 Protein Kinase B

Protein kinase B, referred to as PKB/AKT, is a serine-threonine kinase with a mass of 57 KDa and a downstream substrate for ILK. As well PKB has been implicated in cell survival mechanisms. This protein kinase is highly similar in the kinase domain to protein kinase A and protein kinase C, hence known as protein kinase B (PKB) (Coffer and Woodgett, 1991). PKB has three mammalian isoforms PKBα, PKBβ and PKBγ. PKBβ and PKBγ are approximately 82% identical with the α-isoform, although PKBγ

lacks 23 amino acids at the C-terminus compared with the others. Homologues have also been identified in the nematode worm *Caenorhabditis elegans* and the fruitfly *Drosophila melanogaster,* demonstrating wide evolutionary conservation (Coffer et al, 1998).

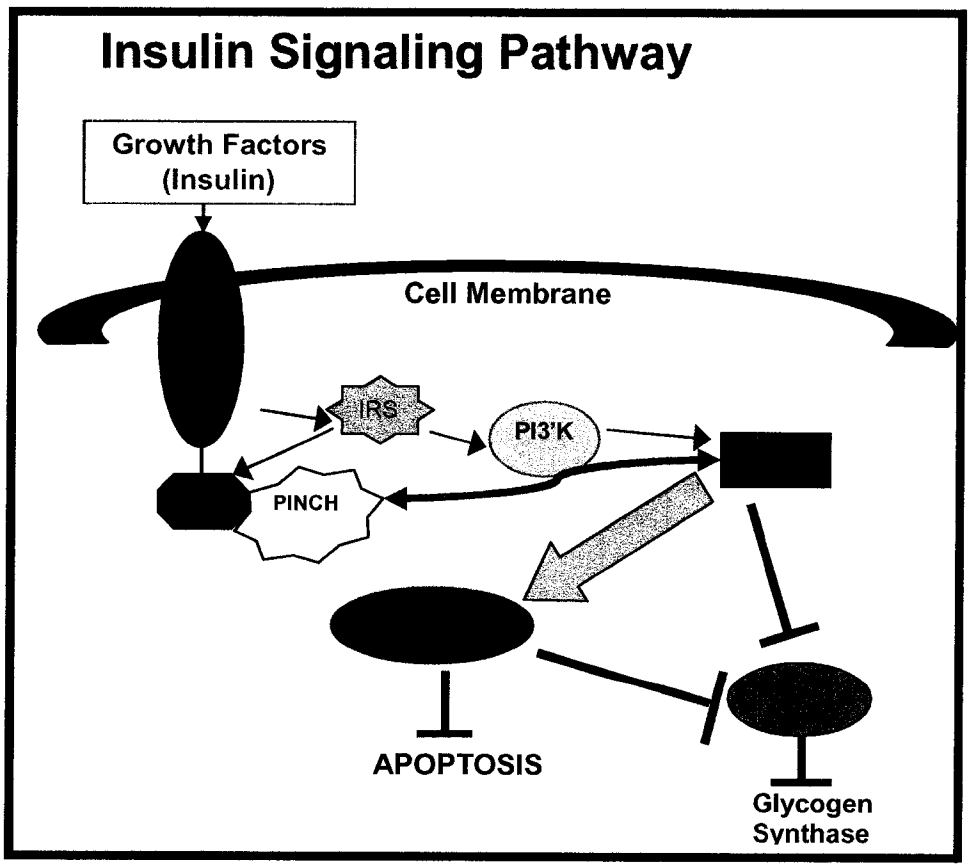
PKB contains a PH domain at the N-terminus that mediates PI3'K dependent cellular processes (Haslam et al, 1993). Its central catalytic domain involved in kinase reactions terminates with a regulatory domain. The C-terminus shows similarity in sequence to the regulatory region present in members of the PKC family of proteins (Bellacosa, et al, 1993).

1.9 Insulin signaling

Integrin linked kinase is also implicated in the growth factor induced cellular pathway which functions through insulin receptors. Insulin receptors are tetrameric proteins composed of 2 α and 2 β subunits, which are linked by disulfide bonds and are present at the surface of the cells (Van der geer et al, 1994). The α subunits remain on the surface of the cell and bind to the signals, but the β subunits span across the membrane and transfer the signal to the interior of the cell. The insulin receptor has an enzymatic activity and is referred to as receptor tyrosine kinase (RTK) because it adds phosphate groups to specific tyrosine residues known as tyrosine motifs. Upon binding of insulin to the α chain of the receptor a conformational change will occur in the β subunit triggering its tyrosine kinase activity. Activated tyrosine kinase functions in auto-phosphorylation of the receptor as well as addition of phosphate groups to insulin receptor substrates (IRS) (Van der geer et al, 1994). The IRS phospho-tyrosine motifs are a key target for the SH2 domain of PI3'K and through such an interaction the signal is transferred to PKB, the immediate downstream target. In cells responding to insulin, the lipid products of PI3'K

immediate downstream target. In cells responding to insulin, the lipid products of PI3'K bind to the PH domain of protein PKB, changing its conformation and making it a suitable substrate to interact with other kinases (Rameh and Cantley, 1999). Activated PKB has several cellular functions: 1) it transfers the glucose transporters to the plasma membrane where they can function in glucose uptake; 2) it increases protein synthesis which is a hallmark of insulin action, and 3) it stimulates glycogen synthase leading to conversion of glucose to glycogen (Fig.5). Integrin linked kinase is supposed to interact with PKB leading to metabolic changes in the cell.

Fig. 5. Insulin signaling pathway (adopted from Dedhar, 2000)



turn phosphorylates GSK-3β leading to increased levels of *cyclin D1* (Coyle-Rink et al, 2002). Cyclin D1 is required for the transition of the G1 phase to S phase, thereby regulating the cell cycle progression (Liang and Slingerland, 2003).

1.10 Function of ILK

In addition to its signaling role as a kinase in various signaling pathways (Delcommenne et al, 1998), ILK has been shown to play a structural role as an adaptor molecule in focal adhesion assemblies (Li et al, 1999).

1.10.1 Kinase activity of ILK

The kinase domain of ILK interacts with the β_1, β_2, and β_3 integrin subunits as well as a family of actin-binding proteins named α-parvin (also named CH-ILKBP or actopaxins) (Nikolopoulos and Turner 2000; Olski et al, 2001; Tu et al, 2001) and β-parvin (also named affixin) (Olski et al, 2001; Yamaji et al, 2001).

As a serine-threonine protein kinase, ILK stimulates many downstream cytoplasmic targets (Hannigan et al, 1996). The activity of ILK was demonstrated within cells upon insulin and fibronectin stimulation, and revealed that ILK interacts in a PI3'K dependent manner. Insulin is known to activate ILK resulting in myelin basic protein (MBP) phosphorylation *in vitro,* and this activation is inhibited by a prior treatment with Wortmanin, a PI3'K specific inhibitor (Delcommenne et al, 1998). In addition, ILK activity is stimulated upon attachment of cells to fibronectin. This transient activation of ILK occurs in a PI3'K dependent manner because it can also be inhibited by addition of another inhibitor of PI3'K, LY294002 (Delcommenne et al, 1998). Since integrin engagement stimulates PI3'K activity and leads to PKB/AKT phosphorylation (Khwaja et al, 1997; King et al, 1997), ILK kinase activity was investigated to determine whether

it is involved in the regulation of PKB/AKT activity. When kinase-deficient ILK was stably transfected into cells, the over-expression resulted in the inhibition of PKB phosphorylation on Ser-473. ILK is likely to regulate phosphorylation directly because purified ILK phosphorylates PKB/AKT on Ser-473 *in vitro* (Delcommenne et al, 1998). Also, ILK inhibits GSK-3 activity *in vitro* through phosphorylation (Delcommenne et al, 1998). This study was carried out through over-expression of ILK whereas, stably expressed kinase deficient ILK or anti-sense ILK cDNA were not able to phosphorylate GSK-3 *in vitro.*

The kinase activity of ILK has been demonstrated to occur in numerous pathways such as regulation of GSK-3 activity in a PKB-dependent manner (Troussard et al, 1999). However, ILK is also capable of phosphorylating GSK-3(β) on Ser-9 in the absence of PKB (Persad et al, 2001b). Although, both ILK and PKB are known to phosphorylate GSK-3 (Delcommenne et al, 1998; Troussard et al, 1999; Cross et al. 1995), GSK-3β is largely regulated by PTEN in an ILK-dependent manner.

Integrin linked kinase activity is regulated through PTEN, a tumor suppressor gene that codes for a phospholipid phosphatase (Persad et al, 2000). This property of PTEN was found through over-expression of PTEN by transfection of wild type PTEN in PTEN null cells and resulted in the inhibition of ILK activity (Persad et al, 2000).

1.10.2 Auto-phosphorylation properties of ILK

The C-terminal domain of ILK is related to the kinase domain of the protein; however, some scientists believe that kinase activity may not be the main function of ILK. Their evidence is that ILK sequence differs from other kinases at some extremely well-conserved sites, such as the aspartic acid in subdomain VIb, which is involved in the

transfer of the phosphate group (Zervas et al, 2001). In contrast several lines of biochemical evidence further confirm that ILK is indeed an active kinase: ILK has been shown to phosphorylate peptides on serine and threonine residues as well as standard kinase substrates such as myelin basic protein (Hannigan et al, 1996; Zervas et al, 2001). Replacement of a conserved residue in the substrate binding loop of the kinase domain (E359K) results in a mutant ILK protein that has lost its *in vitro* kinase activity. Over-expression of mutant ILK in cell culture causes dominant negative effects (Delcommenne et al, 1998; Wu et al, 1998) or loss of wild type over-expression effects (Novak et al, 1998). A mutation in the ATP binding site (K219M) has been shown to eliminate the ability of ILK to stimulate phosphorylation of PKB on Ser-473 (Lynch et al, 1999). However, introducing a second mutation in ILK (S324E), predicted to mimic auto-phosphorylation at a potential site, partially restored PKB phosphorylation despite the mutation in the ILK ATP binding site. This experiment has led to a suggestion that the primary substrate of ILK may be itself, and that the main function of ILK is that of an adaptor rather than a kinase (Zervas et al, 2001).

1.10.3 ILK as an adaptor molecule

Studies in *Drosophila* have demonstrated that integrins are not the only molecules mediating interactions between the plasma membrane and cytoskeleton (Zervas et al, 2001). An intracellular protein which binds the cytoplasmic tail of integrin is integrin linked kinase identified by yeast two hybrid screening (Hannigan et al, 1996). The analysis of the phenotype of a null mutant ILK revealed that ILK is critical for completion of embryogenesis and for wing formation (Zervas et al, 2001). Breakage occurs at the sites of integrin-mediated adhesion (failure in the attachment of actin filaments to the

membrane) in these mutants, although no apparent effect on cellular differentiation was observed. In addition, the wing and cuticle are excellent locations to observe distinct effects from disrupting signaling through, e.g. β-catenin or PKB; nonetheless, the only effects seen were loss of attachments at sites of integrin-dependent adhesion.

Integrin linked kinase is a cytoplasmic component of cell-ECM adhesion structure that plays pivotal roles in the attachment of ECM to actin cytoskeleton (Dedhar et al, 1999; Wu, 1999; Wu and Dedhar, 2001). On the basis of phenotypes observed in the mutational experiments, ILK either forms or assists in the formation of the connection between integrins and actin-binding proteins (Zervas et al, 2001). The protein-protein interactions are mediated by adaptor proteins, a group of non-catalytic proteins specialized in mediating multi-protein complex formation. The linkage between ILK and the actin cytoskeleton seems likely to involve other adaptor proteins such as α-parvin (Nikolopoulos and Turner, 2000; Olski et al, 2001; Tu et al, 2001) and β-parvin (Olski et al, 2001; Yamaji et al, 2001). Integrin linked kinase is required for cell adhesion and controlling actin cytoskeleton accumulation (Sakai et al, 2003). It regulates integrin-mediated cell adhesion, E-cadherin expression and pericellular fibronectins matrix assembly (Hannigan et al, 1996; Wu et al, 1998).

Co-immunopercipitation analysis showed that ILK can interact with the cytoplasmic domain of integrin β1 and β3 subunits and that it is present in some integrin-containing focal-adhesion plaques. ILK most probably interacts with some highly conserved threonine residues present in the cytoplasmic domain of β1and β3 subunits as mutation of these residues to alanine residues abolishes ILK-integrin interactions in co-immunoprecipitation assays (Dedhar and Hannigan, 1996). Studies have indicated a candidate for the interaction with the N-terminal domain of ILK, the LIM domain of

protein PINCH which co-localizes with integrins to focal adhesions (Tu et al, 1999). Since integrins do not interact with PINCH directly, therefore the formation of a ternary complex between integrins, ILK and PINCH (Tu et al, 1999) is important and indicates the role of ILK as an adaptor molecule between PINCH and integrins.

Integrins mediate development via interaction of cells with the cell matrix. Upon ligand binding integrins recruit to focal adhesions and ILK, recruits some adaptor proteins that link integrins to actin cytoskeleton. In mice, the lack of ILK expression leads to failure in epiblast polarization and the formation of cavity which causes death at the peri-implantation stage. These series of events indicate that ILK is crucial for cell adhesion and the process of development.

It has been reported that *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 ILK kinase dead mutant that has been shown to have partial loss of kinase activity was able to rescue the null mutant phenotype (Zervas et al, 2001). The data above demonstrate that the kinase and adaptor properties of ILK function together, in a PI3' kinase-dependent manner, to regulate integrin-mediated cell attachment and signal transduction.

1.10.4 Over-expression of ILK

ILK over-expression leads to oncogenic transformation, which is a significant

phenotypic effect visualized in epithelial cells. These effects are: anchorage independent cell growth (Hannigan et al, 1996), suppression of apoptosis in suspension (Radeva et al, 1997), increased invasion of extracellular matrixes (Novak et al, 1998; Wu et al, 1998) and tumorigenicity in nude mice (Wu et al, 1998). Some of these features are caused by ILK-induced epithelial-to-mesenchymal transformation and loss of E-cadherin expression (Novak et al, 1998), a significant marker of tumor invasion and metastasis (Perl et al, 1998). Over-expression of ILK leads to β-catenin nuclear translocation and transcriptional activation of the complex Lef/β -catenin (Novak et al, 1998). The Lef/β-catenin complex negatively regulates the gene encoding E-cadherin therefore providing a potential mechanism for loss of adhesion (Dedhar, 2000). Over-expression of ILK is common among several types of tumors such as human malignancies and is involved in the regulation of tumor cell survival under stress (Attwell et al, 2000; Zhang et al, 2001).Over-expression of ILK can protect cells from stress induced apoptosis. ILK over-expression promotes anchorage independent tumor growth. ILK has been visualized to be over-expressed in colon and prostate tumors (Marotta et al, 2001; Graff et al, 2001). The potential role of ILK as a survival factor for cancer cells was shown when ILK was up-regulated in hypoxic hepatocarcinoma cells. ILK over-expression can also significantly increase tumor cellular resistance to hyperthermia treatment, a potentially important cancer therapeutic modality that has shown positive results in Phase III clinical trials (Zhang et al, 2003).

