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A ROLE FOR THE STE20-LIKE KINASE, SLK, IN CELL MIGRATION

by

SIMONA M. WAGNER

Thesis submitted to the Faculty of Graduate and Postdoctoral Studies
in partial fulfillment of the requirements for the Ph.D. degree in
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PREFACE

At the time of printing this thesis, an article based on the third chapter has been published in the *Journal of Biological Chemistry* as:

Wagner, S., T.A. Flood, P. O'Reilly, K. Hume, and L.A. Sabourin. 2002. Association of the Ste20-like kinase (SLK) with the microtubule. Role in Rac1-mediated regulation of actin dynamics during cell adhesion and spreading. *J Biol Chem*. 277:37685-92.

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Vicente-Manzanares, M., D.J. Webb, and A.R. Horwitz. 2005. Cell migration at a glance. *J Cell Sci*. 118:4917-9.

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ABSTRACT

The Ste20-like kinase (SLK) is a conserved serine-threonine kinase that is involved in cytoskeletal reorganization and apoptosis (Sabourin and Rudnicki, 1999; Sabourin et al., 2000). Overexpression of SLK in various cell lines was shown to induce the rapid disassembly of actin stress fibres and cell death (Sabourin et al., 2000).

To investigate the physiological role of SLK we used MEF-3T3 fibroblasts spreading and migrating on fibronectin coated substrates.

Using immunofluorescence studies, we report that SLK is redistributed with adhesion components at podosome-like adhesion sites in fibronectin stimulated fibroblasts. Furthermore, we show that SLK is associated with the microtubule network and can be co-precipitated with α-tubulin. However, *in vitro* kinase assays demonstrate that its activity in not modulated following fibronectin stimulation. Using microinjection studies, we show that ectopic expression of activated SLK induces the disassembly of actin stress fibres, a process that can be inhibited by dominant negative Rac1. Furthermore, the overexpression of SLK by adenoviral infection inhibits cell spreading on fibronectin.

As the signalling pathways activated during cell spreading also regulate cell motility we tested the possibility that SLK may play a role in cell migration (Mitra and Schlaepfer, 2006; Webb et al., 2002).

We first investigated the localization of SLK and other cytoskeletal markers following scratch wounding of fibroblasts monolayers, and observed that SLK is recruited at the leading edge of migrating cells with paxillin, Rac1 and the microtubules.

Using short interfering RNA and dominant negative approaches, we show that SLK is required for microtubule-dependent focal adhesion turnover and

cell migration. Scratch wounding of confluent monolayers have been shown to induce polarization and migration (Etienne-Manneville and Hall, 2001). Therefore, we investigated SLK kinase activity following scratch wound induced migration and report that SLK is activated during migration. In addition, we report that SLK activation is dependent on a multitude of signalling pathways such as FAK/c-Src, MAPK, PI3K and RhoGTPases, whereas SLK recruitment to the leading edge is Src-dependent but FAK-independent.

Overall, we have established a role for SLK in cell motility as a novel molecular link between the adhesion-destabilizing activity of the microtubule network and actin-based cytoskeletal remodelling events.

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LIST OF ABBREVIATIONS

Aa	Amino Acid
Ad	Adenovirus
APC	Adenomatous Polyposis Coli
Arg	Arginine (R)
ASEF	APC Associated Nucleotide Exchange Factor
ATH	AT1-46 Homology
ATP	Adenosine Tryphosphate
Bp	Base Pair
BSA	Bovine Serum Albumine
°C	Degree Celsius
CAS	Crk Associated Substrate
cDNA	Complementary Deoxyribonucleic Acid
Ci	Curies
CK2	Casein Kinase II
CRIB	Cdc42/Rac1 Interactive Binding Domain
c-Src	Cellular Src
CSK	C-Terminal Src Kinase
DAPI	4',6-diamidine-2-phenylidole dihydrochloride
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyle Sulfoxide
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
ECM	Extracellular Matrix
EDTA	Ethylenendiaminetetraacetic Acid
EGF	Epidermal Growth Factor

ErbB2(HER2)	Erytorblastic Leukemia Viral Oncogene Homolog 2
ERK2	Extracellular Signal Regulated Kinase 2
F-actin	Fibrilar Actin
FA	Focal Adhesion
FAK	Focal Adhesion Kinase
FAK-/-	FAK Null Cells
FAT	Focal Adhesion Targeting
FBS	Fetal Bovine Serum
FERM	Protein 4.1, Ezrin, Radixin and Moesin Homology Domain
FIP200	FAK Inhibitory Protein of 200 Kilodaltoni
FITC	Fluorescein Isothiocyanate
FN	Fibronectin
GAP	GTPase Activating Protein
GCK	Germinal Center Kinase
GDI	Guanine Nucleotide Dissociation Inhibitor
GDP	Guanince Diphosphate
GEF	Guanine Nucleotide Exchange Factor
GFP	Green Fluorescent Protein
GRB7	Growth Factor Receptor Bound Protein 7
GSK-3β	Glycogen Synthase Kinase 3β
GST	Glutathione S-Transferase
GTP	Guanine Triphosphate
H	Hour
HA	Hemagglutinin
HGF	Hepatocyte Growth Factor
HRPO	Horseradish Peroxidase
IF	Immunofluorescence

IgG	Immunoglobulin G
IP	Immunoprecipitation
JNK	c-Jun amino-Terminal Kinase
KA	Kinase Assay
Kb	Kilobase
KDa	Kilodaltoni
LacZ	Beta-Galactosidase
Ldb1/2	LIM Domain Binding Proteins 1/2
LIMK	LIM kinase
LMW-PTP	Low Molecular Weight Protein Tyrosine Phosphatase
LOK	Lymphocyte Oriented Kinase
Lys	Lysine (K)
LY294002	2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one
µCi	Microcuries
µg	Microgram
µl	Microlitre
µM	Micromolar
MAPK	Mitogen Activated Protein Kinase
MBS	Myosin Binding Subunit
mDia	Mouse *Diaphanous* Protein
MEF-3T3	Mouse Embryonic Fibroblast, strain 3T3
min	Minute
MEK1/2	ERK Kinase 1/2
MLCK	Myosin Light Chain Kinase
Mm	Milimeter
MMP	Matrix Metaloproteinases
MOI	Multiplicity Of Infection

MST1/2	Mammalian Sterile Twenty Kinase 1 and 2
N	Asparagine
ng	Nanogram (10^{-9})
PAGE	PolyAcrylamide
PAK	P21 Activated Kinase
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDGF	Platelets Derived Growth Factor
PDK1	Phosphoinositide Dependent Protein Kinase 1
PFA	Paraformaldhehyde
PH	Pleckstin homology
PI3K	Phosphatidylinositol 3-Kinase
PI(4,5)P2	Phosphatidylinositol 4,5- bisphosphate
PKC	Protein Kinase C
PL	Poly-L-Lysine
PLCγ	Phospholipase C γ
pM	Pico Molar (10^{-12})
PP2	(4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*] pyramidine)
PP3	(4-amino-7-phenylpyrazolo[3,4-*d*]pyramidine)
PTK	Protein Tyrosine Kinase
PTP	Protein Tyrosine Phosphatase
PVDF	Polyvinylidene Difluoride
RIPA	Radioimmune precipitation assay
RNA	Ribonucleic Acid
ROCK	Rho-Associated Kinase

SB203580	4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-imidazole
Ser/Thr	Serine/Threonine
SDS	Sodium Dodecyl Sulphate
SH	Src Homology
Shp2	SH2 Containing Tyrosine Phosphatase 2
shRNA	Short hairpin RNA
siRNA	Short Interfrering RNA
SLK	Ste20- Like Kinase
Ste20p	Sterile 20 Protein
STK	Src Family of Tyrosine Kinase
SYF	Src, Yes, Fyn Triple Knockout
TRITC	Tetrametyl Rhodamine Isothiocyanate
Tyr	Tyrosine
U0126	(1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene)
v-Src	Viral Src
WB	Western Blot
xPlkk1	Xenopuous Polo-Like Kinase Kinase 1

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CHAPTER 1

GENERAL INTRODUCTION

1.1. Cell Adhesion and Migration: an Overview

Cell migration is a highly integrated multistep process that orchestrates embryonic morphogenesis, contributes to tissue repair and regeneration and drives disease progression in cancer, mental retardation, atherosclerosis and arthritis (Ridley et al., 2003). The migrating cell is highly polarized with complex regulatory pathways that spatially and temporally integrate its component processes.

The general model of cell motility divides movement into four sequential steps. Cells extend a lamellipodium at the leading edge (step 1), make new adhesion contacts (step 2), contract to move the cell body forward (step 3) and detach the trailing edge (step 4) (Ridley et al., 2003). Thus forward movement requires that new adhesion contacts form at the leading edge and old ones break in the trailing edge of the cell.

Adhesion of cells to the extracellular matrix (ECM) is mediated by the integrin family of heterodimeric receptors (Burridge and Chrzanowska-Wodnicka, 1996; Burridge et al., 1988). Engagement of integrin receptors with their extracellular ligands leads to the formation of adhesion sites linking the ECM and the actin cytoskeleton. In cultured cells, points of adhesion, termed focal adhesions, are visualized as sites of close membrane contact with the substrate and anchor a highly bundled and crosslinked actin stress fibre network (Craig and Johnson, 1996). It is now clear that sites of ECM-integrin adhesion are in fact dynamic, often heterogeneous structures, varying in size and organization (Zamir et al., 1999; Zamir et al., 2000). In motile cells, sites of adhesion within filopodia and lamelipodia are relatively small and transient and are referred to as "focal complexes". In contrast, the large, stable sites of adhesion underlying the body of cells and localized to the end of actin stress fibers are referred to as "focal adhesions" (Hall, 1998). Signal transduction through focal complexes and focal adhesions has been implicated in the regulation of a number of key cellular processes, including growth factor induced

mitogenic signals, cell survival and cell locomotion (Burridge and Chrzanowska-Wodnicka, 1996; Schwartz et al., 1995).

The dynamic regulation of cell adhesion is of particular importance when cells move in response to a stimulus, either an immobilized ligand, such as ECM proteins (haptotaxic) or a soluble ligand, such as growth factors or cytokines (chemotaxis) (Horwitz and Parsons, 1999; Lauffenburger and Horwitz, 1996). Focal adhesions serve as sites for force transmission for the cell against the rigid substratum, force provided by contraction of the actin-myosin stress fiber network (Burridge and Chrzanowska-Wodnicka, 1996; Horwitz and Parsons, 1999). Newly generated "focal complexes" formed at the leading edge of the cell provide new adhesion contacts and thus provide directionality for cell movement (Horwitz and Parsons, 1999). The coordinated regulation of the formation and turnover of adhesion complexes is central to how cells move in response to different signals. The formation and remodeling of focal contacts is a dynamic process under the regulation of protein tyrosine kinases, protein tyrosine phosphatases and small GTPases of the Rho family **(Fig. 1.1)** (Burridge and Chrzanowska-Wodnicka, 1996; Burridge et al., 2006; Horwitz and Parsons, 1999).

Because migration contributes to several important pathological processes, including cancer, understanding the fundamental mechanisms underlying cell migration holds the promise of effective therapeutic approaches (Ridley et al., 2003).

1.2. Integrin Signalling in Cell Motility

The integrins are heterodimeric receptors consisting of α and β chains with large ligand-binding extracellular domains and short cytoplasmic tails. The binding of ligands to the extracellular portion of integrins leads to conformational changes in the receptors by altering the interactions between α and β chain cytoplasmic

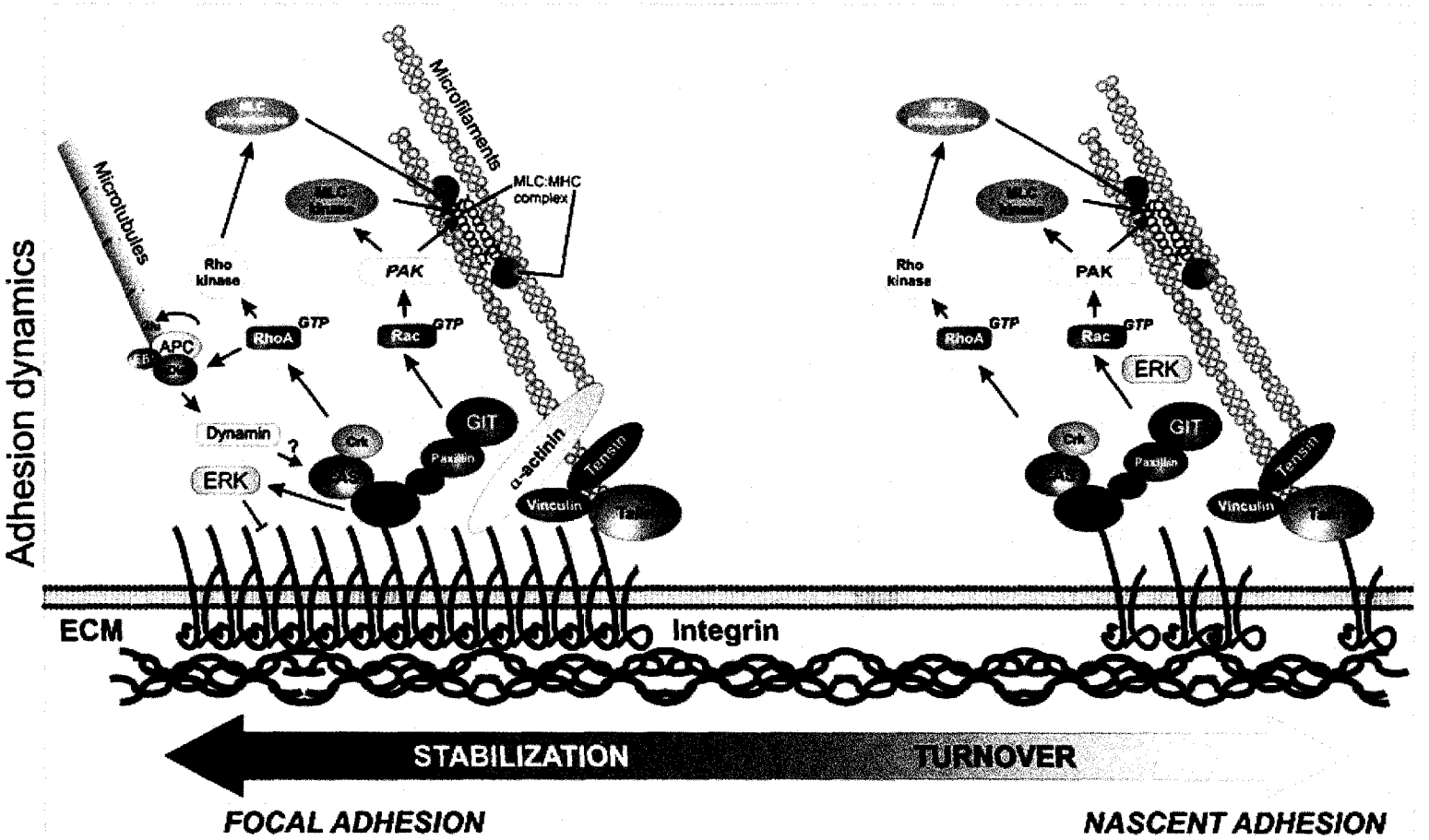


Figure 1.1. Adhesion sites dynamic. Adhesions are point of interaction between the cell and the substrate. They assemble and disassemble in response to extracellular cues and regulate cell motility. APC, adenomatous polyposis coli, CAS, Crk associated substrate; ERK, extracellular regulated kinase; FAK, focal adhesion kinase, GIT, G protein coupled receptor interacting protein; mDia, mouse Diaphanous, MHC, myosin heavy chain; MLC, myosin light chain; PAK, P21 activated kinase (Vicente-Manzanares et al., 2005).

domains and to integrin clustering (Sever et al., 2000). This combination of occupancy and clustering initiates intracellular signals such as protein tyrosine phosphorylation, activation of small GTPases and changes in phospholipid biosynthesis that regulate the formation and strengthening of adhesion sites as well as the organization and dynamic of the cytoskeleton and cell polarity during migration (Ridley et al., 2003). Although integrins themselves do not have any catalytic activity, signals are transmitted through direct and indirect interactions with many partners such as: talin, vinculin, paxillin and α-actinin (Wiesner et al., 2005).

With respect to cytoskeletal organization and cell migration, signalling from integrin-mediated adhesion is typically characterized by two phases. Early adhesion is associated with pathways that stimulate protrusion, whereas mature adhesions are associated with the development of tension. The early phase leads to Rac and Cdc42 activation and to actin polymerization. The later phase leads to Rho A activation, increased contractility and the transmission of tension to the sites of integrin ligation (DeMali et al., 2003). Thus, integrin signalling regulates important biological processes such as cell migration **(Fig. 1.2).**

1.3. Integrin-Regulated FAK-Src Signaling in Motile Cells

1.3.1. Focal Adhesion Kinase (FAK): Structure and Function

FAK is a non-receptor protein tyrosine kinase (PTK) composed of a central kinase domain flanked by N-terminal and C-terminal domains **(Fig. 1.3 A).** The FAK terminal domain was termed FERM because it shares sequence homology to the erythrocyte band 4.1, ezrin, radixin, and moesin proteins (Girault et al., 1999). The central kinase domain shares sequence similarities with other PTKs and harbors tyrosine residues that are important for FAK kinase activity (Ruest et al., 2000). The FAK C-terminal domain is termed FAT (focal adhesion targeting)

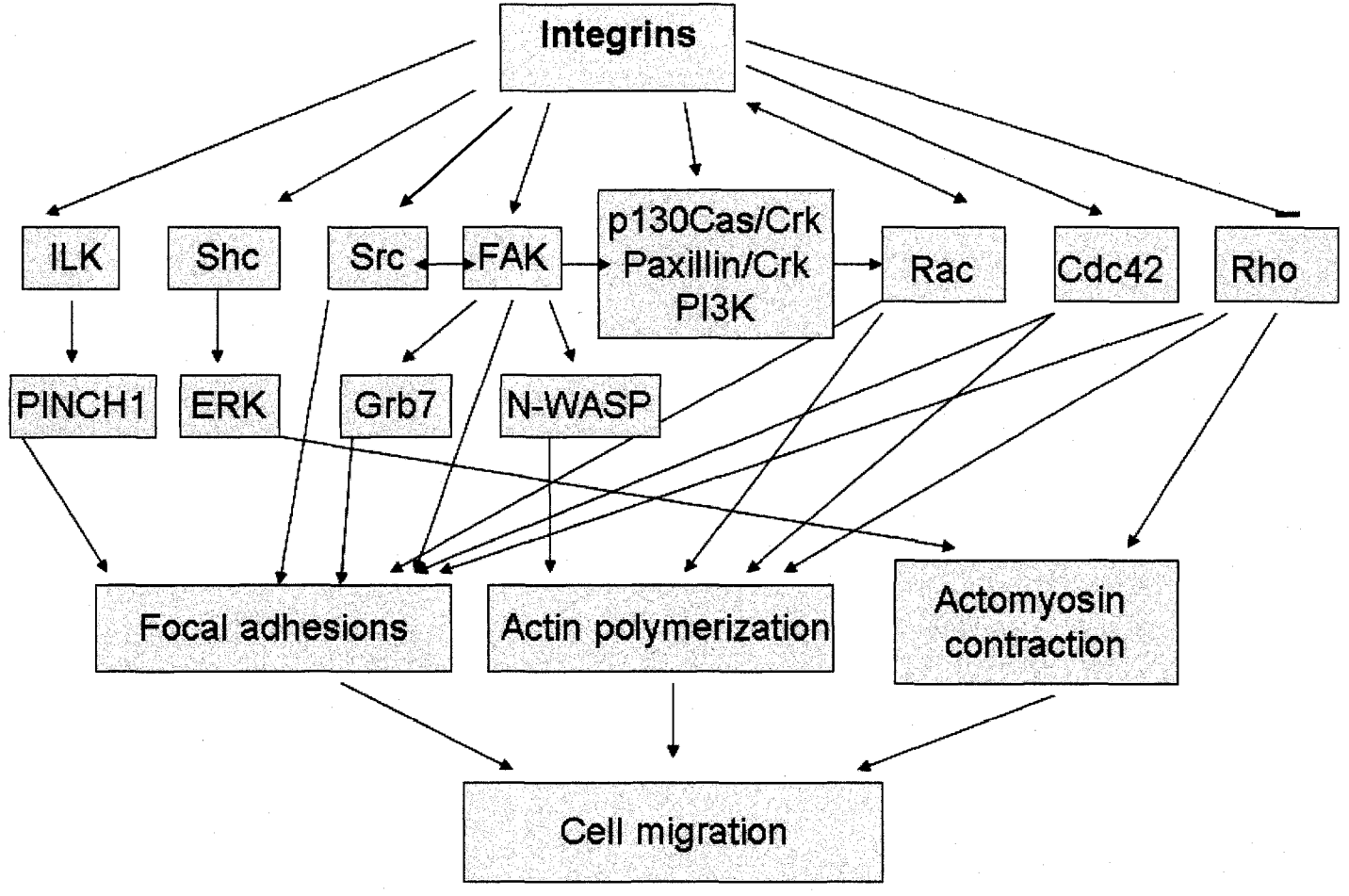


Figure 1.2. Integrin mediated signalling in cell motility. Integrins outside-in signalling can modulate the activity of RhoGTPases and other signalling molecules at focal adhesions, such as FAK, Src, integrin linked kinase (ILK) and Shc, thus regulating cell migration (Li et al., 2005).

and is rich in protein-protein interaction sites that are crucial for FAK localization to adhesion complexes and binding to adaptor proteins such as paxillin, talin, and CAS (p130Cas, Crk associated substrate). (Bertolucci et al., 2005; Shen and Schaller, 1999).

1.3.2. The C-Src Proto-Oncogene: Structure and Function

C-Src is the cellular, non-transforming progenitor of v-Src, the oncoprotein encoded by the chicken, Rous sarcoma virus. At least nine other proteins with similar overall structure are comprised by the Src family of tyrosine kinases (STKs) (Thomas and Brugge, 1997). Some are ubiquitously expressed (c-Scr, c-Yes, Fyn and Lyn), while others are restricted to specific cell types (Lck, Hck and Fgr in hematopoetic cells) (Haskell et al., 2001). All of the Src family members share a similar domain structure which consists of six distinct functional domains: an N-terminal membrane association domain (SH4 domain, SH, Src homology), a "unique" domain, SH3 and SH2 domains, a catalytic domain (SH1 domain) and a negative regulatory domain **(Fig. 1.3 B).** The kinase activity of c-Src is regulated in part by a short domain at the extreme C-terminus of the molecule. This negative regulatory region harbors a tyrosine residue that becomes phosphorylated (Tyr530 in human c-Src, Tyr527 in chicken c-Src) by another tyrosine kinase, termed C-terminal Src kinase or CSK (Okada et al., 1991). Src phosphorylated at Tyr527/ Tyr530 is capable of binding its own SH2 domain in a manner that inhibits kinase activity without physically blocking the catalytic site (Sicheri et al., 1997; Xu et al., 1997).

Like FAK, cellular Src is a non-receptor cytoplasmic tyrosine kinase that plays a key role in regulating multiple cellular functions, including cell migration and proliferation. Cellular Src associates with multiple cell surface receptors, including some integrins, and with the organized actin cytoskeleton, as well as with FAK (Thomas and Brugge, 1997). As with FAK, it has been shown that the activation

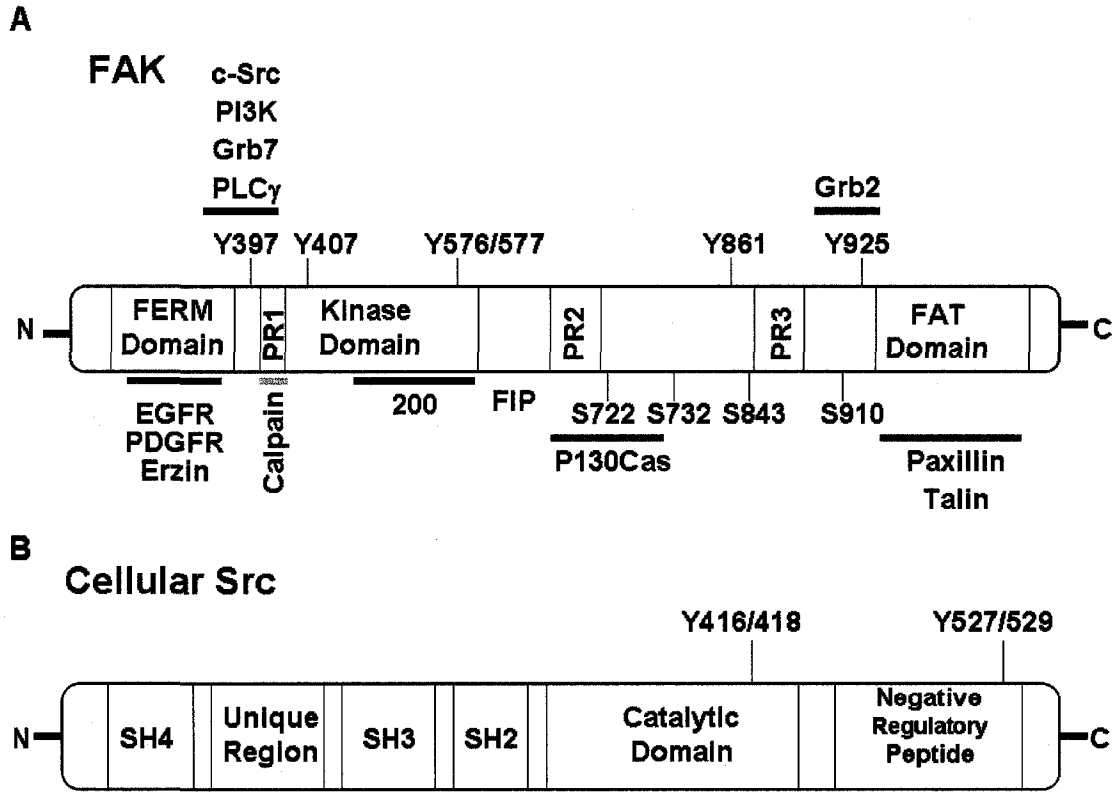


Figure 1.3. Schematic representation of focal adhesion kinase (FAK) and cellular Src.
A. The domains organization of FAK. The FERM domain shares homology to the band 4.1 protein, and the ezrin, radixin, moesin family of proteins. The kinase domain indicates the region of catalytic activity. PR1, PR2 and PR3, denote proline rich regions. FAT denotes the focal adhesion targeting domain. Sites of tyrosine and serine phosphorylation are indicated. Known binding partners of FAK are underlined according to their binding sites. **B. The domains organization of cellular Src**. The catalytic domain contains the autophosphorylation sites Y416/418 in c-Src. The carboxyl-terminal negative regulatory peptide contains Y527/529 which is negatively regulated in mouse/human c-Src.

of cellular Src occurs upon ligand binding and clustering of the integrins (Thomas and Brugge, 1997).

1.3.3. Formation of the Signaling FAK-Src Complex:

The mechanisms regulating integrin-stimulated cell migration are very complex and the activation of protein tyrosine kinases (PTK) plays an important role in these events. Emerging evidence supports the role of both focal adhesion kinase (FAK) and Src-family PTKs in these processes (Mitra and Schlaepfer, 2006).

The activation of FAK requires autophosphorylation of tyrosine 397 (Tyr397, Y397). Such autophosphorylation has been shown to be temporally related to the clustering of integrins receptors in the cell membrane that occurs when these receptors bind their ligand (Hanks et al., 2003). Phosphorylated Y397 is a high-affinity binding site for Src, and the autophosphorylation of FAK results in the recruitment and binding of cellular Src through its SH2 domain (Schlaepfer et al., 1994). Cellular Src can then phosphorylate Y576/Y577 in the kinase domain of FAK, in the second step of the autoactivation loop that is essential for maximal FAK activity (Calalb et al., 1995).

In addition to phosphorylating Y576/577 of FAK, cellular Src can phosphorylate several other tyrosine residues that, when phosphorylated, serve as docking sites for SH2 domain binding molecules (Parsons, 2003; Schlaepfer and Mitra, 2004). Within the FAK-Src complex, Src phosphorylates FAK at Y861, and this is associated with an increase in SH3-domain mediated binding of CAS to the FAK C-terminal proline-rich regions (Lim et al., 2004). Activated Src also phosphorylates FAK at Tyr 925, which creates a SH2- binding site for the GRB2 adaptor protein. GRB2 binding to FAK is one of several connections that lead to the activation of Ras and the extracellular signal-regulated kinase-2 (ERK2)/ mitogen- activated protein kinase (MAPK) cascade (Schlaepfer et al., 2004). ERK2

phosphorylation and the subsequent activation of myosin light chain kinase can modulate focal contact dynamics in motile cells, as well as generate both protrusive and survival signals inside cells (Hanks et al., 2003; Ridley et al., 2003).

To date, the autophosphorylation site (Y397) appears to be the main binding site for the molecules that associate with FAK through an SH2 domain, including Src, Shc, phosphatidylinositol-3 kinase (PI3K), phospholipase Cγ (PLCγ) and Grb7.

Therefore, FAK and cellular Src act to activate each other. Thus, the association of the SH2 (Src homology domain 2) of cellular Src with phosphorylated Y397 of FAK, increases cellular Src activity by opening the closed autoinhibited conformation of cellular Src resulting in increased autophosphorylation (Hanks et al., 2003). These reciprocal roles of FAK and c-Src in mutual activation are supported by studies of FAK-null cells in which reduced c-Src activity is found (Hanks et al., 2003). Maximal activation of Src promotes its interaction and phosphorylation of two major targets, CAS family members and paxillin, which both bind FAK (Hanks et al., 2003). Paxillin and CAS can recruit other molecules to adhesion sites and regulate the organization of the actin cytoskeleton.

Oncogenic v-Src has been shown to phosphorylate Y397 of FAK, suggesting that cellular Src may play also a direct role in phosphorylating Y397 of FAK. Thus, c-Src may function both upstream and downstream of FAK (Cox et al., 2006).

The FAK-c-Src complex is subject to extensive negative regulation. A FAK-inhibitory protein of 200 kDa (FIP200) has been shown to bind the FAK kinase domain and to interact with suppressors of cytokine signaling, thus targeting FAK for poly-ubiquitination and degradation (Abbi et al., 2002). The C-terminal Src kinase (CSK) also mediates c-Src inactivation by phosphorylating Tyr-527 (McGarrigle et al., 2006; Rengifo-Cam et al., 2004). In addition, several protein tyrosine phosphatases (PTP) such as the SH2 containing tyrosine phophatase 2

(Shp2) and the low molecular weight tyrosine phosphatase (LMW-PTP) have been shown to regulate c-Src (Sastry et al., 2002; Schoenwaelder et al., 2000).

