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OVEREXPRESSION OF THE INTEGRIN LINKED KINASE (ILK) PROMOTES ANCHORAGE-INDEPENDENT CELL CYCLE PROGRESSION

by

Galina Radeva

A Thesis submitted in Conformity with the Requirements
for the Degree of Master of Science
Graduate Department of Medical Biophysics
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ABSTRACT

Overexpression of ILK in rat epithelial cells results in decreased cell adhesion and anchorage-independent growth. I hypothesized, therefore, that overexpression of ILK would directly or indirectly affect the cell cycle machinery. Cell adhesion to substratum has been shown to regulate cyclin A expression as well as cyclin D and E-dependent kinases. Here, I demonstrate that stable transfection and overexpression of the Integrin Linked Kinase (ILK) induces anchorage-independent cell cycle progression but not serum-independent growth of IEC18 rat intestinal epithelial cells. ILK overexpression results in increased expression of cyclin D1 and cyclin A, activation of cdk4 and cyclin E-associated kinases, and hyperphosphorylation of the retinoblastoma protein. In addition, p21 and p27 cdk inhibitors display altered electrophoretic mobilities, with p27 having reduced inhibitory activity. These results indicate that, when overexpressed, ILK induces signalling pathways resulting in the stimulation of G1/S cyclin-cdk activities which are normally regulated by cell adhesion and integrin engagement.

I dedicate this work

to my Mother Stoika Stancheva

Acknowledgements

I would like to thank my supervisor, Dr. S. Dedhar, for allowing me to pursue my work in his laboratory and for his help and advice throughout my studies. I would also like to thank my committee advisors Dr. J. Filmus and Dr. J. Woodgett, as well as Dr J. Slingerland for all their insightful input. I acknowledge Teresa Petrocelli and Chungyee Leung-Hagesteijn for their technical help and advice. I acknowledge also Marc Coppolino, Dr G. Hannigan and Dr. Marc Delcommenne for scientific and non-scientific discussions, as well as the enjoyment of a good beer every now and then.

Last, but certainly not least, I give my thanks to my husband, Krassimir, for all his support.

LIST OF DIAGRAMS AND FIGURES

LIST OF ABBREVIATIONS

CAK	cyclin dependent kinase-activating kinase
CDK	cyclin dependent kinase
cDNA	complementary deoxyribonucleic acid
CKI	cyclin dependent kinase inhibitor
Col	collagen
ECM	extracellular matrix
FACS	fluorescence-activated cell sorting
FAK	focal adhesion kinase
FAP	focal adhesion plaques
FCS	fetal calf serum
Fn	fibronectin
Fg	fibrinogen
Grb2	growth factor receptor binding protein 2
IAP	integrin associated protein
ICAM	intercellular cell adhesion molecule
IEC	intestinal epithelial cell
IL	interleukin
ILK	integrin linked kinase
IRS-1	insulin receptor substrate 1
Lm	laminin

MAP kinase	mitogen activated protein kinase
mRNA	messenger ribonucleic acid
Op	osteopontin
PBS	phosphate-buffered saline
PDGF	platelet derived growth factor
PI-3 kinase	phosphatidil inositol 3 kinase
SDS	sodium dodecyl sulphate
SH2	src homology 2
SH3	src homology 3
Tn	tenascin
Vn	vitronectin

CHAPTER I

GENERAL INTRODUCTION

PART I: INTEGRIN MEDIATED ADHESION AND INTEGRIN SIGNAL TRANSDUCTION

1. Role of adhesive interactions

Cell to cell and cell to matrix adhesive interactions are essential for maintaining integrity of multicellular organisms. They play central roles in tissue- and organogenesis during development and in wound healing, immunity, and inflamation in adult organisms. Adhesive interactions regulate diverse cellular processes such as cell motility, growth, differentiation, gene expression and apoptosis. Disruption or abrogation of normal adhesiveness leads to various pathological diseases, including cancer.

2. Integrin family

Integrins comprise a large family of cell surface receptors, which mediate adhesive interactions and transduce signals across the plasma membrane (Hynes, 1992; Juliano and Haskill, 1993; Clarke and Brugge 1995; Dedhar and Hannigan, 1996). They are heterodimeric glycoproteins, composed of α and β subunits (Diagram 1a). The molecular weight of α subunits is in the range of 120 kDa to 180 kDa, whereas β subunits vary between 90 and 110 kDa. Each subunit has three distinct domains : a large extracellular domain, a single membrane spanning region and usually a short cytoplasmic tail. Both subunits have substantial intrachain disulfide bonding. This is consistent with a model of compact, folded structure of integrin domains and explains their resistance to proteolysis when in the native state (Hynes, 1992). All β subunits contain a highly conserved four-fold repeat of a cysteine-rich segment in their membrane proximal extracellular domain. These repeats are believed to be involved in internal disulfide

Diagram 1a. Integrin Structure

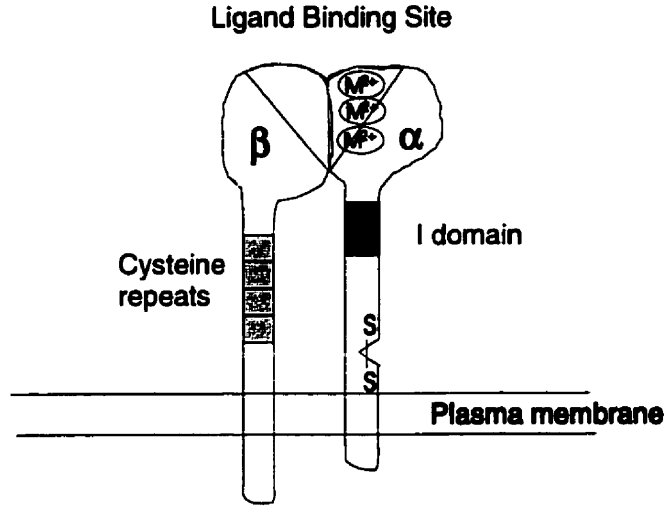
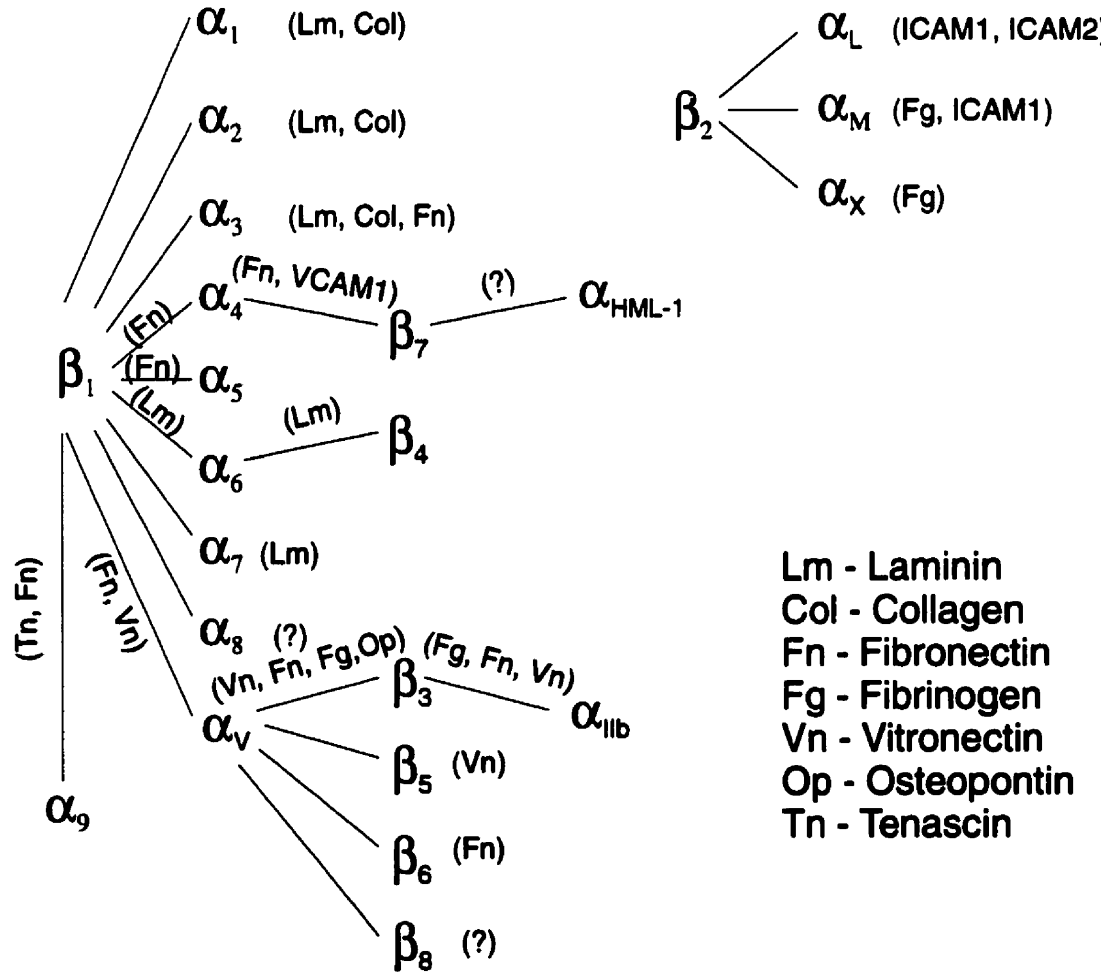


Diagram 1b. Integrin Family



bonding, which further contributes to the overall conformation of β subunits (Calvete *et al.*, 1991). Some α subunits (α_1, α_2) contain a segment of approximately 200 amino acids, known as an I domain, which is inserted before the five homologous repeats of the cation binding region, contained in all the α subunit extracellular domains (Hynes, 1992). The function of the I domain is not clear, but it is believed to contribute to the ligand binding of the integrin receptor. Other α subunits (α_3, $\alpha_{5\text{-}9}$, α_v,and α_{IIb}) are post-translationally cleaved in the extracellular domain to give rise to two chains: a light (~25-30 kDa) membrane spanning chain and a heavy (~120-130 kDa) extracellular chain, which are disulfide-bonded to each other (Hynes, 1992). The α and β subunits associate non-covalently by their extracellular domains to form a ligand binding site and a functional receptor. The pairing between the α and β subunits, and the determination of ligand binding specificity is not dependent on the transmembrane or the cytoplasmic domains (Solowska *et al.*, 1991). The binding of integrins to their ligands and in some cases the association between the α and β subunits is dependent on the presence of divalent cations (Gailit and Ruoslahti, 1988; Kirchhofer *et al.*, 1990, 1991).

Association between fifteen α and eight β subunits can occur in different combinations leading to numerous distinct integrin receptors (Diagram 1b). The α/β association determines the ligand binding specificities of integrin heterodimers for various ECM (Extra-Cellular Matrix) proteins. A further level of complexity and variety is provided by alternatively spliced forms, predominantly in the cytoplasmic tails of either the α or the β subunit (reviewed in Hynes, 1992). Most integrins are expressed on a wide variety of cells, and usually most cells express several different types of integrins. However, β_2 integrin subunits are expressed exclusively on leukocytes along with three leukocyte specific α subunits (α_M, α_L, α_X). These integrins mediate cell-cell interactions (Hynes, 1992). Integrins, containing the β_1 subunit bind components of the

extracellular matrix and are found on a wide variety of cells. Individual integrins can often bind to more than one ligand and single ligands are often recognized by more than one integrin receptor. In some cases, two integrins that share the same ligand will actually recognize different regions of the ligand molecule, as is the case for $\alpha_5\beta_1$ and $\alpha_4\beta_1$ fibronectin receptor, or the $\alpha_1\beta_1$ and $\alpha_6\beta_1$ laminin receptor. One of the well defined recognition sites, which is present in fibronectin, vitronectin, collagen and other adhesive proteins, is the RGD (Arg-Gly-Asp) sequence (Ruoslahti and Pierschbacher, 1987). This tripeptide is recognized by $\alpha_5\beta_1$, $\alpha_{IIb}\beta_3$ and most of $\alpha_v\beta$ integrins. Another identified sequence is the KQAGDV (Lys-Gln-Ala-Gly-Asp-Val) which is specifically recognized in fibrinogen by $\alpha_{IIb}\beta_3$ (Calvete *et al.*, 1992). Other integrins recognize different sequences: $\alpha_2\beta_1$ binds DGEA (Asp-Gly-Glu-Ala) in type I collagen (Staatz *et al.*, 1991), $\alpha_4\beta_1$ binds EILDV (Glu-Ile-Leu-Asp-Val) in fibronectin (Komoriya *et al.*, 1991) and $\alpha_x\beta_2$ binds GPRP (Gly-Pro-Arg-Pro) in fibrinogen (Loike *et al.*, 1991). Although the precise binding sites for other ligands have yet to be determined, it is known that the various laminin receptors recognize specific parts of the laminin molecule (Hall *et al.*, 1990) and the integrins binding the immunoglobulin superfamily counter-receptors recognize specific immunoglobulin-like domains (Staunton *et al.*, 1990; Diamond *et al.*, 1991).

3. Integrin cytoplasmic domains

Integrins provide a physical link between cells or between cells and the surrounding ECM network. They do so by binding other cell-surface receptors or the constituents of the ECM on the extracellular side of the cell, and cytoskeletal components inside the cell (Hynes, 1992). The multiplicity of such bridging interactions leads to the formation of Focal Adhesion Plaques (FAP's), which are the sites where cells attach to the ECM. Integrin-ligand association can

trigger the reorganization of the cytoskeleton and initiate cascades of signalling events (Sastry and Horwitz, 1993; Yamada and Miyamoto, 1995; Clarke and Brugge; 1995). Therefore a number of cytosolic proteins possessing signalling properties can be found in the FAP's in addition to the cytoskeletal proteins. Since neither of the α and β subunits has any intrinsic enzymatic activities, integrins must mediate signalling via coupling their cytoplasmic tails with cytosolic signalling molecules. Mutation analysis and chimeric construction approaches have revealed the absolute requirement of the cytoplasmic domains of integrins for their functions as adhesive and signalling molecules (LaFlamme *et al.*, 1994; Lukashev *et al.*, 1994;). β subunit cytoplasmic tails have been found to be necessary and sufficient for targeting integrins to FAP's and FAP formation (Akiyama *et al.*, 1994), while the α cytoplasmic domains can regulate the specificity of the ligand-dependent interactions (O'Toole *et al.*, 1991; Kawaguchi *et al.*, 1994).

4. Proteins in the Focal Adhesion Plaques

The Focal Adhesion Plaques contain a high concentration of protein constituents of the actin-cytoskeleton as well as cytosolic proteins capable of transducing signalling events.

4.1. Proteins with roles in cytoskeletal reorganization

The cytoplasmic domains of β-subunits can bind directly to α-actinin or talin, which in turn bind to and cross-link actin filaments (Horwitz *et al.*, 1986; Otey *et al.*, 1990). β1 integrin peptides and *in vitro* binding assays have been employed to study the interaction between integrins and α-actinin. The binding sites for α-actinin have been mapped to two distinct regions in the cytoplasmic tail of β1 integrin: one of them is a sequence located in the C-terminus of β1 integrins (amino acids 785-794), while the other one is in the

membrane-proximal region (amino acids 768-778) (Otey *et al.*, 1993). The importance of these specific sequences in facilitating integrin/α-actinin binding and thus directing integrins to focal adhesions is in agreement with *in vivo* data from an earlier study by Reszka *et al.* (1992). The sequences in β integrin which are involved in the direct interaction with talin (amino acids 780-789; 791-799) are different from those observed for α-actinin, even though they overlap to some extent (Tapley *et al.*, 1989; Lewis and Schwartz, 1995). Again, both regions have to be present so that talin can effectively bind to the β cytoplasmic tail. Each region contains an NPXY (Asn-Pro-X-Tyr) sequence that forms a tight turn motif (Reszka *et al.*, 1992). It has been speculated that the two segments are likely to be closely positioned and provide a structural conformation that forms a single binding site for talin. Indeed, removal of 791-799 residues weakens the affinity and prevents association *in vivo* (Lewis and Schwartz, 1995). Alternatively, such a deletion results in abolishing a signal that is necessary for interaction with talin.