ILK over expression leads to up-regulation of Lef-1 protein. These results are specific for ILK since transfection of activated H-*ras* or *v-src* do not lead to activation of Lef/β-catenin complex, thus oncogenic properties of ILK contain Lef/β-catenin regulation (Novak et al, 1998).

1.11 *Xenopus laevis* in developmental studies

Xenopus laevis is a fresh water amphibian which has several advantages for molecular and developmental studies over other known vertebrate models. *Xenopus* can be induced to lay eggs by simple hormone injection. The large size of the embryos allows micromanipulation and microinjection of the embryos, and their rapid rate of development makes them a perfect model system for vertebrate developmental studies. The major disadvantages of *Xenopus laevis* are their long generation time (1-2 years) and pseudo-tetraploidy (Sive et al, 1999).

1.12 My objectives

Previous studies have focused on characterizing ILK in *Drosophila*, mice, human as well as in other organisms. Yet the role of ILK in amphibians, specifically *Xenopus* is unknown. There have been no studies indicating the role of *Xenopus laevis ILK* (*XILK*) gene. Since *Xenopus* is a suitable model system for vertebrate developmental studies, the role of ILK during early amphibian development was studied. The main goals of this study are:

1. To compare the sequence of *X*-ILK cDNA and protein with the sequence of ILK cDNA and proteins of other organisms.

2. To determine the amount of ILK transcript levels during development and how it is regulated in *Xenopus* embryos as well as in adult tissues.

3. To verify the expression of ILK protein during development and its regulation in embryos and in adult tissues.

4. To examine ILK function as a kinase in embryos during early embryogenesis and in adult tissues.

Materials and Methods

2.1 Biological Material and Methods

Xenopus laevis embryos were obtained by *in vitro* fertilization using *Xenopus* purchased from *Xenopus*, Inc. The female frogs were injected with (0.5 ml) HCG (Human Chorionic Gonadotropin hormone, 5000 units) the night before and squeezed to release eggs the day after. The male frog was sacrificed and dissected from the abdominal wall to obtain the testes. Twenty five percent of the testes was minced in 80% Steinberg solution [0.8X Steinberg's A (Steinberg's A (20X stock): 1160 mM NaCl, 13.41 mM KCl, 16.6 mM $MgSO_4$ and 6.7 mM $Ca(NO_3)_2$) and 40 mM Steinberg's B (Steinberg's B stock solution: 1 mM Trizma-HCl)] and the rest was kept in 200% Steinberg (0.2X Steinberg A and 10 mM Steinberg B) solution at 4°C for later use. The eggs were exposed to the sperm for 7 minutes. Once fertilized the zygotes rotated and their black cap (animal cap) was visible. Modified Barth's Saline (MBS) media [0.88 mM NaCl, 0.01 mM KCl, 0.01 mM $MgSO_4$, 0.05 mM Hepes (Sigma-Aldrich, Oakville) and 0.025 mM $NaHCO_3$ was adjusted to pH 7.8 using NaOH] was then added to fertilized eggs. The fertilized eggs were dejellied using 2% L-cysteine in 0.1% MBS. The embryos were incubated at room temperature, stored in 0.1% MBS and collected at the: 4-cell, blastula (stage 8), gastrula (stage 10.5), neurula (stage 21) and tailbud (stage 28) stages.

2.2 RT-PCR

Total RNA was extracted from *Xenopus* embryos (40 embryos per stage) and adult tissues (heart, liver, kidney and muscle) using the Trizol™ method (Invitrogen Life Sciences, Burlington, Ontario). Five μg of RNA was reverse transcribed using Superscript™ II RNase H⁻ Reverse Transcriptase kit (Invitrogen Life Sciences,

Burlington, Ontario). The PCR reaction was carried out using 0.4 µl Taq DNA Polymerase (5U/µl) per reaction, 5 µl 10X PCR buffer [200 mM Tris-HCL (pH 8.4), 500 mM KCL from (Invitrogen Life Sciences, Burlington, Ontario)], 2 µl cDNA, 1 µl primers 5' *X*ILK Eco (50.8 nmol) and 3'*X*ILK Not (96.0 nmol) for test experiment and 1 µl 5' *X*ef-1α (25.8 nmol) and 3' *X*ef-1α (54.0 nmol) primers (for control experiment), 1 µl 10 mM dNTP Mix (1/10 dilution of dCTP) with 5 mM $MgCl_2$ and the reaction volume was then taken up to 50 µl using autoclaved, double distilled water. The RT-PCR reaction was performed using 1.7 µl of ^{32}P-α dCTP to make the product radioactive. The primers used are as follow:

5' Primer (*X*ILK Eco): 5' CCGGAATTCCGGATGGACGACATTTTCG 3'

3' Primer (*X*ILK Not): 5' TTGCGGCCGCAACTATCTCTGCATCTTCTC 3'

5' Primer (*X*ef1-α): 5' CAGATTGGTGCTGGATATG 3'

3' Primer (*X*ef1-α): 5' ACTGCCTTGATGACTCCTA 3'

The *X*ILK PCR reaction was carried out for 27 cycles at temperatures 95°C for 5 min, 92°C for 1 min, 55°C for 1 min, 72°C for 2 min and 72°C for 10 min. The control ef-1α PCR reaction was carried out for 29 cycles at temperatures 94°C for 4 min, 94°C for 45 Sec, 57°C for 1 min, 72°C for 45 Sec and 72°C for 10 min. The PCR product was electrophorsed onto a 1.5% agarose gel which was then transferred to a nylon membrane by Southern blotting. Following transfer, the membrane was exposed to an X-ray film (Kodak distributed by Perkin Elmer, Boston, MA) for a few hours and developed.

2.3 Northern blot

Total RNA was isolated from 0.15-0.2 g of tissue *Xenopus* from a recently sacrificed frog as described above. The tissues were either used fresh or stored at -80°C

and cut into small pieces using a clean scalpel. Tissues were then homogenized in a homogenizer using the Trizol™ solution. Fifteen μg of RNA was quantified using gel electrophoresis and spectrometry. RNA was then electrophoresed onto a 1.2% agarose gel (gel containing 0.6 g agarose, 1X MOPS, 5.92 % Formaldehyde, 0.1% DEPC treated water was used to adjust the final volume up to 50 ml, the agarose was dissolved in DEPC treated water by boiling, after cooled down the rest of the ingredients were added) with 1X MOPS (3-[N-Morpholino] propanesulfonic acid) buffer and subjected to electrophoresis at 70 volts. The gel was rinsed in 0.1% diethyl pyrocarbonate (DEPC) treated water for 1 hour while the marker lane on the gel was removed and stained in DEPC water containing ethidium bromide for one hour. The marker lane was de-stained in DEPC treated water overnight at 4°C. The RNA on the gel was transferred to a nylon membrane (Biodyne B Membrane 0.45 μm) and cross linked to the membrane using a UV cross linker (Biorad-GS Gene Linker™ UV chamber, 150 Joule) for 150 seconds. The membrane was pre-hybridized in the solution containing 0.5X Formamide, 5X SSC, 2.5X Denhardts, 0.25 mg/mL salmon sperm DNA (ssDNA) (boiled for 10 minutes at 95-100°C then cooled for 5 minutes on ice, before added to solution), and the volume was taken up to 20 ml with 0.1% DEPC H_2O. Pre-hybridization was caused over night. The next day hybridization was performed with a probe using a hybridization solution (0.5X Formamide, 4X SSC, 2.5X Denhardts, 0.05% dextran sulfate, 100 uL radioactive probe from the RT-PCR reaction, 0.25 mg/mL ssDNA (boiled for 10 minutes at 95-100°C then cooled for 5 minutes on ice, before added to solution). The final volume was taken up to 20 ml with 0.1 % DEPC treated H_2O and left in the hybridization chamber overnight. The ^{32} P-α-dCTP labeled probe was made using PCR method and purified through a Sephadex G50 column (DNA grade, Sigma, Oakville, Ontario). After hybridization, the membrane

was washed sequentially with buffer I (1 X SSC, 0.1% SDS), buffer II (0.5 X SSC, 0.1% SDS) and buffer III (0.1 X SSC, 0.1% SDS) and dried followed by exposure to an X-ray film. The cassette containing the film and the membrane was kept at -80°C for a minimum of five days and then developed.

2.4 Western blot

Xenopus embryos were homogenized and lysed in 600 µl of lysis buffer (10 mM NaCl, 20 mM PIPES, 0.5% NP40, 0.05% β-mercaptoethanol, 5 mM EDTA, 100 µM sodium orthovanadate, 5 µg/mL leupeptin and aprotinin, 50 mM sodium fluoride, 1 mM benzamidine), and then centrifuged (15 000 X g, for 10 minutes at 4°C). The upper aqueous phase containing the protein was separated. The protein content was quantified using the Bio-Rad assay method (Bradford, 1976). Equal concentrations of protein were denatured in an SDS loading buffer at 95°C for 5 minutes and then separated by SDS-PAGE on a 12% SDS poly-acrylamide gel (containing of 12% resolving gel and 3% stacking gel, the resolving gel containing: 30% acrylamide 29:1, 3.0 M Tris (pH 8.8), 10% SDS, 30% ammonium persulfate and addition of 4µl TEMED and the 3% stacking gel containing: 30% acrylamide, 1.0 M Tris (pH 6.8), 10% SDS, 30% ammonium persulfate and addition of 3 µl TEMED). For the Western blot analysis of tissues the SDS poly acrylamide gel was performed differently [12% resolving gel (Laemmli, 1970) : 1X Lower Tris buffer (contained of:1.5 M Tris-HCl, 0.4% SDS and pH adjusted to 8.8), 12% glycerol, 12% acrylamide, 0.32% ammonium persulfate and addition of a 4 µl TEMED and 3.86% stacking gel: 1.2X Upper Tris buffer (contained of: 0.5 M Tris-HCl, 0.4% SDS and pH adjusted to 6.8), 4.86% acrylamide, 0.13% ammonium persulfate with an addition of 3 µl TEMED]. The protein in the gel was transferred onto a 0.45µm polyvinylidene

fluoride (PVDF) transfer membrane using a semi-dry transfer blot apparatus. The blot was then exposed to 4% Blotto at room temperature for a few hours followed by exposure to the primary antibody solution (1 in 10,000-fold of either Anti-Human ILK or Anti-Human PKB/Akt in 2% Blotto – 2% skim milk powder, 1X PBS, 0.05% Tween) over night at 4°C, with gentle shaking on a nutator. The antibody solutions were removed and the blots were washed 3 times 10 minutes each using fresh 2% Blotto for a total of half an hour. The blot was then exposed to the secondary antibody solution (0.024% Anti-rabbit polyclonal antibody linked to horse radish peroxidase) for three hours at room temperature with gentle shaking. The blots were then washed in 1 X PBS followed by exposure to the chemiluminiscence reagents (Perkin Elmer Life Sciences, Markham). Finally the blots were exposed to autoradiographic film for a time range of 1-5 minutes and then developed as described previously.

The same protocol was followed in assessing the protein levels in various *Xenopus* tissues.

2.5 Western Blot analysis of phospho-proteins

The principle of this experiment is similar to a standard Western blot procedure except the antibody used in this experiment specifically identifies the phosphorylated form of the substrate. The transferred blot was treated with 1X TTBS (50mM Tris-HCl, pH. 7.4, 150mM NaCl and 0.1% Tween) and the blot was then exposed to anti phospho-Ser 473 PKB/Akt antibody diluted with 1X TTBS (1 in 10,000-fold) plus 1% BSA. The blot was then washed with TTBS without BSA and the secondary antibody, which in our case was anti-mouse monoclonal antibody conjugated with horse radish peroxidase, in TTBS

and 1% BSA. The blot was washed with excess of TTBS and TBS (50mM Tris-Cl pH. 7.4 and150mM NaCl) and subjected to a chemiluminescence reagent (Perkin Elmer Life Sciences, Markham). Finally the blot was exposed to an X-ray film for a time range of 1-5 minutes and developed.

2.6 Immunoprecipitation (IP) assay

Proteins were extracted from embryos using the lysis buffer as described above and centrifuged at 15,000 rpm for 15 minutes at 4 °C. Immunopercipitation (IP) of the upper aqueous phase containing 12 μg protein was performed using 1.5 μl anti-human ILK antibody (Upstate Biotechnology, Lak Placid, NY), 20 μl Protein G-Sepharose beads 4 fast flow (Amersham Pharmacia, Baie d' urfe, Quebec) and 150 μl of lysis buffer per sample. After overnight incubation at 4°C on a rotating apparatus, the complex of protein plus antibody conjugated to the beads was centrifuged for 10 sec and washed 3 times each with 1 ml of lysis buffer.

2.7 *In vitro* kinase assay

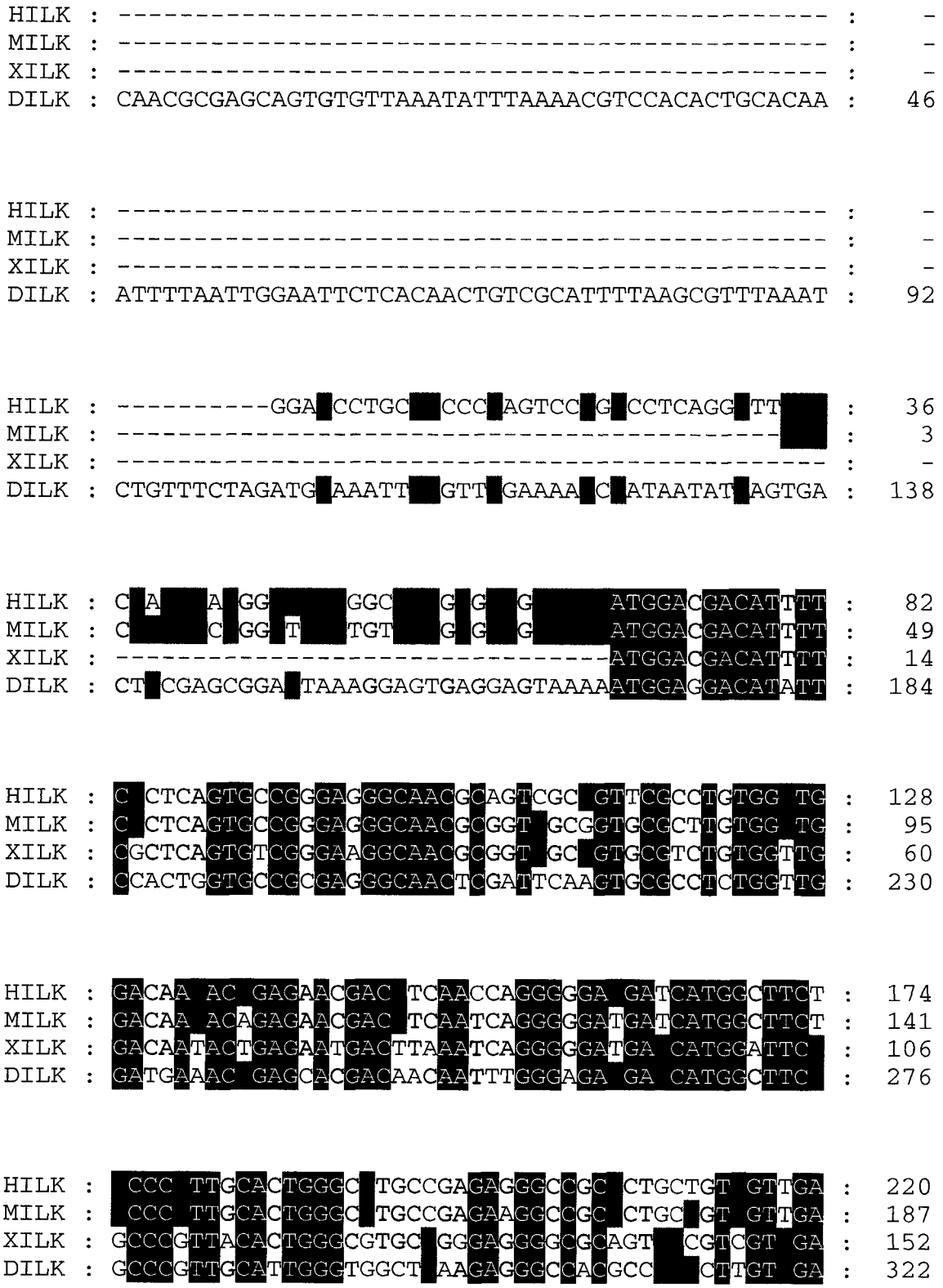
The kinase reaction mixture (ATP 4.7 μM, 3 μCi [γ-^{32}P] ATP, 1 μg GST-tagged PKB (protein kinase B isolated from Hela cells), 10 μl 5X kinase buffer [10 mM $MgCl_2$, 50 mM Tris-Cl, pH 7.5, 1 mM EGTA, pH 7.5] and 12.5 μl ddH_2O) was added and incubated at 30 °C for 30 minutes. The reaction was stopped by addition of 1X SDS sample buffer dye, and the samples were electrophoresed on a 12% SDS polyacrylamide gel. The gel was washed with distilled water, dried in a vacuum oven on 3 MM whatman paper for 1 hour and exposed to an X-ray film over night.

