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GRADE / DEGREE

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FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

Molecular Regulation of the STE20-Like Kinase (SLK) by SRC-Dependent Pathways

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MOLECULAR REGULATION OF THE STE20-LIKE KINASE (SLK) BY SRC-DEPENDENT PATHWAYS

By

Ziad Y. Chaar

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

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Your file Votre référence
ISBN: 0-494-10954-8
Our file Notre référence
ISBN: 0-494-10954-8

ABSTRACT

The Ste20-like kinase (SLK) is a conserved serine/threonine kinase that induces the dissociation of actin stress fibers in a Rac1-dependent manner. Recently, our laboratory has shown that SLK is a microtubule-associated protein regulating migratory components at the cell periphery in membrane ruffles and lamellipodia (Storbeck *et al*; Wagner *et al* unpublished data).

To investigate the role of SLK during cell adhesion and migration, we used the Polyomavirus Enhancer Activator 3-null (PEA3(-/-/)) cell line, known to exhibit migration deficiencies. We observed that SLK redistribution to the cell periphery is altered in the PEA3(-/-) cells and that this phenotype could be rescued by the re-expression of PEA3 cDNA into PEA3(-/-) cells. Further analysis revealed that the PEA3(-/-) cells exhibit impaired c-src activation highlighted by decreased focal adhesion kinase (FAK) cleavage and reduced p130Cas tyrosine phosphorylation following fibronectin stimulation, suggesting that c-src activity and proper adhesion signaling may be required for SLK recruitment to the cell periphery.

To further investigate FAK and c-src involvement in SLK redistribution to the cell periphery, we used FAK- or src, yes and fyn (SYF)-deficient cells. We have shown that SLK redistribution to the cell periphery is also altered in SYF-deficient cells but not in FAK-deficient cells, suggesting that c-src mediates SLK redistribution to the cell periphery independently of FAK.

SLK in vitro kinase assays performed on PEA3(-/-), FAK- and SYF-deficient cells revealed that SLK kinase activity is unaffected. However, v-src and

c-srcY527F transformed cells displayed a 2- to 3-fold decrease in SLK kinase activity. Biochemical analysis revealed that SLK is hyperphosphorylated on serine residues in these cells with the kinase domain being the main target. Further analysis revealed that casein kinase II (CK2) phosphorylates the SLK kinase domain on serine 347/348 to downregulate SLK kinase activity. In addition, we also showed that CK2 kinase activity is dramatically increased in v-src-transformed cells and that the inhibition of CK2 restores SLK kinase activity.

Overall, we showed that SLK redistribution to the cell periphery requires c-src, which can also regulate SLK kinase activity via CK2 to allow the turnover of adhesion sites and cytoskeletal rearrangements.

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LIST OF ABBREVIATIONS

bp	Base Pair
BSA	Bovine Serum Albumin
°C	Degree Celsius
cDNA	Complementary Deoxyribonucleic Acid
Ci	Curies
CSK	C-terminal src kinase
DME	Dulbecco's modified Eagle's
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
F	Phenylalanine
FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
FN	Fibronectin
GAP	GTPase Activating Protein
GDI	Guanine Nucleotide Dissociation Inhibitor
GDP	Guanine Diphosphate
GEF	Guanine Nucleotide Exchange Factor
GTP	Guanine Triphosphate
h	Hour

kb	Kilobase
l	Liter
LB	Luria-Bertani Medium
μCi	Microcuries
μg	Microgram
μl	Microliter
μM	Micromolar
min	Minute
ml	Milliliter
mM	Millimolar
mmol	Millimole
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEA3	Polyomavirus Enhancer Activator 3
PFA	Paraformaldehyde
PH	Pleckstrin Homology
PI3K	Phosphoinositide 3-Kinase
PL	Poly-L-Lysine
PMSF	Phenylmethylsulfonyl Fluoride
PVDF	Polyvinylidene Difluoride
RNA	Ribonucleic Acid
S	Serine
SDS	Sodium Dodecyl Sulphate

Ser	Serine
SH	Src Homology
SLK	Ste20-Like Kinase
TBE	Tris-Borate EDTA
TRITC	Tetramethyl Rhodamine Isothio-Cyanate
Tyr	Tyrosine
Y	Tyrosine

DEDICATION

To my wife, Rouéida, my daughters, Gabriella and Maya and my parents, Mona and Youssef Chaar for their continuous love, inspiration and support.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr Luc A. Sabourin, for giving me the opportunity to pursue my graduate studies in his laboratory. Luc, thank you for your patience, guidance and support, It was great working for you. Thank you to my advisory committee members, Dr J. Bell, Dr A. Hakim and Dr D. Picket, for their support and advice. Also, thank you to Dr C. Thompson, AKA sergeant Thompson, for believing in me and introducing me to Luc. Last but definitely not least, thank you to all my lab colleagues, Dr C. Storbeck, S. Wagner, P. O'Reilly, I. Hester, K. Hume, K. Daniel, M. Mckay and P. Pelletier. You guys are awesome and the best. Cheers

I would like to thank the Canadian Institute of Health and Research in partnership with the K.M. Hunters Institute and the Ontario Graduate Scholarship for the scholarships offered during my graduate studies.

I would also like to thank my parents-in-law, Rosette and Youssef Matar, for their continuous moral support. Yes mom and dad, I am done school. Sisters, you are awesome, thank you for being there and your tremendous love.

CHAPTER 1

GENERAL INTRODUCTION

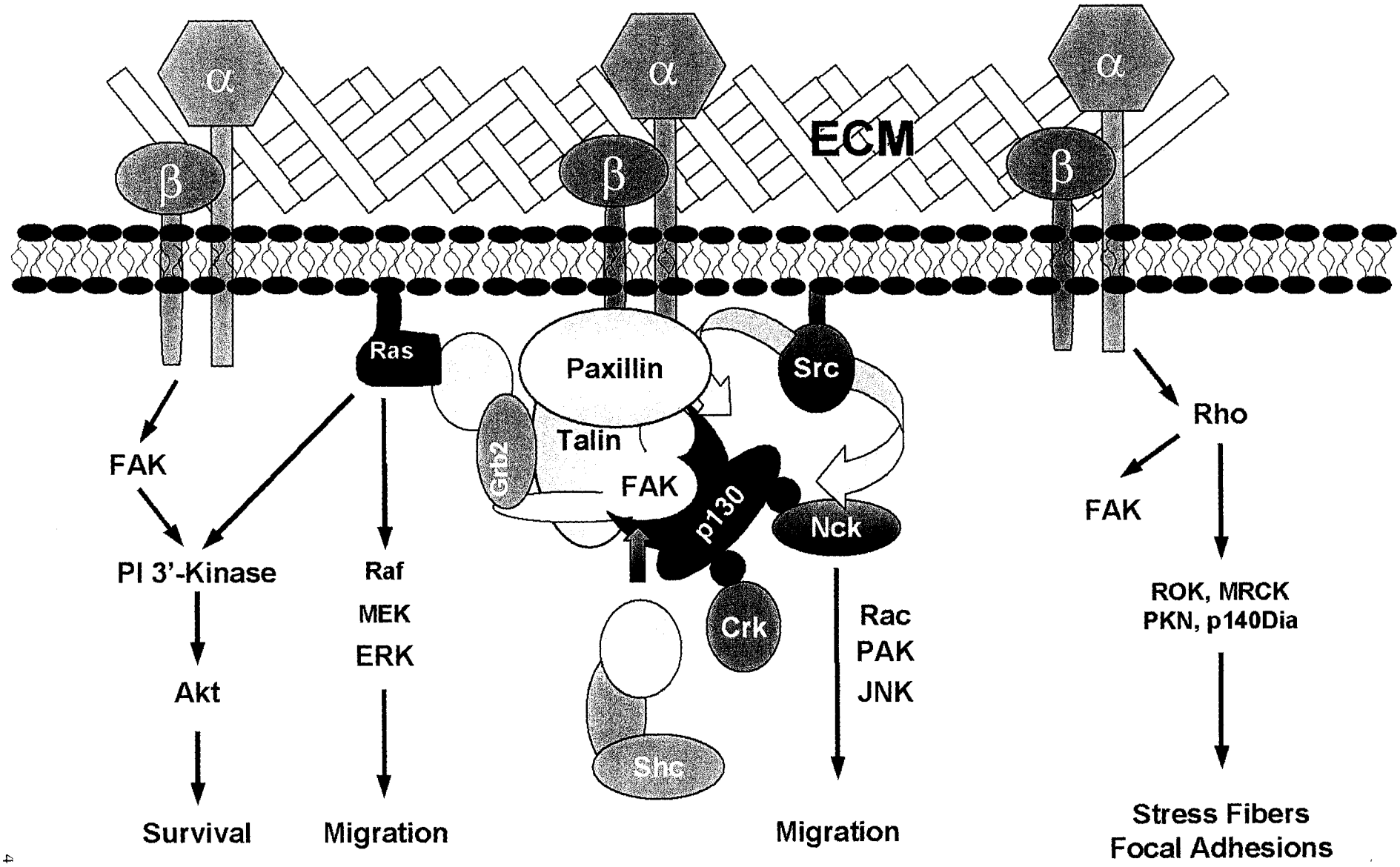
1.1 Cell Adhesion And Migration: An Overview

Cell adhesion and migration is a complex mechanism involved in multiple processes from mammals to plants and even single celled organisms (Cotran et al. 1999). In humans, cell migration is involved in embryonic development, tissue repair and immune surveillance (Cotran, et al. 1999, Gilbert 2003, Ridley et al. 2003). Unfortunately, cell migration also contributes to the development of pathological diseases such as cancer, mental retardation, osteoporosis, inflammatory and vascular diseases (Ridley, et al. 2003).

In order for a cell to migrate, it requires constant reorganization of the actin cytoskeleton as well as the microtubule network, a process that is dependent on cell-extracellular matrix (ECM) or cell-cell interactions. The link between the ECM and the actin cytoskeleton is mediated by adhesion receptors such as the integrin family of transmembrane receptors. ECM binding to the integrins links it to the actin cytoskeleton via the Rho family of small GTPases and their effectors. Interestingly, integrin binding to the ECM is also essential for intracellular signaling such as cell growth and survival.

Upon binding to the ECM, integrin receptors cluster causing the activation and recruitment of adhesion components, such as talin, vinculin, paxillin, focal adhesion kinase (FAK) and src. Integrin signaling regulates important biological processes such as cell migration and survival (Figure 1-1). The crosstalk between integrin receptors and adhesion components will be discussed in the context of Rho-GTPases, FAK and src in this chapter.

Figure 1-1. Schematic representation of the integrin signaling pathways. Integrin receptors, subunits α and β, binding to the extracellular matrix (ECM), cluster generating an intracellular signaling cascade. Integrin receptors signaling was shown to be involved in cell survival, migration and reorganization of the actin network as well as focal adhesion complexes assembly (as discussed in this chapter).


α
β
ECM
Ras
Paxillin
Src
Talin
Crk2
FAK
p130
Nck
Crk
Shc
FAK
PI 3'-Kinase
Akt
Survival
Raf
MEK
ERK
Migration
Rac
PAK
JNK
Migration
Rho
FAK
ROK, MRCK
PKN, p140Dia
Stress Fibers
Focal Adhesions

1.2 Actin Network And Rho-GTPases

Cell adhesion and migration requires dynamic actin cytoskeleton remodeling. The lamellipodium, a meshwork of actin filaments, is firstly generated at the leading edge of a spreading or migrating cell (Small 1981). The lamellipodium is often punctuated with radially oriented bundles of actin fibers termed filopodia or microspikes which extend as finger-like projections beyond the lamellipodia meshwork tip (Small 1981).

In order for a cell to adhere and migrate it requires contact with the ECM at specialized sites termed foci where the largest of which are termed focal contacts or focal adhesions (Burridge et al. 1988, Curtis 1964, Izzard and Lochner 1980). Focal contacts refers to the generation of a foci in the lamellipodia linking the ECM to the actin cytoskeleton via the integrin family of transmembrane receptors (Kaverina et al. 2002, Rottner et al. 1999). Focal adhesions, found at the termini of stress fibers bundles, refer to the scaffolding of substrates at a foci (Kaverina, et al. 2002, Rottner, et al. 1999). Based on these studies, it was suggested that focal contacts are the precursors of focal adhesions.

A key family of proteins involved in the regulation of actin dynamics during cell adhesion and migration is the Rho-GTPase family. The Rho-GTPases are regulated by three family of proteins, the guanine nucleotide exchange factor (GEF), the GTPase-activating protein (GAP) and the guanine nucleotide dissociation inhibitor (GDI). The GEFs promote the activation of the Rho-GTPases by promoting the exchange of GDP to GTP (Schmidt and Hall 2002,

Zheng 2001) whereas the GAPs inhibit the Rho-GTPases activity by enhancing the hydrolysis of GTP to GDP (Moon and Zheng 2003). The GDIs also inhibit the Rho-GTPase activity by sequestering or solubilizing the bound GDP (Zheng 2001).

RhoA, Rac1 and cdc42 Rho-GTPases have been widely explored in relation to their abilities to induce the formation of actin bundles, lamellipodia and filopodia respectively. Studies have shown that RhoA can induce actin stress fiber formation leading to focal adhesion complex assembly through the effectors Rho-kinases (also termed ROCK, ROK) and the mammalian homologue of diaphanous (mDia). The Rho-kinase was found to inhibit the myosin light chain (MLC) phosphatase and to phosphorylate the myosin II regulatory light chain thereby increasing myosin-based contractility (Amano et al. 1996, Kimura et al. 1996). In addition, Rho-kinase was found to phosphorylate *lin-11, isl-1 and mec3* (LIM)-kinase and adducin, leading to increased actin polymerization (Kimura et al. 1998, Maekawa et al. 1999). Rac1 and cdc42 are both required at the leading edge or protrusive end of a migrating cell. Rac1 is involved in generating a protrusive force through localized actin polymerization whereas cdc42 is crucial for directed cell migration through the generation of filopodia (Raftopoulou and Hall 2003). Both Rac1 and cdc42 are potent activators of actin polymerization and are believed to play crucial role in cellular migration. The first common effector for Rac1 and cdc42 is the p21-activated kinase, PAK. PAK is a serine/threonine kinase that promotes the formation of lamellipodia at the leading edge and regulates focal adhesion turnover at the rear of the cell (Manser et al.

1997, Sells et al. 1997). While the exact mechanism of action of PAK is unknown, one interesting substrate of PAK is LIM-kinase. LIM-kinase is activated by PAK and also by Rho-kinase leading to the phosphorylation and the inactivation of cofilin, thus stabilizing the actin filaments (Maekawa, et al. 1999). Another common effector of Rac1 and cdc42 is the actin-related protein 2/3 (Arp2/3) complex, an inducer of actin polymerization. While both, Rac1 and cdc42 are able to stimulate the Arp2/3 complex, the pathways leading to this event diverge.

It is now apparent that the actin network drives migration through a delicate balance between the activation-inactivation of Rho-GTPases. In general, Rac1 and cdc42 activation induces actin polymerization at the lamellipodia and filopodia respectively leading to the formation of focal contacts. Once the focal contacts are established, RhoA activity is enhanced leading to actin stress fibers formation resulting in stable focal adhesion complex formation.

1.3 Focal Adhesion Kinase

The loss of tissue attachment in normal adherent cells leads to growth arrest and anoikis, a form of apoptosis caused by loss of adhesion. Interestingly, elevated protein levels and kinase activity of FAK are observed in a number of human cancers such as primary sarcomas, cervical carcinomas, prostatic carcinomas, breast tumors, ovarian carcinomas and liver metastases of colon cancer (Jones et al. 2000). Thus, understanding the molecular mechanisms

involved in the regulation of FAK and its downstream signaling will result in the design of and more efficient treatment of cancer.

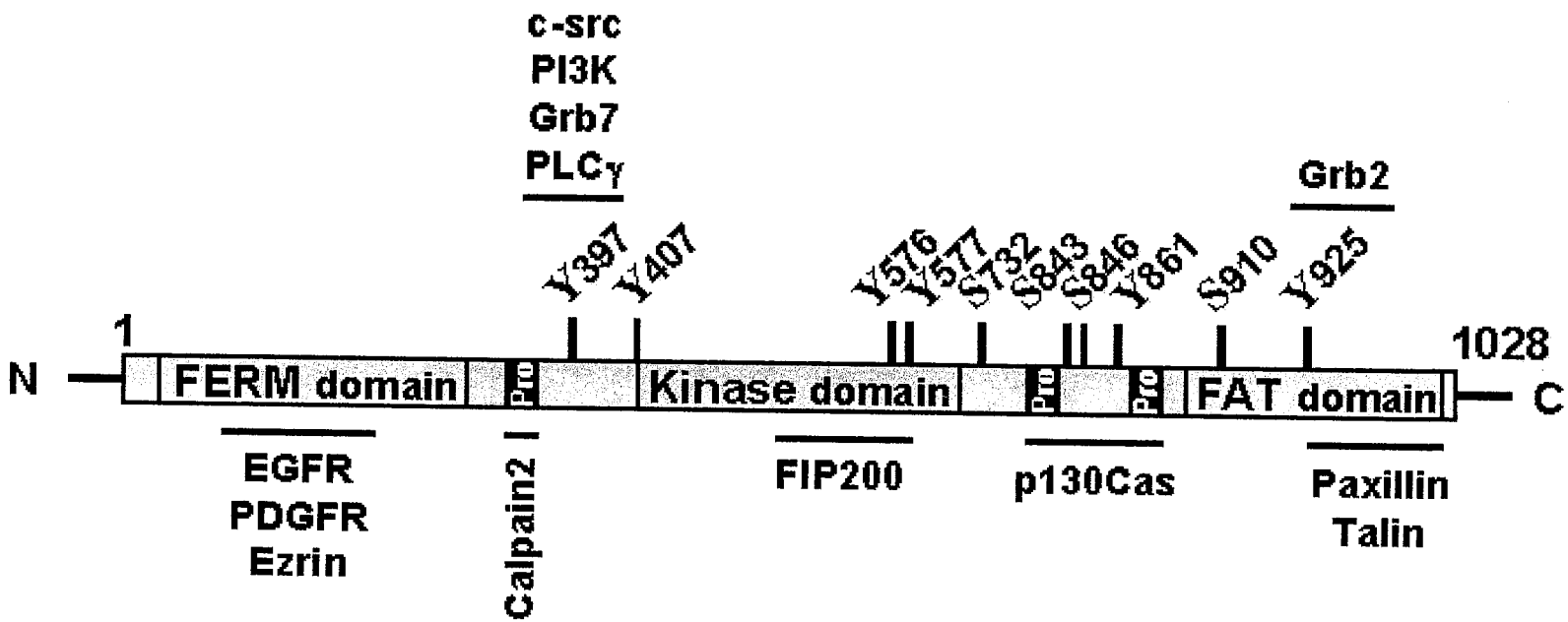
1.3.1 Structure Function

FAK is a non-receptor protein tyrosine kinase (PTK) composed of a central kinase domain flanked by N-terminal and C-terminal domains (Figure 1-2). The FAK N-terminal domain was termed FERM because it shares sequence homology to the erythrocyte band Four point 1(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 (Calalb et al. 1995, Ruest et al. 2000, Toutant et al. 2002). The FAK C-terminal domain is termed FAT for Focal Adhesion Targeting since it 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 p130Cas (Bouton et al. 2001, Martin et al. 2002, Turner 2000).

1.3.2 Adhesion Signaling

Normal cellular function and survival depends on the cell-cell interactions and contacts with the ECM. The integrin family of transmembrane receptors mediate the interaction of cells with ECM proteins to generate intracellular signals mediating cell growth, survival and migration (Ruoslahti 1999). It is widely accepted that integrin binding to the ECM, more specifically FN, results in the localization of FAK to these integrin clusters and subsequently its

Figure 1-2. Schematic representation of focal adhesion kinase (FAK) domains and known binding partners. FAK is composed of an N-terminal FERM domain, a central tyrosine kinase domain and a C-terminal focal adhesion targeting (FAT) domain. It contains three proline rich motifs (Pro) and several tyrosine (Y) and serine (S) residues that are known to be phosphorylated. Known binding partners of FAK are underlined according to their binding sites.


c-src
PI3K
Grb7
PLCγ
Grb2
Y397
Y407
Y576
Y577
S732
S843
S846
Y861
S910
Y925
1
1028
N
FERM domain
PRD
Kinase domain
PRD
SH3
FAT domain
C
EGFR
PDGFR
Ezrin
Calpain2
FIP200
p130Cas
Paxillin
Talin

autophosphorylation on Tyr-397, thus creating a high affinity binding site for the c-src SH2 domain. In addition, Tyr-397 has also been shown to bind other SH2 containing proteins such as PI3K, phospholipase C (PLC)γ and Grb7 (Akagi et al. 2002, Chen et al. 1996, Han and Guan 1999). However, FAK maximal activation is reached upon the formation of a FAK-c-src complex and the subsequent c-src phosphorylation of FAK at Tyr-576, Tyr-577 (Owen et al. 1999, Schaller et al. 1994, Xing et al. 1994).

The c-src proto-oncogene has also been shown to efficiently phosphorylate FAK Tyr-861 and Tyr-925 (Owen, et al. 1999). FAK Tyr-861 is mainly believed to play a role in mediating conformational changes to the FAK molecule since elevated Tyr-397 autophosphorylation levels was observed after c-src-mediated Tyr-861 phosphorylation (Leu and Maa 2002). FAK Tyr-925 phosphorylation was shown to mediate the association with the Grb2 SH2 domain thus promoting signaling to the Erk2/mitogen-activated protein kinase (MAPK) pathway (Katz et al. 2003, Renshaw et al. 1999). In addition, studies have shown that membrane-targeted FAK exhibits an increase in Tyr-925 phosphorylation level and does not localize to sites of focal contacts as the endogenous FAK or the phenylalanine (Phe)-925 mutant FAK, suggesting that Tyr-925 phosphorylation or its subsequent binding to Grb2 may result in FAK dissociation from focal contact (Katz, et al. 2003).

FAK contains four serine phosphorylation sites, Ser-732, Ser-843, Ser-846 and Ser-910. However, the role of phosphorylation on these residues is poorly understood. Ser-732 phosphorylation was shown to be cyclin-dependent kinase

(Cdk) 5 mediated in post mitotic neurons (Xie et al. 2003). Recently, Palazzo *et al* have showed that integrin signaling facilitates the stabilization of microtubule network and correlates with increased FAK phosphorylation level as well as activation of the Rho-GTPase-mDia signaling (Palazzo et al. 2004). On the other hand, Ser-910, located within the FAT domain and in close proximity of paxillin binding site, was shown to be phosphorylated during mitosis and after mitogen stimulation of cells (Hunger-Glaser et al. 2003, Ma et al. 2001). Ser-910 phosphorylation was also associated with decreased paxillin binding (Hunger-Glaser, et al. 2003), suggesting that activated FAK and more specifically Ser-910 phosphorylated FAK may dissociate from the focal contact site. Interestingly, it appears that both Tyr-925 and Ser-910 phosphorylation may mediate FAK dissociation from focal contact through decreased binding to paxillin.

The FAK-c-src complex is also subject to extensive negative regulation. FAK-inhibitory protein of 200 kDa (FIP200) was shown to bind FAK kinase domain and to interact with suppressors of cytokine signaling, thus targeting FAK to poly-ubiquitination and degradation (Abbi et al. 2002). The p50 C-terminal src kinase (Csk) also mediates c-src inactivation by phosphorylating Tyr-527. In addition, several protein tyrosine phosphatases (PTP) such as the SH2 containing tyrosine phosphatase 2 (Shp2) and the low molecular weight tyrosine phosphatase (LMW-PTP) have been shown to regulate c-src (Chiarugi et al. 2003, Von Wichert et al. 2003, Yu et al. 1998).

1.4 The C-src Proto-oncogene

Increased c-src activity, and in some cases protein levels, have been observed in human cancers such as breast cancers (20 fold increase in activity; 7), colon cancers (up to 8 fold increase in activity) and pancreatic cancers (Jones, et al. 2000). The c-src proto-oncogene can directly phosphorylate the β1 integrin subunit, causing a loss of β1 integrin ligand-binding affinity (Akamatsu et al. 1996). In addition, Adams *et al.* showed a reduction in FN synthesis in cells transformed with the Src oncogene (Adams et al. 1977). All together, these changes contribute to reduced adhesiveness of the cell thus increasing its propensity to migrate.

A mutated form of the cellular-src (c-src) proto-oncogene, viral-src (v-src), was first identified in the Rous Sarcoma Virus (RSV) as an inducer of chicken tumors (Rous 1910). Further characterization of the RSV genome showed that it encoded a 60 kDa protein tyrosine kinase (Brugge and Erikson 1977, Hunter and Sefton 1980). It was later established that v-src is a truncated version of c-src rendering it constitutively active.

1.4.1 Structure function

C-src is the founding member of the src family of protein tyrosine kinases (SFKs) which comprises nine members, c-Src, c-Fyn, c-Yes; expressed in most tissues; and Blk, Yrk, Fgr, Hck, Lck and Lyn, which are tissue restricted. SFKs are involved in transducing signals which result in altered cell growth and behavior (Courtneidge 1994, Frame 2002). C-src, a 60 kDa phosphoprotein, is comprised of an N-terminal SH4 domain that contains a myristylation site for

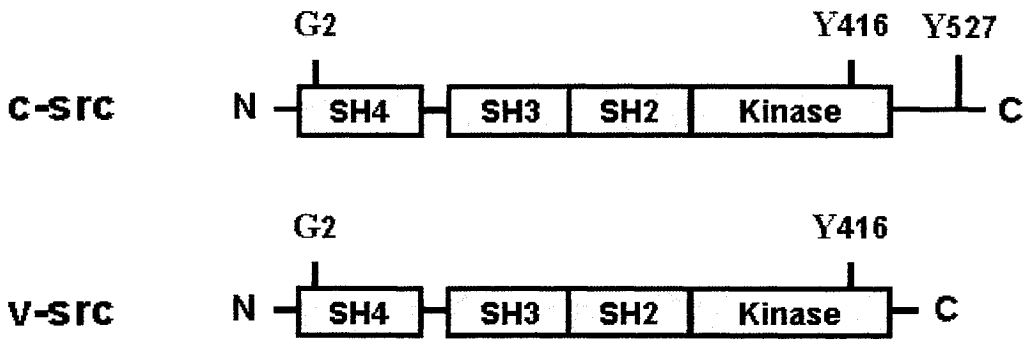
membrane anchoring, an SH3 domain that interacts with proline rich motifs (PXXP), an SH2 domain that binds phosphorylated tyrosine residues, and a C-terminal tyrosine kinase domain, also termed SH1 (Figure 1-3). C-src contains key tyrosine residues that upon phosphorylation regulate its kinase activity as well as its protein-protein interaction capabilities. The Tyr-416 residue is auto-phosphorylated and is required for optimal activation of the kinase (Johnson et al. 1996). C-src, unlike its viral counterpart v-src, contains Tyr-527 residue that is involved in the inactivation of the kinase. Phosphorylation of Tyr-527 residue by Csk leads to the intramolecular binding of Phospho-Tyr-527 to its own SH2 domain, resulting in a closed conformation thus an inactive kinase (Figure 1-3) (Cooper et al. 1986, Okada and Nakagawa 1989, Thomas and Brugge 1997, Xu et al. 1997).

1.4.2 Activation and Substrates

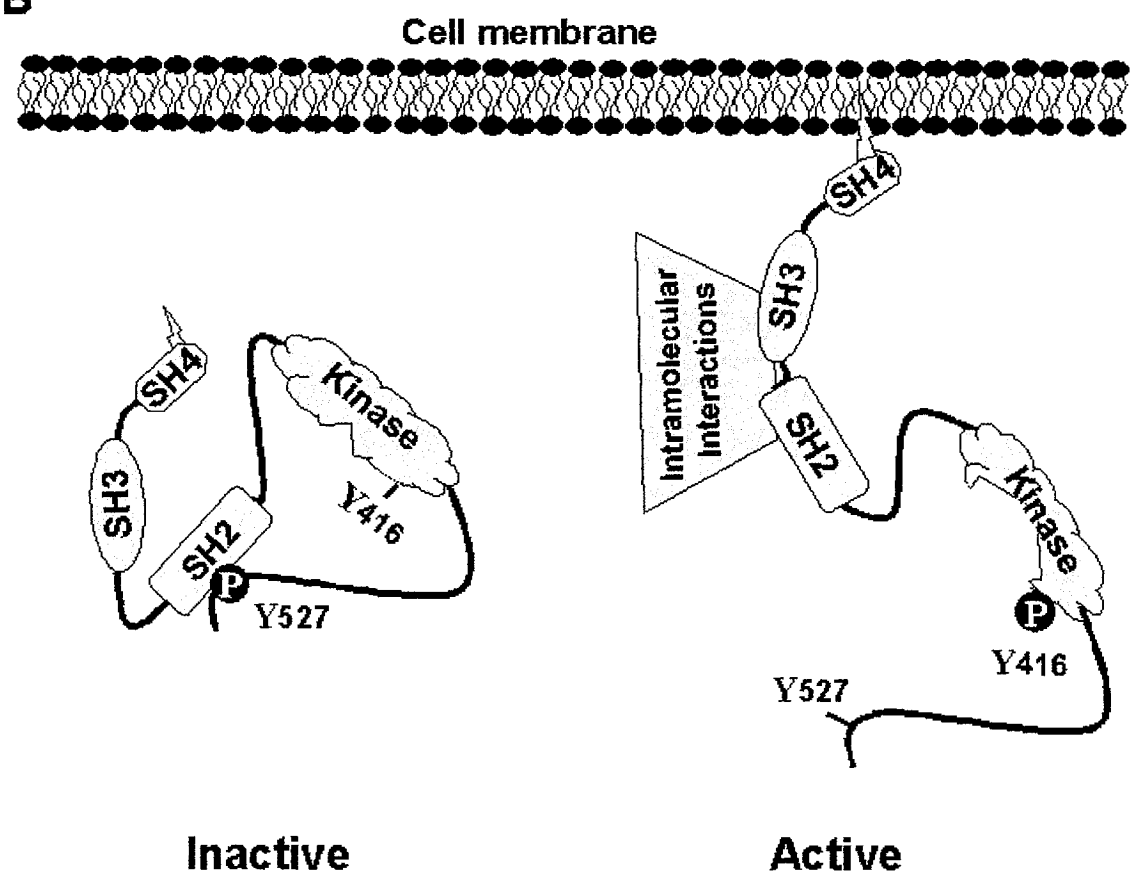
Unlike v-src, which is constitutively active, c-src activation can be achieved in different manners. PTPα, a transmembrane phosphatase, was shown to directly interact with c-src SH2 domain and to cause Tyr-527 dephosphorylation both in vitro and in vivo (Pallen 2003, Ponniah et al. 1999, Sonnenburg et al. 2003, Su et al. 1999). Thus, it is proposed that PTPα contributes to c-src activation by interacting with its SH2 domain and dephosphorylating Tyr-527. Another phosphatase thought to be involved in c-src Tyr-527 dephosphorylation is Shp2. The mechanism of action of Shp2 is still being unraveled. Recent studies have revealed a novel

Figure 1-3. Schematic representation of c- and v-src domains in active and inactive conformations. Both c- and v-src are composed of a an N-terminal src homology (SH)4 domain containing a myristylation site for membrane anchoring at glycine (G) residue located at position 2 (G2), followed by a SH3 then SH2 and a C-terminal kinase domain (panel A). Also, the tyrosine (Y) residue located at position 416 (Y416) and known to be involved in src autophosphorylation for maximal activation is highlighted. In addition, c-src contains a tyrosine residue located at position 527 (Y527) that is involved in the regulation of its kinase activity. As shown, v-src lacks the Y527, thus its constitutively active characteristic. Inactive c-src is mainly perinuclear, upon dephosphorylation of Y527, phosphorylation of Y416 or molecular displacement through interactions with the SH3 and SH2 domains, c-src is unfolded, anchored to the membrane and becomes kinase active (panel B).