Another structural component of FAP's is vinculin, which can bind to α-actinin and talin as well as to other cytoskeletal constituents such as paxillin and tensin (Clarke and Brugge, 1995). In addition, vinculin and tensin can directly bind to actin filaments (Clarke and Brugge, 1995). Recently, an actin-binding protein, filamin, has been found to directly interact with the β_2 integrin cytoplasmic domain (Sharma *et al.*, 1995). The filamin binding site localizes within the N-terminal portion of β_2 cytoplasmic tail (amino acids 724-747). This site is distinct from the α-actinin binding site (amino acids 733-742) even though they overlap. The net effect of all these direct and indirect interactions is the formation of structural protein assemblies that are believed to play important roles in regulating cell adhesion and modulating cell shape and motility. Furthermore, they could provide a framework for association of signalling molecules that mediate integrin-induced signal transduction pathways.

The role of the small GTP-binding proteins Rho, Rac and Cdc42 in the formation of FAP's, as well as the cytoskeletal structures lamellipodia, filopodia and membrane ruffles, has been revealed by microinjection experiments performed in Swiss 3T3 fibroblasts (Nobes and Hall, 1995; Hotchin and Hall, 1995). It was determined that functional Rho is required for both actin stress fiber organization and focal adhesion assembly (Ridley and Hall, 1992; Chrzanowska-Wodnicka and Burridge, 1996), while Rac is necessary for membrane ruffling and lamellipodia formation (Ridley *et al.*, 1992). A third type of actin-based structure found at the cell periphery, filopodia, is promoted by activation of Cdc42 (Nobes and Hall, 1995).

4.2. Cytosolic proteins in the Focal Adhesion Plaques

In addition to the proteins comprising the actin-cytoskeleton, cytosolic proteins that interact directly or indirectly with integrins have been observed to localize to the FAP's. Most of them possess signalling properties and participate in signal transduction cascades leading to integrin-induced changes in cell behaviour. The Focal Adhesion Kinase (FAK) is a tyrosine kinase thought to be a major player in integrin-mediated signalling events. FAK was discovered by a homology-based cDNA cloning approach in a search for new members of the Protein-Tyrosine Kinase (PTK) family (Hanks *et al.*, 1992). This non-membrane spanning protein-tyrosine kinase displays a unique structure that does not contain SH2 or SH3 domains, characteristic of many other members of the PTK family. It was found that FAK accumulates at the FAP's and becomes tyrosine phosphorylated upon integrin engagement (Hanks *et al.*, 1992; Richardson and Parsons, 1995). Clustering of chimeric integrin receptors expressing extracellular portion of the IL-2 receptor and different β cytoplasmic domains (but not α) is sufficient to trigger FAK phosphorylation (Akiyama *et al.*, 1994), suggesting that

integrin-induced phosphorylation of FAK requires the β integrin cytoplasmic domain. A direct interaction between the β cytoplasmic tail and FAK has been proposed. It is not clear, though, which region of the β integrin cytoplasmic tail could be involved in binding FAK. *In vitro* peptide studies indicate amino acids 756-768 (Schaller *et al.*, 1995), which are different from a previously determined region encompassing amino acids 791-799 (Lewis and Schwartz, 1995). It is possible that both regions are essential, where the second segment (791-799) provides a conformational requirement for the first one (756-768) to form the actual binding site. Yet, there is no *in vivo* experimental data in support of this predicted direct association.

Recently, three novel proteins have been reported to directly interact with β-specific cytoplasmic domains: β-endonexin, cytohesin-1 and ILK (Shattil *et al.*, 1995; Kolanus *et al.*, 1996; Hannigan *et al.*, 1996). In all three cases, a yeast two-hybrid screen was used to identify proteins which interact with integrins and possibly mediate integrin-regulated signal transduction pathways. β_3-endonexin is a 12.6 kDa polypeptide that specifically interacts with the β_3 cytoplasmic domain (Shattil *et al.*, 1995). This interaction is dependent on a serine residue since it was reduced by 64% by a single point mutation in the β_3 cytoplasmic tail (S752-P) that also disrupts integrin signalling. The biological function of β_3-endonexin remains to be determined. Cytohesin-1 is a cytoplasmic molecule, which contains a pleckstrin homology (PH) domain (Kolanus *et al.*, 1996). Cytohesin-1 was found to interact specifically with the β_2 cytoplasmic domain and stimulates $\alpha_L\beta_2$ binding to its ligand ICAM-1 (Kolanus *et al.*, 1996). ILK (Integrin Linked Kinase) is a serine-threonine kinase, specifically interacting with β_1 and β_3 cytoplasmic tails *in vivo*, as demonstrated by co-immunoprecipitation (Hannigan *et al.*, 1996). ILK overexpression in rat epithelial cells results in decreased cell adhesion to ECM substrates (Hannigan *et al.*, 1996), which implies a role in "inside-out" signalling. On the other hand,

attachment and spreading of cells on fibronectin leads to altered ILK kinase activity, and overexpression in epithelial cells induces anchorage-independent growth (Hannigan *et al.*, 1996), as well as anchorage-independent cell cycle progression (Radeva *et al.*; submitted, Chapter II). Therefore, ILK may be involved in regulating bidirectional transfer of information mediated by integrins.

Two intracellular proteins have been shown recently to interact directly with the heterodimer molecule of $\alpha_v\beta_3$ integrin (Bartfeld *et al.*, 1993; Vouri and Ruoslahti, 1994). Bartfeld *et al.*, (1993) have found that $\alpha_v\beta_3$ vitronectin receptor co-immunoprecipitates with a tyrosine phosphorylated 190 kDa protein in mouse fibroblasts. The formation of a complex between the two molecules is independent of ligand occupancy, while phosphorylation of the 190 kDa protein was observed only following cell activation by PDGF. Furthermore, exposing cells to vitronectin before PDGF treatment results in an increased amount of tyrosine phosphorylation of the 190 kDa $\alpha_v\beta_3$ associated protein (Bartfeld *et al.*, 1993). Another molecule that associates with $\alpha_v\beta_3$ vitronectin receptor is the insulin receptor substrate-1 (IRS-1), a molecule that functions in growth factor signalling (Vouri and Ruoslahti, 1994). This association can be detected after insulin treatment, where IRS-1 is the major target protein that is phosphorylated on tyrosine by ligand-activated insulin receptor (Vouri and Ruoslahti, 1994). Tyrosine phosphorylated IRS-1 links insulin receptor activation to downstream intracellular signalling cascades through its association with SH2 domains containing proteins such as Grb-2, PI-3 kinase, Syp and Nck (White and Kahn, 1994). At least two of these proteins, Grb-2 and PI-3 kinase, co-immunoprecipitate with $\alpha_v\beta_3$ from insulin-stimulated cells (Vouri and Ruoslahti, 1994). These observations imply a synergistic action of growth factor and extracellular matrix receptors. In addition to the intracellular proteins, integral plasma membrane proteins have also

been demonstrated to interact with integrin heterodimers. IAP (integrin associated protein) contains three or five membrane-spanning domains and associates with the $\alpha_v\beta_3$ integrins (Brown *et al.*, 1990; Lindberg *et al.*, 1993). It regulates integrin ligand binding, since anti-IAP antibody inhibits the binding of vitronectin-coated beads to the $\alpha_v\beta_3$ integrin on human erythroleukemia cells (Lindberg *et al.*, 1993). Another example of integrin-associating integral plasma membrane proteins are the members of the TM4 protein family, CD9 and CD63, which interact with the $\alpha_3\beta_1$ and $\alpha_6\beta_1$ integrins (Berditchevski *et al.*, 1995; Nakamura *et al.*, 1995).

5. Role of ligand occupancy and integrin clustering in cytoskeletal reorganization and FAP formation

Integrins function to mediate cell adhesion, signal transduction and cytoskeletal reorganization. How a single transmembrane receptor co-ordinates multiple functions and still accomplishes specificity in triggering downstream pathways is an intriguing question. One level of specificity could be achieved by recruiting different cytoplasmic molecules that could specifically initiate a given cascade of signalling events. Receptor occupancy and aggregation is another level of specificity (Miyamoto *et al.*, 1995). By using polystyrene beads coated with different ligands, as well as adhesion-blocking or adhesion-stimulating antibodies, Miyamoto *et al.*, (1995) have shown that integrin receptors are able to induce distinct cellular responses to binding of a ligand, to aggregation, or to a combination of both. Binding of a monovalent ligand results in receptor redistribution, but minimal tyrosine phosphorylation or cytoskeletal protein reorganization. On the other hand, aggregation of integrins leads to clustering of tensin and FAK with concomitant signalling involving tyrosine phosphorylation, but no accumulation of other cytoskeletal proteins. Intracellular organization of large cytoskeletal complexes requires

both integrin receptor occupancy and clustering. Large aggregates of accumulated integrins and cytoskeletal molecules often accompany reduced cell migration rates (Miyamoto *et al.*, 1995). Thus, regulation of integrin functions by the above three mechanisms can allow a single integrin transmembrane receptor to function selectively in translocation, signalling, or different cytoskeletal reorganizations depending on the local environmental stimuli.

6. Signal transduction by integrins

In addition to their roles in regulation of cell adhesion and cytoskeletal organization, integrins mediate transmembrane signal transduction. Current understanding of how integrins function as signal transduction molecules, guiding the bi-directional transfer of information, has derived from analysis of biochemical events triggered by integrin engagement as well as identification of proteins associating with focal adhesion complexes.

6.1. Inside-out signalling

As discussed earlier (PART I, Section 2.), combination between the fifteen α and eight β subunits gives rise to a significant variety of heterodimers and determines the specificity of their binding to ligands. Cells can regulate their adhesive properties by selectively expressing certain integrins and thus co-ordinate their behavior toward the surrounding ECM environment. Further complexity in the control of cell adhesiveness is introduced by the fact that cells can modulate the integrin affinity state, a phenomenon called "inside-out" signalling (O'Toole *et al.*, 1991; O'Toole *et al.*, 1994). "Inside-out" signalling is thought to be regulated by changes in the spatial relationships or conformation of integrin subunits, provoked by interactions of cellular factors with integrin cytoplasmic domains (O'Toole *et al.*, 1994). Such a model has been

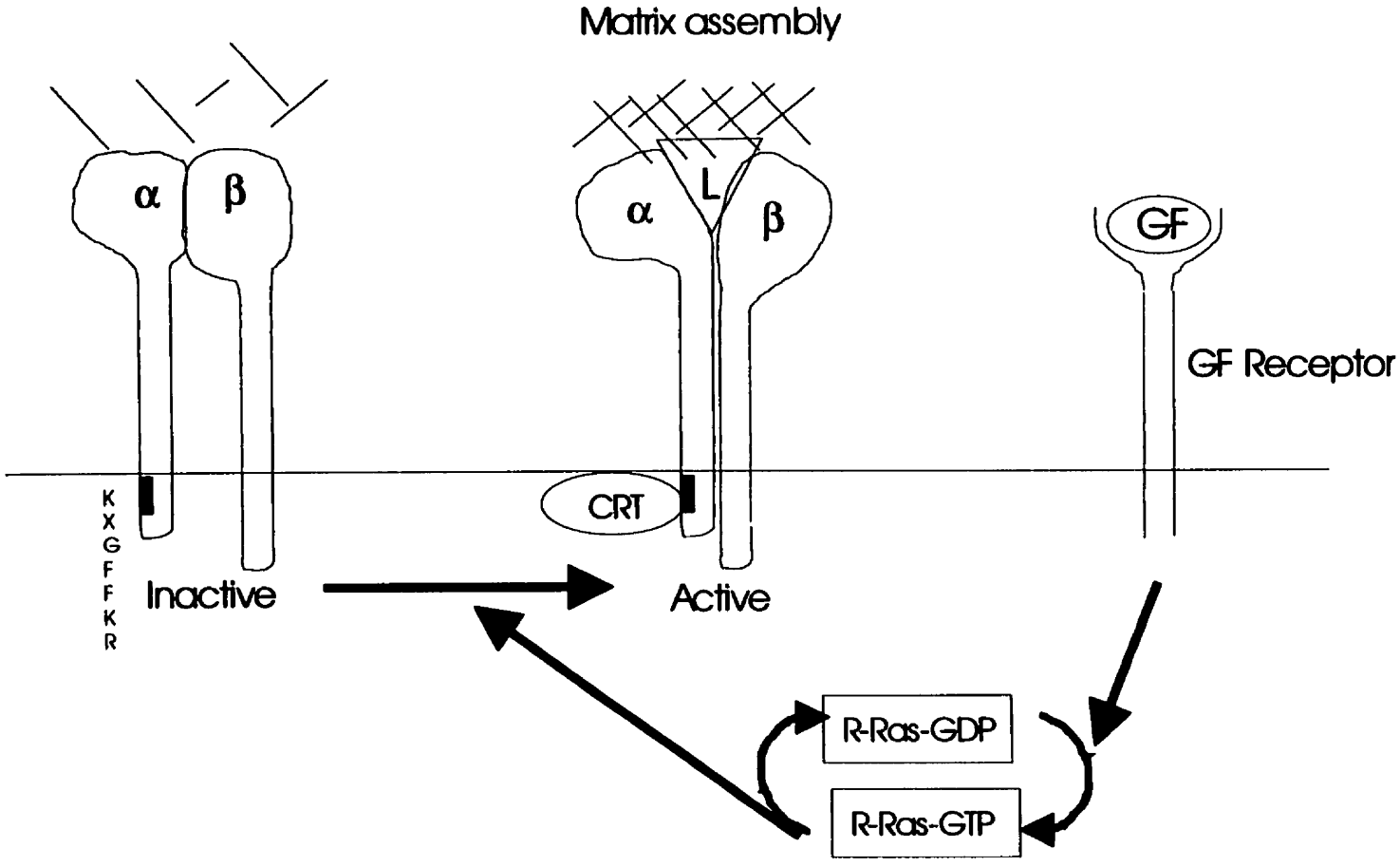
proposed after an extensive study of the $\alpha_{IIb}\beta_3$ integrin, expressed on platelets. Using chimeric constructs, site-directed mutagenesis and deletion analysis, the role of different cytoplasmic domains has been examined and the importance of specific sequences within them was determined (O'Toole *et al.*, 1994). The affinity state of manipulated integrins was assessed by either binding of fibrinogen or the monoclonal antibody PAC1, which recognizes only the active state of $\alpha_{IIb}\beta_3$. It was found that the affinity state of integrins is regulated by cell type-specific cytosolic factors, since $\alpha_{IIb}\beta_3$ chimaera containing cytoplasmic portions of $\alpha_5\beta_1$ would be in a high affinity state, when transfected in CHO cells, but not in K562 cells (O'Toole *et al.*, 1994). Furthermore, integrin affinity is controlled by the α-subunit cytoplasmic domain and this control is α-subunit specific. Indeed, α_2, α_{6A}, α_{6B} cytoplasmic domains conferred high affinity binding of PAC1, when cotransfected with β_3 subunit, while chimaeras containing the cytoplasmic domains of α_M, α_L or α_V did not. Deletion in the cytoplasmic tail of β_3 ($\beta_3\Delta724$) or mutation of Ser to Pro (β_3S752-P) abolished the constitutive PAC1 binding to α_2, α_{6A} or α_{6B} high affinity chimaeras (O'Toole *et al.*, 1994). Therefore, it was concluded that the α-subunit cytoplasmic domain determines the integrin-specific affinity state, whereas the β-cytoplasmic domain is required for stabilization. A highly conserved membrane-proximal motif present in all integrin α-subunits, KXGFFKR (Lys-X-Gly-Phe-Phe-Lys-Arg), was also found to play a role in regulating integrin affinity state. Truncation $\alpha_{IIb}\Delta991$ removes a GFFKR (Gly-Phe-Phe-Lys-Arg) segment and results in constitutive binding of PAC1, whereas a truncation after the GFFKR ($\alpha_{IIb}\Delta996$) does not (Williams *et al.*, 1994, O'Toole *et al.*, 1994). Furthermore, deletion of VGFFK (Val-Gly-Phe-Phe-Lys) residues from the α_L cytoplasmic domain chimaera locks the integrin receptor in a constitutive high affinity state (O'Toole *et al.*, 1994). Point mutation analysis identified the two phenylalanine residues and the terminal arginine residue of GFFKR

in the α subunit as critical for the regulation of the integrin affinity state (Hughes *et al.*, 1996). The requirement for the presence of the GFFKR motif in order to regulate the molecular switches between different affinity states of integrins is cell-type independent (O'Toole *et al.*, 1994). Some understanding of how this highly conserved region may be involved in modulating integrin function comes from the observation that calreticulin binds to the KXGFFKR motif *in vitro* (Rojiani *et al.*, 1991; Dedhar, 1994) and directly associates with integrins *in vivo* (Coppolino *et al.*, 1995). Furthermore, calreticulin was found to associate with the active form of the collagen receptor, $\alpha_2\beta_1$, but not with the inactive form (Coppolino *et al.*, 1995). Introducing anti-calreticulin antibodies into cells prevents $\alpha_2\beta_1$ activation by integrin-activating antibodies or by phorbol esters (Coppolino *et al.*, 1995). These observations suggest that the KXGFFKR sequence sets integrins in a default inactive state and calreticulin binding to KXGFFKR results in a switch to a high affinity state (Diagram 2a).