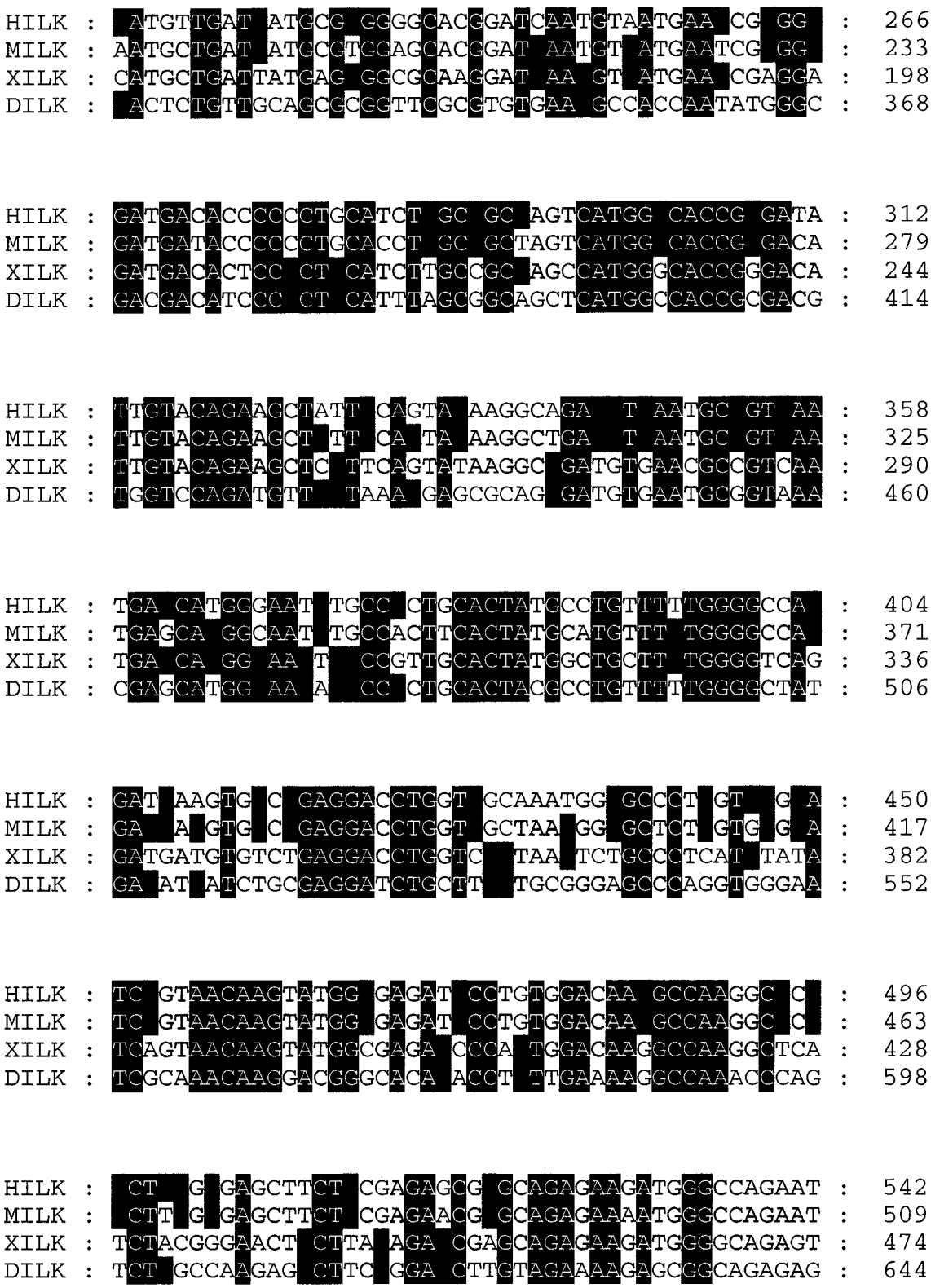
Results

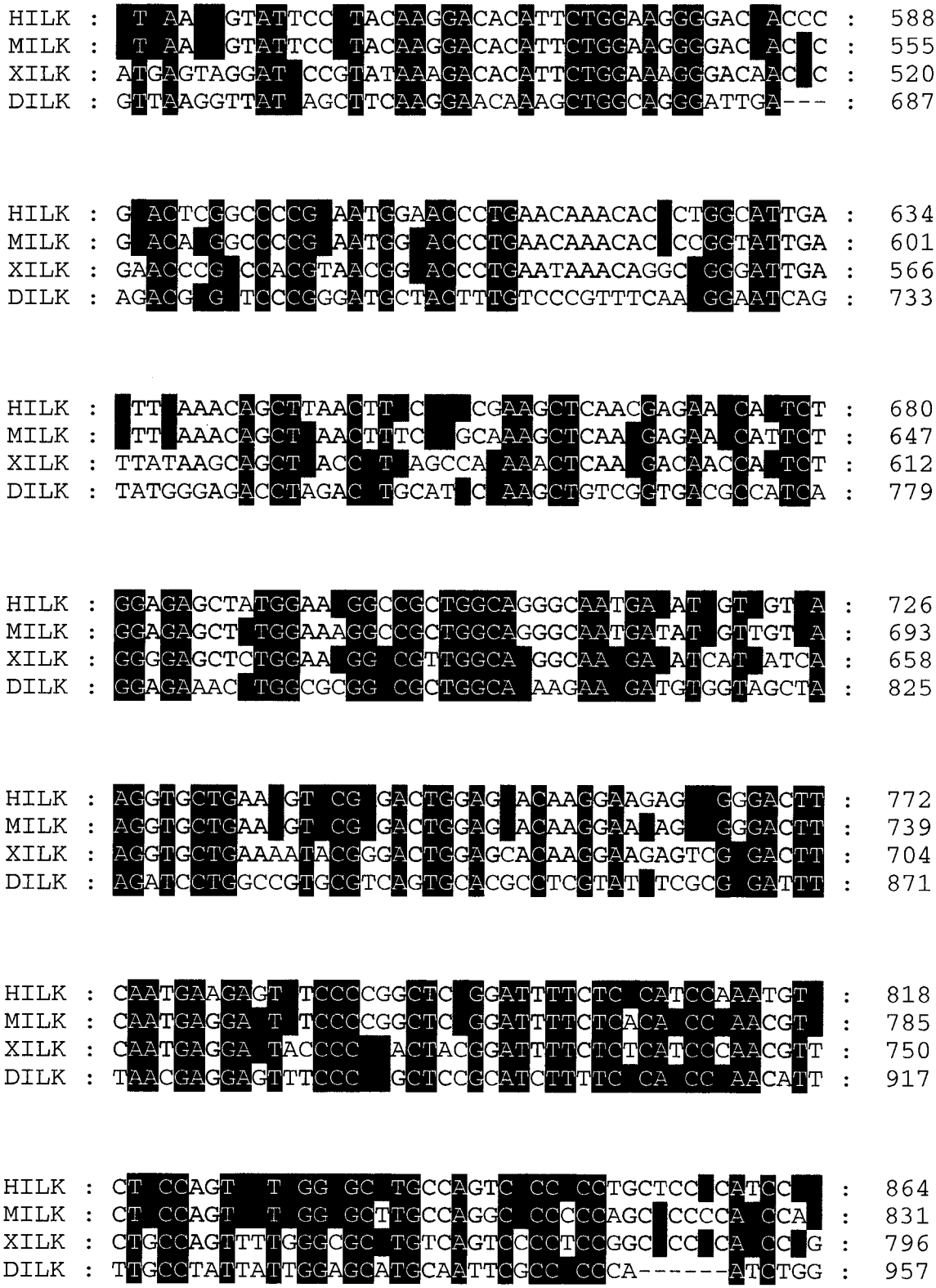
3.1 Comparison of ILK sequences

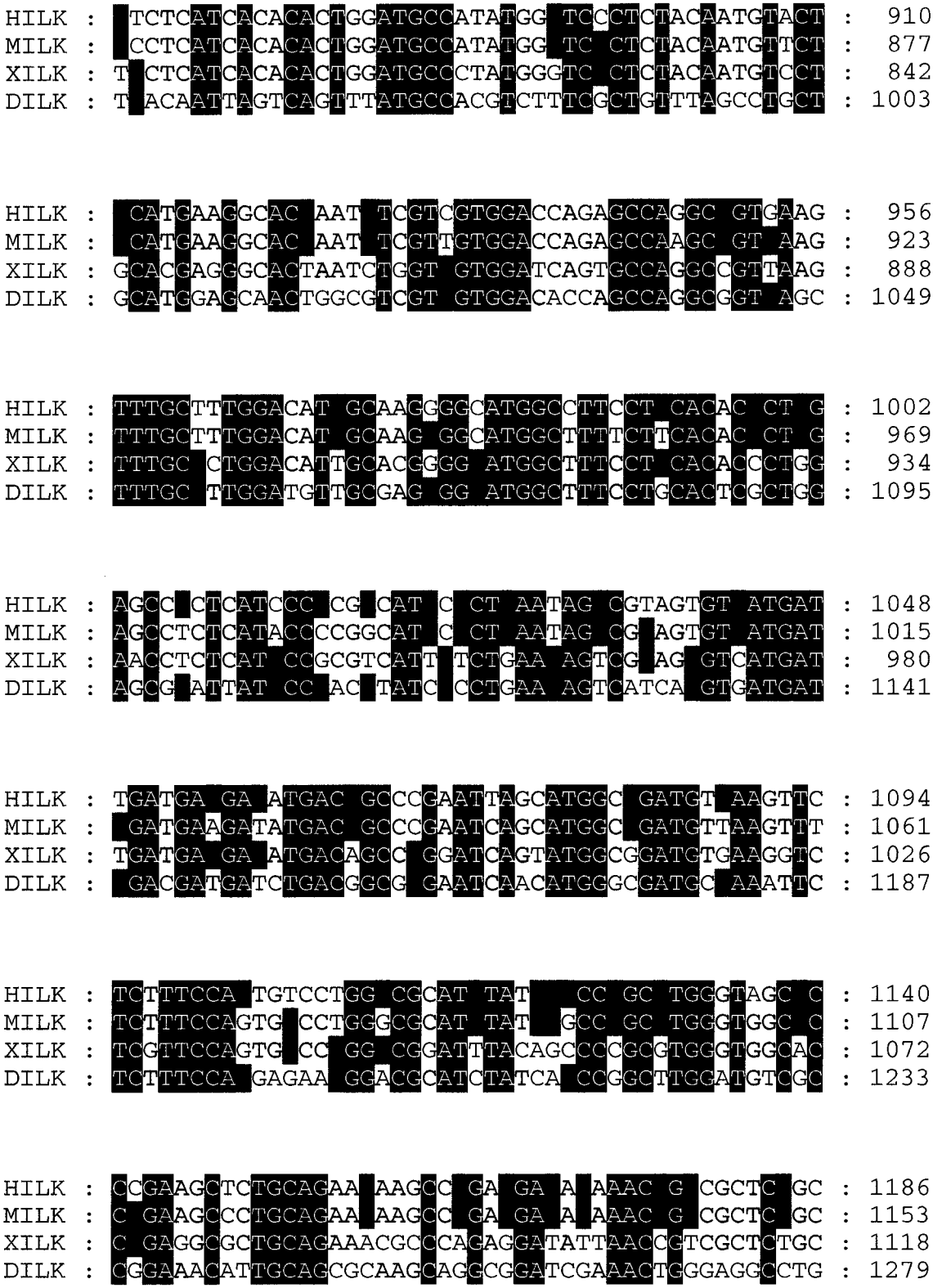
As mentioned in the introduction, the *ILK* gene is present in most if not all eukaryotes. To determine the similarity between different homologues, in the *ILK* gene an alignment between their cDNA and putative amino acid sequences was carried out using Clustal W (http://www.ebi.ac.uk/clustalw/) and the analysis of the aligned sequences such as conservation and score of identity of sequences were performed in GeneDoc [version 2.6.002, (http://www.psc.edu/biomed/genedoc/)]. The cDNA sequences were extracted from NCBI gene bank database [accession numbers of cDNA sequences: HILK: BC001554, MILK: BC003737, *X*ILK: not submitted yet and *D*ILK: AF226669]. The *X*ILK cDNA was cloned and sequenced by Dr. Ali in our lab and its putative amino acid sequence was obtained by DNA star program. Comparisons of the domains in ILK are shown in the following 3 diagrams. Figure 6 shows the alignment of ILK cDNAs from human, mouse, *Xenopus* and *Drosophila.* The degree of similarity (as %) between each two aligned sequences is given in Table 1 using Genedoc program. *Xenopus* ILK cDNA is more similar to mammalian ILK cDNA (59% similar to human and 61% similar to mouse ILK sequences) as compared to *Drosophila* ILK. Figure 7 shows the alignment of ILK protein in human, mouse, *Xenopus* and *Drosophila,* (using protein sequences from NCBI, accession numbers: HILK: Q13418, MILK: AAH03737, *X*ILK: not submitted and *D*ILK: AAF28365) and the similarity (as %) between each two ILK protein sequences is given in Table 2. *Xenopus* ILK protein sequence resembles human and mouse ILK protein sequences (71% identical). Figure 8 shows the alignment of *Xenopus* ILK protein