A


G2
Y416
Y527
c-src
N
SH4
SH3
SH2
Kinase
C
G2
Y416
v-src
N
SH4
SH3
SH2
Kinase
C

B


Cell membrane
SH4
SH3
SH2
Kinase
Y416
P
Y527
Intramolecular Interactions
SH4
SH3
SH2
Kinase
P
Y416
Y527
Inactive
Active

mechanism for Shp2 where it controls the localization of Csk via its regulator PAG/Cbp (Zhang et al. 2004). Furthermore, EGF stimulation was shown to induce Shp2-dependent paxillin dephosphorylation, leading to the detachment of Csk from the paxillin-c-src complex and resulting in the activation of c-src (Ren et al. 2004).

In addition, interactions with various other molecules such as Sin-1 (a p130Cas-related protein) and the β3 integrin subunit, have been shown to activate c-src through molecular displacement of its SH2 and SH3 domains (Arias-Salgado et al. 2003, Thomas et al. 1998, Walter et al. 1999).

C-src appears to be retained at the perinuclear region in a microtubule network dependent manner in its inactive conformation (Fincham and Frame 1998). Activation of c-src, triggers its membrane targeting in a RhoA-dependent mechanism (Fincham et al. 1996). Furthermore, localization of c-src to focal adhesions, lamellipodia and filopodia is specified by RhoA, Rac1 and cdc42 respectively (Timpson et al. 2001).

1.5 Focal Adhesion Turnover

The loss of tissue attachment in normal adherent cells leads to growth arrest and anoikis, a form of apoptosis caused by loss of adhesion. Interestingly, elevated protein levels and kinase activity of FAK are observed in a number of human cancers such as primary sarcomas, cervical carcinomas, prostatic carcinomas, breast tumors, ovarian carcinomas and liver metastases of colon cancers (Jones, et al. 2000). Furthermore, increased c-src activity has been

observed in human cancers such as breast cancers (20 fold increase in activity; 7), colon cancers (up to 8 fold increase in activity) and pancreatic cancers.

Evidence from SYF cells revealed that SFKs are not required for the assembly of focal adhesions (Klinghoffer et al. 1999). However, SYF cells exhibit impaired focal adhesion turnover (Webb et al. 2004). On the other hand, FAK-/- cells display larger focal adhesion and decreased motility (Ilic et al. 1995). It is now well established that the observed decrease in motility is due to an impairment in the focal adhesion turnover (Ren et al. 2000, Webb, et al. 2004). More interestingly, the re-expression of wildtype FAK into FAK-/- cells restored normal adhesion turnover, whereas a FAK mutant defective for c-src binding and activation (FAK Y397F), failed to do so (Webb, et al. 2004).

The delineation of the molecular pathways involved in focal adhesion turnover came mainly from work done using the temperature sensitive oncogene v-src (Ts LA29 v-src). Fincham *et al* showed that transformed primary chicken embryo fibroblasts (CEFs) with the Ts LA29 v-src resulted in a complete loss of focal adhesions and cell detachment (Fincham et al. 1995). Interestingly, transformed CEFs induce turnover of integrin-associated focal adhesion and disruption of the associated actin filaments (Fincham et al. 2000, Fincham and Frame 1998). Furthermore, a correlation was observed between v-src tyrosine phosphorylation of FAK and the subsequent calpain cleavage of FAK (Carragher et al. 2001, Carragher et al. 2003, Fincham and Frame 1998).

1.6 The Polyomavirus Enhancer Activator 3 (PEA3) Transcription Factor

1.6.1 Structure Function

PEA3, also called E1AF or ETV4 (Higashino et al. 1993, Xin et al. 1992), is the founding member of a subfamily of *ets* transcription factors that include an additional two members that are ER81, also called ETV1 (Brown and McKnight 1992, Jeon et al. 1995, Monte et al. 1995), and ERM, also called ETV5 (Monte et al. 1994). The signature of the Ets family is the ETS DNA binding domain, a region of approximately 85 amino acids, which has been widely conserved during evolution (Karim et al. 1990). Ets family members bind to the core sequence 5'-GGAA/T-3' element in the promoter of target genes where they act to regulate transcription (Macleod et al. 1992, Seth et al. 1992, Wasylyk et al. 1993). PEA3 activation domains located at the N-terminal and the ETS DNA binding domain, located at the C-terminal, are flanked by two independent negative regulatory domains (Bojovic and Hassell 2001).

1.6.2 Biological Role And Substrates

Ets proteins are involved in the regulation of the transcription of membrane receptors, growth factors, transcription factors, and extracellular matrix metalloproteinases. Few Ets proteins direct gene targets have been identified. This is mainly due to the fact that Ets proteins are co-expressed in the same cell type and they possess similar DNA binding properties (Kurpios et al. 2003). However, many potential candidates have been proposed based on their Ets binding site (Sementchenko and Watson 2000). *ETS* genes are also implicated in a number of human tumors (Oikawa and Yamada 2003, Sharrocks

2001). The mechanism of *ETS* gene activation involved in human malignancies appear to be mediated either by reciprocal chromosome translocation, such as Ewing's sarcoma and chronic and acute leukemia, or increased transcription of a specific *ETS* gene such as breast, ovarian or prostate cancer (Oikawa and Yamada 2003). Interestingly, PEA3 is overexpressed in the vast majority of human breast and ovarian tumors and in nearly all of the HER2/Neu-positive subclass of such tumors (Benz et al. 1997, Hynes and Stern 1994). Furthermore, Null mutations in the *pea3* allele compromise the capacity of mammary tumors to metastasize in the mouse mammary tumor virus (MMTV)-Neu/HER2 transgenic mice (Kurpios, et al. 2003, Shepherd and Hassell 2001). In contrast, forced expression of PEA3 in MCF7 human breast tumor cells increased their metastatic potential (Kaya et al. 1996), whereas PEA3 antisense RNA reduced the invasiveness of human tumor cells (Hida et al. 1997).

A well studied PEA3 target gene is the human epidermal growth factor receptor 2 (HER2) (also called *neu* or *c-erbB2*) proto-oncogene, a transmembrane tyrosine kinase receptor of 185 kDa structurally related to the epidermal growth factor receptor (c-ErbB-1) (Singleton and Strickler 1992). No ligand has been found for HER2, however activation is thought to occur through homo- or heterodimerization with either itself or other members of the HER family, HER1, 3 or 4 (Belsches-Jablonski et al. 2001). Several studies have demonstrated a link between HER2, integrin, c-src and FAK activation, suggesting that PEA3 targets such as HER2 may ultimately affect cell motility.

Supporting this, PEA3(-/-) cells display a migratory defect (J Hassell, personal communications).

1.7 Casein Kinase II (CK2)

CK2 was discovered in 1954 as a casein kinase with no physiological substrate (Brunett and Kennedy 1954). It took two decades to identify the first physiological target and since then over 300 different substrates of CK2 have been identified (Litchfield 2003, Meggio and Pinna 2003, Pinna 1994, Pinna 1997, Pinna 2002). Nowadays, CK2 is being referred to as "a house keeping enzyme" given its increasing number of substrates. This resulted in CK2 being involved in a wide range of cellular functions and properties starting with cell proliferation (Allende and Allende 1995, Guerra et al. 1999, Litchfield and Luscher 1993, Tawfic et al. 2001), to survival (Ahmed et al. 2002), moving on to differentiation, transformation and tumorigenesis (Ahmed et al. 2000, Tawfic, et al. 2001) as well as apoptosis (Guo et al. 2001).

1.7.1 Structure Function

CK2 is a serine/threonine kinase tetramer complex composed of two catalytical subunits, α and/or α' and/or α'' and two regulatory β subunits. It is capable of utilizing both ATP and GTP as phosphate donors (Allende and Allende 1995). CK2 minimal amino acid consensus phosphorylation sequence is Ser-X-X-Acidic, where the acidic residue could be glutamic acid, aspartic acid, phospho-Ser or phospho-Tyr (Pinna 1990, Pinna and Meggio 1997). However,

the presence of the minimal consensus sequence does not guarantee efficient phosphorylation (Meggio et al. 1994). It is also worth mentioning that CK2 mediated phosphorylation of p53 on Ser-392 does not conform to the above identified CK2 consensus (Meek et al. 1990), suggesting that some exceptions may arise.

1.7.2 Regulation

CK2 has been classified as a messenger-independent kinase and that phosphorylation; redistribution to subcellular compartment or protein interaction may contribute to its regulation. Autophosphorylation of the β subunit on Ser-209 in a cell cycle-dependent manner appear to modulate the stability of the tetramer (Zhang et al. 2002). CK2 has been shown to interact with tubulin (Faust et al. 1999), Fas associated factor (FAF)1 (Jensen et al. 2001) and CK2-interacting protein-1 (CKIP-1) (Bosc et al. 2000) thus targeting of CK2 to specific structures within the cell. CK2 protein interaction also may regulate CK2 activity as with peptidyl-prolyl isomerase 1 (Pin1) (Messenger et al. 2002). Phosphorylated CK2α interaction with Pin1 results in the specific inhibition of topoisomerase IIα threonine 1342 phosphorylation (Messenger, et al. 2002).

1.7.3 CK2 And The Actin Network

On the growing list of CK2 substrates some are cytoplasmic membrane localized and others are cytoskeletal associated. One such substrate is CK2-interacting protein-1 (CKIP-1) (Bosc, et al. 2000). CKIP-1 is a 46 kDa protein

containing a pleckstrin homology (PH) domain at the N-terminal, followed by five PXXP motifs then a leucine zipper at the C-terminal (Bosc, et al. 2000). CKIP-1 was found to interact with CK2α only and not CK2α′ through its PH domain (Olsten et al. 2004). Recently, CKIP-1 was shown to interact with the actin capping protein (CP)α and β subunits (Canton et al. 2005). Moreover, CK2α was shown to phosphorylate CPα Ser-9 residue. Interestingly, CKIP-1 interaction with CPα, an effect that was enhanced by both CK2α and CK2α kinase inactive, caused a decrease in the capping of the barbed (fast growing) ends of the actin filament and an increase in F-actin depolymerization (Canton, et al. 2005). Another intriguing CK2 substrate in relation to the actin cytoskeleton is WASP. WASPs are known to bind the Arp2/3 complex through their VCA domain and to stimulate Arp2/3 actin nucleating activity leading to actin polymerization (Mullins et al. 1998). Cory *et al* showed that CK2 phosphorylates Ser-483 and Ser-484 of WASP VCA domain. This phosphorylation led to an increase in the VCA binding affinity to the Arp2/3 complex and was shown to be required for efficient actin polymerization (Cory et al. 2003).

1.8 The Ste20-Like Kinase (SLK)

1.8.1 Structure Function

The Ste20-like kinase (SLK) is a serine/threonine kinase composed of 1202 amino acids with a predicted molecular mass is of 147 kDa. However, on an SDS-PAGE gel it displays a mobility shift of approximately 220 kDa protein (Sabourin and Rudnicki 1999). It comprises an N-terminal kinase domain

(residues 1-338), a central domain (residues 339-947) and a C-terminal domain (residues 788-1171) of unknown functions (Sabourin and Rudnicki 1999). The kinase domain is a typical serine/threonine catalytical core comprising a conserved lysine residue at position 63 located within the ATP binding site (Hanks and Hunter 1995). The Ste20 signature motif (TPYWMAPE) is present within the kinase domain at position 189. The SLK kinase domain displays high sequence similarities, over 72 %, with LOK and *Xenopus* polo-like kinase kinase 1 (xPlkk1), involved in cell cycle control (Kuramochi et al. 1997, Qian et al. 1998). The SLK kinase domain was also found to be related, up to 30 %, to the human MST1 and MST2 as well as germinal central kinase (GCK) (Creasy and Chernoff 1995, Katoh et al. 1995, Kuramochi, et al. 1997, Schinkmann and Blenis 1997). The SLK central domain stretching from residue 339 to 947 shares a 70 % sequence homology with a microtubule and nuclear associated protein (M-NAP) of an unknown function (the M-NAP domain; (Schaar et al. 1996). Interestingly, the SLK M-NAP domain contains potential SH3 binding sites, a PXXP motif, at position 735 (Pawson and Scott 1997). Furthermore, the SLK M-NAP domain contains a putative caspase 3 cleavage site (DTQD) located at position 436 (Nicholson et al. 1995.). The SLK C-terminal domain is highly homologous to the AT1-46 protein of unknown function (Schaar, et al. 1996).

1.8.2 Role In Cell Adhesion And Migration

Cellular adhesion and migration are tightly regulated mechanisms, involving a constant assembly and disassembly of new focal adhesion at the leading edge.

Our laboratory has focused on the identification of the molecular pathways implicated in SLK-mediated cytoskeletal remodeling. Studies have demonstrated that the SLK kinase domain induces apoptosis whereas the ATH domain induces actin stress fiber disassembly (Sabourin et al. 2000). Similarly, overexpression of the full length kinase, induces actin stress fiber disassembly (Sabourin and Rudnicki 1999). Recently, Wagner *et al* have shown that SLK is redistributed to the cell periphery with vinculin or paxillin upon cell replating on FN matrix (Wagner et al. 2002). SLK recruitment to vinculin containing structures was proposed to be involved in regulating cell spreading through focal adhesion disassembly. This suggestion was supported by the fact that SLK is recruited to vinculin containing structures at the cell periphery following tyrosine phosphorylation of adhesion components and that the overexpression of SLK inhibited cell spreading onto FN matrix (Wagner, et al. 2002). Furthermore, SLK was shown to interact indirectly and colocalize with α-tubulin during cell spreading, suggesting that SLK is recruited to the cell periphery via the microtubule network to regulate cytoskeletal dynamics. Recent studies in our lab have shown that a fraction of SLK localizes to membrane lamellipodia and ruffles during cell spreading and wound closure (Wagner *et al*, unpublished data).

1.9 Thesis Objectives And Hypotheses

Our working hypothesis is that SLK is involved in cell adhesion and migration via its regulatory effects on adhesion disassembly. Data generated from our laboratory support a critical role for SLK in cell adhesion and migration, however the underlying mechanisms are still being explored (Storbeck *et al*; Wagner *et al* unpublished data). Previously published observations show that SLK is recruited to the cell periphery via the microtubule network resulting in stress fiber disassembly. To investigate the role of SLK in cell adhesion and migration, I have used PEA3-deficient (PEA3(-/-)) MEF cell line, provided by Dr Hassell J.A. as a cell system that exhibits uncharacterized migration deficiencies (J. A. Hassell personnel communications). I have characterized wildtype, PEA3(-/-) and PEA3 reconstituted PEA3(-/-) cells, where a PEA3 cDNA was stably re-introduced into PEA3(-/-) cells (PEA3(-/-)+PEA3), in motility assays. A second major objective was to investigate the role of integrin signaling on SLK activity and regulation. Using biochemical and molecular approaches, the regulation of SLK by the FAK-c-src complex was investigated and found to be negatively regulated in part through phosphorylation by CK2. These studies demonstrated a critical role for SLK in cell adhesion and migration.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell Lines and Cell Culture

HEK293, COS1 and 49F cells were purchased from the American Tissue Type Collection. The *pea3* null mouse embryonic fibroblast cell lines, PEA3(-/-)1, 2 and 3, as well as the PEA3(-/-) cells stably expressing PEA3 cDNA and the wild-type cells (WT) were from J.A. Hassell. The mesodermal FAK-/- cells (p53-/-, FAK-/-) and WT counterpart were kindly provided by D. Ilic. The SYF cells, deficient for src, yes and fyn; and SYF cells stably expressing c-src were a generous gift from P. Soriano. All cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO2 in Dulbecco's modified Eagle's medium (DME medium, Bio-Whitaker) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 μg/ml penicillin, and 50 μg/ml streptomycin.

2.2 Plasmids Construction And Transfections

2.2.1 Plasmids

The pBabepuro3 v-src encoding the full length v-src was kindly provided by M. McMahon. HA-tagged pcDNA3 expression vector bearing full length FAK, FAK kinase dead, FAK K454R (lysine to arginine at position 454), FAK Y397F mutant (tyrosine to phenylalanine at position 397), FRNK and FRNK S1084A mutant (serine to alanine at position 1084) were obtained from D. Schlaepfer. The fpgv-1 control vector and the TsLA29 v-src encoding the conditional v-src were provided by M.C. Frame. HA-tagged pRc/CMV expression vectors bearing the CK2 catalytical subunit kinase active, HA-CK2α, or kinase dead (lysine to

methionine at position 68), HA-CK2αK68M, as well as the CK2 regulatory subunit β, HA-CK2β, were generously provided by D. Litchfield.

2.2.2 General PCR And Cloning Procedures

The polymerase chain reaction (PCR) reaction samples consisted in general of 50 ng (1 µl) of DNA template, 100 ng (1 µl) of forward and reverse primers, 5 µl of 10X ThermoPol Reaction buffer supplemented with 100 mM MgSO4 (NEB), 2 mM (2.5 µl) dNTPs, 1 µl of Vent DNA polymerase (NEB) and made up to 50 µl with sterile double distilled H_2O (stddH2O). Reactions were amplified in an Eppendorf Master Cycle Gradient PCR thermocycler as follows: 1 cycle at (96°C for 3 min), 30 cycles at (96°C for 1 min, 55-65°C for 1 min and 72°C for 1.5 min) followed by 1 cycle at (72°C for 7 min) then 4°C until analysis. Excised vectors and inserts were ligated using T4 DNA Ligase (NEB) as per the manufacturer's procedures at a 1:4 ratio of vector to insert in a total volume of 20 µl. Ligation were performed overnight at 16°C and transformed into competent *E. coli* strain DH5α. Ampicillin resistant single colonies were picked and grown overnight in LB broth supplemented with ampicillin in a shaker at 37°C. Plasmid DNA was extracted using the boiling method (Sambrook J 1989). Large scale DNA plasmid preparations were done using the alkaline lysis method (Birnboim and Doly 1979).

All PCR-generated mutants were subjected to sequencing analysis at the Ottawa Health Research Institute. Sequencing data was analysed using EditSeq and MegAlign (DNAStar computer software).

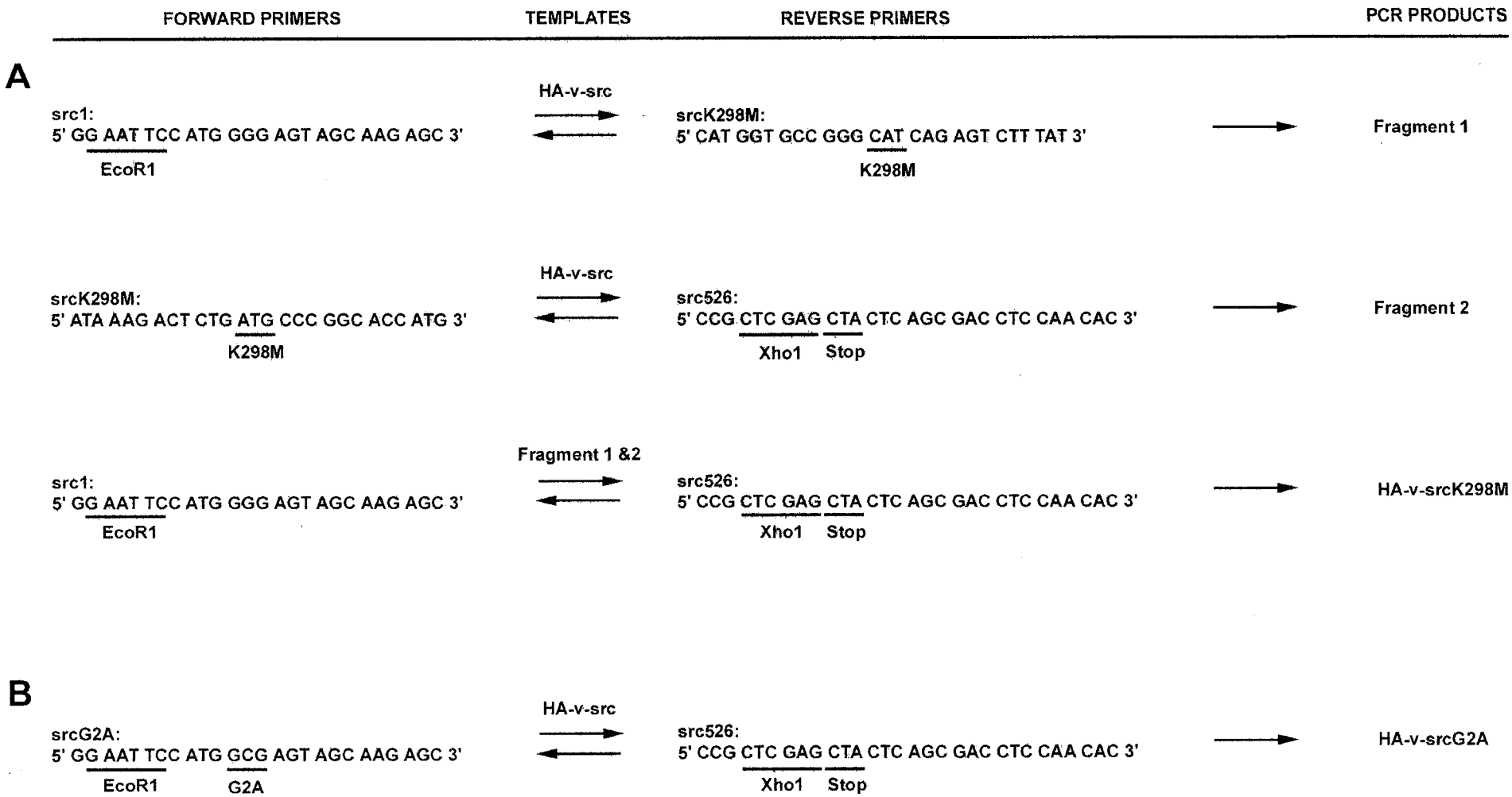
2.2.3 Construction Of HA-Tagged V-src Point Mutants

HA-tagged v-src (HA-v-src) was constructed using standard cloning procedures (Sambrook J 1989). The HA-v-src kinase dead point mutant was generated using PCR-based mutagenesis converting Lysine (K) residue at position 298 to a methionine (M) residue. Briefly, two PCR fragments (1 & 2) were first generated using HA-v-src as a template and src1 forward with srcK298M reverse primers or srcK298M forward with src526 reverse primers. Fragment 1 & 2 are then used as templates with src1 forward and src526 reverse primers to generate v-srcK298M, as illustrated in Figure 2-1 A. The HA-v-src myristylation site mutant, glycine to alanine at position 2, (HA-v-srcG2A) construction was achieved using also a PCR-based approach (Figure 2-1 B). The PCR generated v-srcK298M and v-srcG2A were subcloned into HA-tagged pcDNA3 expression vector (Invitrogen) using standard cloning procedures.

2.2.4 Construction Of Myc-Tagged SLK Kinase Domain Truncations

SLK kinase domain kinase dead (Myc-SLK$^{1-373}$K63R) truncations were generated using PCR-based approach. Myc-SLK$^{1-192}$K63R, containing amino acid residues 1 to 192, was PCR amplified using Myc-SLK$^{1-373}$K63R as DNA template and the T7 (recognizing the T7 promoter region in pcDNA3 vector) with SLK192 (reverse): 5' CCG CTC GAG *CTA* AGC CAT CCA ATA TGG TGT 3'; primers. Myc-SLK$^{1-325}$K63R, containing amino acid residues 1 to 325, was generate with Myc-SLK$^{1-373}$K63R as DNA template and T7 and SLK325 (reverse): 5' CCG CTC GAG *CTA* CAG AGC ATT CTC TGC TTC 3'; primers.

Figure 2-1. Schematic representation of the PCR-based approach used to generate v-src point mutants. HA-v-src kinase dead (HA-v-srcK298M) was generated using by single point mutation of the lysine (K) residue at position 298 to a methionine (M). First, fragments 1 & 2 were PCR amplified with HA-v-src as DNA template and src1 with srcK298M or srcK298M with src526 primers. Fragments 1 & 2 were then used as DNA templates with src1 and src526 primers to generate HA-v-srcK298M (panel A). HA-v-srcG2A mutant, mutation of the glycine residues at position 2 to alanine, was generated with HA-v-src as DNA template with srcG2A and src526 as primers (panel B).


FORWARD PRIMERS
TEMPLATES
REVERSE PRIMERS
PCR PRODUCTS
A
HA-v-src
src1:
5' GG AAT TCC ATG GGG AGT AGC AAG AGC 3'
EcoR1
srcK298M:
5' CAT GGT GCC GGG CAT CAG AGT CTT TAT 3'
K298M
Fragment 1
HA-v-src
srcK298M:
5' ATA AAG ACT CTG ATG CCC GGC ACC ATG 3'
K298M
src526:
5' CCG CTC GAG CTA CTC AGC GAC CTC CAA CAC 3'
Xho1 Stop
Fragment 2
Fragment 1 &2
src1:
5' GG AAT TCC ATG GGG AGT AGC AAG AGC 3'
EcoR1
src526:
5' CCG CTC GAG CTA CTC AGC GAC CTC CAA CAC 3'
Xho1 Stop
HA-v-srcK298M
B
HA-v-src
srcG2A:
5' GG AAT TCC ATG GCG AGT AGC AAG AGC 3'
EcoR1 G2A
src526:
5' CCG CTC GAG CTA CTC AGC GAC CTC CAA CAC 3'
Xho1 Stop
HA-v-srcG2A

The PCR amplified fragments were subcloned into Myc-tagged pcDNA3 expression vector (Invitrogen) using Xba1 (an internal SLK restriction site contained in the PCR fragment) and Xho1 (indicated in bold in SLK192 and SLK325 primers sequence) sites. The Stop codon was inserted in the primers SLK192 and SLK325 as indicated in italic.

2.2.5 Construction Of Myc-Tagged SLK Point Mutants

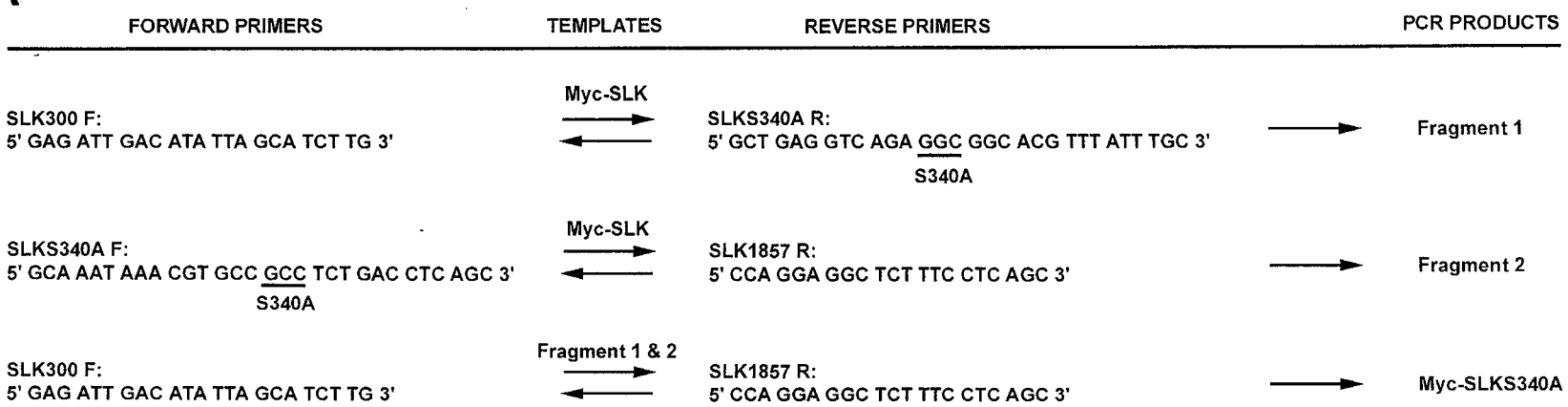
To construct Myc-SLK point mutants we used similar PCR-based as with HA-v-srcK298M. Briefly, PCR fragment 1 was generated using Myc-SLK (Sabourin, et al. 2000) as DNA template and SLK300 F (forward) with either SLKS340A R, SLKSS347/348AA R, SLKS362A R or SLK364A R (reverse) primers. Fragment 2 was also generated using Myc-SLK as DNA template and SLKS340A F, SLKSS347/348AA F, SLKS362A F or SLKS364A F (forward) and SLK1857 R (reverse) primers. Fragments 1 & 2 were then used as DNA templates with SLK300 F (forward) and SLK1857 R (reverse) primers for the final PCR product S340A, SS347/8AA, S362A and S364A (as illustrated in Figure 2-2). Subcloning of the PCR products into Myc-SLK pcDNA3 expression vector was done standard cloning procedures.