1.3.4. FAK-Src Complex and Proteolysis

In addition to signaling events that are associated with the phosphorylation of α-actinin, CAS and paxillin, FAK-Src signaling can affect focal contact dynamics through the regulation of both extracellular and intracellular proteolytic events (Mitra et al., 2005).

Activation of matrix metalloproteinases (MMPs) at the leading edge of migrating carcinoma cells functions to promote matrix proteolysis, which lead to the extracellular release of integrin-matrix contacts and thereby facilitates focal contact turnover (Visse and Nagase, 2003). The influence of FAK on MMP regulation is associated with signaling from Ras to ERK2 and from Rac to Jun N-terminal kinase (JNK) (Hauck et al., 2001) .

The intracellular linkage of focal contacts to the actin cytoskeleton is also regulated by calpain-mediated proteolysis (Bhatt et al., 2002). Calpain is a Ca^{2+}–dependent protease that can cleave constituents of focal contacts, such as talin and FAK. Supporting this, calpain 4 -/- cells have an increase number of peripheral focal contacts (Dourdin et al., 2001). Notably, FAK re-expression in FAK -/- cells promotes the formation of a complex between calpain, ERK2 and activated Src (Carragher et al., 2003). Thus, FAK signaling is connected to the increased turnover of focal contacts through calpain activation. Furthermore, the FAK-calpain linkage might be selectively activated at either cell protrusions or tail retraction sites in motile cells.

1.4. Focal Adhesion Turnover

1.4.1. Lessons from FAK-/- and SHP2-/- Cells.

In analyses monitoring the formation of focal contacts, FAK was found to be one of the first signaling proteins to be recruited to these sites (Kirchner et al., 2003). Although FAK recruitment to focal contacts is associated with increased FAK tyrosine phosphorylation, focal contacts readily form in FAK null (FAK-/-) fibroblasts , which indicates that FAK activity is not essential for the process of focal-adhesion formation (Ilic et al., 1995). However, focal contacts in FAK null cells do not undergo a normal maturation cycle (Sieg et al., 1999). In normal fibroblasts, peripheral immature focal contacts become connected to longitudinal stress fibers in cells and undergo actin contractility-mediated maturation during cell polarization (Zaidel-Bar et al., 2003).

SHP2 -/- cells have increased FAK activity, but they also show an accumulation of immature focal contacts and similar migration defects as FAK-/- cells (Yu et al., 1998). In SHP2 null cells, there is a high level of focal contact turnover, whereas in FAK null cells, focal contact turnover and maturation are inhibited, which indicates the importance of FAK expression and activity in the control of focal contacts dynamic (Ren et al., 2000; von Wichert et al., 2003).

1.4.2. Lessons from Paxillin -/-, p130Cas-/- and Src -/- Cells

Time-lapse analyses showed that the incorporation of fluorescent-labeled paxillin into focal contacts of FAK-/-, paxillin-/-, p130Cas-/-, or SYF-/- (Src-/-, Yes-/-, Fyn-/-) cells did not significantly differ compared to normal fibroblasts (Webb et al., 2004). However, the rate of focal adhesion disassembly was much slower in these null cells. Analyses of FAK-/- cells showed that disassembly was dependent on FAK Tyr397 phosphorylation; SYF-/- cells showed that Src kinase activity was required; and studies with paxillin-/- cells indicated that the integrity of the Tyr31 and Tyr118

paxillin phosphorylation sites were needed to promote focal contact turnover (Webb et al., 2004). As the expression of constitutively active Src in FAK-/- cells can promote focal contact turnover and increased cell motility, these combined analyses support the conclusion that, in normal cells, integrin- and FAK-mediated control of Src activity is a key event that promotes focal contact dynamic (Hsia et al., 2003; Moissoglu and Gelman, 2003).

Overall, the recent study of Webb et al. has shown that FAK, Src, CAS, paxillin, ERK, and myosin light chain kinase (MLCK) are critical for adhesion turnover at the cell front, a process central to migration (Webb et al., 2004). The authors proposed different models for how FAK-Src signaling regulates adhesion disassembly.

One pathway is by modulating actomyosin contractility through ERK and MLCK. Active ERK localizes to adhesions, where it can promote the phosphorylation of MLCK and increase contractility (Fincham et al., 2000). These results implicate ERK and MLCK as key targets of FAK-Src signaling that promote adhesion turnover, but other effectors may contribute to this process. Another recent study proposes a speculative regulatory cycle in which FAK-Src activation and signaling to ERK2 can function first to promote FAK release from existing focal contacts and then, through ERK2-mediated phosphorylation of paxillin, to promote FAK re-binding and activation at new or different focal contacts in a migration cell (Mitra et al., 2005).

Another possibility is through post-translational modification of key adaptors such as paxillin and CAS. And yet another possibility is through the activation of Rac or the inhibition of Rho (Chen et al., 2002; Ren et al., 2000). FAK could also modulate calpain activity by recruiting calpain and ERK to adhesions, where it regulates adhesion disassembly and migration (Carragher et al., 2003; Dourdin et al., 2001). Finally, FAK might influence actin organization in adhesions by

phosphorylating α-actinin on Tyr12, resulting in a decrease in its affinity for actin filaments (Izaguirre et al., 2001).

1.4.3. Microtubules Regulate the Dynamics and Turnover of Cellular Adhesions

The molecular mechanisms by which microtubules contribute to cell migration have been intensely studied. Early experiments with colchicines and other microtubule- depolymerizing drugs demonstrated that an intact microtubule array is essential for directed migration of fibroblasts, endothelial cells and other crawling cells (Vasiliev et al., 1970). Low concentration of drugs, which decrease microtubule dynamic inhibit lamelipodia formation and fibroblast migration (Liao et al., 1995). Similarly, microinjection of antibodies that inhibit kinesin motor activity also inhibit cell protrusion and migration (Rodionov et al., 1993). These and future studies indicated that microtubule dynamics and motor activity contribute to regulating the actin cytoskeleton (Palazzo and Gundersen, 2002).

Geiger's group provided the first demonstration that cytoskeletal modulation, such as microtubule disruption, triggers integrin-dependent signaling in the absence of external growth factor stimulation. Their results reported that the microtubule network, together with the actin system, participates in the control of integrin-dependent signal transduction (Bershadsky et al., 1996). Furthermore, they showed that microtubule disruption induces signal transduction events in quiescent cells, similar to those triggered by a variety of growth factors, including tyrosine phoshorylation of FAK and paxillin, and the assembly of focal adhesion and stress fiber. The authors suggested that the involvement of microtubules in adhesion-dependent signaling is related to microtubule interaction with the contractile actin-myosin system (Bershadsky et al., 1996).

Dynamic microtubules were observed to grow toward focal adhesions, probably guided there by actin stress fibers (Kaverina et al., 1998). This targeting

of microtubules to focal adhesions was temporally correlated with the dissolution or turnover of the targeted focal adhesion at the cell periphery (Kaverina et al., 1999). One mechanism proposed by Small *et al.*, is that the microtubule delivers a "relaxing factor" that stimulates the turnover of targeted focal adhesions (Small et al., 2002a). Alternatively, microtubules may transport factors away from focal adhesions, stimulating focal adhesion disassembly. Taken together, these studies suggest that the plus-end-directed motor kinesin uses microtubules to locally deliver a relaxing factor to dissolve focal adhesions (Small et al., 2002a).

There may be other mechanisms by which microtubules affect focal adhesion, such as sequestering and inhibiting a specific guanine-exchange factor for Rho (GEF-H1 or Lfc) or by providing a track for the kinesin Kif3 to deliver adenomatous polyposis coli (APC), which can then locally activate the Rac specific GEF, APC-associated guanine nucleotide exchange factor (ASEF) (Jimbo et al., 2002; Kawasaki et al., 2000).

The general paradigm demonstrated by these studies is that microtubule growth inhibits contraction and promotes actin polymerization and cell spreading, whereas microtubule depolymerization activates contraction. These effects may be localized to a narrow region near the microtubule plus end (Palazzo and Gundersen, 2002).

Other studies showed an evolutionary conserved pathway for microtubule capture suggesting that the formin mDia (mouse Diaphanous) functions as a scaffold protein for EB1 and APC to stabilize microtubules and promote cell migration (Palazzo et al., 2001; Wen et al., 2004). Recent studies report that integrin-mediated activation of FAK at the leading edge is required for microtubule stabilization by the Rho-mDia signaling pathway in mouse fibroblasts (Palazzo et al., 2004). Furthermore, a new role for mDia in regulating glycogen synthase kinase 3β (GSK3β) was identified through novel atypical PKCs and their role in

microtubule stabilization (Eng et al., 2006).

Recently, a model system was developed, that kinetically separates focal adhesion assembly from focal adhesion disassembly. Using a nocodazole washout system, it was shown that FAK and the GTPase dynamin, a large GTPase necesarry for endocytosis, are required for microtubule-induced focal adhesion disassembly (Ezratty et al., 2005). These data provided evidence that the focal adhesion disassembly defect in FAK-/- fibroblasts results from a reduced recruitment of dynamin to focal adhesion, and that a primary role of FAK in focal adhesion disassembly is to recruit dynamin. Taken together, these results establish that dynamin, FAK and microtubules are important components of a focal adhesion disassembly pathway that function independently of the pathways that regulate focal adhesion formation.

1.5. The Ste20-Like Kinase (SLK)

Ste20p (sterile 20 protein) is a putative yeast mitogen-activated protein kinase (MAP4K) involved in the mating pathway. Its homologs in mammals, *Drosophila melanogaster*, *Caenorhabditis elegans* and other organisms make up a large group of protein kinases, including 28 members in human (Dan et al., 2001b). The Ste20 kinases are further divided into the p21-activated kinase (PAK) and germinal center kinase (GCK) families, which are both, involved in apoptosis, morphogenesis and cytoskeletal rearrangements.

Several members of the Ste20 family of kinases have been identified in mammals. They have been implicated in various biological processes such as stress responses, cell death and cytoskeletal reorganization as well as growth and differentiation (Brown et al., 1996; Dan et al., 2001a; Dan et al., 2001b; Daniels et al., 1998; Fanger et al., 1997).

We and others previously identified a novel Ste20-related kinase termed

SLK (Itoh et al., 1997; Pytowski et al., 1998; Sabourin and Rudnicki, 1999; Sabourin et al., 2000; Yamada et al., 2000). Furthermore, SLK is part of a signaling pathway mediating activation of c-Jun terminal kinase1 (JNK1) and induces apoptosis in cultured fibroblasts (Cybulsky et al., 2007; Hao et al., 2006; Sabourin and Rudnicki, 1999). In addition, recent papers showed that SLK is involved in C2C12 myoblast differentiation, has a role in cell cycle progression and is regulated by casein kinase II (CK2) (Chaar et al., 2006; O'Reilly et al., 2005; Storbeck et al., 2004). SLK is ubiquitously expressed, but during embryogenesis it is highly enriched in muscle and neuronal tissues (Zhang et al., 2002).

However, relatively high levels of SLK protein and activity have been observed in all cell types analyzed, suggesting a functional role for SLK in physiological processes other that apoptosis (Sabourin et al., 2000).

1.5.1. SLK: Structure and Function

The Ste20-like kinase (SLK) is a serine/threonine kinase composed of 1202 amino acids with a predicted molecular mass of 148 kDa. However, on an SDS-PAGE gel it displays a mobility shift of approximately 220 kDa, suggesting that SLK is subject to major post-translational modifications (Sabourin and Rudnicki, 1999). SLK comprises an N-terminal kinase domain (residues 1-338), a central coil domain (residues 339-788) and a C-terminal domain (residues 789-1202) with various functions. The catalytic domain is most similar to lymphocyte oriented kinase (LOK) and mammalian sterile twenty-like 1 and 2 (MST1/2), two Ste20-related kinases (Sabourin et al., 2000). The Ste20 signature motif (TPYWMAPE) is present at position 189 within the kinase domain. **(Fig. 1.4.)**

The central domain of SLK consists of a coiled coil region with unknown function. Analysis of the SLK primary sequence revealed the presence of a caspase3 consensus cleavage site at amino acid residue 436 (DTQD436) and a putative SH3 binding domain (PPEPE) at position 735, suggesting a potential

interaction with SH3 domain-containing proteins (Sabourin and Rudnicki, 1999). The carboxyl-terminal region of SLK displays high homology to LOK, *Xenopous* polo-like kinase kinase1 (xPlkk1) and AT1-46, a cDNA clone encoding a protein of unknown function (Schaar et al., 1996). The C-terminal domain of SLK was termed the ATH (AT1-46 homology) domain (Sabourin et al., 2000). Taken together, these observations suggest that SLK, LOK and xPlkk1 represent members of a new kinase subgroup belonging to the germinal centre kinase 5 subfamily (GCK-V) (Dan et al., 2001b).

1.5.2. Role of SLK in Cell Adhesion and Migration

Overexpression of SLK in various cell lines was shown to induce the rapid disassembly of actin stress fibers and cell death (Sabourin and Rudnicki, 1999; Sabourin et al., 2000; Wagner et al., 2002). These studies have demonstrated that the SLK kinase domain induces apoptosis, whereas the ATH domain induces actin stress fiber disassembly. Overall, these data suggest that enhanced apoptotic response is due to the activation of the kinase domain of SLK (Sabourin et al., 2000). Similarly, anoxia-recovery activates a SLK/p38 dependent apoptotic response (Hao et al., 2006). Interestingly, SLK has also been shown to regulate cell cycle progression, as inhibition of SLK function leads to a cell cycle block in late G2 (O'Reilly et al., 2005).

A recent study from our laboratory showed that viral Src (v-Src) expression leads to down-regulation of SLK kinase activity, a process that requires v-Src translocation to the membrane, kinase activity and membrane anchoring (Chaar et al., 2006). Surprisingly, this down-regulation does not proceed through FAK or by direct tyrosine phosphorylation of SLK, but rather through activation of caseine-kinase II (CK2), which in turn down-regulates SLK by direct phosphorylation. One possibility is that SLK activity is down-regulated in v-Src-transformed cells as part of an anti-apoptotic pathway (Chaar et al., 2006).

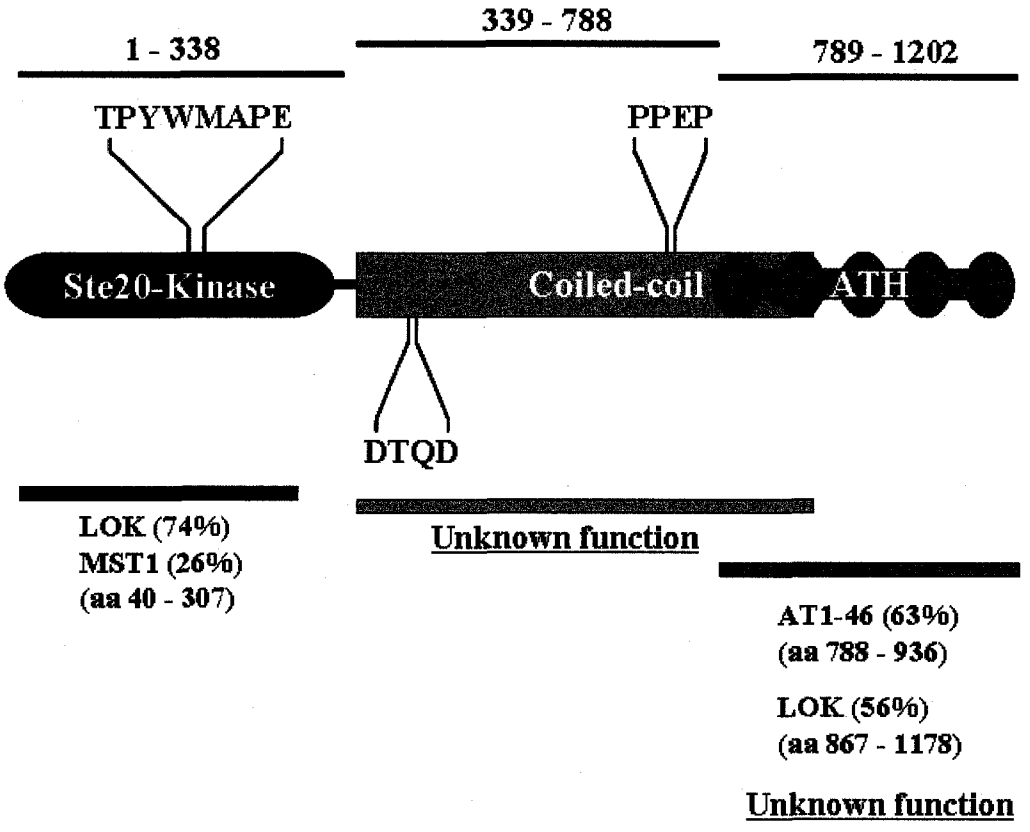


Figure 1.4. Schematic representation of SLK and percent similarities (in parentheses) to other proteins. The catalytic domain (black) is most similar to LOK and MST1/2, two Ste20-related kinases. The central domain of SLK contains a coiled-coiled region with unknown function. SLK is highly homolog to AT1-46 in the carboxyl-terminal region. Numbers in brackets represent SLK amino acid (aa) residues. The Ste20 signature sequence (TPYWMAPE), caspase 3 consensus cleavage site (DTQD), and a putative SH3 binding domain (PPEPE) are shown. Numbers above the different domains denote the amino acid residues representing the boundaries. ATH: AT1-46 homology domain; LOK: lymphocyte oriented kinase; MST1/2 – mammalian sterile twenty kinases 1 and 2.

1.6. Thesis Objectives and Hypotheses

Studies from our laboratory have previously demonstrated that the overexpression of SLK induces actin stress fiber disassembly and cell death (Sabourin et al., 2000). Therefore my initial hypothesis was that SLK is involved in cytoskeletal remodeling during cell adhesion and migration.

As a first step towards understanding the role of SLK, we have analyzed the levels, distribution, and activity of SLK protein during cell spreading on fibronectin (FN), a process that requires extensive cellular remodeling.

Using indirect immunofluorescence (IF) microscopy and biochemical techniques, I have determined that SLK is redistributed to ruffles and lamelipodia during cell spreading on FN and that it is associated with the microtubule cytoskeleton. Furthermore, I have shown that ectopic expression of activated SLK induces the disassembly of actin stress fibers, a process that can be partially inhibited by dominant negative Rac1. Finally, the overexpression of active SLK by adenoviral infection inhibits cell spreading on fibronectin and results in focal complexes at the cell periphery.

A second objective of my project was to asses the role of SLK in MEF- 3T3 fibroblast migration. Using immunofluorescence microscopy, I reported that SLK is recruited to the leading edge of migrating cells with other adhesion signaling proteins, such as Rac1, paxillin and α-tubulin. Furthermore, using dominant-negative SLK constructs and small interfering RNA (siRNA) molecules specific for murine SLK, I demonstrated that SLK deficient cells exhibit impaired migration using both, a transwell assay and a wound closure assay. We also demonstrated that SLK knockdown or expression of a dominant negative version of SLK results in impaired microtubule-dependent adhesion turnover and delayed migration.

A third objective of my thesis was to investigate the role of integrin signaling on SLK activity and translocation. Using scratch-induced migration we demonstrated

that SLK is activated during migration. In addition, we report that SLK activation is dependent on a multitude of signaling pathways such as FAK/c-Src, MAPK, PI3K and RhoGTPases, whereas SLK recruitment to the leading edge is Src-dependent but FAK independent.

Overall our results show that SLK is a novel regulator of focal adhesion turnover and cell migration.

CHAPTER 2

MATERIALS AND METHODS

2.1. Cell Lines and Cell Culture

The mouse fibroblast cell lines MEF-3T3 (MEF Tet-Off, C3018, Clontech) and Swiss 3T3 mouse embryonic fibroblast (ATCC cat no.4925) were used in spreading and migration experiments. The mesodermal FAK-/- cells (p53-/-, FAK-/-) and wild-type counterpart were kindly provided by Dr. D. Ilic. The SYF (Src, Yes, Fyn, triple knock-out) cells and SYF cells stably expressing c-Src were a generous gift from Dr. P. Soriano. All cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO2 in Dulbecco's modified Eagle's medium (DMEM, Bio-Whitaker) supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 50 µg/ml penicillin, and 50 µg/ml streptomycin.

2.2. Plasmids, Short Interfering RNA (siRNA) Specific for SLK and Short Hairpin Adeno-virus Construction

For expression studies and immunolocalization experiments, hemagglutinin (HA) or Myc-epitope-tagged pcDNA3 (Invitrogen) expression vectors bearing full-length or truncated SLK were constructed. The SLK expression plasmids consisted of HA-tagged SLK C-terminal truncation (amino acids1-373), which results in SLK activation (HA-SLK $^{1-373}$). Plasmid HA-SLK $^{1-373K63R}$ is the kinase inactive version of HA-SLK $^{1-373}$ (Sabourin et al., 2000). Briefly, SLK $^{1-373}$ was constructed by deleting amino acids 374 to 1202, leaving the first 373 amino acids of SLK. The kinase-dead version of this construct (SLK$^{1-373K63R}$) was obtained through PCR-based mutagenesis of the ATP-binding site at residue 63 (Lys63→Arg).

The adenovirus vectors used in these studies consisted of a GFP tracking virus and a LacZ expressing virus, used as controls, and two hemagglutinin (HA)-tagged SLK C-terminal truncations, HA-SLK$^{1-373}$ which is a constitutively active form and HA-SLK$^{1-373K63R}$, which is the inactivated form of SLK. These viral vectors were kindly supplied by Dr. Robin Parks laboratory, at the Ottawa Health Research

Institute. For Rho small-GTPase studies we used adenovirus vectors encoding HA tagged-dominant negative constructs for Rac1 and Cdc42 (Rac1N17 and Cdc42N17), which were kindly provided by Steve Callaghan, at the Neuroscience Research Institute.

For SLK knockdown experiments, MEF-3T3 cells plated at a density of 1-3 X 10^5 in 60 mm plates were transfected with 5 or 10 pM SLK siRNA (Dharmacon) duplex (5'- GUUGAGAUUGACAUAUUA) using Lipofectamine 2000 transfection reagent (Gibco) according to manufacturer recommendations. Control siRNAs consisted of Dharmacon's non-targeting duplex or scramble siRNA.

Cells were collected at 48 hours post-transfection and assayed for protein expression by western blot analysis. The short hairpin SLK (shSLK) plasmid was constructed using the psiStrike U6 hairpin cloning vector (Promega) and contained the same siRNA sequence as the SLK siRNA duplex.

2.3. Antibodies and Immunofluorescence

SLK rabbit polyclonal antibodies were as described previously (Sabourin et al., 2000). The primary antibodies used in these studies were as follows: phospho-GSK3β (Ser9) and GSK3β (Cell Signalling), phospho FAK- Y397 (Biosource) and total FAK (Upstate and Santa Cruz), vinculin (clone V11-5 Sigma), paxillin (clone 349, BD Transduction Labs), α-tubulin (Sigma), and Rac1 (Santa Cruz). Hemagglutinin tagged constructs were detected with anti-HA rabbit polyclonal antibody (sc-805, Santa Cruz Biotechnology, Inc.). Actin was detected with tetrametyl rhodamine isothiocyanate (TRITC)-phalloidin (Sigma). For the detection of tyrosine phosphorylation an anti-phosphotyrosine antibody was used (RC20:HRPO; Transduction Laboratories). The nuclei were visualized by staining with 4'-6'-diamidino-2-phenylindole (DAPI) (Sigma).

For immunofluorescence studies, the coveslips were fixed in 4% paraformaldehyde (PFA) for 10 minutes at room temperature, washed in phosphate

buffered saline buffer (PBS), and blocked with 0.1% bovine serum albumin (BSA) and 200 mg/ml ChromaPure Goat IgG (Jackson Immunoresearch Laboratories). Antibodies were detected with either anti-mouse or anti-rabbit secondary conjugated to either fuorescein isothiocyanate (FITC) or TRITC (Sigma). For identification of HA-tagged expression plasmids and adenovirus, we used HA- rabbit polyclonal antibody (sc-805, Santa Cruz Biotechnology, Inc.).

Samples were visualized with a Zeiss Axioskop100 epifluorescence microscope equipped with appropriate filters and photographed with a digital camera (Sony Corporation HBO50) using the Northern Eclipse software package.

2.4. Fibronectin Replating Assays

For fibronectin (FN) replating assays, sub-confluent MEF-3T3 cultures were serum starved in 0.25% FBS-DMEM for 24 hours and harvested by trypsin-EDTA treatment as previously described (Sieg et al., 1999). The trypsin was inactivated using soybean trypsin inhibitor (0.5 mg/ml) and the cells were collected by centrifugation and resuspended in 0.1% BSA-DMEM medium. After 1 hour at 37°C in suspension, the cells were plated onto fibronectin coated plates or coverslips. At various times after replating, the attached cells were rinsed in PBS, prepared by protein extraction and analyzed by western blot (WB) and kinase assay (KA) and immunofluorescence (IF). The culture dishes (or coverslips) were precoated with fibronectin (10 mg/ml, Sigma-Aldrich) or poly-L-lysine (100 mg/ml, Sigma-Aldrich) in PBS overnight at 4°C, rinsed with PBS and warmed to 37°C for one hour prior to replating.

2.5. Drug and Inhibitors Treatments

For cytoskeleton disruption experiments, MEF-3T3 cells were plated on FN-coated (10 µg/ml) plates and then serum starved overnight on 0.25% FBS-

DMEM. The plates were then treated with colchicine (10 µg/ml) or nocodazole (10 µM) for 30 minutes prior to lysis. To disrupt the microtubule network prior to FN stimulation, colchicine was added to the cells in suspension in the final 30 minutes of incubation.

In some scratch-wound induced migration experiments, the MEF 3T3 monolayer were pre-incubated for 60 minutes with 10 µM of PP2, PP3 (EMD-Calbiochem) or U0126 (Cell Signaling), SB203580 (EMD-Calbiochem) and DMSO as control, prior to scratch wounding and kinase assays. *Clostridum difficile* Toxin B (Sigma) was used at a concentration of 40 pg/ml and incubated overnight.

2.6. Microinjection and Adenovirus Infection

For microinjection studies, plasmid DNA in water (100ng/ml) was injected into the nuclei of cells plated on FN-coated coverslips using a semi-automated Eppendorf System equipped with a 5171 Micromanipulator on a Zeiss Axiovert S100 microscope. The SLK expression plasmids consisted of HA-tagged SLK C-terminal truncation which results in SLK activation (HA-SLK$^{1-373}$). Plasmid HA-SLK$^{1-373K63R}$ is the kinase inactive version of HA-SLK$^{1-373}$. For co-injections, FLAG-tagged RacN17 and HA-SLK$^{1-373}$were mixed (each at 100ng/ml) and injected in MEF-3T3 cells. The injected cells were incubated for 3h (or 8h in coinjections) at 37°C, fixed in 4 % paraformaldehyde and processed for indirect immunofluorescence staining. Coverslips were double stained for HA, using anti-HA rabbit polyclonal antibody and anti-vinculin or actin as described above. In spreading assays, MEF-3T3 cells were infected with adenoviruses expressing either of the two HA-tagged truncations of SLK. The cells were serum-starved in 0.25% FBS-DMEM overnight and infected for 90 minutes at a MOI of 100 in serum-free medium and allowed to express the tagged proteins for 5h. The cells were then collected by centrifugation, held in suspension for 1h and plated onto fibronectin coated coverslips and allowed

to spread for 10-20 minutes prior to fixation and immunofluorescence staining.

2.7. Western Blot, Immunoprecipitation and In Vitro Kinase Assays

Protein extracts were made in modified RIPA buffer containing 50 mM Tris-HCl (pH 7.4), 150mM NaCl, 1mM EDTA, 1% Triton X-100, 0.5 % sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS), 1% NP40 and protease inhibitors (Sigma-Aldrich inhibitor cocktail). For SLK expression analysis, 20 µg of total cell lysate was subjected to Western blot with anti-SLK rabbit polyclonal antibodies. Equal amounts of protein (20- 40µg) were electrophoresed on SDS-polyacrylamide gels (SDS-PAGE) and transferred to polyvinylidene difloride (PVDF) membrane (NEN Life Science Products). Membranes were probed with the indicated antibodies overnight at 4°C in 5% BSA or skim milk powder in 1 X TBST (50 mM Tris pH 7.4, 150mM NaCl, 0.05 Tween 20). Reactive proteins were detected by enhanced chemiluminescence using a goat anti-rabbit horseradish peroxidase (HRP)-labeled secondary antibody, and visualized using a chemiluminescence reagent (Perkin Elmer Life Science Inc.).

For immunoprecipitations, 300-400 µg of protein lysate was immunoprecipitated with 2µg of SLK antibody or anti-paxillin and 20µl of protein A sepharose (Amersham Pharmacia) for 2-12 hours at 4°C. Immune complexes were recovered by centrifugation and washed with NETN buffer (20mM Tris-HCl pH 8.0, 1mM EDTA, 150mM NaCl, 0.5% Nonidet P-40). Samples were fractionated on 8-12% SDS-PAGE and transferred to PVDF membranes.

For the detection of focal adhesion kinase (FAK), 50 µg of cell lysate was subjected to Western blot with anti-FAK rabbit polyclonal IgG antibody (Upstate Biotechnology) or with anti-FAK [pY397] phosphospecific rabbit polyclonal antibody. To evaluate the level of paxillin tyrosine phosphorylation, 500 µg of total cell lysate in modified RIPA was immunoprecipitated using 2 µl of anti-

paxillin monoclonal antibody (clone 349, Transduction Laboratories), washed in NETN and transferred to PVDF membranes. The membranes were probed with an anti-phosphotyrosine antibody (RC20: HRPO; Transduction Laboratories) and subjected to chemiluminescence detection.

In co-immunoprecipitation experiments, equal amounts of total cell lysate (400 µg in modified RIPA or Triton-X buffer (50 mM Tris pH7.4, 150 mM NaCl, 1mM EDTA, 1% Triton-X 100) were immunoprecipitated with anti-SLK, washed in NETN, resolved on 8% SDS-PAGE gels, transferred to PVDF membranes and probed for the indicated proteins.