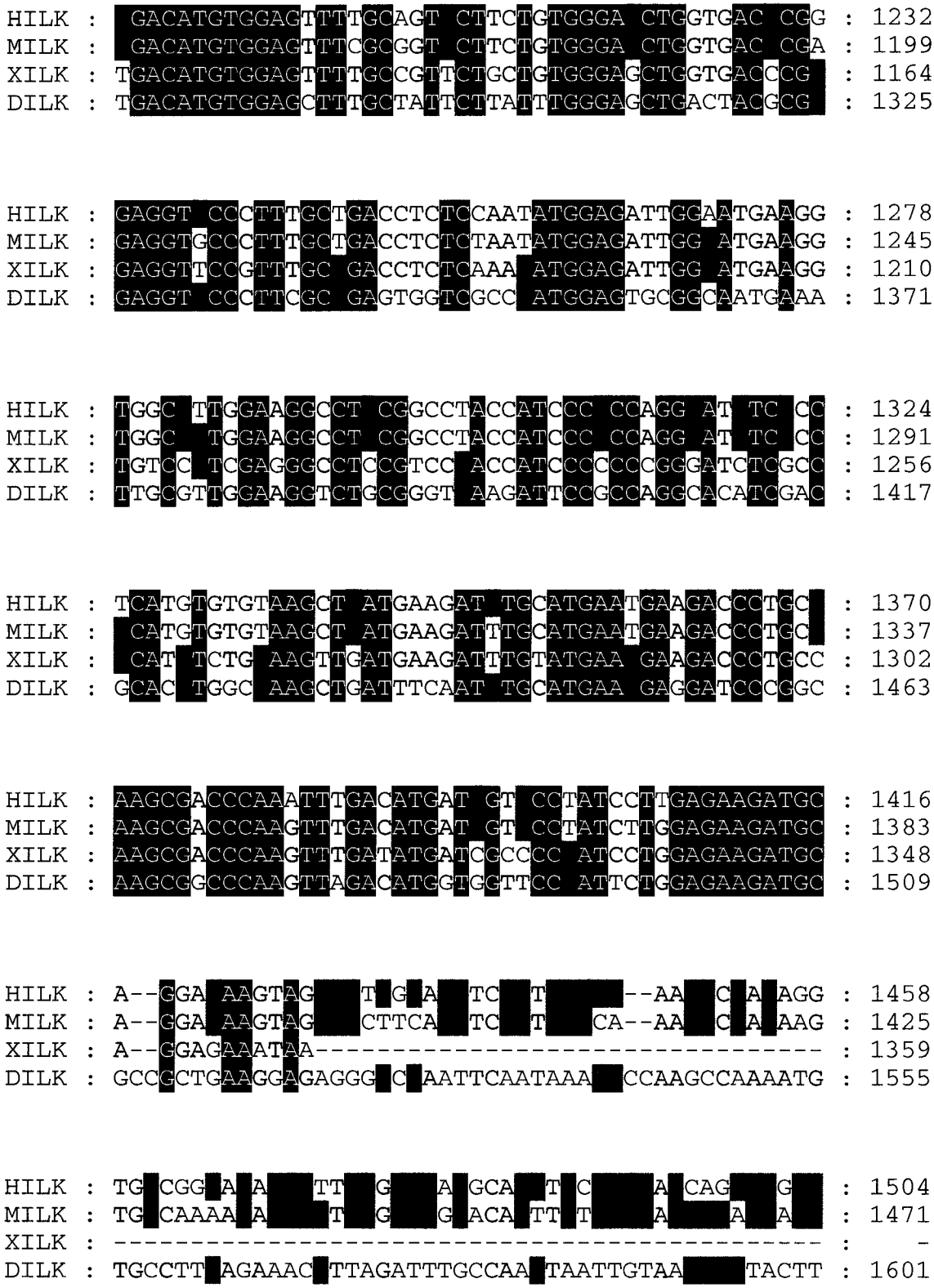
Fig. 6











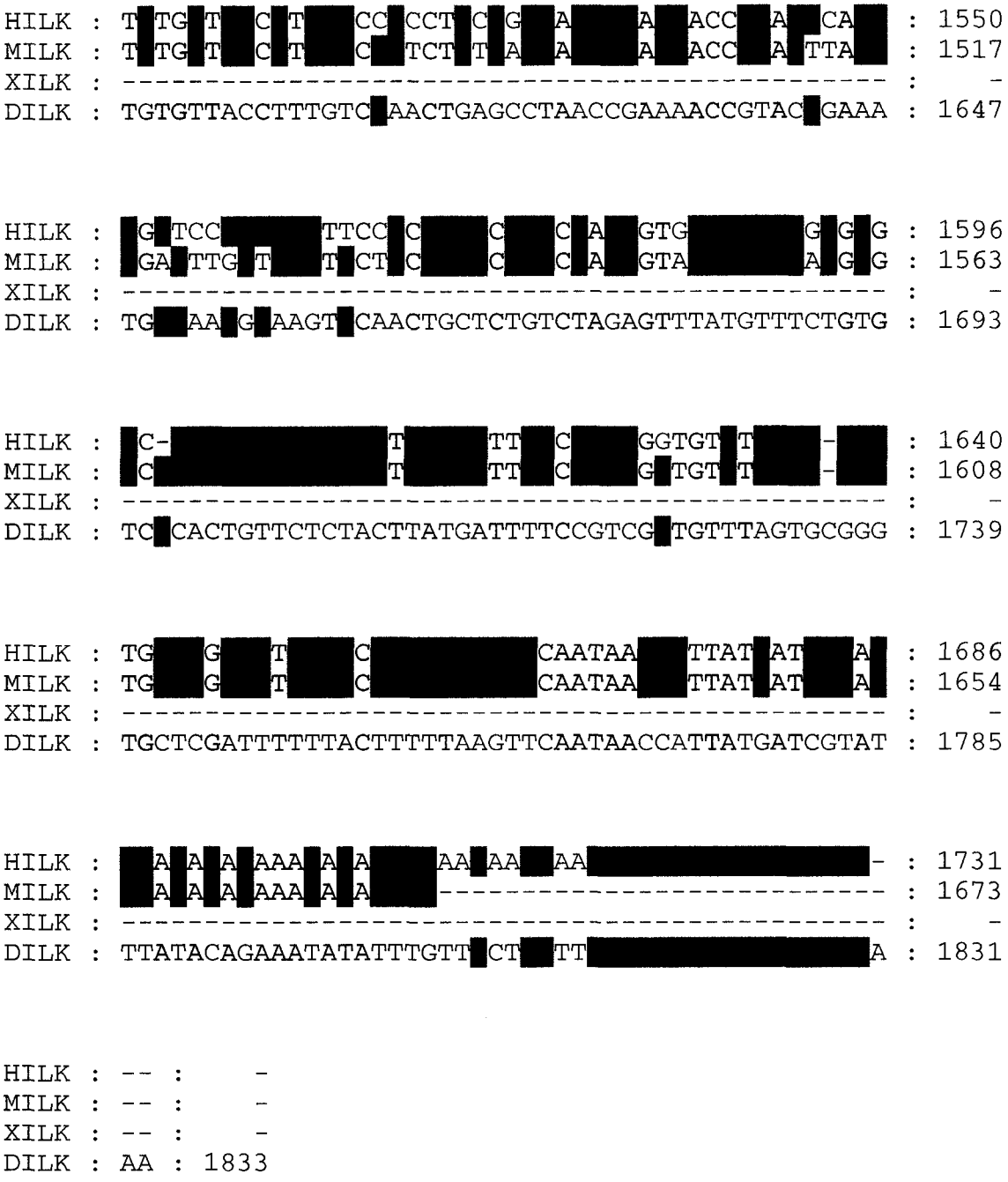
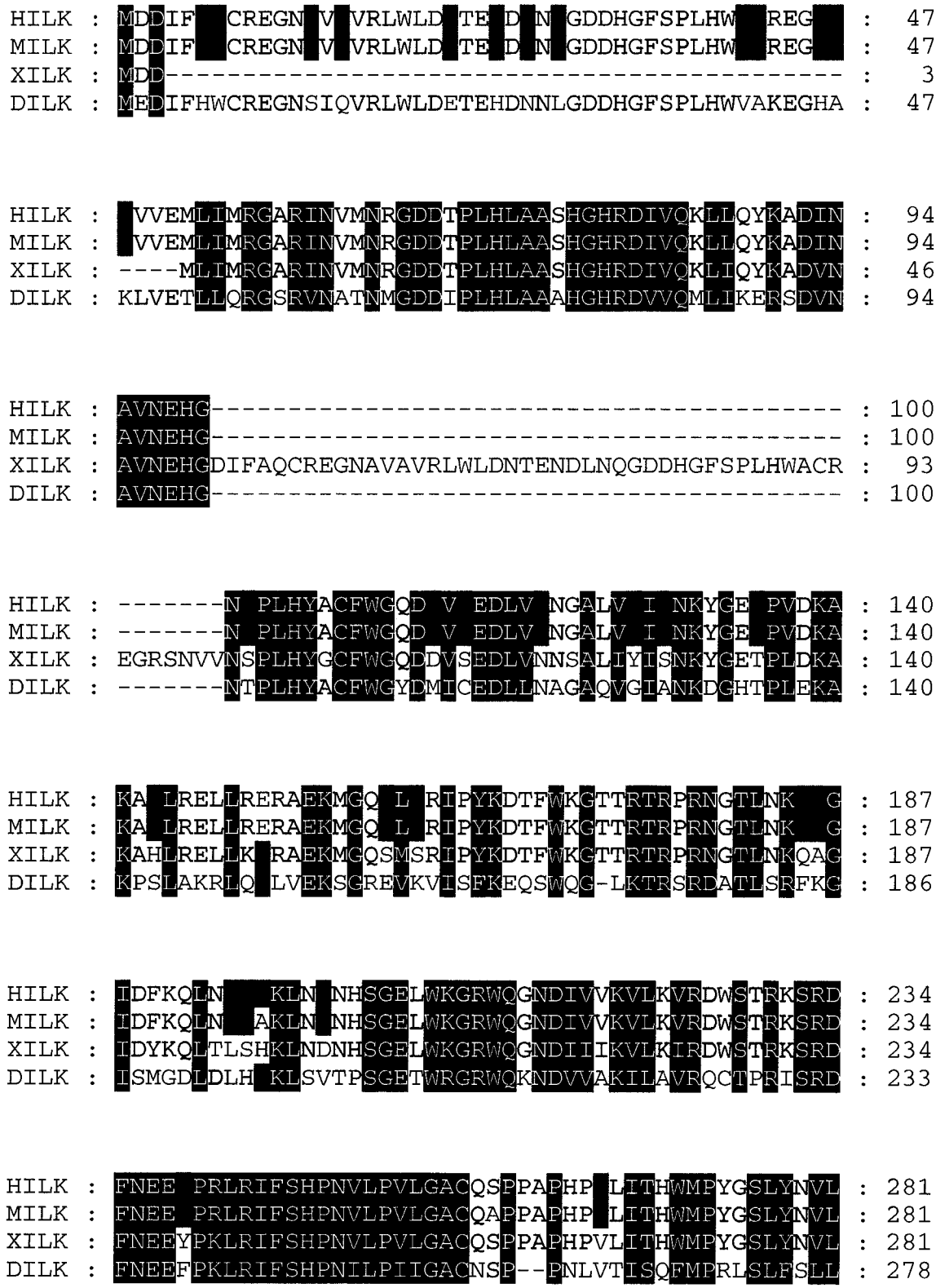


Table 1. Similarity (%) between ILK cDNA from different species. Seq.A is the first sequence to be aligned with Sequence B (Seq.B). Len (nt) stands for length in nucleotides, signifying the length of ILK cDNA in different species. The data represent the amount of similarity (as %) between Seq.A and B. Analysis of identity (Score) of sequences is performed in GeneDoc.

SeqA	Name	Len (nt)	SeqB	Name	Len (nt)	Score (%)
1	Human	1731	2	Mouse	1673	87
1	Human	1731	3	*Xenopus*	1359	59
1	Human	1731	4	*Drosophila*	1020	51
2	Mouse	1673	3	*Xenopus*	1359	61
2	Mouse	1673	4	*Drosophila*	1020	49
3	*Xenopus*	1359	4	*Drosophila*	1020	43