2.2.6 Construction Of Gst-Tagged SLK Kinase Domain Point Mutants

Gst-tagged SLK^{1-373}K63R was constructed using standard cloning procedures as above. Briefly, Myc- SLK^{1-373}K63R was digested with BamH1 and HindIII, a 1.5 Kb fragment was extracted and subcloned into a pGEX-KG

Figure 2-2. Illustration of the PCR-based approach used to generate SLK point mutants. Schematic representation of the approach used to generate Myc-SLKS340A (panel A). List and description of the constructs and primers used to generate the remaining mutants (panel B).

A



B

CONSTRUCTS	DESCRIPTION	PRIMERS SEQUENCE (5'→3')
Myc-SLK SS347/348AA	Mutation of the serine (S) residues at position 347/348 to alanine (A).	Forward: GAC CTC AGC ATT GCC GCC GCT GAA GAA GAT AAA CT Reverse: AG TTT ATC TTC TTC AGC GGC GGC AAT GCT GAG GTC
Myc-SLKS362A	Mutation of the serine (S) residue at position 362 to an alanine (A).	Forward: GCT TGT ATT TTG GAA GCT GTG TCA GAA AGA ACA Reverse: TGT TCT TTC TGA CAC AGC TTC CAA AAT ACA AGC
Myc-SLKS364A	Mutation of the serine (S) residue at position 364 to an alanine (A).	Forward: TGT ATT TTG GAA TCT GTG GCA GAA AGA ACA GAA G Reverse: C TTC TGT TCT TTC TGC CAC AGA TTC CAA AAT ACA

expression vector (Invitrogen) generating Gst-SLK$^{1-373}$K63R. To construct Gst-SLK$^{1-373}$K63R SS347/348AA and Gst-SLK$^{1-373}$K63R S362A, a Myc-SLK SS347/8AA and Myc-SLK S362A fragment was subcloned into Gst-SLK$^{1-373}$K63R.

2.2.7 Transfections

49F fibroblasts stably expressing v-src the temperature sensitive TsLA29 v-src were generated using LipofectAMINE PLUS Reagent as per the manufacturer's instructions (Invitrogen). 2 x 10^6 cells were plated onto 60 mm dishes the day before the transfection. The next day, 5 μg of plasmid DNA was diluted in 237 μl of serum-free DME medium in a 15 ml polystyrene tube, followed by the addition of 8 μl of the PLUS Reagent. In a second tube, 12 μl of lipofectAMINE was diluted in 238 μl of serum-free DME medium. The cells were washed once and the transfection mixture was added to the dishes for 4-6 h at 37°C followed by the addition of 2.5 ml of complete DME medium. V-src transformed 49F cells were generated by puromycine (2μg/ml, Sigma) selection for two weeks. Cells stably expressing fpgv-1 or TsLA29 v-src were obtained by G418 (0.4mg/ml, Life Technologies) selection for two weeks. Prior to SLK kinase assays, cells stably expressing fpgv-1 or TsLA29 v-src (maintained at 37°C) were shifted to 41°C for 24-48 h.

HEK293 were transfected using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instruction. Briefly, the day before the

transfection, 2 x 10^6 cells were plated onto 60 mm dishes in a total volume of 4 ml complete DME medium and transfected as above.

2.3 Fibronectin Replating Assays

Subconfluent cultures were serum-starved in 0.25% FBS-DME medium for 24 h and harvested by trypsin-EDTA treatment as described previously (Sieg et al. 1999). The trypsin was inactivated using soybean trypsin inhibitor (0.5 mg/ml, Sigma) and the cells were collected by centrifugation and resuspended in 0.1% bovine serum albumin-DME (BSA-DME) medium. After 1 h at 37°C in suspension, the cells were plated onto FN or PL pre-coated coverslips. The coverslips were pre-coated with FN (10 μg/ml, Sigma) or PL (10 μg/ml, Sigma) in PBS overnight at 4°C, rinsed with PBS, and warmed to 37°C for 1 h prior to replating.

2.4 Wound Healing Assays

Exponentially growing cells were plated onto FN- or BSA-coated coverslips in complete growth medium and incubated overnight at 37°C. The next day, the monolayer of cells was washed twice with PBS and once with 0.1% BSA-DME medium. The wound healing assay was initiated by manually scratching the monolayer of cells with a pipette tip and allowing migration in 0.1% BSA-DME medium at 37°C for 6 h.

2.5 Migration Assays

The Millicell Boyden chambers from Millipore were used in a modified migration assay as described previously (Sieg et al. 1998). The lower side of the polycarbonate membranes of the haptotaxis chambers were pre-coated with BSA (1% (w/v)) or FN (10 μg/ml) overnight at 4°C, rinsed with PBS, and warmed to 37°C for 1 h prior to the initiation of migration. Serum-starved cells collected and resuspended in 0.1% BSA-DME medium were added to the upper chamber to initiate cell migration. Following migration, 4 h at 37°C, both chambers were rinsed with PBS and the lower side of the membrane was fixed in 4% paraformaldehyde (PFA) and stained with DAPI. The remaining cells in the upper chamber were removed using a cotton tip applicator. The number of migrated cells was determined by counting the DAPI stained cells (cells/field using a X20 objective) on the underside of the membrane.

2.6 Immunofluorescence

Coverslips were fixed in 4% PFA for 10 min at room temperature, washed in PBS, and blocked with 1% BSA or 200 μg/ml ChromaPure Goat IgG (Jackson Immunoresearch Laboratories). SLK was detected using an anti-SLK rabbit polyclonal antibody (Sabourin and Rudnicki 1999) in conjunction with fluorescein isothiocyanate (FITC)-labeled secondary antibodies. Vinculin and tubulin proteins were detected with a mouse anti-vinculin monoclonal antibody (clone VIN11–5, Sigma) and an anti-α-tubulin monoclonal antibody (clone DM1, Sigma) respectively in conjunction with tetramethyl rhodamine isothio-cyanate (TRITC)-labeled secondary antibodies. TRITC-conjugated phalloidin was used to detect

actin stress fibers. Samples were visualized on a Zeiss Axioskop100 epifluorescence microscope equipped with appropriate filters and photographed with a digital camera (Sony Corporation HBO50) using the Northern Eclipse software package. To quantitatively measure the localization of SLK to membrane lamellipodia in the SYF cultures, cells that did not display any SLK/vinculin co-localization were scored as negative. The results were graphed as "Colocalized" or "Not colocalized" as a percentage of the total number of cells counted.

2.7 Immunoprecipitations And Western Blots

Cells were rinsed in PBS and protein extracts were made in modified RIPA buffer containing 50mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1% (v/v) Nonidet P-40, and protease inhibitors (Sigma inhibitor cocktails). 400 μg of total cell lysate was immunoprecipitated using 2 μg of anti-FAK rabbit polyclonal antibody (clone 556368, BD Pharmingen) or anti-paxillin mouse monoclonal antibody (clone 610051, BD Biosciences) and 20 μl of protein A-Sepharose (Amersham Biosciences) for 4 h at 4°C. Immunoprecipitates were washed three times with NETN (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, and 0.1% (v/v) Nonidet P-40) and eluted with 4X SDS sample buffer (containing 200 mM Tris-HCl pH6.8; 400 mM DTT; 8% (w/v) SDS; 0.4% (w/v) bromophenol blue and 40% (v/v) glycerol). Samples were fractionated on 8-12% SDS-PAGE and transferred to polyvinylidene difluoride membranes (PVDF). PVDF membranes

were then probed with anti-p130CAS monoclonal antibody (P27820, Transduction Laboratories), anti-c-src monoclonal antibody (Sigma) or anti-phospho-tyrosine monoclonal antibody (Cell Signaling). Also, 40 μg of total cell lysates were subjected to Western blot with rabbit polyclonal anti-FAK phosphospecific antibodies for phospho-tyrosine residues 397 (pY^{397}) and anti-c-src phospho-tyrosine 416 specific antibody (c-src pY^{416}; Upstate Biotechnology). To evaluate the efficiency of the immunoprecipitation, PVDF membranes were stripped using Re-Blot Plus (Chemicon International) and re-probed with anti-FAK or anti-paxillin antibodies accordingly. Reactive proteins were detected by enhanced chemiluminescence (PerkinElmer Life Sciences) using a goat anti-rabbit or anti-mouse horseradish peroxidase-labeled secondary antibody and visualized using autoradiography.

2.8 In Vitro Kinase Assays

2.8.1 SLK Kinase Assays

400 μg of total cell lysate was immunoprecipitated using 2 μg of anti-SLK or 9E10 antibodies and 20 μl of protein A-Sepharose (Amersham Biosciences) for 4h at 4°C. Immunoprecipitates were washed three times with NETN and once with SLK kinase buffer (20 mM Tris-HCl (pH 7.5), 15 mM MgCl2, 10 mM NaF, 10 mM β-glycerophosphate, and 1 mM orthovanadate). Reactions (20 μl in kinase buffer) were initiated by the addition of 5 μCi of [γ-^{32}P] ATP. After a 30 min incubation at 30°C, reactions were terminated by the addition of 4X SDS sample buffer and 20 μl aliquots were fractionated by 8% SDS-PAGE. Gels were

transferred to PVDF membranes and exposed to x-ray film. PVDF membranes were then probed for SLK to evaluate the efficiency of the immunoprecipitation. For expression analysis, 40 μg of total cell lysates was subjected to Western blot with the appropriate antibodies. Anti-SLK rabbit polyclonal antibodies (Sabourin and Rudnicki 1999) were used to detect SLK, 12CA5 mouse monoclonal antibodies for HA-tagged proteins or 9E10 mouse monoclonal antibodies for Myc-tagged proteins.

2.8.2 Endogenous CK2 Kinase Assays

400 μg of total cell lysate was immunoprecipitated using 2 μg of anti-CK2α goat polyclonal antibodies (clone C-18, Santa Cruz Biotechnology) and 20 μl of protein A-Sepharose (Amersham Biosciences) for 4h at 4°C. Immunoprecipitates were washed three times with NETN and once with modified CK2 kinase buffer (50 mM Tris-HCl (pH 7.5), 10 mM MnCl2, 10 mM NaF, 10 mM β-glycerophosphate and 1 mM orthovanadate) (Donella-Deana et al. 2003). Reactions (20 μl in kinase buffer) were initiated by the addition of 3 μg of dephosphorylated casein (C4032, Sigma) and 5 μCi of [γ-^{32}P] ATP. After a 30 min incubation at 37°C, reactions were terminated by the addition of 4X SDS sample buffer and 20 μl aliquots were fractionated by a gradient gel 8-12% SDS-PAGE. Gels were transferred to PVDF membranes, ponceau stained then exposed to x-ray film. 40 μg of total cell lysates was subjected to Western blot with anti-CK2α goat polyclonal antibodies (C-18, Santa Cruz Biotechnology) to detect endogenous CK2α proteins.

2.8.3 Recombinant CK2 Kinase Assays

E. coli strain BL21 transformed with Gst-SLK$^{1-373}$K63R, Gst-SLK$^{1-373}$K63R SS347/8AA or Gst-SLK$^{1-373}$K63R S362A were grown overnight in 5 ml of LB containing 50 μg/ml of ampicillin at 37°C in a shaker at 250 rpm. The next day, the 5 ml cultures, representing a dilution of 1:10, were transferred into a flask containing 45 ml of LB supplemented with ampicillin and re-incubated at 37°C. After 1 h incubation, 50 μl of 1 M IPTG was added and protein expression was allowed for 2 h at 37°C with shaking. The expression of the Gst fusion constructs in the pGEX-KG vector is under the control of an IPTG-inducible *tac* promoter. The bacterial cultures were then transferred to 50 ml conical tubes and spun down for 5 min at 4000 x g. The bacterial pellet was re-suspended in modified RIPA buffer containing protease inhibitors as described above. The lysates were transferred into 1.5 ml eppendorff tubes, sonicated for 10 sec, incubated on ice for 15 min and spun down at 13,000 x g for 10 min. The Gst fusion proteins were purified from the supernatant using glutathione sepharose bead as described (Pharmacia) for 1 h at 4°C. The beads were washed three times with NETN and once with CK2 kinase buffer. Reactions (20 μl in kinase buffer) were initiated by the addition of 2 μl recombinant CK2 (rCK2) from rat liver (C3460, Sigma) and 5 μCi of [γ-^{32}P] ATP. After a 30 min incubation at 37°C, beads were washed seven times with NETN to remove any traces of rCK2 and once with PBS.

To cleave the Gst peptide of the expressed proteins, beads were incubated in 20 μl PBS containing 5 u/μl of thrombin protease (Amersham Biosciences) overnight at room temperature. The digestion was terminated by

the addition of 4X SDS sample buffer, boiled for 3 min and fractionated on 8% SDS-PAGE. The gels were then stained with coomassie blue, dried and exposed to x-ray film.

For the tryptic digest, beads were incubated in 50 µl of 50 mM NH4HCO3 with 10 u/µl of trypsin from bovine pancreas (T8802, Sigma) overnight at 37°C followed by an addition of another 10 µl of 1 u/µl of trypsin for 4 h at 37°C the next day. The reaction was terminated by adding 4X SDS sample buffer, boiled for 3 min and fractionated on a 20% Tricine-SDS-PAGE. Aliquots of 30 µl were collected initially to show expression of the Gst fusion proteins.

2.9 Phosphoamino Acids Analysis

2.9.1 Orthophosphate Cell Labeling And Acid Hydrolysis

On the day of the assay, exponentially growing cells were washed twice and incubated in phosphate-free DME medium (Gibco-BRL) at 37°C in a humidified atmosphere containing 5% CO2 for 2 h, [^{32}P] orthophosphate (250 µCi/ml) was then added for an additional 4 h. After the labeling period, cells were washed three times with TBS (pre-incubated at 4°C) and lysed using modified RIPA buffer with protease inhibitors as above. Equal amounts of cell lysates was used to immunoprecipitate endogenous SLK using 2 µg of anti-SLK antibodies overnight at 4°C. Immunoprecipitates were then washed three times with NETN, once with TBS and eluted off the beads with 50 µl of re-distilled 5.7 M HCl. The eluted immunoprecipitate were then transferred to a new tube.

Acid hydrolysis was accomplished by capping the tubes with snap-cap clamp and incubating at 110°C for 1 h. Following hydrolysis, 100 µl stddH2O was added and samples were vacuum dried at 65°C. The last step was repeated twice using decreasing amounts of stddH2O, 50 and 10 µl to concentrate the sample at the bottom of the tube. The pellet was then dissolved in 2 µl of pH1.9 buffer containing, 1 mg/ml of xylene cyanol, phospho-serine (PS), phospho-threonine (PT) and phospho-tyrosine (PY) standards (Sigma).

2.9.2 Thin Layer Electrophoresis

Hydrolyzed SLK immunoprecipitates were separated by two dimensions thin layer chromatography (TLC) (100 µm cellulose on polyester; Sigma). Samples were spotted using a micropipetter with frequent cold air stream drying. The first dimension electrophoresis was performed by wetting the TLC plate with pH1.9 buffer (50 ml of 88% formic acid, 156 ml of glacial acetic acid and 1794 ml of ddH2O) (Hardie 1999). The plate was then assembled in the electrophoresis apparatus and a 1.5 kV current was applied for 15 min. The second dimension electrophoresis was performed using pH3.5 buffer (10 ml of pyridine, 100 ml of glacial acetic acid and 1890 ml of ddH2O) (Hardie 1999). The pH3.5 buffer wetted TLC plate was rotated 90° clockwise before assembly in the electrophoresis apparatus and a 1.5 kV current was applied for 15 min. Visualization of PS, PT and PY standards was achieved by dipping the TLC plate into a 0.2 % (w/v) ninhydrin in acetone and baking at 65 °C until purple spots

appears. The colored spots representing the PS, PT and PY were marked and the plate was then exposed to x-ray film.

CHAPTER 3

DELAYED FOCAL COMPLEX TURNOVER AND IMPAIRED RECRUITMENT OF SLK TO MEMBRANE RUFFLES AND LAMELLIPODIA IN PEA3-NULL FIBROBLASTS

3.1 Introduction

PEA3 is the founding member of a subfamily of ETS transcription factors that are involved in the transcriptional regulation of membrane receptors, growth factors, transcription factors, and extracellular matrix metalloproteinases (Higashino, et al. 1993, Xin, et al. 1992). PEA3 proteins have been shown to be overexpressed in the vast majority of human breast and ovarian tumors and in nearly all of the human epidermal growth factor receptor 2 (HER2)/Neu/erbB2-positive subclass of breast tumors (Benz, et al. 1997, Hynes and Stern 1994, Singleton and Strickler 1992). Furthermore, null mutations in the *pea3* allele compromise the capacity of mammary tumors to metastasize in the mouse mammary tumor virus (MMTV)-Neu/HER2 transgenic mice (Kurpios, et al. 2003, Shepherd and Hassell 2001). In contrast, forced expression of PEA3 in MCF7 human breast tumor cells increased their metastatic potential (Kaya, et al. 1996), whereas PEA3 antisense RNA reduced the invasiveness of human tumor cells (Hida, et al. 1997). Interestingly, c-src was shown to be overexpressed and activated in late-stage human ovarian cancer and that src overexpression was frequently associated with HER2 overexpression (Weiner et al. 2003). Moreover, Vadlamudi *et al.* showed that the activation of HER2 results in the phosphorylation of c-src on Tyr-215 resulting in an increase in c-src kinase activity in MCF7 human breast cancer cells (Vadlamudi et al. 2003).

Cell adhesion and migration involves a dynamic assembly/disassembly of adhesion components mediated by the actin and microtubule networks. Fibronectin-stimulated cell adhesion and migration was shown to induce the

formation of an integrin-FAK-c-src complex. This complex will then recruit a number of adaptor molecules such as p130Cas leading to focal adhesion assembly. Furthermore, c-src mediated tyrosine phosphorylation of p130Cas requires FAK as a scaffolding protein (Ruest et al. 2001). Interestingly, focal adhesion turnover appears to be regulated by c-src kinase activity. Indeed, FAK mutant deficient for c-src binding (Y397F) failed to induce focal adhesion disassembly in FAK-deficient fibroblasts (Ren, et al. 2000, Webb, et al. 2004). Furthermore, expression of kinase inactive v-src or the N-terminal domain of c-src in fibroblasts produced larger focal adhesions (Fincham and Frame 1998, Kaplan et al. 1994). Recently, calpain 2, a calcium dependent cysteine protease, was shown to cleave FAK into an N-terminal (95 kDa) and a C-terminal (30 kDa) fragments, releasing the kinase domain from the FAT domain upon v-src-mediated cell transformation (Carragher, et al. 2001). Furthermore, Carragher *et al* have shown that the FAK proline-rich sequence is required for calpain 2 recruitment to focal adhesion and maximal calpain activity (Carragher, et al. 2003).

We have previously shown that SLK can be colocalized with vinculin in membrane ruffles and that it associates with the microtubule network (Wagner, et al. 2002). Furthermore, SLK expression in fibroblasts induces actin stress fibers dissociation in a Rac1-dependent manner (Wagner, et al. 2002).

To gain further insight into the role of SLK in cell adhesion and migration, we investigated SLK cellular distribution and kinase activity in the PEA3(-/-) cells, known to exhibit migration deficiencies (J.A. Hassell personal communications).

However, the mechanisms responsible for this phenotype remain to be elucidated. Our results show that upon FN stimulation, SLK redistribution to the cell periphery is altered in the PEA3(-/-) cells, whereas SLK kinase activity remained unchanged. To further understand the molecular mechanisms involved in SLK redistribution to the cell periphery, we characterized the PEA3(-/-) cells for potential cell adhesion and migration defects upon FN stimulation. We found that PEA3(-/-) cells exhibit migration deficiencies and abnormal distribution of focal adhesions upon stimulation by FN. We further demonstrated that c-src kinase activity and FAK proteolytic cleavage are downregulated in the PEA3(-/-) cells, indicative of a focal adhesion turnover defect. Supporting this, we show that p130Cas remains associated with FAK and is not tyrosine phosphorylated during FN replating assays in the PEA3(-/-) cells. Together, our results show that a subset of PEA3 target proteins regulates c-src kinase activity, which is required for SLK re-distribution to the cell periphery and focal adhesion turnover.

3.2 Results

3.2.1 SLK Redistribution To The Cell Periphery Is Altered In The Pea3(-/-) Fibroblasts

Stimulation of fibroblasts by FN has been shown to induce the formation of adhesion signaling complexes (Schaller and Parsons 1994, Schlaepfer et al. 1999). We have previously shown that SLK can induce actin stress fibers disassembly and that it colocalizes with vinculin containing structures at the cell periphery upon stimulation with FN (Sabourin, et al. 2000, Wagner, et al. 2002).

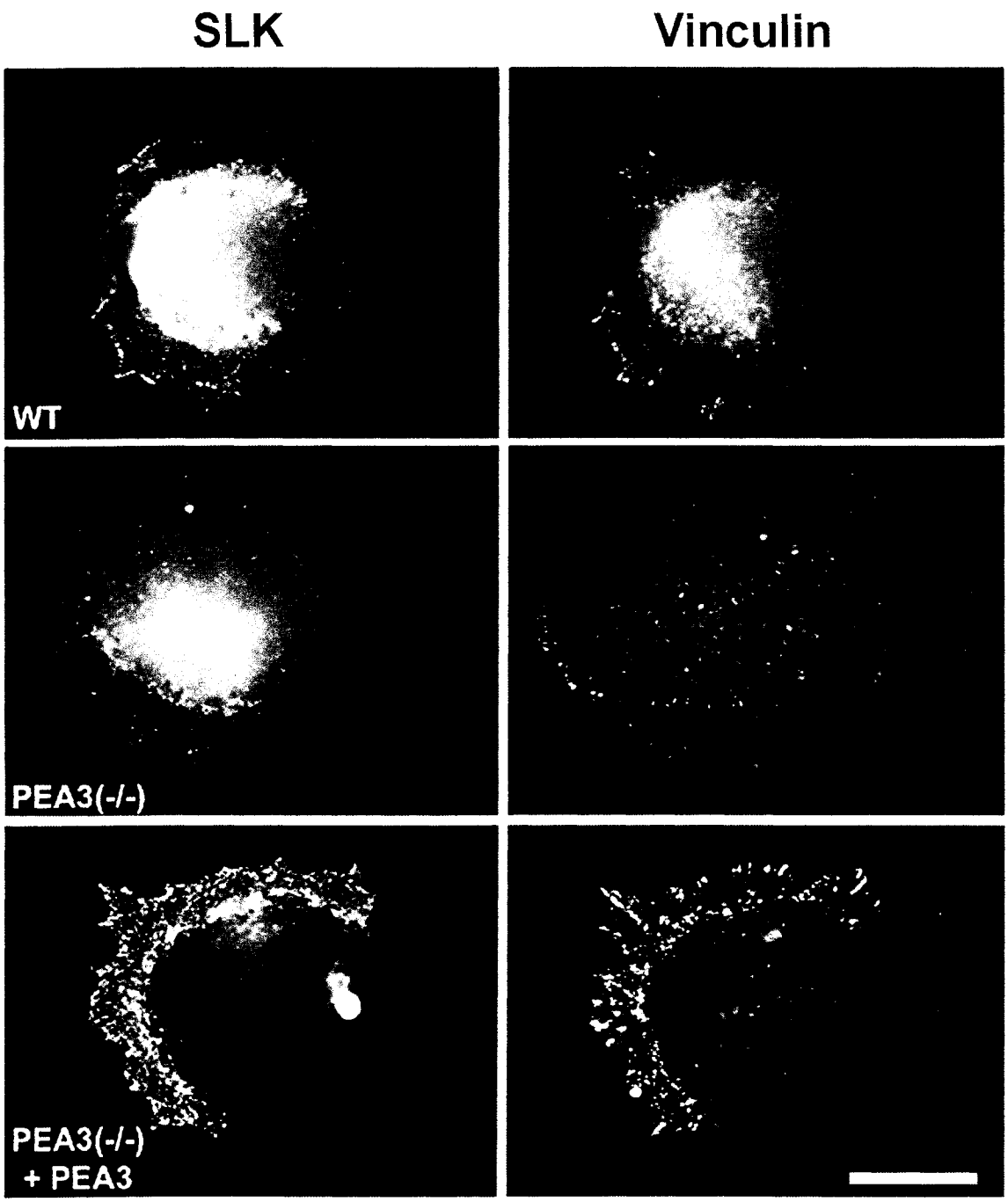
Interestingly, PEA3(-/-) cells were shown to exhibit migration deficiencies, however the mechanisms involved are not known (J.A. Hassell personal communications). Therefore, to gain further insights on the role of SLK in cell adhesion and migration, we have investigated SLK redistribution and kinase activity in PEA3(-/-) cells.

Cultures of PEA3(-/-), PEA3(-/-)+PEA3, where PEA3 cDNA was re-expressed into PEA3(-/-) cells, and wildtype control (WT) cells were held in suspension and replated onto FN-coated substrates followed by co-immunostaining for SLK and vinculin. As previously observed following replating, a fraction of SLK protein was found to be redistributed to the cell periphery and colocalized with the adhesion protein vinculin at the cell periphery in WT and PEA3(-/-)+PEA3 cells (Wagner, et al. 2002). Interestingly, SLK and vinculin colocalization at the cell periphery was absent in the PEA3(-/-) cells (Figure 3-1). Surprisingly, we observed no changes in SLK kinase activity (not shown). Taken together, these results suggest that PEA3 target proteins may be required for proper recruitment of signaling proteins to sites of actin remodeling.

3.2.2 PEA3(-/-) Cells Display Migration Deficiencies And A Polarized Distribution Of Adhesion Complexes

To characterize the migration deficiencies in PEA3-null cells, we first conducted a wound-healing assay onto FN-coated coverslips. We observed that the wildtype cells migrated more efficiently towards the wound than the PEA3(-/-) cells (Figure 3-2). Interestingly, PEA3(-/-) cells did not seem to form any filopodia

Figure 3-1. Altered distribution of SLK in the PEA3(-/-) cells. WT, PEA3(-/-) and PEA3(-/-)+PEA3 cells were stimulated onto FN matrix for 30 min, fixed and co-immunostained for SLK and vinculin proteins. Replating onto FN coated coverslips shows that SLK extends poorly to vinculin containing adhesion sites in the PEA3(-/-) cells. Re-expression of PEA3 re-establishes SLK/vinculin co-localization. Altered SLK redistribution to the cell periphery in the PEA3(-/-) cells was observed in 85 ± 7% of the cells (n=200). The cells were photographed at X630. Scale bar = 10 μm.


SLK
Vinculin
WT
PEA3(-/-)
PEA3(-/-)
+ PEA3

as WT cells (Figure 3-2 E & D), but consisted mainly of lamellipodia. To quantify the observed migratory defect, we performed a haptotaxis migration assay onto FN matrix. Three clones of PEA3(-/-) cells, PEA3(-/-)+PEA3 and WT control cells were subjected to migration assays in Boyden chambers. We observed that all three PEA3(-/-) clones displayed a 4-fold decrease in migration in comparison to WT or PEA3(-/-)+PEA3 cells (Figure 3-3). These results suggest that PEA3 target proteins are involved in some regulatory aspects of cell migration.

The replating of suspended cells onto FN matrix has been shown to induce tyrosine phosphorylation of adhesion components resulting in adhesion complex assembly and the subsequent actin polymerization from adhesion sites (Schaller and Parsons 1994, Schlaepfer, et al. 1999). To further characterize the PEA3(-/-) cells migration defect, we conducted FN-stimulated replating assays and immunostained for actin stress fibers and vinculin, an adhesion complex component, or phospho-tyrosine residues. We observed no major changes in the actin stress fiber network staining following a 30 min replating assay (Figure 3-4 A & B). However, the majority of the PEA3(-/-) cells displayed an enriched lamellipodium with the absence of filopodia structures supporting the wound healing assay observation (Figure 3-4 A & B and 3-2 E & D) Immunostaining for vinculin proteins, an adhesion complex marker, revealed that PEA3(-/-) cells displayed a very polarized focal adhesion complex distribution relative to WT (Figure 3-4 C & D). Supporting this, phospho-tyrosine immunostain also showed an altered distribution of focal adhesion complexes as for vinculin (Figure 3-4 E & F). These results suggest that the migration deficiencies of the PEA3(-/-)

Figure 3-2. PEA3(-/-) cells exhibit migration deficiencies during FN stimulated wound healing assay. Confluent cell layers on FN coated coverslips were scratched with a pipette tip (A & B) and allowed to close the wound for 6 h (C & D). The cells were then fixed and immunostained with phalloidin for actin stress fibers. We observed that Wt cells migrated towards the wound more efficiently than PEA3(-/-) cells. PEA3(-/-) cells form little or no filopodia or microspikes structures, but rather appear to display an enriched lamellipodia with actin bundles. The cells were photographed at X200.