The small GTP-binding protein family can also play a role in integrin "inside-out" signalling. Recently, a non-transforming Ras GTP-binding protein, R-Ras has been shown to activate integrins (Zhang *et al.*, 1996). Expression of constitutively active R-Ras leads to a switch from an integrin low affinity state to a high affinity state and adhesion of suspension cells to ECM. In contrast, expression of dominant-negative R-Ras decreases the ability of cells to adhere, suggesting that endogenous R-Ras is involved in the control of integrin ligand affinity (Zhang *et al.*, 1996). The pathways and the mechanism of such control are currently unknown.

Integrin "inside-out" signalling affects the deposition of the components of the pericellular matrix as well as their assembly (Wu *et al.*, 1995). Activation of integrins by distinct cytoplasmic domain mutations or by activating antibodies was shown to support fibrillogenesis (Wu *et al.*, 1995). This is an important aspect of integrin function, since matrix

Diagram 2a. "Inside-out" Signalling



assembly and fibrillogenesis are vital to vertebrate development, wound healing and tumorigenesis.

Recently it was shown that the urokinase-type plasminogen activator receptor (uPAR) can modify integrin function (Wei *et al.*, 1996). uPAR was found to form stable complexes with integrins, which led to inhibited native integrin adhesiveness and promoted adhesion to vitronectin via a ligand binding site on uPAR (Wei *et al.*, 1996).

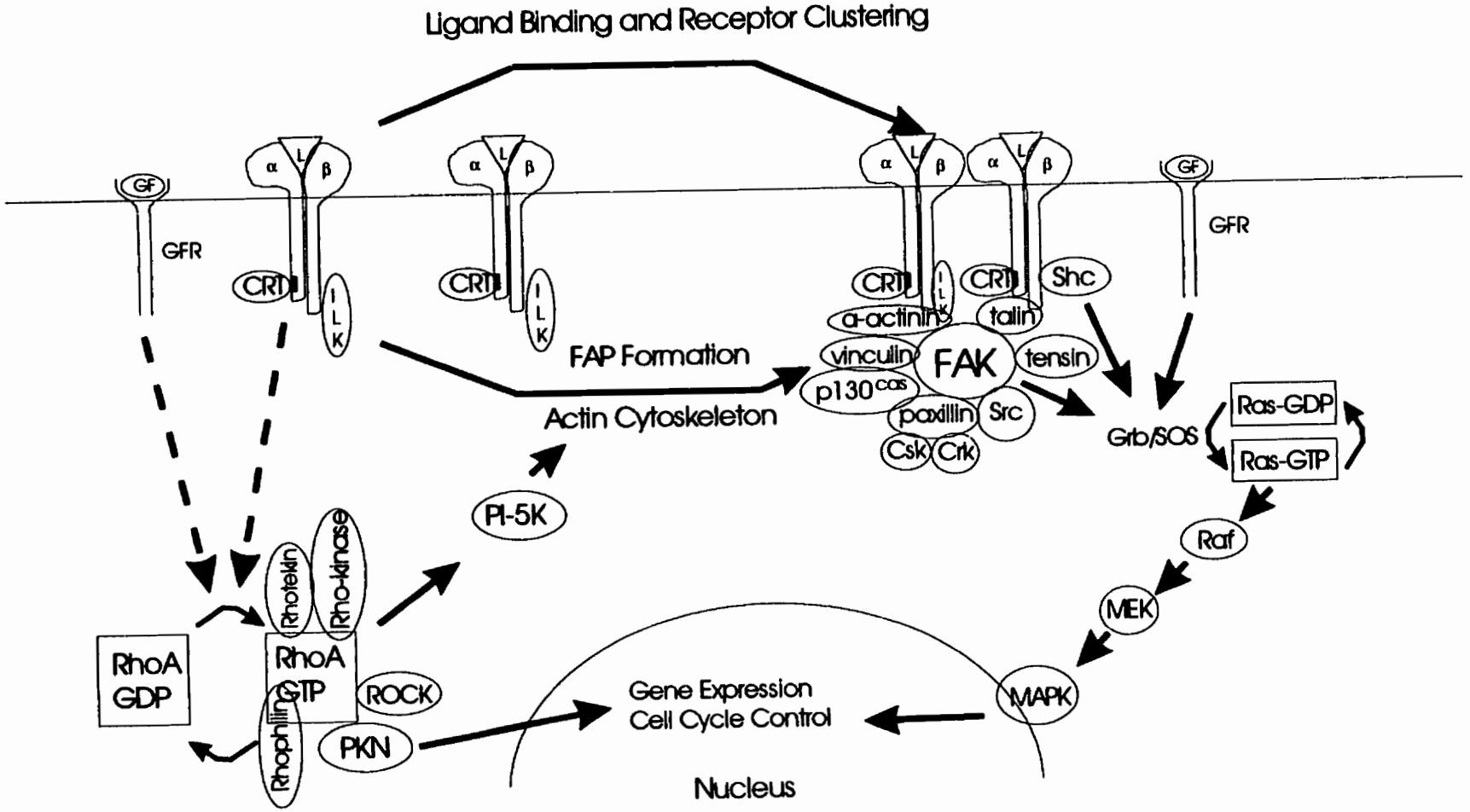
6.2. Outside-in signalling

Binding of integrins to their ECM ligands elicits a series of signalling events such as tyrosine phosphorylation (Schaller *et al.*, 1992; Hanks *et al.*, 1992; Kapron-Bras *et al.*, 1993), activation of Na^+/H^+ antiporter (Schwartz *et al.*, 1991) and Ca^{2+} pump (Schwartz, 1993), stimulation of phospholipid metabolism (McNamee *et al.*, 1993), activation of serine-threonine kinase families such as PKC (Protein Kinase C) (Vuori and Ruoslahti, 1993) and MAPKinase (Mitogen Activated Protein Kinase) (Chen *et al.*, 1994; Zhu and Assoian, 1995), as well as Ras (Kapron-Bras *et al.*, 1993) and other small GTP-ases (Nobes and Hall, 1994; Burbelo *et al.*, 1995). Ultimately these changes lead to modulation of gene expression, regulation of cell cycle progression and/or programmed cell death (Diagram 2b).

6.2.1. Cell cycle progression and apoptosis

Cell attachment to substratum has been shown to be required for progression through the cell cycle as well as for cell survival, since cells prevented from adhering arrest in the G1 phase of the cell cycle and undergo apoptosis (Meredith *et al.*, 1993; Schwartz and Ingber, 1994; Zhu *et al.*, 1996). In this way, regulation of the balance between proliferating cells and cells undergoing apoptosis in an anchorage-dependent manner has an important role in maintaining correct cell number and tissue organization.

Diagram 2b. "Outside-in" Signalling



Integrin engagement is required for cell survival as well (Frisch and Francis, 1994). Cell survival may be mediated through Bcl-2 upregulation (Zhang *et al.*, 1995) or through suppression of interleukin-1 converting enzyme (ICE) upon ligand engagement of certain integrins (Boudreau *et al.*, 1995). In contrast, the cytoplasmic domains of specific β subunits (β_{1C} and β_4) have been shown to trigger cell cycle arrest and apoptosis (Meredith *et al.*, 1995).

6.2.2. Gene expression

Specific gene expression upon integrin-mediated cell attachment has also been observed. β-casein gene expression is ECM-dependent and requires functional β_1-integrins (Roskelley *et al.*, 1994). Increase in the expression of c-fos mRNA and collagen mRNA has been reported following attachment of fibroblasts to a substratum (Dhawan and Farmer, 1990; Dhawan *et al.*, 1991). Engagement of $\alpha_5\beta_1$ integrin induces the expression of metalloprotease genes in fibroblasts, while occupation of $\alpha_v\beta_3$ integrin in melanoma cells induces type IV collagenase (Werb *et al.*, 1989; Seftor *et al.*, 1992). Plating of monocytes on tissue culture plastic or on ECM-coated substrata leads to rapid induction of a number of immediate early (IE) genes such as c-jun, c-fos, I-κB, as well as the cytokines: IL-1β, IL-8 and TNFα (Haskill *et al.*, 1991; Yurochko *et al.*, 1992). Many of the genes induced upon integrin ligation, have NF-κB motifs in their upstream regulatory regions (Juliano and Haskill, 1993), suggesting a role of NF-κB in integrin-stimulated gene expression.

6.2.3. Small GTP-binding proteins

The members of the Rho-family of GTP-binding proteins are essential in integrin-dependent cytoskeletal reorganization and FAP formation. To dissect the signalling pathways in which Rho GTP-ases participate, several groups have focused on identifying

downstream effectors for these molecules. Three serine/threonine kinases (PKN, p160ROCK and Rho-kinase), are able to associate with GTP-bound RhoA. As a result of this association their kinase activities are stimulated (Watanabe *et al.*, 1996; Amano *et al.*, 1996; Ishizaki *et al.*, 1996; Matsui *et al.*, 1996). Two other proteins with yet undefined biological functions can also bind to activated RhoA. These have been named Rhophilin and Rhotekin (Watanabe *et al.*, 1996; Reid *et al.*, 1996). A highly conserved Rho binding domain has been mapped to the N-terminal portion of Rhophilin, PKN and Rhotekin (Watanabe *et al.*, 1996; Reid *et al.*, 1996). It is possible that interactions of PKN, p160ROCK, Rho-kinase, Rhophilin and Rhotekin with the activated Rho recruits them to specific sites at the membrane, such as FAP's, where they are required for facilitating their function or triggering downstream targets. Indeed, Rho is involved not only in the organization of specific actin cytoskeleton structures (Ridley and Hall, 1992), but also in phospholipid metabolism, transcriptional control and cell cycle progression (Chong *et al.*, 1994; Hill *et al.*, 1995; Olson *et al.*, 1995). These observations indicate that Rho may drive multiple signalling pathways. Such a scenario implies multiple Rho effectors in order to achieve specificity.

6.2.4. Phospholipid metabolism

Activation of certain lipid kinases has been shown to be dependent on integrin adhesion. PI-3 kinase, which phosphorylates PI(4)phosphate (PIP) or PI(4,5)bisphosphate (PIP2) to generate PI(3,4)P2 or PI(3,4,5)P3 respectively, associates with integrin-regulated cytoskeletal complexes in platelets (Zhang *et al.*, 1993). In extracts from cells plated on fibronectin, PI-3 kinase co-precipitates with FAK (Chen and Guan, 1994). Inhibition of PI-3 kinase blocks growth factor-induced actin rearrangements, suggesting a similar role for PI-3 kinase in integrin-induced cytoskeletal rearrangements (Wymann and Arcaro, 1994). PI-5 kinase is another lipid

kinase, which phosphorylates PIP to generate PIP2. It has been observed that adhesion of mouse fibroblasts to fibronectin stimulates PI-5 kinase and induces the production of PIP2 (McNamee *et al.*, 1993; Chong *et al.*, 1994). Since PIP2 can regulate actin-binding proteins such as profilin (Theriot and Mitchinson, 1993), the increased amounts of PIP2 induced by integrin engagement could be important for the adhesion-dependent polymerization of actin.

6.2.5. Ca^{2+} signalling

Increase in the intracellular calcium concentration was observed in certain cell types following cell spreading on a specific ECM substrate (Schwartz, 1993). This response was dependent on the presence of extracellular Ca^{2+} and occured independently of the integrin-triggered rise in pH. Furthermore, adhesion-dependent calcium intake by cells is suggested to regulate cell migration, since migration of cells on vitronectin is stimulated by Ca^{2+} in certain systems (Grzesiak *et al.*, 1992). In other instances adhesion-dependent increase in the intracellular Ca^{2+} involves PLCγ-mediated, IP3-induced calcium mobilization from the endoplasmic reticulum (Kanner *et al.*, 1993; Clapman *et al.*, 1995).

6.2.6. Na^+/H^+ antiporter

Spreading of normal fibroblasts on fibronectin has been shown to lead to activation of the Na^+/H^+ antiporter activity and an increase in the intracellular pH (Schwartz *et al.*, 1991). Occupation of the integrin receptor as well as receptor clustering are required for the stimulation of the Na^+/H^+ antiporter. In tumour cells, anchorage-independence correlates strongly with their ability to maintain an alkaline pH, which is independent of cell adhesion to ECM (Schwartz *et al.*, 1990). Furthermore, transfecting tumour cells with a gene encoding for a yeast proton pump (yeast H^+-ATPase), which artificially elevates pH, is sufficient to induce anchorage-independent growth (Perona and Serrano, 1988).

6.2.7. Tyrosine phosphorylation

Adhesion of cells to ECM results in tyrosine phosphorylation of multiple cellular proteins (Schaller *et al.*, 1992; Hanks *et al.*, 1992; Kapron-Bras *et al.*, 1993). Several tyrosine kinases have been implicated in integrin signalling because of their integrin-dependent activation and/or because of their localization to the FAP's. The Focal Adhesion Kinase (FAK) has been most extensively studied. FAK phosphorylates itself as well as cytoskeletal proteins such as paxillin and tensin (Schaller and Parsons, 1994; Richardson and Parsons, 1995). The consequence of FAK autophosphorylation is maximal kinase activity and creation of binding sites for other proteins. Tyr-397 has been shown to be the major autophosphorylation site both *in vivo* and *in vitro* (Calalb *et al.*, 1995; Schaller *et al.*, 1994). Phosphorylation of this residue stimulates FAK kinase activity and directs SH2-dependent binding of $pp60^{src}$ (Calalb *et al.*, 1995; Schaller *et al.*, 1994). Once $pp60^{src}$ is bound to FAK it phosphorylates other FAK tyrosine residues: Tyr-407, Tyr-576, Tyr-577 and Tyr-925. The first three represent additional binding sites for Src-family kinases, whereas Tyr-925 is thought to localize the SH2 domain adaptor protein, Grb-2 (Calalb *et al.*, 1995; Schaller *et al.*, 1994). The latter event is suggested to couple integrins to the Ras-dependent MAPK pathway (Schlaepfer *et al.*, 1994). Indeed, integrin-dependent activation of MAP kinase has been reported by several groups (Chen *et al.*, 1994, Zhu and Assoian, 1995). Interestingly, in some cell systems activation of MAP kinase in response to ECM signal was found to be independent of Ras activation, suggesting an integrin-specific pathway leading to the activation of MAP kinase (Chen *et al.*, 1996). However, in some cases FAK does not participate in the coupling of the ECM signals to the growth-regulatory signalling cascades. Signalling triggered by $\alpha_6\beta_4$ laminin receptor leads to direct association of the adaptor protein Shc with β_4 phosphorylated cytoplasmic domain (Mainiero *et al.*, 1995). Shc in turn binds the

Grb-2 adaptor protein, which could then activate the Ras pathway via Sos (Mainiero *et al.*, 1995). Recently, it has been reported that the recruitment of Shc is specified by the extracellular or transmembrane domain of integrin α subunit and that this process is mediated by caveolin (Wary *et al.*, 1996). Furthermore, association of Shc with integrins couples integrin-dependent adhesion to cell cycle progression (Wary *et al.*, 1996). Another example of FAK-independent integrin signalling comes from monocytes, where Syk is the integrin-responsive tyrosine kinase (Lin *et al.*, 1994).