Fig. 7



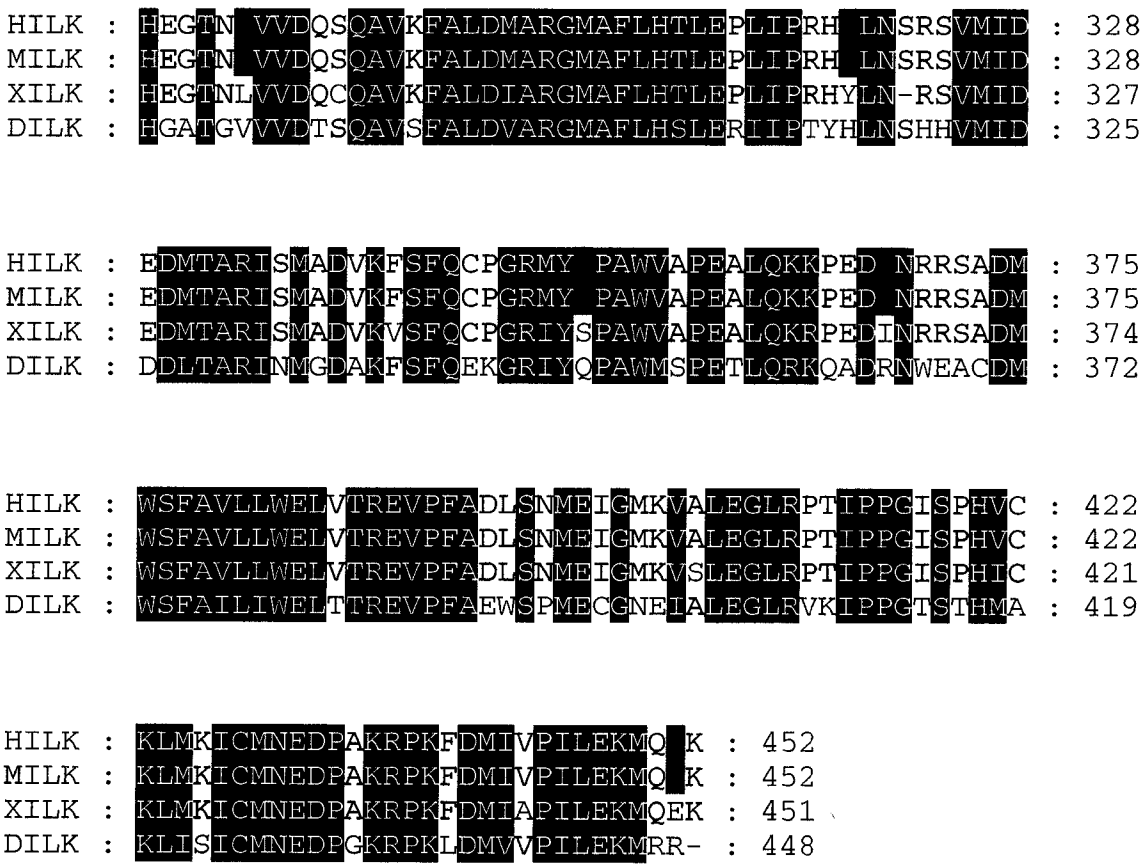
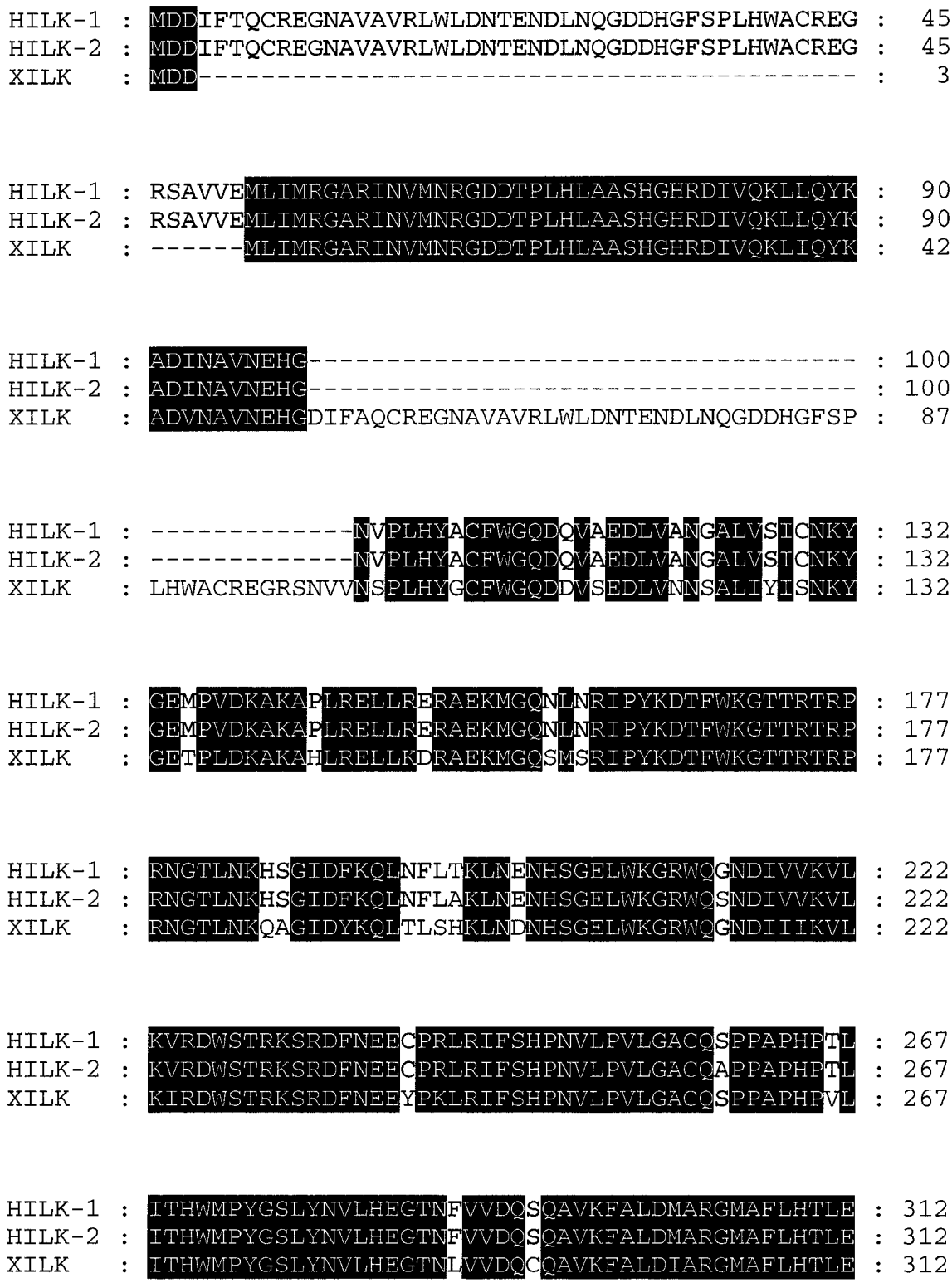
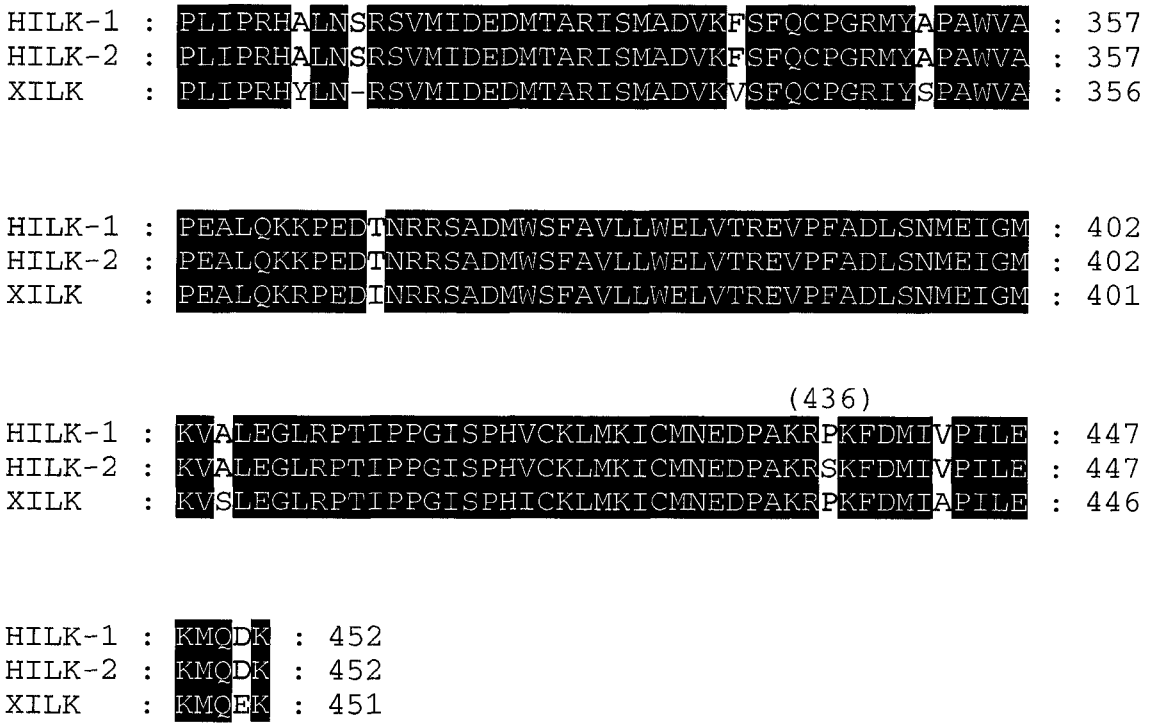


Table 2. Similarity between 2 ILK protein sequences from different species. *Xenopus* ILK is closer in the amino acid sequence to human and mouse ILK than it is to *Drosophila*. The length of the ILK protein in amino acids and percent similarity are shown for region pairs of sequences. The analyses were performed in GeneDoc.

SeqA	Name	Length (aa)	Seq.B	Name	Length (aa)	Score (%)
1	Human	452	2	Mouse	452	99
1	Human	452	3	*Xenopus*	451	71
1	Human	452	4	*Drosophila*	448	59
2	Mouse	452	3	*Xenopus*	451	71
2	Mouse	452	4	*Drosophila*	448	58
3	*Xenopus*	451	4	*Drosophila*	448	46

Fig. 8





with the isoforms of human ILK protein (accession numbers: HILK-1: Q13418 and HILK-2: CAB94832). *Xenopus* ILK protein is more similar to human ILK-1 in sequence (specifically at sites where HILK-1 and 2 differ, which are positions 197, 214, 257 and 436).

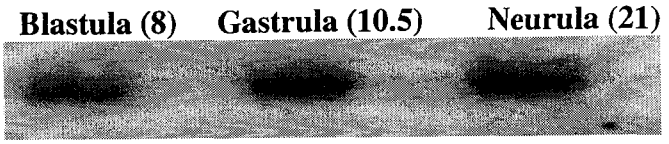
3.2 Expression of *XILK* gene during early development

In order to investigate the regulation of expression of *X*ILK within embryos, RT-PCR analyses were performed on cDNAs derived from mRNA of embryos. The detectable levels of ILK mRNA during early stages of development increased. Figure 9 shows expression of ILK during blastula, gastrula and neurula stages of development. As observed there is an increase in each stage as compared to the previous stage. This increase was measured by GeneSnap (image capture) and GeneTools (image analysis) softwares (SynGene-Synoptics, Cambridge, England) and revealed a corresponding increase during gastrula and neurula stages as compared to the blastula stage of development. The increase was measured by kilo pixels (KP) as the height of the band which stands for the integrated optical density of pixels illuminated in the given area. Elongation factor1-α (ef1-α) was used as a loading control for the experiment.

3.3 *XILK* gene expression in adult tissues

In order to look at the expression of ILK in adult tissues, RT-PCR analysis was carried out on total RNA extracted from several male frog tissues and were either used right away or frozen in liquid nitrogen and stored at -80°C. The RT-PCR product of interest was detected using *X*ILK primers. RT-PCR analysis of heart, liver, kidney and muscle tissues showed that heart had the least amount of detectable ILK mRNA compared to other tissues (Figure 10). This expression was analyzed by GeneTool

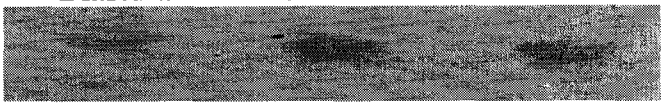
Fig. 9



*X*ILK
1.4 Kb

Height (KP): 1.62 1.69 1.85

RT-PCR analysis of *X*ILK in embryos

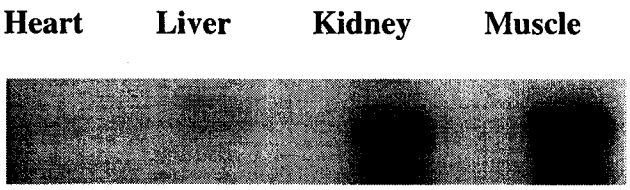


*X*ef1-α
0.5 Kb

Height (KP): 0.65 0.73 0.86

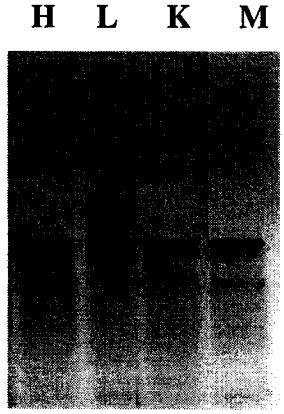
RT-PCR analysis of *X*ef1-α in embryos

Fig. 10



Height (KP):	0.16	0.27	0.68	0.71

Levels of *X*ILK cDNA measured by ChemiGenius^2



Control RNA from tissues

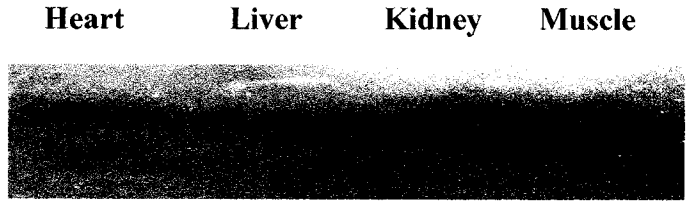
Software (SynGene-Synoptics, Cambridge, England) measuring the amount of pixels illuminated from each band. For the RT-PCR reaction, I used total RNA as a control to show equal amounts of RNA used in the reaction.

To further confirm the results obtained from RT-PCR, a Northern blot experiment was performed (Figure 11). The expression of ILK mRNA in heart was relatively low and this was consistent with the results of RT-PCR analysis. This quantification was carried out by GeneTool (SynGene-Synoptics, Cambridge, England).

3.4 Analysis of *X*ILK protein levels during early development

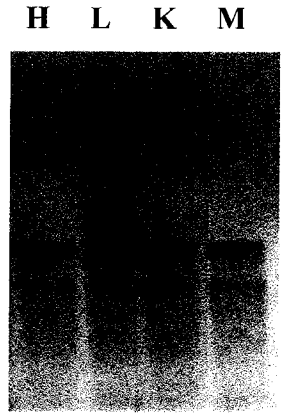
In order to investigate the expression of *X*ILK protein during embryogenesis Western blot analysis was performed (Figure 12). *Xenopus* ILK protein was detected using anti-human ILK antibody since human and *Xenopus* ILK are close in sequence identity (human ILK protein being 71% identical to *Xenopus* ILK protein). The presence of two bands of *X*ILK (approximately 59 KDa and 61 KDa) was detected using this methodology. As shown in Figure 12, the 59 KDa band was expressed at the blastula stage when the zygotic transcription becomes active, whereas the 61 KDa band was expressed throughout all stages from the 4-cell embryos to the tailbud stage of development. The level of expression for the 59 KDa ILK increased as development progressed, but the expression of the 61 KDa ILK protein seemed to be more constant throughout early development. There was an increase in expression of *X*ILK during the gastrula stage. PKB was used as a loading "control" for the experiment and showed a corresponding increase during early development.

Fig. 11



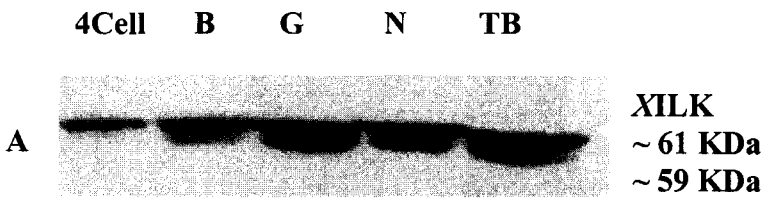
Height (KP):	1.2	1.5	2.4	2.2

Levels of *X*ILK mRNA measured by GeneTool

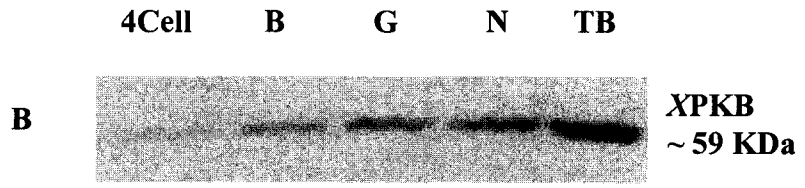


Control RNA from tissues

Fig. 12



*X*ILK protein levels detected using anti-human ILK antibody in embryos



*X*PKB protein levels detected using anti-PKB/Akt antibody in embryos

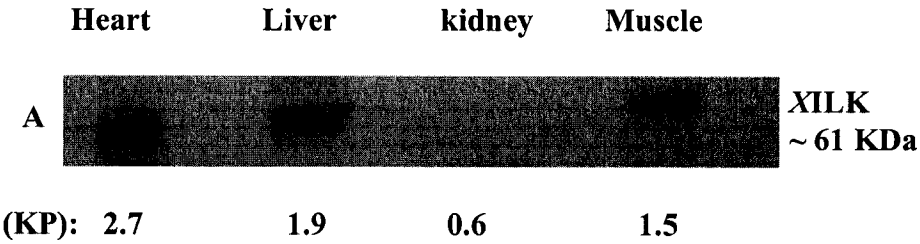
3.5 Analysis of ILK protein levels in *Xenopus* adult tissues

Western blot analysis was also performed to demonstrate the expression of ILK protein in various *Xenopus* adult tissues. Different tissues were collected from male frogs. Approximately 0.15-0.2 g of heart, liver, kidney and muscle tissue was dissected and homogenized using protein lysis buffer. After centrifugation, aliquots were loaded onto a 12% acrylamide gel, following electorphoresis, protein was transferred to PVDF membrane and treated with anti-human ILK antibody for the detection of *Xenopus* ILK protein (Figure13). This analysis showed the presence of one form of ILK protein (approximately 61 KDa) demonstrating that only one band is present in adult tissues in contrast with the results observed from embryonic tissues. *Xenopus* ILK protein was significantly higher in the heart, liver and muscle tissues as compared to kidney tissue.