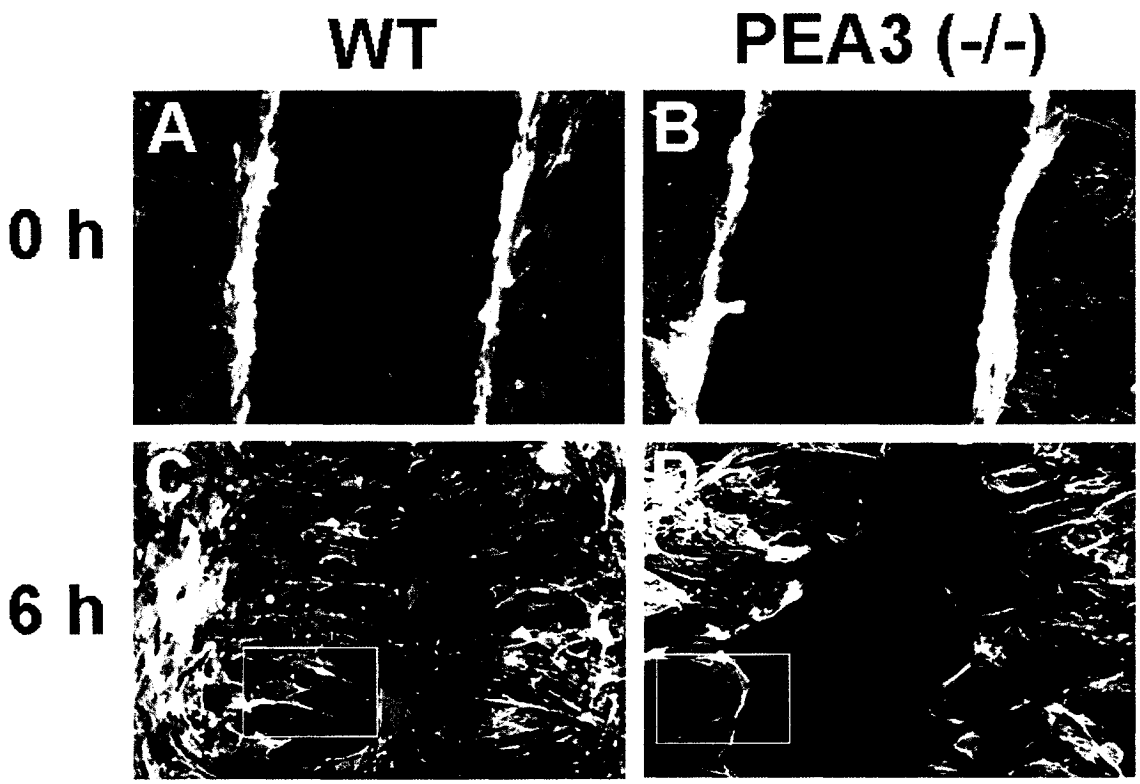

WT
PEA3 (-/-)
A
B
0 h
C
D
6 h

Figure 3-3. PEA3(-/-) cells also show migration deficiencies during FN stimulated haptotaxis migration assay. Wt, PEA3(-/-)-1, -2, -3 and PEA3(-/-)+PEA3 cells were incubated in a Boyden chamber coated with BSA or FN for 4h. Boyden chamber membranes were then peeled off, fixed and stained with DAPI to visualize the migrated cells (A, B & C). Quantification of the cell number positive for DAPI stain after 4h of stimulation onto FN coated membrane (D). The data shown represent averages ± standard errors for three independent experiments performed in triplicates.

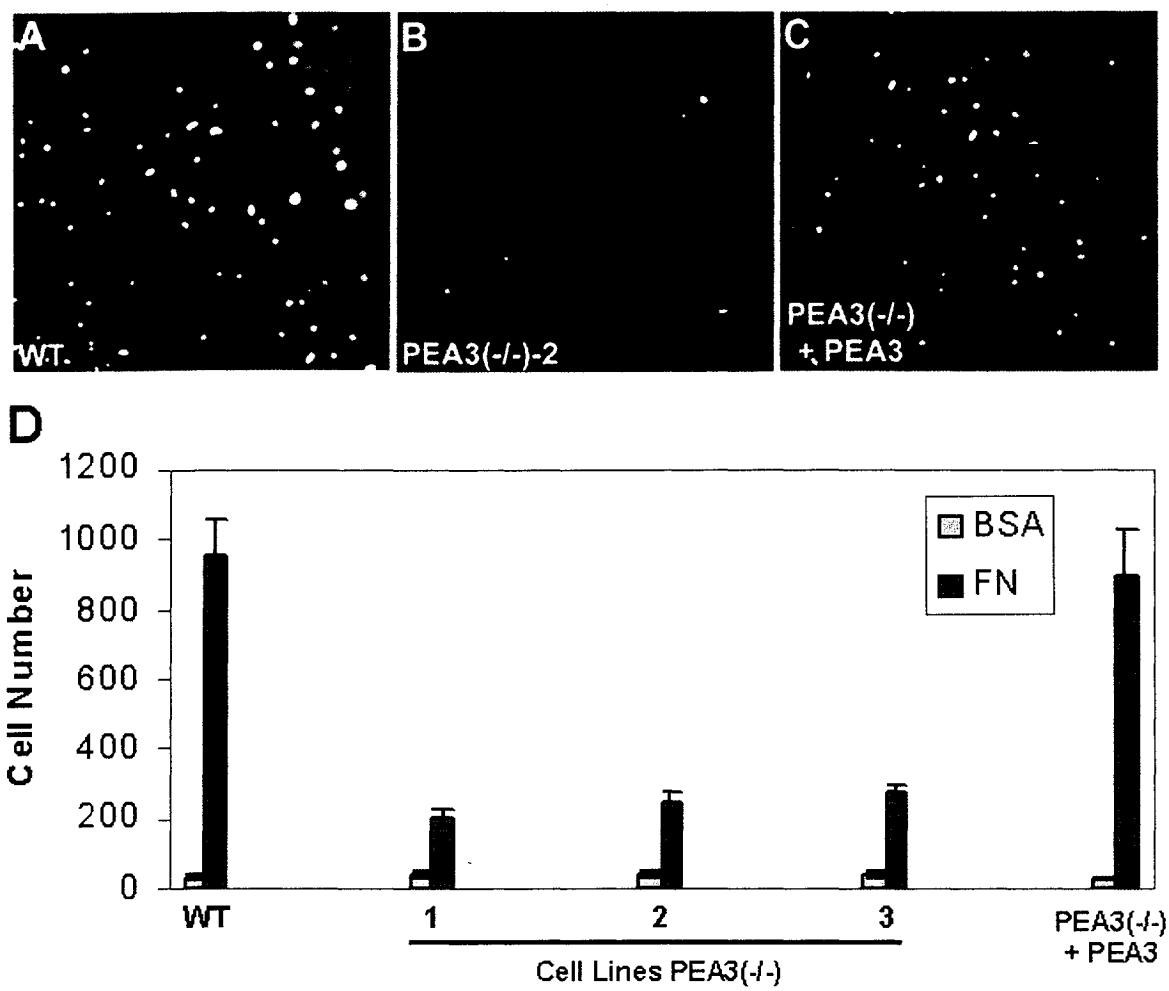
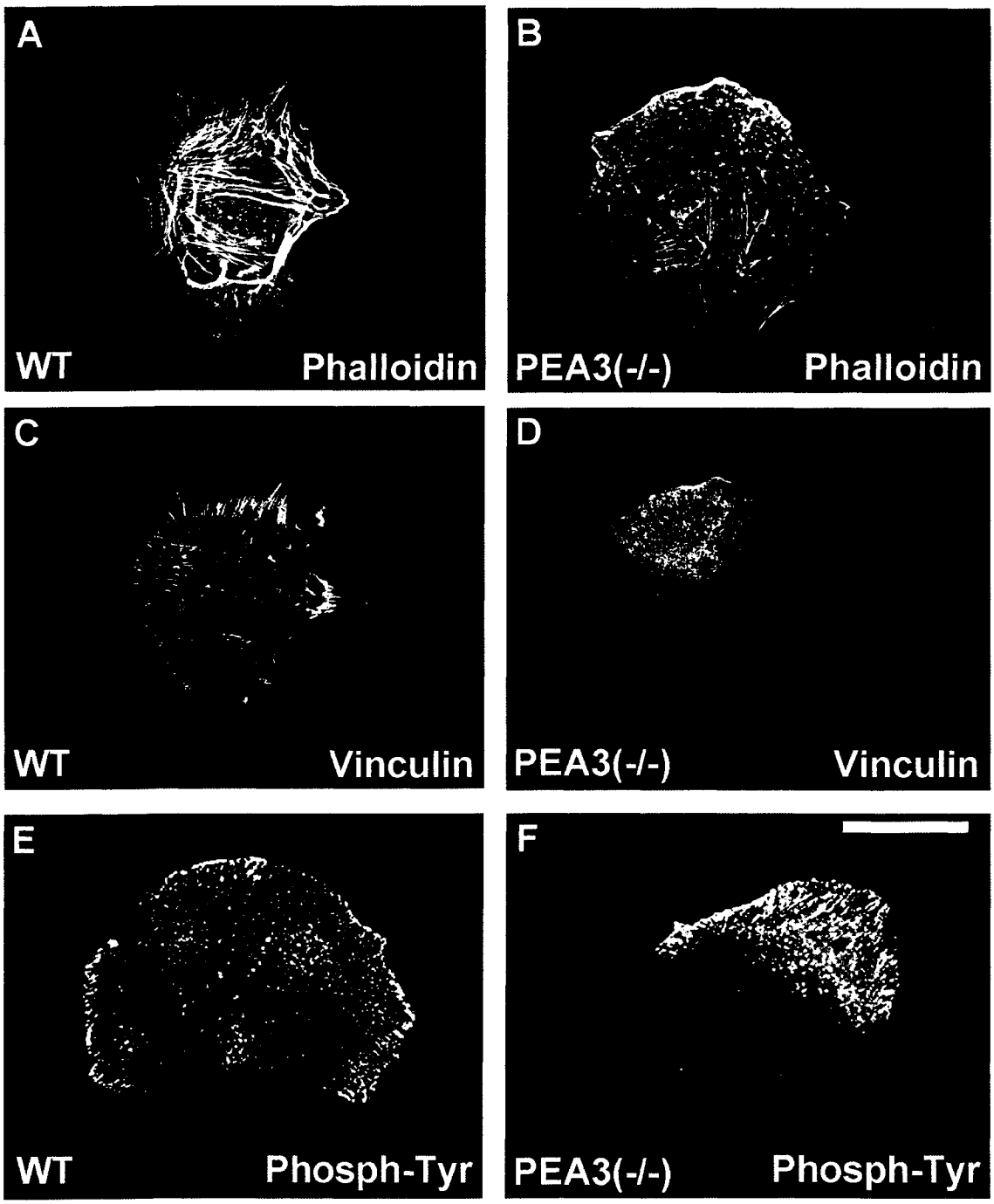

A
WT
B
PEA3(-/-)-2
C
PEA3(-/-)
+ PEA3
D
Cell Number
1200
1000
800
600
400
200
0
BSA
FN
WT
1
2
3
PEA3(-/-)
+ PEA3
Cell Lines PEA3(-/-)

Figure 3-4. PEA3(-/-) cells display a polarized distribution of focal adhesion complexes. Wt and PEA3(-/-) cells stimulated by replating onto FN matrix for 30 min were fixed and co-immunostained for actin stress fibers (phalloidin) and vinculin proteins (panels A to D) or phospho-Tyr (Panels E & F). No major alterations in the actin stress fibers were observed, however it appears that PEA3(-/-) cells exhibit a polarized, unidirectional, distribution of the focal adhesion complexes, as displayed by the vinculin stain (panels C & D) and phospho-tyrosine protein stain (4G10; panels E & F). The cells were photographed at X630. Scale bar = 10 μm.


A
WT
Phalloidin
B
PEA3(-/-)
Phalloidin
C
WT
Vinculin
D
PEA3(-/-)
Vinculin
E
WT
Phosph-Tyr
F
PEA3(-/-)
Phosph-Tyr

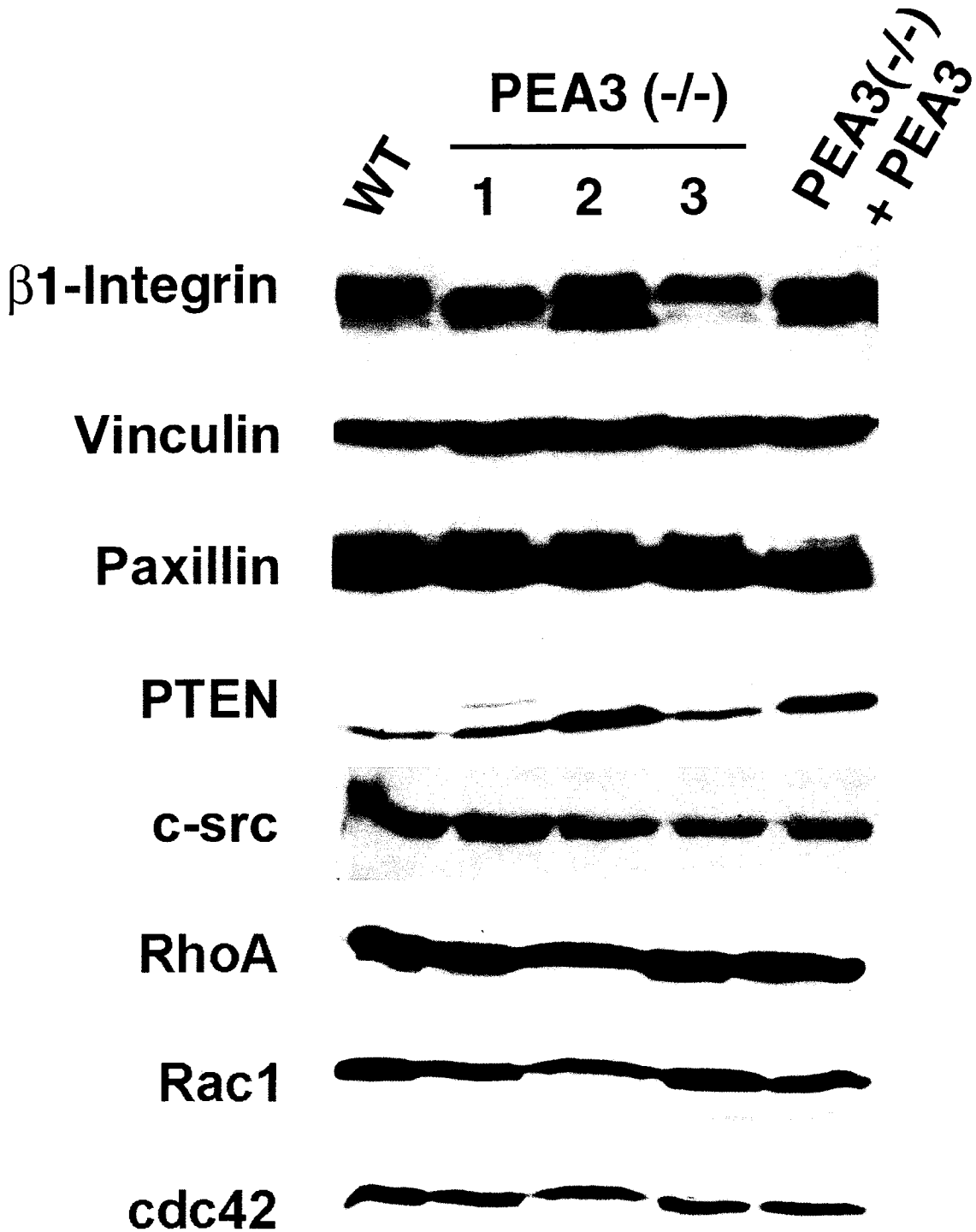
may result from an actin assembly phenotype due to an altered spatial distribution of adhesion complex molecules.

3.2.3 Failure To Activate C-src Leads To Impaired Focal Adhesion Disassembly In PEA3(-/-) Cells

To further investigate the migration defect in PEA3(-/-) cells, we first examined the expression level of key adhesion complex components. Cell lysate collected from three clones of PEA3(-/-) cells, PEA3(-/-)+PEA3 and WT control were resolved on SDS-PAGE, transferred onto PVDF membrane and immunoblotted for β-integrin, vinculin, paxillin, PTEN, c-src, RhoA, Rac1 and cdc42 proteins (Figure 3-5). Our results show that no major differences were observed in the different cell lines.

FN stimulation of fibroblasts has been shown to trigger the activation of FAK, the recruitment and activation of c-src and the formation of adhesion signaling complexes mediating actin rearrangements (Schaller and Parsons 1994, Schlaepfer, et al. 1999). To further test for potential defects at the focal adhesion levels, we conducted replating assay on FN matrix. To assay for FAK activation, we immunoblotted for FAK phospho-Tyr397 (pY397), an autophosphorylation event indicative of FAK activation. Western blot analysis shows that FAK pY397 is transiently detected during the replating assay in WT cells with a low level detected in cells held in suspension for 1 h (S), as previously reported (Schaller and Parsons 1994). Unlike WT cells, high levels of FAK pY397 were detected in all PEA3(-/-) samples throughout the replating time

Figure 3-5. The protein expression level of focal adhesion components is not deregulated in the PEA3(-/-) cells. Equal amounts of WT, PEA3(-/-)-1, -2, -3 and PEA3(-/-)+PEA3 cell lysate were resolved by SDS-PAGE. Proteins are then transferred onto PVDF membrane and immunoblotted for the indicated proteins. No major changes in the protein expression levels were observed.

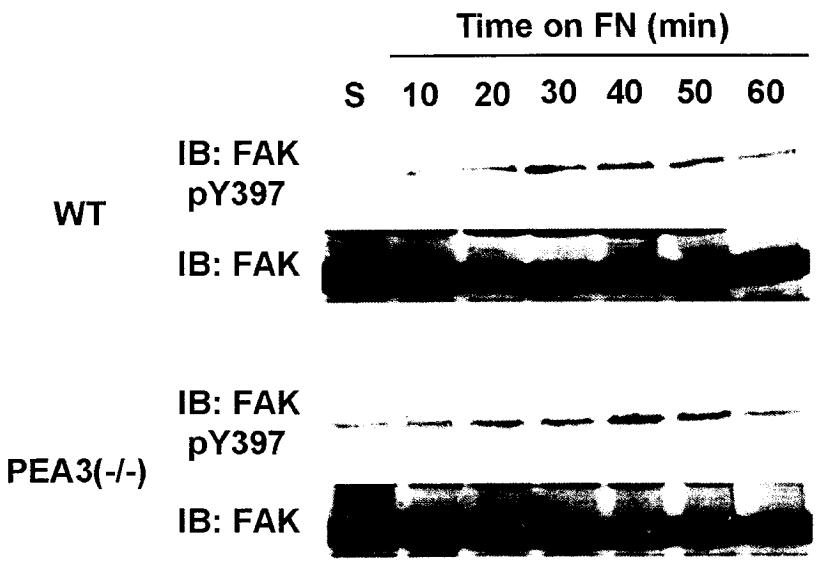

WT
PEA3 (-/-)
1
2
3
PEA3(-/-) + PEA3
β1-Integrin
Vinculin
Paxillin
PTEN
c-src
RhoA
Rac1
cdc42

course, even in cells held in suspension for 1 h (S) (Figure 3-6 A). We then tested for c-src activation following FN replating assays by immunoblotting for c-src phospho-Tyr416 (pY416). Phosphorylation of c-src at Y416 is required for c-src maximal activation (Johnson, et al. 1996). Delayed and reduced c-src pY416 in PEA3(-/-) cells was observed, whereas it was readily detectable in WT and PEA3(-/-)+PEA3 cells (Figure 3-6 B). No differences were observed in the phosphorylation of Y527, involved in c-src inactivation (not shown). These results strongly suggest that PEA3(-/-) cells fail to dephosphorylate FAK Y397 and to fully activate c-src.

Previous studies have showed that v-src-mediated cell transformation leads to FAK cleavage by calpain 2, releasing a 95 kDa N-terminal and a 30 kDa C-terminal fragment, suggesting that adhesion site turnover involves FAK cleavage (Carragher, et al. 2001). Therefore, to investigate whether PEA3(-/-) cells are defective in adhesion site turnover, we conducted a replating assay onto FN matrix and immunoblotted for FAK N- and C-terminals. FN-stimulated replating assays revealed that both WT and PEA3(-/-)+PEA3 cells displayed a transient FAK cleavage that is highlighted by the presence of both 95 and 30 kDa FAK fragments (Figure 3-7). More specifically, maximal FAK cleavage was observed when cells were held in suspension (S). Interestingly, the levels of FAK cleavage products were reduced in the PEA3(-/-) cell lysates, including cells in suspension (S) (Figure 3-7). Taken together, these results suggest that PEA3 target proteins may regulate c-src activation, thus controlling focal adhesion turnover and migration.

Figure 3-6. Dysregulation in FAK and c-src activation in the PEA3(-/-) cells. Time course replating assay on FN was performed and equal amounts of cell lysate were resolved by SDS-PAGE. Proteins are then transferred onto PVDF membrane and immunoblotted for FAK phospho-Tyr397 (FAK pY397), stripped and re-blotted for FAK proteins (A). We observed that FAK pY397 is detected all across the time course of the replating assay in the PEA3(-/-) cells, even after I h in suspension (S). Similarly, cell lysate from time course FN replating assay or 30 min stimulation on Poly-L-lysine (PL) were resolved by SDS-PAGE. PVDF membranes were then immunoblotted for c-src phospho-Tyr416 (c-src pY416), stripped and re-blotted for total c-src (B). Minimal signal of c-src pY416 was detected in the PEA3(-/-) cells unlike the Wt control or the PEA3(-/-)+PEA3 cells.

A


Time on FN (min)
S
10
20
30
40
50
60
WT
IB: FAK pY397
IB: FAK
PEA3(-/-)
IB: FAK pY397
IB: FAK

B

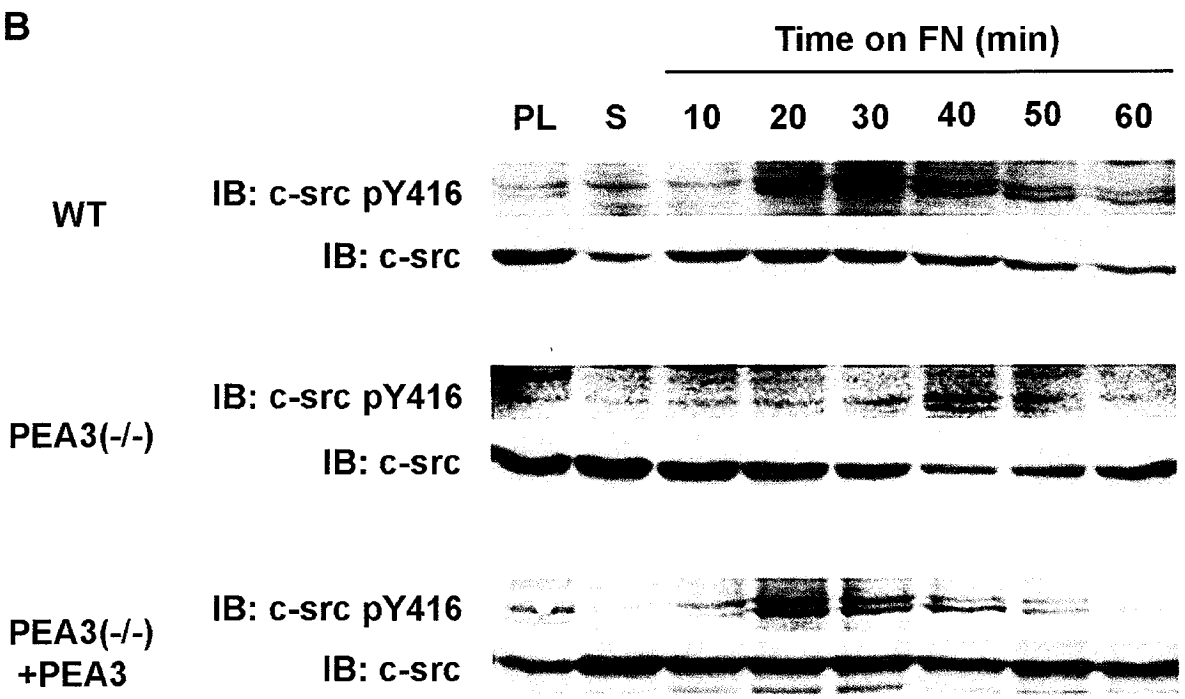
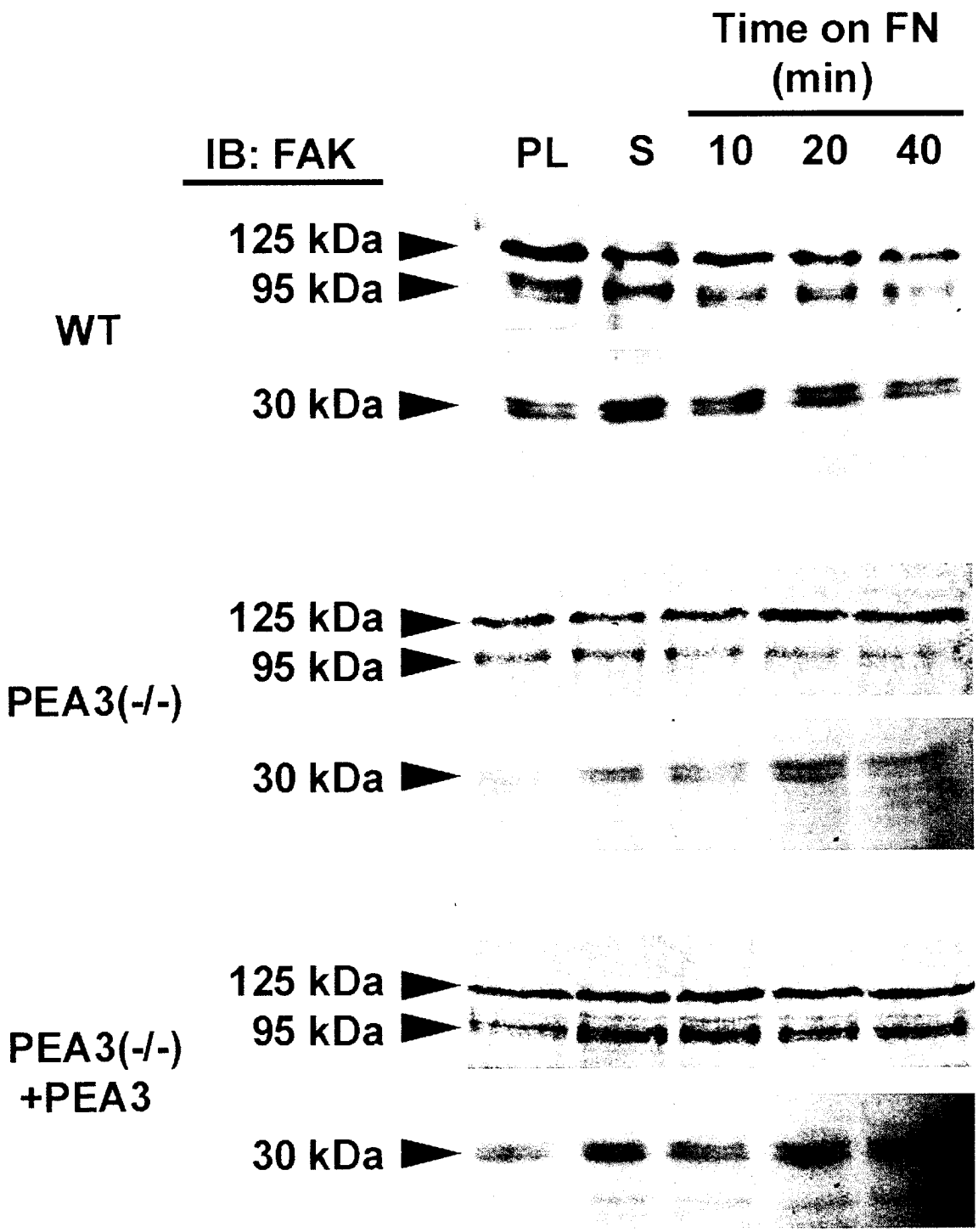

Time on FN (min)
PL
S
10
20
30
40
50
60
WT
IB: c-src pY416
IB: c-src
PEA3(-/-)
IB: c-src pY416
IB: c-src
PEA3(-/-) +PEA3
IB: c-src pY416
IB: c-src

Figure 3-7. Impairment of FAK cleavage during FN stimulated replating assay in the PEA3(-/-) cells. Time course replating assay on FN was performed and equal amounts of cell lysate were resolved by SDS-PAGE. Proteins are then transferred onto PVDF membrane and immunoblotted for FAK N-terminal, to detect a 125 and a 95 kDa fragment, and C-terminal to detect a 30 kDa fragment. We observed that FAK cleavage products, 95 and 30 kDa, are transiently detected during the replating assay for both Wt and PEA3(-/-)+PEA3. We also observed that the strongest FAK cleavage signal was when cells were held in suspension for 1 h (S). Minimal signal of FAK 95 and 30 kDa fragments were detected in PEA3(-/-) cells, even when cells were held in suspension (S).