FAK associates with a number of cytosolic and cytoskeletal proteins, some of which are tyrosine phosphorylated by FAK (Schaller and Parsons, 1995). The cytoskeletal protein paxillin is phosphorylated on tyrosine upon FAK activation (Schaller and Parsons, 1995). In this way paxillin can provide binding sites for SH2 domains of Csk kinase and Crk adaptor protein (Tobe *et al.*, 1996;). Csk (C-terminal Src-related kinase) negatively regulates the kinase activity of c-Src by phosphorylation of a C-terminal tyrosine of c-Src (Tobe *et al.*, 1996). Cells lacking Csk display activated Src kinase localized in the FAP's, suggesting that Csk may regulate both kinase activation and subcellular localization of Src. Recently, another cytosolic protein, $p130^{Cas}$ (Crk associated substrate), was identified in the multimolecular complex of FAP's and shown to bind directly to FAK as well as to pp41/43FRNK (Focal Adhesion Related Non-Kinase) both *in vivo* and *in vitro* (Harte *et al.*, 1996, Vuori *et al.*, 1996). $p130^{Cas}$ has the structural characteristics of an adaptor protein, containing multiple consensus SH2 domain binding sites, an SH3 domain, and a proline-rich domain. Such a structure suggests that $p130^{Cas}$ may act to provide a framework for protein-protein interactions. $p130^{Cas}$ becomes tyrosine phosphorylated upon integrin-mediated cell adhesion (Vuori and Ruoslahti, 1995) and is a potential substrate for Src kinase, since cells in which Src kinases are activated display enhanced phosphorylation of

$p130^{Cas}$ (Vuori *et al.*, 1996).

Another possible target of FAK could be PI-3 kinase, since stable association between these two molecules was observed in mouse fibroblasts (Chen and Guan, 1994). Furthermore, the p85 subunit of PI-3 kinase is tyrosine phosphorylated during cell adhesion and can be directly phosphorylated by FAK *in vitro*. FAK/PI-3 kinase association may be mediated by interaction of FAK phosphotyrosine residues with PI-3 kinase SH2 domains, so that autophosphorylation of FAK upon integrin/ligand binding would lead to recruitment of PI-3 kinase (Chen and Guan, 1994). As a result PI-3 kinase will be translocated to the plasma membrane and juxtaposed to its physiological target.

PART II: PRINCIPLES OF CELL CYCLE REGULATION

Progression through the cell cycle is tightly regulated at the transitions between G1→S, G2→M phases of the cell cycle. Principal regulatory molecules of the cell cycle are the cyclin-dependent kinases (CDK's). The activity of CDK's can be stimulated by binding to a cyclin partner and phosphorylation of a conserved threonine residue in the C-terminus by CDK-activating kinase (CAK) (Pines, 1993; Morgan, 1995). Each cyclin is expressed during a specific stage of the cell cycle and quickly degraded after it's function is accomplished (Pines, 1993; Morgan, 1995) (Diagram 3a). The D-type cyclins are expressed during G1 phase and bind predominantly to cdk4 to form an active Cyclin D-cdk4 complex (Hunter and Pines; 1994). Both Cyclin E and Cyclin A associate with cdk2 and are essential for entry and progression of S phase, respectively. Cyclin E functions during G1/S and early S phase (Dulic *et al.*, 1992), whereas cyclin A levels culminate at S phase and continue to be active during G2 (Hartwell and Kastan, 1994). Cyclin A and B coupled to cdc2 during G2 and early M phase are essential for mitosis (Hartwell and Kastan, 1994). Inhibition of the active cyclin-cdk complexes is achieved by phosphorylation of a conserved threonine-tyrosine pair in the N-terminus of cdk or by binding to CDK inhibitory molecules, CKI's (Cyclin-dependent Kinase Inhibitors). Two families of CKI's can be distinguished based on their structure and specificity. The INK4 family (p15, p16, p18, p19) is specific for cdk4- and cdk6-cyclin complexes and inhibits when one or more inhibitor molecules binds to the complex (Sherr and Roberts, 1995). The second family (p21, p27, p57) of inhibitors bind to all cyclin-cdk complexes and inhibit only when there are two or more molecules of CKI associated with the complex (Sherr and Roberts, 1995).

The commitment of cells to progress through the cell cycle occurs at the restriction (R) point late in G1 phase. Once the cells pass the R point, they are committed to complete cell

Diagram 3a. Cell Cycle Regulation

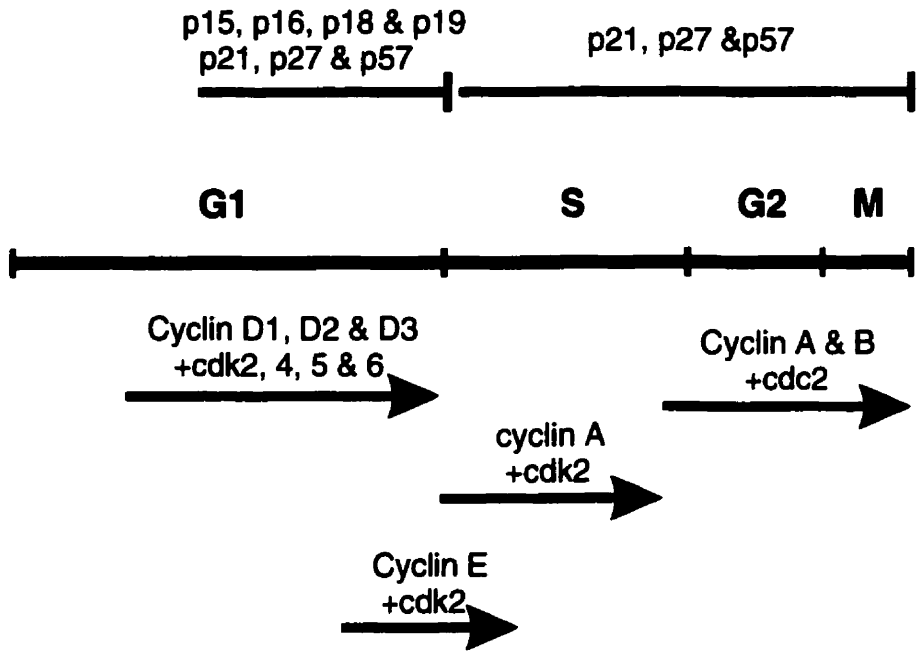
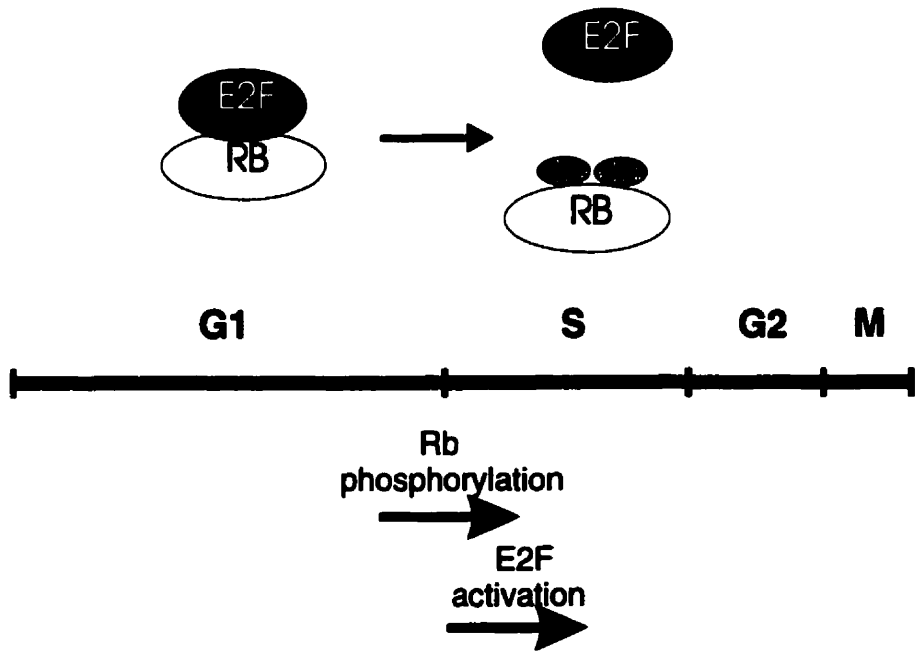


Diagram 3b. Regulation of E2F Activity



division, regardless of the presence of growth factors (Pardee, 1989).

The complexity of molecular events underlying the G1/S transition is not well understood. A key event for cell cycle progression is hyperphosphorylation of the retinoblastoma protein (Rb) (Diagram 3b). CyclinD-cdk4/6 and CyclinE-cdk2 are the principle kinases which phosphorylate Rb (Dulic *et al.*, 1992; Dowdy *et al.*, 1993). Rb is hypophosphorylated in G1 and bound to the transcription factor E2F. Upon phosphorylation of Rb, E2F is released and activates transcription of genes required for the transition into S phase.

Adherent cells require attachment to substratum in order to progress through the cell cycle. Recently, it was shown that cells attached to the ECM contain active cyclin D1-cdk and cyclin E-cdk complexes as well as hyperphosphorylated Rb, which allow them to progress through the G1/S restriction point (Fang *et al.*, 1996; Zhu *et al.*, 1996). In contrast, cells kept in suspension have decreased cyclin D1-cdk and cyclin E-cdk kinase activities, and, as a result of that, hypophosphorylated Rb. The ultimate consequence of the latter events is a G1 phase block and inability to complete a cell cycle. Enforced expression of cyclin D1 rescues Rb phosphorylation and entry into S phase when G1 cells are cultured in the absence of substratum (Zhu *et al.*, 1996). Another cell cycle event that has been linked to adhesion is the expression of cyclin A (Guadagno *et al.*, 1993; Schulze *et al.*, 1996). The appearance of cyclin A mRNA and protein in late G1 was found to be dependent on cell adhesion in NRK and NIH 3T3 fibroblasts (Guadagno *et al.*, 1993). Abrogation of the anchorage-dependent transcription of the cyclin A gene has been shown to be a result of blocking its promoter activity through G0-specific E2F complexes (Schulze *et al.*, 1996). Overexpression of cyclin D1 restores cyclin A transcription in cells in suspension and rescues them from cell cycle arrest (Schulze *et al.*, 1996). The exact pathways via which integrin-mediated adhesion regulate cell cycle control are not well

understood, although there are implications of MAP kinase involvement (Zhu and Assoian, 1995; Lavoie *et al.*, 1996). The receptor proximal, integrin linked kinase (ILK), may mediate these signals, since ILK overexpression in epithelial cells leads to anchorage-independent cell cycle progression and survival (Radeva *et al.*, submitted; ChapterII).

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CHAPTER II

The data contained herein have been submitted for publication as:

OVEREXPRESSION OF THE INTEGRIN LINKED KINASE (ILK) PROMOTES ANCHORAGE-INDEPENDENT CELL CYCLE PROGRESSION

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1. INTRODUCTION

Normal, untransformed epithelial cells require anchorage to a substratum for cell growth and survival. Adhesion to the extracellular matrix (ECM) is required for progression of cells through the G1 and into the S phase of the cell cycle. When forced to remain in suspension such cells become arrested in the G1 phase of the cell cycle and undergo apoptosis (Frisch and Francis, 1994; Meredith *et al.*, 1993; Boudreau *et al.*, 1995). Oncogenic transformation frequently induces anchorage-independent growth, *in vitro*, and is a specific correlate of tumor growth *in vivo* (Shin *et al.*, 1975; Ruoslahti and Reed, 1994).

In fibroblasts, cell adhesion has recently been demonstrated to regulate cell cycle progression by inducing the expression of cyclin D1 (Zhu *et al.*, 1996), the activation of cyclin E-cdk2 (Zhu *et al.*, 1996; Fang *et al.*, 1996), and phosphorylation of Rb (Zhu *et al.*, 1996). Fibroblast adhesion also results in the down-regulation of expression of the cdk inhibitor proteins, p21 and p27 (Zhu *et al.*, 1996; Fang *et al.*, 1996). The combined adhesion-dependent elevation in cyclin D1 and decrease in the expression of p21 and p27 results in the stimulation of cyclin D-cdk4 and cyclin E-cdk2 activities, both of which can phosphorylate Rb. This latter event relieves restriction of the entry of cells into S phase, presumably by the release of the transcription factor E2F from phosphorylated Rb (Johnson *et al.*, 1993; Sherr, 1996). In some cell types the expression of cyclin A is also regulated in an anchorage-dependent manner (Boudreau *et al.*, 1995; Schulze *et al.*, 1996; Kang and Kraus, 1996), and activated Ras-induced anchorage-independent growth has been shown to depend on cyclin A expression (Kang and Kraus, 1996). However cyclin D1 expression and cyclin E-dependent kinase activity are also dependent on Ras activation. Although mitogens can also activate cyclin D- and cyclin E-dependent kinases, cell adhesion *per se* can regulate these activities. The regulation of G1 cdks,

therefore requires the convergence of signals from both growth factors as well as the ECM.

Anchorage of cells to the ECM is mediated to a large extent by integrins, a large family of heterodimeric cell surface receptors (Schwartz *et al.*, 1995; Hynes, 1992). The interaction of integrins with ECM ligands results in the transduction of intracellular signals leading to stimulation of tyrosine phosphorylation (Clark and Brugge, 1995; Dedhar and Hannigan, 1996), turnover of phosphoinositides (McNamee *et al.*, 1993), and activation of the Ras-MAP Kinase (MAPK) pathways (Kapron-Bras *et al.*, 1993; Chen *et al.*, 1994; Clark and Hynes, 1996; Chen *et al.*, 1996). The activation of MAPK by cell adhesion is dependent on the presence of an intact actin cytoskeleton (Zhu and Assoian, 1995), as well as activated $p21^{rho}$ (Hotchin and Hall, 1995). Presumably the adhesion-dependent stimulation of cyclin A expression and the cyclin D1- and cyclin E-cdks is also mediated via integrins, although whether this requires the activation of MAPK is not clear as yet. The cytoplasmic domain of the integrin β_1 subunit is required for many of the integrin mediated signalling events (Schwartz *et al.*, 1995; Akiyama *et al.*, 1994; Lukashev *et al.*, 1994).

Integrin-proximal events involved in the initiation of integrin-mediated signal transduction are still poorly understood. However, a novel ankyrin-repeat containing serine-threonine protein kinase (ILK) has recently been demonstrated to associate with the integrin β_1 and β_3 subunit cytoplasmic domain (Hannigan *et al.*, 1996) and may be involved in regulating integrin-mediated signalling. Overexpression of ILK in intestinal epithelial cells results in an altered cellular morphology, reduction in cell adhesion to ECM and also in the stimulation of anchorage-independent growth in soft agar (Hannigan *et al.*, 1996). Such constitutively ILK overexpressing cells are also tumorigenic in nude mice (Wu *et al.*, submitted).

We now report that overexpression of ILK in rat intestinal epithelial cells (IEC18)

stimulates the expression of cyclin A, cyclin D1 and cdk4 proteins. The activities of both cyclin D1-cdk4 and cyclin E-cdk2 kinases are also elevated, resulting in hyperphosphorylation of the Rb protein. In addition, both p21 and p27 inhibitors of cyclin-cdks have altered electrophoretic mobilities and p27 from ILK overexpressing cells has reduced inhibitory activity as compared to p27 from the parental IEC18 cells. Furthermore, whereas cyclin A and cyclin D1 protein expression, and Rb phosphorylation, are downregulated upon transfer of IEC18 cells to suspension culture, they are constitutively upregulated in ILK overexpressing cells kept in suspension. ILK overexpression in these epithelial cells thus overrides the adhesion-dependent regulation of cell cycle progression through G1 and into S phase, indicating that ILK may be a key regulator of integrin-mediated cell cycle progression.