For *in vitro* kinase assays, 400 µg of total cell lysate was immunoprecipitated using 2 µg anti-SLK antibodies and 20 µL of Protein A sepharose for 2 hours at 4°C. Immunoprecipitates were washed three times with NETN (50 mM Tris-HCl [pH 7.5], 150mM NaCl, 1mM EDTA, 0.1% Nonidet P-40) and once with kinase buffer (20mM Tris-HCl [pH 7.5], 15mM MgCl2, 10 mM NaF, 10 mM ß-glycerophosphate, 1mM orthovanadate). Reactions (20 µl in kinase buffer) were initiated by the addition of 5 µCi of $\gamma[^{32}P]$ ATP. After 30 minute incubation at 30°C, reactions were terminated by the addition of 4 x SDS sample buffer and 20 µl aliquots were fractionated by 8% SDS-PAGE. Gels were transferred to PVDF membranes, exposed to X-ray film and probed for SLK to evaluate the efficiency of the immunoprecipitation and normalization. The kinase assays data were analyzed by densitometry and normalized with the western blots, using Image One software (Bio-Rad).

2.8. Migration Assays: Boyden chamber, Wound Healing and Microtubule-Dependent Adhesion Turnover Assays

For migration assays, MEF 3T3 cells were either infected with adenovirus or treated with control or SLK siRNAs and serum starved overnight (DMEM+0.25% FBS). Cells (1-3 $X10^4$) were then resuspended in DMEM containing 0.1 % BSA

and added to the top of a Boyden chamber (Chemicon) pre-coated with fibronectin (10 µg/ml) and allowed to migrate for 3-6 hours. Residual cells were removed from the top of the chamber and the filter was rinsed in PBS, fixed in 4% PFA for 10 minutes and stained with DAPI (0.5µg/ml). The cells that migrated to the underside of the filter were enumerated from 5 to 10 random fields using DAPI fluorescence. Cell counts were performed in triplicate for three independent experiments.

Wound healing assay was performed as described (Etienne-Manneville and Hall, 2001). The wound was made by scraping a micropipette tip across a coverslip. After 3-6 hours incubation at 37°C, the coveslips were prepared for immunoflourescence and migration of the cells at the leading edge measured by ImageJ software (NIH Image).

For biochemical analysis, MEF 3T3 cells were plated on fibronectin coated (10 µg/ml) dishes and serum starved confluent monolayers were then scratched with a pipette tip until approximately 50% of the monolayer was removed. Cells were then washed with PBS, refed with 0.25% DBS-DMEM and collected at various time points. In some experiments, the monolayers were pre-incubated for 60 minutes with 10µM of PP3, PP2, U0126, SB203580, and scratched wounded in the presence of inhibitors or DMSO control. Toxin B incubation was for 12-24 hours before scratching. The collected cells were analyzed by kinase assays and western blots.

For microtubule dependent adhesion turnover assays (Ezratty et al., 2005), serum-starved subconfluent MEF-3T3 cells were treated with 10µM nocodazole for 3 hours. The cultures were then washed 4 times with serum-free medium and refed with 0.25% FBS-DMEM for the duration of the time course. Cultures were harvested at different time points and surveyed for FAK-pY397 (BioSource) and α-tubulin (Sigma) by Western blotting and immunofluorescence.

CHAPTER 3

A ROLE FOR SLK IN REGULATION OF ACTIN DYNAMICS DURING CELL ADHESION AND SPREADING

3.1. Introduction

Cells reside in a protein network called the extracellular matrix, which they secrete and mold into the intercellular space. The extracellular matrix exerts profound control over the cells. The effects of the matrix are primarily mediated by integrins, a family of cell surface receptors that attach cells to the matrix and mediate mechanical and chemical signals from it. These signals regulate the activities of cytoplasmic kinases, growth factor receptors, and ion channels and control the organization of the intracellular actin cytoskeleton (Giancotti and Ruoslahti, 1999).

Integrin receptor binding to extracellular matrix proteins generates intracellular signals through tyrosine phosphorylation events that are important for cell growth, survival, and migration (Schlaepfer et al., 1999). In various cell types, integrin clustering triggers tyrosine phosphorylation of signaling proteins through the activation of a large number of non-receptor protein tyrosine kinases such as focal adhesion kinase (FAK) and the Src family of kinases. Increased tyrosine phosphorylation of intracellular proteins is one of the earliest responses stimulated by integrin receptor activation when cells contact matrix protein such as fibronectin (FN). Upon replating of cells on fibronectin, FAK becomes activated either through conformational changes or as a result of aggregation mediated by integrin clustering. FAK autophosphorylation at Tyr397 under these conditions promotes the transient association of Src-family protein tyrosine kinases with FAK and the formation of a signaling complex.

In addition, FAK associates with several different signaling proteins such as CAS, Shc, Grb2, phosphatidylinositol 3-kinase, and paxillin. This association enables FAK to function within a network of integrin-stimulated signaling pathways, leading to the activation of targets such as the ERK, JNK and MAPK pathways (Horwitz and Parsons, 1999; Ilic et al., 1997).

For many cellular processes, microtubules and the actin cytoskeleton dynamics must be coordinated. Recent studies suggest that there may be signal transduction systems that integrate the responses of the two systems (Bershadsky et al., 1996; Kaverina et al., 2000; Kaverina et al., 1999; Small et al., 1999a; Small et al., 1999b). Previous data have suggested the existence of a factor associated with the microtubules that can be released and affect assembly of the actin meshwork (Gundersen and Cook, 1999). Interestingly, microtubule breakdown stimulates stress fiber formation in fibroblasts, requires active Rho, and involves elevated myosin light chain phosphorylation, suggesting a coordinate regulation of the actin cytoskeleton and the microtubule network (Cook et al., 1998; Danowski, 1989; Enomoto, 1996). This is accompanied by an assembly of adhesion sites and tyrosine phosphorylation of the adhesion components FAK and paxillin (Bershadsky et al., 1996; Bershadsky et al., 2006).

Tension exerted by microtubules on the actin cytoskeleton has been hypothesized to counteract stress fiber formation. Recently, it has been shown that there is direct physical interaction between the microtubules and adhesion sites, suggesting that relaxation signals are delivered via the microtubules to focal contacts to modulate the development of adhesions in a region-specific manner (Kaverina et al., 1998; Waterman-Storer and Salmon, 1999; Waterman-Storer et al., 1999).

Our laboratory has previously shown that overexpression of SLK in various cell lines induce the rapid disassembly of actin stress fibers and cell death (Sabourin et al., 2000). Relatively high levels of SLK protein and activity have been observed in all cell types analyzed, suggesting a functional role for SLK in physiological processes other than apoptosis. Because of its ability to induce cytoskeletal remodeling when overexpressed in a variety of cultured cell lines, we wanted to analyze the levels, distribution, and activity of SLK protein during cell spreading on

FN, a process that requires extensive cellular remodeling (Schlaepfer et al., 1999; Small et al., 1998).

3.2. Results

3.2.1. SLK Is Redistributed To the Cell Periphery to Podosome-Like Structures during Cell Adhesion and Spreading

To gain insight into the role of SLK during normal cell growth, we first investigated the cellular distribution of endogenous SLK in exponentially growing MEF-3T3 cells. Supporting the observation that SLK can induce stress fiber disassembly, endogenous SLK distribution was found to correlate with an absence of actin stress fibers **(Fig. 3.1. A-B)**, and suggested a role for SLK in the regulation of actin dynamics. Interestingly, the levels of SLK protein were also found to be variable, suggesting that its expression may be regulated throughout the cell cycle.

The replating of suspended cells on FN-coated substrates has been shown to trigger the recruitment of adhesion complexes and subsequent actin polymerization from adhesion sites (Schaller and Parsons, 1994). To investigate the function of SLK in cytoskeletal remodeling events, MEF-3T3 cells were immunostained for SLK during adhesion and spreading shortly after FN stimulation. When MEF cells held in suspension were replated onto FN coated substrates, SLK was found to be redistributed to the cell periphery and to colocalize with the adhesion protein vinculin **(Fig. 3.1. C-D)** and FAK (data not shown). Surprisingly, SLK was observed to colocalize with vinculin at large structures resembling lamellipodia that formed abundantly 20 min post-replating **(Fig. 3.1. E-F)**. A poor association was observed at discrete adhesion complexes that formed in the initial 10 min of spreading **(Fig. 3.1. C-D)**, suggesting that SLK may be associated with adhesion complex disassembly or turnover.

3.2.2. SLK Kinase Activity Is Not Activated During Cell Adhesion and Spreading On Fibronectin

To investigate potential changes in SLK kinase activity during cell spreading on FN, extracts from replated cells were prepared and subjected to Western blot analysis and in vitro kinase assays. Following replating and Western blot analysis, total SLK levels remained unchanged during cell spreading **(Fig. 3.2.A)**. Interestingly, following in vitro kinase assay and normalization to total immunoprecipitated SLK, SLK activity remained unchanged following replating onto FN coated plates **(Fig. 3.2. A)**. Similarly, no changes were observed during cell adhesion (20 min) to poly-L-lysine-coated substrates. Over the same time period, the phosphorylation of FAK at tyrosine 397 and increased paxillin tyrosine phosphorylation did occur, indicating that the adhesion components were activated **(Fig. 3.2. B)** (Schaller and Parsons, 1994). These results suggest that SLK distribution is modulated by adhesion without any detectable changes in its kinase activity.

3.2.3. SLK is Associated with the Microtubules and Adhesion Components during Cell Spreading

The observation that SLK colocalizes with adhesion components during late spreading events raises the possibility that it is implicated in adhesion complex disassembly or turnover. Recently, adhesion site contact by the microtubule network has been shown to promote their dissociation (Bershadsky et al., 1996; Kaverina et al., 1999; Kaverina et al., 1998) . In addition, the disruption of microtubules has been shown to induce the assembly of focal adhesions and to stimulate the formation of actin stress fibers (Bershadsky et al., 1996).

Therefore, we investigated the distribution of SLK and α-tubulin in double immunostaining experiments. As shown in **Fig. 3.3** a very strong colocalization was observed between the microtubule network and endogenous SLK protein in exponentially growing MEF cells (95% of exponentially growing cells). In some

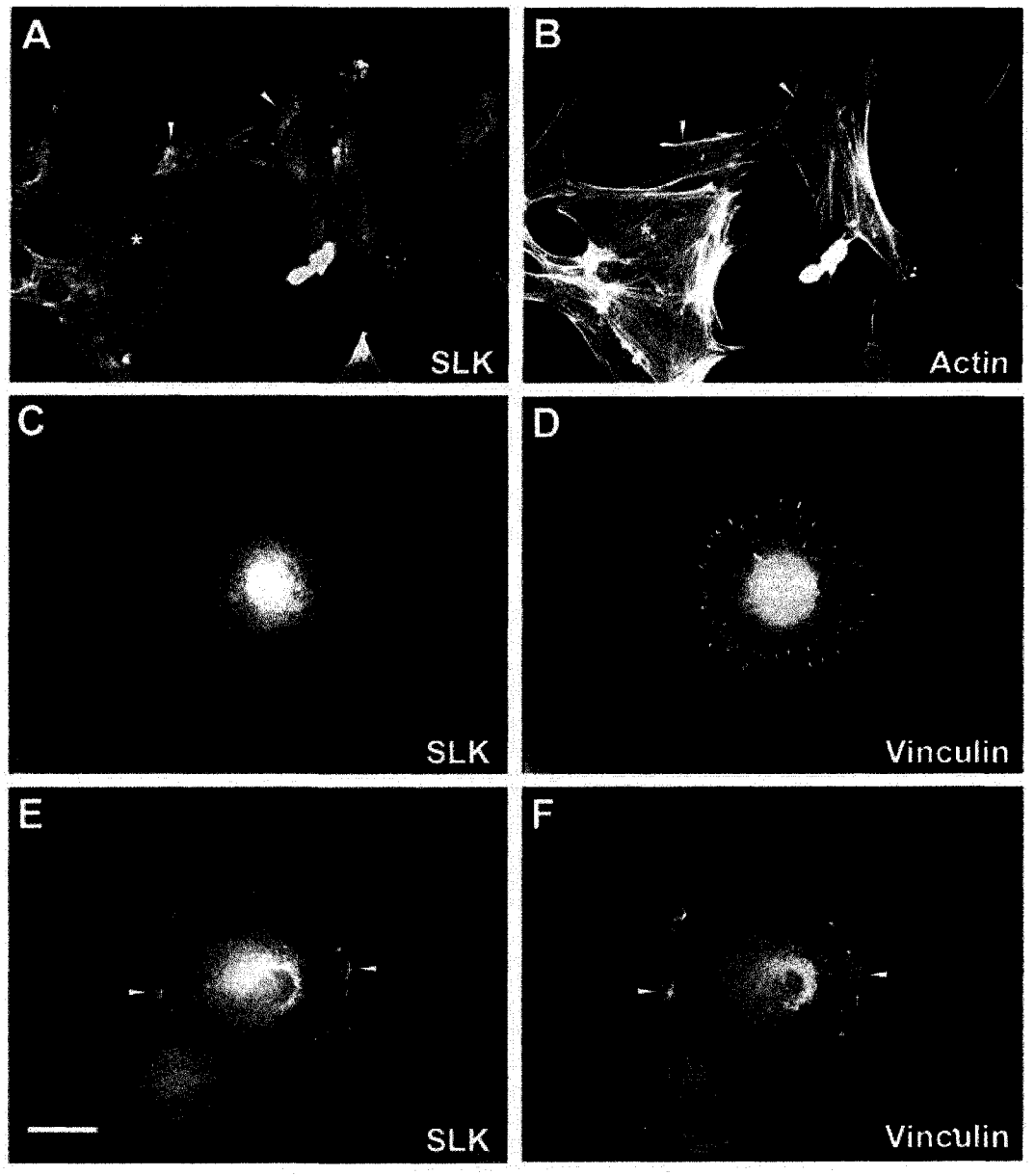


Figure 3.1. SLK localizes to podosome-like structures during cell adhesion and spreading. Exponentially growing MEF-3T3 cells immunostained for SLK **(A)** and F-actin **(B)** and are shown. Following replating on FN (10 min) SLK was found to be predominantly perinuclear (C) and not associated with focal complexes detected by vinculin antibody (D). At later time points (20 min), a proportion of SLK **(E)** was found to be associated with vinculin **(F)** in diffuse podosome-like adhesion structures (arrows). This colocalization was observed in 60 ± 7% cells (n = 200) 20 min after replating. The cells were photographed at X400 **(A and B)** and X1000 **(C–F)**. Scale bar: 10 μm.

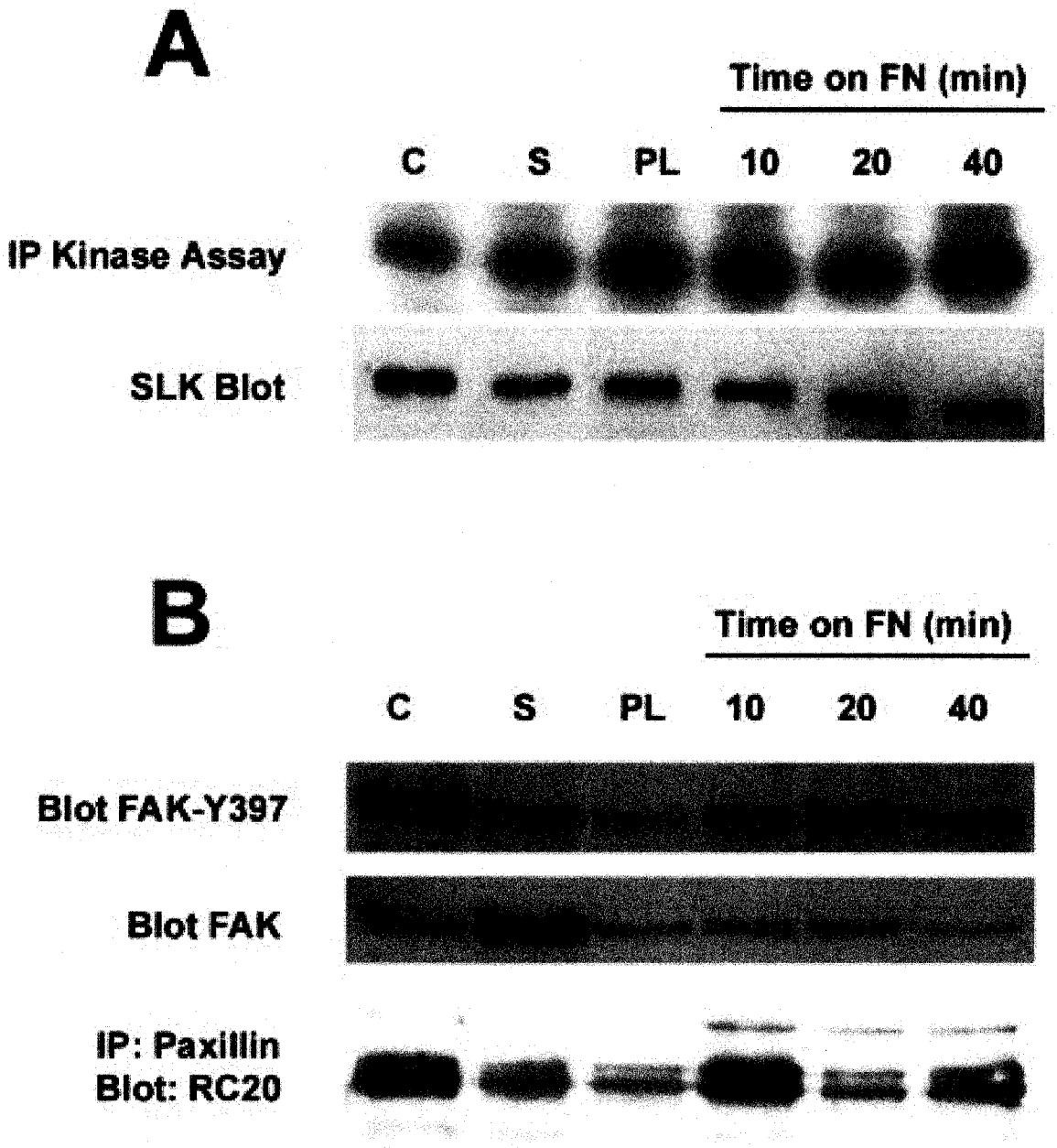


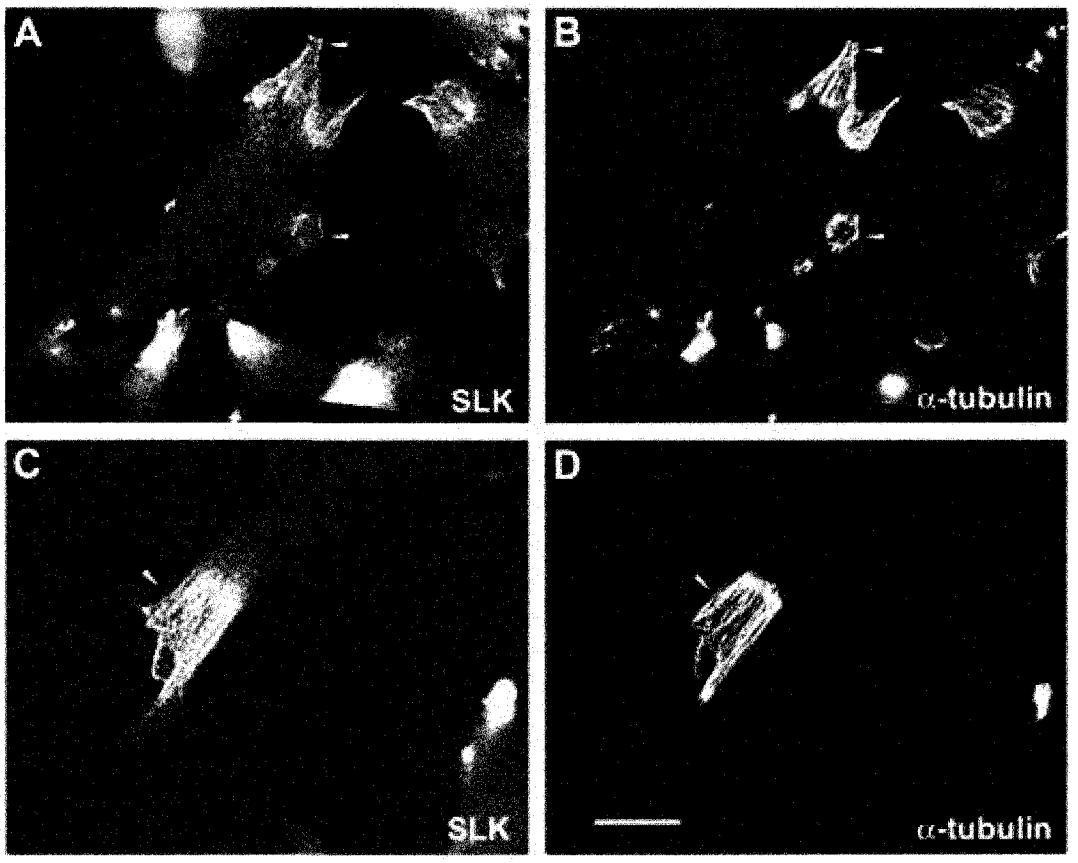
Figure 3.2. SLK is not activated during cell adhesion and spreading on fibronectin. A. Serum-starved MEF-3T3 cells (C) were held in suspension (S), replated on FN-coated dishes or poly-L-lysine (PL), and sampled at timed intervals for SLK expression and activity. **B.** The activation of FAK (determined by Tyr-397 phosphorylation) and tyrosine phosphorylation of paxillin were analyzed for comparison.

areas at the cell periphery, SLK staining presented a "filament-like" pattern **(Fig. 3.3. C-D)** that colocalized with the microtubule network, suggesting that SLK is a microtubule-associated kinase. Interestingly, this association of SLK with the microtubules appeared to be preferentially non-centrosomal. Similarly, non-centrosomal microtubule nucleation has been observed at focal contacts in 3T3 cells during recovery from nocodazole or taxol treatment (Kaverina et al., 1998).

To test whether SLK forms part of a complex with the microtubule, whole cell extracts obtained under different lysis conditions were subjected to immunoprecipitations using anti-SLK followed by Western blot analysis. Our results show that under all of the conditions tested (Triton X-100, NP-40 and RIPA extraction buffers), α-tubulin could be coprecipitated with SLK **(Fig. 3.3. E)**. However, we performed an *in vitro* binding assays using glutathione S-transferase-tubulin (GST-tubulin), and reported that SLK did not interact directly with α-tubulin (data not shown), suggesting that SLK is indirectly associated with the microtubule and that they are part of a higher order protein complex.

3.2.4. Microtubule Disrupting Drugs Modulate SLK Cellular Distribution during Cell Spreading in Fibronectin

The treatment of serum-starved fibroblasts with microtubule disrupting agents has been previously shown to stimulate the formation of large adhesion complexes containing tyrosine phosphorylated FAK and paxillin at the cell periphery (Bershadsky et al., 1996; Liu et al., 1998). To test whether SLK can redistribute to newly formed adhesion sites following microtubule depolymerization, serum-starved MEF cells grown on FN were treated with nocodazole for 30 min and subjected to double immunostaining for SLK and vinculin or α-tubulin. Induction of focal adhesion assembly by nocodazole resulted in the redistribution of a portion of SLK to the cell periphery with vinculin and α-tubulin, suggesting that SLK can be targeted to newly formed adhesion sites **(Fig. 3.4. D-E)**. Interestingly, this can be



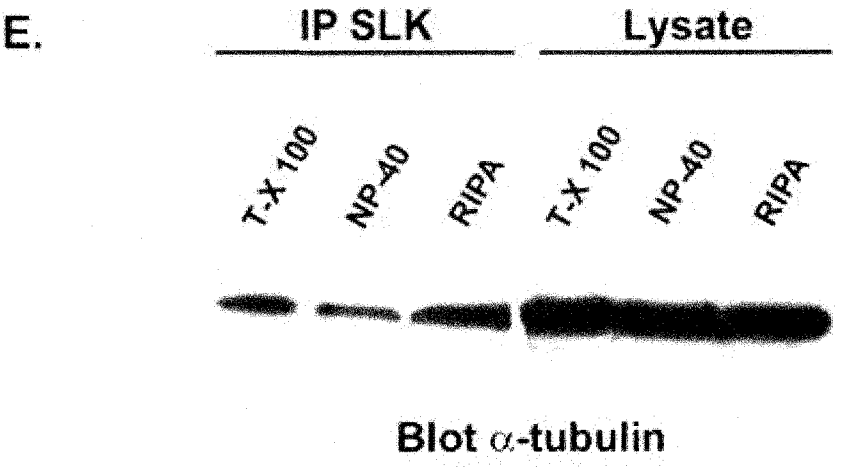


Figure 3.3. SLK co-localizes and co-imunoprecipitates with the microtubule network.

Exponentially growing Swiss-3T3 cells were double-labeled for both SLK **(A and C)** and tubulin **(B and D)**. **E.** Cells exponentially growing on FN were immunoprecipitated with anti-SLK antibody and subjected to Western blot analysis using an anti-α-tubulin monoclonal antibody. Photomicrographs are shown at X630 **(A and B)** and X1000 **(C and D)**. Scale bar: 10 μm.

achieved in the absence of an intact microtubule network **(Fig. 3.4. F-G)**.

Microtubule-disrupting agents have been shown to impair cell spreading and focal adhesion turnover (Kaverina et al., 1999). Therefore, we next examined whether SLK was also associated with the microtubule network during cell adhesion and spreading on FN. The replating of suspended cells on FN-coated substrates resulted in SLK localization to the cell periphery with polymerized microtubules **(Fig. 3.5. A-B)**. Interestingly, in the presence of colchicine, SLK was also colocalized with α-tubulin at the cell periphery, even though the vast majority of the cells failed to spread **(Fig. 3.5. C-D)**. Similarly, SLK was colocalized with large vinculin-containing structures in colchicine treated cells **(Fig. 3.5. E-F).** Overall, these results suggest that SLK is associated with the microtubule network but can also be targeted to adhesion sites independently of polymerized microtubules.

3.2.5. Overexpression of SLK Promotes Cellular Retraction and Inhibits Cell Spreading

To gain insight into the role of SLK during cell adhesion and spreading, MEF-3T3 cells grown on FN coated substrates were microinjected with epitope-tagged SLK expression vectors encoding the activated truncation SLK^{1-373} (or the kinase inactive mutant $SLK^{1-373K63R}$) and analyzed for the presence of actin stress fibers and focal adhesions **(Fig. 3.6 and Fig. 3.7)**. Cells overexpressing activated SLK^{1-373} displayed a retracted morphology (rounded appearance and loosely adherent), a marked reduction in actin stress fibers and extensive actin reorganization at the periphery **(Fig. 3.7. A-B)**. Quantitative analysis showed that approximately 85% (86.6 ± 7%) of cells expressing SLK^{1-373} displayed that phenotype. In contrast, no change in stress fiber density was observed in cells injected with the kinase-inactive $SLK^{1-373K63R}$ (97 ± 2% retained stress fibers, **Fig. 3.7. C-D)**. When SLK^{1-373}-overexpressing cells were surveyed for focal adhesions using anti-vinculin antibodies, peripheral adhesion sites were smaller and fibrillar adhesions were

greatly reduced or absent, consistent with the disassembly of actin stress fibers **(Fig. 3.7. E-H).** Overexpression of $SLK^{1-373K63R}$ had no effect on the density, size, and distribution of vinculin containing adhesion sites in all injected cells **(Fig. 3.7, G-H)**. No obvious differences were observed in the organization of the microtubule network of microinjected cells (data not shown). These results suggest a functional role for SLK in the process of actin stress fiber disassembly rather than a direct destabilization of adhesion structures.

3.2.6. Investigation of the Functional Interaction between SLK and Rac1, a Small RhoGTPase Protein, Involved in Actin Remodeling

The small GTPase Rac1 has been previously shown to induce stress fiber disassembly as well as lamellipodia formation (Van Aelst and D'Souza-Schorey, 1997). Interestingly, Rac1 also binds directly to the microtubule in its GTP-bound form and becomes activated by microtubule growth in fibroblasts (Best et al., 1996; Waterman-Storer et al., 1999). Therefore, we tested the possible involvement of Rac1 in SLK-mediated stress fiber disassembly. As shown in **Fig. 3.8. A-B**, the coinjection of both $HA\text{-}SLK^{1-373}$ and a dominant negative form of Rac1, RacN17, resulted in a marked inhibition of stress fiber disassembly (86% compared with 32% in the presence of RacN17) and subsequent delay in cellular retraction for up to 8 h post-injection **(Fig. 3.8. C)**. Over the same period, cells expressing $HA\text{-}SLK^{1-373}$ alone had completely retracted and displayed extensive blebbing, a hallmark of apoptotic cells. Furthermore, as for vinculin, in 60% (60 ± 5%) of the cells, SLK was found to colocalize with Rac1 following replating of MEF-3T3 cells onto FN **(Fig. 3.9)**. Together, these results suggest a functional interaction between these two proteins and that Rac1 may be a mediator of SLK-induced actin remodeling. Whether SLK directly regulates Rac1 or an upstream guanine exchange pathway remains to be elucidated.