Time on FN
(min)
IB: FAK
PL
S
10
20
40
125 kDa
95 kDa
WT
30 kDa
125 kDa
95 kDa
PEA3(-/-)
30 kDa
125 kDa
95 kDa
PEA3(-/-)
+PEA3
30 kDa

3.2.4 p130Cas Tyrosine Phosphorylation Is Impaired In The PEA3(-/-) Cells

p130Cas, a scaffolding adhesion protein, has been shown to bind the FAK proline-rich domain resulting in its tyrosine phosphorylation by both FAK and c-src (Astier and al. 1997, Cary et al. 1998, Polte and Hanks 1997, Polte and Hanks 1995, Tachibana and al. 1997). Furthermore, studies have shown that p130Cas is tyrosine phosphorylated following FN stimulation of cells in a FAK- and c-src-dependent manner (Schlaepfer et al. 1997, Vuori et al. 1996). Interestingly, c-src-mediated phosphorylation of p130Cas has been shown to induce adhesion turnover and to regulate cell migration (Cary, et al. 1998). To further confirm that the impairment of focal adhesion turnover is due to c-src inactivation, we immunoprecipitated paxillin proteins from equal amount of cell lysates following a replating assays onto FN matrix and immunoblotted for p130Cas, c-src and phospho-Tyr (p-Tyr). Our results show that p130Cas tyrosine phosphorylation (p130Cas/p-Tyr) level is minimal relative to WT and PEA3(-/-)+PEA3 cells (Figure 3-8). Furthermore, we observed that p130Cas did not co-immunoprecipitate with paxillin when cells were replated onto PL matrix in WT and PEA3(-/-)+PEA3 cells unlike the PEA3(-/-) (Figure 3-8), suggesting that disassembly of adhesion complexes is impaired in PEA3(-/-) cells. We next immunoprecipitated FAK proteins from FN stimulated replating assay lysate and immunoblotted for p130Cas. We observed that p130Cas remains associated with FAK for longer periods on FN and when cells were replated onto PL matrix, similar to the results obtained for paxillin immunoprecipitation (Figure 3-9). Taken together, these results further suggest that PEA3(-/-) cells exhibit

Figure 3-8. p130Cas tyrosine phosphorylation is diminished in the PEA3(-/-) cells. Time course FN stimulation was performed and paxillin was immunoprecipitated from equal amounts of cell lysates. Samples were resolved on SDS-PAGE and PVDF membranes are then immunoblotted for phosphorylated tyrosine (p-Tyr) residues, stripped and re-blotted for p130Cas, c-src and paxillin proteins. We observed that p130Cas is detected all across the time course of the replating assay in the PEA3(-/-) cells, even upon replating onto poly-L-lysine (PL) matrix. In addition, p130Cas tyrosine phosphorylation (p130Cas/p-Tyr) level was minimal in comparison with WT or PEA3(-/-)+PEA3. There were no major changes in c-src level.

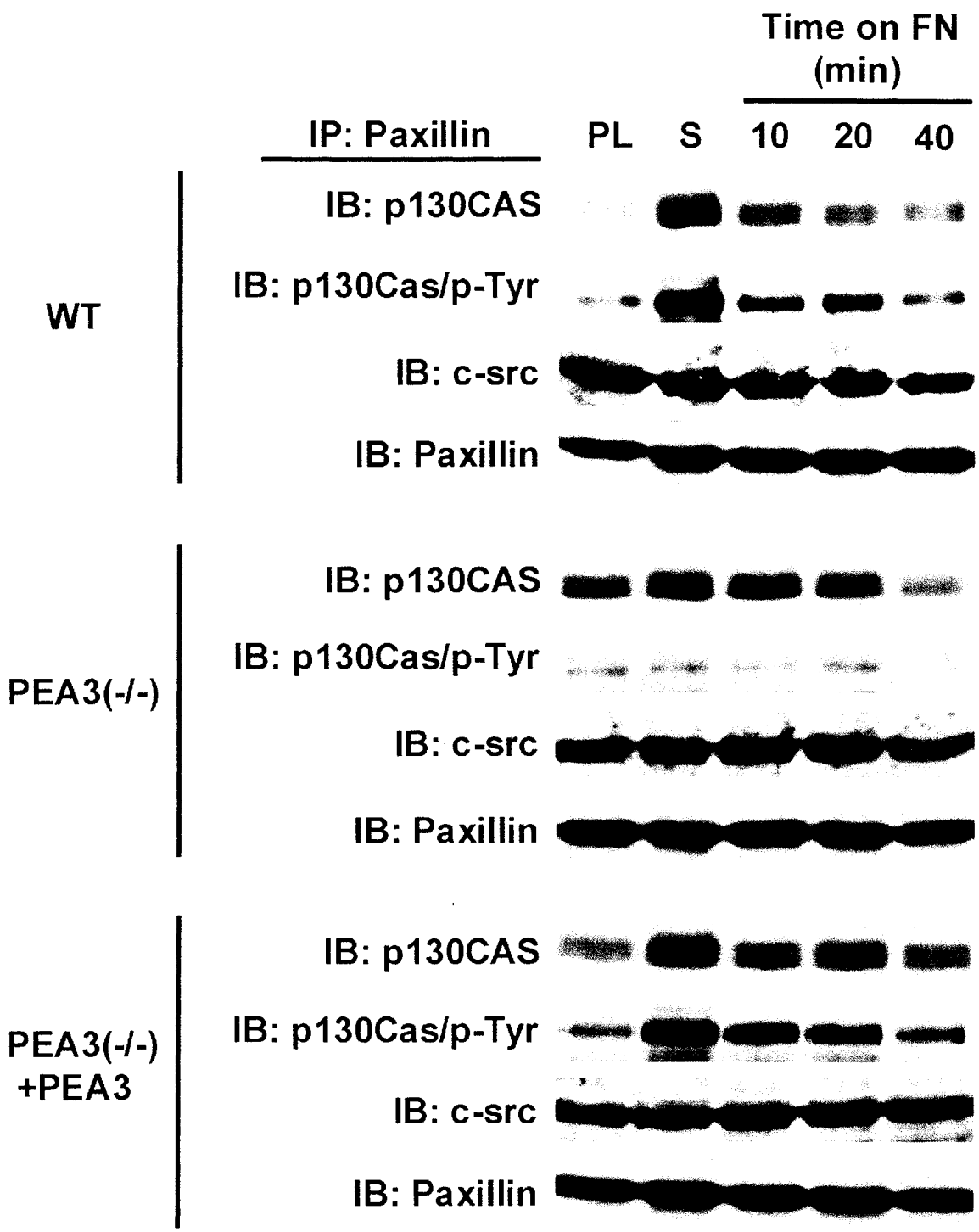
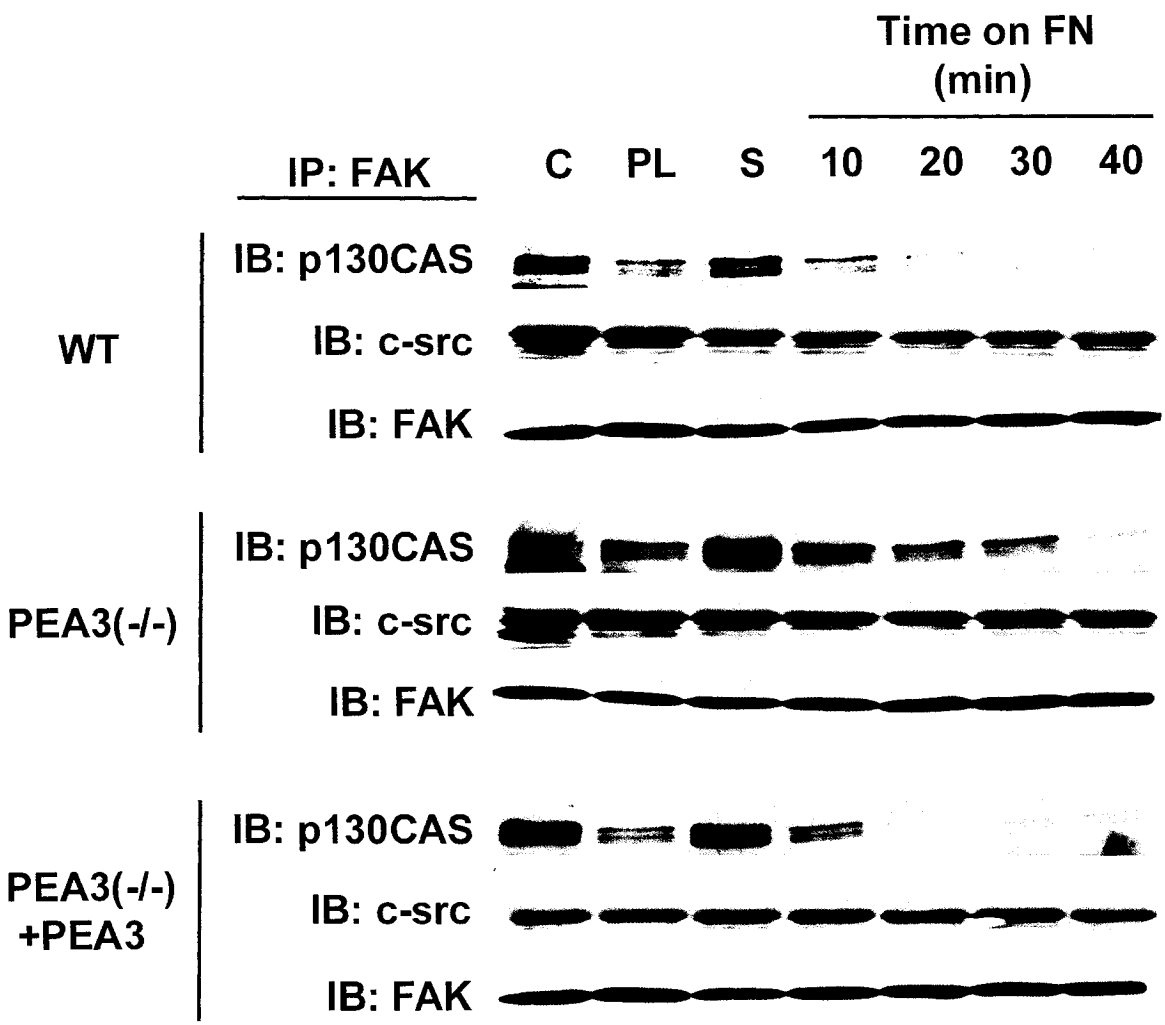

Time on FN (min)
IP: Paxillin
PL
S
10
20
40
WT
IB: p130CAS
IB: p130Cas/p-Tyr
IB: c-src
IB: Paxillin
PEA3(-/-)
IB: p130CAS
IB: p130Cas/p-Tyr
IB: c-src
IB: Paxillin
PEA3(-/-) +PEA3
IB: p130CAS
IB: p130Cas/p-Tyr
IB: c-src
IB: Paxillin

Figure 3-9. Dysregulation in the FAK/c-src/p130Cas complex disassembly in the PEA3(-/-) cells. Time course FN stimulation was performed and FAK was immunoprecipitated from equal amounts of cell lysates. Samples were resolved on SDS-PAGE and PVDF membranes are then immunoblotted for p130Cas and c-src, stripped and re-blotted for total FAK proteins. We observed that p130Cas remained associated with FAK for longer time intervals during FN replating assay. We also observed that p130Cas is associated with FAK upon replating onto poly-L-Lysine (PL) matrix to level equals to that of attached cells (C) in the PEA3(-/-) cells. We did not observe any major differences with c-src association with FAK.


Time on FN
(min)
IP: FAK
C
PL
S
10
20
30
40
WT
IB: p130CAS
IB: c-src
IB: FAK
PEA3(-/-)
IB: p130CAS
IB: c-src
IB: FAK
PEA3(-/-)
+PEA3
IB: p130CAS
IB: c-src
IB: FAK

focal adhesion disassembly deficiencies due to c-src inactivation. Supporting this, c-src association with paxillin or FAK remained unchanged in the different cell lines tested (Figure 3-8 and 3-9).

3.3 Discussion

We have previously shown that the overexpression of SLK induces the rapid disassembly of actin stress fibers and cell death (Sabourin, et al. 2000). Recently, we have demonstrated that SLK is redistributed to vinculin containing complexes during cell spreading on FN (Wagner, et al. 2002).

Here, we show that SLK redistribution to the cell periphery, more specifically to vinculin containing ruffles and lamellipodia, is altered in the PEA3(-/-) cells. In addition, we observed that PEA3(-/-) cells failed to close a wounded cell monolayer and exhibited a delayed migratory phenotype in haptotaxis migration assay. Immunostaining of PEA3(-/-) cells revealed an altered distribution of focal adhesion complexes, as visualized by vinculin and phospho-tyrosine staining following FN stimulation. We also demonstrated that PEA3(-/-) cells exhibit focal adhesion disassembly deficiencies characterized mainly by the failure to efficiently activate c-src resulting in decreased FAK cleavage, p130Cas reduced tyrosine phosphorylation and a prolonged association with FAK. Furthermore, we showed that the impairment in focal adhesion disassembly is not due to dysregulation in c-src association with FAK or paxillin but rather c-src kinase activity.

Recent studies have showed that FAK is cleaved by calpain 2 upon v-src mediated cell transformation (Carragher, et al. 2001). Here, we have shown that FAK is cleaved upon replating onto FN matrix in two fragments of 95 and 30 kDa. Interestingly, the levels of FAK cleavage products were reduced in the PEA3(-/-) cells. In support of this, we also observed that PEA3(-/-) cells failed to efficiently activate c-src through reduced Tyr-416 phosphorylation. Furthermore, Cary *et al* have reported that c-src mediated tyrosine phosphorylation of p130Cas regulates cell migration (Cary, et al. 1998). Our results show that p130Cas is not tyrosine phosphorylated in the PEA3(-/-) cells unlike WT and PEA3(-/-)+PEA3 cells. Interestingly, we did not observe any dysregulation in c-src association with either FAK or paxillin, suggesting that the observed reduction in FAK cleavage or p130Cas tyrosine phosphorylation is due to impaired c-src kinase activity. Taken together, we propose that the absence of the *pea3* gene expression results in the downregulation of c-src regulators, leading to a decrease in FAK cleavage, impaired focal adhesion disassembly and an overall reduced migration.

C-src activation can be accomplished in four manners, dephosphorylation of Tyr-527, decreased CSK activity, phosphorylation of Tyr-416 and intramolecular interactions with SH3 and SH2 binding substrates (Frame 2002). Here, we showed that FAK and paxillin interaction with c-src is not altered in the PEA3(-/-) cells relative to WT and PEA3(-/-)+PEA3 cells, suggesting that c-src activation through intramolecular interactions does not contribute to the observed inactivation of c-src in the PEA3(-/-) cells. We suggest that the observed impairment in c-src activation in the PEA3(-/-) cells could be due to altered

expression or activation of tyrosine phosphatases targeting c-src tyrosine residue 527, such as PTP1B, Shp1 and 2, PTPα, or tyrosine residue 416, such as PTP-BL, PTP-BAS (Roskoski 2005).

Wagner *et al* have previously shown that the expression of SLK in fibroblasts induces actin stress fibers disassembly (Wagner, et al. 2002). Furthermore, SLK was shown to be redistributed to large adhesion complexes during cell spreading on FN and that it is associated with the microtubule network (Wagner, et al. 2002). Interestingly, both c-src and v-src were shown to be implicated in microtubule reorganization (Abu-Amer et al. 1997, Matten et al. 1990, Nakayama et al. 1994). In this study, we demonstrated that PEA3(-/-) cells fail to activate c-src leading to impaired focal adhesion disassembly. Interestingly, we also observed that SLK redistribution to the cell periphery is altered in the PEA3(-/-) cells. Together, these results support a role for c-src in microtubule network organization, potentially regulating SLK localization to the cell periphery. Interestingly, SLK appear to be one of the destabilizing signals delivered by the microtubule network during adhesion disassembly (Storbeck *et al*, unpublished data).

Overall, we have shown that *pea3* gene expression regulates cell adhesion and migration. PEA3(-/-) cells migration deficiencies appear to arise from failure to efficiently activate c-src. Screening for potential PEA3 target proteins mediating c-src activation is underway. This study highlighted potential PEA3-mediated pathway involved in cell adhesion and migration. Understanding

these pathways will help developing efficient treatments for PEA3 positive cancers.

CHAPTER 4

C-SRC IS REQUIRED FOR SLK LOCALIZATION TO MEMBRANE RUFFLES AND REGULATION OF KINASE ACTIVITY

4.1 Introduction

Normal cellular functions and survival are highly dependent upon interaction with adjacent cells and the ECM. Specifically, the interaction of cells with ECM proteins generates intracellular signals mainly mediated through the integrin family of transmembrane receptors (Ruoslahti 1999). Integrin receptor binding to ECM proteins generates intracellular signals through tyrosine phosphorylation events that are important for cell growth, survival, and migration (Akamatsu, et al. 1996). 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 FAK and SFKs implicated in the control of cell spreading and cell migration (Schaller et al. 1999).

The c-src kinase as well as its constitutive active oncogenic counterpart v-src are well characterized members of SFKs. Importantly, increased c-src activity and in some cases protein levels are observed in human cancers such as breast cancer (20 fold increase in activity; 7), colon cancer (up to 8 fold increase in activity) and pancreatic cancer increase in both kinase activity and protein levels (Jones, et al. 2000). Src characterization is mainly based on studies performed on c-src and v-src. The main sequence differences between c-src and v-src is that the latter is about 15 to 20 amino acid residues shorter than c-src. More specifically, v-src lacks Tyr-527 involved in the inactivation of the kinase, thus v-src constitutive activation.

We have recently shown that SLK is redistributed to large adhesion complexes during cell spreading on FN and that it is associated with the

microtubule network (Wagner, et al. 2002). Furthermore, ectopic expression of SLK was shown to induce the disassembly of actin stress fibers, a process that can be inhibited by dominant negative Rac1 (Wagner, et al. 2002).

To gain further insights into the role of SLK in cytoskeletal remodeling, we investigated SLK activity and distribution in cell lines lacking or overexpressing FAK or src, two major regulators of adhesion site turnover, actin reorganization and cell migration. Our results show that SLK redistribution to vinculin rich lamellipodia and membrane ruffles is dependent on c-src during cell spreading on FN but independent of FAK. Further, we demonstrate that SLK kinase activity is reduced in cells expressing the v-src oncogene and that this regulation requires v-src translocation to the cell periphery. The downregulation of SLK activity by v-src was also dependent on its kinase activity and myristylation site. Overexpression of FAK or inhibition of FAK signaling by FRNK expression had no effect on SLK activity. Furthermore, expression of v-src in FAK-null cells also resulted in SLK downregulation, suggesting that v-src-mediated SLK regulation is independent of FAK. Together our results show that src is required for both SLK redistribution and regulation at sites of cytoskeletal remodeling.

4.2 Results

4.2.1 C-src-Dependent Recruitment Of SLK At Membrane Ruffles And Lamellipodia

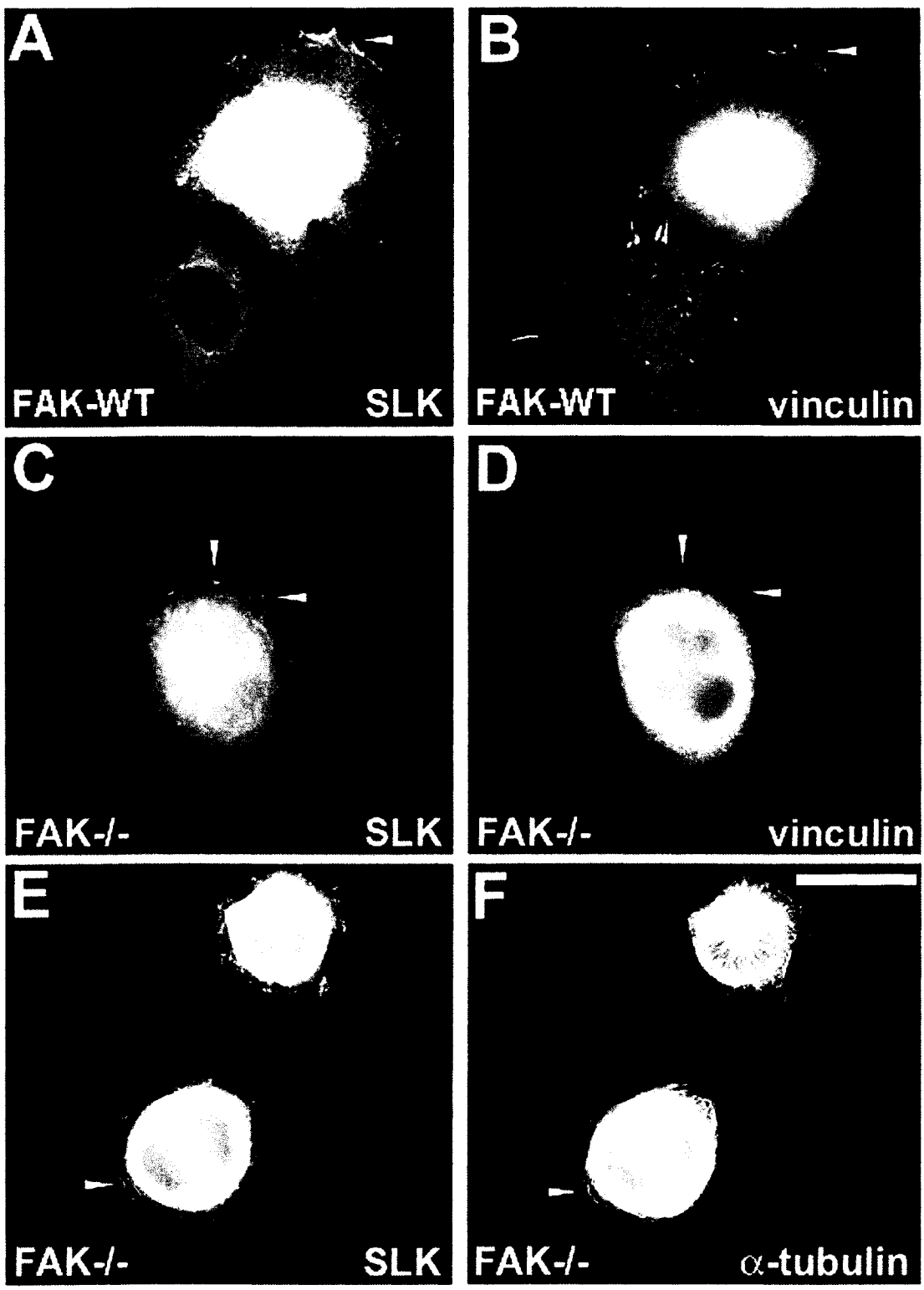
Fibronectin stimulation of fibroblasts has been shown to trigger the activation of FAK, the recruitment of c-src and the formation of adhesion

signaling complexes mediating actin rearrangements (Schaller and Parsons 1994, Schlaepfer, et al. 1999). Furthermore, FAK and c-src appear to be critical for the turnover of these complexes, allowing cell migration. We have previously shown that SLK can induce actin disassembly and that it is redistributed to membrane structures reminiscent of ruffles and lamellipodia during cell spreading on FN (Wagner, et al. 2002). Therefore, to gain further insights into the role of SLK in cytoskeletal remodeling at sites of actin dynamics, we have investigated SLK distribution and activity in FAK- and SYF-deficient cell lines.

Cultures of FAK-/- and WT control were held in suspension and replated onto FN-coated substrates followed by immunostaining for SLK and vinculin. As previously observed, following a 10 to 20 min replating period, a fraction of SLK protein was found to be redistributed to the cell periphery and colocalized with the adhesion protein vinculin at apparent membrane ruffles and lamellipodia in FAK-WT cells (Figure 4-1). Even though the kinetics of cell spreading differ markedly from WT cells, SLK was also found to colocalize with vinculin at ruffle structures in FAK-null cells (Figure 4-1), suggesting that the recruitment of SLK to sites of actin dynamics is independent of FAK. Supporting our previous observations (Figure 4-1 and (Wagner, et al. 2002)), SLK also colocalized with microtubule filaments at the cell periphery in FAK-/- cells.

The proto-oncogene c-src has been previously shown to be recruited to focal adhesion and to be required for downstream signaling and adhesion turnover (Carragher, et al. 2001, Fincham and Frame 1998). Interestingly, previous studies have shown that c-src is still able to translocate to cell periphery

Figure 4-1. SLK distribution to membrane ruffles and lamellipodia is FAK-independent. FAK-Wt and FAK-/- cells were stimulated by FN replating for 20 min and co-immunostained for SLK (A, C, E) and α-tubulin (F) or vinculin (B & D). Replating onto FN-coated coverslips shows a redistribution of SLK at the cell periphery in diffuse lamellipodia-like structures along with some microtubule filaments and vinculin (*arrowheads*) in all cell lines. The cells were photographed at X630. Scale bar = 10 μm.

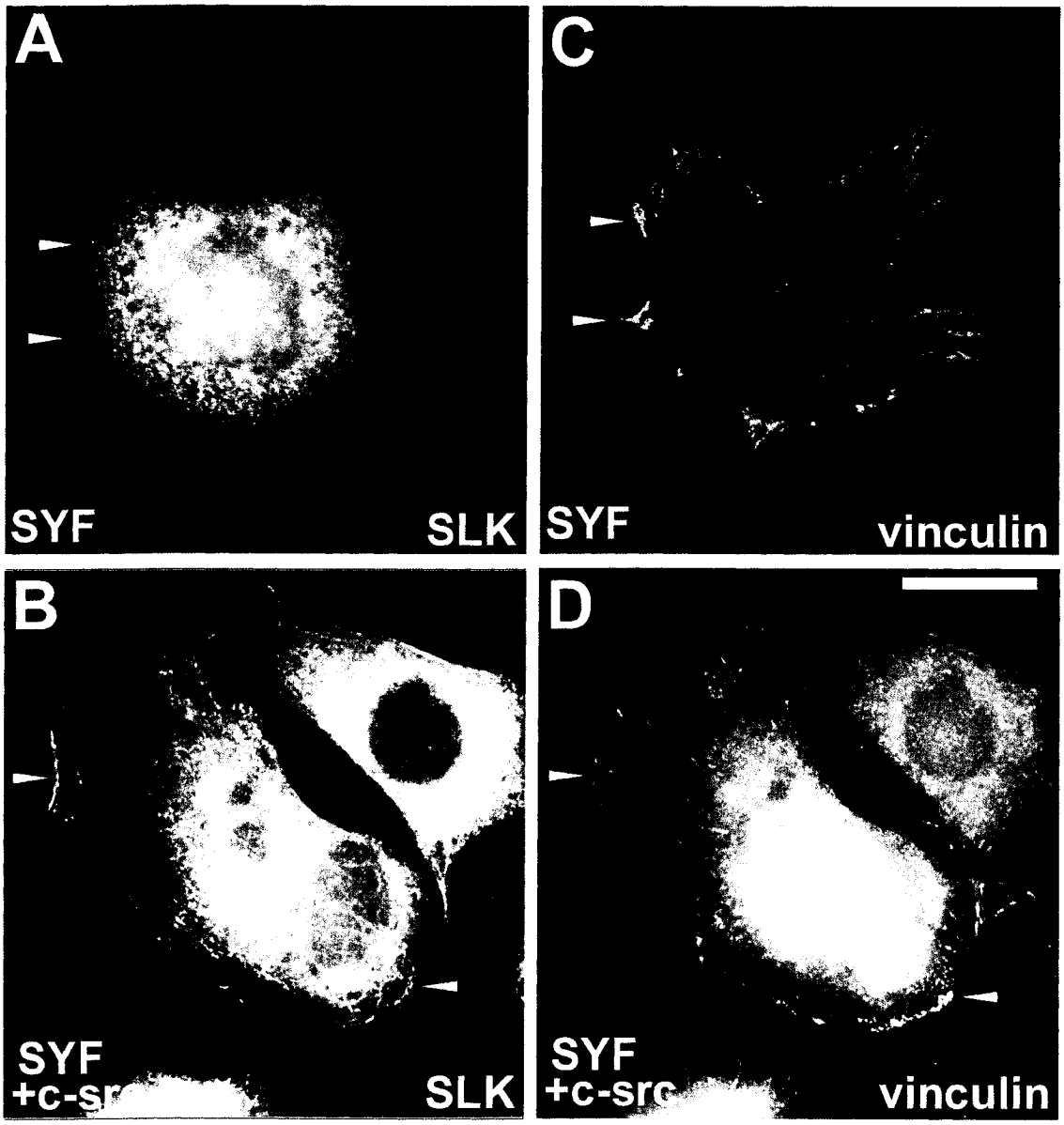

A
FAK-WT
SLK
B
FAK-WT
vinculin
C
FAK-/-
SLK
D
FAK-/-
vinculin
E
FAK-/-
SLK
F
FAK-/-
α-tubulin

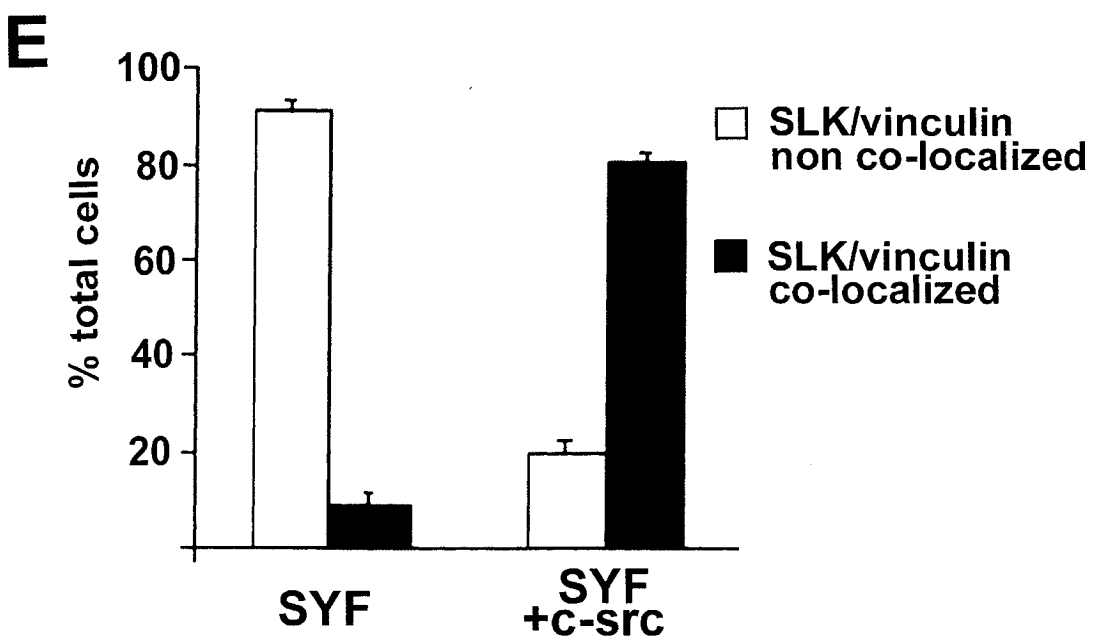
in the FAK-/- cells in a Pyk2-dependent manner (Sieg, et al. 1998). Therefore, we performed similar replating and immunostaining assays using SYF-deficient cells. Interestingly, in the absence of the src family kinases, SLK and vinculin colocalization at the cell periphery was markedly reduced and most often absent (Figure 4-2). Mostly, large mature adhesion complexes were observed, supporting a role for the src kinases in adhesion turnover or disassembly (Fincham and Frame 1998). The redistribution of SLK at the cell periphery was restored by re-expressing c-src into the SYF-deficient cells (Figure 4-2). Taken together, these results suggest that SFKs are required to recruit SLK to membrane ruffles and lamellipodia.