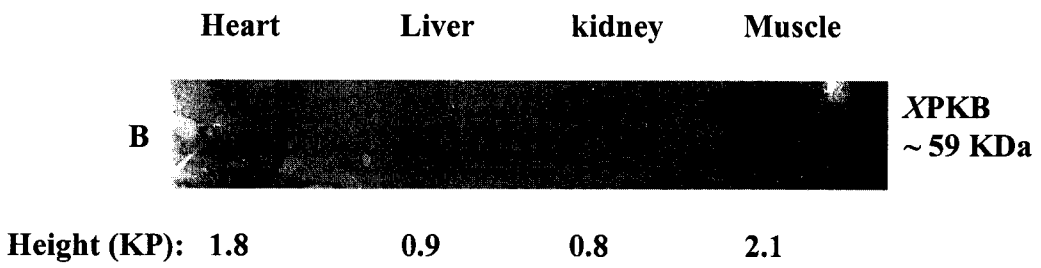
3.6 Analysis of the catalytic activity of *X*ILK during development

The catalytic activity of ILK during embryogenesis was examined in *Xenopus* embryos using *in vitro* kinase assays (Figure 14). For immunoprecipitation Proteins (15 μg) were collected from different stages of development and subjected to anti-human ILK antibody and protein G- Sepharose beads. These beads are capable of attaching to the antibody which can be collected by centrifugation. In the immuno-precipitation reaction, the proteins bind to the antibody and the antibody in turn binds to the beads; this binding reaction was performed overnight at 4°C on a nutator. Next day the *in vitro* kinase reaction was performed by adding the appropriate substrate to the kinase (in this case recombinant PKB/GST protein purified from HeLa cells, which is a specific substrate for ILK kinase), and ^{32}P γ-ATP. After the reaction was complete the products were separated on a12% poly acrylamide gel, which was then washed and dried on a 3 MM whatman paper in a

Fig. 13

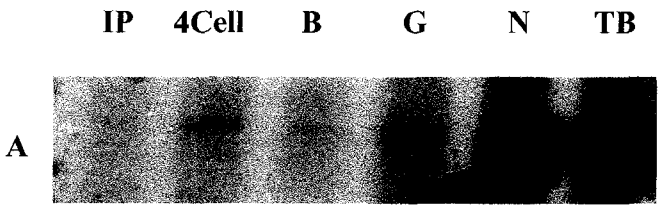


*X*ILK protein levels detected by anti-human ILK antibody in tissues



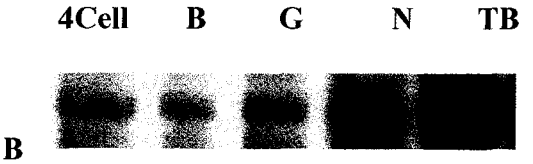
*X*PKB protein levels detected by anti- Akt/PKB antibody in tissues

Fig. 14



Height (KP): 1.0 0.8 1.2 2.0 2.7

^{32}P autoradiograph of P-PKB by *X*ILK



Height (KP): 1.8 1.5 1.8 2.1

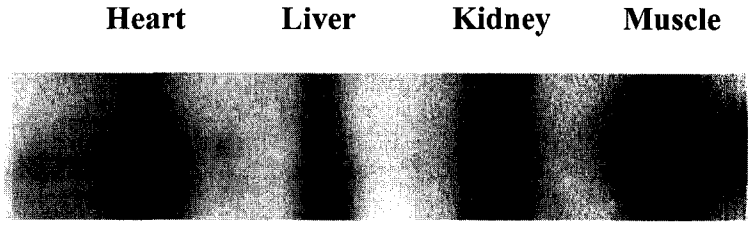
Western blot of P-PKB on Ser-473

vacuum oven. Detection of the kinase reactivity was done by autoradiography. Results in Figure 14 show that the levels of phosphorylated PKB increase during development suggesting that the kinase activity of *X*ILK is increasing since very high levels of activity was present in the nuerula and tailbud stages. The size of the phosphorylated substrate PKB was observed to be approximately 80 KDa (PKB plus GST). The control experiment is a Western blot of phosphorylated PKB using anti phospho-Ser 473 AKT antibody. This experiment was specifically performed to show that the amount of endogenous phosphorylated PKB is increasing during early development and is in accordance with the amount of PKB being phosphorylated by ILK *in vitro* suggesting that ILK may be the kinase responsible for activating PKB.

3.7 Analysis of the kinase activity of ILK in *Xenopus* adult tissues

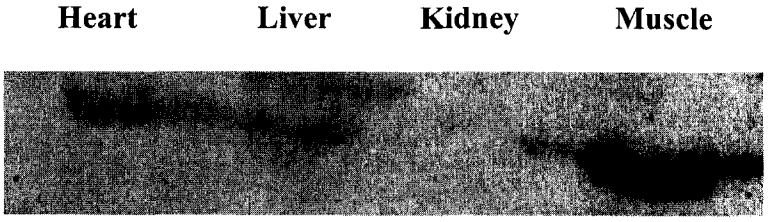
In order to identify the *X*ILK kinase activity in adult tissues, different tissues were dissected and homogenized in lysis buffer to collect protein as described earlier. Anti-human ILK antibody and G-Sepharose protein beads were incubated with the total protein extracted from the tissues (15 μg) overnight at 4°C on a nutator. The *in vitro* kinase reaction was carried out using PKB/GST as a substrate isolated from HeLa cells (mammalian cell line) in the presence of ^{32}P γ ATP. The kinase reaction added radiolabelled phosphate to the substrate PKB which could be detected by autoradiography (Figure 15). The kinase reaction mixture was electrophoresed on a 12% poly acrylamide gel, the gel was dried on 3 MM whatman paper and exposed to an X-ray film. In Figure 15 the kinase reaction showed one major band characteristic of the size of phosphorylated PKB. The catalytic activity of *X*ILK was greatest in heart and muscle tissues, and least in liver and kidney tissues. For control purposes, phospho-Western blot was carried out

Fig. 15



Height (KP): 1.8 1.31 1.36 2.05

^{32}P autoradiograph of P-PKB by *X*ILK



Height (KP): 1.3 0.9 0.4 2.5

Western blot of P-PKB on Ser-473

against PKB. Anti-phospho-AKT-Ser-473 antibody was used to detect the amount of phosphorylated PKB on Ser-473 within *Xenopus* adult tissues.

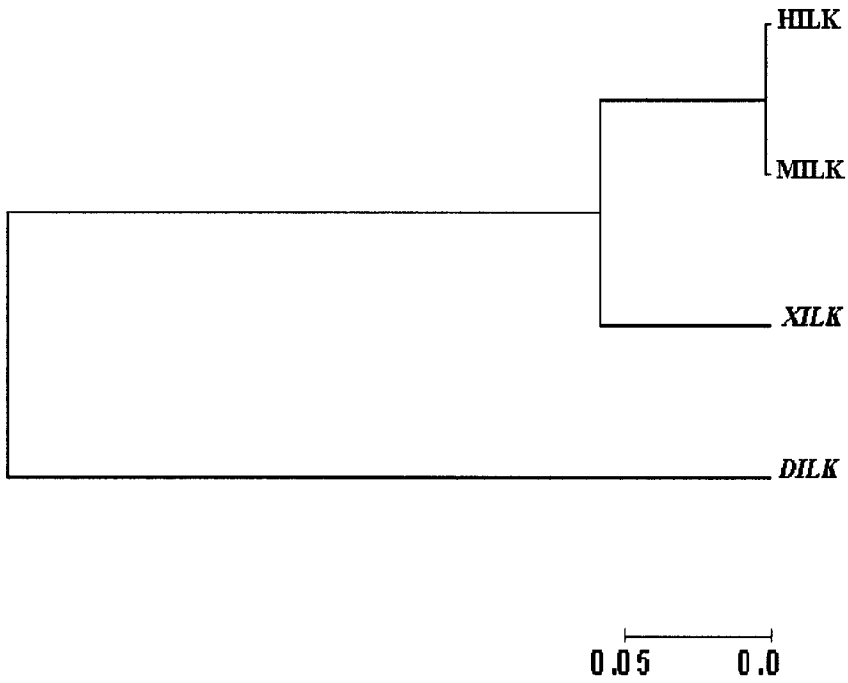
Discussion

4.1 Integrin linked kinase conserved Domains

Integrin linked kinase sequence is conserved among different organisms. *Xenopus* ILK cDNA sequence is 59% identical to human ILK and exhibits 71% identity to protein sequence. All ILK sequences from various species contain the ankyrin (ANK) repeat, in the most N-terminal domain of ILK. In mammalian ILK, 4 ANK repeats are present (Dedhar, 2000), whereas *Drosophila* has only 3 ANK repeats (zervas et al, 2001). *Xenopus* ILK is more comparable to human and mouse ILK containing 4 ANK repeats. These analyses reveal its close similarity to mammals rather than insects as well as its higher position in the evolutionary tree compared to *Drosophila* ILK. The numbers of ANK repeats have increased during evolution indicating a possible greater requirement for this domain in vertebrates. Figure 16 shows the phylogenetic tree of HILK, MILK, *X*ILK and *D*ILK proteins compared to each other. Human and mouse ILK are more similar to each other whereas *Xenopus* and *Drosophila* are more distant from mammalian ILK, although *Xenopus* is still closer to mammals when compared to insects (*Drosophila).* Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 2.1 (Kumar et al, 2001).

Human ILK has been shown to localize in focal adhesions (FAs) (Hanningan et al, 1996) through interaction with PINCH, a binding protein for ILK (Li et al, 1999). The ANK domain of ILK binds to the LIM-1 domain, a cysteine rich domain of PINCH (Li et al, 1999), and is involved in focal adhesion assemblies (Hannigan et al, 1996) as well as

Fig. 16



in insulin pathway (Li et al, 1999). Integrin linked kinase interacts with PINCH through its ANK-1, most N-terminal domain, through amino acids residues 1-66 (Li et al, 1999) shown in green (in appendix, Figure 17). Deletion mutation in the ANK-1 domain of ILK results in a failure to localize and cluster to the focal adhesions suggesting that interaction with PINCH (on ANK-1 domain amino acids 1-66 in mammalian-ILK) is necessary for the focal adhesion assembly (Li et al, 1999). Additionally, PINCH functions as a bridge linking ILK with Nck-2, an SH3/SH2-containing adaptor protein. NcK-2 is associated with components of growth factor receptor kinase signaling pathways (Li et al, 1999) such as, insulin responsive substrate-1 (IRS-1), through its SH3/SH2 domain (Tu et al, 1998). Through these interactions, insulin signal is delivered to ILK. From various studies, it is concluded that growth factor induced ILK signaling is involved in growth and cell cycle progression (Karp, 2002).

The kinase activity of ILK is regulated by growth factors such as insulin and also by extracellular matrix interactions in a PI3'K dependant manner. Integrin linked kinase is implicated in the PI3'K pathway upon binding to phospholipids (Delcommenne et al, 1998). The activity of ILK is stimulated *in vitro* by PtdIns (3,4,5) P3 but not by PtdIns (3,4) P2 or PtdIns(3) P (Delcommenne et al, 1998). This interaction is most possibly mediated by the interaction of PIP3 with ILK (Delcommenne et al, 1998). In mammalian ILK, amino acids 180-212 have been shown to be present in the conserved PH domain; these residues have also been found in other proteins with PH domains such as cytohesin-1 (a β2 integrin cytoplasmic domain-interacting protein referred to CH-1), general receptor for phosphoinositides-1(referred to GRP1, which interacts with PIP3) and PKB/AKT. The amino acids highlighted in blue in Figure 17 (appendix) are the major residues for the binding of phospholipids (Rameh et al, 1997; Delcommenne et al, 1998).

The consensus sequence for phospholipid binding sites in PH domain is: GWLXK--GXXXK.....WKXRW, Which is clearly observed in mammalian ILK and *Xenopus* ILK protein between residues 180-212 (Delcommenne et al, 1998) except for GTLXK at the N-terminal site of this domain instead of GWLXK.

The kinase domain of serine-threonine protein kinases has certain residues being conserved throughout all species studied (Hank and Hunter, 1995). However, ILK kinase sub-domain exhibits a differential display, being variant in those conserved residues. More surprising, the kinase domain of ILK is also variant in different species. In kinase sub-domain I, the kinase consensus is GxGxxG, with the middle glycine invariant, but the same pattern is not seen in ILK sequence in different species and the first two glycines are different among various species (Hank and Hunter, 1995). However different from the consensus, the pattern is similar between *Xenopus* and human ILK for this sub-domain (NxNxxG). The other important site is the aspartic acid in sub-domain VIb which is involved in the transfer of the phosphate group (Hank and Hunter, 1995; Johnson et al, 1996). It is invariant in other kinases, but different in the ILK sequence among different species (Zervas et al, 2001). By contrast, the invariant lysine in sub-domain II, the chief residue in ATP binding, and the motif A/SPE in sub-domain VIII, involved in substrate recognition, are conserved in all the sequences including *X*ILK (Hank and Hunter, 1995; Johnson et al, 1996; Zervas et al, 2001). Thus, it is obvious that the divergence of ILK from other kinases is not a recent phenomenon, but happened evolutionarily probably before the separation of invertebrates and vertebrates (Zervas et al, 2001).

Table 3 shows the consensus of serine-threonine kinase subdomain residues (Zervas et al, 2001) as well as ILK kinase subdomains in different species. ILK kinase

Table 3. Serine-threonine kinase consensus in different species

I	II	III	VIb	VII	VIII	IX	XI	Kinase sub-domains
G-**G**--G...	A-**K**...	E...	**D**L-**N**...	**DFG**...	APE...	**D-G**...	**R**	Kinase consensus *
G-G--G...	A-**K**...	E...	**D**L-**N**...	**DFG**...	APE...	**D-G**...	**R**	*Drosophila* Raf
S-T--G...	V-**K**...	E...	HL-H...	**D**AK...	SPE...	**D**-A...	**R**	*Drosophila* ILK
N-N--G...	I-**K**...	E...	YL-**N**...	**D**VK...	APE...	**D**-A...	**R**	*Xenopus* ILK **
N-N--G...	V-**K**...	E...	AL-S...	**D**VK...	APE...	**D**-A...	**R**	*H. sapiens* ILK

*In ILK protein sequences, the conserved sites are depicted in bold

**Adopted from Zervas et al, 2001 and *X*ILK from Fig.7.

domain is most similar to the serine-threonine kinase domain of protein Raf. Raf protein kinase has retained the consensus of typical kinases in the conserved sites unlike ILK.