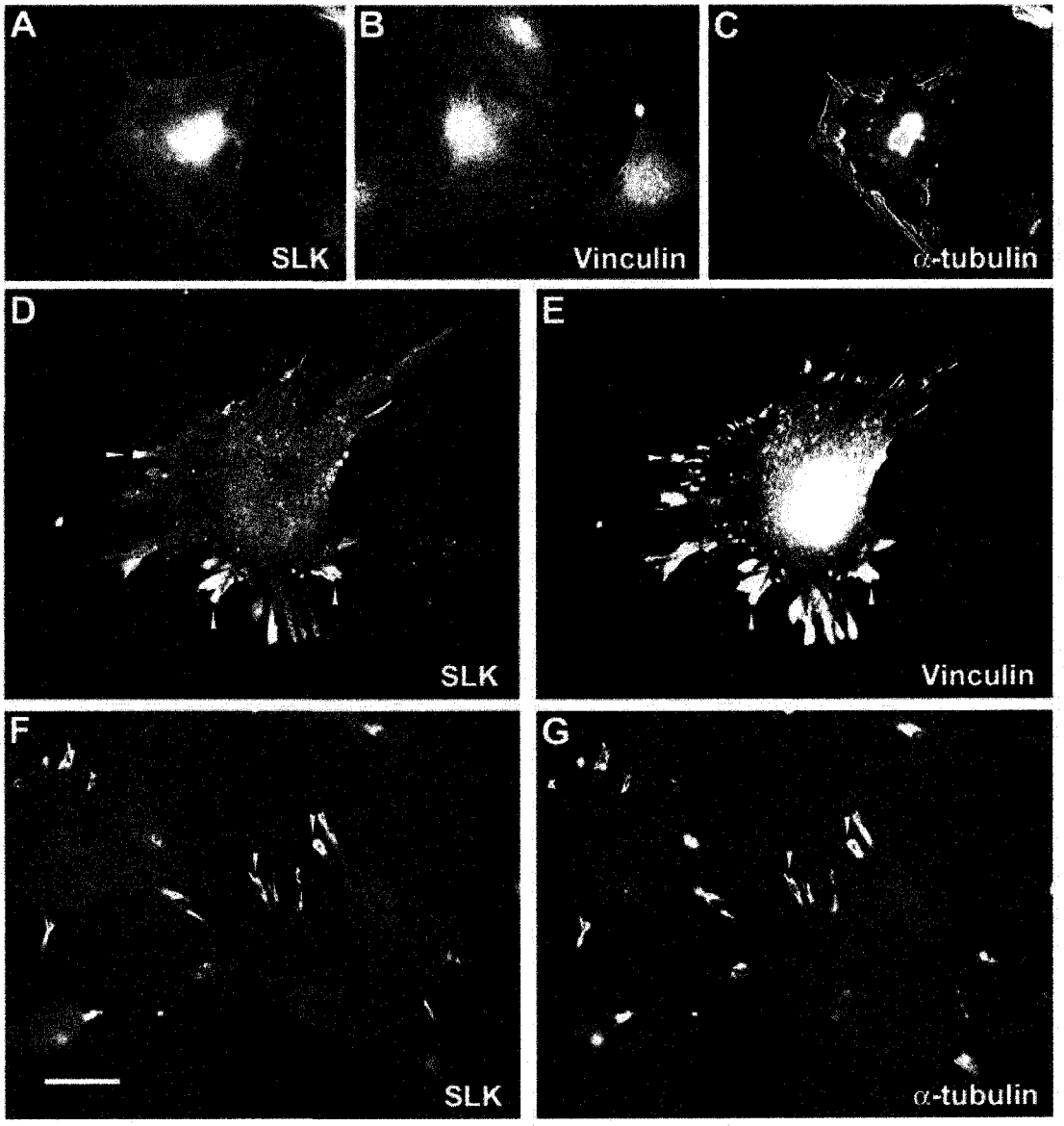


Figure 3.4. SLK is redistributed to adhesion complexes induced by microtubule disruption. Serum-starved cells were immunostained for SLK **(A)**, vinculin **(B)**, α-tubulin **(C)** prior to the addition of nocodazole. The addition of nocodazole to the cultures for 30 min induced the formation of large adhesion complexes **(E)** and the redistribution of SLK to these sites **(D)**. Similarly, SLK was also localized to these sites **(F)** along with depolymerized α-tubulin **(G)**. Photomicrographs are shown at X630. Scale bar: 10 μm.

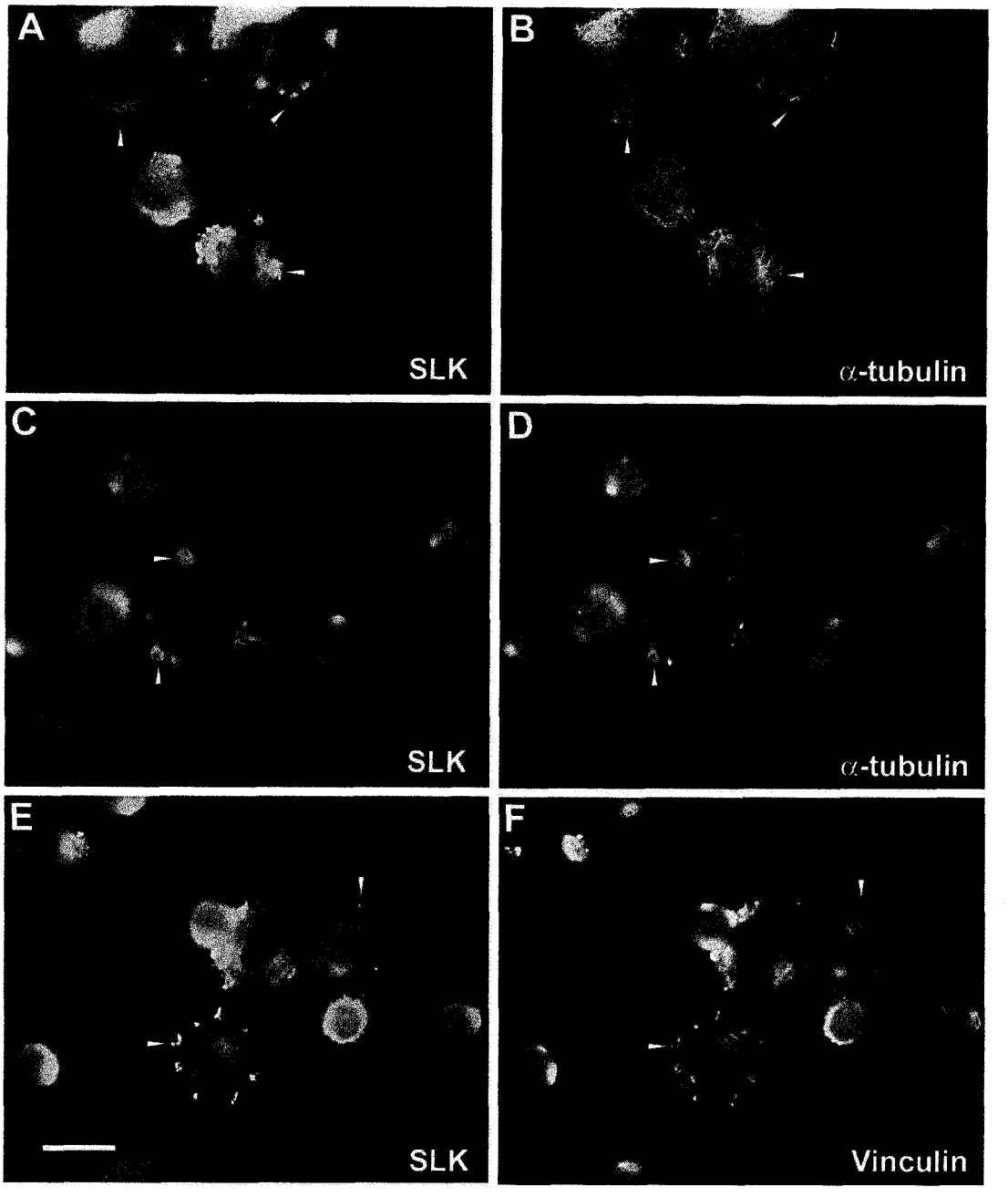


Figure 3.5. SLK colocalizes with the microtubule network during adhesion and spreading. Cells suspended in serum-free medium were replated onto FN-coated coverslips for 20 min in the absence **(A and B)** or in the presence **(C–F)** of colchicine and immunostained for SLK, α-tubulin, and vinculin. Photomicrographs are shown at X630. Scale bar: 10 μm.

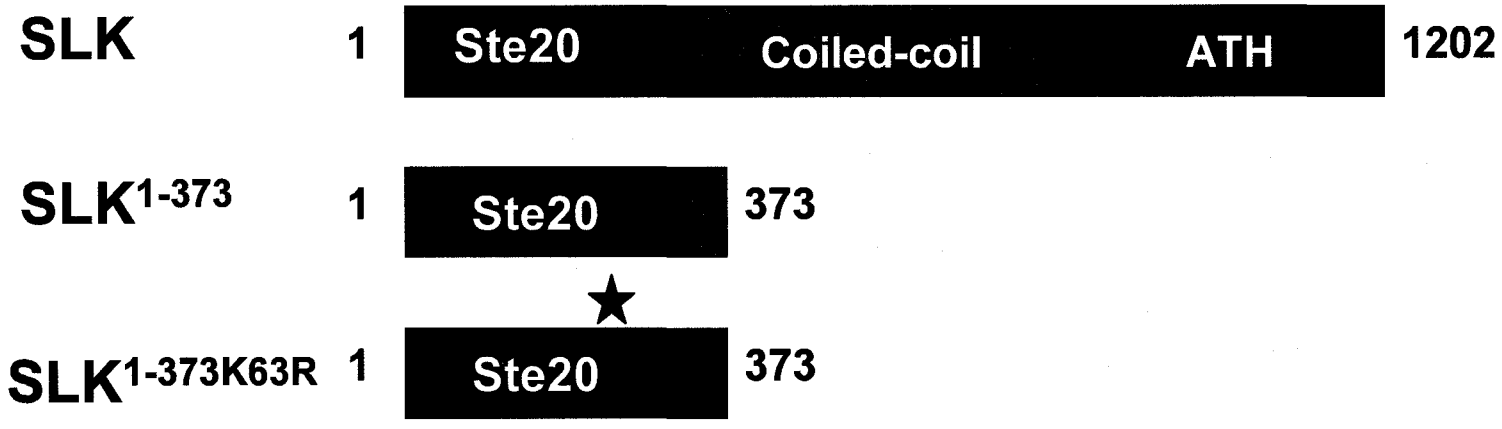


Figure 3.6. Schematic representation of the plasmid expression vectors used in this study. The Ste20 kinase, coiled-coil and ATH domains of SLK protein are indicated. SLK amino acid residues are indicated at the amino- (N) and carboxyl- (C) termini. The SLK^{1-373} truncation was constructed by deleting amino acids 374 to 1202, leaving the kinase domain. The construct $SLK^{1-373K63R}$ was obtained through PCR-based mutagenesis of the ATP binding site at lysine residue 63 (star).

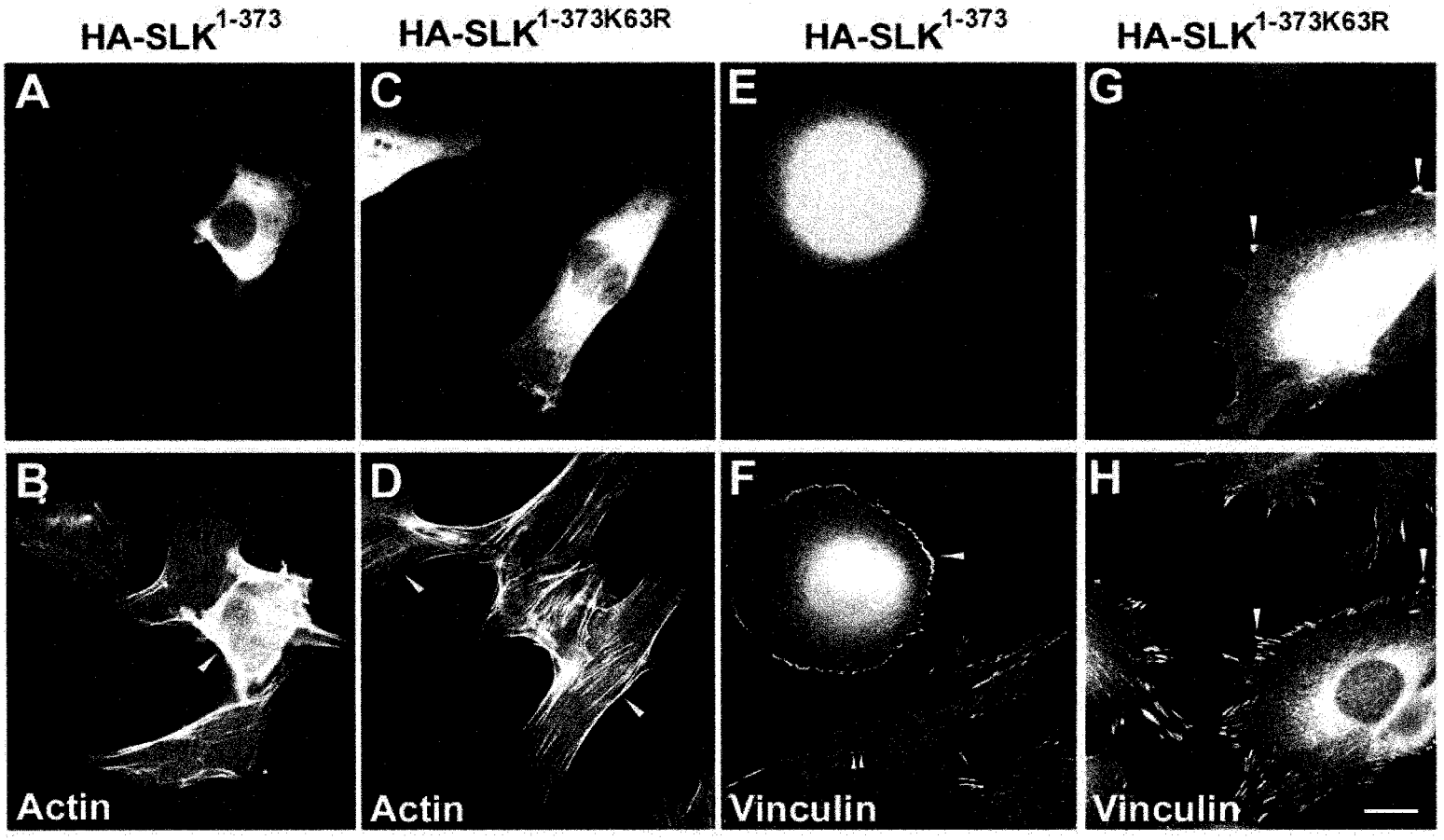


Figure 3.7. Microinjection of activated SLK induces actin stress fiber disassembly.
MEF-3T3 cells plated on FN-coated coverslips were microinjected with HA-tagged SLK^{1-373} and immunostained 3h post-injection. **A and E,** wild-type SLK^{1-373}-injected cells (or the inactive kinase $SLK^{1-373K63R}$, **C and G)** were double-labeled with anti-HA **(A, C, E, and G)** and TRITC-phalloidin **(B and D)** or anti-vinculin **(F and H)**. Photomicrographs are shown at X630. Scale bar: 10 μm.

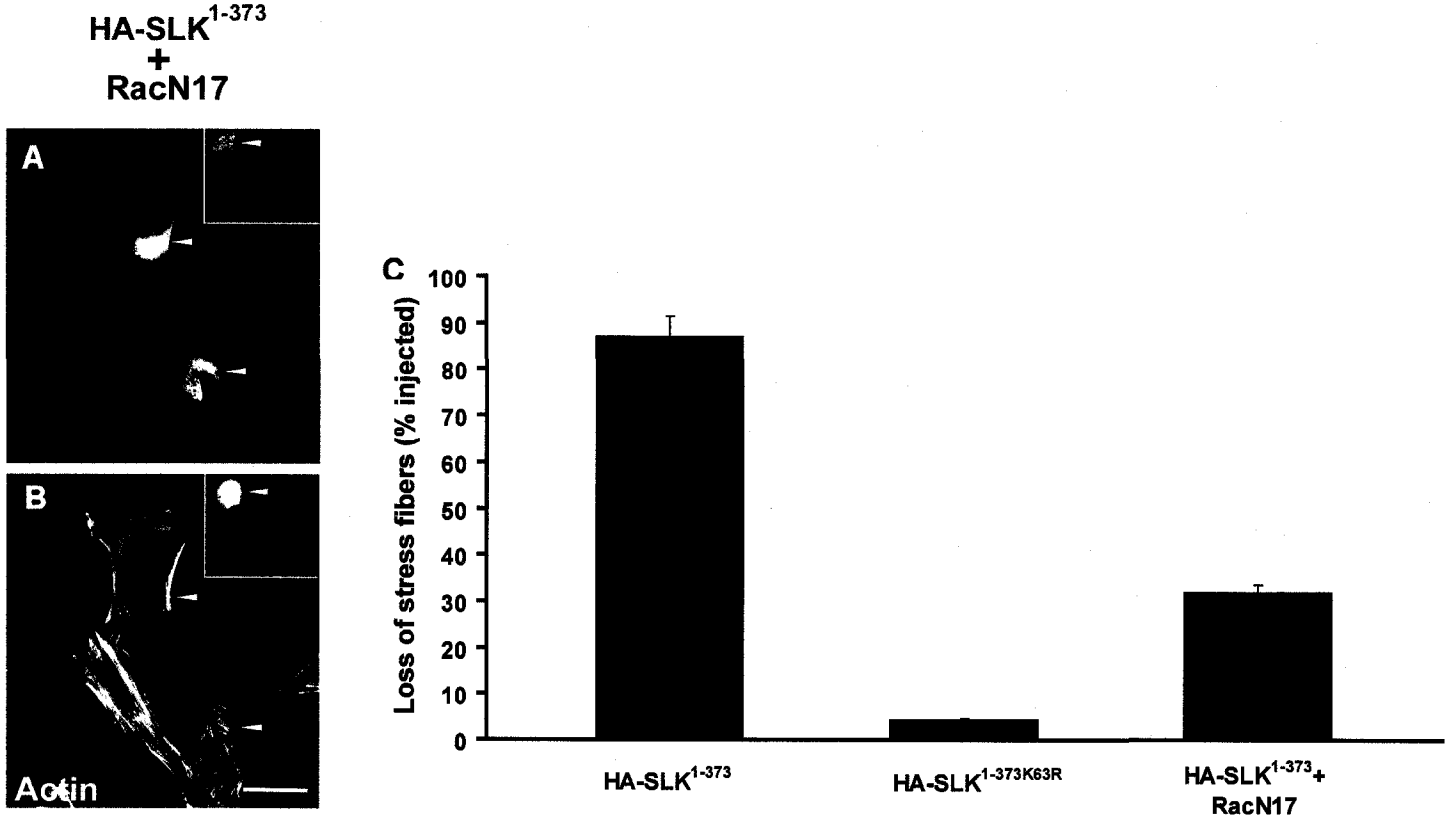


Figure 3.8. Rescue experiment using co-injection of activated SLK and dominant negative form of Rac1 (RacN17). **A-B.** Coinjection of RacN17 with SLK^{1-373} inhibits stress fiber disassembly for up to 8h post-injection. Insets show cells injected with SLK^{1-373} alone and fixed after 8h of expression. **C.** Cells that stained positive for the HA epitope and displayed an absence of cytoplasmic stress fibers were scored. At least 50 injected cells were counted in three separate experiments, and the average is shown. Stress fiber disassembly induced by expression of SLK^{1-373} (87 ± 7% cells) could be inhibited by coinjection with RacN17 (loss of stress fibers in 32 ± 7% of the injected cells following RacN17 coinjection). The data represent averages ± standard errors for three independent experiments performed in duplicates. Error bars represent standard errors (S.E).

3.2.7. Overexpression of Activated SLK Inhibits Cell Spreading on Fibronectin

To test the effect of SLK overexpression on cell spreading, MEF-3T3 cells were infected with adenoviral constructs encoding SLK^{1-373} or $SLK^{1-373K63R}$ and then replated on FN-coated substrates for 10–20 min. Because overexpression of SLK^{1-373} induces a rapid apoptotic response the cells were only allowed to express the HA-tagged constructs for 6 h prior to plating (Sabourin and Rudnicki, 1999; Sabourin et al., 2000). As shown in **Fig. 3.10**, cells expressing the kinase-inactive protein $SLK^{1-373K63R}$ assembled vinculin-containing adhesion sites but displayed an increased stress fiber density and appeared larger than uninjected cells with numerous lamellipodia **(Fig. 3.10. A, B, E, and F)**. This phenotype was observed in 95 ± 5% of HA-positive cells. In contrast, cells expressing activated SLK^{1-373} at relatively low levels (8% HA-positive cells) displayed very small adhesion sites throughout the cell **(Fig. 3.10. C-D)**. Cells (92 ± 6% HA-positive cells) expressing relatively higher levels of SLK^{1-373} failed to spread and assemble actin stress fibers on FN-coated substrates **(Fig. 3.10. G-H)**. The failure to polymerize actin in SLK^{1-373} overexpressing cells may lead to adhesion site instability and an inhibition of contractile mechanisms, leading to impaired cell spreading. These results are consistent with a role for SLK during late spreading events such as adhesion turnover, actin remodeling, and membrane protrusion.

3.3. Discussion

The processes regulating cellular adhesion and migration involve complex-signaling mechanisms, ultimately resulting in cytoskeletal remodeling and changes in cell shape and cell movement (Parsons et al., 1994; Small et al., 1999b).

To investigate the role of SLK in cytoskeletal remodeling during normal cell growth, we analyzed the expression, activity, and cellular distribution of endogenous

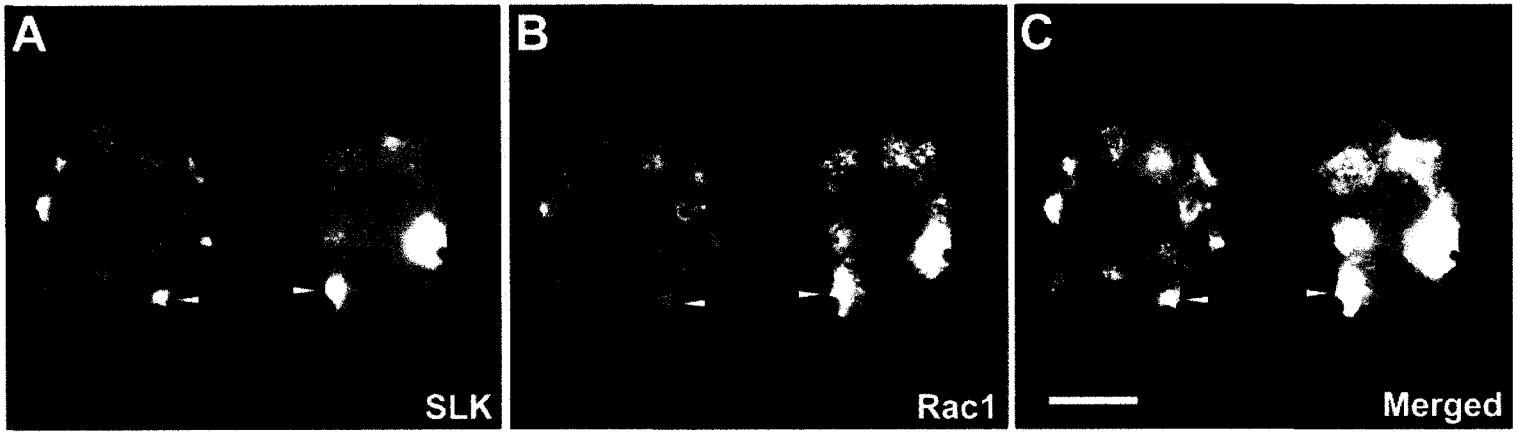


Figure 3.9. Rac1 and SLK co-localization in spreading MEF-3T3 cells.
MEF-3T3 cells held in suspension were replated for 20min and double-labeled for SLK (A) and Rac1 (B). Colocalization was observed at the cell periphery in 60 ± 5% (n = 200) of spreading cells (C). Scale bar: 10 µm.

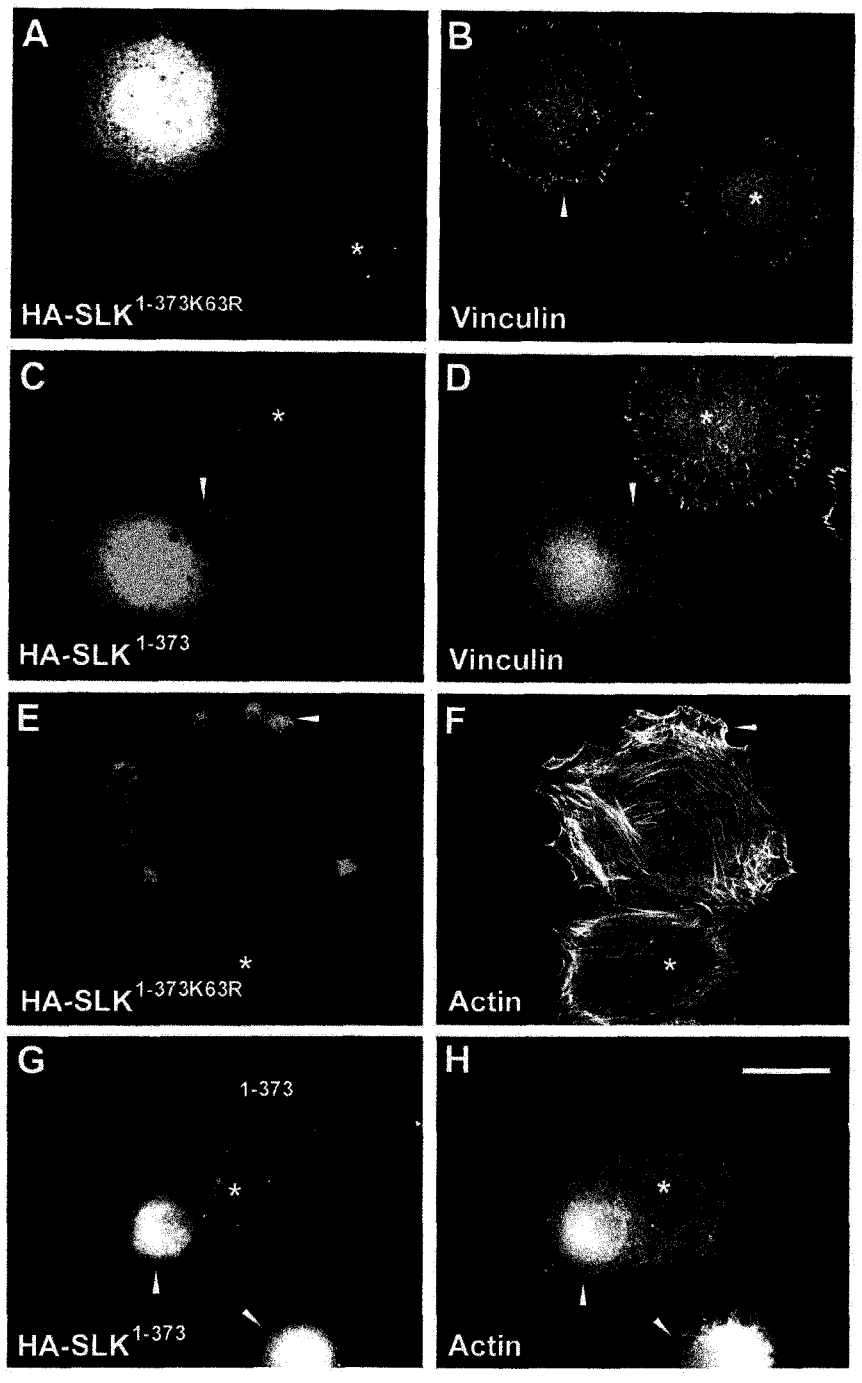


Figure 3.10. Overexpression of activated SLK inhibits cell spreading on FN. MEF-3T3 monolayers in serum-free medium were infected with adenoviral vectors carrying HA-tagged SLK^{1-373} **(C and G)** (or inactive kinase $SLK^{1-373K63R}$) **(A and E)**, held in suspension, and replated on FN-coated coverslips for 20 min. The cells were immunostained using anti-HA and TRITC-phalloidin **(F and H)** or anti-vinculin antibodies **(B and D)**. For both expression plasmids, at least 100 HA-positive cells were counted in three separate experiments. Uninfected cells are marked with an asterisk. Photomicrographs are shown at X630. Scale bar: 10 μm.

and ectopic SLK proteins following FN stimulation and cell spreading.

Our results show that SLK colocalizes with the adhesion protein vinculin at "podosome-like" structures, which formed predominantly during the late phase of cell spreading (20 min) on FN in MEF-3T3 cells. These large vinculin-containing structures are suggestive of loosely assembled adhesion sites resembling lamellipodia, which suggests that SLK may be involved in regulating cell spreading through destabilization of adhesion contacts. Supporting this possibility, no colocalization with vinculin was observed early (10 min) during spreading when focal adhesions are assembling and adhesion components become tyrosine-phosphorylated. In addition, the overexpression of activated SLK in exponentially growing cells or cells that were replated onto FN-coated substrates induced their retraction from the substrate and inhibited cell spreading, respectively. This appeared to be mediated by a loss of actin stress fibers as well as fibrillar adhesions, presumably located along the fibers.

Furthermore, SLK-induced actin fiber disassembly could be inhibited by dominant negative Rac1, RacN17, suggesting a role for Rac1 downstream of SLK-mediated cytoskeletal remodeling events. These results support a role for SLK in actin dynamics during cell spreading, which may contribute to adhesion site destabilization and turnover (Horwitz and Parsons, 1999; Schlaepfer et al., 1999).

Recently, the microtubule network has been clearly implicated in adhesion site destabilization and turnover, a process that requires kinesin motors (Kaverina et al., 1998; Krylyshkina et al., 2002). Interestingly, the Ballestrem group have demonstrated that microtubules are required for cell migration, tail retraction, and modulation of cell adhesion (Ballestrem et al., 2000). Similarly, it has been proposed that regional contractility is modulated by the interplay of microtubule-linked events with focal adhesions (Kaverina et al., 2000). Interestingly, our results show that SLK is associated with a microtubule-containing protein complex in MEF-3T3 cells.

In addition, we find that SLK colocalizes with vinculin-containing structures and the microtubule network during cell spreading. Combined with previous observations, our results suggest that one of the adhesion-destabilizing signals delivered by the microtubule may be SLK. One possibility is that the rapid disassembly of actin stress fibers mediated by SLK may represent a regulatory mechanism for the control of microtubule-mediated suppression of contractility during remodeling events. Alternatively, the depolymerization of actin fibers by SLK may allow more efficient targeting of adhesion sites by the microtubule for further delivery of destabilizing signals (Kaverina et al., 1998). Direct interaction between SLK and α-tubulin was not observed by in vitro binding assays, suggesting an indirect interaction. One possibility is that post-translational modification of one or both proteins is required for their interaction, a signal that could be transduced downstream of adhesion complex formation. Alternatively, an adapter protein is required to recruit SLK to the microtubule.

The Rho family of small GTPases has been implicated as a mediator of cytoskeletal remodeling events such as the formation of stress fibers, lamellipodia, and ruffling (Ridley et al., 1999; Ridley et al., 1992). The small GTPase Rac1 has been shown to be activated following microtubule growth and to promote lamellipodial protrusion (Waterman-Storer et al., 1999). Specifically, GTP-Rac1 can directly bind α-tubulin suggesting that it plays a role in cytoskeletal remodeling events regulated by microtubule growth such as adhesion disassembly (Best et al., 1996).