4.2.2 V-src and C-srcY527F Downregulate SLK Kinase Activity

We have previously demonstrated that the total SLK kinase activity remains unchanged during cell spreading and adhesion complex assembly while its cellular distribution is altered (Wagner, et al. 2002). Because SLK recruitment to the cell periphery was found to depend on the expression of src family kinases, we also analyzed SLK kinase activity in fibroblasts lacking or overexpressing FAK or the src tyrosine kinase. We performed SLK in vitro kinase assays on cell lysates from FAK-WT, FAK-/-, SYF-deficient, SYF+c-src. In addition, 49F cells stably expressing the v-src oncogene or c-srcY527F, a constitutively active mutant of c-src, were used. In vitro kinase assays showed no change in SLK kinase activity in SYF-deficient, SYF+c-src, and FAK-null or FAK-WT cells (Figure 4-3). However, a 2 to 3-fold decrease in SLK kinase activity was

Figure 4-2. Altered distribution of SLK in SYF-deficient fibroblasts. FN stimulated SYF-deficient and SYF-deficient + c-src MEF cells co-immunostained for SLK (A & B) and vinculin (C & D). Endogenous SLK expression was found to be excluded from the cell periphery and lamellipodia in SYF-deficient cells (A & C; *arrowheads*), whereas it was observed to be associated with vinculin in diffuse lamellipodia-like structures in the SYF-deficient + c-src cells (B & D) (*arrowheads*). Panel E is a quantification of SLK/vinculin colocalization. Poor SLK/vinculin colocalization was observed in about 10% of SYF cells, whereas 80% of colocalization was detected in SYF-deficient + c-src cells (n=400) 20 min after replating. The data shown represent averages ± standard errors for three independent experiments. The cells were photographed at X630. Scale bar = 10 μm.


A
SYF
SLK
C
SYF
vinculin
B
SYF
+c-sr
SLK
D
SYF
+c-src
vinculin


E
100
80
60
40
20
% total cells
SLK/vinculin
non co-localized
SLK/vinculin
co-localized
SYF
SYF
+c-src

consistently observed in v-src and c-srcY527F transformed cells (Figure 4-3), suggesting that an activated c-src or its downstream effectors negatively regulates SLK. Supporting a role for c-src in SLK regulation, immunofluorescence staining of exponentially growing cells revealed that c-src and SLK proteins could be colocalized at membrane ruffles and lamellipodia (Figure 4-3).

To further understand the mechanism by which v-src downregulates SLK activity, we generated 49F cells stably expressing TsLA29v-src, a mutant v-src that is temperature-sensitive for translocation to adhesion sites (Fincham and Frame 1998). Shifting the cultures to the permissive temperature (35 ºC) results in the translocation of v-src to the cell periphery. Following incubation of the cells at the permissive temperature and SLK in vitro kinase assay, we observed a 2-3 fold decrease in SLK kinase activity concomitant with the characteristic v-src-induced morphological changes (Figure 4-4). Interestingly, immunoprecipitation of SLK followed by immunoblotting for phosphotyrosine residues showed that SLK is not tyrosine phosphorylated in control or v-src expressing cells (not shown). Furthermore, we observed that SLK and v-src do not co-immunoprecipitate (not shown). Taken together, these results suggest that v-src-mediated SLK downregulation is indirect and that this requires v-src translocation to the membrane and adhesion sites.

Figure 4-3. Inhibition of SLK kinase activity in v-src and c-srcY527F transformed 49F cells. In vitro kinase assay performed on NIH3T3, FAK-Wt, FAK-/-, SYF-deficient, SYF-deficient + c-src, 49F, v-src and c-srcY527F cells are shown. Endogenous SLK was immunoprecipitated from equal amounts of cell lysates and subjected to in vitro kinase assays using γ^{32}P ATP. The reaction mixture was resolved by SDS-PAGE and gels were transferred to PVDF membranes, and exposed to X-ray film. The PVDF membranes were then probed for SLK to evaluate the efficiency of the immunoprecipitation. The extent of SLK autophosphorylation was used as a measure of kinase activity. Downregulation of SLK was observed in v-src and c-srcY527F transformed cells. Supporting a role for src-mediated SLK regulation, double staining of endogenous SLK (B) and c-src (C) shows colocalization in membrane ruffles and lamellipodia (*arrowheads*). The cells were photographed at X630. Scale bar = 10 μm.

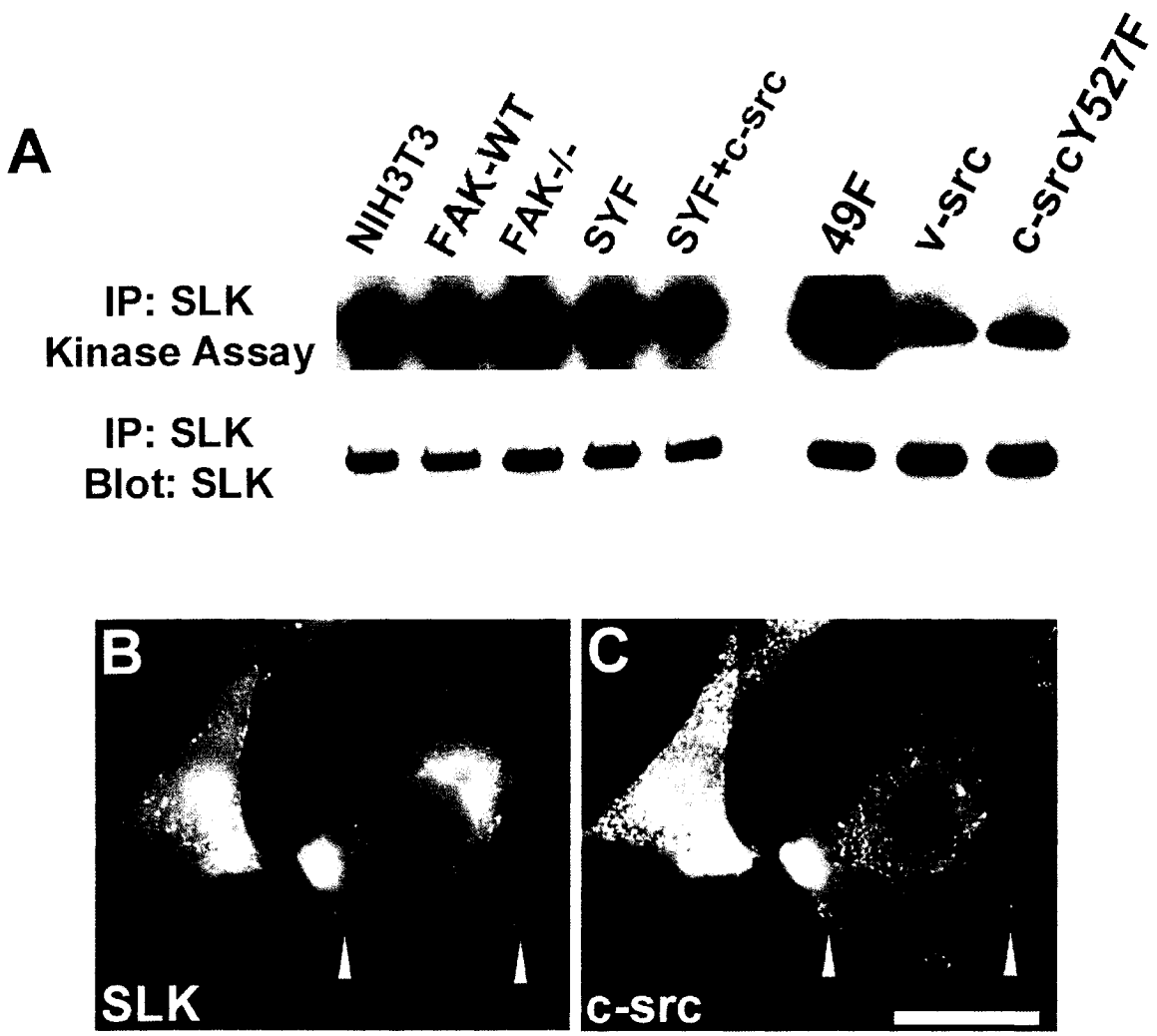
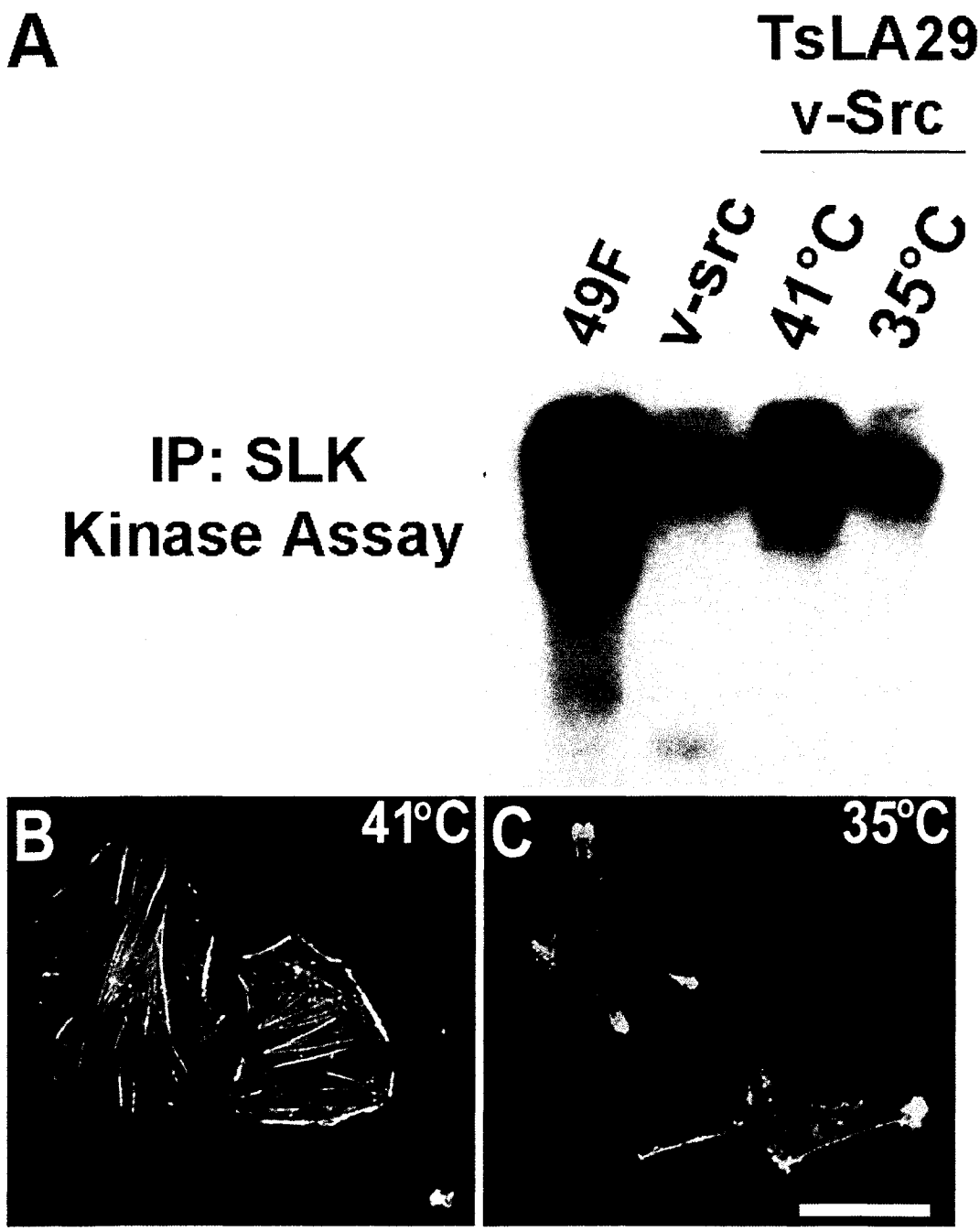

A
NIH3T3
FAK-WT
FAK-/-
SYF
SYF+c-src
49F
v-src
c-srcY527F
IP: SLK
Kinase Assay
IP: SLK
Blot: SLK
B
SLK
C
c-src

Figure 4-4. V-src-mediated downregulation of SLK activity requires translocation to adhesion sites. (A) Cells stably expressing Fpgv29 (Ts v-src) were maintained at the restrictive temperature and then shifted to the permissive temperature for 16-18h. Endogenous SLK was then immunoprecipitated from equal amounts of cell lysate and subjected to in vitro kinase assays using γ^{32}P ATP. The gels were dried and exposed to X-ray film and the extent of autophosphorylation was used as a measure of kinase activity. Translocation of the Ts v-src to adhesion sites by incubation of the cultures at the permissive temperature (35°C) induced the characteristic v-src transformed cell morphology, as highlighted by the phalloidin stain (B & C) and SLK downregulation. The cells were photographed at X630. Scale bar = 10 μm.


A
TsLA29
v-Src
49F
v-src
41°C
35°C
IP: SLK
Kinase Assay
B
41°C
C
35°C

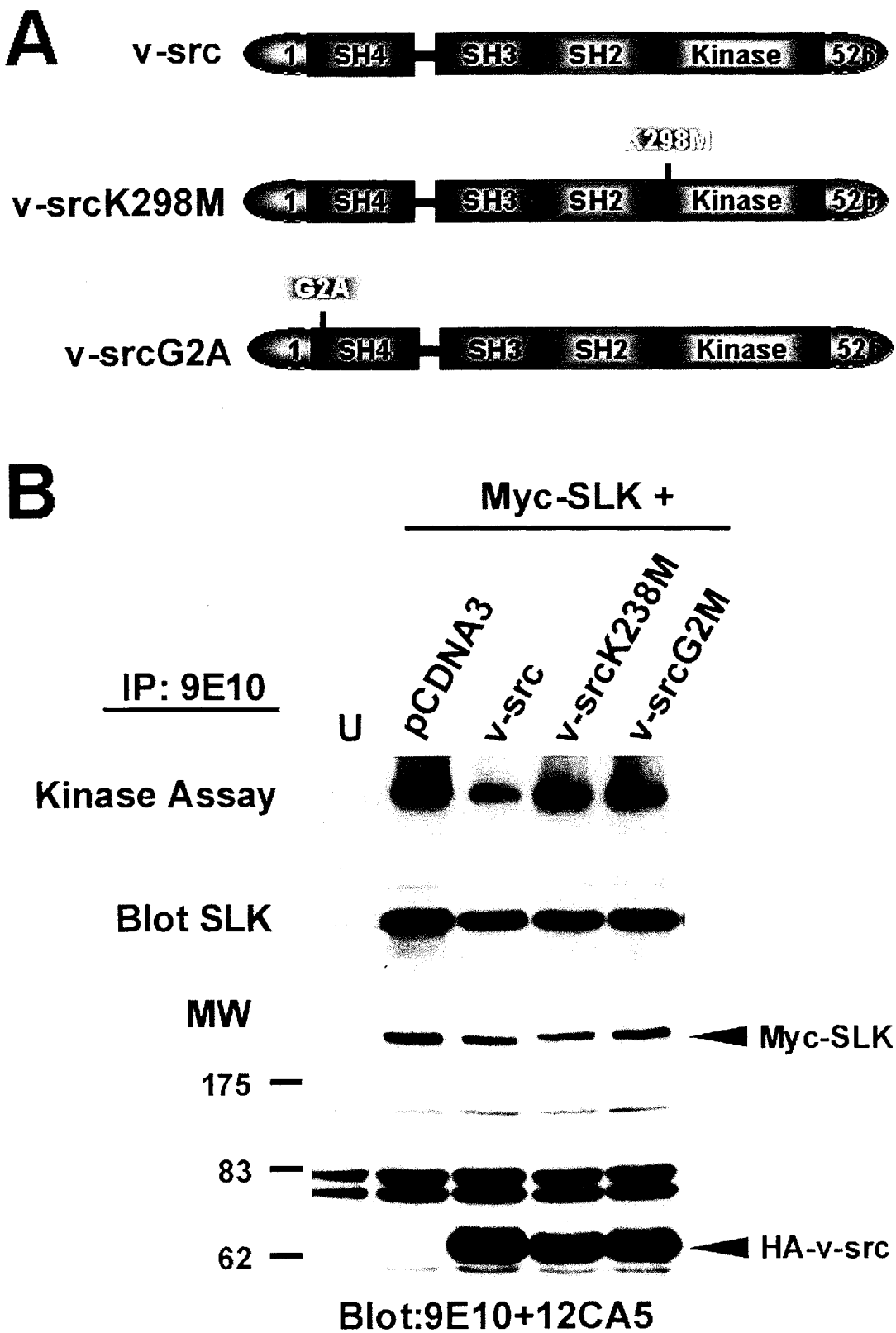
4.2.3 V-Src Kinase Activity And Membrane Translocation Are Required For SLK Downregulation

Previous studies have shown that c-src kinase activity is required for Rac1- and cdc42-induced adhesion remodeling and directed cell migration whereas, src SH3 and SH2 domains are sufficient for its translocation to the cell periphery in a RhoA-dependent manner (Fincham and Frame 1998, Timpson, et al. 2001). Therefore, based on our previous findings (Figure 4-4) and the RhoGTPases studies (Fincham and Frame 1998, Timpson, et al. 2001), we set out to test the potential implication of the v-src kinase activity and myristylation site on SLK downregulation. Two v-src point mutants were engineered, kinase inactive (v-srcK298M) and myristylation defective (v-srcG2A) mutants (Figure 4-5A). These mutants along with the WT counterpart were co-transfected with Myc-SLK in HEK293 cells and SLK kinase activity was assessed. SLK immunoprecipitation and in vitro kinase assays showed that the v-src point mutants rendering it kinase inactive (v-srcK298M) or myristylation defective (v-srcG2A) could no longer downregulate SLK kinase activity in comparison with cells co-transfected with empty vector (Figure 4-5B). Together, these data suggest that both v-src kinase activity and membrane anchoring are required to negatively regulate SLK kinase activity.

4.2.4 V-src-Mediated Downregulation Of SLK Kinase Activity Is Independent Of FAK

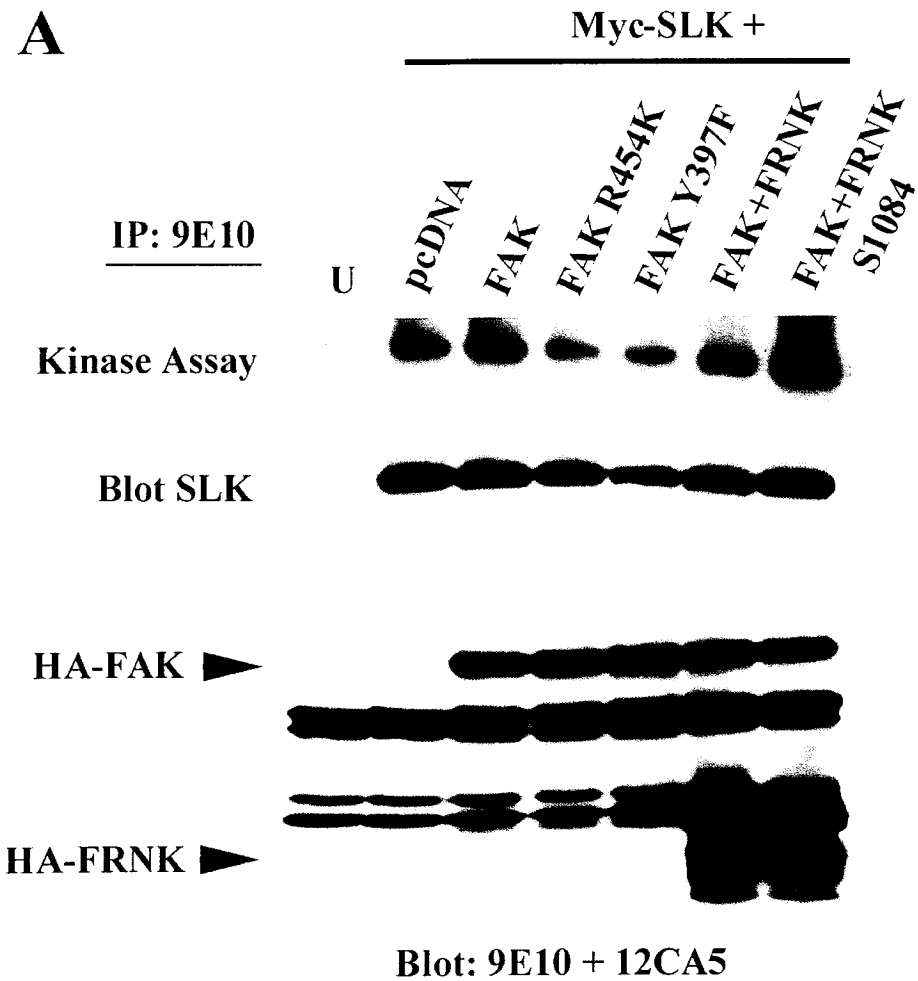
Integrin signaling following FN stimulation proceeds through the

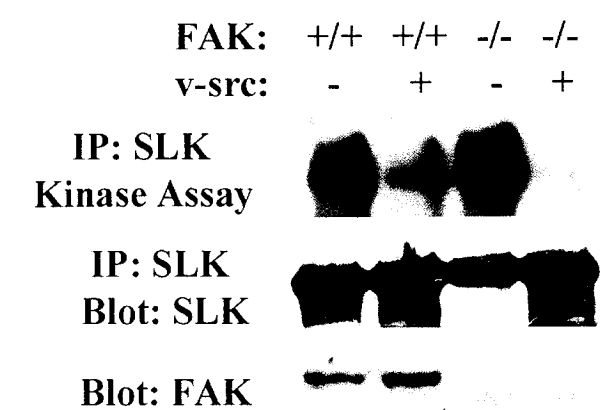
Figure 4-5. V-src kinase activity and membrane localization are required for SLK downregulation. (A) Schematic representation of the HA-v-src point mutants. The v-srcK298M and v-srcG2A point mutations were introduced into the full-length v-src rendering it kinase inactive or myristylation defective, respectively. (B) HEK293 cells were co-transfected with the HA-v-src or mutants in the presence or absence of Myc-SLK. Myc-SLK was immunoprecipitated from cell lysates using 9E10 antibodies and subjected to in vitro kinase assay. The SLK IP was monitored by probing the kinase assay with anti-SLK antibodies. Expression of all mutants and SLK was confirmed by Western blotting of the total cell lysate for Myc and HA (bottom panel).


A
v-src
1
SH4
SH3
SH2
Kinase
526
v-srcK298M
K298M
v-srcG2A
G2A
B
Myc-SLK +
pCDNA3
v-src
v-srcK238M
v-srcG2M
U
IP: 9E10
Kinase Assay
Blot SLK
MW
175
83
62
Myc-SLK
HA-v-src
Blot:9E10+12CA5

recruitment and autophosphorylation of FAK on Tyr-397, causing its transient association with activated c-src and further phosphorylation creating additional SH2 binding sites (Ruoslahti 1997). Similarly, in v-src transformed cells, FAK is hyperphosphorylated, in addition to other adhesion complex proteins (Fincham, et al. 1995, Hanks et al. 1992, Schaller et al. 1992). Therefore, we investigated whether SLK downregulation by v-src required signaling through FAK. To test this, HEK293 cells were co-transfected with Myc-SLK and either wild-type HA-FAK, kinase defective (HA-FAK R454K) or a Tyr397-mutant (HA-FAK Y397F). FAK was shown to induce adhesion signaling and migration upon overexpression (Schaller, et al. 1999). Furthermore, FAK was able to restore focal adhesion turnover and the cellular phenotype in FAK-/- cells, whereas FAK Y397F failed to do so (Webb, et al. 2004). In vitro kinase assays showed that FAK overexpression had no effect on SLK kinase activity (Figure 4-6A). Similarly, interfering with endogenous FAK signaling by overexpression of the dominant negative FRNK molecule, or the non-FAK binding mutant FRNK S1084A (Hauck et al. 2002, Richardson and Parsons 1996), had no effect. In addition, expression of FAK kinase dead or the FAK Y397F mutant did not affect SLK kinase activity. Supporting these observations, SLK kinase activity was still downregulated in FAK-WT or FAK-/- cells stably expressing the oncoprotein v-src (Figure 4-6B) (Moissoglu and Gelman 2003). Together, these results suggest that v-src mediated SLK downregulation does not proceed through FAK and its downstream signaling pathways, but rather by activating an independent signaling system.

Figure 4-6. V-src mediated SLK downregulation is independent of FAK. (A) HEK293 cells were co-transfected with Myc-SLK, HA-FAK, HA-FAK R454K, HA-FAK Y397F, HA-FRNK or HA-FRNK S1084A and subjected to SLK kinase assays. Overexpression of FAK or FRNK had no effect on SLK kinase activity. SLK IP were monitored by reprobing the kinase assay for SLK protein. Expression of FAK was verified by Western blotting (bottom panel). (B) SLK was immunoprecipitated from FAK+/+ or -/- MEFs stably expressing v-src and subjected to in vitro kinase assay. SLK IP was assessed as in (A) and FAK expression was also evaluated by Western blot analysis.


A
Myc-SLK +
pcDNA
FAK
FAK R454K
FAK Y397F
FAK+FRNK
FAK+FRNK S1084
U
IP: 9E10
Kinase Assay
Blot SLK
HA-FAK
HA-FRNK
Blot: 9E10 + 12CA5


B
FAK: +/+ +/+ -/- -/-
v-src: - + - +
IP: SLK
Kinase Assay
IP: SLK
Blot: SLK
Blot: FAK

Overall, our data show that SLK recruitment to lamellipodia and membrane ruffles during cell spreading is dependent on the src family kinases. Furthermore, we have shown that v-src can downregulate SLK activity and that the c-src protein can be colocalized with SLK at lamellipodia and membrane ruffles. Finally, v-src-mediated SLK regulation requires v-src kinase activity and is independent of FAK.

4.3 Discussion

We have previously described that overexpression of SLK in various cell lines induces the rapid disassembly of actin stress fibers and cell death (Sabourin, et al. 2000). Recently, we have demonstrated that SLK is redistributed with vinculin to structures reminiscent of membrane lamellipodia and ruffles during cell spreading on FN (Wagner, et al. 2002).

Here, we have shown that this redistribution of SLK at membrane ruffles requires the src family of tyrosine kinases. We have observed that SLK localization to the cell periphery is altered in SYF-deficient cells, a cell system exhibiting migratory defects. Re-expression of c-src in SYF cells rescued the SLK phenotype, suggesting that c-src is sufficient to induce the recruitment of SLK at the cell periphery. Interestingly, our data shows that v-src expression leads to downregulation of SLK kinase activity, a process that requires v-src translocation to the membrane, kinase activity and membrane anchoring. Surprisingly, this downregulation does not proceed through FAK or by direct tyrosine phosphorylation of SLK, suggesting that v-src modulates the activity of other

signaling systems such as kinases or phosphatases. Alternatively, it may modulate the ability of SLK to associate with a negative regulator.