Mammalian ILK binds to paxillin on the residues 377-396 as shown (in pink) in Figure 17 (appendix). The paxillin binding site in *X*ILK is 100% similar to HILK and MILK PBS (amino acids 377-396). Through this conservation, a potential site for the interaction of *X*ILK with paxillin can be predicted. Since mammalian ILK binds to paxillin through the PBS domain and contributes to focal adhesion assembly, there are possibilities indicating a similar role for *X*ILK. In *Drosophila* ILK, this site is not identical to mammalian ILK, indicating that *Xenopus* ILK is similar in sequence to mammalian ILK as compared to *Drosophila,* and this in itself demonstrates the higher evolutionary position of amphibians in the evolutionary tree.

4.2 ILK isoforms

The presence of isoforms has been observed for a variety of expressed gene families. An isoform for ILK, known as ILK-2, has been identified while studying the effect of TGF-β1 stimulation of HT-144 human melanoma cells (Janji et al, 2000). This newly characterized protein is highly homologous to ILK-1 with the difference of 4 amino acids at the protein level and 102 nucleotides in the cDNA sequence, eliminating the possibility of ILK-2 being an allelic variant of ILK-1. Integrin linked kinase-2 is an isoform of ILK-1, which is expressed in highly invasive tumors but not in normal human adult tissues. Through TGF-β1 stimulation, ILK-2 exhibits a selective over-expression meaning that its expression is regulated through TGF-β1 but not insulin, platelet derived growth factor AB (PDGF-AB) or epidermal growth factor (EGF) induction. Similar to ILK-1, bacterially expressed GST/ILK-2 has the ability of auto-

phosphorylation as well as phosphorylation of myelin basic protein *in vitro*. This protein is also capable of phosphorylating recombinant GST/β_3 integrin cytoplasmic tail peptide. Transfection of ILK-2 cDNA into non-metastatic melanoma cells induced anchorage-independent cell growth and cell proliferation (Janji et al, 2000). The alignment of *X*ILK with both HILK-1 (Hannigan et al, 1996) and HILK-2 (Janji et al, 2000) was performed. *X*ILK is more similar in protein sequence to the HILK-1 as compared to HILK-2 in certain residues. Moreover, HILK-1 varies from HILK-2 in only 4 amino acids at residues 197 (T-A), 214 (G-S), 259 (S-A) and 436 (P-S) (See Fig.8). Interestingly, *Xenopus* ILK cDNA sequence is similar to HILK-1 in these residues except at the position 197 and varies from HILK-2 at all these sites.

4.3 *XILK* gene expression during embryogenesis

Integrin linked kinase has been shown to be involved in the Wnt/Wg signaling, a major cellular pathway involved in early development. The mRNA levels of integrin linked kinase increase during early embryogenesis as shown in Figure 9. This RNA expression is in accordance to the embryonic protein level increase during development (Figure 12). Interestingly, this up-regulation peaks at the gastrula (stage 10.5) and neurula stages (stage 21) at which time different embryonic layers and the neural plate and tube are being developed. ILK levels continue to increase throughout the tailbud stage (stage 28, data not shown).

Integrin linked kinase transcripts appear as only one band on the autoradiograph of the ^{32}P-labeled RT-PCR product from blastula, gastrula and neurula stages, whereas in protein expression studies, two bands are observed (Figure 12). Further experiments, such as Northern blot analysis, hybridization assays and RNase protection assays are required

to confirm these results. In addition, the mRNA bands could be extracted from the gel and eluted, then reverse transcribed into cDNA and cloned into a suitable vector for sequence analysis. This experiment would tell us about the existence of another form (in case it was present but could not be detected through gel electrophoresis analysis).

For the spatial and temporal expression of *X*ILK during development, whole mount *in situ* hybridization needs to be carried out. This experiment will confirm the temporal expression results obtained from the RT-PCR analysis. Although the spatial expression analysis from *in situ* hybridization could be a determining factor in the mRNA expression of adult tissues, the expression pattern may vary after development of the embryo progresses. A preliminary result of *in situ* hybridization of *X*ILK within embryos at different stages of development was gained (by Farhad Khosrowshahian in collaboration with Dr.Crawford's lab) and the data gathered from this experiment exhibited non-specific expression of *X*ILK throughout the whole embryos at different stages studied. No experiment regarding ILK's spatial and temporal expression (such as *in situ*) has been carried out up to date.

For control experiment RT-PCR analysis was performed using *X*ef1-α primers which confirmed the experimental procedure by exhibiting an expected increase in the expression of *X*ef1-α cDNA during embryogenesis. In *Xenopus*, finding a control that is expressed at a constant level throughout early development is difficult.

4.4 *XILK* gene expression in adult tissues

RT-PCR analysis of RNA samples from *Xenopus* adult tissues shows that ILK transcripts are present in all tissues studied. Surprisingly, ILK mRNA levels are lower in heart tissue as compared to other tissues tested (Figure10). This observation is

contradictory to the levels of ILK protein in the heart (Figure 13). The apparent inconsistency may be due to increased stability of ILK protein in heart tissue, due to the need for ILK's vital functional properties. Another possibility may be due to increased translational efficiency of ILK mRNA as a result of the tissue's requirements. A third possibility is a decreased stability of ILK mRNA, resulting in lower amounts of ILK mRNA in the cardiac tissue. As a control, total RNA extracted from tissues using Trizol was analyzed.

In a previous study the transcript levels of heart, liver, kidney and spleen of mice were determined through RT-PCR methodology (Martin and Guenter, 2003). Although protein levels of heart were up-regulated as illustrated by Western blot analysis, no regulation of ILK expression at the transcriptional level was detected (Martin and Guenter, 2003).

In order to confirm the results of RT-PCR, Northern blot analysis was carried out (Figure 11). RNA isolated from heart, liver, kidney and muscle was probed using ^{32}P labeled ILK cDNA. Consistent with RT-PCR results, ILK transcript levels were found to be the lowest in heart tissue, but the expression of ILK transcripts in kidney tissue varied from the RT-PCR result. The observed difference may be due to the lower sensitivity of Northern blot analysis as compared to RT-PCR.

4.5 Protein levels of *X*ILK during early embryonic development

Integrin linked kinase has been demonstrated to be important in morphogenesis and differentiation (Somasiri et al, 2000). In the present study, expression of ILK protein was examined through Western blot analysis (Fig.12). Embryos were collected at 4 cell, blastula (stage 8), gastrula (stage10.5), neurula (stage 21) and tailbud (stage 28) stages.

Xenopus ILK revealed two different size proteins (approximately 61 KDa and 59 KDa). The predicted size of amino acids of *X*ILK protein calculated by protein calculator (www.scripps.edu/~cdputnam/protcalc.html) is 51.4 KDa. The presence of ILK was detected in early embryonic stages, and as the embryos grew and reached the mid-blastula stage, the second ILK band appeared. This suggested that the 61 KDa protein is maternally expressed, while the 59 KDa protein is not expressed until the onset of zygotic transcription.

Since only one form of ILK mRNA was seen during embryogenesis (Figure 9), we can not conclude the reason of the second band (59 KDa). Walsh and Brown (1998) have isolated a second allele (ILK-2) in a screen for genes required for integrin-mediated adhesion in the adult *Drosophila* wing. This mutation was lethal over ILK-1, although a few adults escaped death (<5%). Genetic and cytological analysis revealed that ILK-2 is due to a reciprocal translocation between the second and third chromosomes. The dominant phenotype of ILK-2 is unique and not similar to other ILK mutations (ILK-1 and ILK deficiencies), so it is not evident if ILK-2 is a result of deviation in the ILK gene from its natural form or a second site mutation (Zervas et al, 2001).

The presence of isoforms has been observed for a variety of expressed gene families. Janji et al (2000) have identified a second isoform for ILK, known as ILK-2, while studying the effect of TGF-β1 stimulation of HT-144 human melanoma cells. This newly isolated ILK-2 is highly homologous to ILK-1 with the difference of 4 amino acids at the protein level and 102 nucleotides in the cDNA sequence, eliminating the possibility of ILK-2 being an allelic variant of ILK-1. This contradicts the fact that we only found one cDNA expressed in early *Xenopus* developmental stages within embryos.

The two protein forms visualized from the Western blot analysis could be the

results of a post-translational modification. Post-translational modifications occur in the cell due to acylation, methylation, phosphorylation, sulfation or any other alteration after the translation of a protein. These two forms of ILK may also be expressed from two different mRNAs or variant alleles, however only one mRNA was observed through RT-PCR analysis (Figure 9). This finding could be due to the existence of allelic variants of ILK, unless the primers were not able to function in the synthesis of both isoforms or detection of both isoforms was not possible through gel electrophoresis technique.

Protein expression of ILK in cancer cell lines has also revealed more than one band encoding human ILK gene. An adenovirus encoding the human *ILK* gene was engineered and used to infect TRAMP-C prostate cancer cells (Zhang et al, 2003). This transfection mediated efficient expression of the *ILK* gene. A total of four ILK bands were detected through Western blot analysis. Two major bands at 50 and 59 KDa were more prominent compared to the other bands. In addition RT-PCR, Northern blot and Western blot analyses in HUVEC cells demonstrated that ILK mRNA shows only one band but at the protein expression level, 2 different size bands are evident (50 and 59 KDa) (Zhang et al, 2001). In both studies (Zhang et al, 2001 and 2003) the characteristic of different forms of ILK were not identified.

ILK protein expression increased during embryogenesis. Integrin linked kinase was more abundant at the tailbud stage of embryos compared to the 4-cell stage indicating the importance of ILK in more advanced stages. Integrin linked kinase is an ankyrin repeat containing serine-threonine protein kinase proposed to be implicated in the Wnt/Wg pathway leading to GSK-3 inactivation and *cyclin D1* gene expression regulating cell cycle progression (Novak et al, 1998). Inhibition of ILK suppresses cell growth in culture as well as growth of human colon carcinoma cells in mice (Tan et al, 2001). Therefore,

activity of ILK is required for cell growth being an important process during development. In addition, kinase deficient dominant negative form of ILK induced G1 phase cycle arrest and enhanced apoptosis in PTEN negative prostate cancer cells (Persad et al, 2000). These results show the importance of ILK during cell cycle progression leading to cell survival.

Integrin linked kinase has been demonstrated to be important in morphogenesis and differentiation (Somasiri et al, 2000). One of the examples of ILK function during development is the expression of a dominant negative, kinase dead form of ILK in mouse mammary epithelial cells. This kind of expression subtly altered epithelial mouse mammary morphogenesis. The data gathered from these experiments illustrate that ILK induces a mesenchymal transformation in mammary epithelial cells, at least in part by disrupting cell-cell junctions (Somasiri et al, 2000).

Another example for ILK expression during growth and development is the involvement of ILK in neurite outgrowth (Ishii et al, 2001). ILK is expressed in neuroblastoma cells, N1E-115 cells, and the expression levels of ILK were constant under both normal and differentiating conditions. A stable transfection of a kinase-deficient mutant resulted in inhibition of neurite outgrowth in serum-starved N1E-115 cells grown on laminin. Further a transient expression of wild type ILK stimulated neurite outgrowth. These results suggest that transient activation of ILK is required for neurite outgrowth in serum-starved N1E-115 cells on laminin.

4.6 Protein levels of ILK in *Xenopus* adult tissues

Western blot analysis shows that ILK is expressed at higher levels in cardiac tissue as compared to liver, kidney and muscle (Fig.13).The protein detected was approximately 61 KDa in mass. Kidney ILK showed the least amount of expression although its mRNA

levels were high (Fig. 10). This decreased expression could be due to lower amounts of kidney ILK translation or protein stability as compared to other tissues. Hannigan et al (1996) also performed Western blot analysis to determine the expression of ILK protein in different tissues. Among heart, liver, kidney and spleen, ILK protein expression was similarly the highest in the heart as compared to other organs.

ILK has been shown to be a differentiation marker of myocardial development as well as being involved in post-natal myocardial maturation (Martin and Guenter, 2003). To investigate the role of ILK in heart development, mice hearts were harvested at birth (day 0), at days 1-3 and at 6 and 12 months after birth. After extraction of protein, Western blots and kinase assays were performed. During the phase of post-natal myocardial growth, ILK displayed a distinct pattern of expression and activity. Protein expression and activity of integrin linked kinase in the neo-natal mouse heart were inversely regulated. The expression of ILK was affected by cell density and differentiation of cardiomyocytes. This study highlighted the critical role of ILK during heart development in mice.

Integrin linked kinase has been shown to be involved in cardiomyocyte migration, survival and repair through thymosin β4 (Bock-Marquette et al, 2004). Thymosin β4, a G-actin binding peptide, forms a complex with PINCH and ILK resulting in activation of PKB/AKT. This property of ILK has been used in cardiac repair therapies, where after coronary artery ligation in mice, thymosin β4 treatment led to the up-regulation of ILK and PKB/AKT activation in the heart. This treatment enhanced early myocyte survival and improved cardiac function.

GSK-3β is a ubiquitously expressed constitutively active serine-threonine kinase demonstrated to be an essential negative regulator of cardiac hypertrophy (Hardt and

Sadoshima, 2002). In addition, inhibition of GSK-3β has been shown to be an important mechanism contributing to the development of cardiac hypertrophy. GSK-3β also plays an important role in regulating cardiac development. Integrin linked kinase is one of the up-stream regulators of GSK-3β that inhibits its activity and could also be a possible regulator of GSK-3 activity during cardiac development (Hardt and Sadoshima, 2002).

4.7 The catalytic activity of ILK in *Xenopus* embryos

In order to determine the kinase activity of *X*ILK during development, kinase assays on different stages of embryos were performed. In this experiment the activity of *X*ILK was observed throughout early development from 4-cell stage to tailbud stage embryos. The catalytic activity of *X*ILK significantly increased at neurula stage indicating more requirements for ILK function at the onset of neural plate and tube formation (Fig.14). The kinase activity of ILK was demonstrated using PKB as a substrate. In the regulation of PI3'K pathway, ILK interacts with PKB and leads to cell survival through inhibition of apoptosis and cell metabolism through inhibition of GSK-3. This could be one reason for the higher amounts of ILK at later stages of development. However, this experiment was carried out *in vitro*; such interactions may not necessarily occur *in vivo.* This suggests that the amount of ILK kinase activity is higher at later stages of development, and the substrate for such activity could be either PKB or GSK-3. Therefore an *in vivo* experiment such as *in vivo* labeling in different developmental stages should be attempted to determine the *in vivo* substrate in response to ILK kinase activity.

4.8 The catalytic activity of ILK in *Xenopus* adult tissues

To identify the kinase activity of *X*ILK in adult tissues, kinase assays were performed on tissue extracts. Among heart, liver, kidney and muscle, the activity of *X*ILK

was highest in heart and muscle tissues (Fig.15). Similar to the kinase assays in embryos, the kinase activity of ILK was assessed through PKB substrate phosphorylation *in vitro.*

One reason for this expression pattern could be the involvement of ILK in the regulation of contractile machinery in a Ca^{2+}- independent contraction (Deng et al, 2001 and 2002). ILK phosphorylates 20 KDa myosin light chain (MLC20) at the kinase site. There are two pools of ILK in smooth muscle, one which is associated with myofilaments and is involved in the Ca^{2+}- independent myosin phosphorylation (Deng et al, 2001). In addition ILK phosphorylates myosin light chain phosphatase (MLCP) inhibitor proteins such as protein kinase C (PKC)-dependent phosphatase inhibitor of 17 KDa (CPI-17) and phosphatase holoenzyme inhibitor-1 (PHI-1), two known substrates of PKC (Deng et al, 2002). Therefore, ILK may activate muscle contraction directly or indirectly.