2. MATERIALS AND METHODS

2.1. Cell culture

Three sets of cell lines were used throughout this study: IEC18, ILK13 and ILK14. IEC18 is an immortalized non-tumorigenic rat intestinal epithelial cell line (Quaromi and Isselbacher, 1981), cultured in α-MEM medium supplemented with 2 mM L-glutamine (Gibco/BRL), 3.6 mg/ml glucose (Sigma), 10 μg/ml insulin (Sigma), and 5% FCS (Gibco/BRL). ILK13 cells were engineered to overexpress ILK by stably transfecting the parental IEC18 cells as described previously (Hannigan *et al.*, 1996). ILK14 cells are control transfectants (Hannigan *et al.*, 1996). Both ILK13 and ILK14 cell lines were grown under the same conditions as the parental IEC18 with addition of 200 μg/ml G418 Geneticin (Gibco/BRL) to maintain a selection pressure for ILK or control vector respectively. Two independently derived clones of each ILK13 (A1a3 and A4a) and ILK14 (A2c3 and A2c6) were used.

2.2. Growth curves

IEC18, ILK13 (ILK overexpressing cells) and ILK14 (control transfectants) cells were harvested from tissue culture, counted and 10^4 cells from each cell line were plated in 35mm tissue culture plates (Nunc). Cells were grown in α-MEM medium as described above under different serum concentrations (FCS, Gibco/BRL) for various numbers of days. At each time point, adherent cells were harvested with 5 mM EDTA/PBS (Phosphate Buffered Saline, pH 7.6) and viable cells were quantitated by Trypan Blue exclusion.

2.3. Suspension-maintained cells

Asynchronously growing cells were harvested from monolayer culture using 5 mM

EDTA/PBS and washed twice in PBS. Cells were then resuspended in 5% FCS containing α-MEM medium (see "tissue culture") and transferred to 50 ml rocker tubes. A short burst of CO_2 was given to the cells before tubes were capped. Suspension cells were incubated for 12 hrs, rotating on a nutator at 37°C in 5% CO_2. After that cells were either fixed for FACS analysis or alternatively cell pellets were recovered, washed twice in ice-cold PBS and then lysed in NP-40 lysis buffer (see below).

2.4. Cell cycle analysis

Cells were collected, washed in ice-cold PBS (pH 7.6), fixed in 70% ethanol for 1 hr on ice, rinsed with PBS, and DNA stained with 50 μg/ml Propidium Iodide in PBS containing 10 μg/ml RNase for 30 min. at room temperature. Cell cycle profiles were analyzed by fluorescence activated cell analyzer (FACS) using Becton Dickinson FACScan analyzer and the percentage of cells in the various phases of cell cycle was calculated using CellFit Software.

2.5. Immunoblotting

Cells grown in monolayer or in suspension were lysed in ice-cold NP-40 lysis buffer (1% NP-40; 150 mM NaCl; 50 mM Tris, pH 7.6; 1 mM EDTA) plus inhibitors (0.1 mM PMSF, 20 μg/ml aprotonin, 20 μg/ml leupeptin) or in ice-cold Tween-20 lysis buffer (0.1% Tween-20; 50 mM Hepes, pH 7.5; 150 mM NaCl; 2.5 mM EGTA; 1 mM EDTA) plus inhibitors (1 mM DTT, 0.1 mM PMSF, 20 μg/ml aprotonin, 10 mM β-glycerophosphate, 0.1 mM sodium vanadate, 1 mM sodium fluoride).

Total protein extracts or immune-complexes were resolved on SDS-PAGE and then

separated proteins were transferred to Immobilon-P (Millipore). The membrane was first blocked in 5% Milk in TBST (0.05% Tween-20, Sigma, in Tris Buffered Saline, pH 7.4) and then incubated with the primary antibody of choice. The following antibodies have been used: anti-cyclin D1 (DCS-6, mouse monoclonal, from Dr. J. Bartek, Danish Cancer Society, Denmark), anti-cyclin E (rabbit polyclonal, Santa Cruz), anti-cyclin A (rabbit polyclonal, Santa Cruz), anti-cdk4 (rabbit polyclonal, Santa Cruz), anti-cdk2 (rabbit polyclonal, Santa Cruz), anti-PSTAIRE (mouse monoclonal, a gift from Dr. S. Reed, Scripps Res. Inst., La Jolla, CA), anti-p27 (mouse monoclonal, Transduction Labs), anti-p21 (rabbit polyclonal, Santa Cruz), anti-retinoblastoma (mouse monoclonal, Pharmingen), anti-ILK (affinity purified rabbit polyclonal). Detection was carried out using secondary antibody (either anti-mouse-HRP [Jackson Labs or Pharmingen], anti-rabbit-HRP [Jackson Labs] or protein A-HRP [Amersham Life Sciences]) and enhanced chemiluminescence (ECL) detection system (Amersham Life Sciences).

2.6. Kinase assays

For cdk4-associated kinase activity asynchronous cells growing in monolayer culture were scrape-lysed in ice-cold Tween-20 lysis buffer (0.1% Tween-20; 50 mM Hepes, pH 7.5; 150 mM NaCl; 2.5 mM EGTA; 1 mM EDTA), containing the following inhibitors (1 mM DTT, 0.1 mM PMSF, 20 μg/ml aprotonin, 10 mM β-glycerophosphate, 0.1 mM sodium vanadate, 1 mM sodium fluoride). Cell lysates were then sonicated. Protein A-Sepharose beads (Sigma Immunochemicals Co.), precoated with anti-cdk4 Ab (rabbit polyclonal, Santa Cruz) were used to immunoprecipitate cdk4. Cdk4-associated kinase activity was assayed using the protocol of Matsushime *et al.* (1994).

For Cyclin E kinase assays, cells from asynchronous monolayer culture were lysed in 1%

NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris, pH 7.6) plus inhibitors (0.1 mM PMSF, 20 μg/ml aprotonin, 20 μg/ml leupeptin). Cyclin E was immunoprecipitated with polyclonal anti-cyclin E serum (gift from Dr. S. Reed, Scripps Res. Inst., La Jolla, CA and also from Dr. D. Agrawal, M. Lee Moffit, Cancer Center, Tampa, FL) and complexes collected on protein A-Sepharose beads (Sigma Immunochemicals Co.). Cyclin E-associated kinase reactions were carried out as described previously (Dulic *et al.*, 1996).

For both cyclin D1-cdk4 and cyclin E-cdk2 assays, kinase reaction products were resolved by SDS-PAGE and the incorporation of radioactivity into substrate was visualized by autoradiography (Kodak, X-OMAT AR or Dupont, REFLECTION™) and quantitated by phosphoimager analysis (Molecular Dynamics).

2.7. p27 inhibitory assay

Cell lysates (100 μg) protein were recovered from asynchronously growing IEC18 or ILK overexpressing cells using lysis buffer as for cyclin E-cdk2 kinase assay with NP-40 at 0.1% concentration. Lysates were boiled for 5 min. and clarified by centrifugation. p27 was immunoprecipitated (rabbit polyclonal serum provided by Dr. T. Hunter, Salk Institute, CA) from boiled lysates. Immune-complexes were collected on protein A-Sepharose beads and then washed five times in 0.1% NP-40 lysis buffer. To release bound p27, the beads were resuspended in 200 μl 0.1% NP-40 lysis buffer, containing protease inhibitors (1 mM PMSF and 20 μg/ml each of aprotonin, leupeptin and pepstatin), boiled for 5 min. and supernatants recovered. Cyclin A-cdk2 complexes immunoprecipitated from asynchronous ILK14 cells (control transfected cells) were used as test substrate for inhibition by p27. Heat stable p27 released from immune complexes was incubated at 30^{0}C for 30 min. together with cyclin A-

cdk2. Cyclin A-cdk2 kinase activity was assayed using histone H1 (Boehringer Mannheim) as a substrate and compared to the activity of cyclin A-cdk2 complexes without added immunoprecipitated p27. As a negative control, non-immune serum immunoprecipitates were collected, boiled and supernatant added to active cyclin A-cdk2 test complexes. The p27 antiserum used in these assays does not cross react with p21. Detection of radioactivity in kinase substrate was carried out as described for kinase assays.

3. RESULTS

3.1. ILK overexpression induces adhesion-independent cell growth and survival but not serum-independent growth

Our laboratory has previously shown that overexpression of the Integrin Linked Kinase (ILK) in normal rat intestinal epithelial cells (IEC18) results in a less adherent phenotype and in anchorage-independent growth in soft agar (Hannigan *et al.*, 1996). When maintained in suspension, the IEC18 cells have been demonstrated to undergo programmed cell death (Rak *et al.*, 1995), which was suppressed by mutant *c-H-ras* oncogene expression (Rak *et al.*, 1995). Since we have found that ILK overexpression in IEC18 cells induces anchorage-independent growth as well as tumorigenicity in nude mice (Wu *et al.*, submitted), we wished to determine whether ILK overexpression also suppresses suspension-induced cell death and prevents suspension-induced cell cycle arrest. ILK overexpressing cell clones (ILK13) were capable of anchorage-independent cell growth in soft agar (Hannigan *et al.*, 1996) and were also able to survive in suspension over a longer time period than the control transfected IEC18 clones (ILK14) in which ILK expression levels are similar to the parental IEC18 cells (data not shown). This increased cell survival was reflected in the greater proportion of ILK13 cells which were present in S phase after 12 hours in suspension, as compared to the control ILK14 cells, in which the percentage of cells in S phase fell to 5% (Fig. 1a). Furthermore, a sub-G1 (<2n) peak is present in the control ILK14 cells after 12 hours in suspension, consistent with the presence of apoptotic cells. This population of cells was completely absent in the ILK overexpressing ILK13 clones (Fig. 1a). Although ILK overexpression clearly renders these epithelial cells anchorage-independent for cell growth and survival, we had found that in regular monolayer cultures the ILK13 cells did not appear to have a growth advantage over the parental or control

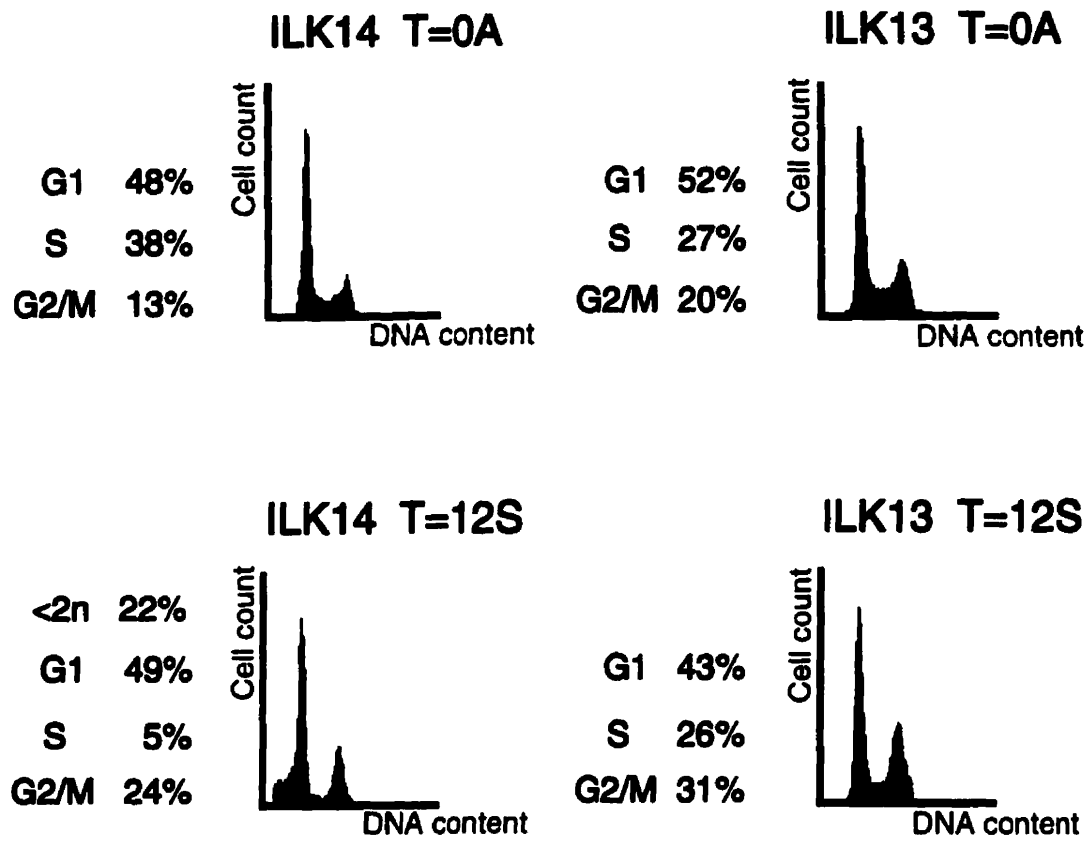

Fig. 1 a) Cell cycle profiles of ILK13 and ILK14 cells maintained in suspension or monolayer culture. Asynchronously growing ILK overexpressing (ILK13) and control transfected (ILK14) cells (Hannigan *et al.*, 1996) were transferred from monolayer to suspension culture for 12 hr as described in Materials and Methods. The cell cycle profiles of the cells in suspension (S) or in monolayer (A) were analyzed by FACScan (see Materials and Methods section) and compared. The numbers on the left represent the percentage of cells in each phase of the cell cycle.

(ILK14) cells. We therefore wanted to determine whether ILK overexpression induced serum-independent growth in monolayer adherent cultures. As shown in Fig. 1b, the growth rate of ILK13 cells was not elevated when compared to the IEC18 or the control ILK14 cells. In fact, the ILK overexpressing clones grew slightly more slowly than the parental IEC18 and the ILK14 control transfected cells (Fig.1b). In addition, ILK13 cells failed to survive in serum-free conditions similar to the IEC18 and control ILK14 cells. These data demonstrate that ILK overexpression selectively induces anchorage-independent growth but not serum (mitogen)-independent growth.