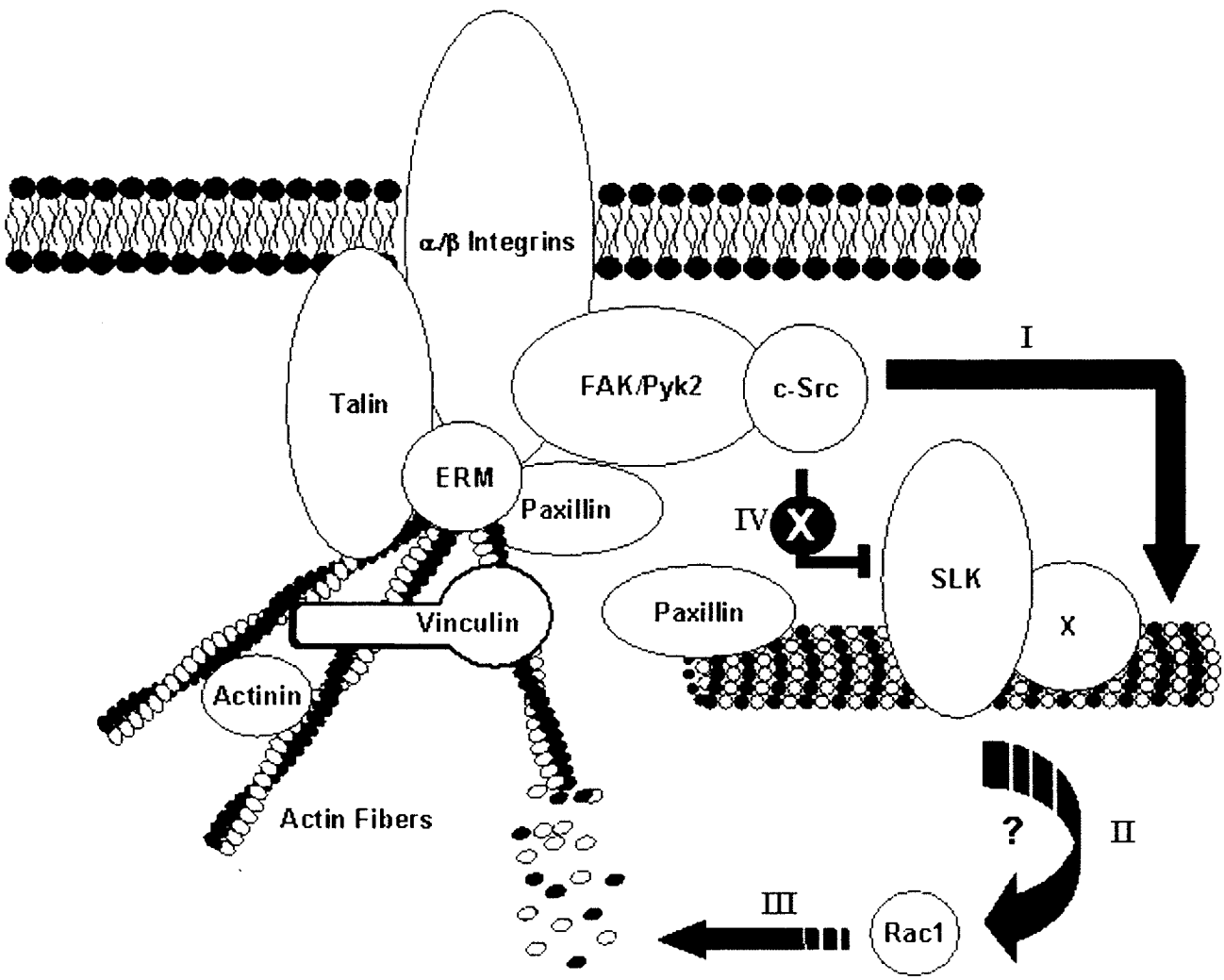
Recently, Timpson et al. showed that c-src kinase activity is required at peripheral adhesion sites for Rac1- and cdc42-induced adhesion remodeling and directed cell migration (Timpson, et al. 2001). Interestingly, we have shown that SLK-mediated actin stress fiber disassembly can be inhibited by the co-expression of a dominant negative version of Rac1 (RacN17). One possibility is that the effects of SLK on actin dynamics are mediated by Rac1 and regulated in part by the c-src kinase. It has been previously reported that c-src kinase activity and myristylation site are required to regulate focal adhesion turnover during cell motility (Fincham and Frame 1998). Therefore, one possibility is that the process of focal adhesion turnover induces SLK recruitment, through the microtubule (Wagner, et al. 2002), in a c-src-dependent manner. This further requires the actin disassembling activities of SLK and Rac1, allowing adhesion site destabilization. Upon assembly of new focal contacts, SLK activity is downregulated, allowing actin polymerization (see Figure 4-7). However, in a v-src expression system, the turnover rate of adhesion sites is markedly increased (Fincham and Frame 1998), resulting in an apparent SLK downregulation. Supporting this, FAK- and SYF-deficient cells, which display reduced migration rates and adhesion turnover, had relatively normal levels of SLK activity.

Recently, the microtubule network has been clearly implicated in adhesion site destabilization and turnover, a process that requires kinesin motors (Ballestrem et al. 2000, Bershadsky et al. 1996, Kaverina et al. 1999, Kaverina et

al. 1998, Krylyshkina et al. 2002). 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 reorganization (Abu-Amer, et al. 1997, Matten, et al. 1990, Nakayama, et al. 1994). Whether, SLK recruitment to membrane ruffles and lamellipodia in a src-dependent manner involves src regulation of the microtubule network remains to be investigated. One attractive possibility is that SLK represents one of the destabilizing signals delivered by the microtubule network during adhesion turnover.

Overall, we have demonstrated that c-src is an upstream modulator of SLK activity and localization to the cell periphery. The identification of potential upstream kinases mediating the link between SLK and v-src will bring further insights into the mechanisms of cell adhesion and migration.

Figure 4-7. Proposed model for SLK regulation by the src-family kinases. Upon FN stimulation or migration, focal adhesions assemble through the activation of the FAK/src complex. SLK is then recruited to sites of actin dynamics, a process that requires the src-family kinases perhaps by modulating microtubule dynamics (I). Following SLK-mediated actin disassembly, which may require Rac1 (II and III), adhesion complex relaxation can occur and SLK becomes downregulated, indirectly by c-src (IV).


α/β Integrins
Talin
FAK/Pyk2
c-Src
I
ERM
Paxillin
IV
X
SLK
Paxillin
X
Vinculin
Actinin
Actin Fibers
?
II
III
Rac1

CHAPTER 5

V-SRC-DEPENDENT DOWNREGULATION OF SLK KINASE ACTIVITY IS MEDIATED BY CK2

5.1 Introduction

Cell growth and differentiation are tightly regulated mechanisms involving a large number of signaling cascades. Dysregulation and accumulation of genetic aberrations in these signaling cascades are key components in the transformation of a normal cell to a cancer cell. Furthermore, a direct correlation has been found between the metastatic potential of cancers and the nature of the observed genetic mutations (Irby et al. 1999, Irby and Yeatman 2000, Jones, et al. 2000, Ruoslahti 1999, Ruoslahti 1997). Indeed, cellular transformation by the src oncogene, a non-receptor tyrosine kinase, results in loss of adherence, invasiveness and metastasis through increased phosphorylation of adhesion proteins and cytoskeletal disorganization.

C-src and its viral counterpart v-src are the most studied members of SFKs. Several studies have illustrated potential src-mediated mechanisms regulating cell survival and apoptosis (Frame 2002). In addition, studies have demonstrated altered c-src kinase activity, and in some cases protein levels, in human cancers such as breast cancer, colon cancer and pancreatic cancer (Jones, et al. 2000). V-src transformed cells have been widely used to elicit the oncogenic effect of a constitutively active c-src. Features characterizing these cancer cell line models include increased cell detachment and migration. More specifically, v-src transformation induces the rate of integrin-associated adhesion turnover and loss of actin stress fibers (Fincham, et al. 2000, Fincham and Frame 1998).

Casein kinase II (CK2) is a serine/threonine kinase tetramer complex composed of two catalytic subunits, α and/or α′ and/or α″ and two regulatory β subunits. CK2 minimal amino acid consensus phosphorylation sequence is Ser-X-X-Acidic, where the acidic residue can be glutamic acid, aspartic acid, phospho-Ser or phospho-Tyr (Pinna 1990, Pinna and Meggio 1997). CK2 is referred to as "a house keeping enzyme" given its increasing number of substrates (over 300). This results in CK2 being involved in a wide range of cellular functions and properties starting with cell proliferation (Allende and Allende 1995, Guerra, et al. 1999, Litchfield and Luscher 1993, Tawfic, et al. 2001), to survival (Ahmed, et al. 2002), moving on to differentiation, transformation and tumorigenesis (Ahmed, et al. 2000, Tawfic, et al. 2001) as well as apoptosis (Guo, et al. 2001). Recently, the CK2α subunit was shown to be phosphorylated by SFKs, c-Fgr and Lyn, resulting in an increase in its catalytic activity (Donella-Deana, et al. 2003). Interestingly, the CK2-interacting protein-1 (CKIP-1) was found to bind CK2α through its PH domain, an interaction that is required for CK2 redistribution to the plasma membrane (Bosc, et al. 2000, Olsten, et al. 2004).

Previous studies in our laboratory have shown that SLK is redistributed to the cell periphery via the microtubule network to induce actin stress fiber disassembly in a Rac1-dependent manner (Wagner, et al. 2002). Recently, we have observed that SLK redistribution to the cell periphery is altered in SYF-deficient cells and that this phenotype could be rescued by the re-expression of c-src (Chapter 4). In addition, we have demonstrated that v-src kinase activity

and membrane targeting are required to downregulate SLK kinase activity and that this process was independent of FAK (Chapter 4).

To further delineate the molecular mechanisms involved in SLK downregulation in a v-src-dependent manner, we examined the phosphoamino acid content of SLK. We observed a dramatic increase in SLK phospho-serine content in v-src and c-srcY527F transformed 49f cells in comparison to parental cells. Further mapping led us to the identification of two serine residues located at position 347/348 (SS347/348) that are phosphorylated by CK2. Mutation of SS347/348 to alanine resulted in a 2- to 3-fold increase in SLK kinase activity. Furthermore, inhibition of CK2 kinase activity in v-src transformed cells rescued SLK kinase activity. Together, our results suggest that SLK hyperphosphorylation on serine residues is partially mediated by CK2 hyperactivity in v-src transformed cells. More importantly, we demonstrated that CK2 phosphorylation of SLK results in the downregulation of its kinase activity, an effect that could be reversed by CK2 inhibitors.

5.2 Results

5.2.1 The SLK Kinase Domain Is Hyperphosphorylated On Serine Residues In V-Src And C-srcY527F Transformed Cells

We have previously shown that SLK kinase activity is downregulated in v-src transformed cells and that this effect is dependent on v-src kinase activity and membrane localization (Chapter 4). To determine whether potential post-translational modifications on SLK were responsible for the downregulation, we

conducted phosphoamino acid analysis. Endogenous SLK protein was immunoprecipitated from ^{32}P labeled 49f, v-src-transformed 49f or 49f cells stably expressing the constitutively active form of c-src, c-srcY527F. SLK immunoprecipitates were then subjected to acid hydrolysis and resolved by two dimensional thin layer chromatography (TLC). Phosphoamino acid analysis revealed that SLK is hyperphosphorylated solely on serine residues in v-src- or c-srcY527F-transformed 49f cells in comparison to 49f cells (Figure 5-1), suggesting that the v-src- or c-srcY527F-mediated effect is indirect. The observed serine hyperphosphorylation could be attributed to a v-src-dependent increase in the catalytic activity of a serine/threonine kinase or a decrease in phosphatase activity. However, treatment of 49F cells with phosphatase inhibitors followed by SLK immunoprecipitation showed no changes in its kinase activity (not shown). This suggests that v-src mediates SLK downregulation through a serine/threonine kinase. To identify potential kinases involved in the downregulation of SLK, we generated Myc-tagged SLK kinase inactive (Myc-$SLK^{1-373K63R}$) constructs (Myc-$SLK^{1-192K63R}$ and Myc-$SLK^{1-325K63R}$) each designed to contain 8 serine residues and encompassing the kinase domain (Figure 5-2). Co-transfection of these constructs with or without v-src in COS1 cells revealed that only the Myc-$SLK^{1-373K63R}$ truncation was hyperphosphorylated when co-transfected with v-src, suggesting a potential phosphorylation occurring within the last 8 serine residues of the kinase domain (Figure 5-2). The peptide sequence containing these last serine residues was analyzed for potential protein phosphorylation sites using ScanProsite from ExPASy. The analysis showed the

Figure 5-1. Phosphoamino acid analysis of endogenous SLK protein shows increased phospho-serine content in the v-src and c-srcY527F transformed cells. Cells were labeled with ^{32}P-orthophosphate, lysed and SLK was immunoprecipitated. The phosphorylated SLK was then hydrolyzed for 90 min at 100°C and spotted onto a thin layer chromatography plate. pH 1.9 and 3.5 buffers were used respectively to separate the serine, threonine and tyrosine residues along with their appropriate standards.

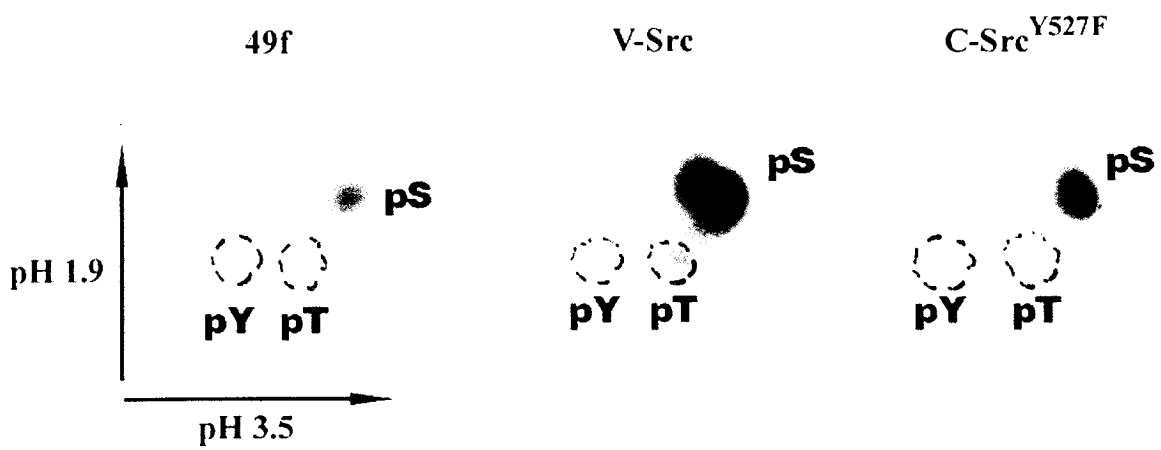
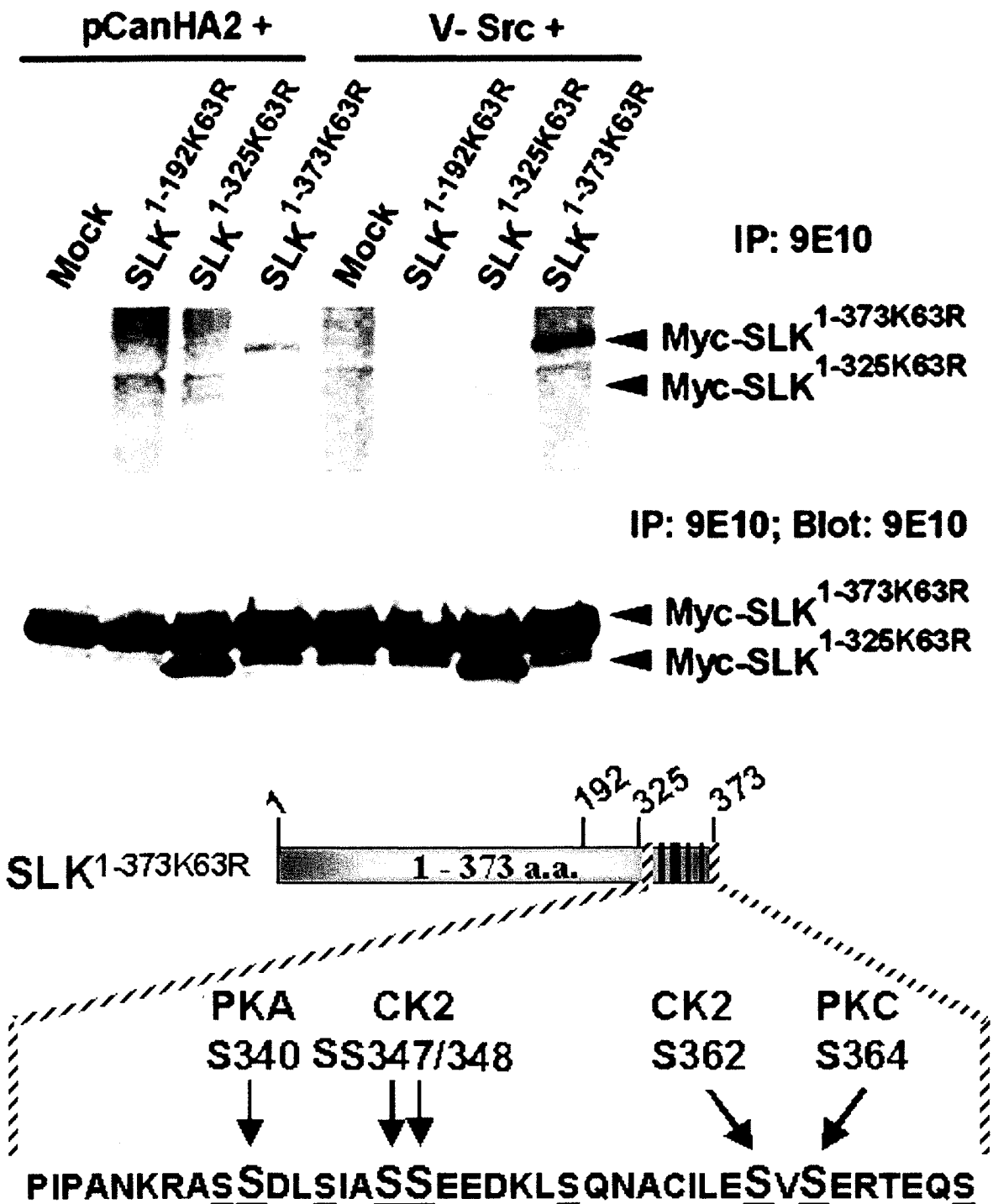

49f
V-Src
C-Src^{Y527F}
pH 1.9
pH 3.5
pS
pY
pT
pS
pY
pT
pS
pY
pT

Figure 5-2. V-src mediates the phosphorylation of SLK at the kinase domain and more specifically within the last 8 serine residues of the catalytical domain. Myc tagged SLK kinase domain kinase inactive truncations, $SLK^{1\text{-}192K63R}$, $SLK^{1\text{-}325K63R}$ and $SLK^{1\text{-}373K63R}$ were co-transfected with or without v-src. Cells are than labeled with ^{32}P-orthophosphate and $SLK^{1\text{-}373K63R}$ truncated constructs were immunoprecipitated with an anti-myc antibody. Samples were resolved by SDS-PAGE and gels were transferred to polyvinylidene difluoride (PVDF) membranes, and exposed to X-ray film. The PVDF membranes were probed with an anti-myc antibody to evaluate the efficiency of the immunoprecipitation. The peptide sequence illustrated highlights potential serine residues that are hyperphosphorylated in v-src transformed cells as well as potential serine kinase.


pCanHA2 +
V- Src +
Mock
SLK 1-192K63R
SLK 1-325K63R
SLK 1-373K63R
Mock
SLK 1-192K63R
SLK 1-325K63R
SLK 1-373K63R
IP: 9E10
Myc-SLK 1-373K63R
Myc-SLK 1-325K63R
IP: 9E10; Blot: 9E10
Myc-SLK 1-373K63R
Myc-SLK 1-325K63R
SLK 1-373K63R
1
192
325
373
1 - 373 a.a.
PKA
S340
CK2
SS347/348
CK2
S362
PKC
S364
PIPANKRASSDLSIASSEEDKLSQNACILESVSERTEQS

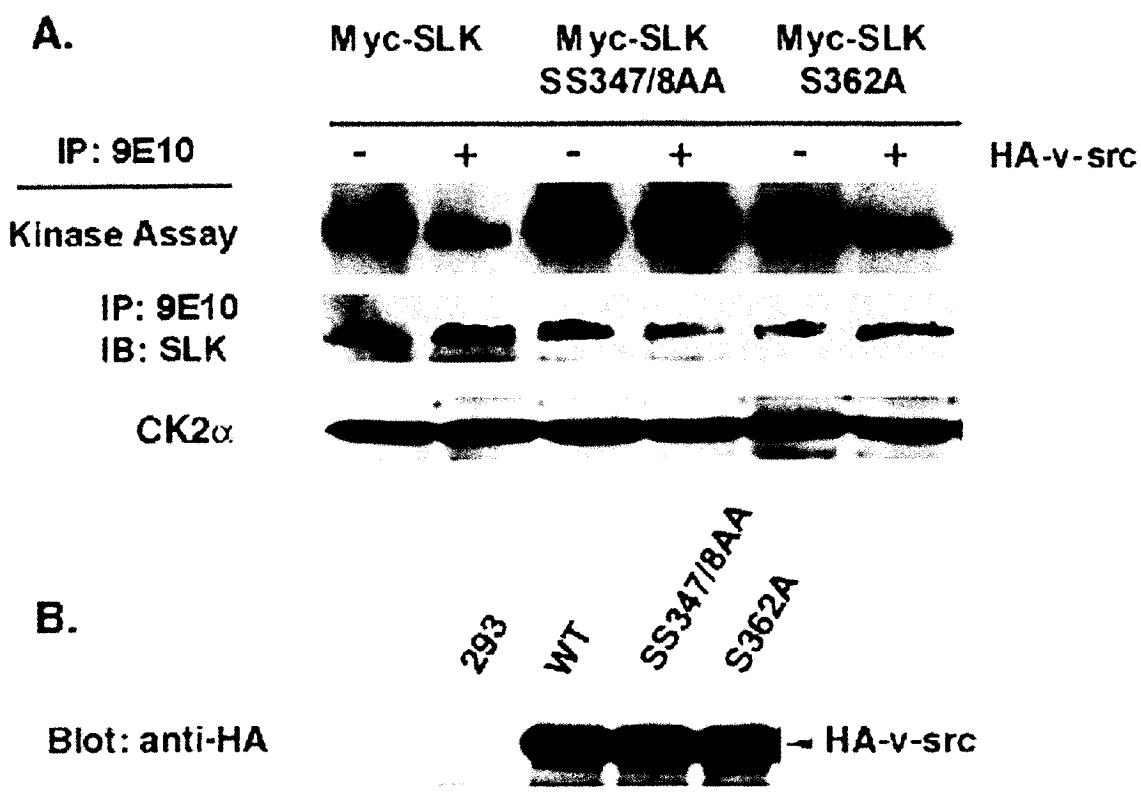
presence of putative PKA, PKC and CK2 phosphorylation sites. To test the role of PKA and PKC on SLK kinase activity, HEK293 cells were transfected with HA-SLK and treated with either dibuturyl-cAMP (dbcAMP) or 12-*O*-tetradecanoylphorbol-13-acetate (TPA). SLK immunoprecipitation and kinase assay showed no changes in SLK kinase activity (not shown). Supporting this, Myc-tagged SLK point mutants S340A and S364A, sites of potential PKA and PKC phosphorylation respectively, showed no changes in SLK kinase activity at the basal level or when co-expressed with HA-v-src (not shown), suggesting that the v-src effect is not mediated by PKA or PKC.

5.2.2 CK2 Phosphorylates SLK And Regulates Its Kinase Activity

To investigate the potential role of CK2 on SLK kinase activity, Myc-SLK point mutants for serine residues located at position 347/348 and 362 were generated (Myc-SLK SS347/348AA and Myc-SLK S362A). HEK293 cells transfected with these constructs revealed that Myc-SLK SS347/8AA displayed a 2- to 3-fold increase in basal kinase activity when compared to Myc-SLK (Figure 5-3 A). Supporting this, co-transfection of Myc-SLK SS347/348AA with HA-v-src did not result in the downregulation of SLK kinase activity (Figure 5-3 B). Interestingly, examination of the CK2α protein content in HEK293 cells revealed that it is highly expressed, suggesting that CK2 may also regulate the catalytic activity of the transfected Myc-SLK (Figure 5-3 A).

We then addressed whether SLK is phosphorylated by CK2 on serine 347/348 or 362. Therefore, the kinase domain mutant were GST-tagged and

Figure 5-3. Mutation of potential CK2 phosphorylation sites increased SLK basal activity. Potential CK2 phosphorylation sites mutants, serine (S) to alanine (A), were generated at residue 347/348 and 362. Myc-SLK, Myc-SLK SS347/348AA and Myc-SLK S362A mutants were transfected into HEK293 cells in the absence or presence of HA-v-src (panel A) and 9E10 immunoprecipitate were subjected to an in vitro kinase assay. Samples were resolved by SDS-PAGE, transferred to PVDF membranes, and exposed to X-ray film. The PVDF membranes were then probed with an anti-SLK antibody to evaluate the efficiency of the immunoprecipitation. Endogenous levels of CK2α expressed in HEK293 cells are shown (panel A). Equal amount of cell lysates resolved on SDS-PAGE and probed with 12CA5 show the efficiency of HA-v-src transfection (panel B).

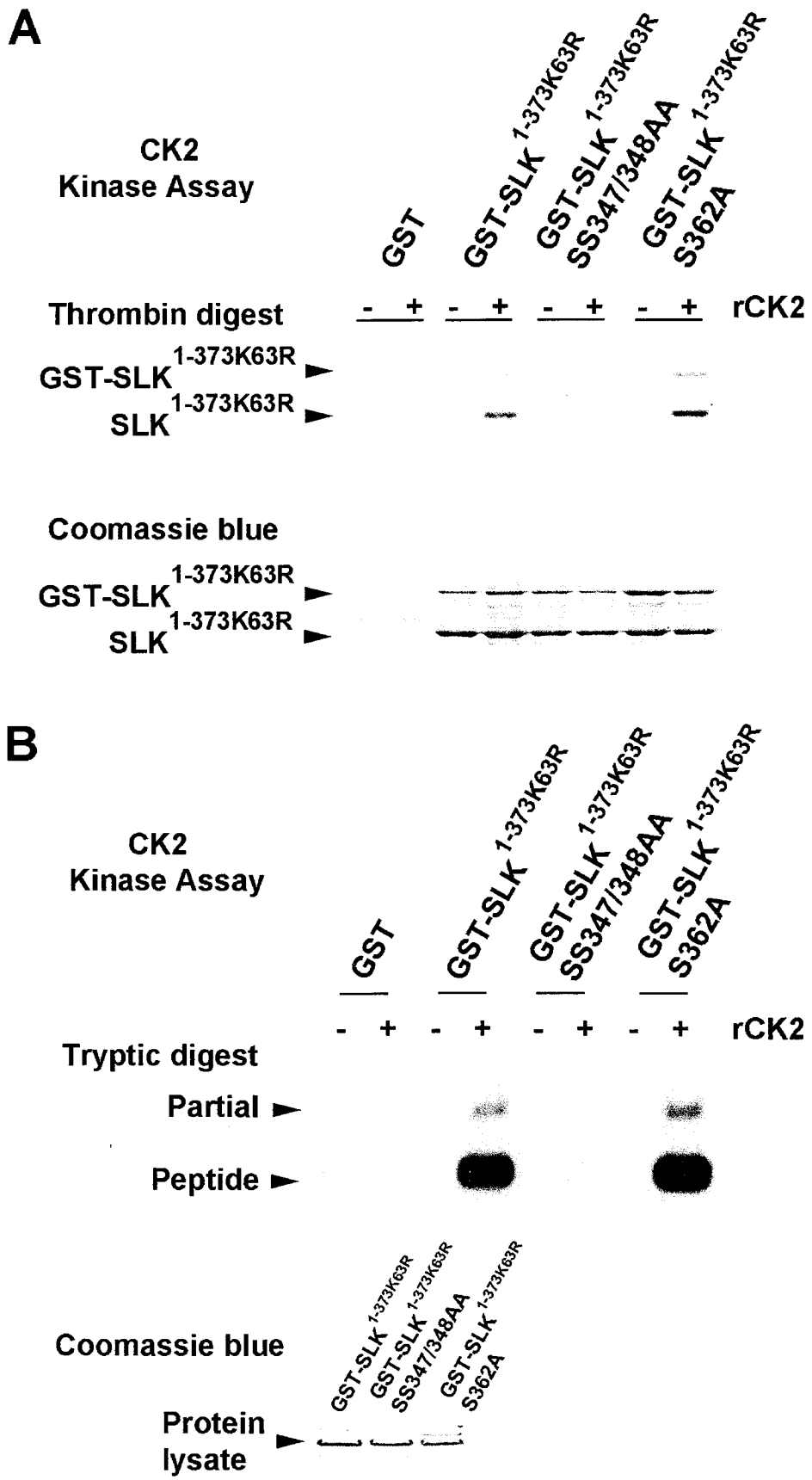

A.
Myc-SLK
Myc-SLK SS347/8AA
Myc-SLK S362A
IP: 9E10
- + - + - +
HA-v-src
Kinase Assay
IP: 9E10
IB: SLK
CK2α
B.
293
WT
SS347/8AA
S362A
Blot: anti-HA
HA-v-src

subjected to an in vitro CK2 kinase assay in the presence or absence of recombinant CK2 (rCK2, Sigma). Following the kinase assay, samples were either digested with thrombin, to remove the GST peptide, or with trypsin to generate a 1.5 kDa peptide of interest containing serines 347/348 or 362 (Figure 5-4). Both thrombin or trypsin digests revealed that the SLK mutant SS347/348AA displayed a marked decrease in its phosphorylation level relative to GST-SLK$^{1\text{-}373K63R}$ or GST-SLK$^{1\text{-}373K63R}$S362A. These results suggest that CK2 phosphorylates SLK directly on serine residues 347/348, contributing to SLK downregulation.