Our results show that SLK and Rac1 localization overlap extensively during cell spreading on FN at membrane protrusions resembling lamellipodia (cellular structures that can be induced by activated Rac1), suggesting that Rac1 may be a downstream effector of SLK. Furthermore, dominant negative Rac1 inhibits the cytoskeletal remodeling events induced by activated SLK, suggesting a functional

interaction between these proteins.

Interestingly, microtubule-disrupting agents induce the formation of adhesion sites and actin stress fibers, supporting the notion that the growing microtubules carry actin and adhesion destabilizing signals. Therefore, we propose a model whereby specific adhesion components through direct interaction with tubulin can target the microtubule network to adhesion sites **(Fig. 3.11)**. Targeting of SLK to adhesion sites by the microtubules can lead to actin network destabilization, a process required for further cytoskeletal remodeling and cell movement.

Overall, we have established a novel molecular link between the adhesion-destabilizing activity of the microtubule network and actin-based cytoskeletal remodeling events.

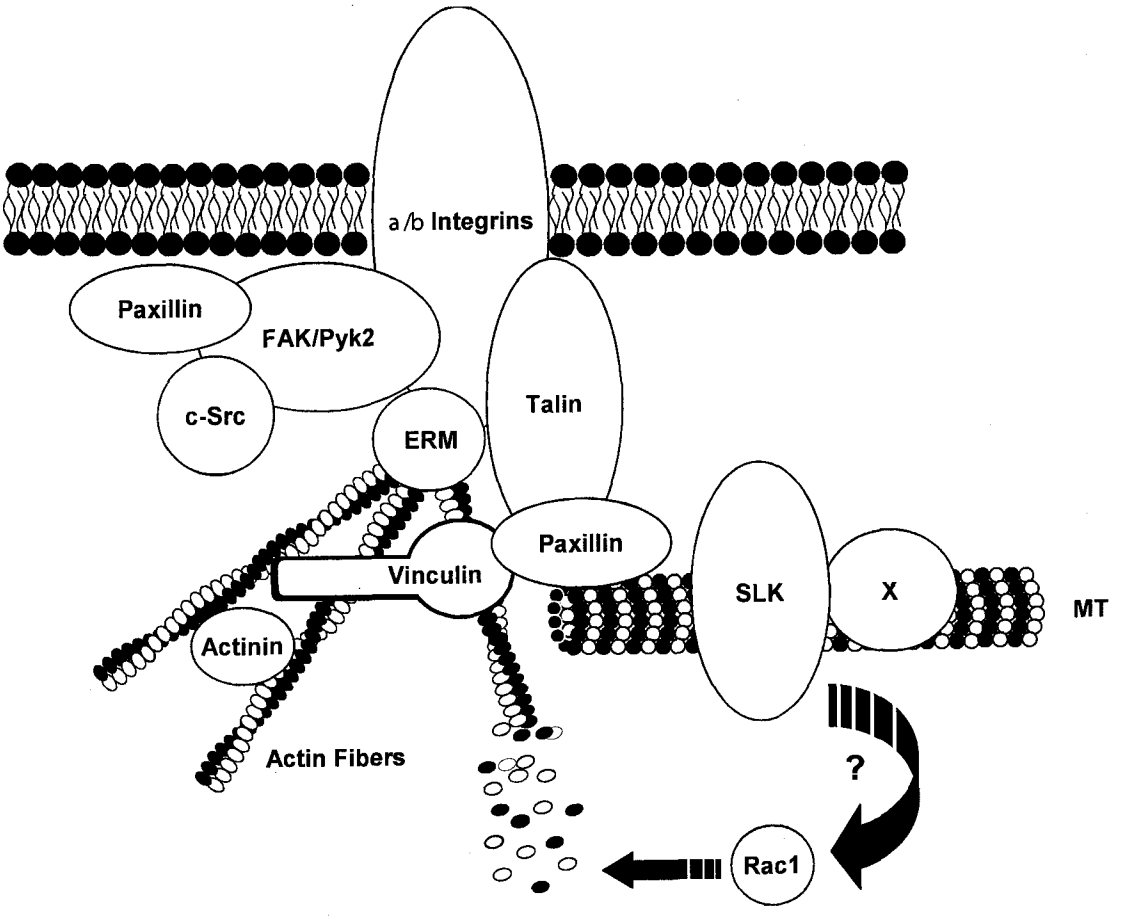


Figure 3.11. Proposed model for SLK recruitment and regulation of actin dynamics at adhesion sites. Following integrin binding, adhesion sites assemble through the recruitment of FAK and structural proteins. During adhesion site turnover and disassembly, microtubule (MT) targeting of the adhesion sites, perhaps by direct interaction with paxillin, recruits SLK to the adhesion sites where it regulates actin dynamics. The disassembly of actin stress fibers induced by SLK may be mediated by Rac1 promoting the relaxation and destabilization of adhesion sites, a process that would probably involve additional regulators. X denotes an interacting binding partener between SLK and tubulin.

CHAPTER 4

ROLE OF SLK IN CELL MIGRATION

4.1. Introduction

Cell migration is a multistep process that involves the formation of a leading edge protrusion and extension of a lamellipodium, followed by the establishment of new focal adhesion sites at the front of the cell and detachment of adhesions at the rear. These events required the dynamic reorganization of the actin cytoskeleton and the turnover of focal adhesions (Horwitz and Parsons, 1999; Lauffenburger and Horwitz, 1996).

Cytoskeleton and cell-ECM adhesions are two molecular machineries involved in signal transduction during cell migration. Although all three types of cytoskeleton (actin microfilaments, microtubules and intermediate filaments) contribute to cell motility, actin cytoskeleton plays the central role. The polymerization of actin filaments provides the driving force for the protrusion of the leading edge, and actomyosin contraction generates the traction force at focal adhesions and induces the retraction at the rear (Li et al., 2005).

Microtubules also play a significant role in the protrusion of the leading edge via their interactions with actin and RhoGTPases (Raftopoulou and Hall, 2004; Rodriguez et al., 2003). Actin and microtubules have mutual influences that may lead to feedback actions. Microtubule growth at the cell front activates Rac1 to induce lamellipodium formation, whereas the inhibition of microtubule growth decreases lamelipodial protrusion, cell spreading and cell migration (Ren et al., 1999; Waterman-Storer and Salmon, 1999; Wittmann et al., 2003). Microtubule disruption increases the assembly of actin filaments, increases contractility by RhoA activation and formation of focal adhesions (Bershadsky et al., 1996). There is also evidence that the plus ends of microtubules can target focal adhesions and cause their disassembly (Kaverina et al., 1999; Small et al., 1998). Microtubule-Rac-actin may form a positive feedback loop to promote the protrusion at the leading edge (Small et al., 2002b).

Focal adhesions are multicomplexes of proteins that form at sites of integrin adhesion to the extracellular matrix. Focal adhesions contain clustered integrins and numerous cytoplasmic proteins, such as FAK, talin, vinculin, paxillin and zyxin that contribute to the integrin connection to the actin cytoskeleton and signal transduction. Focal adhesion formation at the front of the cell and disassembly at the rear are critical for the migration of many adherent cells (Sastry and Burridge, 2000; Webb et al., 2002).

Stimulation of cell migration induces the formation of integrin-FAK-Src complexes required for the recruitment and activation of a number of adaptor molecules leading to focal adhesion turnover and migration (Brown and Turner, 2004; Mitra and Schlaepfer, 2006). Indeed, FAK-null cells assemble large and stable adhesion complexes leading to migratory deficits (Sieg et al., 1999). Similarly, a FAK mutant at tyrosine 397, deficient for c-Src binding, fails to induce focal adhesion disassembly in FAK-null fibroblasts (Hamadi et al., 2005; Ren et al., 2000). Supporting this, Src-family kinase-deficient cells or cell expressing kinase inactive v-Src display larger focal adhesions that fail to disassemble (Fincham and Frame, 1998; Kaplan et al., 1994).

The molecular mechanisms that lead to focal adhesion assembly have been well characterized, with emphasis on the role of Rho family GTPases and integrin engagement (Ridley et al., 2003; Schwartz and Horwitz, 2006). In contrast, few factors other than components of focal adhesion or factors that affect Rho activity have been implicated in the regulation of focal adhesion disassembly (Arthur et al., 2000; Ren et al., 2000; Webb et al., 2004).

Interestingly, a recent study has shown that focal adhesion disassembly involves microtubules, dynamin and FAK, and is not simply the reversal of focal adhesion assembly (Ezratty et al., 2005). These authors developed a model system to dissect the molecular mechanisms of microtubule-induced focal adhesion

disassembly. They postulated that upon microtubule regrowth after nocodazole washout, the focal adhesions disassemble from the centre of the cell outwards, temporally coinciding with the regrowth of microtubule towards cell periphery. Furthermore, focal adhesion reformation was observed two hours after microtubule regrowth, showing that focal adhesion disassembly in this system was reversible (Ezratty et al., 2005).

We have previously shown that SLK can be co-immunoprecipitated with α-tubulin and that it localizes to membrane ruffles at the periphery of spreading fibroblasts. In addition, SLK appears to induce actin stress fiber breakdown through a Rac1-mediated pathway (Wagner et al., 2002). Furthermore, recent results support a role for SLK in actin dynamics during cell spreading, which may contribute to adhesion site destabilization and turnover. As the signaling pathways activated during cell spreading also regulate cell motility, we tested the possibility that SLK may play a role in cell migration (Ridley et al., 2003; Webb et al., 2002).

4.2. Results

4.2.1. SLK Is Recruited to the Leading Edge of Migrating Fibroblasts

We initially investigated the localization of SLK and other cytoskeletal markers in a wound healing assay of MEF 3T3 confluent monolayers. We performed co-immunoflourescence studies using rabbit polyclonal antibody against SLK in conjunction with antibody against actin, tubulin, paxillin and Rac1. Co-immunostaining of SLK with actin stress fibers shows that, in addition to a perinuclear distribution, SLK it is also enriched at the leading edge of migrating cells but not along stress fibers **(Fig. 4.1. A-C)**. Similarly, at the leading edge, SLK was found to co-localize with paxillin, α-tubulin and Rac1 in structures reminiscent of membrane ruffles **(Fig. 4.1. D-L)**. Interestingly, SLK did not localize to large focal adhesions as evidenced by the lack of co-localization with paxillin at these sites

(Fig. 4.1. D-F, arrows). Supporting our previous results demonstrating SLK-tubulin co-precipitation, high magnification images of the leading edge revealed a filament-like distribution of SLK, a proportion of which co-localized with the microtubule network **(Fig. 4.1. M-O)**. Together, these observations suggest that SLK is recruited at the leading edge of migrating cells with other adhesion signaling proteins.

4.2.2. SLK Knock-Down Cells Showed Impaired Directional Cell Migration and an Increase Number of Vinculin Adhesion Sites.

To investigate a role for SLK in directed cell migration we performed a wound healing assay using confluent monolayers of MEF-3T3. First, subconfluent cells were infected for 48 hours with short hairpin adenovirus specific for SLK (shSLK) or scramble control adenovirus in low serum conditions and then subjected to the wound healing assay.

To analyze the efficiency of infection we performed WB using SLK rabbit polyclonal antibodies and we observed that expression of SLK was efficiently downregulated in cells infected with shSLK, compared with scramble infected cells. Total tubulin is shown as a loading control **(Fig. 4.2. A)**.

Next, the confluent monolayer was wounded with a pipette tip (0.5 mm wide gap) and then the cells at the leading edge were monitored during wound closure. The cells migrating on FN-coated coverslips were fixed at 0, 4, and 12 hours after injury and imaged by phase-contrast microscopy **(Fig. 4.2. B)**. By quantifying the dynamic of wound closure at specific time points after wounding, we observed a *60% inhibition of migration of SLK knock-down cells compared to control scramble* infected cells **(Fig. 4.2. C)**.

To further investigate the impaired migration of SLK knock-down cells, we performed double-immunostaining studies using SLK and vinculin antibodies and phalloidin staining. The SLK knockdown and scramble shRNA infected cells were analyzed after 4 hours of migration towards the wound. The staining intensity of

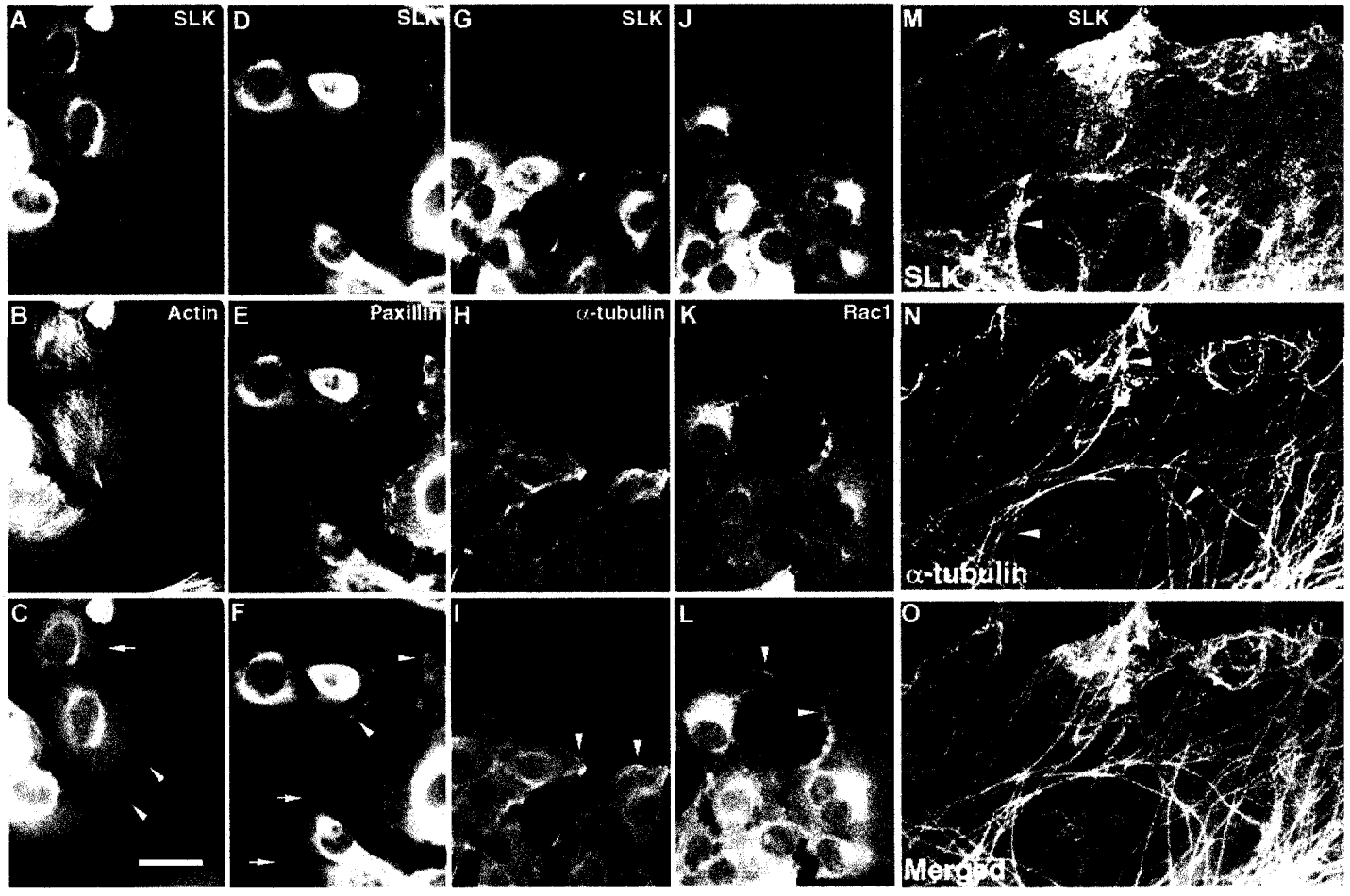


Figure 4.1. SLK is recruited to the leading edge of migrating fibroblasts.
MEF-3T3 monolayers on fibronectin coated coverslips were scratch wounded and allowed to migrate for 2-3 hours. Monolayers were immunostained for SLK in combination with actin **(A-C)**, paxillin **(D-F)**, α-tubulin **(G-I)**, or Rac1 **(J-L)**. All photomicrographs are shown at 400X. **(M-O)** High magnification (1000X) of SLK and α-tubulin double immunostaining at the leading edge. Scale bar: 10μm.

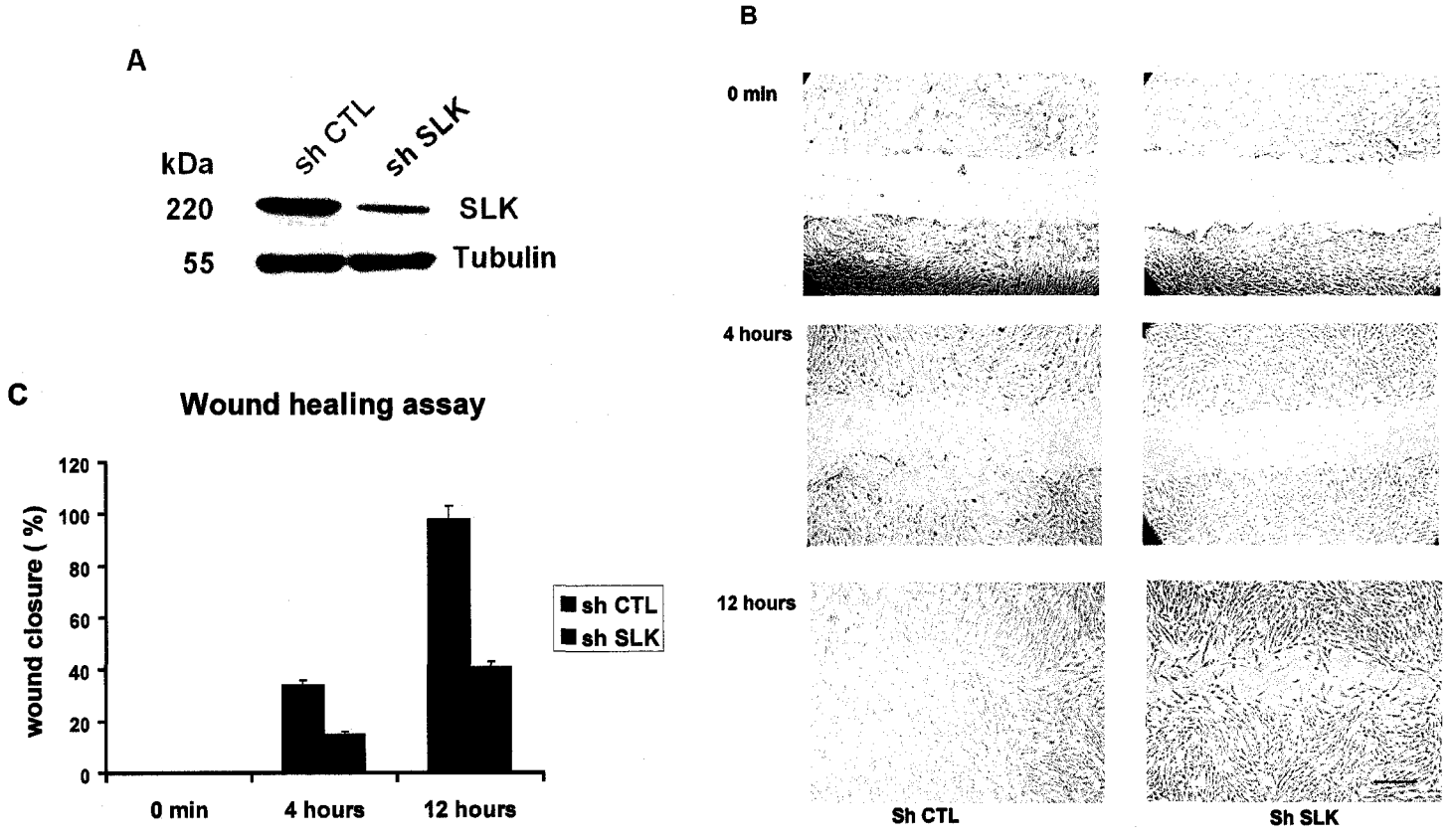


Figure 4.2. SLK knock-down cells show impaired directional cell migration in a wound healing assay.
Samples were collected after 48 hours of infection with short hairpin (shSLK) and scramble adenoviruses and proteins expressions were analyzed by SDS-PAGE and WB. The tubulin levels are shown as loading control **(A)**. The wound healing dynamic was monitored by phase-contract microscopy **(B)**. Panel **C** is a quantification of wound closure at 0, 6 and 12 hours after injury. Three independent measurements were recorded along the wound. The data shown represent averages ± standard errors for three independent experiments. Error bars represent standard errors (S.E). The cells were photographed at x100. Scale bar: 10 µm.

SLK is reduced in SLK knock-down cells compared to control scramble infected cells, supporting our previous biochemical data. Furthermore, by staining the actin cytoskeleton with TRITC-phalloidin, we observed that SLK knock-down cells formed a broad lamellipodia, similar to cells infected with scrambled adenovirus **(Fig. 4.3. E-H)**. Interestingly, using vinculin staining, as a marker for focal adhesion, we observed an enhanced number of vinculin positive sites in the SLK knock-down cells compared to scramble infected cells **(Fig. 4.3. A-D)**. Quantification shows that the number of vinculin sites in SLK knock-down cells is doubled compared to scramble infected cells **(Fig. 4.3. I)**.

Together these results show that SLK knock-down cells display an increase number of focal adhesions and reduced motility, characteristics that were previously correlated with a decrease in focal adhesion turnover (Webb et al., 2002).

4.2.3. SLK Knock-Down Cells Show Impaired Migration in a Haptotaxis Assay on Fibronectin Coated Boyden Chamber Traswells

To extend the wound healing analyses, we performed the Boyden chamber migration assay to evaluate the role of SLK in haptotaxis.

In order to quantify the role of SLK in cell migration, we transfected MEF 3T3 cells with small interfering RNA (siRNA) specific for murine SLK and assayed their migration in a transwell assay. Following siSLK transfection, the levels of SLK protein were efficiently reduced at 5 pM and undetectable at 10 pM compared with control siRNA treated cells **(Fig. 4.4. A)**. An equal number of cells (3x 10^4) were plated in the upper chamber of the transwells, incubated for 4 hours and then cells that migrated through the porous membrane of the transwell were fixed, stained with DAPI and counted. After quantification of the DAPI stained nuclei, we observed that cells treated with SLK siRNA showed a ~60% decrease in migration compared to siRNA control treated cells **(Fig. 4.4. B-C)**. Previous reports have shown that FN is a specific ligand for integrin mediated migration (Guan, 1997;

Ilic et al., 1997). We also used bovine serum albumin (BSA) as a non-specific ECM ligand and demonstrated that cells plated in BSA coated transwells failed to migrate. Together, these data strongly support a role for SLK in the process of integrin induced cell migration.

4.2.4. Cells Expressing an Inactive SLK Mutant (SLK1-373K63R) Show Impaired Migration in a Haptotaxis Assay

We previously demonstrated that the $SLK^{1\text{-}373K63R}$ construct, acts as a dominant negative version of SLK when overexpressed into the MEF 3T3 cell, by blocking the effects of activated SLK on cell spreading on fibronectin (Wagner et al., 2002).

To further investigate the potential role of SLK in cell migration, MEF-3T3 cells were infected with adenoviral vectors expressing the $SLK^{1\text{-}373K63R}$ or a LacZ control, and subjected to transwell migration assays. To evaluate the efficiency of infection, cell lysates from $SLK^{1\text{-}373K63R}$ infected cells were subjected to western blotting using anti-HA antibody which recognizes the epitope-tag encoded by the adenovirus construct. As a loading control we used an anti-Cdc42 antibody, a small RhoGTPase that is ubiquitously expressed in these cells **(Fig. 4.5. A)**.

Cells that migrate through the porous membrane of the transwell were fixed and stained with DAPI. After quantification of DAPI positive stained nuclei, we observed that overexpression of $SLK^{1\text{-}373K63R}$ resulted in a 60-70% inhibition of migration on fibronectin-coated transwell inserts, compared to cells infected with LacZ control **(Fig. 4.5. B-C)**. These results support our SLK short interfering RNA data.

4.2.5. SLK Kinase Activity is Upregulated during Microtubule Growth after Nocodazole Recovery

Efficient cell migration requires focal adhesion turnover at the leading edge of

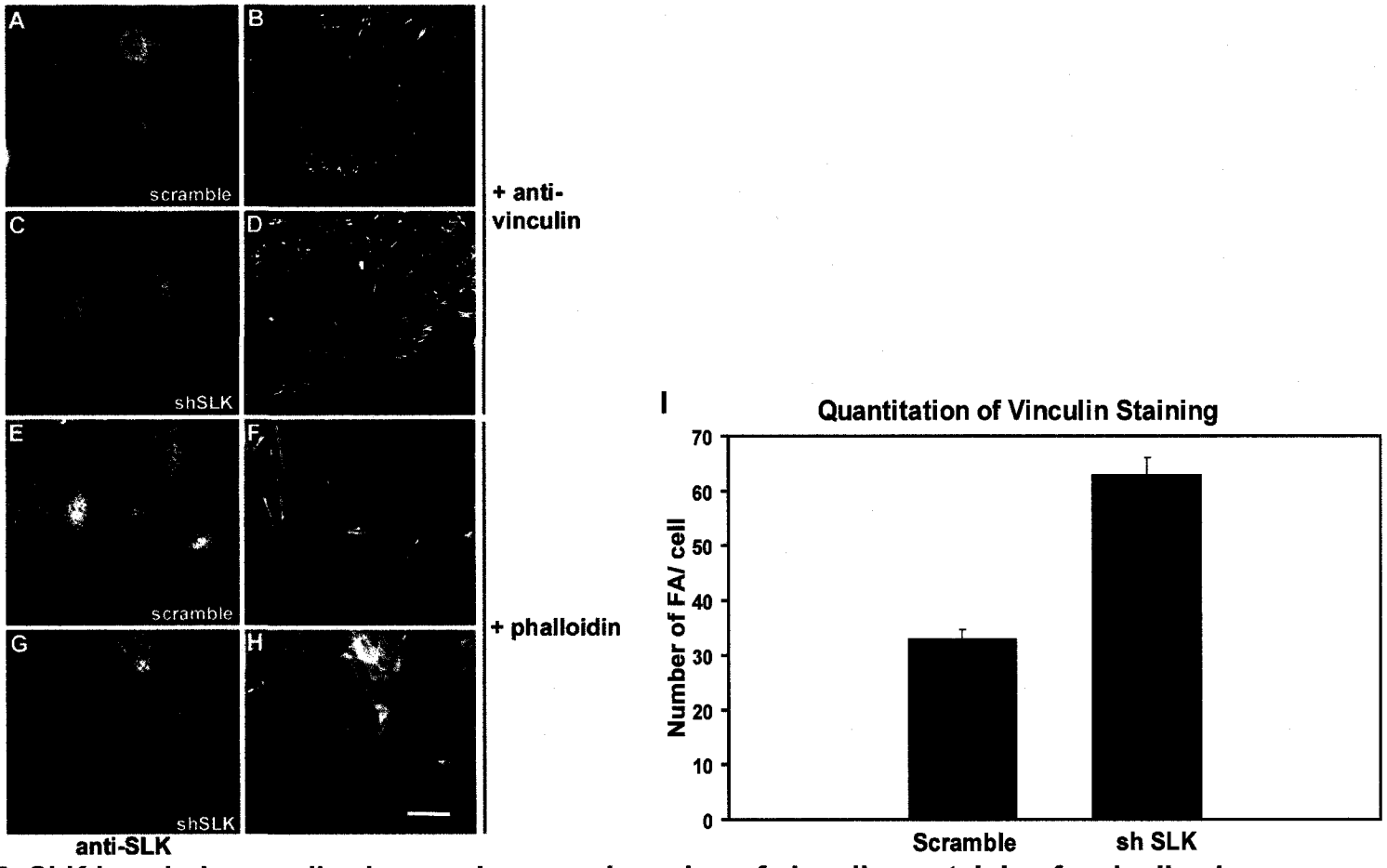


Figure 4.3. SLK knock-down cells show an increased number of vinculin containing focal adhesions. Confluent monolayers of MEF-3T3 cells, previously infected with scramble or SLK short hairpin adenoviruses, were plated on FN, scratch-wounded, and allowed to migrate for 4 hours. Fixed coverslips were immunostained for SLK in combination with vinculin **(A-D)** and phalloidin **(E-H)**. Pictures are taken at X630. Scale bar: 10μm. **Panel I**: Quantification of vinculin positive adhesion sites was obtained using Image J software. The data shown represent averages ± standard errors for three independent experiments. Error bars represent standard errors (S.E).

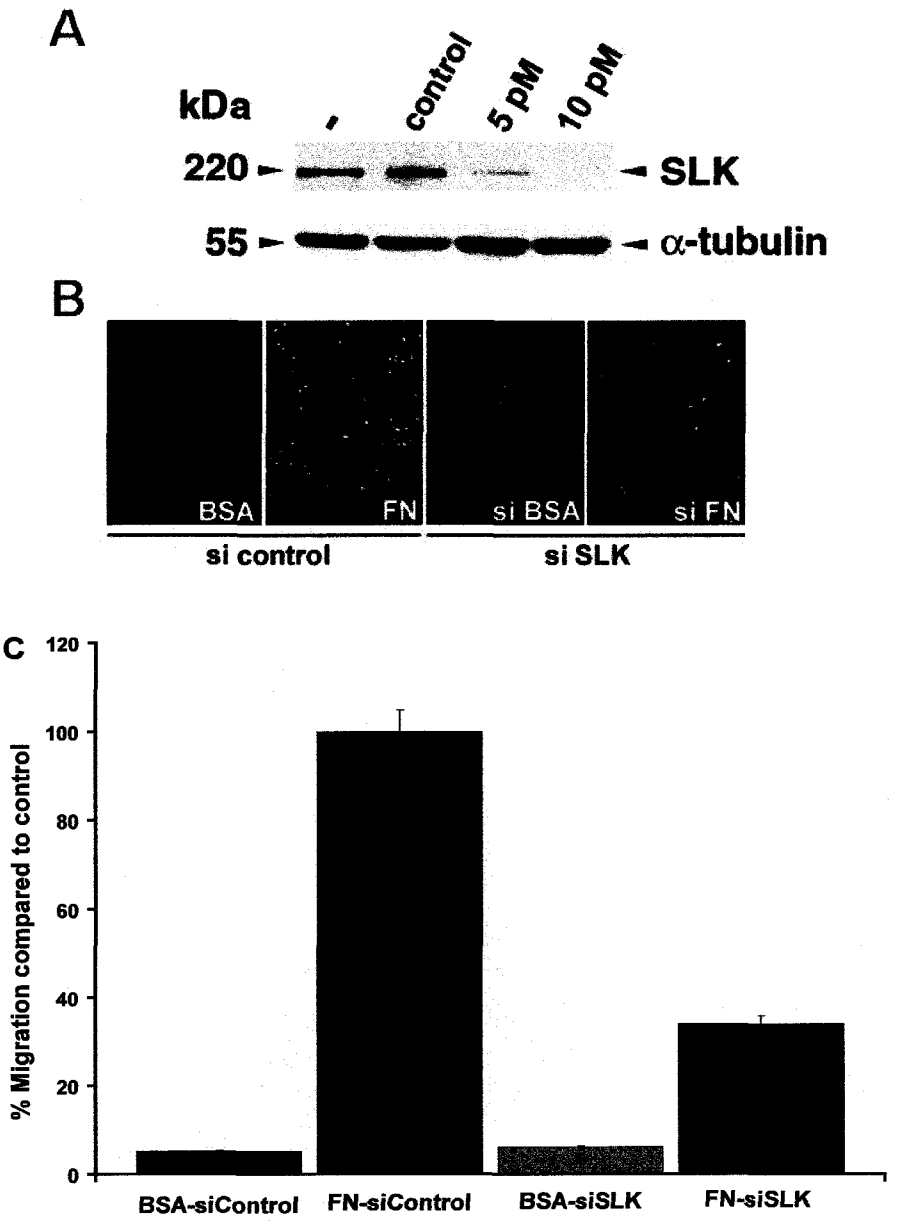


Figure 4.4. SLK knock-down cells showed impaired migration in a haptotaxis assay.