3.2. ILK overexpression alters the expression of cell cycle regulators

Adhesion of fibroblasts to ECM has been shown to induce the expression of cyclin D1 (Zhu *et al.*, 1996). Since overexpression of ILK in epithelial IEC18 cells induces cell survival and cell cycle progression in the absence of adhesion, we wanted to determine whether ILK overexpression altered the expression and/or activity of cell cycle regulators. The expression of various cell cycle regulators was examined in IEC18, ILK13 and ILK14 cells growing under standard tissue culture conditions. As shown in Fig. 2a, ILK overexpressing cell clones (ILK13) (Hannigan *et al.*, 1996) expressed substantially higher levels of cyclin D1 protein than the parental IEC18, or control transfected ILK14 cells. In contrast, the level of expression of cyclin E was not altered in ILK13 cells. The expression of cyclin A was examined as well and was found to be elevated in ILK13 cells (data not shown in Fig. 2, see Fig. 4). Since the cyclins function as a complex with the cyclin dependent kinases, we also determined the expression of cdk4 and cdk2 kinases which complex with cyclin D1 and cyclin E, respectively. Surprisingly, the level of expression of cdk4 protein was also elevated in the ILK13 cells, whereas the level

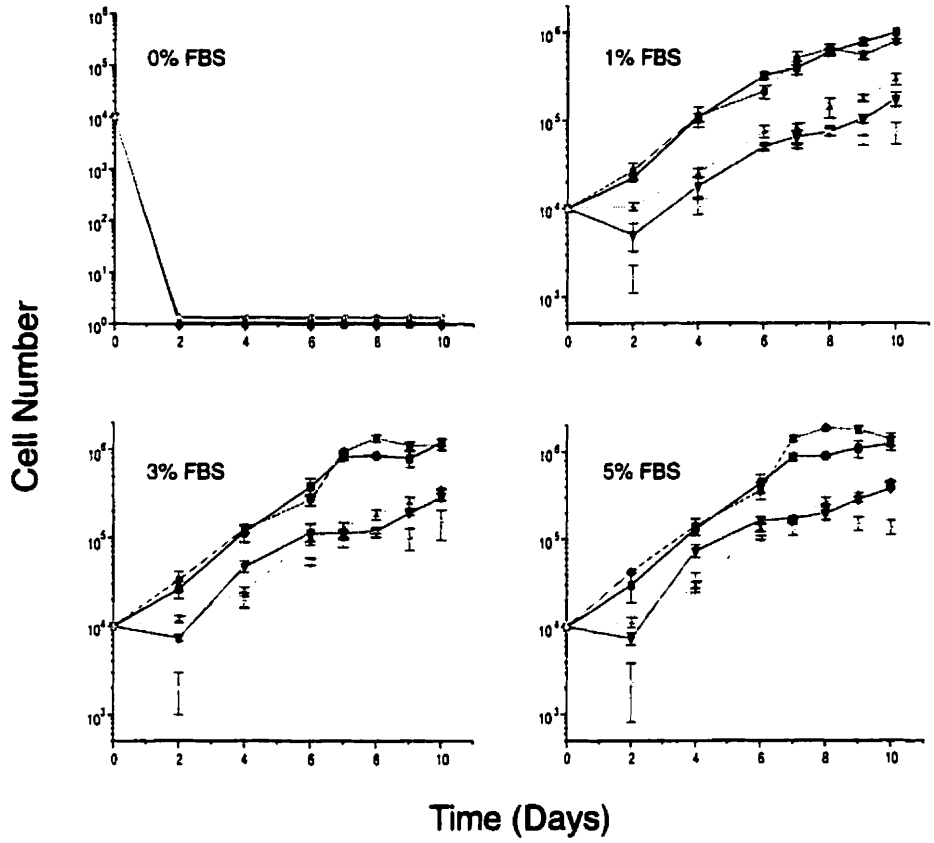

Fig. 1 b) Growth rates of IEC18, ILK13 and ILK14 cells at various serum concentrations. 10^4 cells from each cell line were plated on 35 mm tissue culture plates under various serum concentrations. At different time points adherent cells were harvested and number of viable cells was determined by Trypan Blue exclusion. Cell lines correspond as follows: IEC18-■, ILK14(A2c3)-●, ILK14(A2c6)-△, ILK13(A1a3)-▼, ILK13(A4a)- ◇

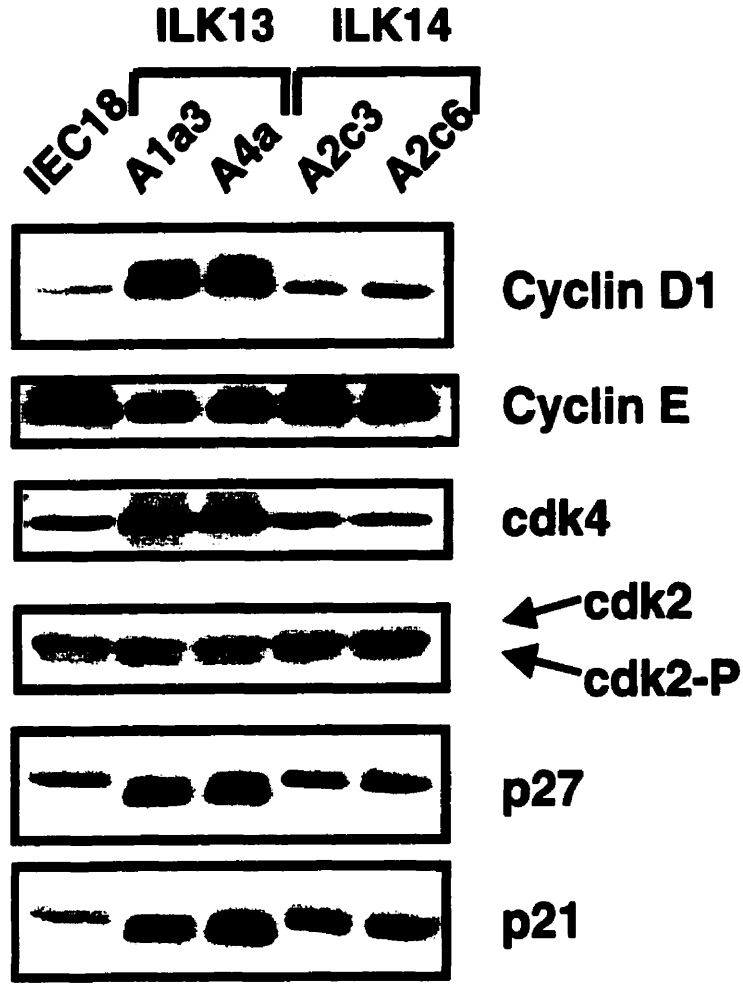

Fig. 2 a) Alteration in the expression levels of the constituents of the G1/S cyclin-cdk complexes. Immunoblot analysis of the various cell cycle regulators was carried out as described in Materials and Methods. Two independently derived ILK overexpressing clones (ILK13: A1a3 and A4a) (Hannigan *et al.*, 1996) and the control transfectants (ILK14: A2c3 and A2c6) (Hannigan *et al.*, 1996) were tested and compared to the parental IEC18 (rat intestinal epithelial cell line). The levels of cyclin D1 and cdk4 proteins were increased in the ILK overexpressing cells, while no difference in the amount of cyclin E and cdk2 proteins was observed. Cyclin-cdk inhibitory proteins, p21 and p27, were found to have an altered mobility in the ILK overexpressing cells.

of cdk2 was not altered (Fig. 2a). The kinase activities of cdk4 and cdk2 are also regulated by inhibitor proteins, p21 and p27, and expression of these inhibitors is known to be enhanced in non-adherent (suspension) cells and decreases upon cell adhesion (Zhu *et al.*, 1996; Fang *et al.*, 1996). In ILK overexpressing cells, the level of expression of both p21 and p27 was increased (Fig. 2a). However, their electrophoretic mobilities were clearly altered in ILK13 clones. The faster migrating forms of p21 and p27 in ILK overexpressing cells may reflect covalent modification, or in the latter, the product of partial proteolytic cleavage (Loda *et al.*, 1996). For p27, at least, this alteration correlates with a decreased inhibitory potential (see Fig. 3b).

In order to demonstrate that the observed changes were mediated by ILK, we transfected IEC18 cells with an ILK expression vector under the control of a metal inducible promoter (Filmus *et al.*, 1992). As shown in Fig. 2b, induction of ILK expression with Zn^{2+}/Cd^{2+} resulted in the stimulation of expression of ILK. Concomitantly, the expression of cyclin D1 was also induced in these cells (Fig. 2b). The data shown were confirmed in two independent clones and the treatment of the parental IEC18 cells with Zn^{2+}/Cd^{2+} had no effect on ILK or cyclin D1 expression (data not shown). These data demonstrate that increased ILK expression can induce expression of cyclin D1 protein.

We next determined whether the complex formation between the cyclins, cdks and the p21/p27 inhibitors was also altered upon ILK overexpression. As shown in Fig. 2c, both cyclin D1 and cdk4 were elevated in cdk4 immunoprecipitates from ILK13 cells as compared to the parental IEC18 and control ILK14 cells. Although the amount of p27 was also higher in the immunoprecipitates from ILK13 cells, quantification clearly demonstrated that the ratio of p27 to cyclin D1 was much higher in IEC18 and ILK14 cells than it was in the ILK13 cells (Table 1), in which cyclin D1 was present in greater amounts. Furthermore, p27 in the cdk4

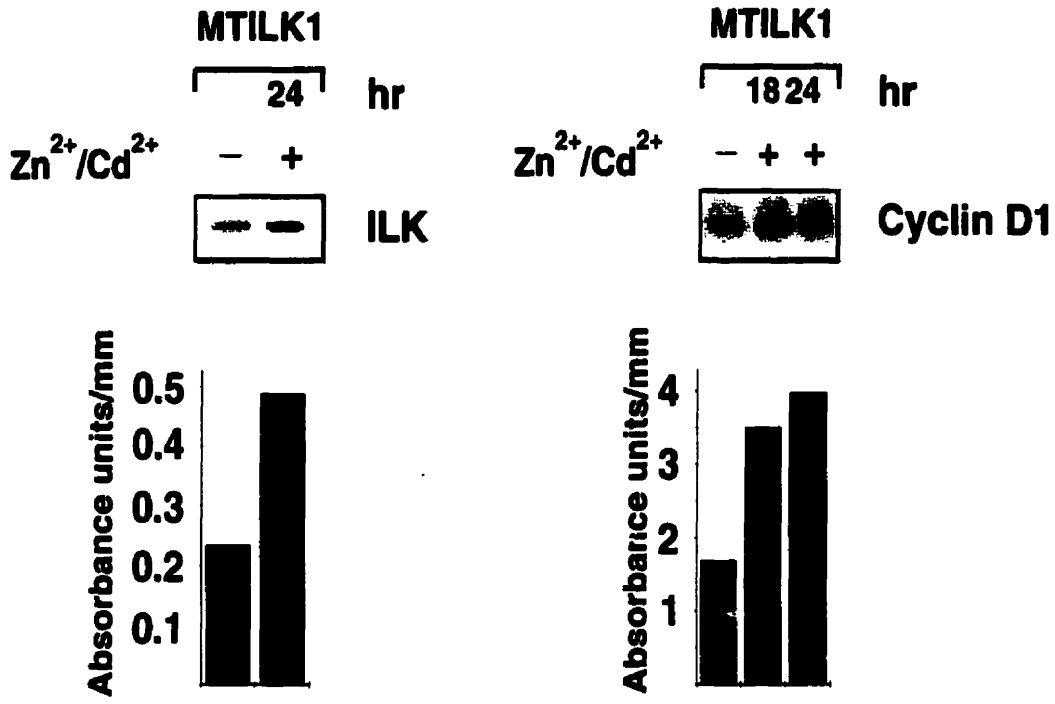

Fig. 2 b) Cyclin D1 overexpression is specifically triggered by ILK overexpression. IEC18 cells were transfected with a metallothionine inducible construct (Filmus *et al.*, 1992) containing the complete ILK gene inserted in-frame (MTILK1). Transfection was performed using Lipofectin, as per the manufacturer's instructions (Gibco/BRL). Transfected cells were cloned by limiting dilution in 96 well tissue culture plates (Nunc). Metallothionine inductions were performed in the presence of serum supplemented with 100 µM $ZnSO_4$ and 2 µM $CdCl_2$ for 18 to 24 hours. After induction, cells were lysed in NP-40 lysis buffer and ILK and cyclin D1 levels screened by immunoblot analysis. ILK expression was induced in the MTILK1 clone (containing plasmid vector with cDNA encoding for ILK) following treatment of the cells with Zn^{2+}/Cd^{2+}. Concomitantly, the expression of cyclin D1 protein was also induced. ILK and cyclin D1 protein levels were quantified by densitometric analysis using a LKB Laser Densitometer (Model 2222-020) using Gelscan XL Software (Pharmacia).

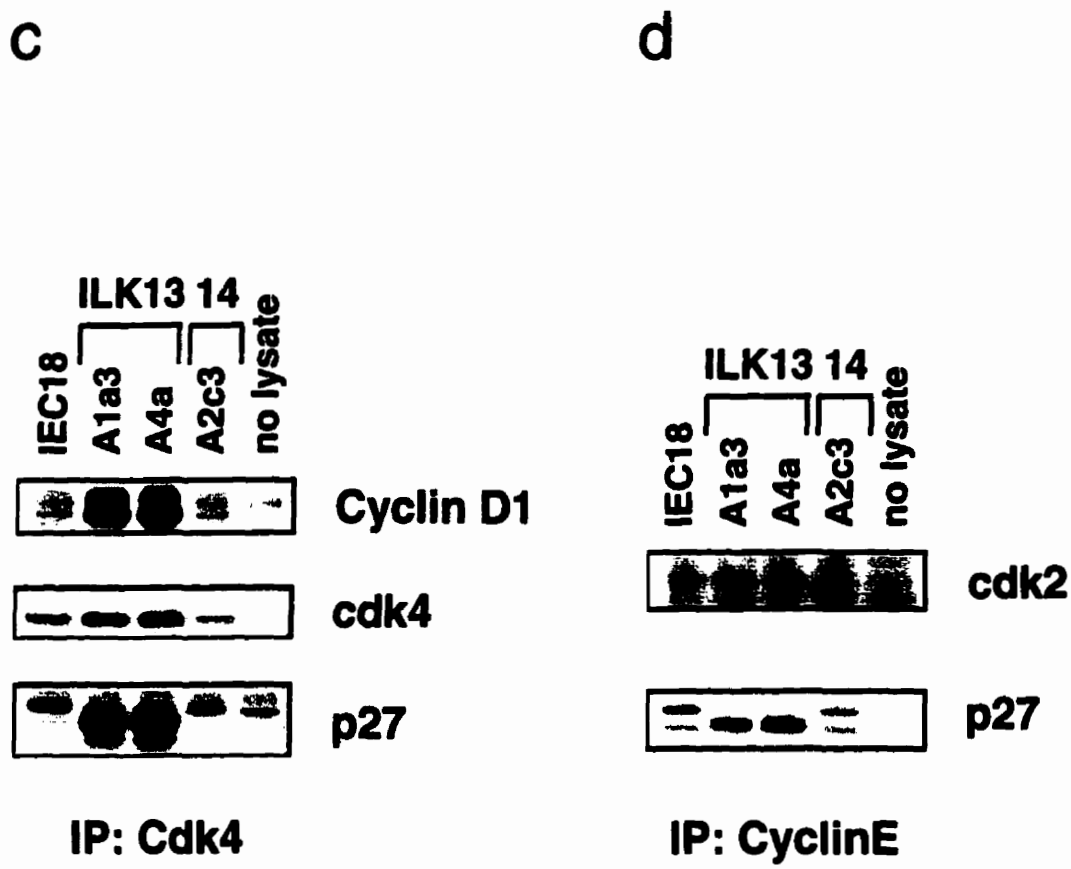

Fig. 2 c) Immunoblot analysis of cyclin D1-cdk4 complex. Cdk4 was immunoprecipitated from each of the cell lines described and then associated cyclin D1, cdk4 and p27 detected by immunoblotting. ILK13 cells show a higher content of cyclin D1, cdk4 and p27 in the immunoprecipitated cyclin D1-cdk4 complex. Fig. 2 d) Immunoblot analysis of cyclin E-cdk2 complex. Cyclin E was immunoprecipitated from each of the cell lines described and then associated cdk2 and p27 were detected by immunoblotting.

	IEC18	ILK13 (A1a3)	ILK13 (A4a)	ILK14 (A2C3)
p27/cyclin D1 Ratio	2.44:1	0.73:1	0.94:1	4.3:1

TABLE 1 The amounts of cyclin D1 and p27 proteins in the cyclin D1-cdk4 complexes were quantitated by densitometry using a LKB Laser Densitometer (Model 2222-020) and Gelscan XL Software (Pharmacia). The densitometric values for a given protein were obtained after subtracting the value present in the negative control (antibody alone lane). The ratios of intensities of p27/cyclin D1 were calculated for each cell line. The exposure of the film on which the scanning was done was in the linear range of ECL.

immunoprecipitates from ILK13 clones had faster electrophoretic mobility (Fig. 2c). The amount of cyclin E-associated cdk2 did not differ between the parental IEC18 and ILK overexpressing, ILK13 cells. However, in the ILK13 cells, although cyclin E-associated p27 was increased, p27 manifested the altered mobility seen in the cdk4 complexes (Fig. 2d).

3.3. ILK overexpression leads to the stimulation of cyclin D1-cdk4 and cyclin E-cdk2 kinase activities

The levels of expression of cyclin D1 and cdk4 were elevated upon ILK overexpression, but those of cyclin E and cdk2 were not (Fig. 2). To determine whether this translated into increased kinase activities we carried out immune complex *in vitro* kinase assays on both cyclin D1-cdk4 and also cyclin E-cdk2 complexes using recombinant Rb and histone H1 as substrates, respectively. As shown in Fig. 3a, the kinase activity of cdk4 was dramatically increased in the ILK overexpressing clones (ILK13). Although protein levels of cyclin E and cdk2 were not elevated (Fig. 2), the kinase activity of cyclin E-cdk2 complex was also enhanced in these cells (Fig. 3a). Cell adhesion in fibroblasts has been shown to stimulate cdk2 activity (Zhu *et al.*, 1996; Fang *et al.*, 1996), without elevations in cyclin E or cdk2 levels (Zhu *et al.*, 1996). This was thought to be brought about by the down-regulation of the cdk inhibitors p21 and p27. In the ILK-overexpressing cells, the increased cdk2 kinase activity could be a result of the decreased inhibitory activity of p27 (see Fig. 3b). The net effect of the increased activities of cyclin D1-cdk4 and cyclin E-cdk2 in ILK13 cells is that the Rb protein is constitutively hyperphosphorylated (Fig. 3a), leading to the progression of the cells through G1/S restriction point.