5.2.3 Inhibition Of CK2 Rescues SLK Kinase Activity In V-src-Transformed Cells

To assess the effect of CK2 on SLK kinase activity in v-src-transformed cells, we used 4,5,6,7-Tetrabromo-2-azabenzimidazole (TBB; Calbiochem), a specific CK2 inhibitor (Sarno et al. 2001). 49f and v-src transformed 49f cells were treated overnight with 50 μM TBB. SLK and CK2 were then immunoprecipitated independently from the same cell lysate and subjected to in vitro kinase assays. Our results show that after TBB treatment, SLK kinase activity in v-src-transformed cells is restored to levels similar to wildtype 49f cells (Figure 5-5 A). Interestingly, we observed a dramatic increase in CK2 kinase activity in v-src-transformed cells, suggesting that v-src transformation results in CK2 activation (Figure 5-5 B). TBB treatment resulted in a decrease in CK2 kinase activity in both cell lines. Supporting a role for CK2 in the regulation of

Figure 5-4. CK2 phosphorylates SLK at serine 347/348 in vitro. GST tagged SLK kinase domain kinase inactive ($SLK^{1\text{-}373K63R}$) harboring mutations to serine residues 347/348 and 362 were generated, $SLK^{1\text{-}373K63R}$ SS347/348AA and $SLK^{1\text{-}373K63R}$ S362A. Bacterially expressed GST constructs were subjected to an in vitro kinase assay in the presence or absence of recombinant CK2 (rCK2). Samples were then digested with thrombin, panel A, or trypsin, panel B, and resolved by SDS-PAGE. Gels were coomassie stained, dried and exposed to X-ray film.


A
CK2
Kinase Assay
GST
GST-SLK 1-373K63R
GST-SLK 1-373K63R SS347/348AA
GST-SLK 1-373K63R S362A
Thrombin digest
- + - + - + - + rCK2
GST-SLK 1-373K63R
SLK 1-373K63R
Coomassie blue
GST-SLK 1-373K63R
SLK 1-373K63R
B
CK2
Kinase Assay
GST
GST-SLK 1-373K63R
GST-SLK 1-373K63R SS347/348AA
GST-SLK 1-373K63R S362A
- + - + - + - + rCK2
Tryptic digest
Partial
Peptide
Coomassie blue
GST-SLK 1-373K63R
GST-SLK 1-373K63R SS347/348AA
GST-SLK 1-373K63R S362A
Protein lysate

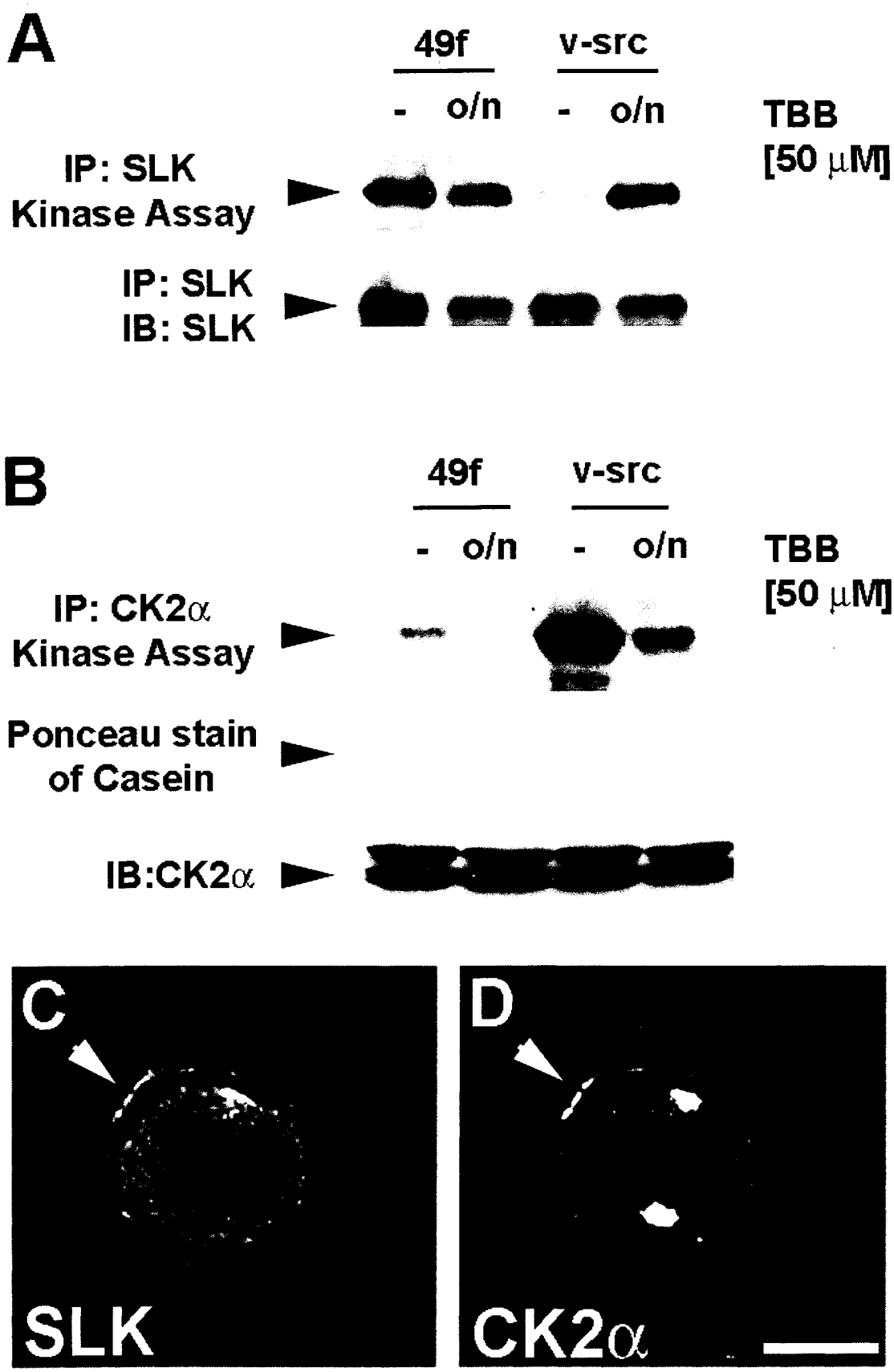
SLK activity, SLK and CK2α could be colocalized at the cell periphery in 49f cells following a 20 min replating assay on FN matrix (Figure 5-5 C & D). Taken together, these results suggest that v-src mediates the downregulation of SLK kinase activity through activation of CK2 that can directly phosphorylate SLK.

5.3 Discussion

We have previously shown that SLK is redistributed to vinculin-containing structures during cell spreading (Wagner, et al. 2002). Recently, we have shown that SLK redistribution to cell periphery is altered in SYF-deficient cells. In addition, we have demonstrated that SLK kinase activity is downregulated in v-src transformed cells, an effect that required both v-src kinase activity and membrane translocation (Chapter 4).

Here, we show that SLK is hyperphosphorylated on serine residues in v-src and c-srcY527F transformed 49f cells. Mutation analysis have led us to the identification of three potential serine/threonine kinases involved in SLK hyperphosphorylation, PKA, PKC and CK2. While both PKA and PKC did not appear to affect SLK kinase activity, SLK mutants for CK2 putative phosphorylation sites (SLK SS347/348AA) exhibited 2- to 3-fold increase in kinase activity. In addition, we have demonstrated that CK2 phosphorylates SLK and that the SLK SS347/348AA mutant displayed reduced phosphorylation levels relative to the wildtype SLK or SLK S362A. Most importantly, we showed that inhibition of CK2 kinase activity in v-src transformed 49f cells restored SLK activity to levels similar to what is observed in control 49f cells.

Figure 5-5. Inhibition of CK2 restores SLK kinase activity in the v-src transformed 49f cells. 49f and v-src transformed 49 f cells were incubated with or without [50μM] of TBB overnight. SLK as well as CK2α were then immunoprecipitated independently from the same cell lysate and subjected to an in vitro kinase assay. SLK kinase activity was assayed based on its level of autophosphorylation (panel A) whereas, dephosphorylated casein was used as a substrate for CK2 activity (panel B). Samples were resolved by SDS-PAGE, transferred to PVDF membranes, and exposed to X-ray film. The PVDF membranes were probed with anti-SLK or with anti-CK2α antibodies to evaluate the efficiency of the immunoprecipitation. Panel C & D, show perfect co-localization of SLK and CK2α during a 20 min replating assay of 49f cells on FN matrix. Scale bar = 10 μm.


A
49f
v-src
- o/n - o/n
TBB
[50 μM]
IP: SLK
Kinase Assay
IP: SLK
IB: SLK
B
49f
v-src
- o/n - o/n
TBB
[50 μM]
IP: CK2α
Kinase Assay
Ponceau stain
of Casein
IB:CK2α
C
SLK
D
CK2α

CK2 minimal amino acid consensus phosphorylation sequence is Ser-X-X-Acidic (Pinna 1990, Pinna and Meggio 1997). Although we have focused on the catalytic region of SLK as the target of v-src-mediated downregulation, truncation analysis of the full length SLK revealed that SLK is also serine phosphorylated on its M-NAP and ATH domains, however at much lower levels (not shown). Therefore, serine phosphorylation of those domains cannot be excluded as other important regulatory sites. Nonetheless, serine 347/348 residues are the main CK2 targets within the SLK kinase domain. Interestingly, mutation of these residues to alanine resulted in an increase in SLK activity in comparison to the WT or S362A mutant, suggesting that SLK activity in HEK293 cells is partially restricted by the endogenous levels of CK2 and that mutation of serine residues 347/348 to alanine causes the release of CK2-regulated conformational constraints.

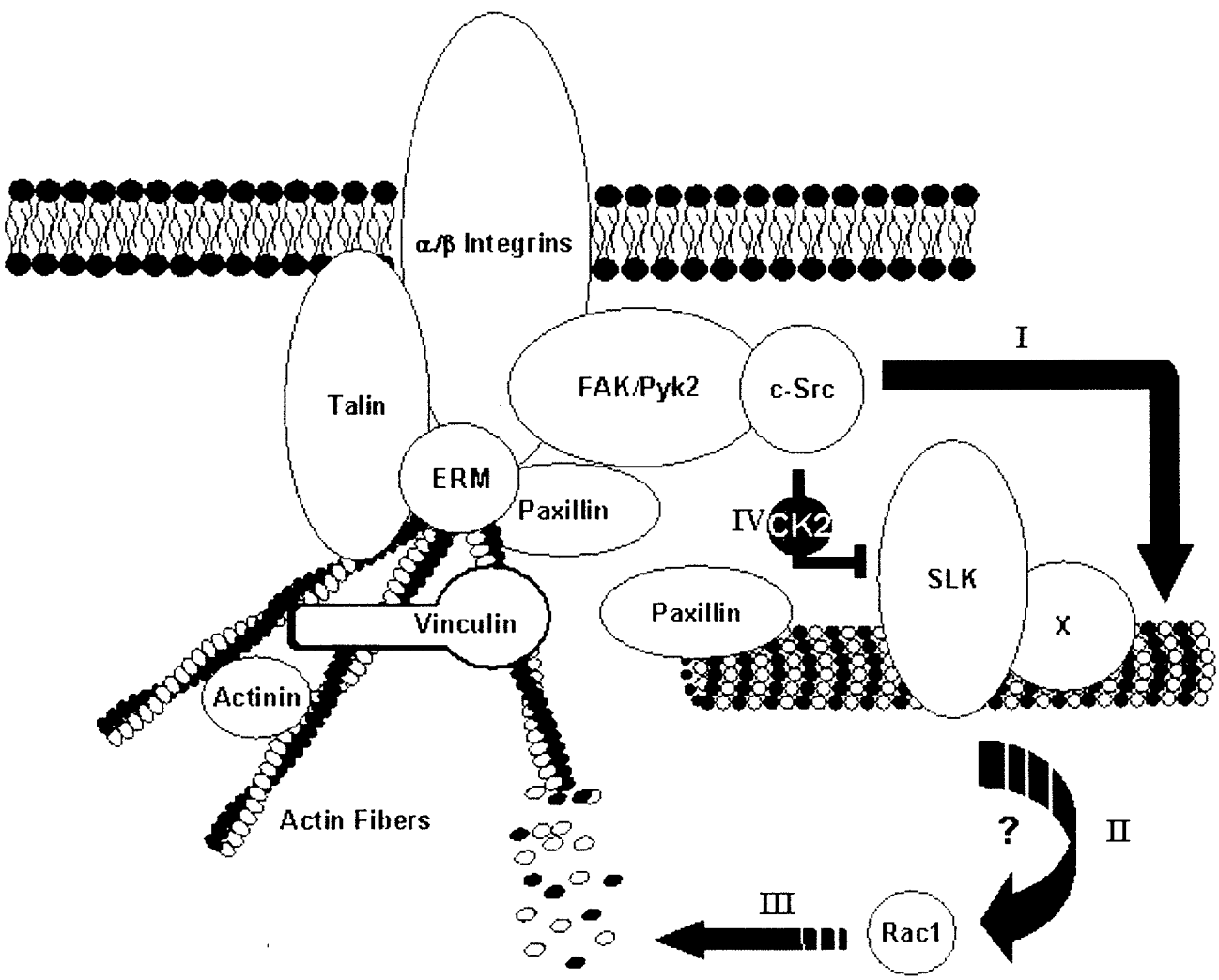
Previous studies have shown that SFKs, Lyn and c-Fgr, are capable of phosphorylating CK2 α subunit leading to an increase in its kinase activity in vitro (Donella-Deana, et al. 2003). Interestingly, we have observed a dramatic increase in CK2 activity in v-src transformed cells that could not be attributed to differences in protein expression levels. Therefore, one possibility is that v-src may directly modulate CK2 activity.

CK2 has been classified as a messenger-independent kinase and that phosphorylation, redistribution to subcellular compartment or protein interaction may contribute to its regulation (Tuazon and Traugh 1991). Recent studies have showed that CK2 interacts with CKIP-1 and that this interaction is required for the

translocation of CK2 to the plasma membrane (Olsten, et al. 2004). On the other hand, we have previously shown that c-src modulates SLK redistribution to the cell periphery whereas v-src kinase activity and membrane translocation are required to downregulate SLK kinase activity. In this study, we show that SLK and CK2 colocalize at the cell periphery during cell spreading on FN. Furthermore, we have demonstrated that the inhibition of CK2 activity in v-src transformed cells restores the catalytic activity of SLK. Combined, these results suggest that v-src upregulates CK2 activity and that CK2 in turn downregulates SLK kinase activity. This cascade suggests that SLK is translocated to the cell periphery in a c-src-dependent manner as part of the microtubule network, an adhesion destabilizing signal (Abu-Amer, et al. 1997, Matten, et al. 1990, Nakayama, et al. 1994). At the cell periphery SLK kinase activity is downregulated by CK2 allowing actin polymerization and the assembly of new focal contacts (as illustrated in Figure 5-6). The signaling mechanisms that induce a src-dependent recruitment and regulation of SLK at sites of cytoskeletal remodeling await further studies.

Overall, we have demonstrated that v-src-mediated downregulation of SLK kinase activity proceeds in part via CK2. In addition we have identified two serine residues 347/348 that upon phosphorylation by CK2 downregulates SLK kinase activity. The implications of SLK downregulation on cell adhesion and migration are being investigated.

Figure 5-6. Proposed model for SLK regulation by the src-family kinases and CK2. Upon FN stimulation or migration, focal adhesions assemble through the activation of the FAK/src complex. SLK is then recruited to sites of actin dynamics, a process that requires the src-family kinases perhaps by modulating microtubule dynamics (I). Following SLK-mediated actin disassembly, which may require Rac1 (II and III), adhesion complex relaxation can occur and SLK becomes downregulated, indirectly by c-src via CK2 (IV).


α/β Integrins
Talin
FAK/Pyk2
c-Src
I
ERM
Paxillin
IV
CK2
SLK
X
Paxillin
Vinculin
Actinin
Actin Fibers
?
II
III
Rac1

CHAPTER 6

GENERAL DISCUSSION

Cell adhesion and migration are highly regulated processes involving dynamic assembly and disassembly of adhesion complexes and constant remodeling of the actin and microtubule networks. Studies have shown that upon replating cells onto FN matrix, the integrin receptors cluster causing the recruitment and activation of FAK and complex formation with c-src. C-src mediated phosphorylation of FAK at multiple residues generates further SH2 binding sites leading to the recruitment of adaptor proteins, such as p130Cas, paxillin, Nck, Grb2 and others.

The Ste20-Like kinase (SLK) is a serine/threonine kinase that was shown to induce actin stress fibers disassembly and apoptosis when overexpressed (Sabourin and Rudnicki 1999). Recently, SLK was found to redistribute to vinculin containing structures at the cell periphery and to be associated with tubulin containing protein complexes (Wagner, et al. 2002). Interestingly, SLK induced actin stress fibers disassembly could be inhibited by a dominant negative Rac1, suggesting that the effect of SLK on actin dynamics may be mediated by Rac1 (Wagner, et al. 2002).

To further characterize the role of SLK during cell adhesion and migration, we used the PEA3(-/-) cell line. Null mutations in the *pea3* allele have been shown to compromise the capacity of mammary tumors to metastasize in the MMTV-Neu/HER2 transgenic mice, whereas forced expression of PEA3 in MCF7 human breast tumor cells increased their metastatic potential (Kaya, et al. 1996, Kurpios, et al. 2003, Shepherd and Hassell 2001). Furthermore, PEA3(-/-) cells were shown to exhibit migration deficiencies, however the pathway involved

remains to be elucidated (J.A. Hassell personal communications). Here we have shown that during FN-stimulated replating assays, SLK did not redistribute to vinculin containing ruffles and lamellipodia in the PEA3(-/-) cells as it did in WT or PEA3(-/-)+PEA3 cells, as previously shown by Wagner *et al* (Wagner, et al. 2002). PEA3(-/-) cells exhibited focal adhesion disassembly defect mainly due to impaired activation of c-src, suggesting that c-src activation is required for SLK recruitment and redistribution to the cell periphery. Supporting this, we also showed that SLK did not redistribute to the cell periphery in the SYF-deficient cells and that this phenotype could be rescued by re-introducing c-src into the SYF cells (SYF+c-src; Chapter 4). Surprisingly, SLK recruitment to the cell periphery was found to be independent of FAK.

Recently, the microtubule network has been shown to mediate adhesion site destabilization and disassembly, a process that requires kinesin motors (Ballestrem, et al. 2000, Bershadsky, et al. 1996, Kaverina, et al. 1999, Kaverina, et al. 1998, Krylyshkina, et al. 2002). It has been postulated that the microtubules deliver adhesion destabilizing signals. Previously, Wagner *et al* 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 the regulation of microtubule reorganization (Abu-Amer, et al. 1997, Matten, et al. 1990, Nakayama, et al. 1994). This led us to propose that the c-src-mediated effect on the reorganization of the microtubule network may mediate the translocation of SLK as well as other substrates to the cell periphery to induce adhesion site turnover.

C-src has been shown to associate with paxillin through its SH2 domain. Furthermore, the FAK-c-src complex was shown to induce tyrosine phosphorylation of paxillin creating a binding site for SH2 containing proteins such as Crk, leading to Rac activation (Bellis et al. 1995, Burridge et al. 1992, Schaller and Parsons 1995). In addition, Brown *et al* have shown that paxillin is serine phosphorylated on the LIM domain 3 and that overexpression of paxillin serine mutants retards adhesion to FN matrix (Brown et al. 1998). Interestingly, our laboratory has recently demonstrated that SLK can phosphorylate paxillin in vitro (O'Reilly *et al* unpublished data). While the exact role of SLK and paxillin in vitro phosphorylation is still under investigation, it is tempting to speculate that SLK redistribution to the cell periphery might be required for the phosphorylation of target proteins thereby inducing the relaxation of adhesion sites.

Previously, we have shown that SLK is recruited to vinculin and paxillin containing structures at the cell periphery following tyrosine phosphorylation of adhesion components and that the overexpression of SLK inhibited cell spreading onto FN matrix (Wagner, et al. 2002). In addition, we have also shown that SLK is found in a complex with α-tubulin (Wagner, et al. 2002). This led us to propose that SLK redistribution to the cell periphery may require c-src and microtubule-association. SLK delivery through the microtubules could regulate focal adhesion disassembly during cell adhesion and migration. Supporting this, SLK RNA interference studies in fibroblasts showed a 3-fold decrease in cell migration during Boyden chamber assays, whereas normal vinculin containing adhesion complexes were observed during FN-stimulated replating assays

(Wagner *et al* unpublished data). Similarly, SYF-deficient and p130Cas-deficient fibroblasts exhibit impaired migration due to focal adhesion disassembly and normal adhesion complex structures (Honda et al. 1999, Klinghoffer, et al. 1999).

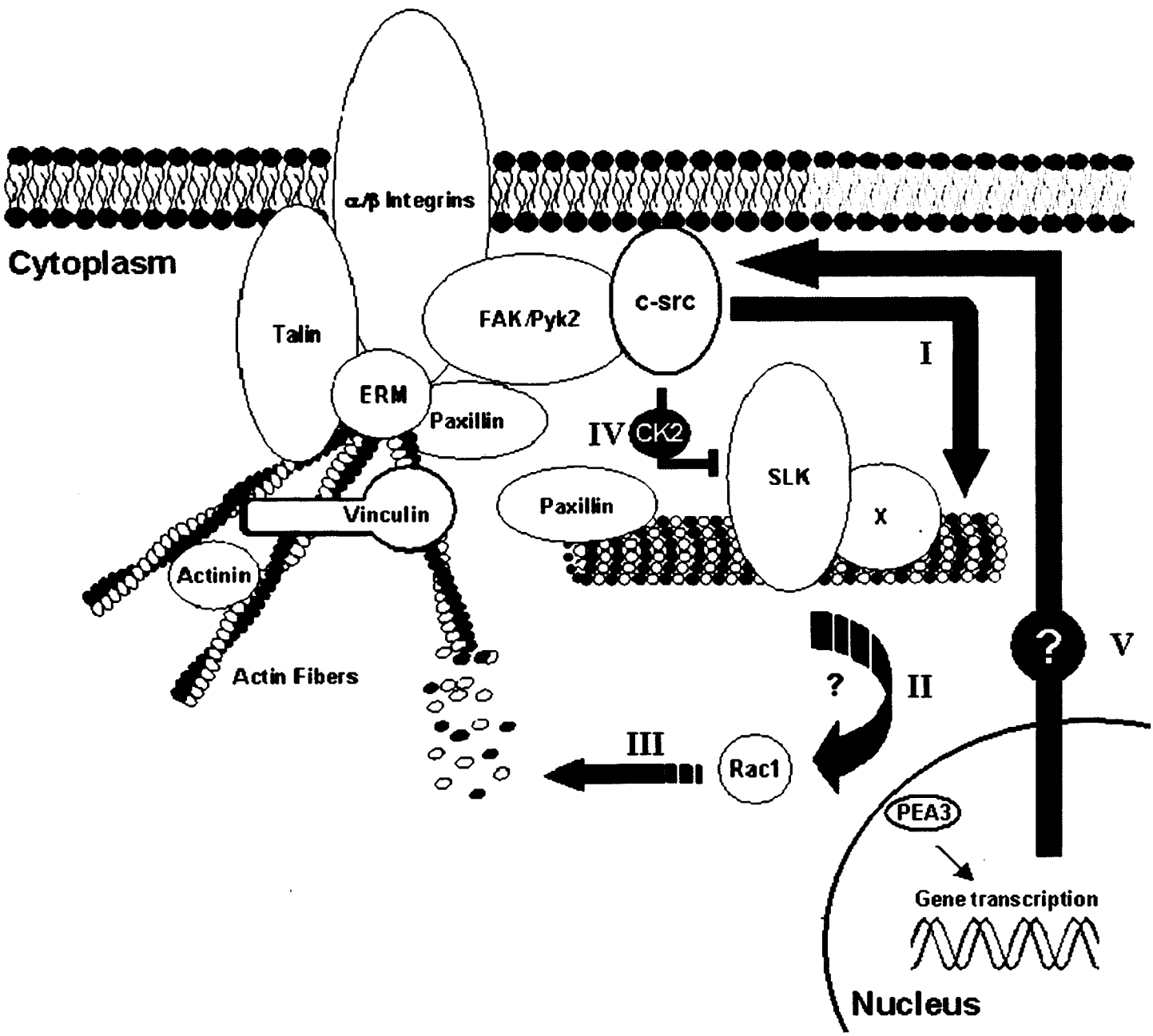
Although SLK distribution was affected, we observed that SLK kinase activity was not altered in SYF-deficient cells. However, a 2- to 3-fold decrease in SLK kinase activity was observed in both v-src and c-srcY527F transformed cells. We also demonstrated that SLK downregulation requires both v-src kinase activity and membrane anchoring. Furthermore, using biochemical and molecular approaches, we demonstrated that SLK is hyperphosphorylated on serine residues mainly in the kinase domain. This led us to the identification of two CK2 phosphorylation sites located on serine 347/348. Mutational analysis revealed that CK2 directly phosphorylates SLK on these two serine residues resulting in SLK downregulation. Interestingly, we also observed that CK2 activity is dramatically increased in v-src-transformed cells and that the inhibition of CK2 restores SLK kinase activity in these cells. These observations extended our model whereby SLK is recruited to sites of cytoskeletal remodeling in a c-src-dependent manner. SLK is then further regulated by a src-CK2 pathway at these sites.

Recently, Timpson et al. showed that c-src kinase activity is required at peripheral adhesion sites for Rac1- and cdc42-induced adhesion remodeling and directed cell migration (Timpson, et al. 2001). Interestingly, we have shown that SLK-mediated actin stress fiber disassembly can be inhibited by a dominant negative version of Rac1 (Wagner, et al. 2002). Here, we showed that both v-src

kinase activity and membrane anchoring are required to mediate the downregulation of SLK, suggesting a role for SLK kinase activity in focal adhesion disassembly. In support of this, Fincham *et al* have reported that c-src kinase activity and myristylation site are required to regulate focal adhesion turnover during cell motility (Fincham and Frame 1998). Therefore, one possibility is that the process of focal adhesion turnover induces SLK recruitment, through the microtubule (Wagner, et al. 2002), in a c-src-dependent manner. This further requires the actin disassembling activities of SLK and Rac1, allowing adhesion site destabilization. Upon assembly of new focal contacts, SLK activity is downregulated, allowing actin polymerization (see Figure 6-1). However, in a v-src expression system, the turnover rate of adhesion sites is markedly increased (Fincham and Frame 1998), resulting in an apparent SLK downregulation. Supporting this, FAK- and SYF-deficient cells, which display reduced migration rates and adhesion turnover, had relatively normal levels of SLK activity.

Recently CK2 was shown to interact with CKIP-1 and that this interaction is required for the translocation of CK2 to the plasma membrane (Olsten, et al. 2004). Furthermore, studies have shown that SFKs, Lyn and c-Fgr, are capable of phosphorylating the CK2 α subunit leading to an increase in its kinase activity in vitro (Donella-Deana, et al. 2003). Here, we have shown that c-src modulates SLK redistribution to the cell periphery whereas v-src kinase activity and membrane anchoring are required to downregulate SLK kinase activity. We also showed that CK2 phosphorylates SLK on serine 347/348 resulting in the

Figure 6-1. Proposed model for SLK recruitment and regulation during FN-stimulated adhesion and migration. Upon FN stimulation or migration, focal adhesions assemble through the activation of the FAK/src complex. SLK is then recruited to sites of actin dynamics, a process that requires the src-family kinases perhaps by modulating microtubule dynamics (I). Following SLK-mediated actin disassembly, which may require Rac1 (II and III), adhesion complex relaxation can occur and SLK becomes downregulated, indirectly by c-src via CK2 (IV). In addition, c-src kinase activity appears to be regulated by PEA3 transcription targets (V).


α/β Integrins
Cytoplasm
Talin
FAK/Pyk2
c-src
I
ERM
Paxillin
IV
CK2
SLK
X
Paxillin
Vinculin
Actinin
Actin Fibers
?
II
?
V
III
Rac1
PEA3
Gene transcription
Nucleus

downregulation of SLK kinase activity. Interestingly, we observed that CK2 activity is dramatically increased in v-src transformed cells and that the inhibition of CK2 activity in these cells restores the SLK kinase activity. In addition, SLK and CK2 colocalized at the cell periphery during cell spreading on FN. Therefore, we propose that during cell adhesion and migration, SLK is recruited to dynamic focal contacts in a c-src-dependent manner via the microtubule network where it modulates actin dynamics and adhesion complex disassembly. SLK kinase activity is then downregulated in a src-mediated manner via CK2 to allow actin polymerization, protrusive forces and the formation of new focal contacts at the leading edge of the cell.

Overall, our data show that SLK recruitment to the cell periphery is c-src-dependent whereas v-src kinase activity and membrane anchoring are required for the downregulation of SLK kinase activity. Furthermore, v-src mediated SLK downregulation is CK2 dependent, in part. Studies in our laboratory aimed at elucidating potential SLK domains involved in the c-src mediated recruitment to the cell periphery are ongoing. In addition, we have identified novel SLK interactors that are clearly involved in cell adhesion and migration. Combined with the RNA interference studies, we have established a role for SLK in cell motility. Recent observations in our laboratory have strengthened these findings by demonstrating a complex formation between the CLIM1/2 adaptor proteins, microtubules, SLK and paxillin (Storbeck *et al* unpublished data). Knockdown experiments for CLIMs showed increased cell motility, suggesting that CLIMs act as SLK "retention" factors or regulators of substrate access. Therefore, c-src

activation may allow the microtubules to deliver the SLK complex to induce adhesion turnover. The identification of SLK substrates will shed light on the cytoskeletal remodeling events that are regulated by SLK.

Interestingly, this study also contributed to the identification of SLK as a potential target for cancer therapeutics. Cancer cells are known to exhibit increased migration and to be highly invasive. Regulating SLK cellular distribution and kinase activity in cancer cells might retard the propagation of cancer cells resulting in a restricted cancer localization thus a more effective extraction.

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