Although ILK kinase activity on PKB substrate was demonstrated *in vitro* using muscle tissues extracts, PKB may not be the target of ILK *in vivo*. Over-expression of ILK in L6 myoblasts resulted in increased ILK kinase activity leading to stimulation of myotube formation and induction of biochemical differentiation markers (Miller et al, 2003). However, known targets of ILK, PKB/AKT and GSK-3β are not involved in the ILK-induced L6 myoblast differentiation. This result is accomplished by inducing insulin and stimulating phosphorylation of PKB on Ser-473 in ILK mutant cells (E359K, kinase deficient ILK). The result of this experiment does not demonstrate any weakness in the phosphorylation levels of PKB, suggesting that PKB is not a myogenic target of ILK. Inhibition of GSK-3β by LiCl blocks L6 myogenesis, indicating that ILK-mediated inhibition of GSK-3β is not implicated in differentiation. My experiments showed that ILK activity is highest in muscle and heart tissues which is consistent with previous studies (Hannigan et al, 1996).

4.9 Directions for future study

In this study our goal was to characterize *Xenopus* Integrin linked kinase (*X*ILK) during early development. The sequence of *X*ILK was analyzed and the level of expression of ILK within *Xenopus* embryos was investigated. The expression of *X*ILK as a kinase was also reported within *Xenopus* embryos and adult tissues. Adult tissues were examined in combination with embryos in order to determine the differences of expression pattern and activity of *X*ILK during development. A summary of my findings is listed below:

1. The sequence analysis of *X*ILK revealed a similar sequence compared to the mammalian ILK (59% identical to HILK, 61% to MILK in the cDNA, and 71 % identical to human and mouse ILK in the protein sequences), indicating that the ILK sequence is conserved throughout evolution.

2. *X*ILK mRNA levels were investigated through RT-PCR technique during early embryonic development and revealed an increase during embryogenesis with a maternal expression pattern.

3. *X*ILK mRNA levels were examined within *Xenopus* adult tissues through RT-PCR and Northern blot analysis and revealed a lower level of mRNA expression in the heart tissue compared to other tissues.

4. *X*ILK protein levels were demonstrated using Western blot analysis within embryos during embryogenesis and the levels increased during development with a maternal expression pattern as well as expression of a second form in these embryos.

5. *X*ILK protein levels were identified within adult tissues using Western blot analysis and demonstrated that ILK protein is most abundant in heart as compared to other tissues.

6. *In vitro* XILK kinase activity was estimated within embryos and revealed an increase during embryogenesis, suggesting the potential importance of ILK during development.

7. *In vitro* XILK kinase activity was assessed within adult tissues and showed that heart and muscle tissues have a higher catalytic activity as compared to other tissues.

In conclusion, ILK is expressed in *Xenopus* with a significant similarity to human ILK in the functional domains, and its protein product may play an important role during early embryonic development as well as in adult tissues.

4.9.1 Future prospects

In the present study, the DNA sequence of XILK from a cDNA library was analyzed. Further research is required on the genomic DNA to determine whether another allele of ILK is present. Experiments of this type will determine whether, the two bands observed in the Western blots are allelic variants or isoforms. According to Janji et al (2000), the second ILK is an isoform in cancer tissues, but in the studies performed by Walsh and Brown (1998) a second allele of ILK in a screen for genes required for integrin-mediated adhesion in *Drosophila* was identified. In our experiments, the presence of one mRNA was observed. However, we can't conclude the identity of the bands (observed in Western blot) until further experiments through Northern blots, RNase protection assays and sequence analysis of XILK are performed.

To further prove the results obtained from the kinase assays, a similar experiment in prokaryotic systems such as bacteria (*E.coli,* Bl-21 gold strain) should be carried out. In this experiment, XILK will be cloned into a GST tagged PGEX vector (using EcoRI and XbaI sites) and transformed into Bl-21 Gold *E.coli* strain. This strain should be used because it doesn't have any proteases. The XILK protein will be expressed

in the bacterial cells using IPTG (isopropyl-β-D-thiogalactopyranoside) and the protein will be extracted from the *E.coli*. The protein of interest (in this case *X*ILK) is bound by GST-beads and purified by affinity chromatography. The protein and beads are denatured (at 95°C) to break down the interaction and separate the beads from the protein. The protein is then electrophoresed on a 12% acrylamide gel to detect its presence. Once the protein has been isolated, the immunoprecipitation (IP) reaction should be carried out the same way as described earlier followed by the *in vitro* kinase assay. This experiment should provide further support that phosphorylated PKB is a result of the kinase activity of ILK and not any other kinase which may be bound to ILK. The results obtained from such experiments will demonstrate the fact that ILK has the capability of phosphorylating PKB.

The kinase assays using embryos (Fig.14) provided an *in vitro* approach to measure kinase activity. To see the *in vivo* effects, radio-labeling experiments need to be performed. In these experiments the substrate PKB or GSK-3 (tagged) will be transfected into the cells or embryos with co-transfection of the radiolabeled inorganic phosphate. Following the induction of PI3'K and Wnt/Wg pathways, the desired protein will be precipitated using its tagged epitope (after lysing the cells and extracting the protein) and electrophoresed on a 12% poly acrylamide gel.

In order to determine the role of *X*ILK in the Wnt pathway during development, a kinase assay on GSK-3 as a substrate using *X*ILK in embryos can also be performed. This would give a better idea about the relation of phosphorylation of GSK-3 upon induction of ILK activity in embryos. To further identify the role of ILK in the Wnt pathway, its interaction with GSK-3 as well as the downstream targets such as β-catenin and transcription factor Lef/Tcf can be explored. A kinase deficient ILK cDNA clone (E359K) can be microinjected into embryos to observe the effects of such dominant mutations.

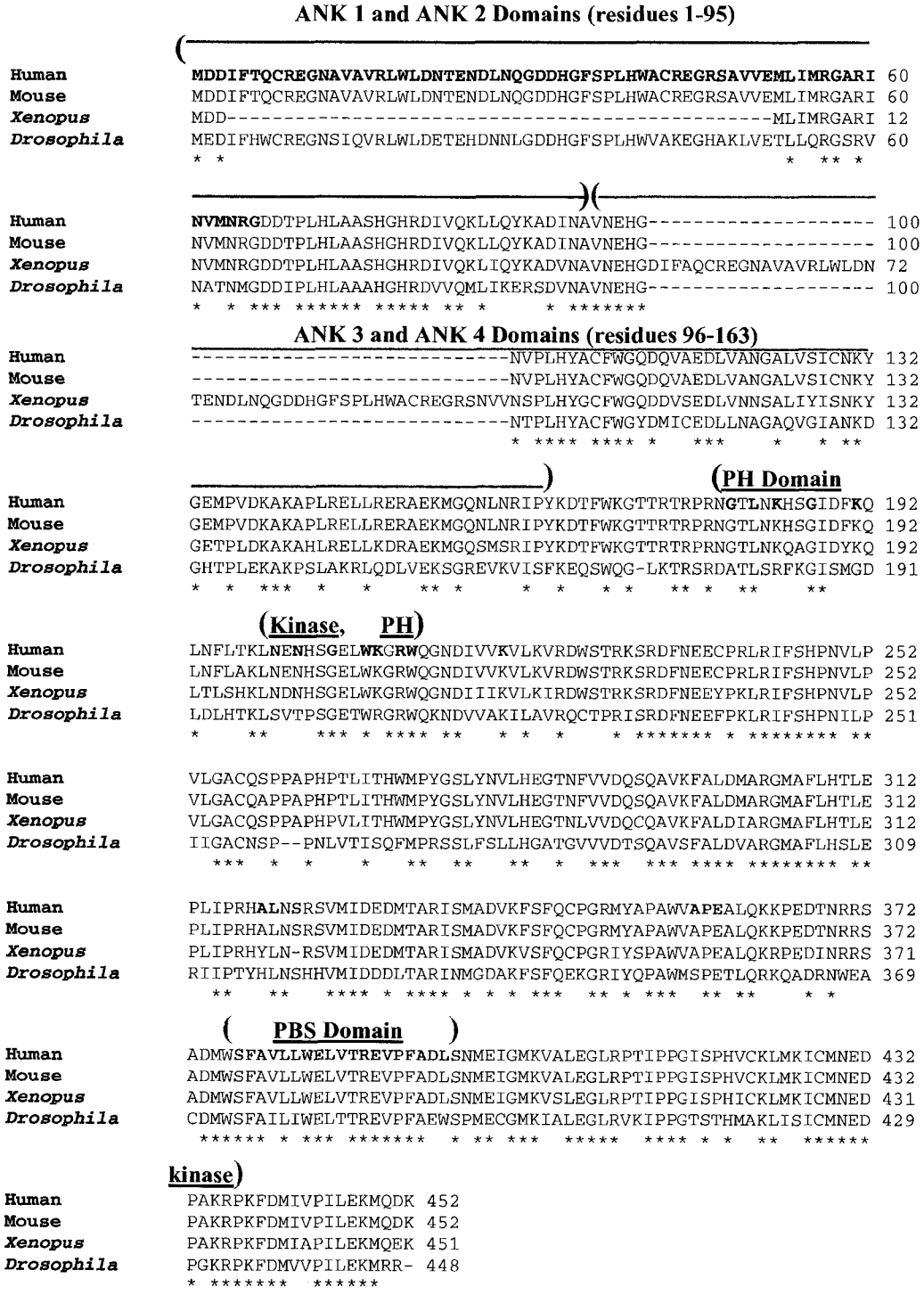
Since ILK phosphorylates GSK-3 and inhibits its activity, we expect to see similar effects as brought by mutation in *X*GSK-3, which inhibits Wnt signaling and leads to dorsal axis formation. Although mutation in the kinase domain of ILK in *Drosophila* (E359K) resulted in lethality of the embryos, it produces wing blisters in the adult wing. Analysis of ILK in *C.elegans*, *Drosophila,* and mammalian systems identified that ILK is an important mediator of integrin function (Zervas et al, 2001; Mackinnon et al, 2002; Huang et al, 2000). The absence of ILK expression in *Drosophila* leads to a severe defect in integrin mediated adhesion. This defect is due to the requirement of ILK to link cytoskeleton to the plasma membrane at sites of integrin mediated adhesion. Such an interaction is required for cell-cell ECM adhesion events such as muscle attachment and adhesion between the two surfaces of the wing cells (Zervas et al, 2001). Since ILK is typically expressed in mesoderm, the defects in mesodermal derivatives were examined. Phalloidin staining, used for actin detection, for stage 17 *Drosophila* ILK mutant embryos showed defects in most of the muscles during development, and the actin molecules were clumped together rather than extended along the length of the muscles as seen in wild-type embryos. In *C.elegans* the homolog of ILK is found in body-wall muscles, where it concentrates together with β-integrin/PAT-3 at focal adhesion-like muscle-attachment sites (dense bodies) (Mackinnon et al, 2002). In this organism, loss of ILK expression leads to a lethal embryonic phenotype called PAT (paralyzed and arrested elongation at the two fold stage). Also, ILK has been shown to be involved in the regulation of myogenic differentiation observed in mice cells (Huang et al, 2000).

The experiments carried out in my project suggest a role of ILK as a kinase molecule. To establish the physiological involvement of ILK in processes such as in cell adhesion and its interaction with other integrin mediated cell adhesion molecules, site

directed mutagenesis should be carried out. In this experiment the important sites of ILK involved in focal adhesions should be investigated and mutated. In a recent study a critical site where ILK interacts with PINCH, a protein involved in focal adhesion assembly, was mutated (Zhang et al, 2002a). The mutation in the ILK site mediating its interaction with PINCH was made on D31→A. This mutation disrupts the interaction of ILK with PINCH and impairs ILK localization to focal adhesions (Zhang et al, 2002a). ILK is capable of binding to PINCH and calponin homology-ILK binding protein (CH-ILKBP), a protein which binds ILK to actin cytoskeleton, resulting simultaneously in the formation of PINCH-ILK-CHILKBP complex (Tu et al, 2001). The formation of PINCH-ILK-CHILKBP complex proceeds integrin mediated cell adhesion and spreading (Zhang et al, 2002a) and its inhibition with dominant negative inhibitors results in defects in cell shape change, migration, proliferation and ECM deposition (Guo and Wu, 2002; Zhang et al, 2002b). An ILK point mutation with F438→A mutant was generated to see whether ILK mediation of PINCH and CH-ILKBP are required for localizing ILK to focal adhesions or other sites are critical. The results of this experiment showed that F438→A point mutation does not inhibit the formation of the PINCH-ILK-CHILKBP complex, but impaired the ability of ILK to localize to focal adhesions (Zhang et al, 2002a). These results demonstrate that the interaction of ILK with PINCH and CH-ILKBP are necessary but not sufficient for mediating ILK localization to focal adhesions. I have already designed the primers for such mutations in the *XILK* gene and have determined the restriction endonuclease enzymes for checking these mutations (the mutations were made based on the restriction sites). It will be interesting to investigate the results of these mutations in *Xenopus* embryos and see how they may effect early development.

Appendix

Fig.17. ILK protein multiple sequence alignment performed by Clustal W program. In this alignment the protein sequences of human, mouse, *Xenopus* and *Drosophila* ILK are included. Different domains of ILK are displayed in color. The first domain which is the Ankyrin domain (ANK domain) is shown in green from 1-66 indicating the first ANK repeat. The first and second ANK repeats are shown with a green line from residues 1-95 (Li et al, 1999). The third and fourth ANK repeats are shown with a purple line from residues 96-163 (Li et al, 1999). Following the ANK domain is the Pleckstrin Homology (PH) domain. The conserved consensus sequences of this domain are shown in blue. In human ILK this domain is between residues 180-212 aa. The conserved consensus sequences of the ILK kinase domain are shown in red starting from aa 200 to 436 in human ILK. The C-terminus domain of ILK contains the Paxillin binding site (PBS) shown in pink in the alignment. The PBS domain lays between residues 377-396 aa.



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Vita Auctoris

Sharareh (Sherry) Savari

Place of Birth: Abadan, Khouzestan, Iran
Date of Birth: January 28, 1978

Education

April, 2005	Master of Science, Department of Biological Sciences, University of Windsor, Windsor, Ontario. Thesis Title: Role of *Xenopus laevis* integrin linked kinase (*X*ILK) during early development Supervisor: Dr. Adnan Ali Co-supervisor: Dr. Alden Warner
May, 2001	Bachelor of Science, Honours General Biology University of Shahid Chamran, Ahvaz, Iran

Publications

Savari, S., Warner, A. and Ali, A. Role of *Xenopus laevis* integrin linked kinase (*X*ILK) during early development (manuscript in preparation).

Abstracts/Posters

Savari, S., Warner, A. and Ali, A. Isolation and characterization of integrin linked kinase (ILK) in *Xenopus laevis.* 48th CFBS Annual Meeting (Second Northern Lights Summer Conference), University of Guelph, Ontario, June 22-25, 2005.