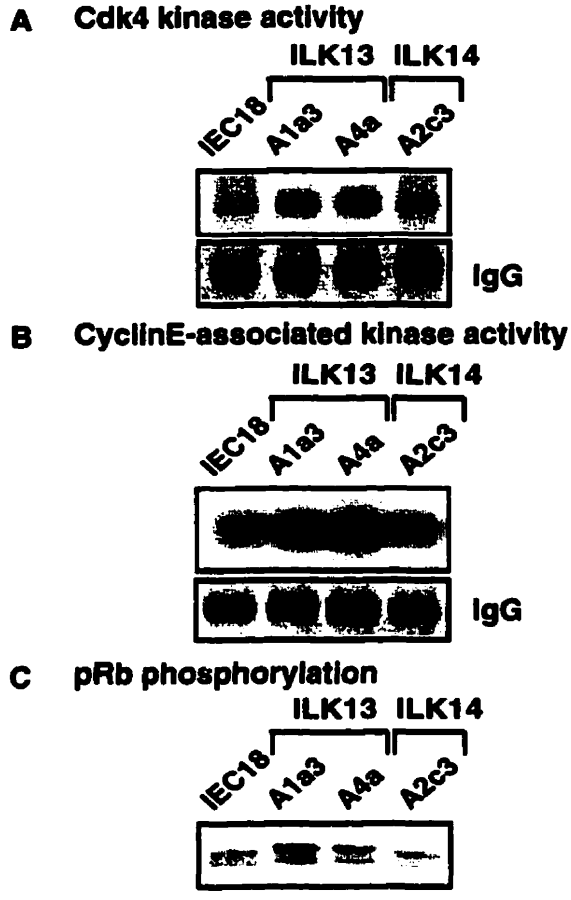

Fig. 3 a) Effect of ILK overexpression on the kinase activities of the G1/S cyclin-cdk complexes. A. Cyclin D1-cdk4 kinase assay. Following immunoprecipitation of cdk4 , an *in vitro* kinase assay was performed using Rb (QED Bioscience Inc.) as substrate. The incorporation of radioactivity in Rb substrate was several-fold higher in ILK13 clones, indicating higher kinase activity of cyclin D1-cdk4 in these cells. B. Cyclin E-cdk2 kinase assay. Cyclin E was immunoprecipitated and the associated cdk2 histone H1 kinase activity was assayed *in vitro*. Cyclin E-cdk2 from the ILK13 cells showed higher kinase activity. The lower panels in A. and B. represent IgG from coomasie Blue Stained gels to confirm equal loading. C. Immunoblot of Rb protein immunoprecipitated from IEC18, ILK13 and ILK14 cells. ILK13 cells (ILK overexpressors) show an increase in the hyperphosphorylated form of Rb.

3.4. p27 from ILK overexpressing cells is altered and has a lower cdk inhibitory potential

It has been demonstrated previously that nonadherent fibroblasts express high levels of p27 cdk inhibitor and that this expression is downregulated upon cell-substratum adhesion resulting in a higher cyclin E-cdk2 activity (Zhu *et al.*, 1996; Fang *et al.*, 1996). Although ILK-overexpression resulted in a more active cyclin E-cdk2 kinase activity, the expression levels of p21 and p27 cdk inhibitors were not decreased. In fact, they appeared to be elevated as compared to the IEC18 and ILK14 control cells (Fig. 2). However, both p21 and p27 from ILK13 cells had an altered electrophoretic mobility (Fig. 2). We therefore determined whether the altered electrophoretic mobility of p27 correlated with an altered inhibitory potential of this protein and hence might contribute to the increased cyclin E-cdk2 kinase activity. To analyze p27 activity, we immunoprecipitated p27 from IEC18 and ILK13 cells and assayed its ability to inhibit test cyclin A-cdk2 kinase complexes. As shown in Fig. 3b, p27 activity from IEC18 cells can be tested according to this assay and inhibited cyclin A-cdk2 in a dose dependent manner. When compared to the p27 from IEC18 cells, equivalent amounts of ILK13 p27 showed consistently less inhibitory activity by this type of an assay (Fig. 3b). The extent of cyclin A-cdk2 inhibition by p27 isolated from ILK13 clones was significantly less, as shown by higher residual cyclin A-cdk2 kinase activity (Fig. 3b), even when 2-fold more p27 was used in the assay (Fig. 3b). Equivalent amounts of p27 were used from each cell line as shown in Fig. 3b. This decreased p27 inhibitory activity could contribute to the higher cyclin E-cdk2 activity present in the ILK13 cells. Thus, although p27 can complex with cyclin E-cdk2 in the ILK13 cells (Fig. 2), its inhibitory potential was reduced, resulting in a net elevation of cyclin E-cdk2 kinase activity.

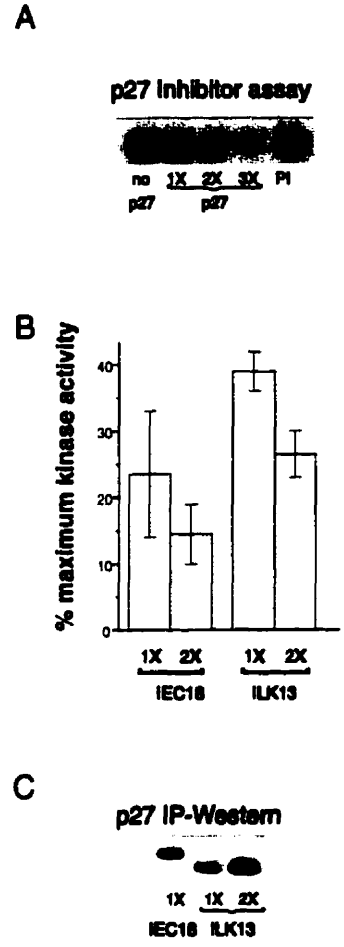

Fig. 3 b) p27 inhibitory activity and immunoprecipitation. A. Inhibitor activity: Increasing amounts of p27 were immunoprecipitated from asynchronous IEC18 cells (1x=100 μg lysate, 2x=200 μg lysate, 3x=300 μg lysate). The ability of p27 to inhibit the test cyclin A-cdk2 kinase activity was assayed (see Materials and Methods section). Cyclin A-cdk2 kinase activity, without any added p27, is shown in the first lane. No cdk2 inhibitory activity was recovered from boiled pre-immune (PI) serum immunoprecipitates. B. p27 inhibitory activity in IEC18 and ILK13 cells. Equal quantities of p27 (1x) were immunoprecipitated from IEC18 and ILK13 cells. p27 was released from protein A- Sepharose beads, added to test cyclin A-cdk2 and the kinase activity was assayed on H1 as a substrate. The results are presented as percent maximum kinase activity in uninhibited cyclin A-cdk2. Comparison of p27 inhibitory activity from equal amounts of p27 from IEC18 and ILK13 (1x and 2x) shows greater inhibition by p27 from IEC18 cells. C. Levels of p27 used in the inhibitor assays (B). p27 was immunoprecipitated from IEC18 (1x=50 μg lysate) and ILK13 cells (1x=15 μg, 2x=30 μg). The quantity of p27 used is shown by resolving complexes by SDS-PAGE and immunoblotting with p27 antibody.

3.5. Adhesion-independent upregulation of cyclin D1 and cyclin A expression, and Rb hyperphosphorylation in ILK overexpressing cells

Non-adherent fibroblasts express low levels of cyclin D1 and low cyclin D1-cdk4 and cyclin E-cdk2 activities. Untransformed fibroblasts and epithelial cells are also growth inhibited in suspension and become arrested in the G0 phase of the cell cycle. Since ILK overexpression in IEC18 cells induces cell survival and promotes cell cycle progression in suspension, we wanted to determine whether the increased levels of cyclin D1 and Rb protein hyperphosphorylation were maintained in suspension. Furthermore, since the expression of cyclin A is regulated in an anchorage-dependent manner in some cells, we also examined adhesion-dependent regulation of cyclin A protein expression in IEC18 and ILK overexpressing (ILK13) cells. Exponentially growing adherent cultures of ILK13 and the control, ILK14 cells were placed in suspension for 12 hours. The cells were then lysed and the expression of cyclin D1, cyclin A and Rb phosphorylation were determined by immunoblotting. As shown in Fig. 4, as expected, the expression of cyclin D1 and cyclin A (Fig. 4) fell with increased duration in suspension ILK14 cells. However, in the ILK13 cells, the elevated cyclin D1 and cyclin A expression was maintained in suspension. Similarly, whereas Rb was rapidly dephosphorylated in control (ILK14) cells in suspension, a substantial proportion of Rb remained hyperphosphorylated in suspension ILK13 cells (Fig. 4). These data indicate that overexpression of ILK overcomes the adhesion dependent regulation of cyclin D1 and cyclin A protein expression and Rb phosphorylation, suggesting that ILK is in the signalling pathway which mediates integrin-dependent regulation of the cell cycle.

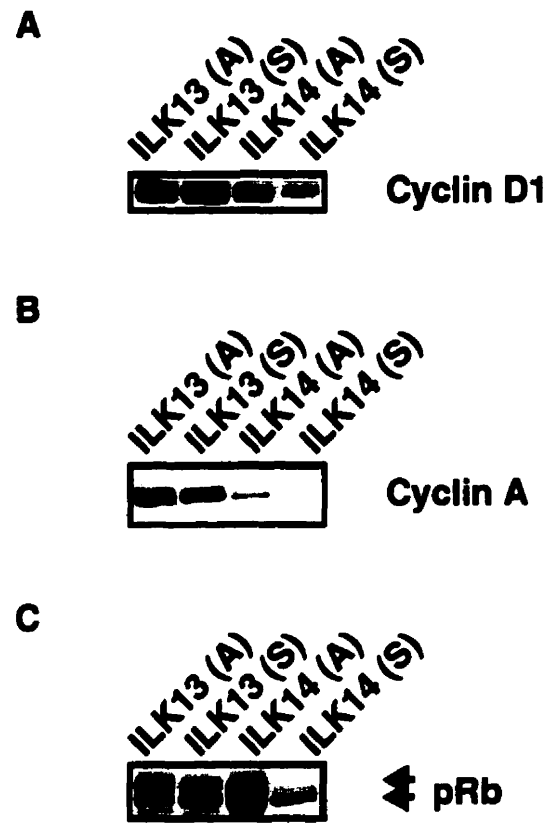

Fig. 4 Adhesion-independent overexpression of cyclin D1, cyclin A and hyperphosphorylation of Rb in ILK overexpressing cells. Adherent ILK13 and ILK14 cells were harvested, transferred into 50 ml tubes and maintained in suspension (S) for 12 hrs. Cell lysates were then recovered from cells in suspension (S) and cells growing in monolayer culture (A). Cyclin D1, cyclin A and Rb proteins were analysed by immunoblotting. A. Each cell line was found to have elevated cyclin D1 protein upon adhesion to substratum in comparison to cells kept in suspension. However, the level of cyclin D1 was constitutively higher in ILK13 cells kept in suspension. B. Cyclin A protein was higher in ILK13 adherent cells than in ILK14 adherent. After transferring cells to suspension, ILK13 cells continued to maintain high cyclin A, while in ILK14 cells cyclin A expression fell dramatically. C. The retinoblastoma protein was hyperphosphorylated in suspension ILK13 cells, but not in control suspension ILK14 cells.

4. DISCUSSION

Cell adhesion to components of the extracellular matrix is a requirement for cell growth and survival for a wide variety of cell types (Frisch and Francis, 1994; Meredith *et al.*, 1993; Shin *et al.*, 1975). Inhibition of cell adhesion results in growth arrest, and many epithelial and endothelial cells also undergo apoptosis (Frisch and Francis, 1994; Meredith *et al.*, 1993; Boudreau *et al.*, 1995). Cell adhesion to the ECM results in the activation of signalling pathways which maintain cell cycle progression from G0 to S phase. The key components of the cell cycle machinery known to be regulated by cell adhesion to ECM are cyclin D1 and cyclin A expression, activation of cyclin D-cdk4 and cyclin E-cdk2 kinases, and Rb protein phosphorylation (Zhu *et al.*, 1996; Fang *et al.*, 1996; Johnson *et al.*, 1993; Boudreau *et al.*, 1995; Schulze *et al.*, 1996; Kang and Kraus, 1996). Determination of the molecular basis of this regulation is clearly important and may be central to our understanding of anchorage-independent cell growth and oncogenic transformation. Cellular transformation by activated Ras results in both serum-independence as well as anchorage-independence, and although cyclin D1 expression and cyclin E-associated kinase activity are induced by Ras, in some cell types the expression of cyclin A seems to be an important factor in Ras-induced anchorage-independent cell growth (Kang and Kraus, 1996). However, Ras-induced cyclin A expression may be a consequence of increased cyclin D1 expression since transfection and overexpression of cyclin D1 induces cyclin A gene expression (Jiang *et al.*, 1993).

It is highly likely that integrins, as receptors for ECM components, initiate signalling events which activate the above mentioned cell cycle parameters. Integrin activation and ligation have been shown to activate MAP Kinase via $p21^{ras}$-dependent (Kapron-Bras *et al.*, 1993; Schlaepfer *et al.*, 1994; Clark and Hynes, 1996), and -independent (Chen *et al.*, 1996) pathways.

Activation of MAPK, in turn, can regulate the transcription (Lavoie *et al.*, 1996), and translation of cyclin D1 mRNA (Lin *et al.*, 1994). The adhesion-dependent increase in cyclin D1 expression is also regulated, in part, at the level of mRNA translation (Zhu *et al.*, 1996), and therefore activation of MAPK may be crucial in adhesion-dependent cell cycle control. Anchorage-dependent expression of cyclin A has been shown to be regulated at the level of gene transcription (Schulze *et al.*, 1996). The integrin-proximal events responsible for the activation of downstream signalling pathways still need to be fully characterized. Integrins can initiate signalling pathways by activating tyrosine kinases such as focal adhesion kinase (FAK), which phosphorylates components of the actin cytoskeleton (Schaller *et al.*, 1992; Schaller and Parsons, 1994). Phosphorylated FAK can also associate with adapter proteins which may activate guanidine nucleotide exchange factors (GEFs) for the Ras, Rho and Rac family GTPases (Chen *et al.*, 1994; Parsons, 1996). The role of FAK in activating these GTPases and in regulating adhesion-dependent cell growth remains unclear although recent reports suggest that, in some cells, FAK can influence adhesion-dependent cell survival (Frisch *et al.*, 1996) and influence cell growth and migration (Gilmore and Romer, 1996). Recent results also demonstrate that integrin ligation and clustering can induce tyrosine phosphorylation of Shc proteins resulting in the activation of Ras via Grb-2/SOS (Giancotti, personal communication).

Our laboratory has recently identified a novel serine/threonine protein kinase (ILK) which can associate directly with the cytoplasmic domain of integrin β_1 and β_3 (Hannigan *et al.*, 1996). Overexpression of this kinase in epithelial cells induces anchorage-independent growth (Hannigan *et al.*, 1996) and oncogenic transformation (Wu *et al.*, 1996). In this chapter, I have demonstrated that, when overexpressed, ILK induces adhesion-independent cell survival of epithelial cells and also stimulates the expression of both cyclin D1 and cyclin A protein levels,

as well as activation of cyclin-dependent kinases. Specifically, I have shown that the induction of ILK expression by stable transfection (ILK13), or by inducible transfection, results in the stimulation of the expression of cyclin D1 protein. Furthermore, the kinase activity of cdk4 is substantially elevated in ILK13 clones compared to the parental IEC18 cells or control ILK14 clones. In contrast, although the expression of cyclin E and cdk2 are unchanged the cyclin E-cdk2 kinase is also more active in the ILK13 clones. The combined activation of cdk4 and cdk2 kinase activities results in the hyperphosphorylation of Rb protein, the phosphorylation of which regulates the entry of cells into S phase (Johnson *et al.*, 1993; Sherr, 1996). Surprisingly, ILK overexpression also seems to increase the levels of both p21 and p27 cdk inhibitors, although, when compared to IEC18 or ILK14 cells, the ratio of p27 to cyclin D1 in complex with cdk4 is substantially higher in the IEC18 and ILK14 cells than it is in the ILK overexpressing ILK13 cells.