MEF-3T3 cells were transfected with SLK siRNA and analyzed for SLK expression. **A**. Western blot analysis of treated cells indicates that SLK siRNA at 10 pM resulted in a marked knockdown of SLK. Reprobing the membrane with α- tubulin antibody was used as a control for loading (lower panel). **B-C.** Treated cells were assayed for migration through a chamber coated with BSA (10 mg/ml) or FN (10 μg/ml). FN-coated membranes were DAPI stained and cells on the underside were enumerated in random fields and expressed as the average from triplicate wells. The data shown represent averages ± standard errors for three independent experiments. Error bars represent standard errors (S.E).

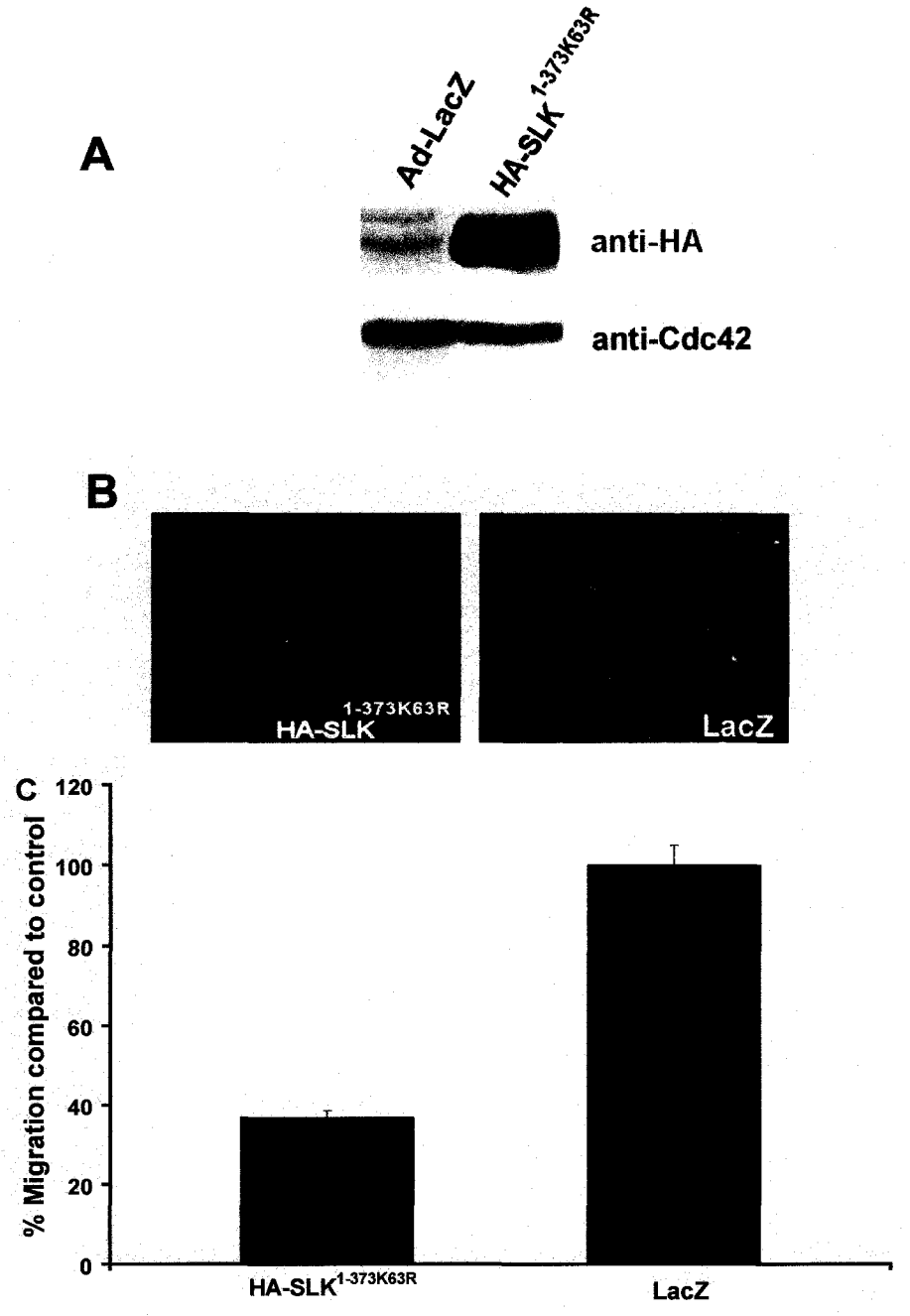


Figure 4.5. Expression of a dominant-negative SLK construct ($SLK^{1-373K63R}$) impaired cell migration in a haptotaxis assay.
Subconfluent MEF-3T3 cells were infected with adenovirus vectors expressing $SLK^{1-373K63R}$ or LacZ control and subjected to FN transwell migration assays. **A.** Western blot analysis of HA-$SLK^{1-373K63R}$ expression. Cdc42 was used as a loading control. **B-C.** FN- coated membranes were DAPI stained and cells on the underside were enumerated in random fields and expressed as the average from triplicate wells. The data shown represent averages ± standard errors for three independent experiments. Error bars represent standard errors (S.E). Anti-HA: hemagglutinin antibody.

migrating cells (Sieg et al., 1999; Webb et al., 2002). This process is dependent on the assembly of a functional FAK-Src complex initiated by FAK autophosphorylation at tyrosine residue 397 and the recruitment of signaling adapter proteins (Schlaepfer et al., 1999). Interestingly, microtubule disruption leads to stable adhesion complex assembly characterized by high levels of both FAK-Tyr397 and actin stress fibers (Bershadsky et al., 1996; Ezratty et al., 2005). Nocodazole wash-out results in focal adhesion turnover and cell migration characterized by cyclical changes in FAK-pY397 levels (Ezratty et al., 2005). Because of its association with the microtubule and the requirement for microtubule cytoskeleton in the process of focal adhesion turnover, we investigated the role of SLK in microtubule-induced adhesion turnover.

First we characterized the dynamic of focal adhesion disassembly in MEF 3T3 fibroblasts, by performing immunofluorescence studies. Serum-starved MEF 3T3 cells were grown on fibronectin coated coverslips and treated with nocodazole (10 μM) for 4 hours to completely de-polymerize microtubules. Next, the drug was washed out with fresh low serum and microtubule re-polymerization was monitored at different time intervals (0, 15, 30, 60, 90 minutes). The coverslips were double-stained for α-tubulin and FAK-pY397, as a marker for focal adhesions. At 15 minutes after nocodazole wash-out, when re-polymerized microtubules have fully extended to the cell periphery, the majority of focal adhesions were disassembled, as indicated by a low intensity of FAK-pY397 staining **(Fig. 4.6)**. Interestingly, focal adhesion re-assembly was observed at 30 and 90 minutes after microtubule regrowth, showing that focal adhesion disassembly was reversible.

Next we performed an *in vitro* kinase assay for SLK using cells collected after nocodazole wash-out, and tested the possibility that SLK kinase activity is modulated during microtubule-induced adhesion sites turnover. We observed a cyclic upregulation of SLK kinase activity in samples collected at different time points

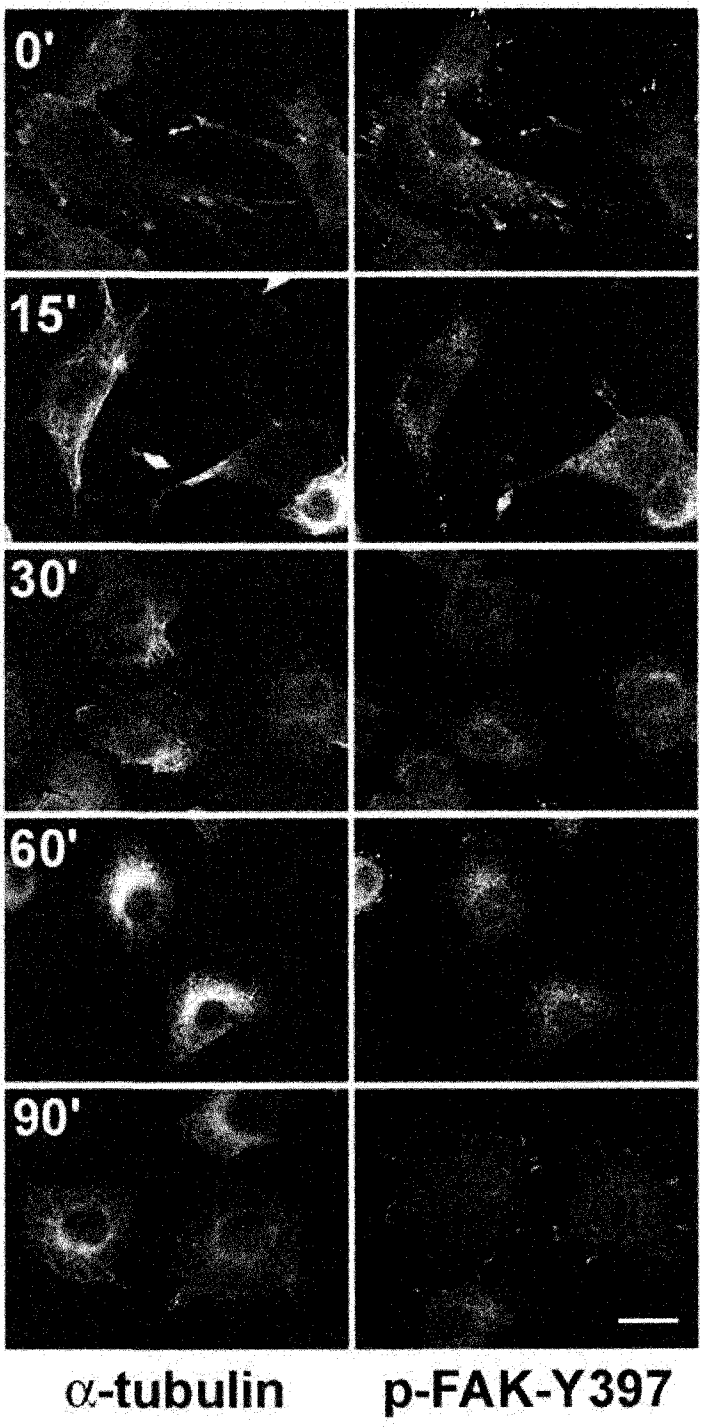


Figure 4.6. Microtubules re-growth after nocodazole wash-out induces focal adhesion disassembly.
Serum starved MEF-3T3 fibroblasts were incubated for 4 hours with 10μM nocodazole. Then the drug was washed out and microtubules were allowed to re-grow for the indicated times (0-90 min). Cells were fixed and immunostained for FAK-pY397, a marker for focal adhesions and α-tubulin. Scale bar: 10 μm.

after nocodazole wash-out **(Fig. 4.7.A)**. The expression level of SLK remained constant during the assay. Densitometric analyses of the kinase assays showed a 2 fold increase in SLK kinase activity at 15 and 60 minutes after nocodazole washout **(Fig. 4.7. B)**. These results suggest that SLK kinase activity is modulated by microtubule growth, after nocodazole wash-out.

Alternatively, upregulation of SLK kinase activity correlates well with active focal adhesion turnover, as indicated by our immunofluorescence data, suggesting that signaling from focal adhesion sites could activate SLK.

4.2.6. SLK Expression Is Required For Efficient Microtubule-Induced Focal Adhesion Turnover

To test if SLK expression is required for microtubule-induced focal adhesion turnover we used dominant negative and knockdown approaches against endogenous SLK. MEF-3T3 cells were infected with adenovirus carrying $SLK^{1-373K63R}$ or transfected with SLK siRNAs and subjected to microtubule-dependent focal adhesion turnover assays. To verify the efficiency of infection, lysates were monitored by western blot for the expression of HA-tagged adenovirus construct **(Fig. 4.8. A)**. To demonstrate the efficiency of SLK siRNA transfection, cell lysates were analyzed by WB for SLK expression levels **(Fig. 4.8. B)**. Furthermore, in both experiments the changes in expression level of FAK-pY397 were normalized to the expression level of total FAK, analyzed by WB.

As shown in **figure 4.8. (A and B)**, following nocodazole wash-out, control treated cells show the cyclical changes in FAK-pY397. However, in cultures where SLK has been efficiently knocked down, adhesion turnover is severely impaired as evidenced by the sustained levels of FAK-pY397, a biochemical marker that correlates with focal adhesion disassembly. Similarly, a marked delay in FAK-pY397 reduction was observed in cultures expressing dominant-negative $SLK^{1-373K63R}$ following nocodazole washout. These results suggest that SLK dependent

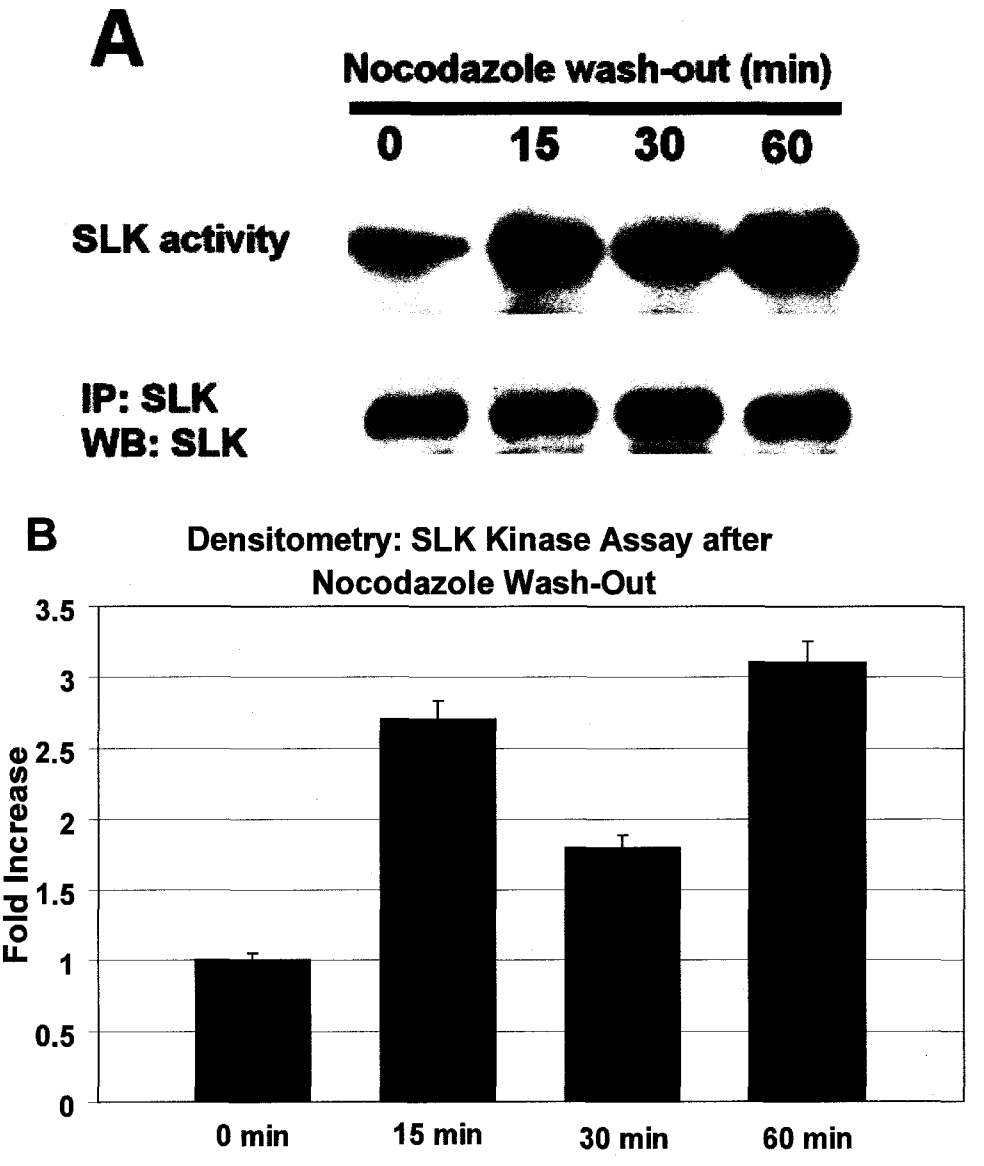


Figure 4.7. SLK kinase activity is upregulated during microtubule-induced focal adhesion turnover.

A. Cells were collected at different time points after nocodazole wash-out (0, 15, 30, 60 minutes) and subjected to *in vitro* kinase assay for SLK. **B.** By densitometric analyses we observed a two fold upregulation of SLK activity at 15 and 60 minutes. The kinase assays were normalized to SLK western blots and the data presented are the average of three independent experiments. Error bars represent standard errors (S.E).

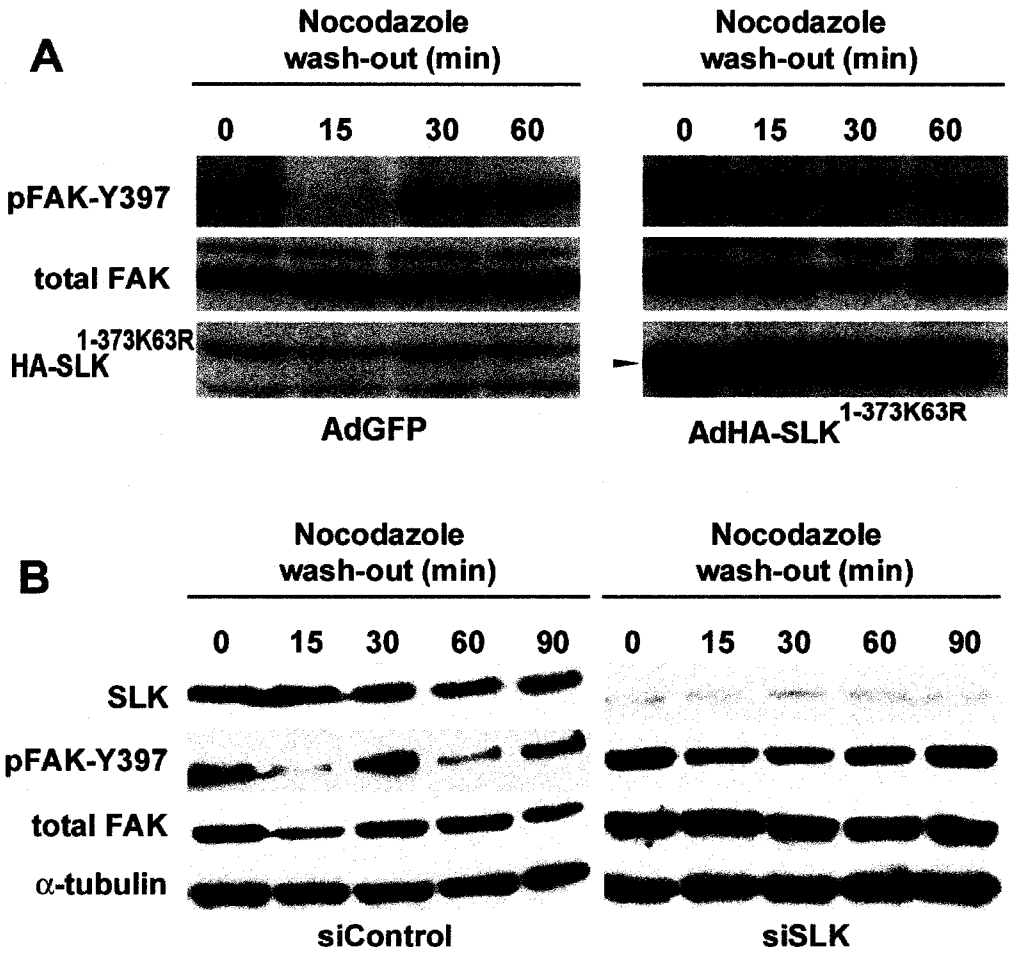


Figure 4.8. SLK expression is required for microtubule-dependent adhesion turnover.

Subconfluent MEF-3T3 fibroblasts were infected with adenoviral constructs **(A)** encoding kinase-defective SLK (HA-SLK$^{1\text{-}373K63R}$) and GFP control or transfected **(B)** with SLK and control siRNAs. Cultures were then treated with nocodazole (10μM) for 4h, washed and surveyed for FAK-pTyr397 levels over time. Expression of HA-tagged SLK or SLK knockdown was confirmed by Western blot analysis.

signals are required to mediate microtubule-dependent focal adhesion turnover. **(Fig. 4.8. A- B).**

4.3. Discussion

One problem in studying the disassembly of focal adhesions is that, under normal conditions, this is an asynchronous event. To solve this problem, a recent paper proposed a focal adhesion disassembly assay that exploits the fact that microtubule target focal adhesion (Ezratty et al., 2005). The strategy was based on earlier reports who noted that growing microtubules would contact focal adhesion transiently and this induced their disassembly (Kaverina et al., 1999). From this early work it was generally assumed that microtubules deliver a "relaxing factor" to focal adhesion that is perhaps locally antagonistic to RhoA and contractility, thereby inducing focal adhesion disassembly (Burridge, 2005). A striking observation made by Erzatty *et al.* was that microtubule-induced focal adhesion disassembly is independent of the Rho GTPases that are known to regulate focal adhesion formation (Ezratty et al., 2005), but depends on focal adhesion kinase and dynamin. During disassembly, dynamin interacts with FAK and colocalizes with focal adhesion. It was proposed that microtubules may be delivering a focal adhesion "relaxing factor" that would stimulate dynamin and FAK activity during focal adhesion disassembly (Ezratty et al., 2005).

Our previous studies have shown that SLK co-localizes with vinculin and Rac1 in the membrane ruffles and lamellipodia of spreading fibroblasts. In addition, we have shown that SLK can indirectly associate with the microtubule network and mediate actin stress fiber dissolution in a Rac1-dependent manner (Wagner et al., 2002).

Now we attempted to characterize the biochemical and physiological defects of SLK knock-down cells by using different migration assays, such as wound

healing, haptotaxis, and microtubule-induced focal adhesion turnover.

We show that SLK can be co-localized with paxillin, α-tubulin and Rac1 at the leading edge of migrating cells. Also, we demonstrate that SLK knock-down fibroblasts display reduced cell migration in both wound healing and haptotaxis assays and they have increased numbers of vinculin positive focal adhesions. These characteristics have been shown to correlate with decrease focal adhesion turnover in FAK, Src and paxillin null cells (Ilic et al., 1995; Mitra et al., 2005; Webb et al., 2004).

In addition, we reported that a reduction in SLK expression levels or kinase activity negatively affects cell migration and microtubule-dependent focal adhesion turnover, suggesting that SLK is required for cell motility. Furthermore, the association of SLK with the microtubule and its requirement for efficient focal adhesion turnover suggests that SLK is a novel microtubule-associated signal required for cell migration.

Recent results from our laboratory showed that SLK could be co-immunoprecipitated with paxillin, suggesting a functional interaction between these two proteins. Paxillin may be a mediator of SLK re-localization at the leading edge during cell migration, as it is reported that paxillin interacts with microtubules (Herreros et al., 2000). Furthermore, recent data from our laboratory defined the LIM domain binding transcriptional cofactor protein Ldb2/CLIM1 as the intermediary protein that links SLK, paxillin and tubulin. In addition, live imaging of SLK knock-down cells overexpressing GFP-paxillin, showed an impaired pattern of microtubule-induced focal adhesion turnover, compared to control siRNA treated cells (C. Storbeck et all, unpublished data).

Thus, we propose a scenario where microtubule growth towards the protruding lamellipodia, recruits the SLK-Ldb2-paxillin complex from the cytoplasm and translocates it to the leading edge. The recruitment of SLK at the leading

edge of migrating cells may be necessary for the destabilization of focal contacts, a process required for further protrusive activity and motility. Focal adhesion and focal contact disassembly would also destabilize the actin network, a phenotype previously reported to be induced by SLK overexpression (Sabourin et al., 2000; Wagner et al., 2002).

Overall, our data suggest that SLK is an important regulator of focal adhesions dynamics.

CHAPTER 5

MECHANISM OF SLK ACTIVATION DURING MIGRATION

5.1. Introduction

The process of cell migration is initiated by protrusion at the leading edge of the cell, the formation of peripheral adhesions, the exertions of force on these adhesions, and finally the release of the adhesions at the rear of the cell (Ridley et al., 2003; Tilghman et al., 2005). Although this model of cell migration is well accepted, the precise mechanisms that regulate these processes remain unclear.

Evidence indicate that multiple signalling molecules, including focal adhesion kinase (FAK), Src, paxillin, CAS and ERK are recruited to newly formed adhesions and contribute to the process of assembly and turnover (Cary et al., 1998; Huang et al., 2004; Ren et al., 2000; Webb et al., 2004). Furthermore, tyrosine phosphorylation of these molecules occurs in cell adhesions, and interference with the phosphorylation or dephosphorylation events results in impaired cell migration (Ballestrem et al., 2006; Burridge et al., 2006). FAK is implicated in the initiation of a tyrosine phosphorylation signalling cascade because it is a tyrosine kinase that is localized to focal adhesions and activated following adhesion to fibronectin (Parsons, 2003). This activation results in the autophosphorylation of FAK on tyrosine 397, which then becomes a binding site for the SH2 domain of Src family of tyrosine kinases (STK) (Schaller et al., 1994). The interaction between FAK and STKs stabilizes the STKs in their active state, allowing the phosphorylation of other focal adhesions proteins in a STK-dependent manner (Schaller et al., 1994).

FAK-promoted cell migration depends on the coordinate regulation of several signalling pathways, involving Src, CAS and PI3K (Hanks et al., 2003; Reiske et al., 1999). One signalling pathway utilizes the CAS family of proteins as downstream effectors of FAK. CAS proteins bind through their SH3 domain to the proline rich regions of FAK or Pyk2, a FAK homolog (Hanks et al., 2003). The relocalization of CAS and signalling to specific CAS family members appear to determine the outcome of this pathway (Cox et al., 2006). Consistent with a key

role for CAS family proteins in cell motility, fibroblasts isolated from CAS null mice exhibit defects in cell migration, such as impaired actin filament assembly (Honda et al., 1998).

It is known that PI3K plays an important role in growth factor-induced migration, by both FAK-dependent and independent pathways (Khwaja et al., 1998; Royal and Park, 1995). The requirement of PI3K signalling in FAK-promoted cell migration was demonstrated by using the PI3K inhibitors, wortmanin and LY294002, which were able to inhibit FAK-promoted migration in a dose-dependent manner (Reiske et al., 1999).

In regard to FAK-independent PI3K signaling pathways, it has been demonstrated that the activity of phosphatidylinositol 3-kinase is required for actin filament remodelling, microtubule stabilization and cell migration in different cell lines (Onishi et al., 2007; Qian et al., 2004). Akt is an essential target of PI3K in the induction of actin filament remodelling and cell migration, and Akt transmits the signal through the activation of p70 S6K1. Furhermore, Rac activity is required for p70 S6K1- mediated actin filament remodelling and cell migration (Qian et al., 2004). A recent paper, reports that the PI3K-Akt signalling pathway plays a pivotal role in growth factor regulation of microtubule stability (Onishi et al., 2007).

Mitogen-activated protein kinases (MAPKs), including ERK, p38MAPK, and JNK, can be activated by integrin-mediated signalling (Huang et al., 2004; Zhu and Assoian, 1995). ERK has been implicated in the migration of numerous cell types (Huang et al., 2004; Klemke et al., 1997). Activated ERK regulates membrane protrusions and focal adhesion turnover by phosphorylating MLCK and promotes focal adhesion disassembly through phosphorylation and activation of calpain (Glading et al., 2001; Liu et al., 2002). Phosphorylation of FAK and paxillin by ERK may regulate focal adhesion dynamics, by influencing the paxillin-FAK interaction (Liu et al., 2002).

Rho GTPases are key players in cell migration. The contribution of Rho, Rac and Cdc42 to the regulation of the actin and microtubule cytoskeletons is essential for membrane protrusion and cell retraction (Etienne-Manneville and Hall, 2002). The polarization of these protrusive and retracting activities in a migrating cell is also under the control of RhoGTPases, in particular Cdc42 (Nobes and Hall, 1999).

To explore the molecular mechanisms controlling polarity establishment in mammalian cells, Etienne-Manneville and Hall, used a relatively simple *in vitro* wound assay with monolayers of primary rat astrocytes (Etienne-Manneville and Hall, 2001). A scratch through the monolayer initiates directional movement of the cells perpendicularly to the wound. The authors reported that activation of Cdc42 upon wounding is mediated by integrin signalling, perhaps involving a Src-like tyrosine kinase pathway. The Src family kinases Fyn and Src are closely associated with integrins and can be activated downstream of integrin activation (Cobb et al., 1994; Shattil, 2005; Wary et al., 1998).