Another interesting consequence of ILK induction is the expression of altered forms of both p21 and p27. These altered forms have faster electrophoretic mobilities as compared to p21 and p27 from the parental IEC18 cells and the control transfected (ILK14) clones. The nature of this alteration is not clear as yet but could result from altered phosphorylation (Gu *et al.*, 1992) or proteolytic degradation (Loda *et al.*, 1996). However, the expression of different isoforms, for example, by alternative splicing, cannot be ruled out. A potential functional consequence of this alteration appears to be decreased inhibitory activity, as demonstrated for p27. This decreased inhibitory activity could account for the increased cyclin E-cdk2 activity observed in ILK overexpressing cells.

Of significant importance to the oncogenic properties of ILK and its role in integrin-mediated signal transduction is the finding that ILK overexpressing cells (ILK13) continue to

cycle in serum-containing suspension cultures, whereas the control transfectant clones (ILK14) undergo cell cycle arrest and apoptosis, as described previously (Rak *et al.*, 1995). In IEC18 and control ILK14 cells, inhibition of adhesion to ECM results in a rapid down-regulation of expression of both cyclin D1 and cyclin A proteins, Rb dephosphorylation, and G1 arrest. This is in marked contrast to ILK13 clones in which cyclin D1 and cyclin A expression as well as Rb phosphorylation are maintained upon transfer to suspension culture and there was no inhibition of cell cycle progression. ILK, like Ras, stimulates the expression of cyclin A and cyclin D1 resulting in Rb phosphorylation. However, unlike Ras, ILK does not induce serum-independent cell growth, indicating that anchorage-independent cell growth can be stimulated independently of serum-independent cell growth. Preliminary data indicate that overexpression of ILK does not activate Ras, but can activate MAPK, thus suggesting that ILK can activate a Ras-independent pathway capable of altering cell cycle control resulting in anchorage-independent cell growth. On the other hand, Ras activates other cellular functions which result in both anchorage-independent and serum-independent cell growth. Our data, therefore, suggest an important role for ILK in integrin-mediated regulation of the cell cycle. Whether the kinase activity of ILK is required for this regulation remains to be determined. Preliminary experiments indicate that ILK overexpression can activate MAP Kinase (Hackam, Behrend and Dedhar, unpublished observations), which in turn has the potential to induce the expression of cyclin D1 protein via transcriptional (Lavoie *et al.*, 1996), or translational control (Lin *et al.*, 1994; Rosenwald *et al.*, 1993). The elevation of cyclin D1 by ILK does not appear to take place at the transcriptional level (Filmus and Dedhar, unpublished results) providing evidence for further translational regulation of cyclin D1 expression by ILK. In contrast, since ILK also induces anchorage-independent cyclin A expression, which is regulated by cell adhesion at the level of

transcription (Schulze *et al.*, 1996), ILK may activate signalling pathways which regulate gene transcription as well as protein translation.

Elevated cyclin D1 expression is quite common in certain types of cancers, especially breast and oesophageal carcinomas (Bartkova *et al.*, 1994; Motokuva and Arnold, 1993; Keyomasi and Pardee, 1993). Although in some cases the increased cyclin D1 expression is due to gene amplification (Keyomasi and Paredee, 1993), for the majority of the cases, the molecular basis of this increased expression is unclear (Buckley *et al.*, 1993). Since *ras* mutations are infrequent in breast carcinomas, it is unlikely that Ras plays an important role in the elevation of cyclin D1. The data presented here suggest that the altered expression of ILK might be involved in this elevated expression and will be the subject of future studies. Finally, these results suggest a key role for ILK in specifically coupling anchorage-dependent growth and cell cycle regulation. Altered expression, and/or kinase activity, of ILK could have an important role in uncoupling cell cycle regulation by cell adhesion and may play a crucial role in pathogenesis of cancer and cardiovascular diseases.

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CHAPTER III

GENERAL DISCUSSION AND FUTURE DIRECTIONS

GENERAL DISCUSSION AND FUTURE DIRECTIONS

In this study I attempted to elucidate the molecular mechanisms leading to transformation upon ILK overexpression. Rat intestinal epithelial cells (IEC18) stably transfected with ILK expression vector were used as a model system (Hannigan *et al.*, 1996). Here, I showed that ILK overexpression results in elevated levels of cyclin D1, cdk4 and cyclin A proteins, increased cyclin D1- and cyclin E-associated kinase activities and hyperphosphorylation of Rb. Furthermore, ILK overexpressing cells maintained constitutively high levels of cyclin D1, cyclin A and hyperphosphorylated Rb when transferred into suspension, while the control cells did not. As a consequence, ILK overexpressing cells were able to survive and proliferate in suspension in contrast to the control cells. We conclude that the integrin receptor proximal kinase ILK is regulating adhesion-dependent integrin signalling involved in cell cycle control, since ILK overexpression leads to anchorage-independent cell cycle progression. The exact pathway(s) and the intermediate effectors participating remain unknown at present.

1. Role for Ras

Oncogenic Ras stimulates the expression of cyclin D1 and cyclin A (Filmus *et al.*, 1994; Kang and Krauss, 1996) inducing both anchorage and serum-independent growth. However, ILK upregulates cyclin D1 and cyclin A expression and induces anchorage-independent growth, but does not bring about serum-independent growth. These findings indicate that the signalling pathways that control anchorage-(in)dependent and serum-(in)dependent growth are regulated separately. Data from our lab (Hannigan and Dedhar; unpublished observation) indicate that Ras is not involved in the signalling cascades initiated in response to ILK overexpression. It is

unlikely, therefore, that the ILK-induced upregulation of cyclin D1 is via a Ras-mediated pathway.

It has been reported that MAP kinase can positively regulate cyclin D1 expression (Lavoie *et al.*, 1996). Preliminary results (Hackam, Behrend and Dedhar) showed that ILK overexpression can stimulate MAP kinase activity in a transient transfection system. These observations imply that ILK can initiate signalling cascades via MAP kinase, but in a Ras-independent manner. Furthermore, work by Chen *et al.* (1996) indicates that activation of MAP kinase in response to integrin engagement can occur without Ras activation. Integrins, therefore, could regulate, via ILK, a Ras-independent signalling pathway involved in the control of cell cycle and anchorage-dependent growth.

2. Role for MAP kinase

2.1 Transient transfection approach

The role of MAP kinase in the ILK signalling pathways involved in the cell cycle regulation and survival control could be investigated in more detail. Co-transfection of ILK and MAP kinase can be performed and the kinase activity of MAP kinase measured. Use of dominant-negative and constitutively active MAP kinase mutants could help determine the requirement for MAP kinase in the cell cycle regulation in our system. If MAP kinase is a downstream effector of ILK, then cells transiently co-transfected with ILK and dominant-negative MAP kinase would not show high levels of cyclin D1 and cyclin A as well as Rb hyperphosphorylation. On the other hand, transient overexpression of dominant-negative ILK mutant (kinase-dead mutant ILK) should not activate MAP kinase and consequently cyclin D1 and cyclin A protein levels, and Rb hyperphosphorylation would be reduced.

2.2 MEK inhibitor effects

Another strategy for revealing the potential role of MAP kinase in mediating anchorage-independent cell cycle progression in ILK overexpressing cells is to use a MAPKK inhibitor (Dudley *et al.*, 1995). 2'-Amino-3'-metoxyflavone is a selective inhibitor of the phosphorylation and activation of MEK (MAPK kinase), which phosphorylates MAP kinase on both threonine and tyrosine residues. Using this inhibitor the role of MAPK in cell cycle regulation in ILK overexpressing cells could be assessed. Preliminary results indicate that inhibition of MEK in the ILK overexpressing cells leads to decreased levels of cyclin A and hypophosphorylation of Rb in suspension cultures (Radeva and Dedhar). The effect on cyclin D1 has not been examined. That means that ILK is no longer able to provide the cell cycle progression/survival signal, because its downstream effector, MAP kinase, is not functioning to send the signals through. The consequence of these events would be inhibition of cell cycle progression. To address this possibility, the following experiment will be performed. ILK overexpressing cells and control cells will be grown in monolayer culture or maintained in suspension in the presence of the MEK inhibitor and then the cell cycle profile of each sample could be analysed by FACScan analysis. My model predicts that suspension ILK overexpressing cells treated with the MEK inhibitor would show a G1 arrest and that would be in agreement with the decrease in cyclin A protein and Rb hyperphosphorylation observed under these conditions. Furthermore, ILK overexpressing cells should not form colonies in soft agar after being treated with MEK inhibitor.

To demonstrate that all of the observed effects of MEK inhibitor are specific, the kinase activity of MAPK will be measured in the cells treated as described above. *In vitro* MAP kinase assay will be performed using MBP as a substrate. ILK overexpressing cells that would not

progress through the cell cycle in response to MEK inhibition, should have inhibited MAP kinase activity as compared to the untreated cells.

3. How does ILK signal?

The study I have done demonstrated that overexpression of ILK leads to specific abrogation of anchorage-dependent growth. It remains unclear, though, which part of the ILK molecule triggers the downstream effects that were observed. There are several experiments that could be done to address this question.

Transient overexpression of dominant-negative ILK (kinase-dead mutant ILK) is expected not to activate MAP kinase. Such a result would imply that the kinase activity of ILK is required for the cell cycle events triggered by ILK initiated signalling cascades. Further confirmation of the role of ILK kinase activity should come from transfection and expression of the kinase domain alone. The prediction is that all the effects I see after ILK (full length) overexpression will be observed following ILK kinase domain overexpression.

If cells transfected with dominant-negative ILK (kinase-dead mutant) still maintain increased cyclin D1 and cyclin A protein levels as well as hyperphosphorylated Rb, then another domain of ILK, possibly the ankyrin-repeats containing domain, is involved in its signalling function. Ankyrin repeats have been identified in a number of proteins such as the INK family of cdk inhibitors and the I-κB inhibitor of the NF-κB transcription factor. These amino acid motifs were initially described in the erythrocyte molecule ankyrin and were shown to mediate protein-protein interactions (Bork, 1993). For example, the inhibitor of NF-κB, I-κB, binds to the transcription factor NF-κB via an ankyrin domain and sequesters it in an inactive form in the cytoplasm (Kerr *et al.*, 1992; Thanos and Maniatis, 1995). ILK contains four ankyrin repeats

in its N-terminus (Hannigan *et al.*, 1996) which suggests additional protein-protein interactions in which ILK could be involved in its function. To search for such candidates, a yeast two-hybrid screen could be employed where the ankyrin repeats of ILK molecule will be used as a "bait" (Chien *et al.*, 1991).

4. Immediate downstream effectors of ILK

4.1. 14-3-3 proteins

ILK contains a consensus sequence for binding to the 14-3-3 proteins. The 14-3-3 family of proteins are found as dimers, forming either homo- or heterodimers (Luo *et al.*, 1995). Raf is one of the molecules found to associate with the 14-3-3 proteins (Freed *et al.*, 1994; Luo *et al.*, 1995). Recent work suggests that activation of Raf can occur independently of Ras. Dimerization of Raf, mediated by 14-3-3 proteins, was found to be sufficient to trigger MEK kinase activation (Ferrar *et al.*, 1996). The immediate downstream effector of MEK is MAP kinase. Since we see MAP kinase activation in response to ILK overexpression independently of Ras, we hypothesize that ILK could signal to Raf directly via 14-3-3 protein interaction. Preliminary results (Behrend and Dedhar) indicate that Raf co-immunoprecipitates with ILK. It would be interesting therefore to investigate further the involvment of 14-3-3 type interactions in ILK signalling. To verify the specificity of this interaction, transient transfection of ILK, 14-3-3 protein and dominant-negative Raf could be performed. If ILK signals to MAP kinase via Raf through a 14-3-3 protein mediated ILK/Raf interaction, then the above transfection should not lead to MAP kinase activation.

4.2 Two-hybrid screen approach

A two-hybrid screen with the ankyrin repeats only (as discussed above) might reveal

some of the immediate downstream targets of ILK, while a two-hybrid screen with the kinase domain of ILK only may identify its kinase substrate(s). Kinase-dead mutant could be used in this screen to stabilize the interaction between ILK and its targets. This investigation could be complemented by co-immunoprecipitation and *in vitro* direct binding experiments.

5. Role of ILK in the inside-out signalling

Overexpression of ILK in normal rat epithelial cells causes altered cell phenotype and decreased adhesion to ECM substrates (Hannigan *et al.*, 1996), suggesting a role for ILK in the regulation of integrin "inside-out" signalling.

Work from other groups shows that mutation of certain serine sites in β cytoplasmic tails leads to disruption of integrin regulation (Hibbs *et al.*, 1991; Chen *et al.*, 1992). We propose that phosphorylation on serine, required for normal integrin function, is provided by the integrin proximal serine/threonine kinase ILK. Indeed, *in vitro* experiments confirm that ILK is able to phosphorylate β_1 cytoplasmic peptides (Hannigan *et al.*, 1996). Furthermore, preliminary results (Leung-Hagesteijn and Dedhar) reveal that mutation or deletion of some of these serine sites in β_1 cytoplasmic tail abrogates integrin/ILK interaction in adherent cells and thus can be important for activation of "inside-out" signalling.

Another consequence of ILK overexpression is an increased assembly of fibronectin matrix around the cell (Wu *et al.*, submitted). This observation is consistent with a model where ILK regulates integrin "inside-out" signalling. Existence of secreted fibronectin matrix around the cells would explain the decreased adherence toward exogenous ECM substrates for ILK overexpressing cells. Such a situation also implies that the effects we observe are a result of integrin receptor occupancy by the assembled fibronectin matrix. In this case ILK will solely

function as an inside-out signal transducer. Typically, integrin receptor activation is followed by FAK activation. We do not see any significant change in FAK kinase activity in ILK overexpressing cells, which argues against activation of integrin receptors in suspension. Nevertheless, to control for such an interpretation of our results I suggest the following experiment. Soluble RGD peptides could be used to compete for integrin/fibronectin binding in ILK overexpressing cells kept in suspension. RGD bound to integrin receptor blocks its ligand binding activity (Miyamoto *et al.*, 1995). In our case RGD will prevent integrin receptor binding to the assembled fibronectin matrix around the cell. As a consequence, the cells would fail to proliferate and survive in suspension. Treatment of ILK overexpressing cells in this way would cause decreased cyclin D1 and cyclin A expression, hypophosphorylation of Rb, cell cycle arrest and apoptosis.

6. Significance of the work

It is well documented that ECM signals via integrins can elicit a diverse range of intracellular signalling events leading to modulation of cell shape, motility, specific gene expression, cell survival and/or apoptosis. Co-ordination and regulation of such a wide variety of events would require multiple integrin-proximal molecules. FAK has been a major focus of studying the integrin-mediated signal transduction and was found to have a major role in integrin-stimulated tyrosine phosphorylation as well as cytoskeletal reorganization (Hanks *et al.*, 1992; Schaller and Parsons, 1994; Richardson and Parsons; 1995). Recently, several new integrin effector molecules, including ILK, have been described (Hannigan *et al.*, 1996). Previous studies have shown that upregulation of cyclin D1 and cyclin A proteins as well as stimulation of cyclin D1-cdk4 and cyclin E-cdk2 kinase activities is adhesion-dependent in

adherent cells (Zhu *et al.*, 1996; Fang *et al.*, 1996). In this study I have shown that overexpression of ILK in epithelial cells abrogates the requirement for cell attachment and allows anchorage-independent cell cycle progression (Radeva *et al.*, submitted; chapter II). My study suggests that ILK co-ordinates the adhesion-dependent regulation of cell cycle progression.

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