Our previous studies showed that SLK overexpression induced a rapid actin stress fibre disassembly that could be partially rescued by co-expression of dominant negative Rac1 (Sabourin et al., 2000; Wagner et al., 2002). Furthermore, previous studies showed that v-Src expression can down-regulate SLK activity and that this process requires v-Src kinase activity and membrane translocation. In addition, SLK downregulation by v-Src is mediated by casein kinase II (CK2) (Chaar et al., 2006).

Based on the involvement of SLK signalling in cell migration, we investigated what migratory signalling pathways are required for SLK activation in a scratch wound induced migration assay. We show that SLK activation is dependent on FAK/c-Src/MAPK signalling during scratch induced migration. Furthermore, PI3K and Rho GTPases pathways seem to be also required for SLK regulation. We

also show that SLK recruitment at the leading edge is Src-dependent, but FAK independent.

5.2. Results

5.2.1. SLK Kinase Activity is Upregulated upon Scratch-Induced Migration of MEF-3T3 Fibroblast Monolayers

Scratch wounding of MEF-3T3 confluent monolayers have been shown to induce polarization and migration (Etienne-Manneville and Hall, 2001; Etienne-Manneville and Hall, 2003). This *in vitro* scratch-induced migration assay allows the investigation of the mechanisms controlling cell migration, cell protrusion and cell polarization (Etienne-Manneville and Hall, 2001). Therefore, we investigated SLK kinase activity at various time points following scratch wound induced migration of MEF3T3 fibroblast monolayers plated on fibronectin coated plates.

Immunoprecipitation and *in vitro* kinase assays show that SLK kinase activity is markedly increased following scratch wounding of confluent fibroblasts, with a peak of activity at 60 minutes followed by a decline at 90 minutes **(Fig. 5.1. A)**. Densitometric analyses showed a three fold increase in SLK kinase activity at one hour after wounding compared to SLK activity in unscratched cells **(Fig 5.1. B)**. Extended time courses up to 120 minutes have shown that SLK activity does not return to basal levels observed at time 0 (not shown). This is likely due to the continued cell migration that occurs following wounding.

As previously reported for wounded astrocyte monolayers, inactivation of glycogen synthase kinase-3β (GSK-3β), monitored by phosphorylation of Ser9 of GSK-3β, also occurred over this time course, indicating the polarization and migration of the MEF3T3 wounded monolayer (Etienne-Manneville and Hall, 2003). Together, these data indicate that SLK is activated during cell migration and suggest a role for SLK in cell migration.

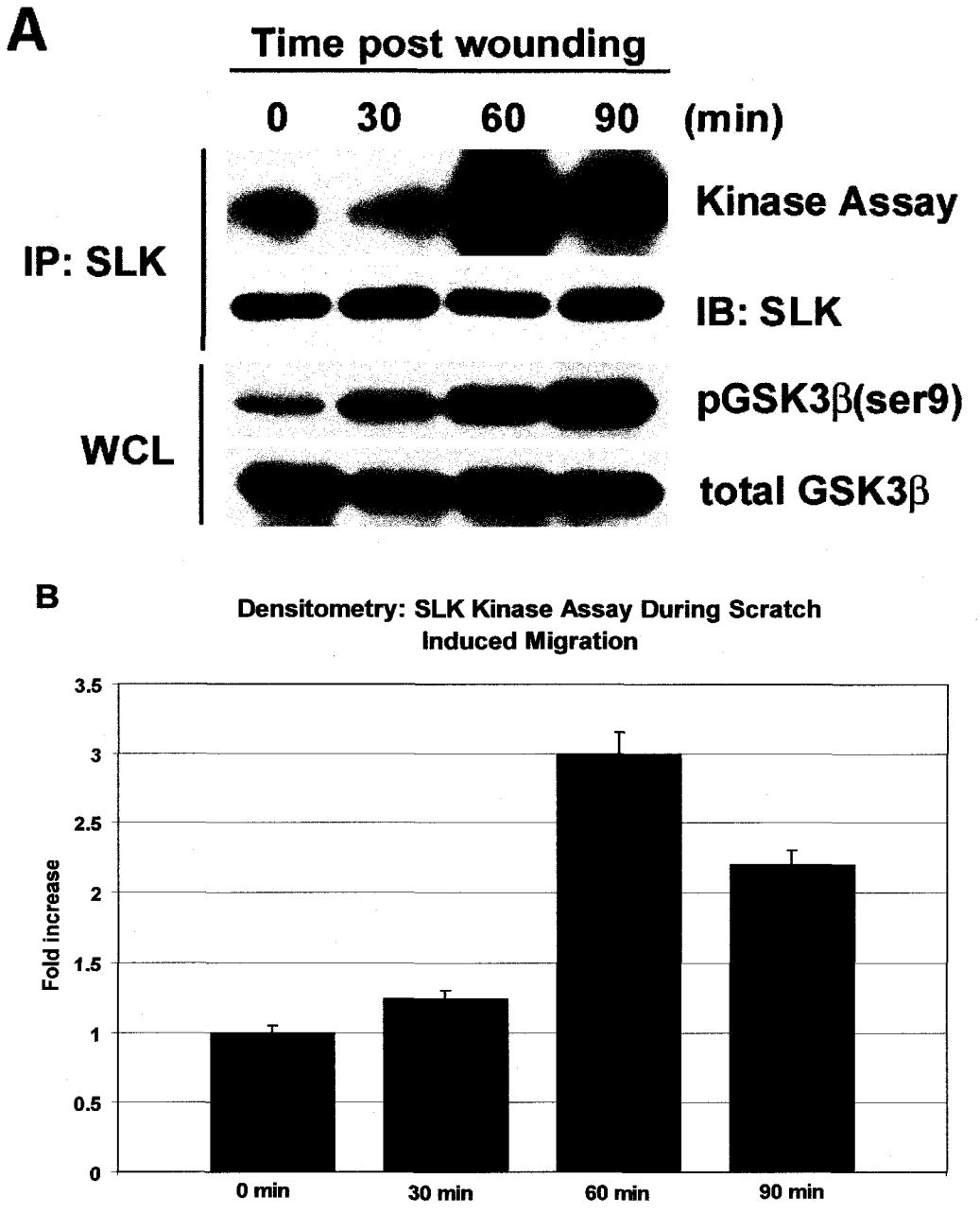


Figure 5.1. SLK is activated by scratch wounding and is required for cell migration. A. Confluent MEF-3T3 fibroblasts plated on fibronectin (10 μg/ml) were stimulated to migrate by scratch wounding. SLK kinase activity was assayed *in vitro* and was found to increase over time reaching a maximum of activity by 60 minutes following wounding. As previously reported, GSK3β was found to be inactivated over the time course indicating that cell migration have been induced. WCL, whole cell lysate. **B.** Quantification of SLK kinase activation by densitometry. The kinase assays were normalized to SLK western blots and the data presented are the average of three independent experiments. Error bars represent standard errors (S.E).

5.2.2. Rho small GTPases Signalling Pathways Regulate SLK Activation

Rho, Rac and Cdc42, three members of the Rho family of small GTPases, each control a signal transduction pathway linking membrane receptors to the assembly and disassembly of the actin cytoskeleton and of associated integrin adhesion complexes. Rho regulates stress fibre and focal adhesion assembly, Rac regulates the formation of lamelipodia protrusion and membrane ruffles, and Cdc42 triggers filopodial extensions at the cell periphery (Hall, 2005; Raftopoulou and Hall, 2004). Even though SLK bears no Cdc42/Rac interactive binding domain (CRIB) its potential role in focal adhesion disassembly prompted us to test the possible involvement of Rho small GTPases in its regulation.

We performed adenovirus infections of MEF-3T3 fibroblasts using HA-tagged dominant negative constructs for Rac1 and Cdc42 (RacN17 and Cdc42N17). Interestingly, MEF-3T3 cells infected with Cdc42N17 adenovirus failed to activate SLK upon scratch induced migration. These results suggest a requirement for Cdc42 and perhaps the polarity pathways for SLK activation. In contrast, the GFP adenovirus infected cells showed a strong activation of SLK after one hour of migration **(Fig. 5.2. A)**. Interestingly, in cells infected with a dominant negative Rac1 construct (RacN17), we noticed an upregulation of SLK in unscratched cells, suggesting that Rac1 negatively regulates SLK in non-motile cells. However, a downregulation to basal levels was observed following wounding, suggesting that Rac1 signalling may also be required downstream of FAK/Src complex to maintain SLK activity **(Fig. 5.2. B)**. In the presence of Toxin B, a general small RhoGTPases inhibitor, the pattern of SLK activation was indistinguishable from that of DNRac1 in a scratch-induced migration assay, suggesting that RhoA signalling may not be required for SLK regulation **(Fig. 5.2. C)**. Thus, we concluded that in motile cells signalling from the Rac1 and Cdc42, and perhaps RhoA, are required for SLK

regulation during cell migration.

5.2.3. Activation of SLK Requires FAK/Src/MAPK Signalling

The activation of SLK following scratch wounding of MEF3T3 monolayers appears to be a relatively late event (60 min; **Fig. 5.1. A**), suggesting that upstream signalling may be necessary prior to SLK activation.

Because of the ultimate requirement for the FAK-c-Src complex in cell migration, we tested whether Src family kinases and FAK were required for SLK activation in a scratch wound induced migration. To test the involvement of Src family kinases in SLK regulation, MEF3T3 monolayers were pre-treated with the Src-family inhibitor PP2 (or PP3 control) and then subjected to wounding in the presence of the inhibitor. As shown in **Fig. 5.3. A**, treatment with the control PP3 resulted in SLK activation at 60 minutes following scratch wounding. Interestingly, treatment with PP2 resulted in an increase in SLK activity in unscratched confluent monolayers that could not be further increased by wounding. The same results were obtained when we used SYF (Src/Yes/Fyn triple knock-out), and SYF re-expressing c-Src (SYF+ c-Src) cell lines and performed scratch induced migration and in vitro kinase assay for SLK (not shown). These results suggest that Src family kinases are required to negatively regulate SLK activity. Supporting these data, our laboratory has previously shown that overexpression of v-Src inhibits SLK kinase activity in a casein kinase II (CK2)-dependent manner (Chaar et al., 2006).

To investigate the role of FAK in SLK regulation, scratch wound induced migration assays were performed on FAK (-/-) fibroblasts and wild type controls. Following wounding of FAK wild-type monolayers, SLK kinase activity is upregulated to levels comparable to that of MEF3T3 cells. However, little or no SLK upregulation was observed following wounding of FAK-null monolayers **(Fig. 5.3. B),** suggesting that FAK signalling pathways are required for SLK activation during scratch wound

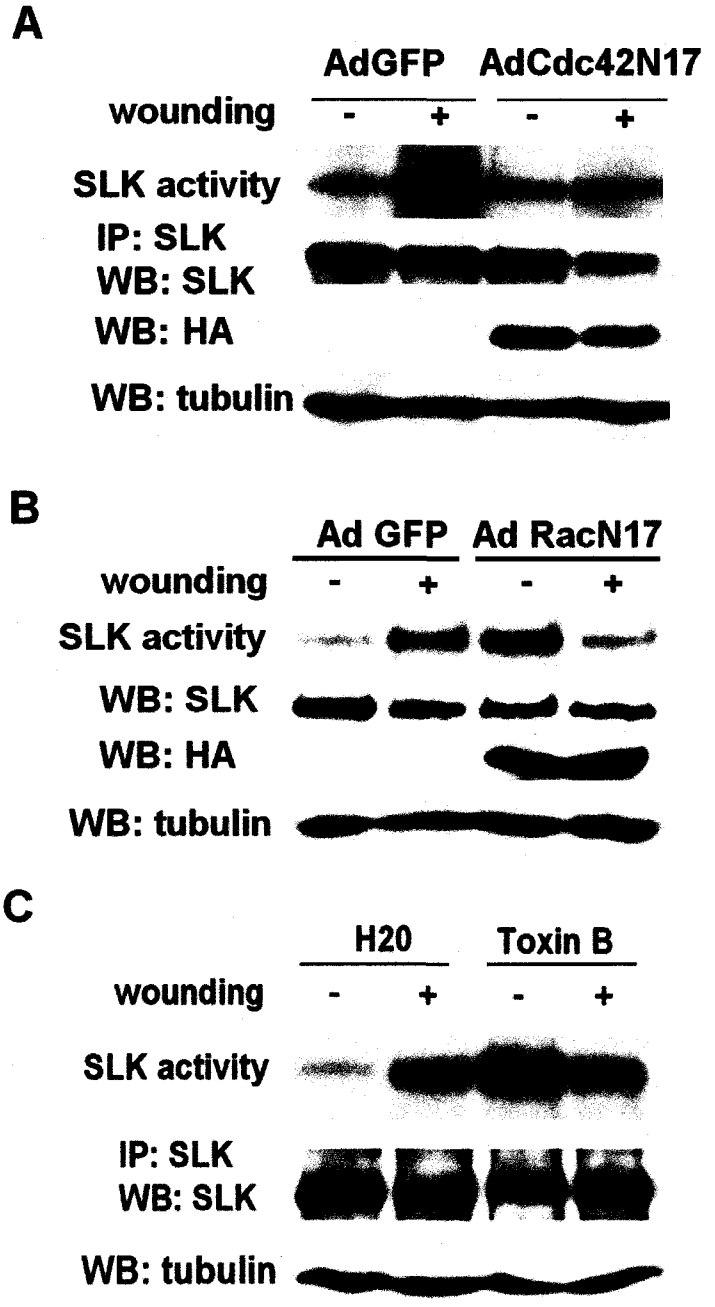


Figure 5.2. SLK activation is dependent of Rho small GTPases signaling. **A. and B.** Subconfluent monolayers of MEF-3T3 fibroblasts were infected with HA-tagged adenoviruses encoding dominant negatives Cdc42N17 and Rac1N17 constructs. After 24 hours we performed scratch induced migration and *in vitro* kinase assays for SLK. To test the efficiency of infection we performed WB using hemagglutinin (HA) antibody. **C.** Cells were incubated overnight in the presence of Toxin B (20-24 h) and we performed scratch induced migration and *in vitro* kinase assay for SLK. Total tubulin blot was used as a loading control. (-): unscratched cells; (+): cells collected after one hour of migration.

induced migration.

Similarly, scratch wounding of MEF3T3 cells in the presence of the MAPK (MEK) inhibitor U0126 prevented SLK upregulation when compared to DMSO control-treated cells **(Fig. 5.3. C)**. During the same time course, scratch wounding resulted in ERK1/2 phosphorylation in DMSO treated cells, whereas ERK1/2 phosphorylation was markedly reduced in the presence of U0126, confirming the specificity of the inhibitor. These data suggest that ERK1/2 MAPK signalling pathways are required upstream of SLK activation.

Similar experiments in the presence of the P38 MAPK inhibitor SB203580 showed no effect on SLK activation, suggesting that P38 MAPK signalling is not required for SLK activation (not shown). Overall, these results suggest that SLK activation by scratch-induced migration requires the FAK/c-Src/MAPK signalling system.

5.2.4. SLK Activation is Dependent on PI3K and Independent of CAS Signalling

Crk-associated substrate (CAS) proteins, through their SH3 domain bind to the proline rich regions of FAK and Pyk2 (Hanks et al., 2003). Cellular Src optimally phosphorylates CAS proteins in the substrate domain when bound to FAK, leading to the recruitment of a Crk family adaptor molecule, activation of a small GTPase, such as Rac1 or Cdc42 and JNK, and the subsequent promotion of membrane protrusion and cell migration (Cho and Klemke, 2002; Klemke et al., 1998).

Cell lacking CAS display impaired actin filament assembly and significantly decreased rates of migration and spreading (Honda et al., 1999; Honda et al., 1998). We used CAS null cells to study the activation of SLK in a scratch induced migration assay. We show that following wounding of CAS null monolayers, SLK kinase activity is upregulated to levels comparable to that of MEF-3T3 cells **(Fig. 5.4. A)**. These data suggest that CAS signalling pathways are not required for SLK

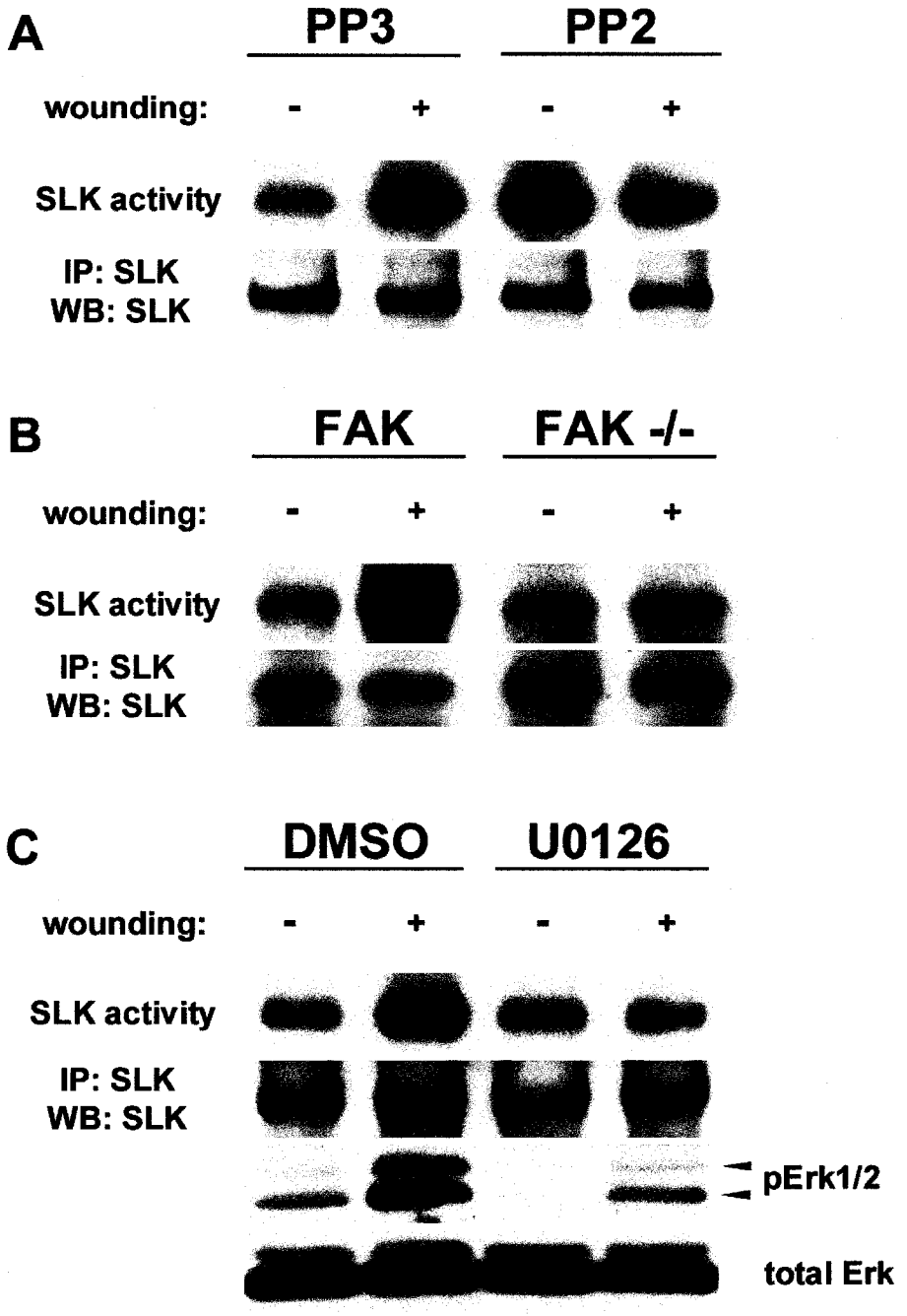


Figure 5.3. SLK activation requires FAK/Src/MAPK signaling.
A. Confluent MEF-3T3 monolayers were pre-incubated (60 min) in the presence of PP2 and PP3 inhibitors and then scratch wounded in the presence of inhibitors. Cells were collected 60 minutes later and analyzed for SLK kinase activity. **B.** FAK-null or wild type cells were subjected to scratch wound assays and assayed for SLK activity. **C.** Treatment with U0126 and DMSO control. Phospho-Erk1/2 western blot is shown as a control for U0126 treatment. (-): unscratched cells; (+): cells collected after one hour of migration.

activation during migration.

Previous studies have shown that the regulatory p85 subunit of PI3K binds to FAK at Tyr397, which leads to PI3K activation by FAK (Chen et al., 1996; Chen and Guan, 1994). Furthermore, it was demonstrated that the PI3K inhibitors, wortmanin and LY294002, were able to inhibit FAK-promoted migration in a dose-dependent manner (Reiske et al., 1999).

Therefore, we performed scratch-wound induced migration assay in the presence of LY294002 (and wortmanin- not shown) and observed that SLK kinase activity was not activated after one hour of migration, compared to DMSO treated cells **(Fig. 5.4. B)**. The efficiency of LY294002 inhibitor was confirmed by analyzing the phosphorylation levels of Akt, a downstream target of PI3K signalling. These data suggest that PI3K signalling is also required for SLK activation during MEF3T3 cell migration.

5.2.5. Recruitment of SLK to the Leading Edge of Migrating cells is Src Dependent, but FAK and MAPK Independent

Cell spreading and scratch wound migration of MEF-3T3 fibroblast monolayers resulted in the recruitment of a proportion of SLK protein at the cell periphery or the leading edge, respectively. These data were supported by recent immunofluorescent studies performed with a new SLK phospho-specific antibody that showed that phosphorylated SLK kinase is predominantly localized at the leading edge of migrating cells (not shown).

Microtubule dynamics and stability have been shown to be regulated, in part, by Src family kinases in various systems, including stabilization by integrin-mediated FAK signalling (Laurent et al., 2004; Palazzo et al., 2004; Simon et al., 1998) . Therefore, we investigated whether SLK recruitment to the leading edge was FAK/c-Src/MAPK dependent. Monolayers of FAK-null or SYF (Src/Yes/ Fyn triple knock-out), as well as wild-type controls, were scratch wounded and

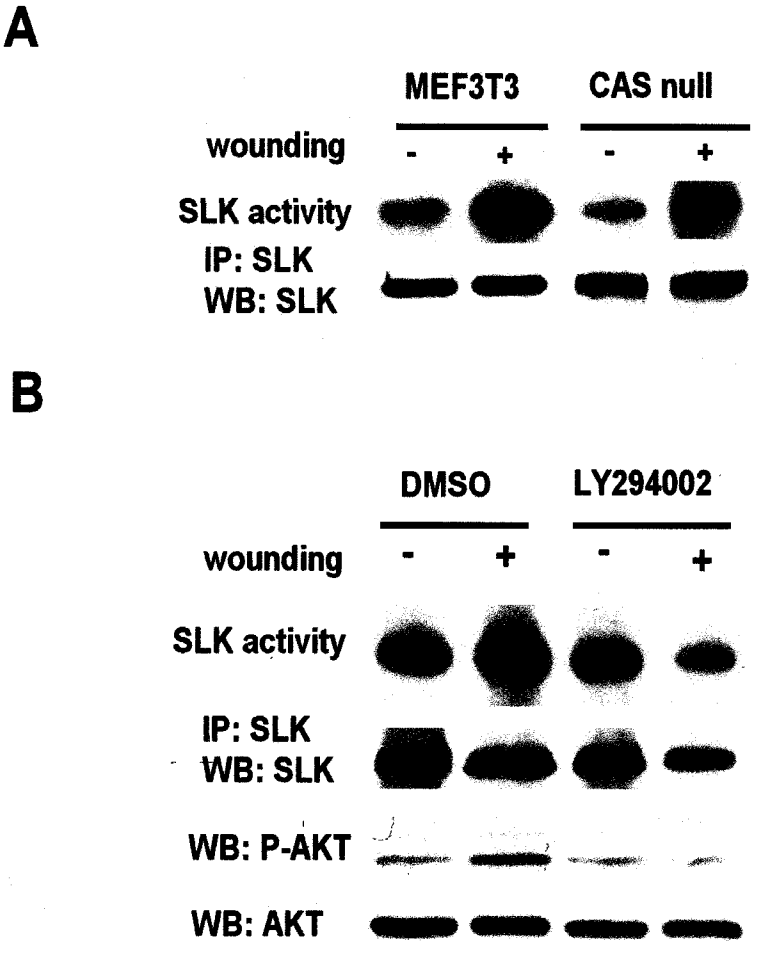


Figure 5.4. SLK activation is independent of CAS signaling but is dependent on PI3K signaling. A. Scratch-induced migration assays in MEF-3T3 and CAS null cells. Cells were collected as confluent monolayer and at 60 minutes after scratch, and analyzed for SLK kinase activity. Total immunoprecipitated SLK levels are shown (lower panel). **B.** Confluent MEF-3T3 cells were pre-incubated (60 min) in the presence of LY294002 or DMSO as a control and then scratch wounded in the presence of the inhibitor or DMSO control. Cells were collected 60 minutes later and analyzed for SLK kinase activity. Phospho-AKT was used as a control for LY294002 treatment. (-): unscratched cells; (+): cells collected after one hour of migration.

co-immunostained for SLK and Rac1, used as a marker for active cytoskeleton rearrangements.

FAK wild-type cells analyzed by immunoflourescence staining one hour after scratch-wound induced migration displayed a partial redistribution of cytoplasmic SLK to the leading edge, a phenotype similar with the one described in MEF-3T3 cells. Although FAK-null cells do not migrate efficiently, SLK was still detected, along with Rac1, at the leading edge **(Fig. 5.5. C-D)**. However, little or no SLK could be detected at the leading edge of wounded SYF monolayers **(Fig. 5.5. G-H).** Similarly, Rac1 distribution was impaired in those cells. The distribution of both Rac1 and SLK was restored when c-Src was re-expressed in SYF cells, suggesting that c-Src expression is sufficient to recruit SLK at the leading edge in migrating cells. The mechanism of SLK recruitment to the leading edge in a c-Src dependent manner awaits further investigations.

Furthermore, MEF-3T3 cells pre-treated with the MEK1 inhibitor U0126 and analysed for SLK and Rac1 localization after scratch wounding, showed a normal pattern in re-distribution of these two proteins at the leading edge of migrating cells **(Fig. 5.5. I-J)**. These results suggest that MAPK signalling pathways are not involved in SLK redistribution at the leading edge of migrating cells. Overall our results show that SLK redistribution to the leading edge is Src-dependent, but is independent of FAK and MAPK signalling pathways.

5.3. Discussion

Our recent data demonstrate that SLK knock-down fibroblasts display reduced cell migration in both wound healing and haptotaxis assays and this impaired migration correlate with decreased focal adhesion turnover in these cells. We then wanted to asses if SLK kinase activity was required for efficient cell migration.

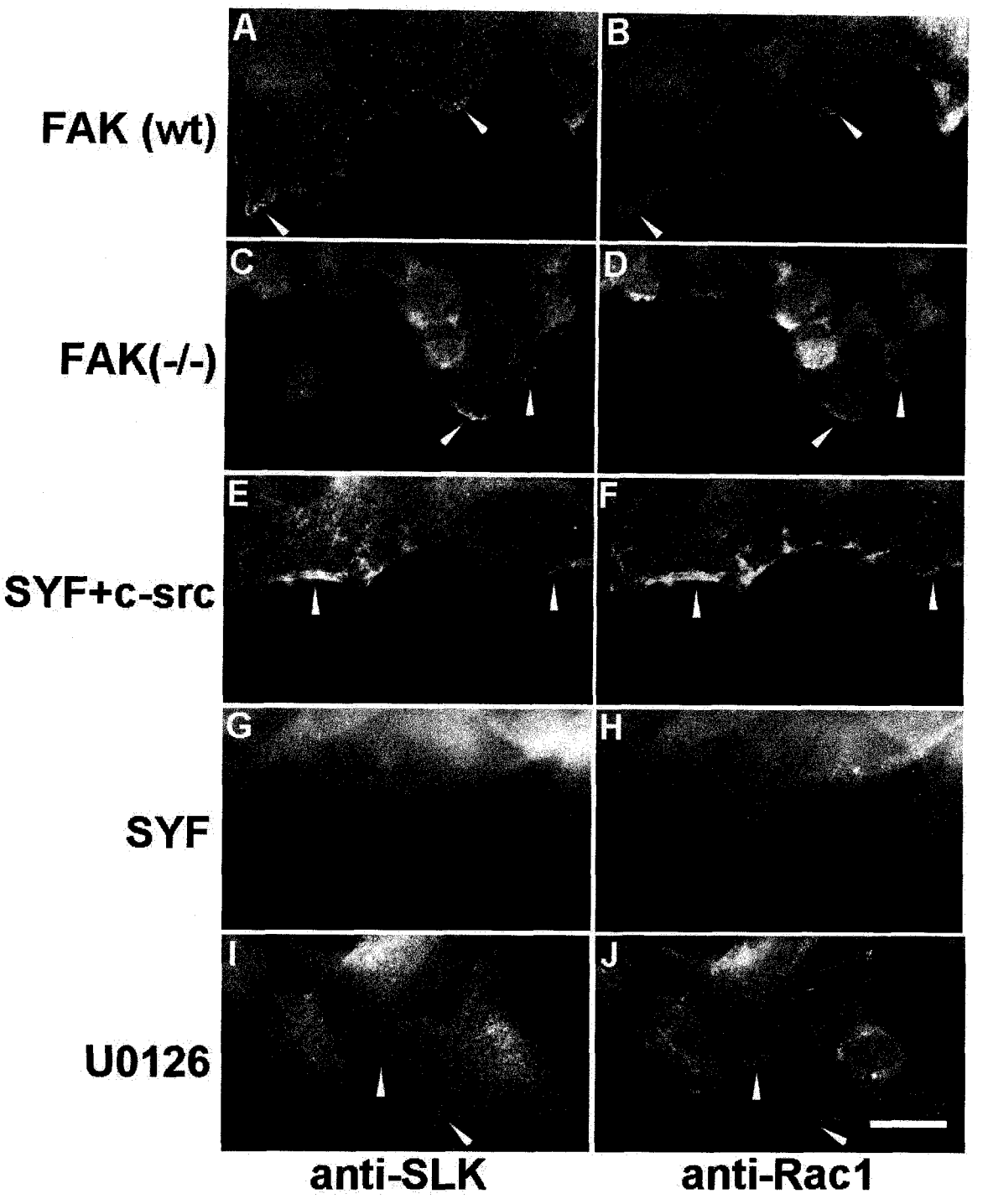


Figure 5.5. Recruitment of SLK at the leading edge is c-Src-dependent. Confluent monolayers of FAK wildtype **(A-B)**, FAK-null **(C-D)**, SYF +c-Src **(E-F)**, SYF **(G-H)** and MEF-3T3 **(I-J)** cells were scratch wounded and immunostained for SLK and Rac1, after one hour of migration. MEF3T3 cells were pre-incubated with U0126 and scratch-wounded in the presence of the inhibitor. Scale bar: 10μm.

Therefore, we investigated SLK kinase activity at various time points following scratch wound induced migration of MEF3T3 fibroblast monolayers. Immunoprecipitation and *in vitro* kinase assays show that SLK kinase activity is markedly increased following scratch wounding of confluent fibroblasts, with a peak of activity at 60 minutes. Based on the requirement for SLK signalling in cell migration, we investigated next, what migratory signalling pathways are required for SLK activation in a scratch wound induced migration assay.

Recent studies have demonstrated that integrin-mediated adhesion to the extracellular matrix regulates the activity of the Rho family of small GTP-binding proteins (del Pozo et al., 2000; Ren et al., 1999). Interestingly, a study has shown that expression of RhoA down-regulates Cdc42 and Rac1 activity, providing a mechanism whereby RhoA may inhibit cell polarization and protrusion (Cox et al., 2001). Furthermore, previous studies have shown that activation of Cdc42 is mediated by integrin signaling, involving a Src-like tyrosine kinase (Etienne-Manneville and Hall, 2001). The recognition of space by integrins appears to be the first polarity signal, which rapidly leads to the activation and localized recruitment of Cdc42. Activated Cdc42 controls a signal transduction pathway that links integrin engagement to the establishment of polarity in migrating cells (Etienne-Manneville and Hall, 2001).

Therefore, we tested if the Rho small GTPases are required for SLK activation by performing adenovirus infections using Rac1 and Cdc42 dominant negative (RacN17 and Cdc42N17) constructs. We report here that by interfering with Rac1 and Cdc42 signalling, SLK activation is impaired upon scratch-induced migration. The involvement of RhoA signalling was indirectly assessed by pre-incubating the monolayer of MEF3T3 cells with Toxin B inhibitor. Our results suggest that RhoA signalling is not required for SLK activation during scratch induced migration, as cells treated with Toxin B are indistinguishable from DNRac1-treated cultures in

their SLK activation pattern. Therefore, this suggests that signalling from Cdc42 and Rac1 GTPases is required for SLK activation during scratch-induced migration.

Because, in normal migrating cells, integrin and FAK mediated control of Src activity is a key event that promotes focal adhesion dynamics (Mitra et al., 2005), we investigated the possible involvement of FAK and c-Src signalling pathways in SLK activation.

We used FAK null, SYF cells and CAS null cells and analyzed the activation of SLK in these cells (Mitra et al., 2005; Webb et al., 2004). FAK null fibroblasts exhibit a decreased rate of migration and spreading and an increase in the number and size of peripherally localized adhesions (Ilic et al., 1995). SYF cells also display reduced motility and spreading and the rate of focal contact disassembly is much slower compared to wild-type cells (Klinghoffer et al., 1999; Webb et al., 2004).

By using FAK null fibroblasts and wild-type control in a scratch induced migration assay we show that SLK activation is FAK dependent. The FAK autophosphorylation site (Y397) is the binding site for the molecules that associate with FAK through an SH2 domain, including Src, Shc, PI3K, Grb7 and PLCγ. In addition, it has been reported that phosphorylation of FAK Y925 is associated with ERK activation in human 293 kidney epithelial cells and with integrin adhesion (Brunton et al., 2005; Schlaepfer et al., 1997). Which pathway downstream of the FAK-Src signaling complex is involved in SLK activation remains to be further investigated, but our data suggest that MAPK and PI3K signaling are also involved **(Fig. 5.6)**.

Therefore, we provide evidence that scratch wounding of MEF3T3 cells in the presence of the MAPK (MEK) inhibitor U0126 prevented SLK upregulation suggesting that MAPK signalling pathways are required for SLK activation during migration. In addition, recent data in our laboratory show that activation of the heregulin receptor (ErbB2/HER2), a member of epidermal growth factor (EGF)

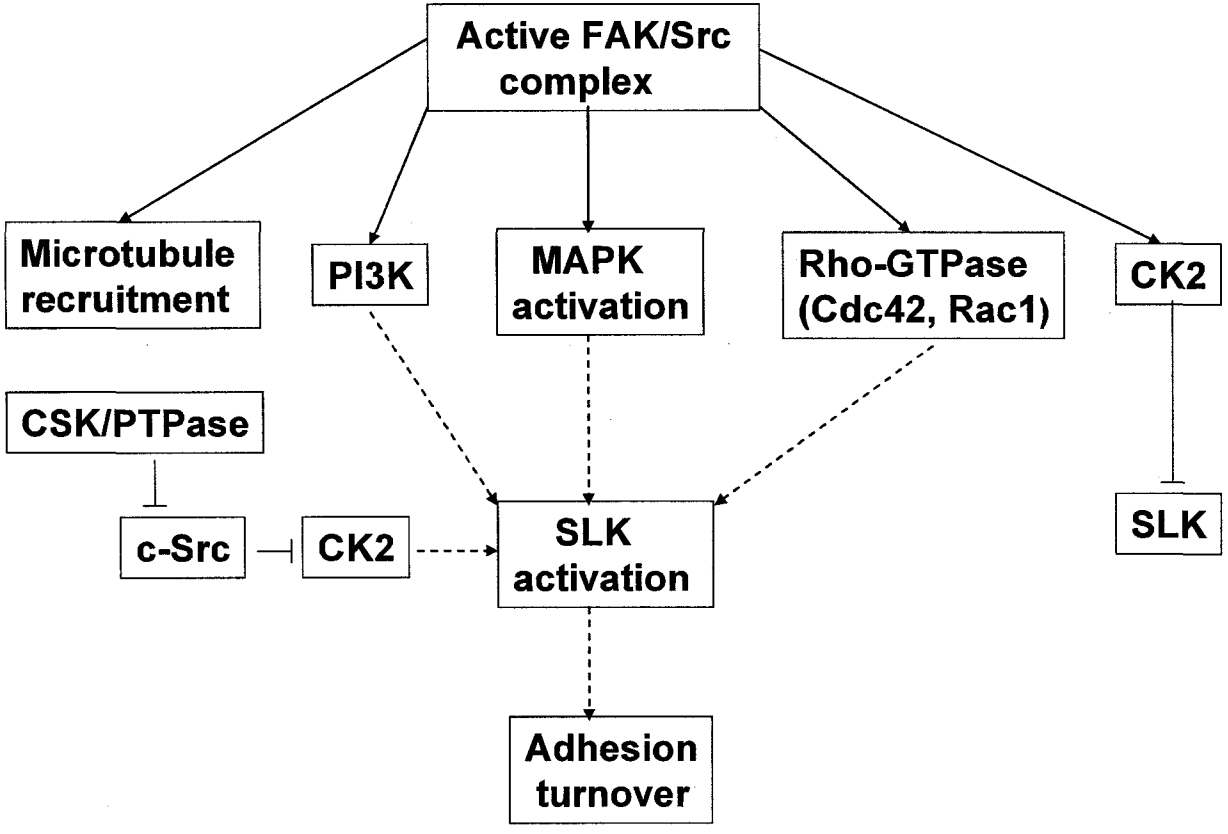


Figure 5.6. A schematic representation of SLK activation and recruitment at the leading edge of migrating cells. SLK activity can be upregulated through a FAK/c-Src/MAPK pathway. Previous results suggest that FAK/c-Src activation during focal contact assembly, negatively regulate SLK through a c-Src-CK2 mediated pathway. Following the inactivation of c-Src by CSK or protein phosphatases, SLK activity can be upregulated through a MAPK dependent pathway. PI3K and Rho small GTPases signaling are also involved in SLK activation. SLK recruitment to the leading edge depends on c-Src signaling and microtubules targeting of focal contacts. Continuous arrows: characterized pathways. Dash arrows: hypothetical pathways. CSK: C-terminal Src kinase; CK2: casein kinase II.

receptor family, leads to upregulation of SLK through MAPK signaling (K. Roovers, unpublished data). These data support our findings of a role for MAPK signaling in SLK activation.

CAS (Crk associated substrate) has been shown to bind the FAK proline-rich domain resulting in its tyrosine phosphorylation by both FAK and c-Src. Furthermore, studies have shown that CAS is tyrosine phosphorylated following fibronectin stimulation of cells in a FAK and c-Src dependent manner (Cary et al., 1998; Sawada et al., 2006; Schlaepfer et al., 1997). Interestingly, c-Src mediated phosphorylation of CAS has been shown to induce adhesion turnover and to regulate cell migration (Cary et al., 1998). By performing scratch-induced migration in CAS null cells we demonstrate that SLK kinase activity was upregulated to levels comparable with that observed in MEF-3T3 cells, suggesting that SLK activation is independent of CAS signaling. In contrast, our scratch induced migration studies performed in the presence of PI3K inhibitor (LY 294002) suggest that PI3K signaling is important for SLK activation during migration. However, the exact molecular mechanisms of regulation remain to be elucidated.

We propose a model for SLK activation and recruitment at the leading edge that briefly describes the multistep process of SLK activation, recruitment at the leading edge and inactivation by a feed-back mechanism **(Fig. 5.6)**.

One possibility is that the initial c-Src recruitment and activation during focal contact assembly negatively regulates SLK. Following the subsequent inactivation of c-Src through CSK or protein phosphatases, SLK activity can be upregulated through a FAK/MAPK pathway in addition with Rho GTPases and PI3K signaling **(Fig. 5.6)** (Angers-Loustau et al., 1999; McGarrigle et al., 2006; Rengifo-Cam et al., 2004). As the recruitment of SLK to the leading edge occurs in FAK-null but not in SYF cells, it is likely that microtubule recruitment through c-Src is FAK independent. One possibility is that, in FAK(-/-) cells, some aspects of the Pyk2/c-

Src signaling complex can compensate for the loss of FAK in the recruitment of SLK (Sieg et al., 1998). However, Pyk2 cannot substitute for FAK activity which appears to be necessary for SLK activation, through a MAP kinase dependent pathway (Huang et al., 2004). Our previous results have shown that SLK is associated with a tubulin containing protein complex in MEF-3T3 cells (Wagner et al., 2002). Furthermore, c-Src and v-Src have been implicated in microtubule association and reorganization during cell migration (Abu-Amer et al., 1997; Matten et al., 1990; Nakayama et al., 1994). Whether the SLK recruitment to the leading edge involves Src regulation of the microtubule network remains to be further investigated.

Overall our data show that SLK is activated by various pathways, such as FAK/c-Src, MAPK, RhoGTPases and PI3K, during cell migration. Its recruitment to the leading edge in a c-Src-dependent manner is required for focal adhesion turnover.

CHAPTER 6

GENERAL DISCUSSION

Previous work has shown that focal adhesions (FA) are highly motile in stationary cells yet stationary in migrating fibroblasts, suggesting that they are highly dynamic structures that turn over rapidly (Smilenov et al., 1999). These findings have led to a model by which migration is initiated by the extension of filopodia, driven by actin polymerization. Actin networks are stabilized through the formation of FA leading to further spreading by the formation of lamelipodia (Giancotti and Ruoslahti, 1999; Horwitz and Parsons, 1999). These nascent FAs grow into larger complexes and serve as traction points as the cell moves. Finally, the release of FAs at the rear of the cell results in net cell displacement. These processes are regulated in part by the Rho family of GTPases and in part by integrin signalling.

During cell migration, newly formed adhesions can either turn over or mature into more organized structures. FAK and Src have a key role in regulating this process (Webb et al., 2004). Recently it was demonstrated that FAK-Src signalling interrupts the maturation of adhesions by promoting their disassembly and thus provides continuous adhesion turnover as the cells protrude and advance. When adhesions mature, they grow in size and molecular complexity (Zamir and Geiger, 2001). Molecules such as paxillin, FAK, and zyxin characterize the nascent, dynamic adhesion, whereas α-actinin is present in more stable adhesion that do not turn over (Webb et al., 2004).

Several members of the Ste20 family of kinases have been identified in mammals. They have been implicated in various biological processes such as stress responses, cell death and cytoskeletal reorganization as well as growth and differentiation (Brown et al., 1996; Dan et al., 2001a; Dan et al., 2001b; Daniels et al., 1998; Fanger et al., 1997).

We and others previously identified a novel Ste20-related kinase termed SLK (Itoh et al., 1997; Pytowski et al., 1998; Sabourin and Rudnicki, 1999; Sabourin

et al., 2000; Yamada et al., 2000). Previous results from our laboratory showed that overexpression of SLK in various cell lines induces the rapid disassembly of actin stress fibers and cell death (Sabourin and Rudnicki, 1999; Sabourin et al., 2000). In addition recent papers showed that SLK is involved in C2C12 myoblast differentiation, has a role in cell cycle progression and is regulated by casein kinase II (CK2) (Chaar et al., 2006; O'Reilly et al., 2005; Storbeck et al., 2004).

To further characterize the role of SLK during adhesion and migration, we used MEF3T3 fibroblasts spreading and migrating on fibronectin coated substrates. The most important findings of our research are summarized below.

We report that SLK is associated with the microtubules and adhesion components during cell spreading on fibronectin (FN). Furthermore, overexpression of SLK by adenoviral infection inhibits cell spreading on FN, supporting a role for SLK in regulating cytoskeletal dynamic during cell spreading. Using short interfering RNA and dominant negative approaches, we showed that SLK is required for focal adhesion turnover and cell migration downstream of the FAK/c-Src complex. In addition, we report that SLK activation is dependent on a multitude of signaling pathways such as FAK/c-Src, MAPK, PI3K and RhoGTPases, whereas SLK recruitment to the leading edge is Src-dependent but FAK independent.

These results suggest that SLK is a part of a microtubule-associated complex that is targeted to adhesion sites and implicated in the regulation of cytoskeletal dynamics. Thus, we propose that SLK represents a novel focal adhesion disassembly signal required for efficient cell migration.

Using immunofluorescence studies, we report that SLK is associated with the microtubule network and can be co-precipitated with α-tubulin. Also, the stimulation of adhesion site formation by microtubule-disrupting agents induces the re-localization of SLK with un-polymerized α-tubulin to large vinculin-containing adhesion complexes.

Direct interaction between SLK and α-tubulin was not observed by in vitro binding assays, suggesting an indirect interaction. Recent work performed in our laboratory has shown that SLK interacts with the LIM domain binding transcriptional co-factor proteins Ldb1 and 2. Interestingly, we have shown that Ldb2 can bind α-tubulin and paxillin, establishing a link between SLK and microtubules. Furthermore, SLK and Ldb2 co-localized with paxillin in lamelipodia and ruffles of migrating fibroblasts (C. Storbeck et al., unpublished data). Thus, SLK is part of a higher order tubulin complex that contains paxillin and the Ldb2 adaptor protein.

SLK was also found to colocalize with paxillin, α-tubulin, and Rac1 in membrane ruffles after scratch-wounding of MEF-3T3 fibroblast monolayer. These results suggest that SLK is recruited to the leading edge of migrating cells with other adhesion signaling proteins. We have found that SLK recruitment to the leading edge of migrating cells is c-Src dependent but FAK independent.

Different lines of evidence suggest that Src kinase activity is involved in regulating focal adhesion turnover. The v-Src temperature sensitive mutant (as well as c-Src in its active conformation) translocates to focal adhesions at the permissive temperature and the kinase activity of v-Src leads to focal adhesion disassembly upon phosphorylation and degradation of FAK (Fincham and Frame, 1998; Kaplan et al., 1994). A substantial proportion of exogenously expressed wild-type c-Src is concentrated around the nucleus in fibroblasts, co-localizing with microtubules and with markers of endosomes and the trans-Golgi network (Kaplan et al., 1992). Furthermore, constitutively active c-Src is targeted to focal adhesions at the cell periphery by a mechanism that requires the amino-terminal half of the molecule, which contains the SH2 and SH3 domains (Kaplan et al., 1994). Further studies showed that, once at the cell periphery, Src is directed to specific adhesion sites by individual members of the Rho family (Timpson et al., 2001). RhoA and mDia have been demonstrated to stabilize the microtubule network by

capping, presumably to stabilize adhesion sites and the actin network (Cook et al., 1998; Hollenbeck, 2001). Interestingly, RhoA participates in Src-regulated tyrosine phosphorylation of multiple substrates, including focal adhesion kinase (FAK) and paxillin, and is required for correct localization of Src (Fincham et al., 1996). The RhoA downstream effector, mDia was found to interact with Src, and inhibition of Src blocked mDia-dependent stress fiber formation (Tominaga et al., 2000). These results strongly suggest that mDia1 is a RhoA effector responsible for actin-dependent delivery of c-Src to focal adhesions (Tominaga et al., 2000). Recent findings suggest that mDia, via its actions on actin and microtubules, recruits critical signaling molecules, such as c-Src, APC and Cdc42 to their respective sites of action for cell polarization and migration (Yamana et al., 2006). At present we did not investigate a possible interaction between SLK and mDia, but mDia it may represent a good candidate in facilitating SLK-microtubule translocation in a c-Src dependent manner.

In our attempt to characterize the role of SLK in cell adhesion and spreading, we performed overexpression studies, using microinjection and adenovirus infections. We report that overexpression of activated SLK induces actin stress fiber disassembly and inhibits cell spreading on fibronectin. Furthermore, we show that a dominant negative form of Rac1 (RacN17) could partially rescue the SLK phenotype of actin stress fiber disassembly, suggesting that Rac1 is a mediator of SLK-induced actin remodeling. It was demonstrated that Rac1 promotes focal adhesion turnover via its antagonism to RhoA (Rottner et al., 1999). Whether SLK directly regulates Rac1 or an upstream guanine exchange pathway remains to be elucidated.

Using short interfering RNA and dominant negative approaches we have shown that SLK is required for focal adhesion turnover and cell migration downstream of a FAK/c-Src complex. Our results show that SLK knock-down cells

and cells expressing the dominant-negative SLK mutant ($SLK^{1-373K63R}$) display normal lamelipodia, but an increased number of vinculin containing sites and reduced rates of directed cell migration. These characteristics were previously correlated with impaired focal adhesion turnover (Ilic et al., 1995). In addition, SLK knock-down cells and cells overexpressing the dominant negative SLK mutant showed a 60% inhibition of migration on fibronectin coated transwell inserts. Together, these data support a role for SLK in the process of cell migration.

By using a recently described microtubule-induced focal adhesion turnover assay, we report that SLK expression and activity are required for efficient focal adhesion turnover (Ezratty et al., 2005). In cultures where SLK expression was knocked down by specific siRNA against murine SLK, adhesion turnover was severely impaired, as evidenced by the sustained level of FAK- pY397. Furthermore, we report that SLK kinase activity is upregulated during nocodazole wash-out, suggesting that microtubule and adhesion dynamic regulates SLK kinase activity.

An important link between microtubules and focal adhesion dynamics has been identified by live-cell monitoring. Specifically, microtubule targeting of focal adhesions is often followed by focal adhesions disassembly, suggesting that microtubules might deliver a "relaxing signal" which destabilizes focal contacts (Kaverina et al., 1999). Interestingly, calpain was reported to be required for the microtubule-mediated turnover of adhesion complex sites after nocodazole wash-out, suggesting that calpain may mediate focal complex disassembly downstream of microtubules (Bhatt et al., 2002). Recent studies in our laboratory showed that SLK and calpain co-immunoprecipitate, suggesting a possible functional interaction between the two proteins. This SLK-calpain interaction could provide a link for SLK requirement in the mechanism of microtubule- induced adhesion turnover.

Paradoxically, calpain activity is also associated with integrin activation and clustering, as well as actin stress fiber formation and cell spreading (Kulkarni et

al., 1999; Potter et al., 1998). Recently it was reported that Src-induced tyrosine phosphorylation of FAK is required for optimal F-actin assembly and cell spreading, at least in part via calpain activity (Carragher and Frame, 2004). Thus, calpain appears to be involved in both assembly and disassembly of actin and focal adhesion dynamic by mediating tyrosine phosphorylation events within the focal adhesion.

By performing replating assays we show that SLK is not activated during cell adhesion and spreading on fibronectin. One possibility is that specific redistribution of SLK leads to localized changes in substrate phosphorylation that are subtle and hard to detect by our biochemical analyses. Another explanation for the lack of detectable SLK activation during cell spreading on fibronectin, could be the absence of cell polarity in spreading cells, a pathway which we report to be important for SLK activation.

In contrast, in a scratch-induced migration assay of MEF3T3 monolayer plated on FN, we show that SLK kinase activity is markedly increased, with a peak of activity at 60 minutes. The activation of SLK following scratch wounding of MEF3T3 monolayer appears to be a late event that requires upstream signaling. Using a scratch wound assay, we tested the role for FAK and c-Src family kinases in activation of SLK during migration. We report that FAK/c-Src signaling is required for SLK activation, but that Src family kinases are required to negatively regulate SLK activity. Previous studies from our laboratory have shown that SLK down-regulation by v-Src is indirect and is accompanied by SLK hyper-phosphorylation on serine residues. Deletion analysis revealed that casein kinase II (CK2) sites at position 347/348 are critical for v-Src-dependent modulation of SLK activity. Further studies showed that CK2 can directly phosphorylate SLK at these positions and that inhibition of CK2 in v-Src transformed cells results in normal SLK kinase activity (Chaar et al., 2006). Overall, these results suggest a mechanism whereby

SLK may be regulated at sites of actin remodeling, such as membrane lamellipodia and ruffles through a Src-CK2 pathway.

By using specific inhibitors for MAPK during scratch-induced migration, we report that MAPK signaling, MEK1/2 signaling, but not p38 signaling, are required for SLK activation during migration. The ERK/MAPK pathway can regulate cytoskeletal dynamics and cell migration through several routes. It was reported that active ERK is targeted to newly forming focal adhesion after integrin engagement and activation of v-Src (Fincham et al., 2000). Furthermore, the ERK/ MAPK cascade is a target for FAK-Src signaling and its local activation can be facilitated by association with paxillin, at adhesion sites (Ishibe et al., 2004; Schlaepfer et al., 1999). Paxillin is constitutively associated with MEK, and in response to hepatocyte growth factor (HGF), also binds Raf1 and ERK/MAPK (Ishibe et al., 2003). ERK/MAPK binding by paxillin requires phosphorylation of Y118 on paxillin by Src. Once bound by paxillin and activated, ERK/MAPK phosphorylates paxillin on Ser83, which promotes the binding of focal adhesion kinase to paxillin and further downstream, the activation of the Rho family GTPase, Rac1 (Ishibe et al., 2004). FAK induces the local disassembly of focal adhesions, whereas Rac initiates migration through the extension of lamelipodia at the leading edge. So paxillin coordinates focal adhesion turnover and migration by functioning as a regulated scaffold for a localized connection between the ERK/MAPK and FAK signaling pathways.

Unpublished data suggests that SLK can phosphorylate paxillin *in vitro* (P. O'Reilly and J. Foucault, unpublished data). It is tempting to speculate that paxillin could serve as a scaffold for SLK activation by MAPK when they are brought in close proximity. Alternatively, paxillin phosphorylation by SLK may induce adhesion destabilization and focal adhesion turnover. The identification of SLK phosphorylation sites in paxillin will provide more insights into the mechanism of

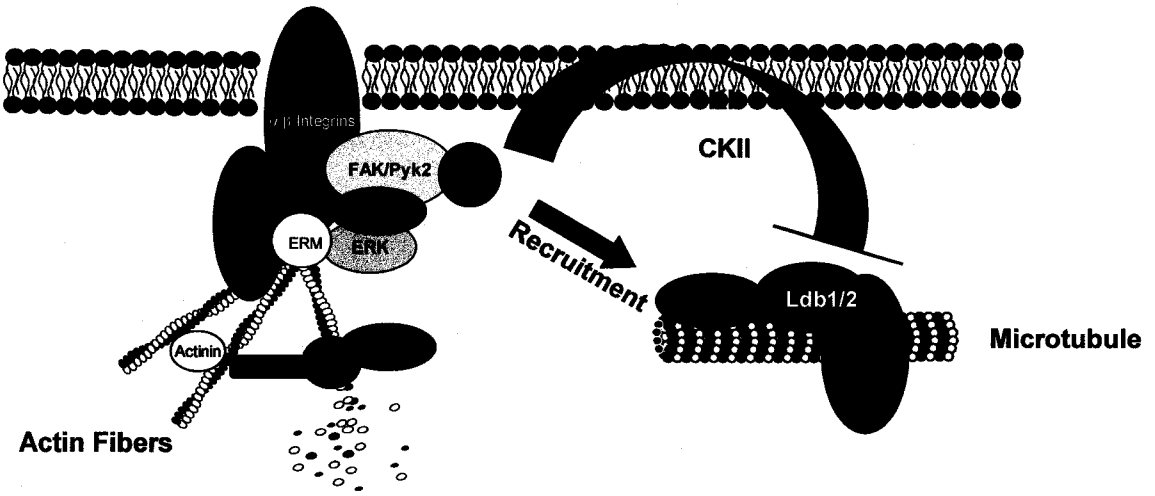
SLK regulation at adhesion sites.

Other pathways that seem to play a role in SLK activation are the PI3K and small RhoGTPases. We reported that signalling from Cdc42 and Rac1 GTPases are required for SLK activation during scratch-induced migration, whereas RhoA activity may not be required.

In addition, our scratch induced migration studies performed in the presence of PI3K inhibitor (LY294002) suggest that PI3K signaling is important for SLK activation during migration. Several studies described a role for PI3K in cell migration. One study has shown that PI3K binding is required for FAK to promote cell migration and that the binding of Src and CAS to FAK may not be sufficient for this event (Reiske et al., 1999). Furthermore, an essential role of the PI3K regulatory subunit p85alpha has been shown in controlling PDGF receptor-induced cytoskeletal changes and cell migration (Jimenez et al., 2000). Recently, it was reported that activation of PI3K activity alone is sufficient to remodel actin filaments to increase cell migration through the activation of Akt and p70S6K1 in chicken embryonic fibroblasts (Qian et al., 2004). Ongoing experiments in our laboratory will test if SLK is a potential substrate for the PI3K and PDK1 kinases.

Overall, our results suggest that one of the adhesion-destabilizing signals delivered by the microtubule at adhesion sites may be SLK. Therefore, we propose a model for SLK recruitment and regulation of adhesion dynamics **(Fig. 6.1)**. During focal adhesion assembly, SLK that is associated with the microtubule network in a complex with Ldb1/2 is inactivated by active Src and casein kinase signaling. This inactivation of SLK coincides with the assembly and signaling of nascent adhesion sites. In contrast, during focal adhesion disassembly, the microtubule-SLK-Ldb1/2 complex is recruited in the proximity of adhesion sites in a c-Src dependent manner, perhaps through an Ldb2-paxillin interaction. When c-Src is inactivated by CSK or other tyrosine phosphatases, SLK can then be activated by upstream signaling

A. Adhesion assembly



B. Adhesion disassembly

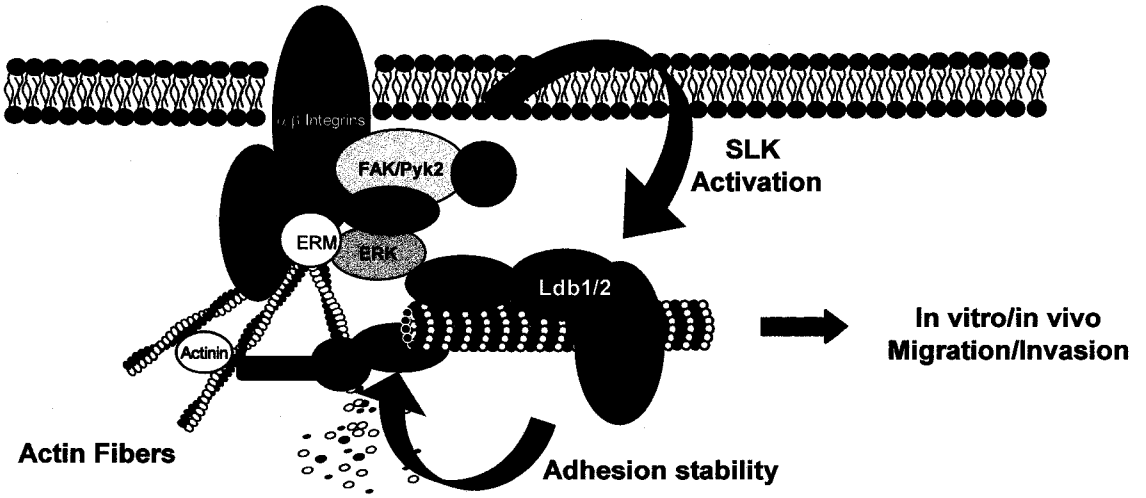


Figure 6.1. Proposed model for SLK recruitment and regulation during FN-stimulated adhesion and migration.

Upon FN stimulation of migration, focal adhesions assemble through the activation of the FAK/c-Src complex. During assembly, SLK is inactivated by Src-CK2 signalling, and it is associated with the microtubule and Ldb1/2 complex.

During adhesion disassembly, the microtubule-SLK-Ldb1/2 complex is recruited in the proximity of adhesion sites in a c-Src-dependent manner, perhaps through an Ldb2-paxillin interaction. When Src activity is inactivated by CSK or other tyrosine phosphatases, SLK is activated by various upstream signalling: MAPK, PI3K and Rho small GTPase. When activated, SLK function to induce actin stress disassembly and paxillin phosphorylation resulting in relaxation of adhesion sites and their turnover. The net result of SLK signalling will induce adhesion turnover and cell migration.

coming from the MAPK, PI3K and RhoGTPase pathways. When activated, SLK functions to induce actin stress fiber disassembly and paxillin phosphorylation, resulting in the relaxation of adhesion sites and their turnover. The net result of SLK signaling will induce adhesion sites turnover and cell migration **(Fig. 6.1)**.

Overall, our data show that SLK is required for efficient cell adhesion and migration on fibronectin substrate. We have established a role for SLK in cell motility, as a novel molecular link between the adhesion-destabilizing activity of the microtubule network and actin-based cytoskeletal remodeling events. In addition we have identified novel members of the SLK network, such as Rac1, α-tubulin, paxillin, and c-Src, proteins that are involved in cell adhesion and migration. This work, in combination with the future characterization of downstream events regulated by SLK will provide potential new targets for therapeutic intervention in highly invasive human cancers.

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